BIOCHEMISTRY OF SPORES OF AEROBIC BACILLI WITH SPECIAL REFERENCE TO GERMINATION

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I. INTRODUCTION

The formation and maintenance of the dormant state, especially as represented by the bacterial endospore, presents an interesting problem in microbial ecology. Authors during the past decade have disagreed on a uniform criterion. The topic of bacterial endospores has been treated in numerous reviews (1 to 6), and need not be summarized here. The resistance to heat, which is of doubtful value as a selective agent in nature for spore formation, nevertheless represents our best current standard for the dormant spore state.

Detailed cytological observations on dormant endospores and during the breaking of dormancy have been reviewed elsewhere (7 to 9), and are beyond the scope of this review. It is necessary, however, to summarize some of these changes occurring during the breaking of dormancy. The bacterial endospore is characterized by its high refractility, resistance to stains and impermeability to substrates. The endospore coat is thought to be composed of two layers (10). A number of enzymes, peptides (11, 12) and fat soluble components are associated with at least one of these layers. With the exception of some components, such as high concentrations of calcium, dipicolinic acid (DPA)[1], and in *Bacillus sphaericus*, α-ϵ-diaminopimelic acid (DAPA), constituents of spores are similar to those of vegetative cells. When dormancy is broken, the loss in refractility of the spore (13) coincides with an imbibition of water. This period is associated with losses in heat resistance, stainability and dry weight, the latter being partly ascribed to a loss of calcium dipicolinate (11). Later the spore coat breaks and a new germ cell emerges from the spore case and eventually matures into a vegetative form. Chromatin material differentiates (14), and changes occur in ribonucleic acid, deoxy-

[1]In addition to the usual abbreviations (DPN, TPN, AMP, ADP, ATP, RNA, DNA, TCA) the following are used in this paper: DPA, dipicolinic acid; DAPA, α,ϵ-diaminopimelic acid; 2KG, 2-ketogluconate; α-KG, α-ketoglutarate; G-6-P, glucose-6-phosphate; F, fructose, F-6-P, fructose-6-phosphate; 6-P-G, 6-phosphogluconate; HMP, hexose monophosphate; HDP, hexose diphosphate; 2KDPG, 2-keto-3-deoxy-6-phosphogluconate; 2K6PG, 2-keto-6-phosphogluconate; FAD, flavin-adenine-dinucleotide; CoASH, reduced coenzyme A; DPT, diphosphothiamine; OAA, oxalacetic acid; PP, pyrophosphate.

ribonucleic acid, and metaphosphate (15) during this entire transition.

At various times the term germination has been applied to one or all of these above changes. For the purposes of the present review, we shall adopt the definition recently proposed by Campbell (6), *i.e.*, "Spore germination may be regarded as the change from a heat resistant spore to a heat labile entity which may not necessarily be a true vegetative cell." Later development, leading eventually to the formation of a mature vegetative cell, is called "outgrowth."

Improvements in the methodology for studying spore germination, as well as the discovery of enzymes inherent to the dormant endospore, have led in the last ten years to a renewal of active interest in the breaking of spore dormancy. It is the purpose of this review to examine some of these findings and attempt to correlate these to the problem of spore dormancy and to the mechanism of germination.

II. GERMINATION

1. Spore Cleanliness

An examination of the literature dealing with bacterial spores emphasizes the need for clearly defining the cleanliness of the various spore stocks employed. Contaminating debris from the medium and vegetative cells, absorbed minerals, and the age and conditions of storage of spores can influence the germination requirements (16). It is difficult, therefore, to evaluate such studies, when the conditions for preparation and storage of the spores are not clearly defined. Microscopic evidence alone, employed as a measure of spore cleanliness, is insufficient. An improvement in the methodology for preparing spores followed from the finding by Lawrence and Halvorson (17) of a heat resistant catalase as an integral part of the resting spore structure, probably attached to the spore wall. Heat labile catalase is, on the other hand, merely adsorbed on the spore wall, probably as a vegetative cell remnant. Removal of heat sensitive catalase, therefore, serves as a criterion of cleanliness. This process frequently involves twelve to fifteen washings of the spore.

Another interesting set of standards for spore cleanliness has been the suggestion of Murty (18) that the germination requirements themselves be used as an index of cleanliness. A freshly harvested spore preparation would be repeatedly washed until it conformed with a standard set of germination requirements. For example, freshly prepared spores of *Bacillus cereus* var. *terminalis* require L-alanine and adenosine for germination. With insufficient washing, either adenosine or L-alanine alone can serve as a germinating agent (19). Presumably either traces of L-alanine or adenosine are retained from the sporulation medium. Similarly, clean aged spores will germinate in the presence of adenosine or L-alanine alone. In these cases, the germinating stimulants may be produced during aging or some of the reactions activated by L-alanine or adenosine obviated.

2. Heat Activation

One of the frequently encountered phenomena in spore germination was the need for a pre-treatment with heat. Incomplete or delayed germination was frequently observed in the early studies on bacterial endospores until Weizmann (20) observed that a treatment of 90 to 100 C for 1 to 2 min stimulated the germination of spores of *Clostridium acetobutylicum*. Present practices employ lower temperatures and longer time intervals (60 to 85 C for 5 to 15 min) for heat activation in spores which either failed to germinate or showed delayed germination in the absence of heat activation. Evans and Curran (21, 22) first systematically demonstrated that spores which did not germinate or whose germination was delayed in the absence of heat, overcame this "dormancy" when heat activated. Furthermore, the extent of heat activation was not only directly related to the rate and yield of germination, but also to the minimal temperature at which germination would take place. Heat activation was lost on storage but the spores could be reactivated by a second heat treatment (23). Schmidt (2) has recently reviewed the literature on this subject.

The resistance of heat activated spores to phenol, formaldehyde and other chemical agents (24) is reduced. A similar reduction in resistance to furaskin (sic), penta-chlorophenol, and vitamin K_5 by pre-treatment with heat was observed by Simidu and Ueno (25). Powell and Hunter (16) observed that unheated spores of *Bacillus cereus* required inosine or a mixture of L-alanine, tyrosine and adenosine for optimal germination. After heat activation or prolonged storage, adenosine alone could meet the requirement for rapid germination. Heat treatment also activates sev-

eral enzymes which are inactive in dormant spores (26, 27), and, paralleling this activation, the spores lose part of their DPA content (28).

There appears to be an interrelationship among activation by heat, by aging, and, in certain cases, by chemicals. For example, furfural (29) and some of its heterocyclic derivatives (30) activate ascospores of *Neurospora*, and furfural was capable of replacing heat as an activator of the germination of spores of some thermotolerant and thermophilic bacilli (31). Also, Falcone (32) has reported that spores of *B. subtilis* lose heat resistance during exposure to H_2O_2. Further studies of this nature will furnish a better understanding of the biochemistry of spore activation.

The duration of dormancy among spores of one species, or even of one strain, is quite variable. Not all spores germinate especially in response to the stimulation of heat, aging, or chemicals (32*a*). For example, although the majority of spores of *B. subtilis* germinate in a few days, some remained dormant for as long as 90 days (33). McCoy and Hastings (34), employing single-cell technique, found that in *C. acetobutylicum*, the germination of 5 out of 100 of freshly harvested spores was delayed from 11 to 117 days and one spore, isolated from a year-old culture, required 222 days to germinate. If the spores are phenotypically heterogeneous with regard to their ability to undergo activation by aging, then such distributions of lag times in germination might be expected. A precedent for this exists in the resistance to ethylene oxide of spores derived from single cells which show a phenotypic distribution against a common genotype (35).

Heat activation may result in (a) the internal production of germinating agents; or (b) the release of an inhibitor of germination whose presence acts in the maintenance of dormancy. Evidence has been advanced in support of each of these theories. Germination requirements can be simplified by activation (16). For example, pyruvate, whose utilization can in certain instances lead to germination (36–38), is present in freshly harvested spores but absent in aged spores in which the pyruvate oxidizing system has been activated. On the other hand, Christian (39) and Stedman *et al.* (40) have presented evidence for the presence in spores and sporulating cultures of inhibitors of germination. The effect of these substances on activation remains to be elucidated.

3. Germination Requirements

Germination, as well as outgrowth, requires the presence of specific chemical agents. The requirements for outgrowth and vegetative growth differ from those required for germination (41 to 43) and probably represent nutritional requirements for synthesis of components designed for vegetative cells. Of particular interest to the present review are those agents which stimulate the germination process. In recent years, numerous reports have appeared characterizing the germinating requirements for various spores (32*a*, 44). Stedman (3) has recently reviewed this literature.

An examination of the germinating agents for spores of aerobic bacilli indicates that an amino acid (usually L-alanine), glucose and adenosine occupy key positions (3). For the purpose of the later discussion, the germination requirements for spores of several species are given in table 1. Although rather simple mixtures can serve to provide optimal germination, many agents permit partial germination or spare the requirements for the primary germinating agents. This situation is illustrated in the case of the germination of spores of *B. cereus* var. *terminalis*. Optimal germination is observed in the presence of 12 μM adenosine and 6mM L-alanine and M/30 phos-

TABLE 1

Chemical stimulants for the germination of spores of Bacillus cereus var. terminalis and B. megaterium

B. cereus var. *terminalis*		*B. megaterium*	
Chemical compounds	References	Chemical compounds	References
L-Alanine	45–47	L-Alanine	15, 37, 46, 48
DL-Alanine	37, 47	Glutamic acid	50
D-Alanine	37	Glucose	15, 46, 48, 50, 51
Glucose	46, 47	Maltose	50
Lactate	36, 37	Acetate	51
Pyruvate	36, 37	Formate	51
Adenosine	45–47	Propionate	51
Inosine	37	Adenosine	15, 51
Xanthosine	37	Phosphate	46
Guanosine	37, 45	Manganese	49, 51
Phosphate	46	Monovalent anions	51
		Spontaneous	16, 50

phate buffer, pH 7.0 (45). Freshly harvested spores show insignificant germination in the presence of L-alanine or adenosine alone. L-Alanine can be replaced by glucose (46), and adenosine by xanthine, guanosine, or inosine (37, 45). Adenosine requirements can be spared by high concentrations of L-alanine (36). Similarly, L-alanine requirements can be spared by lactate, pyruvate, elevated levels of adenosine (36, 37), or extended activation (52). A somewhat similar relationship exists among the chemicals stimulating the germination of spores of *B. megaterium* (table 1).

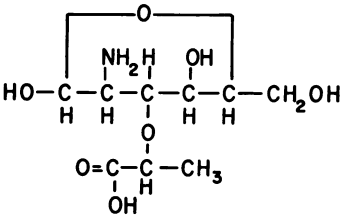
Differences in germination requirements exist among spores of different species. For example, although only L-alanine is active among the amino acids in stimulating germination of spores of *B. cereus* var. *terminalis* (table 1), *B. subtilis* var. *niger* (formerly *B. globigii*) and *B. polymyxa* (46), *B. subtilis* spores can be germinated by at least 20 different amino acids (3, 41) and *B. cereus* and *B. anthracis* spores by L-alanine and L-tyrosine (48).

Spores also vary in their ability to germinate in the presence of various non-nitrogenous compounds. *B. cereus* var. *terminalis* spores are activated by glucose, lactate and pyruvate (table 1), *B. megaterium* spores by glucose, maltose, acetate, formate and propionate (table 1), and *B. subtilis* spores by glucose, galactose, mannose, pyruvate, succinate, fumarate and malate (38). Differences in stimulation by nucleotides and nucleosides (table 1) are also evident between these spore strains.

The common feature of the germination stimulants appears to be their biochemical relationships. Products of hexose metabolism, pyruvate and its normal degradation products, can act as germinating agents. Although the pathways are less understood, the nucleotides and nucleosides may be similarly related. The differences observed between the various spores probably represent differences in the enzyme patterns of the spores. In general, the spores require a source of nitrogen (amino acid), a metabolizable carbon source and a precursor of nucleic acids.

4. Chemical Changes During Germination

Gross physical and chemical changes accompany spore germination. Powell and Strange (11) demonstrated a 30 per cent decrease in the dry weight of *B. subtilis* and *B. megaterium* spores paralleling an increase in the total and amino nitrogen of the medium. The germination exudate contained 13 amino acids, of which glutamic acid and aspartic acid were the major components, and a peptide which represented 10 to 15 per cent of the solid exudate. The peptide had a molecular weight of 15,300 (53), and on hydrolysis was found to contain α-ϵ-diaminopimelic acid, glutamic acid, alanine, acetyl glucosamine and an amino sugar (54) for which the following structure has been suggested (55).



This peptide was present in spore coat preparations from *B. megaterium* and *B. subtilis*, and in much smaller amounts in spore coat preparations from *B. cereus* (56). It was present in extracts from disintegrated resting spores, but in vegetative cells was present only in the insoluble cell-wall fraction (54, 56). An electrophoretic analysis of spores and a cation spectrum of spores and model particles (57) suggested that the peptide of *B. megaterium* was on the surface of the spore with the free acid groups exposed. The peptide was released when spore coats were suspended in water (56), and its release was accelerated by lysozyme, which was ineffective on intact spores. The presence of lytic activity in extracts of sporulating cells (58 to 62) and in spore extracts (60, 63, 64) suggested that spore germination may involve the activation of an intracellular lytic system which permits release of the spore peptides and destroys the permeability barrier. This lytic system was similar to lysozyme in its mode of action and heat stability (60).

Approximately 50 per cent of the total excreted solids was represented by an ultraviolet absorbing material (11, 15) which has been isolated and identified as the calcium salt of DPA (65) and which is probably identical to the substance with an absorption maximum at 2700 A which is released by *B. subtilis* spores when they are incubated with L-alanine (62).

Reported values for the DPA content of aerobic spores varies from 5 to 15 per cent of the spore

dry weight. The DPA released during germination of spores of *B. megaterium* accounts for 12 to 15 per cent of the dry weight of the spore (66, 67). However, spores of *B. cereus* var. *mycoides* contain 4.8 per cent DPA when sporulated in nutrient broth and 5.1 per cent DPA when sporulated in synthetic medium (68). These lower DPA percentages led to the suggestion that part of the DPA released from germinating spores resulted from DPA synthesis (11). Harrell (28) analyzed this possibility during the germination of spores of *B. cereus* var. *terminalis* and found that the total DPA remains constant during germination with L-alanine, although 75 per cent of the DPA was released into the suspending medium. The remaining 25 per cent was solubilized during the early stages of vegetative growth. Heat activation of the spores leads to a release of between 7 and 12.5 per cent of the DPA, depending upon the time of heat treatment. Similar observations have been reported by Murty and Halvorson (27).

Homologous vegetative forms do not contain DPA (11). DPA appears to parallel an increased calcium content during sporulation (59, 69). The high chelating ability of DPA for Ca^{++} (61, 62) suggested that DPA was responsible for the high Ca^{++} content of spores (69). Calcium accumulation by sporulating cells is probably a complex phenomenon. Vinter (70) showed that this accumulation is inhibited by cysteine, cystine and sulfide, which inhibitions can be reversed by an excess of glucose or calcium. DPA did not occur in spores as crystals of the calcium salt (57, 71), but was probably largely bound since analysis of ruptured spores showed a release of only a fraction of the total DPA (28). Acid hydrolysis was required to obtain quantitative yields (28, 68, 71). Perry and Foster recently isolated the monoethyl ester of DPA from spores (71). The release of DPA in parallel with the activation of glucose dehydrogenase (27, 28) indicated that it may be bound to these enzymes and perhaps contributes to their dormant state and heat resistance. Although this has not been demonstrated, it is interesting to note that alanine racemase was heat resistant when bound to large particles (72) having a high absorption maximum at 2700 A. The role of DPA in calcium retention, or in contributing to heat resistance of spores and spore enzymes (27), requires further study on the state of DPA in the spore itself.

In their investigations of the mechanism of the biosynthesis of DPA, a fundamental problem in the committed state of sporulation, Perry and Foster (68) demonstrated that totally C^{14} labeled 2,6-diaminopimelic acid was converted largely to DPA and in part to leucine, aspartic acid, alanine, and valine during endotropic sporulation of *B. mycoides*. However, the routes of conversion, either through direct ring closure of DAPA to DPA, or through initial degradation to precursors of DPA, have not been demonstrated. It is interesting that the lysine formed from DAPA in *Escherichia coli* (73) was not radioactive. As pointed out by Stedman (3), possible dilution by endogenous substrates and impermeability to DAPA must be considered in evaluating these observations. Powell and Strange (59) have also attempted to implicate DAPA as a precursor of DPA. DAPA was associated with the insoluble cell debris in nonsporulating vegetative cells, whereas in sporulating cells and spores, it appeared in the soluble fraction. The change in DAPA distribution was associated with an enzyme which liberated soluble material containing DAPA and hexosamine from the insoluble fraction of vegetative cells. However, an interesting situation is observed in *B. sphaericus* (60) where both DAPA and DPA are found in sporulating cells and spores, but not in vegetative cells. The increase in DAPA during sporulation is paralleled by a decrease in the content of a heat-stable DAPA decarboxylase

The phosphorus (P) changes during germination of *B. cereus* and *B. megaterium* have been studied by Fitz-James (15). When spores were inoculated into medium capable of supporting vegetative cell growth, an initial decrease in protein bound P occurred, which could be accounted for in the acid soluble fraction as labile P. This had some of the properties of polymerized metaphosphate. The incorporation of P into RNA and acid soluble P began almost immediately, although increases in DNA P were not evident until 20 min after the initiation of germination. Exogenous P rose rapidly 10 min after inoculation. A slight decrease in the rate of RNA synthesis which followed the initial DNA increase coincided with the period in which the cells were changing from spheroidal to cylindrical structures. Fitz-James interpreted the early rapid rise in RNA as the synthesis of "nuclear" RNA, and

TABLE 2

Some chemical differences between spores and vegetative cells

Constituent	Spores	Vegetative Cells	References
N*	11.5–12 per cent	6–6.5 per cent	67
Amino acids: total D amino acid†	1.09 per cent	3.55 per cent	76
D-glutamic acid†	42–57 per cent	57–69 per cent	76
Methionine‡	+	−	77
Tyrosine‡	+	−	78
Sulfhydryl‡			
Free amino acids§	0.8 μ moles/100 mg dry wt	9.2 μ moles/100 mg dry wt	75
Phosphorus:§ total acid soluble	80 mg/100 g dry wt	150 mg/100 g dry wt	9
Lipid P	22 mg/100 g dry wt	71 mg/100 g dry wt	9
Total protein bound	157 mg/100 g dry wt	56 mg/100 g dry wt	9
Labile protein bound	72 mg/100 g dry wt	4 mg/100 g dry wt	9
RNA§	578 mg/100 g dry wt	1015 mg/100 g dry wt	9
β-hydroxy butyric lipid*	0 per cent	30 per cent	67
Polyoside (glucose, galactose and uronic acid)*	5 per cent	20 per cent	67

* *Bacillus megaterium.*
† *B. cereus* var. *terminalis.*
‡ *B. globigii.*
§ *B. cereus.*

the later extensive synthesis as "cytoplasmic" RNA.

These rapid synthetic activities of germinating spores suggest that the process may involve protein synthesis as well. If such a process occurs during the early stages, it would serve as an explanation for some of the enzymatic activities which rapidly increase during germination and outgrowth (42, 49, 74), either at the expense of endogenous amino acids or from exogenous nitrogen. Although the free amino acid pool in intact spores is very low (75), germination of spores of *B. subtilis* and *B. megaterium* lead to the liberation of at least 12 amino acids (11). These either exist free within the spore or are liberated by the action of the lytic system during germination. In either case, sufficient precursors are provided for protein synthesis. The suggestion that germination and perhaps activation may involve such synthesis is supported by the observation that amino acid analogs can inhibit the heat activation of glucose dehydrogenase (27), and of germination (75). In the latter case the inhibitions were reversed by the addition of the corresponding homolog.

Further chemical changes occurring during germination are suggested by the comparison of some of the differences between spores and vegetative cells summarized in table 2. These differences are probably also rectified during germination or outgrowth.

In summary, germination is characterized by a burst of degradative reactions: cell wall lysis, liberation of DPA, phosphate, amino acids, and enzymes; and a loss of heat resistance. Outgrowth, however, is more characterized by synthetic reactions: DNA, RNA, protein and polysaccharide synthesis.

III. ENZYMATIC PATTERNS OF BACTERIAL SPORES

1. Active Enzymes in Intact Spores

Until recent years, we were faced with the difficulty of attempting to understand the biochemical changes in dormancy of bacterial spores which were believed to be nearly devoid of enzymic activity. Although early claims had been made for respiratory activity in resting spores (79 to 82), failure to eliminate possible contributions from contaminating vegetative enzymes or germinated spores made these observations open to question. The need for numerous washings to

free spores of contaminating vegetative enzymes (45, 83) has been amply emphasized.

Stewart and Halvorson (45) clearly demonstrated enzyme activity in resting, intact spores. Spores of *Bacillus cereus* var. *terminalis* contain an active alanine racemase (26, 45) which catalyzes the reaction:

$$\text{L-alanine} \underset{50}{\overset{50}{\rightleftharpoons}} \text{D-alanine}$$

The enzyme is specific for alanine, requires pyridoxal phosphate and converts either D- or L-alanine to a racemic mixture. Following spore rupture, a fraction of the enzyme was particulate and resistant to heat and pepsin. However, after solubilization by sonication, a heat and pepsin sensitive enzyme was obtained. The enzyme was widely distributed among the species of *Bacillus*, being present in higher concentrations in the spores than in the vegetative cells. There are, however, conflicting reports on the presence of the enzyme in spores of *B. subtilis* var. *niger* (26, 40). The activity of the enzyme does not appear to play a role in germination in the presence of alanine (46).

Catalase also appears as an integral part of the spores of *B. cereus* var. *terminalis* (17) and of *B. subtilis* (84, 85). In spore extracts, a heat sensitive and a heat resistant catalase were observed. The enzyme in spore extracts is sensitive to F^-, N_3^- and CN^- (17, 84), suggesting that spore enzymes may differ from those found in vegetative cells (86). The heat resistance, high turnover number and wide distribution of the enzyme among bacillus spores have led to its use as a criterion of spore purity (17).

The observation that inosine served as a more effective germinating agent than adenosine in spores of *B. cereus* and *B. anthracis* (87) led to the discovery in these strains of adenosine deaminase.

$$\text{Adenosine} \underset{-\text{HOH}}{\overset{+\text{HOH}}{\rightleftharpoons}} \text{inosine} + NH_3$$

The enzyme precipitated by ethanol had a pH optimum at 8.7, was not stimulated by pyridoxal phosphate or boiled yeast extract and deaminated only adenosine and cytidine. Adenine, guanine, guanosine, and cytosine were not attacked. The enzyme was heat resistant in the intact spore, but in extracts was destroyed in 15 min at 60 C. A similar enzyme has been reported in extracts of *B. cereus* var. *terminalis* (88).

Spores of *B. cereus* (87) and *B. cereus* var. *terminalis* (89) also contained a heat stable, hydrolytic nucleoside ribosidase which cleaves ribosides into the free base and the free sugar.

$$\text{adenosine} \xrightarrow{+\text{HOH}} \text{adenine} + \text{ribose}$$

The specificity of the nucleoside ribosidase varied with strain. *B. cereus* and *B. anthracis* are equally active on adenosine and inosine, weakly active on guanosine and inactive on xanthosine and cytidine. Spores of *B. cereus* var. *terminalis* attack adenosine, guanosine, xanthosine, adenylic acid, cytidine, uridine (37) and 2-aminoadenosine (88). Deoxyinosine, deoxyadenosine, deoxyguanosine and adenine-9-β-D-glucoside were not attacked. The reaction was probably hydrolytic since it was not inhibited by arsenate or stimulated by phosphate. The mechanism was somewhat uncertain since the stoichiometric equivalent of ribose could not be recovered from adenosine (89). Ribose was not oxidized by this strain, therefore other ribose utilizing reactions may be present. The ribosidase, at least in *B. cereus*, was particulate, which probably accounted for its heat stability.

The role of ribosidase in germination is not clear. Since the enzyme was active under conditions of low pH and high temperature which preclude germination, the loss of germinating ability may be related to causes other than the direct inactivation of the ribosidase. The broader substrate specificity of the enzyme than the specificity of germination requirements (37) suggested that the enzyme may have yet another function. However, spores requiring adenosine for germination, *B. cereus* var. *terminalis*, *B. polymyxa*, *B. anthracis*, and *B. cereus* possessed ribosidase activity while spores of *B. subtilis* var. *niger*, which do not require adenosine for germination, were devoid of the enzyme. In these systems the ribosidase may represent an initial reaction in germination.

As a group these enzymes were heat stable, and with the possible exception of adenosine deaminase, were attached to particulates. In the case of nucleoside ribosidase, and possibly others, the enzyme was attached to the cell wall. This may be the explanation for their activity in dormant spores which normally were considered impermeable.

2. Dormant Enzymes in Intact Spores

Investigations of glucose oxidation by intact spores led to the discovery of a second class of enzymes in intact spores. Glucose, which was one of the common germinating agents in bacillus spores, has been used by numerous investigators as an index of respiratory activity. Tarr (81) and Keilin and Hartree (82) reported the oxidation of glucose by spores which were heated to destroy contaminating vegetative enzymes. However, no attempts were made to determine whether this activity could be attributed to oxidation by germinating spores. Employing well cleaned spores of *B. subtilis* and *B. cereus* var. *anthracis*, Crook (80) was barely able to detect respiration with or without glucose using a microrespirometer. Similar results have been reported by Spencer and Powell (90) with spores of *B. subtilis*.

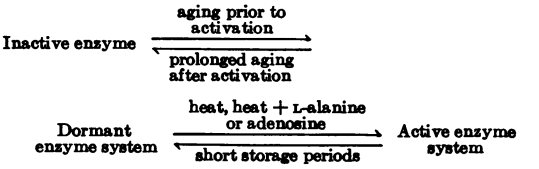
Church and Halvorson (26, 52) and Murrell (84) were the first to demonstrate the oxidation of glucose by intact, heat resistant spores of *B. cereus* var. *terminalis* in the absence of detectable germination. In these studies, extended heat shock treatments of aged spores of *B. cereus* var. *terminalis* were used to activate the oxidation of glucose and endogenous respiration. Freshly harvested spores showed relatively little activity (91); storage at −20 C for 4 months or longer permitted maximal glucose oxidizing activity after heat treatment. The activation by heat was transient and steadily diminished during prolonged storage at 5 C. Following a 24 hr storage after heat activation, the spore retained only 15 per cent of its glucose oxidizing capacity. Full activity could be restored by a second heat treatment. The extent of activation decreased when heat-activated spores were stored for periods longer than 24 hr, eventually being irreversibly lost. The total respiratory activity observed by heat activation was in quantitative agreement with that observed during spore germination (52, 74).

Murrell (84, 85) found that glucose oxidation could be activated in spores of *B. subtilis* by incubating them in the presence of L-alanine. When thick spore suspensions (2×10^9 cells/ml or more) were employed, the concentration of L-alanine controlled the rate of glucose oxidation. Further additions of L-alanine, as the second substrate, increased the rate of oxidation. When spores were first preincubated in L-alanine and then exposed to glucose, the rate of glucose oxidation decreased with increasing preincubation periods with L-alanine. These results, suggesting that L-alanine was consumed during activation, have also been reported in *B. cereus* var. *terminalis* spores (27, 92). In contrast to *B. subtilis*, spores of *B. cereus* var. *terminalis* require heat activation. The duration of heating depends on the concentration of L-alanine and of spores. L-Alanine was required during the second heat treatment, presumably being consumed during the first activation period. *p*-Fluorophenylalanine inhibited the stimulation of glucose activation by L-alanine. DL-Alanine or D-alanine can replace L-alanine during the second heat shock treatment, probably via alanine racemase.

Glucose oxidation, without concomitant germination, could also be activated in non-heated spores of *B. cereus* var. *terminalis* by incubating them in the presence of adenosine and low concentrations of glucose (52). If either higher concentrations of glucose were added or a heat activation applied after 95 min of incubation, rapid germination ensued.

The dormancy of the glucose-oxidizing system was further substantiated in spore extracts. Extracts of non-heat shocked spores contained a DPN linked glucose dehydrogenase (26, 93) which represented only 25 per cent of the activity of activated spores. Heat activation prior to rupture increased the activity in subsequent extracts by 250 per cent. Also, Krask (94) has reproted an acetokinase or CoA kinase, which can be demonstrated only in homogenates from disrupted spores, whose activity increased when spores were heat activated prior to rupture.

The properties of the glucose oxidizing system in intact spores can be described as follows:



The dormant enzyme system is defined as that whose activity is observed in intact, activated spores, and the inactive enzyme system is defined as that whose activity is only recognized in disrupted spore suspensions. The inactive stage was reached predominantly in freshly harvested spores and in aged, heat-activated spores which

had undergone prolonged storage. A deficiency in DPN or some other part of the electron transport system through metabolic loss or poisoning could readily produce the inactive respiratory state observed. Synthesis of these cofactors or the removal of an inhibitor during aging could elevate the glucose oxidizing capacity.

The conversion of dormant enzymes to active enzymes presented a different problem. Heat treatment, heat treatment with L-alanine, or adenosine alone activated the enzyme system. Since these treatments are stimulatory to germination, the reactions involving activation of glucose oxidation may be common to those reactions responsible for germination. An investigation of the chemical structure of the majority of the glucose dehydrogenase, which was inactive in extracts from non-heat treated spores, could throw some light on the mechanism of activation of dormant enzymes.

The presence of dormant enzymes was not limited to glucose oxidation. Heat activation of *B. cereus* var. *terminalis* spores also led to an activation of the oxidation of gluconate, 2-ketogluconate, pyruvate and acetate. However, phosphorylated sugars, maltose, ribose, arabinose, lactose and fructose were inactive (26, 36).

3. Carbohydrate Metabolism in Spore Extracts

a. Glucose oxidation. Activated spores oxidize glucose, 2KG, pyruvate and gluconate while glucose-6-phosphate, fructose-6-phosphate, fructose, hexosediphosphate and acetate are not attacked (93). Dialyzed spore extracts require DPN for the oxidation of glucose and pyruvate whereas gluconate and 2KG are TPN dependent. Extracts are invariably inactive towards members of the Embden-Meyerhof glycolytic system as well as toward arabinose and ribose. When glucose oxidation is carried out in the presence of BEP (1,3-bisethyl heptyl-5-methyl amidio hexahydra pyrimidine), a thiamine antagonist which suppresses pyruvate oxidation, presumably by inhibiting the activation of pyruvate reactions by cocarboxylase, appreciable recoveries of pyruvate are obtained. Thus, these spores possess an active system capable of converting glucose to pyruvate.

The study of carbohydrate metabolism by microorganisms over the past two decades has revealed a number of diverse pathways by which pyruvate can be formed from glucose (95). The Embden-Meyerhof pathway of glycolysis operates in lactobacilli, streptococci and members of the coli-aerogenes group. However, in obligate aerobes and in some facultative aerobes carbohydrate oxidation follows other pathways. One pathway is the hexosemonophosphate oxidative route which diverges at the level of G-6-P, the 6-P-G being converted to pyruvate either by dehydration to 2-keto-3-deoxy-6-phosphogluconate and cleavage to pyruvate and D-glyceraldehyde-3-phosphate or by oxidation to a mixture of ribose-5-phosphate and ribulose-5-phosphate. These are then converted to sedoheptulose-7-phosphate, F-6-P and D-glyceraldehyde-3-phosphate. Another alternate pathway involves a direct oxidation of glucose to gluconate. Gluconate is oxidized to 2KG and phosphorylated to 2K6PG which, by an undefined pathway, is converted to 2 molecules of pyruvate.

Spore extracts are probably devoid of glycolytic activity, being inactive towards intermediates of the Embden-Meyerhof pathway (26, 36, 93). The extracts are devoid of hexokinase, phosphohexokinase, aldolase and triose phosphate dehydrogenase. An analysis of the end products of glucose oxidation by spore extracts failed to reveal the presence of phosphate esters. Furthermore, the oxidation of glucose by translucent spores was resistant to NaF, a normal inhibitor of glycolysis (74).

Several enzymes of the HMP pathway are present in spore extracts. The first is a classical TPN-linked glucose-6-phosphate dehydrogenase (36) which, according to the studies of Cori and Lipmann (96) and Brodie and Lipmann (97), probably involves the two enzymatic steps shown in figure 1. A TPN-linked 6-P-G oxidizing system was also observed in spore extracts (93) which may be identical to the 6-phosphogluconate dehydrogenase of vegetative cells of *B. subtilis* and

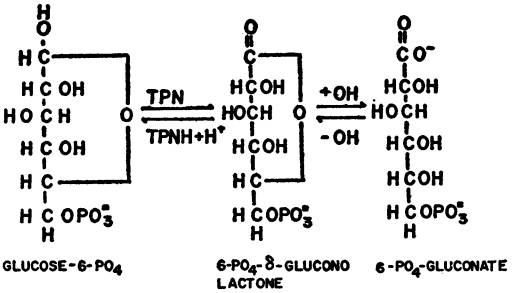


Figure 1. Oxidation of glucose-6-phosphate

B. megaterium (98). Although the mechanism of 6-P-G metabolism has not been studied in these extracts, an end product analysis indicates that pentose is formed from 6-P-G. Pentose might arise by the oxidation of 6-P-G to 2-keto-6-phosphogluconate and cleavage to trioses, which by the action of transketolase yield ribulose-5-phosphate (95). The HMP pathway is, in all likelihood, not operative because of the absence of hexokinase. On the other hand, vegetative cells of the *Bacillaceae* contain all of the enzymes of the HMP system up to the stage of sedoheptulose phosphate formation (99).

Glucose is primarily metabolized by spore extracts via a nonphosphorylated shunt pathway (93). The primary end products are gluconate, 2KG, 2K6PG, and pyruvate. The initial oxidation is phosphate-independent and mediated by a soluble glucose dehydrogenase (36) as shown in the following reaction:

$$\text{Glucose} \underset{\text{DPNH} + \text{H}^+}{\overset{\text{DPN}}{\rightleftharpoons}} \text{Gluconic acid}$$

In contrast with the glucose dehydrogenase of *Pseudomonas fluorescens* (100), which is particulate and uses cytochromes *b* and *c* as hydrogen acceptors, the dehydrogenase of spores (36) and vegetative cells of *B. subtilis* (101) utilizes DPN as the hydrogen acceptor. Gluconate is oxidized to 2KG by a TPN requiring enzyme (93).

$$\text{Gluconic acid} \underset{\text{TPNH} + \text{H}^+}{\overset{\text{TPN}}{\rightleftharpoons}} \text{2-ketogluconate}$$

The extracts are devoid of gluconokinase. 2KG is phosphorylated by a typical Mg^{++} requiring kinase (102) to 2K6PG which is then oxidized by a TPN requiring system.

$$\text{2-ketogluconate} + \text{ATP} \overset{Mg^{++}}{\rightleftharpoons} \text{2-keto-6-phosphogluconate} + \text{ADP}$$

Harrell (unpublished results) has recently observed that glucose oxidation by extracts of *B. cereus* var. *terminalis* spores was stimulated by DPA and ethylene diaminetetraacetate. The DPA was not metabolized, since it was quantitatively recovered at the end of the experiment. Glucose oxidation was inhibited by Ca^{++}, Hg^{++}, Zn^{++}, Sr^{++}, Cr^{++}, and Ba^{++}. The Ca^{++} inhibition was reversed by DPA. These observations suggest a regulatory mechanism for controlling respiration in spores analogous to that observed by Slater in heart preparations (103). He observed that the inhibition of respiration by the calcium of the sarcosomes could be overcome by chelating agents. DPA, which under normal conditions is an excellent chelating agent for calcium, is present initially in spores in a bound form. When it is released during activation or germination, it might release the dormant glucose oxidizing system by chelating the spores' abundant calcium or some other heavy metal and thus liberating a poisoned respiratory system.

The reactions leading to pyruvate from 2K6PG are not well understood. Wood (100) suggested this may occur in *P. fluorescens* via dehydration and cleavage reactions for 2K6PG of the Entner-Doudoroff type leading to 2,4-diketo-3-deoxy-6-phosphogluconate which is cleaved to pyruvate and 3-phosphoglycerate.

b. Pyruvate oxidation. The presence of a system leading to the formation of pyruvate from glucose, as well as the oxidation of pyruvate, provides a basis for a rich supply of energy and biosynthetic intermediates through decarboxylation, carboxylation, clastic and aldehyde transfer reactions.

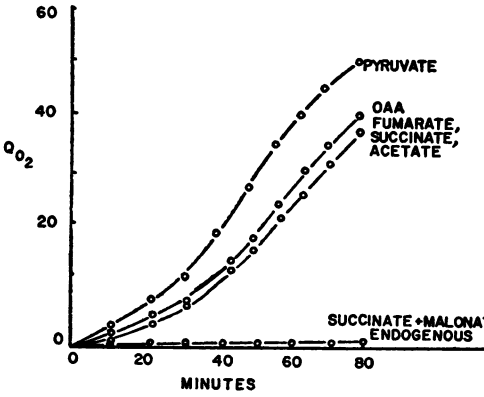


Figure 2. Oxidative activity by particles of spore extracts.

A dialyzed spore extract was centrifuged at 100,000 × G for 1 hr. The pellet was reconcentrated in 0.067 M phosphate buffer pH 7.1. The Warburg flask contents: side arm, 25 μmoles of substrate and 0.05 μmole of OAA; center well, 0.2 ml 30 per cent KOH; main compartment, 1 ml enzyme preparation, 8×10^{-3} M $MnCl_2$, 0.2 ml of 0.05 M ATP, 5×10^{-3} M DPN, 10^{-4} M cocarboxylase, and sufficient phosphate buffer to make a final cell volume of 2.0 ml. The gas phase was air and the temperature 30 C.

The pyruvate oxidizing system is particulate, being completely sedimented at 100,000 × G. (36). Rapid pyruvate oxidation requires DPN, ATP, cocarboxylase and Mn^{++}. Krask (94) has also shown the presence of acetokinase or CoA kinase which, in the presence of Mg^{++}, CoA and ATP, lead to active acetate formation. When the reaction mixture is sparked with oxalacetate, an active oxidation of acetate, succinate, fumarate and OAA (36) is observed (figure 2). Succinate oxidation is competitively inhibited by malonate, indicating the mediation of succinic dehydrogenase. The cofactor and oxidative capacity of these particles indicates that this is the site of a functional tricarboxylic acid cycle operating on the active acetate formed from pyruvate. Such a system is known to be present in vegetative cells of *Bacillaceae*.

On the other hand, Hardwick and Foster (104) observed oxidative activity towards malate, succinate, α-ketoglutarate and pyruvate in extracts of vegetative cells but not of spores of *B. mycoides*. Their failure to detect oxidative activity in spore extracts was probably due to an insufficient endogenous supply of OAA to spark the reaction.

Figure 3 summarizes the reactions of carbohydrates demonstrated thus far in spore extracts. Pyruvate is formed from glucose via gluconate, 2KG and 2K6PG. Pyruvate is probably oxidatively decarboxylated to acetate which is metabolized through a tricarboxylic acid cycle or by other classic type reactions. Several enzymes of the HMP pathway are present but neither this pathway nor a functional glycolytic system are operative. The presence of these latter two pathways in vegetative cells of the *Bacillaceae* (102, 105, 106) must mean that these, as well as other enzyme systems, develop during germination and outgrowth.

c. Electron transport. The pathways of electron transport from substrates to molecular oxygen are, perhaps, as diverse in microorganisms as are the pathways of carbohydrate metabolism. It is, therefore, not unexpected that in our state of meager knowledge about spores, we look upon the electron transport system as fairly complicated.

The cyanide resistant respiration of spores (90), frequently indicative of flavoproteins which react directly with oxygen or through hematin catalysts, indicates that the cytochrome system of the vegetative cells may be partly replaced in spores by flavoproteins. Spores have been found to contain a reserve of hematin compounds (82) and to contain one-third to one-fourth of the flavine adenine dinucleotide content of their corresponding vegetative cells. The evidence on the cytochrome content of spores is contradictory. Keilin and Hartree (82) reported that the cytochrome content of *B. subtilis* spores, which was only visible at liquid air temperatures, was 6 per

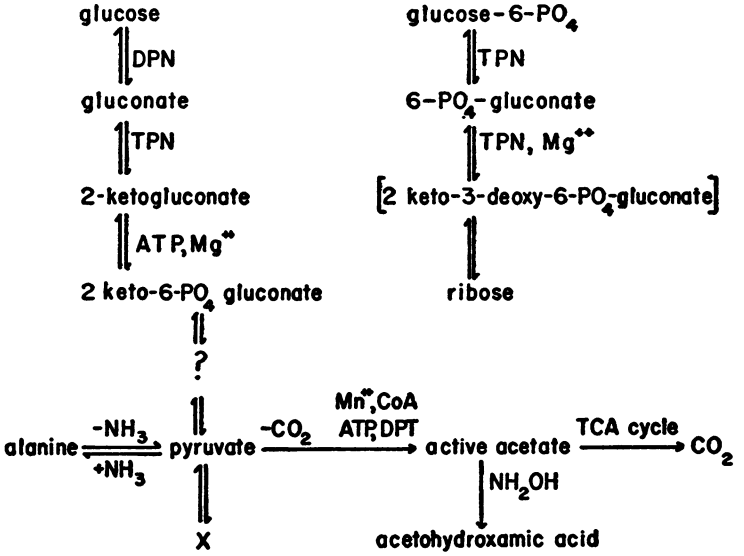
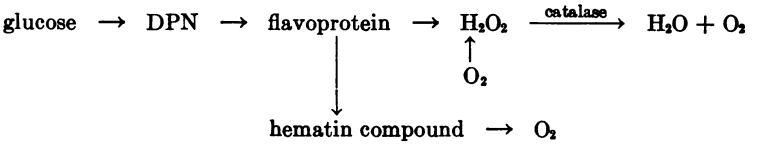


Figure 3. Pathways of carbohydrate metabolism in spores of *Bacillus cereus* var. *terminalis*

cent of that in vegetative forms. Cytochromes could not be detected in *B. cereus* spores (107); however, following germination, cytochrome *b* appeared within 5 hours and cytochromes *a* and *c* within 6 to 8 hours. The presence of cytochrome oxidase (a_3) in spores is also uncertain. Although disrupted *B. subtilis* spores catalyze the oxidation of reduced cytochrome *c* (108), ascorbic acid, hydroquinone and *p*-phenylenediamine (85, 108), the oxidation of the latter two is not stimulated by cytochrome *c* nor sensitive to carbon monoxide (84). Although Spencer and Powell (90) conclude from these findings that an atypical cytochrome-cytochrome oxidase system may be present, Murrell (84) argued that the findings are not incompatible with a normal cytochrome system.

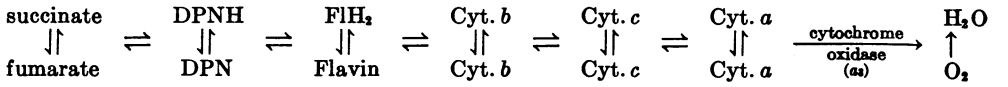
Spencer and Powell (90) attempted to identify the flavoproteins in spores. Although spores of *B. subtilis* and *B. megaterium* are devoid of L-amino acid oxidase, D-amino acid oxidase and xanthine oxidase, they possess an active diaphorase-like enzyme. This enzyme, which couples DPNH oxidation with methylene blue reduction, is heat sensitive and contains combined flavine adenine dinucleotide (FAD). Although its physiological role is obscure, it is interesting that extracts of *B. megaterium* spores contain an active inhibitor of diaphorase which is reversed by DPN but not by FAD. It has been suggested that the diaphorase may represent the cyanide-insensitive respiration of spores (90). Since diaphorase is not auto-oxidizable, the observed respiration might result from a coupling of diaphorase with a cyanide insensitive hematin carrier. Still, the possibility may exist that spores possess a DPNH oxidase of the metallo-hemoflavoprotein variety (109) from which the electrons are transferred to a "built-in" cytochrome component. In the presence of CN^- or CO the electrons are diverted to an external acceptor.

Several pathways of electron transport in spores are suggested by the oxidation of glucose and succinate. Glucose is oxidized in spores (93) and in vegetative cells (101) to gluconate by a DPN linked glucose dehydrogenase. In vegetative cells of *B. subtilis* (101) DPNH is oxidized by a cyanide insensitive flavoprotein. Methylene blue, ferricyanide and 2,6-dichlorophenol-indophenol, in addition to oxygen, serve as hydrogen acceptors from DPNH under anaerobic conditions. H_2O_2 is produced aerobically, as shown by a coupled oxidation of ethanol in the presence of catalase. This has not as yet been studied in spores in which an active catalase is present (17, 84). Therefore, it seems reasonable that either of the two pathways in scheme 1 may mediate the reaction between glucose and molecular O_2.



Scheme 1

The succinic oxidase system probably follows another route. Spore extracts of *B. cereus* possess an active malonate-inhibited succinate-oxidizing system which is probably identical to succinic dehydrogenase (93). This oxidation is coupled to molecular O_2; its pathway in spores has not been studied. The most reasonable assumption is that it follows the sequence of electron transport normally associated with succinate oxidation (103). This is shown in scheme 2. A spectrographic analysis of cytochrome a_3 during succinate, as well as glucose, oxidation by spores would aid greatly in unraveling the problem of electron transport in spores. As the morphogenic changes of germination and outgrowth proceed, an increasing dependence is placed upon a cytochrome carrier system.



Scheme 2

4. Nitrogen Metabolism in Spore Extracts

Two features of spore germination have directed attention to the nitrogen metabolism of the spore: (a) amino acids, notably L-alanine, act as germinating agents, and (b) outgrowth and possibly germination are paralleled by active protein synthesis despite the paucity of internal free amino acids of the spore (75). Thus, the amino acids required for synthesis must be derived either from the germinating agents, spore proteins or endogenous synthesis.

Of the amino acids acting as germinating agents, only L-alanine has been carefully studied. Most aerobic spores contain a pyridoxal phosphate-requiring alanine racemase which converts either D- or L-alanine to a racemic mixture (45, 46). The observation that L-alanine was consumed in the process of activation of spores (27, 84, 92) has led to further studies on alanine metabolism. Harrell and Halvorson (47) attempted to study this problem, employing carboxyl-labelled alanine. They observed that there was sufficient activation by L-alanine during a 45 sec exposure period to permit 40 per cent of the spores to germinate subsequently in the absence of L-alanine. During this period there was a fixation per spore of several hundred molecules of alanine or products derived from alanine. This fixation was not a matter of simple adsorption.

Recently, Falcone (32) reported the production of H_2O_2 and pyruvate from L-alanine by intact spores of *B. subtilis*. In the presence of an inhibitor of pyruvate oxidation, stoichiometric equivalents of NH_3 and pyruvate are observed from D- or L-alanine by intact spores of *B. cereus* var. *terminalis* (93). The presence of catalase in these spores has precluded an examination for H_2O_2 in this reaction. The reaction is specific for alanine. Since these spores contain alanine racemase, the optimal specificity is uncertain. Vegetative cells contain a DPN linked L-alanine dehydrogenase which converts L-alanine to pyruvate and NH_3 (110). NH_3, H_2O_2 and pyruvate can be directly formed from alanine by the action of either D- or L-amino acid oxidases which are coupled either to flavine adenine dinucleotide or riboflavin phosphate (111). L-Amino acid or D-amino acid oxidases could not be detected in crushed spores of *B. subtilis*, *B. megaterium* (90), or *B. mycoides* (104). The alanine deaminating system of *B. cereus* var. *terminalis* spores therefore may represent a new reaction, since dialyzed spore extracts require pyridoxal phosphate as a cofactor for the reaction (93). This reaction can be tentatively described as follows:

$$\text{D-alanine} \underset{\text{L-alanine}}{\overset{\text{pyridoxal phosphate}}{\rightleftharpoons}} NH_3 + (X) \rightleftharpoons \text{pyruvate} + (H_2O_2)$$

The formation of NH_3 from alanine and a functional cycle for triose oxidation provides the N and C skeletons for the synthesis of the aspartic from oxalacetic acid and the glutamic from α-ketoglutaric acid (112). Direct amidation has not, as yet, been demonstrated in spore extracts. Krask (113) has shown amidation and transfer reactions of NH_3 in homogenates and extracts of disrupted spores of *B. cereus* var. *terminalis*. These reactions can be summarized as shown in scheme 3.

$$\text{glutamic acid} + NH_3 + \text{ATP} \underset{\text{glutamine synthetase}}{\overset{Mg^{++}}{\rightleftharpoons}} \text{glutamine} + \text{ADP} + H_3PO_4$$

$$\text{glutamine} + NH_2OH \underset{\text{glutamotransferase}}{\overset{Mn^{++}, \text{ATP}}{\rightleftharpoons}} \text{glutamohydroxamic acid} + NH_3$$

$$X + NH_2OH \underset{X \text{ transferase}}{\overset{\text{adenosine}}{\rightleftharpoons}} X \text{ hydroxamic acid} + NH_3$$

$$\text{Acetate} \underset{\text{acetokinase or CoA kinase}}{\overset{Mg^{++}, \text{ATP, CoA}}{\rightleftharpoons}} \left[\begin{matrix}\text{acetyl phosphate} \\ \text{or acetyl CoA}\end{matrix}\right] \overset{NH_2OH}{\rightleftharpoons} \text{acetohydroxamic acid} + NH_3$$

Scheme 3

The glutamine synthetase and glutamotransferase are present both in disrupted spores and spore free extracts. The acetokinase and the unidentified adenosine activated *X*-transferase are present in disrupted spores but absent in spore free extracts.

Another possible route for amino acid synthesis might be through transamination reactions.

Although vegetative cells of the *Bacillaceae* contain active glutamic-aspartic (104, 106, 111, 114) and glutamic-alanine (104, 111, 114) transaminases, only glutamine-aspartic transaminase has been reported in spore extracts of *B. megaterium* (114). Hardwick and Foster (104) were unable to detect glutamic-alanine transaminase activity in spore extracts of *B. mycoides*. We have confirmed this observation employing extracts of *B. cereus* var. *terminalis* which actively deaminate alanine. Consequently, although some transamination reactions may play a role in amino acid synthesis in spores, their action is not directed towards alanine. The coupled action of alanine deaminase, acetokinase and the interesting adenosine activated *X*-transferase could provide the sequence for the formation of amino acids active in the glutamic-aspartic transaminase system.

A rich supply of amino acids from spore coats and proteins is provided by the action of the Mn^{++}-activated lytic enzyme (64, 65). In *B. megaterium* the amino acids released by the action of this enzyme may furnish the primary stimulants for germination. Germination also accompanies a release of phosphorus compounds (12, 15). Spore coats contain acid and alkali insoluble P, constituting approximately 60 per cent of the total phosphorus of the cell (60). This P is largely lost during germination through the action of a Mn^{++} activated pyrophosphatase (64, 84). Germinated forms lack this pyrophosphatase activity. Levinson (64) has made the interesting suggestion that Mn^{++} activation is responsible for synthesis of coenzymes required in triose metabolism of the spore (93). β-Alanine, possibly formed from L-alanine or released by the lytic system, might be coupled to the synthesis of this coenzyme in the following reactions (115):

$$\text{ATP} + \text{pantoic acid} + \beta\text{-alanine} \rightleftharpoons \text{Pantothenic acid} + \text{AMP} + \text{PP}$$

$$\text{ATP} + \text{CoASH} + \text{acetate} \rightleftharpoons \text{AMP} + \text{acetyl CoA} + \text{PP}$$

Mn^{++} activation of pyrophosphatase would keep the equilibrium of these reactions to the right. The reactions demonstrated in spores of *B. cereus* var. *terminalis* for amino acid, peptide and protein synthesis are summarized in figure 4. The use of the hydroxamates in the reactions shown in figure 4 does not necessarily implicate them as intermediates in N metabolism. The formation of

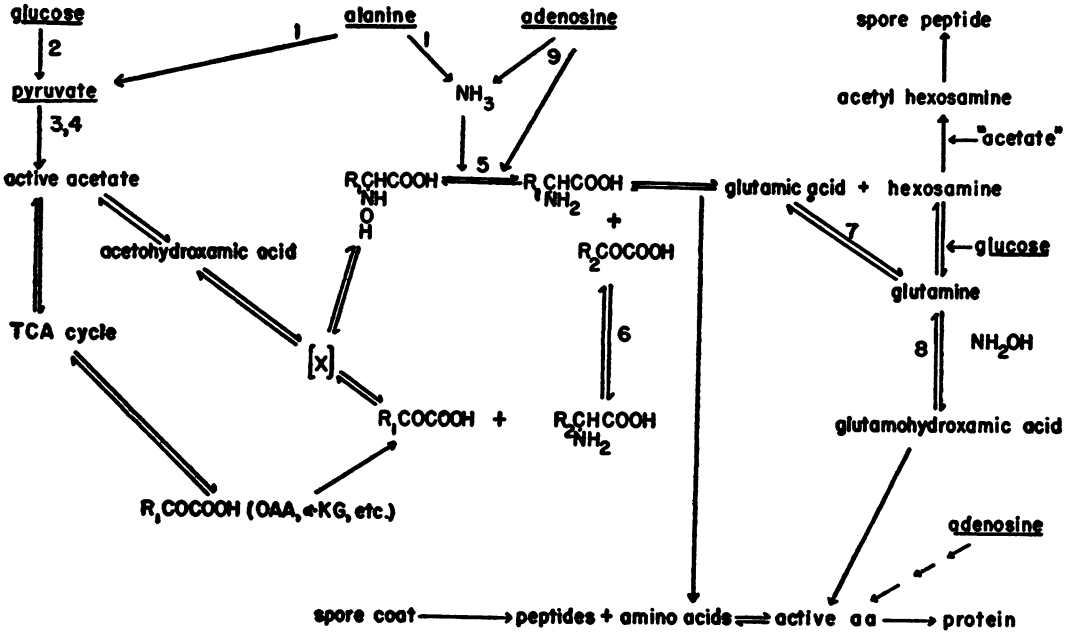


Figure 4. Pathways of nitrogen metabolism in spores of *Bacillus cereus* var. *terminalis*. Enzymatic reactions: 1. alanine deaminase; 2. glucose oxidative system; 3. oxidative decarboxylation; 4. acetokinase or CoA kinase; 5. *X* transferase; 6. glutamic-aspartic transaminase; 7. glutamine synthetase; 8. glutamotransferase; 9. adenosine deaminase.

hydroxamates in hydroxylamine coupled reactions may also indicate analogous activated forms or transferase reactions.

The existence of amino acid synthesizing systems and the amino acid supplied endogenously from spore coats and proteins, when coupled with the energy yielding reactions also present, provide a sufficient supply of energy and amino acids required for protein and enzyme synthesis during germination and the early stages of outgrowth.

IV. SPECULATIONS

The dominant features of spore germination are the loss of heat resistance, paralleling an extensive breakdown of macromolecules, and the appearance of heat sensitive enzymes. The most direct explanation of these observations is that the binding of these enzymes to large structures (protein, bound DPA, etc.) renders them heat resistant and inactive and thereby maintains the dormant state. Their release during germination increases the enzymatic potentialities of the cell and hastens the return to the vegetative state.

The question remaining is how the germinating agents contribute to these changes. The differences in requirements for germination of various spore strains indicate various modes for initiating these changes. The germination of *B. megaterium* spores is Mn^{++} activated. There are permeability barriers in these spores probably partially caused by the protein and phosphorus containing spore coat (64). Through the action of two Mn^{++} activated enzymes, pyrophosphatase and the lytic enzyme, the insoluble residue is degraded into amino acids and phosphorus. The completion of germination could then follow from the entry of water, amino acids and other nutrients into the cell, permitting protein synthesis and the other necessary biochemical reactions.

The germination of *B. cereus* var. *terminalis* spores represents another problem. These spores require L-alanine and adenosine, but not Mn^{++}, for rapid germination. The spores are rich in bound DPA (28). The phosphorus content of their spore coats has not been analyzed. The varied observations on their activation and germination are best explained by the assumption that the process here requires protein synthesis, possibly of some depolymerizing enzyme. L-Alanine serves as a source of both NH_3 and pyruvate through the action of a deaminase system (93). The necessity for pyruvate metabolism for germination, either from alanine or glucose, was seen by the competitive inhibition of germination by an antagonist of thiamine (BEP). Pyruvate serves as a source of C skeletons for amino acid synthesis. Adenosine plays a role in amino acid biosynthesis by activating the *X* transferase, a necessary link from NH_3 to the transaminases of the spores. Since protein synthesis is mandatorily coupled to nucleic acid synthesis (116), the adenosine and the other nucleosides and nucleotides that stimulate germination can serve either as precursors of nucleic acid synthesis or as activators of the carboxyl group of amino acids. By coupling the reaction to hydroxamate formation, Hogland (117) demonstrated the following enzymatic reactions:

$$E + ATP + HOOC{-}CHRNH_2 \rightleftharpoons E{-}AMP{-}CO{-}CHRNH_2 + PP$$

$$E{-}AMP{-}CO{-}CHRNH_2 + NH_2OH \rightarrow E + HONH{-}CO{-}CHRNH_2 + AMP$$

The activated amino acid may serve as a precursor of both nucleic acid and protein synthesis. The reaction requires energy for ATP generation which may possibly be furnished by the polymerized metaphosphate in *B. cereus*.

Spore germination and outgrowth in many ways parallel induced enzyme synthesis (3, 68). Both involve *de novo* synthesis of protein from free amino acids and of nucleic acid. In the induced systems, the inducer stimulates the synthetic capacity of specific enzyme forming systems, thereby qualitatively and quantitatively altering the enzyme pattern. Although such interrelationships have not been demonstrated in germination, a precedent for such relationship exists. Harrell and Halvorson (unpublished results) observed that spores produced from sporulating *B. cereus*, which had been induced to form penicillinase, contained the induced penicillinase; whereas spores from non-induced cells were negative. Furthermore, Pollock and Perret (118) found that the penicillinase synthesizing system persists through the spore state.

One of the interesting features of induced enzyme synthesis is that it is inhibited by the presence of glucose (119). The mechanism of such inhibitions, called "glucose effects," is not well understood. Since bacterial spores germinate in the presence of glucose, they presumably escape these "glucose effects." One possible explanation

is that in systems demonstrating "glucose effects," glycolytic or HMP pathways are operative. If the inhibition is actually from some product of either of these two pathways, these products would be lacking in the glucose metabolism of the spores. Such an escape of "glucose effects" is of selective value to the spore, which must obviously increase at least part of its enzyme pattern during germination and outgrowth.

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