BIOCHEMICAL CHANGES OCCURRING DURING GROWTH AND SPORULATION OF *BACILLUS CEREUS*[1, 2]

HERBERT M. NAKATA[3] AND H. ORIN HALVORSON

Department of Microbiology, University of Illinois, Urbana, Illinois

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It is fairly well established that sporulation is a normal metabolic phenomenon among sporogenic bacteria and that the processes involved are quite different from those associated with vegetative growth. Although a rather large amount of data has been reported concerning this phenomenon in recent years, our understanding regarding the nature of sporogenesis, particularly the physiological changes occurring during the period when multiplication ceases and sporulation begins, is still quite limited. This is partially due to the use of cultures of heterogeneous populations in which the processes of a particular phase of growth or sporulation were often complicated by the presence of cells in another. In addition, vast differences among species and even strains studied have been noted which further minimizes the possibility of correlating these data in hopes of revealing the mechanisms of sporogenesis. Recognizing the advantages of confining these researches to a particular organism, the authors initiated the following investigation on the biochemical changes occurring during growth and sporulation of *Bacillus cereus* strain T.

MATERIALS AND METHODS

Organism and culture medium. Bacillus cereus strain T was used throughout these studies. To avoid undesirable precipitation of some of the components, the culture medium, which satisfied the requirements for germination, growth, and complete sporulation of this organism, was prepared in the following manner.

Stock solutions of glucose, 10 per cent; ether extracted yeast extract, 10 per cent; K_2HPO_4, 5 per cent; $CaCl_2$, 0.8 per cent; and a mixture of other minerals were prepared with distilled water. The stock solution of the mineral mixture was prepared by combining a solution containing $FeSO_4 \cdot 7H_2O$, 0.01 per cent; $CuSO_4 \cdot 5H_2O$, 0.1 per cent; $ZnSO_4 \cdot 7H_2O$, 0.1 per cent; $MnSO_4 \cdot H_2O$, 1.0 per cent; and $MgSO_4$, 4.0 per cent, with an equal volume of $(NH_4)_2SO_4$, 40.0 per cent.

To prepare the medium, a portion from the stock mineral solution was diluted with a predetermined amount of distilled water and autoclaved. The volume of water added was corrected for the subsequent addition of the nutrients and the inoculum. Just prior to inoculation, proper amounts of glucose, ether extracted yeast extract, K_2HPO_4, and $CaCl_2$, which had been sterilized individually, were aseptically added to yield a medium of the following composition: $FeSO_4 \cdot 7H_2O$, 0.00005 per cent; $CuSO_4 \cdot 5H_2O$, 0.0005 per cent; $ZnSO_4 \cdot 7H_2O$, 0.0005 per cent; $MnSO_4 \cdot H_2O$, 0.005 per cent; $MgSO_4$, 0.02 per cent; $(NH_4)_2SO_4$, 0.2 per cent; $CaCl_2 \cdot 2H_2O$, 0.08 per cent; K_2HPO_4, 0.5 per cent; extracted yeast extract, 0.2 per cent; and glucose, 0.1 per cent. Glucose was limited to assure aerobic conditions necessary for complete sporulation. The pH was 7.25 to 7.45 without adjustment.

The yeast extract used routinely in these studies was ether extracted 72 hr in a continuous liquid-liquid extractor at pH 2.5 to remove ether-soluble components which would interfere with subsequent analyses of the culture filtrates. After ether extraction, the yeast extract was adjusted to pH 6.6 to 6.8 and autoclaved.

Preparation of the inoculum. Unless otherwise stated, the active culture method described by Collier (1957), with suitable modifications, was routinely used. Essentially, the method involved the activation of the inoculum by three successive

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[3] Present address: Department of Bacteriology and Public Health, Washington State University, Pullman, Washington.

transfers. The initial culture in this series of transfers was inoculated with about 1×10^6 heat shocked (20 min at 80 C) spores from a stock culture grown on nutrient agar. This culture was then incubated at 30 C on a reciprocating shaker until a near maximal population was obtained. This took about 4 hr. A second flask was inoculated from this initial culture with a volume equal to 10 per cent of the volume of the new culture, and this was incubated on the shaker. The latter procedure was repeated, resulting in the final inoculum for the test flask. Each of the last two transfer cultures was incubated only 2 hr. The determinations reported herein were usually made on samples removed from the test flask at various intervals during rapid growth and simultaneous sporulation of this active culture in which the cells at a given time were about the same physiological age.

Viable and spore counts. Both viable and spore counts were made by plating proper dilutions of samples on nutrient agar. The spore count represented cells which were able to produce colonies after heating the sample at 80 C for 20 min. Usually, at least two dilutions of each sample were plated in duplicate. Often, the spore counts were significantly higher than the viable counts (not heat treated). This discrepancy, also noted by other workers, was attributed to two factors: chain formation characteristic of vegetative growth which gave reduced counts; and clumps of spores which normally dispersed upon heating, thus yielding higher counts.

Turbidimetric and pH measurements. In addition to plate counts, growth and sporulation were also measured turbidimetrically using a Klett-Summerson photoelectric colorimeter with a no. 42 blue filter (400 to 465 mμ). Readings were usually taken at intervals together with the pH, which was estimated on a Beckman pH meter.

Chemical analyses. Acetic acid was estimated by steam distillation according to the method described by Neish (1952) with suitable modifications. The presence of significant amounts of pyruvic acid interfered with the quantitative recovery of acetic acid. To prevent this interference, the pyruvic acid in the sample was converted to the 2,4-dinitrophenylhydrazone by adding 1 ml of the reagent (2 mg/ml in concentrated sulfuric acid) prior to steam distillation.

Pyruvic acid was determined by the method of Friedemann and Haugen (1943). Glucose was estimated according to the Somogyi-Nelson method as described in Neish (1952). Acetoin was determined by the modified Voges-Proskauer method of Sokatch and Gunsalus (1957). Acetone, 2,3-butanediol, ethyl alcohol, glycerol, and lactic acid were estimated using the corresponding methods described in Neish (1952).

Chromatography. Samples for chromatography were prepared by ether extracting 100-ml volumes of supernatant collected at various intervals during growth and sporulation at pH 2.5 for 24 to 36 hr. The acids in the receiving flasks were then neutralized with ammonia, and the ether evaporated off. The residual salts of the acids were diluted to 1 ml, and 0.02- to 0.05-ml samples were spotted on the chromatograms.

The system of Kennedy and Barker (1951), using 95 per cent ethyl alcohol and ammonium hydroxide, was employed for the chromatography of the volatile organic acids. After developing, the spots were located by spraying the paper with acidic bromophenol blue. In an attempt to characterize other organic acids, the descending method of Buch, Montgomery, and Porter (1952), employing *n*-amyl alcohol and 5 M formic acid, was used. The chromatograms were sprayed with alcoholic bromophenol blue adjusted to pH 6.7 with dilute sodium hydroxide.

The procedure for the preparation of the 2,4-dinitrophenylhydrazones of keto acids for chromatography was taken in part from Seligson and Shapiro (1952) and Kvamme and Hellman (1954), in which the hydrazones were extracted with 20 per cent ethyl alcohol in chloroform and extracted back into the aqueous layer with 10 per cent sodium carbonate. The derivatives were chromatographed according to the ascending methods of Meister and Abendschein (1956) and El Hawary and Thompson (1953), and the spots located by spraying with 2 per cent ethanolic KOH.

RESULTS

Changes in pH. Typical changes in pH during growth of *B. cereus* in the presence and absence of glucose are shown in figure 1. In the glucose-containing culture, the pH rapidly decreased during growth, reaching a minimum at about the time exponential growth was complete. The pH then rapidly increased to a level higher than the initial pH of the medium, accompanied by

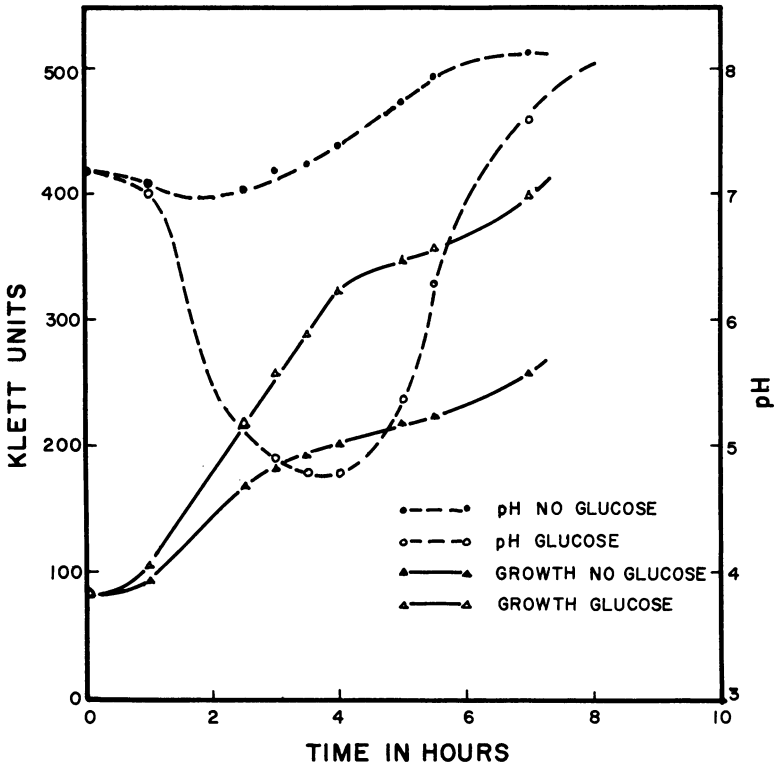

Figure 1. Changes in pH during growth in the presence and absence of 0.1% glucose at 30 C. Limited growth occurs in the absence of glucose. The time when maximal population is reached in each culture is indicated by the break in the linear portion of the respective growth curves as confirmed by the appearance of cells characteristic of the prespore stage in stained preparations. The gradual increase in turbidity after multiplication ceases is due to the increase in refractivity of cells during sporulation.

cytological changes characteristic of the prespore stage as observed in dilute crystal violet stained preparations. On the other hand, the culture lacking glucose failed to show any significant changes in pH during growth. These results indicated that glucose was dissimilated to some organic acid intermediates during growth. These intermediates accumulated in the medium and were subsequently utilized once sporogenesis began. These changes in pH are in agreement with those reported by Knaysi (1945) for *Bacillus mycoides.*

Reversing α-picolinic acid and ethyl malonate inhibition of sporulation. Before proceeding on an extensive investigation regarding the identity of these acid intermediates, it was feasible to determine whether they were in any way implicated with the sporulation process. Gollakota (1959) reported that an analogue of dipicolinic acid, namely α-picolinic acid, and the diethyl ester of malonic acid both completely inhibited sporulation of *B. cereus* but allowed normal growth. Because of the suppression of sporogenesis, the pH of such cultures decreased with growth but remained at the minimal level even after prolonged incubation. These inhibitions were shown to be reversed by the addition of various organic acids.

Assuming that the intermediates accumulating during growth were organic acids commonly associated with end products of incomplete glucose dissimilation, samples of supernatants were acidified to pH 2.5 and ether extracted in a continuous liquid-liquid extractor for 24 hr. The acids collected in the receiving flasks were neutralized with alkali, and the ether was removed by evaporation. The residual salts of these acids were diluted to $\frac{1}{30}$ of the original volume of supernatant collected, adjusted to pH 7.5, autoclaved, and tested for their ability to reverse the

inhibition of sporulation by α-picolinic acid or ethyl malonate.

In addition to the normal constituents of the medium, the 5-ml incubation mixtures contained 2 ml of the ether extractable acid concentrates and the respective inhibitors, α-picolinic acid or ethyl malonate. These were added to give a final concentration of 1.2×10^{-3} M and 1.2×10^{-2} M, respectively. The flasks were inoculated with a drop from a dilute heat shocked spore suspension and incubated on a shaker at 30 C for 24 hr. The results are shown in table 1.

Preparation A, containing acids extracted from a supernatant sample collected when the pH was near minimum (pH 4.9), was able to reverse the inhibition of sporulation by either α-picolinic acid or ethyl malonate, but preparation B, made from supernatant collected when the pH had risen to 6.9, was unable to demonstrate this ability when added in the same amounts. These results led us to believe that the initial decrease in pH of the normal culture was due to the accumulation of organic acids produced

TABLE 1

Reversal of α-picolinic acid or ethyl malonate inhibition of sporulation by ether extractable acids from the supernatant fluid

	Turbidity* (Klett Units)	pH	Stain	Spore† (Count/ml)
Control (no inhibitor)	440	7.7	Spores	1.9×10^8
α-Picolinic control	250	4.8	Vegetative	$<10^3$
With preparation A	475	7.8	Spores	5.3×10^7
With preparation B	250	4.9	Vegetative	$<10^3$
Ethyl malonate control	305	4.9	Granular	3.5×10^5
With preparation A	455	5.5	Spores	3.5×10^7
With preparation B	290	5.1	Granular	$<10^3$

Five ml total incubation mixture. Control flask contained normal medium. α-Picolinic acid and ethyl malonate controls in addition contained the inhibitor in concentrations of 1.2×10^{-3} M and 1.2×10^{-2} M, respectively. Preparations A and B were prepared from supernatants collected at pH 4.9 and 6.9 and concentrated 30×. Two ml were added. Results obtained after 24 hr incubation at 30 C.

* Turbidity measured after heat shocking at 80 C for 20 min to disperse clumps. Values corrected for supernatant color.

† Cells surviving 80 C for 20 min.

TABLE 2

Results of chromatographic analyses of supernatant preparations

	R_F Values			
	Theoretical	Experimental		
A. Volatile acids (Kennedy and Barker, 1951)				
1. Formate	0.31	0.31		
2. Acetate	0.33	0.34		
3. Propionate	0.44	0.42		
4. Supernatant preparation		0.33		
B. Nonvolatile organic acids (Buch et al., 1952)				
1. Citrate	0.23	0.13		
2. Isocitrate		0.14		
3. Succinate	0.61	0.59		
4. Glyoxylate		0.56		
5. Malate	0.32	0.26		
6. Pyruvate		0.61		
7. Supernatant preparation		0.58		
	Meister and Abendschein (1956)		El Hawary and Thompson (1953)	
	Theoretical	Experimental	Theoretical	Experimental
C. 2,4-dinitrophenylhydrazones				
1. Pyruvic acid (brown)*	0.48–0.53	0.49	0.41	0.43
2. Glyoxylic acid (red-brown)*	0.31–0.47	0.39		0.38
3. Supernatant preparation (brown)*		0.49		0.45

* Color after spraying spots with ethanolic KOH.

during growth. The apparent disappearance of these acids once sporulation began suggested that they were indeed necessary for the completion of the sporulation process.

Identification of the ether extractable acids. In the system for the resolution of the volatile organic acids, one mobile spot was observed which upon repeated trials yielded R_F values corresponding to acetic acid. This component was further characterized by steam distillation and by a positive lanthanum nitrate spot test on a drop of the distillate.

One discrete spot appeared in the system used for the tentative identification of the other organic acids whose R_F value closely resembled several common acids selected for reference. These were pyruvic, glyoxylic, and succinic acids. Preliminary color tests with 2,4-dinitrophenylhydrazine indicated the presence of significant amounts of a keto acid in these preparations. Consequently, the 2,4-dinitrophenylhydrazones of pyruvic acid, glyoxylic acid, and the keto acid from several supernatant preparations were purified and chromatographed according to the procedure described in Materials and Methods. After spraying the chromatograms with ethanolic KOH, the color and R_F values of the hydrazones were compared, and the one isolated from the supernatant was identified as pyruvic acid. Further chromatographic analyses of these supernatant preparations failed to yield any organic acids other than pyruvic and acetic acids (table 2).

Production of pyruvic and acetic acids during growth and their utilization during sporulation. A quantitative study was made directly on supernatant samples removed at various intervals during growth and sporulation in an attempt to

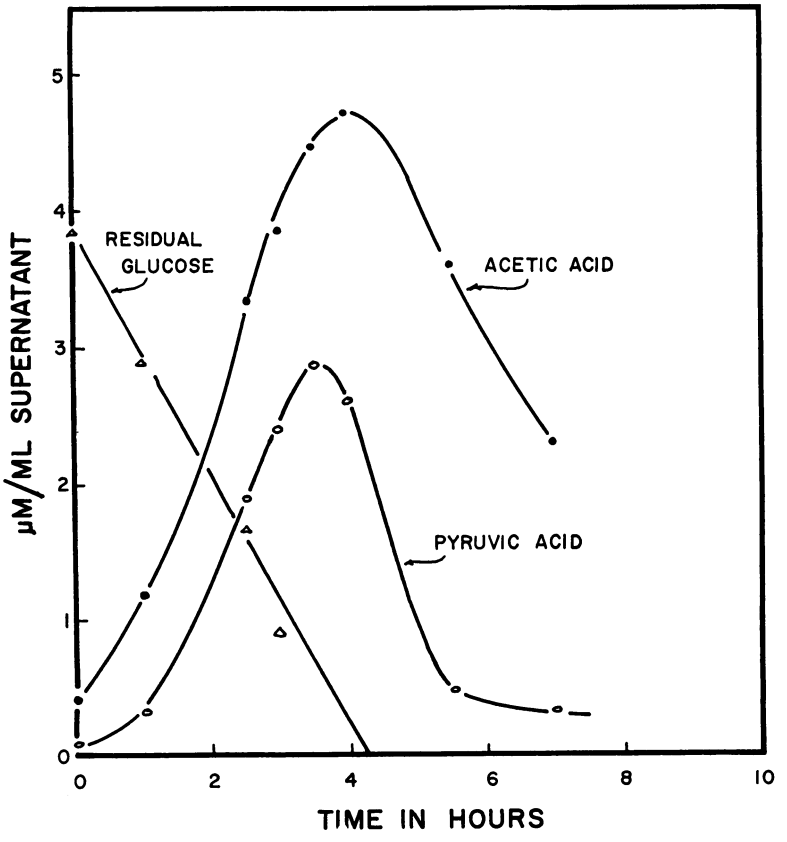

Figure 2. Production of pyruvic and acetic acids from glucose during growth and subsequent utilization of the acids during sporulation at 30 C. Cells characteristic of the prespore stage appeared shortly after 4 hr.

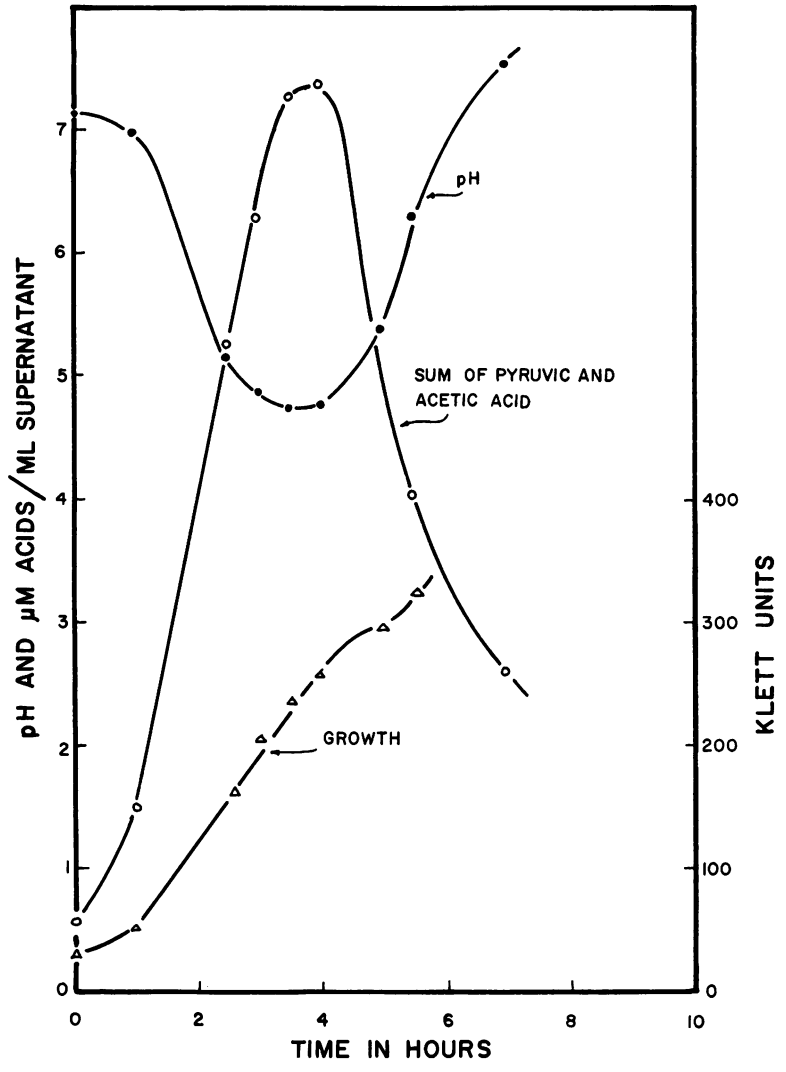

Figure 3. Plot showing that production and utilization of pyruvic and acetic acids are responsible for the characteristic pH changes during growth at 30 C. Sporulation began shortly after 4 hr.

determine whether the production and utilization of these acids were responsible for the characteristic pH changes of the medium.

In figure 2, the low concentration of pyruvic and acetic acids at zero time was attributed to that amount carried over with the inoculum. These acids continued to accumulate during growth until near maximal population was reached, about 3½ hr in this experiment. Pyruvic acid consistently reached a maximal level about ½ hr prior to the maximal acetic acid level, suggesting that pyruvic was the direct precursor of acetate. Once sporulation began, both acids were rapidly utilized. These results support the earlier hypothesis that the acid intermediates were derived from glucose which simultaneously disappeared from the medium. Evidence that these acids were responsible for the pH changes during growth and sporulation is shown in figure 3.

Acid intermediates of cultures inhibited by α-picolinic acid or ethyl malonate. Supernatants collected from cultures in which sporulation was inhibited by α-picolinic acid or ethyl malonate were similarly analyzed for pyruvic and acetic acids. The results are shown in table 3. The

TABLE 3

Pyruvic and acetic acid accumulation in cultures inhibited by α-picolinic acid or ethyl malonate

Inhibitor	Turbidity (Klett Units)	pH	Pyruvic Acid (μmoles/ml Supernatant)	Acetic Acid (μmoles/ml Supernatant)
α-Picolinic acid	305	4.55	0.46	7.29
Ethyl malonate	315	4.75	0.24	5.06

α-Picolinic acid and ethyl malonate were added to give a final concentration of 1.2×10^{-3} M and 1.2×10^{-2} M, respectively. After 24 hr incubation on shaker at 30 C.

principal acid in both α-picolinic acid and ethyl malonate inhibited cultures was acetic acid. The amount accumulated in the presence of α-picolinic acid was comparable to that obtained from normal cultures if all of the pyruvic acid was converted to acetic acid when the pH was at the minimal level. Slightly lower concentration of acetate was accounted for in the culture inhibited by ethyl malonate. Microscopic examination of stained preparations showed that the cells inhibited by α-picolinic acid were typical of normally growing cells whereas those inhibited by ethyl malonate were more advanced, cytologically characteristic of the prespore stage. These data, together with the fact that only small amounts of pyruvic acid were present, suggested that these inhibitors, particularly α-picolinic acid, interfered with the utilization of acetic acid. This latter mechanism appeared to be essential for the completion of the sporulation process.

Decarboxylation of pyruvic acid to acetic acid and CO_2. In the foregoing experiment it was assumed that pyruvate was the direct precursor of acetate during growth. Subsequent studies provided evidence that a typical oxidative decarboxylation mechanism was operative in growing cells of *B. cereus*.

Cells were harvested during the logarithmic phase by centrifugation, washed, and suspended in 0.04 M potassium phosphate buffer at pH 5.2. One ml of this suspension (containing 5.1 mg cells dry weight) was added to each of several Warburg vessels and the oxygen uptake measured manometrically in the presence and absence of alkali, using sodium pyruvate as the substrate. Since earlier results showed that α-picolinic acid effectively inhibited the utilization of acetic acid produced during growth, it was possible to study quantitatively the conversion of pyruvate to acetate by the addition of this inhibitor to the reaction mixture.

From the data given in table 4, the ratio of CO_2 liberated to O_2 taken up per unit time was 2:1, as expected for the oxidative decarboxylation of pyruvate. After 16 hr, the contents of the vessels were analyzed for the presence of acetic acid. Starting with 40 μmoles of pyruvate, 38 μmoles of acetic acid were recovered, accounting for 95 per cent of the initial substrate.

TABLE 4

Oxidative decarboxylation of pyruvic acid to acetic acid and carbon dioxide by cells of Bacillus cereus at 30C

Additions	O_2 Uptake μl:mg dry wt:hr	CO_2 Liberated (μl:mg dry wt:hr)	Acetic Acid Formed (μmoles)
None (endogenous)	0.62	0	0
Pyruvic acid (40 μmoles)	27.5	53.7	38.0

Reaction mixture:

Main compartment: 1.0 ml cells (5.1 mg dry wt/ml); 0.1 ml α-picolinic acid (10 mg/ml); 0.1 ml $MgCl_2$ (0.02 M); 0.7 ml 0.04 M potassium phosphate buffer, pH 5.2.

Side arm: 0.1 ml Na-pyruvate (0.4 M) or 0.1 ml water.

Center well: 0.2 ml KOH (20 per cent) or 0.2 ml water.

Total volume: 2.2 ml.

TABLE 5

Metabolic products of growth and sporulation

Product Formed	Concn (μmoles/ml Supernatant)	
	pH 4.7	pH 7.0
Acetic acid	4.75	2.29
Pyruvic acid	2.65	0.32
Lactic acid	0.24	0.07
Acetoin	0.13	0.42
Glycerol	0.10	0
Acetone	0	0
Ethyl alcohol	0	0
2,3-Butanediol*	0	0.10

* Corrected for acetoin.

Other products of growth and sporulation. Chromatographic analyses of the 2,4-dinitrophenylhydrazone preparations from various supernatant samples failed to demonstrate the accumulation of α-keto glutarate as reported by Powell and Strange (1956) for a different strain of *B. cereus*. Knaysi (1945) reported the accumulation of lactic acid in cultures of *B. mycoides* which was presumably oxidized during sporulation. As indicated in table 5, only a small amount of lactic acid was formed by *B. cereus* when the pH was near minimum, and this amount subsequently decreased as sporogenesis continued.

Since acetic acid was rapidly metabolized during sporogenesis, an attempt was made to determine what new end products were formed during this process. In the literature, a variation was noted among species of bacilli in regard to metabolic end products, and in some studies no indication was given as to whether sporogenic strains were employed or not. Therefore, analyses for the more common end products reported were made.

Two samples of supernatant, one taken at pH 4.7 and the other when the pH had risen to pH 7.0, were examined. Care was taken to collect the latter supernatant before lysis of the sporangia occurred, as this might introduce substances not directly considered end products of acetate metabolism. These were then analyzed for the presence of acetoin, glycerol, acetone, ethyl alcohol, and 2,3-butanediol. The results are shown in table 5, together with the values for pyruvic and acetic acids. With the exception of acetoin, none of these compounds were produced in significant amounts during sporulation.

DISCUSSION

Employing the active culturing technique for rapid growth and simultaneous sporulation of *B. cereus*, it was observed that glucose was rapidly oxidized during growth to pyruvic and acetic acids which accumulated in the medium. Once sporogenesis began, these acids were completely metabolized by the sporulating cells. Accompanying these changes, it was shown earlier (H. Orin Halvorson, 1957) that the oxygen demand abruptly decreased just prior to the initiation of sporulation. Once sporulation began, however, the oxygen demand suddenly increased to a high level; concomitantly, pyruvic and acetic acids disappeared from the medium. These changes observed in the metabolic pattern, together with the appearance of cells characteristic of the prespore stage, are interpreted as the initial stages in sporogenesis.

The accumulation of acetic acid as the end product of glucose oxidation during growth strongly suggests that a complete oxidative mechanism is nonfunctional in the vegetative cells of *B. cereus*, although in the work of Hardwick and Foster (1953), oxidative activities were observed in vegetative cells of *B. mycoides* toward malate, succinate, α-keto glutarate, and pyruvate. This incomplete oxidative activity is supported by the fact that normal growth occurs in cultures where acetate utilization is inhibited by α-picolinic acid. The acetic acid concentration in these cultures prior to sporulation accounts for 60 to 65 per cent of the initial glucose carbon in the medium.

The route of glucose oxidation to acetic acid via pyruvate has not been completely elucidated for *B. cereus*, but evidence available thus far is best explained by an operational hexose monophosphate shunt mechanism. H. Halvorson (1957), Halvorson and Church (1957), and Doi, Halvorson, and Church (1959) have detected the activities of a number of enzymes from spore extracts of this organism which oxidized glucose to pyruvate via gluconate and 2-keto gluconate. Similar observations were made with *Bacillus subtilis* by Gary, Klausmeirer, and Bard (1954) and Keynan, Strecker, and Waelsch (1954). Also, evidence favoring the hexose monophosphate mechanism is provided by the failure to demonstrate hexokinase and glucokinase activities in any of these spore extracts (Doi et al., 1959). Furthermore, no inhibitory effect on growth was observed by the presence of sodium fluoride (Murty and Halvorson, 1957).

The sudden and rapid utilization of acetate from the medium indicates that a new enzyme or enzyme system is induced during the transition from vegetative growth to sporulation. This induction appears to be the first recognizable step in sporogenesis. In cultures where sporulation is inhibited by α-picolinic acid, this induction is suppressed, presumably by preventing the synthesis of the enzyme(s) responsible for acetate utilization (Gollakota and Halvorson, 1960). As a consequence, α-picolinic acid inhibits sporulation.

Among the enzymes associated with acetate metabolism, the most feasible one which may be involved in this induction process is the one

responsible for activating acetate to acetyl phosphate or acetyl coenzyme A. (CoA). In this connection, Krask (1957) reported finding an aceto-CoA kinase-like enzyme in spore extracts of *B. cereus*.

Evidence concerning the fate of acetate taken up by sporulating cells is still lacking. Generally, it can be said that acetate is the substrate for sporogenesis, its metabolism by sporulating cells providing the energy and precursors for the synthesis of spore material. Negative results obtained in the search for the more common end products produced by bacilli and the high oxygen demand during this state of sporulation are suggestive of the presence of a functional tricarboxylic acid cycle in sporulating cells.

Some information regarding a complete terminal oxidation route in bacilli has been reported. Beck and Lindstrom (1955) found that vegetative cell-free extracts of *B. cereus* were capable of oxidizing all the intermediates of the tricarboxylic acid cycle. However, with acetate, no oxidative activity was observed in any of these enzyme preparations. Storck and Wachsman (1957), working with an asporogenous strain of *Bacillus megaterium*, presented evidence for the localization of the tricarboxylic acid cycle enzymes in the ghost and soluble fractions of this organism, further indicating that this cycle may be common among aerobically grown bacilli.

In spore extracts of *B. cereus*, systems capable of oxidizing acetate, succinate, fumarate, and oxalacetate were demonstrated (Halvorson and Church, 1957). Similar results were reported by Nakada et al. (1957) for a different strain of *B. cereus*. No direct evidence is available, however, for the incorporation of acetate into the tricarboxylic acid cycle during sporulation.

The active participation of the tricarboxylic acid cycle in providing precursors for the biosynthesis of dipicolinic acid has been suggested by Martin and Foster (1958). As a result of isotope incorporation and isotope competition experiments, these investigators found several amino acids and carboxylic acids to be efficient precursors of dipicolinic acid. Among the carboxylic acids tested, acetate was most effective, being essentially as efficient as glutamic and aspartic acids, alanine, serine, and proline. Although the specific precursors of dipicolinic acid were not clarified, it was apparent that the incorporation pathway of these components into dipicolinic acid involved a symmetrical intermediate, presumably succinate or fumaric acid.

In *B. cereus* strain T, it was shown that dipicolinic acid synthesis occurred late in the process of sporulation, its synthesis accompanied by the appearance of heat resistant spores (Collier and Nakata, 1958). Gollakota (1959), working with the same organism, has shown that among the compounds found to reverse the inhibition of sporulation by α-picolinic acid, succinic acid is most effective. These and other data are best explained by the glyoxylic acid by-pass mechanism in which succinic acid could be generated as the symmetrical precursor of dipicolinic acid from acetate. The operation of this glyoxylic by-pass mechanism need not be mutually exclusive of the tricarboxylic acid cycle since dipicolinic acid, one of many compounds synthesized during sporulation, could account for only a fraction of the acetate utilized. The possibility of a new mechanism, completely different from the tricarboxylic acid-glyoxylic acid by-pass mechanisms, also cannot be excluded at this time.

SUMMARY

Some of the biochemical changes occurring during growth and sporulation of *Bacillus cereus* strain T were investigated. Employing cultures capable of rapid growth and simultaneous sporulation, it was possible to correlate results obtained at various time intervals with particular phases of growth and sporulation.

During vegetative growth of this organism in a glucose-yeast extract salts medium, large amounts of acetic acid were produced which accumulated in the medium.

After growth was complete, sporulation began, as determined by the appearance of cells typical of the prespore stage described by others. Accompanying this transition from vegetative growth to sporulation, acetic acid rapidly disappeared from the medium. The accumulation of this acid during growth and its utilization during sporulation were shown to be responsible for the characteristic pH changes observed in these cultures.

These results, together with other data, were interpreted to mean that one of the initial changes occurring during the transition stage was the induction of a system capable of oxidizing acetate. This mechanism is believed to be essential for the completion of the sporulation process in a glucose medium.

The relationship of these biochemical changes occurring during growth and sporulation have been discussed.

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