BIOCHEMISTRY OF SPORULATION

I. Metabolism of Acetate by Vegetative and Sporulating Cells[1]

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ABSTRACT

HANSON, RICHARD S. (University of Illinois, Urbana), V. R. SRINIVASAN, AND H. ORIN HALVORSON. Biochemistry of sporulation. I. Metabolism of acetate by vegetative and sporulating cells. J. Bacteriol. **85**:451–460. 1963.—The transition from the vegetative to the sporulating cycle in a sporeformer is marked by a change in the enzymatic machinery of the cell. When vegetative cells of *Bacillus cereus* strain T are grown in a glucose-yeast extract-minerals medium, acetate accumulates until the beginning of the sporulation cycle. The acetate-activating systems are present in the vegetative cells as well as in the cells of the early stages of sporulation, whereas the enzymes necessary for the terminal oxidation of acetate to carbon dioxide are absent in the vegetative stage. The induction of a functional tricarboxylic acid cycle during early sporulation is inhibited by chloramphenicol. α-Picolinic acid also prevents morphological, as well as physiological, changes during the transition.

Investigations on the terminal oxidative pathways in *Bacillus* species demonstrated the presence of components of the Krebs tricarboxylic acid cycle (Beck and Lindstrom, 1955; Halvorson and Church, 1957; Hardwick and Foster, 1953; Gollakota, 1959). Nevertheless, the utilization of acetic acid is prevented in *Bacillus cereus* strain T, possibly because of a block in the terminal oxidative pathways, until the sporulation cycle begins (Gollakota and Halvorson, 1960; Nakata and Halvorson, 1960; Halvorson, 1957).

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Changes occurring during growth and sporulation of *B. cereus* strain T were described by Halvorson (1957). During vegetative growth, acetic and pyruvic acids accumulate and are subsequently utilized when the glucose is exhausted from the medium (Nakata and Halvorson, 1960). This phenomenon is reflected in changes in the pH of the medium, which are characteristic of active cultures of this organism.

Vegetative growth and sporulation have been shown to be distinct in their response to several agents (Charney, Fisher, and Hegarty, 1951; Curran, 1957; Weinberg, 1955; Murrell, 1961; Amaha, Ordal, and Touba, 1956). The work of Gollakota and Halvorson (1960) points out that α-picolinic acid inhibits acetic acid utilization, dipicolinic acid synthesis, and sporulation only if it is added prior to acetate utilization. Other compounds specifically inhibit sporulation by imposing blocks within the tricarboxylic acid cycle, as evidenced by the ability of intermediates of the cycle to reverse the effects of the inhibitors.

We previously reported a citrate-synthesizing system (Hanson, Srinivasan, and Halvorson, 1961) that is active during the sporulation cycle, but not during logarithmic growth. The appearance of citrate-forming activity parallels the disappearance of acetate from the culture medium.

It is the purpose of this paper to describe the function of the Krebs cycle in relation to the growth and sporulation of cultures of *B. cereus* strain T and to offer an explanation of the nature and significance of the observed phenomena in terms of biochemical properties of the cells at different stages of the life cycle.

MATERIALS AND METHODS

Cultural methods. B. cereus strain T, previously referred to as *Bacillus cereus* var. *terminalis*, was used in this study. Active cultures were grown according to the procedure described by Collier (1957), as modified for this organism (Halvorson,

1957). G-medium contained: $FeSO_4 \cdot 7H_2O$, 0.00005%; $CuSO_4 \cdot 5H_2O$, 0.0005%; $ZnSO_4 \cdot 7H_2O$, 0.0005%; $MnSO_4 \cdot 4H_2O$, 0.005%; $MgSO_4$ (anhydrous), 0.02%; $CaCl_2 \cdot 2H_2O$, 0.0025%; K_2HPO_4, 0.05%; $(NH_4)_2SO_4$, 0.2%; yeast extract, 0.2%; glucose, 0.1% (all w/v); final pH, 7.25 to 7.45. Yeast extract used in the G-medium was extracted for at least 72 hr at pH 2.0 in a continuous liquid-liquid extractor to remove organic acids (Gollakota and Halvorson, 1960). All cultures were incubated at 30 C on a rotary shaker. Samples were removed at intervals for optical density and pH determinations. Optical density was determined in a Klett-Summerson colorimeter against a G-medium blank with a no. 66 filter. Sporulation and morphological changes were observed by examination of stained smears and of wet mounts by phase microscopy. The pH of the samples was measured in a Beckman pH meter.

Inhibition studies. To test the effect of inhibitors, a sample was removed from an active culture at the desired time and added to sterile Erlenmeyer flasks containing the appropriate inhibitor adjusted to pH 7.0 to 7.5.

Conversion of C^{14}-acetate to $C^{14}O_2$. A 1.0-ml portion of an active culture was transferred to the growth tube of an aeration train designed to trap the carbon dioxide evolved during growth. The train consisted of a carbon dioxide trap containing 100 ml of 10% NaOH; a wash tube (20 by 2.5 cm) containing 75 ml of demineralized water; a sterile cotton-filled suction flask; a sterile tube (18 by 150 mm) with a side arm, placed in a constant temperature bath at 30 C (culture tube); a second tube containing 0.05 N HCl; and a carbon dioxide trap composed of another similar tube containing 15 ml of an ethanolamine-ethylene glycol monomethyl ether solution (1:2, v/v). The air inlet for each tube consisted of a gas-dispersion tube extending to the bottom; the side arm near the top of the tube served as the outlet. The connecting tubing was loosely packed with dry cotton. The growth tube contained 9.0 ml of G-medium, 1.0 ml of inoculum, and additions as indicated in the experiments. Aeration rates were controlled by placing a bubbler tube at the air inlet and outlet to the train. Air was drawn through the train by suction through the outlet of the final carbon dioxide trap. The pH curve during growth of a culture in the train was determined by removing samples through the side arm of the growth tube covered with a serum stopper, using a 2.0-ml syringe and needle. Samples (3.0 ml) were removed from the carbon dioxide trap and counted as described below. The same amount of trapping solution was then added back to keep a constant volume and to prevent saturation of the trapping solution. Each sample was corrected for the counts/min removed in previous samples. The samples were corrected for the acetate distillation, which was determined in an uninoculated run. After the completion of each experiment, 1.0 ml of 1 N H_2SO_4 was added to the growth tube to release carbon dioxide trapped in the medium.

The ability of cells, harvested at any given time during the growth cycle, to oxidize acetate was determined in the following manner. Portions (100 to 300 ml) of an active culture were removed at the appropriate intervals and chilled to 4 C in a coolant bath at −20 C. The cells were harvested by centrifugation at 12,000 rev/min in a Servall SS-1 centrifuge, washed in a buffered G-medium without glucose (containing chloramphenicol, 10 mg/ml), and suspended in a small amount of the same mixture. A sample containing 6.1 mg (dry wt) of cells in 1.0 ml of the incubation medium was added to the side arm of a 150-ml Warburg vessel. The incubation mixture (9.0 ml) was composed of G-medium (without glucose), 2-C^{14}-acetate, 50 μmoles of sodium acetate, and 100 μg of chloramphenicol. A vial (9 by 35 mm) containing 1.0 ml of the trapping solution was placed in the center well. The flask was sealed with a serum stopper and fastened with a rubber band to ensure a tight seal. At zero time, the cells were tipped into the flask and incubated at 30 C on a rotary shaker for the desired period. The reaction was terminated by adding 1.0 ml of 72% perchloric acid, or 80% trichloroacetic acid, through the serum stopper by means of a syringe and needle. The flask was then placed on a shaker for 15 min and allowed to stand at room temperature for 2 to 3 hr to ensure complete transfer of the carbon dioxide into the trapping solution. Dry weights of each cell suspension were determined by drying the samples for 24 hr at 110 C. $C^{14}O_2$ evolution during runs using standard $BaC^{14}O_3$ in G-medium in the main compartment of the Warburg vessel gave an over-all efficiency for collection and determination of radioactivity of 31.8% in ten trials. The efficiency was not influenced by adding carrier Na_2CO_3 in amounts of up to 100 μmoles.

Controls without cells were used to check 2-C^{14}-acetate distillation. Of 2.5×10^5 counts/min, 32 to 211 counts/min were found to distill over by this procedure. All counts were corrected for acetate distillation and background. The radioactivity of C^{14}-carbon dioxide was measured by a procedure essentially similar to that described by Jaffrey and Alvarez (1961).

Incorporation of C^{14}-acetate into cell components. Cells harvested at different intervals were made up in G-medium (without glucose) and 50 μmoles of 1-C^{14}-acetate (1.5 μc) in a volume of 5.0 ml, incubated at 30 C for 15 min on a rotary shaker, placed in a coolant bath at -20 C to stop incorporation, and cooled to 0 to 4 C. Cell fractionation was carried out as described by Roberts et al., 1955.

Determination of acids accumulating during fluoroacetate inhibition. 2-C^{14}-acetate (2 μc) and 0.005 M neutralized fluoroacetic acid were added to 20 ml of an active culture at 1.5 hr. Culture supernatants from this mixture were assayed for nonvolatile radioactive components at 3.5 hr; 140 ml of acetone and 0.1 ml of 18 N H_2SO_4 were slowly added to the chilled culture as it was being constantly stirred. This acid-acetone treated culture was then centrifuged at 12,000 rev/min for 20 min, washed with 50 ml of acetone, then recentrifuged; 10 μmoles of carrier citric acid were added, and the resulting extract was evaporated to a small volume and brought back to 3.0 ml by adding distilled water. This solution was neutralized with phenol blue and added to a Dowex-1-formate column (0.6 by 10 cm) prepared according to the procedure of Busch et al. (1952). The column was developed with 0 to 3.5 N formic acid gradient and 50 fractions (5 ml each) were collected. Samples (2.0 ml) of each fraction were placed on aluminum planchets, dried under a heat lamp and assayed for radioactivity in a Nuclear-Chicago model D-47 gas-flow counter. Portions of each fraction were also assayed for citric acid by the procedure of Natelson et al. (1948). Identification of radioactive peaks was made by paper chromatography in two solvent systems: a mixture (5:2:1) of ether, 88% formic acid, and water (Lugg and Overall, 1947); and *sec*-butanol, formic acid, and water (85:5:10; Roberts et al., 1955). Strips of paper chromatograms were analyzed for radioactivity in a paper-strip scanner.

Preparation of extracts. Cells, grown in G-medium, were chilled rapidly to prevent further development, harvested, and then washed in 0.05 M phosphate buffer (pH 7.2). The washed cells were suspended in 0.02 M phosphate buffer with 0.005 M sodium thioglycolate at pH 7.2 and broken by passage through a French pressure cell. Cell-free extracts were prepared by centrifugation in a Servall SS-1 centrifuge at 10,000 rev/min for 10 min.

Materials. C^{14} isotopes were purchased from the Nuclear-Chicago Corp. Dilithium acetyl phosphate was prepared according to the method of Avison (1955). Before use, the dilithium salt was passed through a Dowex 50 (H^+ form) column to remove lithium. The acetyl phosphate was neutralized with tris(hydroxymethyl)aminomethane (tris) buffer. The oxalacetic acid was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

It is imperative that the cells used in studies of this nature all be in the same phase of growth. The active-culture method provides a means of obtaining cultures in which most of the cells are of the same physiological age, and sporulate simultaneously. Halvorson (1957) pointed out that not all heat-stable cells disappeared until three 10% transfers were made during logarithmic growth. The importance of multiple transfers is illustrated by the fact that when heat-shocked spores are allowed to germinate, and are then harvested during logarithmic growth, there is four times as much acetate incorporated into the cells and oxidized to carbon dioxide as in cells harvested from the third transfer. By using this technique, one can obtain cultures in which the vegetative growth and the sequential morphological changes

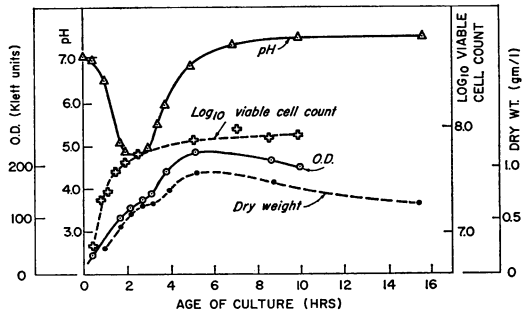

FIG. 1. *Optical density, pH, dry weight, and viable-cell count during growth and sporulation of Bacillus cereus strain T.*

leading to sporulation occur in a synchronous manner. At the same time, it provides a means of obtaining complete sporulation.

Optical density, pH, and oxygen demand curves of synchronously growing cultures are graphically illustrated by Halvorson (1957) and Nakata and Halvorson (1960). (Synchrony, as used here, implies simultaneous morphological and physiological changes, rather than synchronous cell division, as the term is generally applied.) Optical density, dry weight, and pH are plotted in Fig. 1. The apparent increase in the viable-cell count after 2 hr is probably due to chain reduction. It is apparent that the accurate and easily ascertained determinants of culture age are optical density and pH changes. Halvorson (1957) reported two peaks in oxygen demand. One paralleled the increase in cell population, and the second occurred as the pH began to rise, when acetate began to disappear from the medium. The oxygen demand decreased before the pH rise was completed and remained at a low level until sporulation occurred. The utilization of acetate corresponds to granulation of the cells (Nakata, 1962).

Cultures in which the pH is rising respond differently to inhibitors than do those in which the pH is falling (Gollakota and Halvorson, 1960). Glucose has no delaying effect on sporulation when added later than 4.0 hr after inoculation, and it does not support further growth (Nakata, 1959). If added before, however, the pH rise is delayed until the residual glucose is exhausted, and the cells continue to multiply. The cells are irreversibly committed to sporulation when granulation occurs (Hashimoto, Black, and Gerhardt, 1960). The beginning of the morphological changes associated with sporulation occur as the pH begins to rise (Srinivasan and Halvorson, Nature, *in press*). For these reasons, we term cultures in which the pH is rising as sporulating cultures.

TABLE 1. *Oxidation of UC^{14}-glucose and 6-C^{14}-glucose by an active culture of Bacillus cereus strain T**

Compound	$C^{14}O_2$ evolved (counts/min)				
	0–1 hr	1–2 hr	2–3 hr	3–4 hr	4–5 hr
C^{14}-glucose (uniformly labeled).	8,345	8,275	3,731	5,851	9,253
6-C^{14}-glucose.....	38	98	695	5,177	4,596

* Incubation in 150-ml Warburg flasks at 30 C in complete G-medium. Vials containing the CO_2 absorbant were changed at 1.0-hr intervals. Counts/min added = 1.6×10^5.

Since acetate accumulates when glucose is oxidized, glucose is omitted from the medium to prevent dilution of the labeled acetate and to prevent the possibility of a glucose effect on the processes involved in the transition from vegetative growth to sporulation. Furthermore, in order to test its incorporation into products of acetate-assimilating or -oxidizing pathways, C^{14}-acetate cannot be added at the time of inoculation because the dilution of the label during acetate accumulation will cause a manifold decrease in the specific activity of the acetate. The experiments described in this paper were designed so that the specific activity of the C^{14}-acetate remained constant. This constancy was accomplished by placing cells harvested at different physiological ages into a fresh medium that did not contain glucose. This same result could be accomplished by adding 6-C^{14}-glucose to a G-medium, which would cause the accumulation of 2-C^{14}-acetate of a constant specific activity. Blumenthal (1961) found that 6-C^{14}-glucose was not oxidized to $C^{14}O_2$ by germinated spores until after two to three cell divisions occurred; this period can be lengthened by the active-culture procedure, which provides for a longer period of vegetative growth and a definite reproducible transition between the growth phase and the time that oxidation of the acetate begins (Table 1).

The label from 6-C^{14}-glucose was found to accumulate as acetic and pyruvic acids in the culture medium during germination (Blumenthal, 1961). It has been shown that during growth glucose is oxidized to acetic and pyruvic acids as well as CO_2 (Nakata and Halvorson, 1960). Acetic acid is oxidized to CO_2 and also converted to poly-β-hydroxybutyric acid during sporulation (Nakata, 1962). The $C^{14}O_2$ released from uniformly labeled glucose may result from oxidation of 6-phosphogluconate and pyruvate (Doi, Church, and Halvorson, 1957), but cannot be from the oxidation of acetate because of a block in the tricarboxylic acid cycle (Hanson et al., 1961).

Although *B. cereus* strain T will grow and sporulate even in the absence of glucose, the addition of glucose is required for maximal growth and the production of acetic and pyruvic acids (Nakata and Halvorson, 1960). We found that

TABLE 2. *Incorporation of C^{14}-acetate into cell components by vegetative and sporulating cells of Bacillus cereus strain T*

Fraction	1-C^{14}-acetate incorporated*	
	1.5-hr cells	3.5-hr cells
	μmoles	*μmoles*
Whole cells	0.65	2.40
Cold tricarboxylic acid soluble	0.01	0.60
Ethanol soluble (75%)	0.25	0.30
Alcohol-ether soluble	0.01	0.01
Hot tricarboxylic acid soluble	0.06	0.20
Residue	0.20	1.16

* Per 10 mg (dry wt) of cells.

TABLE 3. *Effect of fluoroacetic acid (FAA) on growth and sporulation of Bacillus cereus strain T*

FAA addition*		Analysis after 2.5 hr		Analysis after 22 hr	
Time	pH	pH	Klett reading	pH	Sporulation
hr					%
Control	7.2	4.7	170	8.0	100
0	7.2	4.9	155	8.0	60
1	6.2	4.9	155	5.5	0
2	5.0	4.8	165	5.5	0
3	5.2	—	—	5.2	0
4	6.8	—	—	8.0	100
5	7.0	—	—	8.0	100
6	7.0	—	—	8.0	100
9	7.5	—	—	7.9	100

* FAA = 5×10^{-3} M.

3.9 μmoles of glucose caused the accumulation of 7.3 μmoles of pyruvic and acetic acids when the medium was lightly buffered. Thus, 95% of the theoretical yield of these acids (based on glucose in the original medium) is realized during vegetative growth, indicating that not more than 5% of the acetate available from glucose can be utilized during vegetative growth. Table 1 indicates that less than 1% of carbon-6 of glucose is oxidized to CO_2 before 3.0 hr. It was of interest to determine whether any acetate was incorporated into the cells before this time, and, if so, what fractions of the cell contained the acetate-carbon. Analysis of the various fractions of the cells shows that some is incorporated into lipid material (Table 2).

The disappearance of acetate during the initial stages of sporulation suggested that it might be

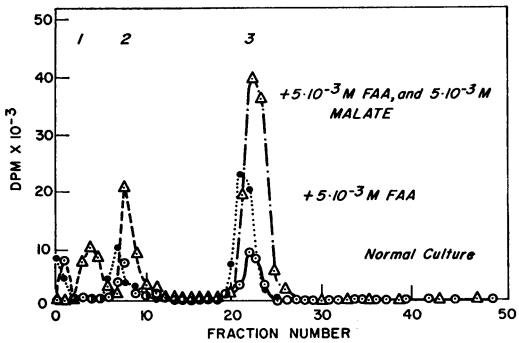

FIG. 2. *Analysis of nonvolatile radioactive acids accumulating in Bacillus cereus strain T cultures at 3.5 hr. Exchanger: Dowex 1-X8 formate 200 to 400 mesh. Solution: formic acid gradient, 0 to 3.5 N. Regions: (1) aspartic and glutamic acids plus an unknown compound; (2) succinic acid; (3) citric acid.*

the major source of energy for sporulation. We tested the effect of fluoroacetic acid (FAA) on growth and sporulation by adding it at different times after inoculation of the culture. The results are shown in Table 3. Vegetative growth is unaffected, even at 10^{-2} M, which is in agreement with previous results obtained by Gollakota and Halvorson (1960).

Acetate utilization, as evidenced by the pH of the medium, is greatly inhibited. Sporulation is also inhibited if the fluoroacetic acid is added before the culture has reached the age of 4.0 hr. The failure of FAA to inhibit when added at zero time is not understood. The lack of inhibition after 4.0 hr suggests that the tricarboxylic acid cycle does not operate or is not necessary after this time. Figure 2 shows that citric acid accumulates in cultures grown in the presence of FAA. There is little or no accumulation of nonvolatile radioactive intermediates from C^{14}-acetate during vegetative growth (Table 2). Citric acid accumulation is enhanced by the addition of malic acid as a carbon-4 source to condense with the active acetate.

The route of citrate formation is via the acetokinase, phosphotransacetylase, and condensing-enzyme catalyzed reactions (Table 4, Fig. 3 and 4).

Acetyl phosphate formation requires Mg^{++}, adenosine triphosphate (ATP), and acetate. Coenzyme A (CoA) inhibits the reaction slightly in many repeated trials. This is probably due to acetyl phosphate utilization via phosphotransace-

TABLE 4. *Cofactor requirements for acetokinase activity in extracts of Bacillus cereus strain T**

Compound	Acetyl hydroxamate formed per 5 min
	μmoles
Control	8.75
− $MgCl_2$	0.80
− Acetate	0.00
− ATP	0.00
+ CoA (30 μg)	8.35
+ EDTA (25 μmoles)	0.00
+ Hydroxylamine (700 μmoles)	0.90
+ Hydroxylamine (700 μmoles) + CoA (30 μg)	0.90

*Reaction mixture: 0.3 ml of substrate [3.2 M K-acetate, 1.0 M $MgCl_2$, 1.0 M HCl buffer (pH 7.4); 25:5:1; v/v], 0.1 M ATP (pH 7.4), water, and extract to make volume 1.0 ml. Reaction stopped by addition of 25 μmoles of ethylenediaminetetraacetic acid. Extract: the cell-free extract was precipitated between 45 and 70% saturated ammonium sulfate, dialyzed overnight vs. 0.05 M potassium phosphate buffer, plus 0.03 M cysteine-HCl (pH 7.4). Acetyl phosphate was determined by the procedure of Lipmann and Tuttle (1945) and expressed as μmoles of acetyl hydroxamate formed.

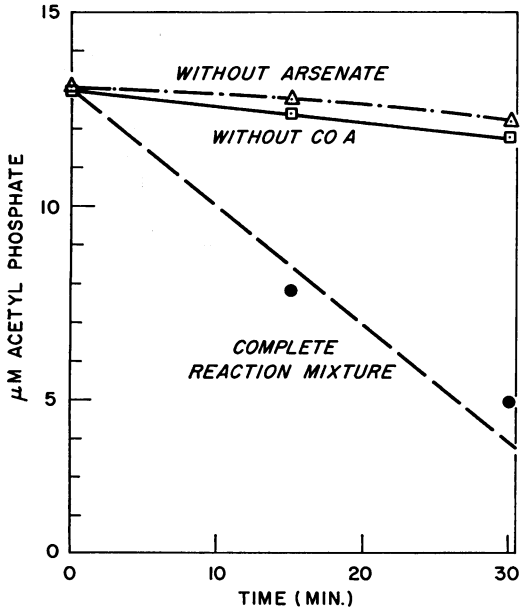

FIG. 3. *Phosphotransacetylase: hydrolysis of acetyl phosphate and its dependence on arsenate and coenzyme A. The reaction mixture contained (in 1.0 ml): tris buffer (pH 7.6); coenzyme A, 0.03 mg; acetyl phosphate; and extract.*

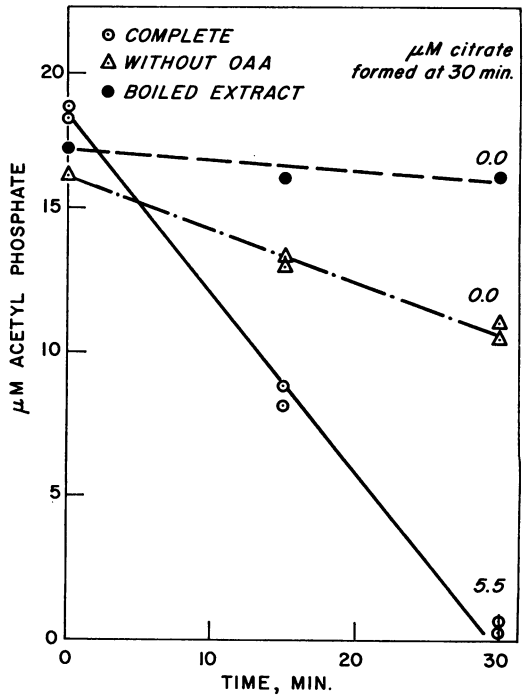

FIG. 4. *Acetyl phosphate hydrolysis in the presence of oxalacetate. The reaction mixture contained (in 1.0 ml): tris buffer (pH 8.0), 50 μmoles; oxalacetic acid (tris salt, pH 7.6); coenzyme A, 0.3 mg; and extract. The cell-free extract was dialyzed for 6 hr in 2 × 10^{-2} M potassium phosphate buffer containing 10^{-2} M 2-mercaptoethanol. Nitrogen was constantly bubbled through the dialysis solution.*

tylase in the presence of CoA. Hydroxylamine prevents acetyl phosphate formation and, thus, acetyl-CoA-kinase could not be assayed for by this procedure. Phosphotransacetylase and condensing-enzyme activities are illustrated in Fig. 3 and 4. Acetyl phosphate utilization requires CoA and arsenate (Fig. 3). Arsenate can be replaced by oxalacetic acid, which yields citric acid as a reaction product (Fig. 4).

Gollakota and Halvorson (1960) suggested that pyridine-6-carboxylic acid (α-picolinic acid) inhibits sporulation by preventing the synthesis of inducible enzymes required for acetate utilization. The mechanism of this inhibition may involve metal chelation since it can be reversed by zinc, cobalt, and nickel as well as by intermediates of the Krebs tricarboxylic cycle. Figure 5 presents evidence that the block by this inhibitor is, indeed, in the Krebs cycle. The oxidation of 2-C^{14}-acetate to $C^{14}O_2$ is inhibited by α-picolinic

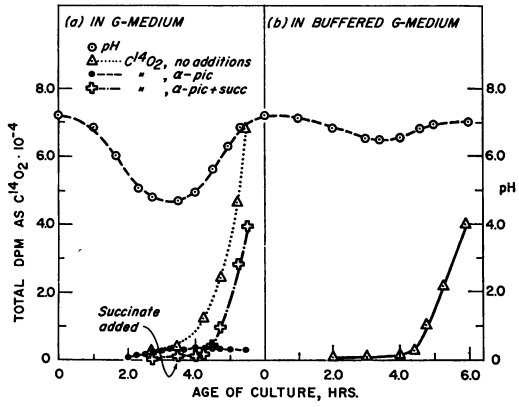

FIG. 5. *Effect of α-picolinic acid and excess phosphate on the oxidation of 2-C^{14} acetate to $C^{14}O_2$ by Bacillus cereus strain T. (a) α-Picolinic acid (6×10^{-4} M) was added at 1.5 hr. Succinate (0.5 mg/ml) was added at 3.5 hr. (b) The effect of a 15-fold excess of phosphate on the pH changes and acetate oxidation during growth and sporulation.*

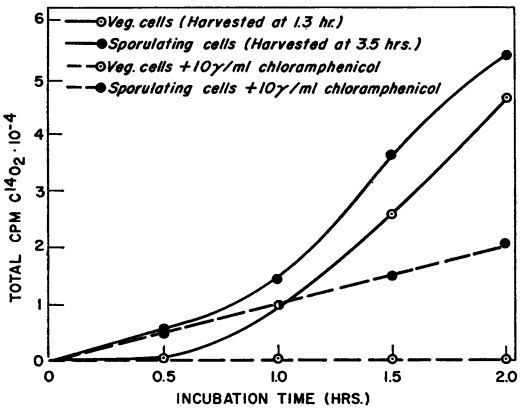

FIG. 6. *Effect of chloramphenicol on the oxidation of 2-C^{14} acetate to $C^{14}O_2$. The cells were harvested from G-medium and treated as described in Materials and Methods.*

acid, and this inhibition is reversed by succinate. Buffering with a 15-fold excess of phosphate does not affect the pattern of acetate oxidation, as shown by the graph on the right in Fig. 5. The pH changes, or lack of orthophosphate, are not responsible for the metabolic shifts.

If vegetative or sporulating cells are incubated with 2-C^{14}-acetate in the presence of chloramphenicol, the rate of $C^{14}O_2$ evolution is linear in sporulating cells and absent in vegetative cells. Chloramphenicol does not inhibit the reaction in sporulating cells that have already formed

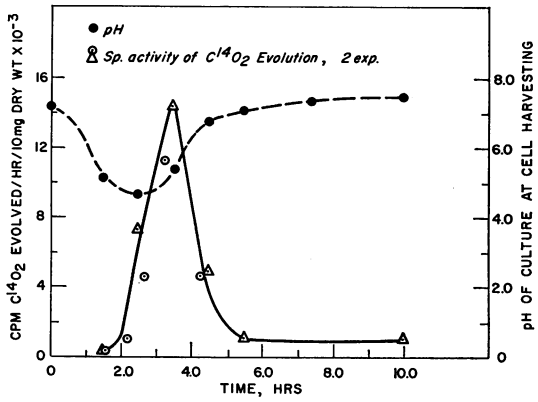

FIG. 7. *Ability of cells harvested at different ages to oxidize C^{14}-acetate to $C^{14}O_2$.*

TABLE 5. *Effect of glucose and chloramphenicol on acetate oxidation by Bacillus cereus strain T cells**

Addition	Concn	Incubation period	Rate of $C^{14}O_2$ evolution†	
			1.5-hr cells	3.5-hr cells
		hr		
None.....	—	0.5	2,050	16,200
None.....	—	1.0	15,000	21,000
None.....	—	1.5	27,000	36,000
Glucose....	0.1%	1.5	2,600	14,000
Chloramphenicol..	10 μg/ml	1.5	285	14,700

* Test medium: G-medium with glucose plus 50 μmoles of 2-C^{14}-acetate (4.25×10^5 counts/min).

† Counts/min of $C^{14}O_2$ per 1.5 hr per 6 mg (dry wt).

these enzyme systems (Fig. 6). This indicates that protein synthesis is required for completion of the acetate-oxidation system in vegetative cells.

In the presence of chloramphenicol, the level of the acetate-oxidation system in cells harvested at any stage of the growth cycle could be measured (Fig. 7). The ability to oxidize acetate begins as the sporulation cycle commences and then decreases at 4.0 hr. This corresponds to the time at which the oxidation of 6-C^{14}-glucose begins, and to the second peak in oxygen demand described by Halvorson (1957).

Glucose also delays the oxidation of acetate when cells are suspended in the incubation medium used to test the level of the acetate-oxidizing system (*see* Table 5).

In experiments with 1.5-hr cells, the added glucose can cause only a onefold dilution of the labeled acetate in this experiment, and thus does not explain the inhibition of $C^{14}O_2$ production. The inhibition is not as effective as that produced by chloramphenicol but it does reduce the amount of $C^{14}O_2$ to one-tenth of that produced in the absence of glucose. Inhibition of $C^{14}O_2$ production in 3.5-hr cells could be explained by dilution of the C^{14}-acetate.

The pH optimum for acetate oxidation was measured by the same method and found to be between 6.0 and 6.5. The rate of the reaction at pH 5.0 and 7.1 was 80% of that at the optimal pH. At pH values below and above these, the rate fell off rapidly. All the cells were incubated in a medium at pH 7.0 to minimize pH effects on the reaction velocity.

Cultures grown in the presence of 2-C^{14}-acetate were tested for their ability to oxidize acetate by the same procedure. Samples (10.0 ml) of a culture growing in the presence of 2-C^{14}-acetate were added to Warburg flasks, and the rate of $C^{14}O_2$ production was compared with that in cells tested after removal from G-medium. The results are essentially the same (Fig. 7). The inability of cells to convert 2-C^{14}-acetate, or endogeneous materials formed from 2-C^{14} after 4.0 hr, indicates that the tricarboxylic acid (TCA) cycle is inoperative after this time. This coincides with the time at which fluoroacetic acid fails to inhibit sporulation, and also with the decreased oxygen demand (Halvorson, 1957).

The experimental approaches used to determine acetate-oxidation patterns in *B. cereus* strain T were applied to other aerobic spore-

TABLE 6. *Effect of α-picolinic acid on acetate utilization and sporulation in some aerobic sporeforming bacilli*

Organism	α-Picolinic acid	OD during growth	Analysis (at 20 hr)	
			pH	Sporulation*
Bacillus subtilis NCTC 2588	None	—	8.0	+
	1.2×10^{-3}	—	4.8	−
B. coagulans	None	66 (1.5 hr)	8.0	+
	1.2×10^{-3}	73 (1.5 hr)	4.8	−
B. cereus albolactis ATCC 7004	None	52 (1.5 hr)	7.8	+
	9×10^{-4}	48 (1.5 hr)	4.7	−
B. megaterium (899)	None	140 (3.0 hr)	6.9	+
	1.2×10^{-3}	157 (3.0 hr)	5.0	−

* Sporulation, by microscopic observation, was greater than 90% in each case.

TABLE 7. *Oxidation of glucose-6-C^{14} by some typical aerobic sporeformers, compared with pH changes*

Organism	$C^{14}O_2$ evolved (hr)					Culture age at pH minimum*
	0–2	2–3	3–4	4–5	5–6	
						hr
Bacillus cereus NCTC 232	31	384	1,531	1,689	—	3.25
B. cereus NCTC 6222	56	125	919	3,723	5,219	4.0
B. cereus var. *mycoides*	48	523	3,724	7,799	—	3.0
B. coagulans 110	45	943	4,405	8,480	—	3.0
B. brevis −106	80	2,325	9,215	8,649	8,596	2.75
B. subtilis NCTC 2591	86	1,319	4,617	16,694	—	2.75

* In each case, growth was 90% complete by the time the pH minimum was reached, except with *B. subtilis* which exhibited a diauxic optical density curve like *B. cereus* strain T (*see* Fig. 1). Conditions were identical with those in Table 1.

formers. The growth and pH curves of all these organisms were qualitatively the same as those discussed in the preceding pages, with only minor exceptions. α-Picolinic acid does not inhibit growth, but inhibits the pH rise and sporulation in all the aerobic sporeformers tested (Table 6). (Growth in *B. cereus* var. *mycoides* was not measurable by optical density because it formed a pellicle.) This is essentially in agreement with results obtained with *B. cereus* strain T (Gollakota and Halvorson, 1960).

B. megaterium and other aerobic sporeformers oxidize glucose-6-C^{14} and uniformly labeled glucose in a manner similar to that observed in active cultures of *B. cereus* strain T (Table 7). Growth is complete by the time the pH minimum is reached. Carbon dioxide evolution from glucose-6-C^{14} begins as the pH of the medium begins to rise.

Fluoroacetic acid prevented sporulation in all the aerobic sporeformers tested. It did not prevent the pH rise in all these organisms. It is possible that alternate pathways of acetate utilization not inhibited by this compound are available in these organisms.

Discussion

The evidence presented establishes a somewhat unified picture of the metabolic events occurring during growth and sporulation in aerobic sporogenic bacteria in a glucose-yeast extract and minerals medium. We can make the following generalizations about the reactions of the two physiological phases of the life cycle.

In a medium containing glucose, vegetative growth continues until the carbohydrate is exhausted. Organic acid products of glucose catabolism accumulate during this process, causing the pH of the medium to drop. Vegetative cells of *B. cereus* strain T incorporate much less acetate into protein than do sporulating cells, although some is assimilated into lipid materials. Acetate is not oxidized to carbon dioxide by rapidly dividing cells that are filamentous and form long chains. α-Picolinic acid and fluoroacetate do not interfere with these processes. A major portion of the energy required for growth is supplied by the oxidation of glucose to organic acids.

As the glucose is exhausted, as growth ceases, and as morphological changes associated with sporulation appear, the cells begin to oxidize acetate to carbon dioxide, and the pH rises. *B. cereus* strain T cells also accumulate poly-β-hydroxybutyric acid during the initial stages of sporulation.

α-Picolinic acid interferes with acetate oxidation if it is added before the pH rise begins. The inhibition of acetate utilization by several other compounds has been shown to block sporulation specifically (Halvorson, 1960). Fluoroacetate prevents sporulation by blocking citrate utilization. Vegetative cells lack condensing enzyme and, therefore, the lethal synthesis of fluorocitrate necessary for this inhibition (Peters, 1957) does not occur.

The inability of the cells to oxidize acetate and the lack of oxygen demand in the latter stages of sporulation are interesting phenomena. The inability of the cells to oxidize acetate after 4.0 hr could be explained by several means: (i) the tricarboxylic acid cycle enzymes formed during the initial stages of sporulation are degraded later; (ii) a repressor of tricarboxylic acid cycle activity is formed; (iii) a permeability barrier is established that does not allow acetate to penetrate into the cell; or (iv) acetate is routed through pathways that do not produce CO_2 as a reaction product.

Hashimoto, Black, and Gerhardt (1960) showed that this organism will sporulate endotropically when granular cells are replaced in distilled water and caldium. The developmental stage at which the cells may be resuspended in the secondary sporulation medium, and sporulation still results, corresponds with 3.5 to 4.0 hr in our experiments. The above-mentioned observations lead to the hypothesis that no oxidative metabolism is required for the completion of sporulation. However, we found that the cells failed to sporulate if placed under nitrogen as late as 1 hr prior to the appearance of heat-stable counts (9.5 to 10 hr). Thus, oxygen is required for sporulation but it is not known how it functions.

This study also points out that energy-providing reactions during growth and sporulation differ considerably. Energy for growth is provided by glucose oxidation (Doi, Halvorson, and Church, 1959; Blumenthal, 1961), whereas acetate oxidation provides energy required during the initial stages of the sporulation cycle.

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