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Metabolism of Poly-β-Hydroxybutyrate and Acetoin in *Bacillus cereus*[1]

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ABSTRACT

KOMINEK, LEO A. (University of Illinois, Urbana), AND H. ORIN Halvorson. Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus*. J. Bacteriol. **90:** 1251–1259. 1965.—The synthesis of poly-β-hydroxybutyrate (PHB) in *Bacillus cereus* strain T begins after the cessation of logarithmic growth. Its accumulation is preceded by the formation of acetoacetyl coenzyme A reductase, an enzyme used for its biosynthesis. Exogenous acetic acid present in the medium owing to incomplete glucose oxidation serves as the carbon source for polymer formation during the initial stages of its synthesis. Pyruvic acid is converted to acetoin by an enzyme system that is formed during vegetative growth. The formation of this enzyme system is dependent on a low *p*H in the medium. As the cells enter the sporulating stage, they lose the ability to form acetoin. The acetoin that accumulates is utilized via the 2,3-butanediol cycle which begins to function late in the sporulation stage. This cycle generates acetic acid which is used for PHB synthesis and is also oxidized to carbon dioxide. PHB accumulation reaches a maximum just prior to the formation of spores, and it is degraded during the process of sporulation. The effect of sporulation inhibitors and *p*H on PHB and acetoin metabolism are discussed.

The development of the "active" culture technique by Halvorson (1957) has permitted the study of growth and sporulation as distinct physiological processes in *Bacillus cereus* strain T. Growth of this organism in a glucose-yeast extract-salts medium resulted in the accumulation of acetic and pyruvic acids due to the incomplete oxidation of glucose (Nakata and Halvorson, 1960). After the exhaustion of glucose from the medium, these acids were utilized during the process of sporulation, and their utilization has been shown to be essential for spore formation (Hanson, Srinivasan, and Halvorson, 1963*a*, *b*; Hanson, Blickarska, and Szulmajster, 1964).

The initial efforts of this investigation were aimed at determining the metabolic fate of these acids and elucidating the pathways of their utilization. During this study, it was found that a portion of these acids was incorporated into poly-β-hydroxybutyrate (PHB), a compound to which a function of carbon and energy storage has been attributed. The implications of the usefulness of such a compound to the endergonic reactions of sporulation stimulated further investigations into the metabolism of this polymeric ester.

[1] This report was taken in part from a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree in microbiology.

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MATERIALS AND METHODS

Strain. B. cereus strain T, formerly referred to as *B. cereus* var. terminalis, was used throughout this investigation.

Cultural methods. The organism was grown in a glucose-yeast extract-salts medium (G medium) by the "active" culture technique as described by Halvorson (1957). All cultures were incubated at 30 C on a rotary shaker.

Samples taken from an "active" culture were routinely checked for turbidity, *p*H, and morphological changes. Turbidity was determined in a Klett-Summerson colorimeter with a no. 66 filter. Measurements of *p*H were made in a Beckman *p*H meter. Sporulation and morphological changes were observed by examination of stained smears or of wet mounts by phase microscopy.

Maintenance of pH. To maintain a *p*H of 6.4 or 7.4, the K_2HPO_4 in G medium was replaced with potassium phosphate buffer to give a final concentration of 0.1 M. To maintain the medium at a low *p*H (4.8 to 5.0), sodium maleate buffer (0.2 M) was added to G medium after the cessation of growth.

Inhibitors. The inhibitors used were adjusted to *p*H 7.0 with NaOH, sterilized, stored at −20 C,

and later added aseptically to the culture to give the desired concentration.

Spore and vegetative-cell counts. Total counts (spores and vegetative cells) were made by plating properly diluted samples on nutrient agar. Spore counts were obtained by heating the sample to 80 C for 30 min prior to plating.

PHB. The concentration of PHB was determined by the spectrophotometric method of Law and Slepecky (1961). The incorporation of radioactive material into PHB was determined by plating a suitable sample of the chloroform extract on an aluminum planchet. The material was dried and assayed for radioactivity in a Nuclear-Chicago model D-47 gas-flow counter.

Colorimetric determinations. Acetoin was determined by the method of Sokatch and Gunsalus (1957). Diacetyl and 2,3-butanediol were determined by the procedures outlined by Neish (1952). Protein was determined by the method of Lowry et al. (1951).

Preparation of cell-free extracts. Cells of the desired physiological age, as determined by turbidity, morphology, and *p*H, were harvested by chilling the culture to 4 C and centrifuging to sediment the cells. The cells were then washed, resuspended in 0.05 M potassium phosphate buffer (*p*H 7.0), and broken in a French pressure cell. Cell-free extracts were prepared by centrifugation at 12,100 × *g* for 10 min in a Servall SS-3 centrifuge.

Enzymatic assays. Of the enzymes examined, all the activity was in the supernatant fraction of the broken-cell extracts after centrifugation. Exact reproduction of the method for extract preparation was strived for so that differences in activity would reflect true differences in the enzyme levels in the intact cells. The specific activity of the enzymes tested was calculated from the slope of the curve resulting from a plot of activity versus milligrams of protein. Specific activity is defined as units per milligram of protein.

Acetoacetyl coenzyme A (CoA) reductase was measured by following the oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) spectrophotometrically at 340 mμ. The reaction mixture (3.0 ml) contained sodium pyrophosphate-HCl buffer of *p*H 7.8 (75 μM), $NADH_2$ (1.0 μM), acetoacetyl CoA (0.25 μM), extract, and water. One unit of enzyme is defined as that amount which causes an initial rate of change in optical density (OD) of 0.001 per min.

Diacetyl reductase and 2,3-butanediol dehydrogenase were assayed in a similar manner. The reaction mixture (3.0 ml) contained potassium phosphate buffer, *p*H 6.3 (300 μM), $NADH_2$ (1.0 μM), diacetyl or acetoin (10 μM), extract, and water. A unit of activity is defined as that amount of enzyme producing an OD change of 0.010 per min.

The formation of acetoin from pyruvate by cell-free extracts was measured colorimetrically. The reaction mixture (1.4 ml) contained potassium phosphate buffer of *p*H 5.5 (100 μM), sodium pyruvate (100 μM), diphosphothiamine (20 μg), extract, and water. One unit of activity is that amount of enzyme producing 1.0 μmole of acetoin per hr.

The enzyme catalyzing the formation of diacetylmethylcarbinol (DAMC) was measured by the procedure of Juni and Heym (1956*b*, 1957*a*). Extracts were prepared in 0.125 M sodium maleate buffer (*p*H 6.4), and the formation of DAMC was followed colorimetrically. One unit of activity is expressed as that amount of enzyme producing 1.0 μmole of DAMC per hr.

Materials. Acetoacetyl CoA was prepared synthetically from diketene and CoA in a manner analogous to that described by Goldman (1954) for the preparation of crotonyl-*S* CoA. Acetoin-*2,3-C*^{14} was prepared enzymatically from pyruvate-*2-C*^{14} by use of whole cells of *B. cereus* T.

C^{14} isotopes were purchased from the Nuclear-Chicago Corp., Des Plaines, Ill. $NADH_2$, CoA, and diphosphothiamine (DPT) were purchased from the Sigma Chemical Co., St. Louis, Mo. Chloramphenicol was obtained from Parke, Davis & Co., Detroit, Mich.

RESULTS

The characteristics of an "active" culture of *B. cereus* T have been described by Halvorson (1957), Nakata and Halvorson (1960), and Hashimoto, Black, and Gerhardt (1960). A description of some of its properties is presented here to establish reference points for the subsequent observations.

During vegetative growth, the *p*H of the culture medium decreases from its initial value of approximately 7.4 to a minimum of 4.6 to 4.9. Logarithmic growth is complete 1.5 to 2.0 hr after inoculation, and its cessation coincides with the approach of the *p*H minimum. After completion of logarithmic growth, the *p*H increases rapidly as sporogenesis progresses. Cells also exhibit characteristic morphological changes at various stages after inoculation. Cells in the logarithmic phase of growth are filamentous, form ong chains, and have a homogeneous cytoplasm.

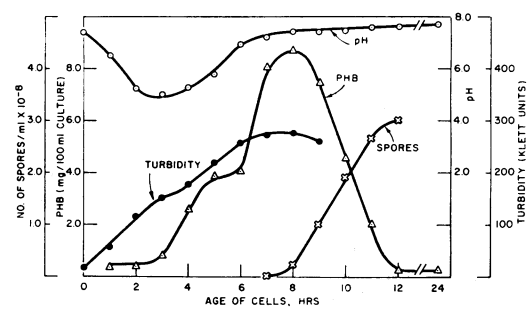


FIG. 1. *Changes in the pH, turbidity, and PHB concentration during growth and sporulation of Bacillus cereus T.*

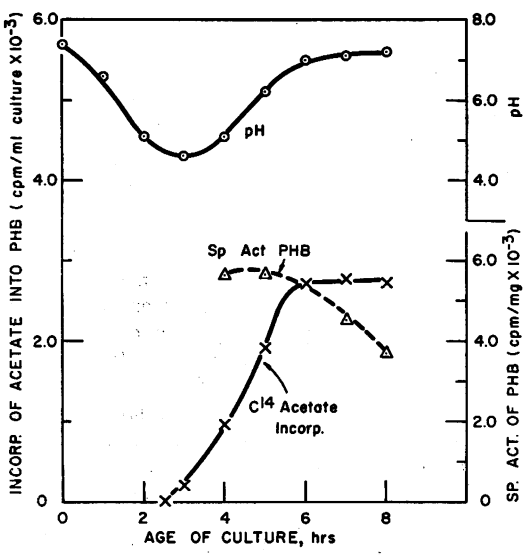


FIG. 2. *Incorporation of acetate into PHB in an "active" culture of Bacillus cereus T.*

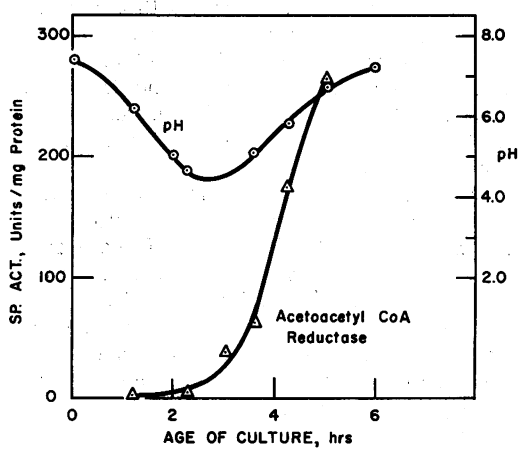


FIG. 3. *Level of acetoacetyl CoA reductase activity in relation to the culture age.*

At the *p*H minimum, the cells become shorter, chain reduction occurs, and the cytoplasm becomes granulated. In all further discussion, filamentous cells in the log phase of growth are referred to as vegetative cells, whereas granulated cells which have become committed to sporulation are referred to as sporulating cells.

Biosynthesis of PHB. The formation of PHB in an "active" culture of *B. cereus* T is illustrated in Fig. 1. Polymer formation begins after logarithmic growth has ceased and the *p*H minimum of the culture medium has been reached. Polymer accumulation continues for several hours, whereupon a lag occurs in its biosynthesis. The accumulation of PHB then continues, reaching a maximum just prior to the onset of sporulation. At this point, PHB accounts for approximately 10% of the dry weight of the cell. The polymer content of the cells then decreases, its disappearance being concomitant with the appearance of mature spores.

TABLE 1. *Effect of α-picolinic acid and chloramphenicol on the formation of acetoacetyl CoA reductase*

Extract from	Specific activity
1.5-hr cells	0
4.0-hr cells	252
4.0-hr cells, grown in the presence of α-picolinic acid (1.2×10^{-3} M)	3
4.0-hr cells, grown in the presence of chloramphenicol (10 μg/ml)	0

Figure 2 shows the incorporation of acetate-2-C^{14} into PHB by cells growing in an "active" culture. The radioactive acetate was added to the culture at 2.5 hr to avoid dilution of the label by glucose catabolism. Cells were harvested at the indicated time intervals and extracted for PHB. Acetate incorporation paralleled polymer accumulation, leveling off at the same time the lag occurred in PHB synthesis. Very little incorporation of acetate took place after this time, and the specific activity of PHB decreased, indicating the further synthesis of polymer from a carbon source other than exogenous acetate.

An enzyme involved in the synthesis of PHB from acetate is acetoacetyl CoA reductase, which catalyzes the reduction of acetoacetyl CoA to β-hydroxybutyryl CoA. Figure 3 illustrates the level of this enzyme in cell-free extracts prepared from cells of different ages. The results show that this enzyme is absent in vegetative cells and that it appears with increasing activity in sporulating cells, indicating the inducible nature of the PHB synthetic mechanism.

α-Picolinic acid has been shown to inhibit sporulation (Gollakota and Halvorson, 1960) and to prevent acetate oxidation due to the inhibition of the induction of aconitase (Hanson et al., 1963*b*). The addition of α-picolinic acid (1.2×10^{-3} M) or chloramphenicol (10 μg/ml) to an "active" culture prevented the synthesis of PHB. The effect of these inhibitors on the formation of acetoacetyl CoA reductase is shown in Table 1. Chloramphenicol was added at 2.0 hr to avoid interference with growth. Cells grown in the presence of these inhibitors showed no increase in the level of the enzyme. α-Picolinic acid had no effect on the reaction velocity of the enzyme when

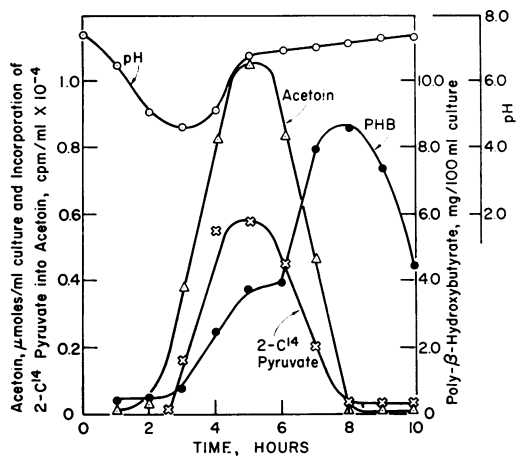


FIG. 4. *Acetoin synthesis from pyruvic acid and the inter-relationship between acetoin utilization and polymer formation.*

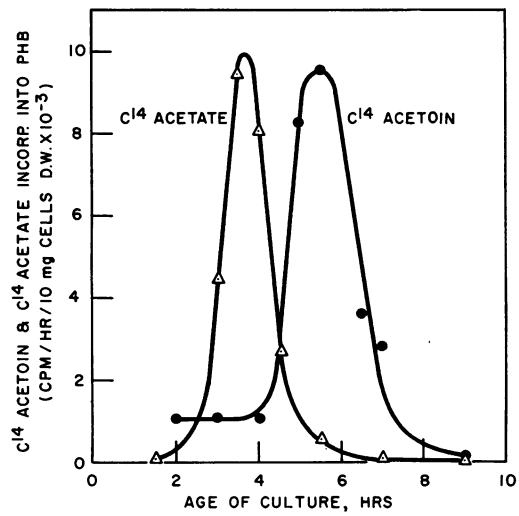


FIG. 5. *Rate of acetate and acetoin incorporation into PHB by cells of different ages. The incubation medium consisted of 10 ml of G medium (without glucose) containing 100 μg of chloramphenicol. In addition, the medium contained either 100 μmoles of sodium acetate (4.3 × 10⁵ count/min) or 50 μmoles of acetoin (8.5 × 10⁵ count/min).*

added to the reaction mixture or preincubated with the crude extract at concentrations to 1.5 × 10^{-2} M.

Acetoin metabolism and its relationship to PHB synthesis. The synthesis of acetoin from pyruvate and its subsequent disappearance is shown in Fig. 4. Pyruvate-*2-C*^{14} (1.3 × 10^4 counts per min per ml of culture) was added to an "active" culture at 2.5 hr. Approximately 50% of the added label was found in acetoin at the point of maximal accumulation which occurred just prior to the lag in PHB synthesis. Acetoin then disappeared from the medium and could not be accounted for as 2,3-butanediol or diacetyl. The correlation between acetoin utilization and further polymer synthesis indicated that acetoin was being used for PHB synthesis.

The ability of cells at various ages to incorporate radioactive acetate or acetoin into PHB is illustrated in Fig. 5. The reaction was started by the addition of cells and, after 30 min of incubation, was stopped by the addition of 1.0 ml of 72% perchloric acid. Acetate was incorporated into PHB at a high rate only during the initial stages of polymer accumulation. As the rate of acetate uptake decreased, the incorporation of acetoin into PHB increased, accounting for the secondary increase in polymer concentration.

The rate of CO_2 evolution from C^{14}-acetate by whole cells of *B. cereus* T at different ages was determined by Hanson et al. (1963*a*). The increase and decrease in the ability of cells to oxidize acetate coincide exactly with the changes in the rate of incorporation of acetate into PHB. The ability of cells to oxidize acetoin was determined in a similar manner, and the results show that the rate of CO_2 evolution from acetoin-*2,3-C*^{14} corresponds with the rate of its incorporation into PHB.

The rate of acetoin synthesis by whole cells of various ages from an "active" culture was determined (Fig. 6). The level of the acetoin-synthesizing system was also determined in cell-free extracts (Table 2). The data from whole cells and cell-free extracts show that maximal activity for acetoin formation occurs when the *p*H minimum of the culture is reached and that this activity decreases on either side of the *p*H minimum.

The fact that acetoin is both oxidized to CO_2 and incorporated into PHB suggested that the 2,3-butanediol cycle as described by Juni and Heym (1956*a*, *b*, 1957*a*, *b*) operates in cells of *B. cereus* T.

Two enzymes which are essential for the operation of this cycle are 2,3-butanediol dehydrogenase and diacetyl reductase. These enzymes were found to be present in sporulating cells, but little activity was seen in vegetative cells (Table 3). These results, which are similar to those mentioned previously for acetoacetyl CoA reductase, illustrate the inducible nature of these enzymes.

The operation of the 2,3-butanediol cycle also includes the formation of DAMC from diacetyl. The ability of *B. cereus* T to catalyze this reaction is illustrated in Fig. 7. The level of activity in

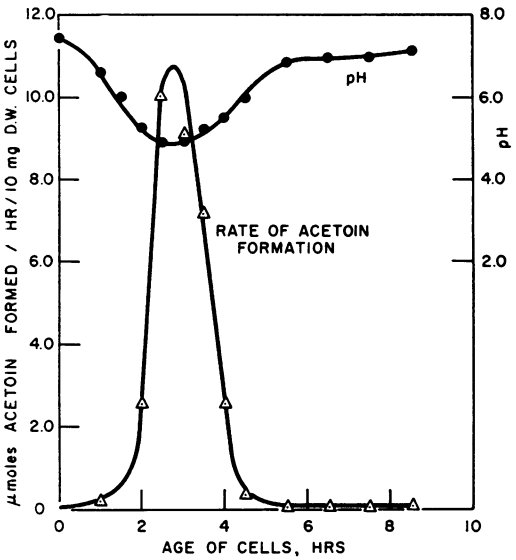


FIG. 6. *Level of acetoin-synthesizing system in cells of different ages. The rate of acetoin formation was determined in a medium containing sodium pyruvate (100 μmoles), chloramphenicol (50 μg), potassium phosphate buffer, pH 6.4 (500 μmoles), cells, and water to a total volume of 5.0 ml.*

TABLE 2. *Acetoin synthesis from pyruvate by cell-free extracts of Bacillus cereus T*

Time of harvesting cells for extract prepn	*p*H of medium at time of harvest	Specific activity
hr		
1.0	6.8	0
3.0	4.7	1.85
6.0	6.7	0

cell-free extracts of vegetative cells is very low and continues to stay at this level even after the transition to sporulating cells has occurred. The specific activity does not begin to increase until the *p*H of the culture medium is approaching neutrality. These results show that the enzyme synthesizing DAMC is inducible, but that it differs from the other enzymes studied in relation to the time of induction.

Effect of pH on PHB, acetoin, and sporulation. In an attempt to determine some of the controlling factors for the numerous biochemical changes occurring during the growth and sporulation of *B. cereus* T in an "active" culture, the effects of *p*H on sporulation, PHB synthesis, and acetoin metabolism were determined.

Nakata (1963), who recently studied the effect of *p*H on the intermediates produced by *B. cereus* T during sporogenesis, found that maximal

TABLE 3. *Level of 2,3-butanediol dehydrogenase and diacetyl reductase in cell-free extracts of Bacillus cereus T*

Time of harvesting cells for extract prepn	Specific activity of	
	2,3-Butanediol dehydrogenase	Diacetyl reductase
hr		
1.5	5	6
3.0	133	175
6.0	224	317

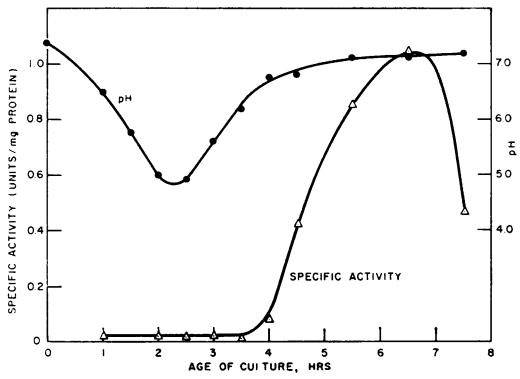


FIG. 7. *Level of the diacetylmethylcarbinol-synthesizing enzyme in cell-free extracts of Bacillus cereus T.*

polymer formation occurred in cultures buffered at *p*H 6.4. He also found that cultures buffered at higher *p*H accumulated very little PHB.

The optimal *p*H for PHB synthesis in whole cells was determined by measuring the rate of acetate-2-C^{14} incorporation into this polyester at various *p*H values. Polymer synthesis showed an optimum at *p*H 6.0 to 6.4 in this experiment.

The effect of *p*H on PHB metabolism in an "active" culture is illustrated in Fig. 8. A culture buffered at *p*H 7.4 accumulated little polymer, whereas the culture buffered at *p*H 6.4 accumulated more polymer and at a faster rate than the control culture. The culture buffered at *p*H 5.0 accumulated approximately 50% of the amount of PHB found in the control, and the utilization of polymer was totally inhibited.

To determine whether the hydrogen ion concentration has an effect on the formation of acetoacetyl CoA reductase, extracts were prepared from cells grown in cultures maintained at a high and a low *p*H. There was essentially no difference in the level of enzymatic activity in cell-free extracts obtained from buffered and unbuffered cultures.

These data are in agreement with those of Nakata (1963) in that PHB synthesis has a *p*H

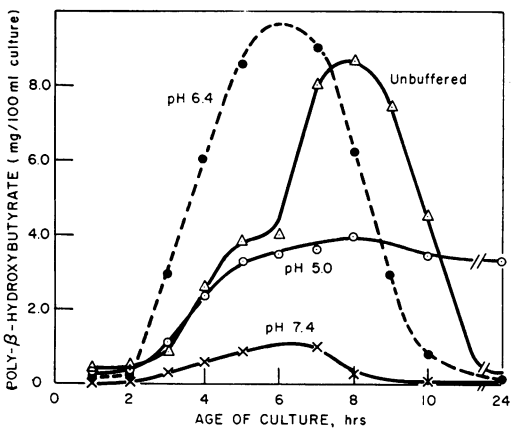


FIG. 8. *Effect of pH on PHB metabolism.*

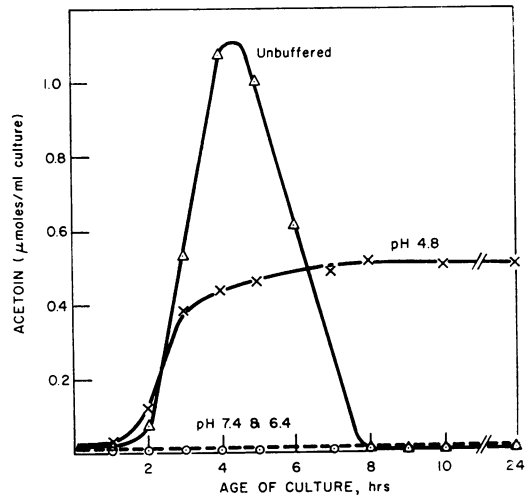


FIG. 9. *Effect of pH on acetoin metabolism.*

optimum around *p*H 6.4 and that the lack of polymer accumulation at higher *p*H values can best be explained by an effect on the degradative enzymes of polymer breakdown, so that PHB is utilized at a rate almost equal to the rate of its synthesis.

The optimal *p*H for acetoin synthesis in whole cells was determined to lie between 6.1 and 6.6. The rate of acetoin synthesis at *p*H 4.8 and 8.0 was approximately 25% of the maximal rate. Buffering of "active" culture at a high *p*H (7.4 or 6.4) completely inhibited acetoin synthesis, whereas buffering at a low *p*H (4.8) produced a reduced accumulation of acetoin and complete inhibition of acetoin utilization (Fig. 9). These results indicated a control by *p*H over the enzymes essential for acetoin synthesis and its utilization.

Figure 10 illustrates the effect of *p*H on the formation of the acetoin-synthesizing system. Cells were harvested at 1.0 hr from an "active" culture, washed, and suspended in water. They were then incubated at 30 C on a rotary shaker for 2.0 hr in a medium containing sodium pyruvate (100 μmoles), yeast extract (14 mg), glucose (33 μmoles), cells (12 mg, dry weight), and water to a total volume of 5.0 ml. The medium also contained sodium citrate buffer (500 μM), sodium maleate buffer (1,000 μM), or sodium phosphate buffer (1,000 μM) for maintenance of the desired *p*H. After 1.75 hr of incubation, 50 μg of chloramphenicol were added and the reaction mixture was incubated for an additional 15 min. The cells were then harvested after chilling to 4 C, washed, and suspended in 5.0 ml of a medium containing sodium pyruvate (100 μM), chloramphenicol (50 μg), sodium phosphate buffer (1,000 μM) of *p*H 6.4, and water. The cells were again incubated at 30 C on a rotary shaker, and samples were removed at intervals for acetoin analysis. This procedure allowed the determination of the effect of *p*H on induction by determining the rate of acetoin synthesis after incubation at various hydrogen ion concentrations. The maximal rate of acetoin formation was obtained with cells incubated at *p*H 5.45. Cells incubated below *p*H 5.0 or above *p*H 6.0 have a very low rate of acetoin formation, indicating that induction takes place only between these limits of *p*H.

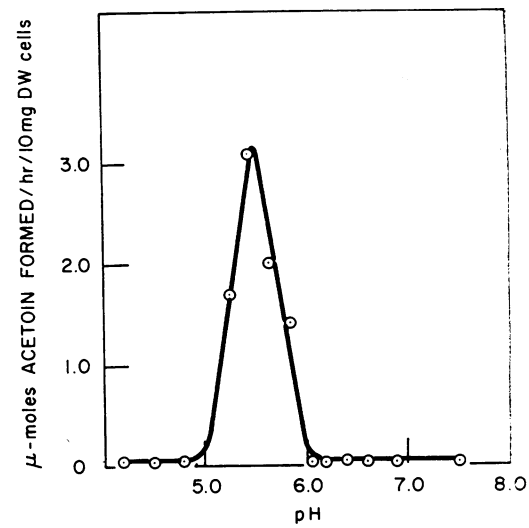


FIG. 10. *Effect of pH on the formation of the acetoin-synthesizing system.*

Table 4 shows the effect of a low *p*H on the enzymes of the 2,3-butanediol cycle. In the case of 2,3-butanediol dehydrogenase and diacetyl re-

TABLE 4. *Effect of buffering at pH 5.0 on the level of 2,3-butanediol dehydrogenase, diacetyl reductase, and the diacetylmethylcarbinol-synthesizing enzyme in cells of Bacillus cereus T*

Enzyme	Cell age at harvest	Specific activity of extracts harvested from	
		Unbuffered media	Buffered media
	hr		
2,3-Butanediol dehydrogenase	1.0	5	3
	5.75	219	189
Diacetyl reductase	1.0	7	5
	5.75	281	241
Diacetylmethylcarbinol-synthesizing enzyme	1.0	0.04	0.05
	5.75	1.01	0.09

TABLE 5. *Effect of pH on sporulation*

*p*H after 24 hr	Viable cells	Heat stable cells/ml	Per cent sporulation
5.3	1.8×10^8	5.2×10^5	0.2
5.8	2.0×10^8	8.9×10^6	4.5
6.05	6.7×10^8	4.0×10^8	60.0
6.45	7.1×10^8	7.1×10^8	100.0
7.9*	6.9×10^8	7.2×10^8	>100.0

* Unbuffered.

ductase, induction took place normally in both the buffered and the control culture. There was very little increase, however, in the level of activity of DAMC synthetase in the buffered culture, indicating that the low level of this enzyme is responsible for the lack of acetoin utilization in cultures buffered at a low *p*H.

The effect of *p*H on spore formation in an "active" culture is shown in Table 5. Sodium maleate buffer (0.2 M) of the desired *p*H was added to the medium at 4.25 hr. Plate counts were made after 24 hr of incubation to determine the viable and heat-stable counts. The results show that spore formation is severely inhibited at *p*H values below 6.0.

DISCUSSION

Acetic acid serves as the source of carbon for the synthesis of PHB during the initial stages of its accumulation, which begins after the cessation of logarithmic growth. The ability of cells to synthesize PHB is closely related to the appearance of acetoacetyl CoA reductase, an enzyme involved in the biosynthesis of polymer. The level of this enzyme is very low during vegetative growth but increases rapidly during the initial stages of sporulation. A similar relationship has been reported for acetate oxidation and the enzymes of the tricarboxylic acid cycle (Hanson et al., 1963*a*, *b*).

α-Picolinic acid has been shown to inhibit sporulation and the utilization of the acids accumulated during glucose catabolism (Gollakota and Halvorson, 1960). Addition of this inhibitor to an active culture prevents the accumulation of PHB and inhibits the formation of acetoacetyl CoA reductase. This same inhibitor has been shown to prevent acetate oxidation and to specifically prevent the induction of aconitase (Hanson et al., 1963*a*, *b*). Chloramphenicol has also been shown to inhibit polymer accumulation and acetate oxidation by preventing the synthesis of the enzymes essential for these processes.

The metabolism of PHB is also affected by the hydrogen ion concentration. Maintenance of the culture medium at higher *p*H values inhibits the accumulation of polymer, whereas buffering at a low *p*H completely suppresses the utilization of this polyester. Under either of these conditions, the formation of acetoacetyl CoA reductase remains unaffected.

These data indicate that the systems involved in acetate utilization are nonfunctional during vegetative growth and are induced during the initial stages of sporulation. Both systems are inhibited by α-picolinic acid due to the inhibition of induction of a specific enzyme. Unlike the acetate-oxidizing system, the ability of cells to accumulate PHB is greatly affected by the *p*H of the medium, and the preliminary data indicate that this effect is primarily on the polymer degradative system.

Pyruvic acid has been shown to be converted to acetoin in "active" cultures of *B. cereus* T. The enzyme system responsible for the synthesis of acetoin from pyruvic acid is inducible, but its induction differs in certain respects from the induction of the acetate-utilizing systems. The ability of cells to form acetoin increases during logarithmic growth and reaches a maximal level when the *p*H minimum of the culture is reached. Further studies showed that the formation of this enzyme system was dependent on the *p*H of the culture medium, with its induction occurring only below *p*H 6.0.

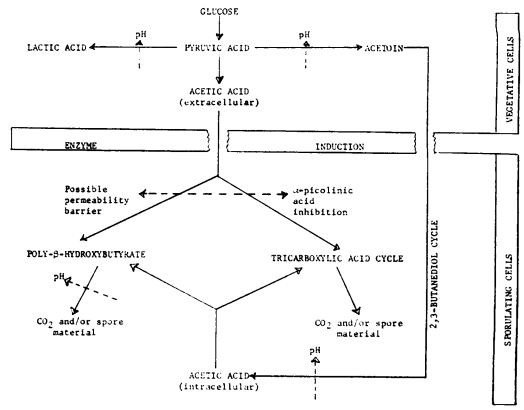
The experimental results present analogies to earlier observations of an increase in the concentration of certain bacterial enzymes as a result of growth in acidified cultures. Silverman and Werkman (1941) showed that the ability of *Aerobacter aerogenes* to form acetoin is dependent on its growth at a low *p*H. Busse and Kandler (1961)

showed that *Leuconostoc citrovorum* does not form acetoin during glucose fermentation in a phosphate buffer. In the presence of pyruvate, however, large amounts of acetoin are formed. These authors suggest that in the presence of a favorable hydrogen donor, such as glucose, the pyruvate is reduced to lactic acid so quickly that the condensation of pyruvic acid to α-acetolactate does not occur, and therefore no acetoin formation takes place. Nakata (1963) showed that cultures of *B. cereus* T, grown in buffered G medium at *p*H 6.4 or above, accumulate lactic acid instead of pyruvic acid. The extrapolation of these facts to the situation that exists in *B. cereus* T suggests a possible explanation for the low *p*H required for the induction of the acetoin-synthesizing system. If the reduction of pyruvic acid to lactic acid occurs rapidly at *p*H values around neutrality, and if this same process is impeded at lower *p*H values, then the presence of the inducer (pyruvic acid) would be determined by the hydrogen ion concentration and, thereby, control the induction of the acetoin-synthesizing system. The fact that spore formation is inhibited at the lower *p*H values suggests that the acetoin-synthesizing system may function as a neutralization mechanism.

It has been shown that acetoin serves as the carbon source for the secondary increase in polymer concentration, and is also oxidized to carbon dioxide during this same period. Hanson et al. (1963*a*, *b*) showed that, although the tricarboxylic acid cycle enzymes are formed and remain at a high level during the entire sequence of events preceding mature spore formation, the ability of cells to oxidize acetic acid is present for only a short period during the initial stage of spore formation. A similar relationship has been shown to exist between the PHB-synthesizing enzymes and the ability of cells to incorporate acetate into polymer. These facts suggest the establishment of a permeability barrier at a time which corresponds to the lag in PHB accumulation to prevent the entrance of exogenous acetate into the cell.

The pathway of acetoin utilization has been shown to proceed by the 2,3-butanediol cycle, which has been described in a number of organisms by Juni and Heym (1956*a*). For every turn of this cycle, two molecules of acetoin yield one molecule of regenerated acetoin and two molecules of acetic acid. The acetate generated in this manner can then be used for further synthesis of PHB and can also be oxidized to CO_2 via the tricarboxylic acid cycle.

The enzymes of the 2,3-butanediol cycle are formed by the cells after the transition to the sporulating stage. The hydrogen ion concentration also has an effect on this system and appears to be concentrated primarily on the DAMC-synthesizing enzyme. The level of DAMC synthetase in cells of *B. cereus* T does not begin to increase until the *p*H of the culture medium is approaching neutrality. At this time, all of the enzymes associated with acetate utilization, and the other enzymes of the 2,3-butanediol cycle which were tested, are already approaching their maximal level of activity. This indicates that *p*H values approaching neutrality are essential for the development of a complete and functional 2,3-butanediol cycle which can be used for the degradation of acetoin.



SCHEMA 1

A schematic summary of some of the pertinent metabolic transitions occurring during the growth and sporulation of *B. cereus* T and the areas which are affected by *p*H is presented in Schema 1.

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