EFFECT OF pH ON INTERMEDIATES PRODUCED DURING GROWTH AND SPORULATION OF *BACILLUS CEREUS*

H. M. NAKATA

Department of Bacteriology and Public Health, Washington State University, Pullman, Washington

Received for publication 9 May 1963

ABSTRACT

NAKATA, H. M. (Washington State University, Pullman). Effect of pH on intermediates produced during growth and sporulation of *Bacillus cereus*. J. Bacteriol. **86**:577–581. 1963.—Cultures of *Bacillus cereus* strain T, grown in an unbuffered glucose-yeast extract-mineral salts medium and in the same medium buffered at pH 6.4, 7.0, or 7.4, were examined to determine the effect of pH on the production and utilization of metabolic intermediates during growth and sporulation. Although the growth rate of the unbuffered cultures decreased as a result of changes in pH, the growth rates of the buffered cultures were constant and similar. Only slight differences were observed in the final number of spores produced, and in the time required for the completion of the sporulation process. Acetic and lactic acids were the chief acidic end products of growth in buffered media, in which more than twice the amount of these acids accumulated than was detected in unbuffered cultures. In the latter cultures, pyruvic acid, rather than lactic acid, was formed together with acetate. Acetoin also accumulated in the unbuffered cultures, but none was detected in any of the buffered cultures. All of these intermediates were rapidly utilized by the cells once sporulation began. Poly-β-hydroxybutyric acid accumulation in the cells during the early stages of sporulation was also influenced by pH, with the greatest accumulation of this polymer occurring in the cells grown at pH 6.2 to 6.4. These results support the hypothesis that high polymer content in the cells is not imperative for the completion of sporulation. However, if any is formed, it is completely utilized during the final stages of spore formation.

The production of acetic and pyruvic acids during growth, and their subsequent utilization during sporulation of *Bacillus cereus* in a glucose-yeast extract-mineral salts medium, was reported earlier (Nakata and Halvorson, 1960). The medium used in the study above, although excellent for the production of homogeneous crops of thermoresistant spores, was not well-buffered, and significant changes in pH were observed during growth and early stages of sporulation. During subsequent investigations regarding the formation of poly-β-hydroxybutyrate by this organism (Nakata, 1962), it was noted that the amount of polymer formed was apparently influenced by the pH of the culture medium. It was of interest then to investigate the effect of pH on the production of various metabolic intermediates during growth and sporulation of *B. cereus* and particularly on the accumulation and disappearance of acetate, pyruvate, and lactate in the culture supernatant fluids, and of poly-β-hydroxybutyric acid in the cells.

MATERIALS AND METHODS

Organism and cultural conditions. B. cereus strain T was cultivated by the active culturing technique described earlier (Nakata and Halvorson, 1960) with one minor modification. In the preparation of the "active inoculum," the initial culture in the series of transfers was replaced by a culture grown on Trypticase Soy Agar (BBL) incubated for 8 hr at 30 C. Growth from this plate, consisting of actively proliferating cells, was then used to inoculate the second flask in the series as described by Nakata and Halvorson (1960). Each inoculum was prepared at the same pH as its corresponding test culture.

In all of these studies, the unbuffered "G" medium was employed with slight changes in the concentration of certain components. The modified medium contained, in g/liter of distilled water: $FeSO_4 \cdot 7H_2O$, 0.0005; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot 7H_2O$, 0.05; $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 0.5; $CaCl_2$, 0.08; glucose, 2.0; and ether-extracted

yeast extract, 4.0. To prevent precipitation of some of the ingredients, this medium was prepared according to the procedure described earlier (Nakata and Halvorson, 1960). The buffered media were prepared by replacing the K_2HPO_4 in the above medium with sterile potassium phosphate buffer (pH 6.4, 7.0, or 7.4) to give a final concentration of 0.1 M. All cultures were incubated at 30 C on a rotary shaker set at 320 rev/min. At regular time intervals throughout growth and sporulation, samples were removed from each culture and examined for certain metabolic intermediates.

Chemical determinations. Acetic acid was estimated on a Beckman GC-2 gas chromatograph using a hydrogen flame detector system and a column containing 8.8% Carbowax (20 M) and 2.2% phosphoric acid. Pyruvic acid was determined colorimetrically as the 2,4-dinitrophenylhydrazone according to the method of Friedemann and Haugen (1943). The procedure described by Neish (1952) was used to determine lactic acid. Acetoin was estimated colorimetrically as described by Sokatch and Gunsalus (1957). Poly-β-hydroxybutyric acid was extracted from the cells, hydrolyzed and dehydrated in hot 90% sulfuric acid, and assayed spectrophotometrically as crotonic acid according to the method described by Law and Slepecky (1961).

RESULTS AND DISCUSSION

Effect of pH on growth and sporulation. Growth and pH curves of *B. cereus* T grown in an unbuffered medium and in media buffered at pH 6.4, 7.0, and 7.4 are shown in Fig. 1. As one would expect, the growth rate of the culture in the unbuffered medium rapidly decreased when the pH approached the minimal level owing to the accumulation of acidic intermediates. On the other hand, little difference was observed among the growth curves of cultures buffered at pH 6.4, 7.0, and 7.4, indicating that there is a rather wide pH range for comparable growth of this organism under the conditions employed. Periodic microscopic examinations of stained smears revealed the simultaneous appearance of spores in all four cultures between 10 and 11 hr of incubation, and nearly 100% sporulation was obtained 2 to 2.5 hr later. Furthermore, only slight differences were noted in the final number of thermoresistant spores produced by each of the cultures, indicating that growth and sporulation processes

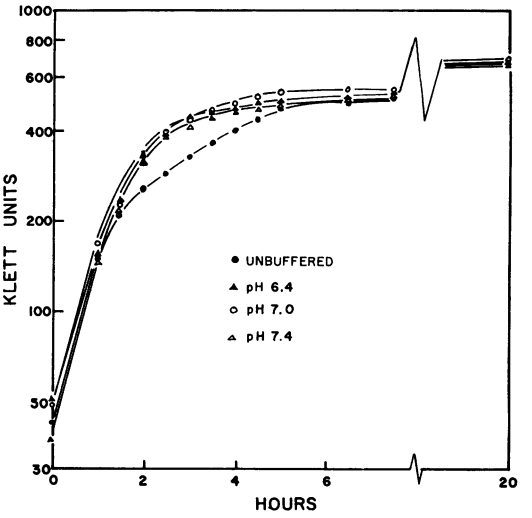

FIG. 1. *Growth curves of Bacillus cereus grown in an unbuffered glucose-yeast extract-mineral salts medium and in the same medium buffered at pH 6.4, 7.0, or 7.4 with 0.1 M potassium phosphate buffer. Cultures were incubated on a rotary shaker at 30 C.*

are not significantly impaired within the pH range tested (Table 1). Results similar to those described in this paper were also obtained with unbuffered cultures maintained at approximately the same pH values given above by the periodic addition of predetermined amounts of alkali or acid.

Effect of pH on production of metabolic intermediates. It was reported earlier that acetic and pyruvic acids are the major acid intermediates produced by *B. cereus* when grown in an unbuffered glucose-yeast extract-mineral salts medium. The accumulation of these acids during growth, and their rapid utilization during sporogenesis, is presumably responsible for the pH changes observed in such cultures. Only small

TABLE 1. *Total viable and spore counts of Bacillus cereus grown for 20 hr at 30 C in unbuffered medium and media buffered at pH 6.4, 7.0, or 7.4*

pH of medium	Viable count/ml	Spore count/ml*
Unbuffered	8.6×10^8	8.7×10^8
6.4	9.7×10^8	8.9×10^8
7.0	9.3×10^8	10.3×10^8
7.4	9.0×10^8	9.5×10^8

* Cells surviving 80 C for 20 min.

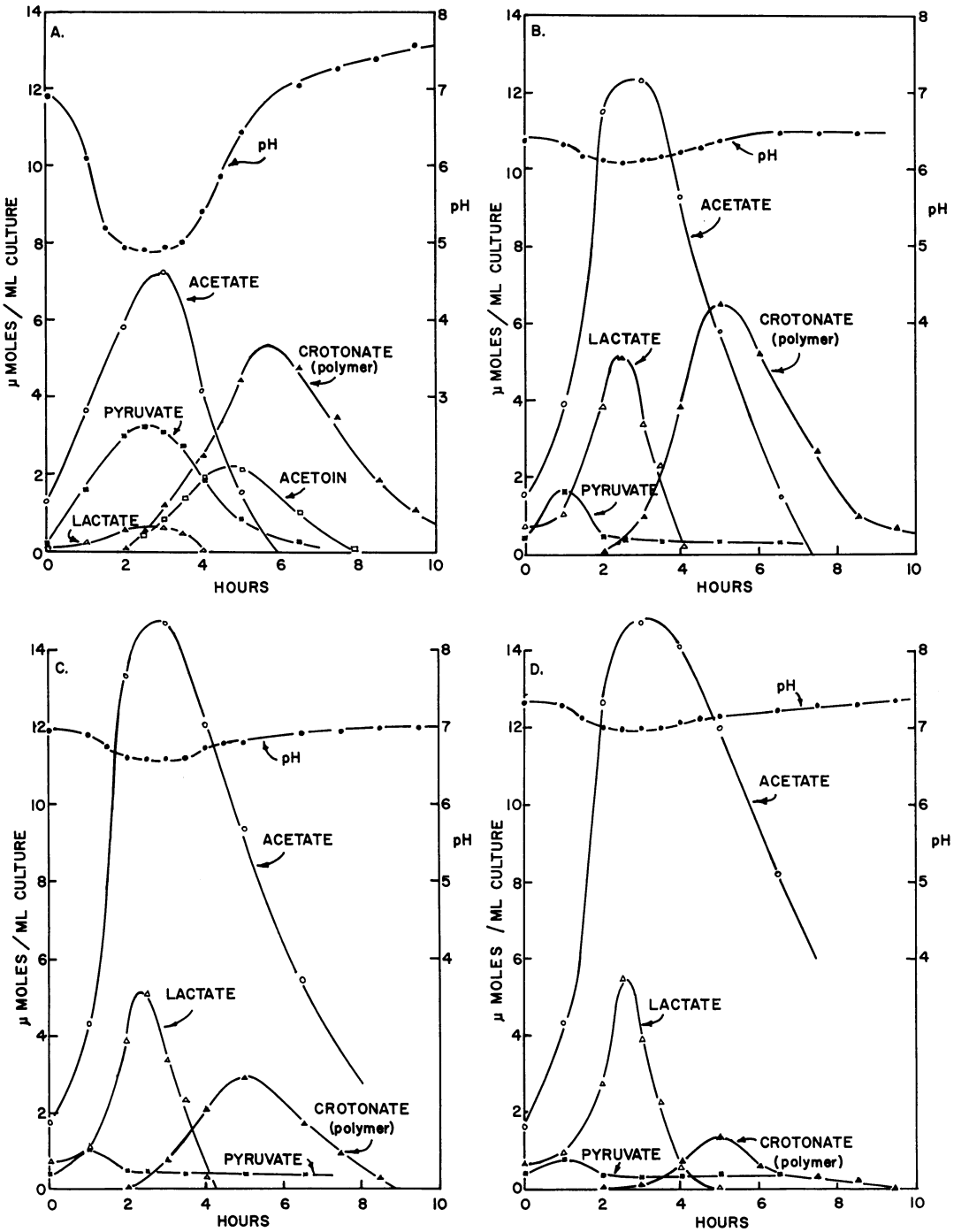

FIG. 2. *Effect of pH and the concentration of acetate, lactate, pyruvate, acetoin, and poly-β-hydroxybutyrate at various time intervals during growth and sporulation of Bacillus cereus at 30 C. A, unbuffered medium; B, pH 6.4; C, pH 7.0; D, pH 7.4. Poly-β-hydroxybutyric acid was hydrolyzed and dehydrated in 90% H_2SO_4 and assayed spectrophotometrically as crotonic acid.*

quantities of lactic acid were produced under these conditions.

However, examination of culture supernatant fluids of *B. cereus* grown in the same medium buffered at pH 6.4, 7.0, or 7.4 revealed significant quantitative differences in the accumulation of metabolic intermediates. Approximately twice the amount of acetate was formed by these cultures than was produced by the cultures grown in the medium lacking the buffer system. The accumulation of pyruvic acid, on the other hand, was less at higher pH values. This is presumably due to the reduction of pyruvic acid to lactic acid which accumulates in larger amounts in the buffered cultures. Accumulation of acetic and lactic acids as the major intermediates at higher pH values was also observed by Goldman and Blumenthal (1960), who suspended cells of the same strain of *B. cereus* in maleate buffer (pH 7.0) containing glucose.

One other metabolic intermediate produced in significant quantities by the unbuffered cultures was acetoin which, like the acid intermediates, was also rapidly assimilated during sporogenesis. Preliminary investigations suggest that the metabolism of acetoin is associated with the metabolism of poly-β-hydroxybutyrate by cells sporulating under these conditions. No acetoin, however, was detected in the supernatant liquids of the buffered cultures. Data summarizing the concentrations of these intermediates at various time intervals during growth and early stages of sporulation under each of the conditions described are shown in Fig. 2.

Effect of pH on accumulation of poly-β-hydroxybutyric acid. The influence of pH on the accumulation of poly-β-hydroxybutyrate in the cells of *B. cereus* is also shown in Fig. 2. In all four cultures, polymer biosynthesis began after 2 hr of incubation, at which time the cells were approaching the stationary phase of growth. The maximal polymer levels were reached several hours later, during what is considered to be the early phase of sporulation for this organism. The polymer content in the cells then diminished as sporogenesis continued, presumably providing carbon and energy for the completion of the sporulation process. No polymer was detected in the mature spores. These results are similar to the observations made by Slepecky and Law (1961) for *B. megaterium* grown under conditions favoring high polymer production and good spore crops.

Although the synthesis of poly-β-hydroxybutyrate occurred concurrently with the rapid disappearance of acetate from the medium, a direct correlation between the amount of acetate utilized and the amount of polymer accumulated by the cells was not observed. Rather, it appears that the factor which influenced polymer content of the cells of these cultures was the pH of the medium. This may be seen by comparing the acetate and polymer levels in Fig. 2. The optimal pH for the accumulation of the polymer was found to be between pH 6.0 and 6.4. The rate at which the polymer disappeared from the cells as sporulation continued was also greatest between pH 6.0 and 6.4. These results are contrary to those of Macrae and Wilkinson (1958), who reported that the optimal pH for polymer biosynthesis and degradation in *B. megaterium* and in a different strain of *B. cereus* is around pH 7.5. Both of these organisms, however, are asporogenic variants.

Further investigations concerning the processes of sporulation are necessary before the significance of poly-β-hydroxybutyrate accumulation in the cells can be adequately discussed. The failure of cells to accumulate large amounts of polymer at pH 7.0 and 7.4 may simply be due to their ability to metabolize the polymer at a rate almost equal to the rate of its formation. Alternatively, the cells may assimilate the major portion of the acetate in the medium via a route which does not involve polymer synthesis.

Nevertheless, whether or not polymer accumulated in large amounts, good yields of thermo-resistant spores were obtained at all pH values tested. These results, coupled with those of Stevenson et al. (1962), provide additional evidence that poly-β-hydroxybutyrate accumulation is not imperative for sporogenesis. Yet, if formed, the polymer is completely utilized during the final stages of spore formation.

ACKNOWLEDGMENTS

The author is grateful to Patricia A. Barry for her capable technical assistance, and to Donald S. May, Division of Industrial Research, for gas chromatography analyses.

This work was supported in part by grant G-18540 from the National Science Foundation.

LITERATURE CITED

FRIEDEMANN, T. E., AND G. E. HAUGEN. 1943. Pyruvic acid. II. The determination of keto

acids in blood and urine. J. Biol. Chem. **147:** 415–442.

GOLDMAN, M., AND H. J. BLUMENTHAL. 1960. Pathways of glucose catabolism in *Bacillus cereus* strain *terminalis*. Bacteriol. Proc., p. 813.

LAW, J. H., AND R. A. SLEPECKY. 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82:** 33–36.

MACRAE, R. M., AND J. F. WILKINSON. 1958. Poly-β-hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. J. Gen. Microbiol. **19:** 210–222.

NAKATA, H. M., AND H. O. HALVORSON. 1960. Biochemical changes occurring during growth and sporulation of *Bacillus cereus*. J. Bacteriol. **80:**801–810.

NAKATA, H. M. 1962. The role of acetate in the sporulation of *Bacillus cereus*. Bacteriol. Proc., p. 47.

NEISH, A. C. 1952. Analytical methods for bacterial fermentations. Natl. Res. Council, Can. Rept. No. 46-8-3.

SLEPECKY, R. A., AND J. H. LAW. 1961. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. J. Bacteriol. **82:**37–42.

SOKATCH, J. T., AND I. C. GUNSALUS. 1957. Aldonic acid metabolism. I. Pathway of carbon in an inducible gluconate fermentation by *Streptococcus faecalis*. J. Bacteriol. **73:**452–460.

STEVENSON, J., K. MILLER, R. STROTHMAN, AND R. A. SLEPECKY. 1962. Occurrence and function of poly-β-hydroxybutyric acid in various *Bacillus* species. Bacteriol. Proc., p. 47.