# BIOCHEMICAL CHANGES OCCURRING DURING SPORULATION OF *BACILLUS CEREUS* T

## II. Effect of Esters of Organic Acids on Sporulation

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### Abstract

GOLLAKOTA, K. G. (University of Illinois, Urbana) AND H. ORIN HALVORSON. Biochemical changes occurring during sporulation of *Bacillus cereus* T. II. Effect of esters of organic acids on sporulation. J. Bacteriol. **85**:1386–1393. 1963.—Sporulation of *Bacillus cereus* T in yeast extract-glucose-minerals medium was specifically inhibited by $\alpha$-picolinic acid (APA), if the acid was added before the pH of the culture began to rise. The effects of APA could be reversed by aspartic acid or asparagine, among the amino acids, and by intermediates of the tricarboxylic acid cycle, with the exception of $\alpha$-ketoglutarate and fumarate. Formate, malonate, and certain other organic acids also possessed this ability. Succinate was the best reversing agent. Fluoroacetic acid (FAA) also inhibited sporulation, but had no effect on vegetative growth or germination of spores of *B. cereus* T. Unlike APA, FAA inhibited sporulation even when added after the pH of the culture had started to rise. Bisulfite was similar to FAA in its effects on sporulation. With the exception of pyruvate, acetate, aspartate, and malate, most of the compounds reversing the effects of APA also overcame the effects of FAA or bisulfite on sporulation. Esters of some of the acids reversing the effects of the above inhibitors were studied for their action on germination, growth, and sporulation. Ethyl pyruvate prevented germination of the spores, slowed down growth, and inhibited sporulation. Ethyl malonate and ethyl succinate inhibited only sporulation. All the above inhibitors prevented the synthesis of dipicolinic acid (DPA) also. When *B. cereus* T was grown in the absence of glucose (in extracted yeast extract-minerals medium), the above inhibitors had no effect on sporulation. Ethyl oxamate permitted sporulation, but the spores produced were heat-sensitive. Ethyl pimelate caused lysis when added before the pH of the culture began to rise. When added after the pH of the culture began to rise, it also permitted sporulation, and the spores were sensitive to heat. (These heat-sensitive spores were refractile and dormant, and did not stain with crystal violet. However, they germinated normally, losing refractibility and became stainable.) The effect of ethyl oxamate and ethyl pimelate could be overcome by DPA. APA, FAA, ethyl malonate, and ethyl succinate also inhibit the sporulation of a number of other bacilli.

In an earlier publication (Gollakota and Halvorson, 1960), we reported that the inhibition of sporulation of *Bacillus cereus* T in glucose-yeast extract-minerals medium by $\alpha$-picolinic acid (APA) can be overcome by increased amounts of yeast extract but not by the ash from yeast extract. It was suggested that APA interferes with the further metabolism of acid intermediates accumulating during vegetative growth, and that yeast extract contains some organic compounds on the metabolic pathway of the acid intermediates past the metabolic block caused by APA.

The present study was undertaken to test our hypothesis (i) by comparing the ability of various amino acids, tricarboxylic acid cycle intermediates, and related compounds to reverse the effects of APA and (ii) by studying the effects of other compounds which may be expected to inhibit sporulation as a result of the reversal studies.

### Materials and Methods

*B. cereus* T was the main organism used in these studies.

Medium and culture methods were described

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in detail in a preceding paper (Gollakota and Halvorson, 1960).

Growth was determined (i) with a Klett-Summerson photoelectric colorimeter using a no. 42 blue filter (400 to 465 mμ) and expressed as Klett units or (ii) as total viable cells, evaluated by plating on nutrient agar.

The degree of sporulation was measured by (i) the number of cells that survived heating for 30 min at 80 C or (ii) the number of cells which remained viable after 15 min of exposure to octyl alcohol (0.01 ml of alcohol/10 ml of suspension) and dilution in octyl alcohol-treated water (prepared by adding 0.06 ml of the alcohol to 100 ml of water and autoclaving).

The pH value of the samples was determined with a Beckman pH meter.

*"Extracted yeast extract" (EYE).* A solution of commercial yeast extract was adjusted to pH 2.5 and continuously extracted with ether for 72 hr in a liquid-liquid extractor. The ether extract, on evaporation, gives the "extractable acids," and the aqueous layer, after adjusting the pH to 7.2, is termed the EYE.

*Chemicals.* The organic esters and fluoroacetic acid (FAA) were purchased from K & K Laboratories, Inc., New York, N.Y. The amino acids were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. All the other chemicals were of reagent grade. Stock solutions of the solid compounds were prepared in distilled water. These were sterilized, after adjusting the pH to nearly 7.2, and stored in a deep freeze. Samples of these were added to the cultures to give the desired concentrations. The liquid esters were added directly to the cultures.

Effects on germination were studied by adding the compound to the medium inoculated with a heat-shocked spore suspension.

Effects on growth and sporulation were studied by adding the compound to an active culture (Gollakota and Halvorson, 1960) immediately after the final transfer was made (referred to hereafter as "0" time). When the effect of time of addition was being studied, the compound was added to an active culture at the denoted time, the age of the culture being the time elapsed between inoculation of the medium and addition of the compound.

*Reversing agents.* The ability of a compound to reverse the effects of an inhibitor was generally tested by adding the inhibitor and the test compound to an active culture at 0 time. To study the effects of time of addition, the inhibitor was added at 0 time and the test compound at the time indicated.

*Effect of glucose.* This effect was studied by adding the inhibitors at 0 time to a culture in EYE-minerals medium. The effect of time of addition of glucose was studied by (i) adding the inhibitor at 0 time and adding glucose at the denoted time, or (ii) by adding glucose and APA singly and in combination, to a normal culture at different times.

Dipicolinic acid (DPA) content of the cells and cultures was determined according to the methods described by Krishna Murty and Halvorson (1957) or by Janssen, Lund, and Anderson (1958).

## RESULTS

Changes in the composition, pH, and oxygen consumption of *B. cereus* T, at various stages during growth in yeast extract-glucose-minerals medium, revealed that the glucose is broken down primarily to pyruvic and acetic acids. These acids accumulate in the medium during vegetative growth but are utilized with the onset of sporulation (Nakata and Halvorson, 1960).

Time studies with APA have shown this compound to inhibit sporulation and DPA synthesis, when added before the organism begins utilizing the above acids (Gollakota and Halvorson, 1960). These findings could be best explained by assuming (i) that, under the conditions of culture used, *B. cereus* T does not possess a pathway for the utilization of acetate during vegetative growth, (ii) that some or all of the enzymes necessary for the above are formed after the glucose of the medium is exhausted and growth ceases, and (iii) that APA prevents the formation of this enzyme system but has no effect on the functioning of the enzymes already synthesized.

Though the effects of APA could be reversed by zinc, cobalt, or nickel, no conclusions could be drawn regarding the pathway of acetate utilization, as these metals appeared to act by chelation with the inhibitor. It was evident that efforts should be made to reverse the effects of APA by specific metabolites to gain an insight into the metabolism of acetate during sporulation. With this in view, the effects of increasing the concentration of yeast extract were studied. That 12 mg/ml of yeast extract reversed the effects of APA, whereas ash from an equivalent amount of yeast extract failed to do so, clearly

TABLE 1. *Effect of time of addition of succinate on the inhibition of sporulation by APA**

| Time of addition | Heat-stable cells (per ml) | |
|---|---|---|
| | At time of addition | After 24 hr of incubation |
| *hr* | | |
| 0 | $<10^2$ | $1.8 \times 10^8$ |
| 1 | $<10^2$ | $2.1 \times 10^8$ |
| 2 | $<10^2$ | $1.6 \times 10^8$ |
| 3 | $<10^2$ | $2.3 \times 10^8$ |
| 4 | $<10^4$ | $1.6 \times 10^8$ |
| 5 | $<10^4$ | $1.4 \times 10^8$ |
| 6 | $<10^4$ | $<10^4$ |

* An active culture was built up in the presence of APA ($1.2 \times 10^{-3}$ M). At regular intervals, samples were removed. Sodium succinate (0.005 M), adjusted to pH 7.2, was added, and the samples were incubated at 30 C on a shaker.

TABLE 2. *Effects of FAA and sodium bisulfite on sporulation*

| pH of culture at time of addition* | Compound added† | After 24 hr of incubation | |
|---|---|---|---|
| | | pH | Heat-stable cells/ml |
| 4.7 ↓ | Bisulfite ($4 \times 10^{-3}$ M) | 4.3 | $10^4$ |
| 5.2 ↑ | Bisulfite ($4 \times 10^{-3}$ M) | 4.8 | $10^4$ |
| 4.7 ↓ | FAA ($10^{-2}$ M) | 4.9 | $1.5 \times 10^4$ |
| 5.2 ↑ | FAA ($10^{-2}$ M) | 5.4 | $1.2 \times 10^4$ |
| — | None (control) | 7.6 | $2 \times 10^8$ |

* Symbols: ↓, falling; ↑, rising.

† The compounds were added to an active culture and incubated at 30 C on a shaker.

implicated the organic constituents of yeast extract for the observed effects. To identify the active fraction of yeast extract, the effects of removing the free, ether-extractable acids of yeast extract were studied. The results of these studies clearly show the ninhydrin-negative acids to be responsible for the ability of yeast extract to reverse the effects of APA. However, the effects of amino acids were studied to confirm that none of them possessed this ability. Only aspartic acid and asparagine, among the amino acids tested, possessed this activity.

The next logical step was to test the intermediates of the tricarboxylic acid and glyoxylic acid cycles. All the intermediates of the tricarboxylic acid cycle reversed the effects of APA, with the exception of α-ketoglutarate and fumarate. The minimal effective concentrations of pyruvate, acetate, and succinate were also determined; whereas $10^{-2}$ M pyruvate or acetate was needed, $5 \times 10^{-3}$ M succinate was sufficient to overcome the effects of APA. Time studies with the minimal effective concentrations of these showed that pyruvate and acetate were inactive unless added at 0 time, but succinate was effective even when added very late (Table 1).

Further studies with some other organic acids also lend support to the above assumption. The ability of propionate and methylmalonate to reverse the effects of APA may be due to the ability of the organism to convert propionate into succinate through methyl malonic acid. The mechanism of reversal with malonate and formate is not clear. It is, however, known that this organism can break formate down to $CO_2$. When $C^{14}$-formate is used, nearly all the label can be recovered in the form of $CO_2$. The inability of oxamate and oxalate, which are known to inhibit lactic dehydrogenase (Quastel and Wooldridge, 1928), to suppress sporulation and the failure of lactate to reverse the effects of APA are consistent with the observation that only traces of lactate can be detected in the culture of this organism (Nakata and Halvorson, 1960).

If the pathway for the utilization of acetate by this organism becomes operative only after growth ceases, FAA, which is known to interfere with acetate metabolism by inhibiting aconitase, should suppress sporulation and DPA synthesis, whether the acetate is utilized through the tricarboxylic acid cycle or the glyoxylic acid cycle. Bisulfite may also behave like FAA by interfering with the metabolism of the carboxyl compounds of the above cycles. FAA and bisulfite have no effect on germination or growth, but inhibit sporulation and DPA synthesis (Table 2). They are effective even when the organism begins utilizing acetate. Reversal studies with these inhibitors have given results somewhat different from those obtained with APA. Among the compounds reversing the effects of APA, it is significant that pyruvate, acetate, aspartate, and malate fail to reverse the effects of these inhibitors (Table 3).

Except for malonate, the compounds which can specifically reverse the effects of APA, FAA, or bisulfite are citrate, *cis*-aconitate, isocitrate, and succinate. Further, α-ketoglutarate and fumarate are unable to reverse the effects of all

the above inhibitors. This led us to believe that succinate may be the key intermediate in the synthesis of DPA and spore material, and that succinate may arise from acetate through the glyoxylic acid cycle. Though this cycle has been found to be absent in cells grown in glucose or $C_4$ acids and present only in cells grown on acetate (Kornberg, Gotto, and Lund, 1958), our assumption that succinate may arise from acetate in sporulating cells does not appear totally inconsistent with these findings. It should be emphasized that during sporulation there is no glucose in the medium and that the cells are actively metabolizing acetate. It is conceivable, therefore, that acetate may induce the enzymes of the glyoxylic acid cycle in sporulating cells. However, attempts to demonstrate unequivocally the presence of the glyoxylic acid cycle in these cells have been unsuccessful.

The pathway of biosynthesis of DPA was studied by Foster and co-workers. Perry and Foster (1955) concluded that very little diaminopimelic acid is converted into DPA and that it must arise in some manner other than by direct ring closure from 2,6-diaminopimelic acid. Martin and Foster (1958) investigated this problem by studying the incorporation of various amino acids into the DPA of sporulating cells; they concluded that DPA is synthesized by the condensation of aspartate with pyruvate or oxalacetate with alanine. However, these investigators also concluded that some of their results could be explained only by assuming that a synthetic intermediate like succinate was involved though, in direct experiments, no succinate was incorporated into the DPA of the cell. Powell and Strange (1959) have shown the nonenzymatic conversion of 2,6-diketopimelic acid to DPA in the presence of ammonia and an electron acceptor like quinone.

As pimelic and $\alpha$-ketopimelic acids reverse the effects of APA, a $C_7$ acid may be inferred to be an intermediate in the synthesis of DPA. This $C_7$ acid may arise by the condensation of a $C_4$ unit with a $C_3$ unit. In such an event, esterification of the carboxyls of the $C_4$ and $C_3$ acids may interfere only with sporulation and DPA synthesis. The effects of the esters of some of the compounds reversing the effects of the above inhibitors were therefore studied.

It is indeed significant that of all the esters tested only ethyl malonate, ethyl oxalacetate, ethyl pyruvate, and ethyl succinate inhibited

TABLE 3. *Effect of some acids on the inhibition of sporulation by sodium bisulfite*

| Compound added* | Heat-stable cells/ml† |
|---|---|
| None (control) | $2 \times 10^8$ |
| Pyruvate | $<10^4$ |
| Acetate | $<10^4$ |
| Citrate | $1.2 \times 10^8$ |
| *cis*-Aconitate | $1.2 \times 10^8$ |
| Isocitrate | $1.5 \times 10^8$ |
| Succinate | $9 \times 10^7$ |
| $\alpha$-Ketoglutarate | $<10^4$ |
| Aspartate | $<10^4$ |
| Malonate | $<10^4$ |
| Propionate | $<10^4$ |
| Methylmalonic acid | $7 \times 10^7$ |
| Malate | $4 \times 10^7$ |
| Formate | $<10^4$ |
| Glyoxylate | $8 \times 10^7$ |

* Except for the control, sodium bisulfite ($4 \times 10^{-3}$ M) was also added in all instances. The compounds were added to an active culture when the pH was 5.1 and dropping, to give a final concentration of $10^{-2}$ M.

† All determinations were made after incubation for 24 hr at 30 C on a shaker.

synthesis of DPA and sporulation. Malonate probably plays an important role in the utilization of acetate, as malonate itself reversed the effects of APA, FAA, and bisulfite, and the ester inhibited sporulation. Time studies with ethyl malonate, ethyl pyruvate, and ethyl succinate showed interesting differences among them.

These esters prevented sporulation when added before or after the pH of the culture began to rise (Table 4). Figure 1 shows the effect of the addition of ethyl malonate at various times. If ethyl malonate was added when the pH of the culture was falling, the pH remained at the low level reached during vegetative growth; when added after the pH began to rise, the pH continued to rise slowly and subsequently came down. Bernlohr and Novelli (1960) have shown ethyl malonate to inhibit sporulation and antibiotic production in *B. licheniformis*. Ethyl pyruvate, like ethyl malonate, seems to inhibit formation of the acetate-utilizing system when added before acetate utilization starts. However, ethyl pyruvate seems to have no effect on the functioning of the acetate-utilizing system once it is formed. Ethyl succinate, on the other hand, seems to exert its effect by interfering only with

TABLE 4. *Effect of time of addition of inhibitive esters on sporulation**

| Ester added | pH of culture | |
|---|---|---|
| | At time of addition | After addition |
| Ethyl malonate ($1.3 \times 10^{-2}$ M) | 5.1 | 4.3 |
| | 5.0 | 4.3 |
| | 5.6 | 4.3 |
| Ethyl pyruvate ($1.5 \times 10^{-2}$ M) | 5.1 | 4.2 |
| | 5.0 | 7.3 |
| | 5.6 | 7.3 |
| | 6.5 | 7.3 |
| Ethyl succinate ($2.0 \times 10^{-2}$ M) | 5.1 | 4.7 |
| | 5.0 | 4.8 |
| | 5.6 | 4.7 |
| | 6.5 | 4.7 |

* An active culture was used. The determinations were made after incubation for 24 hr at 30 C on a shaker. The number of heat-stable cells present in all cases was $<10^4$.

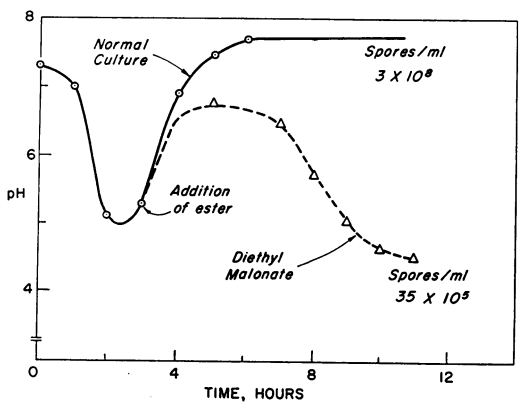



FIG. 1. *Effect of ethyl malonate ($1.3 \times 10^{-2}$ M) on the pH and sporulation of a culture of Bacillus cereus T.*

the activity of the acetate-utilizing system. Whether the ethyl succinate was added before or after the pH began rising, the pH rose slowly from the low level reached during vegetative growth and subsequently fell (Fig. 2). DPA could not be detected in cultures inhibited from sporulating by any of the above inhibitors.

The failure of the organism to utilize intermediates of the tricarboxylic cycle and other reversing agents as carbon sources for growth is understandable in the light of the earlier observations indicating that the pathway for utilization of acetate becomes operative only after growth ceases.

All the inhibitors discussed so far seem to act by preventing the synthesis or functioning of the system involved in the utilization of acetate derived from the glucose of the medium. Omitting glucose from the medium may eliminate or reduce the accumulation of acetate. It was therefore of interest to study the effects of these inhibitors under these conditions. In the absence of glucose, the pH of the medium rose slowly from the beginning, suggesting that no acids were accumulating. It was not surprising that all the above inhibitors failed to prevent sporulation of this organism in yeast extract-minerals medium.

Time studies of the effects of adding glucose to cells growing in yeast extract-minerals-APA medium (Table 5) indicate that there is a

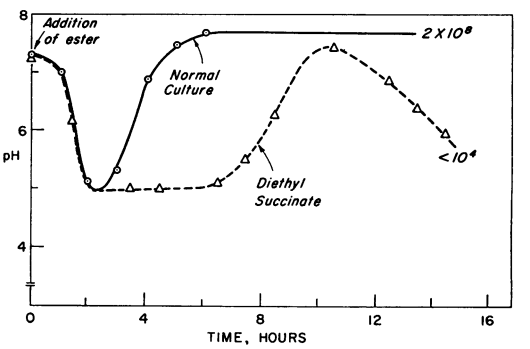



FIG. 2. *Effect of ethyl succinate ($2.0 \times 10^{-2}$ M) on the pH and sporulation of a culture of Bacillus cereus T.*

TABLE 5. *Effect of time of addition of APA and glucose on sporulation of active culture**

| Time of addition | pH | Heat-stable cells/ml | Compound aaded | | | |
|---|---|---|---|---|---|---|
| | | | APA ($1.2 \times 10^{-2}$ M) | | APA + glucose (1 mg/ml) | |
| | | | pH | Heat-stable cells/ml | pH | Heat-stable cells/ml |
| *hr* | | | | | | |
| 3 | 4.9 | $<10^2$ | 5.1 | $<10^3$ | 5.3 | $<10^3$ |
| 4 | 4.9 | $<10^2$ | 7.8 | $1.8 \times 10^8$ | 7.4 | $2.1 \times 10^8$ |
| 5 | 5.8 | $<10^4$ | 7.8 | $1.6 \times 10^8$ | 7.3 | $2.3 \times 10^8$ |

* The active cultures were incubated at 30 C on a shaker.

metabolic transition from vegetative to sporulating cells even in the absence of glucose, and that glucose prevents this transition if added before it occurs. It will be recalled that a similar transition occurs in glucose-grown cells when glucose is added before it occurs. APA, even in conjunction with glucose, has no effect if added after this metabolic transition has taken place. Nakata and Halvorson (1960) have shown that glucose added after this transition to glucose-grown cells merely delays the progress of sporulation until the added glucose is broken down.

The observed effects may be explained by assuming (i) that the metabolism of the organism growing in yeast extract and minerals is different from that in the presence of glucose and (ii) that addition of glucose to cells growing in the absence of glucose changes their metabolism if the glucose is added before the cells are committed to sporulation.

The effects of oxamic acid and its esters were studied in view of the structural similarity of oxamate to pyruvate. Butyl oxamate prevented sporulation when added before or after the pH of the culture began to rise. Normal-looking sporelike bodies were obtained in the presence of ethyl oxamate. These sporelike bodies were refractile, nonstainable with crystal violet, and viable. However, they were sensitive to heat, most of them losing viability on heating for 30 min at 80 C (Table 6). It was evident that the heat-stability test alone was not sufficient to determine the percentage of the sporelike bodies.

During a study of the germination of bacterial spores in milk, we encountered the problem of foaming when making dilutions for plate counts. Several antifoaming agents were tried. During the course of these investigations, we found that octyl alcohol prevented germination of the spores in milk, yeast extract, or a mixture of L-alanine and adenosine. Further studies revealed that octyl alcohol killed germinated spores as well as vegetative cells (Table 7). Halmann and Keynan (*personal communication*) reported that ethyl pyruvate and octyl alcohol inhibited the L-alanine-induced germination of *B. licheniformis* (*B. subtilis* NTCD 9945). It occurred to us that this property of octyl alcohol could be used to estimate the percentage of the heat-sensitive sporelike bodies. The spores produced in the presence of ethyl oxamate were treated with octyl alcohol and the octyl-stable cells were construed as spores. The octyl alcohol-stable cells killed by heating for 30 min at 80 C then represented the heat-sensitive spores in the cultures. Although the sporelike bodies produced in the presence of

TABLE 6. *Effect of time of addition of ethyl oxamate 0.01 M on germination, growth, and sporulation**

| Type of culture | After 24 hr of incubation at 30 C in a shaker | |
|---|---|---|
| | Viable cells/ml | Heat-stable cells/ml |
| Spore inoculum..... | $2 \times 10^8$ | $6 \times 10^5$ |
| Active at 0 time..... | $4 \times 10^8$ | $8 \times 10^5$ |
| Active (pH 5.2 ↓).... | $7 \times 10^8$ | $1.5 \times 10^5$ |
| Active (pH 5.8 ↑).... | $6 \times 10^8$ | $6 \times 10^5$ |
| Active (pH 7.1 ↓).... | $1.5 \times 10^9$ | $1.4 \times 10^8$ |
| Active (pH 7.9 ↑).... | $8 \times 10^8$ | $2 \times 10^8$ |

* For purposes of counting, cells were spun down and resuspended in 0.01 M phosphate buffer (pH 7.2). Symbols ↓, falling; ↑, rising. Staining showed spores in all instances.

TABLE 7. *Effect of octyl alcohol on the viability of spores, germinated spores, and vegetative cells*

| Type of cells | Without treatment | | With treatment* |
|---|---|---|---|
| | Viable cells/ml | Heat-stable cells/ml | Viable cells/ml |
| Spores..... | $3 \times 10^8$ | $2.5 \times 10^8$ | $2.8 \times 10^8$ |
| Germinated spores..... | $1.6 \times 10^8$ | $10^5$ | $10^5$ |
| Vegetative cells. | $6 \times 10^7$ | <100 | <100 |

* Octyl alcohol treatment consisted of shaking the culture with octyl alcohol (0.01 ml of alcohol/10 ml of culture) for 15 min or diluting the suspension with water containing octyl alcohol (0.06 ml of alcohol/100 ml of water).

TABLE 8. *Effect of time of addition of ethyl pimelate (0.01 M) on sporulation*

| pH of culture at time of addition* | After 24 hr of incubation at 30 C on a shaker | | |
|---|---|---|---|
| | Viable cells/ml | Octyl-stable cells/ml | Heat-stable cells/ml |
| 4.9 ↓ | 100 | 100 | 10 |
| 5.3 ↑ | $10^8$ | $1.3 \times 10^8$ | $5 \times 10^6$ |
| 6.3 ↑ | $1.3 \times 10^8$ | $1.7 \times 10^8$ | $2.5 \times 10^6$ |
| 7.3 ↑ | $1.3 \times 10^8$ | $1.5 \times 10^8$ | $1.5 \times 10^6$ |
| 7.8 ↑ | $4 \times 10^8$ | $2.5 \times 10^8$ | $1.5 \times 10^6$ |

* An active culture was used. For purposes of counting, the cells were spun down and resuspended in 0.01 M phosphate buffer (pH 7.2.) Symbols: ↓, falling; ↑, rising.

ethyl oxamate were octyl-alcohol stable, most of them were sensitive to heat.

Oxamate has been shown to inhibit lactic dehydrogenase (Novoa et al., 1959). However, the effects of the oxamic acid esters could not have been due to inhibition of lactic dehydrogenase because (i) oxamate itself has no effect and (ii) other inhibitors of lactic dehydrogenase (oxalate and *meso*-tartrate) also have no effect. The inhibition of growth by oxamic acid after growth ceased and the pH began rising had no effect. Even though the pH dropped immediately, the cells sporulated normally. Further, the pH came back up to neutrality, suggesting that oxamic acid itself might have been metabolized by the sporulating cells. Though the mode of action of the individual esters is not known, the results clearly indicate that the observed effects are due only to the esters and not the free acid.

It will be recalled that pimelate and $\alpha$-ketoglutarate also reversed APA inhibition. Hence, the effects of ethyl pimelate were also studied. Ethyl pimelate caused lysis of the cells when added before the pH of the culture began to rise. If ethyl pimelate was added after the pH of the culture was well on the way up, the culture sporulated (Table 8). However, these spores, like those produced in the presence of ethyl oxamate, were sensitive to heat.

The lysis of growing cultures by ethyl pimelate may be due to competition with 2,6-diaminopimelic acid in the synthesis of cell walls. The production of heat-sensitive DPA-deficient spores by adding ethyl pimelate when growth is complete tends to support the assumption that a $C_7$ may be an intermediate in the synthesis of DPA.

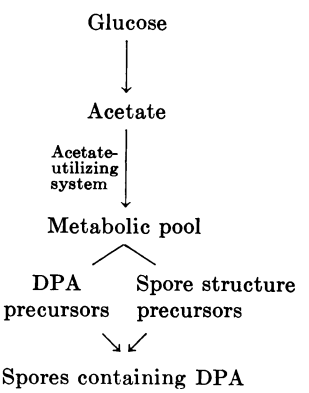
## DISCUSSION

It was generally believed that production of spores (as evidenced by staining), synthesis of DPA, and development of heat resistance occur simultaneously during the sporulation of aerobes (Perry and Foster, 1955; Collier and Nakata, 1958). Halvorson (1961) reported the following sequence of events during the sporulation of the anaerobe *Clostridium roseum*: (i) production of heat-sensitive, sporelike bodies containing no detectable DPA (spores by stain), (ii) synthesis of DPA, and finally (iii) development of heat resistance. Through the agency of ethyl oxamate or ethyl pimelate, it has been possible to separate the production of spores, by the use of stain, from the synthesis of DPA and the development of heat resistance. The DPA content of the spore crops produced in the presence of these esters is about 10% of that of normal spore crops. This value is higher than that calculated by assuming that all heat-sensitive spores contain no DPA and all heat-stable spores contain the normal amount of DPA. This discrepancy may be due to the existence of a threshold value of DPA for dormancy and heat resistance of a spore. This will have to be tested by analysis of the heat-sensitive spores free from heat-stable spores. Church and Halvorson (1959) reported the production of heat-sensitive, DPA-deficient spores of this organism in the presence of phenylalanine.

The heat-sensitive, DPA-deficient spores were refractile and dormant. They germinated normally in a mixture of L-alanine and adenosine. In germination they lost refractility and became sensitive to octyl alcohol and stainable with crystal violet. Ethyl oxamate and ethyl pyruvate seem to have separated dormancy of the bacterial spore from heat resistance and possibly the presence of DPA.

The effects of APA, FAA, ethyl malonate, and ethyl succinate have been studied on the sporulation of a number of other aerobic sporeformers in a glucose-yeast extract-minerals medium. As these inhibitors have been able to prevent the sporulation of most of the organisms tested, it appears as though the same metabolic pathway may be involved in the sporulation of all these organisms.

The results of this investigation lead us to propose the following pathway for the metabolism of sporulating cells of *B. cereus* T and possibly other aerobic sporeformers when grown in a glucose-yeast extract-minerals medium.



In this scheme of metabolic events, APA inhibits only the formation of the acetate-utilizing system; FAA, bisulfite, and ethyl malonate probably suppress both the formation and function of the acetate-utilizing system, depending upon the time of addition; ethyl pyruvate suppresses the systems for utilization of acetate of the metabolic pool, depending upon the time of addition; ethyl succinate prevents only the utilization of the metabolic pool—while ethyl oxamate or diethylpimelate (added after growth has ceased) suppresses the DPA synthesizing system.

ACKNOWLEDGMENTS

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