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Metabolism of *Bacillus thuringiensis* in Relation to Spore and Crystal Formation

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A general pattern of metabolism was determined for *Bacillus thuringiensis* grown in a glucose-yeast extract-salts medium. The pattern did not differ significantly from that of *B. cereus* grown in a similar medium. Acetic acid produced from glucose during exponential growth was further catabolized in the early sporulation phase of growth, at which time the specific activity of aconitate hydratase increased markedly. Fluoroacetate and α-picolinate prevented the removal of accumulated acid, and the resulting low *p*H inhibited spore and crystal synthesis. Neither crystal-related antigens nor insect toxicity was shown by cells whose crystal synthesis was inhibited in this way. α-Picolinate prevented the normal increase in specific activity of aconitate hydratase without inhibiting exponential growth. It also inhibited aconitate hydratase in vitro, but only if preincubated with the enzyme. α-Picolinate did not inhibit the increase in specific activity of aconitate hydratase or spore and crystal synthesis in a medium buffered near neutrality. Chloramphenicol and actinomycin D inhibited crystal enlargement and sporulation when added to cells in which small crystals had already begun to form. Typical messenger ribonucleic acid-dependent protein synthesis, rather than the type associated with peptide antibiotic synthesis, is thus indicated for the synthesis of crystal peptide subunits.

The toxicity of *Bacillus thuringiensis* for larvae of lepidopterous insects is associated with an intracellular parasporal body or crystal (2-4). Upon ingestion of the proteinaceous crystals by susceptible larvae, proteolytic enzymes in the larval gut juice hydrolyze the crystal protein into as yet undefined toxic moieties. The larvae then exhibit gut paralysis, usually followed by general paralysis and eventual death.

Electron micrography (11, 20, 22) and physical and chemical studies (4, 11, 15, 20) have provided a considerable, though by no means definitive, amount of information about the structure and composition of the crystal. There is known to be a close temporal relationship between the synthesis of the crystal and sporulation in *B. thuringiensis*, both events occurring after the cessation of exponential growth. Virtually all of the crystal protein is synthesized de novo from amino acids at this time, and proteins antigenically related to crystal proteins cannot be detected in vegetative cells (17).

Some of the events that accompany sporulation in certain bacilli are known (18, 21). These include the appearance of tricarboxylic acid-cycle enzymes, proteolytic enzyme activity, and antibiotics. There has been no report to date as to whether these changes take place in *B. thuringiensis*, whether the reactions occurring during sporulation are also related to crystal formation, or whether the control mechanisms regulating sporulation and the production of the crystal protein are related. In this report the pattern of metabolic changes occurring in the vegetative and early sporulation phases of growth, the effect of inhibitors of tricarboxylic acid-cycle activity, and the effects of inhibition of protein synthesis on spore and crystal formation by *B. thuringiensis* are presented.

MATERIALS AND METHODS

Medium and inoculum preparation. The culture used throughout this study was a strain of *B. thuringiensis* var. *galleriae*. It was maintained routinely on slants of Nutrient Agar (Difco). Cells for all biochemical studies were grown in a liquid medium of the following composition: 0.1% glucose, 0.2% $(NH_4)_2SO_4$, 0.2% yeast extract, 0.05% K_2HPO_4, 0.02% $MgSO_4$, 0.008% $CaCl_2 \cdot 2H_2O$, and 0.005% $MnSO_4 \cdot H_2O$. Glucose, $MgSO_4$, $CaCl_2 \cdot 2H_2O$, and $MnSO_4 \cdot H_2O$ were added separately as concentrated sterile solutions.

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The *p*H of the $(NH_4)_2SO_4$, K_2HPO_4, and yeast extract solution was adjusted to 7.3 with KOH before autoclaving. Additions to this basic medium (GYS broth) are stated with particular experiments. The inoculum for all experiments was prepared by the active culture method (19). This consisted of an initial 4-hr growth period in which 50 ml of GYS broth in a 250-ml Erlenmeyer flask was inoculated with the cells from a single washed slant. After 4 hr of growth, 2 ml was transferred to a second 50 ml of broth. This 4% (v/v) transfer was repeated two additional times at 2-hr intervals. After the third 2-hr growth period, 5.0 ml was used to inoculate 100 ml of the test system broth in a 500-ml Erlenmeyer single-baffle flask (Kimax no. 26655). Flasks were incubated at 30 C with shaking on a gyratory shaker (288 rev/min; New Brunswick model 653). Inoculum for the fermentor experiments was developed similarly, but the last 2-hr growth period was performed with a 500-ml flask containing 100 ml of GYS broth. The entire 100 ml of broth was used to inoculate 8 liters of medium in the fermentor.

Total viable cell and spore counts were made on Trypticase Soy Agar (BBL). Spores were those colony-forming units which survived heating at 65 C for 30 min.

Preparation of cell-free extracts. Cell-free extracts were prepared by removing cells from 100 ml of the growth medium by centrifugation, washing once with an equal volume of deionized water, and suspending them in 5 ml of 0.02 M potassium phosphate buffer at *p*H 7.4 for disruption. The cells were disrupted by a single pass through a French pressure cell, and debris was removed by centrifugation at 12,000 × *g* for 15 min. Extracts prepared in this manner contained 1 to 3 mg of protein/ml.

Analytical methods. Glucose was determined with Glucostat reagents (Worthington Biochemical Corp., Freehold, N.J.). Protein was determined with biuret reagent (10). Volatile acids were measured with a gas chromatograph (Hewlett Packard, model 810) equipped with a hydrogen flame detector. The 6-ft (1.8 m) column contained 15% Versimide and 3% phosphoric acid.

Aconitate hydratase (EC 4.2.1.3) activity in cell-free extracts was determined by following absorbance changes at 240 nm with a Gilford model 2000 recording spectrophotometer (1). The reaction cuvette contained 0.1 ml of extract and 2.9 ml of 0.3 M potassium citrate adjusted to *p*H 7.4. In preincubation experiments with α-picolinate, 0.1 ml of the enzyme and 0.1 ml of the inhibitor were preincubated in the cuvette for the desired time; the reaction was started by the addition of 2.8 ml of citrate. Increase in absorbance was measured for the 3-min period beginning 1 min after initiating the reaction. The absorbance increase was linear during this period. A unit of activity is defined as that amount of enzyme catalyzing the formation of 1 μmole of *cis*-aconitate per min at 23 C. Specific activity is expressed as units of enzyme per milligram of protein.

Preparation of crystal protein antibody. The crystal protein used to immunize rabbits and as an antigen in gel diffusion plates was prepared by extracting a mixture of spores and crystals with 0.05 M NaOH by the method of Cooksey (5). After removal of spores from the extract by centrifugation, the *p*H was adjusted to 8.5 with acetic acid. The crystal protein obtained by this method retained its toxicity by ingestion for larvae of the cabbage looper (*Trichoplusia ni*). Rabbits were immunized by injecting 4 mg of crystal protein into the marginal ear vein five times at 4-day intervals. Immune serum was obtained 10 days after the last injection.

Toxicity determination. The toxicity of *B. thuringiensis* preparations for the cabbage looper was determined by blending an appropriate amount of the particular preparation into the semisynthetic larval diet described by Ignoffo (16). Fifty larvae, 4 days of age, were placed into individual containers and allowed to feed freely upon the test diet. Larval mortality was determined after 5 days of incubation at 30 C.

Chemicals. Tricarboxylic acid-cycle intermediates were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, with the exception of *cis*-aconitic acid which was obtained from Calbiochem, Los Angeles, Calif. Fluoroacetic acid was purchased from City Chemical Corp., New York, N.Y., α-picolinic acid from Aldrich Chemical Co., Inc., Milwaukee, Wis., chloramphenicol from Sigma Chemical Co., St. Louis, Mo., and gel diffusion plates from Mann Research Laboratories. Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories.

RESULTS

General metabolic pattern. The metabolic pattern of *B. cereus* grown in a glucose-yeast extract-salts medium ("G" medium) is well known (12, 13, 19). The metabolic pattern of *B. thuringiensis* grown in a very similar medium, GYS broth, is presented in Fig. 1A and is essentially the same as found for *B. cereus*. During exponential growth, glucose was catabolized to acetic acid. Upon the completion of exponential growth, the specific activity of aconitate hydratase increased, acetic acid was metabolized, and the *p*H of the medium rose. Aconitate hydratase activity remained high after acetate utilization and declined only slightly as refractile spores appeared. The sequence of events was somewhat slower than found in two of the reports on *B. cereus* (12, 13) but was almost identical to the pattern in a third report (19). *B. thuringiensis* produced refractile spores under these growth conditions at about 12 hr. Small, slightly refractile granules appeared in the cells after about 9 hr of growth. These increased in refractility but did not become as refractile as spores. They assumed typical crystal shape under phase-contrast optics at about the same time the spores acquired refractility (Fig. 2).

Effect of α-picolinate. In *B. cereus*, α-picolinate prevented metabolism of acetate accumulated during growth by interfering with the increase in specific activity of aconitate hydratase (8, 12, 13).

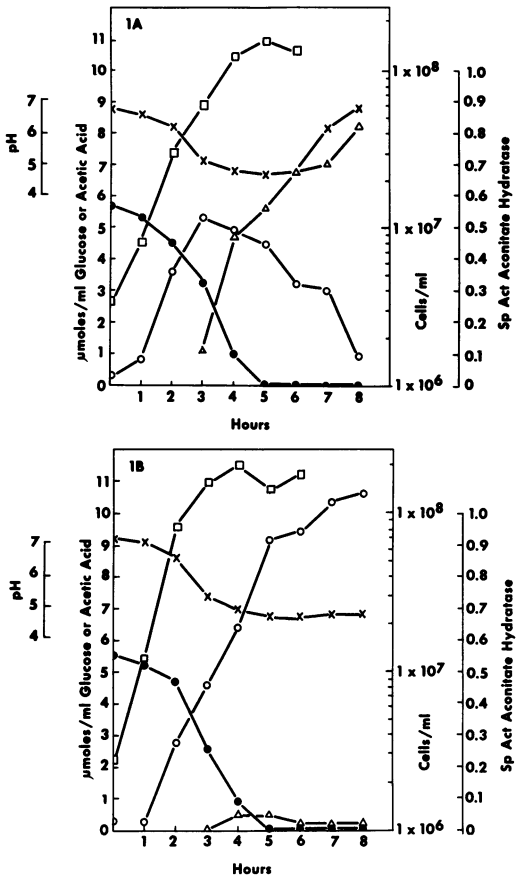

FIG. 1. (A) *Metabolic pattern of Bacillus thuringiensis grown in GYS broth. Symbols:* ●, *glucose;* ○, *acetic acid;* ×, *pH;* △, *specific activity of aconitate hydratase;* □, *cell growth.* (B) *Metabolic pattern of B. thuringiensis grown in GYS broth plus* 10^{-3} M *α-picolinate. Symbols same as A.*

The interference with acetate metabolism resulted in an inhibition of sporulation by an as yet unresolved mechanism. When 10^{-3} M α-picolinate was added to GYS broth at the time of inoculation, *B. thuringiensis* cells developed to a stage of slight granulation but formed neither spores nor crystals. The pattern of metabolic changes found in the α-picolinate-inhibited culture is presented in Fig. 1B. Exponential growth of the organism was not inhibited. In contrast to the pattern observed for uninhibited cells, those grown in the presence of α-picolinate did not exhibit an increase in the specific activity of aconitate hydratase and continued to accumulate acetic acid in the broth. In addition to acetic acid, trace amounts of propionic and butyric acids were detected in inhibited culture broths. As in *B. cereus* (9), the inhibitor was effective only when added prior to

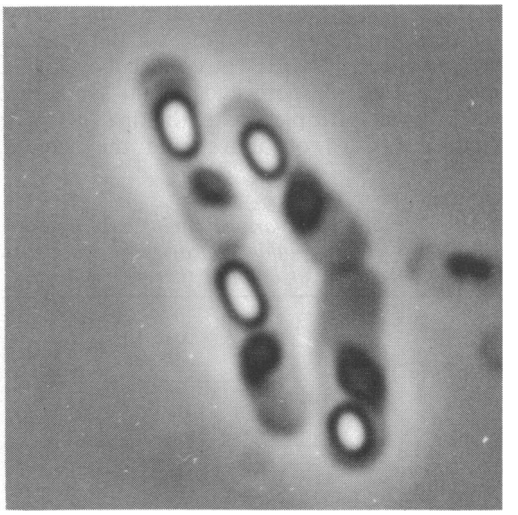

FIG. 2. *Bacillus thuringiensis grown for 16 hr in GYS broth. Phase-contrast micrograph.* ×*1,500.*

the rise in *p*H of the culture (Table 1). It will be shown that this response depends upon the *p*H of the medium rather than upon the enzyme content of the cells at this particular stage.

Relief of α-picolinate inhibition. A previous report stated that a wide variety of compounds was capable of overcoming the inhibitory effect of α-picolinate on sporulation in *B. cereus* (9). Among the most successful were tricarboxylic acid-cycle intermediates. Data obtained when salts of the tricarboxylic acid-cycle intermediates and three amino acids were tested for their effect on α-picolinate inhibition of spore and crystal synthesis in *B. thuringiensis* are given in Table 2. Fumarate, although successful in the trial reported in Table 2, was sometimes unsuccessful in relieving the inhibition. Aspartate, reported to be effective in *B. cereus*, was not effective in the *B. thuringiensis* system. The compounds reported

TABLE 1. *Effect of time of* 10^{-3} M *α-picolinate addition on spore and crystal formation by Bacillus thuringiensis*

Time of α-picolinate addition[a]	3 hr (*p*H)	6 hr (*p*H)	9 hr (*p*H)	22 hr (spores/ml)	22 hr (crystals)
hr					
None added	5.62	4.75	6.80	4.6×10^8	+
3	5.80	4.91	4.80	$<5.0 \times 10^5$	−
6	5.70	5.01	5.05	$<5.0 \times 10^5$	−
9	5.62	4.71	6.70	3.7×10^8	+

[a] α-Picolinate was added as a filter-sterilized solution to a final concentration of 10^{-3} M.

in Table 2 as being effective at 5.0 mg/ml were uniformly ineffective at 0.5 mg/ml. In those cases where the salt of a tricarboxylic acid-cycle intermediate allowed spore and crystal synthesis in the presence of α-picolinate, the final *p*H of the culture was 6.5 or above, and the aconitate hydratase activity of 8-hr cells was markedly above the inhibited level.

TABLE 2. *Ability of various compounds to relieve 10^{-3} M α-picolinate inhibition of spore and crystal formation in Bacillus thuringiensis*

Reversing agent[a] (5.0 mg/ml)	20 hr			Specific activity of aconitate hydratase (8-hr cells)
	*p*H	Spores[b]	Crystals[c]	
No α-picolinate	7.5	+	+	0.38
No reversing agent	4.7	−	−	0.06
Citrate	6.6	+	+	0.22
cis-Aconitate	6.8	+	+	0.47
Isocitrate	7.3	+	+	0.62
L-Glutamate	5.1	−	−	
α-Ketoglutarate	5.4	−	−	0.08
Succinate	7.4	+	+	0.47
Fumarate	7.9	+	+	0.30
Oxalacetate	8.0	+	+	0.21
L-Alanine		−	−	
L-Aspartate		−	−	

[a] All of the compounds tested were introduced into GYS broth as filter-sterilized, concentrated solutions at the time of inoculation.

[b] A negative finding of spores indicates fewer than 5×10^5 spores/ml in a population of about 10^8 cells/ml.

[c] Formation of crystals was determined by microscopic observation.

A possible role for salts of tricarboxylic acid-cycle intermediates as buffers was suggested by the nature of the compounds and by the high final *p*H values found in media containing them. When the *p*H profile of *B. thuringiensis* grown in GYS broth containing 10^{-3} M α-picolinate was followed during the first 8 hr of growth, the presence of any of the successful reversing agents at 5.0 mg/ml prevented the *p*H from falling to the normal low of 4.8 to 5.0. In the case of citrate, for example, the *p*H went no lower than 6.2. Similarly, the presence of 0.1% $CaCO_3$ (but not 0.1% $CaSO_4$) prevented the drop in *p*H and allowed normal spore and crystal synthesis in the presence of 10^{-3} M α-picolinate.

As a further test of the effect of *p*H control on α-picolinate inhibition, the organism was grown for 24 hr at 30 C in 10-liter stirred (400 rev/min) fermentors (New Brunswick) with forced aeration (18 liters/min). Three fermentors were charged with GYS broth made 10^{-3} M with respect to α-picolinate. One fermentor received 0.1% $CaCO_3$; a second had the *p*H automatically controlled at 6.5 by addition of 0.1 N KOH and H_2SO_4; the third received no additional treatment. A fourth fermentor charged only with GYS broth served as a control. The results (Table 3) clearly indicate that α-picolinate was effective in inhibiting sporulation only when the *p*H of the medium was allowed to fall without regulation. In those cases where inhibition of sporulation was relieved by $CaCO_3$ or *p*H control with KOH, inhibition of crystal synthesis was also relieved.

TABLE 3. *Effect of pH control on α-picolinate inhibition of sporulation by Bacillus thuringiensis*

Hr	Additions to GYS broth											
	None			10^{-3}M α-Picolinate			10^{-3}M α-Picolinate (0.1% $CaCO_3$)			10^{-3}M α-Picolinate (*p*H control at 6.5)		
	*p*H	Aconitate hydratase (specific activity)	Spores per ml	*p*H	Aconitate hydratase (specific activity)	Spores per ml	*p*H	Aconitate hydratase (specific activity)	Spores per ml	*p*H	Aconitate hydratase (specific activity)	Spores per ml
0	7.25			6.92			6.90			6.50		
1	7.18			6.80			6.90			6.50		
2	6.95			6.70			6.73			6.35		
3	6.35	0.10		6.30	0		6.25	0.06		6.30	0.04	
4	5.50	0.41		5.75	0.05		6.10	0.07		6.45	0.10	
5	5.50	0.47		5.20	0.05		6.22	0.28		6.43	0.33	
6	6.75	0.59		5.02	0.02		6.92	0.43		6.60	0.43	
7	7.30	0.50		4.95	0.01		7.45	0.51		6.60	0.44	
8	7.58	0.49		4.84	0.01		7.70	0.47		6.60	0.39	
12			2.0×10^8			$<5 \times 10^5$			2.4×10^8			1.8×10^8
20	7.85		3.5×10^8	4.80		$<5 \times 10^5$	7.80		1.9×10^8	6.45		3.1×10^8

Some of the changes which occurred in shake flasks of buffered and unbuffered GYS broth containing 10^{-3} M α-picolinate are recorded in Table 4. In buffered GYS broth containing α-picolinate and in GYS broth not containing α-picolinate, acetic acid utilization was initiated at about the same time, i.e., 4 to 6 hr. Although acetic acid was eventually removed from the unbuffered medium containing α-picolinate, this disappearance began later than the normal time, and the *p*H did not rise. Although aconitate hydratase is undoubtedly involved in the removal of acetic acid from uninhibited cultures, there is no evidence that this enzyme is involved in the late removal of acetate from inhibited cultures. In fact, the failure of the specific activity of this enzyme to rise above a barely detectable level is evidence against its involvement. The failure of the *p*H to rise may indicate conversion of acetic to another acid, quite possibly citric. The 4-carbon compound required for conversion of acetate to citrate might have been provided by amino acid breakdown.

Late addition of α-picolinate. As stated previously, α-picolinate was effective in preventing spore and crystal formation when added prior to the rise in *p*H of the culture (Table 1). This was true even though, during the latter part of the period of low *p*H, the specific activity of aconitate hydratase had already increased significantly. It appeared that the existence of a low *p*H in the broth was of more importance to effective inhibition by α-picolinate than was the level of aconitate hydratase activity at the time of addition. To test this possibility, α-picolinate was added to acidified and nonacidified GYS broth after 10 hr of growth (Table 5). At this time, the *p*H had already risen to above 7.0, small crystals had formed in the cells, and a small amount of acetate was still present. Sporulation was inhibited in the flask in which the *p*H was adjusted to 5.05 and α-picolinate was added. Addition of α-picolinate without *p*H adjustment or *p*H adjustment without inhibitor addition did not appear to restrict spore or crystal synthesis. The low final *p*H of the inhibited system (but not of the system which received acid alone) indicates that the remaining acetate or other organic acids were converted from salts to free acids concomitant with the prevention by α-picolinate of the further catabolism of these acids. The maintenance of a low *p*H was then sufficient to suppress sporulation.

TABLE 4. *Metabolic changes produced by Bacillus thuringiensis grown in GYS broth containing α-picolinate and buffer*[a]

Hr	GYS			GYS + 10^{-3}M α-Picolinate			GYS + 10^{-3}M α-Picolinate buffered at *p*H 6.9		
	*p*H	Glucose (μmoles/ml)	Acetic acid (μmoles/ml)	*p*H	Glucose (μmoles/ml)	Acetic acid (μmoles/ml)	*p*H	Glucose (μmoles/ml)	Acetic acid (μmoles/ml)
0	6.50	6.3	0.7	6.50	6.8	0.8	6.50	6.5	0.7
2	5.60	5.3	2.9	5.55	4.6	3.1	6.91	1.9	5.4
4	4.75	2.0	7.4	4.75		5.7	6.80	0.1	14.8
6	4.88	0	3.3	4.71	0.4	7.5	6.91	0.1	10.1
8	6.70	0	0.5	4.70	0.1	12.8	6.95	0	5.2
10	7.40	0	0.3	4.70	0	8.1	7.00	0	2.8
12	7.58	0	0.5	4.70	0	0.2	7.00	0	1.7
24[b]	7.88			4.80			7.08		

[a] The buffer was 0.1 M potassium phosphate at *p*H 6.9.

[b] Spores and crystals were present at 24 hr in GYS broth and in GYS broth containing buffer and 10^{-3}M α-picolinate.

TABLE 5. *Effect of adding 1 × 10^{-3}M α-picolinate at 10 hr to Bacillus thuringiensis in GYS broth*

Addition to GYS broth	*p*H at 10 hr	Acetic acid (μmole/ml) at 10 hr	*p*H adjusted down to	*p*H at 24 hr	Spores/ml at 24 hr
No addition	7.40	0.34		7.62	3.7×10^8
*p*H lowered	7.10	0.58	5.00	6.80	4.4×10^8
10^{-3} M α-Picolinate	7.15	0.31		7.80	3.4×10^8
*p*H lowered plus 10^{-3}M α-picolinate	7.25	0.31	5.05	5.15	$<5.0 \times 10^4$

Effect of maintaining pH at 5.0 after exponential growth. Inhibition of spore and crystal synthesis by α-picolinate took place when the *p*H remained low throughout the period during which the morphogenic events normally occurred. To determine the effect of maintaining a low *p*H in GYS broth in the absence of inhibitors, the organism was grown for 4 hr in a stirred fermentor with forced aeration. After 4 hr of growth, the *p*H had dropped to 5.0, and automatic addition of HCl was begun to hold the *p*H at 5.0 for another 20 hr. Microscopic examination of the cells after 24 hr showed that neither spores nor crystals had developed.

In vitro effect of α-picolinate on aconitate hydratase. It was observed in *B. cereus* (13), and has now been reported in *B. thuringiensis*, that α-picolinate prevents the increase in specific activity of aconitate hydratase that normally takes place during growth. Since α-picolinate prevented sporulation when added to cells in which a significant level of aconitate hydratase was already present, the existence of direct enzyme inhibition was suggested. Direct inhibition of enzyme activity was demonstrated in *B. subtilis* when the enzyme was preincubated with the inhibitor for 5 min (7). When 10^{-3} M α-picolinate was added to cell-free extracts of *B. thuringiensis* at the time the reaction was initiated, no inhibition of aconitate hydratase was observed. However, when the enzyme was preincubated with the inhibitor for increasing periods of time prior to initiating the reaction by substrate addition, a progressive decrease in specific activity was observed (Table 6). The addition of 10^{-3} M $FeSO_4$ to a 15-min preincubation mixture of enzyme and 10^{-3} M α-picolinate preserved about 75% of the specific activity which was otherwise lost. Low specific activity enzyme preparations from cells grown in 10^{-3} M α-picolinate were not activated by preincubation of the enzyme with Zn^{2+} or Fe^{2+}.

TABLE 6. *In vitro effect of α-picolinate on aconitate hydratase activity*

Min of preincubation	Specific activity of aconitate hydratase		
	No α-picolinate	1.5×10^{-2} M α-picolinate[a]	1.5×10^{-3}M α-picolinate[b]
0	0.418	0.418	0.418
5		0.246	0.402
10		0.172	0.382
15	0.418[c]	0.104	0.329

[a] Concentration of α-picolinate during preincubation. Because of the dilution upon addition of substrate, the concentration was 1.0×10^{-3} M during the 3-min assay.

[b] Concentration of α-picolinate during preincubation; concentration during the 3-min assay was 1.0×10^{-4} M.

[c] Preincubation consisted of 0.1 ml of extract and 0.1 ml of water.

Absence of crystal antigens or toxin in α-picolinate-inhibited cells. Although crystals were not detected microscopically in cells grown in the presence of α-picolinate, it was possible that the crystal proteins were synthesized but failed to crystallize. Two lines of evidence rendered this unlikely.

Ouchterlony gel diffusion plates were prepared with antibody against NaOH-solubilized crystals in the center well. Base-solubilized crystals and cell-free extract prepared from 20-hr α-picolinate-inhibited cells were placed alternately in the peripheral wells. Distinct precipitin lines appeared between the antibody and the solubilized crystal wells, but none appeared between the antibody and the cell-free extract wells. Soluble protein antigenically relatd to crystal protein was evidently not present in inhibited cells.

In a second experiment, 20-hr cells from cultures grown in GYS broth and in this medium with 10^{-3} M α-picolinate or α-picolinate plus 5.0 mg of succinate per ml were fed to 4-day-old cabbage looper larvae. Both intact and disrupted α-picolinate-inhibited (acrystalliferous) cells were tested. The data (Table 7) indicate that toxin was present under conditions which allowed formation of spores and crystals but was absent in the culture inhibited by α-picolinate.

Effect of fluoroacetate. Fluoroacetate is an inhibitor of aconitate hydratase by virtue of its metabolic conversion to fluorocitrate. The addition of 10^{-3} M fluoroacetate to GYS broth at the time of *B. thuringiensis* inoculation inhibited both spore and crystal synthesis. To achieve effective inhibition, it was necessary to add fluoroacetate prior to the normal increase in *p*H of the culture. This inhibitor had no effect on exponential growth of the organism. With the exception of the failure to inhibit the synthesis

TABLE 7. *Effect of α-picolinate on crystal toxin synthesis by Bacillus thuringiensis*

Addition to GYS broth	Spores/ml at 20 hr	Larval mortality[a]
		%
None	1.3×10^9	98
10^{-3} M α-Picolinate	$<5.0 \times 10^5$ [b]	0
10^{-3} M α-Picolinate plus 5.0 mg of succinate per ml	6.3×10^8	100

[a] Larval mortality in a control system which received no bacterial cells was 0%.

[b] This suspension contained 1.08×10^9 cells/ml by Petroff microscopic count.

of aconitate hydratase, fluoroacetate affected the metabolism of *B. thuringiensis* in the same way as α-picolinate. This inhibition of spore and crystal synthesis was reversed by buffering at neutrality. The acetic acid which accumulated during exponential growth in 10^{-3} M fluoroacetate was partially removed from the medium after this growth phase, but the *p*H of the medium remained low.

Actinomycin D and chloramphenicol inhibition. The antibiotics actinomycin D and chloramphenicol inhibit the usual ribosome and messenger ribonucleic acid (mRNA)-dependent protein synthesis. The synthesis of certain polypeptide antibiotics which occurs in bacilli at about the same time as sporulation is not inhibited by these agents. Although the non-mRNA-dependent form of protein synthesis associated with polypeptide antibiotic synthesis is restricted to relatively small molecules, the size of the crystal polypeptide subunits is not known with any certainty. It was therefore of interest to determine the susceptibility of crystal synthesis to chloramphenicol and actinomycin D.

Actinomycin D (4 μg/ml) was added to *B. thuringiensis* growing in GYS broth at 2-hr intervals during the period when the *p*H of the culture normally rose and crystals were first observed. The data in Table 8 indicate that early addition (5 hr) of the antibiotic prevented the synthesis of enzymes responsible for utilization of acids in the medium. By 9 hr, the *p*H had reached neutrality, and at this point the cells would normally have formed crystals and spores. Actinomycin D, however, prevented the formation of either structure. By 11 hr small crystals were visible in the cells. Addition of actinomycin D at that time prevented sporulation and enlargement of the crystals. Figure 3 shows 24-hr cells to which actinomycin D had been added at 11 hr. Similar results were obtained when 100 μg of chloramphenicol per ml was substituted for actinomycin D.

TABLE 8. *Effect of actinomycin D on spore and crystal synthesis*

Hr of actinomycin D addition	At addition			After 24 hr		
	*p*H	Spores[a]	Crystals[b]	*p*H	Spores[a]	Crystals[b]
5	4.90	−	−	5.05	−	−
7	4.95	−	−	6.30	−	−
9	7.00	−	−	7.45	−	−
11	7.50	−	+	7.85	−	+

[a] A negative finding of spores indicates fewer than 5×10^5 spores/ml in a population of about 10^8 cells/ml.

[b] Formation of crystals was determined by microscopic observation.

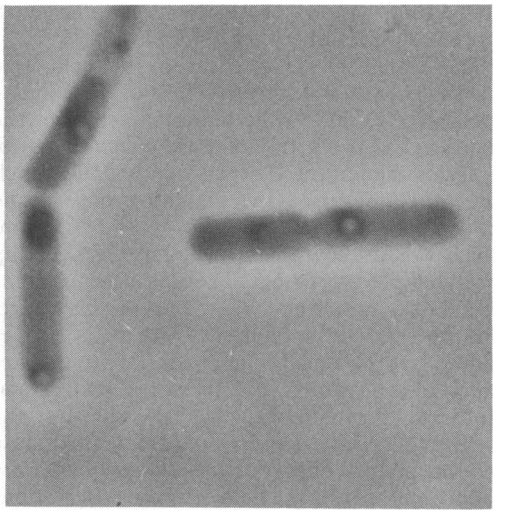

FIG. 3. *Cells (24-hr) of Bacillus thuringiensis to which 4 μg of actinomycin D per ml had been added at 11 hr. Phase-contrast micrograph. ×1,500.*

DISCUSSION

The synthesis of spores and crystals by *B. thuringiensis* occurs at about the same time in the growth cycle; however, the degree to which the two events are metabolically linked is unknown. The four inhibitors whose effects are reported here were not capable of selectively inhibiting spore or crystal synthesis without inhibiting synthesis of the other body. The effectiveness of chloramphenicol and actinomycin D indicate that the formation of the crystal is based upon typical mRNA-dependent protein synthesis. It is possible, of course, that crystal polypeptides are synthesized by both mRNA-dependent and -independent systems, and that interference with one type of synthesis prevented proper crystal enlargement due to lack of a particular subunit.

The earlier reports that α-picolinate (8, 9) and fluoroacetate (12) inhibited sporulation in *B. cereus* were based on results obtained in the lightly buffered "G" medium. This medium differed from the GYS broth used in this study only with respect to a few minor salts. In this type of medium, the catabolism of glucose by *B. thuringiensis* resulted in a drop in *p*H due to accumulation of acetic acid. In the presence of inhibitors of the tricarboxylic acid cycle, α-picolinate or fluoroacetate, the *p*H remained at a low level for the remainder of the incubation period. By growing *B. thuringiensis* in GYS broth to which HCl was automatically added after exponential growth to hold the *p*H at 5.0, it was

shown that the low *p*H was by itself sufficient to suppress spore and crystal development.

The effectiveness of tricarboxylic acid-cycle intermediates in relieving the effect of α-picolinate was probably due to the buffering action of the high level (5.0 mg/ml) of these materials used. It seems significant that all compounds which successfully reversed the inhibition have acidic groups with $_pK_a$ values in the 4.5 to 5.5 region, which would allow them to buffer effectively up to about *p*H 5.5 to 6.5. The unsuccessful compounds do not have $_pK_a$ values in this range. The reversal of inhibition by $CaCO_3$, KOH addition, or phosphate buffer was due to maintenance of a favorable *p*H for spore and crystal synthesis. Furthermore, at a neutral *p*H, the increase in specific activity of aconitate hydratase and the removal of acetate from the medium were not prevented by α-picolinate. These observations indicate that tricarboxylic acid-cycle activity may have been proceeding normally. This is significant in view of the reports of Hanson et al. (14) and Fortnagel and Freese (6) who used *B. subtilis* mutants lacking aconitate hydratase or isocitric dehydrogenase to show that tricarboxylic acid-cycle activity was necessary for sporulation. Since α-picolinate was only effective against aconitate hydratase in vivo at an acidic *p*H (itself capable of inhibiting sporulation) and was ineffective against both the increase in enzyme activity and sporulation at a neutral *p*H, no conclusions can be drawn from these data regarding a link between tricarboxylic acid-cycle activity and spore and crystal formation in *B. thuringiensis*.

The reason for the failure of α-picolinate to prevent the synthesis of aconitate hydratase in buffered GYS broth is unknown. It may be that the cell is not permeable to this agent at a neutral *p*H or that the enzyme is not as easily inactivated at a neutral *p*H as at an acidic *p*H. It has been postulated (7, 8) that α-picolinate exerts its effect as an inhibitor by chelation of required cations. This effect should, however, be more pronounced at a neutral than at an acidic *p*H. The ability of α-picolinate to prevent the normal increase in specific activity of aconitate hydratase and its apparent inhibition of existing enzyme may both be reflections of the same activity. Chelation of a specific cation could prevent proper assembly or activation of the enzyme early in growth and could also inactivate the enzyme after its formation. It must be noted that inhibition of spore and crystal formation by this agent added after the increase in aconitate hydratase activity may have been due to inhibition of any of several other enzymes concerned with catabolism of acids in the medium.

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LITERATURE CITED

1. Anfinsen, C. B. 1955. Aconitase from pig heart muscle, p. 695–698. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
2. Angus, T. A. 1954. A bacterial toxin paralyzing silkworm larvae. Nature 173:545–546.
3. Angus, T. A. 1956. Association of toxicity with protein-crystalline inclusions of *Bacillus sotto* Ishiwata. Can. J. Microbiol. 2:122–131.
4. Angus, T. A. 1956. Extraction, purification, and properties of *Bacillus sotto* toxin. Can. J. Microbiol. 2:416–426.
5. Cooksey, K. E. 1968. Purification of a protein from *Bacillus thuringiensis* toxic to larvae of Lepidoptera. Biochem. J. 106:445–454.
6. Fortnagel, P., and E. Freese. 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. 95:1431–1438.
7. Fortnagel, P., and E. Freese. 1968. Inhibition of aconitase by chelation of transition metals causing inhibition of sporulation in *Bacillus subtilis*. J. Biol. Chem. 243:5289–5295.
8. Gollakota, K. G., and H. O. Halvorson. 1960. Biochemical changes occurring during sporulation of *Bacillus cereus*. J. Bacteriol. 79:1–8.
9. Gollakota, K. G., and H. O. Halvorson. 1960. Biochemical changes occurring in *Bacillus cereus* during sporulation. *In* H. O. Halvorson (ed.), Spores II. Burgess Publishing Co. Minneapolis.
10. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751–766.
11. Hannay, C. L., and P. Fitz-James. 1955. The protein crystals of *Bacillus thuringiensis* Berliner. Can. J. Microbiol. 1:694–710.
12. Hanson, R. S., V. R. Srinivasan, and H. O. Halvorson. 1963. Biochemistry of sporulation. I. Metabolism of acetate by vegetative and sporulating cells. J. Bacteriol. 85:451–460.
13. Hanson, R. S., V. R. Srinivasan, and H. O. Halvorson. 1963. Biochemistry of sporulation. II. Enzymatic changes during sporulation of *Bacillus cereus*. J. Bacteriol. 86:45–50.
14. Hanson, R. S., J. Blicharska, and J. Szulmajster. 1964. Relationship between the tricarboxylic acid cycle enzymes and sporulation of *B. subtilis*. Biochem. Biophys. Res. Commun. 17:1–7.
15. Holmes, K. C., and R. E. Monro. 1965. Studies on the structure of parasporal inclusions from *Bacillus thuringiensis*. J. Mol. Biol. 14:572–581.
16. Ignoffo, C. M. 1963. A successful technique for mass-rearing cabbage looper on a semisynthetic diet. Ann. Entomol. Soc. Amer. 56:178–182.
17. Monro, R. E. 1961. Protein turnover and the formation of protein inclusions during sporulation of *Bacillus thuringiensis*. Biochem. J. 81:225–232.
18. Murrell, W. G. 1967. The biochemistry of the bacterial endospore. Advan. Microbial Physiol. 1:133–251.
19. Nakata, H. M., and H. O. Halvorson. 1960. Biochemical changes occurring during growth and sporulation of *Bacillus cereus*. J. Bacteriol. 80:801–810.
20. Norris, J. R., and D. H. Watson. 1960. An electron microscope study of sporulation and protein crystal formation in *Bacillus cereus* var. *alesti*. J. Gen. Microbiol. 22:744–749.
21. Schaeffer, P. 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol. Rev. 33:48–71.
22. Vanková, J., and O. Králik. 1966. An electron microscope study of protein crystals in different strains of the *Bacillus thuringiensis* group. Zentbl. Bakteriol. Parasitenk. Infektionskr. Hyg. 199:380–389.