Regulation of Dipicolinic Acid Biosynthesis in Sporulating *Bacillus cereus* CHARACTERIZATION OF ENZYMIC CHANGES AND ANALYSIS OF MUTANTS

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Some of the early enzymes in the lysine-biosynthetic pathway also function for dipicolinic acid synthesis in sporulating *Bacillus cereus* T. 1. The first enzyme, aspartokinase, loses its sensitivity to feedback inhibition by lysine. This change occurs before the time of dipicolinic acid synthesis but at a time when diaminopimelic acid is required for spore cortex formation. 2. A possible regulatory change at a branch point in the pathway was studied by examining the properties of a key enzyme, dihydrodipicolinic acid reductase. No alteration in the feedback sensitivity or sedimentation rate of this enzyme could be detected during sporulation. 3. Two mutants producing heat-sensitive spores were analysed. Both produced spores that contained decreased amounts of dipicolinic acid. Although neither was a lysine auxotroph, they both had greatly decreased activities of certain lysine-biosynthetic enzymes in sporulating cells. 4. Starvation of cells for calcium also results in the production of spores that are heat-sensitive and contain less dipicolinic acid than the control. A decreased content of one of the lysine-biosynthetic enzymes, dihydrodipicolinic acid synthetase, in calcium-starved cells could account for the lower concentration of dipicolinic acid in the spores.

Dipicolinic acid is a prominent low-molecular-weight component of bacterial spores that is not present in vegetative cells (Powell, 1953). Neither its function nor its localization is known, but spores deficient in dipicolinic acid as a result of mutation (Aronson *et al.*, 1967; Fukuda & Gilvarg, 1968; Halvorson & Swanson, 1969) or calcium starvation (Black *et al.*, 1960) have increased heat-sensitivity and altered rates of germination. The synthesis of dipicolinic acid occurs very late in sporulation and is correlated with an increased incorporation of calcium into the endospore (Vinter, 1963). The biosynthesis of dipicolinic acid poses some interesting regulatory problems since both mutant analysis (Aronson *et al.*, 1967; Fukuda & Gilvarg, 1968) and studies *in vitro* (Bach & Gilvarg, 1966; Chasin & Szulmajster, 1969) have shown that some of the early reactions in the lysine-biosynthetic pathway (Scheme 1) are essential. On the basis of the lysine pathway as worked out in *Escherichia coli* (Cohen, 1968, and Scheme 1), it would appear that dihydrodipicolinic acid is a pivotal intermediate since oxidation, rather than further reduction, can result in production of dipicolinic acid. There is evidence from experiments *in vitro* for an enzymic activity appearing during sporulation that catalyses this oxidation (Bach & Gilvarg, 1966; Chasin & Szulmajster, 1969).

In addition to the possibility of such a 'shunt', there are some regulatory changes occurring in the lysine pathway, which may be important for the synthesis of dipicolinic acid. In *Bacillus cereus* T the first enzyme in the lysine pathway, aspartokinase (ATP-L-

aspartate 4-phosphotransferase, EC 2.7.2.4), is inhibited only by lysine. During sporulation, however, aspartokinase becomes insensitive to lysine (Aronson *et al.*, 1967) and the last enzyme in the pathway, diaminopimelate decarboxylase (*meso*-2,6-diaminopimelate carboxy-lyase, EC 4.1.1.20), is apparently inactivated (Grandgenett & Stahly, 1968). As a result, a continued flow of carbon atoms into dipicolinic acid and into diaminopimelic acid, another molecule important in the structure of the spore cortex, is ensured even in the presence of an excess of lysine.

The imposition of a new branch point in the biosynthetic pathway could mean that other regulatory mechanisms may be invoked during sporulation to ensure an appropriate flow of carbon atoms to both dipicolinic acid and diaminopimelic acid. We have examined this possibility by (1) studying the properties of dihydrodipicolinate reductase (see Scheme 1) during growth and sporulation and (2) attempting to isolate mutants specifically altered in dipicolinic acid synthesis in the hope of elucidating the relevant control mechanisms. Although we have not succeeded in our original intent, we have characterized mutants with phenotypic alterations relevant to this problem. We have also re-examined the observation by Black *et al.* (1960) that calcium depletion results in an inhibition of dipicolinic acid synthesis and have obtained evidence for a specific effect of calcium starvation on one of the relevant enzymes. Overall, our results help to define better the problem of the control of dipicolinic acid synthesis.

Scheme 1. A portion of the lysine pathway in *E. coli* and related branch points

See Cohen (1968) for a summary of relevant references. Dashed arrow refers to hypothetical conversion of dihydrodipicolinic acid into dipicolinic acid. Enzymes: 1, aspartokinase; 2, aspartic β -semialdehyde dehydrogenase; 3, dihydrodipicolinic acid synthetase (condensing enzyme); 4, dihydrodipicolinic acid reductase; 5, diaminopimelic acid decarboxylase. It should be noted that *B. cereus* T requires leucine, valine, threonine and methionine (Nakata, 1964) and is thus probably missing the branch to homoserine.

Experimental

Organisms and growth

B. cereus T was grown in G medium (Gollakota & Halvorson, 1960) modified by the addition of 0.01 M-tris-HCl, pH 7.6 (G-tris). Cultures were grown at 30°C as described by Levisohn & Aronson (1967). Growth was followed in a Coleman 8 colorimeter by using a 650 nm filter. The stage of sporulation was determined by examination of wet mounts in a Zeiss phase-contrast microscope.

Mutants producing heat-sensitive spores were isolated after treatment of exponentially growing cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) as described by Levisohn & Aronson (1967). After treatment, cells were incubated until free spores were produced. These were plated on a synthetic medium, CDGS agar (Nakata, 1964), and incubated at 30°C until spores were formed (usually 48 h). The plates were replicated and one set was placed in an oven (Fisher Isotemp) at 80°C for 75 min. All presumptive mutants were again tested for heat sensitivity and spore formation. Two mutants designated HS14 and HS29 were selected for further study.

Selection of revertants

Revertants of mutant HS29 were selected by plating dilutions of a culture of spores heated at 80°C for 20 min. Between 0.1 and 0.5% of the original spores survived the heating. After growth and sporulation, the plates were replicated and one set was heated at 80°C for 75 min. About 50% survived and several were picked, grown in G-tris medium until spores were formed; the spores were then assayed for dipicolinic acid content. Of 30 colonies screened, two were found to contain approximately normal amounts of dipicolinic acid.

For mutant HS14 revertants, a double selection technique was found necessary, since simple screening for heat-resistant colonies resulted only in the selection of heat-stable spores still containing small amounts of dipicolinic acid. It had been reported that dipicolinic acid-deficient spores germinate slowly (Halvorson & Swanson, 1969) and slow germination was also found for mutant HS14. Spores purified by centrifugation at 15000g for 15 min in a Sorvall SS-34 rotor (r_{av} , 10.6 cm) through a solution of 33% (w/v) methylglucamine diatrizoate plus 5% (w/v) sodium diatrizoate [38% (w/v) Renografin-76; E. R. Squibb and Sons, New York, U.S.A.] were first activated by heating at 60°C for 15 min (90% survival) and then suspended in a germination medium consisting of 0.05 M-potassium phosphate, pH 8.0, plus 2 mM-L-alanine and 1 mM-adenosine. After incubation

at 27°C for 10 min, there was a 10% decrease in E_{625} for the mutant spores. Wild-type spores would have been almost completely germinated (90% decrease in E_{625}) in this time. The partially germinated suspension of mutant spores was mixed with an equal volume of 38% Renografin, layered on 5 ml of 38% Renografin and centrifuged as described above. The top 1 ml containing germinated spores was removed with a syringe, diluted and plated on G-tris agar. About 0.1% of the original spores were recovered in this layer. The plates were incubated until free spores were formed and then replicated and heated as described for mutant HS29. Colonies appearing on the heated plates within 18 h were selected for further analysis. Two colonies from about a thousand appeared early and contained normal amounts of dipicolinic acid.

Preparation of extracts and enzyme assays

Crude extracts of exponentially growing or sporulating cells were obtained by sonication (Branson no. 4 instrument, at no. 4 setting for 1 min). In one case, extracts were prepared by osmotic lysis of protoplasts. Cells growing exponentially in G-tris medium (E_{650} 0.12) were collected by centrifugation in a Sorvall RC2B SS-34 rotor at 12000g (r_{av} , 10.6 cm) for 8 min, washed once with G-tris medium and resuspended to E_{650} 0.35 in G-tris medium supplemented with 0.3 M-sucrose – 0.01 M-MgCl₂. Lysozyme (100 µg/ml) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added and the cultures incubated on a New Brunswick rotary shaker at 30°C for 45 min. About 75–85% of the cells were converted into protoplasts as judged by phase-contrast microscopy. The protoplasts were collected by centrifugation as described above and the pellet was gently resuspended in 2–3 ml of 0.02 M-tris – HCl – 0.5 mM-MgCl₂, pH 7.8. Deoxyribonuclease (5 µg/ml) (Worthington Biochemical Corp.) was added for 10 min at 27°C to lower the viscosity. The suspension was centrifuged in a Sorvall SS-34 rotor at 15000g for 15 min (r_{av} , 10.6 cm) and the supernatant layered on sucrose gradients for the determination of the sedimentation rate of the dihydrodipicolinic acid reductase activity as described below.

Aspartokinase was assayed as previously described (Aronson *et al.*, 1967), by the procedure of Stadtman *et al.* (1961). After incubation for 10 min at 26°C, the formation of aspartohydroxamic acid was measured at 540 nm and the value subtracted from an appropriate blank. The units used are $\Delta E_{540} \times 1000$. Activity was measured in either crude extracts prepared in 0.02 M-tris – 0.005 M-MgCl₂ – 0.01 M-mercaptoethanol – 0.15 M-KCl – 0.0005 M-L-lysine, pH 7.8, or a 35–60%-(w/v)-(NH₄)₂SO₄ precipitate dissolved in the same buffer. Aspartic semialdehyde dehydrogenase was assayed by the procedure of Black

& Wright (1955) in heated extracts prepared as described by Aronson *et al.* (1967). The dihydrodipicolinic acid synthetase was assayed by the *o*-aminobenzaldehyde method of Yugari & Gilvarg (1965) with either crude extracts or partially purified preparations. For the latter, the procedure of Yugari & Gilvarg (1965) was followed, but after the second acid step the preparation was dialysed overnight at 4°C against 50 vol. of 0.006M-tris-HCl buffer, pH 8.0, and assayed.

Dihydrodipicolinic acid reductase was assayed by the procedure of Farkas & Gilvarg (1965). To generate the unstable substrate, dihydrodipicolinic acid, a preparation of dihydrodipicolinic acid synthetase was purified from *E. coli* ML30 by the procedure of Yugari & Gilvarg (1965). The purification procedure was performed up to the dialysis step after the second acid fractionation. Since there was extensive non-specific NADPH dehydrogenase activity in crude *B. cereus* extracts, it was necessary to purify the reductase partially before assay. Streptomycin (10mg/ml) was added to crude extracts in 0.02M-tris-HCl-0.005M-MgCl₂, pH 7.8, and the mixture was then left on ice for 40 min. After removal of the precipitate by centrifugation in a Sorvall RC2B SS-34 rotor at 12000g (r_{av} , 10.6cm) for 10 min, the supernatant was fractionated with solid (NH₄)₂SO₄. The 25–60% (w/v) precipitate was dissolved in the above buffer and dialysed against 50 vol. of this buffer for 16 h at 4°C. All values reported are specific activities with units determined as in the references cited. Each value ($\pm 8\%$) represents the average of at least two determinations on separate cultures.

Sucrose-gradient centrifugation

The approximate sedimentation coefficient of the dihydrodipicolinic acid reductase was determined by the procedure of Martin & Ames (1961). Extracts, prepared by osmotic lysis of protoplasts or by sonication, were layered on 5–20% (w/v) linear sucrose gradients (sucrose dissolved in 0.02M-tris-HCl-0.005M-MgCl₂, pH 7.8). Portions (each 2mg) of cytochrome *c* (mol.wt. 12400; C. F. Boehringer, Mannheim, Germany) and bovine serum albumin (mol.wt. 69000; Pentex, Miles Lab, Kankakee, Ill., U.S.A.) were mixed with the extract and the tubes centrifuged in an SW50.1 rotor at 189000g (r_{av} , 8.35cm) for 16 h. Samples (12 drops; 0.2ml) were collected by needle puncture and alternate tubes were used for assay of reductase or the marker proteins. The composite results from three separate experiments were combined to determine the sedimentation rates of the reductase preparations. Molecular weights of the markers were taken from Fish *et al.* (1969).

Determination of protein, dipicolinic acid and ⁴⁵Ca uptake

For protein determination, samples were pipetted into 5ml of 7% (w/v) trichloroacetic acid and incubated at 0°C for 30 min, and the pellet was collected by centrifugation in a Sorvall RC2B SS-34 rotor at 12000g (r_{av} , 10.6cm) for 8 min. The pellet was washed once with 7% trichloroacetic acid and dissolved in 1–2ml of 0.2M-NaOH for the colour test (Lowry *et al.*, 1951). For dipicolinic acid determination, 2ml of spores were centrifuged as described above, then suspended in 2ml of deionized water and processed as described by Janssen *et al.* (1958).

Portions (0.1 μ Ci/ml) of ⁴⁵CaCl₂ (New England Nuclear Corp., Boston, Mass., U.S.A.; 10.8mCi/mg) were added directly to G-tris medium at various stages of sporulation. Samples (0.2ml) were removed and pipetted into 4.0ml of G-tris-salts medium at 0°C, i.e. G-tris medium without glucose and containing three times the normal concentration of CaCl₂. The cells were collected on glass-fibre filters (Reeve-Angel 934AH), dried, placed in vials containing 5ml of Omnifluor (Packard Inst., Downers Grove, Ill., U.S.A.) and counted for radioactivity in a Packard 3000 scintillation counter. Under these conditions ⁴⁵Ca is counted with 35% efficiency.

Dipicolinic acid was purchased from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.), a mixture of LL- and meso-diaminopimelic acid from Pierce Chemical Co. (Rockford, Ill., U.S.A.) and DL- α -diaminopimelic acid from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Results

Characterization of the aspartokinase and dihydrodipicolinic acid reductase

Although a loss of sensitivity of the aspartokinase to inhibition by lysine is consistent with the requirements for a 'shunt' in the pathway, the time of the change is somewhat earlier than the commencement of dipicolinic acid synthesis (Table 1). This change is apparently due to an alteration of the vegetative enzyme rather than a new activity, since a lysine auxotroph (which reverts with the frequency expected of a point mutation) with low aspartokinase activity is also defective in dipicolinic acid synthesis (Aronson *et al.*, 1967). One possible explanation for the early change is that another intermediate in the lysine pathway, diaminopimelic acid, is required for spore-cortex formation. Cortex synthesis begins before, but overlaps with, the synthesis of dipicolinic acid (Vinter, 1963; Pitel & Gilvarg, 1970) and the desensitization of the aspartokinase may be necessary to ensure an adequate supply of diaminopimelic acid.

A second lysine-biosynthetic enzyme that could have a role in regulating the flow of intermediates to

Table 1. *Change in lysine sensitivity of B. cereus T aspartokinase during growth and sporulation*

G-tris medium (500ml) in a 2-litre Erlenmeyer flask was inoculated with a final concentration of 10^5 spores/ml. The culture was incubated at 30°C on a New Brunswick rotary shaker. Samples (100ml) were removed at the indicated times for preparation of aspartokinase. Aspartokinase was assayed in the presence and absence of 2mM-lysine in 35–60%-(w/v)-(NH₄)₂SO₄ precipitates as described in the Experimental section. Growth was followed by determining E_{650} in a Coleman 8 colorimeter. The stage of sporulation was based on previous results where [³H]diaminopimelic acid incorporation and amount of dipicolinic acid present had been determined. These stages are only approximate because of asynchrony in the culture.

Cell age (h)	Stage of growth or sporulation	Specific activity of aspartokinase ($\Delta E_{540} \times 1000/\text{mg}$ of protein)	Inhibition by lysine (%)
7	Exponential	7	100
10	End of growth	10	100
12	Early spore formation	15	80
14	Cortex synthesis (diaminopimelic acid incorporation)	28	15
16	Beginning of dipicolinic acid synthesis	32	10

Table 2. *Feedback properties of dihydrodipicolinic acid reductase*

Cells were grown as described in Table 1 and 200ml samples were removed during exponential growth or when >80% of the cells contained phase-white (not refractive in the phase-contrast microscope) endospores. The dihydrodipicolinic acid reductase was partially purified as described in the Experimental section and assayed in the presence of diaminopimelic acid (100–400 μmol of either DL- $\alpha\epsilon$ -diaminopimelic acid or a mixture of LL- and meso-diaminopimelic acid/ml) and dipicolinic acid (100 μmol /ml). The enzyme was incubated at 27°C for 5 min with diaminopimelic acid or dipicolinic acid before assay.

Growth stage	$\Delta E_{340}/\text{min}$ per mg of protein		
	Control	+Diaminopimelic acid	+Dipicolinic acid
Exponential	0.03	0.03	0.03
Phase-white endospores	0.23	0.23	0.23

dipicolinic acid is dihydrodipicolinic acid reductase (see Scheme 1). There is considerable reductase activity in sporulating cells, however, and no feedback inhibition to indicate a site of regulation (Table 2). Regulatory changes could also result from an altered size or state of aggregation of the enzyme, but no such change was found when the sedimentation rate of reductase activity in an extract prepared by gentle lysis of protoplasts was compared with that of the reductase in an extract prepared by sonication of sporulating cells (Fig. 1). There was a high non-specific NADPH oxidation activity in crude extracts (20–25% of the total activity) but the very similar sedimentation profiles of the two preparations (calculated mol.wt. 52000) indicate that neither subunit dissociation nor extensive proteolysis (Sadoff *et al.*, 1970) had occurred.

Characterization of mutants producing heat-sensitive spores

The properties of two mutants studied in detail are summarized in Table 3. They both produce heat-

sensitive spores with a smaller content of dipicolinic acid. There is no requirement for lysine for growth since both mutants were selected on a synthetic medium (see the Experimental section). Since the enzymes of the early part of the lysine-biosynthetic pathway have been implicated in dipicolinic acid synthesis, however (Aronson *et al.*, 1967; Fukuda & Gilvarg, 1968), they were assayed in these mutants (Table 4). In the wild-type all of the activities increased during sporulation, probably owing to a decreased lysine concentration resulting from utilization of the lysine in the medium and inactivation of the diaminopimelic acid decarboxylase (Grandgenett & Stahly, 1968). This de-repression also occurred in the mutants, but with two notable exceptions: (1) the lower activity of dihydrodipicolinic acid synthetase in phase-white mutant HS14 and (2) the lack of increase of aspartokinase in phase-white mutant HS29. In both cases revertants producing normal concentrations of dipicolinic acid could be selected, at about the frequency expected for an initial point mutation (Table 3). Interestingly, there was a much higher reversion frequency in both cases to heat-resistant

Fig. 1. Sedimentation profiles of dihydrodipicolinic acid reductase activities in 5–20% sucrose gradients

(a) A preparation from the osmotic lysate of exponentially growing cells; (b) a preparation by sonication of phase-white sporulating cells. Recovery of activity: (a) 91%; (b) 85%. Arrows indicate position of peak tubes containing bovine serum albumin (BSA) or cytochrome *c* (Cyt.).

spores, which still contained less than the normal amount of dipicolinic acid, but these have not been further studied. One true revertant of each mutant was selected for enzymic analysis and was found to contain normal activities of the relevant enzymes. It is therefore likely that there is a causal relationship

between the altered activities of the biosynthetic enzymes and the phenotypic properties of the mutant. The dihydrodipicolinate synthetase activity did not increase as extensively in HS29 as in the wild-type (Table 4). This difference may be due to differences in the extent of synchrony. The dihydrodipicolinate reductase activity in HS29 is also lower than the wild type although the activity increases tenfold in both strains. The differences in specific activity is not understood but may be a consequence of the mutation in HS29.

Since the aspartokinase of *B. cereus* T is very unstable, no further studies on mutant HS29 were feasible except to show that there was no increase of aspartokinase during sporulation, even in a medium devoid of lysine [a synthetic medium (Nakata, 1964), supplemented with 0.2% (w/v) Difco lysine assay medium].

The changing patterns of dihydrodipicolinic acid synthetase activity in mutant HS14 and the wild-type were further compared (Fig. 2). The activity in the mutant decreased during sporulation rather than showing the increase found in the wild-type. The activity of the two enzymes functioning earlier in the lysine pathway (Scheme 1) did increase (Table 4), so it is unlikely that HS14 is a regulatory mutant. In addition, the partially purified dihydrodipicolinic acid synthetase from the mutant was more heat-sensitive than that from the wild-type (Fig. 3). Results similar to those shown in Fig. 3 were obtained with enzyme purified from exponentially growing cells or cells containing phase-white endospores. In a revertant of mutant HS14, both the amount (not shown) and the heat-sensitivity (Fig. 3) of the dihydrodipicolinate synthetase in sporulating cells were like the wild-type.

The activity of the next enzyme in the lysine pathway, dihydrodipicolinic acid reductase, was also altered in mutant HS14 (Fig. 4), showing an increase during sporulation that was not as great as in the wild-type. Here, secondary effects of the mutation could influence the extent of de-repression of this enzyme.

Effect of calcium depletion on dipicolinic acid synthesis

Both of the mutants discussed above phenotypically resemble the wild-type starved for calcium in that they produce heat-sensitive spores deficient in dipicolinic acid (Black *et al.*, 1960). Spores are relatively rich in calcium and there is a marked increase in the rate of calcium uptake into sporulating cells just before dipicolinic acid synthesis (Vinter, 1962). It appears likely that at least some of the calcium is bound to dipicolinic acid in a chelate complex (Murrell, 1969). If so, it would not be surprising that cells unable to synthesize dipicolinic acid would have a decreased amount of calcium in their spores. Mutants HS14 and HS29 appear to fall into this category, since the uptake of ^{45}Ca into sporulating

Table 3. *Properties of B. cereus T mutants producing heat-sensitive spores*

Growth rates were determined by inoculating 10ml of G-tris medium in 300ml Nephelometer flasks (Bellco Glass Co.) with a final concentration of 10^5 spores/ml (from slants). The flasks were incubated at 30°C in a New Brunswick rotary water-bath shaker and the E_{650} was determined. The flasks were incubated for 28h to produce free spores, which were plated in triplicate on G-tris agar before and after heating to determine final spore yield and % survival. Samples (2ml) of the spores were centrifuged, suspended in deionized water and autoclaved for dipicolinic acid determination. Reversion frequencies are estimates based on the values presented in the Experimental section and are lower limits, since not all possible revertants were scored.

Strain	Growth rate (generations/h)	Survival of spores at 70°C/20min (%)	Final spore concentration (per ml)	Dipicolinic acid (fg/spore)	Reversion frequency
Wild-type	1.10	85–98	7.8×10^8	130	—
HS14	1.05	15	7.5×10^8	25	$1/(5 \times 10^4)$ – $1/10^6$
HS29	1.15	12	7.8×10^8	48	$1/10^5$ – $1/10^6$

Table 4. *Specific activities of lysine-biosynthetic enzymes in B. cereus T wild-type and mutants producing heat-sensitive spores*

For each enzymic assay, 500ml of G-tris medium was inoculated and incubated as described in Table 1. A sample (250ml) was removed during exponential growth (7–8h) and another (200ml) when at least 80% of the cells contained phase-white endospores (15–17h). The cells were washed with 2×50 ml of the buffer required for the assay and broken by sonication. The extracts were then purified as described in the Experimental section except for aspartokinase, which was assayed in crude extracts. Samples were taken for determination of protein and specific activities calculated.

Strain	Growth stage	Aspartokinase ($\Delta E_{540} \times 1000$ per mg of protein)	Aspartic semialdehyde dehydrogenase (nmol of NADPH/min per mg of protein)	Dihydrodipicolinic acid synthetase (ΔE_{540} /min per mg of protein)	Dihydrodipicolinic acid reductase (ΔE_{340} /min per mg of protein)
Wild-type	Exponential	7	40	0.01	0.025
	Phase-white endospores	38	365	0.14	0.25
HS14	Exponential	5.5	38	0.01	0.025
	Phase-white endospores	32	318	0.006	0.055
HS29	Exponential	7	36	0.008	0.01
	Phase-white endospores	6.5	312	0.03	0.10

cells of the mutants differed from the control in the lower plateau values (Fig. 5). The initial rates of uptake were very similar. The final ^{45}Ca content per spore of mutant HS14 was 25–30%, and of mutant HS29 35–40%, of the control.

It is more difficult to understand how calcium deprivation inhibits dipicolinic acid synthesis (Black *et al.*, 1960). Cells were deprived of calcium by subculturing three times in G-tris medium prepared without CaCl_2 . There was no further change in the properties of the cells or spores on continued culturing (five or six transfers) in the calcium-deficient medium. The properties of the cells and spores in the third subculture are summarized in Table 5. Both the

growth rate and final spore concentration were decreased. The spores had about 30% of the control concentration of dipicolinic acid and were relatively heat-sensitive, with about 20% survival (as compared with the control) after heating at 68°C for 30min and 35% survival after heating at 80°C for 20min.

An examination of the lysine-biosynthetic enzymes implicated in dipicolinic acid synthesis in extracts prepared from cells deprived of calcium provides a possible explanation for the lower dipicolinic acid content (Table 6). The dihydrodipicolinic acid synthetase activity in such extracts is markedly decreased, thus mimicking mutant HS14. Exactly how calcium deprivation affects this activity is not clear, since

Fig. 2. Comparison of dihydrodipicolinic acid synthetase specific activities in *B. cereus* T wild-type (▲) and mutant HS14 (●)

G-tris medium (500ml) was inoculated and incubated as described in Table 1. The cells were harvested at the indicated times and the enzyme partially purified as described in the Experimental section. Each point represents the average of at least three determinations ($\pm 5\%$). Arrow (1) indicates end of growth; (2) phase-white endospores in at least 50% of the cells; (3) commencement of dipicolinic acid synthesis.

the enzyme from non-starved cells is not sensitive to EDTA or *o*-phenanthroline, nor does addition of calcium to extracts prepared from starved cells restore activity.

Fig. 3. Heat-sensitivities of partially purified dihydrodipicolinic acid synthetase

The synthetase was prepared from sporulating (9h cultures; see Fig. 2) *B. cereus* T wild-type (●), mutant HS14 (▲) and a revertant of HS14 (○). Samples (2ml) of the enzyme preparations were placed in a water-bath at 65°C and samples (0.2ml) were removed at the indicated times into centrifuge tubes at 0°C. The samples were centrifuged at 12000g for 10min and the supernatants assayed.

Discussion

During sporulation of *B. cereus* T, the lysine-biosynthetic pathway is utilized for the synthesis of two important spore components, dipicolinic acid and diaminopimelic acid. Commensurate with these changing requirements is an alteration in the regulatory and enzymic properties of the pathway. Both the desensitization of the aspartokinase and the increased activity of at least the early biosynthetic enzymes results in a continued flow of precursors at concentrations presumably sufficient to supply both requirements. There may be two or more operons for the lysine-biosynthetic pathway as there apparently are in *E. coli* (Bukhari & Taylor, 1971). The first four enzymes may comprise one unit since they are de-repressed to about the same extent in the wild-type although both mutants are altered somewhat in the extent of de-repression (Table 4 and Fig. 4).

Neither mutant studied appears to possess the regulatory properties originally sought. They both show rather subtle alterations, which only become evident in sporulating cells. Apparently the physical and/or chemical environment changes sufficiently during sporulation to render these altered enzymes

Fig. 4. Change in specific activities of dihydrodipicolinic acid reductase with time for *B. cereus* T wild-type (▲) and mutant HS14 (●)

The cells were grown as described in Table 1 and 500ml was harvested at the indicated times. The enzyme was partially purified and assayed as described in the Experimental section. Each point represents the average of three determinations ($\pm 5\%$). The arrow indicates the time of end of exponential growth; the stages of sporulation are as in Fig. 2.

less active. There are precedents for alterations of a number of enzymes during sporulation (Kornberg *et al.*, 1968; Sadoff *et al.*, 1970) including the desensitization of aspartokinase or even a relative increase in the amount of a lysine-insensitive aspartokinase (Rosner & Paulus, 1971) and the inactivation of the diaminopimelic acid decarboxylase (Grandgenett & Stahly, 1968). The existence of mutants with alterations in lysine-biosynthetic enzymes detected only

Fig. 5. Incorporation of ⁴⁵Ca into sporulating cells of *B. cereus* T wild-type (●), mutants HS29 (▲) and HS14 (○)

G-tris medium (200ml) in a 2-litre flask was inoculated and incubated as described in Table 1. After 50% of the cells contained phase-white endospores, 3ml portions were removed at hourly intervals and placed in sterile 125 ml Erlenmeyer flasks, which were incubated in a New Brunswick water-bath shaker. To each flask, ⁴⁵Ca (0.1 μCi/ml) was added and 0.2ml samples were taken over a 60min period and processed as described in the Experimental section. The results are the maximum rates of incorporation found for each culture.

during sporulation suggests that mutants unable to synthesize dipicolinic acid but without a lysine requirement for growth (Halvorson & Swanson, 1969) may still have alterations in the lysine-biosynthetic enzymes. Mutations in other spore-specific functions should also be carefully examined before implicating a spore-specific enzyme.

The question of regulation at the new branch point is not settled. Since both branches of the pathway must function simultaneously during sporulation, perhaps just an increased flow of precursors is sufficient. The de-repression of the early biosynthetic

Table 5. *Properties of spores produced in B. cereus T starved for calcium*

Cells were grown in 20ml of G-tris or G-tris prepared without CaCl_2 (usually 8mg/ml) in 300ml Nephelometer flasks and the various parameters determined as in Table 3, except that the final spore concentrations are direct counts in a Petroff Hauser chamber of phase-bright spores. A total of at least 300 spores was counted ($\pm 15\%$).

Growth conditions	Growth rate (generations/h)	Final spore concentration (per ml)	Dipicolinic acid (fg/spore)	Survival after heating (%)	
				At 68°C/30 min	At 80°C/20 min
G-tris	1.05	7.5×10^8	110	80	95
Third subculture in G-tris without CaCl_2	0.7	3.9×10^8	35	13	33

Table 6. *Specific activities of lysine-biosynthetic enzymes in B. cereus T wild-type grown in the presence and absence of CaCl_2*

For each determination, spores formed in G-tris medium (with or without CaCl_2) (second subculture) were inoculated into 500ml of G-tris (with or without CaCl_2) and incubated as described in Table 1. The cells were harvested after 17h (some phase-bright spores; dipicolinic acid synthesis beginning), washed twice with the appropriate buffer (40ml each time) and extracts prepared by sonication (see the Experimental section). The specific activities were determined as described in Table 4.

Growth conditions	Specific activities			
	Aspartokinase ($\Delta E_{540} \times 1000$ per mg of protein)	Aspartic semialdehyde dehydrogenase (nmol of NADPH/min per mg of protein)	Dihydrodipicolinic acid synthetase (ΔE_{540} /min per mg of protein)	Dihydrodipicolinic acid reductase (ΔE_{340} /min per mg of protein)
Control (G-tris medium)	31	285	0.13	0.25
Third subculture in G-tris without CaCl_2 added	27	110	0.008	0.20

enzymes coupled with the appearance of dipicolinic acid synthetase activity (Chasin & Szulmajster, 1969; Bach & Gilvarg, 1966) may account for the 'shunt'. Unfortunately, dipicolinic acid synthetase is very unstable (Chasin & Szulmajster, 1969), not yet well characterized and, at least in our experience, very difficult to assay. In addition, there is a rather extensive non-enzymic conversion of dihydrodipicolinic acid into dipicolinic acid (Chasin & Szulmajster, 1969), so other possible regulatory factors could be considered. For example, the availability of NADP (or NAD) as reflected in the intracellular ratio of the oxidized to reduced form of the coenzyme may be critical.

Spores with phenotypic properties similar to the mutants can be produced by growth and sporulation in a calcium-deficient medium. Since calcium/dipicolinic acid ratios in spores are generally constant (about 1.0) and since calcium and dipicolinic acid can form a stable chelate complex (Murrell, 1969), it is not surprising that a block in dipicolinic acid syn-

thesis would result in a lower calcium content of the spore. Indeed, although the initial rates of uptake of ^{45}Ca by the mutants and wild-type are the same (Fig. 5), the amount retained by the mutants is less.

The complementary effect of calcium deprivation on dipicolinic acid synthesis is unexpected but can be explained by the low activity of dihydrodipicolinic acid synthetase in calcium-starved cells (Table 6). We have not been able to show a direct involvement of calcium with this enzyme; neither inhibition by adding EDTA to active extracts nor restoration of activity by adding calcium to extracts from deprived cells was found. A somewhat analogous situation exists with respect to alkaline phosphatase and the metal cofactor zinc. Cells grown in the presence of EDTA have decreased enzymic activity, which is not restored by the addition of zinc (Dvorak, 1968). The lack of restoration is probably due to the instability of the subunits (Schlessinger, 1965).

Although the mutants studied here, and others (Aronson *et al.*, 1967; Fukuda & Gilvarg, 1968), show

a correlation between a lower dipicolinic acid content and heat-sensitivity of the spores, there is no evidence for a causal relationship. In fact, a survey of spores produced by many species shows no correlation between dipicolinic acid content and heat-resistance but there is a suggestive correlation between diamino-pimelic acid content and heat-resistance (Murrell & Warth, 1965). In addition, many of the 'revertants' of mutants HS14 and HS29 selected for production of heat-resistant spores still contained low or intermediate amounts of dipicolinic acid relative to the wild type. Since all of our dipicolinic acid-deficient mutants are altered in lysine-biosynthetic enzymes, it is possible that they are also altered in the diamino-pimelic acid content of the cortex. Further analysis of our heat-resistant low-dipicolinic acid 'revertants' should help to clarify this relationship.

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