Sporulation in *Bacillus subtilis*

BIOCHEMICAL CHANGES

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1. During the course of growth and sporulation of *Bacillus subtilis* in chemically defined media, measurements were made of 16 different parameters, including the specific activities of nine intracellular enzymes. 2. Towards the end of exponential growth, proteolytic activity increased and reached a maximum soon after growth ceased. 3. In the presence of an excess of phosphate the specific activity of alkaline phosphatase increased fivefold at the end of exponential growth. 4. The specific activity of malate dehydrogenase remained at a high constant level throughout sporulation, but the specific activity of fumarase showed a two- to three-fold increase 5–9 hr. after the end of exponential growth. 5. Aconitase activity was barely detectable during exponential growth in a glucose–glutamate medium, but increased rapidly when glutamate was replaced by citrate or when the glucose in the medium was exhausted. 6. The specific activity of alanine dehydrogenase increased threefold 1–5 hr. after the end of exponential growth. 7. The specific activity of soluble NADH oxidase doubled 4–6 hr. after the end of exponential growth. 8. Glucose dehydrogenase was undetectable until 4 hr. after the end of exponential growth, but its specific activity increased 20-fold over the next 3–4 hr. 9. The onset of refractility, the synthesis of 2,6-dipicolinic acid and the appearance of heat-resistance occurred in this order some 6–12 hr. after the end of exponential growth. 10. The significance of these changes is discussed in relation to the morphological development of the spore.

Although a great deal of work has been carried out on the biochemistry of sporulation, the precise nature and sequence of the biochemical changes that occur is still only poorly understood (see Murrell, 1967). Nevertheless a number of biochemical events have been shown to be associated with sporulation, including the production of an anti-biotic (Balassa, Ionesco & Schaeffer, 1963), the production of an exoprotease and a wall-lytic enzyme (Schaeffer, 1967) and an increase in the specific activity of aconitase (Szulmajster & Hanson, 1965). During the later stages of spore formation Ca^{2+} ions are accumulated from the medium and DPA† is synthesized (Powell, 1953; Vinter, 1962). In addition, spores of *Bacillus cereus* contain a number of enzymes that are absent from or present in only very small amounts in vegetative cells. These include a heat-stable catalase (Baillie & Norris, 1963), glucose dehydrogenase (Bach & Sadoff, 1962), a soluble NADH oxidase (Doi & Halvorson, 1961), adenosine deaminase and ribos-

idase (Powell & Hunter, 1956). It has also been shown (Waites, 1968; Mandelstam & Waites, 1968) that new antigens appear during sporulation.

In the present work a number of measurements were made at intervals during sporulation, including determinations of the specific activities of nine different enzymes, the amounts of DPA present and the numbers of heat-resistant spores. A sequence of biochemical events was thus established and an attempt made to correlate this sequence with stages in the morphological development of the spore (see also Kay & Warren, 1968). These results have been reported in a preliminary form (Mandelstam, Waites & Warren, 1967).

MATERIALS AND METHODS

Organism. *Bacillus subtilis* (Marburg strain 168), which requires indole or tryptophan for growth, and two asporogenous mutants, E22 and E21, of this strain (see Mandelstam & Waites, 1968) were used. The first mutant is proteaseless, the second is asporogenous for some other reason.

Incubation and preparation of samples for assay. A loopful of spore suspension (wild-type) or a loopful of cells taken

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† Abbreviation: DPA, 2,6-dipicolinic acid.

from a plate (mutants E21 and E22) was inoculated into a small volume (1–5 ml.) of Difco Penassay Broth (Difco Antibiotic Medium 3) to which additional glucose (0.4%, w/v) had been added, and the culture was shaken for 6 hr. at 37°. Suitable dilutions of this culture were used to inoculate 1 l. amounts of a liquid sporulation medium, medium S, in 5 l. conical flasks. The medium was a 2.5-fold concentration of the medium of Donnellan, Nags & Levinson (1964) and contained FeCl_3 (0.009 mm), MgCl_2 (0.1 mm), MnCl_2 (0.25 mm), NH_4Cl (25 mm), Na_2SO_4 (1.875 mm), KH_2PO_4 (1.25 mm), NH_4NO_3 (3 mm) and sufficient KOH to adjust the pH to 7.1. To the autoclaved medium (900 ml.) was then added aseptically 0.25 M- CaCl_2 (10 ml.), 1 M- MgSO_4 (20 ml.), 0.2 M-sodium glutamate (50 ml.), 0.2% (w/v) L-tryptophan (10 ml.) and 40% (w/v) glucose (10 ml.). On a limited number of occasions two variations of this medium were employed. In one, medium P, the concentration of KH_2PO_4 was raised to 11.25 mm, and in the other, medium C, 0.2 M-sodium glutamate was replaced by 0.2 M-sodium citrate (50 ml.).

The cultures were shaken overnight at 37°, after which the extinction of the cultures at 600 μ varied from 0.1 (early exponential growth, about 40 μg . dry wt./ml.) to about 2.0 (late exponential phase, about 800 μg . dry wt./ml.). Then 3–5 l. of the batches was pooled, samples (500 ml.) were distributed in 3 l. conical flasks and shaking was continued at 37°. The course of growth and sporulation was followed by measurements of extinction at 600 μ and by phase-contrast microscopy.

Unless otherwise stated, all experiments were carried out

with medium S. The assay procedure is summarized in Scheme 1.

Individual flasks were harvested at intervals of 1 hr. or on one occasion 1.5 hr., and samples (50 ml.) removed for extinction measurements, determinations of total protein, DPA determinations and counts of heat-resistant spores. The remainder of the culture (450 ml.) was centrifuged at 5000 g and the pellet at once suspended in buffer A (10 ml.) containing 50 mm-tris-HCl buffer, pH 7.1, 1 mm- MgSO_4 , 1 mm- CaCl_2 and 0.1 mm- ZnSO_4 and cooled in ice. The cell suspension was transferred to a Pyrex bottle with Ballotini no. 12 beads (7 ml.) (Jencons Ltd., Hemel Hempstead, Herts.). The bottle was then shaken in a Braun MSK cell homogenizer (Shandon Scientific Co. Ltd., London, N.W. 10) for 5 min. at 2000 oscillations/min. The homogenate was rapidly filtered through sintered glass to remove the glass beads and the filtrate centrifuged at 10000 g for 15 min.

The supernatant was assayed for protein, protease, catalase and alkaline phosphatase. A sample (0.2 ml.) was also heated at 70° for 10 min., diluted with buffer A (0.4 ml.) and centrifuged at 10000 g for 15 min. The supernatant was assayed for catalase, and the precipitate of coagulated protein was washed with water (1 ml.) and assayed for protein. A portion (6 ml.) of the 10000 g supernatant was centrifuged at 100000 g for 3 hr. and the supernatant (0.4 ml.) so obtained was heated at 65° for 5 min. to destroy lactate dehydrogenase activity and assayed for alanine dehydrogenase. A portion (5 ml.) of the 100000 g supernatant was precipitated by the addition of saturated $(\text{NH}_4)_2\text{SO}_4$, pH 7.0 (30 ml.), and the precipitate was collected

Scheme 1. Flow sheet for assay procedure.

by centrifugation at 7000g for 15 min. and dissolved in buffer A (1.5 ml.). This solution was assayed for aconitase, fumarase, malate dehydrogenase, glucose dehydrogenase and NADH oxidase.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Total protein in a culture was determined by adding a threefold dilution of the culture (1 ml.) to 12% (w/v) trichloroacetic acid (1.5 ml.) and assaying the precipitate. Total protein in the 10000g supernatant was determined by adding the supernatant (0.2 ml.) to 12% (w/v) trichloroacetic acid (1 ml.) and assaying the precipitate.

Assay of DPA. A sample (40 ml.) of the culture was centrifuged, and the pellet was suspended in water (1.5 ml.) and autoclaved for 60 min. at 10 lb./in.². Acetic acid (0.15 ml. of 0.2N) was then added and the debris removed by centrifugation. The supernatant (0.5 ml.) was assayed for DPA by the method of Janssen, Lund & Anderson (1958). Supernatant (0.5 ml.) to which 0.5M-sodium acetate buffer, pH 5.5 (0.1 ml.), had been added was used as blank.

Measurement of refractility. The percentage of cells containing refractile spores was obtained by direct counting in the phase-contrast microscope.

Counting of heat-resistant spores. A threefold dilution of the culture (0.1 ml.) was added to sterile medium (0.9 ml.) and heated at 80° for 10 min. Triplicate drops (0.02 ml.) of appropriate dilutions were placed on nutrient agar plates and incubated overnight at 37°.

Assay of protease. Enzyme (0.1 ml. of cell extract) was added to bovine serum albumin (5 mg./ml.) in buffer A (1.0 ml.). A sample (0.4 ml.) was at once withdrawn and added to ice-cold 8% (w/v) HClO₄ (0.4 ml.). The remainder was incubated at 37° for 30 min. and a sample (0.4 ml.) added to 8% HClO₄ (0.4 ml.). Precipitates were removed by centrifugation and the extinction of the supernatant from the incubated tube was read against that from the unincubated tube at 280 mμ.

Assay of alkaline phosphatase. Sodium *p*-nitrophenyl phosphate (3 μmoles) in 1M-tris-HCl buffer, pH 8.0 (3 ml.), was allowed to equilibrate at 30° in a quartz cuvette and the extinction at 410 mμ recorded at 30 sec. intervals after the addition of enzyme (0.1 ml.).

Assay of catalase. Potassium-sodium phosphate buffer, pH 7.2 (5 ml. of 0.05M), was allowed to equilibrate at 30° in a quartz cuvette. Then 0.5M-H₂O₂ (0.1 ml.) was added and the extinction of the solution measured at 240 mμ at 15 sec. intervals after the addition of enzyme (0.1 ml.) (Beers & Sizor, 1952).

Assays of other enzymes. The following enzymes were assayed as described in the references, with in each case 0.1 ml. of the appropriate extract: alanine dehydrogenase (Wiame, Piérard & Ramos, 1962); aconitase (Anfinsen, 1955); fumarase (Massey, 1955); malate dehydrogenase (Ochoa, 1955); glucose dehydrogenase (Sadoff, Bach & Kools, 1965); NADH oxidase (Doi & Halvorson, 1961).

Determination of bacterial dry weight. This was carried out by measuring the extinction at 600 mμ (see Mandelstam & Waites, 1968).

Enzyme units. In the protease assay, 1 unit of enzyme gives a change in extinction of 0.001 in 30 min. In all other assays, 1 unit of enzyme is defined as giving a change in extinction of 0.001/min. under the conditions described. The specific activities of enzymes are given as units/mg. of protein in the 10000g supernatant, appropriate corrections

being made for the decrease of volume from 5 ml. to 1.5 ml. during the (NH₄)₂SO₄ precipitation.

RESULTS

It was not possible to carry out in a single experiment all the determinations described in the Materials and Methods section. For this reason a number of experiments were performed under similar conditions and different combinations of measurements made. Each parameter (protein, DPA, aconitase activity etc.) was measured on at least two and usually four or five separate occasions.

Growth. Wild-type (*B. subtilis* 168) grew exponentially in the sporulation medium S, with a generation time of approx. 65 min., to a density of about 500 μg. dry wt./ml. During the next 2.5 hr. the density continued to rise at a falling rate to about 1 mg. dry wt./ml. In a high-phosphate medium (medium P) exponential growth was maintained to a density of about 800 μg. dry wt./ml. and a value of about 1050 μg. dry wt./ml. was reached 1.5 hr. later. Fig. 1 shows the changes in extinction, bacterial dry weight and total protein during growth and sporulation in medium P, values for two separate experiments being plotted. Exponential growth ended at 4 hr. and this point is referred to as *t*₀. At 10 hr. (*t*₆) 1–5% of the cells carried refractile spores.

Protease. Intracellular proteolytic activity could be detected in exponentially growing cells at a density of about 200 μg. dry wt./ml. (extinction 0.5), but its specific activity at lower cell densities was difficult to determine accurately in experiments on this scale. The rise in specific activity of the protease towards the end of the exponential phase in a typical experiment is shown in Table 1. Little further increase in specific activity was subsequently observed. On one occasion the specific activity of protease in an extract of mutant E22 harvested at *t*₆ was 5.2% of that of the wild-type. On another occasion the specific activity of an extract of mutant E22 harvested at *t*₁₆ was about 50% of that of the wild-type. Similar extracts of mutant E21 contained protease of about the same specific activity as the wild-type.

Catalase. During the assay the rate of reaction with peroxide fell off rapidly with time, diminishing to about one-third of the initial value after 2 min. For this reason it was difficult to obtain accurate values for the enzyme concentration. Nevertheless in three experiments the specific activity of catalase appeared to show an approximately threefold rise between *t*₂ and *t*₆. The presence of catalase could be demonstrated in the supernatants, obtained after heat treatment of the 10000g supernatant, by the formation of bubbles when a drop of enzyme was mixed with a drop of 1% (v/v) hydrogen peroxide

Fig. 1. Changes in cell density, dry weight and protein during sporulation in *B. subtilis* 168. In two experiments *B. subtilis* 168 was grown in medium S from a small inoculum and measurements were made of extinction at 600m μ and bacterial dry weight. Total protein was determined by the method of Lowry *et al.* (1951). Results from the two experiments are plotted against time on the same Figure. ●, E_{600} ; ▲, dry wt. (mg./ml.); ■, total protein (mg./ml.).

Table 1. Changes in the specific activity of intracellular protease at the end of the growth phase

The specific activity of protease was measured, as described in the Materials and Methods section, in extracts of cells harvested at intervals towards the end of growth and during the early stages of sporulation.

Time (hr.)	Bacterial dry wt. (mg./ml.)	Sp. activity of protease (units/mg. of protein)
t_{-1}	0.435	1360
t_1	0.960	3140
t_2	0.850	3400
t_3	0.980	3030
t_4	0.930	3380

on a microscope slide and covered with a cover slip. Attempts to measure this activity were unsuccessful on all but one occasion, however, possibly because of the relatively low concentrations of the enzyme.

Fig. 2. Changes in the specific activity of alkaline phosphatase during growth and the early phase of sporulation. *B. subtilis* 168 was grown from identical small inocula in medium S, containing 1.25mM-phosphate (○ and ●) and in medium P, containing 11.25mM-phosphate (△ and ▲). Changes in bacterial dry weight (○ and △) and in the specific activity of alkaline phosphatase (● and ▲) are shown as percentages of the maximum values observed (0.85mg./ml. and 3680 units/mg. of protein respectively).

On this occasion the specific activity increased from about 200 units/mg. at $t_{0.5}$ to about 1000 units/mg. at t_5 . Because of the variation in results no conclusions have been drawn about the time of appearance of the enzyme.

Alkaline phosphatase. In a culture grown in medium S, in which the initial phosphate concentration was 1.25mM, a rapid 60-fold rise in the specific activity of alkaline phosphatase was observed towards the end of exponential-phase growth. In medium P, however, which contained 11.25mM-phosphate, the rise was fivefold (Fig. 2). Further increases in phosphate concentration did not prevent the appearance of alkaline phosphatase.

Alanine dehydrogenase and fumarase. Changes in the specific activities of these enzymes are shown in Table 2.

Aconitase. In medium S aconitase showed a rise in specific activity from a barely detectable level of about 5 units/mg. at t_0 to about 500 units/mg. at t_7 (Table 2). On one occasion in which the specific activity of aconitase at t_6 in wild-type was 330 units/mg., that in mutant E21 was 315 units/mg. whereas that in mutant E22 was only 19.8 units/mg.

In another experiment extracts were made from exponentially growing cells of the wild-type and mutant E22, each of which had been grown in both medium S and medium C (in which citrate replaces glutamate). The specific activities of aconitase in these extracts are indicated in Table 3.

Table 2. *Changes in the specific activities of five enzymes during sporulation*

Medium S, which had been inoculated with *B. subtilis* 168, was distributed in 500 ml. lots in 31. conical flasks, and single flasks were harvested at hourly intervals during sporulation. Cells were disrupted and the homogenate was centrifuged at 100000g for 3 hr. A portion (0.1 ml.) of the supernatant was assayed for alanine dehydrogenase, and the remainder (5 ml.) treated with saturated $(\text{NH}_4)_2\text{SO}_4$ (30 ml.). The resulting precipitate was dissolved in buffer A (1.5 ml.) and assayed for aconitase, fumarase, glucose dehydrogenase and NADH oxidase. —, Not determined.

Time (hr.)	Sp. activity (units/mg. of protein)				
	Alanine dehydrogenase	Aconitase	Fumarase	Glucose dehydrogenase	Soluble NADH oxidase
t_1	126.5	63	50.5	< 5	1060
t_2	—	—	50.3	< 5	1035
t_3	240	148	39.5	< 5	912
t_4	—	244	37.7	26.3	—
t_5	380	330	35.6	71	865
t_6	—	325	—	240	—
t_7	410	504	86	246	1065
t_8	—	531	—	308	—
t_9	—	725	115	279	1630

Table 3. *Repression of aconitase by glucose and glutamate in B. subtilis 168 and an asporogenous mutant, E22*

B. subtilis 168 and mutant E22 were each grown to mid-exponential phase in medium S, containing initially 27.5 mm-glucose and 10 mm-glutamate, and in medium C, containing 27.5 mm-glucose and 10 mm-citrate. Bacterial dry weight was calculated from extinctions at 600 m μ and the cultures were harvested in mid-exponential phase. Extracts (5 ml.) of the cells were centrifuged at 100000g for 3 hr. and treated with saturated $(\text{NH}_4)_2\text{SO}_4$ (30 ml.). The resulting precipitate was dissolved in buffer A (1.5 ml.) and assayed for aconitase.

Strain	Medium	Sp. activity	
		Bacterial dry wt. (mg./ml.)	of aconitase (units/mg. of protein)
Wild-type (168)	S (glutamate)	0.237	66.8
Wild-type (168)	C (citrate)	0.235	324.5
Mutant E22	S	0.245	46.75
Mutant E22	C	0.253	365.5

Malate dehydrogenase. Very high activities of malate dehydrogenase were observed throughout sporulation, but the specific activity of the enzyme remained approximately constant at 30000 units/mg.

Soluble NADH oxidase. Changes in the specific activity of this enzyme during a typical experiment are shown in Table 2.

Glucose dehydrogenase. This enzyme could not be detected until t_4 , after which its specific activity rose from 26.3 units/mg. at t_4 to 308 units/mg. at t_8 in a typical experiment (Table 2). The

Table 4. *Appearance of refractility, DPA and heat-resistant spores in the later stages of sporulation*

Portions (500 ml.) of a culture of *B. subtilis* 168 were shaken at 37° in 31. conical flasks and single flasks harvested at hourly intervals during sporulation. Samples of the cultures were examined by phase-contrast microscopy and determinations of DPA and heat-resistant spores carried out as described in the Materials and Methods section.

Time (hr.)	Refractile spores (%)	DPA content (m μ moles/mg. of cells)	10 ⁻⁶ × No. of heat-resistant spores/ml.
t_4	1	4	1.33
t_5	2	12	8.5
t_6	5	10	25
t_7	10	28	183
t_8	25	44	515
t_9	50	57	650
t_{10}	75	88	1100
t_{20}	95	139	2000

specific activity of this enzyme at t_6 in mutant E22 was 18.1 units/mg. and in mutant E21 21.8 units/mg., whereas in the wild-type it had reached 184 units/mg.

Glutamate dehydrogenase. Attempts were made to detect this enzyme by the same method as that employed for assaying glucose dehydrogenase. No activity could be detected whether NAD⁺ or NADP⁺ was used as cofactor.

DPA. This compound could normally be first detected at t_6 and reached a concentration of about 0.06 μ mole/mg. dry wt. (10 μ g./mg.) at t_{10} . However, 12 hr. later a concentration of about 0.14 μ mole/mg. dry wt. was obtained.

Table 5. Increase in the resistance of soluble protein to heat-denaturation during sporulation

Parallel cultures (500 ml.) of *B. subtilis* 168 were shaken at 37° and harvested at intervals. Samples (50 ml.) were taken for DPA determinations and the remainder of the cells removed by centrifugation and disrupted in buffer A (10 ml.). The homogenate was centrifuged at 10000g for 20 min. and two portions (0.2 ml.) of the supernatant were respectively (a) added to 12% (w/v) trichloroacetic acid (1 ml.) and (b) added to buffer A (1 ml.) and heated at 70° for 10 min. In each case the precipitate was removed by centrifugation at 10000g for 20 min., washed once with water (1 ml.) and assayed for protein. The amount of protein precipitated by heat is expressed as a percentage of that precipitated by trichloroacetic acid. DPA is given as a percentage of that found at t_{20} (Table 4).

Expt. no.	Time (hr.)	% of protein precipitated by heat	DPA (% of maximum)
1	t_2	67.3	0
	$t_{3.5}$	74.3	0
	t_5	63.4	0
	$t_{6.5}$	38.0	5.75
	t_8	21.8	13.2
	t_9	3.3	20.3
2	t_5	66.0	0
	t_7	50.0	0
	t_8	51.0	6.3
	t_9	34.0	9.2

Heat-resistance. Increases in refractility, DPA content and numbers of heat-resistant spores are shown for a typical experiment in Table 4.

Proportion of protein precipitated by heat at 70°. This was determined on two occasions and the results are summarized in Table 5. DPA content of the cells is given as a percentage of the maximum value for comparison.

DISCUSSION

To measure accurately the changes in more than a small number of parameters at hourly intervals over a period of some 17 hr. presents considerable difficulties, and in the present work different sets of measurements were made on nine separate occasions, each covering a period of 6–8 hr. during growth and sporulation.

The sequence of changes observed was always consistent and reproducible, though the magnitude of individual changes varied on different occasions. To obtain an overall picture, therefore, for each experiment values for the parameters measured are, except for DPA, expressed as percentages of the maximum values attained, and the percentage for each parameter at a given time is averaged from the values obtained in two to four experiments. The DPA content is expressed as a percentage of that

Fig. 3. Biochemical changes during sporulation. The values for each parameter measured (specific activities for the enzymes) in nine experiments were expressed as percentages of the maximum values attained in that experiment, and the percentages from two to four experiments were averaged. The results are plotted on a semi-logarithmic scale against time. The morphological stages, 0–VI, are taken from Murrell (1967). The curves are identified by numbers as follows: 1, E_{600} or bacterial dry wt.; 2, protease; 3, alkaline phosphatase; 4, soluble NADH oxidase; 5, alanine dehydrogenase; 6, aconitase; 7, glucose dehydrogenase; 8, DPA; 9, refractility; 10, heat-resistant spores.

observed at t_{20} in one experiment (Table 4). All nine overlapping experiments could be 'superimposed' without distortion of their time-scales.

The results are shown in Fig. 3 as a semi-logarithmic plot against time. This scheme may be compared with that of Murrell (1967), who used a linear plot. The advantage of the semi-logarithmic form is that the early synthesis of an enzyme appears as a straight line, which improves the resolution of the order of events.

The production of an exoprotease has previously been linked with sporulation (Schaeffer, 1967), but it has also been shown (Mandelstam & Waites, 1968) that mutations involving a loss of the exoprotease also result in a loss of the ability to degrade intracellular protein and of the ability to form spores. The onset of protein turnover coincides with the synthesis of intracellular protease, and the specific activity of this enzyme in an exoproteaseless mutant, E22, was less than 5% of that of the wild-type. The protease was assayed at pH 7.0, but whether this enzyme is identical with the neutral protease described by McConn, Tsuru & Yasunobu (1964) was not established.

The 60-fold increase in the specific activity of alkaline phosphatase that was observed at the end of growth (t_1-t_2) in medium S (1.25 mM-phosphate) was greatly diminished in medium P (11.25 mM-phosphate), which suggests that the sporulation medium of Donnellan *et al.* (1964) is phosphate-limited. Nevertheless a fivefold increase in the specific activity of alkaline phosphatase was observed even in the higher-phosphate medium, and further experiments are required to establish whether two enzymes are present or whether two independent mechanisms control the activity of a single enzyme.

Very low specific activities of aconitase, but somewhat higher specific activities of fumarase and malate dehydrogenase, were recorded in cells at the end of exponential growth in medium S, which contains both glutamate and glucose. This is consistent with the findings of Hanson & Cox (1967) that the synthesis of aconitase in *B. subtilis* is repressed by glucose plus glutamate, whereas that of fumarase, malate dehydrogenase, succinate dehydrogenase and isocitrate dehydrogenase is not. Further support for this was provided by the observation that cells growing exponentially in medium C, in which glutamate was replaced by citrate, contained aconitase with five to ten times the specific activity found in cells growing in medium S. The rapid rise in aconitase activity starting at time t_0 is presumably due to de-repression of enzyme synthesis when the glucose in the medium is exhausted. It is noteworthy that in mutant E22, which fails to show a rise in aconitase activity in the stationary phase, this enzyme

appears to be de-repressed in the vegetative state in the absence of glutamate to the same extent as in the wild-type (Table 3).

No glutamate dehydrogenase activity could be detected in cell extracts at any time during growth and sporulation.

Heat-stable catalase (Baillie & Norris, 1963), soluble NADH oxidase (Doi & Halvorson, 1961), alanine dehydrogenase (Wax, Freese & Cashel, 1967) and glucose dehydrogenase (Bach & Sadoff, 1962) were investigated because they have either been associated specifically with the spore or are believed to play some part in spore germination. An increase in heat-stable catalase specific activity was recorded during sporulation on one occasion, but the observation was not reproducible and no reliable conclusions can be drawn about its time of appearance. The rise in soluble NADH oxidase specific activity, though significant, was much smaller than the rise in glucose dehydrogenase specific activity, and appreciable activity was observed at time t_1 , whereas the activity of the latter was not detectable at this time. Alanine dehydrogenase also showed 31% of its final specific activity at time t_1 and reached its maximum some 3 hr. before glucose dehydrogenase. The specific activity of glucose dehydrogenase in both mutants E21 and E22 was about 10% of that of the wild-type.

The last changes observed were the synthesis of DPA, the appearance of refractility and finally heat-resistance. The presence of DPA is not essential for refractility in *B. cereus* (Wise, Swanson & Halvorson, 1967), but the same workers showed that spores lacking DPA were not heat-resistant. Thus, though it seems from Fig. 3 that DPA can first be detected before the appearance of refractility, 90% of the DPA is synthesized after the spores have started to become refractile, but before they have acquired heat-resistance, which is consistent with the findings of Wise *et al.* (1967).

The stages described in Fig. 3 occur in the same order as those described by Fitz-James (1965) and by Schaeffer, Ionesco, Ryter & Balassa (1965), but the process takes twice as long as indicated by Murrell (1967). This is due to the conditions of culture. If the organism is grown first in casein-hydrolysate medium and resuspended in a sporulation medium without glucose (Mandelstam & Waites, 1968) the time-scale approximates to that described by Murrell (1967).

The process can then be summarized as follows: stage 0, exponential growth; stages I and II, lytic enzymes are produced (see also Coleman, 1967), protein turnover starts, antibiotic synthesis occurs and the specific activities of enzymes required for the production of energy and carbon skeletons from acetate and acetoin increase; stages III and IV,

enzymes required specifically for the spore, though perhaps not involved directly in its formation, are synthesized; stages V and VI, DPA is synthesized, Ca^{2+} ions are accumulated and the last structural developments occur as the spore matures.

The structural changes that occur during sporulation are discussed in the next paper (Kay & Warren, 1968), but two questions arise from the results discussed above.

The first is which chromosome, i.e. that of the mother cell or that enclosed in the spore, directs the synthesis of the different types of proteins during sporulation? Although it may be accepted that the mother-cell chromosome directs the synthesis of the energy- and carbon skeleton-providing enzymes, the synthesis of, e.g., glucose dehydrogenase occurs during stage IV, when the fore-spore is intact within the mother cell. Since this is a 'spore enzyme', either the enzyme must be synthesized in the mother cell and diffuse into the fore-spore, or, more plausibly, the synthesis of glucose dehydrogenase must be directed by the spore chromosome.

The second question concerns the role of DPA in the heat-resistance of the spore. The results shown in Table 5 indicate the considerable increase in heat-stability of protein extracted from cells during sporulation, which parallels the appearance of DPA. These experiments do not, of course, prove that DPA is directly responsible for the heat-resistance of proteins, though certain salts, including those of DPA, have been shown to exert a stabilizing effect on serum albumin (Mishiro & Ochi, 1966). Further, since most proteins become denatured over a temperature range of only a few degrees, it is possible that the large apparent increase in the heat-stability of intracellular protein indicated in Table 5, which was determined at a fixed temperature of 70°, derives from a relatively small increase in the temperature at which these proteins are denatured. Nevertheless further experiments are needed to discover whether the calcium salt of DPA stabilizes proteins from *B. subtilis* either generally, or in specific instances, and whether the activity of enzymes is protected against the effects of heat by this compound. Further, if DPA were responsible for the stabilizing effect suggested by Table 4, then, in view of the relatively low concentrations of DPA that would be present in cell extracts, it would be interesting to know whether DPA is able to bind specifically to protein, rather than merely contributing to a general 'salt effect'.

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