$\alpha\epsilon$ -Diaminopimelic Acid Metabolism and Sporulation in *Bacillus sphaericus*

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The metabolism of $\alpha\epsilon$ -diaminopimelic acid (DAP) by sporulating cells is of interest on two counts. First, by analogy with the conversion of lysine into piperidine-2-carboxylic acid (Rothstein & Miller, 1953; Lowy, 1953) it seems possible that DAP may be the precursor of pyridine-2:6-dicarboxylic acid (dipicolinic acid). This compound is built up as a calcium salt in the sporulating cell, finally accounting for 10–15 % of the dry weight of the resting spore (Powell & Strange, 1953; Powell, 1953; Powell & Strange, 1956). Secondly, DAP is a constituent of the 'spore peptide', a soluble non-dialysable complex containing DAP, glutamic acid, alanine and hexosamines (Strange & Powell, 1954). This peptide is a component of the spore coat from which it is freed during germination, probably by the activation of an intracellular lytic system similar to lysozyme (Powell & Strange, 1956; Strange & Dark, 1956, 1957). A peptide of the same or similar constitution appears to be widely distributed in bacterial cell walls (Cummins & Harris, 1956; Salton, 1956; Strange & Dark, 1957). We were unable to find a correlation between DAP content and ability to sporulate in *Bacillus subtilis*, *Bacillus megatherium*, *Bacillus anthracis* and *Bacillus pumilus*: all the cultures we examined contained 1–2 % (w/w) of DAP (Powell, Strange & Hunter, unpublished results). *Bacillus sphaericus* proved, however, to be an interesting exception. In an asporogenous strain of this organism (Powell & Hunter, 1955) we could detect no DAP in 30 mg. dry weight of cells. In the parent sporogenous strain vegetative cells contained no DAP, whereas sporulating cells and spores contained normal amounts. This report includes some observations on the general biochemical changes, in particular the build-up of DAP, which occur during sporulation of *B. sphaericus* and two varieties of the species.

ORGANISMS AND METHODS

B. sphaericus, *National Collection of Type Cultures* (N.C.T.C.) 9602. This strain, when tested according to the scheme of Knight & Proom (1950), appeared to be a typical *B. sphaericus*. On the same basis, *B. sphaericus* N.C.T.C. 7582 was classified as var. *fusiformis* and a laboratory strain of *B. sphaericus* isolated by Dr J. L. Peel as var. *rotans*. The asporogenous strain was derived from *B. sphaericus* N.C.T.C.

7582 by repeated subculture as previously described (Powell & Hunter, 1955). Growth and sporulation of *B. sphaericus* N.C.T.C. 9602 was studied in potato-extract medium (Robinow, 1951) enriched with 1/10 vol. of a solution containing acid and trypsin-hydrolysed casein, yeast extract, glycerophosphate and glutamine (CCY; Gladstone & Fildes, 1940). Growth and sporulation of the fusiformis variety was studied in this medium and also in a meat extract-peptone medium (Tarr, 1933). Vegetative cells and spores of the rotans variety were grown in both these media and also in a 10 % yeast-extract medium (Barker & Beck, 1942). Cultures were grown at 37° in shaken 1 l. flasks containing 200 ml. of medium inoculated with 10⁸ spores/ml. Sporulation of all three organisms was usually complete after 24 hr. growth in the potato extract-CCY and meat extract-peptone media. The rotans variety sporulated completely after 48 hr. growth in the yeast-extract medium, but sporulation of the other two organisms was very poor, i.e. less than 10 %. Cells were harvested at various stages of growth and sporulation, washed once with 0.9 % NaCl, then suspended in water and freeze-dried. The method of preparation of soluble and insoluble fractions from these freeze-dried cells after mechanical disintegration (Mickle, 1948) and the determination of their DAP and hexosamine content has been described in detail in an earlier paper (Powell & Strange, 1956). The ultraviolet-absorption spectra of cell extracts and the calcium content of whole cells were also determined as previously described (Powell & Strange, 1956).

Assay of DAP decarboxylase

Cells of the fusiformis variety were harvested at various stages of growth and sporulation in the potato extract-CCY medium, washed twice with water and freeze-dried overnight. They were tested the following day. Gross changes in cell structure and permeability occur during sporulation: we therefore used disintegrated freeze-dried cells in these enzyme tests. Cell suspensions (5–7.5 ml.) containing 10–20 mg. dry wt. of cells/ml. in 0.1 M sodium potassium phosphate, pH 6.9, and one drop of tributyl citrate, were first cooled to 0° then again at 10 min. intervals during disintegration. This usually took 45–60 min. In these early experiments with the fusiformis variety, the semi-quantitative method of DAP decarboxylase assay described by Hoare & Work (1955) was used. The disintegrated cell suspension (1 ml.) was incubated at 37° with 0.2 ml. of 2 mM synthetic DAP and 0.2 ml. of pyridoxal phosphate solution (50 μ g./ml.). The reaction mixture (0.2 ml.) was added to 0.4 ml. of ethanol after 0, 1, 2 and 4 hr. incubation and centrifuged. The protein-free supernatant (0.1 ml.) was spotted on to Whatman no. 1 paper together with standard amounts of DAP, lysine and cadaverine for comparison, and

Cells of *B. sphaericus* N.C.T.C. 9602 ($\times 1500$) from cultures of varying ages in potato extract-CCY medium. Heat-fixed films stained with methylene blue: (1) 6 hr. culture: featureless vegetative cells; (2) 9 hr. culture: first signs of sporulation; appearance of volutin granules; (3) 15 hr. culture: most cells contain a well-developed spore and a volutin granule; (4) 24 hr. culture: mainly free spores.

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run for 18 hr. in the solvent system of Rhuland, Work, Denman & Hoare (1955). The DAP used in these tests was a commercial preparation which appeared to contain mainly the *meso* isomer (see below).

In later experiments with *B. sphaericus* N.C.T.C. 9602, the DAP decarboxylase activities of freshly reaped disintegrated cells and of intact and disintegrated freeze-dried cells were compared. In these tests it was found that the decarboxylase activity of disintegrated cells was increased by adding mm thiolacetic acid (Dewey, Hoare & Work, 1954) to the suspension before disintegration and by substituting octan-2-ol for tributyl citrate as the antifoaming agent. In these experiments with *B. sphaericus* N.C.T.C. 9602, the manometric method of decarboxylase assay was used in an atmosphere of N_2 (Dewey *et al.* 1954). The flasks contained 20–40 mg. (dry wt.) of cells suspended in 2 ml. of 0.1M sodium potassium phosphate, pH 6.9, and 0.1 ml. of pyridoxal phosphate solution (100 μ g./ml.). The side arms contained 0.5 ml. of 25 mm *meso*-DAP and 0.1 ml. of M citric acid. Q_{CO_2} values (μ l. of CO_2 /mg. dry wt. of cells/hr.) were calculated from the initial steady rate of CO_2 production. In most experiments the end products of the reaction were examined by paper chromatography (Antia, Hoare & Work, 1957) after protein precipitation with 2 vol. of ethanol.

Assay of DAP racemase

Freeze-dried cells of *B. sphaericus* N.C.T.C. 9602 were suspended in 0.1M borate buffer, pH 8.5, containing mm thiolacetic acid and octan-2-ol. The cell suspensions (20 mg. dry wt. of cells/ml.) were disintegrated, and 0.8 ml. volumes incubated at 37° with 0.4 ml. of 10 mm *meso*-DAP or with 0.4 ml. of 10 mm LL-DAP. Samples (0.2 ml.) of the reaction mixture were taken after 0, 1 and 18 hr. incubation and examined on a paper chromatogram after deproteinizing with 0.4 ml. of ethanol (Antia *et al.* 1957).

Chemicals. The synthetic DAP used in the early DAP decarboxylase assays was a commercial preparation supplied by L. Light and Co. Examination on paper chromatograms indicated that it contained approximately 10% of the LL isomer. Pure *meso*- and LL-DAP were very kindly provided by Dr E. Work. Calcium dipicolinate was isolated from germination exudates of *B. megatherium* (Powell, 1953). Piperidine-2:6-dicarboxylic acid was obtained by the reduction of dipicolinic acid (Andersson &

Soine, 1950) which was prepared by the oxidation of 2:6-lutidine (Hess & Wissing, 1915). Pyridoxal phosphate was a commercial preparation supplied by Roche Products Ltd.

RESULTS

Whole cells (5 mg.) of the fusiformis variety reaped after 8, 12, 16 and 20 hr. growth in potato extract-CCY medium were examined for their DAP content. No DAP could be detected in the 8 and 12 hr. cultures, whereas the 16 and 20 hr. cultures contained approximately 0.5% of DAP. Similarly, in a series of cultures grown in meat extract-peptone medium, cells reaped after 7 and 10 hr. growth contained no DAP, whereas 15, 20 and 24 hr. cultures contained 0.5–1%. In both these media, the first signs of sporulation appeared after 12–14 hr. growth. At this stage, most cells contained one and occasionally two volutin granules (Powell & Hunter, 1955), and many cells appeared 'tadpole' shaped in heat-fixed stained films (cf. Plate 1, 2). In 15–17 hr. cultures, sporulation was proceeding strongly, and most cells contained a well-defined developing spore as well as a volutin granule (cf. Plate 1, 3). At 24 hr. the cultures in meat extract-peptone consisted almost entirely of free spores. Although growth was heavier in the potato extract-CCY medium, sporulation was incomplete, i.e. 70–80%.

The hexosamine and DAP content of the soluble and insoluble fractions of vegetative and sporulating cells of the fusiformis variety are given in Table 1. In this series is included a 48 hr. sporulated culture washed six times with water to free it as far as possible from vegetative material. During sporulation, which began after about 16 hr. growth, there was a sudden decrease in the hexosamine content of the insoluble fraction of the cell and a corresponding increase in the soluble fraction. This change was most marked in the 48 hr. washed spore suspension. No DAP could be detected in hydrolysates of either

Table 1. *Distribution of hexosamine and DAP in cells of B. sphaericus at various stages of growth and sporulation in potato extract-CCY medium*

Organism	Age (hr.)	Appearance of culture	Hexosamine (g./100 g. dry wt.)			DAP (g./100 g. dry wt.)	
			Whole cells	Insoluble fraction	Soluble fraction	Insoluble fraction	Soluble fraction
<i>B. sphaericus</i> var. <i>fusiformis</i> N.C.T.C. 7582	8	Vegetative	—	12.1	1.2	0	0
	10	Vegetative	—	15.6	1.1	0	0
	12.5	Vegetative	4.2	12.1	1.4	0	0
	15	First signs of sporulation	—	14.1	1.7	0	0
	17.5	Sporulating strongly	—	8.7	2.3	Faint traces	Faint traces
	20	70–80% free spores	—	6.5	3.8	Faint traces	Faint traces
<i>B. sphaericus</i> N.C.T.C. 9602	48	Washed spore suspension	3.4	1.4	4.6	0	0.5–1.0
	6	Vegetative	3.3	9.6	1.6	0	0
	7.5	Vegetative	—	9.1	1.8	0	0
	9	First signs of sporulation	—	10.6	1.7	0	0
	15	Sporulating strongly	—	5.5	4.6	Traces	0.5–1.0
	22	> 95% free spores	6.4	2.1	7.8	Faint traces	1.0–2.0

fraction of the 8, 10, 12.5 and 15 hr. cultures. In the 17.5 and 20 hr. cultures, traces of DAP were detected in both fractions, whereas in the 48 hr. spore suspension the insoluble fraction contained no DAP and the soluble fraction 0.5–1 %.

Similar results were obtained with *B. sphaericus* N.C.T.C. 9602 in potato extract–CCY and in meat extract–peptone medium: no DAP could be detected in vegetative cells, whereas spores contained 1–2 %. Sporulation was more rapid than in the fusiformis variety, 'tadpole' shaped granular cells appearing after 8–9 hr. growth (Plate 1, 2). At 15 hr. (Plate 1, 3) sporulation was practically complete and lysis of the sporangia had begun. At 24 hr. (Plate 1, 4) the culture consisted almost entirely of clean free spores with very little vegetative debris. The DAP and hexosamine contents of the soluble and insoluble cell fractions are included in Table 1. It was very difficult to assess the DAP content of the insoluble fraction of the 22 hr. culture

owing to the presence of an unidentified constituent occupying the same position on the chromatogram as DAP. This unidentified constituent gave a blue colour with ninhydrin, whereas DAP gave an olive-green colour. Probably because sporulation and lysis of the sporangia was more nearly complete in *B. sphaericus* N.C.T.C. 9602 than in the fusiformis variety, the change in distribution of hexosamine and the appearance of DAP in the soluble fraction of the cell at sporulation was more striking. Similarly, the appearance of the characteristic ultraviolet-absorption spectrum of calcium dipicolinate in cell extracts, and the build-up of calcium in the cell during sporulation, was more marked in *B. sphaericus* N.C.T.C. 9602 (Fig. 1).

The DAP content of vegetative cells and spores of the rotans variety grown in potato extract–CCY, meat extract–peptone and yeast-extract media was also determined. Again, we could not detect DAP in vegetative cells, whereas spores contained about 1 %. Vegetative cells of *B. sphaericus* N.C.T.C. 9602 and of the fusiformis variety grown in yeast-extract medium also appeared to contain no DAP. Both these strains sporulated very poorly in yeast extract.

The above results suggested to us the possibility that vegetative cells of *B. sphaericus* might contain (a) an analogue of the DAP spore peptide in which DAP was replaced by lysine, and (b) a DAP decarboxylase system which either disappeared or was inhibited during sporulation. Evidence in support of these suggestions is now presented.

Isolation of non-dialysable peptides containing hexosamine from vegetative cells and spores of B. sphaericus

Non-dialysable peptides were isolated from germination exudates and from spore extracts as previously described by Strange & Powell (1954). We first examined the peptide which appeared in germination exudates from spores of the fusiformis variety. Approximately 80–90 % germination occurred when spores which had been preheated at 60° for 2 hr. were incubated for 1 hr. in 4 mM L-alanine and 20 mM sodium potassium phosphate, pH 7.3 (cf. Powell, 1950). Under these conditions 500 ml. of a suspension containing 2 mg. dry wt. of spores/ml. yielded 21 mg. of peptide. This peptide contained 30 % of hexosamine, and its main constituents were DAP, glutamic acid, alanine, aspartic acid, glucosamine and the substituted hexosamine (SH) for which a structure has recently been suggested by Strange (1956). Most of the common amino acids were present in small amounts and these still persisted after repeated treatment of aqueous solutions of the peptide preparation with chloroform and amyl alcohol (Sevag, 1934). Some purification was effected by paper electrophoresis

Fig. 1. Ultraviolet-absorption spectra of extracts from cells of *B. sphaericus* N.C.T.C. 9602 at various stages of growth and sporulation in potato extract–CCY medium. Extracts contained the equivalent of 1 mg. (dry wt.) of cells/ml. Light path 1 cm. Calcium contents of whole cells are given as g./100 g. (dry wt.) of cells.

and elution of the major component with reference to a paper stained with naphthalene black (cf. Strange & Powell, 1954). This eluted material contained DAP, glutamic acid, alanine, aspartic acid, glucosamine and SH with only faint traces of glycine, serine, valine and leucine. A preparation of nondialysable peptide (5.6 mg.) from disintegrated resting spores (235 mg.) was also examined. This contained 29 % of hexosamine, and its constituents were DAP, glutamic acid, alanine, glucosamine and SH, with faint traces of glycine and serine. Germination exudate peptide from *B. sphaericus* N.C.T.C. 9602 was not examined. A preparation of extract peptide from these spores contained DAP, glutamic acid, alanine, and the two hexosamines together with small amounts of aspartic acid. Its hexosamine content was 35 %.

It was recently shown (Powell & Strange, 1956; Strange & Dark, 1957) that extracts from disintegrated spores of *B. cereus* attacked vegetative cell-wall preparations of this organism and of *B. megatherium* and *B. subtilis*. A non-dialysable peptide containing hexosamine was liberated, similar in composition to that isolated from spore extracts and germination exudates (Strange & Powell, 1954). A similar lytic system was present in extracts from spores of a non-virulent strain of *B. anthracis*, and was also associated with the insoluble fraction of disintegrated *B. megatherium* spores. In an attempt to isolate hexosamine-containing peptides from vegetative cells of *B. sphaericus*, cell-wall preparations from 6 and 8 hr. cultures of *B. sphaericus* N.C.T.C. 9602 were incubated with the soluble and insoluble fractions from disintegrated spores of this organism. The test system (1 ml.) contained 5 mg. of the cell-wall preparation previously heated at 100° for 15 min. (Strange & Dark, 1957) and 10 mg. of the freeze-dried soluble or insoluble fraction from disintegrated spores, suspended in McIlvaine buffer, pH 7.4, and 0.2 mM- CoSO_4 . No liberation of hexosamine-containing material could be detected after incubation for 2 hr. at 60°, or after continued incubation for 18 hr. at 37°. In a similar test system, extracts from *B. cereus* spores did not attack vegetative cell walls of *B. sphaericus*, nor did extracts from *B. sphaericus* spores attack vegetative cell walls of *B. cereus*. Similar negative results were obtained with spores of the fusiformis variety.

Vegetative cell-wall preparations of the fusiformis variety were next treated with lysozyme. This enzyme has been shown to attack vegetative cell walls of *Bacillus* and other species, liberating a number of dialysable and non-dialysable components (Salton, 1953, 1956). The washed, insoluble fraction (80 mg.) from a 10 hr. vegetative culture of the fusiformis variety (560 mg.) was suspended in 10 ml. of 0.4 % NaCl containing 2 mg. of crystalline

egg-white lysozyme. Visible lysis occurred during incubation for 2 hr. at 37° on a rotary shaker. No lysis was apparent in a control suspension incubated without lysozyme. The suspensions were centrifuged and the supernatant was removed and cooled to 0°. Protein was precipitated with 3 % (w/v) trichloroacetic acid and the protein-free supernatant was neutralized with solid NaHCO_3 . After treatment with chloroform and amyl alcohol (Sevag, 1934) the solution was dialysed and freeze-dried; 16 mg. of white solid was obtained. This material was hydrolysed for 3 hr. at 100° in 6N-HCl and examined on two-dimensional paper chromatograms. It contained lysine, glutamic acid, alanine, aspartic acid, glucosamine and SH. Its hexosamine content was 39 %. There was also present in these hydrolysates an unidentified constituent giving a blue colour with ninhydrin and having the same R_f value as arginine in phenol-ammonia and as glycine in collidine-lutidine. It gave no Sakaguchi reaction on the paper (Acher & Crocker, 1952) and no significant colour with *p*-dimethylaminobenzaldehyde. When the time of hydrolysis of the cell-wall component was prolonged to 20 hr. there was a marked increase in intensity of the lysine and aspartic acid spots but no significant decrease of the unidentified component.

A non-dialysable peptide of very similar constitution containing 36 % of hexosamine was obtained from cell-wall preparations of *B. sphaericus* N.C.T.C. 9602 by incubation with lysozyme. Hydrolysates of this material also gave an unidentified spot on paper chromatograms, which occupied a corresponding position to that of the unidentified constituent from the fusiformis variety.

Cell-wall preparations from a 10 hr. vegetative culture of *B. megatherium* in potato extract-CCY medium were also treated with lysozyme. These cell walls contained approximately 5 % (w/w) of DAP. A soluble non-dialysable peptide was isolated from the reaction products. This peptide contained DAP, glutamic acid, alanine, glucosamine and SH. Its hexosamine content was 24 %.

A summary of the experiments with *B. sphaericus* is given in Table 2. There was a small and variable amount of phosphorus present in all the peptide preparations examined. The ultraviolet absorption of a peptide preparation (2.2 mg./ml.) from vegetative cells of *B. sphaericus* N.C.T.C. 9602 indicated that its phosphorus content of 0.32 % was not due to a nucleic acid component. The peptide preparations also contained a variable amount of carbohydrate, excluding hexosamine. This was usually less than 3 %. The peptides liberated by lysozyme gave a positive reaction for *N*-acetylhexosamine (Aminoff, Morgan & Watkins, 1952).

Table 2. Amino acids and amino sugars in peptides from *B. sphaericus*

Organism	Source of peptide	Hydrolysis time (hr.)	DAP	Aspartic acid	Glutamic acid	Alanine	Lysine	Unidentified substance	Hexosamine	SH*	Total amino sugars as glucosamine after hydrolysis for 2 hr. (g./100 g.)	Morgan-Ellson reaction† as N-acetylglucosamine (g./100 g.)
<i>B. sphaericus</i> var. <i>fusiformis</i> N.C.T.C. 7582	8 hr. vegetative cells	16	0	+	+	+	+	+	+	+	32	3.4
	10 hr. vegetative cells†	16	0	+	+	+	+	+	+	+	39	—
	Spore extract	16	+	0	+	+	0	0	+	+	29	0
	Spore exudate	16	+	+	+	+	0	0	+	+	30	0
<i>B. sphaericus</i> N.C.T.C. 9602	6 hr. vegetative cells†	3	0	+	+	+	±	+	+	+	35	2.0
	9 hr. vegetative cells†	20	0	+	+	+	±	+	+	+	37	—
	cells†	20	0	+	+	+	±	+	+	+	35	0
	Spore extract	16	+	±	+	+	0	0	+	+	35	—

* Unidentified hexosamine.

† Peptide heated for 7.5 min. with dilute alkali before addition of Ehrlich reagent.

‡ Peptide obtained by the action of lysozyme on the insoluble fraction of disintegrated cells.

DAP decarboxylase in B. sphaericus

Early semi-quantitative experiments with freeze-dried disintegrated cells of the fusiformis variety indicated that vegetative cells contained a very active DAP decarboxylase system. Lysine appeared as a reaction product, but no cadaverine could be detected. The cells therefore contained no active lysine decarboxylase. Decarboxylation of DAP proceeded more rapidly in the presence of added pyridoxal phosphate. The results of a typical assay are given in Table 3. The activity of sporulating cells was considerably lower than that of vegetative cells, whereas spores showed relatively little decarboxylase activity. The lower activity of spores and sporulating cells might be due to a reduced enzyme content or to the presence of inhibitory substances which are not present in vegetative cells. Calcium dipicolinate was tested as a possible inhibitor of DAP decarboxylase. The addition of 1.5 mg. of this substance/ml. to a suspension of disintegrated vegetative cells containing 10 mg. dry wt. of cells/ml. did not reduce its decarboxylase activity. Calcium dipicolinate is excreted during spore germination (Powell, 1953). We therefore compared the activity of washed germinated spores, obtained as described above, with that of resting spores. In this experiment, the final volume of the germinated-spore suspension was adjusted to allow for the 30% decrease in cell dry wt. which occurs during germination (Powell & Strange, 1953). There was no apparent increase in DAP decarboxylase activity during germination.

The DAP decarboxylase activity of cells of *B. sphaericus* N.C.T.C. 9602 was next determined, by the more accurate manometric method of assay, and with pure *meso*-DAP as substrate. It was first established that under the conditions of assay (see Methods) the enzyme was saturated with substrate and with pyridoxal phosphate. The initial steady rate of CO₂ production was proportional to the dry wt. of cell material present, and the total volume of CO₂ produced during the reaction indicated that

Table 3. Rough assay of DAP decarboxylase activity of freeze-dried disintegrated cells of *B. sphaericus* N.C.T.C. 7582 var. *fusiformis*

Cells (10 mg. dry wt.) were present in 1 ml. of 0.1M sodium potassium phosphate, pH 6.9, with 0.2 ml. of 25 mM synthetic DAP and 0.2 ml. of pyridoxal phosphate solution (50 µg./ml.), and were incubated at 37°. Results are expressed as percentage decarboxylation of DAP.

Age (hr.)	Appearance of cells	Time of incubation (hr.)		
		1	2	4
10	Vegetative	50-75	90	90
15	Sporulating	25	75	80-90
24	70-80% free spores	10-20	10-20	20

Table 4. *DAP decarboxylase activity ($Q_{CO_2}^{N_2}$) of vegetative cells, sporulating cells and spores of B. sphaericus N.C.T.C. 9602*

Age (hr.)	Appearance of culture	Storage at -15° in freeze-dried state (days)	$Q_{CO_2}^{N_2}$	
			Intact cells	Disintegrated cells
7	Featureless vegetative cells	0*	—	24.0
		1	30.0	—
		2	28.2	21.6
		3	27.3	18.6
9	Vegetative cells showing some granule formation	1	27.0	—
		2	19.8	18.0
9	Vegetative cells showing some granule formation	0*	—	21.9
		3	21.0	17.7
		4	20.7	—
15	Sporulating: i.e. most cells containing a well-defined spore	0*	—	12.0
		1	4.2	—
		2	3.0	9.5
15	Sporulating	0*	—	8.6
		1	3.0	8.4
		4	2.8	12.0
15	Sporulating	0*	—	14.4
		1	3.2	12.6
		2	3.0	11.2
24	Washed spore suspension	0*	1.4	4.5
		0*	2.4	6.4
		0*	1.1	2.7

* Freshly reaped cells tested before freeze-drying.

only one carboxyl group of DAP was attacked. Decarboxylation in N_2 by both intact and disintegrated cells proceeded at least twice as rapidly in the presence of added pyridoxal phosphate.

Freeze-dried intact vegetative cells of *B. sphaericus* N.C.T.C. 9602 gave $Q_{CO_2}^{N_2}$ values between 20 and 30. Disintegrated freeze-dried cells and also disintegrated freshly reaped cells gave rather lower $Q_{CO_2}^{N_2}$ values (18–24). There was a slight loss of activity when freeze-dried cells were stored at -15° for several days. Intact freeze-dried sporulating cells, i.e. cells containing a well-defined developing spore, gave relatively low $Q_{CO_2}^{N_2}$ values (3–4), but here disintegration produced a three- or four-fold increase in decarboxylase activity: thus $Q_{CO_2}^{N_2}$ values of 9–14 were obtained with disintegrated freeze-dried sporulating cells and also with disintegrated freshly reaped cells. Spore suspensions gave very low $Q_{CO_2}^{N_2}$ values (1–2.5) when tested intact, and higher values (3–6) after disintegration. These results are shown in Table 4.

Several authors have reported instances of enzymic activity shown by spore extracts but not by intact spores (Murrell, 1952; Powell & Hunter, 1956; Church, 1955). In these instances the enzyme was heat-stable in the intact spore but became heat-labile when the spore was disintegrated. Powell & Hunter (1956) in a study of the heat-stable adenosine deaminase of *B. cereus* spores found that this enzyme also became heat-labile during germination.

We therefore compared the heat stability of DAP decarboxylase in vegetative cells, sporulating cells, resting spores and germinated spores of *B. sphaericus* N.C.T.C. 9602. Suspensions of intact vegetative cells, sporulating cells and resting spores in 0.1M sodium potassium phosphate, pH 6.9, were heated at 60° for 15 min. Their DAP decarboxylase activities were measured before and after disintegration and compared with those of intact and disintegrated unheated cells. The results obtained are shown in Table 5. Vegetative cells tested intact and disintegrated retained very little or no decarboxylase activity after heat treatment. Most of the activity shown by intact sporulating cells and spores was lost during heat treatment, but these heated cells still showed markedly increased decarboxylase activity when disintegrated. This effect was most noticeable with spores which, when disintegrated after heat treatment, showed 50–100% of the activity of the unheated disintegrated suspension. On the other hand, when disintegrated spore suspensions were heated at 60° for 15 min., their DAP decarboxylase activity was completely lost. There was little change in decarboxylase activity during spore germination, whether this took place in 4 mM L-alanine (see above) or in the potato extract-CCY medium supplemented with 4 mM L-alanine. $Q_{CO_2}^{N_2}$ values for washed disintegrated germinated spores obtained in these two media were 5.7 and 4.7. Approximately 90% of this

Table 5. *Heat stability of DAP decarboxylase in vegetative cells, sporulating cells and spores of B. sphaericus N.C.T.C. 9602*

Age (hr.)	Appearance of culture	Unheated cells $Q_{CO_2}^{N_2}$		Cells heated at 60° for 15 min. $Q_{CO_2}^{N_2}$	
		Intact	Disintegrated	Intact	Disintegrated
7	Featureless vegetative cells	28.2	21.6	0	0
9	Vegetative cells showing some granule formation	18.0	15.0	0	0.8
15	Sporulating strongly	3.2	12.6	0.6	3.0
15	Sporulating strongly	3.0	8.4	0.6	3.2
24	Free spores	1.4	4.5	0	4.5
24	Free spores	2.4	6.4	0.2	4.0
24	Free spores	1.1	2.7	—	1.05

activity was lost when the germinated spores were heated at 60° for 15 min. before disintegration.

To determine whether there was a rapid increase in DAP decarboxylase activity during the development of the germinated spore into a vegetative cell, preheated resting spores (2 mg. dry wt. of spores/ml.) were shaken at 37° in the potato extract-CCY medium supplemented with 4 mM L-alanine. Stained films made at 30 min. intervals indicated that, after incubation for 2 hr., at least 90 % of the spores had developed into vegetative cells. The suspension was centrifuged and the cells were resuspended in 0.1 M sodium potassium phosphate, pH 6.9, to give 15 mg. dry wt. of cells/ml. Their DAP decarboxylase activity was determined after disintegration. A $Q_{CO_2}^{N_2}$ value of 5.0 was obtained.

The above experiments with germinated spores of *B. sphaericus* N.C.T.C. 9602 and the early experiments with the fusiformis variety indicated that the low DAP decarboxylase activity of disintegrated resting spores was probably not due to their calcium dipicolinate content. This conclusion was supported by further experiments with *B. sphaericus* N.C.T.C. 9602. The rate of decarboxylation of DAP by disintegrated vegetative cells was unaffected by the addition of equimolar amounts of calcium or sodium dipicolinate. These compounds were not themselves decarboxylated. Piperidine-2:6-dicarboxylic acid was also tested under the same conditions as a possible inhibitor of DAP decarboxylation. Added in equimolar amounts it was not inhibitory and, tested alone, it was not decarboxylated.

The DAP decarboxylase activity of vegetative cells from a 9 hr. culture of the fusiformis variety in potato extract-CCY medium was compared with that of a 9 hr. culture of the asporogenous strain in the same medium. Freeze-dried intact cells of the sporogenous strain gave a $Q_{CO_2}^{N_2}$ value of 18.0, compared with 7.9 for the asporogenous strain. In another pair of 9 hr. cultures in the same medium, $Q_{CO_2}^{N_2}$ values for freshly reaped disintegrated cells of the sporogenous and asporogenous strains were 13.5 and 8.1. The corresponding values for intact freeze-dried cells from these cultures were 15.3 and

8.4. In a single experiment with the rotans variety, a $Q_{CO_2}^{N_2}$ value of 23.5 was obtained with intact freeze-dried vegetative cells from a 18 hr. culture in yeast-extract medium.

Under the conditions of assay, vegetative cells, sporulating cells and spores of *B. sphaericus* N.C.T.C. 9602 showed no DAP racemase activity. The *meso* isomer of DAP was completely decarboxylated to lysine by vegetative cells during 1 hr. incubation, and by sporulating cells and spores during overnight incubation. There was no detectable conversion of the LL into the *meso* isomer, and no lysine formation was apparent with the LL isomer.

DISCUSSION

B. sphaericus appears to be atypical of the *Bacillus* genus (Antia *et al.* 1957) in its high DAP decarboxylase activity and the absence of DAP from its cells during vegetative growth. DAP was present in spores of *B. sphaericus* and, as in other *Bacillus* species (Strange & Powell, 1954), appeared in spore extracts and germination exudates as a constituent of a non-dialysable peptide containing DAP, alanine, glutamic acid and hexosamines. The germination exudate peptide from the fusiformis variety also contained aspartic acid, and traces of aspartic acid were present in the extract peptide from *B. sphaericus* N.C.T.C. 9602. On previous evidence (Strange & Dark, 1956, 1957) it at first seemed likely that these peptides were derived from the spore coat. Extracts from spores and sporulating cells of several *Bacillus* species have been shown to contain a lytic, lysozyme-like system which attacked vegetative cell-wall and spore-coat preparations, releasing peptides of very similar constitution to those just described (Powell & Strange, 1956; Strange & Dark, 1957). It was suggested that this lytic system might be concerned in the lysis of the sporangium and freeing of the spore during sporulation, and also in the initiation of the early stages of the germination process. In the present study, although we isolated similar peptides from spore extracts and germination

exudates of *B. sphaericus*, we failed to demonstrate a lytic system in these spores. We have shown, however, that vegetative cell-wall preparations of *B. sphaericus* were attacked by lysozyme and that a peptide was released containing lysine, aspartic acid, alanine, glutamic acid and hexosamines but no DAP. It is possible that, if present, the lytic system of *B. sphaericus* spores may be relatively feeble. This suggestion is supported by the observation that spores of *B. sphaericus* germinate relatively slowly even after prolonged preheating. It is also possible that the lytic system of *B. sphaericus* spores has greater specificity than that of the other *Bacillus* species examined and that it can only liberate the peptide containing DAP from its particular linkages in the *B. sphaericus* spore. Another possibility, rather less likely in our opinion, is that in *B. sphaericus*, the DAP-hexosamine peptide is not a component of the coat of the intact spore and that it appears in the soluble fraction of the disintegrated spore independently of a lytic system. The peptides released from vegetative cell-wall preparations by lysozyme all contained an unidentified constituent reacting with ninhydrin. The position of this material on paper chromatograms was similar to that of a constituent of cell-wall hydrolysates of certain lactobacilli described by Cummins & Harris (1956). These authors found that, on prolonged hydrolysis, the intensity of the aspartic acid and lysine spots increased while that of the unidentified constituent decreased. They therefore considered the unidentified constituent to be a peptide relatively resistant to acid hydrolysis and composed chiefly of aspartic acid and lysine. Although on prolonged hydrolysis of the vegetative cell-wall peptides from *B. sphaericus* the intensity of the aspartic acid and lysine spots increased, we could not detect any diminution of the unidentified constituent. Guex-Holzer & Tomcsik (1956) have described a constituent of hydrolysed mucopolysaccharide from a *Bacillus* species occupying a similar position on phenol/collidine-lutidine chromatograms to that of the unidentified material from *B. sphaericus*, but giving a positive reaction with *p*-dimethylamino-benzaldehyde. We hope to isolate the unidentified constituent from *B. sphaericus* for further investigation.

Vegetative cells of *B. sphaericus* contained a very active DAP decarboxylase system requiring pyridoxal phosphate as a cofactor. Sporulation was accompanied by the appearance of DAP as a cell constituent and by diminished DAP decarboxylase activity. Although the DAP decarboxylase activity of spores was relatively low, the activity of sporulating cells was still approximately 50 % that of vegetative cells, i.e. there was no sudden disappearance of DAP decarboxylase activity during the

onset of sporulation, although at this stage DAP appeared as a cell constituent. These observations were made with disintegrated cells saturated with pyridoxal phosphate. It is possible that in the living cell, DAP decarboxylase activity might be greatly reduced by a shortage of pyridoxal phosphate, and that the onset of sporulation is associated with a disturbance of pyridoxal phosphate synthesis. The possibility will be investigated. In this connexion, it was interesting to find that an asporogenous strain of *B. sphaericus* derived from the fusiformis variety showed roughly only one-half of the DAP decarboxylase activity of the parent strain. No trace of DAP could be detected in the asporogenous strain.

Freeze-dried intact sporulating cells and spores appeared to be less permeable to DAP than were vegetative cells: the DAP decarboxylase activity of sporulating cells and spores was increased approximately threefold by disintegration. Several examples of the relative inactivity of intact compared with disintegrated spores in enzyme tests have already been given (Murrell, 1952; Church, 1955). The DAP decarboxylase activity of *B. sphaericus* spores is a further example. As in previous instances (Murrell, 1952; Church, 1955; Powell & Hunter, 1956) the DAP decarboxylase of *B. sphaericus* spores was heat-stable in the intact spore, but became heat-labile during disintegration or germination. A possible explanation for this behaviour was discussed in a previous study of the adenosine deaminase system of *B. cereus* spores (Powell & Hunter, 1956).

In sporulating cells of *B. sphaericus*, presumably both the sporangium and the developing spore contained DAP decarboxylase. It seems likely that the decarboxylase activity which survived heat treatment was associated with the developing spore. On the other hand, the difference in decarboxylase activity between intact and disintegrated sporulating cells was considerably greater than the heat-stable activity of the cell (Table 5). This would imply either that (a) the developing spore contained both heat-stable and heat-labile DAP decarboxylase activity, or that (b) the wall of the sporangium itself had become less permeable to DAP. At the moment we have no evidence in favour of either of these possibilities.

The DAP decarboxylase system of disintegrated *B. sphaericus* cells appeared to be less sensitive to inhibition by dipicolinic acid than was the partially purified system from *Aerobacter aerogenes* studied by Dewey *et al.* (1954). This difference might be attributed to the presence of relatively large amounts of inert protein in our system, or to a difference in properties between the enzymes from *B. sphaericus* and *Aero. aerogenes*. It appears that, in *B. sphaericus*, the accumulation of calcium

dipicolinate in the sporulating cell was not responsible for its diminished DAP decarboxylase activity. Germinated spores and 'first-generation' vegetative cells also showed relatively low activity. It seems that the actual amount of enzyme present in the cell decreased to a minimum in the spore and increased to a maximum during the period of rapid vegetative growth.

SUMMARY

1. In *Bacillus sphaericus* and in *Bacillus sphaericus* var. *fusiformis* and *Bacillus sphaericus* var. *rotans* α -diaminopimelic acid (DAP) was present in sporulating cells and spores but not in vegetative cells.

2. As in other *Bacillus* species, pyridine-2:6-dicarboxylic acid was built up as a calcium salt in the developing spore.

3. A peptide containing DAP, aspartic acid, glutamic acid, alanine and hexosamines appeared in spore extracts and germination exudates. A peptide containing lysine, aspartic acid, glutamic acid, alanine and hexosamines was isolated from vegetative cell walls by treatment with lysozyme.

4. Vegetative cells showed strong DAP decarboxylase activity, stimulated by pyridoxal phosphate; $Q_{CO_2}^N$ values in nitrogen were 20-30. Lysine was a reaction product but no cadaverine was detected. Sporulating cells were less active ($Q_{CO_2}^N$, 10-15). Spores showed relatively little activity ($Q_{CO_2}^N$, 3-6). These values were obtained with disintegrated cells. Sporulating cells and spores gave lower $Q_{CO_2}^N$ values when tested intact.

5. The DAP decarboxylase activity of spores was relatively heat-stable. There was no increase in decarboxylase activity during germination and development to the first vegetative cell. During spore germination, the decarboxylase system became less heat-stable.

6. Pyridine-2:6-dicarboxylic acid and piperidine-2:6-dicarboxylic acid were not decarboxylated by vegetative cells of *B. sphaericus*; in equimolar concentration, they did not inhibit the decarboxylation of DAP by these cells.

7. Vegetative cells, sporulating cells and spores showed negligible DAP racemase activity.

8. Some possible relationships between DAP metabolism and sporulation in *B. sphaericus* are discussed.

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