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The Changes in the Total Vitamin B₆ and the Pyridoxal Phosphate Content of Cells of *Bacillus sphaericus* during Growth and Sporulation: their Possible Relationships with $\alpha\epsilon$ -Diaminopimelic Acid Metabolism

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The present study was prompted by the observation that vegetative cells of *Bacillus sphaericus* contained no $\alpha\epsilon$ -diaminopimelic acid (DAP), whereas sporulating cells and spores contained 1–2% (w/w) of DAP (Powell & Strange, 1957). A connexion between DAP metabolism and sporulation was already suspected: from structural considerations and the analogous conversion of lysine into piperidine-2-carboxylic acid (Rothstein & Miller, 1953; Lowy, 1953) it seemed possible that DAP was the precursor of pyridine-2:6-dicarboxylic acid (dipicolinic acid), a substance synthesized in large amounts by sporulating cells (Powell, 1953; Powell & Strange, 1956). In other *Bacillus* species there was, however, no connexion between sporulation and the appearance of DAP as a cell constituent: vegetative cells, sporulating cells and spores all contained approximately equal amounts of DAP. *B. sphaericus* was also exceptional among *Bacillus* species (Antia, Hoare & Work, 1957) in possessing an active DAP decarboxylase system requiring pyridoxal phosphate as a cofactor (Powell & Strange, 1957). A comparison of the DAP decarboxylase activities of vegetative cells, sporulating cells and spores showed that although the decarboxylase activity of *B. sphaericus* cultures decreased at sporulation, the activity of strongly sporulating cells was still 50% of that of vegetative cells. There was therefore no sudden disappearance of the decarboxylase at sporulation to account for the appearance of DAP; nor was its activity in

disrupted cells inhibited by dipicolinic acid (Powell & Strange, 1957). There remained the further possibilities that (a) other unidentified substances synthesized during sporulation inhibited the decarboxylase or (b) that sporulation was associated with a disturbance of total vitamin B₆ or, more particularly, of pyridoxal phosphate synthesis. The present report is chiefly concerned with the latter possibilities and also includes some data on DAP decarboxylase and vitamin B₆ synthesis during spore germination and development.

EXPERIMENTAL

Materials

Organisms. Most of the work was done with *B. sphaericus*, National Collection of Type Cultures (N.C.T.C.) 9602, since this strain usually sporulated strongly and gave 'clean' spore suspensions, i.e. suspensions containing very little vegetative debris, after growth for 24 hr. in the test medium. A few observations were made on a laboratory strain of *Bacillus cereus* which was used in a previous study (Powell & Strange, 1956). Some growth tests were made with *B. sphaericus* N.C.T.C. 7582 var. *fusiformis*, an asporogenous variant of *B. sphaericus* N.C.T.C. 7582 (Powell & Hunter, 1955) and a laboratory strain of *B. sphaericus* var. *rotans*.

Chemicals. Pyridoxal hydrochloride and pyridoxine hydrochloride were supplied by L. Light and Co., pyridoxal phosphate by S. A. F. Hofmann-La Roche and Co., deoxypyridoxine hydrochloride by Nutritional Biochemical Corp. and isonicotinylhydrazine by Ward, Blenkinsop and Co.

Methods

Assays for total vitamin B₆ and pyridoxal phosphate. Cells of *B. sphæricus* N.C.T.C. 9602 and of a laboratory strain of *B. cereus* at various stages of growth and sporulation were produced in an enriched potato-extract medium as previously described (Powell & Strange, 1956, 1957). The cells were harvested by centrifuging, washed once with water and assayed for total vitamin B₆ by an unpublished modification of the method of Atkin, Schultz, Williams & Frey (1943). The modified method was developed in the Department of Biochemistry, Oxford, and was made available through the courtesy of Professor D. D. Woods.

Washed cells of *B. sphæricus* were assayed for pyridoxal phosphate with the manometric method of Umbreit, Bellamy & Gunsalus (1945). The cells were reaped, washed, and mechanically disintegrated for 45–60 min. at 4° (Mickle, 1948) and assayed on the same day. To reduce the risk of pyridoxal phosphate losses during disintegration the cells were suspended (1–2 mg. dry wt./ml.) in 0.2 M-acetate buffer, pH 5.5, and the glass containers were wrapped with black paper. The reaction mixture for the assay contained 2 mg. (dry wt.) of *Streptococcus faecalis* cells, 50–200 µg. (dry wt.) of disintegrated cells of *B. sphæricus* and 50 µmoles of finely ground tyrosine in a total volume of 2.7 ml. of 80 mM-acetate buffer, pH 5.5. The manometers were filled with nitrogen and shaken at 30°. Cells of *B. sphæricus* from cultures at various stages of growth and sporulation were assayed simultaneously and at two cell concentrations. A set of calibration flasks containing 0, 2, 4, 6 and 8 µmg. of pyridoxal phosphate instead of *B. sphæricus* cells was included in each experiment.

The DAP decarboxylase activity of freshly reaped disintegrated cells of *B. sphæricus* was assayed manometrically with unresolved synthetic DAP as substrate as previously described (Dewey, Hoare & Work, 1954; Powell & Strange, 1957).

Determination of the isomeric constitution of the diamino-pimelic acid present in sporulating cells. Freeze-dried sporulating cells of *B. sphæricus* N.C.T.C. 9602 (20 mg.) were hydrolysed in 0.5 ml. of 6N-HCl at 105° for 24 hr. After removal of the HCl *in vacuo*, the hydrolysate was applied as a band on to Whatman no. 4 paper and run with collidine-lutidine-water (60:15:75, by vol.) for 18 hr. A paper spotted with synthetic DAP was run in parallel. The dried papers were examined in ultraviolet light and fluorescent bands in corresponding positions to those taken up by the synthetic DAP were cut out and eluted (Powell & Strange, 1956). A volume of eluate corresponding to 2 mg. of cells was spotted on to Whatman no. 1 paper and run for 18 hr. in pyridine-methanol-conc. HCl-water (10:80:2.5:17.5, by vol.) (Rhuland, Work, Denman & Hoare, 1955). Comparison with reference spots of the DAP isomers (Rhuland *et al.* 1955) indicated that the *meso*- or *D*-isomer or both were present in sporulating cells of *B. sphæricus*. It was possible to distinguish between the *meso*- and *D*-isomers by treatment with DAP decarboxylase, which attacks only the *meso*-isomer (Hoare & Work, 1955). The remainder of the eluate from the collidine chromatogram, containing approx. 180 µg. of DAP, was distributed equally among three small tubes. To one of these tubes, 40 µg. of *meso*-DAP was also added and to another, 40 µg. of *D*-DAP. All three tubes were then evaporated to dryness *in vacuo*. Equal amounts (0.2 ml.) of a suspension of

disintegrated vegetative cells of *B. sphæricus* (10 mg. dry wt. of cells/ml.) of high DAP decarboxylase activity were added to each tube, together with 5 µg. of pyridoxal phosphate. The tubes were incubated at 37° for 1 hr. Ethanol (0.2 ml.) was added to each tube and, after centrifuging, samples (0.2 ml.) of the supernatants were spotted on to Whatman no. 1 paper and run in the solvent system of Rhuland *et al.* (1955) for 18 hr. No trace of DAP was detected in the portions of eluate which had no addition or an addition of *meso*-DAP, whereas the portion of eluate to which *D*-DAP was added gave a strong DAP spot. It therefore appeared that sporulating cells of *B. sphæricus* contained only the *meso*-isomer of DAP.

RESULTS

The total vitamin B₆ content of *B. sphæricus* cells was taken as the mean value from assays at two levels of cell concentration each carried out in duplicate. The mean coefficient of variation within 83 duplicates, which included pyridoxine standards, was 0.032. The mean coefficient of variation between the means of duplicates at two levels of cell concentration was 0.082. The pyridoxal phosphate content of *B. sphæricus* cells was taken as the mean value from single assays at two levels of cell concentration. The mean coefficient of variation between assays at two levels of cell concentration was 0.102. The changes found to occur in the total vitamin B₆ and pyridoxal phosphate content of *B. sphæricus* cells at various stages of growth and sporulation were considerably greater than could be accounted for by experimental error. A relatively small number of vitamin B₆ assays were made in the same way on cells of a laboratory strain of *B. cereus*. The variation between duplicates and between assays at two levels of cell concentration were well within the limits of variation found for *B. sphæricus*.

Sporulation

The results of four separate experiments in which the vitamin B₆ content of vegetative cells, sporulating cells and free spores of *B. sphæricus* was determined are given in Table 1. In Expts. 3 and 4, values for pyridoxal phosphate content are also included. From these experiments, and from scattered observations on cells from cultures of various ages, it was concluded that the total vitamin B₆ and the pyridoxal phosphate content of the cell did not decrease and in some cultures even increased during sporulation, i.e. during the period 13–18 hr. after inoculation while the spore was developing inside the sporangium. Vitamin B₆ assays on vegetative and sporulating cells gave values in the range 300–550 µm-moles/g. dry wt. The pyridoxal phosphate content of vegetative and sporulating cells range between 180 and 330 µm-moles/g. In instances where determinations of the total vitamin B₆ and pyridoxal phosphate

Table 1. *Total vitamin B₆ and pyridoxal phosphate content of vegetative cells, sporulating cells and free spores of Bacillus sphaericus N.C.T.C. 9602*

Age of culture (hr.)	Appearance of culture	Vitamin content (μ m-moles/g. dry wt. of cells)					
		Expt. 1 Vitamin B ₆	Expt. 2 Vitamin B ₆	Expt. 3		Expt. 4	
				Vitamin B ₆	Pyridoxal phosphate	Vitamin B ₆	Pyridoxal phosphate
9	Vegetative cells	480	532	408	227	490	204
16	Strongly sporulating cells	404	550	396	265	580	330
20	Sporulation complete: approximately one-third of spores still enclosed by sporangia	160	—	—	—	—	—
24	Free spores*	101	200	83	72	403*	212*

* Sporulation was incomplete in the 24 hr. culture of Expt. 4 and there were very few free spores.

Table 2. *Total vitamin B₆ content of vegetative cells, sporulating cells and free spores of a laboratory strain of Bacillus cereus*

Age of culture (hr.)	Appearance of culture	Vitamin B ₆ content (μ m-moles/g. dry wt. of cells)	
		Expt. 1	Expt. 2
10	Vegetative cells	162	224
17	Strongly sporulating cells	150	145
24	Mainly free spores	99	73

content of the same batch of cells were made, the pyridoxal phosphate represented 40–60 % of the total vitamin B₆ content. In 24 hr. cultures, which consisted mainly of free spores, the vitamin B₆ and pyridoxal phosphate content of the harvested cells had fallen to, at most, 50 % and usually to 25 % of that of vegetative and sporulating cells. Low values for cells from 24 hr. cultures were obtained only when sporulation had proceeded to completion and the spores were mainly free of sporangial material. In Expt. 4 (Table 1), although growth appeared to have proceeded normally, sporulation was incomplete at 24 hr. and there were very few free spores. The vitamin B₆ content of cells from this culture at 24 hr. was only slightly lower than that at 9 and 16 hr. Similarly, in Expt. 1 (Table 1) at 20 hr., the culture had sporulated well, but approximately one-third of the spores were still enclosed by sporangia: a higher value for the vitamin B₆ content was obtained at 20 hr. than at 24 hr. when all the spores were free. It was thus possible to predict the approximate vitamin B₆ content of harvested cells from a 24 hr. culture by an examination of stained films (cf. plate 1, Powell & Strange, 1957).

The total vitamin B₆ and pyridoxal phosphate content of washed-spore suspensions did not appear to change appreciably during storage at 0° for 4–8 weeks. Vitamin B₆ assays made on intact and on mechanically disintegrated resting spores

gave values which were similar to each other and to those obtained with germinated spores (see below). It therefore seemed unlikely that the low values obtained for the vitamin B₆ content of resting spores could be attributed to the difficulties of extraction associated with their peculiar structure.

The results of vitamin B₆ assays on vegetative cells, sporulating cells and free spores of a laboratory strain of *B. cereus* are given in Table 2. Two cultures of *B. cereus* were examined, and in both cases the vitamin B₆ content of spores was considerably lower than that of vegetative cells. In one culture, sporulating cells reaped after growth for 17 hr. contained significantly less vitamin B₆ than vegetative cells reaped after growth for 10 hr., whereas in the other culture sporulating cells and vegetative cells contained approximately equal amounts of vitamin B₆.

Spore germination and development

The changes in the total vitamin B₆ content of spores of *B. sphaericus* during germination and growth were also studied. Resting-spore suspensions were heated at 60° for 1 hr. to stimulate subsequent germination (Curran & Evans, 1945; Powell, 1950) and added to the enriched potato-extract medium at 37° to give suspensions containing 0.2–1.0 mg. dry wt. of spores/ml. The suspensions were then shaken at 37°. Samples were taken out at various intervals, cooled to 0° and centrifuged and the cells washed once with water before being assayed for vitamin B₆. Stained films indicated that during the first 1 hr. germination was practically complete (Powell, 1950), and there was some swelling of the germinated forms. Development and growth of the first vegetative cells occurred during the following 2 hr. No sign of cell division was seen in these suspensions, and it was noticed that the developing vegetative cells contained one or two terminal volutin granules, in contrast with the uniformly staining, featureless vegetative cells of 6–8 hr. cultures growing from a

small inoculum of 0.05–0.10 μg . dry wt. of spores/ml. The results of these experiments with developing spores are summarized in Table 3. In every experiment synthesis of vitamin B₆ occurred during the first hour of development. This was especially marked in the more dilute suspensions inoculated with 0.2 mg. dry wt. of spores/ml.: here there was an approximately threefold increase in the cellular vitamin B₆. From scattered assays made after incubation for 2 and 3 hr. it appeared that there was a further slight increase in the vitamin B₆ content of the developing vegetative cells, followed by a decrease. The highest values obtained were 200–220 μm -moles of vitamin B₆/g. dry wt. of cells, corresponding to approximately one-half of the values obtained for cells of actively dividing vegetative cultures growing from a small inoculum (Table 3, Expts. 1 and 2). In Expts. 5 and 6 (Table 3) assays made after incubation for 20 min. indicated that a 65% increase in cellular vitamin B₆ occurred during spore germination, i.e. during the period when the spore lost heat resistance and became permeable to stains but had not increased markedly in size (Powell, 1950). This apparent increase was partly false; it was calculated on a basis of cell dry wt. and should be corrected to account for the 30% decrease in spore dry wt. which occurs during germination (Powell &

Strange, 1953). In this connexion, the vitamin B₆ content of spores (0.2 mg. dry wt./ml.) allowed to develop for 1 hr. at 37° in the enriched potato-extract medium was compared with that of spores incubated for 1 hr. in 40 mM-sodium potassium phosphate, pH 7.3, containing 4 mM-L-alanine (Table 4). Germination was complete in the simple amino acid medium (Powell, 1950), but no development into vegetative cells occurred. There was a threefold increase in cellular vitamin B₆ during spore development in the enriched potato-extract medium and only a slight increase in the simple amino acid medium. The slight increase could be entirely accounted for by the change in spore dry wt. during germination.

In a previous study (Powell & Strange, 1957) the DAP decarboxylase activity of cells of *B. sphaericus* from cultures at various stages of growth and sporulation was determined. It was concluded that no DAP decarboxylase synthesis occurred during the development of the spore into a vegetative cell. This conclusion was the result of measurements made on cultures in the enriched potato-extract medium inoculated with 2 mg. dry wt. of spores/ml. The results obtained in the present study suggested that DAP decarboxylase synthesis during spore development might be demonstrable in more dilute suspensions. In Expts. 5 and 6 (Table 3), the DAP

Table 3. *Total vitamin B₆ content of resting, germinated and developing spores of Bacillus sphaericus N.C.T.C. 9602 in enriched potato-extract medium at 37°*

In Expt. no. 1 values in parentheses refer to pyridoxal phosphate (μm -moles/g. dry wt. of cells). The inocula (mg./dry wt. of spores/ml.) were 1.0 and 0.6 for Expts. 1 and 2 respectively and 0.2 for Expts. 3–6.

Time of incubation (hr.)	Appearance of culture	Vitamin B ₆ content (μm -moles/g. dry wt. of cells)					
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
0	Resting spores	95 (57)	83	67	68	83	81
0.33	Germinated spores	—	—	—	—	136	136
1.0	Swollen germinated spores	124 (53)	106	200	188	215	219
2.0	Very short granular vegetative cells	148 (60)	124	—	—	—	—
3.0	Short granular vegetative cells	—	—	158	163	—	—
7.0*	Featureless vegetative cells*	426 (250)	510	—	—	—	—

* Separate cultures grown from a small inoculum (0.05–0.10 μg . dry wt. of spores/ml.) for comparison assay.

Table 4. *Vitamin B₆ content and diaminopimelic acid decarboxylase activity of resting and developing spores of Bacillus sphaericus N.C.T.C. 9602*

The spore inoculum was 0.2 mg. (dry wt.) of spores/ml.

Medium	Appearance of culture after 1 hr. at 37°	Vitamin B ₆ content (μm -moles/g. dry wt. of cells)	DAP decarboxylase activity ($Q_{\text{CO}_2}^{\text{N}}$)
Distilled water	Resting spores	72	1.4
4 mM-L-Alanine in 40 mM-phosphate buffer, pH 7.3	Germinated spores	93	2.0
Enriched potato-extract medium	Short granular vegetative cells	255	4.7

decarboxylase activity of spores (0.2 mg. dry wt./ml.) allowed to develop for 1 hr. in the enriched potato-extract medium was compared with that of resting spores. In Expt. 5, the $Q_{CO_2}^{N_2}$ of resting spores was 3.2 and of developing spores 5.1. In Expt. 6 the corresponding $Q_{CO_2}^{N_2}$ values were 3.2 and 5.4, and in a similar experiment not included in Table 3, 3.6 and 11.5. In the experiment summarized in Table 4 the DAP decarboxylase activity of resting spores was compared with that of spores allowed to develop for 1 hr. in the enriched potato-extract medium and in a buffered solution of L-alanine. The $Q_{CO_2}^{N_2}$ values obtained were 1.4, 4.7 and 2.0. The apparent slight increase in DAP decarboxylase activity during germination in L-alanine could be accounted for by the decrease in spore dry wt. during germination. It should be noted that the DAP decarboxylase assays were made in the presence of an added excess of pyridoxal phosphate.

It seemed possible that first-generation vegetative cells of *B. sphaericus*, having developed under conditions of relatively low vitamin B₆ concentration and DAP decarboxylase activity, might contain DAP in amounts comparable with those in resting spores. In Expts. 2 and 3 (Table 3) resting spores contained 1.5%, and spores incubated for 1 hr. in the nutrient medium 0.2% (w/w) of DAP. After a further incubation for 1 hr. in Expt. 2, and for 2 hr. in Expt. 3, no DAP could be detected in 10 mg. dry wt. of cells.

Effect of pyridoxine and its antagonists on growing cultures of Bacillus sphaericus

Pyridoxine, pyridoxal or pyridoxal phosphate was added to the enriched potato-extract medium to give concentrations up to 0.5 mM. Batches of medium (10 ml.) with and without additions were inoculated with *B. sphaericus*, strains N.C.T.C. 9602, 7582, or a laboratory strain of *B. sphaericus* var. *rotans*. The cultures were shaken at 37° for 18 hr. and examined in stained films. The additions had no apparent inhibitory effect on sporulation and there was normal growth. In a similar series of tests, the addition of deoxypyridoxine (2 mM) or isonicotinyl hydrazide (INH) (10 mM) had no stimulatory effect on the sporulation of *B. sphaericus* N.C.T.C. 7582 or its asporogenous variant (Powell & Hunter, 1955). Vegetative cells from 9 hr. cultures growing in the presence of the inhibitors were examined for their DAP content. No DAP was detected chromatographically (Rhuland *et al.* 1955) in hydrolysates containing 5 mg. dry wt. of cells.

DISCUSSION

In previous studies it was shown that DAP was a constituent of sporulating cells and spores of

B. sphaericus but not of vegetative cells, whereas in other *Bacillus* species vegetative cells, sporulating cells and spores all contained approximately equal amounts of DAP (Powell & Strange, 1956; Strange & Dark, 1956a; Powell & Strange, 1957). On a basis of cell dry wt., the DAP content of *B. sphaericus* cells increased approximately twofold as the sporangia were shed and the spores freed (Powell & Strange, 1957). It therefore seemed likely that DAP was a constituent of the spore, but not of the surrounding sporangium. As in other *Bacillus* species, the DAP of *B. sphaericus* spores was identified as a constituent of a soluble hexosamine-containing peptide liberated during spore germination or mechanical disintegration and derived from the spore coat (Strange & Dark, 1956b; Powell & Strange, 1957; Strange & Dark, 1957). In contrast with other *Bacillus* species (Antia *et al.* 1957) vegetative cells of *B. sphaericus* possessed a very active DAP decarboxylase system ($Q_{CO_2}^{N_2}$, 20–30) requiring pyridoxal phosphate as a cofactor (Powell & Strange, 1957). It is possible that in *B. sphaericus* *meso*-DAP is produced during vegetative growth but immediately decarboxylated. Alternatively both DAP and the structurally related dipicolinic acid may be synthesized from a common precursor when sporulation begins. It was first thought that the appearance of *meso*-DAP in sporulating cells of *B. sphaericus* might be associated with a decrease in their DAP decarboxylase activity, brought about by a failure in the synthesis of the apoenzyme or its cofactor. It was found, however, that the DAP decarboxylase activity of sporulating cells was still approximately 50% that of vegetative cells and that their pyridoxal phosphate content was equal to that of vegetative cells. On the other hand, the DAP decarboxylase activity of free spores corresponded to only 10% and their pyridoxal phosphate to 25% of that of vegetative and sporulating cells. Free resting spores are metabolically inert and their protoplasmic constitution is very different from that of vegetative cells. It is therefore likely that the protoplasm and the metabolic activity of developing spores are different from that of the surrounding sporangia: thus the DAP decarboxylase activity of developing spores of *B. sphaericus* may be considerably lower than the measured overall activity of sporulating cells and may closely approach that of free spores. The incorporation of DAP rather than lysine into the spore peptide might be accounted for on this basis, if, as again seems likely, the construction of the spore coat depends upon the metabolic processes of the developing spore itself. A preparation of spore peptide isolated from germination exudate of *Bacillus megaterium* (Strange & Powell, 1954) was not decarboxylated by a disintegrated suspension of vegetative cells of *B. sphaericus* (J. F. Powell,

unpublished work). It appeared therefore that once incorporated into the spore peptide, DAP was no longer susceptible to attack by the decarboxylase.

It has been shown (Davison, 1956) that INH inhibited certain enzymes which require pyridoxal by combining with the cofactor. Hoare (1956) showed that lysine and DAP decarboxylases are inhibited by INH (0.5 mM). In the present study, vegetative cells of *B. sphaericus* growing in the presence of a concentration of INH (10 mM) slightly lower than that which caused growth inhibition, still contained no DAP. In this connexion it was also interesting that no DAP could be detected in first-generation vegetative cells of *B. sphaericus* which had developed under conditions of relatively low DAP decarboxylase activity.

It appears that no vitamin B₆ or DAP decarboxylase synthesis occurred during spore germination when this proceeded in the presence of the specific germination stimulant alone. Vitamin B₆ and DAP decarboxylase synthesis did occur in the presence of nutrients as a result of the further development and growth of the germinated spore. This is in accord with previous observations on the different natures of the germination process and the subsequent phases of development and growth, readily demonstrated by their widely different susceptibilities to various inhibitors (Powell, 1950, 1951; Roth & Lively, 1956; Murty & Halvorson, 1957). The increase in vitamin B₆ content and DAP decarboxylase activity during spore development was more marked when the size of the spore inoculum was reduced. In the most dilute suspensions used (0.2 mg. dry wt./ml.) the cell concentration was approximately one-eighth of the maximum cell concentration reached in cultures growing freely from a very small inoculum. The microscopic appearance of first-generation vegetative cells developing in suspensions inoculated with 0.2 mg. dry wt. of spores/ml. and their failure to divide during the 3 hr. period of an experiment suggested that the spore inoculum should have been reduced even further to simulate normal growth conditions.

SUMMARY

1. In sporulating cells of *Bacillus sphaericus* N.C.T.C. 9602, $\alpha\epsilon$ -diaminopimelic acid (DAP) was present as the *meso*-isomer.

2. The total vitamin B₆ content of vegetative and sporulating cells of *B. sphaericus* ranged between 300 and 550 μ m-moles and the pyridoxal phosphate content between 180 and 330 μ m-moles/g. dry wt. of cells. The corresponding values for

free resting spores were 70–200 and 50–95 μ m-moles/g. dry wt. of cells.

3. In two cultures of a laboratory strain of *Bacillus cereus* the total vitamin B₆ content of vegetative cells was 162 and 224 μ m-moles/g. dry wt. of cells and that of free resting spores 99 and 73 μ m-moles/g. dry wt. of cells.

4. The vitamin B₆ content and DAP decarboxylase activity of *B. sphaericus* spores increased approximately threefold during development and growth for 1 hr. in a nutrient medium. Although germination occurred there was no increase in cellular vitamin B₆ or DAP decarboxylase activity when spores of *B. sphaericus* were incubated for 1 hr. with L-alanine.

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

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