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Cell Wall Polymers of *Bacillus sphaericus* 9602

II. Synthesis of the First Enzyme Unique to Cortex Synthesis During Sporulation

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The cell wall peptidoglycan of vegetative cells of *Bacillus sphaericus* 9602 contains L-lysine and D-isoasparagine and is devoid of diaminopimelic acid (Dap), whereas the peptidoglycan of its spore cortex is devoid of L-lysine and D-isoasparagine and contains *meso*-Dap. These two structures have a common biosynthetic precursor, uridine-diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamic acid, which accepts either L-lysine or *meso*-Dap, the latter reaction being the first unique to the synthesis of the spore cortex peptidoglycan. L-lysine-adding activity decays at the end of vegetative growth to a level which is maintained until Dap-adding activity appears, when it declines rapidly again. Dap-adding activity is not detectable in refractile spores, in vegetative cells, or in sporulating cells until about 4 hr after the end of vegetative growth, when it increases rapidly for about 1.5 hr in a process dependent on continued protein and ribonucleic acid (RNA) synthesis. This process apparently involves transcription and translation during this period of a "sporulation-specific" gene whose product is essential for and unique to sporulation. It is closely followed by the acquirement of refractility. Another sporulation-specific gene, that for dipicolinate synthase, is apparently transcribed and translated in an overlapping period commencing about 0.5 hr later, although dipicolinate does not accumulate rapidly until 1.5 hr later, when about 75% of the cells are already refractile. Inhibition of protein synthesis with chloramphenicol or of RNA synthesis with streptolydigin inhibited accumulation of these enzymes in sporulating cells; this inhibition could be reversed by washing out the antibiotics after 1.5 hr. Sporulation recommenced with an unaltered sequence of events but with poorer synchrony. There was no evidence for a messenger RNA for either enzyme of lifetime greater than a small fraction of the period of enzyme accumulation, although dilution with 10 volumes of fresh medium failed to prevent synthesis of Dap-adding enzyme in cells which had become terminally swollen, a process preceding enzyme synthesis by about 1.5 hr. The synthesis of this enzyme in *B. sphaericus* is apparently dependent on programmed transcription of the appropriate gene.

The physiology of sporulation in bacteria has been intensively studied (for recent reviews, see 1, 9, 14, 19) since the product, the most resistant, dormant, and long-lived autonomous cell form known, is an important factor in the ecology of microorganisms (especially in the soil), in disease, and in problems of food preservation. Sporulation involves a well-defined sequence of morphological stages which have been documented by electron microscopy in *Bacillus cereus*, *B. subtilis*, and *B. megaterium* and correlated with sequential biochemical events (19). Sporulation has also been studied as an example of cellular differentiation in bacteria, and has been subjected to analysis by the biochemical and genetic techniques to which procaryotic organisms are particularly susceptible. The genetics of sporulation is somewhat confused at present (1), probably because of difficulties in interpretation of some of the biochemical data. It seems clear that sporulation involves the expression of genes which are repressed during vegetative growth, and that the derepression of these genes is triggered by an unknown event coupled to depletion of the medium. The problem is in recognizing and assaying for the products of these genes so that the control of their expression can be investigated. This problem is compounded by the fact that, as in all bacteria, nutrient depletion causes derepression of many inducible enzyme systems related to the supply of

energy and structural components for biosynthesis. Although the supply of these commodities may be essential for sporulation to take place, since sporulation involves synthesis of large quantities of new structural components (coats and cortex) and turnover of at least 85% of the total cell protein (14), several of these enzyme systems can be induced during vegetative growth. A "spore" gene can be recognized if its product (i) is found only in spores or (ii) is necessary for the synthesis of a unique spore component. This conservative definition excludes enzymes inducible during vegetative growth [e.g., some proteases and several enzymes involved in acetate oxidation, including those which complete the tricarboxylic acid cycle (normally incomplete in vegetative bacilli), and NADH (reduced nicotinamide adenine dinucleotide) dehydrogenase (5, 10, 19)]. The only chemically identified candidates for class i spore gene products are the unique spore coat polypeptides (2, 25), whereas only dipicolinate synthase among class ii spore gene products has been investigated (4). Other candidates include those for synthesis of sulfolactic acid in *B. subtilis* (20) or for the synthesis of unique components of spore cortex peptidoglycan, whose structure in *B. subtilis* has only recently been elucidated. This peptidoglycan, and that in the cortex of several other bacilli (30; A. D. Warth, Ph.D. thesis, University of Wisconsin, 1968), contains the same components as does the vegetative cell wall peptidoglycan of *B. subtilis* and most other bacilli, namely glucosamine, muramic acid, L- and D-alanine, D-glutamate and *meso*-α,ϵ-diaminopimelic acid (Dap). It contains *N*-acetylmuramic acid residues substituted by tetrapeptides of sequence identical to that found in *Escherichia coli* cell wall peptidoglycan (6), namely L-alanyl-D-isoglutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine. These tetrapeptides are crosslinked about 20% between D-alanine and (D)-*meso*-diaminopimelic acid residues. Like all known peptidoglycans, its glycan consists of alternating, probably β-1,4-linked residues of glucosamine and muramic acid. However, unlike all other known peptidoglycans, 10 to 20% of its muramic acid residues are substituted by single C-terminal L-alanine residues and another 50% exist as muramic lactam residues (30). The structure of all other known peptidoglycans is consistent with a common nucleotide pentapeptide precursor of sequence uridine diphosphate (UDP)-*N*-acetylmuramyl-(A)-isoglutamyl-(B)-D-alanyl-D-alanine, where A and B are glycine or amino acids with both residues of their L-α-aminoacyl moiety involved in this peptide chain. This precursor is converted to a lipid-bound *N*-acetylglucosaminyl-*N*-acetylmuramic acid pentapeptide, which is converted to a complete subunit of the peptidoglycan, and then polymerized (to give the alternating glycan structure) and subjected to transpeptidation and partial hydrolysis to give the product found in isolated cell walls (27). If the cortex is also synthesized by polymerization of disaccharide-pentapeptide units, subsequent events must include transpeptidation and D-alanine carboxypeptidase action to give the tetrapeptides, endopeptidase action to give the L-alanine C-termini, and either amidase action and transacylation or de-N-acetylation and transamidation to form the lactam residues. This process in *B. subtilis* requires a minimum of two new enzymes, presumably class ii spore genes. However, it has been known for some time that *B. sphaericus* vegetative cell walls are unusual in containing aspartic acid and lysine while being apparently devoid of Dap, whereas their spore peptide contains Dap (21, 22). Since *meso*-Dap is converted directly to L-lysine in *B. sphaericus* by Dap decarboxylase (8, 22, 33, 34), the cells should have Dap available at all times. However, if the vegetative peptidoglycan is indeed devoid of Dap and if the pentapeptide-synthesizing enzymes are specific for a single amino acid (12, 13), cortex peptidoglycan synthesis in *B. sphaericus* should require synthesis of at least one additional new enzyme for production of the Dap-containing nucleotide pentapeptide.

The structure of the vegetative cell wall peptidoglycan of *B. sphaericus* 9602 has been extensively analyzed (11) and found to consist of a glycan of alternating β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, all of whose muramic acid residue are substituted by the peptide N^{α}-(L-alanyl-D-isoglutamyl) N^{ϵ}-(D-isoasparaginyl) L-lysyl-D-alanine, 65% cross-linked between D-alanine and D-isoasparagine. The residual C-terminal peptide subunits are devoid of D-alanine residues (Fig. 1) and the walls of this organism are indeed devoid of Dap. The glycan of the spore cortex peptidoglycan also consists of alternating glucosamine and muramic acid residues, but 54% of the latter are lactamized, whereas 18% carry a single L-alanine residue and only the residual 28% carry a tetrapeptide, identical to that found in *B. subtilis* cortex (Fig. 2) (D. J. Tipper, *in preparation*). The nucleotide pentapeptide precursors of these two peptidoglycans should differ only in the substitution of Dap at position B in the general structure (*above*) in the cortex precursor for L-lysine in the wall precursor, A being L-alanine in both cases. Since the first three amino acids are added sequentially to UDP-*N*-acetylmuramic acid in synthesis of the nucleotide pentapeptide in *Staphylococcus aureus* [which should be identical to that for *B. sphaericus* wall synthesis (13)], these two nucleotide penta-

peptides should have a common precursor, UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid. If this nucleotide dipeptide accepts L-lysine, the product is a precursor of the wall peptidoglycan, whereas addition of Dap constitutes a divergence leading to cortex synthesis. If the lysine and Dap-adding enzymes are specific, the latter will be the first enzyme unique to cortex synthesis, and having no vegetative function, would qualify as a class ii spore product. This paper describes the appearance of such an enzyme activity during sporulation in *B. sphaericus* and the sensitivity of this event to inhibition of protein and ribonucleic acid (RNA) synthesis and to dilution with fresh medium.

MATERIALS AND METHODS

Physiology of B. sphaericus 9602. The organism and the medium have been previously described (11). Cultures were maintained on slopes of 2% agar in the same medium. Primary cultures were prepared in 50 ml of medium inoculated from slopes and grown at 33 C in 250-ml flasks with vigorous aeration. After 9 hr (mid-log phase), samples (10 ml) were inoculated into 1 liter of medium in 2,000-ml flasks with deep-base indentations and grown at 33 C with vigorous aeration. Under these conditions, growth in the secondary culture was rapid, with a generation time of about 50 min, slowing after 3.5 to 4 hr. Cell division ceased after 4 to 5 hr, and subsequent sporulation was relatively synchronous and at least 95% complete. In some experiments, only 2 ml of inocula were used, with a consequent protraction of vegetative growth to about 7 hr. Terminal swelling of *B. sphaericus* cells occurs about 2 hr after cell division ceases and 3 hr before refractility appears. The proportion of terminally swollen cells and refractile prespores (per cent morphology) was estimated by phase-contrast microscopy. Turbidities were determined, on samples diluted 1:5 with 0.02 M KPO_4 (*p*H 7.5), at

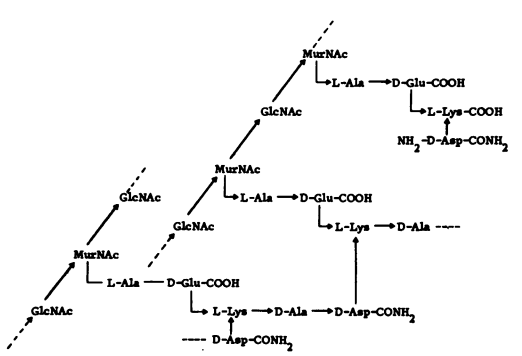

FIG. 1. *Structure of the vegetative cell wall peptidoglycan of B. sphaericus 9602. The lower part of the figure illustrates two glycan chains cross-linked through N^{ϵ}-(D-alanyl-D-isoasparaginyl) L-lysine linkages. The upper part of the figure contains an uncross-linked peptide devoid of D-alanine.*

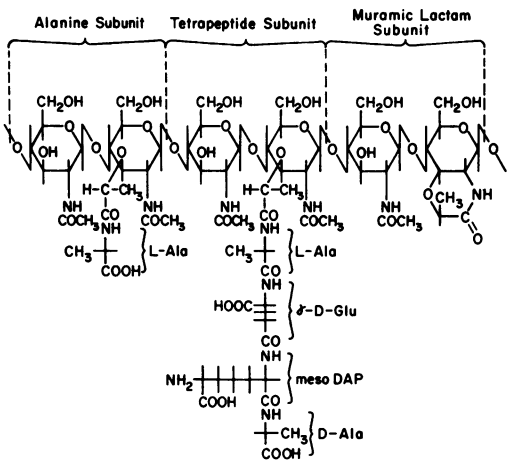

FIG. 2. *Structure of the spore cortex peptidoglycan of B. sphaericus 9602. The linkages between N-acetylglucosamine and N-acetyl-muramic acid and between muramic lactam and N-acetylglucosamine have been identified as β-1,4.*

600 nm by using a Zeiss PMQ2 spectrophotometer. A dispersion of 1 g of wet-cell pellet of *B. sphaericus* cells in 1 liter of medium had a turbidity of 0.11.

B. subtilis culture. A culture of *B. subtilis* 168 was obtained from R. Hanson, Department of Bacteriology, University of Wisconsin. It was cultured and maintained as described for *B. sphaericus*, except that the incubation temperature was 37 C.

Materials. Dap-*U*-^3H was a mixture of LL and *meso* forms, containing about 60% of the latter, and was a gift from J. L. Strominger; it was purified by fractionation on the long column of a Beckman-Spinco amino acid analyzer by using the *p*H 3.25 eluant. The Dap-containing fractions were identified by paper electrophoresis at *p*H 1.9 (20 ml of concentrated formic acid per liter), pooled, and desalted on Dowex-50 eluted first with water and then with 0.5 M ammonia. The product gave a single radioactive spot after electrophoresis at *p*H 1.9 with the mobility of Dap, and was diluted with cold 60% *meso*-Dap to a specific activity of 1.5 mc/mmole. It could then be 55% decarboxylated to lysine by a crude *B. sphaericus* enzyme preparation containing DAP decarboxylase. L-Lysine-*U*-^{14}C (248 mc/mmole) was purchased from New England Nuclear Corp. and was diluted with cold L-lysine to 1.5 mc/mmole.

UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid (UDP-MurNac-dipeptide) was isolated from *S. aureus* cells starved for lysine (12) as follows. A culture of *S. aureus* Copenhagen (24 liters) was grown to 35% of maximum density, harvested, and resuspended in 6 liters of a medium containing 0.8 mM L-alanine, 0.2 mM D-glutamate, 30 mM glucose, and 30 mM phosphate buffer (*p*H 7.8). After 90 min at 37 C, the cells were again harvested, washed twice with water, and boiled for 10 min in water (240 ml). After cooling, the suspension was made 5% in trichloroacetic acid, stirred for 30 min, and centrifuged. The supernatant was extracted five times with ether

at 0 C, neutralized to *p*H 3 with a few drops of 1 M ammonia; after centrifugation, it was fractioned on a column of AG1-X2 resin (2 by 30 cm) in 0.01 N HCl by using a gradient of increasing NaCl concentration. Nucleotide peaks were detected by measurement of absorbance at 260 nm, and peaks were analyzed for *N*-acetyl-hexosamine content by the 7-min Morgan-Elson procedure after hydrolysis for 5 min at 100 C in 0.025 N HCl (12). The amino acid contents of these peaks were identified by acid hydrolysis and paper electrophoresis of the products. The peak containing only muramic acid, alanine, and glutamic acid was pooled and desalted on Biogel P2 in water, which, incidentally, further fractionated the required nucleotide from nonpeptide nucleotides. The final product (40 μmoles) contained equimolar muramic acid, alanine, and glutamate (by amino acid analysis), and no other ninhydrin-positive components. According to its ultraviolet (UV) spectrum, it also contained 1 mole of uridylate per mole of muramic acid.

UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysine was prepared in essentially the same way from mid-log-phase cells of *S. aureus* Copenhagen (10 liters) incubated for 90 min at 37 C in 30 mM KPO_4 (*p*H 7.8), containing 30 mM glucose, 1.8 mM L-alanine, 1.2 mM D-glutamate, 1.2 mM L-lysine and 0.7 mM D-cycloserine (12). The yield, following extraction and fractionation on AG1 and on Biogel P2, was 62 μmoles, containing equimolar uridylate, muramic acid, alanine, glutamic acid, and lysine. The *meso*-diaminopimelic acid (Dap) analogue was a gift from Roland Plapp.

Enzyme preparation and assay: B. sphaericus. Samples of cell suspensions (10 to 100 ml) were centrifuged for 10 min at 20,000 × *g*; the pellets were washed once with 20 ml of cold 0.02 M KPO_4 (*p*H 7.8), resuspended in 1 ml of 0.02 M KPO_4 (*p*H 7.8) in 15-ml Corex tubes, and disrupted for 10 min by using the microtip of a Branson J17V Sonifier (Heat Systems, Inc.). Immersion in an alcohol bath at −10 C maintained an internal temperature of 0 to 4 C, and about 50% cell breakage occurred in 2 min. In cells containing visibly refractile prespores, these remained largely intact after 10 min. The broken-cell suspensions were centrifuged (20 min, 25,000 × *g*) and the supernatants were precipitated for 30 min at 0 C with three volumes of cold saturated ammonium sulfate (*p*H 7.5) containing 10^{-3} M ethylenediaminetetraacetic acid (EDTA). Pellets were redissolved in 0.05 KPO_4 (*p*H 7.8) to give a protein concentration of 10 to 20 mg/ml and stored at −20 C.

The assay for lysine addition contained, in 50 μliters: 50 mM tris(hydroxymethyl)aminomethane-(Tris)-hydrochloride (*p*H 8.5), 4 mM $MgCl_2$, 5 mM adenosine triphosphate (ATP), 1 mM L-aspartate, 0.4 mM L-lysine-*U*-^{14}C (5 × 10^4 counts/min), 0.4 mM MurNac-dipeptide, and enzyme (1 to 10 μliters). After incubation for 30 min at 37 C, assay mixes were heated for 2 min at 100 C, centrifuged, and samples (30 μliters) of the supernatants were spotted on Whatman 3MM paper. Strips were electrophoresed for 45 min at 40 volts/cm in a Gilson model D solvent-cooled apparatus. After drying, radioactive products were detected by autoradiography or by reference to ninhydrin-stained marker strips, or both, and the appropriate areas of paper were cut out, immersed in toluene/2,5-diphenyloxazole/dimethyl-1,4-bis-2-(5-phenyloxazolyl)-benzene scintillator and counted in a Packard Tri-Carb liquid scintillation counter. The assay mixture for Dap addition contained, in 50 μliters: 50 mM Tris-hydrochloride (*p*H 8.5), 4 mM $MnCl_2$, 0.8 mM Dap-*U*-^3H (10^6 counts/min, 60% *meso*, 40% LL), 5 mM ATP, 2 mM isonicotinic acid hydrazide (INH), 1 mM L-aspartate, 8 mM L-lysine, 0.5 mM UDP-MurNac-dipeptide, and enzyme (1 to 10 μliters). The procedure followed that described for lysine addition. The micromoles of amino acid incorporated was calculated from the fraction of the total counts found in the nucleotides. Protein was determined by the procedure of Lowry et al. (18). One unit of specific activity was defined as one nanomole of lysine or DAP added per milligram of protein per hour at 37 C.

Enzyme preparation and assay: B. subtilis. A culture (1,000 ml) of *B. subtilis* 168 was grown to 30% of maximum turbidity. The cells were isolated by centrifugation and broken in a Braun MSK homogenizer (Bronwill Scientific, Rochester, N.Y.) in 20 ml of 0.02 M KPO_4 (*p*H 7.8). The broken cells were centrifuged at 40,000 × *g* and the supernatant was precipitated with ammonium sulfate (75% saturation). The precipitate was redissolved in the same buffer (10 ml, 22 mg of protein/ml). This crude enzyme preparation, assayed for Dap addition under the conditions described for *B. sphaericus* but using 4 mM Mg^{2+} instead of Mn^{2+}, had a specific activity of 490 units.

Quantitation of DPA. The procedure was essentially that described by Lewis (15). Samples (1 ml) of cell suspensions were centrifuged, and the pellets were washed once in 0.01 M KPO_4 (*p*H 7.5) and heated for 1 hr at 100 C in 75% ethanol containing 1% acetic acid (0.4 ml). After centrifugation, a portion (0.3 ml) of the supernatant was dried and redissolved in 0.4 ml of 0.01 M $Ca(OH_2)$. The calcium chelate of DPA has absorption maxima at 263, 269.8, and 277.8 nm and minima at 265.8 and 276.5 nm. Calcium chelate can be quantitated in the presence of a high background of continuous absorbance (as given by pigment in extracts of sporulating cells) by determination of $N = (A\ 277.8 + 2\ A\ 269.8) - (A\ 266.8 + 2\ A\ 276.5)$, where $N = 1$ for 16 μg of DPA in the original 1-ml sample of the culture.

RESULTS

Enzyme preparation. Enzymes involved in the synthesis of UDP-*N*-acetylmuramyl-pentapeptide precursors of cell wall peptidoglycans are found in the 50,000 × *g* supernatant from breakage of several bacterial species (12), and the same is true in *B. sphaericus*. Under the conditions described for disruption, release of protein into the 50,000 × *g* supernatant was maximal in 8 to 10 min, and 10 min was taken as the standard procedure. Further disruption (5 min) of the 50,000 × *g* pellet from disruption of vegetative cells of *B. sphaericus* released only 5% more pro-

tein into the 50,000 × *g* supernatant from recentrifugation, and this supernatant contained only 2% of the total solubilized lysine-adding activity. Ammonium sulfate precipitation was necessary to remove endogenous amino acids and nucleotides, and gave excellent recoveries of enzyme activity. The efficiency of the entire procedure for isolation of soluble proteins is demonstrated by the consistent recovery of 0.35 to 0.4 mg of protein per ml of culture from parallel sporulating cultures and also from sequential samples of single sporulating cultures, and the consistent recovery of enzyme activity is indicated by the relatively constant values found in duplicate culture samples and for sequential samples from chloramphenicol-inhibited cultures.

General assay procedures. The electrophoresis assay procedure described depends on separation, at *p*H 3.9, of negatively charged UDP-MurNac-peptide derivatives from the neutral or positively charged free amino acid. The mobilities, relative to alanine (zero) and lysine (100), were: Dap, −2; glutamate, −38; aspartate, −68; UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysine, −62; and the analogous Dap-containing nucleotide, −80. Radioautography and the counting of strips 1 cm wide indicated that the ^{14}C-lysine and ^{3}H-Dap solutions employed each gave single radioactive spots of unchanged mobility after incubation under the appropriate assay conditions followed by hydrolysis (except for a small amount of lysine formed from Dap). The only significant new radioactive spots found before hydrolysis had the expected mobilities of the nucleotide tripeptides. Incubations contained aspartate, which was sometimes used as a marker for the nucleotide-containing areas after detection with ninhydrin. Duplicate assays were also made by using unstained electrophoretograms, and the quenching and radioactivity losses due to the ninhydrin stain were found to be less than 5%. Areas corresponding to the unchanged amino acid and the nucleotide products were excised for counting, and the proportion of the total radioactivity incorporated into the nucleotide was calculated. The recovery of total radioactivity was always greater than 90%. On two occasions, a charcoal assay procedure (12) was used on duplicate incubations. This procedure depends on the adsorption of nucleotides in 5% trichloroacetic acid solution to charcoal, and subsequent elution by dilute ammonia in 50% ethanol. The reported recovery of radioactivity is 70% (12), and 70 to 75% of the radioactivity found in the nucleotide by the electrophoresis procedure was actually recovered.

Properties of lysine-adding enzyme in B. sphaericus. The properties of the lysine-adding enzyme, UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid, L-lysine ligase, prepared from cells of *B. sphaericus* 3 hr after the end of vegetative growth (90% terminally swollen) were similar to those found for the *S. aureus* enzyme (13). In 50 mM Tris-hydrochloride (*p*H 8.5), optimal Mn^{2+} concentration was 4 mM, but a slightly higher activity was found with 4 mM Mg^{2+}, the condition chosen for the assay, although 10 mM Mg^{2+} gave 30% higher activity. The intracellular concentrations of unchelated divalent metal ions are unknown in *B. sphaericus*, and probably depend on growth phase and medium composition. In 4 mM Mg^{2+}, the optimal *p*H range in Tris-hydrochloride was broad (8.3 to 9). Under the assay conditions, incorporation in 30 min was proportional to the amount of enzyme added over the range of 20 to 200 μg of protein, and incorporation was approximately linear for 60 min, if less than 10% of the nucleotide acceptor was utilized.

In view of the consistent recovery of soluble protein and the proportionality of the assay procedure, it is felt that ratios of measured specific activities are meaningful. Since the in vivo environment cannot be reproduced and probably changes during sporulation, the true in vivo activities are unknown, but hopefully they are proportional and not too different.

meso-Dap decarboxylase activity and its inhibition. The Dap-adding enzymes of *E. coli* and *B. cereus*, like all known enzymes involved in the synthesis of UDP-MurNac-pentapeptide, have an optimal *p*H range of 8 to 9. They are also equally active in 4 mM Mg^{2+} and Mn^{2+} (12). Preliminary attempts to assay for Dap-adding enzyme in *B. sphaericus* under the conditions used for the lysine-adding enzyme resulted only in the conversion of ^{3}H-Dap to lysine. Enzymes (50 μg of protein) from all stages of sporulation caused a maximum conversion to lysine of 57% of added cold Dap (60% *meso*, 40% LL forms) in 30 min at 37 C, and obviously contained high activities of *meso*-Dap decarboxylase (33), as previously reported by Powell and Strange (22). The *E. coli* enzyme has an absolute requirement for pyridoxal phosphate as a cofactor (34) and is inhibited by aldehyde reagents such as D-penicillamine (34).

B. sphaericus enzyme from cells that had just completed the terminal swelling stage of sporulation (200 μg) was incubated with 1 mM ^{3}H-Dap at 37 C for 30 min under the conditions for lysine assay. Conversion to lysine was 99% inhibited by 2 mM phenylhydrazine, 95% inhibited by 2 mM isonicotinic acid hydrazide, 72% inhibited by 2 mM D-penicillamine, and 12% inhibited by 10 mM β-aminopropionitrile or 10 mM L-lysine. These concentrations of phenylhydrazine and

isonicotinic acid hydrazide had no effect on the addition of lysine to UDP-MurNac-dipeptide in these enzyme preparations. Conditions chosen for assay for Dap addition were 1 mM ^{3}H-Dap, 2 mM isonicotinic acid hydrazide, and 8 mM cold L-lysine, the latter to dilute the tritiated lysine (>0.05 mM after 30 min with 200 μg of enzyme) produced by residual decarboxylase activity. This prevents appreciable synthesis of labeled lysine-nucleotide tripeptide which is not well separated from Dap-nucleotide tripeptide by the electrophoresis assay procedure. Because of the relatively low activities of the lysine-adding enzyme during sporulation, this did not result in significant reduction in the UDP-MurNac-dipeptide acceptor concentration during assay for Dap addition.

Properties of B. subtilis and B. sphaericus Dap-adding enzymes. First attempts at demonstrating Dap addition in preparations from sporulating *B. sphaericus* cells under the conditions used for the assay of lysine addition, but in the presence of INH and excess cold lysine, were disappointing. *B. subtilis* contains Dap in both its vegetative cell wall and spore cortex peptidoglycans, and must contain high activities of Dap-adding enzyme during vegetative growth. To test these assay conditions for Dap addition, a preparation of soluble enzymes was made from exponential-phase *B. subtilis* cells and found to be highly active in 4 mM Mg^{2+}, with a broad *p*H optimum (Tris-hydrochloride) between 8 and 9. The dependence of the specific activity of Dap addition of this enzyme on Mn^{2+} and Mg^{2+} concentrations was found to differ considerably from that of a preparation from sporulating *B. sphaericus* cells. The specific activity of the *B. subtilis* enzyme (60 μg of protein, assayed as described in Materials and Methods) increased with Mn^{2+} concentration to a maximum of 430 units at 1 mM, and dropped markedly at higher concentrations. It also increased with Mg^{2+} concentration to a maximum of 490 units at 4 mM, and the activity was maintained at higher concentrations (up to 0.1 M). The *B. sphaericus* enzyme had distinct optimal Mn^{2+} and Mg^{2+} concentrations of 4 mM and 16 mM, respectively, giving specific activities of 240 and 150 units (Fig. 3). Ca^{2+} did not activate. In 4 mM Mn^{2+} in 50 mM buffers, the *p*H optimum of the *B. sphaericus* enzyme was broad and activity was somewhat dependent on the anion. *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-hydrochloride (*p*H 8.1) or Tris-hydrochloride (*p*H 8.5) gave the highest activities. Four mM Mn^{2+} in 50 mM Tris-hydrochloride (*p*H 8.5) was used routinely. Under these conditions, the incorporation of Dap into UDP-MurNac-tripeptide was continuous for 75 min (Fig. 4). A

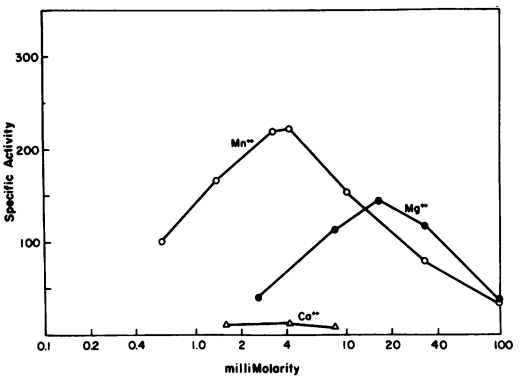

FIG. 3. *Divalent metal ion dependence of B. sphaericus Dap-adding enzyme. Enzyme from sporulating cells (95% swollen, 50% refractile, 102 μg) was assayed as described in the text.*

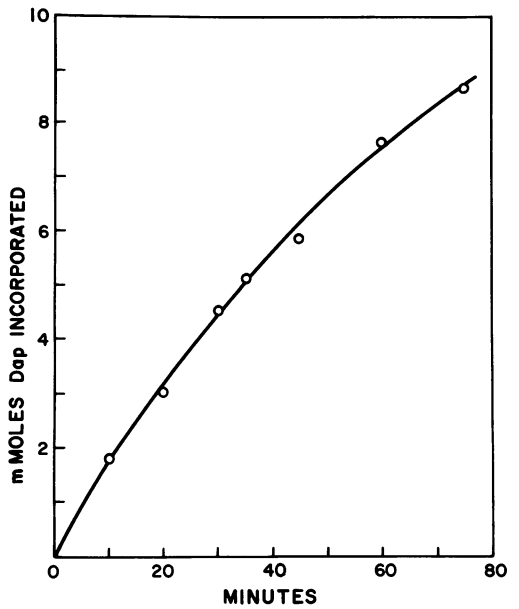

FIG. 4. *Kinetics of Dap addition by the B. sphaericus enzyme. Samples (60 μg) of the enzyme described in Fig. 3 were incubated under the standard conditions for assay of Dap addition except that the time of incubation was varied as indicated. The total amount of limiting substrate (UDP-MurNac-dipeptide) present was 20 nmoles.*

time of 30 min was chosen for the standard assay. With this enzyme, the amount of nucleotide formed in 30 min was proportional to the amount of enzyme protein in the range 10 to 80 μg (Fig. 5).

Variation in activity of lysine and Dap-adding enzyme activities during sporulation in B. sphaericus. Two cultures of *B. sphaericus* (1,000 ml) were simultaneously inoculated with exponential-

phase cells (10 ml) and incubated at 33 C. Measurement of optical densities and observation of dividing cells indicated that vegetative growth in both cultures ceased between 4 and 5 hr (Fig. 6), and a time of 4.25 hr was taken as the start of sporulation. Samples (50 ml) were removed at the intervals indicated in Fig. 6 and assayed for per cent morphology. Morphological data indicate reasonably good synchrony; 50% of the cells were swollen at 6.5 hr and 50% were refractile at 10 hr, by which time there was some loss of synchrony. Yields of soluble protein were similar from both cultures; the yields were constant (±10%) from 5.75 to 9.25 hr, and then decreased slowly to about 75% by 13.25 hr, at which time very little lysis of sporangia had occurred. (This process is frequently inefficient in *B. sphaericus* cultures.) Recoveries of lysine- and Dap-adding activities from the two cultures were similarly consistent. The activity of lysine-adding enzyme (multiplied by 4 in Fig. 6) decreased when exponential growth ceased and cells became shorter. After reaching a minimum of about 10 units at 5 to 6 hr, it increased slowly after terminal swelling occurred, reaching a maximum of about 13 units at 8 to 9 hr, followed by a rapid decline to 6 units as Dap-adding activity appeared. Activity decreased slowly thereafter to 2 units. By contrast, the cells were devoid of Dap-adding activity until 8.25 hr, at which time traces of activity

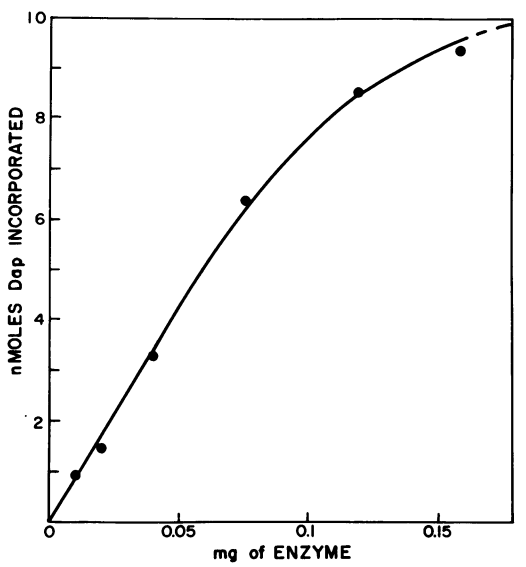

FIG. 5. *Dependence of Dap addition on amount of enzyme. The indicated amounts of the enzyme described in Fig. 3 were incubated under the standard conditions for assay of Dap addition (30 min at 37 C). Each incubation contained 20 nmoles of acceptor nucleotide dipeptide.*

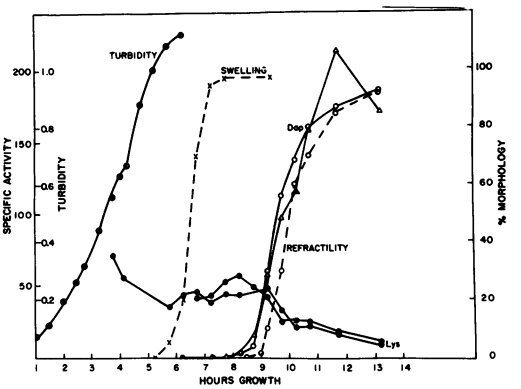

FIG. 6. *Variation in activity of lysine- and Dap-adding enzymes during sporulation in B. sphaericus. Samples of two 1-liter cultures were assayed for turbidity (⊗) and for the presence of dividing cells and the per cent morphology, i.e., the per cent of terminally swollen cells (×) and cells containing refractile prespores (○, dashed curves). These data for the two cultures were very similar and their averages are presented. Soluble enzymes were prepared from samples (30 ml) at the indicated times, and assayed for lysine-adding activities (●) and DaP-adding activities (○ and △) as described in the text. The specific activity data for the two cultures are presented separately. The activities of the lysine-adding enzymes are 25% of those indicated.*

appeared in both cultures. Activity increased rapidly in both cultures, the time of most rapid rise occurring at 9.25 hr, just after the first refractile prespores were seen, and 0.75 hr before 50% of the cells contained refractile prespores. Activity continued to increase to a maximum of 180 to 200 units at about 13 hr, when refractility had reached 90%. Most accumulation of Dap-adding activity occurred during the period from 4.5 to 6.5 hr after vegetative growth ceased, whereas most refractility was acquired during the period from 5 to 7 hr, 0.5 hr later.

Inhibition of protein and RNA synthesis in sporulating B. sphaericus. Chloramphenicol inhibits protein synthesis in bacteria by reversible binding to the 50*S* ribosomal subunit, causing cessation of peptide chain elongation (32). Streptolydigin inhibits RNA synthesis in bacteria by interacting with RNA polymerase (24). The sensitivity of protein and RNA synthesis to inhibition by these antibiotics in sporulating *B. sphaericus* was tested as shown in Fig. 7. Incorporation of uracil and leucine in the control followed identically shaped curves of decreasing slope. Addition of chloramphenicol resulted in immediate and complete cessation of net leucine incorporation, whereas addition of streptolydigin caused an immediate 80% reduction in the rate of net uracil incorporation. The residual 20% incor-

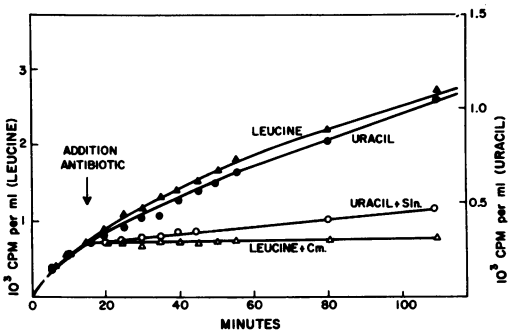

FIG. 7. *Inhibition of protein synthesis by chloramphenicol and of RNA synthesis by streptolydigin in B. sphaericus. Two samples (15 ml) of a culture (160 ml) of sporulating B. sphaericus (75% swollen) were mixed with L-leucine-U-^{14}C (1 μc, 0.1 μmole), two other samples (15 ml) were mixed with uracil-U-^{14}C (1 μc, 0.1 μmole), and all four were incubated at 33 C. After 15 min (arrow), chloramphenicol (Cm) was added to one of the first pair (final concentration 57 μg/ml), and streptolydigin (Sln) was added to one of the second pair (final concentration, 55 μg/ml). At the indicated times, samples (1 ml) were filtered on prewetted membrane filters (pore size 0.45 μm; Millipore Corp.) and washed twice with fresh medium (10 ml). After drying, the filters were immersed in toluene scintillator and counted.*

poration may be into uridine nucleotide, and other non-RNA cellular components.

Effect of chloramphenicol and streptolydigin on appearance of Dap-adding activity. The morphological state of a culture (100 ml) of *B. sphaericus* was followed, and the time when 50% of the cells were terminally swollen was noted. At intervals of 1, 1.75, 2.5, and 3.25 hr thereafter (estimated to cover the period of Dap-adding enzyme synthesis), duplicate 100-ml samples were removed and incubated with chloramphenicol (50 μg/ml) or streptolydigin (100 μg/ml) at 33 C in 250-ml flasks with vigorous aeration. At the same time, 30-ml samples were removed for Dap-adding enzyme assay and 1-ml samples were removed for assay of DPA. Similar samples were removed from the 100-ml cultures containing antibiotics at intervals of 45 min. The results of enzyme assays are shown in Fig. 8, and the DPA data are shown in Fig. 9. Figures 8 and 9 both contain the morphological data for the controls, showing that synchrony was better than that shown in Fig. 6, since swollen cells become refractile in about 2.75 hr, whereas the time of maximum rate of Dap-adding enzyme synthesis occurred only 0.5 hr before 50% of the spores were refractile. Dap-adding enzyme activity had not appeared when the first samples were removed for incubation with antibiotics, and subsequent appearance of activity in these samples was slight, although activity probably appeared shortly thereafter in the control (Fig. 8). The other incubations with antibiotics commenced when activity in the control was 12, 50, and 90% of maximum. In each instance, addition of chloramphenicol prevented all but a small subsequent increase in activity, which remained relatively constant after the first sample (45 min later; Fig. 8). The kinetics of this increase are unknown, but if it proceeded at the control rate, it would be accomplished in about 10 min. Manipulative delays and the delay in inhibition of ribosomal function are probably small (Fig. 7), and this increment may in part correspond to the time of maturation of nascent,

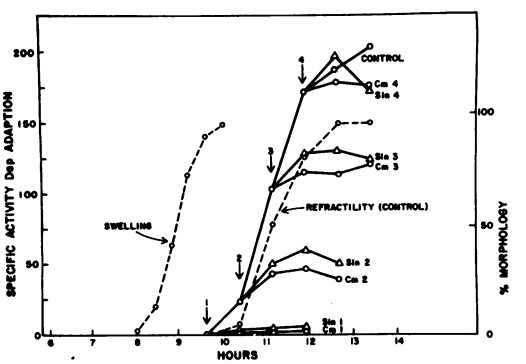

FIG. 8. *Inhibition of synthesis of Dap-adding enzyme in sporulating B. sphaericus. Samples of a sporulating B. sphaericus culture were removed at the four times indicated by the arrows and further incubated with either chloramphenicol (Cm, 50 μg/ml) or streptolydigin (Sln, 100 μg/ml). Cultures were assayed for Dap-adding enzyme at the indicated times. Morphological data are presented only for the control.*

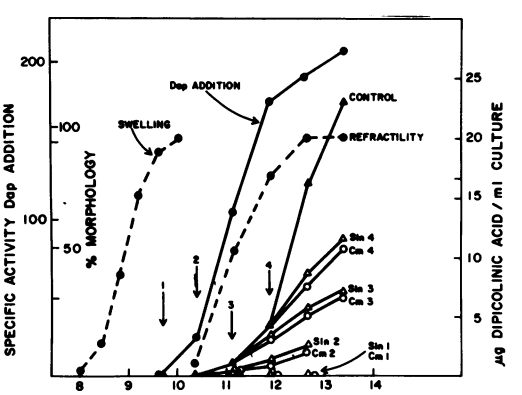

FIG. 9. *Inhibition of accumulation of dipicolinic acid. The same cultures described in the legend to Fig. 8 were assayed at the same intervals for dipicolinic acid content. The morphology and Dap-adding enzyme curves for the control are presented for reference, and the arrows again indicate the four times at which incubation with either chloramphenicol (Cm) or streptolydigin (Sln) commenced.*

completed polypeptides. It is clear that continued protein synthesis is required for the appearance of Dap-adding activity throughout the period of that appearance. Streptolydigin had a similar effect, but the plateaus, which were also reached within 45 min, were somewhat higher, corresponding to a longer delay in inhibition, about 20 min at the control rate of appearance of activity. The same factors plus transcription of preexistent messenger are involved in this delay. Thus continued appearance of activity also requires continued RNA synthesis, and is dependent on a messenger whose lifetime is less than 10% of the period (2 hr) during which activity accumulates rapidly.

The DPA data (Fig. 9) represent the accumulation of the product of DPA synthase, which is presumably synthesized at this time as in *B. subtilis* (4). Both chloramphenicol and streptolydigin prevented all accumulation of DPA when added before accumulation started in the control, and when added subsequently, caused rapid stabilization of the rate of DPA accumulation. If one assumes that this rate is proportional to the activity of DPA synthase, then this enzyme is apparently stable in vivo in the presence of both antibiotics, and these antibiotics rapidly inhibit accumulation of DPA synthase activity. Antibiotic-induced leakage of DPA from cells could not be detected.

Reversal of the inhibitory effects of chloramphenicol and streptolydigin on sporulation in B. sphaericus. The binding of chloramphenicol to ribosomes and of streptolydigin to RNA polymerase are both reversible (24, 32), so inhibited bacteria can be washed free of antibiotic and will eventually resume growth and cell division. These antibiotics were used to inhibit partially sporulated *B. sphaericus* cells to see whether removal of the antibiotics would be followed by resumption of the sporulating process.

Samples were first removed from a sporulating culture of *B. sphaericus* at 8.4 hr when its cells were 85% swollen, 0.5 hr before Dap-adding activity was first detected (Fig. 10). These samples were incubated with chloramphenicol or streptolydigin, which were washed out 1.5 hr later, when the control culture was already 70% refractile and rapidly accumulating DPA (Fig. 10). The washed cells were reincubated in the supernatant from duplicate samples removed from the main culture and centrifuged at the same time (8.4 hr). Fresh medium could not be used because of the possible reversibility of the sporulation process.

In both cultures, accumulation of Dap-adding activity, accumulation of DPA and acquirement of refractility ensued in their normal sequence.

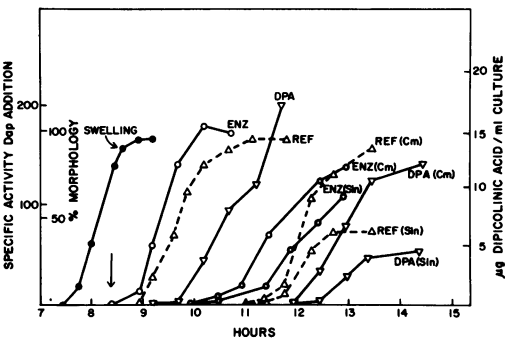

FIG. 10. *Reversible inhibition of sporulation in B. sphaericus. The four left hand curves show the data for the control culture. The other curves are for cultures incubated with chloramphenicol (Cm) or streptolydigin (Sln) from 8.4 hr (arrow) to 9.9 hr. A culture (750 ml) of B. sphaericus was grown at 33 C and samples (10 and 1 ml, respectively) were removed for assay of Dap-adding enzyme and DPA accumulation at the indicated times. Samples (200 ml) were also removed at 8.4, 8.9, and 9.7 hr. Of these samples, half were centrifuged at 2 C and the supernatant was stored at 2 C for subsequent use. The residuel (100 ml) was immediately divided into two 50-ml portions which were incubated at 33 C in the presence of chloramphenicol (5 μg/ml) or streptolydigin (5 μg/ml). After 1.5 hr, these samples were centrifuged at 2 C, and the pellets were washed once with 0.02 M KPO_4 (pH 7.2) at 2 C, then resuspended in 50 ml of the supernatant from the original 200-ml sample, pre-warmed to 33 C. Samples were immediately removed for analysis and the incubation continued at 33 C, further samples being removed at intervals. ENZ, Dap-adding enzyme activity; REF, per cent refractility; and DPA, accumulated dipicolinic acid.*

Synchrony was much poorer than in the control, possibly due to a variable rate of recovery from the effects of the antibiotics. The extents of conversion to refractility indicated at least 90% recovery from chloramphenicol but only about 60% recovery from streptolydigin treatment. Dap-adding activity started accumulating rapidly in the control 0.6 hr after sampling. Rapid accumulation in the chloramphenicol- and streptolydigin-treated cultures did not commence until after 1.0 and 1.5 hr, respectively, of reincubation. The corresponding periods for conversion to refractility are 0.7 hr (control), 1.7 hr (chloramphenicol), and 2.0 hr (streptolydigin), whereas for dipicolinate accumulation the periods are 1.5 hr (control), 2.3 hr (chloramphenicol), and 2.8 hr (streptolydigin). Thus, the normal "program" was not resumed until about 0.7 and 1.2 hr of reincubation had occurred in chloramphenicol- and streptolydigin-treated cells, respectively. However, the three events mentioned then ensued at approximately normal relative intervals.

The second samples were removed at 8.9 hr, when Dap-adding activity had appeared, a small fraction of the cells showed refractility, but no dipicolinate had been accumulated (Fig. 11). They were treated with antibiotics for 1.5 hr and then reincubated in the supernatant from duplicate samples removed at 8.9 hr, as before. In the presence of the antibiotics, about 20% of the cells became refractile, and accumulation of Dap-adding activity and dipicolinate and acquirement of refractility ensued during reincubation in their normal sequence and at approximately normal intervals, but again synchrony was poor. Recovery from both antibiotics was similar in timing and extent in this instance, except for poor accumulation of DPA after streptolydigin treatment. There was no apparent delay in the resumption of Dap-adding enzyme synthesis, but dipicolinate accumulation was delayed about 1 hr in both cultures.

The third samples were removed at 9.7 hr when the cells were 50% refractile, and rapid dipicolinate accumulation was just beginning (Fig. 12). After incubation with antibiotics for 1.5 hr and reincubation as before, accumulation of Dap-adding enzyme recommenced without delay in both cultures. Refractility had increased to 70% during incubation with the antibiotics, but did not increase again until after 0.6 hr of reincubation, again a normal sequence of events. Considerable DPA had accumulated during incubation with both antibiotics, indicating that by the time they took effect (9.8 to 9.9 hr), most of the dipicolinate synthase synthesis had been completed. This is corroborated by the approximately linear rate of dipicolinate accumulation in the control after 9.7 hr (Fig. 12). Apparently no further synthesis of dipicolinate occurred in the reincubated samples (Fig. 12).

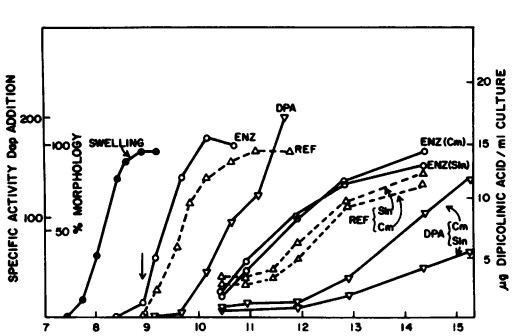

FIG. 11. *Reversible inhibition of sporulation in B. sphaericus. These data are from the same experiment shown in Fig. 10 but are for the cultures incubated with antibiotics from 8.9 (arrow) to 10.6 hr. The control curves are included for reference. Symbols as in Fig. 10.*

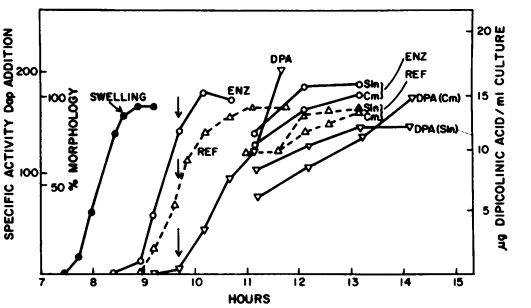

FIG. 12. *Reversible inhibition of sporulation in B. sphaericus. These data are for the same experiment depicted in Fig. 10, but are for the cultures incubated with antibiotics from 9.7 hr (arrows) to 11.2 hr. Symbols as in Fig. 10.*

As in the previous experiment, these data also indicate that continued transcription in the period of appearance of Dap-adding activity (8.8 to 10 hr) was necessary for that appearance, and both transcription and translation necessary for synthesis of DPA apparently also occurred at about the same time, since this was the period of sensitivity of DPA accumulation to both antibiotics.

"Commitment" to synthesis of Dap-adding enzyme in B. sphaericus. A cell is said to be committed to a certain event in its development when the occurrence of that event cannot be prevented by changing the cellular environment to one favoring growth of the initial cell type. Commitment in *B. sphaericus* was tested by diluting samples of sporulating cultures with 10 volumes of fresh, sterile medium, prewarmed to 33 C. Cells which had stopped dividing but had not yet become terminally swollen recommenced cell division after a variable lag phase, whereas most swollen cells (some of which are still motile) formed small loose clumps immediately on dilution. Most of these clumped cells eventually acquired refractility.

The synthesis of Dap-adding enzyme in three samples of a sporulating culture, after dilution with fresh medium, was assayed with the results shown in Fig. 13. The sample which was diluted shortly before terminal swelling was first observed, largely reverted to vegetative growth, but 20% of the control (undiluted) Dap-adding activity was synthesized; accumulation of DPA occurred at the normal time, but again at about 20% of the control rate. Thus, 20% of the cells were apparently committed to synthesis of Dap-adding enzyme and DPA synthase about 2 hr before these syntheses commenced. A culture diluted 1 hr later was already 50% swollen, and acquired 60% of the Dap-adding activity of the control. A third sample diluted 0.5 hr later was 85% swollen, and

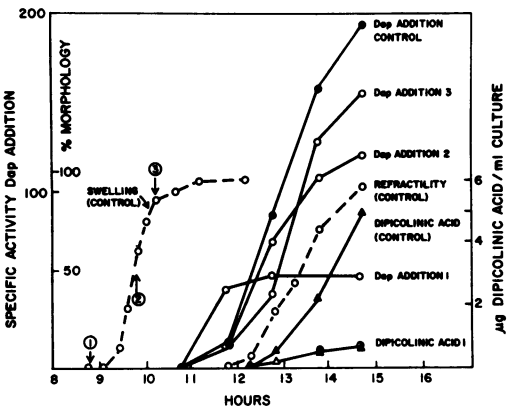

FIG. 13. *Commitment to sporulation in B. sphaericus. A culture (750 ml) of B. sphaericus was incubated at 33 C. At the times indicated by the arrows 1, 2, and 3, samples (20 ml) were removed and immediately added to fresh sterile medium (200 ml) at 33 C, and incubation was continued. Samples (5 and 1 ml, respectively) of the original culture were removed at intervals for assay of Dap-adding enzyme activity (Dap addition) and accumulated dipicolinic acid, as shown. The morphological state of the diluted cultures could not be estimated because of clumping of swollen cells.*

acquired 80% of the Dap-adding activity of the control. "Commitment" to this event apparently coincided approximately with terminal swelling, 2 hr previously, but with poorer synchrony.

DISCUSSION

As predicted from the structure of their cell wall peptidoglycan, vegetative cells of *B. sphaericus* contain an enzyme that adds L-lysine onto UDP-MurNac-dipeptide, and this enzyme is without demonstrable activity with Dap. The layer contiguous with cortex but immediately adjacent to the core membrane of spores of several *Bacillus* species can be differentiated from the cortex in electron micrographs, and persists on germination (when the cortex is solubilized), and apparently develops into the cell wall of the outgrowing cell (19). This layer has therefore been called "germ cell wall," although, apart from its susceptibility to lysozyme in some species (31), no evidence as to its composition is available. In spores of *B. sphaericus* which have been extensively sonicated and incubated with lysozyme to remove residual traces of vegetative cell wall, some peptidoglycan containing lysine and D-aspartic acid is solubilized by lysozyme only after fragmentation, and could be derived from a germ cell wall layer of vegetative-type peptidoglycan (D. J. Tipper, *unpublished data*). Vinter (29) found two peaks of incorporation of ^{14}C-Dap into spores during sporulation in *B. cereus*. Label incorporated during the earlier period (coincident with the appearance of prespores) was mostly stable on germination and may therefore have been incorporated into germ cell wall, whereas label incorporated during the second period was mostly solubilized on germination, and so was presumably incorporated into cortex. Although lysine-adding activity in sporulating *B. sphaericus* cells is lower than in vegetative cells, it has been consistently found to increase 30 to 40% between swelling and the appearance of Dap-adding activity (Fig. 6). Staining of unfixed cells (7) indicates that swelling is coincident with spore septum formation, whereas prespore formation by engulfment occurs subsequently (P. Linnett, *unpublished data*); therefore, lysine-adding activity is maximal at the stage at which germ cell wall synthesis apparently occurs in *B. cereus*, which is consistent with synthesis of a germ cell wall of vegetative-type peptidoglycan early in prespore formation in *B. sphaericus*.

Dap-adding activity is absent from vegetative cells, spores, and sporulating cells of *B. sphaericus* until about 4 hr from the end of vegetative growth. The appearance of this activity is continuously dependent on protein and RNA synthesis, and therefore corresponds closely in time to the transcription and translation of a gene the transcription product of which is short-lived and which is spore-specific, since its translation results in synthesis of a unique spore component. The product could be the Dap-adding enzyme itself, or possibly a very short-lived enzyme which converts an inactive precursor to Dap-adding enzyme. In *B. cereus*, vegetative aldolase is converted to a spore enzyme with very different properties by a limit protease (23, 26; Sadoff and Celikkol, Bacteriol. Proc., p. 24, 1969); it is possible, for example, that lysine-adding enzyme, the activity of which decays sharply as Dap-adding activity appears, is similarly converted to the Dap-adding enzyme. The production of such a specific and short-lived protease seems improbable, but could only be excluded by purification of the Dap-adding enzyme and demonstration that it is a product of de novo synthesis. This decrease in lysine-adding enzyme activity also coincides with the appearance of sonic treatment-resistant prespores and so would be explained if synthesis of this enzyme during sporulation were confined to the prespores, which could be solely responsible for germ cell wall synthesis.

Accumulation of Dap-adding activity ceases after 1.5 to 2 hr, and since the enzyme is relatively stable, at least in the presence of chloramphenicol (Fig. 8), this indicates cessation of

transcription of the appropriate gene. However, when transcription is interrupted for 1.5 hr with streptolydigin, turnoff does not occur until maximal control levels have been reached, 4 to 6 hr later (Fig. 11), indicating that turnoff is related to the physiological state of the bacterium (e.g., depletion of energy stores) and is relatively independent of the time taken to reach that state. The degree of synchrony in the cultures, as indicated by the slopes of the accumulation curves, differs in different experiments. It is not clear whether these curves represent the sum of small sequential increments, each due to the rapid and total accumulation in a single cell, or to a much slower but simultaneous accumulation in all of the cells. The true state must lie between these extremes, and some evidence for asynchrony is given in Fig. 13, where cells whose synthesis of Dap-adding enzyme was not inhibited by dilution at the earliest time (8.7 hr) accumulated this activity earlier than the average in the control. This unresolved problem does not affect the present conclusions.

Chasin and Szulmajster (4) studied synthesis of DPA synthase in *B. subtilis*, and found it to be absent from vegetative cells and to be synthesized between 4 and 6 hr after the end of vegetative growth, although most accumulation of DPA occurred subsequently. In *B. sphaericus*, accumulation of DPA also commenced about 4.5 hr after the end of vegetative growth, and continued at an increasing rate (interpreted as being proportional to DPA synthase concentration) for 1 to 2 hr (Fig. 9), a period almost coincidental with that of Dap-adding enzyme synthesis (Fig. 9). As with Dap-adding activity, increase in the ability to accumulate DPA over the period from 10.5 to 12 hr was dependent on both continuous protein and RNA synthesis and was, therefore, the result of a specific transcriptional event resulting in formation of a messenger of short lifetime as compared with the period of increase. However, since streptolydigin exerted its effect somewhat slower than chloramphenicol, the messenger lifetime may be significant. This messenger could be for DPA synthase, or again could code for a short-lived enzyme that converts an unknown precursor to DPA synthase. In this experiment, accumulation of DPA mostly occurred after the period of sensitivity to antibiotics. Since small amounts of DPA accumulated after addition of antibiotic at 8.9 hr, whereas addition at 9.7 hr was followed by accumulation at 65 to 85% of the control rate, it would again appear that most synthesis of DPA synthase occurred during this period (8.9 to 9.7 hr), coincident with synthesis of Dap-adding enzyme and before most accumulation of Dap occurred (Fig. 10–12).

The acquirement of refractility follows the accumulation of Dap-adding activity, and its extent is correlated with Dap-adding activity in inhibited cultures (Fig. 11, 12). Acquirement of refractility is presumably due to dehydration of the prespore, which would require cortex synthesis according to the contractile cortex theory (16, 31), which is consistent with the present findings. The acquirement of refractility has also been correlated with the maturation of spore coats, an event that involves cysteine incorporation in *B. cereus* (2). If this also occurs during phase-whitening in *B. sphaericus*, another new enzyme may be required, and its synthesis would be approximately coincidental with that of Dap-adding enzyme.

RNA polymerase in several organisms has recently been shown to consist of a core enzyme reversibly associated with "sigma" factors which apparently determine its affinity for different classes of promotors (28, 31). The recent demonstration that an early event in sporulation in *B. subtilis* is the disappearance of a vegetative sigma factor necessary for transcription of phage DNA has led to the suggestion that it may be replaced by a new sigma factor that allows transcription of spore genes (17), which would therefore be under group-positive control. Such an event could account for the synthesis, for example, of those enzymes necessary for peptide antibiotic synthesis. If such an event occurs early in sporulation in *B. sphaericus*, the later synthesis of Dap-adding enzyme and DPA synthase must be under separate or additional control. However, it does appear that these events may be programmed 2 hr before they occur, approximately at the time of terminal swelling, since after this time they cannot be prevented by placing the cells in fresh medium (Fig. 13). This commitment problem has been extensively studied during sporulation in *B. subtilis* (26), where it was found that formation of alkaline phosphatase, development of refractility, accumulation of DPA, and acquirement of heat resistance had a separate commitment time, followed shortly by resistance to actinomycin D. This was interpreted as indicating synthesis of long-lived messengers under translational control. However, except for alkaline phosphatase synthesis, some of the delay in expression of the actinomycin D-sensitive events could be due to their secondary nature, as exemplified by the delay between accumulation of DPA synthase and of its product in *B. subtilis*, where the DPA synthase messenger had an apparent half-life of 15 min (4). Our experiments give no indication of especially long-lived messengers in *B. sphaericus*, but indicate a predetermined sequence of transcriptional events. Although our studies throw little light on the

mechanism by which the program is determined, they do indicate that it can be reversibly interrupted with antibiotic inhibitors of both protein and RNA synthesis, so that unfolding of the program may depend on the physiological state of the bacterium, as previously concluded for turning off of DAP-adding enzyme synthesis.

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