Mucopentptide Metabolism during Growth and Sporulation in *Bacillus megaterium*

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SUMMARY

A mutant of *Bacillus megaterium* lacking the aspartate \( \beta \)-semialdehyde-pyruvate-condensing enzyme and meso-diaminopimelic acid decarboxylase has been used in a study of mucopentptide metabolism during growth and sporulation. The absolute requirement for both diaminopimelic acid and lysine in this mutant guarantees that exogenous labeled diaminopimelic acid is incorporated specifically into mucopentptide greatly facilitating such studies.

During the vegetative growth phase, no turnover of mucopentptide was observed. Upon glucose exhaustion and the initiation of sporulation, vegetative cell mucopentptide synthesis ceased. The mother cell wall remained quiescent throughout the sporulation sequence. Some 3 hours after the end of vegetative cell growth, spore-specific mucopentptide synthesis began and continued for approximately 6 hours. Sporulation was terminated by lysis of mother cell wall which released free spores into the medium.

The mucopentptide component of bacterial cell walls is largely responsible for the shape of bacterial cells and their resistance to osmotic shock. This structure consists of linear polymers of alternating \( \beta \)-linked units of \( N \)-acetylglucosamine and \( N \)-acetylmuramic acid with virtually all the \( N \)-acetylmuramic acid residues substituted on the carboxyl group of the lactyl moiety by a peptide composed of \( D \)- and \( L \)-alanine, and \( D \)-isoglutamine, and either \( L \)-lysine or one of the isomers of \( \alpha \),\( e \)-diaminopimelic acid (1). In general \( \alpha \),\( e \)-diaminopimelic acid is found in most bacterial walls with the exception of the Gram-positive cocci (2).

A rigid structure is achieved through cross-links involving the diaminopimelate or lysine residues (1).

In addition to its occurrence in the cell wall, mucopent peptide is also a constituent of the bacterial spore (3). The spore-specific mucopentptide is located in the cortex, the region between the inner core and outer coat of the spore. Although resembling the vegetative cell wall mucopentptide, evidence is available which suggests that structural differences exist (4). Perhaps the major difference is that spore mucopentptide has muramic \( \delta \)-lactam in place of every second or third \( N \)-acetylmuramic acid residue (5).

The mucopentptide of bacteria is of obvious importance since it is part of the structure-forming element in vegetative cells and in spores. It is therefore of considerable interest to follow its metabolism during the sporulation sequence. Succinctly, endospore formation is a complex differentiation process whereby the vegetative cell undergoes a series of morphological and biochemical changes (6). Initially, the vegetative cell at the end of its growth phase is divided asymmetrically into two compartments. The membranes of the larger compartment then engulf the smaller which creates the double membranated forespore and places it within the cytoplasm of the mother cell. Maturation of the forespore is accompanied by the development of the cortex and the spore envelope. The sporulation process is completed when the mother cell disintegrates releasing the spore.

Early studies by Vinter (7) established that \( ^{14} \)C-labeled diaminopimelic acid was incorporated into spore integuments of *Bacillus cereus* in two peak periods. The first occurred during early sporulation (septation) while the second occurred during the period of increasing refractility of the forespore (maturation). The use of a prototrophic strain in these studies, however, presented several difficulties, i.e., conversion of diaminopimelic acid to lysine resulting in protein as well as mucopent peptide labeling and dilution of the labeled diaminopimelic acid due to endogenous synthesis. The endogenous synthesis is particularly disturbing since, in the absence of a knowledge of pool sizes, it makes possible alternative interpretations of Vinter's data.

We have been able, with the use of a double mutant blocked at two points in the diaminopimelate-lysine pathway, to circumvent these difficulties. In this mutant, exogenous labeled diaminopimelic acid was incorporated without dilution solely into mucopent peptide which meant that cellular radioactivity became synonymous with mucopent peptide. With the use of this mutant we have determined the course of mucopent peptide synthesis and degradation during growth and sporulation in *Bacillus megaterium*.

EXPERIMENTAL PROCEDURE

Bacterial Strains  Experiments were performed with a local strain (wild type) and mutant 46 of *B. megaterium*. Mutant 46

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\( ^{1} \) This is also called peptidoglycan, murein, or glycopeptide.
lacks aspartate $\beta$-semialdehyde-pyruvate-condensing enzyme and mesodiaminopimelic acid decarboxylase.

**Media and Growth Conditions**—The minimal medium (FCG) for growth and sporulation of wild type strain, plates for scoring colony-forming units, procedures for maintaining a vegetative inoculum, and conditions for growth and sporulation of both strains were similar to that described (8). For growth and sporulation of mutant 46, medium FCG was supplemented with diaminopimelic acid (50 $\mu$g per ml) and L-lysine·HCl (20 $\mu$g per ml).

The diaminopimelic acid used routinely in these studies was composed of a mixture of isomers in the following proportions, meso, 80%; LL, 20%. This mixture was obtained from culture filtrates of a lysine auxotroph of *Escherichia coli* (9) and was a gift of the Pfizer Research Laboratory, New London, Connecticut. Comparable sporulation efficiencies were obtained with synthetic mixtures composed of the pure meso and LL isomers of diaminopimelic acid.

Under our conditions vegetative growth of the wild type and mutant strains occurred with a generation time of 65 min and ceased due to glucose limitation at 130 to 140 Klett units at 660 nm. Throughout growth and sporulation the pH of the cultures remained relatively constant within the range of 0.2 to 0.4.

**Sporulation**—For sporulation of mutant 46, dipicolinic acid (pH 7, $1 \times 10^{-3}$ M) and CaCl$_2$·2H$_2$O ($1 \times 10^{-3}$ M) were added to the cultures 1 hour after vegetative growth ceased. Sporulation efficiency was determined by scoring heat-resistant colony-forming units after heating a portion of the culture for 15 min at 60°. In addition, the marked changes in optical density exhibited by our strains of *B. megaterium* during the sporulation sequence, in particular the secondary optical density rise (see Fig. 1), were conveniently used as a measure of sporulation efficiency (8).

**Incorporation of $^{14}$C-Diaminopimelic Acid into Mucopeptide**—To determine the net incorporation of radioactivity from $^{14}$C-diaminopimelic acid into mucopeptide during growth and sporulation of mutant 46, the labeled precursor was added to the medium at the time of inoculation. On the other hand, when the rate of incorporation was desired, 1.0-ml portions of an unlabeled culture were incubated with $^{14}$C-precurser under conditions of vigorous aeration for either 30 or 60 min.

**TABLE I**

**Assay of enzymatic activity**

Enzyme activities were determined in sonic extracts prepared from log phase cells. The aspartate $\beta$-semialdehyde-pyruvate-condensing activity was assayed by the o-aminobenzaldehyde method of Yugari and Gilvarg (14). Mesodiaminopimelic acid decarboxylase was determined by measuring the rate of disappearance of mesodiaminopimelic acid with the use of a specific colorimetric method (15).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Wild type</th>
<th>Mutant 46</th>
<th>Mutant 46</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate $\beta$-semialdehyde-pyruvate-condensing enzyme</td>
<td>$\mu$moles/min/mg protein</td>
<td>$4.5 \times 10^{-5}$</td>
<td>$3.0 \times 10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td>Mesodiaminopimelic acid decarboxylase</td>
<td></td>
<td>$5.9 \times 10^{-4}$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

In all cases, the extent of mucopeptide synthesis was measured by withdrawing 0.2 ml of culture and diluting it with 5 ml of ice-cold 5% trichloroacetic acid. Trichloroacetic acid precipitable material was collected by rapid filtration through a membrane filter (Millipore, 0.45 $\mu$m pore size) and washed with a further 15 ml of ice-cold 5% trichloroacetic acid.

Radioactivity was measured with a Packard model 3000 Tri-Carb liquid scintillation spectrometer after dissolving the membrane filter in 10 ml of the scintillation fluid described by Bray (10).

**Other Methods**—Hydrolysis of cold 5% trichloroacetic acid precipitable material was performed with 6 × HCl for 40 hours at 95°. Samples for chromatography were applied to Whatman No. 1 filter paper and developed in the solvent system described by Rhuland et al. (11).

Chemicals—$\alpha$, $\beta$-Diaminopimelic-1,7-$^{14}$C acid (13 mCi per mm) was purchased from Volk Radiochemical Company, Westwood, New Jersey. The meso and LL isomers of diaminopimelic acid were prepared from the corresponding mixture excreted by the M-26-26 mutant of *E. coli* (12).

**RESULTS**

**Metabolic Lesions of Mutant 46**—Mutant 46 was isolated from the parent strain of *B. megaterium* by a three-stage selection procedure. In the first stage a mesodiaminopimelic acid decarboxylase mutant (3-48) was isolated from the wild type strain after treatment with N-methyl-$N'$-nitro-$N$-nitrosoguanidine (13) by screening for L-lysine, but not diaminopimelic acid, dependence. This dependence was correlated with the loss of the enzyme which catalyzes the decarboxylation of mesodiaminopimelic acid to L-lysine (Table I). An analogous mutant of *E. coli* has been described (16).

The 3-48 isolate was further mutagenized with N-methyl-$N'$-nitro-$N$-nitrosoguanidine and plated on solid, glucose-limiting medium at 0.1% sporulation medium (FCG) supplemented with diaminopimelic acid (50 $\mu$g per ml), L-lysine·HCl (100 $\mu$g per ml), and 4H-pyran-2,6-dicarboxylic acid (5 × $10^{-4}$ M). The technique for isolation of the second series of auxotrophs was based on the fact that if the asporogeneity resulted from an inability to synthesize dipicolinic acid, this could be overcome by exogenously added dipicolinic acid (8). However, a more facile screening procedure was developed in which pyran decarboxylic acid was used as a substitute for dipicolinic acid (17). Here, those colonies which would sporulate only in the presence of added pyran dicarboxylic acid appeared yellow in color. Those colonies which were able to synthesize their own complement of dipicolinic acid appeared white or gray in color and were subsequently discarded.

The yellow colonies therefore represented not only those mutants which lacked the mesodiaminopimelic acid decarboxylase, but in addition, those which contained a metabolic lesion interfering with dipicolinate synthesis. This could occur either by a block in the diaminopimelic pathway prior to the enzymatic reduction of dihydrodipicolinic acid to $\Delta^1$-tetrahydrodipicolinic acid (15) or by block in dipicolinate synthetase (18).

Final screening of the yellow colonies for diaminopimelic acid dependence produced a series of auxotrophs which then specifically lacked the aspartate $\beta$-semialdehyde-pyruvate condensing enzyme (8, 19).

That mutant 46 lacks both the condensing and decarboxylase activity (B. Llanco, unpublished results).
activities is shown by the enzymatic analyses of these enzymes given in Table I. While the prototrophic strain has a full complement of each enzyme, mutant 46 is clearly devoid of both activities.

Growth and Sporulation of Wild Type Strain and Mutant 46—The *B. megaterium* wild type strain exhibits marked changes in optical density during sporulation in medium FCG (Fig. 1). Mutant 46, grown similarly at 32° in medium FCG supplemented with diaminopimelic acid and L-lysine but without dipicolinate shows the initial decline in turbidity after glucose exhaustion. However, during the late period of sporulation, no secondary rise occurs and extensive autolysis of cells follows, which markedly lowers the turbidity of the culture. Under these conditions, few heat-resistant spores are formed (Table II).

The addition of dipicolinic acid and CaCl2·2H2O to the mutant culture 1 hour after vegetative growth ceases, restores the second turbidity rise (Fig. 1). Under these conditions the appearance of refractile bodies, as determined microscopically, parallels that observed in the wild type culture. Therefore, mutant 46 still shows the high degree of sporulation synchrony noted for the parent strain despite its complex requirements. However, it does show a somewhat lower sporulation efficiency (Table II).

Requirement of High Concentrations of Diaminopimelic Acid for Sporulation—An interesting property of mutant 46 is its requirement for high concentrations of diaminopimelic acid for sporulation. Mutant 46 will grow vegetatively to an optical density of 140 Klett units in the presence of 4.5 μg per ml of diaminopimelic acid. A further 1.4 μg per ml is utilized during spore formation. However, for optimum sporulation, at least 50 μg per ml must be provided (Fig. 2).

This property of the mutant could result from the loss of a specific mechanism, possibly located in the membrane component of either the vegetative cell or the forespore, which was required for active diaminopimelic acid uptake. In this event, high concentrations of exogenous diaminopimelic acid would be necessary to facilitate the entry of this compound by a nonspecific permease with poor affinity for diaminopimelate or by diffusion. To test this hypothesis, the rate at which mutant 46 incorporates diaminopimelio acid into mucopeptide was examined as a function of exogenous diaminopimelic acid concentration during exponential growth and at 1 and 7 hours after end of growth.

Cells harvested during the vegetative phase of growth are not dependent on high exogenous concentrations of diaminopimelic acid for mucopeptide synthesis (Fig. 3). However, cells which were harvested during the sporulation cycle are, in fact, dependent on elevated diaminopimelic acid concentrations for the synthesis of spore mucopeptide. These results then offer an explanation for the necessity of using 50 μg per ml of diaminopimelic acid for sporulation studies.

**Table II**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony forming units per ml Before heating</th>
<th>Colony forming units per ml After heating</th>
<th>Heat stable colony forming units %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.7 × 10⁷</td>
<td>3.1 × 10⁷</td>
<td>115.0</td>
</tr>
<tr>
<td>Mutant 46</td>
<td>3.2 × 10⁷</td>
<td>2.4 × 10⁷</td>
<td>75.0</td>
</tr>
<tr>
<td>Mutant 46 minus</td>
<td>2.0 × 10⁷</td>
<td>0.8 × 10¹</td>
<td>0.000004</td>
</tr>
</tbody>
</table>

**Fig. 1.** Sporulation curves of *B. megaterium* wild type and mutant 46. Bacteria were grown in 25 ml of medium FCG in 300-ml Nephelo flasks at 32° with vigorous aeration. In the case of mutant 46, the FCG medium was supplemented with diaminopimelic acid, 50 μg per ml, and L-lysine·HCl, 20 μg per ml. Dipicolinic acid was added to one of the mutant cultures at 1 hour after the end of growth to provide a final concentration of 1.0 × 10⁻⁷ M, and an equimolar amount of CaCl₂·2H₂O was added at the same time. ▲, wild type (WT); ○, mutant 46, no addition; ●, mutant 46 plus dipicolinate.

**Fig. 2.** The relationship of sporulation in mutant 46 and diaminopimelic acid concentration. Bacteria were grown as in Fig. 1, except that the concentration of diaminopimelic acid was varied as shown. Sporulation efficiency was correlated with the extent of the second optical density rise (cf. Fig. 1).
Vegetative cells

DIAMINOPIMELIC ACID CONCENTRATION (\mu g/ml)

one hour post end growth

17 hours post end growth

DIAMINOPIMELIC ACID CONCENTRATION (\mu g/ml)

Vegetative cells

Fig. 3. Rate of diaminopimelic acid incorporation into mucopeptide as a function of exogenous diaminopimelic acid concentrations. Bacteria were grown as in Fig. 1. At an optical density of 30 Klett units, the cells from 20 ml of culture were collected by centrifugation, washed, and resuspended in 20 ml of fresh FCG medium, containing \( \text{-lysine \cdot HCl, 20 \mu g per ml, but lacking diaminopimelic acid. Portions of this suspension (1 ml) were placed in contact with varying concentrations of } ^{14}\text{C diaminopimelic acid (specific activity, 1.0 } \text{Ci per 5.3 pmoles) for 1 hour under conditions of vigorous aeration. Mucopeptide synthesis was measured at the end of this period. Two 20-ml portions were also removed from the same culture as above 1 and 7 hours after growth ceased. The experimental procedure was identical with that described for vegetative cells except that the medium in which the cells were resuspended also lacked glucose. Incorporation was calculated as the number of counts per min of diaminopimelic acid incorporated per ml of culture corrected for the cell density by dividing by the number of Klett units. ●, vegetative cells; ○, cells harvested 1 hour after end of growth; □, cells harvested 7 hours after end of growth.

and Sporulation—The incorporation of radioactivity from \(^{14}\text{C-diaminopimelic acid into mucopeptide during growth and sporulation is shown in Fig. 4. Under these conditions, where the radioactive precursor was added to the medium at the time of inoculation, } ^{14}\text{C-diaminopimelic acid was incorporated throughout vegetative growth. Shortly after the cells reached the stationary phase of growth, the net incorporation of } ^{14}\text{C-diaminopimelic acid abruptly ceased for a period of about 3 hours. Renewed net incorporation was then observed for a period of 6 hours. Subsequent lysis of vegetative cell walls leads to a solubilization of } ^{14}\text{C-mucopeptide as the mother cell disintegrates during the release of free spores into the medium. These results clearly indicate that there are two periods during which diaminopimelic acid is incorporated into a macromolecular component of mutant 46. One occurs throughout vegetative growth, the other is limited to a fraction of the sporulation phase.}

In addition, a unique period of no net incorporation temporally separates these two events. More precise information regarding the localization and fate of the diaminopimelic acid which is incorporated during the two periods will be provided in the following section.

To exclude the possibility that diaminopimelic acid was metabolized to other compounds, which would have vitiated the as-
assumption that incorporated radioactivity represented mucopeptide, the following control experiment was performed. Mutant 46 was inoculated into medium FCG supplemented with \( ^{14} \)C-diaminopimelic acid and cold L-lysine. Two hours after growth of the culture ceased, trichloroacetic acid-precipitable material was collected and hydrolyzed with acid. Paper radiochromatography of a portion of the hydrolysate revealed that more than 99% of the radioactivity was localized in the meso-isomer of diaminopimelic acid. L-Diaminopimelic acid was not detected. Radiochromatography of a portion of the trichloroacetic acid-soluble fraction showed that more than 99% of the radioactivity was also localized in the diaminopimelic acid.

**Stability of Vegetative Cell Wall and Incorporation of Exogenous Diaminopimelic Acid into Spore Mucopeptide during Sporulation** —It was shown above that shortly after growth ceased there was a period of 3 hours during which net mucopeptide synthesis was absent. This was then followed by a 6-hour period of renewed diaminopimelic acid incorporation. Following these observations it became necessary to determine whether the vegetative cell wall undergoes turnover during sporulation and whether exogenous diaminopimelic acid can be incorporated into spore mucopeptide. The experimental approach chosen was to perform the following switch experiment. Two cultures were allowed to grow under identical conditions except that the diaminopimelic acid in one of the cultures was labeled with \( ^{14} \)C at the time of inoculation. Shortly after vegetative growth ceased, the cells were removed from their media by centrifugation and the hot cells were transferred to the analogous cold medium, while the cold cells were transferred to the corresponding hot medium. The results of the experiment are presented in Fig. 5.

**Fig. 6.** Incorporation of radioactivity from \( ^{14} \)C-diaminopimelic acid during growth and sporulation. Bacteria were grown as in Fig. 1. At time indicated a 1.0-ml portion of the culture was withdrawn and immediately placed in contact with \( ^{14} \)C-diaminopimelic acid (0.02 \( \mu \)Ci, 2.0 \( \times \) 10\(^{4} \) cpm per ml) for 30 min under conditions of vigorous aeration. Incorporation of radioactivity into mucopeptide was determined as described under "Experimental Procedure". ○, optical density; ▲, observed incorporation of \( ^{14} \)C-diaminopimelic acid into trichloroacetic acid-insoluble material; △, corrected values derived from Fig. 7.

The mucopeptide component of cells which had incorporated \( ^{14} \)C-diaminopimelic acid during vegetative growth and were then placed in a medium containing cold diaminopimelic acid did not diminish in radioactivity until sporulation. Since the trichloroacetic acid-precipitable radioactivity did not decline until lysis of the mother cell occurred, this indicates that the vegetative cell mucopeptide does not undergo turnover. Moreover, it is also evident that pre-existing mucopeptide was not utilized for spore mucopeptide since the free spores did not contain any radioactivity.

In the corresponding experiment where cold cells were placed in contact with hot medium, it is apparent that the second period of mucopeptide synthesis is due to incorporation of \( ^{14} \)C-diaminopimelic acid from the medium. Since it is the spore mucopeptide which is being formed during this interval, there is virtually no loss of radioactivity on subsequent lysis of vegetative cell walls.

The failure to detect turnover of vegetative cell wall strongly suggests that there is a 3-hour period of complete cell wall stasis following cessation of growth. This separates two periods of diaminopimelic acid incorporation, the first into the vegetative cell wall, the second into the spore.

**Ability of Mutant 46 to Incorporate \( ^{14} \)C-Diaminopimelic Acid at Various Times during Growth and Sporulation** —Vinter (7) has shown that for B. cereus two periods of increased diaminopimelic acid incorporation into mucopeptide occur during sporulation. It was therefore of some interest to determine whether mutant 46 exhibits a similar phenomenon.

During vegetative growth, the mutant organism was able to efficiently incorporate the radioactivity of \( ^{14} \)C-diaminopimelic acid into mucopeptide (Fig. 6). Within the 30 min following the
cessation of growth, the rate of incorporation decreased dramatically to a very low level. This result would be expected since it has been shown that during this period the vegetative cell wall suddenly becomes quiescent. However, this low level of incorporation was maintained throughout the sporulation cycle. Similar results were obtained when the contact period was increased to 60 min. Thus, with this contact method we were unable to demonstrate periods during the sporulation cycle when the organism had an increased ability to incorporate labeled diaminopimelic acid.

These results are indeed surprising since it would be expected that during the second period of diaminopimelic acid incorporation, the microorganism should show an increased ability to incorporate the mucopeptide precursor. An explanation for this apparent dilemma was found by examining the quantity of intracellular diaminopimelic acid during growth and sporulation. Mutant 46 was inoculated into medium FCG supplemented with diaminopimelic acid (50 μg per ml) and L-lysine-HCl (20 μg per ml). At an optical density of 20 Klett units, the cells were centrifuged, washed, and resuspended in 25 ml of fresh FCG medium supplemented with diaminopimelic acid, 50 μg per ml and L-lysine-HCl, 20 μg per ml. The concentration of glucose was increased to 20 g per liter to allow for completely vegetative growth. At intervals after the transfer, the radioactivity associated with mucopeptide was determined. •, optical density; ■, 14C-diaminopimelic acid in trichloracetic acid-insoluble material.

During late vegetative growth the intracellular pool of diaminopimelic acid was maintained at a low, constant level (Fig. 7). However, during the interval of mucopeptide stasis, the pool increased in size 1.5-10. Thereafter the size of the intracellular pool remained relatively constant. It is during this second period that diaminopimelic acid is incorporated into spore mucopeptide. Therefore during sporulation, exogenous labeled diaminopimelic acid must compete with an endogenous pool of unlabeled diaminopimelic acid which had accumulated during cell wall stasis. Because the quantity of diaminopimelic acid in the pool was larger than the amount incorporated into mucopeptide during the contact period, we were unable to detect the expected increase in the rate of uptake during sporulation. If the rate curve (Fig. 6) during sporulation is corrected to account for the change in the size of the pool during this interval, the corrected curve (Fig. 6) does in fact show that the cells have an increased ability to incorporate diaminopimelic acid into spore mucopeptide at that period when the actual net increase in spore mucopeptide was observed.

Cell Wall Turnover during Vegetative Growth—Mauck and Glaser (20) have recently demonstrated that during logarithmic growth of B. subtilis W-23 both the mucopeptide and teichoic acid components of the cell wall undergo extensive turnover.

During the interval of mucopeptide stasis, the concentration of glucose was increased to 20 g per liter to completely suppress sporulation. At intervals after the transfer, the radioactivity associated with cold 5% trichloroacetic acid-precipitable material was determined (Fig. 8). The results clearly indicate that when vegetative cells of mutant 46 are labeled with 14C-diaminopimelic acid and then allowed to grow in a nonlabeled medium, there is no net loss of radioactivity from the mucopeptide component of the cell wall. This demonstrates that there is no turnover of cell wall mucopeptide of mutant 46 during vegetative growth.

**Discussion**

In previous studies, measurement of mucopeptide synthesis necessitated the use of either lengthy extraction procedures (21) or the addition of excess amounts of nonradioactive lysine to dilute or repress endogenous synthesis from labeled diaminopimelic acid (7). The availability of an auxotroph having an absolute requirement for both diaminopimelic acid and lysine presented an opportunity to re-examine mucopeptide metabolism in sporulating cultures of B. megaterium.

Diaminopimelate incorporated during the vegetative phase in R. megaterium is not released indicating that no turnover of mucopeptide occurs during growth. This result contrasts with that of Mauck and Glaser (20) who provided evidence for extensive turnover of B. subtilis W-23 mucopeptide. However, Mauck and Glaser were not able to use specific labels for the mucopeptide and at this point it is not clear to us whether the contradictory
results reflect idiosyncratic species' differences or a difference in experimental technique.

With the beginning of the stationary phase in a sporulating culture a unique period of 3 hours is observed during which time no mucopeptide is synthesized or degraded. In contrast, Vinter (7) observed only a decrease in the amount of diaminopimelic acid incorporated into mucopeptide at the end of vegetative growth and the beginning of stationary phase. Whether the low level of incorporation he observed during this period resulted from culture asynchrony is not known.

As first recorded by Vinter (7), we have confirmed the observation that during sporulation, mucopeptide formed during the vegetative growth phase does not contribute to the spore cortex since it is clear that there is no turnover of mother cell mucopeptide during the entire sporulation sequence. In this connection, a more rigorous test of this nonutilizability is provided in the present study in that mutant 46 will not sporulate when grown in the sporulation medium containing limiting concentrations of diaminopimelic acid. Thus, even under stress conditions, the mother cell mucopeptide is not utilized for spore mucopeptide.

In our system labeled diaminopimelic acid, added at the time of inoculation, is used for both vegetative and spore mucopeptide. The incorporation of radioactivity from diaminopimelic acid into spore mucopeptide is the same whether the labeled precursor is added to cultures of mutant 46 at the time of inoculation or shortly after growth ceases. In contrast, Vinter (7) has shown, with a prototrophic strain of B. cereus, that the carbon-14 content in mucopeptide remains practically constant once vegetative growth ceases, when labeled diaminopimelic acid is added at the time of inoculation. However, he did not establish that the same result would be obtained at higher concentrations of diaminopimelate, and it is possible that in his experiment utilisable diaminopimelate had been exhausted when sporulation commenced.

In Vinter's experiment some 30% of the initially added radioactivity was not incorporated at all. Vinter postulated that this fraction of diaminopimelic acid is bound in the form of peptides during vegetative growth, and these peptides were therefore unavailable as substrates for mucopeptide formation during sporulation. However, these results could be most easily explained by assuming that the synthetic diaminopimelic acid used by Vinter contained 20% of the isomer. It is known that this isomer is not incorporated into mucopeptide in E. coli (22) or B. megaterium. In fact, separate experiments designed to test this peptide hypothesis with the use of mutant 46 revealed that exogenous diaminopimelic acid is not converted during vegetative growth to a metabolically inert component which remains in the supernatant during sporulation.

Vinter (7) has demonstrated that during sporulation of B. megaterium there are two periods of increased diaminopimelic acid incorporation into spore integuments. We have been unable to verify this finding in sporulating B. megaterium. Although exogenous diaminopimelic acid was utilized for spore mucopeptide, there was only one period of incorporation during the sporulation sequence. Some of Vinter's data are similar to that obtained in the present study for surprisingly, when net incorporation of diaminopimelic acid was measured during spore formation of B. cereus a smooth curve was obtained (7). It is therefore possible that his observed period of decreased diaminopimelic acid incorporation during sporulation corresponds to a time of heightened endogenous diaminopimelic acid synthesis rather than decreased mucopeptide synthesis. It should be remembered that a prototrophic strain of B. cereus was employed (7).

The finding that only spore mucopeptide is formed during sporulation strongly suggests the presence of a mechanism which regulates the spatial deposition of mucopeptide. That is, the site of active mucopeptide synthesis must shift from the vegetative cell wall to the spore cortex during the sporulation sequence. This may occur through a structural change in the vegetative cell wall at the time of glucose exhaustion which would prevent its further elaboration. Alternatively, it may require that the enzymes concerned with mucopeptide synthesis are expressed in the membranes of the forespore during early sporulation, while those in the mother cell membrane are repressed as a consequence of glucose exhaustion. In this connection it has been discovered that a significant fraction of the mother cell mucopeptide becomes resistant to lysosome during late vegetative growth and early stationary phase. Although these results do not differentiate between a chemical modification or an inactivation of cell wall enzymes, further work dealing with this question is in progress.

The discovery of a unique period of cell wall stasis during sporulation of B. megaterium has led to studies in which the relationship of mucopeptide synthesis to that of phospholipid synthesis has been investigated in an attempt to provide evidence for a hypothesis on the mechanism of spore septation and engulfment (23).

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