Turnover of the Cell Wall of Gram-positive Bacteria*

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SUMMARY

Previous observations (Mauk, J., and Glaser, L., Biochem. Biophys. Res. Commun., 39, 699 (1970)) on the turnover of the cell wall of Bacillus subtilis W-23 during logarithmic growth have been extended to show that (a) freshly synthesized wall does not become available for turnover for about one-half to one generation; (b) the turnover rates of the cell wall mucoprotein and cell wall teichoic acid are identical; (c) the products of wall turnover can be isolated from the growth medium and are the products of cell wall cleavage by an N-acetylmuramyl-l-alanine amidase. Cell wall turnover has also been observed in Bacillus megaterium strain KM in logarithmic growth phase. Preliminary studies with inhibitors suggest that turnover is dependent on new cell wall deposition. The implications of these observations for the mechanism of cell wall growth are discussed.

In a previous communication (1) we presented evidence that the cell wall of Bacillus subtilis W-23, as well as the Marburg strain of B. subtilis, undergoes extensive turnover during logarithmic growth. We also showed that the rate of turnover, approximately 50% per generation, is independent of the growth rate. Since such turnover has important implications for the mechanism of bacterial cell growth, we examine in the present communication the kinetics of cell wall turnover in B. subtilis W-23 and report on the isolation and characterization of the cell wall fragments released as a result of the turnover into the growth medium. In addition, we present evidence that the cell wall of Bacillus megaterium strain KM also undergoes turnover during logarithmic growth, and that the products of cell wall turnover can be isolated from the medium as with B. subtilis W-23.

These results appear only to be compatible with a model of cell wall growth which allows for the addition of new cell wall material over the whole cell surface, rather than a single growth point. After publication of our previous paper (1), we became aware of the paper by Chaloupka, Rihova, and Kreckova (2), which indicated the possibility of cell wall turnover in B. megaterium and Bacillus cereus. These interesting experiments were carried out with cells labeled on solid medium and transferred to liquid culture, and the possibility of cell lysis appears not to have been adequately controlled. Our results with B. megaterium, however, agree with those of Chaloupka et al. (2).

MATERIALS AND METHODS

The growth medium (3) for B. subtilis W-23 St* (obtained from Dr. P. Schaeffer, Institut Pasteur, Paris, France) and the methods used for isolating cell walls (1) from this organism have been described in detail previously. In these experiments radioactive cells from a fixed volume of culture are added at various times after labeling to a constant quantity of carrier cells, and cell walls are prepared from this mixture. Under these conditions the mass of the cell walls isolated comes from the carrier cells, and in the absence of turnover the specific activity of the cell walls isolated would remain constant. We have provided evidence previously (1) that no selective loss of the radioactive walls occurs under these conditions and that isolated walls are free of radioactive protein or nucleic acid.

B. megaterium strain KM (obtained from Dr. J. B. Ward, of this department) was grown either in rich medium (Difco antibiotic medium 2) or in a defined medium of the following composition per liter: NH₄Cl, 2 g; Na₂HPO₄, 6 g; K₂HPO₄, 3 g; NaCl, 3 g; MgCl₂, 10 mg; Na₂SO₄, 25 mg; MnCl₂, 1.98 mg. Glucose was autoclaved separately and added to a final concentration of 0.4%. The pH of the medium after autoclaving was 6.8. In some experiments B. megaterium was grown in the medium of Pitel and Gilvarg (4) and Fukuda and Gilvarg (5) with 0.4% glucose as carbon source. This medium differs from the above in pH (6.2 instead of 6.8) and in the presence of Ca++. In order to obtain logarithmic growth of B. subtilis W-23 or B. megaterium immediately after filtration of the cells, the cells from a radioactive labeling experiment were collected on a Millipore filter, washed with partially spent medium at 37°, and then suspended in partially spent medium. Partially spent medium was prepared by sterilely filtering a culture of the appropriate organism at OD₆₀₀ₐ₅ of 0.1 and storing the medium at 37° (usually for 1 or 2 hours) until it was used for growth of radioactive cells. Under these conditions no lag phase was observed when labeled cells were transferred into unlabeled medium. Filtration was always carried out with 0.45 μ Millipore filters.

3C-L-Proline, 3HL-L-glutamic acid, 3C-L-glutamic acid, and 3C-L-leucine were obtained from New England Nuclear. 3C- Carboxyl-labeled DAP was obtained from Calbiochem. 3H-Labeled DAP was obtained from Amersham-Searle or was pre-

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The abbreviation used is: DAP, α,γ-diaminopimelic acid.
pared by catalytic exchange by New England Nuclear and was purified by Dowex 50 chromatography followed by paper chromatography with ethanol-concentrated NH₄OH-H₂O (180:10:10) as the solvent (6). Identical results were obtained with all three preparations. Radioactivity was determined in a Packard liquid scintillation counter, equipped with an absolute activity analyzer, with Aquasol (New England Nuclear) or Bray's solution (7) as the counting fluid.

For amino acid and amino sugar analysis, samples were hydrolyzed in sealed ampoules in 6 N HCl at 105° for indicated periods of time (usually 12 hours), dried under vacuum, and analyzed. Total amino sugar in the hydrolysate was determined by the method of Levy and McAllan (8), with N-glucosamine as standard. DAP was determined by the method of Work (9). The amino acid and amino sugar compositions were determined with the Beckman amino acid analyzer run at 46° to separate muramic from glutamic acid. In some cases, highly concentrated samples were hydrolyzed with 6 N HCl in capillary tubes and aliquots analyzed after suitable dilution without drying of the sample. Amino sugar assays were 10 to 15% higher in these samples than in similar samples which had been dried under vacuum.

Ribitol and glucosyl ribitol (the teichoic acid components of *B. subtilis* W-23 (10)) were determined enzymatically after HF degradation (10, 11) or after alkaline hydrolysis as follows. Samples containing approximately 2 μmoles of total ribitol were hydrolyzed in 1 ml of 1 N NaOH for 3 hours at 100°. The hydrolysate was neutralized by passage through a column (0.5 x 5 cm) of Dowex 50-X8, H⁺ form, which was washed with 10 ml of H₂O. The eluate was evaporated to dryness and dissolved in H₂O. For assay of ribitol, an aliquot (0.25 ml) was incubated at 37° for 16 hours with 0.1 mg of *Escherichia coli* alkaline phosphatase (Sigma) in 0.2 M sodium carbonate buffer, pH 9.6, followed by enzymatic assay for free ribitol (12). For determination of glucosyl ribitol, an aliquot (0.25 ml) was incubated in 0.05 M potassium acetate, pH 5.0, with 0.5 mg of β-glucosidase ( Worthington) for 16 hours at 37°. The pH was adjusted to 9.6 with Na₂CO₃, and phosphate monoesters were hydrolyzed with *E. coli* alkaline phosphatase as above.

The difference between total ribitol and free ribitol was considered to be due to glucosyl ribitol. The glucose released by β-glucosidase and determined enzymatically provides an independent determination of this value (10).

Free amino groups were determined with trinitrobenzenesulfonate (13). Protein was measured by a modified biuret assay (14).

**Fig. 1.** Turnover of old and new mucopeptide of *Bacillus subtilis* W-23. *B. subtilis* was grown in 90 ml of minimal medium with 0.67% maltose as a carbon source. At the time indicated, 0.5 mCi of 3H-DL-glutamic acid (5 μmoles) was added. After growth to an ODe₅₀ of 0.3, 100 μCi of 14C-L-glutamic acid (200 mCi per mmole) were added after an additional 40-min incorporation of radioactivity was stopped by the addition of 400 μmoles of DL-glutamic acid. At the arrows, samples were removed and added to carrier cells, and the cell walls were isolated (1). In A is shown the growth curve of the cell and the total trichloracetic acid (TCA)-precipitable counts per ml of culture. In B is shown the radioactivity in the isolated cell wall, as per cent of the radioactivity of Sample 1, as well as the ratio of these two quantities. For 3H, 100% is 78,500 dpm per μmole of carrier wall amino sugar (●-●); 100% for 14C is 8,950 dpm per μmole of carrier wall amino sugar (△-△).

**Fig. 2.** Rate of turnover of mucopeptide and teichoic acid in *Bacillus subtilis* W-23. *B. subtilis* W-23 was grown in 20 ml of minimal medium with 1% glucose and 2 x 10⁻⁴ M P₀. At an ODe₅₀ of 0.10, 0.6 mCi of 3H-DL-glutamic acid and 3 mCi of 32P₀ were added. After 50 min, 1 ml of 1 M potassium phosphate, pH 7.0, and 40 μmoles of DL-glutamic acid were added. After 75 min (ODe₅₀ of 0.3), the culture was filtered, washed with partially spent medium, and rapidly suspended in 95 ml of partially spent medium containing 0.01 M Pi and 1 μmole per ml of DL-glutamic acid. Fifty milliliters were allowed to grow normally, and 10-ml samples were removed at times indicated to isolate cell walls (circles). To the remaining 40 ml (triangles), 400 μg of actinomycin D were added, and 10-ml samples were removed as indicated for the isolation of cell wall. 100% 32P is 7170 dpm per μmole of carrier cell walls amino sugar; 100% 3H is 2023 dpm per μmoles of carrier cell wall amino sugar.
When early time periods after pulse labeling were examined, it appeared that freshly synthesized wall was not subject to turnover. Experiments illustrating this phenomenon are shown in Fig. 1. It should be emphasized that in all of the experiments reported in this paper the radioactivity in the cell wall fraction in a fixed volume of culture is measured, and, after the labeling period, this value would remain constant in the absence of turnover. In the experiment in Fig. 1, the cells were first labeled with \(^{14}\)C-glutamic acid, and after this was exhausted they were labeled with a small pulse of \(^{14}\)C-glutamic acid, followed by chase with cold glutamic acid. Under these conditions, \(^{14}\)C represents old cell wall, and \(^{1}\)H represents new cell wall. It is clear that there is a lag before new cell wall is subject to turnover. That this lag is not due to a pool of \(^{14}\)C-glutamic acid in the cell is shown by the fact that the increase in total trichloracetic acid-precipitable counts is stopped by the addition of cold glutamic acid. Maltose was used as a carbon source in these experiments to slow down growth and allow for a more precise timing. Essentially similar results have been obtained with glucose as a carbon source.

The lag before newly synthesized wall becomes available for turnover accounts for the apparent difference in the turnover of teichoic acid and mucopeptide seen in previous experiments. In previous experiments (1), after labeling with glutamic acid and chasing with cold glutamic acid, we waited for about one-half generation before taking the first sample for the isolation of cell walls. However, in \(^{32}\)P-labeling experiments in which the turnover of teichoic acid was measured, the first sample was taken immediately, and subsequent samples were taken at least one and one-half generations later. In Fig. 2 we show data from a double label experiment in which turnover of the cell wall mucopeptide was measured with \(^{14}\)C-glutamic acid, and turnover of the cell wall teichoic acid was measured with \(^{32}\)P, and they are clearly identical. In other experiments (not shown) we have observed the same lag in teichoic acid turnover as we have observed in mucopeptide turnover.

For construction of a possible model of cell wall biosynthesis, it is important to know whether turnover can proceed as an apparent first order process for a number of generations or whether turnover represents the degradation of cell wall at a fixed time period after its synthesis. In Fig. 3 we show that turnover can occur for at least four generations, strongly suggesting that the segments of the wall removed at any given time are removed at random. One would expect from studies with other organisms that the spread in division times of the various cells in the population would only be \(\pm 0.2\) generation (15), and thus it is likely that the observed random degradation of the wall is not due to heterogeneity in the generation times of individual cells.

It is also important to know what effect lack of growth has on cell wall turnover. Shown in Fig. 2 are data on the effect of actinomycin D addition on cell wall turnover. As can be seen, this turnover is considerably diminished in the presence of this inhibitor. In the presence of actinomycin, incorporation of radioactive glutamic acid into cell wall continues for a period of about one-half a generation. The possible significance of these observations is discussed below. In experiments identical with those in Fig. 2 but in which cell protein was labeled with \(^{15}\)N-L-proline, the total trichloracetic acid-precipitable counts are stable. In addition, it was noted that in the presence or absence of actinomycin D, no \(^{13}\)C from \(^{15}\)N-labeled protein was released.
into the medium, showing that no cell lysis occurred under these conditions.

Isolation of Degradation Products of the Wall of *B. subtilis* W-23

The degradation of the cell wall of *B. subtilis* W-23 during logarithmic growth is quite extensive, and the degradation products of the cell wall have been isolated from the growth medium. In a typical experiment, *B. subtilis* W-23 was grown in 2.4 liters of minimal medium to an optical density at 600 μm of 1.8. The culture was rapidly cooled to 0°C in an ice bath, and the cells were collected by centrifugation at 15,000 × g for 10 min. The supernatant fluid was decanted into a flask at 0°C, filtered through a 0.45-μm Millipore filter, and lyophilized. The dry powder was dissolved in 120 ml of water, heated at 100°C for 5 min, and again filtered through a 0.45-μm Millipore filter. The filtrate was diluted to 200 ml with H2O and chromatographed on a column (5 × 100 cm) of Bio-Gel P-2 equilibrated with 0.1 M pyridine acetate, pH 7.0, and eluted with the same buffer at flow rate of 120 ml per hour. Tubes between the void volume of the column and the position of the salt peak from the medium (determined by conductivity measurement) were pooled, lyophilized, dissolved in 15 ml of H2O, and brought to 10% trichloracetic acid. The precipitate which formed was removed by centrifugation and was found to contain 20 mg of protein.

An aliquot of the solution containing 44 μmoles of amino sugar and 22 μmoles of DAP was chromatographed on a column (1 × 8 cm) of Dowex 50-X8, H+ form, 200 to 400 mesh. The column was washed with 50 ml of H2O and then eluted with a linear gradient of 150 ml of H2O to 150 ml of 4 M HCl at a rate of 12 ml per hour, and 2-ml fractions were collected. The elution profile of the column is shown in Fig. 4. In Table I is shown the analysis of the Dowex 50 water eluant, as well as the analysis of the major peaks of the acid eluant from the column. All the amino sugar recovered from the column, 32 μmoles, was removed in the water eluant which appears to contain the carbohydrate backbone of the mucopeptide and the teichoic acid but which is essentially devoid of all wall amino acids. The HCl eluate of the Dowex 50 column contains no amino sugar but contains the characteristic cell wall amino acids, specifically DAP, alanine, and glutamic acid. The total recovery of DAP in this experiment was 14.4 μmoles out of the 22 μmoles put on the column.

Essentially the same data have been obtained in three separate preparations in different growth stages. The analysis of muramic acid in the *B. subtilis* W-23 wall deserves special comment. As can be seen, the data in Table I indicate that approximately one-half of the total amino sugar behaves as glucosamine in the amino acid analyzer, about 25% behaves as muramic acid, and the remainder is unaccounted for. This is not due to destruction of muramic acid, since mixtures of muramic acid, glucosamine, galactosamine, glutamic acid, alanine, and DAP subjected to acid hydrolysis under the same conditions do not show selective destruction of muramic acid, nor was selective destruction of muramic acid observed when cell walls were hydrolyzed for different time periods.

In Table II we show the analysis of the cell wall of *B. subtilis* W-23 compared to *B. subtilis* NCTC 3610 (strain Marburg) and *Bacillus licheniformis* ATCC 9945, as well as *B. subtilis* W-23 grown under conditions of phosphate limitation (3, 17). All of these organisms show close to the expected 1:1 ratio of glucosamine to muramic acid, and suggest the presence in *B. subtilis* W-23 of a substituted muramic acid which is not detected in the

| Table I
| Analysis of cell wall fragments obtained from medium of *B. subtilis* W-23

For details, see text. Peaks 3, 5, and 6 are those of Fig. 4.

<table>
<thead>
<tr>
<th>Cell wall fragments</th>
<th>Dowex 50 H2O eluate</th>
<th>Dowex 50 acid eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Total amino sugar</td>
<td>32.4</td>
<td>—</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>15.5</td>
<td>—</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>4.8</td>
<td>—</td>
</tr>
<tr>
<td>DAP</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Ribitol</td>
<td>6.4</td>
<td>—</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>8.5</td>
<td>—</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.0</td>
<td>—</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.84</td>
<td>0.38</td>
</tr>
<tr>
<td>Serine</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>Threonine</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>—</td>
<td>0.03</td>
</tr>
<tr>
<td>Leucine</td>
<td>—</td>
<td>0.17</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>Trace</td>
<td>—</td>
</tr>
</tbody>
</table>

| Table II
| Comparative analysis of cell walls of *B. subtilis* strains and *Bacillus licheniformis*

Analysis was carried out as described in the text after 12 hours of hydrolysis in 0.1 M HCl at 100°C. The Dowex 50 H2O eluate from the growth medium of *B. subtilis* W-23 is a different preparation than that shown in Table I. All values are relative to glucosamine taken as 1. The large amount of galactosamine in *B. subtilis* W-23 walls from cells grown under conditions of phosphate limitation is due to the presence of teichuronic acid in these walls (17). All the samples were analyzed without drying after acid hydrolyses.

<table>
<thead>
<tr>
<th>Cell wall fragments</th>
<th><em>B. subtilis</em> W-23</th>
<th><em>B. subtilis</em> NCTC 3610</th>
<th><em>B. licheniformis</em> ATCC 9945</th>
<th>Dowex 50 H2O eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.45</td>
<td>0.88</td>
<td>0.89</td>
<td>0.81</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.82</td>
<td>0.86</td>
<td>0.97</td>
<td>1.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.52</td>
<td>1.44</td>
<td>1.60</td>
<td>1.50</td>
</tr>
<tr>
<td>DAP</td>
<td>0.82</td>
<td>0.91</td>
<td>0.97</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>trace</td>
<td>3.08</td>
<td>0.089</td>
<td>0.5</td>
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</table>

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Kibitol

DAP

Galactosamine

Glucosamine

Total amino sugar

Muramic acid

DAP

Alanine

Glutamic acid

Ribitol

GlcNAc

Glucose

Glycine

Serine

Threonine

Aspartic acid

Glu

GluNAc

Galactosamine

trace
Fig. 5. Chromatography of polysaccharide fraction on Bio-Gel P-100. The medium polysaccharide from Bacillus subtilis W-23 eluted from Dowex 50 with H2O was lyophilized and placed on a column (1 X 60 cm) of Bio-Gel P-100 in 0.1 M pyridine acetate, pH 7.0. Fractions (2 ml) were collected at the rate of 6 ml per hour. Amino sugar expressed as OD542 mp (8) was determined in fractions after acid hydrolysis. Fractions were pooled as indicated, and analyses are given in the text. Arrow 1 indicated the position of dextran blue peak, and Arrow 2 indicated the position of KCl peak in a separate calibration run of the column.

Fig. 6. Turnover of cell wall of Bacillus megaterium KM. B. megaterium KM was grown in 20 ml of rich medium containing 2 μmoles per ml of L-lysine and 10 μCi of 3H-DAP (200 mCi per mmole) and 10 μCi of 14C-1-proline (213 mCi per mmole). At an OD of 0.3, the cells were filtrated, washed, and suspended in previously spent medium containing 2 μmoles per ml each of L-lysine and L-proline and 4 μmoles per ml of DAP. At intervals, 1-ml samples were removed, mixed with an equal volume of 10% trichloracetic acid, filtered, washed with 10% trichloracetic acid, and counted. The 14C counts in all samples were between 95 and 100% 14C. 100% 14C count is 12,780; 100% 3H count is 12,780; 14C was 1,840 dpm per ml initially and 1,720 dpm per ml in the final sample. The generation time was 35 min. This experiment is denoted by ••. ▲—▲, illustrates a similar experiment in defined medium, generation time 50 min. In this experiment 100% 3H is 99,000 dpm per ml.

Fig. 7. Chromatography of an acid hydrolysate of DAP-labeled Bacillus megaterium. B. megaterium was labeled with 3H-DAP as in Fig. 6 but at higher specific activity. After two generations in cold medium, a sample of cells (64,000 dpm) was acid-hydrolyzed in 6 N HCl for 12 hours at 100° and, after drying in vacuum, it was applied to Whatman No. 3MM paper and chromatographed by descending paper chromatography with ethanol-concentrated-NH4OH-H2O (90:5:5) as the solvent for 46 hours. The chromatogram was scanned with a Vanguard paper chromatogram scanner. Elution of the chromatogram with 0.1 M pyridine acetate, pH 7.0, yielded 45,200 dpm in the DAP region and 4,900 dpm in the L-lysine region; no counts were recovered from the rest of the chromatogram.

Fig. 8. Chromatogram of Bacillus megaterium medium on Bio-Gel P-30. In Experiment A, B. megaterium was grown in rich medium as in Fig. 6, and 30 ml of the final medium (2.5 X 10^8 dpm) were lyophilized, dissolved in 1 ml of H2O, and chromatographed on a column (0.9 X 57 cm) of Bio-Gel P-30 in 0.1 M pyridine acetate, pH 7.0. Fractions (0.9 ml) were collected. In B, the same experiment on a larger scale was carried out in defined medium, and 1.6 X 10^8 dpm were placed on a column (85 X 2.5 cm) of Bio-Gel P-30. Fractions (3 ml) were collected.

amino acid analyzer, or by the method of Perkins and Rogers (not shown) (18). In at least one other published analysis of the walls of B. subtilis W-23, a low muramic acid content of the wall was noted (19). The problem has not been investigated further.
TABLE III

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glu-star culture</th>
<th>TCA precipitable counts</th>
<th>Isolated cell walls</th>
<th>Theoretical H-13C ratio (no turnover)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H-13C</td>
<td>H-13C X 10^{-6}</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.375</td>
<td>2.44</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.650</td>
<td>2.39</td>
<td>3.37</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.900</td>
<td>2.11</td>
<td>4.77</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.50</td>
<td>2.00</td>
<td>7.60</td>
<td></td>
</tr>
</tbody>
</table>

* TCA trichloroacetic acid.

For the purpose of the present communication, it appears clear that the cell wall degradation products seen in the medium are consistent with an N-acetylmuramic acid L-alanine amidase known to be present in this organism (20) being responsible for cell wall hydrolysis.

The fraction eluted with water from Dowex 50 (H+) is clearly heterogeneous. Fig. 5 shows the chromatography of this fraction on Bio-Gel P-100. The ratio of amino sugar to ribitol to glucosyl ribitol is Fraction 1, 1:0.44:0.71; Fraction 2, 1:0.61:1; and Fraction 3, 1:0.11:0.09, showing that trichloroacetic acid is enriched in the fractions of higher apparent molecular weight.

Turnover of Cell Wall Mucoprotein in B. megaterium KM- In Fig. 6 we show that when B. megaterium KM is labeled with DAP in the presence of an excess of cold L-lysine and then transferred to a nonradioactive medium containing cold DAP and cold L-lysine, the radioactivity from DAP decays about 40 to 45% per generation, whereas the radioactivity in protein measured with 14C-proline remains constant. Acid hydrolysis of cells labeled with DAP in the presence of lysine shows that 90 to 95% of the label is in DAP (Fig. 7), and the remaining chromatographs with lysine. Since polymeric DAP occurs only in the cell wall, the data in Fig. 6 show that the cell wall of B. megaterium KM also turns over during logarithmic growth, both in rich medium as well as in defined medium. The rate of turnover, about 30 to 35% per generation, is about the same in rich medium (generation time 35 min) as in defined medium (generation time 55 min).

The radioactivity lost from the cells can be quantitatively recovered in the growth medium. Fig. 8 shows a chromatogram on Bio-Gel P-30 of the medium in which B. megaterium KM, previously labeled with DAP, was grown. In rich medium, an average of about 50% of the counts in the medium are low molecular weight and appear to be mostly free DAP (70%), and the remainder can be converted to DAP by acid hydrolysis. In defined medium, only a high molecular weight fraction is seen. Acid hydrolysis of the high molecular weight fraction in either case yields only radioactive DAP; no radioactive L-lysine is seen in any of the fractions. Incubation of the medium after removal of the cells by filtration does not change the product distribution, indicating that these changes are not due to secondary degradation of the cell wall fragments by enzymes present in the growth medium.

The curious difference in degradation products of the cell wall of B. megaterium depending on growth medium suggests that the type of wall hydrolytic activity present in B. megaterium is influenced by growth condition. Dr. C. Gilvarg has kindly made available to us the manuscript of a paper (4) in which no turnover of the cell wall was seen in a different medium with a different strain of B. megaterium. We have grown B. megaterium KM in the medium used by Pitel and Gilvarg with 0.4% glucose and in partially spent medium to suspend the radioactive cells after labeling. In three apparently identical experiments, we have seen 35% turnover per generation, 15% turnover per generation, and no turnover during a two and one-half generation period. This suggests that in B. megaterium growth is possible without cell wall turnover, although this clearly needs further investigation. In Table III we show that, in B. subtilis W-23, cell wall turnover occurs when the cells are grown in the medium of Pitel and Gilvarg (4).

In a number of experiments we have seen no cell wall turnover of the mucoprotein in E. coli Hfr 139. When B. megaterium approaches stationary phase and the rate of growth decreases, the rate of wall turnover decreases, suggesting, as did the experiment with B. subtilis inhibited with actinomycin (Fig. 2), that turnover only occurs during cell wall growth.

In Fig. 9 we show that when B. megaterium KM is incubated with chloramphenicol and penicillin, cell wall turnover continues for a period of time but then stops, again indicating that in the absence of cell wall synthesis turnover of the wall is regulated.

In all cases we observe that a certain fraction of the wall is destroyed before turnover stops, suggesting among other possibilities that at any given time during logarithmic growth a certain fraction of the cell wall is irreversibly committed to be hydrolyzed. In the control experiment, no DAP was incor-

1 For these experiments, E. coli Hfr 139, an E. coli K-12 strain obtained from Dr. P. R. Vagelos (from this department), was grown in antibiotic medium 3 and labeled with radioactive DAP in the presence of a large excess of nonradioactive L-lysine. Under these conditions, as with B. megaterium, 90 to 95% of the trichloroacetic acid precipitable radioactivity is in the cell wall.

* The data in Fig. 7 are taken from cells after two generations in unlabeled medium, at which time 50% of the DAP has been lost from the cell wall. In the original cells, only 5% of the label was in lysine.
Chloramphenicol and, after 20 min, 5 mg of potassium penicillin. To 50 ml of the culture were added 5 mg of chloramphenicol and, after 20 min, 5 mg of potassium penicillin. At the times indicated, 1-ml samples were precipitated with 10% trichloroacetic acid, filtered, washed with 10% trichloroacetic acid, and counted. Less than 5% of the total 3H trichloroacetic acid-precipitable counts were found in the medium, indicating that there was little cell lysis under these conditions.

It is assumed that in Model a the site of cell wall synthesis and degradation are fixed with respect to each other. If their relative positions change continuously during a single generation then Model a cannot be excluded.

Within the accuracy of our measurements no fraction of the cell wall is immune from turnover, thus excluding the possibility that wall at the cell poles is not subject to degradation.

Although it was originally assumed that cell wall hydrolysis enzymes are concerned with the insertion of new cell wall material (21) at the growing point, our evidence that wall turnover does not involve newly synthesized wall would suggest that at least in B. subtilis the N-acetylmuramyl-L-alanine amidase described by Young (20) is largely not located at the growing point.

The lag observed with B. subtilis W-23 before newly synthesized wall becomes available for turnover is in striking contrast with data by Pooley and Shockman (22) which suggest that, when Streptococcus faecalis cells are broken, newly synthesized wall is degraded first, as well as electron microscopic data that suggest that the same is true during starvation of intact cells (23). Although these observations were made with a different organism, they suggest that the location or activity, or both, of the cell wall autolytic enzymes is different during normal growth, as compared to autolysis either during starvation or after breakage of the cell. Furthermore, great caution should be exerted in equating the first point of cell wall lysis with the growing point of the cell wall (24).

We have previously pointed out (1) that fluorescent antibody experiments (25) to locate the growing point of a cell may specifically focus on septum formation which, by necessity, is new wall. On the other hand, the uniform thickening of the cell wall observed in the absence of protein synthesis both in B. subtilis (26) and most thoroughly investigated in S. faecalis (21) is best interpreted in the assumption of multiple cell wall synthesis sites, and indeed recent electron microscopic studies of Higgins and Shockman (27) have been interpreted as indicating multiple cell wall synthesis sites, as well, however, as equatorial growth (28).

Recently Mindich (29) has shown turnover of the membrane lipid of B. subtilis at approximately the same order as the cell wall turnover reported in this paper. On the other hand, Morrison and Morowitz (30) have reported no turnover of the membrane lipids of B. megaterium KM, an organism in which we also observe cell wall turnover under some conditions. It is possible, therefore, that cell membrane and cell wall growth may not be tightly coupled, although the precise site of cell wall synthesis in these organisms needs to be determined.

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