The Synthesis of Peptidoglycan in an Autolysin-Deficient Mutant of *Bacillus licheniformis* N.C.T.C. 6346 and the Effect of β -Lactam Antibiotics, Bacitracin and Vancomycin

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The synthesis of peptidoglycan by cell-free membrane and membrane+wall preparations from an autolysin-deficient, β -lactamase-negative mutant of *Bacillus licheniformis* N.C.T.C. 6346 was studied. The membrane preparation synthesized un-cross-linked polymer, the formation of which was not inhibited by β -lactam antibiotics. Release of D-alanine by the action of D-alanine carboxypeptidase was inhibited variably according to the antibiotic. This inhibition was reversed by neutral hydroxylamine but not by the action of β -lactamases or by washing. Bacitracin inhibited peptidoglycan synthesis, but not the D-alanine carboxypeptidase. Examination of peptidoglycan synthesized in the presence of excess of bacitracin showed that synthesis was not restricted to the addition of one disaccharide-pentapeptide unit at each synthetic site, an average of 2–3 disaccharide-pentapeptide units being added. Peptidoglycan synthesis was three- to four-fold more sensitive to vancomycin than was the release of D-alanine by the action of the carboxypeptidase. Incorporation of newly synthesized peptidoglycan into pre-existing cell wall was studied in membrane+wall preparations. This incorporation was catalysed by a benzylpenicillin- and cephaloridine-sensitive transpeptidase. The concentrations of these antibiotics giving 50% inhibition of incorporation were almost identical with those required to inhibit growth of the bacillus. Inhibition of the transpeptidase was reversed by treatment with β -lactamase or by washing.

The mechanism of biosynthesis of the peptidoglycan fraction of bacterial cell walls has been elucidated for a number of organisms, including *Bacillus megaterium* (Reynolds, 1971*a,b*; Wickus & Strominger, 1972*a*), *Staphylococcus aureus*, *Micrococcus lysodeikticus* and *Escherichia coli* (Strominger, 1970; Araki *et al.*, 1966*a,b*). Membrane-bound enzymes transfer *N*-acetylmuramyl (pentapeptide) 1-phosphate and *N*-acetylglucosamine from their respective uridine nucleotide precursors to a lipid intermediate, the structure of which in *S. aureus* and *M. lysodeikticus* is undecaprenyl phosphate (Higashi *et al.*, 1967). The disaccharide (pentapeptide)-pyrophosphate-undecaprenol may then be modified by addition of amino acids involved in the formation of cross-bridges in some organisms. The disaccharide peptide is then transferred from the lipid intermediate to the growing glycan chain. In *Bacillus licheniformis* this transfer occurs at the reducing terminal (Ward & Perkins, 1973). The undecaprenyl pyrophosphate released becomes available for further use after dephosphorylation to yield the alcohol phosphate (Siewert & Strominger, 1967).

The final reactions involved in synthesis of the peptidoglycan are the formation of cross-bridges in the newly synthesized material. Cross-bridge

formation is mediated by a transpeptidation reaction and probably occurs on the outside of the cytoplasmic membrane, where energy sources such as ATP are generally considered to be absent. The terminal D-alanyl-D-alanine bond is split and the penultimate D-alanine residue is cross-linked either to the free amino group of the dibasic amino acid of a second peptide side chain or to the *N*-terminus of a cross-bridging peptide, with the release of the terminal D-alanine residue. This transpeptidase reaction has proved less available to investigation than the earlier stages of the biosynthetic cycle. Membrane preparations from *S. aureus* and *M. lysodeikticus* that synthesize peptidoglycan *in vitro* show no concomitant release of alanine (Strominger, 1970). However, preparations from *B. megaterium* catalyse the transpeptidation reaction, although the results are complicated by the presence of a D-alanine carboxypeptidase (Reynolds, 1971*a,b*; Wickus & Strominger, 1972*a,b*). These last two enzymes are inhibited by the penicillins at concentrations similar to those required to inhibit growth of the bacillus (Wickus & Strominger, 1972*b*). More recently the release of D-alanine by penicillin-sensitive transpeptidation has been demonstrated in membrane-wall (crude cell-wall) preparations of *S. aureus* and

M. lysodeikticus (Mirelman & Sharon, 1972; Mirelman *et al.*, 1972). The present paper describes the effects of various antibiotics on the biosynthesis of peptidoglycan in membrane and membrane+wall preparations of a mutant of *B. licheniformis* and establishes the reversible nature of the inhibition of its transpeptidase by benzylpenicillin and cephaloridine.

Experimental

Materials

All chemicals used were analytical-reagent grade unless otherwise stated. Benzylpenicillin (Crystapen) and cephaloridine (Ceporin) were purchased from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Vancomycin hydrochloride (Vancocin HCl), cephalosporin C (potassium salt) and 6-aminopenicillanic acid were gifts from Eli Lilly and Co. Ltd., Basingstoke, Hants., U.K., I.C.I. Ltd., Alderley Park, Cheshire, U.K., and Beechams Research Laboratories Ltd., Brockham Park, Betchworth, Surrey, U.K., respectively. Bacitracin, D-cycloserine and UDP-*N*-acetylglucosamine were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Chloramphenicol was from Parke-Davis, Hounslow, Middx., U.K., lysozyme (EC 3.2.1.17) from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. and penicillinase (EC 3.5.2.6) from Koch-Light Laboratories, Colnbrook, Bucks., U.K. UDP-*N*-acetyl[U-¹⁴C]glucosamine (270mCi/mmol), 2,6-diamino[G-³H]pimelic acid (300mCi/mmol), D-[U-¹⁴C]alanine (30mCi/mmol), NaB³H₄ (590mCi/mmol) and D-[U-¹⁴C]glucose (292mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. D-Alanyl-D-[U-¹⁴C]alanine (30mCi/mmol), synthesized as described by Nieto *et al.* (1973), was a gift from Dr. H. R. Perkins. *Streptomyces N*-acetylmuramidase was purified as described previously from culture filtrates of *Streptomyces griseus* (Ward & Perkins, 1968) and β -lactamase [penicillin amido- β -lactamhydrolase (EC 3.5.2.6)] was purified by the method of Dale & Smith (1971) from *E. coli* containing the resistance factor R-1818. The final preparation had an activity of 21.2 μ mol of cephalosporin C hydrolysed/min per mg of protein.

Organisms

The organisms used in this study were *B. licheniformis* N.C.T.C. 6346 *his* and three poorly lytic (Lyt⁻) mutants Lyt⁻-3, Lyt⁻-4 and Lyt⁻-5 previously obtained (Forsberg & Rogers, 1971) from the above strain. These mutants are deficient in the major autolytic enzyme of *B. licheniformis*, the *N*-acetylmuramyl-L-alanine amidase and in phosphoglucosmutase (Forsberg *et al.*, 1973).

A penicillinase-negative mutant (strain 94) was

obtained from Lyt⁻-3 after mutagenesis with 1-methyl-3-nitro-1-nitrosoguanidine as described below.

Media and cultural conditions

B. licheniformis 6346 *his*, Lyt⁻-3 and the penicillinase-negative mutant (strain 94) were kept as spores in water at 4°C, while Lyt⁻-4 and Lyt⁻-5 were maintained in a dried form (Forsberg *et al.*, 1973). Cultures (50ml) were grown with shaking at 35°C on a casein hydrolysate medium supplemented with inorganic salts (Janczura *et al.*, 1961). These cultures were used to inoculate larger batches (2–4 litres) of the same medium supplemented with Bacto-tryptone (0.5%), Bacto-yeast extract (0.5%) (Difco Laboratories Inc., East Molesey, Surrey, U.K.), glucose (0.8%) and MgSO₄ (3mm). Organisms were harvested in mid-exponential phase of growth, 2.5–3.5h after addition of a 2.5% (v/v) inoculum.

Preparation of substrates

UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid was isolated from lysine-deprived *S. aureus* H (Strominger & Threnn, 1959). UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-meso-diaminopimelyl-D-alanyl-D-alanine, referred to as UDP-*N*-acetylmuramyl-pentapeptide, which was labelled with radioactivity in diamino[³H]pimelic acid (300mCi/mmol), was prepared enzymically as described previously (Ward & Perkins, 1973). UDP-*N*-acetylmuramyl-pentapeptide labelled in either the terminal D-alanine residue or the D-alanyl-D-alanine dipeptide was prepared by the enzymic addition of the radioactive dipeptide to UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-meso-diaminopimelic acid obtained from cultures of *B. licheniformis* Lyt⁻-3 inhibited by D-cycloserine and chloramphenicol essentially as described by Ito *et al.* (1966). The enzyme preparation used was partially purified from *B. licheniformis* (Ward & Perkins, 1973).

UDP-*N*-acetyl[¹⁴C]muramyl-pentapeptide was prepared from *B. licheniformis* Lyt⁻-3 inhibited by vancomycin in the presence of D-[U-¹⁴C]glucose. Cells in the mid-exponential phase of growth (1litre) were harvested at room temperature and rapidly transferred to an incubation medium (500ml) of the following composition: 0.05M-potassium phosphate buffer, pH7.0, containing DL-alanine, DL-glutamic acid, DL-aspartic acid, glycine (all at a concentration of 0.04%); MgCl₂ (10mm); chloramphenicol and vancomycin (both 50 μ g/ml final concentration); glucose (0.04mm, 10mCi/mmol). After incubation at 35°C with shaking for 90min, the cells were harvested and extracted with ice-cold 5% (w/v) trichloroacetic acid for 30min at 0°C. The extracted cells were removed by centrifuging, and trichloroacetic acid was extracted from the supernatant with diethyl ether. UDP-*N*-acetylmuramyl-

pentapeptide was freed from complexed vancomycin by selective adsorption of the antibiotic on to purified cell walls of *M. lysodeikticus* (Best *et al.*, 1970) as follows. After concentration, the soluble material (5ml) was mixed with cell walls (5mg) at 4°C. The cell walls were removed by centrifugation and washed with water (2ml) and the combined supernatants were submitted to column chromatography on Sephadex G-25 and paper chromatography in solvents A and B as described by Garrett (1969) to recover the UDP-*N*-acetylmuramyl-pentapeptide. The yield was 2.75 μ mol, having a specific radioactivity of 6.7mCi/mmol. A sample of the radioactive precursor was hydrolysed with 4M-HCl at 100°C for 4h. After removal of the HCl *in vacuo* over NaOH pellets the residue was chromatographed on Whatman 3MM paper in solvent A (see below). Radioactivity on the chromatogram was determined by using a Packard Radiochromatogram Scanner and was located in one major and several minor peaks. The major peak, corresponding in position to authentic muramic acid, contained 78% of the total radioactivity on the chromatogram. The identity of this radioactive material as muramic acid was confirmed by subsequent elution and chromatography on Whatman no. 1 paper in solvent C (see below). Non-radioactive UDP-*N*-acetylmuramyl-pentapeptide was prepared from *B. licheniformis* Lyt⁻³ by accumulation in a medium lacking Mg²⁺ ions (Garrett, 1969).

Source of marker compounds

Cross-linked dimer [bis-(disaccharide-heptapeptide)] and the monomer fractions, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-meso-diaminopimelic acid (disaccharide-tripeptide) and the same compound with an additional D-alanine residue (disaccharide-tetrapeptide) were prepared from peptidoglycan of the parent bacillus, as described by Hughes (1968) but by using the *Streptomyces N*-acetylmuramidase. Cell walls (50mg) were incubated in 0.1M-sodium acetate buffer, pH4.8 (5ml), containing 500 μ g of enzyme protein at 35°C for 16h. Under these conditions more than 95% of the *N*-acetylmuramyl- β -(1-4)-*N*-acetylglucosaminyl linkages were hydrolysed as shown by measuring residual muramic acid on the amino acid analyser after reduction of free reducing groups with NaBH₄ and acid hydrolysis (4M-HCl at 100°C for 4h). The solubilized material was dialysed against water (3 $\times$ 50ml), and the diffusible material was pooled, concentrated and chromatographed on Whatman 3MM in solvent A for 48-60h. In a typical experiment the cross-linked dimer moved 3.3cm, the mono-amidated cross-linked dimer 4.6cm, the disaccharide-tripeptide 7.2cm, the mono-amidated disaccharide-tripeptide 11.0cm, the disaccharide-tetrapeptide

8.6cm and the mono-amidated disaccharide-tetrapeptide 14.0cm. However, as noted previously (Ward & Perkins, 1973), the absolute, but not the relative, mobilities are subject to some variation.

Under the conditions of growth used for isolation of membrane and membrane+wall preparations, the cell walls of *B. licheniformis* Lyt⁻³ contained 58% of their diaminopimelic acid as cross-linked dimer and 37% as disaccharide-tripeptide. The remaining diaminopimelic acid was present as disaccharide-tetrapeptide.

Lactyl-peptides were obtained from these preparations by NH₃ treatment as described previously (Ward & Perkins, 1973).

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Amino sugars and amino acids were determined with a Beckman-Spinco automatic amino acid analyser after hydrolysis of the samples in 4M-HCl for 4h at 100°C for amino sugars and 6M-HCl for 16h at 105°C for amino acids. *N*-Acetylhexosamines and hexosamines were determined by the methods of Reissig *et al.* (1955) and Levvy & McAllan (1959) respectively.

Radioactivity on paper chromatograms was located by radioautography or 1cm strips were cut out and counted directly in a Packard Tri-Carb liquid-scintillation spectrometer model 3375, by using a toluene-based scintillation fluid [toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene]. With ³H the samples containing polymeric peptidoglycan were incubated at 35°C for 4h with a lysozyme solution (0.2ml; 1mg/ml). This procedure ensured that radioactivity was distributed throughout the paper, as was the case with lower-molecular-weight material, so that the counting efficiency in all samples was comparable (J. B. Ward, unpublished work). After drying, these samples were counted for radioactivity as described above. Radioactive areas located by radioautography were cut out and counted directly.

Samples in solution were counted with a dioxan-based scintillation fluid [dioxan containing 10% naphthalene, 1% 2,5-diphenyloxazole and 0.06% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene]. Wall and membrane preparations containing ³H were solubilized with lysozyme as a routine, as described above, before measurement of radioactivity.

Paper chromatography and electrophoresis

Whatman 3MM paper was used unless otherwise stated and was washed extensively with 2M-acetic acid and water before use. Descending paper chromatography was carried out in the following solvent systems: A, isobutyric acid-0.5M-NH₃ (5:3, v/v); B, ethanol-1M-ammonium acetate, pH7.5

(5:2, v/v); C, butan-1-ol-acetic acid-water (3:1:1, by vol.). Electrophoresis was carried out at 40V/cm on Whatman 3MM paper in the following buffer systems: D, pyridine-acetic acid-water (1:10:989, by vol.), pH 3.5; E, pyridine-acetic acid-water (25:1:474, by vol.), pH 6.5.

Nucleotides were located by absorption of u.v. light, and amino acids and amino sugars were located with ninhydrin.

Preparation of enzymes

Membrane preparations were isolated by a method based on that of Anderson *et al.* (1965) as described previously (Ward & Perkins, 1973). Crude cell wall+membrane preparations were isolated from the same cell-alumina mixture described and were the fraction sedimenting between 3000 and 12000g. The sedimented material was washed three times with 50mM-Tris-HCl buffer, pH 7.3, containing 30mM-MgCl₂ and 1mM-dithiothreitol and resuspended in the same buffer to give a final protein concentration of 4-6mg/ml and 25-35mg dry wt. of cell walls per ml. This suspension was used as the enzyme source when required.

Determination of enzyme activities

Membrane preparations. Incubation mixtures contained, in a final volume of 50 μ l, 0.75mM-UDP-*N*-acetylmuramyl-pentapeptide [labelled with both diamino-³H]pimelic acid (15mCi/mmol) and D-[¹⁴C]-alanine (1.2mCi/mmol), 0.9mM-UDP-*N*-acetylglucosamine and enzyme suspension (25 μ l), or 1mM-UDP-*N*-acetylmuramyl-pentapeptide and 0.6mM-UDP-*N*-acetylglucosamine (1.4mCi/mmol) and enzyme as above. After incubation at 24°C for 30min the reaction was terminated either by heating at 100°C for 2min or by the addition of 1ml of ice-cold 0.3M-HClO₄. The heated samples were applied directly to Whatman 3MM paper, chromatographed in freshly prepared solvent A for 18-24h and dried at 50°C before location of radioactivity. The samples precipitated by HClO₄ were centrifuged and the precipitate was then washed successively with 3 $\times$ 2ml of cold HClO₄ solution and with 0.02M-ammonium acetate. Radioactivity in the precipitated material was determined as described above.

Membrane+wall preparations. The reaction mixture contained, in a final volume of 150 μ l, 0.6mM-UDP-*N*-acetylmuramyl-pentapeptide labelled with either [¹⁴C]muramic acid (5.2mCi/mmol) or diamino-³H]pimelic acid (45mCi/mmol) and D-[¹⁴C]alanine (3.1mCi/mmol) in the terminal residue, 0.67mM-UDP-*N*-acetylglucosamine and enzyme preparation (100 μ l). Reaction mixtures were incubated at 24°C for 30min, then ice-cold water (0.5ml) was added and the mixture was centrifuged at 12000g for 5min. The supernatant was removed and analysed for released D-[¹⁴C]alanine either by paper chromatography

in solvent A or by paper electrophoresis in buffer system D. The cell-wall pellet was washed with sodium lauryl sulphate (2%, 3 $\times$ 2ml), NaCl (1M; 2 $\times$ 2ml) and water (3 $\times$ 2ml) before its radioactivity was determined as described above. All determinations were corrected for the radioactivity either in zero-time samples or in boiled-enzyme controls.

Isolation of the penicillinase-negative mutant *B. licheniformis* 94

The isolation of a penicillinase-negative mutant of *B. licheniformis* Lyt⁻3 was based on the method of Dubnau & Pollock (1965) where organisms producing no detectable penicillinase were isolated from constitutive mutants. In this way the complication of enzyme induction was avoided. The procedure adopted for isolation of such a mutant from *B. licheniformis* Lyt⁻3 was as follows.

Cells in exponential growth were harvested, washed with 50mM-potassium phosphate buffer, pH 6.5, and resuspended in the same buffer (pre-warmed to 35°C) to give a concentration of 200 μ g dry wt./ml. 1-Methyl-3-nitro-1-nitrosoguanidine was added to a final concentration of 20 μ g/ml and the suspension shaken at 35°C for 30min. Cultures were plated directly on to Andrade agar (Kogut *et al.*, 1956) and incubated at 35°C overnight. The plates were then flooded with a solution of benzylpenicillin (20mg/ml) and mutant colonies constitutive for penicillinase production were selected by their immediate production of a red coloration. One of these isolates was subjected to a second mutagenesis and replica plates were flooded with penicillin as described above. In this case penicillinase-negative mutants were selected as colonies that remained white, whereas the rest were red. The mutant selected had no detectable penicillinase activity whether subject to inducing conditions or not (Dubnau & Pollock, 1965). The inhibitory effects of benzylpenicillin, cephalosporin C and cephaloridine on growth of the organism were studied in liquid cultures containing approximately threefold dilutions of the antibiotics. Under these conditions the minimum concentration to inhibit overnight growth of the organism was as follows: benzylpenicillin, 0.01 μ g/ml; cephalosporin C, 10 μ g/ml; cephaloridine 0.03 μ g/ml.

Results

Synthesis of peptidoglycan by membrane preparations of B. licheniformis 94

Preliminary experiments with enzyme prepared from *B. licheniformis* 6346 demonstrated small amounts of peptidoglycan synthesis. Nevertheless, the newly synthesized material was sensitive to lysozyme and to the major autolytic enzyme of bacilli, the *N*-acetylmuramyl-L-alanine amidase. Hence the

small amount of peptidoglycan synthesized may have been due to autolytic activity known to be associated with membranes of this organism. In consequence, enzyme was prepared from poorly lytic mutants previously obtained (Forsberg & Rogers, 1971) from *B. licheniformis* 6346. Of these organisms membranes from *Lyt*⁻³ and *Lyt*⁻⁵ showed considerable activity in peptidoglycan synthesis. On the other hand, *Lyt*⁻⁴, in which the activity of autolysin, although substantially decreased from that found in the parent organism, is some fourfold higher than either *Lyt*⁻³ or *Lyt*⁻⁵, more closely resembled the biosynthetic activity of enzyme prepared from the parent organism.

In view of the interest in the action of β -lactam antibiotics on peptidoglycan synthesis a penicillinase-negative mutant (strain 94) was isolated from *B. licheniformis* *Lyt*⁻³, as described in the Experimental section, and the results described below were obtained with enzyme prepared from this organism.

Initial experiments showed that incubation of these membrane preparations with UDP-*N*-acetylmuramyl-pentapeptide (labelled with diamino[³H]-pimelic acid and D-[¹⁴C]alanine) and UDP-*N*-acetylglucosamine resulted in the formation of polymeric peptidoglycan which remained on the origin when chromatographed in solvent A. Radioactivity was also located in alanine (R_f 0.65) and much smaller amounts in lipid intermediates (R_f 0.85) (Anderson *et al.*, 1967). Synthesis of peptidoglycan showed an absolute requirement for UDP-*N*-acetylglucosamine and the product was sensitive to lysozyme, less than 10% of the radioactivity remaining as polymeric material on the origin of the chromatogram after treatment (Table 1). The reaction was less sensitive to the addition of *N*-acetylmuramyl-L-alanine amidase, prepared from autolysates of *B. licheniformis*

Fig. 1. Effect of temperature on peptidoglycan synthesis by membrane preparation from *B. licheniformis* 94

The assay mixture was identical with that described in Table 1. After incubation for 30 min at the temperatures indicated, synthesized peptidoglycan was determined after chromatography in solvent A.

Table 1. Peptidoglycan biosynthesis by a membrane preparation from *B. licheniformis* 94

The assay system contained, in a total volume of 50 μ l, 0.75 mM-UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[³H]pimelic acid (15 mCi/mmol), 0.9 mM-UDP-*N*-acetylglucosamine and enzyme (25 μ l) prepared as described. Reaction mixtures were incubated at 24°C for 30 min. The amount of polymer synthesized was determined after chromatography of the assay mixture in solvent A.

System	Peptidoglycan synthesis (nmol of diamino[³ H]- pimelic acid incorporated/ h per mg of protein)
Complete	15.5
- UDP- <i>N</i> -acetylglucosamine	0.7
+ Lysozyme (25 μ g/ml)	1.4
+ <i>B. licheniformis</i> 6346 amidase (200 μ g/ml)	6.3

6346 (Forsberg & Ward, 1972). The optimum temperature was 24°C, when synthesis proceeded linearly for at least 60 min. At higher temperatures incorporation into peptidoglycan was decreased with increasing temperature and synthesis was not maintained for the same period of time (Fig. 1). The complete system had a broad pH optimum from 7.3 to 8.0 and required Mg^{2+} for maximum activity. The optimum Mg^{2+} concentration was determined on membranes washed extensively in the absence of Mg^{2+} and also on membranes that had been washed with sodium pyrophosphate buffer (0.15 M, pH 7.5) (Anderson *et al.*, 1972). As shown in Fig. 2 this latter procedure resulted in a significant decrease in the activity of enzyme preparation, with an increase in the optimum Mg^{2+} concentration from 30 mM for enzyme prepared in Tris-HCl buffer to 60 mM for pyrophosphate-washed membranes; higher Mg^{2+} concentrations were inhibitory. The optimum concentration for both nucleotide substrates was approx. 1.5 mM (Figs. 3 and 4). The K_m for UDP-*N*-acetylmuramyl-pentapeptide calculated from these data was 5.4×10^{-4} M and that for UDP-*N*-acetylglucosamine was 4.2×10^{-4} M.

The membrane preparation also contained a D-alanine carboxypeptidase activity, assayed as described for peptidoglycan synthesis but in the

Fig. 2. Effect of Mg^{2+} ions on peptidoglycan synthesis by a membrane preparation from *B. licheniformis* 94

The assay mixture contained, in a volume of 50 μ l, 1 mM-UDP-*N*-acetylmuramyl-pentapeptide, 0.6 mM-UDP-*N*-acetylglucosamine (1.4 mCi/mmol), enzyme (25 μ l) prepared as described in the text and $MgCl_2$ as indicated. Mixtures were incubated at 24°C for 30 min, when synthesized peptidoglycan was determined after precipitation with 0.3 M- $HClO_4$. Enzyme washed with 0.15 M-sodium pyrophosphate buffer, pH 7.5 (▲); enzyme washed with 0.05 M-Tris-HCl, pH 7.5 (●); both buffers contained 1 mM-dithiothreitol.

Fig. 3. Effect of concentration of UDP-*N*-acetylmuramyl-pentapeptide on peptidoglycan synthesis by a membrane preparation from *B. licheniformis* 94

The assay mixture contained 6 mM-*N*-acetylglucosamine, enzyme (25 μ l) and UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[³H]pimelic acid (15 mCi/mmol) as indicated, in a total volume of 50 μ l. Mixtures were incubated at 24°C for 30 min and synthesized peptidoglycan was determined after chromatography in solvent A.

Fig. 4. Effect of concentration of UDP-*N*-acetylglucosamine on peptidoglycan synthesis by a membrane preparation from *B. licheniformis* 94

The assay mixture contained 3 mM-UDP-*N*-acetylmuramyl-pentapeptide, enzyme (25 μ l) and UDP-*N*-acetylglucosamine (1.4 mCi/mmol) as indicated in a total volume of 50 μ l. Mixtures were incubated at 24°C for 30 min and synthesized peptidoglycan was determined after chromatography in solvent A.

absence of UDP-*N*-acetylglucosamine. Free [¹⁴C]-alanine was separated by chromatography in solvent A or by electrophoresis at pH 3.5.

Freezing resulted in an immediate loss of 50% of the peptidoglycan-synthesizing activity, but no further decrease was observed during storage at -20°C over a period of several weeks. The D-alanine carboxypeptidase activity was almost completely resistant to freezing. Freshly prepared enzyme was used in the experiments described unless otherwise stated.

Effects of antibiotics on synthesis of peptidoglycan and release of alanine

(a) *Bacitracin*. When the complete system was incubated in the presence of increasing concentrations of bacitracin, inhibition of both peptidoglycan synthesis and the formation of lipid intermediates occurred (Fig. 5), with a maximum of 75% inhibition at a concentration of 50 μ g/ml of the antibiotic. The release of alanine was unaffected even at the highest concentration tested.

In similar experiments with *Staph. aureus* Siewert & Strominger (1967) found that bacitracin produced a maximum of 76% inhibition of peptidoglycan synthesis, and showed that the antibiotic specifically inhibited the dephosphorylation of undecaprenyl pyrophosphate. These authors reasoned that the residual peptidoglycan synthesis found in the presence of bacitracin represented the amount of undecaprenyl phosphate originally available in the preparation, and hence that in the absence of antibiotic the extra synthesis occurring corresponded to

Fig. 5. Effect of bacitracin on the incorporation of *N*-acetylmuramyl-pentapeptide into peptidoglycan and lipid intermediates and on the release of alanine by a membrane preparation from *B. licheniformis* 94

Incubation mixtures (50 µl) contained 0.75 mM-UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[³H]-pimelic acid (15 mCi/mmol) and D-[¹⁴C]alanine (1.2 mCi/mmol), 0.9 mM-UDP-*N*-acetylglucosamine, enzyme (25 µl) and bacitracin to give the concentrations indicated. The mixtures were incubated for 30 min at 24°C. Incorporation of radioactive diaminopimelic acid into peptidoglycan (●) and lipid intermediates (■) and the release of radioactive alanine (▲) were determined after chromatography in solvent A. Enzyme incubated as described in the absence of antibiotic incorporated 6.5 nmol of diamino[³H]-pimelic acid/mg of protein.

additional 'rounds' of biosynthesis, i.e. successive addition of disaccharide-pentapeptide units. If one applies this argument to the present experiments, the maximum inhibition of 75% observed in the presence of bacitracin would imply that an average four 'rounds' of synthesis occur at each synthetic site during 30 min. This value was not in agreement with a glycan chain length of 17–22 disaccharide units for newly synthesized material found previously with membrane preparations from the same organism (Ward & Perkins, 1973). If the hypothesis of Siewert & Strominger (1967) were correct then radioactivity incorporated in the presence of bacitracin, at concentrations of antibiotic producing maximum inhibition, would all be present as single disaccharide-peptide units. In fact, these units would be at the reducing terminal of the glycan chain (Ward & Perkins, 1973). To test this, radioactivity was incorporated into peptidoglycan in the presence of a maximally inhibiting concentration (100 µg/ml) and in the absence of bacitracin. Peptidoglycan synthesis was inhibited in this experiment by 64%. Unincorporated precursor was removed from the membrane preparations by washing repeatedly with 0.3 M-HClO₄ (4 × 2.0 ml) and finally with water. The peptidoglycan reducing end groups were released by mild acid hydrolysis and degraded to yield lactyl-peptide units as described by Ward & Perkins (1973).

The lactyl-peptides were then separated from polymeric material by chromatography in solvent A. In both the control and bacitracin-treated samples the major lactyl-peptide corresponded to a marker of lactyl-tetrapeptide with lesser amounts of lactyl-penta- and -tri-peptides. The proportion of the total radioactivity present as lactyl-peptides was 13.6% in the control and 34.9% in the bacitracin-treated samples. All the remaining radioactivity was present as polymeric material on the origin of the chromatogram. Omission of the hydrolysis step prevented the subsequent β-elimination reaction in both preparations. Thus in the presence of excess of bacitracin not more than 35% of the newly synthesized peptidoglycan was present as single disaccharide units.

These results were confirmed by a second experiment, in which peptidoglycan was synthesized in the presence and absence of bacitracin, from UDP-*N*-acetyl[¹⁴C]muramyl-pentapeptide. In the presence of the antibiotic (100 µg/ml) peptidoglycan synthesis was inhibited 67%. After removal of unincorporated precursor and hydrolysis as described above, the proportion of [¹⁴C]muramic acid present as reducing terminals was determined after reduction with NaB³H₄ (Ward, 1973). Purified cell walls of *B. licheniformis* (3.2 mg) were added as carrier before the reduction, and after hydrolysis in 4 M-HCl at 100°C for 4 h and removal of acid *in vacuo* the radioactive amino sugar alcohols and muramic acid were fractionated on a column (0.8 cm × 20 cm) of Dowex 50 as described previously (Ward, 1973). In the peptidoglycan synthesized in the absence of bacitracin [¹⁴C]muramitol accounted for 12.8% of the total radioactivity present in this and [¹⁴C]muramic acid. Synthesis in the presence of the antibiotic resulted in this proportion increasing to 39.3%. [¹⁴C]Muramitol was not present in samples which had not been subjected to the initial mild acid hydrolysis (0.1 M-HCl at 60°C for 2 h).

(b) *Vancomycin*. Fig. 6 shows the inhibition of peptidoglycan synthesis and the release of alanine by increasing concentrations of vancomycin. The amount of antibiotic included in the reaction mixture is given as the molar proportions relative to the amount of UDP-*N*-acetylmuramyl-pentapeptide. These calculations are based on a molecular weight for vancomycin of 1800 (Nieto & Perkins, 1971a). Peptidoglycan synthesis was more sensitive to the presence of the antibiotic than was the release of alanine, 50% inhibition being obtained with molar proportions of 0.08 and 0.32 respectively. Lipid intermediates also accumulated, the maximum amount being found with a molar proportion of 0.12. Increasing the amount of vancomycin approximately ninefold did not affect the amount of lipid intermediate that accumulated.

(c) *Penicillins and cephalosporins*. The results

Vancomycin: UDP-N-acetylmuramyl-pentapeptide ratio

Fig. 6. Effect of vancomycin on the incorporation of N-acetylmuramyl-pentapeptide into peptidoglycan and lipid intermediates and the release of alanine by a membrane preparation from *B. licheniformis* 94

Incubation mixtures (50 μ l) contained 0.75 mM-UDP-N-acetylmuramyl-pentapeptide labelled with diamino[3 H]-pimelic acid (15 mCi/mmol) and D-[14 C]alanine (1.2 mCi/mmol), 0.9 mM-UDP-N-acetylglucosamine, enzyme (25 μ l) and antibiotic to give the final concentrations indicated, based on a molecular weight for vancomycin of 1800 as described in the text. Mixtures were incubated at 24°C for 30 min. Incorporation of radioactive diaminopimelic acid into peptidoglycan (●) and lipid intermediates (■) and the release of radioactive alanine (▲) were determined after chromatography in solvent A. Enzyme incubated in the absence of antibiotic (i.e. control preparations) incorporated 6.4 nmol of diamino[3 H]pimelic acid/mg of protein.

obtained on the addition of benzylpenicillin, 6-aminopenicillanic acid, cephalosporin C and cephaloridine to the membrane preparation are given in Table 2. The substrate was UDP-N-acetylmuramyl-L-alanyl-D-isoglutamyl-meso-diamino[3 H]pimelyl-D-[14 C]-alanyl-D-[14 C]alanine, enabling the release of alanine, in either the presence or the absence of UDP-N-acetylglucosamine, and also synthesis of polymeric peptidoglycan to be followed. In both cases the release of alanine was strongly inhibited by the addition of benzylpenicillin and to a lesser extent by 6-aminopenicillanic acid; the inhibition by the cephalosporins was much less effective. Under the conditions used the concentrations of benzylpenicillin and 6-aminopenicillanic acid required to inhibit alanine release by 50% were 3 and 20 μ g/ml respectively.

The inhibition of alanine release obtained in the presence of the higher concentrations of benzylpenicillin was accompanied by an increase in the amount of [14 C]alanine incorporated into the peptido-

Table 2. Effect of benzylpenicillin, 6-aminopenicillanic acid, cephalosporin C and cephaloridine on the incorporation into peptidoglycan and the release of alanine by membrane preparations from *B. licheniformis* 94

Antibiotic	Assay system	Benzylpenicillin			6-Aminopenicillanic acid			Cephalosporin C			Cephaloridine		
		Complete		Alanine incorporated into peptidoglycan released	Complete		Alanine incorporated into peptidoglycan released	Complete		Alanine incorporated into peptidoglycan released	Complete		Alanine incorporated into peptidoglycan released
		Alanine incorporated into peptidoglycan released	Alanine released		Alanine incorporated into peptidoglycan released	Alanine released		Alanine incorporated into peptidoglycan released	Alanine released		Alanine incorporated into peptidoglycan released	Alanine released	
Antibiotic concn. (μ g/ml)													
100		—	—	—	178	9	8	169	58	69	114	87	84
30		211	2	0.2	155	25	31	163	92	100	98	89	95
10		177	11	8	132	70	80	120	100	102	94	92	98
3		132	63	49	116	103	98	113	97	103	104	92	98
1		105	93	84	103	98	101	115	97	98	97	94	100
0.3		101	99	103	104	100	104	106	99	101	99	101	103

Table 3. *Effect of preincubation with cephalosporin C on the inhibition of peptidoglycan synthesis and the release of alanine*

Assay mixtures contained 0.9 mM-UDP-*N*-acetylglucosamine, enzyme (25 μ l) and cephalosporin C at a final concentration of 100 μ g/ml. The mixtures were preincubated at 24°C for the times indicated, with (+) or without (–) cephalosporin C, after which time 0.75 mM-UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[3 H]pimelic acid (15 mCi/mmol) and D-[14 C]alanine (1.2 mCi/mmol) was added and the mixture was incubated for a further 30 min at 24°C. Peptidoglycan synthesized and alanine released were determined after chromatography in solvent A.

Time of preincubation (min)	Diamino[3 H]pimelic acid incorporated (c.p.m./mg of protein)			[14 C]Alanine released (c.p.m./mg of protein)		
	Cephalosporin C		Inhibition (%)	Cephalosporin C		Inhibition (%)
	–	+		–	+	
0	6806	5962	12	8114	4793	41
15	4505	4172	7	7518	1704	77
30	4347	4134	6	8113	745	91
60	3830	3612	6	8457	318	96

glycan. The molar ratio of alanine to diaminopimelic acid incorporated into the polymeric material on the origin of the chromatogram rose from 1.12 to 2.08. This increase in the amount of alanine incorporated, characteristic of the formation of un-cross-linked peptidoglycan, was not accompanied by the formation of the spreading product previously found in penicillin-treated *E. coli* (Izaki *et al.*, 1968). The radioactivity, located by radioautography, remained closely associated with the area of application of the membranes and could not be eluted from the chromatogram without prior hydrolysis or lysozyme digestion. Synthesis of peptidoglycan, as judged by incorporation of diamino[3 H]pimelic acid into material remaining associated with the membranes, was not inhibited by the concentrations of antibiotics tested. Indeed, a concentration of benzylpenicillin of 500 μ g/ml even increased incorporation by 27% over that of the control.

Preliminary experiments had demonstrated the presence in the membranes of D-alanine carboxypeptidase activity. In these circumstances the alanine released in the peptidoglycan-synthesizing system, described above, could arise either by transpeptidation or by the action of such a carboxypeptidase. To determine the effect of the antibiotics on the release of alanine by this enzyme the assays were repeated in the absence of UDP-*N*-acetylglucosamine, when peptidoglycan synthesis cannot occur. The inhibition of alanine release under these conditions essentially matched that found with the complete system (Table 2). Blumberg & Strominger (1971) have reported the time-dependence of the inhibition of D-alanine carboxypeptidase from *B. subtilis* by penicillins and cephalosporins. To determine whether the inhibitory action of cephalosporin C was of this nature membrane preparations were preincubated with the antibiotic before peptidoglycan synthesis and the release of alanine was deter-

mined. Although preincubation of the preparation, even in the absence of antibiotic, caused a rapid decrease of its ability to synthesize peptidoglycan, cephalosporin C, with or without preincubation, had very little effect on this process. On the other hand, alanine release was unaffected by preincubation of the membranes but cephalosporin C produced a 40% inhibition without preincubation which was increased to 96% inhibition when the antibiotic had been present 60 min before the assay began (Table 3).

The reversibility of the observed inhibition by preincubation with cephalosporin C was studied by the addition of β -lactamase, at a concentration sufficient to hydrolyse 100 times the amount of cephalosporin C present, or by extensive washing of the membrane preparation, neither of which procedures had any effect (Table 4). Treatment with neutral hydroxylamine (0.8 M) for 10 min at 30°C resulted in almost complete reversal of the inhibition. Thus in these respects the sensitivity of the D-alanine carboxypeptidase to β -lactam antibiotics is similar to that previously reported in *B. subtilis* (Lawrence & Strominger, 1970).

Characterization of the material synthesized by membranes of B. licheniformis 94

In view of the presence of a penicillin-sensitive D-alanine carboxypeptidase, the fact that penicillins and cephalosporins inhibited alanine release could not be taken to indicate the occurrence of transpeptidation. To demonstrate the latter activity, cross-linked fragments were sought. Newly synthesized peptidoglycan remained associated with membranes and could not be removed by repeated washing with water or dilute buffer. This was also true of material synthesized in the presence of penicillin. After removal of alanine and unincorporated precursor from the membrane preparation peptidoglycan synthesized during a 2 h incubation was digested with

Table 4. *Effect of β -lactamase, washing and neutral hydroxylamine on inhibition of alanine release by cephalosporin C*

Assay mixtures were set up and the preincubation with cephalosporin C (100 μ g/ml) carried out as described in Table 3. Samples were then either treated with β -lactamase, washed or treated with neutral hydroxylamine (0.8M) as described in the text. Then 0.75 mM-UDP-*N*-acetylmuramyl-pentapeptide was added and the mixtures were incubated as described in Table 3. Alanine released was determined after chromatography in solvent A.

System	[¹⁴ C]Alanine released (c.p.m./mg of protein)		Inhibition (%)
	Cephalosporin C		
	—	+	
No preincubation	7607	5344	29.7
30 min preincubation	7158	1536	78.5
30 min preincubation+ β-lactamase	8033	2348	70.8
30 min preincubation+ washing	5054	1578	68.8
30 min preincubation+ neutral NH ₂ OH	6937	6215	10.4

Streptomyces N-acetylmuramidase. Carrier material, prepared by digestion of cell walls as described in the Experimental section, was added and the mixture was separated by chromatography in solvent A and located by radioautography.

In several preparations examined the major component accounted for approx. 65–75% of the incorporated diamino[^3H]pimelic acid and had the mobility of disaccharide-tetrapeptide. This identity was confirmed by electrophoresis at pH 6.5 after conversion into the corresponding lactyl-peptide (Ward & Perkins, 1973) and by the ratio of radioactive diaminopimelic acid to alanine. Minor components were identified as disaccharide-tripeptide and the cross-linked dimer. The latter component, arising by transpeptidation, did not account for more than 15% of the incorporated diamino[^3H]pimelic acid. Radioactivity incorporated in the presence of penicillin (10 and 25 μ g/ml) was located predominantly in disaccharide-pentapeptide with lesser quantities in disaccharide-tetrapeptide. No radioactivity was found in either the cross-linked dimer or disaccharide-tripeptide. Evidently the peptidoglycan synthesized by membrane preparations was mainly un-cross-linked and in this respect contrasted with material synthesized by membrane+wall preparations (see below).

Peptidoglycan synthesis in membrane+wall preparations of B. licheniformis 94

Although measurable transpeptidase activity was absent from membrane preparations from *M. lysodeikticus* and *Staph. aureus* (Katz *et al.*, 1967; Strominger, 1970) crude cell-wall preparations from these organisms have recently been shown to perform penicillin-sensitive transpeptidation of radioactive peptidoglycan precursors (Mirelman & Sharon, 1972; Mirelman *et al.*, 1972). The results obtained with similar preparations from *B. licheniformis* 94 are described below.

Experiments were carried out by using the same assay system as described for membrane preparations.

Fig. 7. *Effect of benzylpenicillin and cephaloridine on the incorporation of diamino[^3H]pimelic acid into cell walls and membranes of B. licheniformis 94*

Incubation mixtures (150 μ l) contained 0.6 mM-UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[^3H]pimelic acid (45 mCi/mmol), 0.67 mM-UDP-*N*-acetylglucosamine, enzyme preparation (100 μ l) and antibiotic to give the final concentrations indicated. The mixtures were incubated at 24°C for 30 min. Samples were either treated as described in the Experimental section with HClO_4 to determine incorporation into cell walls+membranes or with sodium lauryl sulphate to determine incorporation into cell walls alone. The effect of benzylpenicillin on incorporation into cell walls+membranes (Δ) and cell walls ($\blacktriangle$) and cephaloridine on incorporation into cell walls ($\circ$) is shown. Enzyme preparations incubated in the absence of antibiotic incorporated 9150 and 7850 c.p.m. respectively into cell walls+membranes and cell walls alone.

Incorporation of either diamino[^3H]pimelic acid or *N*-acetyl[^{14}C]glucosamine, from the appropriate precursors, proceeded in a linear manner for 30–45 min and uptake continued for a further 60 min. This incorporation was completely dependent on the presence of the other unlabelled precursor.

Table 5. Effect of penicillinase and washing on the inhibition by benzylpenicillin of the release of alanine and peptidoglycan synthesis in membrane+wall preparations of *B. licheniformis* 94

Membrane+wall preparations were preincubated with benzylpenicillin (10 µg/ml final concn.) at 24°C for 15 min. Penicillinase (100 units) was then added and the incubation continued for a further 5 min before the preparation was washed as described in the text. Assay mixtures contained 0.6 mM-UDP-*N*-acetylmuramyl-pentapeptide, labelled with diamino[³H]pimelic acid (45 mCi/mmol) and D-[¹⁴C]alanine (3.1 mCi/mmol) in the terminal residue, 0.67 mM-UDP-*N*-acetylglucosamine and enzyme preparation (100 µl) in a total volume of 150 µl. Mixtures were incubated at 24°C for 30 min. Alanine was determined after electrophoresis in buffer system D and peptidoglycan synthesized was measured as radioactivity remaining associated with the cell walls after treatment with sodium lauryl sulphate as described in the Experimental section.

System	Radioactivity incorporated into cell wall (c.p.m.)		Alanine released (c.p.m.)
	Diamino[³ H]pimelic acid	[¹⁴ C]Alanine	
(1) Control	7150	179	64207
(2) -UDP- <i>N</i> -acetylglucosamine	282	15	52653
(3) +Penicillin	517	66	1290
(4) +Penicillin - UDP- <i>N</i> -acetylglucosamine	66	25	899
(5) As (3) + penicillinase and washing	5018	331	1469
(6) As (4) + penicillinase and washing	122	31	891

Addition of benzylpenicillin or cephaloridine to reaction mixtures strongly inhibited the incorporation of radioactivity from the precursor into cell-wall material. Fig. 7 shows the effect of increasing concentrations of these antibiotics on the incorporation of diamino[³H]pimelic acid into HClO₄-precipitable (cell walls and membranes) and sodium lauryl sulphate-resistant (cell walls only) material. Amino acid analysis of the cell walls remaining after extraction with sodium lauryl sulphate and extensive washing, as outlined in the Experimental section, revealed only the expected amino sugars and amino acids; any contamination with other material was less than 5%. The amount of benzylpenicillin and cephaloridine required to inhibit the incorporation of diamino[³H]pimelic acid into cell-wall material by 50% was 0.02 and 0.03 µg/ml respectively and the incorporation was inhibited by more than 90% by 0.1 µg of benzylpenicillin/ml and 0.5 µg of cephaloridine/ml. In the absence of antibiotic, the cell walls contained 86% of the total radioactivity incorporated into cell walls and membranes together, whereas under conditions of maximum inhibition, i.e. 0.5–1 µg of benzylpenicillin/ml, this value had decreased to 22% (Fig. 7). Compared with the membrane preparations previously described, the incorporation of diamino[³H]pimelic acid was 0.97 nmol/h per mg of protein into the combined cell walls and membranes and 0.83 into the purified cell walls.

Inhibition of the incorporation of diaminopimelic acid by benzylpenicillin was reversible (Table 5). Cell wall-membrane preparations were incubated with benzylpenicillin (10 µg/ml) for 15 min at 24°C, when 100 units of penicillinase were added and the incubation was continued for a further 5 min. The enzyme preparation was then washed with Tris-MgCl₂-

dithiothreitol buffer (3×20 ml). Normal assays were set up with these penicillin-treated preparations with UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[³H]pimelic acid and D-[¹⁴C]alanine in the terminal residue as the substrate. In the presence of benzylpenicillin (1 µg/ml) the incorporation of diaminopimelic acid into the cell walls was inhibited by 93%. Pretreatment of the enzyme preparation with penicillinase and washing restored much of the activity, the inhibition being decreased to 29%.

The enzyme preparation also contained D-alanine carboxypeptidase activity releasing free alanine from the substrate, UDP-*N*-acetylmuramyl-pentapeptide, and newly synthesized peptidoglycan. This activity was also inhibited by benzylpenicillin, at 10 µg/ml, the inhibition being 98%. In the absence of benzylpenicillin essentially all the terminal D-alanine was removed from the peptidoglycan synthesized. The inhibition of D-alanine carboxypeptidase by benzylpenicillin was not reversible as described above for the effect of cephalosporin C on membrane preparations. Thus the amount of alanine incorporated into the cell wall after removal of inhibition, by treatment with penicillinase and washing, was increased by 88%. The inhibition of incorporation of diamino[³H]pimelic acid by cephaloridine was also reversed by a similar procedure (Table 6). Enzyme preparations incorporated only 12% of the radioactivity of the control after treatment with the antibiotic (10 µg/ml at 24°C for 15 min). After washing, but without treatment with β-lactamase, the incorporation increased to 58%. The release of alanine by D-alanine carboxypeptidase was not significantly inhibited by this treatment and no increase in the amount of alanine incorporated into the cell wall was observed.

Table 6. Effect of washing on the inhibition by cephaloridine of alanine release and peptidoglycan synthesis in membrane+wall from *B. licheniformis* 94

Membrane+wall preparations were preincubated with cephaloridine (10 µg/ml final concn.) at 24°C for 15 min. The enzyme preparation was then washed as described in the text. Alanine release and peptidoglycan synthesis were determined as described in Table 5.

System	Radioactivity incorporated into cell wall (c.p.m.)		Alanine released (c.p.m.)
	Diamino[³ H]pimelic acid	[¹⁴ C]Alanine	
(1) Complete	3970	152	53 220
(2) -UDP- <i>N</i> -acetylglucosamine	279	10	49 870
(3) +Cephaloridine	472	27	51 210
(4) +Cephaloridine-UDP- <i>N</i> -acetylglucosamine	142	12	50 730
(5) As (3) +washing	2177	84	47 130
(6) As (4) +washing	257	33	45 610

Fig. 8. Fractionation of the free reducing groups and hexosamines in hydrolysed cell walls of *B. licheniformis* 94 after incorporation of [¹⁴C]muramic acid

Cell walls were purified, by treatment with sodium lauryl sulphate and washing as described in the Experimental section, after incorporation of [¹⁴C]muramic acid from the appropriate precursor. The labelled walls were hydrolysed in 4M-HCl (1.0 ml) at 100°C for 4 h. The acid was removed *in vacuo* and the hydrolysate applied to a column (0.8 cm × 20 cm) of Dowex 50 (× 2; 200–400 mesh) equilibrated with 0.1 M-pyridine-acetate buffer, pH 2.8. Fractions (2.0 ml) were eluted with the same buffer (70 ml total) and then with 0.133 M-pyridine-acetate buffer, pH 3.85. Samples (0.1 ml) of the fractions were analysed for ³H (○), ¹⁴C (▲) and hexosamines (■). Key: 1, [1-³H, ¹⁴C]muramitol; 2, [¹⁴C]muramic acid; 3, [1-³H]glucosaminol; 4, glucosamine.

Characterization of the newly synthesized peptidoglycan

A cell-wall fraction containing newly synthesized material was purified from membrane+wall incubation mixtures and digested with *Streptomyces N*-acetylmuramidase as described in the Experimental section. The products were examined by paper chromatography (solvent A); 39% of the incorporated diamino[³H]pimelic acid chromatographed with cross-linked dimer. The remaining radioactivity

migrated in positions corresponding to disaccharide monomers. The recovery of radioactivity from the chromatogram was 87%. The isolated products were also characterized as the corresponding lactyl-peptides by rechromatography in solvent A and paper electrophoresis at pH 6.5.

The length of the newly synthesized glycan chain was determined by reduction of the free reducing group of muramic acid with NaB³H₄ and subsequent

separation of the resulting [^3H]muramitol and unchanged muramic acid (Ward, 1973). Peptidoglycan was synthesized from UDP-*N*-acetyl[^{14}C]-muramyl-pentapeptide and purified by the normal procedure. The cell walls including incorporated [^{14}C]muramic acid were treated with an excess of NaB^3H_4 for 6 h at 4°C . Excess of reagent was removed by acidification and washing and the reduced cell walls were hydrolysed. The hydrolysate was fractionated on a column (0.8 cm $\times$ 20 cm) of Dowex 50. ^3H was located in muramitol and to a smaller extent in glucosaminol. ^{14}C was found in both muramitol and unchanged muramic acid (Fig. 8). Appropriate samples were pooled and the average chain length of the newly synthesized glycan was calculated from the ratio of [$1\text{-}^3\text{H}$, ^{14}C]muramitol to [^{14}C]muramic acid. In addition the average chain length of the glycan present in the complete cell-wall preparation could be obtained from the ratio of [$1\text{-}^3\text{H}$]muramitol to muramic acid present. These values were 20.8 and 104.5 disaccharide units respectively. Similar experiments in which the newly synthesized peptidoglycan was labelled with *N*-acetyl[^{14}C]glucosamine failed to reveal the presence of [^{14}C]glucosaminol.

Discussion

Preparations have been obtained from a penicillinase-negative, poorly lytic mutant of *B. licheniformis* 6346 which synthesizes peptidoglycan and incorporate newly synthesized material into the bacterial cell wall. Their activity is relatively high compared with many similar systems from other organisms, but still represents only 1–2% of the rate of synthesis in the growing organisms. The maximum activities obtained were similar to those reported previously for *B. megaterium* (Reynolds, 1971a). Several attempts to obtain preparations from the parent *B. licheniformis* 6346 with similar activity were unsuccessful.

The effect of various antibiotics interfering with various stages of peptidoglycan synthesis (Strominger, 1970) has also been determined by using the membrane preparation. Vancomycin, known to combine strongly with peptides having a terminal D-alanyl-D-alanyl structure (Perkins, 1969; Nieto & Perkins, 1971b), inhibits both peptidoglycan synthesis and the release of alanine mediated by D-alanine carboxypeptidase. The former is some three- to four-fold more sensitive to the antibiotic, and lipid intermediates accumulate at concentrations of vancomycin similar to those inhibiting peptidoglycan biosynthesis. These results suggest that the affinity of vancomycin for some intermediate in peptidoglycan synthesis is greater than the affinity for the precursor UDP-*N*-acetylmuramyl-pentapeptide. This intermediate may be either the *N*-acetylmuramyl-pentapeptide-pyrophosphate-undecaprenol, the disaccharide

(pentapeptide)-pyrophosphate-undecaprenol, or the growing glycan chain, which previous work (Ward & Perkins, 1973) has suggested remains attached by a glycosyl link between the reducing terminal of a muramic acid residue and undecaprenyl pyrophosphate. High affinity of vancomycin for one or more of these intermediates would correlate with the observation that a high molar proportion of the peptide diacetyl-L-diaminobutyryl-D-alanyl-D-alanine to vancomycin was required to reverse the inhibitory action of the antibiotic on peptidoglycan synthesis in *B. megaterium* (Nieto *et al.*, 1972).

From the residual peptidoglycan synthesis observed in the presence of excess of bacitracin Siewert & Strominger (1967) concluded that their membrane preparation from *M. lysodeikticus* was performing four to six rounds of disaccharide-pentapeptide incorporation at each biosynthetic site during 60 min incubation. The present results show conclusively that two or three rounds of disaccharide-pentapeptide addition occur even in the presence of bacitracin. Hence the argument proposed by Siewert & Strominger (1967) cannot indicate with certainty the length of the newly synthesized chain in a given membrane preparation. The latter value can only be arrived at by direct measurement. The present results suggest either that some of the synthetic sites are resistant to the action of bacitracin or that a pool of undecaprenyl phosphate exists in the membrane sufficient to allow the addition of several disaccharide units at each site.

The membrane preparation from *B. licheniformis* 94 synthesizes linear peptidoglycan, which is mainly un-cross-linked. This process is insensitive to both penicillins and cephalosporins. Alanine is released from the nascent peptidoglycan by the action of a penicillin-sensitive D-alanine carboxypeptidase. In the membrane+wall preparations these reactions also occur, together with the synthesis of peptide cross-bridges which presumably attach newly synthesized material to the cell walls. The formation of these covalent linkages is mediated by the action of a transpeptidase, sensitive to both penicillin and cephaloridine. The concentrations of these antibiotics inhibiting the transpeptidase by 50% are essentially the same as those inhibiting growth of the organism. The inhibition of the transpeptidase is reversible, in direct contrast with the situation in *E. coli* (Izaki *et al.*, 1968), whereas inhibition of the D-alanine carboxypeptidase is not. The latter enzyme resembles a particulate D-alanine carboxypeptidase from *B. subtilis* (Lawrence & Strominger, 1970; Blumberg & Strominger, 1971).

The inhibition of transpeptidase activity by both penicillin and cephaloridine occurs at concentrations very similar to those inhibiting growth of the organism. This suggests that inhibition of the transpeptidase may well be the killing reaction in *B. licheniformis*.

In *B. megaterium* (Wickus & Strominger, 1972b) the concentration of several penicillins which inhibited the incorporation of diaminopimelic acid by transpeptidation by 50% also caused a similar inhibition of growth. However, attempts to demonstrate a similar correlation with the cephalosporins were not successful.

The almost complete inhibition of the incorporation of newly synthesized peptidoglycan into the pre-existing cell wall by low concentrations of both antibiotics precludes transglycosylation as a route for this incorporation. This contrasts with the findings in *M. lysodeikticus* (Mirelman *et al.*, 1972) where some 30% of newly synthesized material is incorporated by this mechanism. In the present work the relatively high degree of cross-bridge formation and the length of the newly synthesized glycan chains show that transpeptidation and glycan synthesis both occur during the course of the incubation. The results obtained in the presence of penicillin show that some peptidoglycan synthesis continues in the complete membrane+wall preparation, but hardly any product becomes attached to the wall (Fig. 7). Presumably, this material resembles the linear un-cross-linked peptidoglycan synthesized by membrane preparations in the absence of cell wall. In membrane+wall preparations much newly synthesized material cannot be removed by sodium lauryl sulphate and therefore presumably becomes attached to the pre-existing cell wall by direct formation of cross-bridges between newly synthesized peptidoglycan and the 'old' cell wall. Alternatively, it could be rendered insoluble by cross-linking between chains of newly synthesized material.

Biochemical (Mauck & Glaser, 1972) and electron-microscopic (Frehel *et al.*, 1971) evidence suggests that the insertion of newly synthesized peptidoglycan occurs at multiple sites in existing wall in both *B. subtilis* and *B. megaterium*. Attachment by means of transpeptidation could occur via the penultimate D-alanine residue of the pentapeptide chain of the repeating unit and a free amino group of suitable configuration in the pre-existing cell wall. In *B. licheniformis* and *B. subtilis* this would be a free amino group at the D-centre of diaminopimelic acid (Schleifer & Kandler, 1972). Such free amino groups are normally found in the cell walls of these bacilli where 30–40% of the diaminopimelic acid is present in un-cross-linked tripeptide substituents of the glycan chain (Hughes, 1970). However, there is no available evidence as to the ability of these un-cross-linked peptides to act as acceptors in a transpeptidation reaction.

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