

The Mechanism of Wall Synthesis in Bacteria

THE ORGANIZATION OF ENZYMES AND ISOPRENOID PHOSPHATES IN THE MEMBRANE

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1. The synthesis of peptidoglycan and teichoic acids by cell-free preparations from *Bacillus licheniformis* A.T.C.C. 9945 and *Bacillus subtilis* N.C.T.C. 3610 has been studied under a variety of conditions. 2. It was shown that poly(glycerol phosphate) is synthesized through a lipid intermediate, and it is concluded from this and other work that all major bacterial wall polymers are formed in a similar manner through such intermediates. 3. Close interrelation between the synthesis of peptidoglycan and teichoic acids was demonstrated, and inhibition studies confirm that the polyprenol phosphate molecules participating in the synthesis of peptidoglycan are shared with the systems that synthesize teichoic acids. 4. Nucleotides for the synthesis of one polymer are inhibitory towards synthesis of the other, and these effects can be enhanced or diminished by preincubation of the enzyme system with appropriate nucleotide precursors. 5. It is concluded that the return of undecaprenol phosphate to a common pool occurs only after the completion of polymer chains, and not after each cycle in the attachment of individual repeating units. This and other observations support a model for bacterial wall synthesis in which the multi-enzyme systems for each polymer are closely aligned in the membrane, with a molecule of undecaprenol phosphate located between them in a manner that enables it to be shared. The general mechanisms of wall synthesis and its control are discussed.

The two major wall polymers in Gram-positive bacteria, peptidoglycan and teichoic acid, are synthesized by enzyme systems located mainly in the cytoplasmic membrane. In the formation of peptidoglycan the nucleotide precursors, UDP-*N*-acetylglucosamine and the appropriate UDP-*N*-acetylmuramyl peptide, transfer residues first to an undecaprenol phosphate and hence to the polymer chain. The characterization of the isoprenoid group in these lipid intermediates was achieved by direct chemical examination of isolated material (Higashi *et al.*, 1967, 1970*b*). Although acid-labile lipid intermediates have been shown to participate in the synthesis of teichoic acids (Douglas & Baddiley, 1968; Brooks & Baddiley, 1969), these lipids have not been characterized by direct chemical examination. Nevertheless, the identity of one of these lipids has recently been established by an indirect method (Watkinson *et al.*, 1971). The method made use of a membrane preparation from *Staphylococcus lactis* I3 with which it was shown that the synthesis of wall teichoic acid was markedly inhibited by bacitracin or vancomycin only when an adequate supply of peptidoglycan precursor nucleotides was maintained. It was concluded that the isoprenoid monophosphate was a common component of the two systems, and that inhibition of synthesis of teichoic

acid was the consequence of a decrease in the amount of this lipid arising from the blockage of its return from peptidoglycan synthesis; it is known that bacitracin powerfully inhibits the mono-dephosphorylation of lipid pyrophosphate to lipid monophosphate, an essential step in the biosynthetic cycle for peptidoglycan (Siewert & Strominger, 1967). It follows that the same undecaprenol phosphate molecules participate in the biosynthetic routes for both peptidoglycan and teichoic acid. Although these observations were conclusive, the system from *S. lactis* I3 was not highly efficient in the synthesis of completed peptidoglycan, and labelled residues from the nucleotide precursors were found predominantly in the lipid intermediates for this polymer. In an attempt to overcome this and to extend the study, preparations from *Bacillus licheniformis* A.T.C.C. 9945 and *Bacillus subtilis* N.C.T.C. 3610 have been examined.

Membrane preparations from this strain of *B. licheniformis* synthesize from CDP-glycerol a poly(glycerol phosphate), and if UDP-glucose is also present a wall teichoic acid with the structure $\text{-(glycerol-phosphate-glucose)-}_n$ is produced (Burger & Glaser, 1964, 1966). Lipid intermediates participate in the synthesis of this wall teichoic acid (Hancock & Baddiley, 1972). It was found that an

enzyme preparation from *B. licheniformis* was highly efficient in the synthesis of complete peptidoglycan (results in the present paper); this latter ability is presumably an indication that the complex of enzymes concerned in peptidoglycan synthesis is in a relatively undisturbed state. The *B. subtilis* synthesizes a poly(glycerol phosphate) and a glucosylated poly(glycerol phosphate) that occurs in its wall. The glucose residues in this teichoic acid are not a part of the main polymer chain (Burger & Glaser, 1964; Glaser & Burger, 1964).

The experiments described in the present paper were designed to study the effect of the nucleotide precursors for teichoic acid synthesis on the synthesis of peptidoglycan in cell-free systems, and conversely the effect of nucleotide precursors for peptidoglycan synthesis on the synthesis of teichoic acids. The effect of bacitracin on the synthesis of teichoic acids was also examined under a variety of conditions.

The results with the membrane preparations from the bacilli confirm the finding with the staphylococcus that the undecaprenol phosphate is shared between the two syntheses. Moreover, the studies suggest that the synthesis of polymer chains must be completed before the lipid phosphate becomes available for synthesis of the other polymer. These and related findings support a model for the membrane in which the synthesis of the wall polymers is achieved by fairly rigidly orientated enzyme complexes in close proximity to each other with undecaprenol phosphate located between them. This model provides a meaningful role for the lipid intermediates, and helps to explain the mechanism whereby intermediates on one side of the membrane produce polymers on the other side.

Experimental

Materials

Vancomycin hydrochloride was purchased from Eli Lilly and Co. Ltd., Basingstoke, Hants., U.K. Bacitracin was purchased from Therapham Ltd., Kingston-on-Thames, Surrey, U.K. [$1-^{14}\text{C}$]Glycerol, [$\text{G}-^{14}\text{C}$]glucose 1-phosphate, [^{14}C]acetic anhydride and [$\text{G}-^3\text{H}$]2,6-diaminopimelic acid dihydrochloride, were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Other biochemicals were purchased from Sigma Chemicals, St. Louis, Mo., U.S.A. The solvents and chemicals used in the investigation were of analytical grade, and were obtained from BDH Chemicals, Poole, Dorset, U.K.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951). *N*-Acetyl amino sugars were determined by the method of Reissig *et al.* (1955). Phosphate was determined by the method of Chen *et al.* (1956).

Quantitative amino acid and amino sugar analyses were done with a Technicon Autoanalyser. Radioactive material on paper was located with a radiochromatogram scanner (Baird Atomic Inc., Cambridge, Mass., U.S.A.); quantitative measurements of radioactivity were done by cutting out the appropriate areas of paper and counting these in a Beckman LS-150 liquid-scintillation counter, with a scintillant of the following composition: toluene, 2 litres; 2,5-diphenyloxazole, 8 g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.2 g. Water-soluble samples were counted in this scintillant mixed with Triton X-100 (2:1, v/v) (Patterson & Greene, 1965). All samples were counted in glass vials with 10 ml of scintillation fluid. The counting error was 1–2%. The counting efficiency of ^{14}C samples on paper was 45% and for water-soluble samples was 70%.

Other methods

Paper chromatography. This was done on Whatman no. 1 paper in the following descending solvent systems: *A*, 1M-ammonium acetate-ethanol, pH 3.6 (5:2, v/v) (Paladini & Leloir, 1951); *B*, isobutyric acid-aq. 0.5M-NH₃ (5:3, v/v) (Krebs & Hems, 1953); *C*, propan-1-ol-NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949). The products were detected with the following reagents: alkaline AgNO₃ for sugars and polyols (Trevelyan, *et al.*, 1950); molybdate for phosphoric esters (Hanes & Isherwood, 1949) and ninhydrin for amino acids (Consden & Gordon, 1948).

Culture conditions. A 200 ml culture of *B. licheniformis* A.T.C.C. 9945 or *B. subtilis* N.C.T.C. 3610, inoculated from a nutrient agar slope, was grown overnight at 37°C with shaking in a medium of the following composition: nutrient broth no. 2 (Oxoid), 12.5 g; yeast extract (Difco), 2.5 g; K₂HPO₄, 2.5 g; glucose, 1 g; water, 1 litre. This culture was then used to inoculate a batch (8 litres) of medium containing polypropylene glycol 2000 (2 ml) anti-foaming agent. Incubation was continued with forced aeration at 37°C for 3–3.5 h (mid-exponential growth phase), when the organisms were harvested by using the continuous-flow attachment on a Sorvall refrigerated centrifuge and then washed with cold 0.6% NaCl.

Preparation of enzyme. Particulate enzyme was prepared by a method similar to that of Anderson *et al.* (1965). Washed cells (10 g wet wt.), mixed with type 305 alumina (30 g) (Sigma), were ground gently for 5 min in a pre-cooled mortar at 4°C. The cell-alumina mixture was then suspended in 150 ml of 0.05 M-tris-HCl buffer, pH 7.5, containing 0.1 mM-MgCl₂ and 1 mM-2-hydroxyethanethiol, and was then centrifuged at 4000g for 5 min. The pellet of alumina and unbroken cells was discarded and the residual suspension was centrifuged at 15000g for 5 min. The supernatant was centrifuged at 105000g for 1 h. The

sedimented gel was washed twice with 0.15M-sodium pyrophosphate buffer, pH7.5, and once with the above tris-HCl buffer and finally suspended in this buffer (1:2, w/v) to give a protein concentration of approx. 15mg/ml. This suspension was used as enzyme in the investigations.

Preparation of substrates. CDP-glycerol was synthesized from the morpholidate of CMP (Roseman *et al.*, 1961). CDP-[^{14}C]glycerol and UDP-*N*-[acetyl- ^{14}C]acetylglucosamine were prepared enzymically by the methods described by Baddiley *et al.* (1968). UDP-[^{14}C]glucose was prepared by the method of Hancock & Baddiley (1972). The peptidoglycan precursor, UDP-MurAc*-L-Ala-D-Glu-Dap-D-Ala-D-Ala (UDP-MurAc-pentapeptide), was isolated from *B. subtilis* N.C.T.C. 3610, the growth of which had been inhibited by vancomycin, as follows (Reynolds, 1961; Jordan, 1961). The organism was grown at 37°C in the culture medium (2 litres) as described above. Cells from an exponentially growing culture were harvested and resuspended in 500ml of 0.5% sodium phosphate buffer, pH7.2, containing 1% of glucose, 0.2mM-uracil, 1mM-alanine, 1mM-glutamic acid; 1mM-diaminopimelic acid and vancomycin (20mg). The cell suspension was incubated with shaking for 45min at 37°C and then the cells were collected by centrifugation at 10000g in a Sorvall refrigerated centrifuge. The cells were suspended in water (20ml) and heated in a boiling-

* Abbreviations (in sequences): Mur, muramic acid; MurAc, *N*-acetylmuramic acid; Dap, 2,6-diaminopimelic acid.

water bath for 5min. The suspension was then cooled in an ice bath and trichloroacetic acid was added to give a final concentration of 10% (w/v). After 30min the suspension was centrifuged and the nucleotides were recovered from the supernatant by adsorption to and elution from Norit A activated charcoal (Baddiley *et al.*, 1956). The charcoal eluate was subjected to chromatography on Whatman 3MM paper in solvent *A*. Three major u.v.-absorbing zones were visible and material from these was eluted with water. Samples of the eluates were hydrolysed with 4M-HCl at 100°C for 6h and subjected to quantitative analysis. The zone with R_{UMP} 0.10-0.15 was UDP-MurAc-pentapeptide (Found: Glu:Ala:Dap:Mur:Uridine:P, 1.00:2.81:1.24:0.8:0.92:2.05). The yield of this material was usually about 15-20 μmol . Other zones were tentatively identified as UDP-MurAc and UDP-MurAc-L-Ala-D-Glu; these were present in much smaller amounts. The quantity of accumulated nucleotides was related to the logarithm of the concentration of vancomycin, a relationship noted before by Reynolds (1961) by using vancomycin with *Staphylococcus aureus*.

UDP-MurAc-L-Ala-D-Glu-[G- ^3H]Dap-D-Ala-D-Ala was prepared similarly, [G- ^3H]2,6-diaminopimelic acid dihydrochloride (1mCi) being added to the buffered medium. The specific radioactivity of the labelled precursor was usually about 1.5×10^6 c.p.m./ μmol .

Determination of enzyme activities. Routine reaction mixtures for the synthesis of peptidoglycan contained UDP-MurAc-pentapeptide (0.1 μmol)

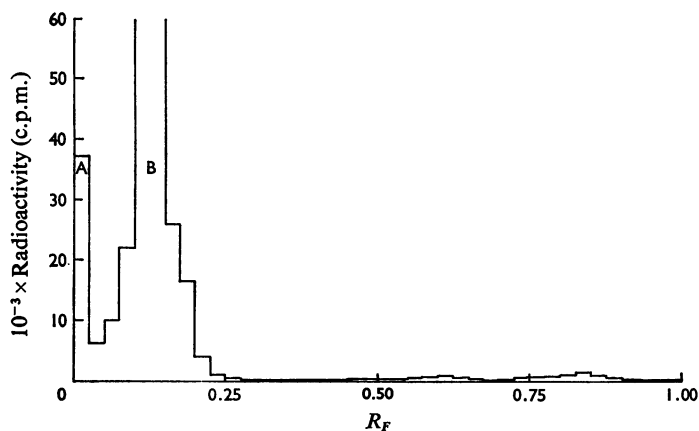


Fig. 1. Chromatography of peptidoglycan synthesized by an enzyme preparation from *B. licheniformis*

The incubation mixture contained UDP-*N*-[acetyl- ^{14}C]acetylglucosamine (0.01 μmol ; 39.5×10^6 c.p.m./ μmol), UDP-MurAc-pentapeptide (0.1 μmol), MgCl_2 (4 μmol) and 0.1ml of enzyme suspension in a total volume of 0.14ml. The reaction mixture was incubated at 30°C for 1h. Reaction was terminated by the addition of butan-1-ol (0.05ml) and the mixture was subjected to chromatography in solvent *C* for 18h. Radioactivity was determined as in determination procedure B.

or UDP-MurAc-[³H]pentapeptide (0.1 μmol; 1.5×10^6 c.p.m./μmol), UDP-*N*-acetylglucosamine (0.1 μmol) or UDP-*N*-[acetyl-¹⁴C]acetylglucosamine (0.1 μmol; 2.47×10^6 c.p.m./μmol), MgCl₂ (7.5 μmol) and 0.06 ml of enzyme suspension in a total volume of 0.12 ml. Reaction mixtures were incubated for 1 h at 20°C. Radioactively labelled polymer was then determined in one of the two ways.

Determination procedure A. The reactions were terminated by the addition of 1 ml of ice-cold 0.3M-perchloric acid and the mixture was kept for 10 min. The residue, containing synthesized polymer, was centrifuged and washed three times with cold 0.3M-perchloric acid (0.5 ml). The supernatant and washings were discarded, then the residue was transferred with the aid of 1 ml of distilled water to counting vials containing scintillation fluid, and the radioactivity was determined. Radioactivities were corrected for a boiled enzyme control.

Determination procedure B. The reactions were terminated by the addition of 0.05 ml of butan-1-ol, then the mixtures were applied as bands to Whatman no. 1 paper and subjected to chromatography in solvent C for 18 h. The chromatograms were cut into bands (1 cm wide) and the radioactivity was located and measured by counting each band.

Reaction mixtures for the synthesis of poly(glycerol phosphate glucose) contained UDP-[¹⁴C]-glucose (0.1 μmol; 1.3×10^6 c.p.m./μmol), CDP-glycerol (0.2 μmol), MgCl₂ (1.2 μmol) and 0.06 ml of enzyme suspension in a total volume of 0.12 ml. Reaction mixtures were incubated for 1 h at 37°C and synthesis of polymer was determined by procedures A or B. When procedure A was used the residue was extracted (×2) with 0.1 ml portions of butan-1-ol to remove glycolipids known to be synthesized by the organism (Hancock & Baddiley, 1972).

Reaction mixtures for the synthesis of poly(glycerol phosphate) contained CDP-[¹⁴C]glycerol (0.1 μmol; 4.44×10^6 c.p.m./μmol), MgCl₂ (5 μmol) and 0.06 ml of enzyme suspension in a total volume of 0.12 ml. Reaction mixtures were incubated for 1 h at 37°C and synthesis of polymer was determined by procedures A or B. The amounts of the various polymers synthesized under these experimental conditions were peptidoglycan, 3.5 nmol; poly(glycerol phosphate glucose), 12 nmol; poly(glycerol phosphate), 50 nmol.

Results

Synthesis of peptidoglycan and lipid intermediate(s) in particulate enzyme from B. licheniformis A.T.C.C. 9945

Optimum conditions for the synthesis of peptidoglycan by the membrane preparation were established in the following manner. Preliminary experiments

indicated that polymeric material (Fig. 1; baseline peak A) was synthesized from UDP-*N*-[acetyl-¹⁴C]-acetylglucosamine and UDP-MurAc-pentapeptide. Similar insoluble radioactive material was observed in the incubation mixtures with determination procedure A. The identity of this material with peptidoglycan was established by examining its behaviour towards lysozyme as follows. The residue was washed with 30 mM-potassium phosphate buffer, pH 7.0 (4 ml), and then suspended in this buffer (0.5 ml) together with lysozyme (2 mg/ml). The suspension was incubated at 37°C for 1 h and the reaction was stopped by the addition of 1 ml of ice-cold 0.3M-perchloric acid. The suspension was then centrifuged and the supernatant, after neutralization with KOH, was evaporated to dryness and examined by paper chromatography in solvent A. About 90% of the incorporated radioactivity now appeared in the soluble products; no soluble radioactive products were obtained from a residue carried through this procedure in the absence of lysozyme. The insoluble material was thus sensitive towards lysozyme and could be regarded as peptidoglycan. The soluble

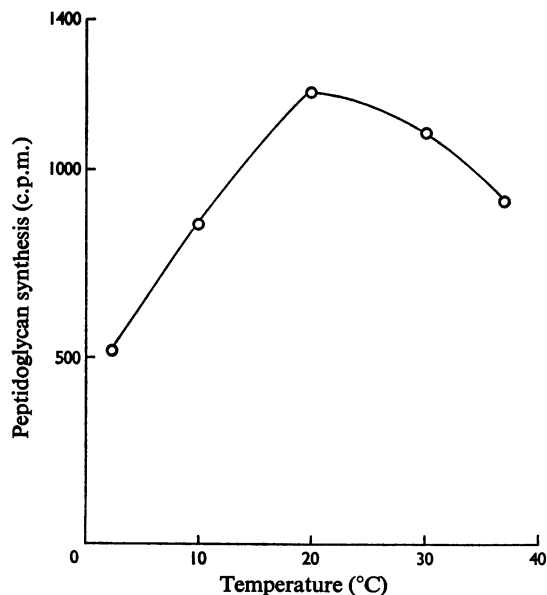


Fig. 2. Effect of temperature on peptidoglycan synthesis by an enzyme preparation from *B. licheniformis*

The incubation mixtures contained UDP-*N*-[acetyl-¹⁴C]acetylglucosamine (0.01 μmol; 2.47×10^6 c.p.m./μmol), UDP-MurAc-pentapeptide (0.1 μmol), MgCl₂ (4 μmol) and 0.1 ml of enzyme suspension in a total volume of 0.14 ml. Reaction mixtures were incubated at the temperatures indicated for 1 h. Synthesized polymer was determined by procedure A.

products comprised two major components with R_F values of 0.52 and 0.67, believed to be the disaccharide pentapeptide and decapeptide, although direct comparison with standard preparations was not attempted. There were two uncharacterized minor components with R_F values of 0.15 and 0.25.

The optimum temperature for the synthesis of peptidoglycan was 20°C (Fig. 2). Mg^{2+} ions were required, the optimum concentration being 65 mM (Fig. 3); concentrations greater than this were inhibitory. The activity of the enzyme was maximum immediately after preparation and decreased to half this value during storage for 3 days at -20°C; no further decrease in activity was observed during several weeks at this temperature. The optimum concentrations for both substrates, in the period of stable enzyme activity, were about 0.8 mM (Figs. 4

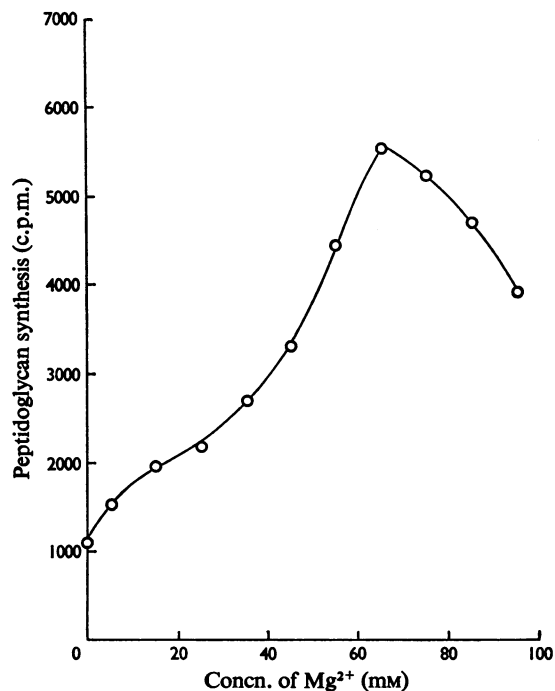


Fig. 3. Effect of magnesium ions on peptidoglycan synthesis by an enzyme preparation from *B. licheniformis*

The incubation mixtures contained UDP-*N*-[acetyl- ^{14}C]acetylglucosamine (0.1 μ mol; 2.47×10^6 c.p.m./ μ mol), UDP-MurAc-pentapeptide (0.1 μ mol), 60 μ l of enzyme suspension and $MgCl_2$ as indicated in a total volume of 0.12 ml. Reaction mixtures were incubated at 20°C for 1 h. Synthesized polymer was determined by procedure A.

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and 5). Higher concentrations of substrates were required to saturate freshly prepared enzyme.

Peak D (Fig. 1) had an R_F value of 0.82. This fast-running material was identified as lipid intermediate(s) in the synthesis of peptidoglycan by examining the kinetics of its formation (Fig. 6) which, together with the kinetics for the synthesis of peptidoglycan, are characteristic of an intermediate and product (Anderson *et al.*, 1967); 95% of the incorporated radioactivity was located in peptidoglycan and 5% in the lipid intermediate(s) after incubation for 1 h.

Synthesis of teichoic acids by particulate enzyme from *B. licheniformis* A.T.C.C. 9945

Particulate enzyme preparations isolated from *B. licheniformis* have been shown to synthesize poly-(glycerol phosphate) from CDP-glycerol (Burger &

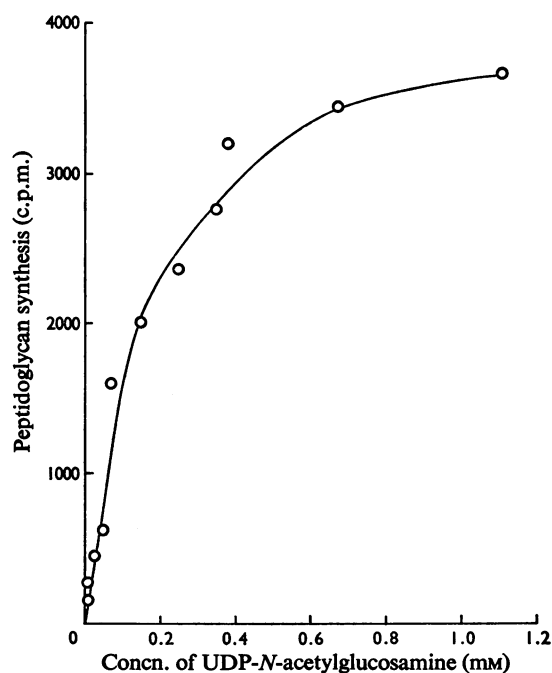


Fig. 4. Effect of concentration of UDP-*N*-acetylglucosamine on peptidoglycan synthesis by an enzyme preparation from *B. licheniformis*

Incubation mixtures contained UDP-MurAc-pentapeptide (0.3 μ mol), $MgCl_2$ (12 μ mol), 0.1 ml of enzyme suspension and UDP-*N*-[acetyl- ^{14}C]acetylglucosamine (2.47×10^6 c.p.m./ μ mol) as indicated, in a total volume of 0.2 ml. Mixtures were incubated at 20°C for 1 h and synthesized polymer was determined by procedure A.

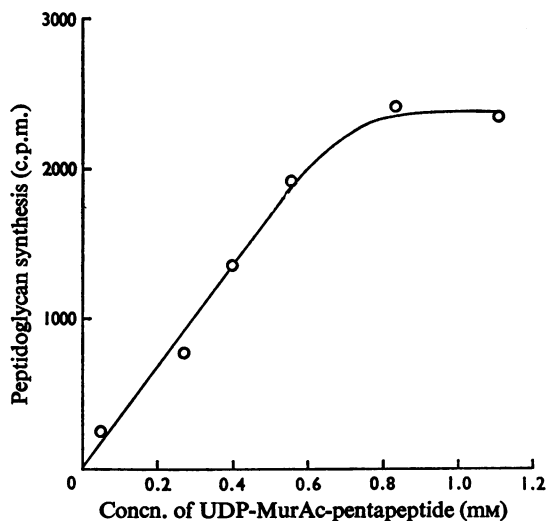


Fig. 5. Effect of concentration of UDP-MurAc-pentapeptide on peptidoglycan synthesis by an enzyme preparation from *B. licheniformis*

Incubation mixtures contained UDP-*N*-acetylglucosamine (0.25 μ mol), MgCl₂ (12 μ mol), 0.1 ml of enzyme suspension and UDP-MurAc-[³H]pentapeptide (1.5 $\times 10^6$ c.p.m./ μ mol) as indicated in a total volume of 0.2 ml. Reaction mixtures were incubated at 20°C for 1 h, and synthesis was determined by procedure A.

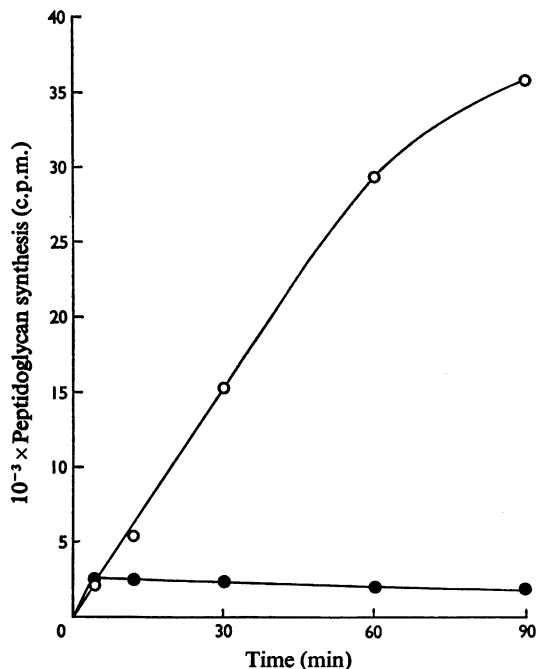


Fig. 6. Rate of formation of lipid intermediates and peptidoglycan by an enzyme preparation from *B. licheniformis*

Incubation mixtures contained UDP-*N*-[acetyl-¹⁴C]-acetylglucosamine (0.01 μ mol; 39.5 $\times 10^6$ c.p.m./ μ mol), UDP-MurAc-pentapeptide (0.1 μ mol), MgCl₂ (4 μ mol) and 0.1 ml of enzyme suspension in a total volume of 0.14 ml. Incubations were done at 20°C for the times indicated and then lipid and polymer were determined by procedure B. ●, Incorporation of ¹⁴C into lipid; ○, incorporation of ¹⁴C into polymer.

Glaser, 1964). In order to confirm the activity of our enzyme system, incubations were done with CDP-[¹⁴C]glycerol as the only substrate. Polymer was formed, which remained on the baseline of the paper chromatogram, and two radioactive products with higher chromatographic mobilities than glycerol were detected. The R_{glycerol} values of these two fast-running products, lipids I and II, were respectively 1.30 and 1.54 in solvent *B*, and 1.17 and 1.31 in solvent *C*. The rates of formation and 'turnover' of these products were examined by a pulse-labelling technique (Fig. 7). Synthesis of polymer showed the expected decrease in its rate after the addition of unlabelled substrate (Fig. 7*a*). However, labelling of lipid I reached a maximum value very rapidly (within 2 min) and then remained nearly constant throughout the incubation (Fig. 7*b*); these are the kinetics of formation usually displayed by a lipid intermediate in polymer synthesis. Moreover, on addition of unlabelled CDP-glycerol, the radioactivity of this product decreased rapidly to a value that was then maintained throughout the remainder of the incubation. These results were consistent with a continuous 'turnover' of lipid I, suggesting that it was indeed an intermediate in polymer synthesis.

Synthesis of the other labelled product (lipid II) was proportional to the time of incubation, and showed none of the characteristics of an intermediate (Fig. 7*c*).

Samples of lipid I were hydrolysed with 0.1 M-HCl in 50% (v/v) methanol for 10 min at 100°C and then subjected to chromatography in solvents *A* and *C*; a radioactive product that co-chromatographed with glycerol 1-phosphate was produced. Although the hydrolysis was not complete (60–80% hydrolysis), even partial hydrolysis of lipid I under these mild acidic conditions is consistent with it being a lipid intermediate of the undecaprenol phosphate type. Lipid II failed to hydrolyse under these conditions and its nature has not been determined. The radioactive polymeric product at the origin of the chromatogram was hydrolysed (1 M-HCl for 3 h at 100°C) and products were examined by chromatography in

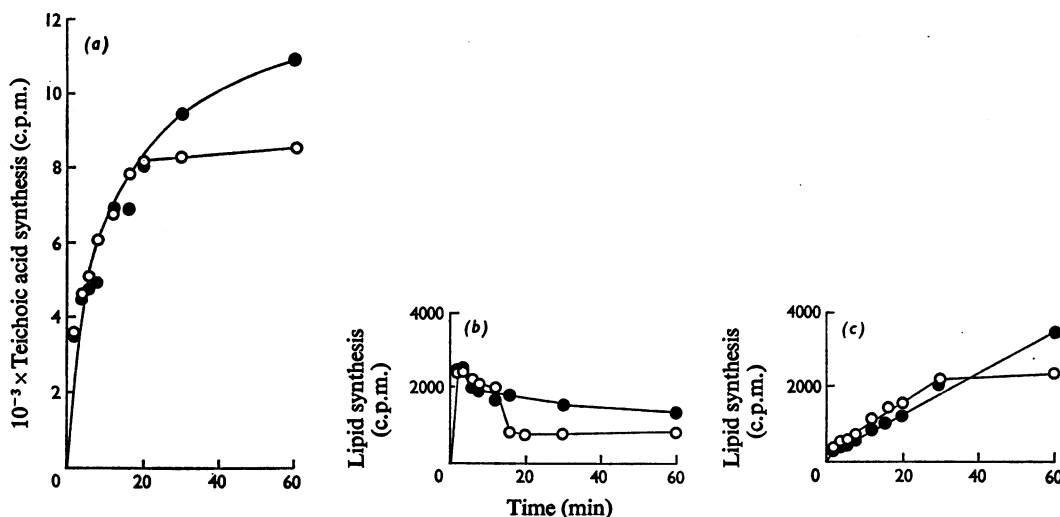


Fig. 7. Pulse-labelling of lipid intermediate and poly(glycerol phosphate)

Incubation mixtures contained CDP- $[^{14}\text{C}]$ glycerol ($0.1 \mu\text{mol}$; 4.44×10^6 c.p.m./ μmol), MgCl_2 ($8 \mu\text{mol}$) and enzyme suspension from *B. licheniformis* (0.18 ml) in a total volume of 0.2 ml . Incubations were done at 37°C . After 12.5 min unlabelled CDP-glycerol ($0.6 \mu\text{mol}$) was added to one incubation mixture and buffer to the control. Incubation was continued. Samples ($20 \mu\text{l}$) were removed at the times indicated and the formation of polymer and labelled lipids was determined by procedure B. (a) Polymer: ●, control; ○, CDP-glycerol added after 12.5 min . (b) Lipid I: ●, control; ○, CDP-glycerol added after 12.5 min . (c) Lipid II: ●, control; ○, CDP-glycerol added after 12.5 min .

solvent C; there were three radioactive products identified as glycerol, glycerol monophosphates ($R_{\text{glycerol}} 0.36$) and glycerol diphosphates ($R_{\text{glycerol}} 0.14$). These products are those expected from acid hydrolysis of a poly(glycerol phosphate) (Kelemen & Baddiley, 1961).

The synthesis of the wall teichoic acid, poly(glycerol phosphate glucose), from UDP-glucose and CDP-glycerol has been investigated by Burger & Glaser (1966) and by Hancock & Baddiley (1972); it has been shown by the latter workers that synthesis proceeds via lipid intermediates. Incubation conditions of temperature and Mg^{2+} ion concentration similar to those of Hancock & Baddiley (1972) were used in the present experiments and found to be satisfactory with our enzyme preparation.

Synthesis of peptidoglycan and teichoic acid by particulate enzyme preparations from *B. subtilis* N.C.T.C. 3610

The conditions used for the synthesis of peptidoglycan by particulate enzyme preparations from *B. subtilis* were the same as those used for the synthesis of this polymer by preparations from *B. licheniformis*. The system was again found to synthesize polymer and lipid intermediate(s); peptidoglycan was again

the major product accounting for 97% of the incorporated radioactivity. Digestion of the synthesized material with lysozyme gave soluble products that accounted for over 90% of the radioactivity of the polymer.

Synthesis of the glycerol teichoic acid of *B. subtilis*, by using CDP- $[^{14}\text{C}]$ glycerol, was examined as described above for *B. licheniformis*. Again material corresponding to a lipid intermediate was detected on chromatography of the incubation mixtures in suitable solvents, and this material was hydrolysed under controlled acid conditions to yield glycerol monophosphate. From these results it is concluded that the synthesis of poly(glycerol phosphate) proceeds through a lipid intermediate.

Effect of teichoic acid precursors on peptidoglycan synthesis

The results in Table 1 show that the synthesis of peptidoglycan by particulate enzyme preparations was markedly inhibited when teichoic acid precursors were present in the incubation mixtures.

CDP-glycerol and UDP-glucose at concentrations (1.6 mM and 0.8 mM respectively) that permit good synthesis of poly(glycerol phosphate glucose) and poly(glycerol phosphate) decreased the synthesis of

Table 1. *Inhibition of the synthesis of peptidoglycan by teichoic acid precursors and bacitracin*

Particulate enzyme suspension (60 μ l) was incubated with UDP-*N*-[acetyl- 14 C]acetylglucosamine (0.1 μ mol), UDP-MurAc-pentapeptide (0.1 μ mol) and MgCl₂ (7.5 μ mol or 1.2 μ mol). UDP-glucose (0.1 μ mol), CDP-glycerol (0.2 μ mol) and bacitracin (40 μ g/ml) were added as indicated. The total volume was 0.12 ml. Incubation was at 20°C or 37°C for 1 h. Synthesized polymer was determined by procedure A. Values in parentheses are the numbers of samples measured.

Expt. no.	Additives	Inhibition of peptidoglycan synthesis (%)	
		MgCl ₂ concn. and temperature optimum for peptidoglycan synthesis	MgCl ₂ concn. and temperature optimum for teichoic acid synthesis
A. Enzyme from <i>B. licheniformis</i>			
1	None (control)	0	0
2	UDP-glucose + CDP-glycerol	51.5 \pm 2.76 (20)	64.0 \pm 2.55 (14)
3	CDP-glycerol	45.0 \pm 2.36 (20)	53.0 \pm 1.88 (14)
4	UDP-glucose	16.0 \pm 2.15 (20)	23.5 \pm 2.12 (14)
5	Bacitracin	45.0 \pm 1.03 (6)	—
B. Enzyme from <i>B. subtilis</i>			
6	None	0	—
7	UDP-glucose + CDP-glycerol	47.5 \pm 2.16 (10)	—
8	CDP-glycerol	46.0 \pm 1.94 (10)	—
9	UDP-glucose	3.0 \pm 1.17 (10)	—

peptidoglycan by particulate enzyme preparations from *B. licheniformis* by 51.5% (Table 1, expt. 2). This was only a little greater than the inhibition (45%, Table 1, expt. 3) caused by CDP-glycerol alone, when only poly(glycerol phosphate) synthesis was possible. When the Mg²⁺ ion concentration and temperature of the incubation mixtures were altered to favour synthesis of teichoic acid, the inhibition was increased (Table 1, expts. 2 and 3). Similar results were obtained with enzyme preparations from *B. subtilis* (Table 1, expts. 7 and 8).

That this inhibition was specific, and not observed with other nucleotides unrelated to the synthesis of wall polymers, was indicated by the results of expts. 4 and 9 in Table 1. Inhibition of synthesis of peptidoglycan by UDP-glucose differed markedly, depending upon which enzyme preparation was used; with preparations from *B. licheniformis*, where UDP-glucose is an intermediate for the production of poly(glycerol phosphate glucose), inhibition was about 16% (expt. 4, Table 1), whereas with preparations from *B. subtilis* the inhibitory effect was only 3% (Table 1, expt. 9).

Bacitracin, an established inhibitor of the synthesis of peptidoglycan, inhibited the synthesis of the polymer by 45% (Table 1, expt. 5) when present at a concentration of 40 μ g/ml. This is very similar in degree to the inhibition caused by the teichoic acid precursors.

The amount of isotope in lipid intermediates for the synthesis of peptidoglycan decreased in the

presence of teichoic acid precursors. In incubation mixtures that contained UDP-glucose and CDP-glycerol the incorporation of label into lipid intermediate was decreased by 42%.

Effect of peptidoglycan precursors on teichoic acid synthesis

The synthesis of teichoic acids by particulate enzyme preparations from *B. licheniformis* was greatly inhibited by the peptidoglycan precursors, UDP-MurAc-pentapeptide and UDP-*N*-acetylglucosamine. Synthesis of poly(glycerol phosphate glucose) was inhibited by UDP-MurAc-pentapeptide and by a mixture of this nucleotide and UDP-*N*-acetylglucosamine to much the same extent, 52 and 45.5% inhibition respectively (Table 2, expts. 2 and 3). When the Mg²⁺ ion concentration and the temperature were altered to favour synthesis of peptidoglycan the inhibition was increased (66 and 60%; Table 2, expts. 2 and 3). The synthesis of poly(glycerol phosphate) was similarly affected, the amount of inhibition when conditions favoured the synthesis of peptidoglycan being 70 and 66% (Table 2, expts. 7 and 8).

It is interesting that the extent of inhibition of one biosynthetic pathway by the precursors of another was similar, irrespective of which pathway was being investigated, e.g. synthesis of poly(glycerol phosphate glucose) was decreased to 54.5% by a mixture of the two nucleotides for peptidoglycan synthesis, and synthesis of peptidoglycan was decreased to 48.5%

Table 2. *Inhibition of the synthesis of teichoic acid by peptidoglycan precursors*

Particulate enzyme suspension from *B. licheniformis* (60 μ l) was incubated with UDP-[14 C]glucose (0.1 μ mol), CDP-glycerol (0.2 μ mol) and MgCl₂ (1.2 or 7.5 μ mol) in expts. 1–5, or with CDP-[14 C]glycerol (0.1 μ mol) and MgCl₂ (7.5 μ mol) in expts. 6–8. UDP-MurAc-pentapeptide (0.1 μ mol), UDP-*N*-acetylglucosamine (0.1 μ mol) and bacitracin (40 mg/ml) were added as indicated. The total volume was 0.12 ml and incubation was for 1 h at 37°C or 20°C. Synthesized polymer was determined by procedure A.

Expt. no.	Additives	Inhibition of teichoic acid synthesis (%)	
		MgCl ₂ concn. and temperature optimum for teichoic acid synthesis	MgCl ₂ concn. and temperature optimum for peptidoglycan synthesis
A. Poly(glycerol phosphate glucose) synthesis			
1	None (Control)	0	0
2	UDP-MurAc-pentapeptide	52.0 \pm 3.33 (12)	66.0 \pm 2.80 (16)
3	UDP-MurAc-pentapeptide + UDP- <i>N</i> -acetylglucosamine	45.5 \pm 1.7 (12)	60.0 \pm 1.92 (16)
4	Bacitracin	—	4.0 \pm 1.14 (6)
5	Bacitracin + UDP-MurAc-pentapeptide + UDP- <i>N</i> -acetylglucosamine	—	76.5 \pm 1.66 (8)
B. Poly(glycerol phosphate) synthesis			
6	None	—	0
7	UDP-MurAc-pentapeptide	—	70.0 \pm 2.13 (10)
8	UDP-MurAc-pentapeptide + UDP- <i>N</i> -acetylglucosamine	—	66.0 \pm 1.74 (10)

by a mixture of UDP-glucose and CDP-glycerol and to 55% by CDP-glycerol alone. The incorporation of radioactive isotope into lipid intermediates for both poly(glycerol phosphate glucose) and poly(glycerol phosphate) was decreased in the presence of UDP-*N*-acetylglucosamine and UDP-MurAc-pentapeptide by 65 and 53% respectively when conditions of Mg²⁺ ion concentration and temperature favoured the synthesis of peptidoglycan.

Effect of bacitracin on the synthesis of teichoic acid

The synthesis of poly(glycerol phosphate glucose) by particulate enzyme preparations from *B. licheniformis* (when the Mg²⁺ ion concentration and the temperature were favourable to synthesis of peptidoglycan) was inhibited by a mixture of UDP-MurAc-pentapeptide and UDP-*N*-acetylglucosamine (60%; Table 2, expt. 3). When bacitracin was added this inhibition was increased (76.5%; Table 2, expt. 5). Bacitracin alone had very little effect (4% inhibition). Thus only under conditions when the synthesis of peptidoglycan occurred did bacitracin inhibit the synthesis of teichoic acid.

Factors affecting the synthesis of wall polymers in the presence of inhibitors

The synthesis of poly(glycerol phosphate glucose) by enzyme preparations from *B. licheniformis* was

affected by preincubation of the preparations with the inhibitory nucleotides and bacitracin before the addition of the teichoic acid precursors (Table 3). This preincubation increased the inhibition. Similarly, the inhibition of synthesis of peptidoglycan by teichoic acid precursors was increased slightly by preincubation with UDP-glucose and CDP-glycerol (Table 4). Although these enhanced inhibitory effects are not large compared with the effects produced without preincubation, they are nevertheless significant and are well outside the limits of experimental error.

If preincubation with inhibitory nucleotides enhances the inhibition, then it seemed possible that preincubation with nucleotides required for synthesis would decrease this inhibitory action. The results of such an experiment are given in Fig. 8, in which it is seen that the inhibitory action of CDP-glycerol and UDP-glucose on the synthesis of peptidoglycan was significantly decreased when the enzyme preparation had first been incubated with nucleotides for synthesis of peptidoglycan; an increase of 35% in synthesis of peptidoglycan was observed in the preincubated preparations.

Figs. 9, 10 and 11 show the effect on polymer synthesis of increasing the substrate and inhibitor concentration. When the substrate:inhibitor ratio was increased, synthesis of both peptidoglycan (Fig. 9) and poly(glycerol phosphate glucose) (Fig. 10) by

Table 3. *Effect of preincubation with peptidoglycan precursors on the synthesis of teichoic acid*

Particulate enzyme suspension from *B. licheniformis* (60 μ l) was incubated with UDP-[14 C]glucose (0.1 μ mol), CDP-glycerol (0.2 μ mol) and MgCl₂ (7.5 μ mol). UDP-MurAc-pentapeptide (0.1 μ mol), UDP-*N*-acetylglucosamine (0.1 μ mol) and bacitracin (40 μ g/ml) were added as indicated. The total volume was 0.12 ml. Preincubation with inhibitors was for 30 min at 20°C. Teichoic acid precursors were then added and the mixtures were further incubated for 1 h at 20°C. The synthesized polymer was determined by procedure A.

Expt. no.	Additive	Inhibition of teichoic acid synthesis (%)	
		With preincubation	Without preincubation
1	None	0	0
2	Bacitracin	—	4 \pm 1.14 (6)
3	UDP-MurAc-pentapeptide + UDP- <i>N</i> -acetylglucosamine	70.5 \pm 2.5 (4)	60.0 \pm 1.92 (16)
4	UDP-MurAc-pentapeptide + UDP- <i>N</i> -acetylglucosamine + bacitracin	83.0 \pm 4.0 (4)	76.5 \pm 1.66 (8)
5	UDP-MurAc-pentapeptide	—	66.0 \pm 2.80 (16)

Table 4. *Effect of preincubation with teichoic precursors on the synthesis of peptidoglycan*

Particulate enzyme suspension from *B. licheniformis* (60 μ l) was incubated with UDP-MurAc-pentapeptide (0.1 μ mol), UDP-*N*-[acetyl- 14 C]acetylglucosamine (0.1 μ mol) and MgCl₂ (7.5 μ mol). UDP-glucose (0.1 μ mol) and CDP-glycerol (0.2 μ mol) were added as indicated. The total volume was 0.12 ml. Preincubation was for 15 min at 20°C. Incubation after addition of peptidoglycan precursors was for 30 min at 20°C. Synthesized polymer was determined by procedure A.

Expt. no.	Additive	Inhibition of peptidoglycan synthesis (%)	
		With preincubation	Without preincubation
1	None	0	0
2	UDP-glucose + CDP-glycerol	59.0	53.5

particulate enzyme preparations from *B. licheniformis* increased, indicating alleviation of the inhibition. Decrease of this ratio for peptidoglycan synthesis caused increasing inhibition (Fig. 11).

Discussion

The experiments that make use of CDP-[14 C]-glycerol to examine the biosynthesis of poly(glycerol phosphate) in particulate membrane preparations from *B. licheniformis* and *B. subtilis* establish that these syntheses proceed through lipid intermediates. It is not known whether the resulting teichoic acid from *B. licheniformis* is located in the wall or the membrane. From this and the published work on lipid intermediates in the synthesis of teichoic acids it can now be stated that polyprenol phosphate participates in the synthesis of all known types of wall teichoic acid, i.e. polymers of polyol phosphates, sugar 1-phosphates and glycosylpolyol phosphates. It appears that for many bacteria, all of the major wall components are synthesized in this manner, with

polyprenol phosphate as a common component of the system. The present study has also shown that the biosynthetic routes do not operate independently but are closely interrelated. In the absence of knowledge of the precise location of the poly(glycerol phosphate) synthesized by *B. licheniformis* membrane preparations, it is not yet possible to state whether membrane teichoic acid is synthesized through lipid intermediates.

Watkinson *et al.* (1971) showed that in *S. lactis* I3 the polyprenol phosphate that participates in the synthesis of peptidoglycan in the wall also participates in the synthesis of the wall teichoic acid, and that the same lipid phosphate molecules are shared by both systems. The present study extends these conclusions to *B. licheniformis*, where the inhibitory action of bacitracin on the synthesis of teichoic acid only in the presence of precursors for the synthesis of peptidoglycan is interpreted in the same manner as for the staphylococcus, i.e. as an indirect effect due to the depletion of common undecaprenol phosphate resulting from the

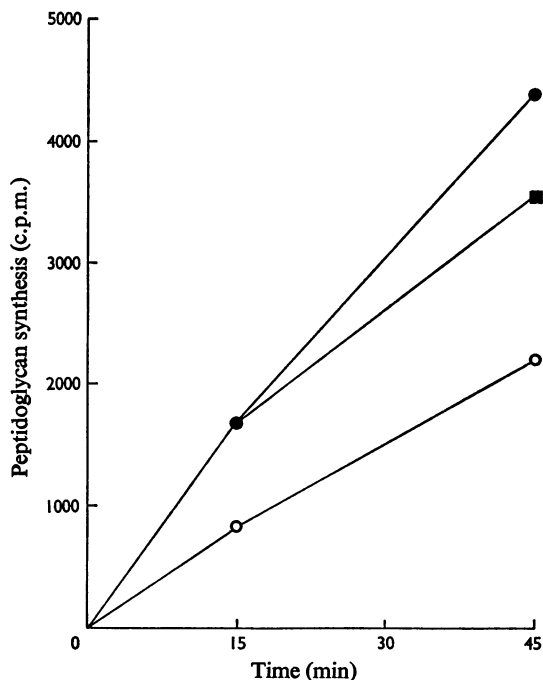


Fig. 8. Effect of preincubation with peptidoglycan precursors on the inhibition of peptidoglycan synthesis by UDP-glucose and CDP-glycerol

Incubation mixtures contained UDP-*N*-[acetyl-¹⁴C]-acetylglucosamine (0.1 μmol), UDP-MurAc-pentapeptide (0.1 μmol), MgCl₂ (7.5 μmol) and enzyme suspension from *B. licheniformis* (50 μl). UDP-glucose (0.1 μmol) and CDP-glycerol (0.2 μmol) were added as indicated at zero time or after 15 min at 20°C. The total volume was 0.12 ml. The mixtures were incubated at 20°C for 45 min. Peptidoglycan synthesis was determined by procedure A. ●, Control without UDP-glucose and CDP-glycerol; ○, +UDP-glucose and CDP-glycerol from zero time; ■, +UDP-glucose and CDP-glycerol after 15 min.

blockage of the synthetic cycle for peptidoglycan and consequent accumulation of the lipid pyrophosphate in this blocked cycle. Although the effect of bacitracin on the synthesis of poly(glycerol phosphate) by *B. licheniformis*, and on the synthesis of the glucosylated poly(glycerol phosphate) by *B. subtilis*, was not investigated the inhibition of the synthesis of these polymers by peptidoglycan precursor nucleotides indicates that undecaprenol phosphate is shared in the synthesis of both of these polymers and of peptidoglycan. The synthesis of teichoic acids and of peptidoglycan through common undecaprenol phosphate is outlined for *B. licheniformis* in Scheme 1.

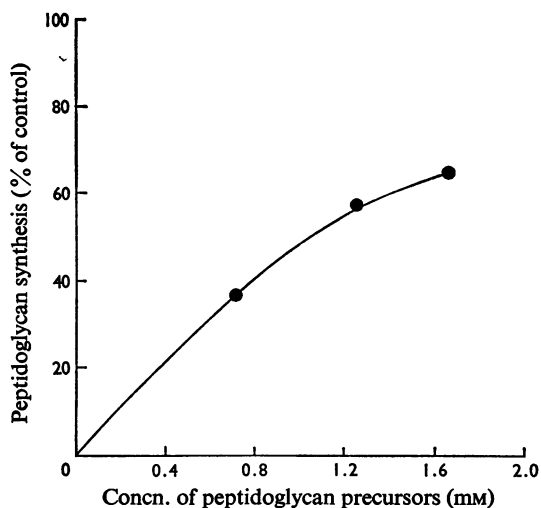


Fig. 9. Effect of increasing the concentration of peptidoglycan precursors on the synthesis of peptidoglycan in the presence of teichoic acid precursors

Incubation mixtures contained UDP-glucose (0.2 μmol), CDP-glycerol (0.2 μmol), MgCl₂ (7.5 μmol), enzyme suspension from *B. licheniformis* (60 μl) and UDP-*N*-[acetyl-¹⁴C]acetylglucosamine (2.47 × 10⁶ c.p.m./μmol) and UDP-MurAc-pentapeptide as indicated, in a total volume of 0.12 ml. Reaction mixtures were incubated at 20°C for 1 h. Peptidoglycan was determined by procedure A. Control mixtures did not contain UDP-glucose and CDP-glycerol.

The polyprenol involved in peptidoglycan synthesis in *S. aureus* and *Micrococcus lysodeikticus* has been shown to be undecaprenol (Higashi *et al.*, 1967, 1970b). If it can be assumed from this that undecaprenol participates in the biosynthesis of peptidoglycan in all micro-organisms we can conclude that the polyprenol in teichoic acid biosynthesis is also undecaprenol.

The participation of a common isoprenoid lipid phosphate in the synthesis of all the major wall components provides a possible control for the rate of wall synthesis by an organism. If this lipid is in limited supply, then the rate of wall production would depend upon the rate of supply of lipid. The composition of the wall would then depend upon the availability of the nucleotide precursors for the respective wall polymers and their affinities for the enzymes, as well as upon the relative affinities of the different enzymes for the lipid phosphate.

The inhibition of synthesis of teichoic acid by the precursors of peptidoglycan occurs because there is competition for a common intermediate (lipid

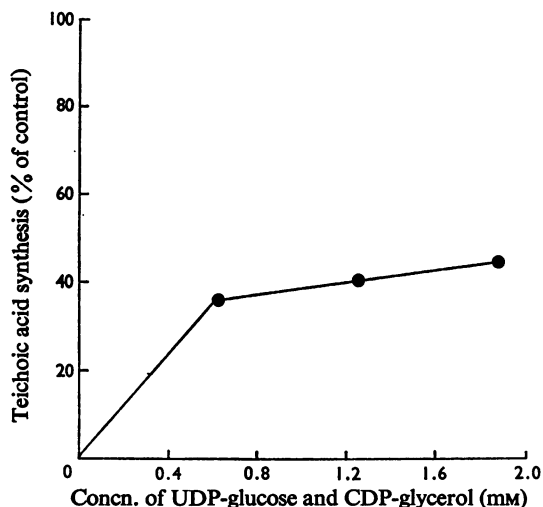


Fig. 10. Effect of increasing the concentration of teichoic acid precursors on the synthesis of teichoic acid in the presence of peptidoglycan precursors

Incubation mixtures contained UDP-*N*-acetylglucosamine (0.1 μ mol), UDP-MurAc-pentapeptide (0.1 μ mol), MgCl₂ (7.5 μ mol), enzyme suspension from *B. licheniformis* (60 μ l) and UDP-[¹⁴C]glucose and CDP-glycerol as indicated in a total volume of 0.12 ml. Incubation was for 1 h at 20°C. Teichoic acid was determined by procedure A. Control mixtures did not contain UDP-*N*-acetylglucosamine and UDP-MurAc-pentapeptide.

phosphate) that is in limited supply. If UDP-*N*-acetylglucosamine is omitted then synthesis of peptidoglycan cannot proceed; lipid phosphate can still be used to form *N*-acetylmuramyl-pentapeptide undecaprenol pyrophosphate but cannot be recycled. Hence lipid phosphate is removed and no longer becomes available for synthesis of teichoic acid. A similar situation occurs if bacitracin is added to the system that is synthesizing both polymers, lipid phosphate being 'trapped' as lipid pyrophosphate. Under these conditions increased inhibition of the synthesis of teichoic acid would be expected. If lipid phosphate is returned to the common pool after each turn of the teichoic acid cycle and becomes available to both polymer systems, then most, if not all, of the lipid phosphate should be 'trapped' in the peptidoglycan system after omission of UDP-*N*-acetylglucosamine or addition of bacitracin. However, the results suggest that lipid phosphate is not returned to the pool after each turn of each cycle. Omission of UDP-*N*-acetylglucosamine or addition of bacitracin increased the inhibition of teichoic acid synthesis but not greatly (by 6% and 16.5% respectively). Pre-

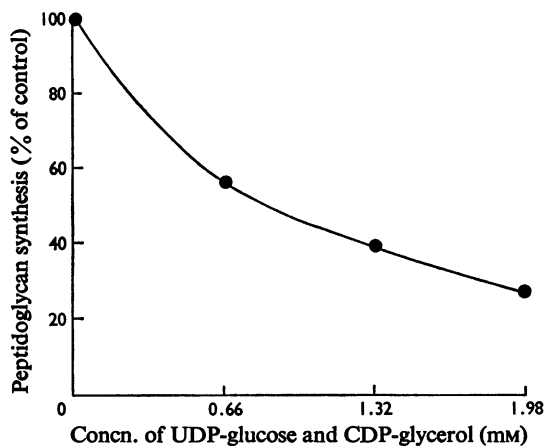
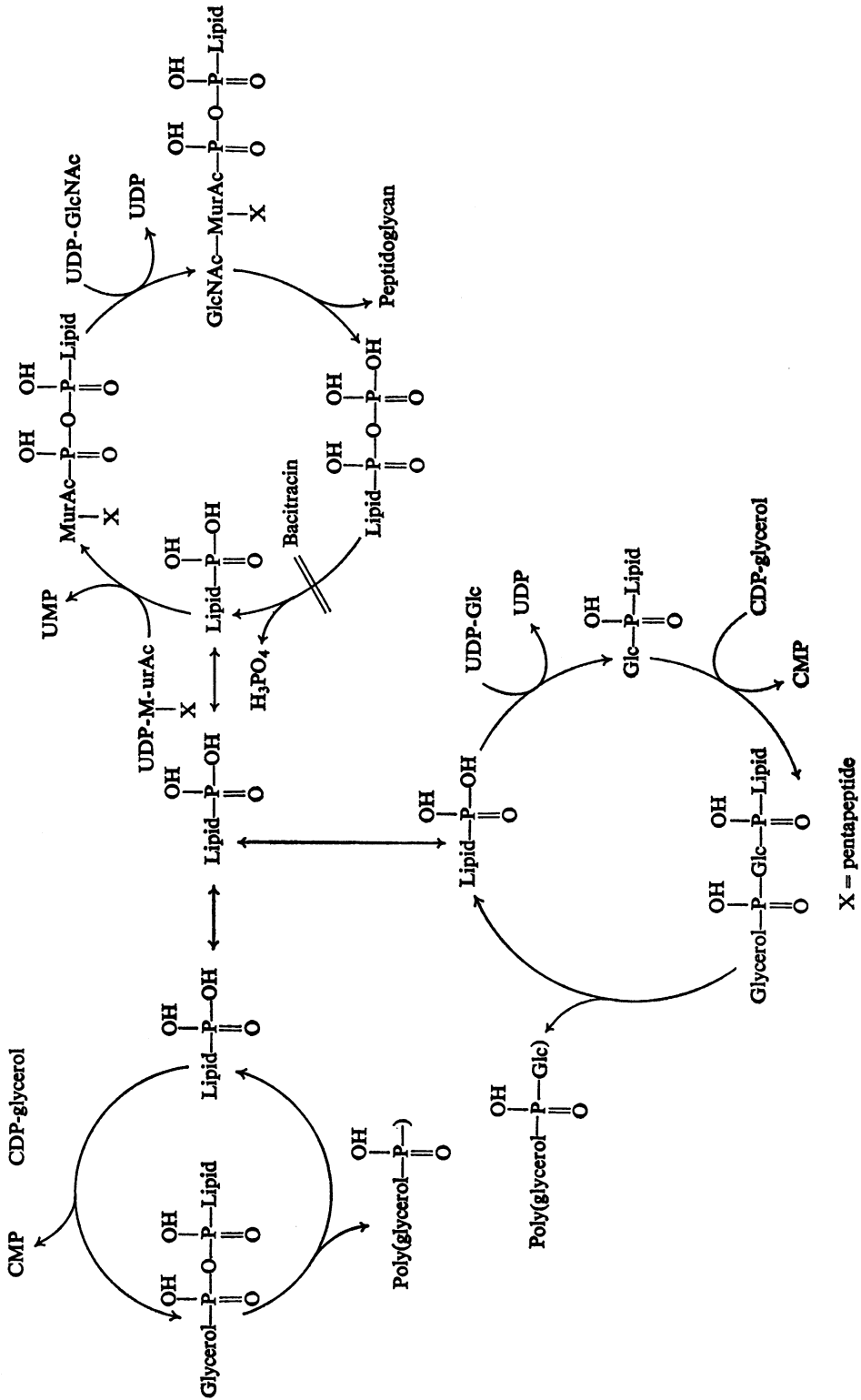


Fig. 11. Effect of increasing the concentration of teichoic acid precursors on the synthesis of peptidoglycan

Incubation mixtures contained UDP-*N*-[acetyl-¹⁴C]-acetylglucosamine (0.1 μ mol), UDP-MurAc-pentapeptide (0.1 μ mol), MgCl₂ (7.5 μ mol), enzyme suspension from *B. licheniformis* (60 μ l) and UDP-glucose and CDP-glycerol as indicated in a final volume of 0.12 ml. Incubation was for 1 h at 20°C. Peptidoglycan was determined by procedure A.

incubation of particulate enzyme preparations with substrates or inhibitors decreased or increased respectively the inhibition observed. This would not be expected if the lipid phosphate is returned to the pool after each turn of each cycle. We conclude therefore that the undecaprenol phosphate is not returned immediately to a common pool after the completion of each synthetic cycle, but that its return is restricted and sharing between the several routes is thereby under a restraint. The following proposals for a model of the process of synthesis of wall polymers by the cytoplasmic membrane are consistent with the above observations.

The enzymes for wall synthesis exist in the cytoplasmic membrane in an ordered manner. They are assembled as 'wall synthesizing units', each unit comprising a multi-enzyme complex for the synthesis of each wall polymer. The multi-enzyme complexes must be close to each other and must have a limited amount, perhaps only one molecule, of undecaprenol phosphate located between them in such a manner that it can become available to each. In the presence of its nucleotide substrate(s), the complex for the synthesis of a particular polymer is able to bind the undecaprenol phosphate, and the production of that polymer proceeds. The undecaprenol phosphate, however, remains bound to that complex until the



Scheme 1. Biosynthesis of teichoic acids and peptidoglycan in *Bacillus licheniformis*

synthesis of a molecule of polymer has been completed. Only then does the lipid phosphate dissociate from the enzyme complex and thereby become available for use by one of the other enzyme complexes in the unit. Such an organized system would prevent the formation of only partly completed polymers that might confer undesirable properties upon the newly forming wall. It is interesting that V. M. Reusch & F. C. Neuhaus (personal communication) have found that the enzymic introduction of D-alanine ester residues on to membrane teichoic acid in *Lactobacillus casei* A.T.C.C. 7469 requires a ligase that is effective only with teichoic acid that is already bound in the membrane and is ineffective with isolated teichoic acid. These authors suggest that the polymer attached to the membrane may be in a highly orientated state, and that the ligase operates only under such conditions. This explanation would agree well with our view that synthesis of the polymers occurs in a spatially organized manner.

Although the large effects caused by inhibitory nucleotides, the action of bacitracin and the preincubation studies are not easily explained on the basis of simple enzyme kinetics or allosteric properties of the component enzymes, it is nevertheless possible that allosteric effects operate within the proposed units. Thus the choice of which enzyme complex will combine with the undecaprenol phosphate and therefore which polymer is synthesized on a given occasion will depend upon the nucleotides present, as well as on possible allosteric activation of the process of lipid-enzyme combination and allosteric inhibition at various stages by nucleotide precursors of the other syntheses.

There has been a growing belief that the function of undecaprenol phosphate in biosynthesis is concerned in some unexplained manner with vectorial transportation of monomer units inside the membrane to polymer chains on the outside, a process that could include movement through a lipid phase. Such a function for the undecaprenol phosphate is unlikely, especially as it is possible that lipid molecules associated with proteins in a membrane are mainly in a rather rigid orientated state and freedom of movement is severely restricted. Vectorial transportation across a membrane would require considerable movement of the lipid intermediates, and seems unlikely with molecules that have their general shape and charge. The function of undecaprenol phosphate is, however, readily understood in terms of our model. It controls the synthesis of wall components with respect to their precise structure as regularly repeating polymers, their relative amounts and their location, as well as ensuring the accurate completion of each chain; it thus determines that the wall is produced in an ordered manner. The occurrence of isoprenoid alcohol phosphokinase and of an enzyme that dephosphorylates C₅₅-ficaprenol phos-

phate in *S. aureus* has been reported by Higashi *et al.* (1970a). It was suggested by these authors that these enzymes might be involved in controlling the concentration of polyprenol phosphate, which in turn might regulate the amount of wall synthesis. This is supported in the present work, where in the cell-free system the availability of undecaprenol phosphate is a rate-controlling factor in the synthesis of wall polymers.

The relative rigidity of the organization of enzymes in wall synthesis discussed here, and the present suggestions regarding the function of lipid phosphate, would favour a mechanism for the synthesis of wall polymers in which considerable vectorial movement of intermediates does not occur. It is possible then that the multi-enzyme systems, and their undecaprenol phosphate, operate from the inner surface of the membrane, and newly synthesized polymer chains are extruded through the membrane. Such a mechanism would require little movement of lipids, the main motion being that of polymer chains. The chemical and physical properties of undecaprenol phosphate are well suited to the requirement that it should be correctly aligned in the bilayer structure of the membrane and thereby participate in reactions with the two or more multi-enzyme complexes.

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