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# The Direction of Glycan Synthesis in a Bacterial Peptidoglycan

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A cell-free membrane preparation from a poorly lytic mutant of *Bacillus licheniformis* was used to synthesize radioactive peptidoglycan. The product was apparently un-cross-linked. When UDP-*N*-acetyl[$^{14}$C]glucosamine was used and the final peptidoglycan subjected to Smith degradation, no radioactive glycerol was found. On the other hand, when peptidoglycan labelled with *meso*-diamino[$^{14}$C]pimelic acid was first hydrolysed in 0.1M-HCl at 60°C for 2h and then subjected to alkaline conditions, radioactive lactyl-peptides were eliminated. The proportion of radioactive lactyl-peptide decreased with increasing time of incorporation. It is concluded that the glycan chains grow by extension at their reducing ends while remaining attached by some linkage labile to mild acid, such as a glycosyl link to undecaprenol pyrophosphate.

The sequence of reactions involved in the biosynthesis of the peptidoglycan found in the cell walls of bacteria is now well established. The involvement of a lipid intermediate was first observed by Anderson *et al.* (1965), Struve & Neuhaus (1965) and Struve *et al.* (1966), and the lipid portion was characterized as a polyisoprenyl phosphate by Higashi *et al.* (1967) at the same time as a similar compound was implicated in the biosynthesis of the lipopolysaccharide of Gram-negative bacteria (Wright *et al.*, 1965, 1967). In the former reactions *N*-acetylmuramyl(pentapeptide) 1-phosphate was transferred from UMP to undecaprenyl phosphate, with formation of a pyrophosphate bond, and $\beta$-*N*-acetylglucosamine was then added at the 4-position of the muramic acid to yield a disaccharide(pentapeptide)–pyrophosphate–undecaprenol. After addition of cross-bridging amino acids the lipid-bound disaccharide was transferred to the acceptor present in the cell-free preparation used (Matsuhashi *et al.*, 1967). The undecaprenyl pyrophosphate released lost the terminal phosphate residue it had acquired in the biosynthetic process and was then available for reuse (Siewert & Strominger, 1967).

In the partially analogous synthesis of the polysaccharide portion of lipopolysaccharide, it was found that the chains, consisting of multiple trisaccharide repeating units, grew by addition of these units at the reducing terminal of the lengthening chain; in other words, the reducing terminal of the growing chain was transferred from its phosphate link with undecaprenol to the non-reducing terminal of the new trisaccharide unit, which was itself linked to another molecule of lipid. Thus the chain grew at its reducing end and yet remained linked to lipid (Bray & Robbins, 1967; Robbins *et al.*, 1967). The direction of growth of the glycan chains in peptidoglycan synthesis was unknown, but the results below suggest that, in *Bacillus licheniformis* at least, the mode of growth resembles that in the biosynthesis of lipopolysaccharide.

## Materials and Methods

### *Organism*

The strain used was a penicillinase-negative mutant obtained from one of the poorly lytic mutants, Lyt-3, previously derived from *B. licheniformis* 6346 (Forsberg & Rogers, 1971).

### *Media and cultural conditions*

The bacilli were maintained as spores in water at 4°C. Cultures (50ml) were grown overnight in a casein-hydrolysate medium supplemented with inorganic salts (Janczura *et al.*, 1961) and used to inoculate batches (3 litres) of the same medium supplemented with Bacto-tryptone (0.5%), Bacto-yeast extract (0.5%) (Difco Labs.) and glucose (0.2%). Cultures were harvested in mid-exponential phase (after incubation for 2.5–3.5h at 35°C with shaking) and the cells were washed once with 50mM-Tris–HCl buffer, pH7.3, containing 30mM-$MgCl_2$ and 1mM-dithiothreitol.

### *Preparation of enzyme*

The method was based on that of Anderson *et al.* (1965). Washed cells were ground in a pre-cooled mortar at 2°C with three times their wet weight of levigated alumina (Norton, Welwyn Garden City, Herts., U.K.). The cells–alumina mixture (1g) was suspended in 4ml of the Tris–$MgCl_2$–dithiothreitol buffer and centrifuged at 3000*g* for 5min. The pellet of alumina and unbroken cells was discarded and the supernatant was centrifuged at 12000*g* for 5min. The supernatant from this step was then centrifuged

at 40000*g* for 30min and the sedimented material was washed twice with buffer and dispersed in the buffer to a final protein concentration of approx. 10mg/ml. This suspension was used as enzyme in the subsequent experiments.

*Precursors of peptidoglycan*

The precursors were UDP-*N*-acetylglucosamine [from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.], labelled where appropriate by the addition of UDP-*N*-acetyl[U-$^{14}$C]-glucosamine (The Radiochemical Centre, Amersham, Bucks., U.K.), and UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine, which was labelled with radioactivity in the diaminopimelic acid and is referred to as UDP-*N*-acetylmuramyl-pentapeptide. The latter compounds were prepared enzymically by addition of radioactive diaminopimelic acid and D-alanyl-D-alanine to UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid isolated from lysine-deprived *Staphylococcus aureus* (Strominger & Threnn, 1959).

Exponential-phase cells of *B. licheniformis* 6346 grown in the medium described above were harvested and washed with 50mM-potassium phosphate buffer, pH7.8, containing 0.1mM-EDTA. The washed cells were disrupted in a French pressure cell and debris was removed from the broken cell suspension by centrifuging at 40000*g* for 30min. The supernatant was fractionated by precipitation with $(NH_4)_2SO_4$. Protein soluble at 50% saturation but precipitated at 70% saturation of $(NH_4)_2SO_4$ was dissolved in 25mM-Tris–HCl buffer, pH8.0, containing 0.1mM-EDTA and 0.1mM-$\beta$-mercaptoethanol and dialysed overnight at 4°C against several changes of the same buffer. The dialysed material was further fractionated in two batches on a column (2.5cm × 35cm) of DEAE-cellulose equilibrated with 25mM-Tris–HCl buffer, pH8.0, containing 0.1mM-$\beta$-mercaptoethanol and eluted with a linear gradient of 0–0.4M-KCl. The appropriate fractions were pooled and protein was concentrated by addition of $(NH_4)_2SO_4$ to 80% saturation. The precipitated protein was finally dissolved in 25mM-Tris–HCl buffer, pH8.0, containing 0.1mM-$\beta$-mercaptoethanol (J. B. Ward, unpublished work).

The reaction mixture contained, in a total volume of 3.0ml, 125$\mu$mol of Tris–HCl buffer, pH8.9, 25$\mu$mol of $MgCl_2$, 25$\mu$mol of KCl, 10$\mu$mol of ATP, 4.8$\mu$mol of D-alanyl-D-alanine, 2$\mu$mol of UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid, 1$\mu$mol of diamino[1-(7)-$^{14}$C]pimelic acid (11.2mCi/mmol) (Calbiochem Ltd., London W.1, U.K.) and 2.0ml (13.8mg) of the enzyme preparation from *B. licheniformis* described above. After incubation at 37°C for 2h protein was precipitated with trichloroacetic acid, 5% (w/v) final concn., and removed by centrifuging. After removal of trichloroacetic acid by ether extraction the nucleotides were fractionated on a column (1.5cm × 25cm) of Dowex 1 (X2; $Cl^-$ form; 200–400 mesh) equilibrated with 10mM-HCl. Nucleotides were eluted with a linear gradient of 0–0.45M-NaCl (800ml). Fractions containing labelled nucleotide were pooled and salt was removed by filtration on a column (1.8cm × 50cm) of Sephadex G-25 eluted with water. The fractions containing nucleotide were pooled and concentrated. On average the amount of *meso*-diamino[$^{14}$C]pimelic acid recovered in the final product was 70–80% of the theoretical yield.

The preparation of *meso*-[$^3$H]diaminopimelic acid-labelled nucleotide was carried out essentially as described above by using *meso*-[G-$^3$H]diaminopimelic acid (300mCi/mmol; The Radiochemical Centre).

*Biosynthetic system for peptidoglycan*

A typical reaction mixture for synthesis of peptidoglycan contained 2mM-UDP-*N*-acetylglucosamine, 0.4mM-UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[$^{14}$C]pimelic acid (11.2mCi/mmol) and enzyme suspension (25$\mu$l) in a total volume of 50$\mu$l, or 0.4mM-UDP-*N*-acetyl[$^{14}$C]glucosamine (10mCi/mmol), 2mM-UDP-*N*-acetylmuramyl-pentapeptide and enzyme suspension as above. Reaction mixtures were incubated at 24°C for the various times indicated and the reaction was terminated by the addition of 1ml of ice-cold 0.3M-$HClO_4$. The precipitated enzyme containing synthesized polymer was washed with 3 × 2ml of 0.3M-$HClO_4$ and the supernatants were discarded. In early experiments the residue was then washed twice with 1ml of 0.3M-$HClO_4$ containing unlabelled precursor (2mM). This procedure was subsequently discontinued when it was found that no further radioactivity was removed from the precipitated sample. The residue was then resuspended in 0.5ml of water and extracted with an equal volume of butan-1-ol–6M-pyridinium acetate, pH4.2 (2:1, v/v), to remove lipid intermediate (Anderson *et al.*, 1967). After mixing, the solvent phase was removed and the aqueous phase re-extracted with water-saturated butan-1-ol (0.5ml). The residue was recovered from the aqueous phase by centrifuging and investigated further as described below.

In experiments in which the initial incubation with UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[$^{14}$C]pimelic acid was followed by incubation with UDP-*N*-acetylmuramyl-pentapeptide labelled with [$^3$H]diaminopimelic acid, the procedure was as follows. Enzyme (0.05ml) was incubated at 24°C with UDP-*N*-acetylglucosamine (0.2$\mu$mol) and UDP-*N*-acetylmuramyl-[$^{14}$C]pentapeptide (0.023$\mu$mol, 6.9mCi/mmol) for 5min, after which time UDP-*N*-acetylmuramyl-[$^3$H]pentapeptide (0.184$\mu$mol, 55

mCi/mmol) was added. The reaction was allowed to proceed for the times indicated and the polymerized material was purified as described above, except that the extraction with butan-1-ol–6M-pyridinium acetate to remove lipid intermediate was omitted.

*Smith degradation of the glycan chain*

The non-reducing terminal *N*-acetylglucosamine of the peptidoglycan was degraded as follows. After biosynthesis and washing of the product as described above, the sample was treated with 25mM-$NaIO_4$ (0.5ml), pH4, and left at 2°C in the dark for 6h. The pH value remained unchanged and excess of periodate was shown to be present at the end of the oxidation period. Remaining periodate was destroyed by addition of glycol, an excess of $NaBH_4$ was added, and the mixture was left at 2°C overnight. Excess of $NaBH_4$ was destroyed and the mixture adjusted to pH1 by the addition of dil. HCl and stored at room temperature overnight. By this procedure C-4–C-6 of non-reducing *N*-acetylglucosamine would be converted into glycerol. Carrier glycerol (1$\mu$mol) was added and $Na^+$ ions were removed on a small column of Zeo-Karb 225 ($H^+$ form). Then boric acid was removed by repeated drying with methanol under vacuum. Finally the sample was transferred to Whatman no. 1 paper for chromatography overnight in ethyl acetate–pyridine–water (5:2:7, by vol., upper phase) along with markers of glycerol. The area corresponding to the glycerol markers was cut out, eluted and the radioactivity determined by counting in a Packard liquid-scintillation spectrometer with a dioxan-based scintillation fluid [dioxan containing 1% 2,5-diphenyloxazole, 0.06% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 10% naphthalene]. The recovery of carrier glycerol was measured enzymically as described by Ward (1973) and was on average 70–80%.

*Degradation of the reducing terminal N-acetylmuramyl peptide unit*

The reducing terminal was first released from any possible pyrophosphate linkage to undecaprenol by treatment with 0.1M-HCl at 60°C. The period used as a routine was 2h (see the Results section). After this hydrolysis the sample was frozen to −80°C and water and acid were removed under vacuum over NaOH pellets. The sample was then treated with 4M-$NH_3$ and kept in a stoppered vessel at 37°C for 6h. It was then transferred as a 2cm streak to the origin of a chromatogram on Whatman no. 3 paper, which had been previously washed by irrigation with 1M-ammonium acetate and then exhaustively with water. The chromatogram was developed by the descending method with freshly prepared isobutyric acid–0.5M-$NH_3$ (5:3, v/v) for 24h. After thorough drying at about 50°C the paper was either subjected to radioautography, or 1cm strips were cut out and placed in scintillation vials for measurement of radioactivity by using 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 origin regions that contained the sample and polymeric peptidoglycan were first moistened with a lysozyme solution (1mg/ml) and incubated at 37°C for 4h. They were then dried and treated like the other samples. This precaution ensured that the radioactivity was distributed throughout the paper as with the lower-molecular-weight compounds, and the counting efficiency was therefore comparable (J. B. Ward, unpublished work).

Lactyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine, hereafter referred to as lactyl-pentapeptide, and the corresponding compounds lacking one or both the terminal D-alanine residues were used as marker compounds, the positions of which were detected with ninhydrin. In a typical experiment the lactyl-peptide markers move as follows: pentapeptide, 15cm; tetrapeptide, 14cm; mono-amidated tetrapeptide, 19.5cm; tripeptide, 12.4cm; mono-amidated tripeptide, 17.3cm. However, as frequently observed with this solvent system, the absolute, but not the relative, mobilities showed considerable variation.

The lactyl-peptides were further characterized by elution from the chromatogram and subsequent paper electrophoresis at pH6.5 (pyridine–acetic acid–water, 25:1:474, by vol.; 80V/cm, 20min) and comparison with authentic markers (see below). Under these conditions the non-amidated markers moved 18–22cm towards the anode.

*Source of marker compounds*

UDP-*N*-acetylmuramyl-pentapeptide was prepared from *B. licheniformis* by accumulation in a medium lacking bivalent cations (Garrett, 1969), and converted into *N*-acetylmuramyl-pentapeptide by brief acid hydrolysis (0.1M-HCl, 100°C, 5min). The corresponding lactyl-pentapeptide was then prepared by incubation in 4M-$NH_3$ at 37°C for 6h (Tipper, 1968). For preparation of the lactyl-tetrapeptide the lactyl-pentapeptide (0.4$\mu$mol) was dissolved in 10$\mu$l of water and to this solution were added 0.1M-sodium phosphate buffer, pH8 (1$\mu$l), and the concentrated enzyme from *Streptomyces* strain R39 (1$\mu$l) (Leyh-Bouille *et al.*, 1972). The mixture was incubated at 37°C for 4h, by which time all the *C*-terminal D-alanine had been removed. The lactyl-tripeptide was prepared from UDP-*N*-acetylmuramyl-tripeptide by the method used for the pentapeptide. Samples of amidated lactyl-tri- and tetra-peptide were prepared by $NH_3$ treatment as

above from a mixture of *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanine and the same compound without the terminal D-alanine obtained from the cell walls of *B. licheniformis* Lyt-3 after digestion with *Streptomyces* muramidase (Ward & Perkins, 1968). This produced a mixture of lactyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelic acid, with or without one amidated carboxyl group (major components) and lactyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanine, also with or without one amidated carboxyl group (minor components). The chromatographic behaviour of these components is listed above.

## Results

A membrane preparation that would biosynthesize peptidoglycan, i.e. incorporate radioactively labelled *N*-acetylmuramyl-pentapeptide from its UDP-activated precursor into material that remained on the origin of a chromatogram, provided that UDP-*N*-acetylglucosamine was also present, was isolated from exponential-phase cells of a lytic-negative mutant of *B. licheniformis*. Incorporation into immobile material was entirely prevented by the presence of lysozyme; this has usually been considered a good criterion of peptidoglycan synthesis. On the other hand, incorporation was unaffected by benzylpenicillin (500$\mu$g/ml), which was taken as an indication that the polymeric material produced was mostly un-cross-linked.

The direction of glycan synthesis was studied as follows. If disaccharide units consisting of *N*-acetylglucosaminyl-$\beta$-(1$\rightarrow$4)-*N*-acetylmuramyl-pentapeptide were added at the non-reducing terminal of preformed acceptor chains, then at any stage of biosynthesis the non-reducing terminal sugar would be a newly added glucosamine residue. Hence when biosynthesis was conducted with all the components unlabelled except for radioactive *N*-acetyl[$^{14}$C]glucosamine, the terminal residues should be radioactive. To demonstrate whether or not this was so the product was subjected to Smith degradation with periodate, followed by $NaBH_4$ and mild acid, under which conditions the non-reducing terminal glucosamine residue would yield, from C-4–C-6, glycerol, which would be radioactive like the sugar from whence it came. None of the other *N*-acetylhexosamine residues in the $\beta$-(1$\rightarrow$4)-linked polymer would be attacked by periodate. This particular mutant of *B. licheniformis* does not produce a teichuronic acid (Forsberg *et al.*, 1973) and hence does not contain hexosamine in any polymer other than peptidoglycan.

The experimental procedure for detecting radioactive glycerol was checked in two ways. First, radioactively labelled UDP-*N*-acetylglucosamine was treated by the same method, and 40–50% of the total radioactivity was recovered in chromatographically isolated glycerol (almost the theoretical yield). Secondly, non-radioactive peptidoglycan preparations from the same organism were subjected to the same procedure, except that $NaB^3H_4$ was used. Under these conditions the expected quantity of [$^3$H]glycerol was recovered on the final chromatogram (Ward, 1973).

The same procedure was applied to preparations in which peptidoglycan had been synthesized from UDP-*N*-acetyl[$^{14}$C]glucosamine. Incubation was continued for 15 and 30min and samples containing 8000 and 13000d.p.m. were obtained. After Smith degradation, less than 0.1% of the radioactivity was found in the chromatographic position of glycerol, far less than could conceivably have occurred if growth of the glycan chain took place at the non-reducing terminal. The validity of the procedure was also checked by using preparations from which the lipid intermediate had not been removed. In that case, one would expect that the glucosamine still present as lipid intermediate would yield radioactive glycerol. The proportion of total radioactivity found as glycerol after Smith degradation of samples incubated for 15 and 30min was 8.3 and 5.5%, corresponding to 16.6 and 11.0% of the glucosamine at non-reducing terminals if conversion into glycerol had been quantitative. Hence one can conclude that the absence of radioactive glycerol from the degradation products of purified peptidoglycan was significant. The almost complete lack of labelled glycerol implies that in the membrane preparation as isolated, sites for peptidoglycan synthesis were already primed before radioactive precursors were added. Thus the reducing ends of all the new chains were non-radioactive.

Since glycan chain elongation appeared not to occur at the non-reducing end of the growing chains, we sought a method for studying growth at the reducing end. For this purpose we used the fact that in alkaline conditions *N*-acetylmuramyl(peptide) with a free reducing group will undergo $\beta$-elimination of the lactyl-peptide side chain (Perkins, 1967; Ghuysen *et al.*, 1967; Tipper, 1968). Thus if *N*-acetylglucosaminyl-$\beta$-(1$\rightarrow$4)-*N*-acetylmuramyl-[$^{14}$C]pentapeptide were added at the reducing terminal, then provided the reducing group were free $\beta$-elimination should give rise to radioactive lactyl-pentapeptide. Another feature of this method of biosynthesis is that the growing glycan chain would remain attached by its reducing group to undecaprenol pyrophosphate. We therefore studied elimination of lactyl-peptides before and after acid hydrolysis for various periods in 0.1M-HCl at 60°C.

The precursor used for these experiments was UDP-*N*-acetylmuramyl-pentapeptide labelled in the diaminopimelic acid residue with either $^{14}$C or $^3$H.

Table 1. *Proportion of peptide chains in newly synthesized peptidoglycan removed by β-elimination after mild acid hydrolysis*

A membrane preparation was incubated for various times with peptidoglycan precursors as described in the text, with UDP-*N*-acetylmuramyl-pentapeptide labelled with *meso*-diamino[$^{14}$C]pimelic acid. After thorough washing and removal of 'lipid intermediate' the preparation was hydrolysed in 0.1M-HCl for 2h at 60°C, dried and incubated with 4M-$NH_3$ at 37°C for 6h. Lactyl-peptides were separated by paper chromatography and radioactivity in them and in material that remained on the origin was measured. If the acid-hydrolysis step was omitted there was no release of radioactive lactyl-peptides.

| Incubation time (min) | Total radioactivity in sample (d.p.m.) | Radioactivity in lactyl-peptides (d.p.m.) | Percentage of total radioactivity in lactyl-peptides |
|---|---|---|---|
| 2 | 5820 | 2033 | 35.0 |
| 5 | 13000 | 1783 | 13.8 |
| 15 | 25200 | 1487 | 5.9 |
| 30 | 34900 | 1567 | 4.5 |

After biosynthesis with a membrane preparation as before, and thorough washing to remove unchanged precursor and lipid intermediates, samples were hydrolysed in 0.1M-HCl at 60°C and, after removal of the acid, incubated with 4M-$NH_3$ at 37°C for 6h (Tipper, 1968). Liberated lactyl-peptides were separated from polymeric material by chromatography in isobutyric acid–aq. $NH_3$ and their radioactivity was measured. In some experiments, in which $^{14}$C labelling was used, radioautographs were prepared. In this way it was shown that the lactyl-peptides corresponded to the markers of lactyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine, with lesser amounts of the corresponding lactyl-tetra- and tri-peptide. The presence of these shortened peptides was expected, since the preparation was known to contain a carboxypeptidase I, which would remove the terminal D-alanine, and a carboxypeptidase II, which would remove the next residue. There was hardly any lactyl-peptide if the acid hydrolysis step were omitted, but when the step was included a proportion of the total incorporated radioactivity was recovered as the lactyl-peptides (Table 1). The peptides could not have arisen from trapped precursor, since in various experiments with the same amount of radioactive precursor the radioactivity in lactyl-peptides always represented the same proportion of the total incorporation, which differed widely in different preparations. The experiment in Table 1 shows that, as would be expected for chain elongation at the reducing end, the proportion of radioactivity recovered in lactyl-peptides decreased with increasing time of incorporation. Thus at 2min the proportion represented 35% of the total activity, indicating that on average about three disaccharide units had been added to each chain in that time. After incubation for 30min the proportion was 4.5%, corresponding to an average length for each piece of newly synthesized glycan chain of about 22 repeating units. These calculations assume a quantitative yield of lactyl-peptide from the reducing terminals. The release of free reducing groups of muramic acid and hence of

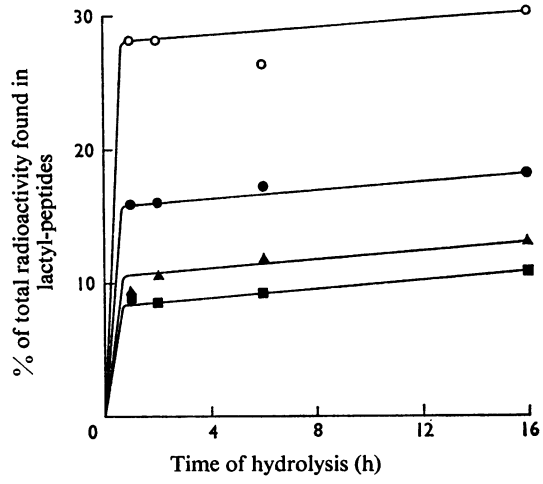


Fig. 1. *Effect of varying the time of hydrolysis on the subsequent elimination of lactyl-peptides*

Membrane preparations were incubated with suitable radioactive precursors as described in the text for 2(○), 5(●), 15(▲) and 30min(■). After extensive washing to remove unincorporated precursor and 'lipid intermediate' the preparations were hydrolysed in 0.1M-HCl at 60°C for the times indicated. Lactyl-peptides were eliminated in 4M-$NH_3$ at 37°C for 6h and separated by paper chromatography as described. The radioactivity of the peptides and the material on the origin of the chromatogram was then measured.

lactyl-peptides was not due to indiscriminate hydrolysis of the glycan chain for the following reasons. First, the total radioactivity released in this form remained essentially the same, regardless of the times of incorporation and the total radioactivity incorporated. Secondly, in experiments in which various times of hydrolysis in 0.1 M-HCl at 60°C were used, very little further release occurred after the first 1–2h (Fig. 1). For this reason, in later experiments a standard hydrolysis time of 2h was used.

The results so far suggested that the glycan chains were being extended at their reducing ends. If this were correct then, provided biosynthesis continued at the same sites, newly biosynthesized material should be buried deeper within the chains with increasing time of incorporation. Thus if the radioactive label were changed then the label found in the lactyl-peptides should soon change also. Membrane preparations were incubated for 5min with precursor labelled with diamino[$^{14}$C]pimelic acid and the precursor was then diluted eightfold with precursor labelled with [$^{3}$H]diaminopimelic acid, after which time further samples were taken at 2, 5, 15 and 30min. The proportions of $^{14}$C and $^{3}$H radioactivity recovered in the lactyl-peptides is shown in Fig. 2. In this particular experiment extraction of lipid intermediate was omitted and hence the proportion of the total radioactivity found in lactyl-peptide at a particular incubation time was higher than in Table 1. The $^{14}$C label rapidly disappeared from the lactyl-peptides after dilution with $^{3}$H-labelled precursor. The fact that the theoretical asymptotic value was not quite achieved probably indicates that some biosynthetic sites had ceased to function by the time the dilution was made.

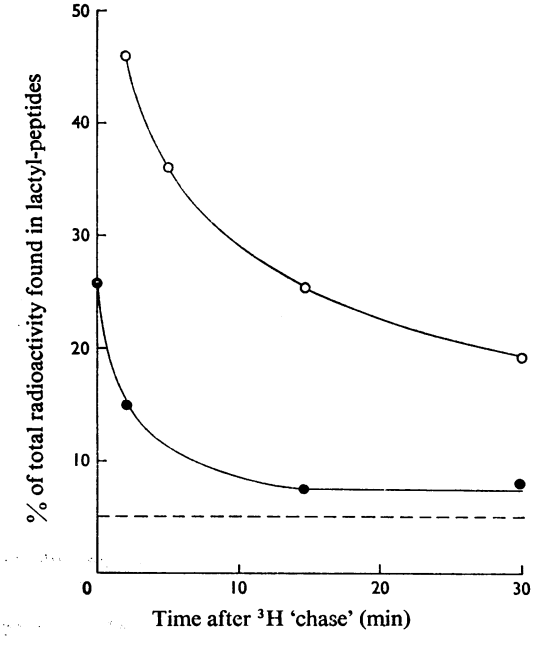


Fig. 2. *Replacement of $^{14}$C-labelled peptide chains linked at the glycan reducing terminal by $^{3}$H-labelled chains during further incubation*

Membrane preparations were incubated for 5min with UDP-*N*-acetylmuramyl-pentapeptide labelled with diamino[$^{14}$C]pimelic acid. The precursor was then diluted eightfold with precursor labelled with [$^{3}$H]diaminopimelic acid. Samples were taken after further incubation for 2, 5, 15 and 30min and washed to remove unincorporated radioactivity. Lactyl-peptides were eliminated as described in the text after hydrolysis of the sample in 0.1 M-HCl for 2h at 60°C. After separation by paper chromatography the proportion of lactyl-peptide labelled with $^{14}$C (●) and $^{3}$H (○) was determined. The theoretical asymptote for a 1:8 dilution is shown (----).

## Discussion

The results indicate that during biosynthesis of peptidoglycan by a membrane preparation from a poorly lytic mutant of *B. licheniformis*, the glycan chains grew by addition of disaccharide-peptide units at the reducing end. Further, this reducing end remained attached by some linkage that was labile to dilute acid at 60°C. From previous knowledge of the biosynthetic pathway of peptidoglycan synthesis it seems likely that this linkage was to undecaprenol pyrophosphate, although at present we have no direct evidence on this point. This method of synthesis would allow the synthetic enzymes to remain in the cytoplasmic membrane, the growing chains being pushed out into the region of preformed cell wall, as proposed by Anderson *et al.* (1972). One would then envisage that final incorporation into the finished wall structure would occur by cross-linkage of the peptide chains to pre-existing peptidoglycan. This is consistent with the observations of Mirelman & Sharon (1972) and Mirelman *et al.* (1972), who found that incorporation of radioactive peptidoglycan precursors into a crude wall preparation was highly penicillin-sensitive, and therefore presumably took place by cross-linkage. Our results would also fit the general model for the transfer of peptidoglycan into the cell wall proposed by Mirelman *et al.* (1972).

The method of glycan chain elongation shown here is consistent with that originally demonstrated for the lipopolysaccharide O-antigen of *Salmonella anatum* (Bray & Robbins, 1967; Robbins *et al.*, 1967). The poly(glycerol phosphate) of *Bacillus*

*subtilis*, on the other hand, has been shown to grow at the glycerol rather than the glycerol phosphate terminus (Kennedy & Shaw, 1968). Although this product is not a polysaccharide, it may be compared in the sense that individual units of glycerol phosphate are being transferred from the precursor CDP-glycerol in such a way that the newly added glycerol can be attacked by periodate. Hence the part of the molecule that was bound to the precursor is now bound to the growing chain, which is the reverse of the situation in O-antigen synthesis and in the present experiments.

Lipid intermediates that are probably the same as those involved in peptidoglycan synthesis are also used in the biosynthesis of the teichoic acids of the walls of *B. licheniformis*, but in this case the direction of synthesis has not yet been shown (Anderson *et al.*, 1972; Hancock & Baddiley, 1972). On the other hand, Fiedler *et al.* (1974) were unable to find any evidence for involvement of a polyprenol phosphate intermediate in the synthesis of the wall teichoic acids of *Bacillus subtilis* and *Staphylococcus aureus*. The direction of chain extension of a bacterial cell wall polymer containing a repeating unit of D-glycerol 1-phosphate attached to the 3-position of *N*-acetylglucosamine 1-phosphate (*Staphylococcus lactis* I3) and another made up of *N*-acetylglucosamine 1-phosphate units (*S. lactis* 2102) has also been studied (Hussey *et al.*, 1969). In each case chain extension occurred in the same direction as in the synthesis of the *B. subtilis* polymer (Kennedy & Shaw, 1968).

Anderson *et al.* (1972) proposed that synthesis should continue at the inner face of the membrane and that the growing chains should be pushed through leaving the lipid-bound end always on the inside. The preparation used in the present experiments can offer no answer to this type of question but the observed direction of glycan synthesis could fit their model.

It has been customary to implicate an 'acceptor' for peptidoglycan synthesis and in the past this was assumed to be pre-existing peptidoglycan (Strominger *et al.*, 1967). The present mechanism requires that any 'acceptor' for glycan chain elongation can only be chains that are still attached to lipid intermediate. These could conceivably be as short as another molecule of disaccharide(pentapeptide)-pyrophosphate undecaprenol, the implication being that once a tetrasaccharide had been formed it would be preferred as the growth point for further addition of disaccharide units at the reducing end, or in other words long chains are more likely to be transglycosylated to the non-reducing terminal of disaccharide units than vice versa. Such a situation could account for the fact that a preparation of membranes from staphylococcal L-forms, which apparently contained no peptidoglycan acceptor, would synthesize polymeric peptidoglycan *in vitro* (Chatterjee *et al.*, 1967). The same argument would apply to the other results of Reynolds (1971) for peptidoglycan synthesis in washed protoplast membranes from *Bacillus megaterium*.

At present there is no evidence about how the glycan chain finally becomes detached from its site of synthesis and liberated into the cross-linked wall. In a growing cell cross-linking may well occur while glycan synthesis is still proceeding at the membrane. Certainly the length of the glycan chains as synthesized in *B. licheniformis* is considerable, amounting to some 150 disaccharide units (Ward, 1973).

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## References

Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 881–889

Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1967) *J. Biol. Chem.* **242**, 3180–3190

Anderson, R. G., Hussey, H. & Baddiley, J. (1972) *Biochem. J.* **127**, 11–25

Bray, D. & Robbins, P. W. (1967) *Biochem. Biophys. Res. Commun.* **28**, 334–339

Chatterjee, A. N., Ward, J. B. & Perkins, H. R. (1967) *Nature (London)* **214**, 1311–1314

Fiedler, F., Mauck, J. & Glaser, L. (1974) *Ann. N.Y. Acad. Sci.* in the press

Forsberg, C. W. & Rogers, H. J. (1971) *Nature (London)* **229**, 272–273

Forsberg, C. W., Wyrick, P. B., Ward, J. B. & Rogers, H. J. (1973) *J. Bacteriol.* **113**, 969–984

Garrett, A. J. (1969) *Biochem. J.* **115**, 419–430

Ghuysen, J.-M., Bricas, E., Leyh-Bouille, M., Lache, M. & Shockman, G. D. (1967) *Biochemistry* **6**, 2607–2619

Hancock, I. C. & Baddiley, J. (1972) *Biochem. J.* **127**, 27–37

Higashi, Y., Strominger, J. L. & Sweeley, C. C. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 1878–1884

Hussey, H., Brooks, D. & Baddiley, J. (1969) *Nature (London)* **221**, 665–666

Janczura, E., Perkins, H. R. & Rogers, H. J. (1961) *Biochem. J.* **80**, 82–93

Kennedy, L. D. & Shaw, D. R. D. (1968) *Biochem. Biophys. Res. Commun.* **32**, 861–865

Leyh-Bouille, M., Nakel, M., Frère, J.-M., Johnson, K., Ghuysen, J.-M., Nieto, M. & Perkins, H. R. (1972) *Biochemistry* **11**, 1290–1298

Matsuhashi, M., Dietrich, C. P. & Strominger, J. L. (1967) *J. Biol. Chem.* **242**, 3191–3206

Mirelman, D. & Sharon, N. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1909–1917

Mirelman, D., Bracha, R. & Sharon, N. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 3355–3359

Perkins, H. R. (1967) *Biochem. J.* **102**, 29C–32C
Reynolds, P. E. (1971) *Biochim. Biophys. Acta* **237**, 255–272
Robbins, P. W., Bray, D., Dankert, M. & Wright, A. (1967) *Science* **158**, 1536–1542
Siewert, G. & Strominger, J. L. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 767–773
Strominger, J. L. & Threnn, R. H. (1959) *Biochim. Biophys. Acta* **36**, 83–92
Strominger, J. L., Izaki, K., Matsuhashi, M. & Tipper, D. J. (1967) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**, 9–22
Struve, W. G. & Neuhaus, F. C. (1965) *Biochem. Biophys. Res. Commun.* **18**, 6–12
Struve, W. G., Sinha, R. K. & Neuhaus, F. C. (1966) *Biochemistry* **5**, 82–93
Tipper, D. J. (1968) *Biochemistry* **7**, 1441–1449
Ward, J. B. (1973) *Biochem. J.* **133**, 395–398
Ward, J. B. & Perkins, H. R. (1968) *Biochem. J.* **106**, 69–76
Wright, A., Dankert, M. & Robbins, P. W. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 235–241
Wright, A., Dankert, M., Fennessey, P. & Robbins, P. W. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 1798–1803