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Biosynthesis of the Wall Teichoic Acid in *Bacillus licheniformis*

By I. C. HANCOCK and J. BADDILEY
Microbiological Chemistry Research Laboratory, School of Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

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1. The biosynthesis of the wall teichoic acid, poly(glycerol phosphate glucose), has been studied with a particulate membrane preparation from *Bacillus licheniformis* A.T.C.C. 9945. The precursor CDP-glycerol supplies glycerol phosphate residues, whereas UDP-glucose supplies only glucose to the repeating structure of the polymer. 2. Synthesis proceeds through polyprenol phosphate derivatives, and chemical studies and pulse-labelling techniques show that the first intermediate is the phosphodiester, glucose polyprenol monophosphate. CDP-glycerol donates a glycerol phosphate residue to this to give a second intermediate, (glycerol phosphate glucose phosphate) polyprenol. 3. The glucose residue in the lipid intermediates has the β configuration, and chain extension in the synthesis of polymer occurs by transglycosylation with inversion of anomeric configuration at two stages.

The participation of CDP-glycerol, CDP-ribitol and nucleoside diphosphate sugars as precursors in the biosynthesis of teichoic acids has been known for some time (Archibald *et al.*, 1968; Baddiley, 1970). The more detailed mechanism of the synthetic route occurring in the cell membrane of at least some bacteria resembles in outline that of the other wall components, peptidoglycan and lipopolysaccharide, in that residues are first transferred from the nucleotide precursors to a polyisoprene phosphate in the membrane and hence to the growing polymer chain (Douglas & Baddiley, 1968; Brooks & Baddiley, 1969). During our studies it has been shown in several laboratories that other polysaccharides in bacteria and glycoprotein of liver are synthesized through isoprenoid phosphate carriers. The very small amount of lipid intermediates for teichoic acids normally found in membrane preparations have prevented direct characterization of the lipid component of these molecules by conventional methods, but it has been demonstrated with a staphylococcus that the same lipid phosphate molecules are used for the synthesis of both peptidoglycan and teichoic acid (Watkinson *et al.*, 1971); thus it is concluded that the common lipid is the isoprenoid, undecaprenol, that has been characterized for the synthesis of peptidoglycan and lipopolysaccharide in other organisms.

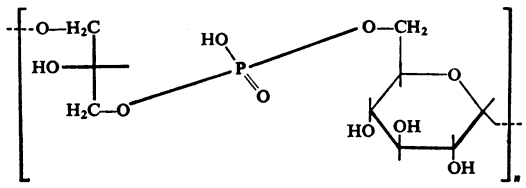
The demonstration of lipid intermediates in the synthesis of teichoic acids has hitherto been confined to those organisms in which the teichoic acids contain acetylamino sugar 1-phosphate residues, where the sugar 1-phosphate is transferred as an intact unit from nucleotide to lipid phosphate and then to polymer. In a number of teichoic acids, and in many of the related pneumococcal capsular substances, the sugar residues occur in the main chain and are glycosidically attached to other sugars or to glycerol or ribitol. As the mechanism of synthesis of such polymers must clearly differ considerably from that of polymers containing sugar 1-phosphate residues, and from that of the simpler polymers of polyol phosphates, it was of interest to determine whether lipid intermediates occurred in such cases. A suitable organism for our studies was *Bacillus licheniformis* A.T.C.C. 9945, in the walls of which a teichoic acid comprising glucose, glycerol and phosphate has been found (Burger & Glaser, 1966). The structure of this polymer is known; D-glucose is in the α-glycosidic linkage with the 3-position of D-glycerol 1-phosphate, the phosphate of which is attached to the 6-position of its neighbouring glucose (I). The teichoic acid could thus be regarded as a polymer of α-(1$\rightarrow$3)-glucosylglycerol phosphate or of (glycerol phosphate glucose). It is also known that cell-free membrane preparations from the organism catalyse the synthesis of the polymer from CDP-glycerol and UDP-glucose.

The work described in the present paper establishes that lipid intermediates participate in the biosynthesis of the teichoic acid in the wall of *B. licheniformis*, and a mechanism for the synthesis is proposed.

Experimental

Materials

UDP-glucose (sodium salt), lysozyme, calf-spleen alkaline phosphatase and cytidine phosphoromorpholidate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. CDP-glycerol



(I)

(lithium salt) was prepared from CMP morpholidate by the general procedure described by Roseman *et al.* (1961).

[1-^{14}C]Glycerol, α-D-[G-^{14}C]glucose 1-phosphate, [^{32}P]P_i and [γ-^{32}P]adenosine 5'-triphosphate were supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

CDP-[^{14}C]glycerol was synthesized enzymically as described by Baddiley *et al.* (1968), from [1-^{14}C]-glycerol. The [β-^{32}P]CDP-glycerol was prepared in the same way, by using [γ-^{32}P]ATP to phosphorylate the glycerol (H. Hussey & J. Baddiley, unpublished work). Both preparations were used at a concentration of 20μmol/ml and their specific radioactivities were 1μCi/μmol for ^{14}C, or 1.33×10^7c.p.m./μmol for ^{32}P on the day of use.

UDP-[^{14}C]glucose was prepared from [G-^{14}C]-glucose 1-phosphate and UTP by using a crude preparation of UDP-glucose pyrophosphorylase from baker's yeast. The enzyme was prepared as follows: air-dried yeast was allowed to autolyse as a suspension at 50g/100ml in 0.1M-$NaHCO_3$ at 37°C for 5h. Cell debris was removed by centrifugation at 8000*g* for 15min at 0°C. The supernatant was fractionated by precipitation with ammonium sulphate at 0°C; the fraction of protein precipitating between 30% and 60% saturation contained most of the pyrophosphorylase. The preparation was suspended in 0.1M-tris–HCl–5mM-EDTA–5mM-$MgCl_2$ at pH8.0 and dialysed against the same buffer overnight at 5°C. The resulting material (50ml from 150g dry wt. of yeast) was used as the enzyme. It was stable for at least 6 months when frozen at −20°C. The enzyme was used to prepare UDP-[^{14}C]glucose as follows: [G-^{14}C]glucose 1-phosphate (50μCi; 1μCi/μmol) and 70μmol of UTP (trisodium salt) were dissolved in 1ml of water and the pH was adjusted to 8.0 with dilute aq. NH_3; 5ml of yeast enzyme was added and the mixture was incubated for 2h at 37°C. Protein was precipitated by the addition of an equal volume of boiling ethanol and the precipitate was washed three times with cold 50% (v/v) ethanol. The supernatant from the original reaction mixture was combined with the washings and evaporated to dryness at room temperature under reduced pressure. The product was purified by chromatography on Whatman 3MM paper in solvent (ii) for 20h. It was used at a final concentration of 10μCi/ml (1μCi/μmol).

[^{32}P]UDP-glucose was prepared from glucose 1-[^{32}P]phosphate by the method described above for UDP-[^{14}C]glucose. Glucose 1-[^{32}P]phosphate was obtained by the phosphorolysis of starch in the presence of [^{32}P]P_i by potato starch phosphorylase. The potato enzyme was prepared as described by McCready & Hassid (1955), and the reaction mixture for synthesis of the glucose 1-phosphate contained 0.3ml of 0.67M-potassium phosphate buffer, pH6.6, 0.4ml of 2.5% (w/v) soluble starch solution, 0.2ml of enzyme and 0.2μmol of [^{32}P]P_i (500μCi/μmol). The mixture was incubated at room temperature for 24h, then the reaction was stopped by heating in a boiling water-bath for 5min and the mixture was adjusted to pH8.5 with aq. NH_3. The remaining phosphate was precipitated by the addition of 0.06g of magnesium acetate and the supernatant was evaporated to a small volume, then applied to a column (8cm×1cm) of Dowex-1 (Cl^- form) resin; after washing the resin with water the glucose phosphate was eluted in 20ml of 2% (w/v) LiCl in 3mM-HCl. The product was precipitated with acetone and washed free of LiCl with acetone–ether (6:1, v/v). The glucose 1-[^{32}P]phosphate was adjusted to a final concentration of 20μmol/ml. It was converted into [^{32}P]UDP-glucose as described above and the product had a specific activity of 10^6c.p.m./μmol at a concentration of 10μmol/ml at the time of use.

Methods

Chromatography. Paper chromatography was done on Whatman no. 1 or no. 4 paper. The solvents were: (i) propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949); (ii) ethanol–0.1M-ammonium acetate, pH7.5 (5:2, v/v) (Paladini & Leloir, 1952); (iii) butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes *et al.*, 1951). The following detection reagents were used: alkaline $AgNO_3$ for sugars and polyols (Trevelyan *et al.*, 1950); molybdate for phosphoric esters (Hanes & Isherwood, 1949).

T.l.c. of lipids was done on 0.4mm layers of silica gel G (Merck) on 5cm×20cm plates. When hydrolysis of the lipid in the presence of silica gel was necessary, similar layers of silica gel H were used. The two types of silica gave identical separations. The solvent used was anhydrous methanol–chloroform (5:16, v/v) (Nichols, 1964).

Radioactivity measurements. Solutions of lipids (0.1ml) in chloroform–methanol (1:1, v/v) were counted in 10ml of scintillant that contained 2,5-diphenyloxazole (8g), and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.2g) in 2 litres of toluene. The efficiency of counting was about 70%.

Aqueous samples (1.5ml) were counted in 10ml of scintillation fluid consisting of the above solution mixed with Triton X-100 (2:1, v/v), with an efficiency of about 70%. The radioactivity of paper strips (1cm × 4cm), and of 1cm bands of silica gel from thin-layer plates, was counted in 10ml of the first scintillant with an efficiency of 30% for ^{14}C on silica gel, 45% for ^{14}C on paper and 65% for ^{32}P on paper. The instrument used was a Beckman L 150 liquid scintillation spectrometer. Counts were accumulated to a counting error of ±2% or less.

Growth of bacteria and preparation of the enzyme. *B. licheniformis* A.T.C.C. 9945 was grown for 3h at 37°C with forced aeration in a medium containing 12.5g of Oxoid no. 2 nutrient broth, 2.5g of Difco yeast extract, 2.5g of K_2HPO_4 and 1g of glucose in 1 litre. The cells were harvested in the continuous-flow attachment of a Sorval RC2 centrifuge at 10000rev./min, and washed once with cold 0.05M-tris–HCl buffer at pH7.5. Enzyme was prepared from the washed bacteria by treatment with lysozyme essentially as described by Burger & Glaser (1966). The resulting particulate membrane fraction was resuspended in 0.1M-tris–HCl that contained 5mM-sodium thioglycollate, pH7.5, at a concentration of 1ml/litre of the original culture.

Incorporation of radioactivity into lipid and polymer. The composition of reaction mixtures is described with the individual experiments. Lipid was extracted from reaction mixtures into three equal volumes of butan-1-ol for 30min at 4°C. The combined butanol extracts were washed twice with equal volumes of water saturated with butanol at 4°C and the washed extracts were evaporated to dryness at room temperature under reduced pressure. The lipid was redissolved in a measured volume of chloroform–methanol (1:1, v/v) and samples were taken for t.l.c. or scintillation counting. The residue of the reaction mixture after extraction of lipids was mixed with an equal volume of methanol and applied as a band (2.5cm) to the origin of a chromatogram on Whatman no. 4 paper. The chromatogram was developed in solvent (ii) for 20h. The polymer remained at the origin of the chromatogram and radioactivity was measured by scintillation counting of this region of the paper.

When measurement of incorporation of radioactivity from CDP-glycerol was studied, an alternative method had to be used to distinguish between poly(glycerol phosphate) and poly(glycerol phosphate glucose). In this case the residue after extraction of lipid was mixed with an equal volume of methanol and centrifuged at 12000*g* for 5min at 0°C. All of the radioactive polymer sedimented with the protein under these conditions. The pellet was suspended in 1ml of 1M-NaOH and heated on a boiling-water bath for 3h. After cooling, the hydrolysates were adjusted to pH8.5 with acetic acid and mixed with 0.5ml of alkaline phosphatase (5mg/ml). The mixture was incubated at 37°C overnight, then was evaporated to a small volume under reduced pressure at room temperature and chromatographed on Whatman no. 1 paper in solvent (iii) for 20h. This treatment produced glucosylglycerol from poly(glycerol phosphate glucose), and this product was well separated from those formed by hydrolysis of poly(glycerol phosphate). Radioactivity in glucosylglycerol was measured by scintillation counting of the appropriate region of the paper chromatogram.

Hydrolysis of lipids. Acid hydrolysis of lipids was done at the required concentration of HCl in aq. 30% (v/v) methanol at 100°C. For routine acid hydrolysis of lipid intermediates on silica gel H from thin layer plates, the gel was suspended in 0.05M-HCl in aq. 30% (v/v) methanol and heated at 95°C for 15min with frequent shaking. Silica was removed by centrifugation and the supernatant was neutralized with 0.5M-NH_3 and was then evaporated under reduced pressure at room temperature to a small volume before paper chromatography.

Alkali hydrolysis of lipids on silica gel H was done with 0.5M-KOH in water-saturated butan-1-ol at 48°C for 2h. The butan-1-ol was extracted with two equal volumes of water and the aqueous extracts were combined and then evaporated to a small volume for paper chromatography.

Isolation of polymer and its hydrolysis with alkali. Polymer required for chemical examination was obtained from the residue of synthesis mixtures after extraction of lipids. The residue was mixed with 0.5ml of aq. 0.15M-NH_3 and 0.5ml of aq. 90% (w/v) phenol at 4°C for 30min. The phases were separated by gentle centrifugation and the aqueous layer was removed, washed with an equal volume of chloroform and then dialysed overnight against running tap water. The dialysis sac contained polymeric material free of substrate.

Polymer obtained in this way was completely degraded by hydrolysis in 0.5M-NaOH at 100°C for 3h. At least 90% of the poly(glycerol phosphate glucose) was converted into glucosylglycerol phosphate, whereas poly(glycerol phosphate) yielded mainly isomeric glycerol phosphates, with smaller amounts of glycerol diphosphates and glycerol. The hydrolysis products were separated by chromatography on Whatman no. 1 paper in solvent (i) overnight.

Results

Properties of the enzyme system

The enzyme preparation behaved qualitatively like that described by Burger & Glaser (1966). It differed from theirs in having an optimum Mg^{2+} ion requirement of 10mM, whereas they reported an optimum

requirement of 15mM for their preparation. Our enzyme was significantly stabilized by 5mM-sodium thioglycollate, so that it retained 50% of its activity after storage at −20°C for 3 months. Repeated freezing and thawing, or prolonged incubation at 37°C, inactivated the preparation, and for this reason it was stored in small batches when not intended for immediate use. At least a part of the loss of activity occurred through disaggregation of the membrane system. It was found that 70mM-$MgCl_2$ caused the enzyme to reaggregate to a particulate form that sedimented with the original membrane and retained enzymic activity; consequently, during experiments all centrifugation steps at which retention of enzymic activity was essential were done in the presence of 70mM-$MgCl_2$.

The enzyme preparation was highly active in the synthesis of poly(glycerol phosphate) from CDP-glycerol. Thus in all complete incubations CDP-glycerol was simultaneously converted into both poly(glycerol phosphate) and poly(glycerol phosphate glucose). These two teichoic acids cannot be separated readily, and therefore incorporation of [^{14}C]glycerol phosphate from CDP-glycerol into an individual polymer could not be measured directly.

Origin of the phosphate groups in poly(glycerol phosphate glucose)

Studies by Burger & Glaser (1966) did not indicate whether the phosphate group of the repeating unit in the polymer originated from CDP-glycerol or UDP-glucose, although structural considerations would suggest the former origin. To determine the source of the phosphate, CDP-glycerol and UDP-glucose labelled with ^{32}P in the phosphate group adjacent to the glycerol and glucose were prepared. In experiments with these labelled nucleotides reaction mixtures contained 0.1ml of enzyme, 10mM-$MgCl_2$, 0.2ml of UDP-[^{14}C]glucose (10μCi/ml; 1μCi/μmol), 0.02ml of [^{32}P]UDP-glucose and 0.02ml of 20mM-CDP-glycerol, or 0.02ml of [^{32}P]CDP-glycerol. After incubation for 1h at 37°C polymer was isolated by phenol extraction and dialysis, and was then subjected to hydrolysis in alkali followed by paper chromatography in solvent (i). In both cases glucosylglycerol phosphate, the degradation product of poly(glycerol phosphate glucose), contained all of the ^{14}C from UDP-glucose, but it was only labelled with ^{32}P when [^{32}P]CDP-glycerol was present in the reaction mixture. Hence UDP-glucose donates only glucose residues to the polymer, whereas CDP-glycerol provides glycerol phosphate residues.

Incorporation of glucose residues into the polymer via a lipid

Incubation of the enzyme preparation with [^{32}P]UDP-[^{14}C]glucose in the presence or absence of CDP-glycerol led to the incorporation of ^{14}C, but not of ^{32}P, into lipids that could be extracted into butan-1-ol. The overall incorporation of [^{14}C]glucose residues into lipid in 30min appeared to be much less dependent upon the presence of CDP-glycerol than was polymer synthesis (see Table 1). The labelled lipids from this experiment were subjected to t.l.c. on silica gel in chloroform–methanol (16:5, v/v). They separated into a labelled phospholipid fraction (*A*, R_F 0.2) and two labelled neutral lipid fractions (*B*, R_F 0.62; *C*, R_F 0.9) (see Fig. 1). Incorporation into fraction *A* was decreased by 50% in the presence of CDP-glycerol, but incorporation into the neutral lipids was not affected.

The chromatographic properties of the neutral

Table 1. *Incorporation of* [^{14}C]*glucose from UDP-glucose into teichoic acid and lipids*

Mixtures contained 0.4ml of enzyme, prepared as described in the Experimental section, 0.1ml of 0.1M-tris–HCl that was 5mM with respect to sodium thioglycollate and 10mM in $MgCl_2$ at pH7.5, 1μmol of UDP-[^{14}C]glucose and 1μmol of CDP-glycerol or 0.1ml of water, in a total volume of 0.8ml. Incubation was done at 37°C for 30min. Lipids and polymers were extracted and their radioactivity was measured as described in the Experimental section.

	[^{14}C]Glucose in polymer (c.p.m.)	[^{14}C]Glucose in lipid (c.p.m.)
With CDP-glycerol	51110	11300
Without CDP-glycerol	11570	13770

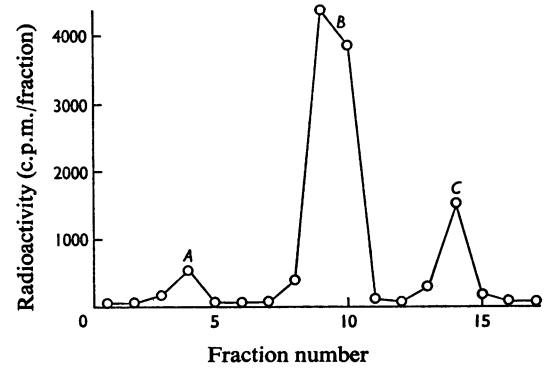


Fig. 1. *T.l.c. separation of lipids containing incorporated* [^{14}C]*glucose*

Reaction mixtures were those given for Table 1. Lipid was extracted and t.l.c. done as described in the Experimental section.

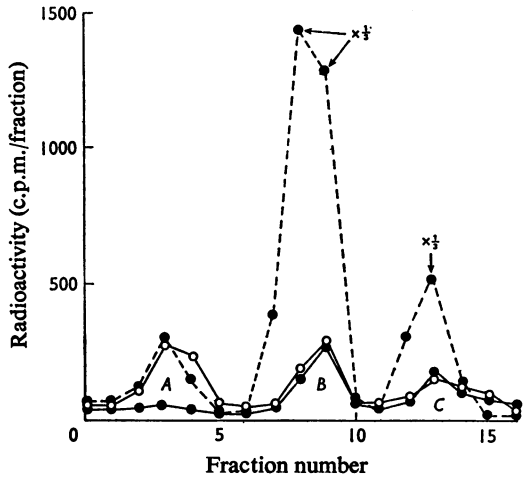


Fig. 2. *Effect of preincubation with UDP-glucose on incorporation of UDP-[^{14}C]glucose*

The initial reaction mixture contained 0.8ml of enzyme, 1 μmol of UDP-glucose and 10mM-$MgCl_2$ in a total volume of 1.0ml. The control mixture contained 0.4ml of enzyme in 10mM-$MgCl_2$. They were incubated for 1h at 37°C and the enzyme was recovered by centrifugation at 27000*g* for 15min at 0°C. The enzyme was resuspended as two equal parts in 0.3ml of 0.1M-tris–HCl that was 5mM with respect to sodium thioglycollate (pH7.5); the control was suspended in 0.3ml of the same buffer. Each part was incubated with 1 μmol of UDP-[^{14}C]glucose and 1 μmol of CDP-glycerol or 0.1ml of water; the solution was made 10mM with respect to $MgCl_2$ in a total volume of 0.6ml; incubation was for 30min at 37°C. Lipids were extracted and chromatographed as described in the Experimental section. ●----●, Not preincubated with UDP-glucose; o——o, preincubated with UDP-glucose, reincubated with CDP-glycerol; ●——●, preincubated with UDP-glucose, reincubated without CDP-glycerol.

lipids *B* and *C* were consistent with their being a diglucosyldiglyceride and a monoglucosyldiglyceride respectively (Nichols, 1964). Glycolipids of this type are known to occur in related *Bacillus* species (Shaw & Baddiley, 1968; Shaw, 1970) and their biosynthesis proceeds by the sequential addition of glucose residues from UDP-glucose to the 3-hydroxyl group of endogenous 1,2-diglyceride (Smith, 1969). The dependence of lipid synthesis upon endogenous acceptor in the enzyme preparation was therefore tested by preincubating the preparation with unlabelled UDP-glucose and washing the particulate fraction, which was then incubated with UDP-[^{14}C]-glucose either in the presence or absence of CDP-glycerol. Fig. 2 shows that the preincubation greatly decreased subsequent incorporation of [^{14}C]glucose into all the lipids in the absence of CDP-glycerol. Subsequent incubation in the presence of CDP-glycerol and UDP-[^{14}C]glucose, however, led to incorporation into the phospholipid *A*, although it had no effect on the neutral lipids. These results indicated that all three labelled lipid fractions were formed by the addition of glucose residues to endogenous acceptors, but that whereas the neutral lipids, once formed, were stable the phospholipid fraction *A* 'turned over' in the presence of CDP-glycerol.

Table 2. *Chromatography of hydrolysis products from labelled lipids*

Paper chromatography of samples of alkali hydrolysates of lipid fractions was done in solvents (ii) and (iii) (see the Experimental section).

	R_{Glc} in solvent (iii)	R_{Glc} in solvent (ii)
Hydrolysate of *A*	0.0	0.26; 0.39
Glucose 1-phosphate	0.0	0.3
Glucose 1,2-cyclic phosphate	0.05	0.42
Hydrolysate of *B*	0.51	
Glucosyl-(1→6)-glucosyl-(1→1)-glycerol	0.49	
Hydrolysate of *C*	1.00	
Glucosyl-(1→1)-glycerol	0.94	

The three lipid fractions, formed in the absence of CDP-glycerol, were hydrolysed with alkali as described in the Experimental section, and then subjected to chromatography of the water-soluble products in solvent (iii). The lipids were completely hydrolysed under these conditions, over 95% of the initial radioactivity becoming water-soluble after hydrolysis. The chromatographic properties of the products are shown in Table 2, together with the R_F values of standard materials. Lipids *B* and *C* gave products identical with the deacylation products of the diglycosyldiglyceride and the monoglucosyldiglyceride from *Bacillus subtilis* (Shaw, 1970). The products of hydrolysis of lipid *A* did not migrate from the origin of the chromatogram in solvent (iii), and therefore appeared to be acidic. They were rechromatographed in solvent (ii) in which they separated into two components as shown in Table 2; these components were indistinguishable from glucose 1-phosphate and glucose 1,2-cyclic phosphate, both of which are products of hydrolysis of UDP-glucose with alkali (Paladini & Leloir, 1952). Hydrolysis of the mixture in 0.1M-HCl for 15min at 100°C, followed by treatment with alkaline phosphatase at pH8.5, yielded only glucose and P_i. Lipid *A* was resistant to 0.1M-LiOH in chloroform–methanol

(1:4, v/v) at room temperature for 30min. Lipids *B* and *C* were completely deacylated under these conditions.

Controlled acid hydrolysis, as described in the Experimental section, gave the same products from lipids *B* and *C* as did hydrolysis with alkali, but the reaction was incomplete. Lipid *A* was completely hydrolysed to glucose in 0.01M-HCl at 100°C in 15min in the absence of silica gel, but when the experiment was done in the presence of the gel the more vigorous conditions described in the Experimental section were required for complete hydrolysis.

Catalytic hydrogenolysis of an undecaprenol pyrophosphate sugar causes cleavage of the bond between the polyprenol and the pyrophosphate group (Wright *et al.*, 1967). This hydrogenolysis reaction has been employed to yield glucose 1-phosphate from glucose undecaprenol monophosphate, which is an intermediate in the glucosylation of the side chains of a salmonella lipopolysaccharide (Nikaido & Nikaido, 1971). We examined the effect of hydrogenation on the lipid intermediate *A* from *B. licheniformis*. The reaction was done as described by Nikaido & Nikaido (1971), with platinum catalyst freshly prepared by reduction of chloroplatinic acid with sodium borohydride, and lipid *A* labelled with ^{14}C in the glucose moiety, isolated by t.l.c. from 5ml of the incubation mixture as described for Fig. 1. The enzyme used was free of UDP-glucose 4′-epimerase, as indicated by its inability to reduce NAD^+ in the presence of UDP-galactose and UDP-glucose dehydrogenase. After the lipid had been hydrogenated for 3h at room temperature and atmospheric pressure the reaction products were partitioned between the organic and aqueous phases of a chloroform–methanol–water mixture (Bligh & Dyer, 1959). The aqueous phase was neutralized, and was then evaporated to dryness at room temperature under reduced pressure and redissolved in water; the partitioning procedure was then repeated. From the 720c.p.m. originally in lipid *A*, 660c.p.m. were recovered in the aqueous phase. The water-soluble products were subjected to chromatography on Whatman no. 4 paper in solvent system (i) overnight, when 70% of the radioactivity co-chromatographed with glucose 1-phosphate. This material yielded glucose on treatment with alkaline phosphatase, indicating that the 1-position of the sugar was protected by the phosphate group during reduction. The other 30% of the radioactivity was located in glucitol, presumably formed by breakdown of the lipid to glucose during the hydrogenation process.

Hydrogenolysis of the glucose polyprenol monophosphate from the salmonella yielded β-glucose 1-phosphate (Nikaido & Nikaido, 1971). In an attempt to determine the configuration of the anomeric linkage in the lipid *A* the effect of phosphoglucomutase on the glucose 1-phosphate from hydrogenolysis of the lipid was examined. The glucose 1-phosphate was incubated with phosphoglucomutase, glucose 6-phosphate dehydrogenase, glucose 1,6-diphosphate, 10mM-$MgCl_2$ and $NADP^+$; after 30min, authentic α-glucose 1-phosphate was added and rapid reduction of the $NADP^+$ was observed spectrophotometrically, showing that the system was active under these conditions. Incubation was continued at room temperature for a further 2h and then the products were subjected to chromato-

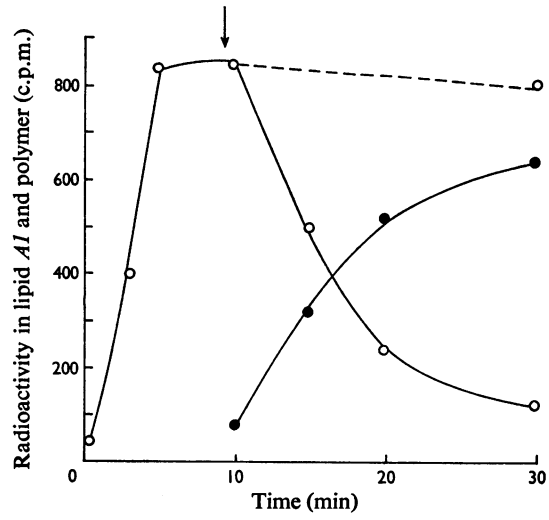


Fig. 3. *Measurement of lipid A turnover by incorporation of UDP-[^{14}C]glucose*

The initial reaction mixture contained 2ml of enzyme, 2μmol of UDP-[^{14}C]glucose, and was 10mM with respect to $MgCl_2$ in a total volume of 2.5ml; it was incubated at 37°C for 10min. Enzyme was recovered by centrifugation as for Fig. 2 and was then resuspended in 0.5ml of tris–thioglycollate, pH7.5, with 2μmol of UDP-glucose, 2μmol of CDP-glycerol and 20μmol of $MgCl_2$ in a total volume of 2.0ml and incubated at 37°C. At the time-intervals shown, 0.4ml samples were withdrawn and lipid and polymer extracted as described in the Experimental section; the lipids were fractionated by t.l.c. The counts shown have been corrected for differences in counting efficiency for lipid and polymer, and apply to the initial total enzyme volume. The arrow indicates the start of the incubation with unlabelled substrates. o——o, Counts in lipid fraction *A*; o----o, counts in lipid from sample reincubated without substrates; ●——●, counts in poly(glycerol phosphate glucose). In this experiment 1000c.p.m. of radioactivity is equivalent to the incorporation of 1nmol of glucose or glycerol into lipid or polymer.

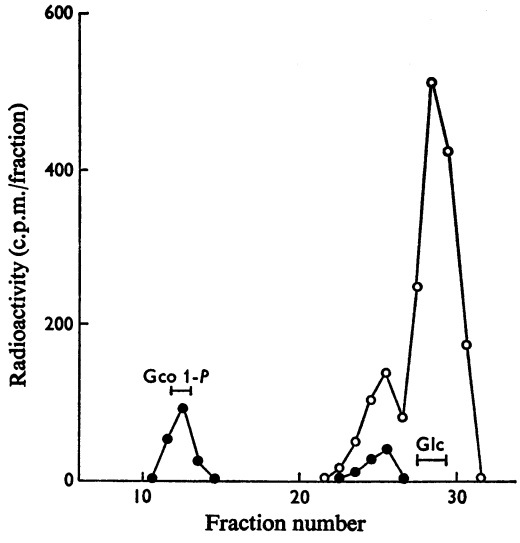


Fig. 4. *Separation by paper chromatography of the products of acid hydrolysis of doubly-labelled lipid A*

The reaction mixture contained 0.5ml of enzyme, 1μmol of UDP-[^{14}C]glucose and 1μmol of [^{32}P]-CDP-glycerol, all in 10mM-$MgCl_2$, and was incubated at 37°C for 1h. Lipids were extracted and fractionated by t.l.c. on silica gel H as described in the Experimental section. The lipid fraction *A* was subjected to controlled acid hydrolysis on the gel and the water-soluble products were separated by paper chromatography on Whatman no. 4 paper in solvent (i). Radioactivity on the chromatogram was measured as described in the Experimental section, in 1 cm bands of the paper. Gco 1-*P*, glycerol 1-phosphate. ●, ^{32}P; o, ^{14}C.

graphy on Whatman no. 4 paper in solvent system (i). All of the radioactivity remained in glucose 1-phosphate, although the authentic α-glucose 1-phosphate had been converted almost entirely into 6-phosphogluconate ($R_{\text{glucose 1-phosphate}}$ 0.69). This indicates that the glucose 1-phosphate formed by hydrogenolysis of the lipid does not have the α-configuration, and the lability of the lipid to hydrogenolysis strongly supports the conclusion that the glucose is linked through a monophosphate group to a polyprenol, the terminal group of which is unsaturated, as in other characterized bacterial polyprenols.

The results indicate a structure for phospholipid *A* in which the glucose residue is attached through its 1-position in the β-configuration to a phosphate group on an acceptor polyprenol monophosphate. As ^{32}P was not incorporated into the lipid from [β-^{32}P]UDP-glucose the phosphate group must

Table 3. *Chromatography of products of controlled acid hydrolysis of lipids A1 and A2*

	R_{Glc} in solvent (i)	R_{Glc} in solvent (ii)
Glucose 1-phosphate	0.3	0.26
(Glucose-6)(glycerol-1)-phosphate	0.88	0.52
(Glucose-1)(glycerol-1)-phosphate*	0.77	0.50
Glucose	1	1
Product of *A1*	1.0	1.0
Product of *A2*	0.90	0.50

* Kindly provided by Dr. N. Shaw.

originate in the acceptor lipid. The glucose 1-phosphate linkage in lipid *A*, and its turnover in the presence of CDP-glycerol, clearly distinguish it in structure and metabolism from the glycolipids *B* and *C*.

The turnover of lipid *A* was examined in more detail. The enzyme preparation was preincubated with UDP-[^{14}C]glucose, then the labelled substrate was removed by centrifugation before the preparation was incubated with CDP-glycerol and a relatively large amount of unlabelled UDP-glucose. Radioactivity in lipid *A* and teichoic acid was measured at intervals and the results are shown in Fig. 3. Incorporation into lipid *A* rapidly reached a maximum in the absence of CDP-glycerol, and in the presence of unlabelled substrates labelled glucose disappeared from the lipid and appeared in polymer. The glucose-containing phospholipid *A* therefore appeared to be an intermediate in the synthesis of the teichoic acid.

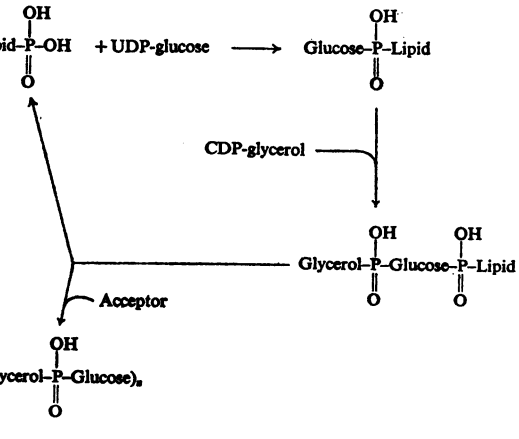
Incorporation of glycerol phosphate into lipid and polymer

The possibility that the glycerol phosphate residue of the teichoic acid was also incorporated through a lipid intermediate was examined. Incubation of the enzyme preparation with UDP-[^{14}C]glucose and [^{32}P]CDP-glycerol yielded a phospholipid *A* fraction containing ^{32}P and [^{14}C]glucose in a fraction giving a single spot on t.l.c.; no ^{32}P appeared in the neutral lipid fractions. Controlled acid hydrolysis of the doubly-labelled lipid *A* gave three labelled products that were separated by paper chromatography as shown in Fig. 4. The material labelled only with ^{32}P behaved like glycerol phosphate(s) and yielded P_i on treatment with alkaline phosphatase. The product labelled only with ^{14}C co-chromatographed with glucose and is the expected hydrolysis product of the phospholipid containing glucose described above. The third, doubly-labelled, component contained

^{32}P and [^{14}C]glucose in the molar ratio of 1:1, calculated from the specific radioactivities of the compounds used. It was unaffected by alkaline phosphatase and migrated faster on chromatography than would be expected for a monophosphate. These properties suggest a structure in which glycerol is attached through a phosphodiester to a position other than 1 on glucose. The chromatographic properties of the material were compared with those of the phosphodiesters (glucose-6)(glycerol-1)phosphate and (glucose-1)(glycerol-1)phosphate in solvents (i) and (ii) (see Table 3). In both systems it co-chromatographed with (glucose-6)(glycerol-1)phosphate, the repeating unit of the structure (I).

In the presence of CDP-glycerol, therefore, an additional component of the phospholipid *A* fraction appeared, with the structure expected for the phospholipid-bound repeating unit of the polymer; the repeating unit in this component is attached to the phosphate group of the lipid through the 1-position of its glucose residue. The phospholipid containing only glucose will therefore be designated component *A1* and the phospholipid containing the repeating unit will be called component *A2* in the following discussion of pulse-labelling experiments. The structures of these lipids are consistent with the biosynthetic route for the teichoic acid given in Scheme 1. To examine this further the enzyme preparation was preincubated with UDP-[^{14}C]glucose to produce labelled lipid *A1*. The preparation was recovered by centrifugation, washed and then incubated with unlabelled CDP-glycerol. The result is shown in Fig. 5. Within 30min, 36% of the radioactivity of lipid *A1* was lost, and appeared in lipid *A2*. No polymer synthesis could be detected in this experiment. This may have been due to loss of enzyme activity during the washing procedure, which was not done at as high a concentration of Mg^{2+} ions as usual. Enzyme preparation that had been incubated in the absence of CDP-glycerol still contained only lipid *A1* at the end of the experiment. It therefore appeared that lipid *A2* was formed by the reaction of lipid *A1* with CDP-glycerol.

The turnover of lipid *A2* was studied in the pulse-labelling experiment shown in Fig. 6. Enzyme preparation that contained labelled lipid *A2* was produced by incubation with UDP-glucose and CDP-[^{14}C]glycerol, and this was then incubated with unlabelled substrates. Label in lipid *A2* was rapidly transferred to polymer. Enzyme that had



Scheme 1. *Participation of isoprenoid intermediates in the biosynthesis of wall teichoic acid in B. licheniformis*

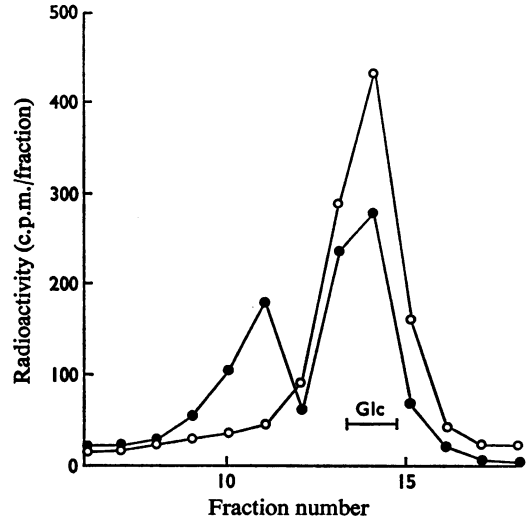


Fig. 5. *Biosynthetic route of formation of lipids A1 and A2 (Scheme 1)*

The initial reaction mixture contained 1.0ml of enzyme and 1.0μmol of UDP-[^{14}C]glucose, all in 10mM-$MgCl_2$, and was incubated for 10min at 37°C. The enzyme was recovered by centrifugation, and was washed twice with 0.15M-sodium pyrophosphate, pH7.5. A portion (0.5ml) of the suspension was then incubated with 1μmol of CDP-glycerol in 10mM-$MgCl_2$ in a total volume of 0.8ml, for 30min at 37°C. The other 0.5ml of enzyme was incubated with 10mM-$MgCl_2$ alone, also in a total volume of 0.8ml. Lipid was extracted, then was fractionated by t.l.c., and the component *A* was degraded by controlled acid hydrolysis as described in the Experimental section. The water-soluble products were chromatographed on Whatman no. 1 paper in solvent (i) and radioactivity was measured in 1cm bands of the paper. o, Reincubated without CDP-glycerol; •, reincubated with CDP-glycerol.

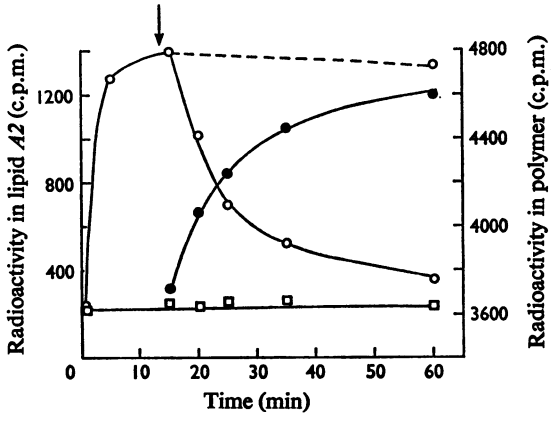


Fig. 6. *Examination of lipid A2 turnover by pulse-labelling with CDP-[^{14}C]glycerol*

The initial reaction mixtures contained 2ml of enzyme, 2.0μmol of CDP-[^{14}C]glycerol, 2.0μmol of UDP-glucose (or 0.2ml of water in the control), all in 10mM-$MgCl_2$, and was incubated at 37°C for 15min. Enzyme was recovered by centrifugation and was then resuspended in 1.5ml of tris–HCl–thioglycollate buffer, pH7.5, and was incubated with 2.0μmol of UDP-glucose and 2.0μmol of CDP-glycerol in 10mM-$MgCl_2$ in a total volume of 2.0ml at 37°C; 0.5ml samples were taken at the time-intervals shown for extraction of lipid and polymer as described in the Experimental section. The total lipid was degraded by controlled acid hydrolysis and the water-soluble products were separated by chromatography on Whatman no. 1 paper in solvent (i). Labelled products were located, and their radioactivity was measured by counting 1cm bands of the paper chromatograms. The figure shows the counts calculated for the starting volume of enzyme. There was no synthesis of labelled poly(glycerol phosphate glucose) in the control. o——o, Radioactivity in lipid *A2*; o----o, radioactivity in lipid *A2* from sample reincubated without substrates; ●——●, radioactivity in polymer; □——□, radioactivity in lipid *A2* in control. In this experiment 1000c.p.m. of radioactivity is equivalent to the incorporation of 1nmol of glucose or glycerol into lipid or polymer.

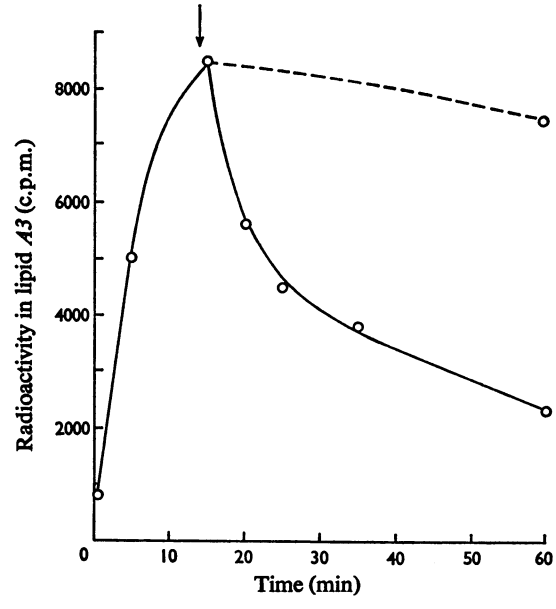


Fig. 7. *Examination of lipid A3 turnover by pulse-labelling with CDP-[^{14}C]glycerol*

The results are from the experiment illustrated in Fig. 6. A control, identical with the experimental reaction mixture of Fig. 6, except that in the second incubation UDP-glucose was omitted, gave an identical curve. Incorporation into poly(glycerol phosphate) during the first incubation was too high to permit accurate measurement of further teichoic acid synthesis during the incubation with unlabelled substrates. o——o, Radioactivity in lipid *A3*, calculated for the starting volume of enzyme; o----o, radioactivity in lipid *A3* from sample reincubated without substrates. In this experiment 1000c.p.m. of radioactivity is equivalent to the incorporation of 1nmol of glucose or glycerol into lipid or polymer.

been preincubated without UDP-glucose contained no labelled lipid *A2* and formed no labelled teichoic acid during the subsequent incubation with unlabelled substrates. An experiment with [^{32}P]CDP-glycerol had shown that a third phospholipid (lipid *A3*), carrying only a glycerol phosphate residue, was formed from CDP-glycerol. Fig. 7 shows that lipid *A3*, formed in the experiment illustrated by Fig. 6, also lost its radioactivity during the subsequent incubation. However, when the second incubation was done in the absence of UDP-glucose the rate of loss of radioactivity was unchanged. This provides strong evidence that lipid *A3* is not the source of the glycerol phosphate residues in lipid *A2*, a conclusion that is supported by the observation that labelled lipid *A3* was formed when the enzyme preparation was incubated with CDP-[^{14}C]glycerol but without UDP-glucose, and turned over normally in the subsequent incubation with unlabelled CDP-glycerol and UDP-glucose, without even the transient appearance of labelled lipid *A2*, as illustrated in Fig. 6.

The results indicate that lipids *A1* and *A2* are

consecutive intermediates in the synthesis of poly-(glycerol phosphate glucose). Lipid *A3* is not involved in the synthesis of this wall teichoic acid. It does turn over, however, in the presence of CDP-glycerol and is presumably an intermediate in the synthesis of poly-(glycerol phosphate). This conclusion has been established by Anderson *et al.* (1972).

Discussion

The participation of lipid intermediates in the synthesis of the poly(glycerol phosphate glucose) in *B. licheniformis* is interesting, since this is the first example of such a biosynthetic route for a teichoic acid in which glycosyl residues rather than glycosyl phosphate residues form a part of the main polymer chain. As the formation of the polymer chain must at some stage include the synthesis of glycosidic as well as phosphodiester linkages, the mechanism of its synthesis was not obvious. The present work has shown that the nucleotide precursors UDP-glucose and CDP-glycerol contribute glucosyl and glycerol phosphate residues respectively to the polymer, and that lipid intermediates containing these residues participate in the synthesis. Pulse-labelling experiments showed that there were two intermediates, one containing glucose and the other containing the repeating unit of the polymer, (glycerol phosphate glucose) (I). Despite the simultaneous glucosylation of diglycerides in the membrane preparation, it could be shown that the first intermediate in the synthesis of the polymer was a glucosylated lipid, and the second intermediate was a lipid that contained the complete repeating unit.

The observation that only glucose, and not its 1-phosphate, was transferred from UDP-glucose to the lipid intermediates excluded a mechanism, analogous to those for the synthesis of peptidoglycan or lipopolysaccharide, in which UDP-glucose would donate glucose 1-phosphate to a lipid monophosphate, giving (glucose pyrophosphate)-lipid. The alternative route, in which UDP-glucose donates a glucosyl residue to a lipid monophosphate giving (glucose phosphate)-lipid, is supported by a study of the structure of the lipid intermediates and by the failure of bacitracin to inhibit polymer synthesis (see Anderson *et al.*, 1972).

Hydrolysis of the two lipid intermediates with acid gave respectively glucose and (glycerol phosphate glucose), whereas hydrogenolysis of the glucosyl lipid with a platinum catalyst (Nikaido & Nikaido, 1971) gave glucose 1-phosphate. These observations confirm the conclusion that the intermediates are phosphodiesters rather than pyrophosphates. The structure of these intermediates, and the results of pulse-labelling experiments, suggest that the wall teichoic acid is synthesized by the route outlined in Scheme 1. The first intermediate, resulting from interaction between a lipid monophosphate and UDP-glucose, is the (glucose phosphate)-lipid. This reacts directly with CDP-glycerol to give the (glycerol phosphate glucose phosphate)-lipid that contains the repeating unit of the polymer. Although there is evidence for the formation of an additional lipid that contains glycerol phosphate, this was shown not to participate in the synthesis of the wall teichoic acid, and it is believed that this lipid derivative is a precursor of the poly(glycerol phosphate) that occurs in both the wall and membrane of this organism (Anderson *et al.*, 1972). The final transfer of repeating unit to the polymer chain effects regeneration of lipid monophosphate.

The nature of the lipid has not been established by direct examination, in view of the very small amounts of these intermediates in the membrane preparations. Nevertheless the ease of removal of lipid from its phosphorylated derivatives by either gentle acid hydrolysis or hydrogenolysis is consistent with the presence of an unsaturated centre in a β position to the phosphate, as required for a polyisoprenoid. If it can be assumed, by analogy with other bacterial systems, that the lipid intermediate in peptidoglycan synthesis in this organism is undecaprenol phosphate, then it can be concluded from the demonstration that the identical lipid phosphate molecules are shared in the biosynthesis of peptidoglycan and poly(glycerol phosphate glucose) (Anderson *et al.*, 1972) that the polyprenol phosphate described in the present work is undecaprenol phosphate.

Although the wall teichoic acid contains glycosyl and phosphodiester linkages in its chain, unlike the syntheses of other teichoic acids studied so far, the process of chain extension is a transglycosylation and not a transphosphorylation; the linkage formed in the attachment of repeating units to the growing chain is one between glucose and glycerol. For this reason we prefer to regard the polymer as a poly-(glycerol phosphate glucose), and not a poly-(glucosylglycerol phosphate). Transglycosylation through lipid intermediates has been described for the synthesis of lipopolysaccharides (Wright *et al.*, 1967), mannan (Scher *et al.*, 1968) and other polysaccharides from Gram-negative bacteria (Troy *et al.*, 1971; Sutherland & Norval, 1970). In the mannan synthesis the intermediate is mannose undecaprenol phosphate, and a glucose undecaprenol phosphate has been identified in the incorporation of glucose into lipopolysaccharide (Nikaido & Nikaido, 1971). In both this latter intermediate and the lipid intermediate *A1* from *B. licheniformis* the glucose 1-phosphate linkage appears to be in the β configuration, whereas in UDP-glucose and in the final polymers the glucose has the α configuration. The process of glucosylation via a lipid monophosphate glucose intermediate thus involves an inversion of configuration of the glucose at both glucose transfer steps.

The nature of chain initiation is not known. Although teichoic acids could not be detected in the clean membrane preparations (Hughes *et al.*, 1971), it is possible that very small amounts were present and these could participate as acceptors for chain elongation. The direction of chain extension is similarly unknown. In this connexion it is established that polysaccharide chains in a lipopolysaccharide extend by addition of glycosyl residues at their 'reducing end' (Wright *et al.*, 1967), whereas for poly-(glycerol phosphate) teichoic acids (Kennedy & Shaw, 1968) and for teichoic acids possessing sugar 1-phosphate groups in the chain, extension is at the opposite end.

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