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The Linkage of Sugar Phosphate Polymer to Peptidoglycan in Walls of *Micrococcus* sp. 2102

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1. Protein-free walls of *Micrococcus* sp. 2102 contain peptidoglycan, poly-(*N*-acetylglucosamine 1-phosphate) and small amounts of glycerol phosphate. 2. After destruction of the poly-(*N*-acetylglucosamine 1-phosphate) with periodate, the glycerol phosphate remains attached to the wall, but can be removed by controlled alkaline hydrolysis. The homogeneous product comprises a chain of three glycerol phosphates and an additional phosphate residue. 3. The poly-(*N*-acetylglucosamine 1-phosphate) is attached through its terminal phosphate to one end of the tri(glycerol phosphate). 4. The other end of the glycerol phosphate trimer is attached through its terminal phosphate to the 3- or 4-position of an *N*-acetylglucosamine. It is concluded that the sequence of residues in the sugar 1-phosphate polymer–peptidoglycan complex is: (*N*-acetylglucosamine 1-phosphate)$_{24}$-(glycerol phosphate)$_3$-*N*-acetylglucosamine 1-phosphate-muramic acid (in peptidoglycan). Thus in this organism the phosphorylated wall polymer is attached to the peptidoglycan of the wall through a linkage unit comprising a chain of three glycerol phosphate residues and an *N*-acetylglucosamine 1-phosphate, similar to or identical with the linkage unit in *Staphylococcus aureus* H.

The major components of the cell walls of most Gram-positive bacteria are teichoic acid and peptidoglycan (cf. Baddiley, 1972), and it has been known for a considerable time that these polymers are covalently attached to each other (Strominger & Ghuysen, 1963; Button *et al.*, 1966). In the earlier studies (Button *et al.*, 1966; Munoz *et al.*, 1967) muramic acid phosphate was reported after degradation of walls containing teichoic acid, and it therefore seemed possible that the linkage was through the terminal phosphate of the teichoic acid and a muramic acid residue in the peptidoglycan. Such a direct linkage, however, is not consistent with the ease of extraction of teichoic acids with acid and alkali.

Evidence for the nature of the linkage between the two polymers was obtained by Heckels *et al.* (1975) in a mutant of *Staphylococcus aureus* H lacking the usual *N*-acetylglucosaminyl substituents on the poly(ribitol phosphate) teichoic acid. It was found that the teichoic acid was attached through its terminal phosphate to a linkage unit containing a chain of three glycerol phosphate residues. Walls of the parent strain (Coley *et al.*, 1976) were shown to possess an identical unit. Subsequently, it was shown (Hancock & Baddiley, 1976; Bracha & Glaser, 1976*a*) that a precursor for the biosynthesis of this linkage unit in isolated membranes was CDP-glycerol, but that UDP-*N*-acetylglucosamine was also required. Moreover, the synthesis was inhibited by tunicamycin (Hancock *et al.*, 1976; Bracha & Glaser, 1976*b*), a known inhibitor of the biosynthesis of isoprenoid lipid intermediates containing *N*-acetylglucosamine (Takatsuki *et al.*, 1975). Thus it was possible that the linkage unit possessed an *N*-acetylglucosamine residue. This was supported by studies with [^{32}P]UDP-*N*-acetylglucosamine in wall-membrane preparations from *Bacillus licheniformis* (Wyke & Ward, 1977), where it was found that ^{32}P was incorporated into muramic acid phosphate. Wall lysates of *S. aureus* contain an excess of glucosamine over muramic acid (Bracha & Glaser, 1976*b*) consistent with the presence of 1.5–2.0 *N*-acetylglucosamine residues in the linkage unit. Subsequently in this laboratory it has been shown that the linkage unit in *S. aureus* H contains one *N*-acetylglucosamine residue interposed between the glycerol phosphate trimer and muramic acid phosphate (Coley *et al.*, 1977).

Micrococcus sp. 2102 contains in its wall a poly-(*N*-acetylglucosamine 1-phosphate) in which the position of attachment between repeating structures involves the phosphate residue and the 6-position of a neighbouring glucosamine residue (Archibald & Stafford, 1972). These walls contain small amounts of glycerol phosphate (Stafford, 1972), and toluene-treated whole cells incorporated into the wall glycerol

phosphate from CDP-glycerol (Hancock & Baddiley, 1976). The present paper shows that there is a linkage unit in *Micrococcus* sp. 2102, similar to that in *S. aureus* H, in which the wall polymer is attached to a tri(glycerol phosphate), which is in turn linked through an *N*-acetylglucosamine 1-phosphate residue to muramic acid in the peptidoglycan.

Experimental and Results

Materials

Flavobacter L-11 enzyme was a gift from Dr. S. Kotani, Department of Microbiology, Osaka University Dental School, Osaka, Japan. DEAE-cellulose was purchased from H. Reeve Angel, London E.C.4, U.K. Calf intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Biochemica glycerol test combination was obtained from Boehringer, Mannheim, Germany.

Paper chromatography and electrophoresis

Paper chromatography was carried out on Whatman no. 1 paper. For preparative work Whatman 3MM paper was washed with 1M-acetic acid and water before use. Development was by descending chromatography at room temperature (21°C) in the following solvent systems: A, propan-1-ol/aq. NH_3 (sp.gr. 0.88)/water (6:3:1, by vol.); B, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). Electrophoresis was carried out in either water/acetic acid/pyridine (43:2:5, by vol., pH5.3) or water/acetic acid/pyridine (225:1:25, by vol., pH6.5) with a potential gradient of 42V/cm for 1h.

Compounds were detected with the following spray reagents: molybdate for phosphate esters (Hanes & Isherwood, 1949); alkaline $AgNO_3$ for reducing compounds (Trevelyan *et al.*, 1950); periodate/Schiff for α-glycols (Baddiley *et al.*, 1956); ninhydrin for amino compounds (Consden & Gordon, 1948).

Analytical methods

Both total phosphate and P_i were determined by the method of Chen *et al.* (1956). Glycerol was determined enzymically with the Biochemica test combination or by the method of Hanahan & Olley (1958), which was also used to determine formaldehyde formed after oxidation with periodate. Hexosamines were determined by the method of Ghuysen *et al.* (1966); individual hexosamines were determined with a JEOL 5AH autoanalyser after hydrolysis at 100°C in 4M-HCl for 4h.

Growth of bacteria and isolation of cell walls

The bacteria were grown for 16h under the conditions described previously (Archibald & Stafford, 1972). Cells were disrupted by shaking for 4min with no. 11 Ballotini beads in a Braun disintegrator. Cell walls were recovered by centrifugation at 17000*g* for 30min. The walls were freed from membrane and other contaminants by treatment with trypsin, aq. 80% (w/v) phenol (Archibald & Stafford, 1972) and then pouring the wall suspension into an equal volume of boiling 2% (w/v) sodium dodecyl sulphate solution. The walls were finally washed five times with water; they contained 4.1% of phosphorus.

Oxidative removal of sugar phosphate polymer from the cell wall

Cell walls (650mg) were suspended in 0.1M-sodium acetate buffer, pH4.7 (250ml), containing 0.01M-$NaIO_4$ and stirred in the dark at room temperature (20°C) for 150h. Samples were removed for the determination of total phosphate. The wall residue was collected by centrifugation at 25000*g* for 30min and washed twice with water. The sediment was suspended in 0.25M-glycine buffer, adjusted to pH10.6 with NaOH, for 5h. After centrifugation at 25000*g* for 30min, the material was washed twice with water and analysed for phosphate. The sediment (380mg) contained 18% of the phosphate originally present in the wall.

Isolation of the glycerol phosphate derivative, compound A, from oxidized walls

A sample of the oxidized cell walls (100mg) was suspended in 0.5M-NaOH (25ml) and stirred at room temperature for 4h. Analysis of the supernatant obtained after centrifugation at 25000*g* for 30min showed that 73% of the phosphate had been solubilized by the treatment with alkali. Na^+ ions were removed from the supernatant by passage down a column (100mm×15mm) of Dowex 50 (NH_4^+ form), followed by rotary evaporation to dryness. Examination of the products by electrophoresis at pH5.3 showed a phosphate, $m_{\text{glycerol 1-phosphate}}$ 1.4 (compound A).

In an alternative procedure the wall residue (340mg) was suspended in 0.02M-Tris/HCl buffer, pH7.05 (50ml), containing 0.1% NaN_3 and *Flavobacter* L-11 enzyme (40mg) was added. The suspension was incubated at 37°C for 20h. The supernatant solution was diluted to 200ml with water and applied to a column (300mm×25mm) of DEAE-cellulose (Cl^- form). The column was washed with 2 bed vol. of water to remove any unadsorbed material, and products were eluted with a linear gradient of 0–0.7M-NaCl (500ml total volume); fractions (5ml) were collected and analysed for phosphate. All of the phosphate was represented as a single peak (0.4–0.55M-NaCl). Appropriate fractions were combined and the salt was removed by gel filtration on a column (600mm×15mm) of Sephadex G-25 with water as eluent. The glycan phosphate complex migrated on electrophoresis at pH5.3 as a single component, $m_{\text{glycerol 1-phosphate}}$ 0.69.

Samples of the above glycan phosphate complex were treated with 0.5M-NaOH (20ml) at room temperature for 2h. The solution was passed through a column (100mm×20mm) of Dowex 50 (NH_4^+ form) to remove Na^+ and evaporated to dryness *in vacuo*. Electrophoresis at pH5.3 gave a substance containing phosphate with $m_{\text{glycerol 1-phosphate}}$ 1.4 (compound A), which was purified by preparative electrophoresis.

Analysis of compound A

A sample (2μmol of phosphate) was hydrolysed in 2M-HCl at 100°C for 3h. Acid was removed over KOH *in vacuo*, and the products were examined by chromatography in solvent A and by electrophoresis at pH5.3; glycerol monophosphate(s) and glycerol diphosphate(s) were detected, together with a small amount of glycerol. Analysis of the acid hydrolysate, after enzymic dephosphorylation with alkaline phosphomonoesterase in 0.02M-$(NH_4)_2CO_3$, gave glycerol and phosphate in the molar ratio 0.77:1.0.

Treatment of a sample (2μmol of phosphate) with phosphomonoesterase in 0.02M-$(NH_4)_2CO_3$ (5ml) at 37°C for 16h yielded 48% of the phosphate as P_i. The product, on electrophoresis at pH5.3, had $m_{\text{glycerol 1-phosphate}}$ 1.2 and gave a positive reaction with the periodate/Schiff reagents. $(NH_4)_2CO_3$ was removed by rotary evaporation, and the residue dissolved in water. Samples were treated with 0.1M-$NaIO_4$ (0.2ml) for 10min at room temperature, further oxidation was stopped by the addition of 10% (w/v) sodium metabisulphite (0.2ml), and formaldehyde and phosphate were determined. The molar ratio of formaldehyde to phosphate was 0.96:1.0.

A further sample (5μmol of phosphate) was treated with the phosphomonoesterase, the solution diluted to 60ml with water and applied to a column (100mm×10mm) of DEAE-cellulose (acetate form). The products were eluted with a linear gradient of 0–1.0M-pyridine/acetate buffer, pH5.3 (150ml total volume); fractions (3ml) were collected and analysed for phosphate. Two peaks corresponding to phosphate were observed, the first representing P_i and the second organic phosphate. The fractions corresponding to the second peak were combined and the buffer was removed by rotary evaporation. This material was dissolved in water (5ml) containing 0.01M-$NaIO_4$ and the solution kept in the dark at room temperature for 1h. Ethylene glycol (10mg) was added and the solution was kept at room temperature for a further 1h to destroy the excess of $NaIO_4$. A 1% solution of freshly distilled dimethylhydrazine (1ml) was added, the pH adjusted to 6.0 with 1M-acetic acid and the solution incubated at 37°C for 16h. The solution was shaken with chloroform (10×10ml) and the aqueous phase was passed through a small column of Dowex 50 (H^+ form) and evaporated to dryness *in vacuo*. The material in the aqueous phase co-chromatographed with an authentic sample of glycerol diphosphate(s) both on electrophoresis and on paper chromatography in solvent B.

Formation and isolation of compound B

The sugar phosphate polymer was extracted from cell walls (500mg) with 0.5M-NaOH (40ml) at room temperature for 4h. The wall residue was removed by centrifugation at 25000*g* for 30min, and the supernatant solution was neutralized with 1M-HCl. Salt was removed by dialysis for 6h against several changes of water. The non-diffusible material was evaporated to dryness (rotary evaporation) and hydrolysed with 1M-NaOH (5ml) for 24h at 100°C. Na^+ ions were removed on a column (150mm×20mm) of Dowex (NH_4^+ form) and the solution was evaporated to dryness *in vacuo*. The residue was dissolved in water (5ml) and saturated $NaHCO_3$ solution (1ml) and 5% (v/v) acetic anhydride (1ml) were then added to *N*-acetylate the material. After standing at room temperature for 20min, acetic anhydride was destroyed by heating on a boiling-water bath for 4min. Na^+ was removed on a column of Dowex 50 (NH_4^+ form), the solution was diluted to 200ml and applied to a column (200mm×15mm) of DEAE-cellulose (acetate form). The column was washed with water (200ml) and a linear gradient of 0–1.0M-pyridine/acetate, pH5.3 (500ml total volume), applied. Fractions (5ml) were collected and analysed for phosphate (Fig. 1). Examination of material corresponding to peak 3 by electrophoresis at pH5.3 showed the presence of two phosphate-containing components, $m_{\text{glycerol 1-phosphate}}$ 1.0 and 1.2. The

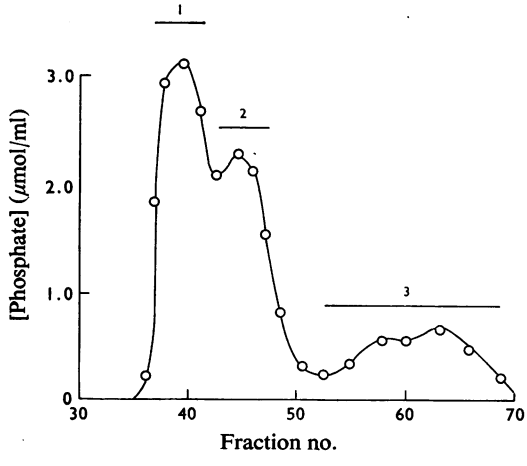

Fig. 1. *Fractionation of the products produced by alkaline hydrolysis of the sugar phosphate polymer*
The phosphates were eluted from DEAE-cellulose with pyridine/acetate buffer as described in the text.

material with the greater mobility, compound B, was isolated by preparative electrophoresis. Hydrolysis with 0.1M-HCl at 100°C for 15min gave *N*-acetylglucosamine 6-phosphate and glycerol diphosphate. After treatment with phosphomonoesterase and chromatography in solvent A, the products were P_i and material with $R_{glycerol\ 1\text{-}phosphate}$ 2.0 that contained phosphorus and reacted rapidly with the periodate/Schiff reagents giving a purple colour characteristic of formaldehyde. This product was isolated by preparative paper chromatography in solvent A. Hydrolysis in 0.1M-HCl at 100°C for 15min, followed by paper chromatography in solvent *B*, gave *N*-acetylglucosamine and glycerol monophosphate(s).

Controlled hydrolysis of walls with acid: isolation of compound C

Walls (590mg) were hydrolysed in 0.1M-HCl at 100°C for 15min, wall residue was removed by centrifugation at 17000*g* for 30min and the pellet washed twice with water. The pellet contained 3.9% of the phosphate in the walls before hydrolysis. The combined supernatant solutions were diluted to 100ml with water, applied to a DEAE-cellulose column (250mm×15mm, acetate form) and material was eluted with a linear gradient of 0–1.0M-pyridine/acetate, pH5.5. The phosphate elution profile is shown in Fig. 2. The fractions corresponding to peaks 1, 2, 3 and 4 were combined appropriately and evaporated to dryness *in vacuo*. These fractions account for 88.4, 2.4, 4.9 and 3.8% respectively of the total extractable phosphate.

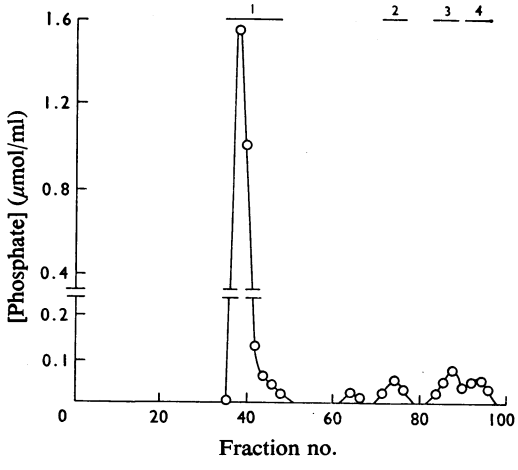

Fig. 2. *Fractionation of the phosphates produced on controlled acid hydrolysis of walls*
The phosphates were eluted from DEAE-cellulose with pyridine/acetate buffer as described in the text.

Electrophoresis on paper at pH6.5 showed the major fraction (fraction 1) to correspond to authentic *N*-acetylglucosamine 6-phosphate, with a trace of glycerol monophosphate(s). Fraction 2 contained only one phosphate, $m_{glycerol\ 1\text{-}phosphate}$ 0.70, whereas fractions 3 and 4 were mixtures of two phosphates, $m_{glycerol\ 1\text{-}phosphate}$ 0.80 and 1.14. These two fractions were combined and the components resolved by preparative paper electrophoresis. The component with the lower mobility is referred to as fraction 3 and that with the higher mobility as fraction 4. None of the fractions gave a ninhydrin reaction.

Hydrolysis of the four products in 2M-HCl at 100°C for 3h, removal of the acid over KOH pellets *in vacuo*, and subsequent electrophoresis at pH 6.5 showed the only phosphates from fractions 2, 3 and 4 corresponded to glycerol mono- and diphosphates. Fraction 1 yielded a ninhydrin-positive neutral phosphate corresponding to glucosamine 6-phosphate, and a trace of glycerol phosphate(s) and P_i. Although the spray reagents for phosphates revealed no neutral phosphates from fractions 2 and 3, the more sensitive ninhydrin reagent detected traces of neutral ninhydrin-positive material in these fractions, together with a major basic component corresponding to glucosamine and a trace of another ninhydrin-positive product with $m_{glucosamine}$ 1.22. The identity of the glucosamine was confirmed with the amino acid analyser and no other basic compounds were present. The faster-moving material was probably an acid-reversion product, since it was not seen in hydrolysates of fractions that had been reduced previously with KBH_4. A small amount of glucosamine was detected from fraction 1, but none of the ninhydrin-positive components were obtained from fraction 4, nor was glucosamine detected in hydrolysates of this fraction by the amino acid analyser. The following analysis shows that fraction 3 corresponds to compound C.

Samples of fractions 1, 2 and 3 (7.7–16.1 μmol of P) were reduced with 0.6ml of 0.1M-KBH_4 at room temperature during 4h. Acetic acid was added to adjust the pH to about 5, after which K^+ ions were removed on a Dowex 50 (NH_4^+ form) column (100mm×10mm) and the borate was removed by rotary evaporation four times with methanol *in vacuo*. The reduced samples were then hydrolysed in 2M-HCl at 100°C for 3h. Electrophoresis of the fractions at pH6.5 revealed a major product containing phosphate at the origin from fractions 1, 2 and 3. This product was ninhydrin-positive and rapidly yielded a purple colour with the periodate/Schiff reagents (formaldehyde production); it co-chromatographed with glucosaminitol phosphate in solvent A. A minor product with the mobility of glucosamine, but which also gave a purple colour rapidly with the periodate/Schiff reagent, was detected in all fractions.

The molar proportions of glycerol, phosphate and

glucosamine were determined in the four fractions (7–16 μmol of P) after hydrolysis with 2M-HCl and enzymic dephosphorylation in 0.2ml of 50mM-Tris/HCl buffer, pH8.0, at 37°C for 1h. The values are given in Table 1.

Examination of the wall residue after extraction of polymer and linkage unit with acid

The above controlled acid treatment removed 96.1% of the phosphate from the walls. The remaining phosphate was examined after hydrolysis of the residual wall (85mg) with 4M-HCl (5ml) at 100°C for 1h. Acid was removed over KOH pellets *in vacuo*, and the products were applied to a DEAE-cellulose column (250mm×15mm; acetate form) and eluted with a linear gradient of 0–1.0M-pyridine/acetate, pH5.5. About 95% of the phosphate was eluted between 0.55 and 0.7M-buffer. This fraction was evaporated to dryness *in vacuo* and a sample was examined by paper electrophoresis at pH6.5. The major phosphate reacted strongly with ninhydrin reagent and had $m_{\text{glycerol 1-phosphate}}$ 0.13; it was presumably incompletely hydrolysed glycan phosphate. The remainder of the fraction (2 μmol of P) was *N*-acetylated by dissolving it in 180 μl of water, to which was added 10 μl of saturated $NaHCO_3$ solution and 10 μl of acetic anhydride. After being kept at room temperature for 10min, excess of acetic anhydride was destroyed by heating at 100°C for 3min. The sample was then evaporated to dryness and hydrolysed in 4M-HCl at 100°C for 4h, the acid removed and the sample examined by paper electrophoresis at pH6.5. About equal amounts of two phosphates were observed, one at the origin and one with $m_{\text{glycerol 1-phosphate}}$ 0.69. After treatment with alkaline phosphatase, the latter product gave muramic acid, identified on the amino acid analyser; it comprised 43% of the phosphate in the fraction. The neutral phosphate reacted strongly with the ninhydrin reagent.

Table 1. *Molar proportions of components of fractions from controlled acid hydrolysis*

For experimental details see the text. The proportions are expressed relative to phosphate.

Fraction	Phosphate	Glycerol	Glucosamine
1	1.0	0.02	0.97
2	1.0	0.65	0.36
3	1.0	0.72	0.25
4	1.0	0.79	—

Discussion

The demonstration that in the walls of *Micrococcus* sp. 2102 there is a linkage unit joining the sugar phosphate polymer to the peptidoglycan follows from the isolation of such a unit, the determination of its structure and the proof that sugar phosphate polymer and peptidoglycan are attached to its opposite ends. The occurrence in this unit of a linear chain of three glycerol phosphate residues follows from the oxidative destruction of the main polymer chain with periodate, in a manner similar to that used in the earlier studies on *Staphylococcus aureus* H (Heckels *et al.*, 1975; Coley *et al.*, 1976). In the present case, however, the removal of the oxidized sugar phosphate polymer from the peptidoglycan required incubation at pH10.5 to achieve a β-elimination of the oxidized sugar residues.

The oxidation was carried out both on intact walls and after solubilization of the walls with *Flavobacter* L-11 peptidase (Kato *et al.*, 1962) to destroy peptide cross-linkages (Kato & Strominger, 1968). In both cases the glycerol phosphate residues remained attached to the glycan, but were readily removed as a homogeneous oligomer by treatment with 0.5M-NaOH at room temperature. This oligomer, compound A, had the structure shown in Fig. 3 and accounted for 73% of the phosphorus remaining in the oxidized walls after the β-elimination. It contained glycerol and phosphate only, in the molar ratio 0.77:1.0. On treatment with a phosphomonoesterase 50% of its phosphate was converted into P_i, and oxidation of the product with periodate indicated that it contained equal numbers of oxidizable glycerol and phosphate residues. When this oxidized product was treated with dimethylhydrazine in a Barry degradation (LeCocq & Ballou, 1964), glycerol diphosphate was produced. It follows that compound A must comprise a chain of three glycerol residues and four phosphate groups in which two

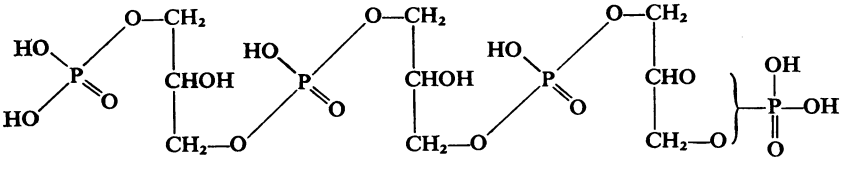

Fig. 3. *Compound A, tri(glycerol phosphate), obtained by oxidation of walls with IO_4^- and treatment with alkali at pH10.5*

The production of this demonstrates the presence of a chain of three glycerol phosphate residues in the linkage unit.

phosphates are monoesters and two are diesters. The diesters must be attached to primary hydroxy groups of two glycerol residues to account for the oxidation by periodate.

When the walls of this organisms were treated with 0.5M-NaOH at room temperature for 4h, the sugar phosphate polymer was removed, together with the glycerol phosphate, without degradation of the polymer chain. This behaviour is closely similar to that observed for the ribitol teichoic acid in parent and mutant strains of *S. aureus* H, and suggests that the glycerol phosphate oligomer is covalently attached to the sugar phosphate polymer. The attachment was examined directly by vigorous alkaline hydrolysis of the extracted polymeric material. It is well known that phosphodiesters are not hydrolysed in alkali unless there is a suitably placed hydroxy group in at least one of the substituent residues of the diester. In such cases alkali catalyses an intramolecular cyclization and simultaneous hydrolysis of one of the phosphate ester linkages; the resulting cyclic phosphate undergoes further hydrolysis to give a mixture of stable isomeric phosphomonoesters. If the *P*-terminus of the sugar phosphate polymer were attached to the tri(glycerol phosphate) through a phosphodiester linkage, then most of those linkages would be hydrolysed in alkali by cyclization on to the 2-hydroxyl group of the glycerol. However, the other phosphodiester linkages in the tri(glycerol phosphate) would also be hydrolysed by cyclic phosphate mechanisms, and during the alkali treatment a proportion of the glycerol residues should become substituted with an alkali-stable phosphomonoester residue at the 2-position. This would prevent further hydrolysis of the phosphate at the *P*-terminus of the sugar phosphate polymer. On the other hand, it is already known (Archibald & Stafford, 1972) that under vigorous conditions alkali degrades the polymer chain, apparently through a 4,6-cyclic phosphate intermediate. Thus hydrolysis in alkali should cause substantial chain degradation and hydrolysis to glycerol phosphates, together with the production of a small amount of a stable phosphodiester with the structure shown in Fig. 4.

In fact compound B, a minor product of alkaline hydrolysis, was isolated, and after re-*N*-acetylation the product was shown to have the structure given in Fig. 4. Extensive analysis was not possible on the small amount available, but controlled acid hydrolysis under conditions suitable for the hydrolysis of sugar 1-phosphates gave *N*-acetylglucosamine 6-phosphate and glycerol diphosphate(s). A part only of the phosphate in compound B could be converted into P_i with the phosphomonoesterase, and the product of this treatment, like the similar product previously isolated after appropriate degradation of the wall teichoic acid of *Staphylococcus lactis* 13 (Button *et al.*, 1966) gave *N*-acetylglucosamine and glycerol

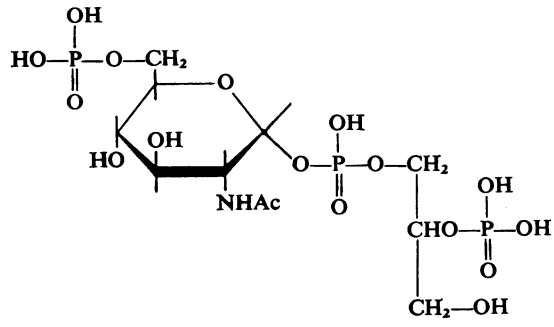

Fig. 4. *Compound B, obtained by vigorous hydrolysis of the walls with alkali, followed by N-acetylation*
This product shows the attachment of the *P*-terminal glucosamine phosphate residue of the polymer chain to glycerol in the linkage unit.

monophosphate(s) on hydrolysis in acid. This, and the behaviour of the compounds towards the periodate/Schiff reagents, confirm the structure of this degradation product. It follows that the glucosamine residue in compound B represents the *P*-terminal glucosamine in the sugar phosphate polymer, and this confirms the linkage of the polymer chain to the glycerol at one end of the linkage unit.

The ease with which the poly-(*N*-acetylglucosamine 1-phosphate)–tri(glycerol phosphate) complex is removed intact from the cell wall or from the soluble glycan is noteworthy. Teichoic acid–tri(glycerol phosphate) complexes are removed similarly by alkali in *S. aureus* and its mutant, where it has been shown (Coley *et al.*, 1977) that the alkali-labile linkage is that between a phosphate group on glycerol in ester linkage with the 4-hydroxyl group of an *N*-acetylglucosamine. Such a structure, in which the tri(glycerol phosphate) is attached to an *N*-acetylglucosamine, therefore seems likely in the present case, and would be consistent with the requirement for UDP-*N*-acetylglucosamine in the biosynthesis (Hancock & Baddiley, 1976). The presence of such an *N*-acetylglucosamine residue on the glycerol phosphate moiety furthest removed from that bearing the sugar phosphate chain was demonstrated by the isolation of compound C after hydrolysis of walls in 0.1M-HCl at 100°C for 15min. After this treatment 3.9% of the phosphate originally present remained bound to the wall. It is known (Archibald & Stafford, 1972) that the sugar phosphate polymer contains 25 repeating units and, together with the glycerol phosphate, this would account for 28 P atoms. Thus the phosphate remaining in the wall corresponds closely to that expected for one P atom (3.5%).

Chromatography of the products of the above acid treatment gave four fractions. The major component

(fraction 1) was *N*-acetylglucosamine 6-phosphate, arising from hydrolysis of the sugar phosphate polymer (see Table 1), together with a trace only of glycerol phosphate; it represented 88.4% of the extracted phosphate. The next in abundance (compound C, fraction 3, representing 4.9% of the extracted phosphate) contained *N*-acetylglucosamine, phosphate and glycerol in the molar proportions 1:4:3. The reducing group of the sugar residue in this compound was unsubstituted, and only one sugar residue was present. Reduction of compound C with borohydride and subsequent acid hydrolysis gave glycerol phosphates, glucosaminitol phosphate and a small amount of glucosaminitol, but no glucosamine. In contrast, acid hydrolysis of compound C itself gave glycerol phosphates and glucosamine, but no trace of glucosamine phosphate. This is the hydrolysis behaviour expected for the structure given in Fig. 5. Stereochemical arguments indicate that acid hydrolysis must occur preferentially through the fission of the P-O linkage between the 3- or 4-position on the sugar, with exclusive retention of phosphate on glycerol, whereas after reduction of the sugar residue to a linear *N*-acetylglucosaminitol, hydrolysis through a cyclic mechanism could occur in either direction and glucosaminitol phosphate would be an expected major product.

The hydrolysis properties of compound C and its reduction product demonstrate that the glycerol phosphate chain is attached to a hydroxyl group of a reducing *N*-acetylglucosamine. As this sugar residue had survived the periodate oxidation, it is likely that the tri(glycerol phosphate) substitutes the 3- or 4-hydroxyl group. It is noteworthy that in the linkage unit of *S. aureus* the tri(glycerol phosphate) occupies the 4-hydroxyl group, and this accounts for the observed alkali lability of the linkage between the sugar hydroxyl and phosphate (Coley *et al.*, 1977). The ease with which the sugar phosphate polymer–tri(glycerol phosphate) complex is removed from the walls of *Micrococcus* sp. 2102 suggests that this unusually labile P-amino sugar linkage also involves the 4-hydroxyl group; but the stability of a glycerol phosphate substituent at the 3-position on the

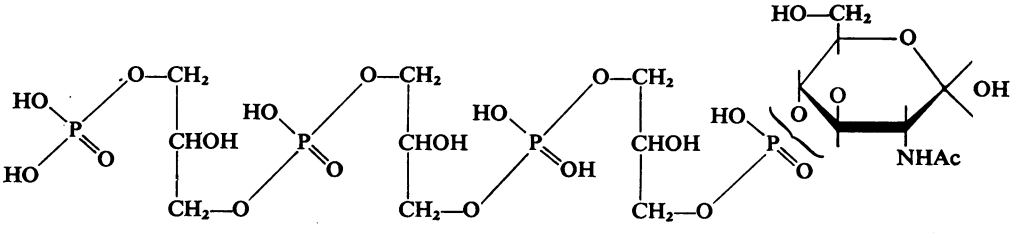

Fig. 5. *Compound C, obtained by controlled acid hydrolysis of walls, showing the attachment of the glycerol phosphate trimer to a reducing N-acetylglucosamine moiety*

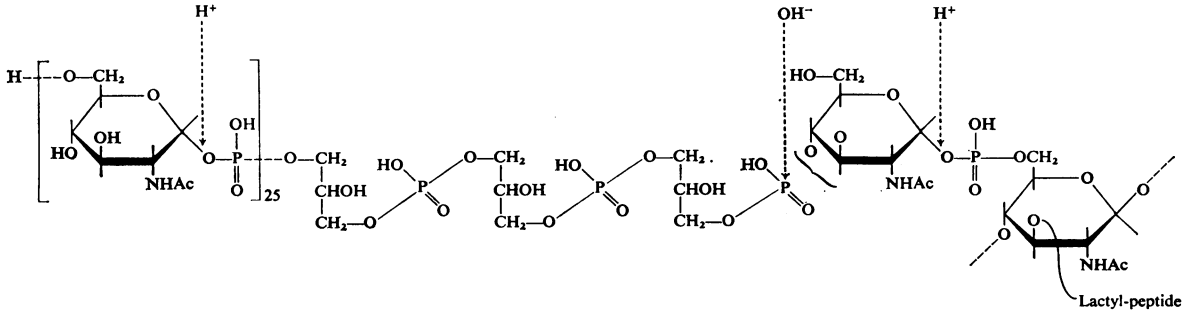

Fig. 6. *Arrangement of sugar phosphate polymer and linkage unit, and attachment to peptidoglycan in Micrococcus* sp. 2102
The phosphate linkages that are most labile towards acid (H^+) and alkali (OH^-) are marked by vertical lines.

amino sugar is not known, and this alternative position of attachment cannot at present be excluded.

Two other products (fractions 2 and 4, representing 2.4 and 3.8% respectively of the extracted phosphate) were obtained from the controlled acid hydrolysis of the cell walls. Fraction 2 contained *N*-acetylglucosamine, phosphate and glycerol in the proportions 1:3:2; it generally resembled compound C, but contained one less glycerol phosphate moiety. It is likely that this represents a partial degradation product of compound C, and its formation is in agreement with the presence of a small amount of glycerol phosphate as contaminant of Fraction 1. Fraction 4 contained no amino sugar and gave glycerol mono- and di-phosphates on acid hydrolysis. Although the characterization of this fragment was incomplete, its analysis and properties suggest that it is probably compound A, having arisen from compound C by loss of *N*-acetylglucosamine.

It is established then that the sugar phosphate polymer is attached through its *P*-terminus to a linear tri(glycerol phosphate), which is in ester linkage at its other end to the 3- or 4-hydroxyl group of an *N*-acetylglucosamine. The reducing group of this must be involved in linkage to the peptidoglycan, and the acid lability of this linkage, together with the observation that the wall residue after removal of this complex with acid still contains one phosphate group, suggests that the *N*-acetylglucosamine is attached as its 1-phosphate to the peptidoglycan (Fig. 6). In agreement with this it was shown that, after acid extraction of the sugar phosphate polymer–linkage unit complex, the residual walls gave muramic acid phosphate on vigorous acid hydrolysis; no glycerol phosphate was detected in such hydrolysates, and it is concluded that *N*-acetylglucosamine in the linkage unit is attached to muramic acid in the peptidoglycan through a phosphodiester linkage involving the 1-position of the *N*-acetylglucosamine and probably the 6-hydroxyl group of a muramic acid.

The conclusion that the structure of the wall complex is as shown in Fig. 6 is supported by quantitative data on the amount of phosphorus retained in the walls and recovered as linkage-unit components after acid extraction and after periodate oxidation. After the acid treatment the phosphate in fractions 2–4 represents 11.1% of the total phosphate extracted by the acid from the wall. This is, of course, a minimum value, since a little decomposition of linkage unit occurs giving glycerol phosphate found in fraction 1. The theoretical value for a linkage unit containing four phosphate groups in the structure as shown is 14.3%. Similarly, after the periodate-buffer treatment of the wall the proposed structure would result in five phosphate groups being retained in the wall, i.e. 17.2% of the original phosphate. In fact 18.0% of the phosphate remained, and 73% of this remainder was removed by alkali.

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