Studies on the Linkage between Teichoic Acid and Peptidoglycan in a Bacteriophage-Resistant Mutant of *Staphylococcus aureus* H

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1. In addition to poly(ribitol phosphate) the walls of a bacteriophage-resistant mutant of *Staphylococcus aureus* H contain glycerol phosphate residues that are not removed on digestion with trypsin or extraction with phenol. 2. The glycerol phosphate is present in a chain, containing three or four glycerol phosphate residues, which is covalently attached to the peptidoglycan through a phosphodiester linkage to muramic acid; this linkage is readily hydrolysed by dilute alkali. 3. The degradative studies described suggest that the poly(ribitol phosphate) chains of the wall teichoic acid may be attached to the wall by linkage to this glycerol phosphate oligomer.

Although a great deal is now known about the structure and biosynthesis of teichoic acid and peptidoglycan, there is still little information on the nature of the linkage between these polymers. It has long been known that teichoic acids are attached to the wall by covalent linkage to the glycan moiety of peptidoglycan (Strominger & Ghuyssen, 1963; Ghuyssen *et al.*, 1965; Button *et al.*, 1966; Grant & Wicken, 1968), and it seems likely that this attachment involves a linkage through a terminal phosphate group of teichoic acid to a muramic acid in the peptidoglycan, since muramic acid phosphate has been obtained by degradation of walls containing teichoic acid (Ågren & De Verdier, 1958; Button *et al.*, 1966; Liu & Gottschlich, 1967; Munoz *et al.*, 1967; Grant & Wicken, 1968).

Good evidence has been presented showing that certain wall polysaccharides are attached to peptidoglycan through a sugar 1-phosphate linkage between the reducing terminal sugar of the polysaccharide and a muramic acid moiety of the peptidoglycan (Knox & Hall, 1965; Knox & Holmwood, 1968; Hughes, 1970). So far, however, direct evidence for the nature of the linkage between teichoic acid and peptidoglycan has been obtained only for walls of *Staphylococcus lactis* I 3 (Button *et al.*, 1966). This teichoic acid was shown to be attached to the wall by covalent linkage between a glycerol phosphate residue and muramic acid. Demonstration of the nature of the linkage between ribitol teichoic acids and the cell wall has been complicated by the presence in such walls of small amounts of glycerol phosphate, the nature and origin of which was unknown. The bulk of the ribitol teichoic acid can be removed from cell walls by oxidation with periodate (cf. Hay *et al.*, 1965), but subsequent reduction of the residual wall with borohydride, as described in the present paper,

would convert the expected three-carbon fragment, derived from the oxidation of the phosphate-terminal ribitol residue, into a glycerol residue, and glycerol would also be formed from carbons 4, 5 and 6 of the sugar attached to the 4-position of the ribitol residue. To avoid this complication and to enable the identification of the glycerol phosphate component present in the wall, the present study has been carried out on a mutant of *Staphylococcus aureus* H, isolated and characterized by Dr. L. Julia Douglas, from which the usual *N*-acetylglucosaminyl substituents on the ribitol residues of the wall teichoic acid (Baddiley *et al.*, 1961; 1962*a,b*) are absent. Oxidation of this teichoic acid should give only two-carbon fragments which would be reduced to ethylene glycol and not to glycerol. As a further simplification we have also carried out the reduction with KB^3H_4 to ensure that any glycerol formed by reduction could be distinguished from that originally present in the wall. The present paper describes the characterization of the glycerol phosphate present in the walls of the mutant staphylococcus.

Materials and Methods

S. aureus H $\text{gol}^{-1}\phi^R 71$, a glycerol-requiring mutant resistant to bacteriophage 52A, was obtained from Dr. L. J. Douglas, Department of Microbiology, University of Glasgow, Glasgow, U.K. *Flavobacter* L-11 enzyme was a gift from Dr. S. Kotani, Department of Microbiology, Osaka University Dental School, Osaka, Japan. DEAE-cellulose, DE 52, and ECTEOLA-cellulose, ET-11, were purchased from H. Reeve Angel, London E.C.4, U.K. DEAE-Sephadex, A-50, was purchased from Pharmacia, Uppsala, Sweden. Calf intestinal phosphomonoesterase was purchased from Sigma Chemical Co.,

St. Louis, Mo., U.S.A. Biochemica Glycerol Test Combination was purchased from Boehringer, Mannheim, Germany.

Paper chromatography and electrophoresis

Paper chromatography was carried out on Whatman no. 1 paper. Development was by descending chromatography at room temperature in the following solvent systems: A, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.); B, butan-1-ol–ethanol–water–aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol., organic phase); C, ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.); D, butan-1-ol–acetic acid–pyridine (43:2:5, by vol., pH 5.3) with a potential gradient of 50 V/cm for 1 h.

Compounds were detected with the following 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).

Analytical methods

Phosphate was determined by the method of Chen *et al.* (1956). Hexosamines were determined by a modified Elson–Morgan method (Ghuysen *et al.*, 1966). Individual hexosamines and amino acids were determined with a Jeol-5AH autoanalyser after hydrolysis at 100°C in 4M-HCl for 4 h and in 6M-HCl for 16 h respectively. No correction was made for destruction during hydrolysis. Periodate was determined by the method of Aspinall & Ferrier (1957), and formaldehyde formed after oxidation was determined by the method of Hanahan & Olley (1958). Glycerol was determined enzymically with the Biochemical Test Combination.

Solutions containing radioactive materials were adjusted to 1.5 ml with water, and radioactivity was measured in a Beckman LS-150 liquid-scintillation counter in a scintillant (10 ml) composed of toluene (2 litres), 2,5-diphenyloxazole (8 g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.2 g). The counting efficiency, determined by using a $[^3\text{H}]$ -lysine standard, was 25%. The specific radioactivity of the KB^3H_4 solution was determined by reduction of a standard solution of glucose 6-phosphate, isolation of the radioactive glucitol phosphate and determination of the ratio $\text{P} : ^3\text{H}$.

Growth of bacteria and isolation of cell walls

The staphylococci were grown in Tryptic Soy Broth (Difco, Detroit, Mich., U.S.A.) containing 0.01% of glycerol, with forced aeration at 37°C for 18 h. A 10-litre batch inoculated with 500 ml of an overnight culture gave 30 g wet wt. of cells which were disrupted by shaking for 4 min with no. 11 Ballotini beads in a Braun disintegrator. Cell walls were recovered by centrifugation at 17000g for 30 min, suspended in water and heated on a boiling-water

bath for 15 min to inactivate autolytic enzymes. The walls were recovered by centrifugation, digested with trypsin and extracted with aq. 40% (w/v) phenol (Archibald & Stafford, 1972). The 'purified' cell walls contained 3.5% of phosphate.

Walls were prepared in a similar manner from the original strain of *S. aureus* H and from *Bacillus subtilis* W 23 grown in a chemostat under K^+ limitation at a dilution rate of 0.1 [see Tempest *et al.* (1968) for the medium].

Examination of cell walls

Cell walls (3.0 mg) were hydrolysed in 2M-HCl (1.0 ml) at 100°C for 3 h. After removal of acid by evaporation *in vacuo* over KOH, chromatographic examination showed that in addition to peptidoglycan components the major products were ribitol phosphates, anhydrosorbitol and P_i .

A further sample of cell walls (4 mg) was suspended in 0.5M-NaOH (0.5 ml) and heated on a boiling-water bath for 3 h. Na^+ ions were removed by passage through a column (20 mm $\times$ 10 mm) of Dowex 50 (NH_4^+ form) resin. After evaporation to dryness *in vacuo* the residue was dissolved in 0.1M- $(\text{NH}_4)_2\text{CO}_3$ solution (0.5 ml), containing phosphomonoesterase (0.2 mg), and incubated at 37°C for 16 h. The solution was evaporated to dryness and the residue chromatographed in solvents B and C. The only major product detected with the periodate–Schiff and AgNO_3 reagents was ribitol.

Oxidation of cell walls with sodium metaperiodate

Cell walls (50 mg) were suspended in 0.1M-sodium acetate buffer, pH 4.5, (25 ml) containing 0.01M- NaIO_4 and stirred in the dark at room temperature (20°C). Samples were removed at intervals and

Fig. 1. Extraction of phosphate and reduction of periodate during oxidation of walls in 0.01M- NaIO_4 .

Conditions were as described in text. □, % of total phosphate bound to walls; ●, mol of periodate reduced/mol of total phosphate.

Fig. 2. Fractionation of the phosphates produced on acid hydrolysis of oxidized walls

The phosphates were eluted from DEAE-cellulose with pyridine-acetate buffer as described in the text.

Fig. 3. Fractionation of the soluble products produced by the action of *Flavobacter* L-11 enzyme on oxidized and reduced walls

The products were eluted from ECTEOLA-cellulose with NaCl as described in the text. □, Phosphate; ○, hexosamine.

centrifuged at 25000g for 30 min. The supernatant solutions were analysed for periodate and the residues were washed twice with water and analysed for phosphate (Fig. 1). Oxidation was complete after 6 h when 2.3 mol of periodate had been reduced for each mol of phosphate. The wall residue contained 10% of the original phosphate.

A larger sample of cell walls (898 mg) was oxidized under similar conditions for 6 h. The cell wall residue was recovered by centrifugation, washed with water and freeze-dried. The oxidized cell walls (555 mg) contained 11% of the original phosphate.

Acid hydrolysis of oxidized cell walls

A sample of oxidized cell walls (250 mg) was hydrolysed in 2M-HCl (25 ml) at 100°C for 3 h. Acid

was removed *in vacuo* over KOH, the residue was dissolved in water (100 ml) and applied to a column (15 mm × 400 mm) of DEAE-cellulose (acetate form). Material was eluted with water (200 ml) followed by a linear gradient of 0–0.5M-pyridine-acetate buffer, pH 5.3 (700 ml). Fractions (14 ml) were collected and analysed for phosphate. Two major peaks corresponding to organic phosphates were observed (Fig. 2). Peak 1 represented 61% of the total phosphate and chromatography of this fraction in solvents A and C indicated that it contained components with the mobility and colour reactions of glycerol monophosphate(s) and muramic acid phosphate. Enzymic dephosphorylation followed by chromatography in solvents B and C showed that glycerol and muramic acid were the only products. Analysis of the dephosphorylated material indicated that glycerol and hexosamine were present in the molar ratio 1.0:0.6.

Peak 2 represented 34% of the original phosphate, and chromatography of this fraction revealed a single component with the mobility and colour reactions of glycerol diphosphate(s). Enzymic dephosphorylation gave glycerol. Thus acid hydrolysis of the oxidized cell walls gave glycerol monophosphate(s), muramic acid phosphate and glycerol diphosphate(s) in the molar proportions 1.0:0.60:0.45.

Enzymic digestion of oxidized cell walls

A sample of cell walls was oxidized with NaIO₄ as described above. The insoluble residue was stirred with 0.1M-NaBH₄ (40 ml) at 4°C for 4 h and recovered by centrifugation. No phosphorus was solubilized by the reduction procedure.

The reduced material was suspended in 0.02M-Tris-HCl buffer, pH 7.0 (50 ml), containing 0.1% (w/v) sodium azide, and *Flavobacter* L-11 enzyme (70 mg) was added. The suspension was incubated at 37°C for 30 h when the value of ϵ_{550} had fallen to 7% of its initial value. The insoluble residue was removed by centrifugation at 38000g for 1 h. The supernatant solution was diluted with water to 200 ml and applied to a column (400 mm × 15 mm) of ECTEOLA-cellulose (Cl⁻ form) which was then washed with water (200 ml) to remove material which was not absorbed. This material did not contain phosphate but gave both amino sugars and amino acids on hydrolysis in acid. The column was then washed with a linear gradient of 0–0.7M-NaCl (1 litre), fractions (10 ml) were collected and samples analysed for phosphate and for hexosamine after acid hydrolysis. Appropriate tube contents were combined (Fig. 3), dialysed against distilled water and freeze-dried to give three fractions representing 73% of the phosphate originally present in the cell wall residue. Fraction A contained hexosamine whereas

Table 1. *Compositions of the fractions obtained on enzymic hydrolysis of oxidized cell walls*

The units are $\mu\text{mol/g}$ of original walls. Further details are given in the text.

	Oxidized cell walls	Peak A	Peak B	Peak C
Lys	349	14.7	33.8	46.6
Mur	253	21.4	56.2	97
Glu	322	10.4	27.1	40.8
Gly	1524	48.1	103.3	121.6
Ala	734	25.2	50.8	87.6
Glucosamine	263	14.3	51.4	105.6
P	124	—	15.2	76.0

fractions B and C contained both phosphate and hexosamine. Samples from each fraction were hydrolysed and used for analysis of amino acids (Table 1). Fraction B before dialysis contained some P_i corresponding to 24% of the phosphate applied to the column, one-half of which originated from the enzyme preparation.

Further purification of fractions B and C

Fractions B and C were rechromatographed on DEAE-Sephadex as follows. The material was dissolved in 0.1M-NaCl (10ml) and applied to a column (200mm $\times$ 20mm) of DEAE-Sephadex A-50 that had been equilibrated with the same concentration of NaCl. Material was eluted with 40ml of 0.1M-NaCl followed by a linear gradient of 0.1–0.5M-NaCl (500ml). Fractions (10ml) were collected and analysed for phosphate and for hexosamine after acid hydrolysis. The elution profiles obtained are shown in Figs. 4(a) and 4(b). Fraction B was further resolved into two fractions. The first was combined with the glycopeptide fraction obtained from the ECTEOLA-cellulose column to give a single glycopeptide (fraction G) and the second gave a glycopeptide containing phosphate which represented 89% of the phosphate applied to the column (fraction GP1). Fraction C could not be resolved further and gave a second glycopeptide (fraction GP2) containing phosphate.

Composition of the glycopeptide fractions containing phosphate

Samples of fractions GP1 and GP2 (1 μmol of phosphate) were hydrolysed in 2M-HCl at 100°C for 3h. Acid was removed *in vacuo* and samples were chromatographed in solvents A and C. In both cases glucosamine, muramic acid, glycerol, glycerol mono- and di-phosphates and smaller amounts of two phosphates with the mobility of muramic acid phosphate and ethylene glycol phosphate were detected. The identities of the latter two phosphates were confirmed by electrophoresis in pyridine–

acetate buffer, pH5.3, at a potential gradient of 50 V/cm for 1h, with authentic samples as controls.

Further samples (1 μmol) were heated in 0.5M-NaOH (1ml) at 100°C for 3h. Na⁺ ions were removed by passage through a small column of Dowex 50 (NH₄⁺ form) resin followed by evaporation under reduced pressure. The residues were dissolved in 0.1M-(NH₄)₂CO₃ (0.5ml) and incubated with alkaline phosphatase (0.1mg) at 37°C for 16h. The solutions were evaporated to dryness three times, dissolved in water and chromatographed in solvent C. The only product observed from either fraction GP1 or GP2 was glycerol; no ethylene glycol was detected. A similar amount of authentic ethylene glycol phosphate was dephosphorylated by the same procedure; no ethylene glycol was observed, it presumably being lost owing to its relatively high volatility.

A further sample of each fraction was hydrolysed in 4M-HCl at 100°C for 4h. Acid was removed *in vacuo* and samples were analysed for phosphate, hexosamines and also for glycerol after enzymic dephosphorylation. Fraction GP1 contained phos-

Fig. 4. *Chromatography of fraction B (a) and fraction C (b) on DEAE-Sephadex under the conditions described in the text*

□, Phosphate; ○, hexosamine.

Table 2. *Composition of the glycopeptide fractions containing phosphate obtained after digestion of periodate-oxidized cell walls with Flavobacter L-11 enzyme*

Results are expressed in molar proportions.								
	P	Glycerol	Lys	Mur	Glu	Gly	Ala	Total hexosamine
Fraction GP1	1.00	0.92	1.27	2.65	1.10	4.80	2.65	5.70
Fraction GP2	1.00	0.85	0.51	1.68	0.42	1.92	—	3.50

Table 3. *Proportions of amino sugars, amino sugar alcohols and ³H-labelled neutral compounds produced on acid hydrolysis of labelled fraction GP1 and GP2 and of cell walls which had been reduced with KB³H₄*

Components were separated and analysed as described in the text. Results are quoted as molar proportions. N.D., Not detectable.

Sample	Unretarded material	Muramitol	Muramic acid	Glucosaminitol	Glucosamine	Chain length of glycan (as measured by the ratio total amino sugar alcohol/glucosamine)
³ H-labelled fraction GP1	0.043	0.029	0.962	0.073	1.00	10.3
³ H-labelled fraction GP2	0.084	0.029	0.810	0.061	1.00	11.6
Cell walls	N.D.	0.025	0.930	0.076	1.00	10.4

phate, glycerol, muramic acid and glucosamine in the molar proportions 1.00:0.92:2.65:2.50 respectively; fraction GP2 contained these components in the proportions 1.00:0.85:1.68:1.63 respectively. Both fractions also contained amino acids (Table 2).

A sample of each fraction (0.3 μ mol of phosphate) was dissolved in 0.1 M-(NH₄)₂CO₃ containing phosphomonoesterase and incubated at 37°C for 16 h. No detectable P_i was produced during this time.

Incorporation of ³H into oxidized cell walls

To label the aldehyde groups formed during periodate oxidation, a sample of oxidized cell walls was treated with KB³H₄ as follows. Oxidized cell walls (2.5 g) were suspended in 0.1 M-KB³H₄ (300 ml, 0.90 μ Ci/ μ mol) and stirred at 0°C for 4 h. Excess of borohydride was destroyed by the dropwise addition of acetic acid until the pH of the suspension had fallen to 6.5. The suspension was equilibrated for a further hour and the cell wall residue was recovered by centrifugation, washed three times with water and freeze-dried. A sample of cell walls (50 mg) that had not previously been oxidized was reduced in a similar manner.

The ³H-labelled cell wall residue was then digested with *Flavobacter* L-11 enzyme and fractions G, GP1 and GP2, containing ³H, were isolated by chromatography on ECTEOLA-cellulose and DEAE-Sephadex as described above. Analysis showed that the molar ratio of phosphate/reduced groups was 1.60:1 in fraction GP1 and 2.77:1 in fraction GP2.

Samples of each labelled fraction were subjected to electrophoresis in a potential gradient of 50 V/cm at pH 5.3 for 1 h. Fraction G gave a single radioactive spot with $m_{\text{glycerol 1-phosphate}}$ 0.20. Fractions GP1 and GP2 gave a single spot containing both ³H and phosphate with $m_{\text{glycerol 1-phosphate}}$ 0.52.

Composition of labelled fractions GP1 and GP2

Samples of fractions GP1 and GP2 containing 1–3 μ mol of hexosamine were hydrolysed in 4 M-HCl at 100°C for 4 h, and the acid was then removed *in vacuo* over KOH. The residue was dissolved in water (1 ml) and fractionated on a column (160 mm $\times$ 20 mm) of Dowex 50W (X8; 200–400 mesh) by the method of Hughes (1970). Samples were analysed for ³H and hexosamine. Fractions were combined appropriately and the relative amounts of muramitol, muramic acid, glucosaminitol and glucosamine were determined. Radioactive components unretarded by the column were also determined. The proportions present in fractions GP1, GP2 and in untreated cell walls are shown in Table 3. The identity of the amino sugar alcohols was confirmed by chromatography in solvent D.

Samples of the material that was not retarded by the column were chromatographed in solvents A and C and examined by electrophoresis at pH 5.3. The only radioactive product detected in fraction G was glycerol. Fractions GP1 and GP2 gave, in addition, ethylene glycol phosphate and a small amount of ethylene glycol.

Fig. 5. Chromatography of the products of gentle alkali hydrolysis of glycopeptide fractions GP1 (a) and GP2 (b) containing phosphate

Conditions were as described in the text. ○, Hexosamine; △, ^3H ; □, phosphate.

Action of dilute alkali on glycopeptides containing phosphate (GP1 and GP2)

Samples of fractions GP1 and GP2 labelled with ^3H were treated with 0.5M-NaOH (20ml) at room temperature for 30min. The solutions were passed through columns (60mm×20mm) of Dowex 50 (NH_4^+ form) resin to remove Na^+ and evaporated *in vacuo*. The residues were dissolved in water and applied to columns (80mm×15mm) of DEAE-cellulose (acetate form). The columns were washed with water (100ml), and material was eluted with a linear gradient of 0–0.5M-pyridine-acetate buffer, pH 5.3, (400ml). Fractions (8ml) were collected and analysed for ^3H , phosphate and also for hexosamine after acid hydrolysis (Figs. 5a and 5b). In each case a sharp peak corresponding to both phosphate and ^3H

was obtained, together with a broader peak representing hexosamine and ^3H . Appropriate fractions were combined to give two phosphate-containing fractions, P1 from GP1 and P2 from GP2.

Electrophoresis of both fractions at pH 5.3 gave a spot representing both phosphate and ^3H with $m_{\text{glycerol 1-phosphate}}$ 1.4 and a smaller amount of a radioactive product with $m_{\text{glycerol 1-phosphate}}$ 0.25.

Composition of fractions containing phosphate (P1 and P2)

Samples of fractions P1 and P2 (2μmol of phosphate) were hydrolysed in 2M-HCl at 100°C for 3h. Acid was removed *in vacuo* over KOH and then the residues were dissolved in water and subjected to chromatography in solvents A and C and to electrophoresis at pH 5.3. In both cases glycerol, glycerol mono- and di-phosphates and ethylene glycol phosphate were detected. Ethylene glycol phosphate was the major radioactive product, together with some ethylene glycol.

Further samples (1μmol of phosphate) of P1 and P2 were hydrolysed in 0.5M-NaOH (0.5ml) at 100°C for 3h. Na^+ was removed by passage through a small column of Dowex 50 (NH_4^+ form) resin followed by evaporation *in vacuo*. The residue was dissolved in 0.1M- $(\text{NH}_4)_2\text{CO}_3$ (0.5ml) containing phosphomonoesterase (0.1mg) and incubated at 37°C for 16h. The solutions were dried *in vacuo* three times, dissolved in water and chromatographed in solvent A. The only product detected with periodate-Schiff and AgNO_3 reagents was glycerol. No radioactive products were observed.

Sample of fractions P1 and P2 were analysed for phosphate, ^3H and also for glycerol after acid hydrolysis and enzymic dephosphorylation. The respective molar proportions were 1.0:0.37:0.72 for P1 and 1.0:0.31:0.70 for P2.

Both fractions (1.5μmol of phosphate) were treated with phosphomonoesterase (0.1mg) in 0.1M- $(\text{NH}_4)_2\text{CO}_3$ (0.5ml) at 37°C for 16h. $(\text{NH}_4)_2\text{CO}_3$ was removed by evaporation *in vacuo* and the residue was dissolved in water. Analysis showed that 22 and 31% of the phosphate had been converted into P_i in fractions P1 and P2 respectively. Samples of the dephosphorylated fractions were subjected to electrophoresis at pH 5.3; in both cases, in addition to a small amount of starting material, a single product was observed with $m_{\text{glycerol 1-phosphate}}$ 1.2 containing phosphate and ^3H and which, unlike the original material, rapidly gave a purple colour with the periodate-Schiff reagents (production of formaldehyde).

Samples of each fraction, before and after treatment with phosphatase, were oxidized in 0.05M- NaIO_4 (0.1ml) for 10min at room temperature. The oxidation mixtures were then analysed for formaldehyde; fractions P1 and P2 gave respectively 0.10

and 0.09 μmol of formaldehyde/ μmol of phosphate. After treatment with phosphatase, oxidation of fractions P1 and P2 gave respectively a further 0.93 and 0.89 μmol of formaldehyde/ μmol of P_i formed.

Samples of both fractions were freed in the following manner from the small amount of contaminating glycopeptide material from which they were not resolved by chromatography on DEAE-cellulose. The samples were subjected to electrophoresis as bands at pH 5.3 for 1 h at a potential gradient of 50 V/cm. The radioactive material was located and that with $m_{\text{glycerol 1-phosphate}}$ 1.4 was eluted from the paper with water. Analysis showed that phosphate and ^3H were present in the molar ratios 3.9:1.0 and 3.7:1.0 in the fractions derived from P1 and P2 respectively.

Determination of the chain length of the teichoic acid

The chain length of the teichoic acid was determined from the amount of formaldehyde produced on oxidation of cell walls with periodate. Preliminary experiments showed that untreated cell walls were capable of combining with formaldehyde, and so walls were first treated with 1-fluoro-2,4-dinitrobenzene to substitute the free amino groups that were responsible for this effect.

Cell walls (20 mg) were suspended in water (5 ml) containing NaHCO_3 (50 mg), and a 5% (v/v) solution of 1-fluoro-2,4-dinitrobenzene in ethanol (10 ml) was added. The suspension was stirred at 20°C for 5 h, cell walls were recovered by centrifugation, washed twice with acetone, six times with ether and dried *in vacuo*.

A sample of dinitrophenylated walls (4.9 mg) was suspended in water (4.9 ml) and samples were removed for determination of phosphate. Further samples (1.5 ml) were treated with 0.1 M- NaIO_4 (0.3 ml) at room temperature for 15 min. A solution (0.45 ml) of 30% (w/v) SnCl_2 in 0.1 M-HCl was added and the mixture was shaken well and centrifuged. Samples (0.4 ml) of the supernatant solution were placed in the outer cell of a Conway diffusion cell, and 0.2% chromotropic acid in 15 M- H_2SO_4 (0.8 ml) was placed in the inner well. The cell was covered and kept overnight for distillation of the formaldehyde. The contents of the inner well were then heated at 100°C for 30 min and value ε_{570} was compared with erythritol standards similarly treated. The molar ratio of phosphate/formaldehyde was 1.0:0.025, indicating an average chain length of 40 units.

Examination of the cell walls of S. aureus H and B. subtilis W23

Cell walls of *S. aureus* H (5 mg) were heated in 1 M-NaOH at 100°C for 3 h. Na^+ was removed by passage through a column (12 mm $\times$ 40 mm) of Dowex 50 (NH_4^+ form) resin. The eluate was dried *in vacuo* and the residue dissolved in 0.1 M- $(\text{NH}_4)_2\text{CO}_3$

(1.0 ml) containing phosphomonoesterase (0.2 mg) and then incubated at 37°C overnight. Ions were removed by passage through columns (12 mm $\times$ 30 mm) of Dowex 50 (NH_4^+ form) and Dowex 2 (CO_3^{2-} form) resins followed by evaporation *in vacuo* three times. The residue was dissolved in water (50 μl) and samples (10 μl) were chromatographed in solvent C. The periodate-Schiff spray gave a strong spot corresponding to glucosaminylribitol and a weaker spot corresponding to glycerol.

Cell walls of *B. subtilis* W 23 treated under the same conditions produced a large amount of glucosyl-ribitol together with a small amount of glycerol.

Discussion

The approach adopted in the present study was to remove the ribitol teichoic acid from the cell walls selectively by oxidation with sodium metaperiodate so that the remaining phosphorylated components might be more readily isolated and identified. Unless special attention is paid to the removal of membrane contaminants wall preparations often contain substantial amounts of membrane teichoic acid. Accordingly the walls used in the present study were digested with trypsin and then extracted with aqueous phenol. This treatment greatly decreased the amount of glycerol phosphate compounds in the wall, although approx. 7.5% of the phosphate in the treated walls was still present as glycerol phosphate. Small amounts of glycerol phosphate were also found in similarly treated walls of *S. aureus* H and *B. subtilis* W 23 and have previously been found in walls of *S. lactis* 2102 and of other bacteria that contain ribitol teichoic acids (Archibald & Stafford, 1972; Stafford, 1972; Davey, 1968). The significance of this phosphate associated with glycerol has been uncertain, since it might have been present as a separate glycerol teichoic acid component of the wall or as membrane teichoic acid that had been incompletely removed from the walls. The present study has shown that the glycerol phosphate residues in trypsin- and phenol-digested walls of *S. aureus* Hg $^{ol-1}$ ϕ^R 71 are covalently attached to the peptidoglycan component of the wall.

The walls contain 3.5% of phosphorus, most of which is present as poly(ribitol phosphate). The chain length of the teichoic acid was determined by measurement of the amount of formaldehyde produced by oxidation with periodate. The only residue in the ribitol teichoic acid which could give formaldehyde is the terminal ribitol residue and on the assumption that this is the only residue in the wall which can give formaldehyde, the teichoic acid must contain 40 alditol phosphate units. This value is greater than that found by Hay *et al.* (1965) for *S. aureus* H but closely similar to that found by Ghuysen *et al.* (1965) for the wall teichoic acid of *S. aureus* Copenhagen.

After oxidation with periodate the walls contain 10–11% of the original phosphate, corresponding to approximately four residual phosphates for each teichoic acid chain. Acid hydrolysis of these oxidized walls gave only the mono- and di-phosphates of glycerol and muramic acid phosphate.

A sample of oxidized walls was reduced with NaBH_4 and then digested by incubation with the *Flavobacter* L-11 peptidase (Kato *et al.*, 1962), which hydrolyses peptide cross-linkages in the peptidoglycan (Kato & Strominger, 1968), to give a mixture of soluble products which was fractionated by ion-exchange chromatography. A sharp separation of products was not obtained (Fig. 3) but the material which was not retained by the column and the first fraction (A) eluted with buffer both contained glycopeptides that did not possess phosphate groups. The organic phosphate was eluted in a broad range containing a narrow fraction of P_i . Two fractions, B and C, were collected (Fig. 3). About one-half of the P_i originated from the *Flavobacter* peptidase preparation; the remainder of the P_i , about 12% of the total phosphate in the oxidized walls, may have been produced by hydrolysis of phosphomonoester residues by the phosphomonoesterase activity present in the *Flavobacter* enzyme preparation. It is noteworthy that acid hydrolysis of oxidized walls gave almost twice as much muramic acid phosphate as would be expected from the structure shown in Fig. 7. Possibly the walls contain a small amount of muramic acid phosphate residues in which the phosphate is present as phosphomonoester.

After dialysis the glycopeptide fractions containing organic phosphate (B and C, Fig. 3) were further fractionated by ion-exchange chromatography which resolved fraction B into phosphate-free glycopeptide and glycopeptide containing phosphate (fraction GP1, Fig. 4a). No phosphate-free glycopeptide was obtained by further chromatography of fraction C which showed a broad tailing peak; tube contents were combined to give a second glycopeptide fraction containing phosphate (fraction GP2, Fig. 4b). Acid hydrolysis of both of these fractions (GP1 and GP2) gave glycerol and its mono- and di-phosphates, muramic acid phosphate, ethylene glycol phosphate and the glycopeptide components shown in Table 2. In neither fraction could phosphomonoester groups be detected and the component(s) containing glycerol phosphate and ethylene glycol phosphate residues are presumably attached to the glycopeptide by linkage through muramic acid phosphate.

Further information on the structure of these glycopeptide fractions containing phosphate was obtained by reduction of oxidized walls with KB^3H_4 ; this permits the identification and quantification of the reduced aldehydic groups. After enzymic dissolution and fractionation as before two radioactive glycopeptide fractions containing phosphate were

isolated. These correspond to fractions GP1 and GP2 and on acid hydrolysis they gave tritium-labelled glucosaminitol and muramitol (Table 3), derived from the reducing ends of the glycan chains, tritium-labelled ethylene glycol and ethylene glycol phosphate, derived from glycolaldehyde phosphate formed during periodate oxidation, and tritium-labelled glycerol. No tritium-labelled glycerol phosphate was formed and so it is likely that the radioactive glycerol was formed by reduction of a glyceraldehyde residue which was not directly linked to phosphate; it is likely that this glyceraldehyde residue is that which would be formed on oxidation of the non-reducing terminal glucosamine residues of the glycan. On paper electrophoresis at pH 5.3 phosphate-free glycopeptide migrated as a single component with $m_{\text{glycerol 1-phosphate}}$ 0.2, whereas the glycopeptide fractions containing phosphate each migrated as single components containing both phosphate and ^3H with $m_{\text{glycerol 1-phosphate}}$ 0.52.

Gentle treatment of the glycopeptide fractions containing phosphate with dilute NaOH resulted in hydrolysis of the phosphate from the glycan. Electrophoresis of the products (P1 and P2) from both fractions revealed a small amount of a radioactive component with $m_{\text{glycerol 1-phosphate}}$ 0.25; this did not contain phosphate and corresponds to glycopeptide. The major component in both cases had $m_{\text{glycerol 1-phosphate}}$ 1.4 and contained ^3H and all of the phosphate. This phosphorylated component was isolated from both glycopeptide fractions by ion-exchange chromatography when sharp peaks were observed; these were, however, incompletely separated from the broad glycopeptide peaks (Figs. 5a and 5b). Acid hydrolysis of the phosphorylated material from both fractions gave ethylene glycol phosphate and a small amount of ethylene glycol; no other radioactive products were detected. Glycerol and its mono- and di-phosphates were also produced, but no muramic acid phosphate was detected and only traces of components characteristic of glycopeptide were produced. The phosphorylated material from each fraction gave a single component with $m_{\text{glycerol 1-phosphate}}$ 1.4 on electrophoresis, and after incubation with phosphomonoesterase each gave a single component with $m_{\text{glycerol 1-phosphate}}$ 1.2 which, unlike the starting material, rapidly gave a purple colour with the periodate-Schiff reagent (a characteristic of formaldehyde production). These observations show that treatment of the glycopeptide fractions containing phosphate (GP1 and GP2) with dilute alkali removes all of the phosphate as a discrete component and that this same component is released from the two fractions.

Since the phosphates obtained from the glycopeptide fractions containing phosphate were contaminated by a small amount of glycopeptide, samples of each were purified by preparative electro-

phoresis in order to determine accurately the amounts of ethylene glycol and phosphate present. Phosphate P1 contained ethylene glycol and phosphate in the molar ratio 1:3.9 and phosphate P2 contained ethylene glycol and phosphate in the molar ratio 1:3.7. On incubation with phosphatase, phosphates P1 and P2 gave 22 and 31 % of P_i respectively. These values, together with the other analytical data, are consistent with the structure shown in Fig. 6, in which ethylene glycol phosphate is attached to a chain containing three glycerol phosphate residues and which terminates in a phosphomonoester residue. This phosphomonoester group is produced by the action of alkali on the glycopeptide phosphates and, since muramic acid phosphate is produced on acid hydrolysis of the glycopeptide containing phosphate but not on acid hydrolysis of the products of alkali cleavage (P1 and P2), it follows that this phosphomonoester group is formed by hydrolysis of a phosphodiester linkage between the terminal glycerol residue and the muramic acid. The position of substitution of the phosphodiester linkages on adjacent glycerol residues has not been determined. However, the terminal glycerol residue must be attached to the rest of the chain by a phosphodiester linkage to a primary hydroxyl group since formaldehyde is formed on periodate oxidation of the oligomer after enzymic removal of its phosphomonoester group.

Since phosphates P1 and P2 represent all of the organic phosphorus in the *Flavobacter* digest of oxidized walls, and since this is present in alditol phosphate chains that contain four phosphate groups, it follows that no phosphorylated glycan molecule in the *Flavobacter* digest can be attached to fewer than four phosphate groups. Independent support for this conclusion was obtained by analysis of the glycopeptide fractions containing phosphate. Thus the glycopeptide fraction containing phosphate (GP1) contains phosphate and *N*-acetylglucosamine in the ratio 1:2.5. Measurement of the reducing terminal glucosamine and muramic acid residues in this fraction shows that the glycan chains contain 10 disaccharide units. Each glycan chain is thus associated with four phosphate groups.

The glycopeptide fraction containing phosphate (GP2) contains more than four phosphates in each chain and presumably consists of a mixture of glycan chains containing one unit of four phosphates and of glycan chains containing two or more such units.

The structural sequence containing the phosphate attached to glycan chains comprises one ethylene glycol phosphate residue combined with three glycerol phosphate residues (Fig. 6). The ethylene glycol is formed by reduction of a glycoaldehyde residue formed on oxidation of the walls with periodate. The present results do not permit the direct identification

Fig. 6. Structure of the phosphate isolated after gentle alkali hydrolysis of the glycopeptide fractions containing phosphate

Fig. 7. Possible scheme for linkage of ribitol teichoic acid to peptidoglycan which would account for the labelled ethylene glycol-glycerol phosphate oligomer isolated from the glycopeptide fractions that contain phosphate

of the component which gives rise to this ethylene glycol and which is therefore attached to the glycerol phosphate chain. The component might be a fourth glycerol residue and hence the walls would contain a chain of four glycerol phosphate residues in addition to a separately attached poly(ribitol phosphate). In this case, however, during treatment of walls with periodate, oxidation of this fourth glycerol residue would give an amount of formaldehyde equal to most or all of that actually observed to be produced on oxidation of the wall, though formaldehyde should also be produced on oxidation of the terminal ribitol unit of the ribitol teichoic acid. An alternative possibility is that the ethylene glycol attached to the glycerol phosphate oligomer is derived from the oxidation of the phosphate-terminal ribitol unit of the poly(ribitol phosphate). Provided that the component to which this is linked is not itself removed from the wall by periodate oxidation then ethylene glycol phosphate would be formed from this terminal ribitol phosphate unit. Since the only ethylene glycol phosphate found in the oxidized and reduced walls is that associated with the glycerol phosphate chain, it seems possible that the poly(ribitol phosphate) is attached directly to the chain containing three glycerol phosphate residues (Fig. 7). Thus the chain of three glycerol phosphate residues would be interposed between the ribitol teichoic acid and the glycan.

It is perhaps significant that a glycerol phosphate residue has been reported at the point of linkage between C-polysaccharide and peptidoglycan in walls of *Streptococcus pyogenes* (Heymann *et al.*, 1967) and between teichoic acid and peptidoglycan in walls of *S. lactis* I 3 (Button *et al.*, 1966). Fiedler & Glaser (1974) have found that a lipoteichoic acid carrier is involved in the biosynthesis of poly(ribitol phosphate) teichoic acid in *S. aureus* H. It is thought that after assembly the poly(ribitol phosphate) is transferred from the lipoteichoic acid carrier (which possesses a glycerol phosphate chain) to the glycan. One explanation of our finding that the walls of a mutant of this organism contain covalently bound glycerol phosphate could be that some of the glycerol phosphate residues are transferred to the glycan together with the poly(ribitol phosphate).

The phosphodiester linkage between muramic acid and the terminal glycerol moiety of the glycerol phosphate chain in walls of *S. aureus* is preferentially hydrolysed on treatment with dilute alkali. Although we do not know why this phosphodiester linkage to muramic acid is more labile towards alkali than are the other phosphodiester linkages in the glycerol phosphate chain, it has been shown that the alkalilability of phosphodiesters varies greatly according to their environment. Thus we have found that the phosphodiester linkage between glycerol and the 4-hydroxyl group of *N*-acetylglucosamine in the wall

teichoic acid of *S. lactis* I 3 is much more labile towards alkali than is the phosphodiester linkage between the 1-position of *N*-acetylglucosamine and glycerol in this same teichoic acid; it is also more labile than those in poly(ribitol phosphate) and poly(glycerol phosphate) teichoic acids (Archibald *et al.*, 1971). These differences might well arise through steric factors and a similar explanation could be advanced in the present case.

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