The Molecular Structure of Bacterial Walls

THE SIZE OF RIBITOL TEICHOIC ACIDS AND THE NATURE OF THEIR LINKAGE TO GLYCOSAMINOPEPTIDES

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1. Ribitol teichoic acids prepared by fractional precipitation of trichloroacetic acid extracts of bacterial cell walls are essentially undegraded and have similar chain length to the teichoic acid originally present in the walls. 2. The chain length of teichoic acid can be determined directly, without prior extraction from the wall. Accurate values have been obtained by measurement of the formaldehyde produced by oxidation of walls with periodate. Less accurate values have been derived from the amount of inorganic phosphate formed by heating walls at pH 4. 3. The relative amounts of *N*-acetylglucosaminyribitol and its mono- and di-phosphates produced by heating walls of *Staphylococcus aureus* with alkali agree with the amounts calculated for the hydrolysis of teichoic acid having the chain length determined by other methods. 4. Chemical considerations indicate that the linkage between teichoic acid and the wall may involve a phosphoramidate bond between the terminal phosphate of the teichoic acid and one of the amino groups in the glycosaminopeptide.

Studies on the products of enzymic degradation of cell walls of *Bacillus subtilis* and *Staphylococcus aureus* indicate that the ribitol teichoic acid in these organisms is held to the wall by a covalent linkage involving the terminal phosphate group of the teichoic acid chain (Strominger & Ghuyssen, 1963; Young, Tipper & Strominger, 1964). The teichoic acid prepared by enzymic degradation of walls from *S. aureus* appeared to be considerably larger than that prepared by extraction of the walls with acid, and it seemed possible that the conditions of extraction might always cause some degradation of the teichoic acid chain. As no phosphomonoester group was detected in the enzymic product, it appears that the linkage to the wall must involve the terminal phosphate group of the teichoic acid, and that there is therefore only one such linkage to each molecule. It is clearly desirable to establish the molecular size of the teichoic acid in the wall so that information can be obtained on the number and nature of linkages to be expected between the two major wall polymers. We have therefore re-examined the evidence on which were based the published values for chain lengths of the ribitol teichoic acids studied previously in these Laboratories. In addition, methods have been developed for the direct examination of teichoic acids in intact cell walls; the results are in accord with the values

previously reported. In the course of this work the reaction of walls to acid and alkali has been studied, and it is possible to suggest the chemical nature of the linkage between teichoic acid and the glycosaminopeptide in the cell wall. A preliminary account of part of the work on the chain length of teichoic acid has been published (Hay, Davey, Archibald & Baddiley, 1965).

EXPERIMENTAL AND RESULTS

Preparation of cell walls. *Lactobacillus arabinosus* 17-5 and *Staphylococcus aureus* H were grown, and cell walls prepared, as previously described (Archibald, Baddiley & Buchanan, 1961; Baddiley, Buchanan, RajBhandary & Sanderson, 1962).

Analytical methods. Phosphate was determined by the method of Chen, Toribara & Warner (1956). Reducing sugars were determined by the method of Park & Johnson (1949). Formaldehyde was measured colorimetrically either directly (Hough, Powell & Woods, 1956) or by Conway diffusion (Hollander, DiMauro & Pearson, 1951). Calf-intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Paper chromatography. Paper chromatography was carried out on Whatman no. 1 or 4 paper. The following solvent systems were used: *A*, propan-1-ol-ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, butan-1-ol-ethanol-water-ammonia (sp.gr. 0.88) 40:10:49:1, by vol.) (organic phase) (Hirst, Hough &

Jones, 1949); *C*, butanol-acetic acid-water (4:1:5, by vol.) (top layer) (Partridge, 1948); *D*, butanol-pyridine-water-benzene (5:3:3:1, by vol.) (top layer) (Gaillard, 1953).

The products were detected by the following spray reagents: periodate-Schiff for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); molybdate for phosphoric esters (Hanes & Isherwood, 1949); alkaline silver nitrate for sugars and polyols (Trevelyan, Procter & Harrison, 1950). When quantitative measurements were carried out on materials eluted from paper chromatograms appropriate determinations on equivalent paper extracts were made.

Extraction of cell walls. The rate of extraction of phosphorus was studied with various reagents. In some cases walls were pretreated with aq. 2*N*-ammonia solution (2 hr., 28°) and washed with water; this removed the alanine ester residues. In most experiments walls were suspended (5 mg. of wall/ml. of reagent), shaken gently and then samples were removed for phosphorus analysis. Further samples were centrifuged to remove wall residues and portions of the supernatant solutions were analysed for phosphorus.

With saturated lithium thiocyanate solution at 28° 4-7% of the total phosphorus was extracted during 24 hr. from the walls of both organisms before or after removal of alanine residues. With 8*M*-urea solution, 1-3% of the total phosphorus was removed during 24 hr. With 10% (w/v) trichloroacetic acid at 4° the phosphorus extracted from untreated walls of *L. arabinosus* and walls from which alanine had been removed was 26.2 and 36.4% of the total during 24 hr., 46.8 and 61.6% during 48 hr. and 65.7 and 72.0% during 5 days. A further sample of walls was treated as above with trichloroacetic acid after prolonged (6 hr.) treatment with aq. 2*N*-ammonia solution; the rate of extraction was the same as that observed after treatment with ammonia for 2 hr. Similar small increases in the rate of extraction with trichloroacetic acid were observed after pretreatment of walls with buffers at pH 8.3, 9.3 and 10.0; no increase was observed after pretreatment with 1% sodium chloride solution, 1% EDTA or acetate buffer, pH 4.6.

L. arabinosus walls were suspended in water at 100°. The amount of phosphorus extracted was 5% of the total after 5 min., 11% after 30 min. and 19% after 90 min. The rate of extraction from walls from which alanine ester groups had been removed was the same as from untreated walls. Phosphorus was slowly extracted during the treatment with aq. 2*N*-ammonia solution; amounts extracted were, after 3 hr. 4.4%, after 6 hr. 7.4%, after 30 hr. 20.4%, after 48 hr. 26.5% and after 72 hr. 32.1% of the total.

Prolonged treatment of teichoic acid with trichloroacetic acid. Teichoic acid (equivalent to 1 mg. of phosphorus) from *S. aureus* was dissolved in 10% (w/v) trichloroacetic acid (4 ml.) and the solution was kept at 4° for 5 days. Trichloroacetic acid was then removed by repeated extraction with ether, the pH was adjusted to 6.5 with dilute ammonia solution and the solution was freeze-dried. The residue was dissolved in water (5 ml.) and a portion (4 ml.) was mixed with an equal volume of aq. 4*N*-ammonia solution and kept for 2 hr. at 28° to remove the alanine ester residues. The solution was evaporated to dryness *in vacuo* and the residue was dissolved in water (0.5 ml.) and passed through a column of Dowex 50 W (H^+ form) resin (2 ml.) which was then washed with water until the eluate was neutral. Combined eluate and washings (20 ml.) were titrated with 0.1*N*-sodium

hydroxide (carbonate-free), giving the ratio primary:secondary acid groups as 4.6:1. A portion (0.5 ml.) of this solution was mixed with sodium metaperiodate solution and the formaldehyde produced was determined. Complete oxidation (24 hr.) resulted in the production of 1.0 mol.prop. of formaldehyde to 7 mol.prop. of phosphorus. The original teichoic acid produced on oxidation 1.0 mol.prop. of formaldehyde to 7 mol.prop. of phosphorus, and on titration gave the ratio primary:secondary acidic groups as 7.7:1.

Oxidation of teichoic acid and cell walls with sodium metaperiodate. The alanine ester groups in the teichoic acid and cell-wall samples were removed by treatment with ammonia; teichoic acid solutions were then passed through columns (4 ml.) of Dowex 50 W (H^+ form) resin and cell walls were washed three times with aq. 1% sodium chloride solution. Cell walls were then treated with fluorodinitrobenzene (Ingram & Salton, 1957) to prevent Schiff-base formation between the liberated formaldehyde and free amino groups present in the wall. Samples of teichoic acid and of cell walls were suspended in 0.01*M*-sodium metaperiodate (10 μ g. of phosphorus/ml.) and shaken gently in the dark at room temperature for 24 or 48 hr. Samples (0.1 ml.) were removed for phosphate analysis. Further samples (1 ml.) were mixed with a solution (0.3 ml.) of stannous chloride in 0.1*N*-hydrochloric acid (2 g./5 ml.), centrifuged and portions (0.4 ml.) of the supernatant solution removed for analysis of formaldehyde by Conway diffusion. Formaldehyde production was complete after 24 hr. In control experiments oxidation of walls from which all teichoic acid had been extracted and of purified samples of extracted polysaccharide was found to produce little or no formaldehyde.

Untreated walls of *L. arabinosus* gave, on complete oxidation 1 mol.prop. of formaldehyde for 12.0 mol.prop. of phosphate, after removal of alanine 1 mol.prop. of formaldehyde for 10.9 mol.prop. of phosphate and after dinitrophenylation 1 mol.prop. of formaldehyde for 10.0 mol.prop. of phosphate.

On complete oxidation of intact cell walls 90% of the phosphorus was converted into soluble products and the insoluble residue, which had the same shape as the original cell wall, contained no detectable degradation products of teichoic acid. Samples (3 mg.) of the residual walls were hydrolysed in 6*N*-hydrochloric acid (1 ml.) at 100° for 16 hr. and analysed for amino acids; the results are shown in

Table 1. *Composition of walls of Lactobacillus arabinosus before and after oxidation with periodate*

	Intact walls (% by wt.; uncorrected)	Oxidized walls (% by wt.; uncorrected)
Alanine	6.5	10.2
Glutamic acid	4.6	11.1
Diaminopimelic acid	6.5	15.1
Aspartic acid	0.57	1.2
Serine	0.54	0.75
Threonine	0.87	1.17
Glycine	0.53	0.66
Lysine	1.15	0.82
Muramic acid	8.0	19.2
Glucosamine	6.0	12.6

Table 1. Further samples (3.3 mg.) were hydrolysed in 2*N*-hydrochloric acid at 100° for 2 hr. and analysed for glucosamine and muramic acid (Perkins & Rogers, 1959). Unoxidized walls were similarly analysed.

Hydrolysis at pH 4. A solution (4 ml.) of teichoic acid or a suspension of cell walls (about 100 µg. of phosphorus/ml.) was mixed with 0.2*M*-sodium acetate-acetic acid buffer, pH 4.0 (12 ml.). Portions (0.5 ml.) of the mixture were transferred to glass tubes which were sealed and then heated at 100°. Tubes were removed at intervals and the contents adjusted to 4 ml. In the early stages these samples were used for the analysis of inorganic phosphate whereas smaller portions (0.2 ml.) were used in the later stages; cell walls were removed by centrifugation before analysis. Typical results are given in Fig. 1.

Hydrolysis of teichoic acid with alkali. A solution of teichoic acid from *S. aureus* (containing 100 µg. of phosphorus) with chain length 7.7 in 2*N*-sodium hydroxide (1 ml.) was heated in a sealed Pyrex tube at 100° for 3 hr. The solution was then passed through a column of Dowex 50 (NH₄⁺ form) resin (5 ml.) and evaporated *in vacuo* to dryness. Methanol was added and the mixture was boiled to dryness; this was repeated several times to remove borate originating from the Pyrex glass. The mixture was then selectively *N*-acetylated with acetic anhydride in sodium carbonate (Roseman & Ludowig, 1954), passed through a column of Dowex 50 (H⁺ form) resin (5 ml.) and freeze-dried. The material was then subjected to descending chromatography in solvent *A*. Examination of chromatograms with the molybdate spray reagents showed the presence of two phosphates, *R*_{α-glycerophosphate} 1.01 and 0.50 respectively. Areas corresponding to these phosphates were cut out and material was eluted with water. The eluates were centrifuged to remove cellulose fibres, freeze-dried and the residues were dissolved in water (5 ml.);

Fig. 1. Formation of inorganic phosphate from walls of *L. arabinosus* at pH 4. For experimental details see the text (*a*, 100 µg. of phosphorus/ml.).

the solutions of mono- and di-phosphates contained respectively 144 and 44 µg. of organic phosphate. Confirmation of their identity was obtained by measurement of the formaldehyde formed on oxidation with sodium metaperiodate after enzymic removal of the phosphomonoester residues (see below).

Examination of phosphomonoesters from the alkali hydrolysate. Portions (0.45 ml.) of the above solutions of phosphates were mixed with 0.1*M*-ammonium carbonate (0.15 ml.) and phosphatase solution (0.075 ml., 500 µg. of enzyme/ml.). Inorganic and total phosphate were determined after incubation at 37° for 16 hr. The amount of inorganic phosphate produced was 99 and 102% of the total phosphorus from the mono- and di-phosphates respectively. Chromatographic examination of the products of dephosphorylation showed the presence in each case of a single compound indistinguishable from *N*-acetylglucosaminylribitol.

Portions (0.1 ml.) of the solution of monophosphates and appropriate control were added to 5*N*-hydrochloric acid (0.5 ml.), and portions (0.2 ml.) of the solution of diphosphates and control were added to 6*N*-hydrochloric acid (0.4 ml.) and the mixtures were heated at 100° for 3 hr. After neutralization with sodium hydroxide the volumes were adjusted to 3.0 ml. with water and assayed for glucosamine.

The glucosamine:phosphate ratios were 1.0:1.0 (monophosphates) and 0.52:1.0 (diphosphates).

The remainder of the solutions of mono- and di-phosphates were heated in 2*N*-hydrochloric acid for 2 hr. and examined chromatographically in solvents *A*, *C* and *D*; glucosamine was detected in both hydrolysates. The chromatogram of the monophosphates revealed spots corresponding to ribitol monophosphates, which gave the characteristic rapidly developing purple and yellow colours with the periodate-Schiff reagents together with some ribitol and anhydrosorbitol. The diphosphate fraction contained principally a mixture of ribitol diphosphates, *R*_{α-glycerophosphate} 0.65 in solvent *A*.

Examination of the neutral products of alkali hydrolysis. A further sample of teichoic acid (containing 60 µg. of phosphorus) was hydrolysed and *N*-acetylated as before. Chromatography in solvent *B* showed the presence of *N*-acetylglucosaminylribitol, *R*_{ribitol} 0.44; it rapidly gave a purple colour with the periodate-Schiff reagents. A large amount of material remained at the origin of the chromatogram; this slowly gave a blue colour and corresponds to a mixture of *N*-acetylglucosaminylribitol mono- and diphosphates. The relative amounts of these substances were determined by analysis of the appropriate eluates from a chromatogram. The amount of *N*-acetylglucosaminylribitol was determined in two ways. (a) A sample was oxidized with sodium metaperiodate and the amount of formaldehyde produced was determined as before. (b) A further sample was hydrolysed in 4*N*-hydrochloric acid and glucosamine was determined. This was compared with the total phosphorus in the original sample. Thus the ratio phosphorus:free *N*-acetylglucosaminylribitol was 7.3:1 (from formaldehyde) and 7.8:1 (from glucosamine).

Alkaline hydrolysis of teichoic acid after partial removal of the terminal phosphomonoester group. Teichoic acid (containing 111.8 µg. of phosphorus) in water (1 ml.) was treated with a solution (1.5 ml.) of phosphatase in 0.05*M*-ammonium carbonate buffer, pH 9.3 (70 µg. of enzyme/ml.),

and the mixture was incubated for 24 hr. at 37°. Under these conditions 7.3% of the phosphorus was converted into inorganic phosphate; this corresponds to the removal of about 50% of the terminal phosphomonoester groups. The solution was evaporated to dryness *in vacuo* and the residue was hydrolysed in alkali and products were fractionated chromatographically as before. Eluates from the monophosphate and diphosphate areas contained respectively 78.7 and 13.7 $\mu\text{g.}$ of organic phosphate.

Hydrolysis of cell walls with alkali. *S. aureus* cell walls (9.4 mg.) were heated for 1 hr. at 100° in 0.1N-sodium hydroxide (1 ml.) in a sealed Pyrex tube. This removed all of the phosphorus corresponding to teichoic acid. Wall residues were removed by centrifugation and the supernatant solution was heated with 2N-sodium hydroxide (1 ml.) for 3 hr. at 100° in a sealed tube. The hydrolysate was then passed through a column of Dowex 50 (NH_4^+ form) resin (5 ml.), the eluate evaporated to dryness *in vacuo* and the residue *N*-acetylated and fractionated chromatographically as before. Two phosphate areas were observed and these corresponded to the mono- and diphosphates of *N*-acetylglucosaminylribitol, which were characterized as before. The eluates from one chromatogram contained 133 $\mu\text{g.}$ of phosphorus as monophosphates and 46.5 $\mu\text{g.}$ of phosphorus as diphosphates.

A second sample of walls (4.95 mg.) was hydrolysed and acetylated as described previously; the organic phosphates and *N*-acetylglucosaminylribitol were separated by chromatography and the eluates analysed by the procedures already described. The sample contained 103 $\mu\text{g.}$ of phosphorus and gave 13.9 $\mu\text{g.}$ of formaldehyde and 81.0 $\mu\text{g.}$ of glucosamine, corresponding to the ratio phosphorus:*N*-acetylglucosaminylribitol 7.4:1 and 7.3:1 respectively.

DISCUSSION

Strominger & Ghuysen (1963) have reported that the teichoic acid prepared from the walls of *Staphylococcus aureus* strain Copenhagen by extraction with trichloroacetic acid had a much lower molecular weight than that obtained by enzymic degradation of the cell walls. These authors suggested that the teichoic acid is attached covalently through a phosphate group to the glycosaminopeptide of the wall, and that this phosphate group, and also some of the inter-ribitol phosphodiester groups, are hydrolysed during extraction in the presence of acid. Similarly, the teichoic acid liberated from *Bacillus subtilis* by autolysis of the isolated walls remains attached to glucosamine-containing components of the wall, to which it is linked apparently through its terminal phosphate group (Young *et al.* 1964). Covalent linkage between teichoic acid and the other components of the wall would explain the slow rate of extraction of teichoic acid with cold trichloroacetic acid and would also account for the present observation that only very small amounts of teichoic acid are extracted by treatment of cell walls with 'hydrogen-bond splitting' reagents such as 8M-urea and saturated lithium thiocyanate. It has previously been shown that linkage to the wall

does not involve the D-alanine ester residues and that multiple linkages are not present (Archibald, Armstrong, Baddiley & Hay, 1961; Rogers & Garrett, 1963).

It is interesting to consider the chemical consequences of a structure in which the teichoic acid is attached to the wall by covalent linkage through its terminal phosphate residue. If this linkage were of comparable stability towards acid as are the phosphodiester linkages between the ribitol units in the teichoic acid, then extraction with trichloroacetic acid would be accompanied by significant hydrolysis of the inter-unit phosphodiester groups so that the liberated teichoic acid would have undergone considerable degradation and be appreciably smaller than the polymer in the untreated wall. If, on the other hand, the extracted teichoic acid was of comparable chain length to that present in the untreated wall, it follows that the linkage between the terminal phosphate and the wall must be appreciably more labile towards acid than are the inter-unit phosphodiester groups. In either case it is clearly important to establish the size of the 'native' teichoic acids.

The chain lengths of the ribitol teichoic acids studied in these Laboratories were determined by potentiometric titration and by oxidation with sodium metaperiodate. In each case the number of phosphomonoester groups (by titration) was the same as the number of terminal ribitol units having a free primary hydroxyl group susceptible to periodate oxidation with concomitant production of formaldehyde. It follows that each chain has a phosphomonoester substituent at the 5-position of one terminal ribitol unit and that no phosphomonoester groups are attached to positions 1 or 2 of the terminal unit at the other end of the chain. Since acid hydrolysis of a phosphodiester having a neighbouring free hydroxyl group occurs by a mechanism requiring intermediate formation of a cyclic phosphate (Brown, Magrath, Nielson & Todd, 1956), the resulting phosphomonoester residue accompanies the alkyl group which has the neighbouring free hydroxyl group. Thus it would be expected that acid hydrolysis of the phosphodiester residues in a ribitol teichoic acid would lead to smaller molecules in which the terminal phosphomonoester residue is attached to positions 1 or 2 of ribitol. The phosphomonoester residue could not be attached to the 5-position since the neighbouring 4-position is substituted by a glycosidic residue. The mechanism of such acid-catalysed hydrolysis for a hypothetical teichoic acid containing only two units is outlined in Scheme 1. Phosphomonoester substituents at positions 1 or 2 would prevent oxidation by periodate at these positions so that the formaldehyde produced during such oxidations would not be equivalent to the amount

Scheme 1. Acid hydrolysis of a teichoic acid containing 2 units (alanine ester groups omitted). The phosphates produced will include small amounts of isomeric 2,5- and 3,5-diphosphates, which do not affect significantly the quantitative relationships between phosphomonoester and formaldehyde. Glyc, glycosyl.

of phosphomonoester. The validity of this conclusion has been confirmed by the observation that although the amount of phosphomonoester to total phosphate in a sample of ribitol teichoic acid from *S. aureus* H increased from 1 in 7 to 1 in 4.6 on treatment for 5 days at 4° with trichloroacetic acid, the amount of formaldehyde produced by oxidation with sodium metaperiodate remained constant at 1mol.prop. to 7.0mol.prop. of phosphate. Thus the results obtained previously on the chain length of teichoic acids from *B. subtilis* (1mol.prop. of formaldehyde to 8.8mol.prop. of phosphorus and 1mol.prop. of phosphomonoester to 8.9mol.prop. of phosphorus; Armstrong, Baddiley & Buchanan, 1960), *S. aureus* (8.05 units by oxidation, 8.1 by titration; Baddiley, Buchanan, Martin & RajBhandary, 1962) and *Lactobacillus arabinosus* (7.6 units by oxidation and 7.8 by titration; Archibald *et al.* 1961) indicate that the samples examined had not undergone significant fragmentation and were presumably similar in size to the teichoic acid present in the walls. It follows that the linkage between the terminal phosphate group

and the wall must be more labile to acid than are the phosphodiester groups joining adjacent units in the chain.

Further evidence on the chain length of teichoic acid in walls has been obtained by studies on cell walls themselves. On oxidation with periodate, walls containing ribitol teichoic acid yielded formaldehyde, whereas oxidation of walls from which teichoic acid had been extracted gave only very small amounts of formaldehyde. Determination of the much larger amounts of formaldehyde produced on oxidation of the intact walls therefore gives the chain length of the teichoic acid in the wall. In this way chain lengths of 10, 9 and 10 were observed for the teichoic acids in intact walls of respectively *L. arabinosus*, *S. aureus* H and *B. subtilis*. All of the teichoic acid was removed from the wall during such oxidation and the residue had the composition (Table 1) of mucopeptide and the shape of the original cell wall.

Determination of the terminal phosphomonoester groups was more difficult. As expected phosphomonoesterase had no action on walls themselves,

consequently the formation of inorganic phosphate by heating teichoic acid or walls at pH 4 was studied. It is known that under these conditions phosphomonoester groups are hydrolysed much more rapidly than are phosphodiester groups by a mechanism that does not involve cyclic esters (Butcher & Westheimer, 1955). When teichoic acid was heated at pH 4, inorganic phosphate was first produced fairly rapidly and then more slowly. The graph $\log(a-x)$ against time (a , total phosphate; x , inorganic phosphate formed at time t) becomes linear corresponding to the rate of hydrolysis of the phosphodiester residues. Thus extrapolation of the linear part of the graph to zero time gives the amount of phosphomonoester originally present. After the initial stages, the rate of inorganic phosphate production was linear and the zero time value for x is 17–14.5%. This is thus a measure of the phosphate which is labile at pH 4, and corresponds to a chain length of 6.9–8.3, in good agreement with the value obtained by potentiometric titration. Similar studies with teichoic acid from *S. aureus* gave a chain length of 7–9, which agrees reasonably well with the value obtained by titration. Although the method appears to give good results, possible errors in the extrapolation render it less accurate than the other procedures used in this investigation. Moreover, it is not possible in this procedure to allow for the small amount of phosphorus in walls that is not associated with teichoic acid (Archibald *et al.* 1961). The nature of this phosphorus and its behaviour at pH 4 are unknown, and consequently the procedure is only useful if results are assessed together with those from other analytical methods.

Very similar values were obtained for the chain length of teichoic acids in walls when hydrolysis at

pH 4 was employed. The graph relating phosphate produced with time again became linear (Fig. 1), and by extrapolation values of about 10% and 8.8% of the total phosphorus of walls of *L. arabinosus* and *S. aureus* were found to be labile. Consequently, if this labile phosphorus represents the terminal phosphate group of the teichoic acid, values of 10 and 11.4 are obtained for the chain lengths of these polymers in walls of *L. arabinosus* and *S. aureus* respectively. The lability at pH 4 of the terminal phosphate group of the teichoic acid in the wall indicates that the linkage between this group and the glycosaminopeptide is very labile to acid, but is not inconsistent with there being such a covalent linkage. For example, it has been observed (D. Button, unpublished work) that large amounts of inorganic phosphate (40–50% of the total phosphorus) are produced rapidly when the teichoic acid from walls of a strain of *S. lactis* (Archibald, Baddiley & Button, 1965) is heated at pH 4. About half of the phosphodiester groups in this atypical polymer are joined to the reducing group of glucosamine residues, and are consequently very labile to acid.

Hydrolysis of teichoic acids in alkali also proceeds by a mechanism involving cyclic phosphates at the positions 1 and 2 of the ribitol units. Details of the mechanism have been discussed previously (Armstrong, Baddiley & Buchanan, 1961) and the expected products of hydrolysis of a regular ribitol phosphate polymer in which a glycosidic substituent occurs at each 4-position of ribitol and phosphate joins positions 1 and 5 in adjacent units are therefore (a) glycosylribitol 1,5- and 2,5-diphosphates from the terminal unit bearing the phosphomonoester, (b) glycosylribitol 1- and 2-monophosphates from the internal units of the

Scheme 2. Hydrolysis of a ribitol teichoic acid in alkali. For a chain of $n+2$ units the proportions of products will be diphosphates:monophosphates:glycosylribitol 1:n:1.

chain and (c) free glycosylribitol from the end unit, which does not bear the phosphomonoester group (see Scheme 2). Thus a ribitol teichoic acid comprising 8 units (Scheme 2, $n = 6$) should give on hydrolysis in alkali diphosphates, monophosphates and free glycoside in molar proportions 1:6:1. Consequently, determination of the amount of diphosphates or of free glycoside produced would give a measure of the chain length of the teichoic acid. To test the validity of this reasoning a sample of teichoic acid from the walls of *S. aureus* was hydrolysed in alkali and the products were separated, after selective *N*-acetylation, by paper chromatography. There were three major products: 4-*O*-(*N*-acetylglucosaminy)-*D*-ribitol, identified chromatographically; the 1- and 2-phosphates, identified by enzymic dephosphorylation and chromatography of the resulting *N*-acetylglucosaminide, and by measurements of the ratio sugar:phosphorus; the diphosphates, identified from the ratio sugar:phosphate, and enzymic dephosphorylation. The ratio phosphorus present as diphosphate:phosphorus present as monophosphate was 1:3.27, corresponding to a chain length of 8.5 units for the teichoic acid. The *N*-acetylglucosaminyribitol was determined from the formaldehyde produced on oxidation with periodate and also from the glucosamine produced on acid hydrolysis. The ratio glucosaminyribitol:total phosphorus was 7.55:1, corresponding to a chain length of 7.55 units. These results agree well with values found by other methods. The method was checked by partial removal of the terminal phosphomonoester from a sample of teichoic acid from *S. aureus* with phosphomonoesterase until 7.3% of the total phosphorus had been converted into inorganic phosphate; this corresponds to the removal of about 50% of the terminal phosphomonoester residues. Subsequent degradation with alkali gave a decreased proportion of diphosphate (phosphorus as diphosphate:phosphorus as monophosphate, 1:5.8).

When intact walls of *S. aureus* were similarly

treated with alkali, diphosphates, monophosphates and *N*-acetylglucosaminyribitol were again produced. They were separated and identified as before. The ratios diphosphates:monophosphates and *N*-acetylglucosaminyribitol:total phosphorus were 1:2.86 and 1:7.35, corresponding to a chain length of 7.7–7.4 units, in good agreement with previous values.

Thus on several grounds it is apparent that the purified samples of ribitol teichoic acids examined here have similar chain lengths to the teichoic acids in the intact walls, and have molecular weights in the range 4000–5000. Quantitative considerations of the number of linkages between teichoic acids and glycosaminopeptides in the wall must be based on the amount of teichoic acid present and on its molecular weight.

Evidence from enzymic degradation of bacterial walls strongly suggests that in both *S. aureus* and *B. subtilis* the teichoic acids are held to the wall by covalent linkage through the terminal phosphate group. The chemical nature of this linkage has not yet been established unequivocally but the present work shows that in all cases examined the linkage is characterized by high lability to acid. Bonds which might be expected to exhibit such lability include those involving the hemiacetal hydroxyl group of a sugar (sugar 1-phosphate), an anhydride or a phosphoramidate. Teichoic acids are extracted only very slowly and incompletely into aqueous 2*N*-ammonia solution and so it is unlikely that attachment is through an anhydride linkage. The increased rate of extraction of teichoic acid into trichloroacetic acid after walls have been treated with aqueous ammonia solution is appreciable, but may be due to one or more of a number of reasons and may not be a consequence of alkali lability of the covalent linkage between teichoic acid and the glycosaminopeptide. That this linkage is labile towards more vigorous treatment with alkali is, however, apparent from the formation of theoretical amounts of glycosylribitol diphosphates on hydrolysis of walls of *S. aureus* in alkali.

Scheme 3. Suggested linkage between teichoic acid and glycosaminopeptide in bacterial walls. X, H or lactyl peptide; R, H or MeCO; Glyc, glycosyl; Ala, *D*-alanyl.

This lability cannot arise through the formation of a cyclic phosphate with a neighbouring hydroxyl group at the point of attachment to the wall, since the terminal phosphate group of the teichoic acid would then become detached from ribitol and remain associated with the wall. Moreover, glycosidic linkages to phosphate are stable towards alkali and it is therefore unlikely that a sugar 1-phosphate linkage is involved. Attachment through a phosphoramidate bond would account for the observed acid lability and also the lability to alkali if the amino group involved were suitably activated. Thus the linkage shown in Scheme 3, involving an amino group of the amino sugar backbone of the glycosaminopeptide, would be broken during the degradation of the sugar in alkali. Such a linkage would also account for the liberation of teichoic acid when cell walls are treated with hydrazines (Archibald & Baddiley, 1965).

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