Further Studies on the Glycerol Teichoic Acid of Walls of *Staphylococcus lactis* I3

LOCATION OF THE PHOSPHODIESTER GROUPS AND THEIR SUSCEPTIBILITY TO HYDROLYSIS WITH ALKALI

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1. The teichoic acid from walls of *Staphylococcus lactis* I3 is readily degraded in dilute alkali. 2. Degradation proceeds by selective hydrolysis of that phosphodiester group attached to an alcoholic hydroxyl group of the *N*-acetylglucosamine and gives a repeating unit in high yield. 3. Further studies on a different repeating unit isolated by partial acid hydrolysis have shown that the glycerol diphosphate is attached to the 4-hydroxyl group of the *N*-acetylglucosamine and not to the 3-hydroxyl group as was proposed earlier. 4. The susceptibility towards hydrolysis by alkali of other structural types of teichoic acid has been examined and found to vary markedly according to their structure.

Direct hydrolysis of cell walls with alkali has been used in structural studies on teichoic acids, since repeating units can be isolated without danger of hydrolysis of acid-labile glycosidic substituents (Hay, Archibald & Baddiley, 1965; Archibald, Baddiley & Heptinstall, 1969a). Hughes & Tanner (1968) found that treatment of walls of *Bacillus subtilis* 168 with 0.1M-sodium hydroxide at 37°C caused rapid and almost complete extraction of teichoic acid which was isolated in polymeric form. These authors also reported that samples of teichoic acid that had been extracted in this way from walls of *B. lichenformis* N.C.T.C. 6346 and *B. subtilis* W23 were not extensively degraded. Archibald, Coapes & Stafford (1969b) found that polymeric teichoic acids could be isolated under similar conditions from walls of *Lactobacillus plantarum* C106 and *Staphylococcus lactis* 2102. Subsequent studies have shown that the teichoic acid in walls of *S. lactis* I3 (Archibald, Baddiley & Button, 1968a) is also rapidly extracted in dilute alkali, but in this case extraction is accompanied by extensive hydrolysis of phosphodiester groups and results in the formation of a repeating unit in which *N*-acetylglucosamine is attached through a sugar 1-phosphate linkage to glycerol diphosphate.

In the earlier work on the structure of the wall teichoic acid from *S. lactis* I3 it was concluded that the polymer possesses a repeating unit in which 6-*O*-D-alanyl- α -*N*-acetylglucosamine 1-phosphate is attached to the 3-position on D-glycerol 1-phosphate; the phosphodiester linkage between repeat-

ing units was from glycerol to what was thought to be the 3-hydroxyl on *N*-acetylglucosamine. Part of the evidence for the location of the phosphodiester group at the 3-hydroxyl group of *N*-acetylglucosamine was the formation of glucometasaccharinic acid on degradation with alkali (Archibald *et al.* 1968a). The formation of this saccharinic acid is consistent with 3 rather than 4 substitution but, as pointed out in the earlier publication, if hydrolysis of the phosphodiester precedes the formation of the saccharinic acid then the resulting *N*-acetylglucosamine would give the metasaccharinic acid whatever had been the original location of the phosphate. In view of the present observation that this phosphodiester group is very readily hydrolysed in alkali it was desirable to investigate further its location. In the present study it is concluded that the 4-hydroxyl of *N*-acetylglucosamine is the position of phosphodiester linkage, and the teichoic acid thus possesses the structure (I) (Scheme 1).

The lability of the phosphodiester groups in other structural types of teichoic acid has also been examined under similar conditions.

MATERIALS AND METHODS

Walls of *Lactobacillus buchneri* N.C.I.B. 8001 (Shaw & Baddiley, 1964), *L. plantarum* C106 (Adams, Archibald, Baddiley, Coapes & Davison, 1969), *Staphylococcus aureus* H (Baddiley, Buchanan, Martin & RajBhandary, 1962b; Baddiley, Buchanan, RajBhandary & Sanderson, 1962a), *S. lactis* 2102 (Archibald, Baddiley, Button, Heptinstall &

Scheme I. Formation of repeating units by hydrolysis of teichoic acid from *S. lactis* I3 with acid or alkali. Ac, acetyl.

Stafford 1968b) and *S. lactis* I3 (Archibald *et al.* 1968a) were prepared as previously described. Whatman DEAE-cellulose was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Calf intestinal phosphatase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Paper chromatography. This was carried out on Whatman no. 4 paper. In preparative work the paper was first washed with 2M-acetic acid and water. The following solvent systems were used: *A*, propan-1-ol-NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.); *B*, butan-1-ol-ethanol-water-NH₃ (sp.gr. 0.88) (40:10:49:1, by vol.) (organic phase); *C*, butan-1-ol-pyridine-water (6:4:3, by vol.); *D*, butan-1-ol-acetic acid-water (5:1:2, by vol.). Compounds were detected by using: (1) molybdate reagents for phosphoric esters (Hanes & Isherwood, 1949); (2) periodate-Schiff reagents for glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); (3) AgNO₃ reagent for reducing compounds (Trevelyan, Procter & Harrison, 1950).

Analytical methods. Phosphates, formaldehyde, periodate and reducing sugars were determined as described by Archibald *et al.* (1968a).

RESULTS

Action of 0.5M-sodium hydroxide on walls of *Staphylococcus lactis* I3. Walls (50mg) were

suspended in 0.5M-sodium hydroxide (50ml) and stirred at 25°C. At suitable intervals portions were removed, the extinction of the suspension was measured at 550nm and the insoluble residue was recovered by centrifugation at 15000g for 20min. The residue was then washed three times with water, suspended in water and then analysed for phosphate. The results (Table 1) show that although the walls eventually dissolved completely, extraction of phosphate occurred much more rapidly than did dissolution of the peptidoglycan.

A further sample of wall (100mg) was extracted with 0.5M-sodium hydroxide (4ml) for 4h at 25°C. The extract obtained by centrifugation was passed through a column (100mm × 15mm) of Dowex 50 (NH₄⁺ form) resin to remove Na⁺. A portion (3 μmol of phosphate) was treated with phosphatase (0.5mg) in 0.05M-ammonium carbonate (1.0ml) for 16h at 37°C, when 59% of the phosphate was converted into P_i. A further portion of the extract was examined chromatographically in solvent *A*; it contained three major products together with a small amount of material that remained at the origin. The three major products were isolated by preparative paper chromatography and identified

Table 1. *Rate of extraction of teichoic acid and solubilization of walls of S. lactis I3*

Walls were suspended in 0.5M-NaOH at 25°C and the extinction at 550nm and the percentage of the total phosphorus remaining in the insoluble wall material were measured at intervals. Experimental procedures are given in the text.

Time (h)	Extinction at 550nm	Phosphate remaining in wall residue (%)
0	0.579	100
1	0.539	10.6
2	0.505	10.0
3	0.472	8.7
6	0.368	6.9
8	0.368	4.7
24	0.063	3.8
48	0.032	0

as: (a) *N*-acetylglucosamine; this had chromatographic properties (solvents *A* and *B*) identical with an authentic sample: (b) glycerol diphosphates; these accounted for 16% of the total phosphate, had chromatographic properties identical with an authentic sample of isomeric glycerol diphosphates and gave glycerol and P_i on enzymic dephosphorylation: (c) the non-reducing repeating unit (structure III); this accounted for 63% of the total phosphate and had the same chromatographic mobility as did this repeating unit isolated previously (Archibald *et al.* 1968a). This compound was characterized by methods similar to those used in the earlier structural work. Thus hydrolysis in M-hydrochloric acid at 100°C for 3h gave glucosamine and glycerol diphosphates, whereas hydrolysis in 0.1M-hydrochloric acid at 100°C for 10min gave *N*-acetylglucosamine and glycerol diphosphates. Prior treatment with sodium borohydride had no effect on the compound; it gave glucosamine and no glucosaminitol on acid hydrolysis, whereas reduction of the reducing repeating unit (structure II), isolated by partial acid hydrolysis of the teichoic acid, converted the glucosamine residue into a glucosaminitol residue.

The non-reducing repeating unit isolated here gave P_i (49% of total P) on treatment with phosphomonoesterase, and the product had the same chromatographic mobility ($R_{\text{glycerol 1-phosphate}}$ 1.9 in solvent *A*) and colour reactions as found previously (Archibald *et al.* 1968a). Thus, the main product of hydrolysis of the teichoic acid with alkali is characterized as the mixture of isomeric phosphates (structure III). The well-established mechanism of hydrolysis of hydroxyl-substituted phosphodiester in alkali proceeds through an intermediate cyclic phosphate that subsequently hydrolyses further to a mixture of isomeric phosphomonoesters. Although we conclude that the product (structure III)

must be a mixture of two isomers, their ratio has not been determined. The formation of these phosphates in high yield shows that hydrolysis of the phosphodiester linkage attached to the alcoholic hydroxyl group of *N*-acetylglucosamine occurs more rapidly than does that of the phosphodiester group at the glycosidic hydroxyl group. Under the conditions described hydrolysis is sufficiently selective to permit isolation of the repeating unit in good yield. The formation of *N*-acetylglucosamine and glycerol diphosphates shows that a few of the other phosphodiester groups in the polymer are also hydrolysed. In view of these results it appears that the nature of the saccharinic acid formed during degradation of the teichoic acid under vigorous alkaline conditions is unlikely to be structurally significant since *N*-acetylglucosamine itself may be formed first and subsequently degraded to saccharinic acid.

Comparison of the action of alkali on walls of various bacteria and on diglycerol phosphate. Walls (50mg) of *L. buchneri* N.C.I.B. 8001, *L. plantarum* C106, *S. aureus* H, *S. lactis* 2102 and *S. lactis* I3 were suspended in 0.5M-sodium hydroxide (2ml) and shaken at 25°C for 4h. The suspensions were then centrifuged and the insoluble residue was washed (three times) with water and then analysed for residual phosphorus. The supernatant solutions were desalted on Dowex 50 (NH_4^+ form) resin, evaporated to dryness *in vacuo* and dissolved in water (1.0ml). Samples were treated with phosphatase as described above and P_i was determined. In each case between 93% and 97% of the phosphate was extracted from the wall but the extracted teichoic acids contained different proportions of phosphomonoester groups. The most stable teichoic acid was that from *S. lactis* 2102 in which 9% of the phosphate was present as monoester. Those from *L. plantarum* C106, *S. aureus* H and *L. buchneri* N.C.I.B. 8001 contained respectively 13, 21 and 27% of their phosphate as monoester but the teichoic acid from *S. lactis* I3 was extensively hydrolysed and contained 60% of phosphomonoester groups. Further samples (100mg) of walls of *S. lactis* I3 and *S. aureus* H were suspended in alkali (4ml) and samples were withdrawn at intervals for analysis. Results are given in Table 2.

Diglycerol phosphate (8 μ mol) was incubated at 25°C in 0.5M-sodium hydroxide (0.4ml) and the resulting solution was analysed for phosphomonoester groups. Conversion into phosphomonoester was 22% after 4h and 36% after 8h incubation in alkali, and chromatographic examination of a sample showed the presence of glycerol and its monophosphates as well as unchanged diglycerol phosphates.

It appears that teichoic acids are extracted in good yield from walls of the five species examined but the

Table 2. Rate of extraction of teichoic acid and of its hydrolysis on treatment of walls with 0.5M-sodium hydroxide at 25°C

Hydrolysis was measured by enzymic determination of the phosphomonoester content of the extracted teichoic acid.

Time (min)	Phosphate extracted (%)		Monoester (% of total P in solution)	
	<i>S. lactis</i> I3	<i>S. aureus</i> H	<i>S. lactis</i> I3	<i>S. aureus</i> H
30	79	82	54	11
60	91	91	60	14
120	93	93	63	19
240	94	94	64	21

extent of degradation varies considerably. They are extracted at similar rates from the walls of both *S. lactis* I3 and *S. aureus* H but hydrolysis of phosphodiester groups occurs more rapidly with the former teichoic acid, which is considerably more labile towards alkali than are the other teichoic acids studied. This greater lability is presumably due to steric factors favouring cyclic phosphate formation, since there is no other obvious explanation for the greater stability of the teichoic acid in walls of *L. plantarum* C106 which contains phosphodiester groups linking glycerol to the 6-hydroxyl group of glucose, a structural feature comparable in some respects with the alkali-labile linkage in the polymer in *S. lactis* I3.

Isolation of the reducing repeating unit (II) of the wall teichoic acid of S. lactis I3. After hydrolysis of wall of *S. lactis* I3 in 0.1M-hydrochloric acid at 100°C for 30 min, chromatography of the soluble products in solvent A showed that the teichoic acid had been degraded almost completely to a single phosphate ($R_{\text{glycerol 1-phosphate}}$ 0.74). This gentle hydrolysis affects only the sugar 1-phosphate groups. Large amounts of this reducing repeating unit were obtained by heating walls (1g) in 0.1M-hydrochloric acid (30ml) at 100°C for 30 min, and fractionating the products by chromatography on a column (300mm × 20mm) of DEAE-cellulose (acetate form). The hydrolysate was adjusted to 300ml with water, applied to the column and neutral products were eluted with water (500ml). Phosphates were eluted in a linear gradient (2l) of 0–0.5M-acetic acid adjusted to pH 5.0 by the addition of pyridine, and fractions (10ml) were collected at the rate of 0.5ml/min. Three peaks of phosphates were obtained, the second of which (fractions 56–110) accounted for 46% of the phosphate applied to the column and consisted of the pure reducing repeating unit (II). The first peak (fractions 20–55, 32% of the phosphate) consisted mainly of the D-alanyl ester of the repeating unit. This compound was hydrolysed on chromatography in solvent A and gave repeating unit, alanine and alanine amide: the ester linkage was stable in solvent D, however, and a single ninhydrin-positive phosphate spot was observed.

Thus over 70% of the total phosphate is recovered as the reducing repeating unit or as its D-alanyl ester. The third peak (fractions 111–160, 16% of the phosphate) consisted of glycerol diphosphates. A sample (3μmol of P) of the repeating unit recovered from the second peak was treated with 0.5M-sodium hydroxide (0.4ml) for 4h at 25°C and then examined chromatographically after removal of sodium ions on Dowex 50 (NH₄⁺ resin). N-Acetylglucosamine and glycerol diphosphates were produced together with some unchanged material. Thus even in the isolated repeating unit, hydrolysis of the phosphodiester group occurs more rapidly than does the saccharinic acid degradation.

Characterization of the reducing repeating unit (II). Sodium borohydride (20mg) was added to a sample (17μmol of P) of the material in water (1ml) and reduction was allowed to proceed in the dark at room temperature. After 16h excess of borohydride was destroyed by dropwise addition of M-acetic acid until the pH was 6.5. Sodium ions were then removed on a column (10mm × 60mm) of Dowex 50 (NH₄⁺ form) resin and the eluate was concentrated *in vacuo* to a small volume. Chromatographic examination (solvent A) showed a single product ($R_{\text{glycerol 1-phosphate}}$ 0.61) which, unlike the original material ($R_{\text{glycerol 1-phosphate}}$ 0.74) gave a purple colour rapidly with the periodate-Schiff reagents (reagent 2). The remainder of the product was applied, as a band, to a preparative paper chromatogram that was developed in solvent A. Material was located by spraying test strips and was then eluted with water. A sample (4μmol of P) of the reduced material was hydrolysed in 2M-hydrochloric acid (0.5ml) for 3h at 100°C. Acid was removed *in vacuo* over potassium hydroxide pellets and the products were chromatographed in solvent A and detected with reagents 1, 2 and 3. Glycerol mono- and diphosphates, glucosaminitol and glucosaminitol phosphate were the only products detected.

Further samples of the reduced material (0.103μmol of P) were oxidized with sodium metaperiodate (0.2μmol) in water (0.4 and 2.4ml). Oxidation in the dilute solution was followed spectrophotometrically and was complete after 24h,

when $0.060\mu\text{mol}$ of periodate had been reduced. Water (2ml) was added to the more concentrated solution after incubation at room temperature for 40h, and the amount of periodate remaining in the solution was determined. The same amount of periodate had been reduced in both solutions (0.58mol of periodate/mol of phosphate). A further sample ($0.31\mu\text{mol}$ of P) was oxidized in 0.1M -sodium periodate whereupon $0.146\mu\text{mol}$ of formaldehyde was formed.

The reduced material thus reduces 1.16mol of periodate and gives 0.94mol of formaldehyde/ 2mol of P. In the previous study this reduced compound was found to reduce 2mol of periodate/ 2mol of P.

A further sample ($5\mu\text{mol}$ of P) was dissolved in 0.067M -sodium periodate (1.0ml) and incubated in the dark at room temperature for 16h. A saturated solution of barium hydroxide was added dropwise until the pH reached 9.5 and then the precipitated barium periodate and iodate were removed by centrifugation. Sodium borohydride (10mg) was then added to the supernatant solution. After incubation for 16h excess of borohydride was destroyed by dropwise addition of M -acetic acid to pH 6.5; sodium ions were removed on Dowex 50 (H^+ form) resin and borate was removed as methyl borate by repeated evaporation *in vacuo*. The reduced product was hydrolysed in 0.1M -sodium hydroxide (0.4ml) at 100°C for 1h. The solution was cooled, passed through a small column of Dowex 50 (NH_4^+ resin) and incubated for 16h with the phosphatase, when 84% of the phosphorus was converted into P_i .

Chromatographic examination of the dephosphorylated material showed the presence of two major neutral components. One of these had the properties of glycerol and the other ($R_{\text{glycerol}} 0.59$ in solvent B) gave positive reactions with reagents 2 and 3. The two products were separated by preparative paper chromatography and eluted with water. The compound having $R_{\text{glycerol}} 0.59$ was dissolved in water (2ml) and examined by the following procedure. A sample (0.25ml) was diluted with water (2.25ml) containing $0.2\mu\text{mol}$ of sodium periodate. The reduction of periodate was complete within 7h when $0.116\mu\text{mol}$ of periodate had been reduced. Further samples (0.5ml) were mixed with 0.05M -sodium periodate (0.5ml) and incubated at room temperature, when $0.092\mu\text{mol}$ of formaldehyde was produced. Thus periodate is reduced and formaldehyde is formed in the molar ratio 2:1.18. This agrees with the conclusion that this reduction product is a 2-acetamidopentitol that would arise from a 4-substituted *N*-acetylglucosaminitol rather than a 2-acetamidotetritol that would be formed from a 3-substituted *N*-acetylglucosamine. The chromatographic mobility of the fragment in solvent B ($R_{\text{glycerol}} 0.59$) is also con-

sistent with this, since the following mobilities (R_{glycerol} values) were obtained for reference compounds: glucitol, 0.41; *N*-acetylglucosaminitol, 0.48; ribitol, 0.55; erythritol, 0.74; 2-deoxy-2-acetamidoglycerol, 1.12.

Reducing power of the reducing repeating unit (II). The reducing power of samples (0.1ml) of a solution of the substance ($1.55\mu\text{mol}$ of P/mol) was determined by the procedure of Park & Johnson (1949) and found to be equivalent to 0.43mol of *N*-acetylglucosamine/ 2mol of phosphate. However, the colour yield with the Morgan-Elson reagent, by using the modified procedure described by Reissig, Strominger & Leloir (1955), was equivalent to only 0.0184mol of *N*-acetylglucosamine/ 2mol of phosphate. This low value is not consistent with 3-substitution, and we conclude that the phosphate group is attached to the 4-hydroxyl of *N*-acetylglucosamine in the repeating unit and in the teichoic acid.

DISCUSSION

In continuation of our study of the action of alkali on bacterial walls (Archibald *et al.* 1969b; Archibald, Baddiley & Goundry, 1970) we have observed considerable differences in the rates of hydrolysis of the inter-unit phosphodiester groups in teichoic acids. Thus the teichoic acid in walls of *L. plantarum* C106 was almost completely extracted with 0.5M -sodium hydroxide for 4h at room temperature (20 – 22°C), although in the extract only 6% of the phosphate was present as monoester, thereby indicating that relatively little degradation of the polymer chain had occurred. Similarly, the phosphorylated polysaccharide in walls of *S. lactis* 2102 was almost completely extracted under these gentle conditions and the purified polysaccharide contained no detectable (by incubation with phosphatase) phosphomonoester groups. In contrast, extraction of teichoic acid from walls of *S. lactis* I3 (Archibald *et al.* 1968a) was accompanied by extensive formation of phosphomonoester groups. Thus treatment of the walls from this organism with 0.5M -sodium hydroxide at 25°C solubilized 79% of the phosphate in 30 min, and 54% of this extracted phosphate was present as phosphomonoester (Table 2). After 4h 94% of the phosphate was in solution and 60–64% of this was present as monoester (Table 2). In contrast only 9% of the phosphate in the unpurified alkali extract of walls of *S. lactis* 2102 was present as monoester after 4h. Corresponding values for extracts of other walls are given above where it is seen that hydrolysis of the teichoic acid from *S. lactis* I3 is substantially more extensive than that of the others; after the initial formation of about 60% of monoester the remaining diester groups are more stable, and

further hydrolysis for 4 h in 0.5 M-sodium hydroxide gave a total of only 66% of phosphomonoester. The phosphodiester groups in this teichoic acid occur in two different locations in the molecule (Archibald *et al.* 1968a), and the rapid hydrolysis of slightly more than half of them probably arises through a preferential and complete hydrolysis at one of the two environments. The degradation products resulting from treatment of walls for 4 h with alkali were therefore fractionated on DEAE-cellulose. The major product was the previously characterized non-reducing repeating unit (III) that arises by hydrolysis of the phosphodiester group connecting glycerol to the alcoholic hydroxyl group of *N*-acetylglucosamine. The structure of this repeating unit was confirmed as before; it readily gave *N*-acetylglucosamine and glycerol diphosphates on hydrolysis in dilute alkali, and its *N*-acetylglucosamine moiety could not be reduced by treatment with sodium borohydride.

The above results show that the phosphodiester group connecting glycerol to the alcoholic hydroxyl of *N*-acetylglucosamine is readily hydrolysed in alkali, and that the phosphodiester group attached to the glycosidic position of *N*-acetylglucosamine is more stable. It follows that further hydrolysis with alkali would give *N*-acetylglucosamine, so that saccharinic acid formed during degradation of the polymer with M-sodium hydroxide at 100°C is probably derived directly from the acetamido sugar rather than from its phosphate.

In an earlier study it was reported that the saccharinic acid formed from the teichoic acid from *S. lactis* I3 was the metasaccharinic acid. The formation of metasaccharinic acid is consistent with the presence of phosphate at the 3-hydroxyl of the *N*-acetylglucosamine residues in the teichoic acid and was earlier used in support of such a location. The complete structure of the teichoic acid deduced from the earlier studies is one analogous to that given as (I) in Scheme 1, but with a phosphodiester substituent at position 3 rather than 4 on the acetylglucosamine residue. However, it was pointed out at the time that the argument about the location of the phosphate on the sugar is invalid if, as has now been shown to be the case, the phosphate substituent becomes detached from the acetamido sugar before degradation to saccharinic acid. This means that the only evidence supporting the attachment of the phosphodiester substituent to position 3 rather than 4 on the acetamido sugar is that obtained from oxidation and reduction studies on the product of gentle acid hydrolysis of the teichoic acid. This product, the reducing repeating unit, was given a structure analogous to that of (II) in Scheme 1, except that the phosphodiester linkage was at position 3 rather than 4 on the acetamido sugar. The evidence supporting this was that after

reduction with sodium borohydride the product reduced 2 mol of periodate/2 mol of phosphate. Such heavy dependence on a single quantitative periodate oxidation is unsatisfactory and the structure of the reducing repeating unit has therefore been investigated further. Accordingly, the product of gentle acid hydrolysis was prepared by partial acid hydrolysis of walls under conditions suitable for the hydrolysis of sugar 1-phosphate linkages. The products were separated by chromatography on DEAE-cellulose and elution with a linear gradient of pyridinium acetate at pH 5.0. The major peak corresponded to the previously described reducing repeating unit, and the material gave glucosamine and glycerol diphosphates on acid hydrolysis. Reduction with sodium borohydride gave a single product that was isolated by preparative paper chromatography. Hydrolysis of this in 2 M-hydrochloric acid at 100°C for 3 h gave glycerol mono- and di-phosphates, glucosaminitol and glucosaminitol phosphate. The reduced repeating unit was incubated with four molar proportions of 0.08 mM-periodate, and oxidation was complete within 24 h when 1.16 mol. prop. of periodate had been reduced/2 phosphate groups, and during the oxidation 0.94 mol. prop. of formaldehyde was formed.

The present results are not consistent with the presence of a 3-substituted *N*-acetylglucosaminitol and indicate that the phosphate substituent is attached to position 4. The reducing repeating unit thus possesses the structure (II). Further evidence for this was obtained by reduction of the oxidized product with borohydride followed by hydrolysis with alkali and enzymic dephosphorylation, when glycerol and a second neutral product were obtained; this product reduced periodate and yielded formaldehyde in the ratio 2:1.18, and had the chromatographic mobility expected of an acetamidopentitol rather than of an acetamidotritol. These properties are those expected of 2-acetamido-2-deoxy-xylitol which would be derived from a 4-substituted *N*-acetylglucosaminitol, rather than 2-acetamido-2-deoxythreitol which would be derived from a 3-substituted *N*-acetylglucosaminitol.

Acetamido sugars carrying substituents at position 3 or 4 may be distinguished readily by their behaviour with the Morgan-Elson reagents; the former readily form chromogen, whereas the latter give very low colour values. Under the standard conditions the reducing repeating unit (II) gave colour equivalent to only 0.0184 mol of *N*-acetylglucosamine/2 mol of phosphate.

We conclude that the teichoic acid from the walls of *S. lactis* I3 possesses the structure (I), and that the earlier values obtained in the quantitative periodate oxidation studies were in error or that migration of phosphate between hydroxyl groups at

positions 3 and 4 of the glucosaminitol is responsible for the different results now obtained. There is, however, no direct evidence for such migration, nor is there any apparent reason why the two studies should have yielded different results, although it is perhaps relevant that in the earlier study borate was removed from the reduced repeating unit by distillation as methyl borate after passage through Dowex 50 (H^+ form) resin. In the present study, carried out on an appreciably larger scale, this treatment was shown to cause substantial hydrolysis of the compound and so was avoided, Na^+ being exchanged for NH_4^+ and the product being desalted by preparative paper chromatography. Because these earlier conditions cause noticeable hydrolysis of the phosphodiester linkage it is also possible that they would bring about acid-catalysed phosphate migration. Moreover, the earlier results might have been influenced by the presence of degraded material in the reduced repeating unit. It is unlikely that the structure (II) could be formed artifactually in appreciable amounts by phosphate migration during the hydrolysis of the teichoic acid in 0.1 M-hydrochloric acid, and the low colour yield given by the Morgan-Elson reagents indicates that little or none of the 3-substituted isomer can be present. The reason for the relatively high lability of the phosphodiester group at the 4-position in alkali is not obvious, but the differences in lability described here, although appreciable, are sufficiently small to be capable of explanation in terms of molecular shape and could be accounted for in terms of relatively small differences in ease of formation of cyclic phosphate intermediates.

It is clear that, although alkali extraction may be generally useful for obtaining fragmented teichoic acid for structural analysis of subunits, it cannot serve as a general method for the isolation of poly-

meric material. Indeed in view of the structural diversity of teichoic acids it is unlikely that any single chemical treatment could serve as a general procedure for their isolation in polymeric form.

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