Some Structural Features of the Teichuronic Acid of *Bacillus licheniformis* N.C.T.C. 6346 Cell Walls

By R. C. HUGHES AND P. F. THURMAN

National Institute for Medical Research, Mill Hill, London N.W. 7, U.K.

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A teichuronic acid, containing glucuronic acid and *N*-acetylglactosamine, was purified from acid extracts of *Bacillus licheniformis* 6346 cell walls as described by Janczura, Perkins & Rogers (1961). After reduction of the carboxyl function of glucuronic acid residues in the polysaccharide the reduced polymer contains equimolar amounts of *N*-acetylglactosamine and glucose. Methylation of the reduced polysaccharide by the Hakamori (1964) technique showed the glucose residues to be substituted on C-4. A disaccharide, 3-*O*-glucuronosylgalactosamine, was isolated from partial acid hydrolysates of teichuronic acid. After *N*-acetylation the disaccharide produces chromogen readily on heating at pH 7, in agreement with C-3 substitution of the reducing *N*-acetyl amino sugar. Teichuronic acid also produces chromogen under the same conditions, with concurrent elimination of a modified polysaccharide from C-3 of reducing terminal *N*-acetylglactosamine residues of the teichuronic acid chains. The number-average chain lengths of several preparations of teichuronic acid were estimated from the amounts of chromogen produced in comparison with the *N*-acetylated disaccharide. The values obtained are in good agreement with the weight-average molecular weight determined by ultracentrifugal analysis. The reducing terminals of teichuronic acid are shown to be exclusively *N*-acetylglactosamine by reduction with sodium boro[³H]hydride. The number-average chain lengths of the teichuronic acid preparations were estimated by the extent of incorporation of tritium and are in agreement with values obtained by the other methods.

The cell walls of *Bacillus licheniformis* N.C.T.C. 6346 contain a polysaccharide, teichuronic acid, which consists of glucuronic acid and *N*-acetylglactosamine in approximately equimolar amounts (Janczura, Perkins & Rogers, 1961). The polysaccharide appears to be of relatively large molecular weight and has a superficial similarity to chondroitin (Meyer, Linker, Davidson & Weissman, 1953). However, teichuronic acid has a high positive optical rotation and is resistant to hyaluronidase, in contrast with chondroitin (Janczura *et al.* 1961), suggesting that the bacterial polysaccharide contains a high proportion of α -glycosidic bonds. Evidence was obtained for the presence of 3-*O*-substituted *N*-acetylglactosamine residues from the behaviour of partial acid hydrolysates of the polysaccharide in the Elson-Morgan test for hexosamine. No information was available about the position of substitution of the glucuronic acid residues.

The present paper describes a further study of the structure of teichuronic acid. In particular, the molecular size of teichuronic acid extracted

from cell walls under various conditions was examined. The reducing ends of the polysaccharide chains were found to be exclusively *N*-acetylglactosamine residues that were substituted on C-3 with glucuronic acid. A disaccharide, glucuronido-(1 $\rightarrow$ 3)-*N*-acetylglactosamine, was obtained from the polysaccharide after mild acid hydrolysis, in agreement with the conclusions of Janczura *et al.* (1961). Methylation analysis of the carboxyl-reduced polysaccharide showed the C-4 substitution of the glucuronic acid residues.

METHODS

Glucuronic acid, phosphorus and total hexosamine were determined as previously described (Hughes, 1968). Glucose was determined either with the anthrone reagent (Dreywood, 1946) or with glucose oxidase (Bergmeyer & Bernt, 1963). Hexosamine fractions containing pyridine-acetate buffers, pH 2.8 or 3.85, were analysed by a modification of the Elson-Morgan reaction. Samples (0.2 ml) containing hexosamine material (up to 0.2 μ mol of glucosamine equivalents) were diluted with 1.25 M- Na_2CO_3 (0.3 ml) and acetylacetone reagent (1.0 ml) and heated at

100°C for 40 min. Then ethanol (1.25 ml) and the Elson-Morgan reagent (1.0 ml) (Gardell, 1958) were added and the colour at 530 nm was read after 1 h. This modification was necessary to ensure an alkaline reaction during treatment with acetylacetone.

Radioactive scintillation counting of aqueous samples (0.5 ml) was carried out in 10 ml of dioxan containing naphthalene (18%, w/v), 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%). Paper strips were counted in 2 ml of toluene containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%).

Chromatograms were run on Whatman no. 3 or 3MM paper in the following solvents: solvent I, butan-1-ol-pyridine-water (6:4:3, by vol.); solvent II, butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase); solvent III, butan-1-ol-acetic acid-water (10:3:7, by vol., upper phase); solvent IV, butan-1-ol-ethanol-water (4:1:5, by vol.).

High-voltage electrophoresis was carried out on Whatman no. 3 or 3MM paper in: solvent V, m-acetic acid at 2000 V for 3 h; solvent VI, 0.1 M-pyridine-acetate buffer, pH 4.7, at 1500 V for 1 h; solvent VII, 0.1 M-sodium molybdate, pH 5.0 (Mayer & Westphal, 1968), run at 2000 V for 50 min.

Reducing sugars were detected with alkaline AgNO_3 , amino sugars with ninhydrin or an Elson-Morgan reagent, and substituted amides were detected with a starch-iodide reagent. In each case a dip technique was used (Smith, 1960).

Gas-liquid partition chromatography was carried out with a column of 10% Carbowax (40 M) supported on silicone-treated Gas-Chrom CLH (80-100 mesh) (Applied Science Laboratories, State College, Pa., U.S.A.). The column was operated at 165°C, with a nitrogen flow rate of 60 ml/min; a flame ionization detector was used.

Mass spectra were obtained by Dr D. Calam of this Institute, using an Hitachi Perkin-Elmer mass spectrometer with direct insertion at an ionizing potential of 80 eV and a temperature of 120-130°C.

RESULTS

Purification of teichuronic acid. *Bacillus licheniformis* N.C.T.C. 6346 was grown and the walls were prepared as described by Janczura *et al.* (1961). Teichuronic acid was prepared from insoluble walls by using two different methods. In Preparation I, cell walls (2.5 g) were extracted overnight at 35°C with 5% (w/v) trichloroacetic acid to remove teichuronic acid and teichoic acid. Trichloroacetic acid was removed from the soluble fraction by ether extraction, and the aqueous layer was freeze-dried. The yield was 1.28 g. Teichuronic acid was separated from teichoic acid by chromatography of the acid extracts on DEAE-cellulose. The purified teichuronic acid was passed through a column (2 cm $\times$ 140 cm) of Sephadex G-50, run in 0.1 M-pyridine-acetate buffer, pH 5.1. The bulk of the teichuronic acid, as measured by glucuronic acid analysis, was recovered in the excluded peak. This fraction was passed through a column (2 cm

$\times$ 40 cm) of Dowex 50 (X4; H^+ form; 200-400 mesh) to prepare the free acid form of the polysaccharide. In a typical experiment fractionation of 1 g of acid extract yielded 203 mg of polysaccharide.

In Preparation II, the teichoic acids were selectively removed from insoluble walls (2.75 g) by extraction with 0.1 M-sodium hydroxide (500 ml) under nitrogen at 35°C for 18 h (Hughes & Tanner, 1968). The alkali-insoluble residue (1.53 g), containing the teichuronic acid and mucopeptide components, was extracted with 0.1 M-hydrochloric acid (150 ml) at 35°C for 14 h, followed by 48 h at 2°C. The soluble teichuronic acid (T1) was isolated by centrifugation, evaporated to a small volume, passed through Dowex 50 and freeze-dried. The yield was 90 mg. The insoluble residue was extracted further with 0.1 M-hydrochloric acid at 35°C overnight, followed by 2 days at room temperature. The soluble teichuronic acid fraction (T2) was treated with Dowex 50 as before and freeze-dried. The yield was 189 mg. Finally the insoluble residue remaining after the second acid treatment was extracted with 0.1 M-hydrochloric acid at 90°C for 7.5 min. The soluble fraction (T3), after treatment with Dowex 50, was freeze-dried and weighed 67 mg. The insoluble residue (925 mg) contained the mucopeptide component and less than 5% of the glucuronic acid content of intact cell walls.

The analyses of the teichuronic acid preparations are shown in Table 1. Each preparation contained approximately equal amounts of glucuronic acid and galactosamine. In addition, Preparation I contained 0.12% of phosphorus, which was probably derived from contamination with teichoic acid. No phosphorus was detected in the fractions from Preparation II, probably because of the very efficient removal of teichoic acids from the walls with alkali.

The positive optical rotations of the preparations were very similar to the values reported by Janczura *et al.* (1961).

Reduction of the carboxyl groups of teichuronic acid. Conversion of glucuronic acid residues within the polysaccharide into glucose was achieved by reduction of the methyl ester derivative (Aspinall, 1965). Teichuronic acid from Preparation I (200 mg) was dissolved in 50% (v/v) methanol (10 ml), cooled in ice and treated with an ethereal solution of diazomethane until the solution became turbid. The ether was removed on a water pump and more diazomethane was added until a yellow colour appeared for an appreciable time. The ether was removed by evaporation. The aqueous layer was diluted to 25 ml with water and treated with sodium borohydride (300 mg) dissolved in 0.2 M-sodium borate buffer, pH 9.7 (25 ml), at room temperature for 5 h, followed by 24 h at 2°C. The solution was then dialysed, passed through a column (2 cm $\times$ 40 cm)

Table 1. *Composition and properties of teichuronic acid fractions*

Teichuronic acid fractions were prepared from cell walls as described in the Results section. The analyses refer to the free acid form of the polysaccharide. Calculated values are 47.2% of galactosamine, 51.2% of glucuronic acid. N.D., Not done.

Preparation	Glucuronic acid (%)	Galactosamine (%)	$[\alpha]_D^{25}$	Glucuronic acid/galactosamine molar ratio
I	43.4	36.2	N.D.	1.12
II T1	47.4	48.0	+37°	0.93
T2	47.4	51.4	+39	0.87
T3	43.2	42.8	+35	0.95
Data of Janczura <i>et al.</i> (1961).	44.8	43.9	+38 to +40	0.93

of Dowex 50 (H⁺ form) and freeze-dried. The whole procedure was repeated four times until a large proportion (see below) of the glucuronic acid content had disappeared. The overall yield of hexosamine was approx. 38%.

The product was purified by chromatography on a column (2 cm × 140 cm) of Sephadex G-50 run in 0.1M-pyridine-acetate buffer, pH 5.1. Fractions (2.5 ml) were collected and portions (0.25 ml) were analysed, after acid hydrolysis, for galactosamine. A single peak that was excluded from the gel was obtained and freeze-dried. The fraction contained hexose (determined by the anthrone reagent) and galactosamine (determined after acid hydrolysis) in the molar ratio 0.94:1.00. After acid hydrolysis the hexose was specifically identified as D-glucose with glucose oxidase. The preparation contained less than 0.1 mol. prop. of glucuronic acid relative to galactosamine. Therefore at least 90% of the carboxyl groups present in teichuronic acid had been reduced. Samples of the carboxyl-reduced derivative (containing 0.4 μmol of galactosamine equivalents) were hydrolysed in M-hydrochloric acid at 100°C for 3 h. Chromatography in solvents I and II showed the presence of compounds with the mobilities of galactosamine and glucose.

A sample (containing 0.4 μmol of galactosamine equivalents) of carboxyl-reduced polysaccharide was examined by electrophoresis in M-acetic acid. A single neutral band was detected by chlorination. In contrast a teichuronic acid standard moved 5 cm towards the anode during electrophoresis. These results confirmed that reduction of carboxyl groups was essentially complete.

Methylation of the reduced polysaccharide. The Hakamori technique was applied in which the methylsulphonyl anion is used to generate the polysaccharide alkoxide before treatment with methyl iodide (Hakamori, 1964; Sandford & Conrad, 1966). A sample of carboxyl-reduced teichuronic acid (containing 4 μmol of galactosamine equivalents) was dissolved in dry dimethyl sulphoxide (2.5 ml) at 40°C. The solution was cooled to room temperature and treated with an excess of

the Hakamori reagent (2.5 ml) at room temperature for 4 h. Then methyl iodide (0.25 ml) was added in small portions over 10 min at a temperature below 25°C. At the end of the reaction the methylated product was dialysed against water, and the non-diffusible fraction was freeze-dried and dissolved in water (2.0 ml) for analysis. Hydrolysis (2M-hydrochloric acid at 100°C for 8 h) of a small sample (0.25 ml) followed by chromatography in solvents I and IV showed the absence of glucose and galactosamine. The aniline phthalate reagent detected in each solvent a fast-moving spot (R_{Glc} 1.98 in solvent I; R_F 0.73 in solvent IV), identical with 2,3,6-trimethylglucose, and two spots moving more slowly. In solvent IV the major slowly-moving spot (R_F 0.34) was intermediate between 3-O-methylglucosamine (R_F 0.20) and 3,4,6-tri-O-methylglucosamine (R_F 0.54) standards, as expected for di-O-methylhexosamines. The minor spot had R_F 0.25. Methanolysis in methanolic 2% hydrochloric acid at 85°C for 4 h, followed by g.l.c., gave peaks corresponding exactly to the α- and β-anomers of methyl 2,3,6-tri-O-methyl-D-glucopyranoside and traces corresponding to the anomers of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside.

These results unequivocally placed the glycosidic linkage on C-4 of glucuronic acid residues in teichuronic acid. The traces of 2,3,4,6-tetra-O-methylglucose detected in the reduced polysaccharide after methylation were presumably derived from non-reducing terminal residues. Unfortunately no standards of methylated galactosamines were available for comparison. Identification of the methylated hexosamines was further complicated because of the tendency of the Hakamori methylation technique to form N-methyl derivatives of N-acetylated hexosamines (Jeanloz, 1966).

Partial acid hydrolysis of teichuronic acid. Samples (0.2 ml) of a 0.25% solution of preparation T2 (Table 1) in M-hydrochloric acid were heated in a boiling-water bath for various times. The hydrolysates were examined by chromatography in solvent I. Increasing amounts of galactosamine,

glucuronic acid and glucuronolactone were formed with time of hydrolysis. The earlier hydrolysates also contained *N*-acetylgalactosamine. Throughout the hydrolysis compounds that moved more slowly than glucuronic acid during chromatography were detected with alkaline silver nitrate. The main compounds had R_{GalN} values of 0.22 and approx. 0.07 respectively. The concentration of these compounds, presumed to be oligosaccharides, reached a maximum after approx. 4 h of hydrolysis.

In a large-scale experiment, preparation T2 (100mg) was heated in *m*-hydrochloric acid (40ml) at 100°C for 3 h. The hydrolysate was evaporated to dryness, dissolved in *m*-sodium chloride (4ml) and applied to a column (2.2cm × 138cm) of

Fig. 1. Sephadex G-25 chromatography of a partial acid hydrolysate of teichuronic acid. Teichuronic acid (100mg) was hydrolysed in *m*-HCl at 100°C for 3 h. The column (2.2cm × 138cm) was eluted with *m*-NaCl and fractions (2.0ml) were collected. Samples (0.1ml) were analysed for glucuronic acid (○), Elson-Morgan reactive material without prior acid hydrolysis (●) and total hexosamine (----).

Sephadex G-25. The column was equilibrated and run in *m*-sodium chloride (Flodin, Gregory & Roden, 1964). Fractions (2.0ml) were collected and portions (0.1ml) were analysed for glucuronic acid and for material reactive with the Elson-Morgan reagent with and without prior acid hydrolysis (Fig. 1). The glucuronic acid and hexosamine material was eluted in a number of broad, poorly resolved peaks. The amount of hexosamine equivalents present in each peak was increased after a vigorous acid hydrolysis except in the tubes under peak S4. The fractions from the column were pooled as indicated in Fig. 1 and analysed (Table 2). Control experiments showed that the high concentration of sodium chloride (1*M*) did not interfere in the carbazole or Elson-Morgan reactions. The yield of hexosamine material recovered from the column was at least 78%. The low galactosamine contents of fractions S1, S2 and S3 are probably due to an increased resistance of the oligosaccharides to further acid hydrolysis after de-*N*-acetylation, as discussed below.

Fraction S1 accounted for a minor proportion of the recovered material and was not examined further. Fraction S2 was a complex mixture of oligosaccharides containing reducing galactosamine residues, and fraction S4 contained free galactosamine and smaller amounts of free glucuronic acid and glucuronolactone. Treatment of fraction S4 with 1% (w/v) sodium borohydride at 35°C overnight destroyed 95% and 83% respectively of the galactosamine and uronic acid content.

Isolation of a disaccharide. Fraction S3, accounting for approx. 14% of the total recovered hexosamine, was concentrated, and then desalted by passage through a column (1.6cm × 115cm) of Sephadex G-10. The column was run in 0.1*M*-pyridine-acetate buffer, pH 5.1. Fractions (2.0ml) were collected. Analysis of the column fractions showed a single sharp peak containing uronic acid, obtained in quantitative yield and free from salt. Chromatography in solvent I gave a major component (R_{GalN} 0.20), detected with alkaline silver

Table 2. Analysis of fractions obtained by Sephadex G-25 chromatography of a partial acid hydrolysate of teichuronic acid

The fractions from Fig. 1 were analysed as described in the Methods section. Galactosamine was estimated after acid hydrolysis in 4*M*-HCl at 100°C for 4 h.

Fraction	Total (μmol)		% of total recovered galactosamine	Glucuronic acid/galactosamine molar ratio
	Glucuronic acid	Galactosamine		
S1	13.1	7.8	4	1.68
S2	99.0	64.0	33	1.54
S3	32.7	26.4	14	1.26
S4	24.4	96.0	49	0.25
Total	169.2	194.2	—	—

nitrate or the Elson–Morgan reagent and corresponding to the faster of the two oligosaccharide bands. Minor components were also present (R_{GalN} values 0.07 and 0.31) and were removed by preparative paper chromatography in solvent I.

The purified material gave a single spot (R_{GalN} 0.52) in solvent III and was neutral on electrophoresis at pH 4.7 in solvent VI. The molar ratio of galactosamine, estimated after acid hydrolysis (4M-hydrochloric acid at 100°C for 4h), to glucuronic acid was 0.83:1.00. The low value for galactosamine was expected, since paper chromatography of the hydrolysate showed the presence of a small amount of unchanged material, indicating a considerable resistance of the glycosidic linkages to hydrolysis. When the fraction was analysed without further acid hydrolysis by the Elson–Morgan reaction, the product obtained showed an absorption maximum at 510nm. This behaviour is characteristic of 3-*O*-substituted hexosamines (Cifonelli, Ludowieg & Dorfman, 1958). After acid hydrolysis in 4M-hydrochloric acid at 100°C for 8h, followed by the Elson–Morgan reaction, the absorption spectrum was identical with that for galactosamine, as expected if hydrolysis of the 3-*O*-substituent had occurred.

The fraction was *N*-acetylated before further characterization. A solution of the material (8.7μmol) in 50% methanol (2.0ml) was stirred vigorously at room temperature during addition of five portions (each 0.05ml) of acetic anhydride at intervals of 30min. The mixture was kept stirring for 30min after the last addition, evaporated to dryness, dissolved in water (1.0ml) and examined by electrophoresis at pH 4.7 in solvent VI. The neutral compound, staining red with the Elson–Morgan reagent, had almost completely disappeared and a new spot moving 6cm towards the anode was detected. The new compound stained mauve with the Elson–Morgan reagent and weakly with silver nitrate. In addition, a faint spot moving 9.3cm to the anode was detected with the silver nitrate and the Elson–Morgan reagents. The remaining *N*-acetylation reaction mixture was streaked on a sheet of Whatman 3MM paper and the major component was purified by electrophoresis at pH 4.7. The main component was eluted from the paper with water and freeze-dried.

Analysis showed the presence of equimolar amounts of galactosamine, measured after acid hydrolysis, and glucuronic acid. The overall yield of material in the main band was 71% of the total equivalent hexosamine taken for the *N*-acetylation.

The *N*-acetylated derivative, isolated from the paper, was homogeneous on chromatography in solvent I (R_{GalN} 0.28), solvent III (R_{GalN} 0.75) and electrophoresis in solvent VI, and was indistinguishable from an authentic sample of *N*-acetyl-

chondrosine (3-*O*-β-D-glucuronosyl-*N*-acetyl-D-galactosamine). A sample (0.44μmol) was treated with 0.6% sodium borohydride (0.5ml) overnight at room temperature and analysis showed that the galactosamine content, determined after acid hydrolysis, had decreased to 5.7% of the starting value. The glucuronic acid content was unchanged.

Samples of the disaccharide from teichuronic acid (0.17μmol) and *N*-acetylchondrosine (0.36μmol) were treated with a mixture of hexamethyldisilazane (0.1ml) and trimethylchlorosilane (0.05ml) dissolved in dry pyridine (0.5ml) at room temperature for 5min. Comparison of the mass spectra of the silylated derivatives indicated very similar structures for the two disaccharides. In particular, the peaks at *m/e* 477, 447, 365, 305, 198 and 173 were present in the same relative intensities. The peaks at *m/e* 305 and 173 are suggestive of a (1→3)-glycosidic linkage, these fragments arising by pathways aB₂ and bH₁ respectively in the nomenclature of Kotchekov & Chizhov (1966).

These findings, together with the behaviour of fraction S3 (Table 2) in the Elson–Morgan test, indicated that the purified material was a disaccharide, glucuronosyl-(1→3)-*N*-acetylgalactosamine. The overall yield of disaccharide from the partial acid hydrolysate of teichuronic acid was approx. 12%.

Formation of chromogen from disaccharides at pH 7 and 100°C. Substituents on C-3 of reducing *N*-acetylated hexosamines are readily eliminated on treatment with alkali, chromogen being formed at room temperature and pH 10.8 (Kuhn & Kruger, 1956; Kuhn, Baer & Gauhe, 1954). Knox & Hall (1965) showed that *O*-β-D-glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine produced chromogen on heating at pH 7 and 100°C. The maximum yield was obtained after 45min. The chromogen produced by *N*-acetylgalactosamine in 45min was only 30% of the maximum value that was reached after several hours. The observed yield of chromogen from the disaccharide was 18 times that for free *N*-acetylgalactosamine and eight times that for free *N*-acetylglucosamine. On a molar basis, *O*-β-D-galactosyl-(1→6)-*N*-acetyl-D-glucosamine yielded the same amount of chromogen as did *N*-acetylglucosamine (Knox & Hall, 1965).

The *N*-acetylated disaccharide obtained from teichuronic acid, and *N*-acetylchondrosine, were therefore examined under the same conditions. Samples of the disaccharides and *N*-acetylgalactosamine were heated in 0.05M-potassium dihydrogen phosphate-sodium hydroxide buffer, pH 7.0, (0.5ml) in a boiling-water bath. At various times the samples were cooled in ice-water and the amount of chromogen formed was measured by addition of acetic acid (3.5ml) and the Morgan–Elson reagent (0.5ml) (Gardell, 1958). The extinction of each

Fig. 2. Morgan-Elson chromogen produced by heating samples of *N*-acetylchondrosine ($0.14\mu\text{mol}$), *N*-acetyl-disaccharide from teichuronic acid ($0.175\mu\text{mol}$) or *N*-acetylgalactosamine ($0.13\mu\text{mol}$) in 0.05M -potassium phosphate buffer, pH 7, at 100°C for various times. —, *N*-Acetylchondrosine; ----, *N*-acetyl-disaccharide from teichuronic acid; ●, *N*-acetylgalactosamine.

solution was read at 550nm after 20min at 37°C . The chromogen produced by the disaccharides reached a maximum within 60min of heating (Fig. 2). In the same time *N*-acetylgalactosamine produced a negligible amount. The molar colour yields for the disaccharide isolated from teichuronic acid and *N*-acetylchondrosine were 2.46×10^6 and 2.12×10^6 extinction units respectively.

These results were consistent with the structure assigned to the *N*-acetylated disaccharide isolated from teichuronic acid.

Formation of chromogen from teichuronic acid at pH 7 and 100°C . Chromogen was rapidly produced on heating samples (containing approx. $1\mu\text{mol}$ of glucuronic acid equivalents) of the polysaccharides (Table 1) in buffer (0.5ml) at pH 7 and 100°C and reached a maximum after approx. 1h. This showed that at least a proportion of the teichuronic acid chains were terminated with a reducing 3-*O*-substituted *N*-acetylgalactosamine residue.

Preparation 1 (Table 1) containing $10.6\mu\text{mol}$ of glucuronic acid was heated in 0.05M -potassium dihydrogen phosphate-sodium hydroxide buffer, pH 7, (5.0ml) at 100°C for 45min. Analysis showed that 72% of the expected chromogen had been formed. A part of the heated sample (4.0ml) was passed through a column ($2.5\text{cm} \times 100\text{cm}$) of Sephadex G-25. The column was equilibrated and run in 0.1M -pyridine-acetate buffer, pH 5.1, and fractions (2.5ml) were collected. Separate portions (0.5ml) were analysed for glucuronic acid with the carbazole reagent, and for preformed chromogen

Fig. 3. Isolation of Morgan-Elson chromogen and modified polysaccharide by chromatography on Sephadex G-25. Teichuronic acid ($8.7\mu\text{mol}$), previously heated at pH 7 and 100°C for 45min, was applied to the column ($2.5\text{cm} \times 100\text{cm}$) and eluted as described in the text. Fractions (2.5ml) were analysed for preformed chromogen (○) and for uronic acid (●).

by the addition of acetic acid (3.5ml) and the Morgan-Elson reagent (0.5ml). Teichuronic acid was eluted in the void volume from the column and was well separated from chromogenic material (Fig. 3). The peak fractions were pooled and freeze-dried. The chromogenic fraction on chromatography in solvent I gave a single spot, detected by the direct development of the paper with a *p*-dimethylamino-benzaldehyde reagent (Perkins, 1960), that was chromatographically identical with the compound produced from *N*-acetylgalactosamine by alkali treatment. A similar result was obtained by Janczura *et al.* (1961). The teichuronic acid recovered from the column (Fig. 3) was heated for 45min at pH 7 and 100°C and the amount of chromogen obtained was 29% of the maximum amount of chromogen produced under the same conditions by an equivalent amount of untreated polysaccharide. This value was almost identical with the amount (28%) expected to be produced by the residual *N*-acetylgalactosamine reducing terminals of the treated polysaccharide.

These results are consistent with elimination of a teichuronic acid chain from C-3 of a reducing terminal *N*-acetylgalactosamine residue by treatment at pH 7 and 100°C . The eliminated teichuronic acid chain would be no longer terminated with a reactive *N*-acetylgalactosamine residue and presumably carried a terminal glucuronic acid residue.

Chain lengths of teichuronic acid preparations. The number-average chain lengths of the polysaccharides (Table 1) were calculated from the maximum amount of chromogen produced after heating at pH 7 and 100°C for 60min, and using the colour yield obtained for the disaccharide (Fig. 2).

Values of 18, 14 and 8 disaccharide units were calculated for the chain lengths of polysaccharides T1, T2 and T3 (Table 3) respectively. A similar experiment, carried out with teichuronic acid from preparation I (Table 1), gave a value of 23 disaccharide units (Table 3). The chain length of this last preparation was also estimated by ultracentrifugation by Dr P. A. Charlwood of this Institute. Teichuronic acid was dissolved in water and the refractive increment was measured in a differential refractometer. The stock solution was diluted to final concentrations of 0.25% or 0.50% in buffers of the following composition: (a) phosphate buffer, pH 6.5 and *I* 0.05, containing 0.5M-sodium chloride or (b) phosphate buffer, pH 6.3 and *I* 0.5, containing 1.0M-sodium chloride. The solutions were ultracentrifuged either at 17980 or 25980 rev./min with a Spinco model E ultracentrifuge at about 20°C; Yphantis short-column experiments were carried out. The partial specific volume was taken as 0.66 (Silpananta, Dunstone & Ogston, 1968). Within experimental error the results at the two speeds were indistinguishable, indicating that teichuronic acid was not greatly inhomogeneous. The weight-average molecular weight was calculated to be approx. 10500–11000. This value indicates that each chain is made up of 25 disaccharide units. The close correspondence of this value and the result obtained by chromogen formation suggests strongly that all of the teichuronic acid chains in preparation I are terminated at the reducing end with C-3-substituted *N*-acetylgalactosamine residues, and also indicates that the chains are unbranched.

Reduction of teichuronic acid with sodium borohydride. The reducing terminal of teichuronic acid was identified by reduction with sodium boro[³H]hydride and characterization of the [1-³H]galactosaminol produced.

Samples (0.2ml) of teichuronic acid preparations T1, T2 and T3 (containing 4 μmol of glucuronic acid equivalents) were reduced overnight at 15°C with 1% (w/v) sodium boro[³H]hydride (0.2ml; 2 μCi/μmol; The Radiochemical Centre, Amersham Bucks., U.K.) in 0.1M-tris-hydrochloric acid buffer, pH 8.4. The mixtures were acidified with 12M-hydrochloric acid (0.2ml), hydrolysed at 100°C for 4h and dried several times from 4M-hydrochloric acid to remove excess of tritium as far as possible. The hydrolysates were applied to separate columns (0.84cm × 22cm) of Dowex 50 (X8; 200–400 mesh), equilibrated with 0.1M-pyridine-acetate buffer, pH 2.8 (Brendel, Roszel & Wheat, 1967). Fractions (2.0ml) were collected. Elution with the same buffer (70ml) removed no Elson–Morgan-reacting material. Subsequent elution with 0.133M-pyridine-acetate buffer, pH 3.85, eluted [³H]galactosaminol (peak tube 51) that was well separated from unchanged galactosamine (peak tube 63). The [³H]galactos-

aminol was identified by electrophoresis in solvent VII in comparison with a standard prepared by reduction of galactosamine with sodium borohydride. Radioactivity was also eluted unretarded from the column of Dowex 50 at peak tube 3 and was similarly eluted during chromatography on columns (0.84cm × 24cm) of Dowex 1 equilibrated and run in 0.1M-pyridine-acetate buffer, pH 5.1, indicating that the fraction was neutral. That the radioactivity was not due to the reduction of teichuronic acid was shown by methods described below. Neutral radioactive material was present in all of the reduction mixtures described, after acidification and drying, as well as in control mixtures containing only sodium boro[³H]hydride and treated in the same way. It was concluded that the material was due to incomplete exchange of tritiated water from the acidified reduction mixtures or alternatively to a radioactive impurity in the reagent. These results confirmed that the polysaccharide chains carried exclusively *N*-acetylgalactosamine residues at the reducing end.

The reducing end of the polysaccharide that was exposed by the elimination reaction at pH 7 and 100°C was shown to be glucuronic acid. The modified polysaccharide (Fig. 3) was treated with sodium boro[³H]hydride and examined as before by chromatography on Dowex 50. The amount of [³H]galactosaminol relative to galactosamine was greatly decreased, as expected. Radioactive material obtained unretarded from Dowex 50 gave radioactivity migrating during paper chromatography in solvent I mainly with standard L-[6-³H]gulono-γ-lactone (*R*_{Glc} 1.48) and, to a smaller extent, with L-[6-³H]gulonic acid (*R*_{Glc} 0.25). L-[6-³H]Gulonic acid (Wolfson & Anno, 1952) was obtained by reduction of D-glucuronic acid with sodium boro[³H]hydride. The compound was purified by chromatography on Dowex 1 at pH 5.1 and was eluted at 34ml of effluent. The γ-lactone was formed from L-[6-³H]gulonic acid by acid treatment (M-hydrochloric acid at 100°C for 2h). A small part of the radioactivity obtained unre-

Table 3. *Estimated average chain lengths of teichuronic acid preparations*

The values were derived as described in the text and are expressed as number of disaccharide units per chain. N.D. Not determined.

Method	Preparation			
	I	II		
		T1	T2	T3
Chromogen formation	23	18	14	8
Reduction by NaB ³ H ₄	N.D.	19	12	7
Ultracentrifugal analysis	25	N.D.	N.D.	N.D.

tarded from Dowex 50 was detected in the glucose region of the chromatograms. This result could only be due to the reduction of a few carboxyl groups of glucuronic acid during treatment of the modified teichuronic acid with sodium borohydride although apparently no reduction took place of the glucuronic acid carboxyl groups of unheated teichuronic acid.

The amounts of [^3H]galactosaminol produced by reduction of polysaccharides T1, T2 and T3 were used to obtain independent values for the number-average chain lengths of these polysaccharides. The sodium borohydride reagent was standardized (Nordin, Hasegawa, Smith & Kirkwood, 1966) by reduction of known amounts ($5.6\text{ }\mu\text{mol}$) of galactosamine followed by Dowex 50 chromatography and isolation of [^3H]galactosaminol. The ratio of galactosaminol to unchanged galactosamine in the hydrolysates of reduced polysaccharides T1, T2 and T3 gave chain-length values (Table 3) that were in reasonable agreement with results obtained by the other methods.

DISCUSSION

Teichuronic acid is firmly linked to the mucopeptide in *B. licheniformis* cell walls (Hughes, Pavlik, Rogers & Tanner, 1968; Hughes, 1970). The covalent linkages are easily broken in dilute acids and appear to be phosphodiester bonds involving the reducing ends of the teichuronic acid chains. These reducing terminals are shown in this present paper to be exclusively *N*-acetylgalactosamine residues.

Prolonged treatment with dilute acid breaks glycosidic bonds in the interior of the teichuronic acid chains. The methods of preparation of teichuronic acid described are therefore certain to produce partially degraded molecules. For example, the apparent chain lengths of polysaccharides T1, T2 and T3, extracted from the cell walls by acid, decreased with increasing time of acid treatment (Table 3). The largest number-average chain length obtained was 23 disaccharide units (Table 3). The native polysaccharide in the cell walls was calculated to have a number-average chain length of at least 35 disaccharide units (Hughes, 1970).

The acid-sensitive links within the teichuronic acids were shown to be exclusively the *N*-acetylgalactosaminyl residues. Thus no free reducing groups of glucuronic acid residues were detected in polysaccharides T1, T2 and T3. The relative stability of the glucuronosyl glycosidic bonds of the polysaccharide to acid hydrolysis is probably due to the carboxyl groups of these residues, which shield the linkages from attack by H_3O^+ ions. Thus the oligosaccharides produced by acid hydrolysis largely carried reducing hexosamine residues. The resis-

tance of these oligosaccharides to further acid hydrolysis is shown in the relatively low yield of galactosamine, measured after further acid hydrolysis (Table 2). This behaviour is probably due to rapid de-*N*-acetylation during partial acid hydrolysis, followed by electrostatic shielding of the glucosaminidic linkages by the free amino groups, which carry positive charges in acid solutions (Moggridge & Neuberger, 1938). The disaccharide 3-*O*-glucuronosylgalactosamine was isolated from partial acid hydrolysates of teichuronic acid in about 12% overall yield. This value is comparable with the recovery (20%) of hyalbiuronic acid (3-*O*- β -glucuronosylglucosamine) after acid hydrolysis of hyaluronic acid (Weissman, Rapport, Linker & Meyer, 1953). The higher oligosaccharides produced by partial acid hydrolysis of teichuronic acid also contained reducing 3-*O*-substituted galactosamine residues, as shown by the absorption spectra of the products in the Elson-Morgan test. These results confirm an earlier suggestion (Janczura *et al.* 1961) that the *N*-acetylgalactosaminyl residues of teichuronic acid are predominantly C-3-substituted.

The relatively good yield of disaccharide also suggests an alternating sequence of glucuronic acid and *N*-acetylgalactosamine residues in teichuronic acid. This finding is in agreement with the biosynthetic evidence for a strict alternation of these residues (Hughes, 1966) and with the identification of glucuronic acid as the residue contiguous to the *N*-acetylgalactosamine reducing terminals of the polysaccharide chains. However, a rigorous proof of this alternating sequence was not obtained in the present work, and will probably depend on the availability of an enzyme capable of degrading teichuronic acid to some simple, identifiable, oligosaccharide in high yield.

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