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The teichuronic acid from the walls of *Bacillus licheniformis* A.T.C.C. 9945

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The teichuronic acid of *Bacillus licheniformis* A.T.C.C. 9945 grown under phosphate limitation was isolated from the cell walls and purified by ion-exchange and Sephadex chromatography. The detailed structure of the polysaccharide was established by methylation analysis, periodate oxidation and partial acid hydrolysis. The polymer is composed of tetrasaccharide repeating units with the structure $[GlcA\beta(1\rightarrow4)GlcA\beta(1\rightarrow3)GalNAc\beta(1\rightarrow6)GalNAc\alpha(1\rightarrow4)]_n$. ^{13}C n.m.r. analysis has confirmed most of the structural features of the polysaccharide and, in particular, the anomeric configurations and linkage positions of substituents. The teichuronic acid from glucose-limited cells was identical with that from cells grown under phosphate limitation.

The cell walls of the bacilli, in common with those of other Gram-positive organisms, contain teichoic acids of different structure when grown in batch culture or in a chemostat with glucose, K^+ or Mg^{2+} as the growth-limiting nutrient. Under phosphate limitation the bacilli replace their teichoic acid with teichuronic acid. *Bacillus licheniformis* A.T.C.C. 9945 is unusual in containing three cell-wall teichoic acids, namely poly(glucosylglycerol phosphate), poly(galactosylglycerol phosphate) and poly(glycerol phosphate) (Burger & Glaser, 1966). Furthermore, when grown in a chemostat under glucose limitation, this organism contains both teichoic acids and a teichuronic acid. It is therefore probable that a teichuronic acid is present under all growth conditions and may have an essential role in the functioning of the organism. *Bacillus subtilis* species var. *niger*, W23 and 168 also contain different wall teichoic acids when grown under phosphate-rich conditions. When grown under conditions of phosphate limitation, each organism replaces its teichoic acid(s) with a teichuronic acid, similar in both chemical and physical properties to the teichuronic acid in *B. licheniformis* A.T.C.C. 9945 (Ellwood & Tempest, 1972). The changeover has been used to study the assembly of newly formed cell wall material (Archibald, 1976) and to study the mechanism of control of teichoic and teichuronic acid biosynthesis (Hussey *et al.*, 1978; Rosenberger, 1976).

The teichuronic acid from *B. licheniformis* N.C.T.C. 6346 grown in batch culture is a polymer containing equimolar amounts of glucuronic acid and *N*-acetylgalactosamine, apparently in a simple disaccharide repeating unit (Janczura *et al.*, 1961; Hughes & Thurman, 1970). The authors proposed that the glucuronic acid residues were linked at C-4, the *N*-acetylgalactosamine residues at C-3, and further that the linkages were in the α-D-configuration because of the optical rotation of the polymer($[\alpha]_D$ +35–40°). The teichuronic acid of *B. subtilus* W23 grown under conditions of phosphate limitation has also been investigated (Wright & Heckels, 1975) and it was concluded that its structure is identical with that proposed for the teichuronic acid found in walls of *B. licheniformis* N.C.T.C. 6346.

The purpose of the present study was to compare the teichuronic acid from cell wall of *B. licheniformis* A.T.C.C. 9945, grown in a chemostat under conditions of glucose limitation, with the corresponding polymer from phosphate-limited growths. It would then be possible to establish whether the teichoic acids produced under glucose limitation are replaced in the wall by the same, or by a different, teichuronic acid. However, the study of the teichuronic acid grown under the latter conditions showed that the repeating unit proposed by other workers (Janczura *et al.*, 1961; Hughes & Thurman, 1970; Wright & Heckels, 1975) was inadequate for

Abbreviations used: g.l.c.–m.s., gas-liquid chromatography–mass spectrometry.

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the polymer from A.T.C.C. 9945. The primary consideration therefore became the structural elucidation of the teichuronic acid from phosphate-limited cells of A.T.C.C. 9945.

Experimental

Materials

Bacillus licheniformis A.T.C.C. 9945 was maintained on nutrient agar plates and grown in either glucose-limited or phosphate-limited medium as previously described (Hussey *et al.*, 1978). *Streptococcus pneumoniae* A.T.C.C. 12213 was maintained on blood/agar slopes and subcultured monthly. It was grown in batch culture as previously described (Poxton & Leak, 1977). Crude hyaluronidase was prepared essentially by the method of Rapport *et al.* (1951).

1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate, chondroitin-4-sulphate, hyaluronic acid, amylose, cellobiose, and testicular hyaluronidase were purchased from Sigma Chemical Co., Poole, Dorset, U.K. The glucose oxidase reagents were purchased from Cambrian Chemicals, Croydon, Surrey, U.K. and sodium boro[^{3}H]hydride from The Radiochemical Centre, Amersham, Bucks, U.K.

Analytical methods

Phosphate was determined by the method of Chen *et al.* (1956), nucleic acid spectrophotometrically at 260nm, uronic acid by the method of Bitter & Muir (1962) and also by the method of Blumenkrantz & Asboe-Hansen (1973) (for reduced teichuronic acid preparations only), glucose by the method of Dubois *et al.* (1956) and also by the glucose oxidase reagents, *N*-acetylhexosamine by the method of Ghuysen *et al.* (1966) and hexosamine by the method of Rondle & Morgan (1955). Amino sugar analyses were carried out on a JEOL autoanalyser. Periodate was determined by the method of Dixon & Lipkin (1954) with the correction factor of Aspinall & Ferrier (1957). Trimethylsilyl derivatives were prepared by the method of Sweeley *et al.* (1963) and were examined directly in a Perkin–Elmer model F17 gas chromatograph with a glass column (2m × 3.0mm) of 10% SE-30 at 290°C and by combined g.l.c.–m.s. on a VG-70-70F instrument with a glass column (1.52m × 4mm) of 3% OV-1 at 270°C.

^{13}C n.m.r. spectra were obtained with complete proton decoupling in ^2H_2O solutions; approx. concentrations, 100mg·ml^{-1} at 32°C and 22.63MHz with external tetramethylsilane as reference. The ^{1}H n.m.r. spectrum was obtained at 270MHz in a ^2H_2O solution; concentration, 4mg·ml^{-1} at 57°C with dioxan as reference. Chemical shifts relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) were calculated with the formula $\delta_{dioxan} = \delta_{DSS} + 3.71$.

Methylation analysis

Methylation was performed by the procedure of Hakomori (1964) as developed by Jansson *et al.* (1976). The mixture of partially methylated sugars was analysed as the alditol acetates by using a Packard model 417 Becker gas chromatograph. Glass columns (2m × 3mm) of 3% ECNSS-M at 180°C (for neutral components) and 3% OV-17 at 180°C (for amino sugar components) were used. Partially methylated alditol acetates were also analysed by using a Varian 1400 gas chromatograph linked to a Micromass model 12B2 mass spectrometer at an ionization potential of 70eV. Glass capillary columns coated with SP-1000 (for neutral components) or OV-1 (for amino sugar components) were used.

Alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose (*a*), 2,3,6-tri-*O*-methyl-D-glucose (*b*), 2,3-di-*O*-methyl-D-glucose (*c*), 2,6-di-*O*-methyl-D-glucose (*d*), 3,6-di-*O*-methyl-D-glucose (*e*) and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxy-D-galactose (*f*) were prepared as follows: (*a*) methyl α-D-glucopyranoside was methylated, hydrolysed (2M-HCl, 1h, 100°C) and converted into the alditol acetate; (*b*)–(*e*) amylose was partially methylated after incomplete dissolution in dimethyl sulphoxide, hydrolysed (2M-HCl, 3h, 100°C) and converted into alditol acetates (components were identified from the mixture by g.l.c.–m.s.); (*f*) chondroitin 4-sulphate was desulphated by the method of Kantor & Schubert (1957) and reduced as described for the teichuronic acid preparations. The product was methylated and partially methylated alditol acetates were prepared as described for reduced teichuronic acid. The major amino sugar component (*f*) was identified by g.l.c.–m.s.

An authentic sample of 1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxy-D-galactitol was a gift from Dr. D. Powell, Department of Organic Chemistry, University of Stockholm, Sweden.

Paper chromatography

The descending technique was used with the following solvent systems; A, ethyl acetate/pyridine/acetic acid/water (5:5:1:3 by vol.); B, butan-1-ol/pyridine/water (6:4:3 by vol.); C, propan-1-ol/ammonia (sp.gr. 0.88)/water (6:3:1 by vol.); D, butan-1-ol/pyridine/water (4:1:1 by vol.).

The detecting reagents used were as follows: (*a*), alkaline silver nitrate reagent for reducing compounds (Trevelyan *et al.*, 1950); (*b*), ninhydrin reagent for amino compounds (Consden & Gordon, 1948); (*c*), periodate/Schiff reagent for *vic*-glycols

(Baddiley *et al.*, 1956). Routine analytical work was carried out on Whatman no. 1 paper whereas preparative separations were performed on Whatman 3MM paper previously washed with 1 M-acetic acid followed by water.

Preparation of cell walls

Cells were harvested by centrifugation of hourly collections (600ml) of bacteria from the chemostat. Washed cells were suspended in 0.9% (w/v) NaCl to a concentration of 10% (w/v) and disrupted with no. 11 Ballotini beads for 2.5min in a Braun disintegrator. The beads were removed on a no. 1 glass sinter and the filtrate was centrifuged at 15000*g* for 20min at 4°C. The walls were separated from whole cells, washed twice in 0.9% NaCl and suspended in 0.1 M-phosphate buffer, pH 7.8. Autolytic enzymes were inactivated by boiling for 15min, and after cooling, trypsin (1mg/ml) was added together with a drop of toluene of inhibit bacterial growth. The suspension was incubated with stirring for 16h at 37°C and the walls were recovered by centrifugation at 15000*g* for 20min. The walls were suspended in water and an equal volume of hot 4% (w/v) sodium dodecyl sulphate was added. Stirring was continued for 6h at room temperature. The walls were washed by centrifugation at 15000*g* for 20min in six changes of distilled water at room temperature and lyophilized.

Isolation and purification of teichuronic acids

Walls from phosphate-limited cells were suspended in 5% (w/v) trichloroacetic acid to a concentration of about 1% and were extracted by gentle stirring at 37°C for 16h to solubilize the teichuronic acid. The insoluble cell wall residue was removed by centrifugation at 15000*g* for 20min at 4°C. Trichloroacetic acid was removed from the soluble fraction by ether extraction and the aqueous layer was concentrated and applied to a column (2.6cm × 34cm) of DEAE-52 cellulose; elution was with a linear gradient of 0–1.0 M-pyridine acetate buffer, pH 5.3. The teichuronic acid was eluted as a single peak (0.4–0.7 M). Nucleic acids were removed from the crude teichuronic acid by fractionation on a stacked column (1cm × 86cm) of Sephadex G-25 and G-75 as previously described (Poxton *et al.*, 1978). The fraction containing the polysaccharide was desalted by dialysis at 4°C, and conversion to the free acid form of the polysaccharide was effected by passage through a column (2.42cm × 20cm) of Dowex 50 (X8, H^+ form, 50–100 mesh). The purified teichuronic acid corresponded to about 58% of the dry weight of the wall.

The teichuronic acid from glucose-limited cultures was prepared by the method described above after selective extraction of teichoic acids with alkali [0.2 M-NaOH containing 2% (w/v) $NaBH_4$ for 16h at 37°C under N_2] (Hughes & Tanner, 1968). The purified teichuronic acid corresponded to about 30% of the dry weight of the wall.

Preparation of reduced teichuronic acid

Reduction of the uronic acid residues in teichuronic acid preparations was achieved by the method of Taylor & Conrad (1972). In a typical experiment a solution of the teichuronic acid (130μmol) was stirred during addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide or 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate (1mmol). As the reaction proceeded the pH of the solution was kept at 4.75 by titration with 0.1 M-HCl. After 150min, uptake of acid had ceased and $NaBH_4$ solution (2 M, 20ml) was added dropwise to the reaction mixture at room temperature. The pH rose rapidly to 7 which was maintained by titration with 4 M-HCl. After 30min, the solution was heated to 50°C and $NaBH_4$ solution (2 M, 10ml) was added during a further 30min. The product was dialysed for 48h at 4°C against six changes of distilled water and lyophilized. The yield was quantitative. Under these conditions uronic acid analysis indicated about 70% reduction of carboxyl groups. The procedure was repeated three times until more than 90% of the carboxyl groups in the polymer had been reduced. The final product was purified on a stacked column of Sephadex G-75 and G-25 and was eluted with 0.2 M-NaCl. A single sharp carbohydrate-containing fraction was obtained which was dialysed against distilled water and lyophilized.

Results and discussion

Composition of the teichuronic acid from phosphate-limited cells

Teichuronic acid (4mg) was hydrolysed in 4 M-HCl for 4h at 100°C in a sealed, evacuated tube. Acid was removed by evaporation *in vacuo* over KOH pellets and the residue was examined by paper chromatography in solvent systems A and B; products were located with reagents (*a*) and (*b*). Glucuronic acid and its lactone and galactosamine were detected. Acid hydrolysis in 1 M-HCl at 100°C for varying times (1–8h) produced *N*-acetylgalactosamine in early hydrolysates.

A sample of the polymer was analysed for uronic acid. A second sample was analysed for hexosamine after hydrolysis in 4 M-HCl for 4h at 100°C. Teichuronic acid had molar proportions of uronic acid and galactosamine of 1.00:0.96. On the basis that all amino sugar residues are *N*-acetylated in the polysaccharide, these two components accounted for 93% of the dry weight of teichuronic acid. Phosphate and nucleic acid were absent. The polysaccharide had $[\alpha]_D^{23}$ +37.1° (*c* 1.08 in water).

Values of +34–40° (Janczura *et al.*, 1961; Hughes & Thurman, 1970; Ellwood & Tempest, 1972; Wright & Heckels, 1975) were reported for the teichuronic acid from *B. licheniformis* N.C.T.C. 6346 and *B. subtilis* W23.

Composition of reduced teichuronic acid from phosphate-limited cells

Reduction of the uronic acid residues in the teichuronic acid to the corresponding neutral sugars was achieved by the method of Taylor & Conrad (1972). The reduced polysaccharide (2mg) was hydrolysed in 2M-HCl for 3h at 100°C. After removal of acid, the hydrolysate was examined by paper chromatography in solvent systems A and B with reagents (*a*) and (*b*) for detection. Galactosamine and glucose were major components; glucuronic acid and its lactone were present in traces.

Reduced teichuronic acid contained uronic acid, galactosamine and glucose (by phenol/H_2SO_4) in the molar proportions 0.09:1.00:0.59. Acid hydrolysis of the polysaccharide was performed to establish conditions for maximum yield of galactosamine and glucose; galactosamine (3M-HCl, 6h, 100°C) and glucose (2M-HCl, 2–5h, 100°C; by glucose oxidase) were present in the molar ratio 1.00:0.48. The galactosamine/glucose ratio was determined in several different preparations of the reduced polymer by two different methods, and we consistently obtained values of 1:0.5–0.6. This ratio is increased to 1:0.6–0.7 after allowing for the loss (10–15%) of glucose under the hydrolytic conditions [this loss is based upon observations (M. R. Lifely, unpublished work) of the effect of 2M-HCl (2–4h, 100°C) on an equimolar mixture of glucose and *N*-acetylgalactosamine]. By making further allowances for incomplete reduction of carboxyl groups, the glucose content of the 'reduced' polymer approaches 80% of the expected value. Further acid hydrolysis of the polysaccharide [0.06% (w/v), 1ml, 2M-HCl for 3h at 100°C] was followed by re-*N*-acetylation (0.5ml of methanol, 10μl of pyridine, 50μl of acetic anhydride; Kozulić *et al.*, 1979), reduction with $NaBH_4$ [2% (w/v), 0.5ml], passage through a short column of Dowex 50 (H^+ form) and removal of borate by evaporation with methanol. The residue was trimethylsilyated and examined by g.l.c. with a column of SE-30. Trimethylsilyl derivatives of *N*-acetylgalactosaminitol and glucitol were present in the molar ratio 1.00:0.57. However, this ratio was increased to 1.00:0.71 by acid hydrolysis under gentler conditions (0.5M-HCl, 4h, 100°C) and further increased to 1.00:0.83 by summation of glucitol and cellobiitol, which is also a product of mild acid hydrolysis of reduced teichuronic acid.

The polysaccharide had $[\alpha]_D^{23}$ +55.6° (*c* 1.08 in water).

Methylation

Methylation as described by Jansson *et al.* (1976) was carried out on the reduced teichuronic acid from phosphate-limited cells to minimize side reactions (Björndal *et al.*, 1970; Stellner *et al.*, 1973). Complete methylation could usually be achieved in one step, as determined by analysis of the mixture of partially methylated sugars as their alditol acetates by using g.l.c. Neutral components were identified by comparison with standards of partially methylated glucose derivatives on a column of ECNSS-M and complementary information was obtained by g.l.c.–m.s. with a capillary column of SP-1000. The major neutral component corresponded to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, thereby confirming C-4 as the linkage position to glucuronic acid residues in the teichuronic acid. A minor component, arising from the terminal non-reducing end of the polymer, corresponded to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. The two derivatives were present in the molar ratio of 19:1 (assuming that molar response is the same for all neutral partially methylated alditol acetates; Björndal *et al.*, 1970).

The determination of partially methylated amino sugar derivatives presented more difficulty than did that of the neutral components, due partly to their poor yield after acid hydrolysis, and partly to their thermal lability and low volatility (Stellner *et al.*, 1973). Analysis of the amino sugar derivatives by g.l.c. on a column of OV-17 gave a complex profile from which quantitative data were difficult to obtain. The mixture was more effectively resolved by g.l.c.–m.s. with a capillary column of OV-1. The most diagnostic ion for alditol acetates of *N*-methylated amino sugars is that at *m/e* 158, caused by primary fission between C-2 and C-3, and only components containing this ion were registered on the instrument. The two major amino sugar components from reduced teichuronic acid corresponded precisely to standards of 1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxy-D-galactitol and the analogous 3,4-di-*O*-methyl ether. Mass spectra of each of the components were recorded and comparison of the data confirmed the presence of both the 3,4- and 4,6-di-*O*-methyl ether derivatives from reduced teichuronic acid. This establishes the presence of both 3-*O*-substituted and 6-*O*-substituted *N*-acetyl-D-galactosamine residues in the teichuronic acid. Other minor components, however, were detected; these could not be identified although their mass spectra showed the diagnostic *m/e* 158 and *m/e* 116 ions. It seems likely that these products are formed either by degradation during hydrolysis of the methylated polymer or by subsequent degradation during the g.l.c.–m.s. procedure.

Periodate oxidation

In preliminary experiments, samples of reduced teichuronic acid from phosphate-limited cells were oxidized with aqueous 0.01 M-sodium metaperiodate solution in the dark at room temperature and at 4°C, reduction of periodate being measured at intervals during the reaction. Although serious overoxidation occurred at room temperature, this was prevented by oxidation at 4°C (Fig. 1*a*). All further oxidations were carried out at 4°C in 0.1 M-acetate buffer, pH 4.5. Teichuronic acid from phosphate-limited cells reduced about 0.5 mol of periodate/mol of galactosamine during the first 10 h (Fig. 1*b*). Overoxidation of teichuronic acid however seemed to occur. Under the same conditions, chondroitin 4-sulphate was almost resistant to periodate (Fig. 1*b*). The reduced polysaccharides of teichuronic acid and desulphated chondroitin 4-sulphate also showed differences in their rates of periodate reduction, the former being oxidized at more than twice the rate of the latter (Fig. 1*c*); no overoxidation was apparent.

Reduced teichuronic acid (20 mg) was analysed in more detail after oxidation for 24 h, when 0.98 mol of periodate/mol of galactosamine had been reduced. The reaction was stopped by addition of ethylene glycol and the oxidized polysaccharide was dialysed for 24 h at 4°C. The reaction mixture was concentrated and treated with $NaBH_4$ (50 mg) for 16 h at room temperature. The pH was adjusted to 7 by the addition of 1 M-acetic acid, the solution was dialysed for 48 h at 4°C, passed through a short column of Dowex 50 (H^+ form) and lyophilized. The yield was 13 mg. A sample of the oxidized polysaccharide was hydrolysed in 2 M-HCl for 3 h at 100°C and examined by paper chromatography in solvent systems A, B and C. Galactosamine and glucose were present, together with erythritol and glycerol. The polysaccharide was analysed for galactosamine after acid hydrolysis (4 M-HCl, 4 h, 100°C) and for hexose after removal of glycolaldehyde by treatment with 2.5% methanolic HCl at 60°C for 3 h, neutralization with lead carbonate, filtration, evaporation to dryness and repeated distillation with methanol; 1.64 μmol of galactosamine and 0.79 μmol of hexose were present in 1 mg of oxidized polysaccharide. Thus about 40% of the galactosamine residues had been destroyed, consistent with substitution at C-6. Some of the hexose residues had been destroyed, although quantitative information was difficult to obtain due to the low colour yield of reduced teichuronic acid in the phenol/H_2SO_4 reaction. The fact that glucose could be detected after acid hydrolysis of the oxidized polymer is not indicative of substitition at C-3 of these residues, since the modified chondroitin 4-sulphate polymer also showed resistance of glucose residues to periodate. A combination of

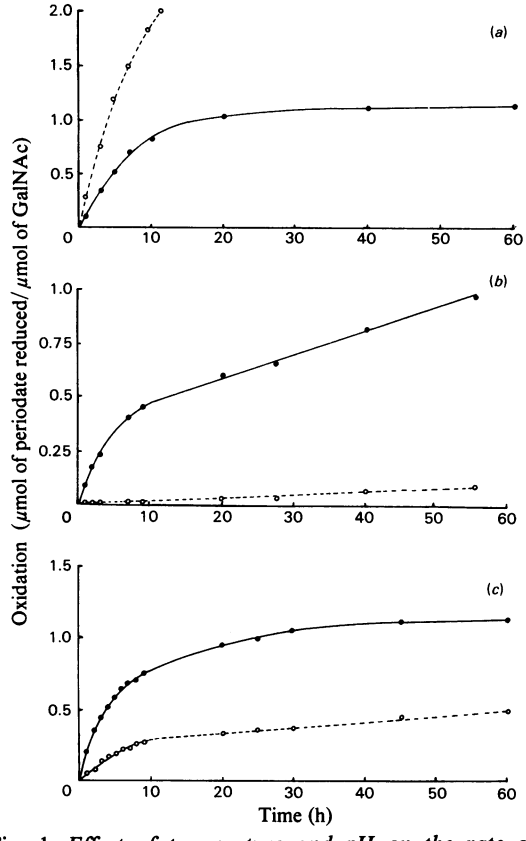


Fig. 1. *Effect of temperature and pH on the rate of oxidation of teichuronic acid and its reduced derivative and comparison with chondroitin 4-sulphate and its reduced and desulphated derivative*

(*a*) Reduced teichuronic acid in aqueous solution at 4°C (●) and at room temperature (○); (*b*) teichuronic acid (●) and chondroitin 4-sulphate (○) at 4°C and pH 4.5; (*c*) reduced teichuronic acid (●) and reduced and desulphated chondroitin 4-sulphate (○) at 4°C and pH 4.5.

factors may explain the stability of glucose residues, such as the unexpectedly low periodate consumption of many polysaccharides, including alginate (Drummond *et al.*, 1962), dermatan sulphate (Fransson, 1974) and amylose (Painter & Larson, 1970), which had been ascribed to inter- and intra-chain hemiacetal formation between oxidized and unoxidized uronic acid (or hexose) residues.

A further sample of reduced teichuronic acid (10 mg) was more completely oxidized by treatment with 0.05 M-periodate for 65 h, when the polymer had reduced 1.18 mol of periodate/mol of galactosamine. The polyaldehyde was reduced with NaB^3H_4 for 4 h at room temperature, the solution was neutralized, dialysed and passed through a short

column of Dowex 50 (H^+ form) and lyophilized. The yield was 6.6mg. A sample (3mg) was hydrolysed in 2M-HCl for 3h at 100°C. After removal of acid, the residue was dissolved in water (0.5ml), and a sample (0.2ml) was analysed by paper chromatography in solvent B. The radiochromatographic profile showed the presence of two major components corresponding to erythritol and glycerol in about equimolar amounts. Both components were eluted from the paper with water, evaporated to dryness and chromatographed separately in solvent D, the radiochromatograms confirming the identity of the products as erythritol and glycerol.

The results of periodate oxidation are in accord with the presence in the reduced polymer of 4-*O*-substituted glucose residues (to give erythritol), 6-*O*-substituted *N*-acetylgalactosamine residues (to give glycerol) and 3-*O*-substituted *N*-acetylgalactosamine (resistant to oxidation), although quantitative interpretation of the results is made difficult by incomplete oxidation of the polymer. Small amounts of glycerol would presumably arise from the terminal non-reducing residues of the polymer.

Enzymic hydrolysis with hyaluronidases

The action of testicular and pneumococcal hyaluronidases on the teichuronic acid from phosphate-limited cells was compared with that on hyaluronic acid. Samples of teichuronic acid and hyaluronic acid were converted to their sodium salts by passage through a short column of Dowex 50 (Na^+ form). Solutions of the substrates ($0.4\,mg \cdot ml^{-1}$) in 0.1M-acetate buffer, pH 6.0, containing 0.15M-NaCl were prepared. Solutions of crude pneumococcal hyaluronidase ($1\,mg \cdot ml^{-1}$) and testicular hyaluronidase ($0.25\,mg \cdot ml^{-1}$) in the same buffer were prepared and used immediately. The substrates (0.8ml) were incubated with enzyme (0.2ml) at 37°C in the presence of a trace of toluene. The reducing *N*-acetylhexosamine was determined. Hyaluronic acid was extensively hydrolysed (45 and 90%) with both testicular and bacterial hyaluronidases respectively after incubation for 24h; teichuronic acid underwent some hydrolysis (13 and 17%) with both testicular and pneumococcal enzymes. Hyaluronidases are known to hydrolyse the 3-*O*-β-D-hexosaminyl linkages in hyaluronic acid (Linker *et al.*, 1956) and it might be these bonds that are hydrolysed in the teichuronic acid.

Reduced teichuronic acid from phosphate-limited cells was not attacked by either testicular or pneumococcal hyaluronidase, even after incubation for 48h.

Partial acid hydrolysis

Hughes & Thurman (1970) isolated a disaccharide in low yield after acid hydrolysis of the teichuronic acid from batch-grown *B. licheniformis* N.C.T.C. 3646, as did Wright & Heckels (1975) from the teichuronic acid of *B. subtilis* W23 grown under phosphate limitation. In both cases this disaccharide was shown to have the structure 2-amino-2-deoxy-3-*O*-(D-glucopyranuronyl)-D-galactose. However, in neither case was the anomeric configuration of the linkage established.

In this present study, preliminary hydrolyses of the reduced teichuronic acid from phosphate-limited cells were performed on samples (1ml) of a 0.1% solution in 0.5M-HCl at 100°C for various times. Examination of the hydrolysates by paper chromatography by using reagents (*a*) and (*b*) showed the presence of a component with R_{GalN} 0.77 in solvent A and R_{GalN} 0.64 in solvent B. In a large-scale experiment, the reduced polysaccharide (100mg) was heated in 0.5M-HCl (10ml) at 100°C for 1.5h. The hydrolysate was neutralized with 0.5M-NaOH (10ml) and evaporated to dryness. The residue was dissolved in 1M-pyridine acetate buffer, pH 5.1, applied to a previously equilibrated column (2.6cm × 100cm) of Sephadex G-10 and eluted with the same buffer. Fractions (2ml) were collected and analysed for hexose (phenol/H_2SO_4) and for hexosamine (Elson–Morgan) (Fig. 2). Three broad peaks were observed; peak III corresponded to glucose and galactosamine and accounted for 42% of the carbohydrate. Fractions corresponding to peaks I and II were examined by paper chromatography in solvent B. The component with R_{GalN} 0.64 was absent from peak I (which consisted of a complex series of oligosaccharides) but was present as the major component of peak II. Attempts to purify this product by preparative paper chromatography in solvent A resulted in some degradation, yielding minor amounts of glucose and an unidentified component, R_{GalN} 0.52. Identical results were obtained in the isolation of the disaccharide, 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyl)-D-galactose from reduced and desulphated chondroitin 4-sulphate. It is known that substituents at C-3 of reducing carbohydrates readily undergo β-elimination on treatment with base, a chromogen being formed (Knox & Hall, 1965). The major oligosaccharide from reduced teichuronic acid was indistinguishable from 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyl)-D-galactose by paper chromatography in solvents A, B and C, and after acid hydrolysis in 2M-HCl for 3h at 100°C the only components observed by paper chromatography in solvent B were glucose and galactosamine.

Samples of the disaccharide (0.75μmol) from both polysaccharides were examined on a JEOL autoanalyser. In both cases single ninhydrin-positive peaks were observed having identical retention times. After acid hydrolysis (2M-HCl, 3h, 100°C) galactosamine was produced.

A sample of the disaccharide (0.5μmol) from

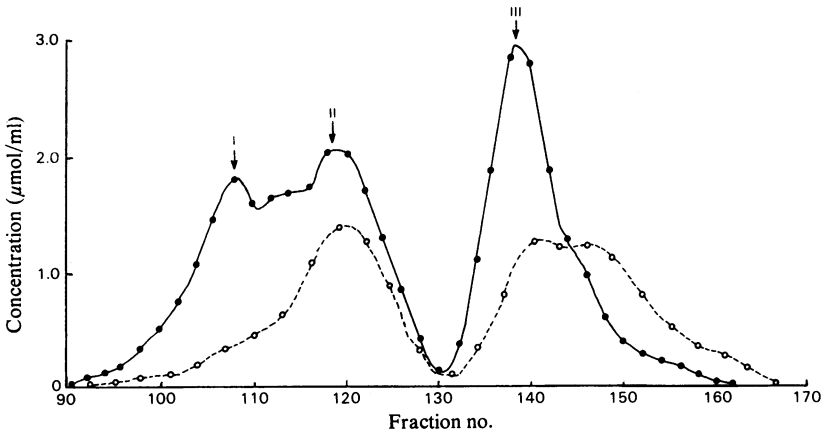


Fig. 2. *Sephadex G-10 chromatography of a partial acid hydrolysate of reduced teichuronic acid*
Fractions (2ml) were collected from the column and analysed for carbohydrate (●) and for hexosamine without prior acid hydrolysis (○). Three peaks (I–III) were observed.

reduced teichuronic acid was examined by the Elson–Morgan reaction; an absorption maximum at 510nm, characteristic of 3-*O*-substituted hexosamines (Cifonelli & Dorfman, 1958) was observed. The ratio of absorption at 540nm to that at 510nm was 0.42. A sample of 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyl)-D-galactose gave a ratio of 0.38. 4-*O*- or 6-*O*-substituted hexosamines give a ratio greater than 1 in this reaction (Cifonelli & Dorfman, 1958).

The two samples of disaccharide were *N*-acetylated by the method of Kozulić *et al.* (1979), and the products were passed through a short column of Dowex 50 (H^+ form) to remove non-*N*-acetylated disaccharide. The resulting components were converted to their alditols by reduction in aqueous 0.5% (w/v) KBH_4 at room temperature. Since no significant degradation of the disaccharides occurred, reduction presumably is faster than β-elimination. The two *N*-acetylated disaccharide alditols were examined as their trimethylsilyl derivatives by g.l.c. on a column of SE-30. Identical chromatograms were obtained in which trimethylsilylated D-glucitol was a minor component.

The β-configuration of the glycosidic linkage in the disaccharide from reduced teichuronic acid was confirmed by passage through a short column (0.9cm × 6cm) of concanavalin A–Sepharose 4B and elution with 0.1M-acetate buffer containing 1M-NaCl and 1mM-$CaCl_2$, $MgCl_2$ and $MnCl_2$. The single carbohydrate-containing fraction was desalted on a column (1.5cm × 40cm) of Sephadex G-10 and lyophilized. The yield was quantitative. These findings show that the component isolated in about 10% yield from reduced teichuronic acid was a disaccharide, 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyl)-D-galactose.

Preferential formation of this disaccharide may be rationalized by consideration of the relative rates of acid-catalysed hydrolysis of simple glycosides of 2-acetamido-2-deoxy sugars and the corresponding 2-hydroxy sugars. The rate for hydrolysis of the glycosidic bond of the former is about 4 times that of the latter (and about 100 times that of protonated 2-amino-2-deoxy analogues); hydrolysis of the glycosidic bond of acetamido sugars tends to precede de-*N*-acetylation (Foster *et al.*, 1957). Increased lability of 2-acetamido-2-deoxy pyranosides in heteropolymeric sytems has been reported on several occasions. For example, *O*-glycosyl-2-amino-2-deoxy aldoses are observed as relatively major products from selective acid hydrolysis of blood-group-A substance (Schiffman *et al.*, 1962) and from a desulphated, carboxyl-reduced, *N*-acetylated heparin derivative (Wolfrom *et al.*, 1964). The isolation of 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyl)-D-galactose as the major disaccharide from the partial acid hydrolysis of carboxyl-reduced teichuronic acid is therefore to be expected.

To identify further oligosaccharide fragments from reduced teichuronic acid, hydrolysis was performed with a 0.1% (w/v) solution of the polymer in 0.5M-HCl (1ml) at 100°C for 30min. Acid was removed *in vacuo*, the hydrolysate was re-*N*-acetylated (1.0ml of methanol, 20μl of pyridine, 100μl of acetic anhydride) and then reduced with $NaBH_4$ (0.6% w/v, 1ml) at room temperature for 17h. After removal of borate by passage through Dowex 50 (H^+ form) and evaporation with methanol, the sample was trimethylsilylated and examined by g.l.c. on a column of SE-30 (Fig. 3).

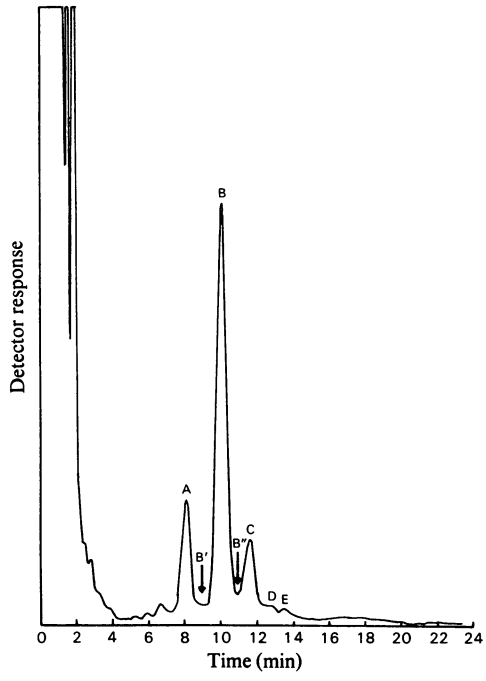


Fig. 3. *G.l.c. of trimethylsilyl derivatives of a partial acid hydrolysate of reduced teichuronic acid with a glass column (2 m × 3 mm) of 10% SE-30 at 290°C*

Table 1. *Partial acid hydrolysis products from reduced teichuronic acid of phosphate-limited cells*
Samples were analysed by g.l.c. as their trimethylsilyl alditols on a column of 10% SE-30 at 290°C.

Component	Retention time (cellobiitol = 1)	Proposed structure
A	1.00	Glcβ(1→4)Glcol
B	1.25	Glcβ(1→3)GalNAcol
C	1.43	GalNAcα(1→4)Glcol

Trimethylsilyl derivatives of glucitol and *N*-acetylgalactosaminitol were well separated from three major components A, B and C (Fig. 3) having retention times typical for trimethylsilylated disaccharides. These components were identified by g.l.c.–m.s. with a column of OV-1. The mass spectrum of A had, *inter alia*, fragments at *m/e* 204, 205, 361, 451, 525, 595 and 685 indicative of a neutral disaccharide alditol (Kärkkäinen, 1969). An authentic sample of cellobiitol had retention time and mass spectrum identical with those of A, thereby confirming its structure (see Table 1). The major component, B, had, *inter alia*, fragments at *m/e* 361, 451, 494, 522, 612, 756, 768, 858 $[M-103]^+$ indicative of a glucosylgalactosaminitol analogue,

Table 2. *Calculated and observed ^{13}C chemical shifts (in p.p.m. from external tetramethylsilane) with assignments for teichuronic acid and reduced teichuronic acid*
A, B, C, D, E and F refer respectively to β-D-glucuronyl(1→3), β-D-glucuronyl(1→4), β-*N*-acetyl-D-galactosaminyl, α-*N*-acetyl-D-galactosaminyl, β-D-glucosyl-(1→3) and β-D-glucosyl(1→4) residues. The carbonyl and methyl carbon atoms of the actamido functions are denoted by 7 and 8 respectively.

		Chemical shift																												
	Assignment	... D7	C7	A6	B6	A1	B1	C1	D1	A4	B4	C3	A5	B5	C5	A3	B3	A2	B2	D5	D4	D6	D3	C4	C6	C2	D2	C8	D8
Teichuronic acid	Calculated		176.1	175.8	177.5	177.5	104.4	104.4	103.5	99.2	82.3	82.3	81.3	76.5	76.5	76.3	76.1	76.1	74.5	74.5	70.6	69.7	69.2	68.6	68.0	62.2	52.9	50.4	23.4	23.2
	Observed		176.0	175.8		175.1		105.3	103.4	102.7	98.7	81.6	81.4	77.4		76.9	76.0	75.2	74.6	73.6	69.5	69.0	68.6	68.1	67.6	62.3	52.3	51.0	23.5	23.2
	Assignment	... D7	C7	E1	F1	C1	D1	C3	E4	F4	E5	F5	E3	F3	C5	E2	F2	D5	D4	D6	D3	C4	E6	F6	C6	C2	D2	C8	D8
Reduced teichuronic acid	Calculated		176.1	175.8	104.1	104.1	103.5	99.2	81.3	80.2	80.2	76.3	76.3	76.3	76.3	74.6	74.6	70.6	69.7	69.2	68.6	68.0	62.4	62.4	62.2	62.2	52.9	50.4	23.4	23.2
	Observed		175.8		105.4	103.6	102.7	99.7	81.6	79.7		77.5		76.0		75.3	74.8	73.8	71.2	69.4		68.7		62.2		61.6	52.4	51.1	23.6	23.3

and furthermore the ions at *m/e* 756, 768, 624 and 522 were characteristic of a 1→3 linkage (Finne *et al.*, 1977). The structure of B was confirmed by comparison with an authentic sample of 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyl)-D-galactitol, which is in fact the disaccharide (*N*-acetylated and reduced) that was isolated from the large-scale partial acid hydrolysis of the reduced teichuronic acid. C had, *inter alia*, fragments in its mass spectrum at *m/e* 330, 420, 510, 576, 666, 685 and 946 $[M-15]^+$ indicative of an *N*-acetylgalactosaminylglucitol analogue (Kärkkäinen, 1969). No standards were available for comparison, but the relatively long retention time indicates that the linkage has the α-D-configuration, and C is possibly the trimethylsilyl derivative of 4-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-glucitol. Four very minor components were also observed, giving two pairs of similar mass spectra B′ and B″ and D and E, which were indicative of the anomers of a glucosyl(1→6)glucitol and a glucosyl(1→6)-galactosaminitol analogue respectively. It is probable that these components are artifacts produced by acid-catalysed reversion.

The results are summarized in Table 1 and, taken together with the findings for the methylation analysis (i.e. 4-*O*-substituted glucose residues and both 3-*O*- and 6-*O*-substituted *N*-acetylgalactosamine residues), enable a tetrasaccharide structure (I) to be proposed for the teichuronic acid from phosphate-limited growths. The experiments described above do not allow the assignment of configuration to the glycosidic linkage between the two *N*-acetylgalactosamine residues.

There is an inconsistency in the molar ratio of galactosamine/glucose in reduced teichuronic acid determined by different methods, the values being about 1.0:0.5 (glucose oxidase), 1.0:0.6 (phenol/H_2SO_4) and 1.0:0.7 (acid hydrolysis). The best approximation to the equimolar ratio expected is by acid hydrolysis (0.5M-HCl, 4h, 100°C) and subsequent analysis of the alditols by g.l.c. With this procedure a more accurate value of the glucose content is obtained by summation of the free glucitol and the cellobiitol, giving a molar ratio of galactosamine/glucose of 1.00:0.83. The low glucose values obtained by using the glucose oxidase reagents and by the phenol/H_2SO_4 method may be due to incomplete reduction of glucuronic acid residues and to the relative instability of glucose in strong acid media.

^{13}C *n.m.r. spectroscopy*

The proton-decoupled ^{13}C n.m.r. spectra of teichuronic acid from phosphate-limited cells and reduced teichuronic acid were determined in ^2H_2O solution. Spectra obtained at 22.63MHz are shown in Figs. 4 and 5; Table 2 gives the assignments of the resonances together with their observed and calculated chemical shift values. The latter are ob-

Table 3. ^{13}C *n.m.r. chemical shifts (in p.p.m. from external tetramethylsilane) of components of teichuronic acid and reduced teichuronic acid with increments for glycosylation, thereby giving the calculated chemical shift values for (i) teichuronic acid and (ii) reduced teichuronic acid*

Increments are given the following values: +7p.p.m. for the formation of a glycosidic bond at an anomeric or primary carbon atom; +9p.p.m. for the formation of a glycosidic bond at secondary carbon atoms; −1p.p.m. for the introduction of an adjacent bond (Hamer & Perlin, 1976; Usui *et al.*, 1973). Values for the reported shifts are from Hamer & Perlin (1976) for β-D-glucuronic acid from Usui *et al.* (1973) for β-D-glucose and from Bundle *et al.* (1973) for *N*-acetylgalactosamine.

	Chemical shift							
Carbon atom ...	1	2	3	4	5	6	7	8
β-D-Glucuronic acid (A and B)								
Reported	97.4	75.5	77.1	73.3	77.5	177.5		
Increment	+7	−1	−1	+9	−1	0		
Calculated	104.4	74.5	76.1	82.3	76.5	177.5		
β-*N*-Acetyl-D-galactosamine (C)								
Reported	96.5	54.9	72.3	69.0	76.3	62.2	23.4	175.8
Increment	+7	−2	+9	−1	0	0	0	0
Calculated	103.5	52.9	81.3	68.0	76.3	62.2	23.4	175.8
α-*N*-Acetyl-D-galactosamine (D)								
Reported	92.2	51.4	68.6	69.7	71.6	62.4	23.2	176.1
Increment	+7	−1	0	0	−1	+7	0	0
Calculated	99.2	50.4	68.6	69.7	70.6	69.2	23.2	176.1
β-D-Glucose (E and F)								
Reported	97.1	75.6	77.3	71.2	77.3	62.4		
Increment	+7	−1	−1	+9	−1	0		
Calculated	104.1	74.6	76.3	80.2	76.3	62.4		

tained from the shifts reported for monomeric units of the polymer after allowing for the effects of *O*-glycosylation (see Table 3).

The spectrum of teichuronic acid (Fig. 4) possesses 25 individual resonances. This is compatible with a tetrasaccharide repeating structure comprising two residues each of uronic acid and acetamido sugar (allowing for coincidence of 3 of the 28 expected resonances). In keeping with a tetrasaccharide structure is the observation of four separate, equally intense, signals within the region 102 ± 4 p.p.m. from tetramethylsilane and these may readily be ascribed to four different anomeric carbon atoms. Furthermore, the chemical shifts of these signals suggest that three of the residues possess β-D- and one residue possesses α-D-anomeric configuration.

The presence of two 'different' hexosamines was clear from the pairs of diagnostic signals observed for *N*-acetyl carbons (approx. 23 and approx. 175 p.p.m.) and amino substituted carbons (approx. 52 p.p.m.). These latter two resonances were very close to the calculated chemical shifts for C-2 of 3-*O*-glycosylated-β-D- and 6 (or 4)-*O*-glycosylated-α-*N*-acetyl-D-galactosamine residues. The observed shifts are significantly different from those, expected from calculation, for other possible modes of linkage. Recent studies (Shashkov *et al.*, 1979) have shown that for methylated derivatives of *N*-acetyl-D-glucosamine the C-2 resonance falls within a narrow chemical shift range which is indicative of both anomeric configuration and position of *O*-methylation (and by inference *O*-glycosylation). The chemical shifts of these carbons of teichuronic acid therefore strongly support the presence of 3-*O*-glycosylated-β- and 6-*O*-glycosylated-α-*N*-acetyl-D-galactosamine residues (a 4-*O*-glycosylated-α-residue can be ruled out by the results from the periodate-oxidation study). Two of the signals observed for anomeric carbons may now be assigned; that at 98.7 p.p.m. to the α-hexosamine and that at 102.7 p.p.m. to the β-hexosamine. The

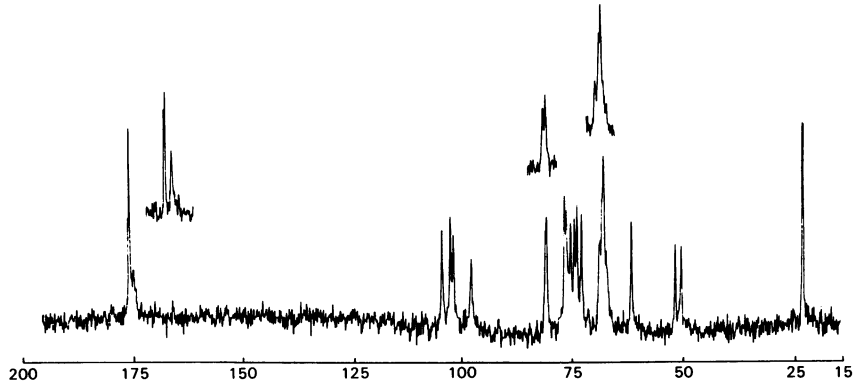


Fig. 4. *Proton-decoupled ^{13}C n.m.r. spectrum of teichuronic acid from phosphate-limited cells at 23.63 MHz in ^2H_2O*
The insets are expansions of regions approx. 175, 80 and 67 p.p.m. from tetramethylsilane.

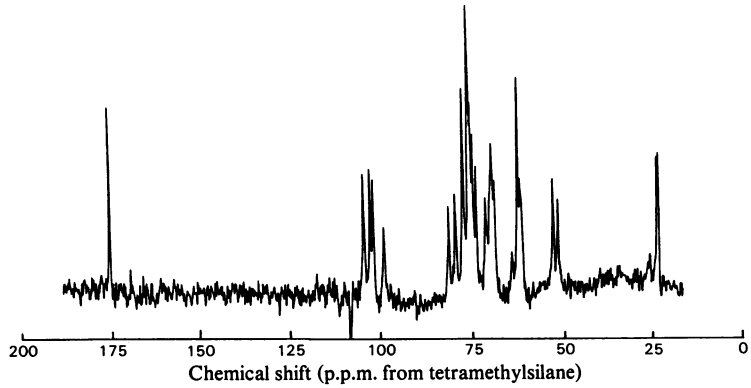


Fig. 5. *Proton-decoupled ^{13}C n.m.r. spectrum of reduced teichuronic acid at 22.63 MHz in ^2H_2O*

latter assignment is made on the basis of an almost identical value which has been reported for C-1 of 3-*O*-glycosylated-β-*N*-acetyl-D-galactosaminyl moieties of chondroitins A and C (Hamer & Perlin, 1976).

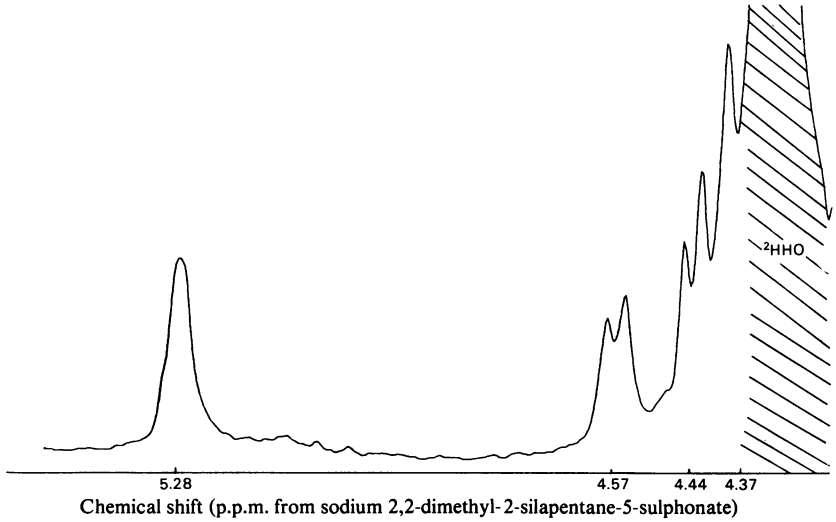
Methylation studies described earlier showed that the uronic acid residues are linked through *O*-4 and this is in keeping with the ^{13}C n.m.r. spectroscopy data since, after the anomeric carbons, the next signal to higher field was that observed at 81.6p.p.m. Chemical shifts 2–4p.p.m. greater than this would be expected from glycosylation of positions other than 4 in β-D-glucuronic acid residues. Observation of two resonances for the β-anomeric carbons for these residues is indicative of their different modes of linkage. The signal at 105.3p.p.m. is in good agreement with that expected from an involvement with *O*-3 of β-*N*-acetylgalactosamine (cf. chondroitins A and C; Hamer & Perlin, 1976) and has been assigned accordingly. The other signal (103.4p.p.m.) is therefore due to that carbon atom involved in linkage through *O*-4 of an adjacent glucuronic acid residue. A difference in chemical shifts from these two similar anomeric carbons is not unexpected, since spectra obtained, for example, from α-glucans containing the linkage sequence 1→4, 1→4, 1→6 gave three separate resonances for their anomeric carbons (Colson *et al.*, 1974).

Other points of note in the spectrum of the teichuronic acid are the two isolated resonances at 81.6 and 81.4p.p.m. (glycosylated carbons) in accord with 4-*O*-substitution of the uronic acid and 3-*O*-substitution of the β-hexosamine. In addition, the sharp singlet at 62.3 p.p.m. suggests the presence of only one hydroxymethyl group in each repeating unit (compare this region in Fig. 5). The remaining resonances have been assigned to give as close a fit as possible to the proposed structure; these assignments are therefore tentative, although the agreement between the calculated and observed shifts is good and there are no major discrepancies.

The ^{13}C n.m.r. spectrum of reduced teichuronic acid (Fig. 5) has, as would be expected, many features in common with the parent polymer. For example, two sets of resonances for acetamido carbon atoms are observed which can be assigned to 3-*O*-glycosylated-β- and 6-*O*-glycosylated-α-*N*-acetyl-D-galactosamine residues. Again there are characteristic signals for three β- and one α-anomeric carbons; it is interesting that of these four signals only the chemical shift for the α-anomeric carbon is altered (1p.p.m. downfield) relative to those observed for the teichuronic acid itself. The resonances observed at 81.6 and 79.7p.p.m. are similarly consistent with 3-*O*-glycosylation of β-*N*-acetyl-D-galactosaminyl residues and 4-*O*-glycosylation of β-D-glucosyl residues. The observed chemical shift values (Table 2) are in agreement with those calculated for the proposed structure of this polymer.

^{1}H *n.m.r. spectroscopy*

L-Iduronic acid, although present in some polysaccharides, is difficult to identify in hydrolysates, and therefore the possibility of it contributing to the structure of the teichuronic acid was considered. A repeating unit comprising D-glucuronic acid:L-iduronic acid:*N*-acetyl-D-galactosamine (1:1:2) could explain some of the analytical data, especially the apparent composition of the reduced



Chemical shift (p.p.m. from sodium 2,2-dimethyl-2-silapentane-5-sulphonate)

Fig. 6. *Region 4.3–5.5p.p.m. from tetramethylsilane of ^{1}H n.m.r. spectrum of teichuronic acid at 270MHz in $^{2}H_2O$*

polymers. Furthermore such a structure could also be partially accommodated within the observed ^{13}C n.m.r. spectra presented here. For example, the anomeric carbon resonance for teichuronic acid observed at 103.4 p.p.m. is similar to that reported for C-1 of the α-L-idosyluronic acid residue in chondroitin B (Hamer & Perlin, 1976) although resonances at approx. 70.4 and 72.0 p.p.m. should also be present. These latter regions of the spectrum of the teichuronic acid were however devoid of resonances which would suggest the absence of the L-acid in the present case. To help clarify the situation the ^{1}H spectrum of the teichuronic acid from phosphate-limited cells was determined and Fig. 6 shows the portion 4.3–5.5 p.p.m. from sodium 2,2-dimethyl-2-silapentane-5-sulphonate of the 270 MHz spectrum of the polymer in $^{2}H_2O$ solution. This area is the resonance region for anomeric and other strongly deshielded protons, such as H-5, of α-L-iduronic acid derivatives. A characteristic signal (4.7–4.8 p.p.m.) for this latter proton is observed in the spectra of a number of mucopolysaccharides containing α-L-iduronic acid residues (Perlin *et al.*, 1970) and it can be seen from Fig. 6 that such a signal is not observed in the present case. Its absence therefore confirms that α-L-iduronic acid is not part of the repeating structure of the teichuronic acid.

The resonances which are observed are typical for four anomeric protons. Their chemical shifts have been correlated (Table 4) wherever possible with the reported values for anomeric protons in systems related to the proposed structure of the teichuronic acid. The unresolved (therefore low coupling constant) resonance at lowest field (5.28 p.p.m.) is characteristic of an α-glycoside and is therefore assigned to H-1 of the α-*N*-acetylgalactosamine residue, whereas the two doublets at 4.57 and 4.44 p.p.m. have both chemical shifts and coupling constants in accord with H-1 of β-glycosides; a third doublet partially obscured by the ^{2}HHO resonance is also indicated in the spectrum. These observations are in complete accord with the ^{13}C n.m.r. spectroscopy data.

Analysis of the teichuronic acid from glucose-limited cells

The original purpose of this study was to compare the teichuronic acids from both phosphate-limited and glucose-limited growths. The purified teichuronic acid from glucose-limited cells contained uronic acid and galactosamine in the molar ratio 1.00:1.04 and accounted for 95% of the dry weight of the polysaccharide. Phosphate and nucleic acid were absent. The polysaccharide had $[\alpha]_D^{23} + 35.5°$ (*c* 1.28 in water).

After reduction by the method of Taylor & Conrad (1972) the reduced polymer contained uronic acid, glucose (phenol/H_2SO_4) and galactosamine in molar proportions 0.08:0.62:1.00. After acid hydrolysis of the polymer, galactosamine (3 M-HCl, 6 h, 100°C) and glucose (2 M-HCl, 2 h–5 h, 100°C, determined with glucose oxidase) were present in the molar ratio 1.00:0.52 which increased to 1.00:0.7 after correction for destruction of glucose under the conditions of hydrolysis. Partial acid hydrolysis of the reduced teichuronic acid [0.1% (w/v) solution in 0.5 M-HCl (1 ml) at 100°C for 30 min] followed by re-*N*-acetylation, reduction with $NaBH_4$, trimethylsilylation and subsequent analysis by g.l.c. and g.l.c.–m.s. indicated similar products to those found for the reduced polymer from phosphate-limited growths. In addition, hydrolysis of the reduced polymer for 4 h followed by the same work-up procedure gave a galactosamine/glucose ratio 1.00:0.74. Summation of glucitol and cellobiitol increased this ratio to 1.00:0.86. The reduced polysaccharide had $[\alpha]_D^{23} + 54.8°$ (*c* 1.03 in water).

The ^{13}C n.m.r. spectrum of the native teichuronic acid from glucose-limited growths was identical with the spectrum of the teichuronic acid from phosphate-limited growths presented in Fig. 4. Thus the teichuronic acid of *B. licheniformis* A.T.C.C. 9945 is identical under conditions of both glucose- and phosphate-limitation.

Structure of teichuronic acid

The results of partial acid hydrolysis and

Table 4. *^{1}H n.m.r. chemical shifts and coupling constants observed for teichuronic acid in $^{2}H_2O$ solution at 270 MHz and 57°C*

Chemical shifts are in p.p.m. relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate; coupling constants (in parentheses) are in Hz.

		Chemical shift and coupling constant			
Assignment ...		D1	C1	A1	B1
Observed		5.28	4.57 (7.2)	4.44 (6.2)	4.37
Reported			4.63*	4.46 (8.0)†	

* Chondroitin A, 220 MHz at 70°C (Perlin *et al.*, 1970).
† Chondroitin A, 270 MHz at 70°C (Gatti *et al.*, 1979).

methylation analysis of the reduced teichuronic acid enabled us to propose a tetrasaccharide repeating structure (I) for the native teichuronic acid. ^{13}C and ^{1}H n.m.r. data were in good agreement with this structure and, in particular, established the anomeric configuration of the glucuronic acid and *N*-acetylgalactosamine residues. The combined results are compatible with a tetrasaccharide repeating structure (II) for the teichuronic acid.

$$[GlcA\beta(1\rightarrow4)GlcA\beta(1\rightarrow3)GalNac?(1\rightarrow6)GalNAc\alpha(1\rightarrow4)]_n$$
(I)

$$[GlcA\beta(1\rightarrow4)GlcA\beta(1\rightarrow3)GalNAc\beta(1\rightarrow6)GalNAc\alpha(1\rightarrow4)]_n$$
(II)

$$[GlcA\alpha(1\rightarrow3)GalNAc\alpha(1\rightarrow4)]_n$$
(III)

Our findings are in contrast with the repeating structure for the teichuronic acid proposed by Janczura *et al.* (1961) and Hughes & Thurman (1970) from *B. licheniformis* N.C.T.C. 6346 and by Wright & Heckels (1975) from *B. subtilis* W23. These authors concluded that the polysaccharide consisted of a disaccharide repeating unit (III) with most or all of the linkages in the α-D-configuration. However, the composition of these teichuronic acids, the specific rotation ($[\alpha]_D^{23}$ +34–40°) and the more limited degradation studies carried out on them do not exclude the possibility that they might be identical with that from *B. licheniformis* A.T.C.C. 9945 with the structure (II). Clearly, the rotation reported is not consistent with a structure in which all glycosidic linkages have the α-configuration, and n.m.r. data on these teichuronic acid preparations have not been obtained.

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