Structural studies of a mannitol teichoic acid from the cell wall of bacterium N.C.T.C. 9742

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Degradative and n.m.r.-spectroscopic studies have been carried out on a novel mannitol teichoic acid extracted from the cell wall of bacterium N.C.T.C. 9742, for which the name *Brevibacterium iodinum* has been proposed. The backbone of the polymer is a poly(D-mannitol phosphate) containing 1→6 phosphodiester linkages. In most residues, pyruvic acid is acetal-linked to positions 4 and 5 of the mannitol. About half of the mannitol residues carry a β-D-glucopyranosyl substituent at position 2. The glucosylmannitol was isolated and thoroughly characterized. At least 24 products were detected by ion-exchange chromatography and paper electrophoresis after alkaline hydrolysis of the polymer. Not all of these products could be identified. The main mechanistic pathways for depolymerization by the cleavage of phosphodiester linkages during alkaline hydrolysis involved (a) participation by the 2-hydroxy group and a cyclic phosphodiester intermediate (leading to a series of mannitol-based products) and (b) participation by the 3-hydroxy group in the cyclization of mannitol (leading to a series of products based on 1,4-anhydromannitol). The presence of glycerol phosphates in hydrolysates could be ascribed either to a linkage unit or to a separate glycerol teichoic acid. The mannitol teichoic acid was absent from the cell walls of *Brevibacterium linens* and *Brevibacterium epidermis* (one strain of each was examined).

The Gram classification and the generic affiliation of the organism represented by strain N.C.T.C. 9742, initially designated '*Chromobacterium iodinum*', have long been controversial and the organism was omitted from the 'Approved Lists of Bacterial Names' (Skerman *et al.*, 1980). However, the ultrastructure and coryneform morphology of the organism (Colwell *et al.*, 1969), taken with the results of numerical-taxonomic studies (Jones, 1975) and the application of chemotaxonomic criteria (Colwell *et al.*, 1969; Collins *et al.*, 1980), point to a Gram-positive type of cell envelope and a close relationship with *Brevibacterium linens*. Both species have the same type of peptidoglycan (Wilkinson, 1968; Fiedler *et al.*, 1970; Schleifer & Kandler, 1972) and diagnostically similar patterns of fatty acids, polar lipids and menaquinones (Collins *et al.*, 1980). Thus it has been suggested that *Chromobacterium iodinum* be renamed *Brevibacterium iodinum* (Collins *et al.*, 1980).

Our own studies of wall components in bacterium N.C.T.C. 9742 have indicated the presence of novel anionic carbohydrate polymers slowly extractable with cold aqueous trichloroacetic acid (Wilkinson, 1968; Anderton & Wilkinson, 1980). One of these polymers appeared to represent a new class of teichoic acid with a poly(mannitol phosphate) backbone to which β-D-glucopyranosyl substituents and pyruvic acid residues (acetal-linked) were attached. Here we report the results of further structural studies of this polymer and its absence from the walls of two other *Brevibacterium* species. Since completion of this study, the presence of similar mannitol teichoic acids in unnamed strains related to *Brevibacterium linens* has been described (Fiedler *et al.*, 1981).

Experimental

Materials

Alkaline phosphatase (EC 3.1.3.1) was type 1 from calf intestinal mucosa (Sigma). Lactate dehydrogenase (EC 1.1.1.27), glucose oxidase (EC

Abbreviations used: c.i., chemical ionization; e.i., electron impact.

1.1.3.4), and β -glucosidase (EC 3.2.1.21) were Boehringer products. DEAE-cellulose was the Whatman product DE-32. Samples of the D-glucosyl-D-mannitols linked $\beta(1\rightarrow1)$ or $\beta(1\rightarrow3)$ were supplied by Professor B. Lindberg (University of Stockholm). Other compounds used for reference or calibration purposes were the best grades commercially available.

Organisms, preparation of cell walls and isolation of the mannitol teichoic acid

Bacterium N.C.T.C. 9742 was grown in nutrient broth (Oxoid CM67) for 24 h at 30°C with aeration of 20 litre batches at 20 litres/min. The mannitol teichoic acid was extracted from isolated cell walls with cold 10% (w/v) trichloroacetic acid, and purified as described previously (Anderton & Wilkinson, 1980). Cell walls were also prepared from *Brevibacterium epidermis* strain 2285 grown under identical conditions, and from *Brevibacterium linens* strain 739 grown similarly (48 h at 30°C).

Chromatographic and electrophoretic methods

Paper chromatography and high-voltage paper electrophoresis were carried out with Whatman papers (no. 1 for analytical separations and washed no. 1 or 3 MM for preparative separations). Solvent systems used were: A, ethyl acetate/pyridine/water (5:2:5, by vol., upper phase); B, butan-1-ol/ethanol/water/aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol., upper phase); C, propan-1-ol/aq. NH_3 (sp.gr. 0.88)/water (6:3:1, by vol.); D, propan-2-ol/aq. NH_3 (sp.gr. 0.88)/water (7:1:2, by vol.). Buffer systems used for paper electrophoresis were: pH 5.3, pyridine/acetic acid/water (5:2:43, by vol.); pH 2.8, pyridine/formic acid/acetic acid/water (2:3:20:180, by vol.). T.l.c. for polyols was carried out on layers of silica-gel 60F₂₅₄ (Merck) impregnated with NaH_2PO_4 with propan-2-ol/acetone/0.2M-lactic acid (6:3:1, by vol.) as the solvent (Kremer, 1978). T.l.c. purification of permethylated hexosylhexitols was carried out on silica-gel 60F₂₅₄, with hexane/acetone (3:2, by vol.) as the solvent. Detection reagents used extensively were the periodate/Schiff reagents (Baddiley *et al.*, 1956) and the reagent of Hanes & Isherwood (1949) for phosphates; other reagents used have been summarized (Wilkinson, 1977). For g.l.c., glass columns of the sizes indicated were packed with the following stationary phases: I, 3% (w/w) Silar 10C on Gas Chrom Q (2 mm $\times$ 1.6 m); II, 30% (w/w) SE-52 on Gas Chrom Q (3 mm $\times$ 2.4 m); III, 3% SE-52 on acid-washed Celite (3 mm $\times$ 0.5 m). G.l.c.-m.s. of methylated alditol acetates and of polyol acetates at the Physico-Chemical Measurements Unit (Aldermaston) was carried out by the e.i. method using stationary phase I. Similar analyses using both e.i. and c.i. methods were

carried out using a Finnigan 4021 instrument and a fused-silica capillary column (25 m) of SF-2100. Both types of mass spectra were also obtained for a permethylated glucosylmannitol by using a Kratos MS25/DS55 system and a glass capillary column (20 m) of OV1. NH_3 was used as the reagent gas for all c.i. mass spectra.

N.m.r. spectroscopy

A Bruker WH-400 spectrometer was used to obtain ^1H -, ^{13}C -, and ^{31}P -n.m.r. spectra for the mannitol teichoic acid (NH_4^+ salt) dissolved in $^2\text{H}_2\text{O}$ (^1H - and ^{13}C -n.m.r. spectra) or in 75% (v/v) $^2\text{H}_2\text{O}$ containing 0.1 M-EDTA (^{31}P -n.m.r. spectra). ^1H -n.m.r. spectra were recorded at 85°C with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as an external reference. ^{13}C -n.m.r. spectra (with complete proton-decoupling or with gated decoupling) were recorded at 40°C with tetramethylsilane as the external reference. Proton-decoupled ^{31}P -n.m.r. spectra were recorded at ambient temperature with 85% orthophosphoric acid as the external reference. Proton-decoupled ^{13}C -n.m.r. spectra were also obtained by using Jeol spectrometers (FX 90Q and FX 200) for solutions at ambient temperature.

Quantitative analyses

Methods used for the quantitative analysis of phosphorus, total carbohydrate, glucose, mannitol, anhydromannitol, glycerol, and pyruvic acid were essentially those described previously (Anderton & Wilkinson, 1980). In general, g.l.c. of the acetates was used for the analysis of mixtures of polyols released by acid hydrolysis (1 M-HCl, 105°C, 4 h) and/or enzymic dephosphorylation of samples, as appropriate. For the determination of pyruvic acid, portions of the acid hydrolysate small enough to be tolerated by the assay buffer used with lactate dehydrogenase were used directly. When the hydrolysates were neutralized with Dowex 50 (Na^+ form) resin and dried before analysis, losses of pyruvate were erratic and often substantial.

Alkaline hydrolysis and fractionation of the products

The polymer (150 mg) was hydrolysed with 1 M-NaOH at 105°C for 3 h under nitrogen in a poly(tetrafluoroethylene) ('PTFE') tube. The hydrolysate was passed down a column of Dowex 50 (NH_4^+ form) resin, dried, and applied in water to a column (1.5 cm $\times$ 12 cm) of DEAE-cellulose (CO_3^{2-} form). Elution was carried out with a linear gradient of $(\text{NH}_4)_2\text{CO}_3$ (0–0.15 M) at a flow rate of 50 ml/h over 24 h by using a Dialagrad (Instrumentation Specialities Co., Lincoln, NE, U.S.A.). Fractions (8 ml) were screened for their content of phosphorus and carbohydrate (phenol/ H_2SO_4 method). The contents of appropriate tubes were

pooled to give fractions F1–F5, which were dried repeatedly on a rotary evaporator.

Other analytical methods

For the selective hydrolysis of phosphomonoesters, samples were dissolved in 0.05M-(NH₄)₂CO₃, pH 9.6, and incubated at 37°C for 16 h under toluene with alkaline phosphatase. Similarly, β -glucopyranosides were hydrolysed by treatment overnight at 37°C under toluene with β -glucosidase. Selective removal from the mannitol teichoic acid of acetal-linked pyruvic acid was attempted by two methods (Duckworth & Yaphe, 1970; Elsässer-Beile *et al.*, 1978).

Methylation analysis of the polymer and of its glucosylmannitol component was carried out by standard procedures (Jansson *et al.*, 1976). The method of Ohashi & Yamakawa (1973) was used for trimethylsilylation of the glucosylmannitol and reference disaccharide alditols. Periodate oxidation of the teichoic acid and derived fractions was carried out in the dark at 4°C with 50mM-NaIO₄. The consumption of periodate was monitored by the method of Avigad (1969). The excess of NaIO₄ was destroyed with ethylene glycol, and the oxidation products were reduced with NaBH₄.

Results

Composition of the polymer from bacterium N.C.T.C. 9742

As reported previously (Anderton & Wilkinson, 1980), the polymer released slowly from isolated cell walls on treatment with cold aqueous trichloroacetic acid was rich in phosphorus (6–7%), D-mannitol (21–24%), pyruvic acid (12–13%), and D-glucose (13–14%). A backbone of phosphodiester-linked mannitol residues was indicated by the presence in acid hydrolysates of both mono- and di-phosphate derivatives of the hexitol. The approximate molar ratio of 2:2:1 for mannitol, pyruvic acid and glucose suggested that most mannitol residues were also substituted by pyruvic acid, but only about half of them by glucose. Glycerol phosphates and D-galactosamine (minor components of the extracts) could arise from contamination by a conventional glycerol teichoic acid (e.g. Endl *et al.*, 1983) or the presence of an oligomeric 'linkage unit' similar to those described for other teichoic acids (e.g. Coley *et al.*, 1978; Kojima *et al.*, 1983; Kaya *et al.*, 1984).

Location of glucose residues

The ¹H-n.m.r. spectrum of the polymer contained a single anomeric signal $\{\delta \text{ (p.p.m.) } [^2\text{H}_2\text{O}] 4.68 [d, J_{1,2} 9 \text{ Hz}]\}$ indicative of a β -D-glucopyranosyl residue. This inference was confirmed by an anomeric signal in the ¹³C-n.m.r. spectrum at δ

(p.p.m.) $[^2\text{H}_2\text{O}] 102.78$ (Fig. 1). In the spectrum recorded with gated decoupling, this signal appeared as a doublet with ¹J_{CH} 163 Hz, again as expected for a β -linkage (Bock & Pedersen, 1974). All of the glucose was destroyed on oxidation of the polymer with NaIO₄, and methylation analysis with g.l.c.-mass spectrometry confirmed the presence of an unsubstituted glucopyranosyl residue. No evidence for glucose phosphates was obtained by acid hydrolysis (1M-HCl, 105°C, 4h) of the polymer, followed by enzymic assay for glucose before and after dephosphorylation of the hydrolysis products. Attempts were therefore made to isolate and characterize the expected glucosylmannitol component of the polymer.

Initially, the polymer was subjected to mild acid hydrolysis to remove the acetal-linked pyruvic acid residues. Hydrolysis with 0.02M-oxalic acid at 105°C for 3 or 4 h (Duckworth & Yaphe, 1970) seemed better than hydrolysis with 0.01M-trifluoroacetic acid at 100°C for 1.5 h (Elsässer-Beile *et al.*, 1978). After neutralization with CaCO₃, the products were depolymerized (1M-NaOH, 105°C, 3h), dephosphorylated (alkaline phosphatase), and deionized. Paper chromatography of the neutral products (solvent A) showed the presence of mannitol, 1,4-anhydromannitol, and a compound with *R*_{mannitol} 0.71. The latter compound reduced alkaline AgNO₃ and gave a purple colour (strengthening and changing to blue on standing) with the periodate/Schiff reagents, but did not react with aniline hydrogen oxalate. The compound gave glucose and mannitol (molar ratio 1:1) on treatment with β -glucosidase. Confirmation of its identity as a glucosylmannitol was provided by g.l.c. (column III) and mass spectrometry of the per-*O*-methyl derivative. The c.i. mass spectrum with NH₃ as the reagent gas gave high-mass ions at *m/z* 488 (*M*+NH₄)⁺ and 471 (*M*+H)⁺, as well as fragment ions at *m/z* 439, 253, and 235, as expected for a permethylated hexosylhexitol (Chizhov *et al.*, 1976). The e.i. mass spectrum of the derivative contained peaks at *m/z* 219 and 235 derived from the hexosyl and hexitol moieties respectively, as well as the expected peaks from further fragmentation (Kärkkäinen, 1970).

Acid hydrolysis of the permethylated hexosylhexitol, followed by conversion of the products into methylated alditol acetates, gave two derivatives. The less volatile derivative was identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol by its retention time on g.l.c. (column I), by its c.i. mass-spectral peaks at *m/z* 340 (*M*+NH₄)⁺ and 263 (*M*+H–60)⁺ (Geyer *et al.*, 1983), and by the primary fragments at *m/z* 205, 161, and 117 in the e.i. mass spectrum. The more volatile derivative was identified as 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylmannitol by its c.i. mass-spectral peaks at

Fig. 1. ^{13}C -N.m.r. spectrum of the mannitol teichoic acid from bacterium N.C.T.C. 9742

The spectrum for the sample in $^2\text{H}_2\text{O}$ at room temperature was obtained at 50 MHz with complete proton-decoupling and tetramethylsilane as an external reference using a Jeol FX200 spectrometer. The inset signal due to the carbonyl carbon of pyruvate residues has δ (p.p.m.) 178.88.

m/z 312 ($M + \text{NH}_4$) $^+$ and 235 ($M + \text{H} - 60$) $^+$, and by the primary fragments at m/z 249, 205, 161, 133, 89, and 45 in the e.i. mass spectrum. Thus the hexosylhexitol could be identified as 2-*O*- β -D-glucopyranosyl-D-mannitol. Consistent with this inference, 2.05 mol of glycerol/mol of glucose was produced after periodate oxidation, borohydride reduction and acid hydrolysis of the glucosyl-mannitol.

Although a reference sample was not available, the compound from the mannitol teichoic acid could be differentiated from the isomeric β -D-glucopyranosyl-D-mannitols linked 1 $\rightarrow$ 1 or 1 $\rightarrow$ 3. The 1 $\rightarrow$ 1-linked compound had a lower mobility on paper chromatography (solvent A), and its trimethylsilyl derivative had a relatively high retention time (1.41) on g.l.c. (column III). Neither chromatographic method resolved the 1 $\rightarrow$ 2- and 1 $\rightarrow$ 3-linked compounds, but only the latter gave the yellow colour characteristic of malonaldehyde derivatives on treatment with the periodate/Schiff reagents (Hardy & Buchanan, 1963).

Location of pyruvic acid residues

The ^1H -n.m.r. spectrum of the polymer contained a sharp singlet with δ (p.p.m.) [$^2\text{H}_2\text{O}$] 1.62, diagnostic for an acetal-linked pyruvic acid residue. In the ^{13}C -n.m.r. spectrum (Fig. 1), pyruvate-derived signals were seen at δ (p.p.m.) [$^2\text{H}_2\text{O}$]

178.88, 107.68, 23.37, and 23.17. The last two signals were of similar intensity, and this duplication of the resonance for the methyl carbon may be caused by the absence of a glucosyl substituent from about half of the mannitol residues, or possibly by a difference in acetal configuration (e.g. Gorin *et al.*, 1982). The chemical shift for the acetal carbon (107.68 p.p.m.) points strongly to the presence of a five-membered (1,3-dioxolan) ring (e.g. Gorin *et al.*, 1982; Buchanan *et al.*, 1982), i.e. pyruvic acid acetal-linked to a vicinal diol unit. As expected from the molar proportions of mannitol and pyruvic acid in the polymer, further studies confirmed that this diol unit was part of mannitol rather than glucose.

As described in the following section, alkaline hydrolysis of the polymer gave small amounts of anionic products containing mannitol or 1,4-anhydromannitol, but no phosphorus or glucose, and much larger amounts of these compounds could be obtained by deglycosylation and/or dephosphorylation of the phosphate esters preponderant in the hydrolysate. These anionic products could be identified as pyruvate acetals of the polyols and were the major components (F1B, F1C) of fraction F1 (Figs. 2 and 3). For reasons discussed below, accurate quantitative data for fraction F1 could not be obtained, but analyses of acid hydrolysates gave the following molar propor-

tions of components: mannitol, 1.00; 1,4-anhydromannitol, 0.32; pyruvic acid, 1.40; glucose, 0.17. Fraction F1 was subjected successively to periodate oxidation, borohydride reduction, and acid hydrolysis (1M-HCl, 105°C, 4h), followed by g.l.c. and mass spectrometry of the peracetates of the polyols produced. The major product was erythritol (differentiated from threitol by g.l.c. of the tetra-acetate using column I), obtained in an apparent molar proportion (g.l.c.) of 1.38 based on the mannitol content of fraction F1. The c.i. mass spectrum contained a strong peak at m/z 308 ($M + \text{NH}_4$)⁺, and the e.i. mass spectrum showed primary fragments at m/z 217 and 145. However, g.l.c.-mass spectrometry also showed that the peak corresponding to erythritol tetra-acetate had merged with that for another component of slightly shorter retention time. The M_r (260) established from the c.i. mass spectrum [peak for ($M + \text{NH}_4$)⁺ at m/z 278] and the fragment ions in the e.i. mass spectrum at m/z 187, 140, 127, 98 and 85 showed that the compound was the triacetate of a 1,4-anhydropentitol (Szafranek & Wiśniewski, 1980). The production of erythritol in the above degradation is consistent with the presence in fraction F1 of mannitol with pyruvate acetal-linked to positions 4 and 5 (equivalent to 2 and 3), as shown in structure (I). The 1,4-anhydropentitol (presumably 1,4-anhydro-D-lyxitol) would similarly be produced by degradation of the 1,4-(3,6)-anhydromannitol derivative (structure II). The degradation of fraction F1 also produced a little glycerol, expected to arise from the small amount of glucose present.

Alkaline hydrolysis of the polymer

The results described so far in this and the previous paper (Anderton & Wilkinson, 1980) indicate that the polymer from bacterium N.C.T.C. 9742 is constructed from phosphodiester-linked units of structure (I), about half of which carry a β -D-glucopyranosyl substituent at position 2 of mannitol. The formation of 1,4-anhydromannitol derivatives was expected to be an artefact of hydrolytic breakdown of the polymer, especially under alkaline conditions. Thus the mannitol content of the polymer determined by acid hydrolysis (1M-HCl, 105°C, 4h), followed by treatment with alkaline phosphatase and g.l.c. of the mannitol hexa-acetate, was about twice the value obtained when the polymer was first treated with alkali (1M-NaOH, 105°C, 3h). The glucose content was the same for both types of hydrolysate, whereas 1,4-anhydromannitol was only a trace component of acid hydrolysates but a significant one (20–30% of the mannitol value) when the polymer was treated first with alkali. The formation of 1,4-anhydromannitol from either mannitol or mannitol 1-phosphate was almost insignificant

under the acidic and the alkaline conditions used for hydrolysis of the polymer.

In order to gain further information about the primary structure of the polymer and the likely mechanism(s) of alkaline hydrolysis, a preparative hydrolysis was carried out and the products were fractionated by chromatography on DEAE-cellulose (Fig. 2). The recovery of phosphorus was about 85%, and the distribution of the recovered phosphorus in the five fractions collected was as follows: F1, 0%; F2, 6%; F3, 70%; F4, 16%; F5, 8%. As judged by the phenol/H₂SO₄ reaction, carbohydrate was absent or minimal in fractions F2 and F4. The colour of the reaction products from the assay for fraction F1 and the later tubes

Fig. 2. Chromatography on DEAE-cellulose of products obtained on alkaline hydrolysis of the mannitol teichoic acid from bacterium N.C.T.C. 9742

The products obtained after hydrolysis of the polymer (150mg) with 1M-NaOH at 105°C for 3h were applied to a column (1.5cm × 12cm) of DEAE-cellulose (CO₃²⁻ form). Elution was carried out with a linear gradient of (NH₄)₂CO₃ (0–0.15M) at a flow rate of 50ml/h over 24h. Fractions (8ml) were screened for phosphorus content (---, A₈₃₀) and carbohydrate content (—, A₄₉₀). F1–F5 represent the pooled fractions.

containing fraction F3 ($\lambda_{\max}$, 475nm) was consistent with pyruvic acid rather than glucose as the major reacting species (Anderton & Wilkinson, 1980). Further examination of the fractions by paper electrophoresis (Fig. 3) showed that all were complex. Although few subfractions could be obtained pure by preparative electrophoresis, sufficient information about the composition and relationships of most of them was obtained for reasonable structures to be inferred.

Fraction F1

Fraction F1 had five components detectable by paper electrophoresis at pH 5.3, three of which (F1B, F1C, F1D) reacted rapidly with the periodate/Schiff reagents. Fractions F1B and F1C gave purple spots (indicating the formation of formaldehyde), with fraction F1C giving the more intense and durable colour. Samples of both fractions only slightly cross-contaminated were isolated and analysed. On acid hydrolysis, fraction F1B gave mannitol and pyruvic acid (molar ratio 1.19:1.00) and, as discussed above, structure (I) has been inferred for this product. Fraction F1C was much more resistant to acid hydrolysis (about half was

unchanged after treatment with 1M-HCl at 105°C for 4h), but the major products were identified as 1,4-anhydromannitol (paper chromatography and g.l.c. of the tetra-acetate) and pyruvic acid (paper electrophoresis and enzymic assay). Structure (II) has been inferred for fraction F1C.

Of the minor components of fraction F1, one (F1D) rapidly gave a yellow colour with the periodate/Schiff reagents, indicating the formation of a malonaldehyde derivative. On paper electrophoresis at pH 2.8, fraction F1D migrated more slowly than either F1B or F1C (m_{F1C} 0.50), in contrast with its behaviour at pH 5.3 (Fig. 3). This result indicated that fraction F1D had a higher pK_a value than either of the characterized pyruvic acid acetals. Insufficient material was available for structural or compositional studies of the product. The other two products (F1A and F1E) appeared on electrophoretograms as faint blue spots several hours after spraying with the periodate/Schiff reagents. Subsequent studies of fraction F3 (see below) indicated that fraction F1A was the 2-*O*-glucosyl derivative of the anhydromannitol acetal F1C. The other trace product (F1E) was not studied further.

Fraction F2

Four phosphorus-containing spots were detected on paper electrophoresis in addition to P_i (fraction F2F). The three major phosphates (fractions F2A, F2B, and F2E) all gave formaldehyde on oxidation with periodate and released the phosphorus quantitatively on treatment with alkaline phosphatase. Fraction F2E was identified as a mixture of glycerol monophosphates on the basis of the following results: (a) paper electrophoresis at pH 5.3; (b) paper chromatography (solvent C); (c) enzymic hydrolysis to glycerol and P_i (molar ratio 1.00:1.03).

Fraction F2A had the electrophoretic mobility of mannitol 1-phosphate, and both mannitol and 1,4-anhydromannitol were released on dephosphorylation of the mixed fractions F2A-D. Analysis of the mixture gave a molar ratio (mannitol/1,4-anhydromannitol/phosphorus) of 0.66:0.11:1.00. Thus about 80% of the mixture could be accounted for as monophosphates of the two polyols. The major products obtained on oxidation of the mixture with periodate, followed by reduction with $NaBH_4$, were ethylene glycol monophosphate and glycerol 2-phosphate, indicating that the mixture included both mannitol 1- and 2-phosphates. Although fraction F2C was not detected with the periodate/Schiff reagents, and fraction F2D was not detected with the reagent of Hanes & Isherwood (1949) for phosphates (but gave a yellow colour with the periodate/Schiff reagents), both of these unidentified trace components were de-

Fig. 3. Composite paper electrophoretogram of products obtained on alkaline hydrolysis of the mannitol teichoic acid from bacterium N.C.T.C. 9742

Fractions F1–F5 obtained by anion-exchange chromatography (Fig. 2) were examined by electrophoresis at pH 5.3. Anionic electrophoretic mobilities are given relative to glycerol 1-phosphate. Arrows indicate the relative mobilities of mannitol 1-phosphate (W), glycerol 1-phosphate (X), mannitol 1,6-diphosphate (Y), and glycerol 1,3-diphosphate (Z). ○, Compounds giving a positive reaction only with the periodate/Schiff reagents; ●, those which also react with the Hanes–Isherwood (1949) reagent for phosphates; ⊕, those which only react with the Hanes–Isherwood (1949) reagent.

stroyed on treatment of the mixture with alkaline phosphatase.

The identity and origin of the compound isolated as fraction F2G (and also as fraction F3E) remain enigmatic. The compound was not detected with the Hanes–Isherwood (1949) reagent, alkaline AgNO_3 or aniline hydrogen oxalate. With the periodate/Schiff reagents it rapidly gave a faint purple spot; on standing, the colour changed to blue and intensified. Whereas the compound migrated more rapidly than glycerol 1-phosphate on electrophoresis at pH 5.3 (Fig. 3), the value of 0.38 for $m_{\text{glycerol 1-phosphate}}$ at pH 2.8 is consistent with repression of the ionization of a carboxylic acid at the lower pH. On the other hand, fraction F2G had $m_{\text{glyceric acid}}$ values of about 1.13 at both pH 5.3 and 2.8. As glyceric acid is the smallest and simplest monocarboxylic acid with a positive periodate/Schiff reaction, this observation could suggest that fraction F2G contained a stronger acid. On the other hand, the compound migrated more slowly than either F1B or F2B on electrophoresis at pH 2.8 (m_{F1B} 0.74, m_{F1C} 0.62). It therefore seems more likely that fraction F2G contained a dicarboxylic acid. This view seemed to be supported by the values for $m_{\text{tartaric acid}}$ of 0.81 (pH 5.3) and 0.57 (pH 2.8), and those for $m_{\text{glucaric acid}}$ of 1.06 (pH 5.3) and 1.05 (pH 2.8).

Fraction F3

The components of this fraction accounted for the bulk of the phosphorus in the alkaline hydrolysate. The non-phosphorus-containing component (F3E) has been discussed above.

Fractions F3C and F3D could not be cleanly and efficiently separated by preparative electrophoresis, but analysis of a mixture gave a molar ratio (phosphorus/pyruvic acid/mannitol/1,4-anhydromannitol) of 1.00:0.58:0.61:0.17 (no glucose was present). On enzymic dephosphorylation, F3C gave F1B, and F3D gave F1C. Thus F3D can be assigned the partial structure (III), consistent with the lack of a periodate/Schiff reaction. From the elongated shape of the spot usually found for F3D (Fig. 3), it is likely to consist of a mixture of the two isomeric monophosphates, and in fact two closely-spaced spots could sometimes be discerned. The

acid resistance of the bicyclic acetal probably explains the lack of stoichiometry in the quantitative data for the mixture with fraction F3C. The latter is expected to contain monophosphate(s) of the mannitol acetal with structure (I) (F1B). The rapid purple produced with the periodate/Schiff reagents points to a location of the phosphate residue at position 3 or 6 of the mannitol, but as discussed below, such esters are unlikely to be major products in the alkaline hydrolysate. Tentatively, the observed reaction is ascribed to the phosphate ester with structure (IV), even though formaldehyde is not generated during oxidation with periodate.

Fractions F3A and F3B were also examined as a mixture (slightly contaminated by fraction F3C), giving a molar ratio (phosphorus/pyruvic acid/mannitol/1,4-anhydromannitol/glucose) of 1.00:0.47:0.43:0.11:0.84. Both fractions reacted only slowly with the periodate/Schiff reagents. Hydrolysis with alkaline phosphatase gave two new products, one with the mobility of fraction F1A on electrophoresis at pH 5.3 and the other less mobile ($m_{\text{glycerol 1-phosphate}}$ 0.50). Like the parent compounds, both dephosphorylation products reacted only slowly with the periodate/Schiff reagents. On treatment with β -glucosidase, the dephosphorylation products gave fractions F1B and F1C. Thus fraction F3A should be the 2-*O*-glucosyl derivative of the phosphate ester F3C, probably with the phosphate residue at position 1 of mannitol as in structure (IV). Similarly, fraction F3B should be the 2-*O*-glucosyl derivative of fraction F3D, with the phosphate residue necessarily at position 1.

Fraction F4

From its position in the elution profile (Fig. 2), this fraction was expected to contain diphosphates. The major phosphorus-positive fraction F4D was identified as mixed glycerol diphosphates by (a) paper electrophoresis at pH 5.3 and 2.8, (b) paper chromatography (solvents C and D), (c) enzymic dephosphorylation giving P_i and glycerol (molar ratio 2.00:0.80).

The second major phosphate (F4B) could not be fully resolved from the trace products F4A and F4C, but had the electrophoretic mobilities of mannitol 1,6-diphosphate. At least 93% of the phosphorus in the mixture F4A–C was released on treatment with alkaline phosphatase, and P_i was the only phosphorus-containing product detected in the enzymic hydrolysate. The hydrolysate also contained (paper chromatography, g.l.c. and e.i. mass spectrometry) free mannitol and 1,4-anhydromannitol in amounts sufficient to account for 47 and 9% of the total phosphorus as the respective diphosphates. The mixture F4A–C also contained

small amounts of glucose and pyruvic acid (molar proportions 0.36 and 0.41, respectively, relative to phosphorus, 2.00), and two anionic periodate/Schiff-reactive compounds were detected in the enzymic hydrolysate. One had the mobility of fraction F1C and gave a slow reaction, whereas the other migrated slightly faster (similar to F1D) and rapidly gave a weak purple colour. It is therefore evident that the mixture F4A-C contains products other than simple polyol diphosphates.

Fraction 5

All of the phosphorus in fraction F5C (the most abundant phosphate in this mixture) was released on treatment with alkaline phosphatase, to give fraction F1B and another (unidentified) periodate/Schiff-reactive product with a marginally higher electrophoretic mobility at pH 5.3. Fraction F5C probably includes, therefore, a diphosphate of the pyruvate-linked mannitol (structure I). Analytical data for fraction F5C (molar ratios: phosphorus, 2.00; pyruvic acid, 0.60; mannitol, 0.48; 1,4-anhydromannitol, trace; glucose, absent) did not provide real clues about the possible identity of the second component.

It was also difficult to draw inferences about the identities of the minor fractions F5A and F5B. Analysis of a mixture gave the molar ratio (phosphorus/pyruvic acid/mannitol/glucose) 2.00:1.05:0.71:1.42. Only 60% of the phosphorus was released on treatment with alkaline phosphatase. Fraction F5B was not detected after this treatment, but a spot in the position of F5A on electrophoresis at pH 5.3 (rapid purple with the periodate/Schiff reagents, intensifying on standing) made it impossible to determine whether any F5A (slow periodate/Schiff reaction) remained. However, it seemed likely that most or all of fraction F5A had been converted into another compound (m_{F5A} 0.82 at pH 5.3) giving a slow blue colour with the periodate/Schiff reagents (indicative of the presence of glucose). Whatever their identities, fractions F5A and F5B may be expected to include products of incomplete hydrolysis of the polymer.

Polymeric structure

Depolymerization of the mannitol teichoic acid in both acid and alkali, the presence of both monophosphates and diphosphates in the hydrolysis products, the formation of 1,4-anhydromannitol derivatives under alkaline conditions, and the resistance of the polymer to alkaline phosphatase (Anderton & Wilkinson, 1980) all point to the involvement of phosphodiester linkages. The ^{31}P -n.m.r. spectra of the polymer confirmed this view. At pH 4.2, the spectrum (Fig. 4a) contained two major signals with δ (p.p.m.) 1.07 and 0.97. Similar

Fig. 4. ^{31}P -N.m.r. spectra of the mannitol teichoic acid from bacterium N.C.T.C. 9742

Proton-decoupled spectra for the sample in 75% (v/v) $^2\text{H}_2\text{O}$ containing EDTA (0.1M) at room temperature were obtained at 162 MHz with 85% orthophosphoric acid as an external reference with a Bruker WH-400 spectrometer. The spectra were recorded at apparent pH 4.2 (a) and 9.0 (b).

chemical shifts have been reported (Egan *et al.*, 1982) for phosphodiester-linked polymers from *Haemophilus influenzae*, which incorporate ribitol 1-phosphate residues. The presence of two major signals in our spectrum may be a consequence of glucosylation of only about half of the backbone units. As expected, the chemical shifts for the two major signals and most of the minor ones were almost unchanged at pH 9.0 (Fig. 4b), although a shoulder appeared on the major downfield signal. One minor signal shifted about 4 p.p.m. downfield on making the solution alkaline, indicating that it could be assigned to monoester phosphorus.

Leaving aside glycerol phosphates (assumed to

arise from a linkage unit or a separate teichoic acid) and derivatives of 1,4-anhydromannitol (shown to be artefacts of hydrolysis), and assuming a regular backbone structure for the polymer, the results of the degradative studies described above are essentially consistent with a 1→6-phosphodiester-linked polymer of mannitol residues, most of which carry pyruvic acid at positions 4 and 5, and about half of which carry glucose at position 2. Thus a polymer containing similar proportions of two major units (structures V and VI) is proposed. Small proportions of units lacking pyruvic acid (possibly lost during the prolonged extraction of the polymer with trichloroacetic acid) are apparently present, as indicated by the formation of mannitol mono- and di-phosphates on alkaline hydrolysis (fractions F2A, F2B, and F4B).

One indication that position 1(6) of mannitol was involved in the phosphodiester bridge was the identification of fraction F2A as mannitol 1-phosphate. Although migration of the phosphate via a cyclic intermediate is to be expected during the alkaline hydrolysis of phosphodiesters such as 1→6-linked (or 2→5-linked) poly(mannitol phosphate), migration of a phosphate residue to the 1(6) position from the 2(5) position is obviously impossible when the latter position(s) are otherwise substituted (as in structure V). Even more informative was the evidence that fraction F3B was the 1-phosphate of a 3,6-(1,4)-anhydromannitol residue substituted by glucose at position 2 and by pyruvic acid at positions 4 and 5. Such a product must be derived from a residue with structure (V), probably flanked by similar residues. Facile cleavage of the phosphodiester linkage at position 6 could then only occur by intra-residue nucleophilic attack at C-6 by the free hydroxy group at position 3. The phosphomonoester group displaced by C–O bond fission would be found as the 1-phosphate in an equivalent product from the adjacent residue. Thus the position of the residual phosphate marks one end of the phosphodiester bridge as C-1, and cyclization of the mannitol marks C-6 as the other end.

Confirmation that all of the primary hydroxy groups of the mannitol residues participate in phosphodiester linkages was provided by further analysis of the ^{13}C -n.m.r. spectrum (Fig. 1). The spectrum contained only one signal, δ (p.p.m.) [$^2\text{H}_2\text{O}$] 61.82, in the region for unsubstituted hydroxymethyl carbons, and this signal must be assigned to C-6 of glucose. Values reported recently for the chemical shift of C-1(6) in mannitol are δ (p.p.m.) 63.7, for a solution in [$^2\text{H}_6$]dimethyl sulphoxide (Schnarr *et al.*, 1979), and 64.6, for a solution in $^2\text{H}_2\text{O}$ (Angyal & Le Fur, 1980), whereas a reference spectrum obtained during the present study for the hexitol in $^2\text{H}_2\text{O}$ gave the value 63.27. However, in a proton-coupled ^{13}C -n.m.r. spectrum of the teichoic acid, the signal at δ (p.p.m.) 66.39 (probably 3C) was clearly seen as a triplet, showing that it corresponded to methylene carbons. Although less clear cut, there was also an indication that one carbon atom contributing to the signal at δ (p.p.m.) 68.27 (resolved into two signals in a spectrum recorded at 100.62 MHz) also gave a triplet in the proton-coupled spectrum. The 3C signal can be attributed to C-1 and C-6 of the mannitol residue in structure (V) and C-6 of the mannitol residue in structure (VI). In each case, a downfield shift of 3.5–4.5 p.p.m. due to the α -effect of phosphorylation and a smaller upfield shift due to the β -effect of glucosylation or acetylation can be applied to the parent signals in free mannitol (e.g. Egan, 1980; Tarelli & Coley, 1979; Wedmid *et al.*, 1980). The absence of a glucosyloxy group would produce a signal at lower field, i.e. δ (p.p.m.) 68.27, for C-1 of the mannitol residue in structure (VI). Although there was some evidence of signal broadening for the above methylene carbon atoms, the expected two-bond ^{31}P - ^{13}C coupling could not be observed. However, splitting of the signals at δ (p.p.m.) 79.24 (J8.6 Hz), 78.19 (J6.5 Hz), and 71.92 (J8.1 Hz) could be ascribed to three-bond ^{31}P - ^{13}C coupling (recording the spectra at different field strengths from 22.5 to 100.62 MHz confirmed that the signals were doublets and not closely spaced singlets). The coupled signal at lowest field, δ (p.p.m.) 79.24 (1C), can be assigned to the β -glucosyloxy C-2 of structure (V), and the signal at δ (p.p.m.) 78.19 (2C) to the C-5 atoms of structures (V) and (VI). The other coupled signal at δ (p.p.m.) 71.92, assigned to C-2 of structure (VI), was at unexpectedly low field. Chemical shifts reported for C-2 of mannitol are δ (p.p.m.) 71.3 (Schnarr *et al.*, 1979) and 72.2 (Angyal & Le Fur, 1980), and the value of 71.02 was obtained in the present study. An upfield shift of about 1.6 p.p.m. expected as a result of phosphorylation at C-1 (Tarelli & Coley, 1979) would therefore give a predicted value for C-2 in structure (VI) in the range δ (p.p.m.) 69.4–70.6, in

marked contrast with the assigned value of 71.92. However, the preferred structure for mannitol is an extended zigzag conformation, on the basis of which its ^{13}C -n.m.r. spectrum can be rationalized (Schnarr *et al.*, 1979; Angyal & Le Fur, 1980). The conformational change (180° rotation around the C-4–C-5 bond) needed to produce the 1,3-dioxolan derivative, and other possible conformational changes consequential on this or on phosphorylation at C-1, may mean that the use of reference data on mannitol itself is inappropriate.

Reasonable assignments can be made for other major signals in the ^{13}C -n.m.r. spectrum of the polymer on the basis that it is constructed essentially from units with structure (V) or (VI) (Table 1). Minor signals in the spectrum can probably be attributed either to units lacking pyruvate, or to residues of glycerol and galactosamine.

The presence in the polymer of roughly equal numbers of units with structure (V) or (VI) suggested that these might alternate in a regular polymer. To test this hypothesis, the polymer was oxidized with NaIO_4 . As expected, all of the glucose and 58% of the mannitol were destroyed. Erythritol was obtained after treatment of the

oxidised polymer with NaBH_4 , followed by acid hydrolysis and enzymic dephosphorylation. The mannitol destroyed and erythritol produced should correspond to residues with structure (VI). If this alternated with structure (V), the oxidation product should consist entirely of degraded 'dimeric' units of low M_r , as a result of cleavage of the backbone at every unit with structure (VI). However, chromatography of the oxidation product on Sephadex G-25 showed the presence of polymeric material virtually excluded from the gel, as well as fractions of lower M_r (Fig. 5). Although incomplete, these data rule out a regular polymer with alternating units (V) and (VI).

Table 1. Assignment of signals in the ^{13}C -n.m.r. spectrum of the mannitol teichoic acid from bacterium N.C.T.C. 9742

Mannitol I is that in the residue with structure (V), and mannitol II that in the residue with structure (VI). Interchangeable assignments are indicated by an asterisk. Chemical shifts are given in p.p.m. downfield from external tetramethylsilane.

Carbon atom	Chemical shift			
	Mannitol I	Mannitol II	Glucose	Pyruvic acid
C-1	66.39	68.27	102.78	178.88
C-2	79.24	71.92	74.54	107.68
C-3	68.27	69.14	76.95*	23.37, 23.17
C-4	76.47*	76.81*	70.99	
C-5	78.19	78.19	76.95*	
C-6	66.39	66.39	61.82

Fig. 5. Fractionation on Sephadex G-25 of periodate-oxidised mannitol teichoic acid from bacterium N.C.T.C. 9742

The polymer (12 mg) was oxidized with 50 mM- NaIO_4 (1 ml) at 4°C for 70 h (reaction essentially complete in 24 h). After destruction of the excess of NaIO_4 (ethylene glycol) and reduction (NaBH_4), the oxidized polymer was applied to a column (2 cm $\times$ 80 cm) of Sephadex G-25. Elution was carried out with pyridine/acetic acid buffer (10 ml of pyridine + 4 ml of acetic acid/litre; pH 5.4) at a flow rate of 5 ml/h. Fractions (4 ml) were screened for phosphorus content. Arrows indicate the elution volumes of Blue Dextran (A) and P_i (B).

Table 2. Composition of cell walls of *Brevibacterium* species

Total carbohydrate, determined by the phenol/ H_2SO_4 method, is expressed as glucose. Polyol phosphates are those detected in acid hydrolysates

Component	<i>Brevibacterium linens</i> 739	<i>Brevibacterium epidermis</i> 2285
Phosphorus	2.8%	1.2%
Total carbohydrate	3.1%	17.0%
Neutral sugars	Galactose, glucose	Galactose, glucose
Polyol phosphates	Glycerol mono- and di-phosphates	Glycerol mono- and di-phosphates

Scheme 1. Major pathways for alkaline degradation of the mannitol teichoic acid
Key to variable substituents: R', Me; R'', CO₂H; R''', β-D-Glcp.

Cell walls of *Brevibacterium* species

In view of the suggestion (Collins *et al.*, 1980) that bacterium N.C.T.C. 9742 should be reclassified as *Brevibacterium iodinum*, cell walls from single strains of *Brevibacterium linens* and *Brevibacterium epidermis* were examined for the presence of a mannitol teichoic acid. Analytical data for the cell walls are summarized in Table 2. Although there was evidence for the presence of a glycerol teichoic acid in both cases, neither mannitol nor 1,4-anhydromannitol was detected in acid hydrolysates (with or without enzymic dephosphorylation of the hydrolysis products).

Discussion

Difficulties experienced in this structural study stemmed partly from the complexity of the alkaline hydrolysate of the mannitol teichoic acid (Fig. 3). Even when apparently homogeneous products could be isolated, it was often not possible to obtain satisfactory stoichiometric ratios for the components identified. Other workers have experienced problems of low recoveries of mannitol, e.g. when used as an internal standard for acid methanolysis of carbohydrates (Jamieson & Reid, 1974; Pritchard & Todd, 1977; Rickert & Sweeley, 1978), particularly as a result of adsorption on Ag_2CO_3 , and in the present study the recovery of mannitol was as low as 6% if alkaline hydrolysis of samples was carried out in glass. Possibly more relevant to this study is the possibility of forming cyclic derivatives other than 1,4-anhydromannitol either directly from the polymer or as a result of acid-catalysed dehydration of the mannitol (Barker, 1970; Szafranek & Wiśniewski, 1978; Bock *et al.*, 1981). Although check experiments and the literature (e.g. Gerwig *et al.*, 1984) suggested that acid-catalysed dehydration was unlikely to be significant under the conditions used, unidentified peaks were sometimes found on g.l.c. of the polyol acetates from hydrolysis products. Nevertheless, the main obstacle to stoichiometry seemed to be the surprising resistance to acid hydrolysis of the pyruvate acetal of 1,4-anhydromannitol.

Although the failure to identify some of the products of alkaline hydrolysis and the incomplete characterization of others leave scope for modifications in the future, a reasonable structure for the mannitol teichoic acid from bacterium N.C.T.C. 9742 can be proposed. The mechanistic pathways expected to lead to the major products of alkaline hydrolysis are indicated in Scheme 1. Assuming that cleavage of phosphodiester bonds occurs either by nucleophilic attack at phosphorus in the 1-phosphate residue by the 2-hydroxy group, or by nucleophilic attack at C-6 by the 3-hydroxy group, the finding of fractions F3A, F3B, F3C, and F3D

as major products is evidence in favour of a structure of the type indicated in Scheme 1, in which units of structure (V) or (VI) are distributed randomly or in blocks. Fraction F3C could not arise from a regular structure of alternating units, and fraction F3A would not be produced if the material was a mixture of two homopolymers each composed of unit (V) or (VI). The results of periodate oxidation (Fig. 5) also tend to support the type of structure proposed.

The preliminary data on the composition of cell walls from *Brevibacterium linens* and *Brevibacterium epidermis* (Table 2) are in accord with those described for Group II strains of the former species by Fiedler *et al.* (1981). Thus these strains appear to contain glycerol teichoic acids whereas other strains of *Brevibacterium linens* placed in Group IV appear to contain both glycerol and ribitol teichoic acids (Fiedler *et al.*, 1981). Structural diversity of teichoic acids, and the occurrence of more than one type in the same organism, is well established for species of *Staphylococcus* (Endl *et al.*, 1983, 1984). Although teichoic acids are of uncertain chemotaxonomic significance, it is nevertheless remarkable that poly(mannitol phosphate) polymers have so far only been found in organisms related to *Brevibacterium linens* (the present study; Fiedler *et al.*, 1981) and in a strain of the Gram-positive anaerobe *Bifidobacterium bifidum* subspecies *pennsylvanicum* (Veerkamp *et al.*, 1983). Pyruvic acid was not reported as a component of the mannitol teichoic acids found by Fiedler *et al.* (1981), but a rhamnosyl substituent seemed to be present in two of the polymers. The product from *Bifidobacterium bifidum* appeared to be a 1-3-linked oligomer of mannitol phosphate. These findings should encourage wider surveys for mannitol teichoic acids, which may eventually be useful in deciding on the most appropriate classification for bacterium N.C.T.C. 9742.

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