The Structure of C-Polysaccharide from the Walls of *Streptococcus pneumoniae*

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The well-known immologically active component of pneumococci, C-polysaccharide, is a teichoic acid that can be isolated from the cell walls and purified by Sephadex and ion-exchange chromatography. Further details of the structure of C-teichoic acid were established by chemical degradation, including hydrolysis in acid and alkali, treatment with HF, periodate oxidation and methylation. In addition, the use of ^{13}C n.m.r. has confirmed some of these structural features and resulted in a proposal for the order of substituents, the location of positions of substitution and the configuration of anomeric centres in the repeating unit of the polymer.

The C-teichoic acid (C-polysaccharide) of *Streptococcus pneumoniae* is receiving renewed attention in immunology. It is mitogenic for T lymphocytes; thus, as it is a widespread contaminant in most pneumococcal capsular polysaccharide preparations, its presence might affect the use of the preparations in studies on their effect on the immune response (J. H. Humphrey, personal communication). It possesses choline phosphate haptenic groups and binds both to idiotypic determinants on immunoglobulins produced against other molecules possessing choline phosphate residues, and to certain myeloma proteins with binding sites specific for choline phosphate (Brown & Crandall, 1976; Glaudemans *et al.*, 1977). C-reactive protein, an acute-phase protein that is found in human serum during several pathological conditions including tissue injury, carcinoma and the febrile stages of infections with various micro-organisms, is precipitated by C-teichoic acid. This again probably involves the choline phosphate determinant and complement is consumed *in vivo* (Kaplan & Volanakis, 1974). An homology has been noted between human and rabbit C-reactive proteins, immunoglobulins and histocompatibility antigens by amino acid sequencing (Osmand *et al.*, 1977).

The physiological importance of choline phosphate residues in the cell wall of the pneumococcus is also noteworthy and well documented. The biosynthetic replacement of choline with analogues such as

ethanolamine affects the organism in a number of ways; the cells are unable to separate, genetic transformation is inhibited, and the organism becomes resistant to autolysis, phage infection and the lytic action of penicillin and other cell-wall antibiotics. These altered properties are probably the results of defects in the autolytic system of the organism (Tomasz *et al.*, 1975). A knowledge of the structure of C-teichoic acid is therefore important in understanding the nature of its association with immunoglobulin, myeloma, C-reactive protein and also the autolytic enzyme system of the pneumococcus.

Since the first description of a species-specific somatic antigen (fraction 'C') of the pneumococcus by Tillett & Francis (1930) several structural studies have been made, notably by Goebel *et al.* (1943) and Gotschlich & Liu (1967). Brundish & Baddiley (1968) first showed the polymer to be a teichoic acid comprising a repeating unit containing ribitol phosphate, *N*-acetylgalactosamine, *N*-acetyldiaminotri-deoxyhexose, choline phosphate and glucose residues. More recently, Watson & Baddiley (1974) studied the action of HNO_2 on the polymer and proposed a partial structure. In the present paper we describe an improved method of preparation and further work on the structure of C-teichoic acid resulting in the clarification of most of the structural features of the molecule.

Experimental

Materials

Streptococcus pneumoniae A.T.C.C. 12213 was grown in 15-litre batches as previously described (Poxton & Leak, 1977). HF (60%, w/w) was purchased from Hopkin and Williams, Chadwell Heath,

Abbreviation used: Dnp, 2,4-dinitrophenyl.

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Essex, U.K. The glucose oxidase reagents were from Sigma Chemical Co., London KT2 7BH, U.K., alkaline phosphomonoesterase was from Boehringer Corp., London BN7 1LG, U.K., and KB^3H_4 from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were purchased from local suppliers.

Analytical methods

Phosphate was determined by the method of Chen *et al.* (1956), choline by the method of Appleton *et al.* (1953), glucose by the method of Dubois *et al.* (1956) and also by the glucose oxidase reagents, hexosamine by the method of Strominger *et al.* (1959) and amino groups by the ninhydrin method (Rosen, 1957). Amino sugar analyses were carried out on a JEOL amino acid analyser.

Methylation analysis, by preparing partially methylated alditol acetates by the method of Jansson *et al.* (1976), was investigated in a Varian 1400 all-glass gas-liquid chromatograph and a Micromass (V. G. Micromass, Altrincham, Cheshire, U.K.) model 12B2 mass spectrometer operating at 70 eV and 4 kV accelerator voltage. Capillary columns were used, coated with SP1000 resin for derivatives of neutral sugars and SE30 resin for derivatives of amino sugars (both liquid phases were supplied by Phase Separations, Queensferry, Clwyd, U.K.).

Proton-decoupled ^{13}C -n.m.r. data were determined at 22.63 and 50.3 MHz in $^2\text{H}_2\text{O}$ solution at 32°C. External tetramethylsilane was used as reference.

Immuno-gel diffusion on agarose plates was by the method of Ouchterlony against C-antiserum kindly given by Dr. Michael Heidelberger (New York University Medical Center, New York, NY, U.S.A.).

Paper chromatography and electrophoresis

Both were carried out on Whatman no. 1 paper or, on a preparative scale, on Whatman 3MM paper (washed with 2M-acetic acid and water). Descending paper chromatography was used with the following solvent systems: A, propan-1-ol/aq. NH_3 (sp.gr. 0.88)/water (6:3:1, by vol.); B, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.).

Electrophoresis was carried out with a Shandon Southern (Camberley, Surrey, U.K.) apparatus, model L24, with the following buffers: C, 8.0% (v/v) formic acid, pH 1.8; D, pyridine/acetic acid/water (1:1:38, by vol.), pH 4.7; E, pyridine/acetic acid/water (5:2:93, by vol.), pH 5.3.

Compounds were detected by the following methods: reducing sugars by the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950), α -glycols by the periodate/Schiff spray reagents (Baddiley *et al.*, 1956), amino compounds by ninhydrin,

phosphate esters by the acid molybdate spray (Hanes & Isherwood, 1949) and choline and choline phosphate by the Dragendorf spray reagent (Bregoff *et al.*, 1953).

Preparation of C-teichoic acid

Washed bacteria were suspended in ice-cold water to a concentration of about 30% (w/v) and disrupted for 2.5 min in a Braun homogenizer with no. 11 Ballotini beads. Walls and unbroken bacteria were collected by centrifugation at 10000g for 30 min at 4°C. The walls were carefully removed from above the pellet of unbroken cells and suspended in water. The suspension was heated rapidly to 80°C and kept at that temperature for 3 min to destroy autolytic enzymes. The walls were then washed three times in water. Protein and membrane components were removed by pouring a 30% (w/v) suspension of cell walls into an equal volume of boiling 5% (w/v) sodium dodecyl sulphate and stirring for 4 h at room temperature (20°C). The cell walls, which were still slightly contaminated, were washed by centrifugation at 10000g for 20 min in six changes of water at 20°C.

Cell walls were suspended in ice-cold water to a concentration of about 2.5% (w/v) and trichloroacetic acid was added to a final concentration of 10% (w/v); C-teichoic acid was extracted by stirring for 48 h at 4°C. The cell walls were removed by centrifugation at 10000g for 30 min at 4°C and the trichloroacetic acid was removed from the supernatant fluid by six extractions with equal volumes of diethyl ether. Water was removed by rotary evaporation and nucleic acids by fractionation on a stacked column of Sephadex G-25 and G-75 similar to that described by Slabyj & Panos (1973), except that 0.2M-NaCl was used instead of LiCl. The fractions containing phosphorus, but which did not absorb at 260 nm, were combined, decreased in volume and desalted on a Sephadex G-25 column (60 cm $\times$ 1.5 cm). A final purification was carried out by ion-exchange chromatography on a column (30 cm $\times$ 1.5 cm) of DEAE-cellulose (Whatman DE-52), eluting with a continuous gradient (0–1 M) of pyridinium acetate, pH 5.3. C-teichoic acid was eluted at 0.4 M; this was freeze-dried.

Results and Discussion

Acid hydrolysis

C-teichoic acid (2 mg) was hydrolysed in 2M-HCl for 3 h at 100°C in a sealed tube. Acid was removed *in vacuo* over NaOH and the products were examined by paper chromatography in solvents A and B. The following were identified: ribitol, anhydrosorbitol, galactosamine, glucose, galactosamine phosphate

and isomeric ribitol phosphates. Electrophoresis in buffer C revealed choline, but not choline phosphate.

Molecular proportions of the constituents were determined with both hydrolysed and unhydrolysed material. After hydrolysis (2M-HCl at 100°C for 3 h), the proportions of phosphorus/hexosamine/glucose/choline/amino groups were 2:1.8:0.2 (by glucose oxidase):0.66:2.6. Unhydrolysed material had a molar ratio phosphorus/hexose (phenol/H₂SO₄ method) of 2:0.2. A more accurate value of phosphorus/galactosamine was obtained from the amino acid analyser. C-teichoic acid (5mg) was dissolved in 1.2ml of water; 0.5ml samples were adjusted to a final concentration of 4M-HCl and hydrolysed *in vacuo* for 6 and 24h at 100°C. After removing the HCl over NaOH, samples were examined in the analyser. Total galactosamine was calculated by summation of the free galactosamine and the galactosamine phosphate. The remaining 0.2ml of sample was used for phosphorus determination. Molar ratios of phosphorus/galactosamine were 2:0.87 after 6h and 2:0.95 after 24h. This showed that the true ratio of phosphorus/galactosamine was 2:1 and the anomaly in the hexosamine value was probably due to the diaminohexose, which is a known constituent, being decomposed to a pyrrole that reacts with hexosamine reagents. The presence of three amino groups/two atoms of phosphorus agrees with this conclusion.

Alkaline hydrolysis

C-teichoic acid (20mg) was dissolved in 4ml of 1M-NaOH, sealed in a plastic tube and hydrolysed for 3h at 100°C, cooled, desalted through 20ml of Dowex 50 (NH₄⁺ form) resin, evaporated to dryness and redissolved in 2ml of water. Less than 5% of the total phosphorus was present as P_i. A portion (1ml) was treated for 16h at 37°C under toluene with alkaline phosphomonoesterase (10µg of Boehringer suspension) at pH9 [adjusted with (NH₄)₂CO₃]. In several experiments, 50–60% of the phosphorus was converted into P_i. Thus about half of the phosphorus was phosphomonoester after alkaline hydrolysis.

The product of alkaline hydrolysis was hydrolysed in acid (2M-HCl, 3h at 100°C) before and after the phosphatase treatment. With solvents A and B, it was found that before phosphatase treatment ribitol, anhydribose, galactosamine, ribitol phosphates and galactosamine phosphate were present. After phosphatase treatment, all but ribitol phosphates were present and the amount of ribitol appeared to have increased. This suggests that the ribitol is attached to a phosphomonoester and galactosamine to a diester after the alkaline hydrolysis.

If choline had been produced in the alkaline hydrolysis, it would have been bound to the Dowex

50 resin during desalting. In a control experiment, choline phosphate was treated with 1M-NaOH for 3h at 100°C. After cooling and neutralization with dilute HCl, both organic phosphate and P_i were determined. No P_i was produced, but electrophoresis of the products showed that choline phosphate had been destroyed and a smell resembling trimethylamine was noted. Presumably choline phosphate undergoes a Hofmann elimination to trimethylamine and possibly vinyl phosphate under the experimental conditions. Similarly, when C-teichoic acid was hydrolysed with alkali a characteristic smell of a tertiary amine was observed. The phosphate associated with the choline residue remained as a diester bound to galactosamine. When the hydrolysate was subsequently hydrolysed with acid, chromatography in solvent B revealed a fast-moving product that was not characterized, but was probably derived from the vinyl group attached through the phosphodiester to galactosamine.

Reaction with 1-fluoro-2,4-dinitrobenzene

To detect the diaminohexose, C-teichoic acid was treated with fluorodinitrobenzene to dinitrophenylate the free amino groups. The reaction was carried out by the method of Distler *et al.* (1966); 12mg of material was used and the final purification was by dialysis in several changes of water for 24h. The resultant dinitrophenylated material (Dnp-C), and C-teichoic acid itself, were examined with C-antisera on a gel-diffusion plate; a sharp yellow precipitin band was obtained continuous with the band given by the C-teichoic acid.

The number of dinitrophenyl groups was determined at A₃₆₀ and compared with a standard curve derived from *ε*-dinitrophenyl-lysine. A molar ratio of phosphorus/dinitrophenyl groups of 2:0.76 was found, i.e. about one free amino group to two phosphates. Acid hydrolysis (4M-HCl, 4h at 100°C) of Dnp-C (2mg) and electrophoresis in buffer D for 30min at 55 V·cm⁻¹ gave a single yellow product that stained with the ninhydrin reagent and migrated about twice as far as dinitrophenyl-lysine. This was similar to the properties observed by Distler *et al.* (1966) for the Dnp-diaminohexose.

Degradation with HF

HF (60%, w/w) converts phosphomono- and phosphodi-esters into P_i and phosphorofluoridates without significant hydrolysis of glycosidic linkages (Lipkin *et al.*, 1969). To protect the sensitive diaminohexose, the C-teichoic acid (20mg) was dinitrophenylated as described above and a dry sample in a plastic tube was treated with 1ml of 60% (w/w) HF at 0°C for 16h. After neutralization with 60ml of Dowex 2

(CO₃²⁻ form) resin by the method of Anderson *et al.* (1977), the resulting material was examined by electrophoresis in buffer E; a single yellow spot remained at the origin, and a spot corresponding to choline was detected by the Dragendorf spray. On paper chromatography in solvent A a single yellow spot, R_{ribitol} 1.3, was seen; in solvent B it had R_{glucose} 1.6. The yellow material was purified by preparative electrophoresis in buffer E on Whatman 3MM paper, where it remained at the origin. It was eluted in water and hydrolysed with acid. Under vigorous conditions (4M-HCl for 6h at 100°C) a yellow spot was observed on electrophoresis in buffer D corresponding to Dnp-diaminohexose. Galactosamine, ribitol, anhydrosorbitol and glucose were also observed on paper chromatograms. Under less vigorous conditions (2M-HCl for 3h at 100°C) an additional yellow spot was detected, migrating 1.5 times as far as the Dnp-diaminohexose; this was a disaccharide of galactosamine and the diaminosugar, both of which had been de-*N*-acetylated. This disaccharide is discussed below.

When the yellow product obtained by treatment of Dnp-C with HF was treated with 0.1M-HCl at 100°C for 30min and the mixture examined by paper chromatography, ribitol and glucose were detected together with two yellow products in roughly equal amounts; one had R_{glucose} 1.1 (solvent B), which was the same as the starting material, and the other had R_{glucose} 1.5. This second product was eluted from the paper and a portion hydrolysed in 2M-HCl at 100°C for 3h; the major products were Dnp-diaminohexose (R_{glucose} 1.7), galactosamine (in solvent B) and a yellow component with R_{glucose} 1.3, which was de-*N*-acetylated starting material (this was identical with the disaccharide described above). After more vigorous hydrolysis (4M-HCl for 6h at 100°C) only galactosamine and Dnp-diaminohexose were observed.

Reduction of the disaccharide with KB³H₄

The disaccharide (1mg) prepared as above was treated with 0.2ml of 0.2M-KBH₄ [containing 14.8 MBq (0.4mCi) of ³H] for 2h in a fume cupboard. Excess of BH₄⁻ was destroyed by adding 50% (v/v) acetic acid dropwise until the pH was below 5. The solution was freeze-dried, the products were treated with 2M-HCl for 3h at 100°C and, after desiccation over NaOH, dissolved in 0.5ml of water, desalted through Dowex 50 (H⁺ form) resin and eluted with 20ml of 1M-NH₄OH. Borate was removed by distillation with methanol (5 times) and Cl⁻ and reducing sugars were removed by passage through 2ml of Dowex 2 (OH⁻ form) resin. The colourless solution contained a radioactive alditol that was indistinguishable from galactosaminitol in solvent A and on electrophoresis in buffer E. This confirmed that

galactosamine was the reducing component of the disaccharide.

Order of substituents in the repeating structure

The present results, together with those obtained before (Brundish & Baddiley, 1968; Watson & Baddiley, 1974) enable a partial structure to be derived for the main repeating unit, i.e.:

The diaminosugar and ribitol were known to be in the polymer chain because of their respective destruction by HNO₂ (Watson & Baddiley, 1974) and by periodate (Brundish & Baddiley, 1968), causing simultaneous fission of the chain. Moreover, *N*-acetyl galactosaminyl ribitol was characterized as a degradation product (Watson & Baddiley, 1974). The isolation in the present work of a disaccharide containing the diaminosugar attached to a reducing galactosamine confirms the above sequence.

Although the study by Watson & Baddiley (1974) and the present work demonstrate the presence of glucosyl substituents in the polymer, the amount is insufficient for one glucose to each repeating unit. It is likely then that either only a few of the repeating units are glucosylated or that the glucose is localized at the end of the chain. It is not attached through a phosphodiester at its 1-position because it remains attached to the unit obtained by HF treatment, and reduction of this with B³H₄⁻ gives no labelled glucitol residue. Watson & Baddiley (1974) suggested that the glucosyl substituent might be associated with phosphate, since a product tentatively identified as glucitol phosphate had been detected, but it now seems likely that this identification was incorrect. Although the location of glucose is still inadequately established, the methylation and n.m.r. studies described below indicate the presence of a glucosyl-glucosyl (β-isomaltosyl) substituent.

Periodate oxidation

Substitution of the galactosamine residue was examined by the action of periodate. Aqueous solutions (1ml) of polymer and the Dnp/HF product (1mg of each) were mixed with 5mM-NaIO₄ (1ml) in 0.1M-acetate buffer at pH4.5. After 16h at room temperature under toluene, excess of periodate was destroyed by adding ethylene glycol (5μl), solvent removed *in vacuo* and the residue hydrolysed in 4M-HCl for 6h at 100°C. After removal of acid over NaOH *in vacuo* galactosamine was determined with the autoanalyser. In neither experiment was a decrease in aminosugar detected after the periodate

treatment, whereas galactosamine itself was completely destroyed under similar conditions.

It follows that the diaminohehexose must be attached to the 3- or 4-position on the *N*-acetylgalactosamine residue. If it had occupied the 6-position then the removal of the choline phosphate residue by HF from the 3- or 4-position would have created an oxidizable glycol structure.

There is strong justification for the assumption that the phosphodiester substituent on ribitol must occupy the D-5-position. All of the many ribitol teichoic acids examined are derivatives of D-ribitol 5-phosphate that, in the form of CDP-ribitol, is the biosynthetic precursor of these compounds. It follows that, as the ribitol residues in the polymer are destroyed by periodate, causing chain fission, the *N*-acetylgalactosaminyl substituents should occupy the D-1- or -2-positions on ribitol.

Preparation of *N*-acetyl/HF product

For the following methylation and ^{13}C -n.m.r. studies the HF product was prepared from fully *N*-acetylated C-teichoic acid. This was preferable to using dinitrophenylated polymer.

The C-teichoic acid (120mg) was dissolved in 10ml of water and 2ml of saturated NaHCO_3 solution was added. This was cooled in ice and 2ml of fresh ice-cold aq. 5% (v/v) acetic anhydride was added. Incubation was for 25 min at room temperature and then excess of acetic anhydride was destroyed by heating to 100°C for 3 min. The *N*-acetylated polymer was first desalted through a 20ml column of Dowex 50 (NH_4^+ form) resin, decreased in volume, then further purified by passage down a column (60cm $\times$ 1.5cm) of Sephadex G-25 and finally freeze-dried in a plastic tube. A yield of 114mg was obtained. This was treated with 60% (w/w) HF as for the Dnp-polymer. After HF treatment, the products were again *N*-acetylated as above. The oligosaccharide products were fractionated on a column (60cm $\times$ 1.5cm) of Sephadex G-10 and the highest-molecular-weight fraction was freeze-dried. A yield of 25mg of pure *N*-acetylated HF product was obtained, which contained ribitol, *N*-acetylgalactosamine, *N*-acetyldiaminohehexose and glucose.

Methylation studies

The position of substitution on ribitol and the nature of the glucosyl substituents were studied by methylation followed by hydrolysis, reduction, acetylation and examination by g.l.c.-mass spectrometry. The method of Hakomori (1964) as developed by Jansson *et al.* (1976) was used with minor modifications. The *N*-acetyl/HF product (4mg) from C-teichoic acid was dissolved by ultrasonic treatment in dry dimethyl sulfoxide (2ml) under N_2 . Prep-

aration of the partly methylated alditol acetates was as described (Björndal *et al.*, 1967; Jansson *et al.*, 1976), except that hydrolysis was with 2M-HCl for 3h at 100°C , followed by removal of acid over NaOH *in vacuo*. The partly methylated alditol acetates of neutral sugars were examined on a column of SP1000 resin, the results being given in Table 1. Products (C)-(F) were identified by comparing their mass spectra with the published data on authentic standards (Jansson *et al.* 1976). Product (A) had a similar retention time (approx. 7 min) to the isomeric 4-*O*-acetyl-1,2,3,5-tetra-*O*-methylribitol and ions of *m/e* 71, 89, 101, 117, 145 and 161 were identified. These were formed as follows:

The mass spectrum was significantly different from that of the above-mentioned isomer. Product (B) had a slightly longer retention time than did authentic 2,3,5-tri-*O*-methyl-1,4-anhydriitol, and both the molecular ion (*m/e* 204) and the ion from the ring (positions C-1 to C-4) with *m/e* 131 were identified. It follows that in the *N*-acetyl/HF product ribitol is monosubstituted at a terminal position, and consequently the *N*-acetylgalactosaminyl substituent must occupy that position.

The structure of the glucitol derivatives arising from the glucosyl substituents was also established. 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol must represent a glucose residue at a non-reducing terminus, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol represents a glucose substituted at its 1- and 6-positions. These results agree with the presence of a glucosyl(1 $\rightarrow$ 6)glucosyl substituent in the polymer and this is supported by the n.m.r. data.

Although partially methylated derivatives from the amino sugars might have been expected, in fact g.l.c.-mass spectrometry studies of these proved unsatisfactory. They are known to be difficult to investigate by this method and a very complex profile was obtained, probably because of their poor volatility and complex fragmentation in the mass spectrometer arising from their thermal instability.

Methylation of C-teichoic acid itself seemed less

Table 1. *Partially methylated products from neutral sugars obtained from C-teichoic acid and its N-acetyl/HF product*
 Samples were examined on a capillary column of SP1000 resin and the temperature was controlled between 100 and 200°C at 2°C/min. Abbreviations: +++, >30%; ++, 10–30%; +, 5–10%; tr, <5% of the sum of peak heights.

Derivative	Present in:		Relative retention time
	C	N-Ac-HF	
(A) 5- <i>O</i> -Acetyl-1,2,3,4-tetra- <i>O</i> -methylribitol	—	+++	0.39
(B) 5- <i>O</i> -Acetyl-2,3-di- <i>O</i> -methyl-1,4-anhydroribitol	+++	tr	0.56
(C) 1,4-Di- <i>O</i> -acetyl-2,3,5-tri- <i>O</i> -methylribitol	++	tr	0.73
(D) 1,5-Di- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methylribitol	tr	tr	0.76
(E) 1,5-Di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylglucitol	+	++	1.00
(F) 1,5,6-Tri- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methylglucitol	+	+	1.25

Fig. 1. Proton decoupled ^{13}C n.m.r. spectrum of C-teichoic acid at 50.3 MHz in $^2\text{H}_2\text{O}$

attractive, since the presence of hydroxy groups in the vicinity of the phosphodiester linkages might be expected to facilitate cyclization during methylation and consequent fission of diesters. This problem has been long recognized in nucleic acid chemistry. Nevertheless, an unpublished report cited by Conrad (1972) suggested that methylation by the Hakomori (1964) method might leave phosphodiester groups intact. Consequently, C-teichoic acid (7mg) was methylated and products were hydrolysed as described for the *N*-acetyl/HF product. The hydrolysate was treated with alkaline phosphatase (10 μg in 1 ml) under toluene after adjustment to pH 9 with aq. NH_3 . After 16 h at 37°C, all of the phosphorus had been converted into P_i . Reduction and acetylation was followed by examination with g.l.c.-mass spectrometry. Results are given in Table 1; these suggest that fission of phosphodiester linkages might well have occurred, giving anhydroribitol derivatives. However, even in this case the glucitol derivatives were easily detected, suggesting the presence of the glucosyl-(1 $\rightarrow$ 6)glucosyl substituent in the polymer.

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

Proton-decoupled ^{13}C n.m.r. spectra of C-teichoic acid and of its *N*-acetyl/HF product were determined

in $^2\text{H}_2\text{O}$ solution. Fig. 1 shows the spectrum (50.3 MHz) of C-teichoic acid and Table 2 gives the calculated and observed chemical shifts relative to internal tetramethylsilane together with the assignments for both the polymer and the *N*-acetyl/HF product. Chemical-shift values were calculated from the reported values of the individual units by allowing increments for the formation of glycosidic and phosphodiester bonds (see Table 3). The spectrum of the polymer, although not fully interpreted (owing in part to the lack of data for 2-acetamido-4-amino-2,4,6-trideoxyhexose), nevertheless provided important structural information, including positions of glycosylation, anomeric configurations and confirmation of the nature of the amino functions.

Two separate signals were observed at low field (approx. 175 p.p.m.) and high field (approx. 23 p.p.m.) portions of the spectrum of the polymer, readily attributable to the carbonyl and methyl carbons of two acetamido functions. In addition, three signals characteristic of the $-\text{C}-\text{N}-$ group were observed at 49.3, 50.7 and 52.0 p.p.m., that at 49.3 p.p.m. being assigned to C-4 of the diaminotrideoxyhexose on the basis of the difference observed for this region in the spectrum of the repeating unit in which the amino

Table 2. Calculated and observed ¹³C chemical shifts (in p.p.m. from external tetramethylsilane) with assignments for: (i) C-teichoic acid polymer, concentration 100 mg/ml, and (ii) C-teichoic acid repeating unit, concentration 23 mg/ml

The observed chemical shifts are from spectra measured at 22.63 MHz in ²H₂O solutions at 32°C. C, D, G, I and R refer respectively to choline, 2-acetamido-4-amino-2,4,6-trideoxyglucose, N-acetylgalactosamine, isomaltose and ribitol moieties; a, unsubstituted with choline phosphate at C-6 of GalN or with isomaltose at C-3 of diaminotrideoxyhexose; b, substituted with choline phosphate at C-6 of GalN or with isomaltose at C-3 of diaminotrideoxyhexose; ³¹P-¹³C coupling constants were not determined.

(i) C-teichoic acid polymer																				
		I1	G1	I1'	D1	G4	I3	D3b	I5	G5a	I3'	I2	G5b	I5'	I2'	R3	R1	R2	I4	
Assignment	>C=O																			
Calculated	~175	104.7	103.5	99.4	—	78.0	77.7	—	75.9	75.3	75.0	74.9	74.8	73.8	73.3	72.9	72.2	71.9	71.3	
Observed	175.9	175.5	102.5	99.6	94.8	—	78.1	76.8	76.5	—	76.1	75.5	74.0	—	—	—	—	72.6	71.7	
Assignment	I4'	R4	G3	R5	I6	C2	G6b	D5a	D5b	I6'	G6a	C1	(CH ₃) ₃ N ⁺	G2	D2	D4	CH ₃ —C(=O)	D6		
Calculated	71.3	71.3	71.3	67.8	67.4	66.7	65.6	—	—	62.5	62.2	61.2	54.8	53.9	—	—	~23	23.3	16.7	
Observed	70.2	—	—	68.0	—	67.4	56.7	65.0	64.4	62.3	—	60.8	55.9	55.2	52.0	50.7	49.3	23.5	23.3	16.7
(ii) C-teichoic acid N-acetyl/HF product																				
		I1	G1	I1'	D1	G4	I3	D3b	I5	G5	I3'	I2	I5'	I2'	R3					
Assignment	>C=O																			
Calculated	~175	104.7	103.5	99.4	—	78.0	77.7	—	75.9	75.3	75.0	74.9	73.8	73.3	72.9					
Observed	176.4	175.8	175.5	102.4	99.6	94.9	—	78.1	—	76.8	76.4	74.1	—	73.1	—					
Assignment	R4	R1	R2	I4	I4'	G3	I6	D5a	D5b	R5	I6'	G6	D4	G2	D2	CH ₃ —C(=O)	D6			
Calculated	72.9	72.2	71.9	71.3	71.3	71.3	67.4	—	—	63.2	62.5	62.2	—	53.9	—	~23				
Observed	—	71.7	70.7	—	69.6	—	67.6	67.3	64.9	63.6	62.2	62.2	54.3	51.9	50.5	23.1	16.6			

Table 3. ^{13}C n.m.r. chemical shifts (in p.p.m. from tetramethylsilane) of components of *C*-teichoic acid with increments expected for substitution, thereby giving the calculated chemical shift values for (i) the polymer and (ii) the *N*-acetyl/HF product

Increments are given the following values: +7 p.p.m. for the formation of a glycosidic bond at the anomeric carbon, +9 p.p.m. for the formation of a glycosidic bond at other carbons, -1 p.p.m. for the introduction of an adjacent bond (Hamer & Perlin, 1976); +4.6 p.p.m. for the formation of a phosphodiester; -1.6 p.p.m. for adjacent phosphodiester in ribitol (Branefors-Helander *et al.*, 1977); +3.4 p.p.m. for the formation of a phosphodiester at position C-6, -0.5 p.p.m. for an adjacent phosphodiester in hexopyranosides (Bundle *et al.*, 1974). (a) Unsubstituted with choline phosphate at C-6 of GalN; (b) substituted with choline phosphate at C-6 of GalN. Values for the reported shifts were from Usui *et al.* (1973) for isomaltose, Branefors-Helander *et al.* (1977) for ribitol, Bundle *et al.* (1973) for *N*-acetylglucosamine and Johnson & Jankowski (1972) for choline chloride.

Isomaltose (I)											
Carbon	1	2	3	4	5	6	1'	2'	3'	4'	5' 6'
Reported shift	97.7	75.9	77.7	71.3	75.9	67.4	99.4	73.3	75.0	71.3	73.8 62.5
Increment	+7	-1	0	0	0	0	0	0	0	0	0
Calculated shifts	104.7	74.9	77.7	71.3	75.9	67.4	99.4	73.3	75.0	71.3	73.8 62.5
Ribitol (R)											
Carbon	1	2	3	4	5						
Reported shift	63.2	72.9	72.9	72.9	63.2						
Increment	+9	-1	0	-1.6	+4.6						
Calculated shift	72.2	71.9	72.9	71.3	67.8						
Increment	+9	-1	0	0	0						
Calculated shift	72.2	71.9	72.9	72.9	63.2						
<i>N</i>-Acetylgalactosamine (G)											
Carbon	1	2	3	4	5	6	>C=O	CH ₃ -			
Reported shift	96.5	54.9	72.3	69.0	76.3	62.2	175.8	23.4			
Increment	+7	-1	-1	+9	(a)-1 (b)-1.5 (a) 75.3 (b) 74.8	(a) 0 (b)+3.4 (a) 62.2 (b) 65.6	0	0			
Calculated shift	103.5	53.9	71.3	78.0			175.8	23.4			
Increment	+7	-1	-1	+9	-1	0	0	0			
Calculated shift	103.5	53.9	71.3	78.0	75.3	62.2	175.8	23.4			
Choline chloride (C)											
Carbon	1	2	CH ₃								
Reported shift	56.6	68.3	54.8								
Increment	+4.6	-1.6	0								
Calculated shift	61.2	66.7	54.8								

function was acetylated. The highest field signal (16.7 p.p.m.) was clearly due to the 6-deoxy group of the same moiety. In the anomeric region of the spectrum, four separate signals were observed; two of these (105.0 and 99.6 p.p.m.), of equal area, were about 0.6 times that of either of the others (102.5 and 94.8 p.p.m.). (Quantification of components on the basis of peak area may be inaccurate because of variations in saturation effects, nuclear Overhauser enhancement etc.) The former pair of resonances were in close agreement with the expected values (104.7 and 99.4 p.p.m.; Usui *et al.*, 1973) for C-1 and C-1' of a β -isomaltoside but significantly different from those (103.8 and 100.3 p.p.m.) expected for an α -gentiobioside and were assigned accordingly. Of the remaining anomeric signals, that at 102.5 p.p.m. was close to the calculated chemical shift of a β -*N*-acetylgalactosaminyl group, and indeed C-1 of such a group has been observed at this value in several polysaccharides (Hamer & Perlin, 1976;

R. Lively, E. Tarelli & J. Baddiley, unpublished work) The remaining signal in this region was assigned to the anomeric carbon of the diaminotriideoxyhexose, the chemical shift (94.8 p.p.m.) supporting the α -configuration.

Resonances caused by the choline phosphate substituent could be assigned to those observed at 55.2, 55.9, 60.8 and 68.0 p.p.m., in good agreement with the calculated values, the signals in the region of 55 p.p.m. being assigned to the trimethylammonium group, as has been proposed (Bennett & Bishop, 1977). This set of resonances was essentially absent from the spectrum of the *N*-acetyl/HF product.

The intermediate section of the spectrum (70.2–78.1 p.p.m.) was difficult to assign unequivocally to individual carbon atoms; however, it was significant that after the anomeric signals the next highest chemical shift observed for either the polymer or the *N*-acetyl/HF product was that at 78.1 p.p.m. Two important points arise from this. (i) Position C-3 of

the *N*-acetylgalactosamine residue cannot be involved in a glycosidic linkage since, had this been so, a signal for this carbon at chemical shift 81–82 p.p.m. would have been present; this value would be expected both from calculation and from studies with chondroitins A, B and C and similar polysaccharides containing (1→3)-linked β -*N*-acetylgalactosamine residues (Hamer & Perlin, 1976; R. Lively, E. Tarelli & J. Baddiley, unpublished work). Therefore in C-teichoic acid the linkage of the diaminotri-deoxyhexose must be to the C-4 position of *N*-acetylgalactosamine (bearing in mind the results obtained from the periodate oxidation study). (ii) Ribitol is most probably substituted at its C-1 position by *N*-acetylgalactosamine, since if a secondary carbon of the pentitol were glycosylated then a signal for this carbon atom would have been expected at a chemical-shift value above 80 p.p.m., both from calculation and from studies on similar systems (Bränfors-Helander *et al.*, 1977). Thus 4-*O*- β -D-glucopyranosyl-ribitol and related teichoic acids give respectively resonances at 81.7 and 80.1 p.p.m. attributable to position C-4 of the ribitol moiety. The signals observed in the region 70.2–78.1 p.p.m. can then be assigned generally as shown in Table 2.

Structural conclusions from chemical and n.m.r. data

It is possible to propose the following detailed structure for the main chain in C-teichoic acid:

From the chemical evidence already presented, the choline phosphate could occupy either position C-3 or C-6 of *N*-acetylgalactosamine. It is probable, however, that this group is substituted at position C-6 of the amino sugar since a resonance was observed at 65.7 p.p.m. in the spectrum of the polymer, which is in accord with the chemical shift expected for a phosphorylated primary carbon of a hexose (Bundle *et al.*, 1974). Further, a signal at this value was absent from the spectrum of the *N*-acetyl/HF product that, in addition, gave a resonance of increased relative intensity at 62.2 p.p.m. (unsubstituted hydroxymethyl carbon), as would be expected after removal of the phosphodiester.

The remaining feature is the location of the β -isomaltose residue. The low glucose content of the polymer suggests that the residue does not contribute to the main repeating structure, but might be located at one end of the chain. The substitution of isomaltose at a secondary position on ribitol is unlikely for reasons similar to those proposed above in connection with the *N*-acetylgalactosaminylribitol linkage. More-

over, the methylation study indicates that the ribitol in the *N*-acetyl/HF product is only monosubstituted. A more likely possibility is that position C-3 of the chain terminal diaminotri-deoxyhexose is glycosylated with β -isomaltose, although in the absence of ^{13}C chemical-shift data on this residue no more than a tentative suggestion can be made. Nevertheless, if a glycosidic linkage of this nature were present, then the chemical shift of the carbon involved in the linkage would be expected to be sufficiently low (owing to the shielding effect of the adjacent amino function) as to be within the range of chemical shifts observed. A further possibility is that position C-6 of those galactosamine residues that do not have a choline phosphate substituent is substituted by an isomaltose residue. The present data do not enable us to distinguish between these possibilities.

The spectrum of the *N*-acetyl/HF product (Table 2) agrees with the structure proposed for the polymer; the differences are accounted for by the absence of phosphate substituents in the former. It is noteworthy that both give signals corresponding to four anomeric centres in glycosyl substituents; these are the two amino sugars and two glucose residues.

Disregarding the isomaltose residue, and assuming that the stereochemistry of the ribitol phosphate is the same as that in CDP-ribitol, the structure of C-teichoic acid is given in Fig. 2. The chain length of the polymer prepared by us has been examined by measuring the choline phosphate-binding sites with a specific antiserum (Glaudemans *et al.*, 1977). It was concluded that there were 4–5 binding sites/molecule, and as about 66% of the *N*-acetylgalactosamine residues in the polymer are substituted with choline phosphate the chain would comprise about 8 repeating units. From the amount of glucose in the polymer a chain of this length would possess about 1 isomaltose residue. It is possible, however, that in the wall itself the teichoic acid might be larger, as the preparations examined were extracted from walls with trichloroacetic acid and this might well have caused a decrease in chain length through hydrolysis.

There is an apparent anomaly in the molar ratio of glucose/phosphorus, the published values being 0.36:2 (Brundish & Baddiley, 1967), 0.74:2 (Brundish & Baddiley, 1968) and 0.2:2 (the present paper), whereas the ^{13}C -n.m.r. data might seem to suggest a yet higher value for glucose. This last evidence should be discounted, however, as peak area is not necessarily proportional to the amount of that carbon atom present owing to differences in shielding of different carbon atoms. In many determinations of the glucose/phosphorus ratio in several different preparations of C-teichoic acid, and by two different methods, we consistently obtained values of about 0.2:2. The present method for the preparation of C-teichoic acid gives a much purer product than hitherto and this might be reflected in the values obtained for

Fig. 2. Repeating structure in *C-teichoic acid*
The isomaltose substituent is not shown.

glucose. It is also possible that culture conditions might influence the amount of substitution by glucose.

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