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Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures

A reinvestigation of teichoid acid (C polysaccharide)

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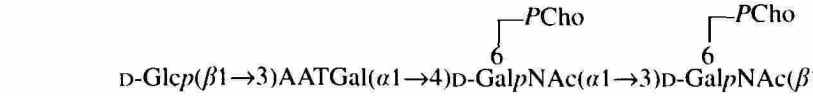
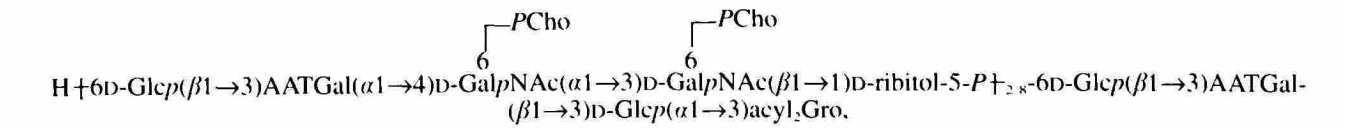
Teichoic acid (C polysaccharide) was extracted and purified from *Streptococcus pneumoniae* R6 with standard procedures except that lipoteichoic acid was extracted first. The dephosphorylated repeating unit was isolated after hydrolysis with 48% (by mass) HF, the bis(phosphocholine)-containing repeating unit was isolated by alkali hydrolysis, anion-exchange chromatography and phosphomonoester cleavage. On the basis of compositional analysis, fast-atom-bombardment mass spectrometry and NMR spectroscopy the following structure is proposed:



where AATGal is 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose. The repeating units are linked to each other by phosphodiester bonds between O5 of the ribitol and O6 of the glucopyranosyl residue of adjacent units. This chain structure is identical with that previously established for pneumococcal lipoteichoic acid [Behr, T., Fischer, W., Peter-Katalinić, J. & Egge, H. (1992) *Eur. J. Biochem. 207*, 1063–1075]. This represents a unique situation because in other Gram-positive bacteria teichoic and lipoteichoic acids are structurally unrelated.

Although the lipoteichoic acid of *Streptococcus pneumoniae* (pneumococcal Forssman antigen) was the first lipocarbohydrate detected in Gram-positive bacteria [1], it was not until recently that the structure was solved and proposed to be [2]:



where AATGal is 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose. It became evident that the repeating unit is similar to that reported for pneumococcal polysaccharide-C substance [3], which in systematic nomenclature has been classified as a ribitol teichoic acid [4]. In the latter, only one phosphocholine residue was observed linked to the ribitol-bound galactosaminyl residue and the amino group of this galactosaminyl residue was non-acetylated. The absolute configuration of the sugar substituents had not been established.

Teichoic acid is covalently linked by an unknown linkage region to the peptidoglycan layer of the cell wall [4, 5]. It participates in the action of the homologous autolytic enzyme, *N*-acetylmuramyl-L-alanine amidase, which is necessary during growth for cell separation [5]. The lipoteichoic acid is hydrophobically anchored in the cytoplasmic membrane [6, 7] by its ester-linked fatty acids [1, 4]. *In vitro* micellar dispersions of lipoteichoic acid proved inhibitory to the autolytic enzyme [8, 9], whereas monomeric forms showed binding rather than inhibitory properties [10]. These observations suggested that *in vivo* lipoteichoic acid might regulate the activity or cellular distribution of the autolytic enzyme. For the interaction with the autolytic enzyme, the phosphocholine residues of both polymers are essential and cannot be replaced by phosphoethanolamine [7, 11].

Owing to their ubiquitous occurrence among pneumococci, teichoic acid and lipoteichoic acid are pneumococcal common antigens, which suggested that antibodies against

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Abbreviations. AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose; COSY, correlation spectroscopy; FAB, fast atom bombardment; HMQC, heteronuclear-multiple-quantum-coherence spectroscopy.

the phosphocholine immunodominant determinant might confer species-specific protection against pneumococcal infections [12]. The phosphocholine component of teichoic and lipoteichoic acid is also responsible for the interaction of pneumococci with C-reactive protein, a major acute-phase reactant of serum [13], and the agglutination of pneumococci by certain myeloma proteins [8, 14].

In pneumococcal infections, teichoic acid and lipoteichoic acid may have a pathophysiological role. They have been reported to act as chemotaxins in the induction of meningeal inflammation [15], to stimulate the production of interleukin-1 in human monocytes [16] and activate the alternative complement pathway [17, 18], whereby lipoteichoic acid becomes active only on the surface of erythrocytes [18].

In the present study, we isolated the teichoic acid from the same strain of *S. pneumoniae* R6 from which we previously extracted lipoteichoic acid. Structural studies revealed that the repeating unit and the complete chain are identical with that of pneumococcal lipoteichoic acid [2]. This observation may explain the high similarity of both polymers in some reactions and suggests that the cause for divergent properties could be that isolated teichoic acid forms monomolecular solutions, whereas isolated lipoteichoic acid adopts micellar supramolecular structures.

EXPERIMENTAL PROCEDURES

Materials and bacteria

DEAE-Sephadex 25-A was purchased from Pharmacia LKB. Lipoteichoic acid from *S. pneumoniae* and its dephosphorylated and phosphorylated repeating unit were obtained as before [2]. D-Quinovosamine was prepared as described [2]. *S. pneumoniae* R6, a derivative of strain R36A (Rockefeller University) was kindly supplied by Dr R. Hakenbeck.

Preparation of teichoic acid

Pneumococci were grown and harvested as described [2], suspended in 0.05 M sodium acetate, pH 4.0 (0.5 g wet mass/ml), and disintegrated by ultrasonication in ice for three 3-min periods (Branson sonifier cell disruptor B15, 20 Hz, 150 W). After adjusting the pH to 5.5 with 1 M $NaHCO_3$, 2 vol. MeOH and 1 vol. $CHCl_3$ were added and the mixture was stirred at room temperature for 3 h. After centrifugation, the supernatant was withdrawn and the pellet was resuspended in 4 vol. 0.05 M sodium acetate pH 5.5/MeOH/$CHCl_3$ (0.8:2.0:1.0, by vol.), stirred overnight and centrifuged. The supernatants contained total lipoteichoic acid and membrane lipids [2]. The pellet was washed with MeOH and acetone, and air dried. The dry material was extracted five times with 75 ml 10% (mass/vol.) trichloroacetic acid at 4°C with stirring for 24 h. After centrifugation (3000 × *g*, 20 min) the supernatant was withdrawn, filtered through celite and fractionated with ethanol and acetone as described by Baddiley and Brundish [4]. The collected material (2.0 g), containing 2.5 mmol nucleic-acid phosphorus and 3.5 mmol total phosphorus, was dissolved in 50 ml 0.1 M sodium acetate, pH 5.0, and treated with nucleases as described [19]. After phenol/water (2:3, by vol.) extraction and dialysis against water, the mixture contained 0.3 mmol nucleic-acid phosphorus and 1.3 mmol total phosphorus. Purification of the teichoic acid was achieved by chromatography on DEAE-Sephadex.

Analytical procedures

Carbohydrate [20], choline [21], glucose [22], hexosamine [23], nucleic acids [24], periodate [25], formic acid [26], formaldehyde [27], and phosphorus [28] were measured as described in each reference. Ribitol and anhydroribitol were quantified as acetates by GLC using mannitol as internal standard [2]. Galactosamine and glucosamine were identified by HPLC. For compositional analysis, teichoic acid was *N*-acetylated [29], dephosphorylated with 48% (by mass) aqueous HF (2°C, 36 h) and, after drying over KOH *in vacuo* (2°C), hydrolysed with 2 M HCl (100°C, 2.5 h). Glucose was enzymically identified [22] and quantified with an anthrone procedure [2, 20]. For rapid determination of choline, hydrolysis with 48% (by mass) HF was performed at room temperature for 3 h. For quantification, amino sugars were released from the HF hydrolysate by hydrolysis with 4 M HCl at 100°C for 18 h. After derivatisation in a mixture of 9-fluorenylmethyl chloroformate in acetone (1 mg/ml)/0.3 M lithium borate, pH 9.3 (1:1, by vol.), the samples were analysed by HPLC using a LiChrosphere RP 18e column (4 mm × 250 mm), which was eluted at a flow rate of 1.5 ml/min with a linear gradient of 20–50% (by vol.) acetonitrile in 80 mM acetic acid. Amino sugars were detected by fluorescence (excitation at 260 nm, emission at 310 nm) and identified as anomeric peaks with retention times of 9.55 and 10.42 for galactosamine, 9.79 and 10.42 for glucosamine, and 10.93 and 12.13 min for muramic acid. Amino acids were quantified after acid hydrolysis (6 M HCl, 155°C, 1 h) using HPLC [30].

GLC was performed as described [2]. For TLC, silica-gel plates (Merck 60) were used with the following solvents: (A) propan-1-ol/25% (by mass) ammonia/water (6:3:1, by vol.); (B) $CHCl_3$/MeOH (9:1, by vol.). Carbohydrates were visualised with 1-naphthol/H_2SO_4 [31], peracetylated oligosaccharides were visualised with iodine vapor.

N-Acetylation

N-Acetylation of teichoic acid was performed as described [29].

Determination of the absolute configuration of the sugar components

Teichoic acid (fraction I, 3 μmol phosphorus) was deaminated with HNO_2 [2], hydrolysed (2 M HCl, 100°C, 3 h) and the hydrolysate (100 μl) was neutralized with Ag_2CO_3. Water (400 μl) and acetic anhydride (15 μl) were added, and the mixture was allowed to stand for 3 h. After centrifugation, the supernatant was passed through celite overlaid with cation-exchange resin H^+, and taken to dryness. After addition of (*R*)-2-butanol or (*S*)-2-butanol (0.2 ml) and acetyl chloride (0.025 ml), samples and standards (*N*-acetyl-D-galactosamine, *N*-acetyl-D-quinovosamine, D-Glc) were heated (100°C, 3 h), evaporated and acetylated (0.1 ml acetic anhydride, 0.025 ml pyridine; 100°C, 1 h). The products were partitioned between $CHCl_3$ and water, and the $CHCl_3$ layer was analysed by GLC (HP5 fused silica-gel capillary column, 20 m, inner diameter 0.25 mm, film thickness 0.25 μm, operated at 185°C).

Oxidation of teichoic acid with $NaIO_4$ and β elimination

N-acetylated teichoic acid (fraction I, 8 μmol phosphorus) was oxidised with 0.05 M $NaIO_4$ in 0.05 M sodium

acetate, pH 4.2 (0.5 ml), at 5°C in the dark. The reaction was followed spectrophotometrically [25]. At different time intervals samples were withdrawn for the determination of formic acid [26] and formaldehyde [27]. After incubation for 18 h, periodate was reduced with ethylene glycol and the pH was adjusted to 7 with NaOH. After addition of 0.05 M sodium borate, pH 9.5, and alkaline phosphatase (5 U), the mixture was incubated overnight at 37°C and analysed for inorganic phosphate and total phosphorus.

Fast-atom-bombardment mass spectrometry

FAB-MS was performed in positive-ion mode as reported previously [32].

NMR spectroscopy

NMR spectra were determined using a Bruker AMX 500 spectrometer and standard acquisition software. Samples were repeatedly exchanged against 99.96% $[^2H]_2O$ by freeze drying (at least three times). For measurement, the sample was dissolved in 0.5 ml 99.96% $[^2H]_2O$ containing 5% [$^2H_6$]acetone as a lock signal and a small amount of sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propionate as reference. Spectra were recorded at 30°C.

$^1H^1H$-correlation-spectroscopy experiments ($^1H^1H$-COSY) were recorded with a 2K block size in F_2 and 512 increments, each with 96 scans in F_1, followed by sine-squared multiplication without phase shifting, zero filling of 2K*2K matrix and finally matrix symmetrization.

Two-dimensional NOE spectroscopy experiments were performed using the pulse sequence contained in the standard software, with a mixing time of 250 ms. A block size of 2K in F_2 and 512 increments each with 256 scans in F_1 was used in time-proportional-phase-increment mode, followed by zero filling of the 2K*2K matrix, processing with a $\pi/2$ phase-shifted sine filter and phase correction in F_2 and F_1.

^{31}P-NMR spectra (202.459 MHz) were recorded using a sweep width of 614 Hz. For calibration, 85% H_3PO_4 was used as external reference. $^{31}P^1H$-COSY experiments were performed using the pulse program for CH-COSY optimised for J_{PH} 7 Hz. A block size of 1K in F_2 and 512 increments, each with 128 scans in F_1, was used, followed by zero filling of the 1K*1K matrix, processing with a $\pi/2$ phase-shifted filter.

The $^1H^{13}C$-heteronuclear-multiple-quantum coherence (HMQC) spectra were recorded using a pulse sequence with bilinear-rotation-decoupling pulse to suppress signals from protons connected to ^{12}C [33]. A block size of 2K in F_2 and 1024 increments, each with 32 scans in F_1, were used in time-proportional-phase-increment mode, followed by zero filling of the 2K*2K matrix, processing with a $\pi/2$ phase-shifted sine filter and phase correction in F_2 and F_1.

RESULTS

Isolation of teichoic acid and compositional analysis

Teichoic acid was released, using trichloroacetic acid [4], from disintegrated cells of *S. pneumoniae* R6 from which lipoteichoic acid and lipids had previously been extracted [2]. The crude preparation was fractionated with ethanol and acetone [4], treated with nucleases [19] and, after dialysis, chromatographed on DEAE-Sephadex. The teichoic acid eluted as non-homogeneous material and three fractions were collected (Fig. 1). Analysis of fraction I and II indicated choline/total phosphorus in molar ratios of 0.65 and 0.59, respectively. Ribitol, D-Glc, galactosamine and non-phosphocholine phosphorus were found in molar ratios of 1:0.9:2.0:1 and 0.75:0.8:1.7:1 in fractions I and II, respectively. The presence in both fractions of 2-acetamido-4-amino-2,4,6-trideoxygalactose (AATGal), a well established component of pneumococcal teichoic acid [3, 4], was indicated by the formation of quinovosamine after deamination with HNO_2 and subsequent acid hydrolysis [2]. Since in previous studies, the absolute configuration of the sugar components had not been addressed [3], we converted glucose and the *N*-acetylated hexosamines into (*S*)- and (*R*)-2-butyl glycosides [34] and analysed the acetylated derivatives by GLC [35]. A comparison with the retention times of standards indicated D-Glc, *N*-acetyl-D-glucosamine and D-quinovosamine, the presence of the latter establishing the D configuration for the AATGal residue.

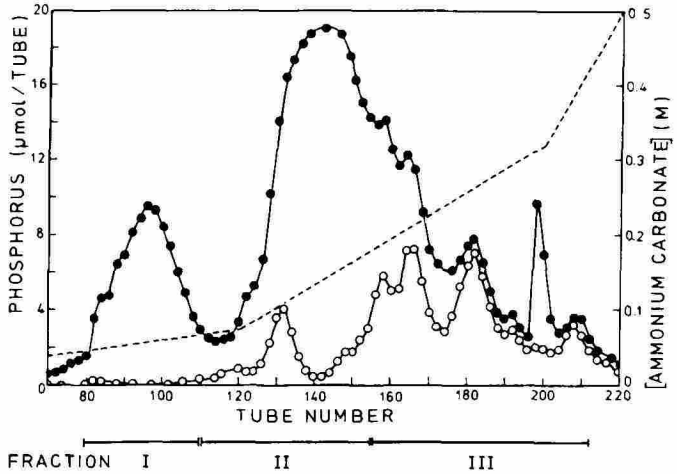

Fig. 1. Purification of teichoic acid by anion-exchange chromatography. A crude preparation containing 0.3 mmol nulceic-acid phosphorus and 1.3 mmol total phosphorus, was dissolved in water and pH adjusted with NH_4OH to 9.3. The mixture was applied to a column (0.8 cm × 16 cm) of DEAE-Sephadex A-25 which had been washed with 0.5 M and 0.05 M ammonium carbonate, pH 8.2, and water (25 ml). The column was eluted with ammonium carbonate, pH 8.4, as indicated in the figure. The flow rate was 60 ml/h. Samples of 4 ml were collected and analysed for phosphorus (●) and nucleic acid phosphorus (○). The salt concentration in the effluent was measured conductometrically. The effluent was collected in three fractions (I–III) which contained 193 (I), 525 (II) and 404 (III) µmol phosphorus. Fraction III was not analysed further. Ammonium carbonate concentration is shown (———).

Fraction I was essentially free of cell-wall material. Fraction II contained, as shown after acid hydrolysis, components of peptidoglycan [36], namely alanine, glutamic acid, lysine, *N*-acetylglucosamine and muramic acid in molar ratios to phosphorus of 0.15, 0.07, 0.04, 0.27 and 0.27, respectively. Phosphomonoester was present in both fractions in a molar ratio to total phosphorus of 0.04.

The dephosphorylated repeating unit

Dephosphorylation with 48% (by mass) HF (2°C, 36 h) of fraction I and II resulted in partial cleavage of the labile *N*-acetyl-β-galactosaminyl bond to ribitol [2, 3]; approximately 50% hexosamine could be detected with the Elson-Morgan reaction [23] without further hydrolysis, and an equimolar

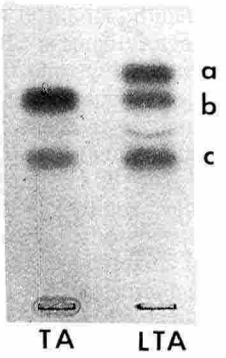

Fig. 2. Dephosphorylation products of teichoic acid (TA) and deacylated lipoteichoic acid (LTA). Teichoic acid (fraction I and II, 1:1 by vol.) and lipoteichoici acid were dephosphorylated with HF. TLC was performed using solvent A with visualisation using 1-naphthol/H_2SO_4. Identification was as described in [2]: a, D-Glc*p*(β1→3)AATGal(β1→3)D-Glc*p*(α1→3)Gro (deacylated lipid anchor); b, D-Glc*p*(β1→3)AATGal(α1→4)D-Gal*p*NAc(α1→3)D-Gal*p*NAc; c, D-Glc*p*(β1→3)AATGal(α1→4)D-Gal*p*NAc(α1→3)D-Gal*p*NAc(β1→ 1)D-ribitol.

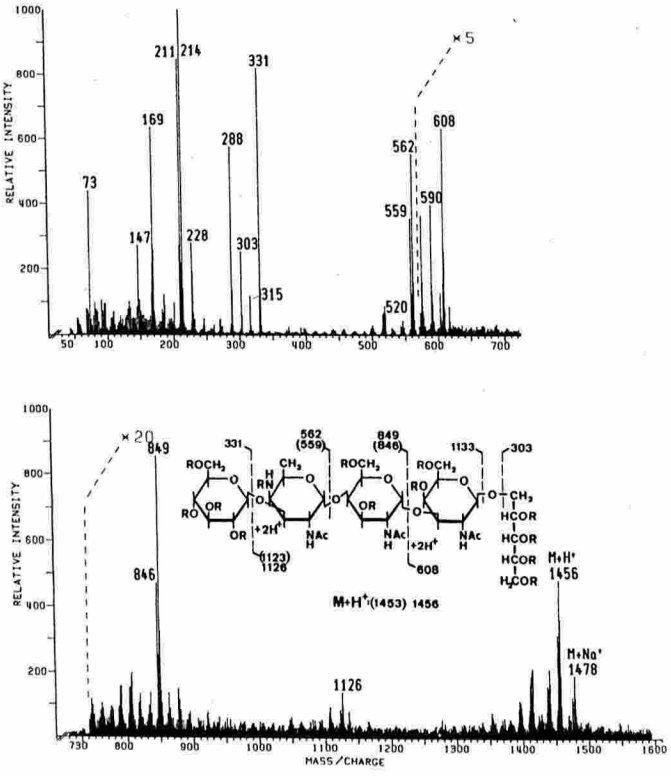

Fig. 3. Structure of the dephosphorylated pentamer (R = H) and positive-ion FAB mass spectrum of the peracetylated derivative (R = Ac). Peracetylation was preceded by *N*-acetylation with a 1:2 mixture of $(CH_3CO)_2O$ and $(C^2H_3CO)_2O$. Values and values in brackets refer to analogues carrying *N*-[^{2}H]acetyl and *N*-acetyl groups, respectively.

amount of ribitol was detected by GLC. The same amount of galactosamine was detected when *N*-acetylation was omitted during the assay procedure, which indicates that the ribitol-linked galactosamine was *N*-acetylated in the native state. For the HF hydrolysate, TLC revealed two oligosaccharides which chromatographed with the same R_f values as the oligosaccharides obtained from pneumococcal lipoteichoic acid (Fig. 2).

The oligosaccharides were treated with $NaBH_4$ [2] and *N*-acetylated, using a 1:2 mixture of $(CH_3CO)_2O$ and $(C^2H_3CO)_2O$, to locate free amino groups by FAB-MS. After subsequent peracetylation with $(CH_3CO)_2O$, the peracetylated pentamer repeat and its peracetylated tetritol derivative were isolated by preparative TLC (solvent B) as described [2].

The purified pentamer contained D-Glc, *N*-acetylgalactosamine and ribitol in molar ratios of 0.85:1.9:1. The positive-ion FAB mass spectrum of its peracetylated derivative is shown in Fig. 3. The double signal of the molecular ion [M + H$^+$] at *m/z* 1456 (*N*-[^{2}H] 1453), indicates the presence of a single *N*[^{2}H]acetyl group. The sugar sequence from the non-reducing terminus, is documented by the ions at *m/z* 331→169 (Glc$^+$), 562 (559)→214 (211) (Glc-AATGal$^+$), and 849 (846)→288 (Glc-AATGal-GalNAc$^+$), and from the non-reducing end by *m/z* 303 (ribitol$^+$), 608 (ribitol-GalNAc-OH) · H$^+$, 590 (ribitol-GalNAc$^+$), and 1126 (1123) (ribitol-GalNAc-GalNAc-AATGal-OH) · H$^+$. The daughter ion at *m/z* 288, of high abundance and free of ^{2}H label, must arise from the trisaccharide *m/z* 849 (846) by cleavage of the α1→4 glycosidic bond and reprotonation. The fragments at *m/z* 562 (559) and 214 (211) locate the single *N*-[^{2}H]acetyl group unequivocally at the AATGal residue. Accordingly, the fragments at *m/z* 288 and *m/z* 608 confirm that both galactosaminyl residues are in the native state *N*-acetylated.

The bis(phosphocholine)-containing repeating unit

Samples of fractions I and II were treated with 0.3 M NaOH at 100°C for 2 h to hydrolyse the phosphodiester bonds between the repeating units, which occurs through cyclic phosphodiester intermediates at O4 and O5 of the ribitol residues [2]. As previously observed with lipoteichoic acid [2], approximately 30% total phosphorus was converted into phosphomonoester and approximately 90% of choline remained unchanged and phosphodiester-linked to the repeat. After passing the hydrolysates through cation-exchange resin (NH_4 form) and subsequent *N*-acetylation, TLC revealed the same hydrolysis products as obtained from lipoteichoic acid, except that the deacylated lipid anchor was absent (Fig. 4). The alkali-hydrolysis products were separated by column chromatography using DEAE-Sephadex A-25 as described [2]. Measurements of choline/phosphate molar ratios throughout the elution profile (data not shown) indicated that phosphocholine-free and putative monophosphocholine-containing derivatives (Fig. 4) were 2% and 8% of the bis(phosphocholine)-containing pentamer.

Column fractions containing the bis(phosphocholine)-containing pentamer (62% total phosphorus) were collected, treated with phosphomonoesterase and purified by ultrafiltration and anion exchange chromatography [2]. The purified repeat contained choline, ribitol, glucose, galactosamine and phosphorus in molar ratios of approximately 2:1:1:2:2. Glucosamine and amino acids were not detected.

The results of NMR analyses for the bis(phosphocholine)-containing pentamer are summarised in Table 1. Using 500-MHz ^{1}H^{1}H-COSY (Fig. 5), it was possible to assign unequivocally all ^{1}H resonances for the four hexopyranosyl residues. Signals for α-anomeric protons are observed at 4.99 ppm ($^3J_{HH}$, 4.0 Hz, B) and 5.21 ppm ($^3J_{HH}$, 3.9 Hz, C) and for the β-anomeric protons at 4.61 ppm ($^3J_{HH}$, 7.8 Hz, A) and 4.67 ppm ($^3J_{HH}$, 8.6 Hz, D). The 6-methyl group resonance at 1.07 ppm, belonging to AATGal (Fig. 6, B), with its

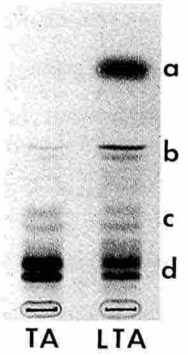

Fig. 4. Alkali-hydrolysis products of teichoic acid (TA) and lipoteichoic acid (LTA) after desalting and *N*-acetylation. The alkali hydrolysate of teichoic acid was obtained from fraction I and II (1:1, by vol., Fig. 1). TLC and visualisation were performed as in Fig. 2. Identification was as described in [2]: a, deacylated-lipid anchor; b–d, phosphomonoester derivatives of the pentamer repeating unit (b), its bis(phosphocholine) derivative (d) and presumably its monophosphocholine derivative (c). Double spots b–d contain isomers which were formed from 4,5-phosphodiester intermediates and have the phosphomonoester at O4 and O5 of the ribitol residue. Analogues are also present in which the choline residues were converted into the vinyl derivatives. They are readily separated from the fragments with intact choline residues by chromatography on DEAE-Sephadex [2].

associated anomeric proton at 4.99 ppm, differentiated the two α-galactopyranosyl spin systems. The two β-anomeric residues were distinguishable by the absence of the glycosylation shift and the 9 Hz $^3J_{HH}$ coupling of H2, H3, H4, H5 protons for the glucopyranose ring. Assignment of the resonances for ribitol (Fig. 6, E) was achieved by comparison with the spectrum of free ribitol 5-phosphate (data not shown).

^{13}C chemical shifts were obtained and assigned using a ^{1}H^{13}C-HMQC experiment. The glycosidic substitution of residues B, C and D (Fig. 6) at O3, O4 and O3, respectively, and of ribitol (Fig. 6, E) at O1 became evident from both ^{1}H and ^{13}C chemical shifts. The monosaccharide sequence depicted in Fig. 6 is derived from the cross-peaks observed in the two-dimensional NOE spectrum (Fig. 5). The anomeric proton of the β-glucopyranosyl residue (Fig. 6, A) shows a dipolar correlation to H3 of AATGal (Fig. 6B), the α-anomeric proton of the AATGal residue shows a dipolar coupling to H4 and H5 of α-GalNAc (Fig. 6, C) and the α-anomeric proton of α-GalNAc shows a dipolar correlation to H3 and H4 of β-GalNAc (Fig. 6, D).

The location of the two phosphocholine residues at O6 of the two *N*-acetylgalactosaminyl residues (Fig. 6, C and D) was possible using ^{31}P^{1}H-COSY (data not shown).

The linkage between the repeating units

For analysis of the linkage between the repeating units, *N*-acetylated teichoic acid (fraction I) was oxidized with $NaIO_4$ as described in Experimental procedures. After 4 h, when the oxidation was complete, approximately 4 mol $NaIO_4$ had been consumed/mol non-choline-phosphate phosphorus with the concomitant formation of 2 mol formic acid and negligible amounts of formaldehyde. Subsequent β elimination at pH 9.5 converted the non-choline-phosphate phosphorus completely into phosphomonoester. These results indicate a phosphodiester bond between O5 of ribitol (Fig. 6, E) and O6 of the glucopyranosyl residue (Fig. 6, A) of adjacent units. 4-*N*-Acetylated teichoic acid (fraction I) was also studied by ^{31}P^{1}H-COSY. The results (data not shown) independently proved the presence of phosphodiester bonds between O5 of ribitol and O6 of glucopyranosyl residues.

Table 1. Chemical-shift assignment for the bis(phosphocholine)-containing pentamer repeating unit. For shift assignments see text, for designation of the residues see Fig. 6.

Assignment	Chemical shift for residue				
	A	B	C	D	E
	ppm				
H1	4.61	4.99	5.21	4.67	3.93; 4.00
H2	3.28	4.38	4.38	4.16	4.00
H3	3.51	4.33	3.95	3.92	3.75
H4	3.37	4.55	4.13	4.23	3.83
H5	3.46	4.68	4.08	3.88	3.68; 3.83
H6a	3.70	1.07 (CH_3)	4.07	4.11	
H6b	3.92				
C1	104.49	98.79	93.63	101.56	70.98
C2	73.31	49.38	49.72	50.89	70.87
C3	76.06	75.55	67.22	74.90	72.31
C4	69.67	53.63	76.71	63.44	72.24
C5	76.05	66.48	71.09	73.80	62.81
C6	61.25	15.88	64.00	64.68	
PO_4			−0.461	−0.031	
H, choline CH_3N			3.30	3.30	
H, choline OCH_2			4.35	4.39	
H, choline NCH_2			3.74	3.75	
C, choline CH_3N			54.26[a]		
C, choline OCH_2			59.75[a]		
C, choline NCH_2			66.30[a]		

[a] The ^{13}C-signals of the phosphocholine residues attached to rings C and D (Fig. 6) cannot be distinguished.

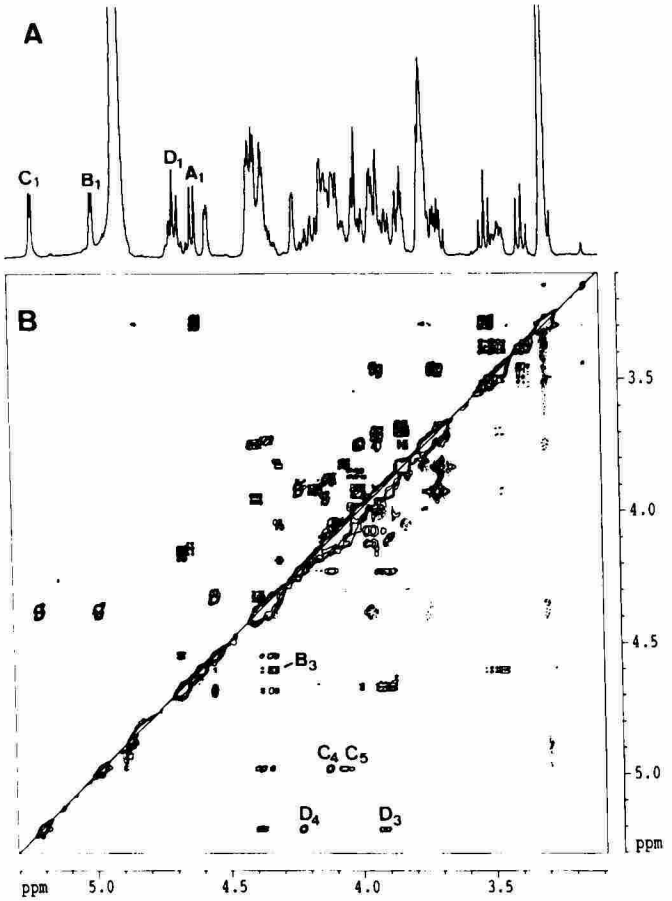

Fig. 5. Partial one-dimensional 500-MHz ^{1}H-NMR spectrum (A), ^{1}H^{1}H-COSY spectrum (B, upper left) and ^{1}H^{1}H-NOE spectrum (B, lower right) of the bis(phosphocholine)-containing pentamer. For structure and designation of the hexosyl residues (A–D) see Fig. 6.

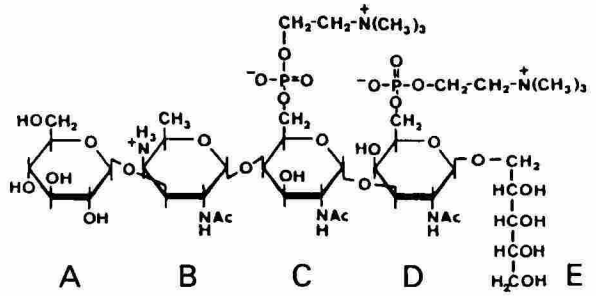

Fig. 6. Structure of the bis(phosphocholine)-containing pentamer repeating unit. In teichoic acid, the repeating units are linked by phosphodiester bonds between O5 of ribitol and O6 of the glucopyranosyl residue of adjacent units [3]. A, D-Glc*p*; B, AATGal; C, D-Gal*p*NAc; D, D-Gal*p*NAc; E, D-ribitol.

DISCUSSION

The teichoic acid of *S. pneumoniae* was extracted by a standard procedure [4], with the modification that lipoteichoic acid had been previously extracted by a Bligh-Dyer monophase system [2]. The purified teichoic acid was free of detectable amounts of lipoteichoic acid, as documented by the absence of the deacylated-lipid anchor among the hydrolysis products obtained with HF (Fig. 2) and alkali treatments (Fig. 4). The fractionation of the extracted material into two peaks by anion-exchange chromatography reflects differences in particle size because fraction II contained peptidoglycan components. Both fractions yielded, after hydrolysis with HF and alkali, identical repeating units of teichoic acid.

Several studies have been devoted to the structure of pneumococcal teichoic acid [3, 4, 29, 37]. The stereochemical configuration of the sugar components had, however, not been clarified and the most recent published structure [3] deviates from the present one (Fig. 6) in two ways. We found two phosphocholine residues/repeating unit and both galactosaminyl residues were *N*-acetylated, whereas in the previous structure there was only one phsophocholine residue attached to the ribitol-linked galactosaminyl residue and the latter was found to be non-acetylated. The reason for these differences is unknown. Most of the previously analysed samples were prepared from the culture medium rather than from cells [3] and might have been exposed to degradative enzymes. Notable in this context is a phosphodiesterase of *S. pneumoniae* which has the capacity of releasing a limited fraction of phosphocholine residues from teichoic acid [38].

The differences between the structures may be functionally important because conformational studies into pneumococcal lipoteichoic acid suggests a considerable influence of the two phosphocholine residues and the intrachain charges on the conformation of the chain (Klein, R. A., Hartmann, R., Egge, H., Behr, T. and Fischer, W., unpublished results). Conformation and charges may be relevant for the interaction of the teichoic acid with the various proteins noted in the introductory remarks.

Compositional, sequence and linkage analyses indicate that the repeating unit of pneumococcal teichoic acid is identical with that of lipoteichoic acid [2]. The connection of the repeating units in both polymers, by phosphodiester bonds between O6 of the glycosyl residue and O5 of the ribitol residues, completes the evidence for identical chain structures. This situation is unique among Gram-positive bacteria because usually teichoic acids and lipoteichoic acids have different structures [39–41]. Even in those organisms in which both teichoic acid and lipoteichoic acid possess poly(glycerophosphate) chains, the glycerophosphate residues are enantiomers and, therefore, are of different metabolic origin (for references see [42]). In an earlier search for a metabolic relationship between teichoic acid and lipoteichoic acid, pneumococci were pulse-labelled with [^{3}H]choline and the distribution of the radiolabel between both polymers was determined in a subsequent chase period [43]. The invariable distribution of the label was taken as evidence that the completed polymers are separate metabolic entities. This result does not exclude, however, the possibility that the two polymers might share a common biosynthetic precursor, e.g. an activated, possibly lipid linked, form of the bis(phosphocholine) pentamer.

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