Structure of Intracellular Teichoic Acids from Group D Streptococci

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Although considerable attention has been given to the isolation and structure of glycerol teichoic acid and ribitol teichoic acid from bacterial walls (for review see Baddiley, 1962), those that occur in underlying regions of the cell appear to be much more widespread (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley & Davison, 1961; A. L. Davison, unpublished work). They have been described for convenience as 'intracellular' teichoic acids, but in the two cases so far studied it is shown that they are probably located in the region between the wall and the protoplast membrane (Hay, Wicken & Baddiley, 1963). They are glycerol phosphate polymers, usually bearing sugar residues, and D-alanine ester groups are always present. Despite the relatively small amounts found in bacteria, and the considerable difficulties encountered during their isolation and purification, structures have been proposed for two found in lactobacilli (Kelemen & Baddiley, 1961; Critchley, Archibald & Baddiley, 1962).

The detailed chemical structure of teichoic acids is of particular interest because of their serological reactions (Baddiley & Davison, 1961; Haukenes, Ellwood, Baddiley & Oeding, 1961; Sanderson, Juergens & Strominger, 1961). In streptococci the group D antigen has been identified as the intracellular teichoic acid by Wicken, Elliott & Baddiley (1963), who suggested that differences in the serological behaviour of group D antigens from different strains of organism might be a consequence of differences in their chemical composition. Particular attention was given to *Streptococcus faecalis* strain 39 and a *Streptococcus* strain 8191 (N.C.I.B.) which is intermediate in character between *S. faecium* and *S. durans*; both organisms are in serological group D and the intracellular teichoic acids are glycerol phosphate polymers containing glucose.

The present work shows that the intracellular teichoic acid from strain 39 is a glycerol phosphate polymer with kojibiose [α -D-glucopyranosyl-(1 $\rightarrow$ 2)-O-D-glucose] in α -glycosidic linkage to glycerol, whereas that from strain 8191 has kojitriose [α -D-glucopyranosyl-(1 $\rightarrow$ 2)-O- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-O-D-glucose] in α -glycosidic linkage to glycerol. Both possess D-alanine ester residues which, unlike

those in other teichoic acids, are attached to sugar hydroxyl groups; moreover, that from strain 8191 also contains a small proportion of L-lysine ester groups. The high sugar content, the presence of amino acid esters other than D-alanine and the attachment of ester residues to the sugar are features that have not previously been observed in teichoic acids. The association between intracellular teichoic acid and ribosomal RNA in these and other organisms is discussed.

MATERIALS AND METHODS

Paper chromatography. The following solvent systems were used: *A*, propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949), Whatman no. 4, ascending; *B*, butan-1-ol-ethanol-water-aq. ammonia (sp.gr. 0.88) (40:10:49:1, by vol.) (Foster, Horton & Stacey, 1957), Whatman no. 4, descending; *C*, pyridine-ethyl acetate-water-acetic acid (5:5:3:1, by vol.) (Fischer & Nebel, 1955), Whatman no. 1, descending; *D*, butan-1-ol-methanol-water (2:2:1, by vol.) (Hardy, Holland & Nayler, 1955), Whatman no. 1, descending; *E*, phenol saturated with water in an atmosphere containing ammonia (Consden, Gordon & Martin, 1944), Whatman no. 1, ascending; *F*, phenol saturated with water in an atmosphere containing HCN (Consden *et al.* 1944), Whatman no. 1, ascending; *G*, butan-2-ol-2-methylpropan-2-ol-butan-2-one-water (4:4:8:5, by vol.), with aq. ammonia (sp.gr. 0.88) added to give a final concentration of 5% (v/v); *H*, butan-1-ol-pyridine-benzene-water (5:5:3:1, by vol.), upper layer (Bull, 1962), Whatman no. 4, descending; *I*, propan-1-ol-ethyl acetate-water (7:1:2, by vol.) (Baer & Bull, 1953), Whatman no. 4, descending; *J*, ethyl acetate-pyridine-water (10:4:3, by vol.) (Goldstein & Whelan, 1962), Whatman no. 1, descending.

Whatman no. 4 paper was washed before use with 2N acetic acid solution and then water. The following spray reagents were used where appropriate: periodate-Schiff reagent for polyols and glycosides (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956); perchloric acid-molybdate for phosphates (Hanes & Isherwood, 1949); alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) and aniline phthalate (Partridge, 1949) for sugars; ninhydrin for amino acids (Consden & Gordon, 1948). Other spray reagents are described in the text.

Analytical methods. Inorganic and total phosphorus were determined by the method of Chen, Toribara & Warner (1956), alanine by Rosen's (1957) procedure after neutralization of acid hydrolysates to pH 7 with sodium hydroxide solution, and glucose on similar neutralized hydrolysates

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by the method of Park & Johnson (1949) or directly on the polymer by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). The production of free glucose was maximal after 3 hr. in 2*N*-hydrochloric acid at 100°; values are corrected for the destruction of glucose under comparable conditions. Nucleic acid was determined from the extinction at 260 m μ (an extinction of 2.8 is given by 100 μ g. of nucleic acid/ml.).

Growth of organisms and isolation of teichoic acids. The streptococcal strains 8191 and 39 were grown and intracellular teichoic acids isolated by the use of Sephadex columns as described by Wicken *et al.* (1963). That from strain 39 required no further purification, but that from strain 8191 sometimes contained ribitol teichoic acid from the walls. It was purified further by chromatography on diethylaminoethylcellulose (DEAE-cellulose) as described below.

Chromatography on diethylaminoethylcellulose. The impure teichoic acid from strain 8191 contained about 30% of RNA. A sample (420 mg.) was dissolved in 2*N*-ammonia solution (10 ml.) and, after 5 hr. at room temperature, excess of ammonia was removed *in vacuo*. The solution was passed through a column (1 cm. $\times$ 10 cm.) of Dowex 50 (H⁺ form) resin, and the eluate was neutralized with ammonia and then freeze-dried. Amino acids and their amides were eluted with *N*-ammonia solution; they were recovered by freeze-drying and examined by the methods described below.

Material (380 mg.) from which amino acids had been removed was dissolved in water (15 ml.) and dialysed (Visking membrane) against distilled water (3 $\times$ 1.5 l. during 36 hr.). Teichoic acid (290 mg.) was recovered by freeze-drying; none was detected in the diffusate. The column was prepared from a thin slurry of DEAE-cellulose (12 g.) activated by washing with 0.1*N*-sodium hydroxide and then 0.1*M*-sodium hydrogen carbonate. The cellulose was extruded from the column and fine material removed by repeated decantation with distilled water. The column (2 cm. $\times$ 30 cm.) was re-formed from an aqueous slurry of the cellulose and washed with water until no further contraction occurred. The impure ammonium salts (290 mg.) in water (5 ml.) were applied to the column, followed by water (2 $\times$ 5 ml.). Linear-gradient elution was effected with 0.6*M*-ammonium carbonate solution (2 l.) in the reservoir and water (2 l.) in the mixing vessel, fractions (10.4 ml.) being collected automatically. Total phosphate and the extinction at 260 m μ were determined on 0.1 ml. samples from every third tube. From an elution diagram (not shown), samples were combined in the following manner: fraction A (tubes 1–60) containing mainly nucleotides; fraction B (tubes 61–104) (53 mg.); fraction C (tubes 105–150) (152 mg.); fraction D (tubes 151–200) (30 mg.). The fractions were freeze-dried and ammonium carbonate was removed by repeated evaporation from small amounts of water at 30°.

Samples (2 mg.) of each fraction were hydrolysed in 2*N*-hydrochloric acid at 100° for 3 hr. and examined by paper chromatography. Glycerol and its phosphates were detected in fractions B, C and D, but anhydrosorbitol was found only in fraction B. Fraction C was purified further by chromatography on a column (2.5 cm. $\times$ 56 cm.) of Sephadex G75 as described by Wicken *et al.* (1962); this gave glycerol teichoic acid (110 mg.) containing less than 0.24% of material that absorbed light at 260 m μ . This material corresponded on

analysis to that obtained previously by repeated chromatography on Sephadex G75.

Extensive purification of the teichoic acid from strain 39 was not necessary, as in this case no contamination with wall material was observed. The material from this strain was prepared by the method described by Wicken *et al.* (1963).

RESULTS

Complexes between teichoic acid and ribonucleic acid

Material from Sephadex column. The first fraction from Sephadex chromatography of material from strain 8191 (Wicken *et al.* 1963) was dissolved in water (15 ml.) and the opalescent solution centrifuged for 1 hr. at 100 000*g* in a Spinco centrifuge. The supernatant was decanted and the sediment was resuspended in water and centrifuged as before. The supernatants were combined and freeze-dried; the dried sediment (3 mg.) and supernatant (21 mg.) were analysed for phosphorus and nucleic acid. Both contained teichoic acid and RNA; the sediment contained 1.48 moles of teichoic acid for each statistical tetranucleotide.

Preparation and study of ribosomes. Strain 8191 was grown in the usual manner for 16.5 hr. at 37°. Cells (74 g.) were harvested in a Sharples refrigerated centrifuge and washed twice with 0.01*M*-phosphate buffer, pH 7.6, containing magnesium sulphate (5 mM) and sodium chloride (0.05*M*). Disruption in the same buffer was effected with an International shaker head. A 25% suspension was completely disrupted in 30 min. at 1400 rev./min. Walls were centrifuged at 15 000*g* in an International refrigerated centrifuge for 20 min. Ribosomal material possibly containing membrane fragments was then collected at 145 000*g* in a Spinco centrifuge during 90 min.; this was stored frozen.

The ribosomal gel was suspended in the above buffer (50 ml.) and the pH adjusted to 5.6 with dilute hydrochloric acid. The resulting precipitate (92 mg.) was collected at 2000*g* in a refrigerated centrifuge, washed once with buffer at pH 5.6, twice with ethanol and then once with ether, and dried. The supernatant was dialysed (Visking membrane) against three changes of distilled water at 5°, then freeze-dried.

A sample of the precipitate was hydrolysed in 2*N*-hydrochloric acid at 100° for 3 hr. and the products were examined in solvent A. Protein, RNA and a trace of glycerol teichoic acid were present. The remaining material was suspended in water (5 ml.) and applied to a column (2 cm. $\times$ 35 cm.) of Sephadex G75. Elution with water was followed by determination of the total phosphorus and extinction at 260 m μ and 280 m μ ; no peak corresponding to a RNA-teichoic acid complex was observed.

The freeze-dried supernatant (850 mg.) was extracted with 10% (w/v) trichloroacetic acid solution at 5° (50 ml. for 48 hr. and then 50 ml. for 4 days). An equal volume of ethanol was added to the extract and the precipitated teichoic acid (212 mg.) was collected in the usual manner. Further purification by extraction with ice-cold water (5 ml.), centrifuging and reprecipitation from the supernatant gave material (160 mg.) containing most of the intracellular teichoic acid.

Structural investigations

Products of acid hydrolysis. Hydrolyses were carried out with 2N-hydrochloric acid at 100° for 3 hr. After evaporation *in vacuo*, the products were examined by chromatography in solvents *A*, *B* and *C*; they were identified by reference to known compounds. The results are given in Table 1. Obvious differences between material from the two strains were the larger amount of glucose and the presence of lysine in the teichoic acid from strain 8191.

Configuration of glucose. The glucose in acid hydrolysates from both preparations was identified as D-glucose by chromatography in solvent *C*, drying and steaming the paper, and spraying with buffered glucose oxidase containing *o*-dianisidine (Glucostat reagents; Worthington Biochemical Corp., Freehold, N.J., U.S.A.). A pink spot corresponding to D-glucose appeared rapidly (Salton, 1960); galactose, hexosamines and pentoses gave no reaction.

Analysis. The ammonium salt of the teichoic acid from strain 8191 had $[\alpha]_D^{20} + 127^\circ$ (c in water, 1.4), and $[\alpha]_D^{20} + 132^\circ$ (c in water, 2.1), measured respectively on batches 1 and 3 after removal of amino acid residues. Insufficient material was available from strain 39 for accurate measurement of rotation, but rough measurements gave high positive values.

Quantitative analysis for glucose, amino acids and phosphorus on three different batches from strain 8191 and one from strain 39 gave the following molar proportions (the value for alanine from

strain 8191 includes lysine; no comparable values were obtained for that from batch 3 as amino acid residues had been removed from this teichoic acid before analysis): strain 8191, glucose:alanine:phosphorus 2.76:0.88:1 (average values); strain 39, glucose:alanine:phosphorus 1.43:0.41:1.

Enzymic dephosphorylation of glycerol mono- and di-phosphate. Teichoic acid (12 mg.) from strain 8191 was hydrolysed with acid in the usual manner and products were separated by chromatography with solvent *A*. Glycerol mono- and di-phosphate were eluted with water from appropriate regions of the paper and the eluates were freeze-dried. Half of each phosphate sample was treated with calf-intestinal phosphomonoesterase (Sigma Chemical Co., St Louis, Mo., U.S.A.) (1 ml. of a 1% solution in 0.05M-ammonium carbonate buffer, pH 9.3) for 16 hr. at 37°. Chromatography in solvents *A* and *B* showed that before enzyme treatment the samples contained glycerol mono- and di-phosphate, whereas incubation with the enzyme caused complete dephosphorylation to inorganic phosphate and glycerol.

Identification of amino acids. The hydrolysis of teichoic acid from strain 39 gave alanine, identified by paper chromatography, and no other amino acid. It was removed by treating the polymer with ethanol-aq. ammonia (sp.gr. 0.88) (1:1, v/v) for 5 hr. at room temperature; the products were alanine and its amide (R_F 0.70 in solvent *A*). Alanine methyl ester was converted into alanine and its amide under comparable conditions, but neither the methyl ester nor the teichoic acid was greatly affected by ammonia under milder conditions that ammonolysed other teichoic acids.

To determine the configuration of the alanine samples from the teichoic acid together with those of authentic D- and L-alanine were run on paper chromatograms with solvent *A*. The dried papers were sprayed with a solution of D-amino acid oxidase (5mg./ml.) (L. Light and Co. Ltd., Colnbrook, Bucks.) in 0.05M-pyrophosphate buffer, pH 8.2, kept at 37° for 6 hr. in an atmosphere of moist oxygen, and dried. Authentic D-alanine, and that from the teichoic acid, were completely decomposed under these conditions, whereas authentic L-alanine was unaffected. The production of ketones was demonstrated with 2,4-dinitrophenylhydrazine in hydrochloric acid (Newcombe & Reid, 1953): yellow spots were observed in the place formerly corresponding to alanine; the yellow changed to reddish brown after spraying with dilute sodium hydroxide solution.

Acid hydrolysis of the teichoic acid from strain 8191 gave alanine and a compound with the paper-chromatographic properties of an amino acid (see Table 1). Ammonolysis as described above gave four products detected by the ninhydrin reagent;

Table 1. *Chromatographic identification of the products of acid hydrolysis of teichoic acids from group D streptococci*

Experimental details, chromatographic conditions and spray reagents are described in the text.

	Strain 8191	Strain 39	R_F in solvent <i>A</i>	R_{glycerol} in solvent <i>B</i>
Glycerol	+	+	0.75	1.00
Glucose	+	+	0.61	0.38
Alanine	+	+	0.63	0.28
Lysine	+	—	0.49	—
Glycerol phosphates	+	+	0.33	—
Inorganic phosphate	+	+	—	—
Glycerol diphosphates	+	+	0.12	—

their R_{alanine} values in solvent *A* were 0.82, 1.0, 1.13 and 1.38. Those with the values 1.0 and 1.13 were respectively alanine and its amide, and it was likely that the compound with R_{alanine} 1.38 was the amide of the amino acid with R_{alanine} 0.82. Visual estimation indicated that the unidentified amino acid represented not more than 10% of the total, and consequently further work was carried out on accumulated amino acids from the ion-exchange purification of the teichoic acid previously treated with ammonia. This material would also be expected to contain small amounts of amino acid from ribitol teichoic acid from the wall, but as this is known to be entirely D-alanine the procedure is justified. Chromatography of the mixture from the Dowex 50 column on Whatman no. 3 paper in solvent *A* gave four separated areas as shown by the ninhydrin reagent. These were separately eluted with water and freeze-dried. The compound with R_{alanine} 0.82 in solvent *A* was identified as lysine by comparison with authentic lysine in several solvents. The R_{alanine} values in solvents *A*, *D*, *E*, *F* and *G* were respectively 0.82, 0.21, 1.35, 0.86 and 0.38. No other common natural amino acid behaved similarly. From the alanine area a small sample of alanine hydrochloride was obtained by the method described by Baddiley *et al.* (1962*b*); it had m.p. 193–195°, undepressed by mixing with authentic D-alanine hydrochloride. The compounds with R_{alanine} 1.13 and 1.38 were identified as alanine amide and lysine amide by hydrolysis to the amino acids in 2*N*-hydrochloric acid at 100° for 3 hr., followed by chromatography in solvents *A* and *E*.

The configuration of the lysine was established by the action of L-lysine decarboxylase. Samples of authentic L-lysine and that from the teichoic acid were treated with the enzyme solution (1 ml.) in 0.1*M*-phosphate buffer (10 mg./ml.) at 30° for 16 hr. with shaking. After being centrifuged, portions were examined by paper electrophoresis (Whatman no. 1 paper, 8 v/cm., in 0.05*M*-ammonium acetate buffer, pH 9.0, for 4 hr.). L-Lysine and that from the polymer were almost completely converted into a compound with mobility –25.0 cm., whereas untreated lysine had mobility –12.2 cm. Although no authentic sample of cadaverine was available, such a compound would be expected to move much faster towards the negative pole than would lysine under the conditions employed.

The presence of traces of a compound with the chromatographic properties of glycine was observed in amino acids accumulated from samples of teichoic acid from strain 8191. However, the amounts were extremely small and this was not in ester linkage (no glycine amide resulted from ammonolysis). It is concluded that it represents a

minor contaminant in unpurified teichoic acid; it was not detected in the purified polymer.

Titration of teichoic acid from strain 8191. Polymer (100 mg.) from which amino acids had been removed was titrated with sodium hydroxide solution by using a micrometer syringe. Two peaks were observed when $\Delta\text{pH}/\Delta V$ was plotted against *V*, corresponding to the proportions of primary and secondary acidic groups in the molecule. It appeared that about 21–22 units occurred in the polymer chain. Traces of amino acid were still attached to the polymer and a slight error may have been introduced in this way.

Partial acid hydrolysis. Preliminary experiments on the partial acid hydrolysis of teichoic acids from both strains indicated that the most suitable conditions for the formation of di- or tri-saccharides employed 0.33*N*-sulphuric acid for 15 min. at 100°. Hydrolysates were passed through short columns of Dowex 3 (OH[–] form) resin and eluates were evaporated to dryness *in vacuo* over phosphoric oxide, then examined by chromatography in solvents *B*, *H*, *I* and *J*.

The teichoic acid from strain 8191 gave under these conditions a heavy spot corresponding to glucose, and less intense spots corresponding to a disaccharide and trisaccharide. The disaccharide was chromatographically indistinguishable from kojibiose, and gave a yellow colour with the periodate–Schiff spray reagents in the same manner as both authentic kojibiose and sophorose. The trisaccharide behaved similarly, whereas all the other sugars examined gave blue colours.

Under similar conditions the teichoic acid from strain 39 gave glucose and kojibiose; no other di- or tri-saccharides were found. The chromatographic properties of these and related sugars and colour reactions with the periodate–Schiff reagents are given in Table 2.

Identification of kojibiose. Partial acid hydrolysis of teichoic acid (25 mg.) from strain 8191 was followed by paper chromatography with solvent *I*. The trisaccharide (2.3 mg.) and kojibiose (3.2 mg.) were eluted and freeze-dried. Hydrolysis of samples of both gave only glucose. The trisaccharide (2 mg.) in water (2 ml.) was reduced with sodium borohydride (tetrahydroborate) (5 mg.) at room temperature overnight. After acidification with acetic acid and passage through a column (1 cm. × 2 cm.) of Dowex 50 (H⁺ form) resin the eluate was freeze-dried and borate was removed by the repeated addition and evaporation of methanol. Total reducing sugar was determined by the phenol–sulphuric acid method (Dubois *et al.* 1956) on reduced and unreduced trisaccharide (polyols do not interfere in this method). The value found for the reduced trisaccharide was 68% of that for the trisaccharide (calculated value 66.7%).

Table 2. *Chromatography of di- and tri-saccharides from teichoic acids of group D streptococci compared with authentic compounds*

Experimental details, chromatographic conditions and spray reagents are given in the text. The rate of development of the colour with periodate-Schiff reagent is given in parentheses in the last column.

Solvent	...	...	...	...	R_{glucose}				Colour with periodate-Schiff reagent
					B	H	I	J	
Disaccharide from teichoic acids of strains 39 and 8191					0.46	0.53	0.63	0.52	Yellow
Trisaccharide from teichoic acids of strain 8191					0.25	0.27	0.37	0.28	Yellow
Kojibiose					0.46	0.53	0.62	0.52	Yellow
Sophorose					—	0.60	0.65	0.55	Yellow
Cellobiose					—	0.52	0.52	—	Blue (slow)
Maltose					0.49	0.56	0.55	0.59	Blue (slow)
Gentiobiose					—	0.41	0.48	0.44	Blue (slow)
Laminaribiose					—	0.74	0.71	0.72	Blue (slow)
Raffinose					0.26	0.27	—	0.29	Blue (slow)
Maltotriose					0.25	0.32	0.30	0.36	Blue (slow)

A partial acid hydrolysate (20 min. at 100° in 0.33N-sulphuric acid) of the trisaccharide examined in solvent *H* showed the presence of glucose, kojibiose and unhydrolysed material. A similar hydrolysate of the reduced trisaccharide examined in solvent *I* showed the presence of glucose, glucitol (R_{glucose} 1.01), reduced trisaccharide and an incompletely separated mixture of kojibiose and kojibitol (R_{glucose} 0.64 and 0.68 respectively). The periodate-Schiff reagents distinguished between the incompletely separated products; reduced trisaccharide, glucitol and kojibitol all rapidly gave a magenta colour, whereas kojibiose gave a yellow and glucose slowly gave a blue colour.

Periodate oxidation of teichoic acids. Material from both strains was treated with aq. ammonia (sp.gr. 0.88) at 37° for 16 hr. to ensure complete removal of amino acid ester residues. Ammonia was evaporated *in vacuo* and the solutions were passed through columns of Dowex 50 (H^+ form) resin; the eluates were neutralized with aq. ammonia and freeze-dried. The products were oxidized with 1% (w/v) sodium metaperiodate solution for 72 hr. at room temperature (1 mg. of teichoic acid in 1 ml. of periodate solution.) Excess of periodate was destroyed and aldehydes were reduced by adding sodium borohydride (5 mg.). After 16 hr. at room temperature the solutions were acidified with acetic acid, passed through columns of Dowex 50 (H^+ form) resin and freeze-dried, and the borate was removed by the repeated addition and evaporation of methanol. In both cases the residues contained much glycerol, a very faint trace of glucose and no erythritol or other polyols.

Similar experiments on teichoic acid with amino acid ester residues intact yielded appreciable amounts of glucose in the final products; smaller amounts of glucose were detected when the amino acid residues had been partially removed.

Quantitative oxidation was carried out on 1–2 mg. samples with 0.01M-sodium metaperiodate solution; total sugar was determined by the phenol-sulphuric acid method (Dubois *et al.* 1956) and periodate by the spectroscopic method (Dixon & Lipkin, 1954) after correction for iodate absorption (Aspinall & Ferrier, 1957). Oxidation was complete in 72 hr. The material from strain 8191 required 3.9 mol.prop. of periodate for 3 mol.prop. of glucose (the kojibiose residue should require 4 mol.prop. of periodate for 3 mol.prop. of glucose). The material from strain 39 required 2.92 mol.prop. of periodate for every 2 mol.prop. of glucose (the kojibiose residue should require 3 mol.prop. of periodate for every 2 mol.prop. of glucose).

Formic acid produced during oxidation of the material from strain 8191 was determined by Whelan's (1962) method, modified according to information kindly provided by Dr Whelan. The polymer (20 mg.), without amino acid ester residues, in 10 ml. of water was oxidized with 0.05M-sodium metaperiodate solution (6 ml.) for 72 hr. at room temperature. Periodate was destroyed by adding redistilled ethylene glycol (2 ml.), and after 30 min. water (to 25 ml.) was added. Portions (10 ml.) were mixed with 10% (w/v) potassium iodide solution (2 ml.) and iodine was titrated with 5 mM-sodium thiosulphate solution; 0.7 mol.prop. of formic acid was produced from each trisaccharide unit (kojibiose residues should give 1 mol.prop. of formic acid for each trisaccharide unit).

DISCUSSION

The isolation and purification of intracellular teichoic acid from the streptococcal strains 8191 and 39 have been described by Wicken *et al.* (1963), who showed that these compounds are serologically identical with the group D antigens. More recent

batches of the teichoic acid from strain 8191 have been contaminated with a small amount of ribitol teichoic acid from the wall, and additional chromatographic purification on a DEAE-cellulose column has been required for its removal. Contamination with wall teichoic acid is not surprising, since it can be extracted from isolated walls of this organism with relative ease (J. J. Armstrong, unpublished work). In the serological work it was shown that during chromatography of impure material on Sephadex an early fraction contained RNA associated with intracellular teichoic acid. Centrifuging of this fraction yielded a sediment comprising RNA and a high proportion of teichoic acid. This is surprising, as teichoic acids are not sedimented by high-speed centrifuging under normal conditions; the preliminary extraction with trichloroacetic acid should have separated teichoic acid and RNA if the association between them was weak. It is likely then that the two compounds form strongly associated complexes.

The occurrence of complexes between RNA and intracellular teichoic acid was observed by Critchley *et al.* (1962), who showed that nearly all the intracellular teichoic acid in the cell-contents fraction of *Lactobacillus arabinosus* 17-5 could be sedimented with RNA and some protein; similar observations have been made with many other organisms in this Laboratory. Thus many of the bacterial ribosome preparations described in the literature probably contain intracellular teichoic acid. We have repeated with strain 8191 the preparation and purification of streptococcal ribosomes described by Utsonomia & Hess (1961) for group A organisms and find that, although the pH 5.6 precipitate contains very little teichoic acid, the purified ribosomes contain a considerable amount. However, it is not suggested that ribosomes in intact cells contain teichoic acid, as this is now thought to be largely located between the wall and protoplast membrane (Hay *et al.* 1963), and there is as yet no evidence that this region of the cell contains RNA. The formation of these complexes may occur during cell disruption.

The products of acid hydrolysis of both teichoic acids included glucose, alanine, glycerol and its mono- and di-phosphate. In addition, that from strain 8191 gave a little lysine (about 10% of the total amino acid). The presence of glycerol and its diphosphates in the hydrolysate is characteristic of glycerol teichoic acids and indicates the presence of a chain in which glycerol residues are joined together through phosphodiester linkages (Kelemen & Baddiley, 1961; Critchley *et al.* 1962; Ellwood, Kelemen & Baddiley, 1963).

The alanine has the expected D-configuration. Lysine from strain 8191 was identified by paper chromatography in a number of solvents; its

identity with L-lysine follows from its ready decarboxylation with L-lysine decarboxylase from *Bacillus cadaveris* (Gale, 1944) (kindly provided by Dr D. A. Kerridge and Professor E. F. Gale, F.R.S.). Both amino acids are in ester linkage with hydroxyl groups in the polymers, as they were removed by ethanol-aq. ammonia, giving a mixture of the amino acids and their respective amides; the amides were isolated by paper chromatography and hydrolysed to the appropriate amino acids.

The presence of glycoside residues attached to glycerol or ribitol has been directly demonstrated in other teichoic acids by alkali hydrolysis to a mixture of polyol glycosides and their monophosphates, which, on treatment with a phosphatase, give the polyol glycoside. Phosphodiester groups in a teichoic acid are hydrolysed with alkali only if an unsubstituted hydroxyl group occurs adjacent to the phosphate group; hydrolysis requires the intermediate formation of cyclic phosphates involving the adjacent hydroxyl group. In glycerol teichoic acids, the presence of a glycosyl substituent on glycerol prevents that glycerol residue from participation in cyclic phosphate formation; thus, in a 1,3-linked glycerol phosphate polymer in which sugar residues are present at the position 2 on each glycerol, the molecule would be unaffected by hot alkali (apart from the loss of amino acid ester groups). Analysis indicates that the polymer from strain 8191 contains nearly three glucose residues for each phosphorus atom present, and that from strain 39 contains about two glucose residues for each phosphorus atom; consequently, hydrolysis through cyclic phosphates would be highly restricted or even impossible. In fact the phosphodiester linkages were unaffected by hot alkali, and all attempts to isolate glycerol glycosides failed.

The nature of the glucose substitution in these polymers was established by partial acid hydrolysis. Preliminary experiments with formic acid, ion-exchange resins and hydrochloric acid under various conditions were unsatisfactory, but 0.33N-sulphuric acid at 100° gave optimum yields of oligosaccharides after 15 min. The teichoic acid from strain 39 gave glycerol and its phosphates, glucose and a single disaccharide, identified chromatographically as kojibiose [α -D-glucopyranosyl-(1 $\rightarrow$ 2)-O-D-glucose], whereas that from strain 8191 gave kojibiose and the trisaccharide kojitriose [α -D-glucopyranosyl-(1 $\rightarrow$ 2)-O- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-O-D-glucose]. The periodate-Schiff spray reagents are particularly useful in the identification of these two oligosaccharides: like sophorose, they give a yellow colour and thus differ from all the other mono- and disaccharides examined. The production of yellow colours in this test has often been observed in this Laboratory, and it has been confirmed (F. E. Hardy, unpublished work) that this is attributable

to the intermediate formation of derivatives of malondialdehyde. W. K. Roberts, J. G. Buchanan & J. Baddiley (unpublished work) have considered the significance of this behaviour in the di- and tri-saccharides, and have concluded that it occurs when the reducing sugar unit in an oligosaccharide bears a substituent at position 2; this structural feature exists in kojibiose, sophorose and kojitriose.

The structure of kojitriose was established by hydrolysis to glucose and kojibiose, and by reduction with sodium borohydride and partial acid hydrolysis; the products from the reduced compound were glucose, kojibiose, kojibi-itol and glucitol. Unlike the di- and tri-saccharides, the reduced compounds gave rapidly developing magenta spots on paper chromatograms sprayed with the periodate-Schiff reagents; this is the usual behaviour of compounds containing acyclic polyol residues (cf. Baddiley, Buchanan, Mathias & Sanderson, 1956).

Structure (I) is suggested for the intracellular teichoic acid from strain 39, and structure (II) for that from strain 8191 (amino acid ester groups have been omitted). They are supported by quantitative periodate oxidation after removal of amino acid residues, when the expected consumption of periodate and formation of formic acid occurred. The glycerol residues in both teichoic acids were apparently unaffected by the periodate, whereas glucose was totally destroyed. The presence of α -D-glucosidic linkages is consistent with the high positive rotations of these compounds; lack of material prevented accurate measurement of the rotation of the material from strain 39, but that from strain 8191 had a rotation consistent with the presence of three α -glucose linkages.

The quantitative analytical evidence would not exclude the possible occurrence of a few tetra-saccharide residues in the polymer from strain 8191, particularly if a similar number of disaccharide

(R = kojibiosyl or kojitriosyl)

residues were also present. However, no traces of tetrasaccharides were detected in partial acid hydrolysates, and it is unlikely that such substituents are present in this compound. Similarly, no trace of kojitrise was detected in hydrolysates of the teichoic acid from strain 39.

Although the stability of the phosphodiester linkages towards alkali has prevented the direct demonstration of a 1,3-linked glycerol phosphate polymeric structure with the sugar substituents attached to position 2 on glycerol, such a structure is most likely, by analogy with other glycerol teichoic acids. Moreover, all teichoic acids studied so far can be regarded as being derived by regular condensation of glycerol or ribitol phosphate units, and so will possess a phosphomonoester group at one end of the chain but not at the other; this is consistent with experimental observation and with the suggested mechanism of biosynthesis from CDP-ribitol (Armstrong *et al.* 1958; Armstrong, Baddiley & Buchanan, 1960; Kelemen & Baddiley, 1961; Baddiley, Buchanan, Martin & RajBhandary, 1962a; Ellwood *et al.* 1963; Critchley *et al.* 1962). The only alternative regular structure to those suggested in formulae (I) and (II) which would conform to the pattern of a glycerol monophosphate polymer is shown in structure (III) where R = kojibiosyl or kojitriosyl. However, structure (III) would be untenable for either of the streptococcal teichoic acids, as it would be hydrolysed in alkali through stepwise loss of glycosylglycerol phosphate units; the hydrolysis of 1,2-linked polymers of glycerol phosphate has been discussed by Kelemen & Baddiley (1961).

The presence of a phosphomonoester group in the teichoic acid from strain 8191 was demonstrated by potentiometric titration and by the action of a phosphomonoesterase. From the ratio of primary to secondary titratable acidic groups it was shown that the molecule is composed of about 20 glycosylglycerol phosphate units. This value is similar to those found with other glycerol teichoic acids, but could not be confirmed by quantitative hydrolysis of terminal phosphate by the enzyme, as its action on the polymer was very slow and incomplete. It is likely that the bulky sugar substituents inhibited the enzymic action, a phenomenon observed by Armstrong *et al.* (1960) and by Baddiley *et al.* (1962a) in similar circumstances. Because of the small amounts of material available, similar studies on teichoic acid from strain 39 could not be carried out.

The location of the amino acid ester residues has not been decided with certainty. Clearly, as no glycerol hydroxyl groups are available, they must be attached to the sugar residues. In this respect these teichoic acids are exceptional, as in all others so far examined the alanine ester residues are attached to the polyols. This unusual structural

feature is reflected in differences in their behaviour towards ammonia. The alanine ester residues in other teichoic acids are very labile towards alkali and ammonia, owing to the presence of phosphate or suitably placed hydroxyl groups or both adjacent to the ester linkages (Shabarova, Hughes & Baddiley, 1962). However, if the amino acid ester residues in the compounds from the two streptococci are attached to glucose units, neither a phosphate nor a hydroxyl group would occur in sterically suitable proximity to the ester linkages. Consequently, hydrolysis or ammonolysis should be relatively slow, as was found.

Periodate oxidation of the compound from strain 8191 before and after removal of amino acid groups strongly suggests that the presence of these groups prevents the oxidation of some glucose residues. This indicates that amino acid ester groups must occur at position 3 or 4 of one or more glucose residues. An ester residue at position 4 in glucopyranosides will readily migrate to position 6 under slightly alkaline conditions; consequently, if position 4 is involved in ester linkage in the teichoic acid, partial removal of these groups with alkali should be accompanied by migration from position 4 to position 6 of all those not removed. However, after partial removal of amino acid ester groups with ammonia from this teichoic acid, some glucose residues were still stable towards periodate. Although further work is required, it is likely that at least some of the ester residues are attached to position 3 on glucose.

The overall similarity between the structures proposed for the two streptococcal teichoic acids is consistent with their serological properties. The differences observed in their reaction with group D antisera (Wicken *et al.* 1963) would be explained by the occurrence of additional glucose residues in that from strain 8191. It is unlikely that the presence of lysine in one of them would influence its serological properties, as in other cases the amino acid ester groups are not involved in these reactions (Haukenes *et al.* 1961). During serological work with these antigens, the lability of the glucosyl residues under moderate acidic conditions should be considered. Thus Lancefield's method for the extraction of antigens (pH 2 with heating) could cause degradation, and reports on the serological identity of group D antigens from different strains of streptococci (Jones & Shattock, 1960) may be due to partial loss of glucose residues during their isolation.

SUMMARY

1. Intracellular glycerol teichoic acids (group D antigens) have been isolated from the cell contents of two streptococcal strains (39 and 8191). They form complexes with the RNA of the cell and may

be sedimented in the ribosomal fraction. A preparation of purified ribosomes from group A *Streptococcus* has been shown to contain teichoic acid.

2. The compounds from both strains are glycerol phosphate polymers in which all or nearly all glycerol units possess sugar substituents. D-Alanine ester residues are attached to sugar in both compounds, and that from strain 8191 also possesses a small number of L-lysine ester residues.

3. The compound from strain 39 has kojibiose [α -D-glucose-(1 $\rightarrow$ 2)-D-glucose] attached to glycerol, whereas kojitrise occurs in that from strain 8191. The di- and tri-saccharides were obtained by partial acid hydrolysis, and identified by chromatography and colour reactions before and after reduction with sodium borohydride.

4. Structures (I) and (II) are proposed for the teichoic acids from strains 39 and 8191 respectively; the latter is composed of about 20 glycosylglycerol phosphate units. The possible location of the amino acid ester residues is discussed.

5. It is concluded that differences in the serological properties of these two antigens are associated with the chemical differences in their sugar substituents.

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