The Wall Teichoic Acids of *Lactobacillus plantarum* N.I.R.D. C 106

LOCATION OF THE PHOSPHODIESTER GROUPS AND SEPARATION OF THE CHAINS

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1. The identities of the component glycerol glucosides of the wall teichoic acids of *Lactobacillus plantarum* N.I.R.D. C 106 have been confirmed by methylation analysis. These glucosides are α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol, α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol and α -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol. 2. These units are connected by phosphodiester groups attached to the 3(L)-hydroxyl group of glycerol and the 6-hydroxyl group of the non-reducing terminal glucose residues in the adjacent unit. 3. Concanavalin A forms a precipitate with the teichoic acid and the material so precipitated contains only the α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol component. This unit is therefore present in a homogeneous polymer so that the teichoic acid is a mixture of this and of other possibly homogeneous chains containing the other two components.

On the basis of their metabolic behaviour, lactobacilli have been divided (Sharpe, 1962) into seven groups (A–G). The species *Lactobacillus plantarum* belongs to group D. Serological studies have shown that the group D specific precipitinogen is a glucosyl-ribitol wall teichoic acid (Sharpe, Davison & Baddiley, 1964). However, certain strains of *L. plantarum* lack the group D precipitinogen and we have previously reported (Adams, Archibald, Baddiley, Coapes & Davison, 1969) that one of these strains, *L. plantarum* N.I.R.D. C 106, contains a glucosylglycerol teichoic acid in its wall. Evidence was presented showing that phosphate-sugar linkages were present in the teichoic acid and we now report confirmatory studies in which the precise location of these linkages is determined.

MATERIALS AND METHODS

Chemicals. Cells of *L. plantarum* N.I.R.D. C 106 were grown at 37°C as described by Adams *et al.* (1969). ³H-labelled sodium borohydride was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Calf intestinal phosphomonoesterase, DL-glyceric acid, gentiobiose, maltose, crude yeast hexokinase, ATP and NADH were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Laminarin, D-amino acid oxidase and catalase were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Glucostat reagent was purchased from Worthington Chemical Corp., Freehold, N.J., U.S.A. Concanavalin A was purchased from Calbiochem Ltd., London W.1, U.K. DEAE-celluloses were purchased from H. Reeve Angel and Co. Ltd., London, U.K., and Sephadex gels were purchased from Pharmacia, Uppsala, Sweden.

Paper chromatography. Paper chromatography was carried out on Whatman no. 1 and 3MM papers, the chromatograms being developed by descending chromatography at room temperature in the following solvent systems: A, propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.); B, butan-1-ol-pyridine-water (6:4:3, by vol.); C, butan-1-ol-ethanol-water-aq. NH₃ (sp.gr. 0.88) (40:10:49:1, by vol., organic phase).

Products were detected by using the following methods. (1) The periodate-Schiff reagent for glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956). (2) The molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949). (3) The aniline phthalate reagent for reducing sugars (Partridge, 1949).

Assays. Total hexose was determined by using the procedure of Dubois, Gilles, Hamilton, Rebers & Smith (1956) and D-glucose was determined with the Glucostat reagents. Protein was determined by using the procedure of Lowry, Rosebrough, Farr & Randall (1951) and phosphate, amino acids, reducing sugars, glycerol, formaldehyde and periodate were determined as described by Archibald, Baddiley & Button (1968).

Determination of radioactivity. Radioactive materials were adjusted to 1.5 ml with water and the radioactivity was counted in a Beckman LS-150 scintillation counter in a scintillant (10 ml) composed of toluene (A.R.) (2 litres), 2,5-diphenyloxazole (8 g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.2 g). The counting efficiency, determined by using a [³H]lysine standard, was 25%. Samples were counted to a counting error of less than 2%.

RESULTS

Isolation of teichoic acids. Cells of *L. plantarum* C 106 were grown for 18 h at 37°C in the glucose-Tryptone medium described by Adams *et al.* (1969).

An 18-litre batch, inoculated with 1 litre of an overnight culture, gave 120 g (wet wt., equivalent to approx. 24 g dry wt.) of cells. These were disrupted by shaking a suspension (50% wet wt./vol.) of cells in water with an equal volume of no. 11 Ballotini beads in a Braun disintegrator for 1 min. Walls were recovered by centrifugation at 17000g for 30 min, washed six times with 0.9% sodium chloride and six times with water and then freeze-dried, yielding about 2.0 g of wall from 120 g (wet wt.) of whole cells. Such walls contained 2.6% P, although this amount varied slightly with different preparations. Teichoic acid was isolated by extraction of walls with trichloroacetic acid as described by Adams *et al.* (1969) or with 0.5M-sodium hydroxide (Archibald, Coapes & Stafford, 1969), the yields of crude teichoic acid/g of wall being 156 and 464 mg respectively. Extraction with alkali gave teichoic acid (containing 4.75% P), which was purified by chromatography on DE 52 cellulose (HCO_3^- form) which removed the free D-alanine, resulting from alkali hydrolysis of the D-alanyl ester residues, and traces of peptidoglycan components. Elution with a linear gradient of 0–0.7M-ammonium carbonate gave a single peak of phosphate at between 0.25 and 0.35M-ammonium carbonate. This material ($[\alpha]_D^{21} +142^\circ$, c 0.4 in water) was recovered by freeze-drying and contained 90% of the phosphate applied to the column. It contained 5.47% P, about 6% of which was present as monoester (determined by incubation with phosphatase) and small amounts of peptidoglycan components. Extraction with trichloroacetic acid gave teichoic acid ($[\alpha]_D^{21} +104^\circ$, c 1.2 in water), which contained 5.6% P, about 5% (by titration and by incubation with phosphatase) of which was present as monoester. The molar proportions of the major components in each preparation were: (a) teichoic acid isolated by extraction with trichloroacetic acid, phosphate:D-alanine:glycerol:D-glucose, 1.00:0.63:0.95:1.78; (b) teichoic acid isolated by extraction with sodium hydroxide, phosphate:glycerol:D-glucose, 1.00:0.95:1.60.

Location of the ester-linked D-alanine. All of the ester-linked alanine was removed from walls by incubation for 2 h in 0.1M-sodium hydroxide at 22°C. The alanine was likewise removed from teichoic acid isolated by extraction with trichloroacetic acid and this led to the disappearance of the characteristic absorption at 1745cm^{-1} in the i.r. spectrum of the teichoic acid. Teichoic acid (20.6 mg) was dissolved in water (10 ml) and samples (0.5 ml containing $1.1\mu\text{mol}$ of alanine) were incubated with salt-free 0.1M-hydroxylamine (0.5 ml, pH 7.2) (Bainert *et al.* 1953) for various times at 45°C. Alanine methyl ester was used for comparison and at suitable times tubes were removed, the reaction was stopped by addition of M-hydrochloric acid (1 ml) and M-ferric chloride (1 ml) was added and the extinction read at 540 nm, alanine hydroxamate being used as standard. The results (Table 1) show that the alanyl ester residues in the teichoic acid form hydroxamate much more readily than does alanine methyl ester. This lability is characteristic of D-alanyl ester residues which are adjacent to free hydroxyl or phosphate groups.

Further characterization of the component glucosides. Walls (1 g) were homogenized in M-sodium hydroxide (40 ml) and hydrolysed at 100°C for 3 h. Wall debris was removed by centrifugation and washed with water. The combined supernatant solutions were evaporated to a small volume and passed through a column (25 mm $\times$ 300 mm) of Dowex 50 (NH_4^+ form) resin. The hydrolysis products were eluted with water and then incubated at 37°C in 0.05M-ammonium carbonate (200 ml) containing 10 mg of phosphomonoesterase. After 48 h all of the phosphate was present as P_i and the mixture was taken to dryness by rotary evaporation at 30°C. The products were dissolved in water (50 ml) and passed through a column (20 mm $\times$ 280 mm) of Dowex 2 (CO_3^{2-} form) resin. Neutral products were eluted with water (500 ml) and the eluate was repeatedly rotary evaporated to dryness to remove ammonium carbonate. The residue was dissolved in water (10 ml) and applied to a column (30 mm $\times$ 400 mm) of Dowex 1 (OH^-

Table 1. *Formation of alanine hydroxamate from teichoic acid and from alanine methyl ester*

Experimental details are given in the text.

Time (min)	Alanine hydroxamate formed			
	From teichoic acid		From alanine methyl ester	
	($\mu\text{mol}/0.5\text{ ml}$)	(% of theory)	($\mu\text{mol}/0.5\text{ ml}$)	(% of theory)
30	0.70	64	0.21	9
60	0.81	75	0.33	15
105	0.92	84	0.41	19
225	1.02	91	—	—
24 h	—	—	1.70	80

Table 2. *Fractionation of the products of alkali and phosphatase hydrolysis by chromatography on Dowex 1 (OH⁻ form) resin*

Experimental details are given in the text.

Fraction	Elution volume (ml)	Yield of material (mg)	Identity
1	100-170	22.6	Glycerol + unidentified optically active material of similar paper-chromatographic mobility to α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol
2	190-250	31.5	α -D-Glucopyranosyl-(1 $\rightarrow$ 1)-glycerol
3	250-375	91.7	α -D-Glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol.
4	675-1000	74.0	α -D-Glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol.

Table 3. *Chromatographic mobilities and yields of the methylated glucosides*

Experimental details are given in the text.

Permethylether	$R_{\text{permethylglucose}}$ in light petroleum (b.p. 40-60°C)-acetone	Yield (mg)
α -D-Glucopyranosyl-(1 $\rightarrow$ 1)-glycerol	0.87	9.3
α -D-Glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol	0.75	8.6
α -D-Glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol	0.64	10.7

form) resin (Austin, Hardy, Buchanan & Baddiley, 1963). Products were eluted with carbonate-free water (2l); the effluent from the column was passed through a constant-flow polarimetric cell and then collected in 5ml fractions. The polarimeter cell was placed in a Bendix-NPL automatic polarimeter which was connected to a chart recorder. Four peaks of optical activity were obtained and appropriate tubes were combined to give four fractions, the elution volumes, yields and identities of which are given in Table 2.

The identities of the three previously characterized glucosides were further confirmed by methylation analysis. Sodium hydride (10mg) was added to solutions of the glucosides (10mg) in *NN*-dimethylformamide (0.6ml). The mixtures were shaken at room temperature before the addition of freshly distilled methyl iodide (0.5ml). The reaction mixtures were set aside at room temperature (22°C) for 24h. Methanol (1ml) was then added and each solution was taken to dryness by rotary evaporation at 70-80°C. The residues were partitioned between chloroform (5ml) and water (5ml). The separated organic layers were further washed with water and concentrated by rotary evaporation to yellow oils. The methyl ethers were dissolved in chloroform and their purity was checked by t.l.c. on a 0.4mm layer of silica gel (kieselguhr PF 254+366) with light petroleum (b.p. 40-60°C)-acetone (3:2, v/v) as solvent. Permethylether was also chromatographed as a standard. Each

methylated glucoside gave a single spot together with a small amount of base-line material. The mobilities of the methylated glucosides are shown in Table 3.

The methyl ethers were purified on thin layers of silica gel, the appropriate areas being removed and eluted with chloroform. Methanolysis of a sample (5mg) of each methyl ether was carried out in methanol containing 5% (w/v) hydrochloric acid (2ml) for 16h at 60°C. The solutions were evaporated to dryness and the last traces of hydrochloric acid were removed under reduced pressure over potassium hydroxide pellets. The products were examined by gas-liquid chromatography on a column of 15% polybutanediol succinate on Celite (80-100 mesh) maintained at 175°C with a Pye-104 model 4 gas chromatograph. The results (Table 4) show that the monoglucosylglycerol gives rise to methyl 2,3,4,6-tetra-*O*-methylglucoside and a more volatile compound, with a retention time relative to methyl 2,3,4,6-tetra-*O*-methyl- β -D-glucoside of 0.25. This latter compound corresponds to 2,3-di-*O*-methyl-D-glycerol (Brundish, Shaw & Baddiley, 1967). α -D-Glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol gives peaks corresponding to di-*O*-methylglycerol, methyl 2,3,4,6-tetra-*O*-methylglucoside and methyl 3,4,6-tri-*O*-methylglucoside, whereas α -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol gives di-*O*-methylglycerol, methyl 2,3,4,6-tetra-*O*-methylglucoside and methyl 2,4,6-tri-*O*-methylglucoside. These

Table 4. *Gas-chromatographic analysis of the products of methanolysis of the methylated glucosides*

Experimental details are given in the text.

Sample	Retention times relative to methyl 2,3,4,6-tetra- <i>O</i> -methyl- β -D-glucoside
Methyl 2,3,4,6-tetra- <i>O</i> -methyl-D-glucoside	1.00, 1.43
Methyl 2,4,6-tri- <i>O</i> -methyl-D-glucoside	3.31, 4.88
Methyl 2,3,4-tri- <i>O</i> -methyl-D-glucoside	2.59, 3.70
Methyl 2,3,6-tri- <i>O</i> -methyl-D-glucoside	3.32, 4.48
Methyl 3,4,6-tri- <i>O</i> -methyl-D-glucoside	3.12, 3.73
Products from α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol	0.25, 1.00, 1.43
Products from α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol	0.24, 1.00, 1.43, 3.09, 3.68
Products from α -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol	0.24, 1.00, 1.43, 3.28, 4.83

results confirm that the linkages in the glucosides are as determined earlier by periodate oxidation studies (Adams *et al.* 1969).

Configuration of the glycerol residues in the glucosides. A sample (25mg) of α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol was placed in a round-bottomed flask (25ml) and freeze-dried over phosphorus pentoxide. The freeze-dried material was then oxidized with nitrogen dioxide (0.2ml) in apparatus similar to that described by Nevel (1963). Oxidation was allowed to proceed at room temperature for 24h and the system was then evacuated for 15min to remove excess of nitrogen dioxide. The oxidized material was dissolved in a small amount of water and then transferred to a graduated tube, the volume being adjusted to 1ml with repeated washings from the flask. Sodium periodate (1ml, 0.2M, pH 7.0) was then added and the mixture was kept in the dark at room temperature for 24h. Ethylene glycol (1ml, 0.1M) was added and after 30min a freshly prepared aqueous solution (1ml) of 1% (v/v) 1,1-dimethylhydrazine, pH 6.0, was added. The mixture was kept at 37°C for 16h and then extracted with several portions of chloroform until no more red colour was removed. The solution was passed through a column (1ml) of Dowex 50 (NH₄⁺ form) resin and the eluate was evaporated to a small volume and chromatographed as a band in solvent B together with glyceric acid. The glyceric acid was detected on marker strips (reagent 1) and recovered from the paper by elution with 0.01M-ammonia.

The amount of glyceric acid in the eluted sample was determined by measurement of the formaldehyde formed on oxidation of a sample with sodium periodate. The configuration of the glyceric acid was determined by oxidation of NADH in the presence of a D-specific enzyme from rabbit muscle (Archibald, Baddiley & Buchanan, 1961). Authentic DL-glyceric acid oxidized 1.1mol. prop. of NADH whereas the glyceric acid from the glucoside oxidized 1.8mol. prop. of NADH, so that

82% of the glyceric acid has the D-configuration. This must be derived by oxidation of a 1-L-substituted glycerol so that the parent glucoside is therefore α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol. Similar studies on α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol gave 74% of the glyceric acid in the D-configuration. The isomeric diglucosylglycerol cannot be degraded in this way but by analogy with the two other glucosides we assume that the stereochemistry of the glycerol moiety is the same.

Origin of the unsubstituted glycerol formed on hydrolysis of the teichoic acid with alkali and phosphatase. Alkali hydrolysis of extracted teichoic acid (sodium hydroxide or trichloroacetic acid) and of intact cell walls gave small amounts of material that formed glycerol on enzymic dephosphorylation. This cannot be formed from a regular polymer in which glucosylglycerol units are held together by phosphodiester bridges between glycerol and glucose moieties of adjacent units. The proportion of glycerol formed varied with different preparations of wall and it was found possible to obtain, by extensive washing with saline, wall preparations which gave little or no glycerol. It appears from this that the glycerol is derived from a polymer other than the poly(glucosylglycerol phosphate) teichoic acid(s) and its removal from the wall by extensive washing with saline suggests that it is a membrane teichoic acid, present as a contaminant in wall preparations which have not been extensively washed. The presence of membrane teichoic acid in the organism was confirmed by extraction (trichloroacetic acid) under standard conditions (cf. Archibald *et al.* 1968) of the particulate fraction obtained by centrifugation (100000g, 1h) of the cytoplasmic fraction of disrupted cells. Addition of acetone to the extract gave a precipitate which on hydrolysis in acid gave glycerol and its mono- and di-phosphates. These products are characteristic of the simple (1 $\rightarrow$ 3)-polyglycerol phosphate teichoic acids found as intracellular or 'membrane' teichoic acids in almost all Gram-

positive bacteria examined and we conclude that the glycerol formed on alkali and phosphatase hydrolysis of walls is derived from this teichoic acid, some of which remains with the wall and is removed only by extensive washing. In all studies reported below, walls used were washed until alkali and phosphatase gave no detectable glycerol.

Location of the phosphodiester residues. Teichoic acid was prepared by extraction of walls with 0.5M-sodium hydroxide and purified by chromatography on DEAE-cellulose as described above.

(a) Periodate oxidation. Sodium metaperiodate (10ml, 0.05M) was added to a solution of teichoic acid (53mg) in water (10ml) and the mixture was kept in the dark at room temperature. Samples (0.1ml) were withdrawn at intervals, diluted to 50ml with water and assayed for periodate. Oxidation was complete after 72h when 2.3 molar proportions of periodate had been reduced/mol of phosphate. Further samples (0.2ml) of the oxidation mixture were withdrawn at intervals, diluted with water (initial samples were diluted to 2ml, final samples to 1ml) and portions (0.2ml) were assayed for hexose by the method of Peplow & Somers (1969). At the end of the oxidation the teichoic acid contained 0.41mol of hexose/mol of phosphate. Thus 23% of the glucose residues in the teichoic acid was resistant to oxidation by periodate. After 72h samples (0.4ml) of the oxidation mixture were analysed for formic acid by using the method of Barker & Somers (1966) and found to contain 1.1mol of formic acid/mol of phosphate. Ethylene glycol (0.6M, 1ml) was added to the remainder of the oxidation mixture which was incubated for a further 3h at room temperature and then freeze-dried.

(b) Action of bases on oxidized teichoic acid. Periodate-oxidized teichoic acid (14 μ mol of P) and unoxidized teichoic acid (13 μ mol of P) were separately dissolved in 0.1M-sodium hydroxide (2ml) and incubated at 37°C for 16h. The solutions were then passed through columns (5ml) of Dowex 50 (NH₄⁺ form) resin, evaporated to dryness and then dissolved in 0.05M-ammonium carbonate, pH 9.0 (5ml). Samples (0.1ml) were analysed for P_i and then phosphatase (0.5mg) was added to each solution, which was then incubated at 37°C for 24h and analysed for inorganic and total phosphate. No P_i was present in either sample before incubation with phosphatase but after incubation 68% of the phosphate in the oxidized sample was P_i whereas the unoxidized sample gave only 6% P_i.

A further sample (10mg) of oxidized teichoic acid was dissolved in 0.25M-glycine buffer, pH 10.5 (10ml), and incubated at room temperature. At intervals samples (1ml) were removed, passed

through Dowex 50 (NH₄⁺ form) resin and incubated with phosphatase as above. After 5h in glycine buffer, 78% of the phosphorus was present as phosphomonoester; this amount did not increase on further incubation.

(c) Reduction with sodium boro[³H]hydride. Oxidized teichoic acid (9.3mg) was desalted by passage through a column (void volume 46ml) of Sephadex G-50. Elution with water gave a single phosphate-containing peak at elution volume 52ml. Fractions containing this phosphate were combined, freeze-dried, dissolved in water (0.2ml) and added to a freshly prepared solution of sodium boro[³H]-hydride (130 μ mol, approx. 900 μ Ci) in water (0.7ml). Reduction was allowed to proceed at room temperature for 20min and the solution was then dialysed against water (250ml) for 6h at which time no further evolution of hydrogen gas was visible. The non-diffusible material was then passed through the column of Sephadex G-50 as described above. Elution with water again gave a single phosphate peak (elution volume 48ml), which coincided with a peak of radioactivity. This material was combined, freeze-dried and dissolved in water (1ml). A sample (0.5ml) was mixed with 2M-hydrochloric acid (0.5ml), allowed to stand at room temperature for 30min, heated at 60°C for 1h and finally heated at 100°C for 2h. This process was used to ensure selective hydrolysis of the more acid-labile groups before cleavage of the phosphodiester linkages. The hydrolysate was then taken to dryness *in vacuo* over potassium hydroxide pellets, dissolved in water (1ml) and applied to a column (5mm $\times$ 30mm) of DE 52 cellulose (previously equilibrated against 0.5M-pyridine acetate buffer, pH 5.3). Neutral products were eluted with water (20ml) and phosphates were eluted with a linear gradient (0–0.5M) of pyridine acetate, pH 5.3. Phosphate was eluted in a single peak, coincident with the single peak of radioactivity, at 0.09–0.12M-pyridine acetate. Fractions containing phosphate were combined, freeze-dried and then dissolved in water (0.5ml). The neutral products were also freeze-dried and dissolved in water (1ml). Both fractions were assayed for phosphate and for radioactivity. Samples (100 μ l) of each fraction were examined by paper chromatography (solvent A) against standards of glycerol and glycerol 1-phosphate. The chromatograms were cut into 1cm strips which were eluted and the eluates were assayed for radioactivity. Almost all of the radioactivity in the phosphate fraction was present in a single spot which had the same chromatographic mobility as glycerol monophosphate, and almost all the radioactivity in the neutral fraction had the same chromatographic mobility as glycerol. The distribution of the radioactivity in the various fractions is shown in Table 5, where it is seen that

Table 5. Products derived from oxidized teichoic acid after reduction with NaB^3H_4

Experimental details are given in the text.

Sample	Radioactivity (d.p.m.)	Treatment	Method of separation of products	Products	Radioactivity (d.p.m.)	Radioactivity/ μmol of phosphate (d.p.m.)
A Oxidized teichoic acid	—	Reduction with NaB^3H_4	—	Reduced teichoic acid	7.2×10^7	1.2×10^7
B Reduced teichoic acid from A	4.3×10^7	m-HCl , 2 h at 100°C	Ion-exchange chromatography	Glycerol monophosphate	6.1×10^6	1.8×10^6
C Glycerol mono-phosphate from B	6.6×10^4	Enzymic dephosphorylation	Paper chromatography	Glycerol (+ glycolaldehyde)	3.7×10^7	—
D Reduced teichoic acid from A	1.8×10^5	0.1 M-HCl , 10 min at 100°C	Paper chromatography	Glycerol	6.2×10^4	—
				Base-line material	1.2×10^4	—
				Glycerol monophosphate	1.6×10^4	—
				Compound of $R_{\text{glycerol phosphate}}$ 1.7	1.9×10^3	—
E Glycerolphosphoryl-glycerol from D	9.0×10^3	m-HCl , 3 h at 100°C	Paper chromatography	Glycerolphosphorylglycerol	2.7×10^4	—
F Glucosamine 6-phosphate	—	Reduction with NaB^3H_4	Ion-exchange chromatography	Glycerol	5.8×10^4	3.7×10^6
				Glycerol monophosphate	5.1×10^3	—
				Glycerol	4.8×10^3	—
				Glucosaminitol 6-phosphate	1.1×10^7	3.7×10^6

after acid hydrolysis of the reduced material, 14% of the ^3H was associated with a phosphate having the chromatographic mobility of glycerol phosphate and 86% of the incorporated label was associated with neutral compounds which co-chromatographed with glycerol (solvent B).

Dephosphorylation of [^3H]glycerol monophosphate.

A sample (0.2 ml) of the [^3H]glycerol monophosphate was added to 0.05 M-ammonium carbonate, pH 9.0 (1.8 ml containing 0.2 mg of phosphatase), and incubated at 37°C overnight. Chromatographic examination of the products showed that over 90% of the radioactivity was associated with material having the chromatographic mobility of glycerol (solvent B).

Partial acid hydrolysis of the reduced polymer.

(a) With formic acid. A sample (0.25 ml, $15 \mu\text{mol}$ of P) of the reduced teichoic acid was added to 60% (v/v) formic acid (0.6 ml) and heated at 60°C for 1 h. A sample (0.01 ml) was chromatographed in solvent A against standards of glycerol monophosphate, glycerolphosphorylglycerol and glycerol. The major peak of radioactivity (26–29 cm from the origin) corresponded to glycerol and a faster-moving peak (32–36 cm) may be a mixture of C_2 fragments and formyl esters. A peak corresponding to glycerol monophosphate (8–9 cm from the origin) was also present together with two small peaks at approx. 13 and 19 cm from the origin. The former was tentatively identified, on the basis of hydrolytic studies, as glucosylglycerolphosphorylglycerol and the latter was coincident with the reference glycerolphosphorylglycerol. Only a small amount of this last material was present, however, and a further sample of reduced teichoic acid was hydrolysed with hydrochloric acid to obtain sufficient glycerolphosphorylglycerol for structural studies.

(b) With hydrochloric acid. A sample (0.25 ml, $15 \mu\text{mol}$ of P) of the reduced teichoic acid was mixed with 0.1 M-hydrochloric acid (0.5 ml), heated at 100°C for 10 min and then immediately freeze-dried over potassium hydroxide pellets. Chromatographic examination (solvent A) showed the presence of radioactive compounds with the same mobility as markers of glycerol monophosphate (7 cm from origin), glycerol (23 cm), small amounts of material at the base-line and at 12 cm, together with an increased amount of material having the mobility (15 cm) of glycerolphosphorylglycerol. This latter component was isolated by preparative paper chromatography and assayed for total phosphate and for formaldehyde produced after periodate oxidation. Two molar proportions of formaldehyde were formed per mol of phosphate. Acid hydrolysis of the material gave equal amounts of radioactive glycerol and radioactive glycerol monophosphate and so the material is character-

ized as glycerylphosphorylglycerol in which the phosphodiester group is attached to a primary hydroxyl group in each glycerol residue.

Determination of the activity of the sodium boro- $^{[3]}\text{H}$ hydride solution used in the reduction of the oxidized teichoic acid. A solution of glucosamine 6-phosphate (3.15 μmol) in water (0.10 ml) was mixed with a portion (0.10 ml) of the sodium boro- $^{[3]}\text{H}$ hydride solution used to reduce the teichoic acid. Reduction was allowed to proceed for 20 min at room temperature before the addition of 0.5 M-acetic acid (0.10 ml) to destroy excess of reagent. After repeated evaporation to dryness the residue was applied to a column (5 mm $\times$ 20 mm) of DE 52 cellulose which had been equilibrated with 0.5 M-pyridine acetate buffer, pH 5.3. Neutral products were eluted with water and the phosphate was eluted by using a linear gradient (0–0.5 M) of pyridine acetate (100 ml). The fractions of the single phosphate peak were combined and assayed for phosphate and for radioactivity. The specific radioactivity of the glucosaminitol 6-phosphate was 1.68 $\mu\text{Ci}/\mu\text{mol}$.

Interaction of the teichoic acid with concanavalin A. Teichoic acid (12.8 mg) was dissolved in water (2 ml) and added to a solution (2 ml) of concanavalin A (20 mg) in 0.01 M-sodium acetate buffer, pH 5.8 (half-saturated with sodium chloride), and the mixture was allowed to stand at room temperature for 30 min. A flocculent precipitate formed which was removed by centrifugation before addition of a further portion (20 mg, in solution as before) of concanavalin A. A second precipitate formed which was removed by centrifugation.

The combined precipitates were washed once with acetate buffer and suspended in a solution (2 ml) of α -methyl glucopyranoside (0.96 mg/ml) whereupon the precipitate dissolved. Analysis showed that 13% of the teichoic acid phosphate had been precipitated and that the precipitate contained 65% of the added concanavalin. The protein was precipitated by addition of cold 40% (w/v) trichloroacetic acid (0.5 ml) and removed by centrifugation. Trichloroacetic acid was removed from the supernatant solution by extraction with ether and the supernatant was then dialysed against water overnight. The non-diffusible material was freeze-dried, dissolved in 2 M-sodium hydroxide (2 ml) and heated at 100°C for 3 h. The hydrolysate was dephosphorylated enzymically and examined by paper chromatography (solvent B, reagent 1) against a similar hydrolysate of the teichoic acid remaining in the supernatant solution after removal of the precipitate formed by addition of concanavalin A and of unfractionated teichoic acid. The precipitated teichoic acid gave only one product on hydrolysis with alkali and enzyme. This was α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-

glycerol, which must therefore occur in a homogeneous polymer containing only this glucoside. The amount of this glucoside in the unprecipitated teichoic acid was considerably decreased, the major components now being the isomeric diglucosylglycerol and the monoglucosylglycerol.

DISCUSSION

Alkali hydrolysis of the wall teichoic acid(s) of *L. plantarum* C106 results in its quantitative conversion into the monophosphates of its three component glycosides. The structures previously assigned to these glucosides have been confirmed by methylation analysis and, in addition, the stereochemistry of the substitution of the glycerol moieties in two of the glucosides has been determined by enzymic assay (cf. Archibald *et al.* 1961) of the glyceric acid derived (cf. Brundish *et al.* 1967) by oxidation with nitrogen dioxide and periodate. These glucosides are therefore α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol ('glucosylglycerol'), α -D-glucopyranosyl-(1 $\rightarrow$ 2)- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol ('kojibiosylglycerol') and α -D-glucopyranosyl-(1 $\rightarrow$ 3)-glucopyranosyl-(1 $\rightarrow$ 1)-L-glycerol ('nigerosylglycerol'). On the basis of the yields of the glucosides isolated after alkali hydrolysis of walls they occur in the molar proportions 12:21:17 respectively. Teichoic acid containing only these units in these proportions would have a glucose/phosphorus ratio 1:1.76. The discrepancy between this and the value (1:1.60) found for teichoic acid isolated by extraction of wall with alkali may imply variability in composition of different wall samples or may be simply due to losses during isolation of the glucosides.

Oxidation of the teichoic acid with sodium metaperiodate resulted in the reduction of 2.3 mol of periodate and the liberation of 1.1 mol of formic acid/mol of phosphate and the destruction of 77% of the glucose. Oxidation of a mixture of the above glucosides in the proportions found in the teichoic acid would result in reduction of 3.42 mol.prop. of periodate, liberation of 1.0 mol.prop. of formic acid and destruction of 81% of the glucose. Since in the teichoic acid the theoretical destruction of glucose occurred, the decreased reduction of periodate implies that the glycerol residues are protected against oxidation. The phosphate groups must therefore be located either exclusively on glycerol [i.e. (2 $\rightarrow$ 3)-linked as in structure (I)] or on glycerol and the primary hydroxyl group of glucose [as in structures (II)–(V) (Fig. 1)]. Teichoic acids of the former structural type have been reported to be present in walls of *Actinomyces antibioticus* 39 (Naumova & Zaretskaya, 1964) and *Bacillus stearothermophilus* B 65 (Wicken, 1966), whereas the wall

Fig. 1. Location of the phosphodiester groups in the teichoic acids.

teichoic acids of *B. licheniformis* 9945 (Burger & Glaser, 1966) have been shown to have the latter type of structure in which the sugar residues form an integral part of the polymer chain. Both types of structure would hydrolyse completely in alkali to a mixture of the monophosphates of the component glucosides (for discussion of the alkali hydrolysis of teichoic acids see Archibald & Baddiley, 1966) and a distinction between these two structural types has been made by further study of the products of periodate oxidation of the teichoic acid. These were found to undergo ready depolymerization in the presence of base whereas the unoxidized teichoic acid was stable under similar conditions. Thus treatment of oxidized teichoic acid in 0.1M-sodium hydroxide for 16h at 37°C led to the formation of 68% phosphomonoester groups whereas the monoester content of unoxidized teichoic acid (6%) did not increase perceptibly under these conditions. Degradation was even more extensive in glycine buffer, pH 10.5, when 78% of the phosphate was present as monoester after 5h at 22°C. Oxidation of a 1-substituted (2→3)-linked polyglycerol phosphate (I) should not significantly increase the lability of the phosphodiester groups to base whereas elimination of phosphate from C-6 of an oxidized glucose residue (carrying an aldehydic function at C-4) would proceed readily under these conditions (Scheme 1). These results, together with the previously reported formation of glucose phosphate on acid hydrolysis of the teichoic acid (Adams *et al.* 1969), support a structure of type (II) (Fig. 1). Failure of sodium hydroxide to effect complete elimination of phosphate has also been encountered in studies on oxidized RNA and has been ascribed to the intervention of Cannizzaro reactions (Fry, Wilson & Hudson, 1942). The difficulty can be overcome to some extent by effecting β -elimination in glycine buffer rather than sodium hydroxide (Brown & Todd, 1955) and in the present study this resulted in 78% conversion into monoester.

On the basis of the periodate oxidation studies the phosphate attached to the glucose moiety of the kojibiosylglycerol and the nigerosylglycerol must be attached to C-6 but may be on either of the two glucose moieties in each glycoside. Elimination would proceed readily from either of these positions in the oxidized kojibiosyl residue but only from the outer glucose moiety of the nigerosyl residue, since the inner glucose moiety (i.e. that attached to glycerol) is substituted at C-3 and so is resistant to oxidation by periodate. If in the teichoic acid the phosphate was attached to the inner glucose moiety, as in structure (V), 34% of the phosphodiester groups should not be susceptible to β -elimination and the maximum value for conversion into monoester should therefore be 66%.

Release of 78% monoester by glycine buffer suggests therefore, especially in view of the difficulty of obtaining quantitative elimination, that the phosphodiester group is unlikely to be attached to the inner glucose residue of the nigerosylglycerol unit.

Further evidence for the location of the phosphodiester groups was obtained by reduction of the oxidized teichoic acid with sodium boro[³H]hydride. Analysis showed that approx. 3.2 aldehyde groups were reduced per phosphate (theory 2.8). Acid hydrolysis of the teichoic acid gave labelled glycerol and labelled glycerol phosphate. The latter can arise only by reduction at C-4 of an oxidized glucosyl residue which carried a phosphate group at C-6, and so its formation confirms the presence of glucose in the chain as shown in Scheme 1. Since each phosphodiester group should have an equal chance of hydrolysis on to the glycerol moiety to which it is originally attached and on to the glycerol moiety formed on reduction, it follows that a structure in which phosphate is attached to the outer glucose residue should give glycerol phosphate in which the glycerol is derived equally from that originally present in the teichoic acid and that formed by reduction of the oxidized glucose moiety, i.e. 50% of the glycerol phosphate should be derived from carbon atoms 4, 5 and 6 of glucose. A structure in which the phosphate is attached to the inner glucose moiety would produce a smaller amount of labelled glycerol phosphate since that formed by hydrolysis of the phosphate connecting glycerol to the inner (i.e. unoxidized) glucosyl moiety of a nigerosylglycerol residue would be unlabelled. Teichoic acid with this type of structure would therefore give glycerol phosphate of which only 33% of the glycerol moiety is derived from carbon atoms 4, 5 and 6 of glucose. The specific radioactivity of the glycerol phosphate formed from the teichoic acid shows that 48% of it is derived from the oxidized and reduced glucose so that the phosphate in the original teichoic acid must be attached to the outer glucose residue of the nigerosylglycerol units. The above results do not permit distinction of inner and outer glucose residues in the kojibiosylglycerol residues but the phosphate groups are shown in structure (III) attached to the outer residues by analogy with the other units.

The glycerol phosphate moiety in teichoic acids is derived biosynthetically by transfer from CDP-glycerol and so one would expect the phosphate to be attached to the 3 (α) position of glycerol in the teichoic acid. This has been confirmed experimentally by isolation of glycerylphosphorylglycerol by partial acid hydrolysis of the reduced products of oxidation of the teichoic acid. The isolated glycerylphosphorylglycerol had the same chromatographic

Scheme 1. Degradation of the teichoic acid by oxidation with periodate and reduction with NaBH_4 .

mobilities as an authentic sample and gave equivalent amounts of glycerol and glycerol monophosphates on acid hydrolysis. The specific radioactivity of the compound was consistent with the presence of one glycerol moiety derived by reduction of oxidized glucose so that the second glycerol moiety represents that originally present in the polymer. The material reduced 2 mol.prop. of periodate so that the phosphate is attached to a primary hydroxyl group of each glycerol residue. Since in the teichoic acid the 1 (L) position of glycerol is glycosidically substituted it follows that the phosphate is attached to the 3 (L) position as in CDP-glycerol.

The point of attachment of the D-alanyl ester substituents to the teichoic acid has not been established, although it has been shown that they exhibit the high lability towards base which is characteristic of alanyl esters that are adjacent to phosphate or to free hydroxyl groups. They may therefore be attached to the secondary hydroxyl groups of a proportion of the glycerol residues, as in the simple (1→3)-polyglycerol phosphate teichoic acids, but attachment to a secondary hydroxyl group of glucose cannot be excluded. Isolated teichoic acid has an average chain length, calculated from its phosphomonoester content, of about 18 units; that in the wall may be of similar size but there does not at present appear to be any direct method for the investigation of this.

The remaining problem is that of the distribution of the various subunits in the polymer. These units do not occur in simple integral proportions so that a regular polymer containing all three subunits is unlikely. It is possible therefore that like the teichoic acids in walls of *B. subtilis* W 23 (Chin, Burger & Glaser, 1966) and *Staphylococcus aureus* Copenhagen (Torii, Kabat & Beyer, 1966) the different units occur in separate chains. Information about this has been obtained by the use of the lectin concanavalin A. This interacts specifically with α -D-glucopyranosyl and α -D-mannopyranosyl residues with unsubstituted hydroxyl groups at C-3, C-4 and C-6 (Goldstein, Hollerman & Smith, 1965b). Concanavalin A gives a precipitin reaction with neutral polysaccharides such as glycogens (Manners & Wright, 1962), amylopectins (Goldstein, Hollerman & Merrick, 1965a), dextrans, levans and yeast mannans (Goldstein & So, 1965). We have found (Coapes, 1970) that concanavalin A also gives precipitin reactions with certain teichoic acids and acts as an agglutinin for certain bacteria and bacterial walls. Initial studies showed that whole cells and cell walls of *L. plantarum* C 106 were agglutinated with concanavalin A and that purified wall teichoic acid gave a single precipitin band on double diffusion in agar gel against the lectin.

Consideration of the structures of the repeating

units in the teichoic acid shows that the glucosyl-glycerol units are substituted at C-6 of glucose by phosphate and so should not interact with concanavalin A. The non-reducing terminal glucose moiety of the nigerosylglycerol units also carries a phosphate substituent on C-6 and the inner glucose residue is substituted at C-3 so that neither glucose residue should interact with concanavalin A. The non-reducing terminal glucose moiety of the kojibiosylglycerol unit is also substituted at C-6 but the inner glucose moiety, being substituted at C-2, has unmodified hydroxyl groups at C-3, C-4 and C-6 and so ought to interact with concanavalin A. The precipitate which is formed with concanavalin A should therefore contain this unit. The other units will also be present only if they are in the same polymer chain. The precipitated teichoic acid was recovered, hydrolysed with alkali and phosphatase and examined chromatographically. The sole product was kojibiosylglycerol so that the precipitated teichoic acid is a homogeneous polymer containing only this repeating unit. The teichoic acid which did not precipitate with concanavalin gave glucosylglycerol, nigerosylglycerol and a decreased proportion of kojibiosylglycerol, the presence of which may be due to incomplete precipitation with the concanavalin. Thus at least 30%, and possibly all, of the kojibiosylglycerol is present in chains which contain only this unit. The other units may also be present in homogeneous chains but experimental evidence for this has not been obtained. The small but variable amount of glycerol produced on alkali and enzyme hydrolysis of certain wall preparations is probably derived from a (1→3)-polyglycerol phosphate teichoic acid. The organism contains a membrane teichoic acid of this type and this may be present as contaminant in some wall preparations since preparations that give glycerol also give glycerol diphosphate on acid hydrolysis. Extensive washing with saline and water removes most or all of this material from the wall and acid hydrolysis of teichoic acid isolated from such walls gives little or no glycerol or its diphosphate. Walls of *L. plantarum* C 106 thus contain teichoic acids in which glucosyl residues form an integral part of the polymer chain. They are related structurally to the teichoic acids in walls of *B. licheniformis* A.T.C.C. 9945, which comprise a 3-O- β -D-galactopyranosyl-D-glycerol 1-phosphate polymer and a 3-O- α -D-glucopyranosyl-D-glycerol 1-phosphate polymer in both of which the phosphodiester linkage is attached to position 6 of glucose (Burger & Glaser, 1966). If, as seems likely, the three glucosides in walls of *L. plantarum* C 106 occur in separate chains it follows that both organisms contain an identical teichoic acid composed of 3-O- α -D-glucopyranosyl-D-glycerol 1-phosphate units. Biosynthesis of this teichoic acid in *B. licheniformis*

A.T.C.C. 9945 utilizes CDP-glucose and CDP-glycerol and has been shown (Stow, Starkey, Hancock & Baddiley, 1971) to proceed by transfer of glucose to a lipid intermediate which then accepts a glycerol phosphate moiety, the glycerol phosphate-glucose then being transferred to the growing polymer chain. This is the sole example, so far, of involvement of lipid intermediates in the biosynthesis of teichoic acids other than those containing *N*-acetylhexosamine 1-phosphate residues and it would be of interest to see whether the same biosynthetic path is used for the biosynthesis of the teichoic acid in *L. plantarum* C106. The presence of diglucosylglycerol teichoic acids in the latter organism presumably arises by the intermediary of diglucosyl lipid since it is clear from the location of the phosphodiester groups that glucosylation must precede polymerization of the units.

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