

The Intracellular Teichoic Acid from *Lactobacillus arabinosus* 17-5

By P. CRITCHLEY, A. R. ARCHIBALD* AND J. BADDILEY

Department of Chemistry, King's College, University of Durham, Newcastle upon Tyne

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It has been shown that extracts of certain bacteria contain both glycerol teichoic acid and ribitol teichoic acid, although the walls of these organisms contain only the ribitol compounds (Armstrong, Baddiley, Buchanan, Cars & Greenberg, 1958). The conclusion that glycerol teichoic acids occur in an unspecified region beneath the wall was substantiated in a more extensive survey (Baddiley & Davison, 1961; and unpublished work) in which separated walls and cell contents of many bacteria were examined. It was found that glycerol or ribitol teichoic acids may occur in considerable amounts in a number of walls, but in some cases neither compound is present. On the other hand, smaller amounts of glycerol teichoic acids were isolated from the cell contents of very nearly all the organisms studied in these Laboratories.

It is now thought that a small amount of glycerol teichoic acid may well occur in the cell contents of all Gram-positive bacteria. In the many cases examined, the only method used so far for the reliable detection of these compounds is actual isolation, followed by hydrolysis and identification of hydrolysis products. The isolation procedure is difficult and probably not quantitative; consequently, in the few cases where no teichoic acid was observed it is possible that the amount is small or that other substances interfere with the isolation. The general term 'intracellular teichoic acid' is applied to these compounds on the understanding that they occur in a region enclosed by the cell wall.

The structure of only one of the intracellular compounds has been studied in detail. That from *Lactobacillus casei* A.T.C.C. 7469 was isolated in an impure state from the cell contents of the organism and was identified as a glycerol 1,3-phosphate polymer bearing D-alanine ester residues at position 2 on most of the glycerol residues (Kelemen & Baddiley, 1961). Unlike the ribitol teichoic acids, this compound apparently contains no sugar residues, although the presence of considerable amounts of carbohydrate impurities would have prevented the detection of small numbers of sugar residues attached to the teichoic acid.

The increasing recognition that teichoic acids possess serological properties (Baddiley & Davison, 1961; Haukenes, Ellwood, Baddiley & Oeding,

1961; Sanderson, Juergens & Strominger, 1961; Elliott, 1962; cf. also McCarty, 1959), and the possibility that their widespread occurrence within the cell might indicate a metabolic role, have justified more extensive studies on the structure of both wall and intracellular types. In this paper the isolation and purification of the intracellular glycerol compound from *Lactobacillus arabinosus* 17-5 is described, and evidence for its structure is discussed.

EXPERIMENTAL AND RESULTS

Materials. Sephadex G-25 and G-75 were purchased from Pharmacia, Uppsala, Sweden, ECTEOLA and diethylaminoethylcellulose ion-exchangers from H. Reeve Angel and Co Ltd., London, Glucostat reagent from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and calf-intestinal phosphomonoesterase from Sigma Chemical Co., St Louis, Mo., U.S.A. The glucosidase was supplied by Dr W. J. Whelan and was specific for β -glucoside.

Paper chromatography. Whatman papers nos. 1, 4 and 3 MM were used after washing with 2*N*-acetic acid and then with water. The following solvent systems were employed: (A) propan-1-ol-ammonia (sp.gr. 0.88)-water (6:3:1) (Hanes & Isherwood, 1949); (B) butan-1-ol-ethanol-water-ammonia (sp.gr. 0.88) (40:10:49:1, organic phase; Foster, Horton & Stacey, 1957). Compounds were detected on papers by the following reagents wherever appropriate: periodate-Schiff reagents for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), molybdate reagents for phosphoric esters (Hanes & Isherwood, 1949), aniline phthalate for reducing sugars (Partridge, 1949) and ninhydrin for amino acids (Conden & Gordon, 1948).

Isolation of teichoic acid from whole cells. The organism was grown and harvested as described by Archibald, Baddiley & Buchanan (1961*b*). Fresh washed cells were treated successively with acetone, ethanol and ether at room temperature. The dry, fat-free cells (70 g.) were stirred with 10% trichloroacetic acid solution (500 ml.) for 12 hr. at 4°. After centrifuging, the supernatant was mixed with 2 vol. of ethanol and kept at 4° for 2 days. The yellowish precipitate was centrifuged and dried with acetone and ether. The cell debris was again extracted with 10% trichloroacetic acid solution (500 ml.) for 24 hr. at 4°, and the supernatant treated as before. A third trichloroacetic acid extraction was carried out for 40 hr. at 4° and the material again precipitated with ethanol. The supernatants from the three ethanol precipitations were separately mixed with 2 vol. of acetone and kept for 2 days at 4°. Precipitates obtained in this way were washed with acetone and then ether.

* Frank Schon Fellow.

Samples (2 mg.) of each precipitate were hydrolysed with 2N-hydrochloric acid in sealed tubes for 3 hr. at 100°. After evaporation *in vacuo* over potassium hydroxide, the residues were examined by paper chromatography in solvent (A). Results are given in Table 1. A similar procedure of acid hydrolysis and paper chromatography was applied to the identification of compounds obtained from the various fractions obtained during the purification procedures described later.

The combined material (6.7 g.) precipitated by ethanol from the second and third trichloroacetic acid extracts was stirred with 10% trichloroacetic acid solution (50 ml.) for 15 hr. at 4° and insoluble material was centrifuged. Ethanol (2 vol.) was added to the supernatant and the mixture was kept at 4° for 2 days. The precipitate (4.6 g.) was collected by centrifuging and dried with acetone and ether. This material contained about 30% of polynucleotide, much of which was removed by triturating with ice-cold water and centrifuging; the insoluble residue (0.81 g.) obtained at this stage contained no teichoic acid. The supernatant was freeze-dried, giving a solid (3.78 g.). Dialysis of a solution of this material against distilled water at 4° through a Visking membrane removed much polynucleotide and its degradation products and a little ribitol teichoic acid; nearly all the glycerol teichoic acid remained in the dialysis tube.

Impure glycerol teichoic acid (2 g.) was recovered by freeze-drying. It was dissolved in aq. N-ammonia solution (20 ml.) and kept for 5 hr. at room temperature to remove alanine ester residues and to precipitate a further amount of polynucleotide. After centrifuging and dialysis the resulting pale-brown solution was freeze-dried to a powder (1.43 g.).

Electrophoresis. Samples of the impure teichoic acid prepared in the manner described above were examined by electrophoresis on Whatman no. 1 paper in 0.05M-veronal-hydrochloric acid buffer, pH 9.0, for 5 hr. with a potential of 8V/cm. Elution of areas containing phosphorus, followed by acid hydrolysis and paper chromatography, showed that the glycerol compound had the greatest mobility, but separation of components was incomplete. In a continuous-electrophoresis assembly with side electrodes (Shandon Scientific Co. Ltd., London, S.W. 7) with Whatman no. 4

paper, separation was still unsatisfactory and only a small proportion (about 2%) of the teichoic acid in a sample was obtained pure.

Sephadex gel filtration. Sephadex G-25 (25 g.) was allowed to swell for 30 min. in 0.05M-sodium chloride solution, poured into a tube (40.5 cm. × 2 cm.) and then washed with water until free from Cl⁻ ions. Impure teichoic acid (49 mg.) was dissolved in water (5 ml.) and a sample (0.1 ml.) was removed and diluted to 2 ml. for analysis. The remaining solution was passed through the column at a rate of 1.5 ml./min., and this was followed by a quantity of water. Fractions (2.5 ml.) were collected automatically. The total phosphate (Chen, Toribara & Warner, 1956) and extinction at 260 m μ of the contents of each tube were determined. Results are given in Fig. 1.

A similar experiment with a column of Sephadex G-75 was carried out on 51.7 mg. of teichoic acid in 6 ml. of water. The rate of flow through the column was 0.5 ml./min. Results are shown in Fig. 2.

Chromatography on ECTEOLA cellulose. The cellulose ion-exchanger had a capacity of 0.5 m-equiv./ml. and was prepared and packed in a column by a procedure similar to that described by Tener, Khorana, Markham & Pol (1958). Impure teichoic acid (410 mg.) prepared by the solvent-extraction procedure described above was dissolved in water (15 ml.), adjusted to pH 4.6 with hydrochloric acid and made to 25 ml. with water. A sample (0.2 ml.) was removed for analysis and the remainder was applied to a column (45 cm. × 1.8 cm.) of ECTEOLA cellulose (chloride form). Elution was effected with a linear gradient; the mixing vessel contained water (5 l.) and the reservoir contained 0.5M-lithium chloride solution (5 l.) adjusted to pH 4.6 with hydrochloric acid. Fractions (25 ml.) were collected automatically at a flow rate of 0.6 ml./min., each tube in the fraction collector containing 0.5 ml. of aq. 2N-ammonia solution; the fractionation was carried out at 4°. In a control experiment glycerol 1(3)-phosphate in 0.5M-lithium chloride solution at pH 4.6 was kept at 4° for the same time (5 days) as was required for the fractionation. The phosphorus content and *E* at 260 m μ of each fraction are shown in Fig. 3. Appropriate fractions were combined and evaporated to dryness at 32° in a rotary evaporator. The lithium chloride was removed by

Table 1. *Hydrolysis products of trichloroacetic acid extracts from whole cells*

Samples obtained by ethanol or acetone precipitation were hydrolysed in 2N-hydrochloric acid and examined in solvent (A).

	First extract		Second extract		Third extract	
	Ethanol ppt.	Acetone ppt.	Ethanol ppt.	Acetone ppt.	Ethanol ppt.	Acetone ppt.
Products with <i>E</i> at 260 m μ	Trace	+	+	+	+	+
Glycerol	Trace	-	+	-	+	-
Anhydroribitol	+	-	+	Trace	+	Trace
Ribitol	+	-	+	-	+	-
Glucose	+	+	+	+	+	Trace
Ribitol and/or glycerol phosphates	+	-	+	Trace	+	Trace
Anhydroribitol phosphate	+	-	+	Trace	+	-
Inorganic phosphate	+	+	+	+	+	+
Ribitol and/or glycerol diphosphates	+	+	+	+	+	Trace
Rhamnose	+	+	Trace	+	-	Trace
Ribose	Trace	+	+	+	+	+
Alanine	+	Trace	+	+	+	Trace
Other amino acids	Trace	Trace	Trace	Trace	Trace	-

extraction with ethanol-acetone (1:1, v/v), followed by dialysis in water, and material was recovered by freeze-drying. Samples (2 mg.) were hydrolysed in acid and products were identified by chromatography in solvents (A) and (B). Fractions 1-90 contained polynucleotide, 91-160 about equal amounts of ribitol teichoic acid and polynucleotide, 161-200 ribitol teichoic acid (70%) and polynucleotide, 201-240 ribitol teichoic acid (80%) and polynucleotide, 241-280 glycerol teichoic acid (75%) and ribitol teichoic acid and a trace of polynucleotide, and 281-400 glycerol teichoic acid.

In a larger-scale experiment a column (42 cm. \times 3 cm.) of the chloride form of the cellulose was equilibrated with 0.25M-lithium chloride solution at pH 4.6 and 4°. The teichoic acid (1.65 g.) in 0.25M-lithium chloride solution (50 ml.) at pH 4.6 was applied to the column and 5 l. of the equilibration solution was passed through at a rate of 1.2 ml./min. Gradient elution was effected with 0.25M-lithium chloride solution (5 l.) in the mixing vessel and 0.6M-lithium chloride solution (5 l.) in the reservoir, both at pH 4.6. Fractions (40 ml.) were collected automatically at a flow rate of 1.2 ml./min. in tubes containing ammonia solution, and analyses and recovery of material were carried out as described above. The first 5 l. of solution yielded 1.22 g. of polynucleotide and ribitol teichoic acid (P:glucose, 1:0.91); fractions 1-55 gave 109.7 mg. of

glycerol teichoic acid (90%) and ribitol teichoic acid (P:glucose, 1:0.26); fractions 56-105 gave 143 mg. of glycerol teichoic acid (98%) and ribitol teichoic acid (P:glucose, 1:0.14); fractions 106-150 gave 49 mg. of pure glycerol teichoic acid (P:glucose, 1:0.09). The course of the fractionation is shown in Fig. 4.

Chromatography on diethylaminoethylcellulose. The cellulose column was prepared by the procedure of Tener *et al.* (1958), 0.1N-sodium hydroxide and 0.1M-sodium hydrogen carbonate solutions being used. Teichoic acid (116 mg.) in water (5.0 ml.) was applied to the column (13 cm. \times 1.3 cm.) after removal of a sample (0.1 ml.) for analysis. After washing with water (100 ml.), elution was effected with a linear gradient by using water (1 l.) in the mixing vessel and 0.5M-ammonium carbonate solution

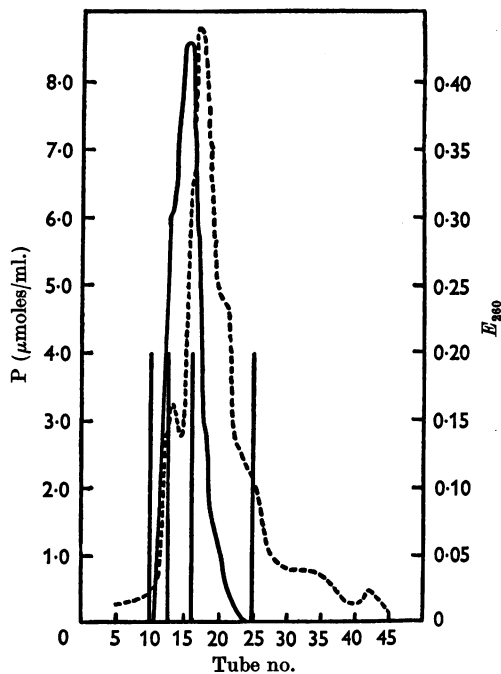


Fig. 1. Fractionation of teichoic acids and polynucleotides on Sephadex G-25. Alanine ester residues had been removed from the preparation by treatment with dilute ammonia (see text). The column (40.5 cm. \times 2 cm.) was prepared and used as described in the text. —, μ moles of phosphorus/ml.; - - -, E at 260 $m\mu$. In Figs. 1-5 and 8 the vertical lines indicate the way in which the fractions were pooled.

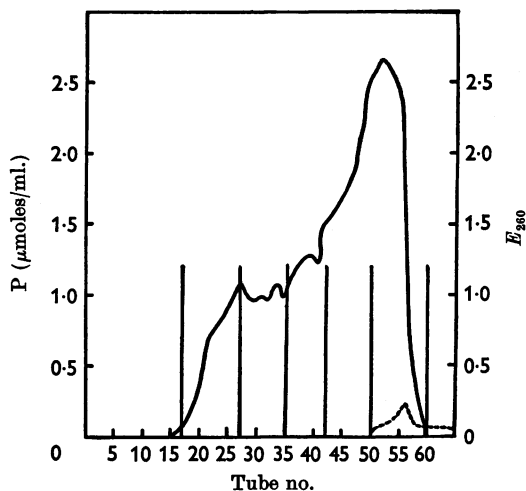


Fig. 2. Fractionation of teichoic acids and polynucleotides on Sephadex G-75. Alanine ester residues had been removed from the preparation by treatment with dilute ammonia (see text). A column (58 cm. \times 2.5 cm.) was used. —, μ moles of phosphorus/ml.; - - -, E at 260 $m\mu$. See legend to Fig. 1.

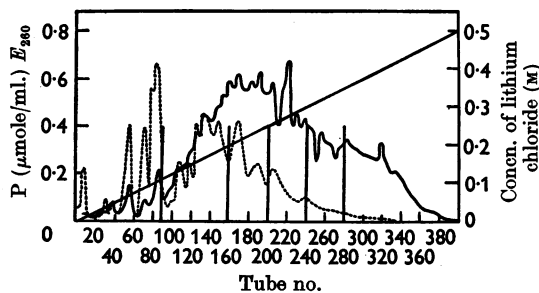


Fig. 3. Chromatography of impure teichoic acids on ECTEOLA cellulose. A column (45 cm. \times 1.8 cm.) of the chloride form of the cellulose was used; elution was effected with a linear gradient of lithium chloride. Alanine residues had been removed. —, μ moles of phosphorus/ml.; - - -, E at 260 $m\mu$. See legend to Fig. 1.

(1 l.) at pH 8.4 in the reservoir. Fractions were collected in the usual manner and ammonium carbonate was removed by repeated evaporation at 37°. Results were very similar to those obtained with the ECTEOLA columns.

Isolation of teichoic acid from cell contents. Cells (150 g. wet wt.) were grown and disrupted by the method described by Archibald *et al.* (1961*b*). Walls and cell contents were separated by centrifuging at 10 000*g* in a Spinco model G ultracentrifuge, and the supernatant with water washings (total 1 l.) filtered through Celite 545. 40% Trichloroacetic acid solution (100 ml.) was added with stirring, and after 12 hr. at 4° protein was removed in the centrifuge. Acetone (2 vol.) was added to the supernatant,

and after 2 days at 4° the precipitated impure teichoic acid (148 mg.) was centrifuged and dried with acetone and ether. The solid was stirred with 10% trichloroacetic acid solution (7 ml.) at 4° for 16 hr.; insoluble protein and polynucleotide were removed by centrifuging and ethanol (2 vol.) was added to the supernatant. After 2 days the precipitate (73 mg.) was collected and extracted with water (2 × 2 ml.). Combined extracts were freeze-dried (55 mg.). The insoluble material was mainly polynucleotide and a little ribitol teichoic acid. The freeze-dried material contained glycerol teichoic acid and smaller amounts of polynucleotide and ribitol teichoic acid.

Further purification was achieved on a Sephadex G-75 column as described for teichoic acid from which alanine had been removed (Fig. 5). Fractions 17–30 (21.5 mg.) contained glycerol teichoic acid, about 4% of the ribitol compound and a trace of polynucleotide; fractions 31–41 (41 mg.) contained glycerol teichoic acid and 2% of the ribitol compound; fractions 42–45 (9 mg.) contained almost pure glycerol teichoic acid; fractions 46–70 (28 mg.) contained glycerol teichoic acid and about 70% of polynucleotide.

Analysis and degradation of teichoic acid

Analysis. Purified material (1 mg.) from cell contents was hydrolysed in 2*N*-hydrochloric acid at 100° for 4.5 hr. The hydrolysate was neutralized (pH 7.0) with sodium hydroxide and the volume adjusted to 3 ml. Phosphorus, glucose (Park & Johnson, 1949) and alanine (Rosen, 1957) were determined: the ratio P:glucose:alanine was 1:0.33:0.885. Material from whole cells purified by cellulose ion-exchange contained phosphorus and glucose in the ratio 1:0.11. The reducing sugar was 97% D-glucose, determined enzymically with Glucostat reagents.

Hydrolysis and paper chromatography of products. Acid hydrolysis was carried out in 2*N*-hydrochloric acid for 3 hr. at 100° followed by evaporation to dryness *in vacuo*.

A sample of pure material (8 mg.) was hydrolysed with *N*-sodium hydroxide solution (0.5 ml.) for 3 hr. at 100° in a sealed tube. The solution was passed through a column of Dowex 50 (H⁺ form) (1 ml.) and the eluent was neutralized with ammonia and then freeze-dried.

Alkali hydrolysis and enzymic dephosphorylation were carried out on a slightly larger scale. Products of alkali hydrolysis were separated by chromatography as a band on Whatman paper no. 3MM; compounds were eluted from appropriate areas and recovered by freeze-drying. To a sample of the phosphates (7 mg.) in 0.05*M*-ammonium acetate solution (5 ml.) at pH 9.3 was added phosphatase (1 mg.) and the mixture was kept at 37° for 5 hr. After freeze-drying, the sample was examined by paper chromatography (Table 2).

Potentiometric titration. Pure teichoic acid (50 mg.) without alanine residues was dissolved in water (4 ml.) and passed through a Dowex 50 (H⁺ form) column (1 ml.). After elution with water (12 ml.) the solution was titrated with 0.1*N*-sodium hydroxide solution. Two peaks were observed in the curve $\Delta \text{pH}/\Delta V$ against *V*, where *V* is the volume of alkali used. The ratio of the two maxima was 17:1, corresponding to primary and secondary acidic groups respectively.

Periodate oxidation. Purified teichoic acid (25 mg.) from which alanine groups had been removed was dissolved in water (10 ml.), 0.133*M*-sodium metaperiodate solution

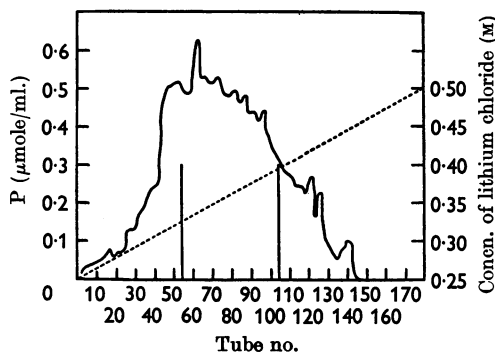


Fig. 4. Large-scale chromatography of teichoic acids on ECTEOLA cellulose. A column (42 cm. × 3 cm.) of the chloride form of the cellulose was used, and linear-gradient elution was carried out after elution to equilibrium with 0.25*M*-lithium chloride solution. —, μ moles of phosphorus/ml.; - - -, concentration of lithium chloride (M). See legend to Fig. 1.

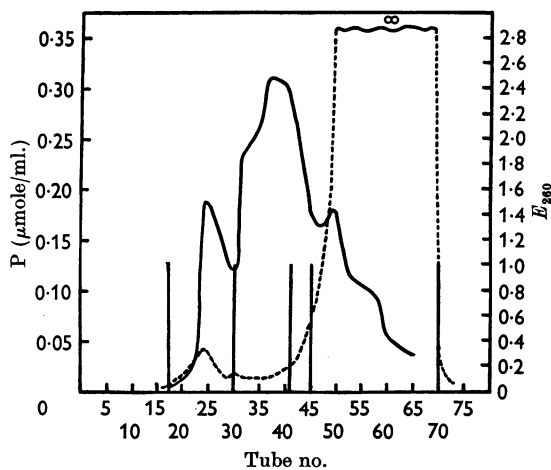


Fig. 5. Fractionation of glycerol teichoic acid from cell contents on Sephadex G-75. The teichoic acid possessed alanine ester residues. A column (60 cm. × 2.5 cm.) was used. —, μ moles of phosphorus/ml.; - - -, *E*₂₆₀. See legend to Fig. 1.

Table 2. *Products of hydrolysis by acid, alkali and phosphatase*

	Acid hydrolysis	Alkali hydrolysis	Alkali hydrolysis followed by enzymic dephosphorylation	R_{Glucose}	
				In (A)	In (B)
Glycerol	+	+	+	1.29	2.20
Alanine	+	+	+	1.05	0.86
Glucosylglycerol	-	+	+	1.10	1.15
Glucose	+	-	-	1.00	1.00
Diglycerol phosphate	-	-	+	0.95	.
Glycerol phosphates	+	+	-	0.54	.
Glucosylglycerol phosphate	-	+	-	0.52	.
Glycerol diphosphates	+	+	-	0.27	.
Diglycerol triphosphate	-	+	-	0.25	.
Inorganic phosphate	Trace	-	+	0.38	.

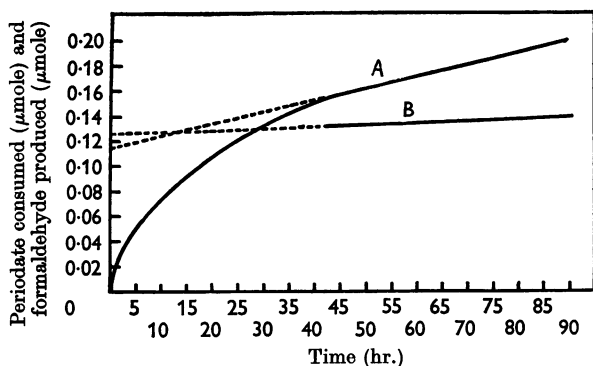


Fig. 6. Reaction of teichoic acid with periodate. *A*, Periodate consumed (μ moles). *B*, Formaldehyde produced (μ moles).

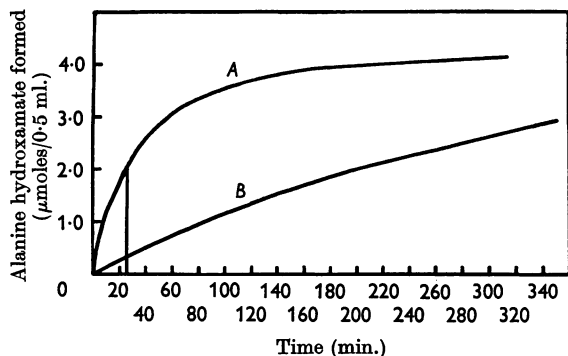


Fig. 7. Reaction of teichoic acid with hydroxylamine. *A*, Formation of alanine hydroxamate (μ moles) from teichoic acid. *B*, Formation of alanine hydroxamate (μ moles) from alanine methyl ester.

(10 ml.) was added and the mixture was kept in the dark at room temperature. The periodate was determined spectrophotometrically at intervals (Dixon & Lipkin, 1954) and formaldehyde was determined by two methods (Hough, Powell & Woods, 1956; Hanahan & Olley, 1958). Results

and the extrapolation to zero time are given in Fig. 6. The phosphorus content of the sample was determined (Chen *et al.* 1956), and after 50 hr. the ratio of periodate used to phosphorus was 1:14.5; after the same time the ratio of formaldehyde produced to phosphorus was 1:17.7.

Action of phosphatase, periodate and phenylhydrazine. Teichoic acid (6.5 mg.) lacking alanine was dissolved in 0.05M-ammonium acetate solution (25 ml.) at pH 9.3 to which phosphatase (0.8 mg.) had been added. The solution was kept at 37° and inorganic phosphate was determined at intervals. After 1.5 hr. the ratio inorganic P:total P was 1:16.2. The mixture was dialysed in water for 24 hr. and the residual material was oxidized with periodate as described above, but a large excess (10–12 times the calculated amount) of periodate was used since the phosphatase consumed periodate. The ratio of periodate consumed (corrected for the amount consumed by the enzyme) to phosphorus was 2:17.3. Oxidation of the enzyme produced no formaldehyde, so the ratio of formaldehyde to phosphate (2:17.8) in the oxidation experiment is probably more reliable.

The oxidation mixture was dialysed with water for 2 days and freeze-dried. The residue (3 mg.) was dissolved in 45% ethanol (5 ml.), and freshly distilled phenylhydrazine (0.3 ml.) was added. The pH was adjusted to 6.0 with acetic acid and the solution was refluxed at 90° for 1 hr. After cooling, osazones were removed by centrifuging and the supernatant was made alkaline with ammonia and exhaustively extracted with ether. The aqueous layer was evaporated to 2 ml. at 37° *in vacuo* and passed through a column of Dowex 50 (H^+ form) resin (2 ml.) to remove phenylhydrazine. The aqueous eluent and washings (16 ml.) were neutralized with ammonia and evaporated to dryness at 37° *in vacuo*. The reaction with phosphatase was repeated and the ratio of inorganic phosphate to total phosphate was 2:11.8.

Reaction between alanine ester residues and hydroxylamine. A sample (10 mg.) of teichoic acid with alanine ester residues still intact was dissolved in water (5 ml.) and portions (0.5 ml.) were mixed with 0.1M-hydroxylamine solution (0.5 ml.) at pH 7.4 and 37°. Reaction was terminated by adding *n*-hydrochloric acid (1 ml.), and alanine hydroxamate was determined at 540 $m\mu$ with M-ferric chloride solution (1 ml.). The rate of hydroxamate formation compared with that for alanine methyl ester is shown in Fig. 7. At least 90% of the alanine was present as reactive ester.

Isolation and identification of hydrolysis products from teichoic acid

Alanine. Purified material (125 mg.) from cell contents was dissolved in aq. *N*-ammonia solution and kept at room temperature overnight. The solution was evaporated to 3 ml. *in vacuo* and then passed through a column of Dowex 50 (H^+ form) resin (7 ml.). The glycerol phosphate polymer in the eluate and water washings (70 ml.) contained no alanine; this solution was neutralized with *N*-sodium hydroxide solution and used in the alkali-hydrolysis studies described below. Alanine was recovered by elution from the column with *N*-hydrochloric acid. Fractions (2.5 ml.) were collected during 3 hr. and appropriate ones combined and evaporated to dryness over sodium hydroxide. The alanine hydrochloride (34.5 mg.) contained no alanine amide. Its configuration was determined by the method described by Armstrong, Baddiley & Buchanan (1960); at least 92% of the material had the *D*-configuration.

Separation of alkali-hydrolysis products. The neutralized water eluate from the Dowex 50 column used in the experiment on the isolation of alanine was freeze-dried, and the residue was heated at 100° for 3 hr. in *N*-sodium hydroxide solution (2 ml.). After passage of the solution through a column of Dowex 50 (NH_4^+ form) resin (6 ml.), water was removed by freeze-drying and the concentrate adjusted to 25 ml. with water. A portion (0.2 ml.) was removed for analysis and the remainder was applied to a column (60 cm. \times 2 cm.) of diethylaminoethylcellulose (HCO_3^- form). Water (2 l.) was passed through the column and gradient elution was carried out with water (2 l.) in the mixing vessel and 0.15*M*-ammonium carbonate solution (2 l.) at pH 8.4 in the reservoir. Fractions (25 ml.) were collected automatically at a flow rate of 1 ml./min. Fractions were analysed for phosphorus, and material was recovered by repeated evaporation of ammonium carbonate at 37° *in vacuo*. The aqueous eluate contained glycerol and glucosylglycerol; these were purified further by chromatography on Whatman paper no. 3MM in solvent (B) and then on short charcoal-Celite columns (see below). The chromatography of phosphates is shown in Fig. 8. Peak 1 corresponded to glycerol phosphates, peak 2 to glucosylglycerol phosphate, peak 3 to glycerol diphosphates, and peak 4 to diglycerol triphosphate.

Glycerol. The material (13.8 mg.) eluted with water (200 ml.) from a charcoal-Celite column (8 cm. \times 1.5 cm.) was identified chromatographically as glycerol. It was dried over phosphoric oxide and dissolved in anhydrous pyridine (1 ml.) and then benzoylated with benzoyl chloride (0.2 ml.) in the usual manner. The product (44.8 mg., 74%) formed long needles; recrystallized from aqueous ethanol it had m.p. 74°, undepressed on mixing with authentic tri-*O*-benzoylglycerol, m.p. 74°. The infrared spectra of the two samples were identical.

2-O- α -D-Glucopyranosylglycerol. Material from the paper chromatography of the neutral fraction (see above) was adsorbed on a small charcoal-Celite (1:1) column. After washing with water, the glycoside was eluted with 5% ethanol. The eluate was evaporated to dryness *in vacuo*, water was added and the solution was clarified by centrifuging. The pure glycoside (2.7 mg.) was recovered by freeze-drying. It had $[\alpha]_D + 119 \pm 15^\circ$ (c 0.155 in water), and a ratio glycerol:glucose of 1:1. A sample was incubated at 37° with β -glucosidase (0.1% solution) for 24 hr. No

reducing sugar was detected before or after incubation. The hexa-*O*-(*p*-nitrobenzoyl) derivative was prepared from the glycoside (2.7 mg.) in pyridine (1 ml.) and *p*-nitrobenzoyl chloride (30 mg.) at 80° for 1 hr. It crystallized from acetone by addition of ethanol and had m.p. 187–188°. Recrystallization by the same procedure gave material with variable m.p. (160–190°). Similar variation in m.p. was observed with a sample of synthetic material provided by Mr P. W. Austin (unpublished work) and a sample from Dr A. S. Perlin. No depression in m.p. below 160° was observed on mixing together any of the above preparations. The infrared spectra of the three samples were identical.

The glycoside (0.15 mg.) in water (0.2 ml.) was mixed with 12 *mm*-sodium metaperiodate solution (0.5 ml.) and kept at room temperature in the dark. Portions were removed at intervals, and after suitable dilution the periodate consumption was determined by the method of Dixon & Lipkin (1954). After 48 hr. oxidation was complete, 1.9 mol.prop. of periodate having been consumed; no formaldehyde was detected. The oxidation mixture was passed through a column of Dowex 1 (acetate form) resin (1 ml.) and the eluate and water washings were freeze-dried. The residue was heated with 2*N*-hydrochloric acid at 100° for 24 hr. Solvent was evaporated over sodium hydroxide and products were examined by chromatography in solvent (A). Glycerol was detected but sugar was absent.

Diglycerol triphosphate. The teichoic acid (15 mg.) was hydrolysed with *N*-sodium hydroxide solution (0.3 ml.) for 3 hr. at 100° and the resulting solution was passed through a column of Dowex 50 (H^+ form) resin (1 ml.) which was then washed with water (10 ml.). Eluate and washings were combined and titrated with 0.1*N*-sodium hydroxide solution (carbonate-free). Two peaks were obtained by plotting $\Delta pH/\Delta V$ against *V*. The ratio of primary to secondary acidic groups was 1.17:1, indicating 7% of residual diester phosphate. A sample of the hydrolysed teichoic acid (8.2 mg.) was dissolved in 0.05*M*-ammonium

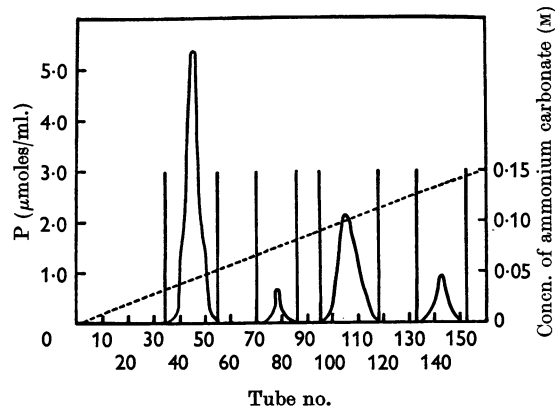


Fig. 8. Chromatography of alkali-hydrolysis products of glycerol teichoic acid on diethylaminoethylcellulose. A column (60 cm. \times 2 cm.) of the HCO_3^- form of the cellulose was used. Elution was carried out with a linear gradient of ammonium carbonate. —, μ moles of phosphorus/ml.; - - -, ammonium carbonate (M). See legend to Fig. 1.

acetate solution (10 ml.) at pH 9.3 containing phosphatase (0.8 mg.). The formation of inorganic phosphate at 37° was determined at intervals. After 25 hr. 8% of the total phosphate was still organically bound (diglycerol phosphate) and this did not decrease after further incubation.

The material (8.4 mg.) corresponding to the fourth peak from the diethylaminoethylcellulose column (Fig. 8) was shown by chromatography before and after phosphatase treatment to be diglycerol triphosphate. The ratio total phosphorus to inorganic phosphate liberated by the enzyme was 3.16:2 after 2 hr. The mixture was then freeze-dried and oxidized with 1.0 mM-sodium metaperiodate solution at room temperature in the dark. The ratio phosphorus (organic) to formaldehyde was 1:2.0.

DISCUSSION

Two methods have been used for the isolation of the intracellular teichoic acid from *L. arabinosus*: direct extraction from whole fat-free cells and extraction from cell contents after removal of walls. Washed walls contain a ribitol teichoic acid but no glycerol derivatives (Armstrong *et al.* 1958; Archibald *et al.* 1961*b*), whereas the cell contents or whole cells contain the glycerol teichoic acid. Thus it is permissible to use whole cells for the isolation of the intracellular compound.

The compounds extracted from whole cells and precipitated by either ethanol or acetone are mainly teichoic acids, polynucleotide and a polysaccharide containing rhamnose. Their relative ease of removal from the cell has been studied by examining acid hydrolysates of successive trichloroacetic acid extracts. The results in Table 1 indicate that the first compounds removed are ribitol teichoic acid and the rhamnose polysaccharide, both of which are in the wall. Later extracts contain larger amounts of glycerol teichoic acid and polynucleotide. These results are consistent with the view that the glycerol compound is intracellular. Moreover, treatment of cell contents under comparable conditions with trichloroacetic acid readily yielded the glycerol compound. Consequently, the rate-controlling processes in the removal of teichoic acids from walls and through walls are those of diffusion and ion-exchange, perhaps also of hydrogen-bond fission. This supports the conclusion of Archibald, Armstrong, Baddiley & Hay (1961*a*) that teichoic acids are held in walls by a combination of electrovalent linkage and hydrogen bonding, but could not be readily explained if the suggestion (Mandelstam & Strominger, 1961) that teichoic acids are chemically (covalently) bound to wall mucopeptide were correct.

High-speed centrifuging (20 000–100 000*g*) of cell contents yielded a gelatinous yellowish sediment that contained much RNA, some protein and the glycerol teichoic acid. The significance of this

aggregate and its relation to ribosomes are unknown, but similar complexes from other bacteria are under investigation. Most of the teichoic acid used in this present work was obtained by direct extraction from whole cells.

The second and third trichloroacetic acid extracts of whole cells contained most of the glycerol teichoic acid. It was recovered by precipitation and purified further by repeated dissolution and precipitation, followed by dialysis to remove compounds with low molecular weights. The main impurities at this stage were polynucleotide and some ribitol teichoic acid. Much of the nucleotide material could be precipitated by adding *N*-ammonia solution, and dialysis effected further purification. The labile alanine ester groups were removed during the ammonia treatment, but for most purposes this was unimportant. Unless otherwise stated, for the remainder of the discussion on isolation the term teichoic acid refers to the polymer lacking alanine residues.

Paper electrophoresis of material at this stage was not satisfactory. Under several conditions poor resolution and streaking were observed: the glycerol compound had the greatest mobility but true separation was not observed. In a preparative paper-electrophoresis apparatus giving a linear field across the paper better resolution was achieved, but this method was still unsuitable for large-scale work.

Filtration experiments with Sephadex gel (Figs. 1 and 2) were interesting but this procedure was of little use for purification purposes. Poor retention of teichoic acid was observed with Sephadex G-25, one bed-volume of eluent being sufficient for complete elution. Repetition with G-75 effected slight fractionation but 30% of the teichoic acid was eluted in one bed-volume of eluent. The manufacturers of these materials state that neutral dextrans having molecular weights up to 40 000 are retained on G-75, whereas the teichoic acids that were not retained have average molecular weights of only 3200. This apparent discrepancy may be explained by the high charge and extended shape of the teichoic acids (*cf.* Gelotte, 1960).

Purification on cellulose ion-exchange columns was more satisfactory. With ECTEOLA cellulose at pH 4.6, and with lithium chloride and hydrochloric acid, resolution was incomplete (Fig. 3). Loss of terminal phosphomonoester groups at this pH value was checked in a control experiment with glycerol phosphate: this loss was negligible when the experiment was carried out at 4° after preliminary elution of unwanted material with 0.25 M-lithium chloride, all fractions from the column being neutralized with ammonia (Fig. 4). As considerable cross-contamination of products was observed, analysis of each fraction was necessary.

Rechromatography did not effect further purification. Although chromatography on diethylaminoethylcellulose at pH 8.4 gave very similar results, these conditions caused no dephosphorylation and the procedure offered advantages in the ease of recovery of material. The fine-structure of the elution diagrams (Figs. 3 and 4) was largely reproducible, but it is not known whether this indicates fractionation of molecular species of differing sizes or sugar distribution.

For some purposes it was necessary to obtain teichoic acid without loss of alanine residues. The purification of this material was difficult, and to avoid as much as possible contamination with the ribitol compound it was necessary to isolate the teichoic acid from the separated cell contents. Cellulose ion-exchange chromatography was avoided to preserve ester linkages, but separation from polynucleotides was achieved by filtration through Sephadex G-75 (Fig. 5). The final product contained 2-4% of ribitol teichoic acid, possibly arising from the walls before centrifuging.

All samples of this teichoic acid contained glucose residues, and the purest samples prepared by cellulose ion-exchange chromatography gave consistently a ratio phosphorus:glucose of 1:0.11. Higher values for glucose (ratio 1:0.33) were obtained with material that had not been subjected to this chromatographic procedure, probably because of the presence of the ribitol teichoic acid, which is relatively rich in glucose (Armstrong *et al.* 1958; Archibald *et al.* 1961b). Phosphorus (15.3%) was present and the only amino acid was D-alanine, the configuration of which was established by quantitative oxidation with a kidney D-amino acid oxidase (cf. Armstrong *et al.* 1960). The ratio phosphorus:alanine in the best preparations was 1:0.89, but the amount of alanine was smaller in samples prepared under less gentle conditions.

In other teichoic acids it has been shown that the alanine is in ester linkage with hydroxyl groups on the polyol units (Armstrong *et al.* 1958; Armstrong *et al.* 1960, 1961; Kelemen & Baddiley, 1961; Archibald *et al.* 1961b); moreover, these esters are remarkably labile towards alkali and amines. Similarly, alanine ester linkages are present in the intracellular teichoic acid from *L. arabinosus*, as alanine and its amide were produced readily when the compound was treated with cold dilute aqueous or ethanolic ammonia. The rate of reaction between these ester groups and hydroxylamine is illustrated in Fig. 7. The reactivity of the ester linkages is comparable with that for other teichoic acids and closely resembles that found for a model compound in which an alanine ester residue is adjacent to a phosphate (Shabarova, Hughes & Baddiley, 1962). This indicates that all the alanine is attached to glycerol hydroxyl groups rather

than to glucose residues. The smoothness of the rate curve in Fig. 7 supports this conclusion; moreover, there is insufficient glucose in this teichoic acid to accommodate all the alanine.

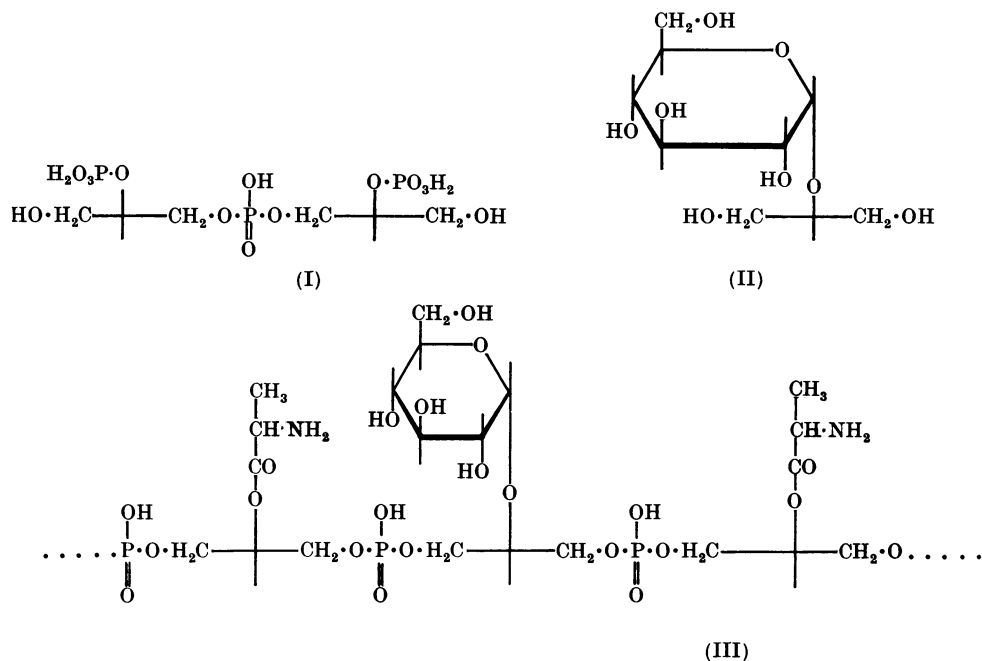
Acid hydrolysis of pure material gave alanine together with the compounds in Table 2. The formation of glycerol and its diphosphates, in addition to glycerol phosphates, shows that glycerol units are joined together through phosphodiester linkages; potentiometric titration confirmed this conclusion. When the teichoic acid was hydrolysed with hot alkali most of the phosphodiester bonds were destroyed; the mechanism of this hydrolysis, which involves intramolecular phosphorylation giving intermediate cyclic phosphates, has been discussed previously (Kelemen & Baddiley, 1961). The main products were monophosphates of glycerol, together with some glycerol and its diphosphates.

The alkali-hydrolysis products were examined by chromatography on diethylaminoethylcellulose. Phosphates were eluted with a linear gradient of ammonium carbonate, whereupon four fractions were obtained. Paper chromatography indicated that the first fraction contained the isomeric glycerol monophosphates, together with a little inorganic phosphate. The second fraction contained glucosylglycerol phosphate, the significance of which is discussed below. The third fraction contained the isomeric glycerol diphosphates, and the fourth contained the diglycerol triphosphate (I) previously detected during alkali hydrolysis of *L. casei* glycerol teichoic acid (Kelemen & Baddiley, 1961).

In the earlier work with material from *L. casei*, degradative evidence for structure (I) was obtained with impure material; in the present case, the cellulose and paper chromatography gave a pure compound. The ratio of total phosphate to inorganic phosphate released by the action of phosphomonoesterase was 3.16:2, the resulting diglycerol monophosphate was identified from its R_{glucose} value, by alkali hydrolysis to glycerol and glycerol phosphates and by periodate oxidation, whereupon 2 mol.prop. of periodate was consumed and 2 mol.prop. of formaldehyde was formed.

The amount of diglycerol triphosphate formed during hydrolysis of the teichoic acid was determined by two methods: potentiometric titration of the hydrolysate gave the proportion of unhydrolysed phosphodiester groups, and dephosphorylation with phosphomonoesterase gave the proportion of phosphomonoesters. Good agreement between the two methods was obtained and it was concluded that 7-8% of the phosphorus in the teichoic acid remained as phosphodiester after alkali hydrolysis.

The formation of the alkali-stable diglycerol triphosphate (I) during alkali hydrolysis of a glycerol



phosphate polymer is important, since it established the presence of 1,3-phosphodiester linkages between glycerol residues. The argument leading to this conclusion, and the mechanism of the formation of such a stable phosphodiester, have been discussed previously.

Alkali hydrolysis of the teichoic acid, followed by treatment with a phosphatase, gave glycerol, inorganic phosphate and a glucosylglycerol, together with a little diglycerol phosphate. After removal of acidic products with a cellulose ion-exchange column, the neutral compounds were separated on charcoal and by paper chromatography. Glycerol was characterized as its crystalline tri-*O*-benzoyl derivative.

The glucosylglycerol was a non-reducing gum with R_f slightly greater than that of glucose in two solvent systems. It was composed of equal parts of D-glucose and glycerol, and reacted slowly with the periodate-Schiff spray reagents on paper to give a blue-grey colour; in this last respect it resembles α -methyl glucoside and the α -glucosylribitol from *L. arabinosus*-wall teichoic acid (Archibald *et al.* 1961*b*). This shade of colour is often observed with α -glucosides; moreover, the absence of a rapidly developing magenta colour suggested that no formaldehyde was evolved in the periodate oxidation, and it follows that the glucosyl residue must be attached through an α -glycosidic linkage to the 2-hydroxyl group of glycerol.

Confirmation of the structure 2-*O*- α -D-glucopyranosylglycerol (II) for the glucoside was obtained

from periodate oxidation, rotation and enzymic studies. The high positive rotation and stability towards a β -glucosidase confirmed the presence of an α -glycosidic linkage. Moreover, it consumed 2 mol.prop. of periodate, giving no formaldehyde, and so must be a 2-*O*-glucopyranosylglycerol; the glycerol residue resisted periodate oxidation and was readily detected in acid hydrolysates of oxidation mixtures. A glucosylglycerol with structure (II) has been prepared synthetically (Charlson & Perlin, 1956; Charlson, Gorin & Perlin, 1957). The hexa-*O*-*p*-nitrobenzoyl derivative of the synthetic compound (kindly supplied by Dr A. S. Perlin) was indistinguishable from that of the glycoside from the teichoic acid.

Most of the glucose in the polymer is released as glucosylglycerol through the action of alkali. This is consistent with a structure (III) for this teichoic acid, in which most of the glucosylglycerol residues occur within the chain. As alkali hydrolysis of the phosphodiester groups must proceed through cyclic phosphate intermediates, it follows that glucosylglycerol units within the chain must always lose both of their attached phosphate groups. Moreover, as no larger fragments were observed in which two glucosylglycerol units are attached together through a stable phosphodiester linkage, it follows that the number of occasions when two glucose residues are attached to adjacent glycerol residues is negligible. The presence of a small amount of glucosylglycerol phosphate in alkali hydrolysates indicates that occasionally a

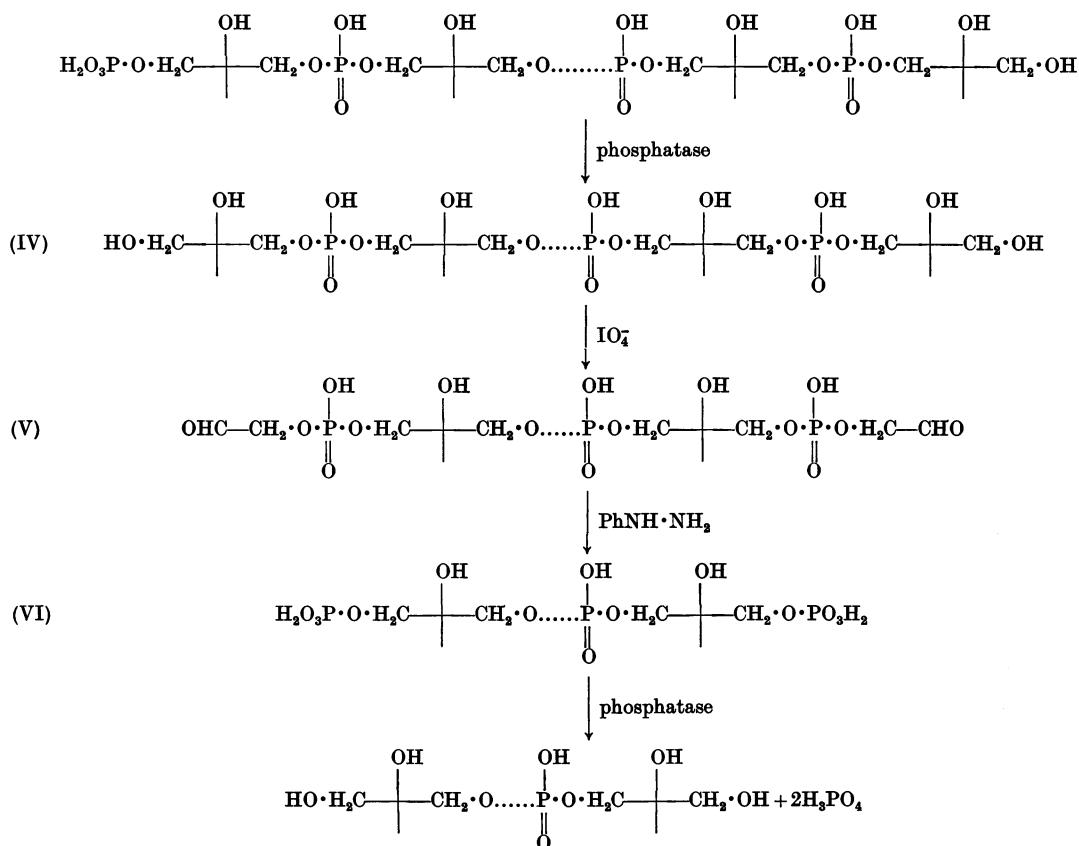
glucose residue occurs at the phosphate-terminal end of a molecule.

It has been suggested previously that teichoic acids are synthesized biologically by the transfer of polyol phosphate residues from nucleotides. Thus a glycerol teichoic acid should be a polymer of the common natural isomer *L*-glycerol 3-phosphate (the residue which occurs in cytidine diphosphate glycerol), and would terminate with a phosphomonoester residue at one end and a glycerol residue at the other end. It has not yet been possible to demonstrate the stereochemical point, but the end groups have been shown to be in accord with these suggestions.

Periodate oxidation of the polymer, after removal of alanine, should give 1 mol.prop. of formaldehyde from the end bearing a glycerol residue, and on treatment with a phosphomonoesterase 1 mol.prop. of inorganic phosphate should be released from the phosphorylated end. The ratio of total phosphorus to formaldehyde produced on oxidation decreased steadily with time

(Fig. 6), indicating a gradual increase in the amount of formaldehyde formed. Previous experiments showed that under these conditions (0.1 mM-periodate) the glucosyl residues were slowly oxidized, giving formic acid but not formaldehyde. It is likely that the consequent increase in acidity would cause hydrolysis of glycolaldehyde residues formed by oxidation of terminal glycerol groups; the free glycolaldehyde would then oxidize further to formaldehyde. In a control experiment with glycerol phosphate, the amount of formaldehyde produced in 91 hr. was 9.3% higher than the theoretical value, compared with a value of 5% for polymer in the same time. Consequently, this error was eliminated by extrapolating to zero time in the curves relating uptake of periodate and production of formaldehyde with time (Fig. 6). The ratio total phosphorus to periodate consumed was 20:1, and total phosphorus to formaldehyde formed was 18.9:1.

The presence of a phosphomonoester at the other end of the polymer was demonstrated by



* Scheme 1

titration and by the action of a phosphatase. The ratio primary to secondary acidic groups was 17:1, and the ratio total phosphorus to inorganic phosphate released by the enzyme was 16:2:1. The somewhat low value for the ratio of total to inorganic phosphate arose through hydrolysis of some phosphodiester linkages; the enzyme preparation hydrolysed phosphodiesters slowly. The conclusion that the teichoic acid is a glycerol 1,3-phosphate polymer containing about 18 glycerol phosphate units is supported in the following manner. Teichoic acid lacking its alanine was treated with phosphomonoesterase (Scheme 1). The product (IV) was oxidized at both ends of the chain with periodate; the ratio of phosphorus to formaldehyde produced was 17:8:2, indicating the presence of a glycol group at both ends of the chain. It follows that the phosphate-terminal end of the original teichoic acid must bear phosphate residues at the positions 1 and 3 on the glycerol. The oxidation product (V) was treated with phenylhydrazine at pH 6 (Brown, Hall & Letters, 1959; Barry & Mitchell, 1954), whereupon the two glycolaldehyde residues were removed, leaving a polymer (VI) containing two less glycerol residues than did the original compound and terminated at both ends by phosphomonoester groups. These phosphate groups were removed with the phosphatase, and the ratio total phosphate in (VI) to inorganic phosphate released was about 12:2, but the presence of phenylhydrazine prevented the determination of formaldehyde produced on further oxidation.

Structure (III) represents a part of the teichoic acid. The end-group analysis and titration studies described here indicate an average of 18 glycerol phosphate residues in the polymer, and two of these bear glucosyl substituents, most of the remainder bearing alanine ester groups. It is not known whether the chain length, the glucose content or the glucose distribution differ in different molecules.

SUMMARY

1. The intracellular teichoic acid from *Lactobacillus arabinosus* 17-5 has been isolated from whole organisms or cell contents and purified by solvent extraction and chromatography on cellulose ion-exchange columns. Its behaviour on electrophoresis and Sephadex gel filtration has been studied. It sediments as a complex with ribonucleic acid and protein on high-speed centrifuging of cell contents.

2. The polymer is composed of glycerol, phosphate, D-glucose and D-alanine. The alanine is in ester linkage with hydroxyl groups in glycerol residues; the reactivity of these ester groups towards hydroxylamine is similar to those in other teichoic acids and to that in a model compound

where phosphate and amino acid ester residues are adjacent.

3. The structure of the compound has been established by acid and alkali hydrolysis. An important product of alkali hydrolysis is the diglycerol triphosphate (I), identified by chromatography, titration and enzymic hydrolysis to diglycerol phosphate. About 7-8% of the phosphorus in the teichoic acid remained as phosphodiester after alkali hydrolysis.

4. A further product of alkali hydrolysis is 2-O- α -D-glucopyranosylglycerol (II), identified by hydrolysis, periodate oxidation, optical rotation and comparison with synthetic material.

5. The occurrence of phosphodiester linkages joining positions 1 and 3 in glycerol residues follows from the formation of the triphosphate (I), and is confirmed by degradation of the polymer by successive treatment with the following reagents: phosphatase, periodate, phenylhydrazine, phosphatase. The reaction sequence is outlined in Scheme 1.

6. The above and other evidence indicates that this teichoic acid has the structure (III), in which about 18 glycerol phosphate residues are joined through 1,3-linkages. α -D-Glucopyranosyl residues occur at position 2 on two of the glycerols, and most of the remaining glycerol residues bear D-alanine ester residues at their position 2.

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The Degradation of Heavy Meromyosin by Trypsin

BY H. MUELLER* AND S. V. PERRY

Department of Biochemistry, University of Birmingham

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Myosin was one of the first proteins with which it could be shown that appreciable proteolytic digestion of the molecule could occur without destruction of its biological activity (Gergely, 1950, 1953; Perry, 1950, 1951). Subsequent extension of these observations led to the isolation of the heavy and light meromyosins (Mihalyi & Szent-Gyorgyi, 1953; Szent-Gyorgyi, 1953; Gergely, Gouvea & Kariban, 1955) and the demonstration that the actin-combining and the adenosine-triphosphatase properties are associated with the former fragment which represents the major portion of the molecule (Lowey & Holtzer, 1959). Clearly a study of the fragments obtained on controlled digestion is of fundamental importance for relating the biological activities of myosin to the ultrastructure of the molecule, yet until studies by Mueller & Perry (1961*a*) heavy meromyosin was considered to be the only fragment which retained the adenosine-triphosphatase activity of myosin. Kakol, Gruda & Rzyzsko (1961) have produced evidence confirming the findings (Mueller & Perry, 1960, 1961*a*) that heavy-meromyosin preparations contain smaller fragments which possess adenosine-triphosphatase activity.

The present paper, which represents an extension of the investigation by Mueller & Perry (1961*a*), is a study of the tryptic digestion of heavy

meromyosin and provides evidence that the ability to combine with actin and to hydrolyse adenosine triphosphate can survive further tryptic digestion. These properties have been shown to be associated with a smaller readily-identifiable fragment which sediments at a slower velocity than does the original heavy meromyosin.

Some aspects of this study have been reported in a preliminary communication (Mueller & Perry, 1961*b*).

METHODS

Preparation of heavy meromyosin. L-Myosin, prepared as described by Perry (1955), was digested with trypsin at 23° for 10 min. by the method of Szent-Gyorgyi (1953) and immediately dialysed against 10 vol. of 6.7 mM-phosphate Sørensen buffer, pH 7.0. The precipitated light meromyosin was separated by centrifuging; the heavy meromyosin was salted out by $(\text{NH}_4)_2\text{SO}_4$ in the range 40–55% saturation, dissolved in 0.15M-KCl–20 mM-tris-HCl, pH 7.6, and stored at 1–2°.

Preparation of F-actin. Actin was prepared from rabbit skeletal muscle by the method of Straub (1943); it was purified by isoelectric precipitation with 10 mM-sodium acetate buffer, pH 4.7, or by ultracentrifuging according to the method of Mommaerts (1951).

Digestion of heavy meromyosin. Solutions containing 13.4–14.8 mg. of heavy meromyosin/ml. in 0.15M-KCl–20 mM-tris-HCl, pH 7.6, were generally used and digestion was carried out in a water bath at 25°. Trypsin (salt-free and twice crystallized; Worthington Biochemical Corp.) solutions (10–40 mg./ml.) in 5 mM-HCl were prepared fresh 1–2 hr. before each experiment and appropriate volumes

* Present address: Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pa., U.S.A.