prostatic phosphomonoesterase. The phosphodiester (V) was hydrolysed with alkali to glycerol and its monophosphates. It follows that the teichoic acid is a polymer in which glycerol residues are joined through phosphodiester linkages at positions 1 and 3 in the glycerol units.

4. The alanine from the polymer is readily oxidized with a D-amino acid oxidase, and thus has the D-configuration.

5. Alanine is joined to the polymer through ester linkages at the 2-position of each glycerol residue. The structure (VI) is assigned to this intracellular teichoic acid.

6. The alanine ester groups are highly reactive towards alkali and amines. They react with hydroxylamine at about the same rate as do amino acids in combination with ribonucleic acid.

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Further Studies on the Teichoic Acid from *Bacillus subtilis* Walls

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The ribitol teichoic acid from the walls of *Bacillus subtilis* is a polymer in which about nine β-D-glucosylribitol phosphate units are joined together through phosphodiester linkages involving the 1- and 5-positions of the ribitol residues. Most of the units bear D-alanine in ester linkage with a hydroxyl of either the glucose or ribitol (Armstrong, Baddiley & Buchanan, 1960).

Alkali hydrolysis of this teichoic acid yields a mixture of glucosylribitol phosphates, which in the previous work were partially characterized without separation. These have now been separated from each other and their structures established. The glucosylribitol diphosphates arising from one end of

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the chain have also been studied in greater detail. Periodate oxidation studies have been made in efforts to locate the alanine ester linkages.

MATERIALS AND METHODS

Growth of organism and isolation of teichoic acid. *B. subtilis* was grown with aeration in a peptone (2%)–sodium chloride (0·5%) liquid medium and teichoic acid was extracted from whole defatted cells with trichloroacetic acid as described by Armstrong *et al.* (1960).

The periodate oxidation studies on teichoic acid were carried out on neutral material. This was prepared by the prolonged cold extraction procedure, and the precipitated teichoic acid (100 mg.) was dissolved in water (25 ml.), neutralized (pH 6·0) by adding dilute ammonia with vigorous stirring and freeze-dried. Only a trace of free alanine was detected in this material by paper chromatography in butan-1-ol–water.

Chromatography. Paper chromatography was carried out on Whatman no. 1 or no. 4 paper previously washed with 2N-acetic acid then water. The solvent systems used were: *A*, propan-1-ol–ammonia soln. (sp.gr. 0·88)–water (6:3:1), (Hanes & Isherwood, 1949); *B*, *n*-butan-1-ol–ethanol–water–ammonia soln. (sp.gr. 0·88) (40:10:49:1) organic phase (Foster, Horton & Stacey, 1957).

Compounds were detected by the improved periodate–Schiff reagents for glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), the molybdate reagents for phosphoric esters (Hanes & Isherwood, 1949), the aniline phthalate reagent for reducing sugars (Partridge, 1949) and the ninhydrin reagent for amino acids (Consden & Gordon, 1948).

Charcoal chromatography was carried out with columns containing a mixture of charcoal/Celite–silica (2:1, w/w) (Zilliken, Rose, Braun & György, 1955). The Norit A charcoal (40 g.) was previously treated with boiling 20% acetic acid solution (300 ml.), filtered, washed by decantation with water (4 × 4 l.), filtered, washed with water (2 l.) and dried at 140° for 24 hr.

Quantitative analyses. Inorganic and total phosphate was determined by the method of Chen, Toribara & Warner (1956). Periodate oxidation was determined volumetrically by the use of 0·01N-sodium thiosulphate (Hough, Powell & Woods, 1956) or by measuring the decrease in ultraviolet-light absorption at 223 mμ (Dixon & Lipkin, 1954).

Periodate oxidation of teichoic acid. Neutral teichoic acid (22 mg.) was dissolved in water (3·0 ml.) and the solution was kept in the frozen state until required.

Ammonia (sp.gr. 0·88) (0·15 ml.) was added to a sample (1·0 ml.) of the teichoic acid solution and, after 5 hr. at room temperature, solvent was removed by evaporation *in vacuo*. The residue was dissolved in water (4·0 ml.), 0·2M-sodium acetate buffer, pH 5·8 (5·0 ml.) and 0·2M-sodium metaperiodate solution (0·6 ml.) were added and the volume was adjusted to 10 ml. with water.

To a further sample (1·0 ml.) of the teichoic acid solution was added 0·2M-sodium acetate buffer, pH 5·8 (5·0 ml.) and 0·2M-sodium metaperiodate solution (0·6 ml.) and the volume was adjusted to 10 ml. with water. A control mixture without teichoic acid was also prepared.

The solutions were kept in the dark at room temperature and samples (2·0 ml.) were removed at intervals for determination of periodate (Hough *et al.* 1956). The preparation that had had alanine ester linkages destroyed by addition of ammonia had ceased consuming periodate after 60 hr., whereas oxidation of the intact teichoic acid was still proceeding slowly after this time. Unused periodate was destroyed at this stage by saturation with sulphur dioxide, solvent was removed *in vacuo*, the residues were dissolved in 2N-hydrochloric acid (0·2 ml.), and the solutions were heated at 100° for 24 hr. in sealed tubes. After evaporation over potassium hydroxide the residues were dissolved in water and passed through small columns of Dowex 50 (H^+ form) and then Dowex 1 (Cl^- form) resins. The eluates were evaporated and examined by paper chromatography in solvent *A*. Intact teichoic acid gave anhydroribitol (R_F 0·75) and its 5-phosphate (R_F 0·29), whereas the polymer in which alanine ester linkages had been destroyed yielded neither of these products.

In a second study samples (0·1 ml.) were removed for phosphate determination; the ratio IO_4^- consumed: organic phosphate for intact teichoic acid and that with alanine removed was 3·32 and 2·71 respectively after 60 hr. oxidation. The corresponding results after 132 hr. oxidation were 3·69 and 2·92. The ratio expected from previous work is 2·97 for pure material. Even after oxidation for 132 hr., treatment with sulphur dioxide and acid still gave anhydroribitol and its 5-phosphate from intact teichoic acid.

Oxidation experiments were also carried out in the absence of buffer. After 60 hr. the polymer without alanine ester groups had consumed slightly more periodate than had the intact material. Destruction of periodate and acid treatment as before resulted in the formation of very small amounts of anhydroribitol and its 5-phosphate from the oxidized intact teichoic acid, but neither product was obtained from the teichoic acid which had had its alanine ester linkages destroyed.

Periodate oxidation of alanine and its methyl ester. Alanine methyl ester hydrochloride (21·7 mg.) and alanine (13·4 mg.) were each dissolved in 0·2M-sodium acetate buffer, pH 5·8, (12·5 ml.), 0·2M-sodium metaperiodate solution (1·5 ml.) was added to each and the volumes were adjusted to 25 ml. with water. A control solution without amino acid or ester was also prepared. Solutions were kept in the dark at room temperature and samples (2·0 ml.) were removed periodically for periodate determination. After 132 hr. alanine had consumed 0·114 and the methyl ester 0·025 mol.prop. of periodate.

Alkali hydrolysis and enzymic dephosphorylation of teichoic acid from whole cells. Material used in hydrolysis studies was isolated by extraction of whole defatted cells with trichloroacetic acid at 50° and consequently contained a small amount of the intracellular glycerol teichoic acid present in this organism (Armstrong *et al.* 1959). A sample (520 mg.) of this impure preparation was hydrolysed with alkali followed by dephosphorylation of products with prostatic phosphatase (Armstrong *et al.* 1960). The main product, glucosylribitol (157 mg.), was isolated by crystallization according to previous procedures (Found: C, 40·8; H, 7·2. Calc. for $C_{11}H_{22}O_{10},\frac{1}{2}H_2O$: C, 40·9; H, 7·2%). Anhydrous material was obtained by recrystallization with scratching from dry methanol–ether (Found: C, 41·7; H, 7·0. Calc. for $C_{11}H_{22}O_{10}$: C, 42·0; H, 7·1%).

The crystallization mother liquors were evaporated *in vacuo*, the residue (150 mg.) was dissolved in water and adsorbed on a charcoal (10 g.)–Celite (5 g.) column. Elution

of products was achieved by successive development with water (600 ml.), 2% ethanol (250 ml.), 5% ethanol (500 ml.) and 10% ethanol (250 ml.). The fraction from 90–180 ml. of water contained glycerol (10 mg.), that from 250–550 ml. of water ribitol (7 mg.); the fraction from 0–100 ml. of 2% ethanol contained ribitol and anhydroribitol (3 mg.), that from 100–500 ml. of 5% ethanol glycosylribitol (122 mg.), and that from 10% ethanol a trace of glucosylribitol.

The glycerol had R_F 0·75 in solvent *A* and $R_{ribitol}$ 1·47 in solvent *B*, reacted rapidly with the periodate–Schiff reagents and the resulting magenta spot faded slowly in a manner characteristic of acyclic polyols. It gave a crystalline tribenzoate (19 mg.), m.p. 74°, undepressed when mixed with authentic tri-*O*-benzoylglycerol (Found: C, 71·1; H, 5·1. Calc. for $C_{24}H_{20}O_6$: C, 71·3; H, 5·0%).

The ribitol had R_F 0·65 in solvent *A* and $R_{ribitol}$ 1·0 in solvent *B*. It was heated at 100° in 2N-hydrochloric acid for 17 hr. and the products were examined by paper chromatography. Anhydroribitol (R_F 0·75 in solvent *A* and $R_{ribitol}$ 1·38 in solvent *B*) was formed and a trace of unchanged ribitol was detected.

The glucosylribitol from the charcoal column was crystallized by concentrating the appropriate fractions to 5 ml., centrifuging to clarify and evaporating to dryness *in vacuo*. The residue was dissolved in aqueous alcohol and solvent was evaporated slowly. The material had m.p. 135–140°, $[\alpha]_D^{21}$ −21·3° in water (*c*, 1·5). The infrared spectrum (KBr disk) of this compound was identical with that given by the glucosylribitol obtained previously, and which is now believed to be a hemi-hydrate.

Preparation and isolation of glucosylribitol phosphates. The teichoic acid was hydrolysed with N-sodium hydroxide solution for 3 hr. at 100°, and the resulting mixture of organic phosphates was isolated as barium salts by adding barium acetate then ethanol (Armstrong *et al.* 1960). The ion-exchange separation conditions are similar to those used by Hara (1959).

A solution containing the mixture of barium salts (94 mg., 162 μmoles of P) in water (200 ml.) was passed through a column of Dowex 50 (NH_4^+ form) resin. The column was washed with water (500 ml.), and the combined eluate and washings were evaporated *in vacuo* to 100 ml. The pH was adjusted to 8·5 with ammonia and the solution was passed through a column (30 cm. × 0·9 cm.) of Dowex 1X2 (Cl^- form). After the column was washed with water (500 ml.) and 0·001N-ammonia (200 ml.), elution was effected by successive passage of 1 l. of each of the following solutions through the column: (1) 25 mM-ammonium chloride and 0·01M-sodium tetraborate, (2) 25 mM-ammonium chloride, 2 mM-ammonia and 1 mM-sodium tetraborate, (3) as (2) but containing 0·1 mM-sodium tetraborate, (4) as (2) but containing 10 μM-sodium tetraborate, (5) as (2) but containing no sodium tetraborate. Fractions (25 ml.) were collected automatically at a flow rate of 1·0 ml./min. and examined for phosphorus and ultraviolet-light absorption at 260 mμ. Finally the column was washed with water and diphosphates were eluted with 0·1M-lithium chloride solution at pH 5·0.

Two fractions, I and II, rich in phosphate, were eluted with solution (3), but it was shown that the first fraction had started to emerge from the column before completion of the passage of solution (2). These fractions contained the glucosylribitol monophosphates. A very small fraction, III, followed closely after II and another minor fraction, IV, was eluted with solution (5). All fractions up to this stage were free from material which absorbed ultraviolet light. Fractions III and IV may have contained glycerol mono- and di-phosphates. Fractions I and II were separately evaporated to 150 ml.; I contained 55 μmoles of P and II contained 43 μmoles of P. The discarded fraction collected between these two main fractions contained 14 μmoles of P and represented a mixture of the isomeric monophosphates. Fraction III contained 8 μmoles of P, most of which probably represented monophosphate from II. Fraction IV contained 0·5 μmole of P.

Elution with lithium chloride gave three fractions. Fraction V contained material which absorbed ultraviolet light. Fraction VI overlapped fraction VII, and was separated mainly in order to obtain this last fraction free from nucleotide impurities. Fraction VII contained 16 μmoles of P, 12% of which was inorganic. Examination of fraction VII by paper chromatography in solvent *A* showed that it contained largely the diphosphate (R_F 0·12), a trace of the monophosphate (R_F 0·28) and some inorganic phosphate. It was adjusted to pH 7 with lithium hydroxide solution and then freeze-dried. The solid was treated with acetone (100 ml.) and acetone–ethanol (3:7) until washings were free from chloride ions. The residue was dissolved in water (15 ml.) and kept in the frozen state (−20°).

Charcoal chromatography of glucosylribitol phosphates. Fractions I (150 ml.) and II (500 ml.) were passed through charcoal (6 g.)–Celite (3 g.) columns, which were then washed with water (2·5 l.), and the eluates (pH 5·8) examined for phosphates The washings from fraction I contained inorganic phosphate (9·0 μmoles) and organic phosphate (8·5 μmoles). Fraction II washings contained inorganic phosphate (9·0 μmoles) and no organic phosphate. The presence of organic phosphate in the washings from fraction I probably arose through the high concentration of salts in that fraction.

Elution of organic phosphates was carried out with 15% ethanol until 95–96% of the total phosphate was present in the eluate. The pH of the eluate fell from 5·8 to 3·8 during the elution. Ammonia soln. (sp.gr. 0·88) (1·0 ml.) was added to each and solvent was removed *in vacuo* (30°). The residues were examined by paper chromatography in solvents *A* and *B*. Both fractions contained glucosylribitol, inorganic phosphate and glucosylribitol phosphate (R_F 0·28 in solvent *A*). Phosphate analysis indicated that about 35% of the organic phosphate had been hydrolysed during the charcoal adsorption and elution. Similar decomposition was observed when columns were prepared from charcoal which had been washed with 2N-ammonia.

The solid residues from the charcoal column eluates were dissolved in the minimum quantity of water, a solution of lithium chloride (20 mg.) in ethanol (5·0 ml.) was added to each and acetone–ethanol (1:1) (200 ml.) was added. After 48 hr. at 0° the precipitated solids were collected by centrifuging, washed with ethanol (2 × 10 ml.) and dissolved in water (5·0 ml.), insoluble material was removed by centrifuging, and the clear supernatants were stored at −20°. Paper chromatography of both solutions in solvent *A* showed the presence of the glucosylribitol monophosphates and inorganic phosphate but no trace of glucosylribitol. The preparation from fraction I contained 16·0 μmoles of organic phosphate and 1·6 μmoles of inorganic phosphate; that from fraction II contained 9·5 μmoles of organic phosphate and 1·6 μmoles of inorganic phosphate. Mixtures of the

glucosylribitol phosphates were unresolved by chromatography in propan-2-ol–ammonia (sp.gr., 0·88)–water (7:1:2) or *tert.*-butyl alcohol–picric acid–water (80 ml.:2 g.:20 ml.).

Glucosylribitol monophosphates

Action of prostatic phosphatase. Samples of solutions (Li salts) from the charcoal treatment of fraction I (0·2 ml.) and fraction II (0·4 ml.) were mixed with 0·4M-ammonium acetate buffer, pH 5·5 (50 μl.) and prostatic phosphatase solution (50 μl.). After incubation at 37° for 40 hr. under toluene the hydrolysates were passed through columns of Dowex 50 (H^+ form) resin and evaporated over potassium hydroxide. The residues were examined in solvents *A* and *B* and were shown to contain only glucosylribitol and inorganic phosphate.

Oxidation with periodate. Samples of the solutions obtained by charcoal chromatography of fraction I (0·5 ml.) and fraction II (1·0 ml.) were each diluted to 2·8 ml. with water. To each were added 0·1M-sodium acetate buffer, pH 5·8 (1·0 ml.) and 0·06M-sodium metaperiodate solution (0·2 ml.), and the solutions were kept in the dark at room temperature. A control solution containing no phosphoric ester was also prepared. Samples (0·1 ml.) were removed at intervals, diluted to 4 ml. with water and their periodate contents determined spectroscopically. Total phosphate and inorganic phosphate were determined in further samples (0·2 ml. and 0·5 ml. respectively). Oxidation was complete after 67 hr. and no inorganic phosphate was released. The ratio periodate consumed: organic phosphate was 2·86:1 for the compound in fraction I (glucosylribitol 1-phosphate) and 1·89:1 for the compound in fraction II (glucosylribitol 2-phosphate).

Sulphur dioxide was passed through samples (0·8 ml.) of the oxidation mixture and the solutions were evaporated to dryness. The residues were dissolved in 2N-hydrochloric acid (0·2 ml.) and heated at 100° for 24 hr. After evaporation to dryness over potassium hydroxide the residues were dissolved in water and passed through small columns of Dowex 50 (H^+ form) and Dowex 1 (Cl^- form) resins. The eluates were examined in solvent *A*. The 2-phosphate (fraction II) gave anhydroribitol and anhydroribitol 5-phosphate, whereas the 1-phosphate (fraction I) gave neither of these products.

Glucosylribitol diphosphates

Periodate oxidation and alkali treatment. A sample (5·0 ml.) of the lithium salt solution obtained from fraction VII was mixed with 0·2M-sodium acetate buffer, pH 5·8 (3·0 ml.) and 0·2M-sodium metaperiodate solution (0·48 ml.), and the mixture was kept in the dark at room temperature for 67 hr. Ethylene glycol (0·1 ml.) was added and the solution was kept in the dark for 1 hr. Ammonia (sp.gr., 0·88) (0·2 ml.) was added and the solvent was evaporated *in vacuo*. The residue was dissolved in N-sodium hydroxide solution (0·3 ml.) and heated for 2 hr. at 100°. The solution was passed through a small column of Dowex 50 (NH_4^+ form) resin and applied as a band to a paper strip. The chromatogram was developed in solvent *B* (descending front) for 30 hr. Phosphates were detected in an area extending for about 5 cm. from the origin. These were eluted and run as a band in solvent *A*. In addition to inorganic phosphate a ribitol diphosphate (R_P 0·12) was detected. It was indistinguishable in R_F from the ribitol diphosphate observed in acid hydrolysates of the teichoic acid from *B. subtilis* and from a synthetic ribitol diphosphate (prepared by Mr D. A. Applegarth).

The diphosphate was eluted and a sample was treated with 0·5% purified, salt-free, calf alkaline phosphatase solution (Sigma Chemical Co., St Louis, Mo., U.S.A.) in 0·05M-ethanolamine–hydrochloric acid buffer, pH 9·5, for 6 hr. at 37°. The solution was passed through small columns of Dowex 50 (H^+ form) and Dowex 2 (acetate form) resins, and the eluate was evaporated to dryness *in vacuo* over potassium hydroxide. The residue was examined in solvent *B* and only ribitol was detected.

A sample of the ribitol diphosphate was heated at 100° for 24 hr. in 2N-hydrochloric acid. After evaporation over potassium hydroxide the residue was examined in solvent *A*. Anhydroribitol and its 5-phosphate were formed.

Action of alkaline phosphatase. A 0·5% solution of alkaline phosphatase in 0·1M-ethanolamine–hydrochloric acid buffer, pH 9·5 (0·3 ml.) was added to a sample (1·0 ml.) of the lithium salt solution from fraction VII and the mixture was kept at 37° for 18 hr. The hydrolysate was passed through small columns of Dowex 50 (H^+ form) and Dowex 2 (acetate form) resins, and the eluate was evaporated to dryness *in vacuo* over potassium hydroxide and then examined in solvent *B*. The main product was glucosylribitol, but a small amount of ribitol and a trace of glycerol were detected.

DISCUSSION

In the earlier work on the ribitol teichoic acid from *B. subtilis* it was shown that the polymer was readily hydrolysed by alkali to a mixture composed largely of two isomeric 4-*O*-(β-D-glucopyranosyl)-D-ribitol phosphates. Although only preliminary evidence for the location of the phosphate groups was obtained they were readily removed by the action of a phosphatase, and the structure of the resulting 4-*O*-(β-D-glucopyranosyl)-D-ribitol was established by chemical and enzymic methods. This structure has been confirmed recently by unambiguous chemical synthesis (Baddiley, Buchanan & Hardy, 1961). In the present work it is shown that all the glucosylribitol units in the polymer are identical.

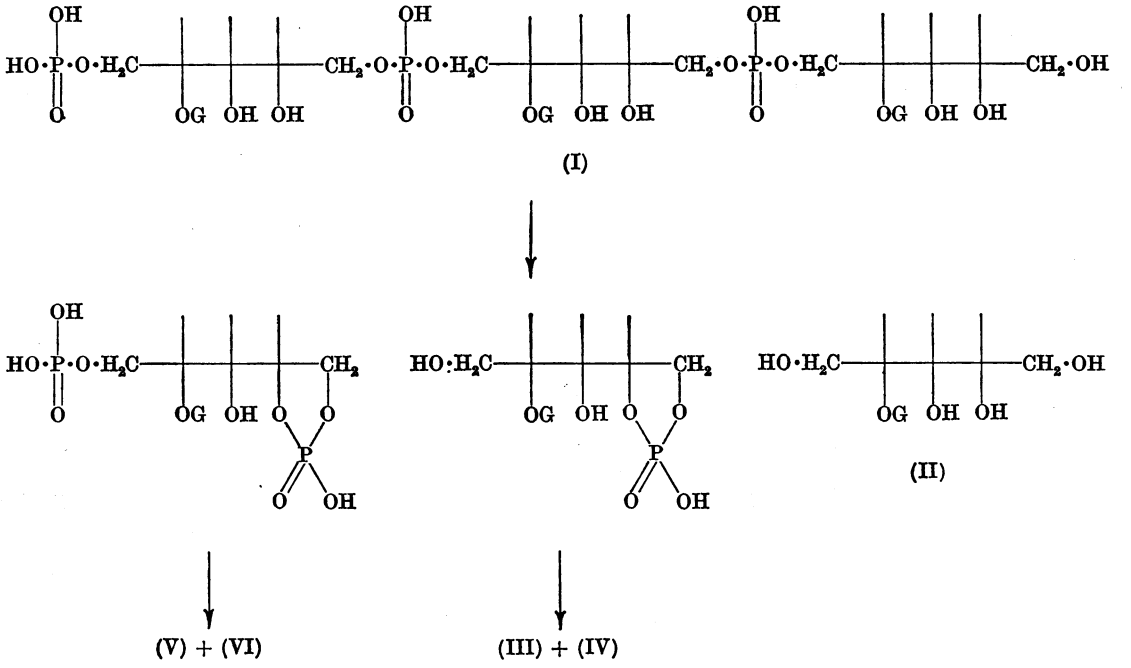
The formation of two phosphates is typical of the alkali hydrolysis of phosphodiesters that have a hydroxyl group on the carbon atom adjacent to that bearing the phosphodiester linkage. The accepted mechanism for such hydrolyses requires the intermediate formation of cyclic phosphates, which hydrolyse further to mixtures of isomeric phosphomonoesters. The course of the alkali hydrolysis is illustrated in Fig. 1; for convenience, the teichoic acid (I) is represented as a polymer of only three units and alanine residues have been omitted.

The hydrolysis must be unidirectional, i.e. fission of phosphodiester bonds must occur between phosphorus and the oxygen attached to the 5-position of the ribitol residues. Only in this way is it possible to form intermediate 5-membered cyclic phosphates. The relative amounts of the different

products will be determined by the size of the polymer, since glucosylribitol (II) and the diphosphates (V and VI) are only formed from units at each end of the chain. With a polymer composed of about 9 units, the main products would be the isomeric monophosphates (III and IV).

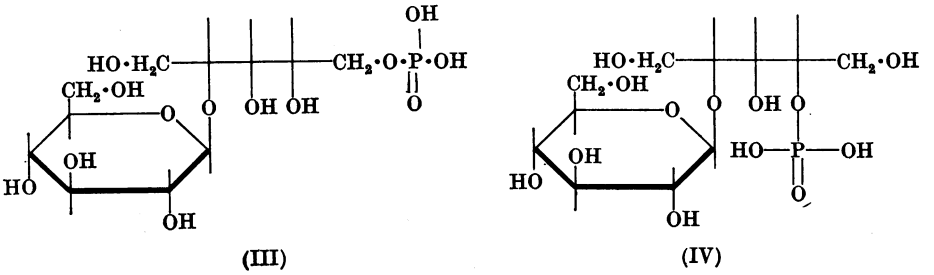
In the earlier work the mixture of monophosphates (III and IV) was not resolved, but it was shown that the two were hydrolysed in part by a β-glucosidase to glucose and a mixture of ribitol 1- and 2-phosphates. The available glucosidase preparations contained a phosphomonoesterase, and even in the presence of fluoride considerable dephosphorylation occurred. Although the structure assigned to the teichoic acid does not depend solely on the evidence for the structure of the glucosylribitol monophosphates, it was clearly desirable that these compounds should be examined more closely. On the other hand, the phosphate groups in compounds of this type would migrate readily at pH values below 4, and conclusions from these studies could not be used in discussions on teichoic acid structure unless acidic conditions had been avoided at all stages in the work.

The mixture obtained by alkali hydrolysis of the teichoic acid was subjected to ion-exchange chromatography on a column of Dowex 1 (Cl^- form) resin by using stepwise elution with solutions containing ammonia, ammonium chloride and sodium borate. This method is similar to that developed by Khym & Cohn (1953) for the separation of mixtures of sugar phosphates. The isomeric monophosphates



G represents β-D-glucopyranosyl

Fig. 1. Course of alkali hydrolysis of teichoic acid.



were readily separated from each other and from other hydrolysis products, but no attempt was made to develop the procedure for preparative purposes. The 1-phosphate (III) was eluted before the 2-phosphate (IV), and the homogeneity of the fractions was demonstrated by paper chromatography. Salts were removed from appropriate fractions by adsorption of the products on charcoal, washing with water and elution with 15% ethanol. Unfortunately, partial hydrolysis of the phosphate esters occurred during the charcoal treatment and the products were contaminated with inorganic phosphate and a corresponding amount of glucosylribitol. The glucosylribitol was removed by converting the phosphates into their lithium salts and precipitating them with ethanol. This unexplained hydrolysis was not prevented by the use of charcoal which had been washed previously with ammonia. The extent of hydrolysis was greater than would be expected to occur through spontaneous loss of phosphate at pH 4 in the same time.

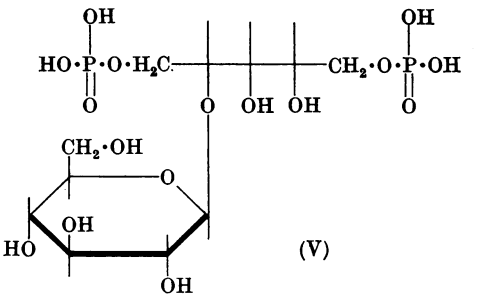
Both phosphates yielded inorganic phosphate and 4-*O*-(β-D-glucopyranosyl)-D-ribitol (II) on treatment with a phosphatase, and on paper chromatography in several solvents they were almost indistinguishable. In the propan-1-ol–ammonia solvent the isomer (II) had a very slightly higher R_F than had the other.

The two isomers were distinguished from each other by their behaviour towards periodate. That which was eluted first from the ion-exchange column consumed 3 mol.prop. of periodate for each mol.prop. of organically bound phosphorus. After removal of excess of periodate the oxidation mixture was heated with acid. Under these conditions, ribitol derivatives give 1:4-anhydroribitol which is easily detected by paper chromatography (Baddiley, Buchanan & Carss, 1957). No anhydroribitol was formed in this way from the oxidation mixture, and it follows that the ribitol residue in the original phosphate had been oxidized by periodate. Consequently, the structure (III) is assigned to this phosphate, since this contains an unsubstituted α-glycol in its ribitol residue.

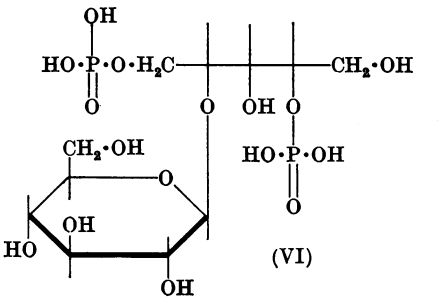
The isomer which was eluted later from the ion-exchange column consumed 2 mol.prop. of periodate for each mol.prop. of organically bound phosphorus. Moreover, acid treatment of the oxidation mixture gave a considerable amount of anhydroribitol, indicating the absence of an α-glycol system in the ribitol residue. It follows that this isomer must have the structure (IV).

In the earlier work on the nature of alkali hydrolysis products from the *B. subtilis* teichoic acid, evidence was obtained for the presence of glucosylribitol diphosphates in the mixture. These would arise from one end of the polymer chain, and were assumed to be the 1:5- and 2:5-diphosphates (V and VI) arising through the opening of an intermediate cyclic phosphate. A small broad peak of organic phosphates was observed in the later stages of development of the ion-exchange chromatogram. A part of this peak corresponded to material that absorbed ultraviolet light and presumably contained nucleotides. These arose through hydrolysis of the small amount of nucleic acid present in the teichoic acid preparation. The rest of the peak corresponded to the mixture of diphosphates. The later fractions within the peak were free from nucleotides, and paper chromatography showed that they contained the unresolved mixture of diphosphates (V and VI).

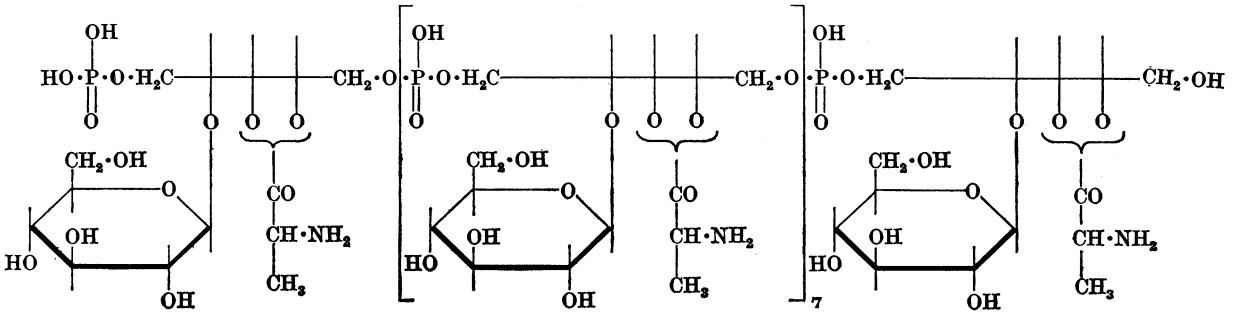
The small amount of diphosphates obtained precluded a detailed study, but qualitative oxidation experiments with periodate supported the structures (V) and (VI). These compounds should behave towards periodate in a manner similar to that already described for the monophosphates. Thus, whereas the ribitol residue in the 1:5-diphosphate (V) would be destroyed, that in the 2:5-diphosphate (VI) would survive the oxidation. The presence of a periodate-resistant ribitol residue in one of the diphosphates was demonstrated by treating the mixture with periodate, removing the excess of reagent and adding alkali. It is known that, under slightly alkaline conditions, the aldehydic fragments from the sugar residues of glycosides become detached from the oxygen formerly involved in glycosidic linkage (Mitra & Perlin, 1957). Consequently, the isomer (VI) should yield D-ribitol 2:5-diphosphate by this procedure. Paper chromatography of the reaction mixture revealed the



(V)



(VI)



(VII)

presence of a compound with the R_F expected for a ribitol diphosphate and which was converted by hot acid into a mixture of inorganic phosphate, anhydroribitol and anhydroribitol phosphate. This type of oxidative degradation was first applied successfully to a teichoic acid by Dr A. R. Archibald (unpublished work) in studies on the ribitol teichoic acid from *Lactobacillus arabinosus*.

The direct demonstration of a ribitol diphosphate amongst the degradation products from the teichoic acid from *B. subtilis* provides strong support for the structure (I) for this polymer. This, together with the detection of traces of ribitol diphosphates in acid hydrolysates (Armstrong *et al.* 1960), confirms the conclusion that ribitol residues are joined directly through phosphodiester linkages in the polymer.

The D-alanine residues in teichoic acids are attached through their carboxyl groups in unusually labile ester linkage with hydroxyl groups. It has been shown that in the intracellular glycerol teichoic acid from *Lactobacillus casei* A.T.C.C. 7469 these ester linkages involve the 2-hydroxyl group of glycerol residues, no sugars being present in this compound (Kelemen & Baddiley, 1961). A partial solution of the more difficult problem of the location of these linkages in the teichoic acid from *B. subtilis* has been achieved by oxidation studies.

If in structure (I) an alanine residue is attached to either the 2- or 3-hydroxyl group in each ribitol residue, then these ribitol residues would not be oxidized by periodate. The presence of unoxidized ribitol residues is readily demonstrated by destruction of excess of periodate followed by acid treatment to give anhydroribitol. In fact, the ribitol residues in the teichoic acid were oxidized gradually with periodate in the absence of buffer. After 60 hr. oxidation was almost complete and only a trace of anhydroribitol was detected after treatment of the product with acid. The rate of destruction was comparable with the rate of spontaneous hydrolysis of alanine ester linkages and it is likely that oxidation of ribitol residues occurred subsequent to loss of alanine. The amount of periodate consumed in this experiment was close to the theoretical value of 3 mol.prop. per mol.prop. of phosphorus.

More satisfactory results were obtained from a similar oxidation in an acetate buffer at pH 5·8. It was shown qualitatively that even after 60 hr. in the presence of periodate most of the ribitol residues were unoxidized. All the ribitol residues were oxidized under comparable conditions in a sample of this teichoic acid in which the alanine ester linkages had been hydrolysed before adding the periodate. It follows that as the alanine ester linkages protect the ribitol residues from oxidation with periodate, they must be associated with the 2- or 3-position in these residues, and the structure (VII) must represent the ribitol teichoic acid from the walls of *B. subtilis*. The amount of periodate consumed in the buffered oxidation was unexpectedly large for the teichoic acid which bore alanine ester linkages, and was larger than the amount consumed by that which had had the ester linkages removed. Under comparable conditions neither alanine nor its methyl ester consumed significant amounts of periodate. No explanation is offered at this stage for this unusual observation.

The above experiments do not enable a distinction to be made between positions 2 and 3 in the ribitol residues for the site of the alanine ester linkages. Attempts to solve this difficult problem would not be worth while on the samples of teichoic acid available at present. It is likely that the acidic conditions employed during the extraction of material from cell walls would bring about migration of ester groups between the 2- and 3-positions in the ribitol residues.

SUMMARY

1. The ribitol teichoic acid from the walls of *Bacillus subtilis* has been hydrolysed with alkali and the products were separated by ion-exchange chromatography.

2. The main hydrolysis products were 4-*O*-(β-D-glucopyranosyl)-D-ribitol 1-phosphate (III) and its isomeric 2-phosphate (IV). These were separated from each other and their structures determined by periodate oxidation.

3. Minor hydrolysis products included the 1:5- and 2:5-diphosphates (V and VI) of glucosylribitol. The structures assigned to these followed from periodate oxidation. The oxidation product from the 2:5-diphosphate gave a ribitol diphosphate when treated with alkali.

4. The structure previously suggested for this ribitol teichoic acid is confirmed by these studies.

5. Oxidation of the teichoic acid with buffered periodate before and after removal of alanine ester residues has shown that the amino acid is attached to a hydroxyl at either the 2- or 3-position in the ribitol residues.

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Formation of Bence-Jones Protein and Myeloma Protein *in vitro* by the Plasma-Cell Tumour MPC-2

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Malignant diseases of plasma cells are associated with high concentrations of myeloma proteins in the serum or Bence-Jones protein of smaller molecular weight in the urine. Both types of anomalous proteins are often present in cases of multiple myeloma. The origin of these proteins and the relationship of the urinary protein to the larger-molecular-weight myeloma protein has not been certain. Strong evidence has pointed to the malignant plasma cells as the sites of synthesis of serum myeloma protein. Studies both *in vivo* (Nathans, Fahey & Potter, 1958) and *in vitro* (Askonas, 1961) have shown that the mouse-plasma-cell neoplasm 5563 is the site of synthesis of the γ-myeloma protein found in high concentrations in serum of mice bearing the 5563 tumour. Meyer (1957) has reported briefly that bone-marrow aspirate from a patient with multiple myeloma incorporated radioactive amino acid into the γ-globulin fraction of urine with solubility characteristics of Bence-Jones protein.

Observations on the Bence-Jones proteins of human disease have been reviewed recently by Putnam (1960). In general, urinary Bence-Jones proteins have about one-third to one-quarter the molecular weight of serum myeloma globulins or normal γ-globulins; they are related immunologically to the serum γ-globulins and myeloma proteins and therefore may represent a portion of the larger molecules. This has raised the question whether the Bence-Jones proteins are precursor molecules of the larger-molecular-weight globulins which accumulate in neoplastic cells. Putnam & Miyake (1958) have shown that, after administration of radioactive amino acids to patients with multiple myeloma, the Bence-Jones protein in the urine

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