## 882. *Isolation and Structure of Ribitol Phosphate Derivatives (Teichoic Acids) from Bacterial Cell Walls.*

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The cell walls of three Gram-positive bacteria have been shown to contain, as a major component, polymers of ribitol phosphate, called teichoic acids. In addition to ribitol phosphate, the teichoic acids from *Lactobacillus arabinosus* and *Bacillus subtilis* contain α-glucosyl residues joined to ribitol, and *O*-alanyl groups probably linked to glucose. In the teichoic acid from *Staphylococcus aureus* H, an *N*-acylglucosaminyl residue is present in the place of glucose. Trichloroacetic acid extracts of defatted whole cells of *L. arabinosus* and *B. subtilis* contain, in addition to teichoic acids, a polymer of glycerophosphate. The location of the latter within the cells has not been determined.

The accumulation of the nucleotide cytidine diphosphate ribitol by *S. aureus* under conditions favouring accumulation of uridine diphosphate derivatives suggests that the cytidine compound is involved in the biosynthesis of teichoic acids.

A preliminary announcement of some of these results has been made.*

The two nucleotides, cytidine diphosphate glycerol (CDP-glycerol) and cytidine diphosphate ribitol (CDP-ribitol), were first isolated from *Lactobacillus arabinosus* [1] but are now believed to be more widely distributed in bacteria. CDP-glycerol, whose structure has recently been confirmed by synthesis,[2] contains an L-α-glycerophosphate residue,[3] whereas CDP-ribitol contains a D-ribitol 5-phosphate residue.[4,5] Although the discovery of these nucleotides gave no information about their biological function, it was probable from analogous cases that they would be concerned in the metabolism of glycerophosphate and ribitol phosphate.

Glycerophosphate and its derivatives occur widely in living cells, and it has been suggested already that CDP-glycerol may participate in the biosynthesis of phospholipids [3] and related compounds.[6] It is noteworthy, however, that no experimental evidence has been advanced in support of these suggestions, and even the presence of the nucleotide has not been demonstrated in the biological systems under consideration. The function of CDP-ribitol was even more obscure than that of the glycerol compound, as ribitol

* Armstrong, Baddiley, Buchanan, and Carss, *Nature*, 1958, **181**, 1692.

[1] Baddiley and Mathias, *J.*, 1954, 2723; Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, **64**, 599.
[2] Baddiley, Buchanan, and Sanderson, *J.*, 1958, 3107.
[3] Baddiley, Buchanan, Mathias, and Sanderson, *J.*, 1956, 4186.
[4] Baddiley, Buchanan, Carss, and Mathias, *J.*, 1956, 4583.
[5] Baddiley, Buchanan, and Carss, *J.*, 1957, 1869.
[6] Benson and Maruo, *Biochim. Biophys. Acta*, 1958, **27**, 189.

phosphate had not been detected hitherto in Nature, and even the free polyol has only been isolated from a few species of plants.

Both CDP-glycerol and CDP-ribitol clearly belong to the group of nucleotide coenzymes, exemplified by uridine diphosphate glucose, in which a phosphoric ester substrate becomes an integral part of the coenzyme. One important function of such coenzymes is in the biosynthesis of macromolecules. Examples of this have been provided recently in the enzymic formation of cellulose [7] and glycogen,[8] where uridine diphosphate glucose participates in the transfer of glucose units, and in chitin synthesis [9] where uridine diphosphate acetylglucosamine serves a similar purpose. Consequently, it was possible that a large-molecular compound containing glycerophosphate or ribitol phosphate residues might occur in *L. arabinosus* and other bacteria.

Freshly grown *L. arabinosus* 17-5 was de-fatted with acetone and extracted with dilute trichloroacetic acid at 0°. Addition of an equal volume of alcohol to the extract, followed by storage at 0°, precipitated a phosphoric ester. The ester was purified further by extraction of the solid with water, followed by precipitation with alcohol, yielding material which was free from ribonucleic acid and gave a clear solution in water.* Evidence described below suggests that this material is not homogeneous, and consequently discussion of the molecular weight of such preparations is of limited value. We believe, however, that the method of isolation, the intractable nature of the material on paper chromatography and electrophoresis, and its very slow rate of dialysis through Cellophane membranes suggest that the molecular weight of the material is not small.

Later work also indicates that a major component is a polymer of ribitol phosphate which bears both glucose and alanine residues and is thus a member of a new group of natural polymers. Before discussing the structure of these remarkable substances some consideration of their biochemical and cytological significance is desirable.

Park and Johnson [10] have demonstrated the accumulation of three nucleotides in *Staphylococcus aureus* during treatment with penicillin: one is uridine diphosphate acetylmuramic acid,[11,12] and the others are derivatives of this bearing either an alanine residue or a small peptide composed of lysine, glutamic acid, and alanine. A connection between the nucleotides and the cell wall followed, since muramic acid is a characteristic component of many bacterial cell walls.[13] Alanine, lysine, and glutamic acid are, moreover, usually found among the relatively small number of amino-acids released by hydrolysis of cell walls.[14,15] It is known that penicillin seriously affects the walls of bacteria during the earlier stages of its action. This is consistent with the observation that the accumulation of the uridine derivatives is also an early effect of penicillin's action.[12,16] It is likely that the nucleotides are involved in the transfer of muramic acid and its peptides into the macromolecular structure of the wall. Penicillin, in interfering with the normal cell-wall structure, presumably causes the accumulation of some of the precursors.

It seemed possible that a similarity in relationship might exist between the uridine derivatives and the muramic acid-peptide residues in cell walls on the one hand, and CDP-ribitol and the ribitol phosphate polymer on the other. Techniques are now available for the isolation of bacterial cell walls uncontaminated by cellular contents.[17] Walls of

* A preliminary account of the isolation of this substance has been published (Baddiley, Buchanan, and Greenberg, *Biochem. J.*, 1957, **66**, 51P).

[7] Glaser, *Biochim. Biophys. Acta*, 1957, **25**, 436.
[8] Leloir and Cardini, *J. Amer. Chem. Soc.*, 1957, **79**, 6340.
[9] Glaser and Brown, *Biochim. Biophys. Acta*, 1957, **23**, 449; *J. Biol. Chem.*, 1957, **228**, 729.
[10] Park and Johnson, *J. Biol. Chem.*, 1949, **179**, 585.
[11] Park, *ibid.*, 1952, **194**, 877, 885.
[12] Park and Strominger, *Science*, 1957, **125**, 99.
[13] Strange, *Biochem. J.*, 1956, **64**, 23P; cf. Work, *Nature*, 1957, **179**, 841.
[14] Salton, *Biochim. Biophys. Acta*, 1953, **10**, 512.
[15] Cummins and Harris, *J. Gen. Microbiol.*, 1956, **14**, 583.
[16] Strominger, *J. Biol. Chem.*, 1957, **224**, 509.
[17] Salton and Horne, *Biochim. Biophys. Acta*, 1951, **7**, 177.

*L. arabinosus* prepared in this way were kindly supplied by Dr. M. R. J. Salton. Hydrolysis of a sample with hot acid, followed by paper chromatography, showed the presence in the hydrolysate of ribitol phosphates, inorganic phosphate, and 1 : 4-anhydroribitol, together with other compounds expected from the hydrolysis of a polymer of ribitol phosphate (see below).* Moreover, direct extraction of the cell walls with cold trichloroacetic acid, followed by addition of alcohol to the extract, yielded a compound apparently identical with the ribitol-containing component of the material obtained earlier from whole cells. Closely related compounds of very similar composition are present in the cell walls of *S. aureus* H (kindly provided by Dr. J. T. Park) and *Bacillus subtilis*. We have been unable to detect derivatives of ribitol phosphate in the walls of *Micrococcus lysodeikticus* and *Escherichia coli*. The general name " teichoic acid " (Greek τεῖχος = wall) is suggested for these polymers of ribitol phosphate with or without other substituents. Although the presence of teichoic acid in other bacteria has not yet been examined, it is likely that it will be found fairly widely. Its function in cell walls is not known, but its absence from the spore walls of *B. subtilis* suggests that it may be concerned in the metabolism of actively growing cells.

By visual estimation from paper chromatograms, the amount of inorganic phosphate liberated on acid hydrolysis of cell walls corresponds on a molar basis to the amount of 1 : 4-anhydroribitol formed. These two materials are known to be the main products of decomposition of ribitol phosphates under strongly acidic conditions.[4,18,19] The only organic phosphates detected in hydrolysates of walls are those expected from teichoic acid; consequently, from a determination of the phosphorus content of the walls, their teichoic acid content can be calculated. In this way it was found that 40—60% of the walls of *S. aureus* and *B. subtilis* consists of teichoic acid. The amount present in *L. arabinosus* may even exceed 60%. It follows that this is a major wall component in all these bacteria.

Although teichoic acid is freely soluble in water, the washing techniques employed in the isolation of walls had failed to remove it. On the other hand, about one-third of the total amount present in the walls examined so far is readily extracted by cold trichloroacetic acid. Apparently the extractable fraction is held in the wall by electrovalent linkage. It is not known whether the remainder is chemically bound to other wall components.

It is highly probable that CDP-ribitol is concerned in the successive condensation of ribitol phosphate residues during the biosynthesis of teichoic acid. Although the details of the enzymic synthesis have not been examined, a close similarity in function between CDP-ribitol and Park's nucleotides is apparent. Moreover, a cytidine derivative accumulates in *S. aureus* during growth in the presence of chloramphenicol (personal communiccation from Dr. J. T. Park). We have identified this nucleotide as CDP-ribitol by comparison with authentic material on paper chromatograms, and by acid hydrolysis to cytidine-5' phosphate, ribitol phosphates, and their decomposition products. Similarly, when *S. aureus* is grown in the presence of Crystal Violet a cytidine derivative accumulates. A sample of this nucleotide (kindly provided by Dr. J. L. Strominger) has been identified as CDP-ribitol by Dr. A. R. Sanderson in this laboratory. It seems that penicillin, chloramphenicol, and certain other inhibitors, by interfering either directly or indirectly with cell-wall formation, cause the accumulation of the uridine precursors and in some cases the cytidine precursors.

Acid-hydrolysis of teichoic acid obtained from the walls of *L. arabinosus* yielded the following compounds (see Table 1), all identified by comparison on paper chromatograms with authentic substances: alanine, glucose, ribitol and its isomeric monophosphates, 1 : 4-anhydroribitol, 1 : 4-anhydroribitol 5-phosphate, and inorganic phosphate. No other sugars, amino-acids, polyols, or organic phosphates were detected in the hydrolysate.

* A preliminary account of this observation has been published by Baddiley, Buchanan, and Carss, *Biochim. Biophys. Acta*, 1958, **27**, 220.

[18] Baddiley, Buchanan, and Carss, *J.*, 1957, 4058.
[19] *Idem, ibid.*, p. 4138.

Confirmation of the identity of the organic phosphates was obtained by enzymic hydrolysis to the respective polyols and inorganic phosphate. 1 : 4-Anhydroribitol 5-phosphate, which has not been described previously, gave the slow-developing blue colour reaction, typical of cyclic glycols, on paper chromatograms sprayed with the periodate–Schiff reagents. It yielded anhydroribitol on enzymic dephosphorylation, and was indistinguishable from a synthetic sample prepared in these laboratories by Mr. R. Cowling. When teichoic acid from whole bacterial cells was hydrolysed in acid, the products included all

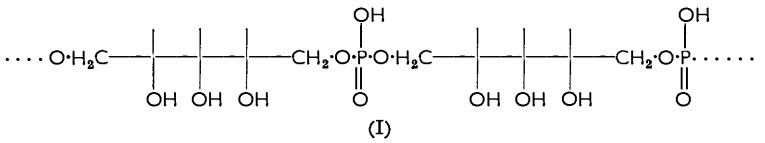
TABLE 1. *Products of acid hydrolysis of teichoic acid from different bacteria.*

| | *L. arabinosus* | *B. subtilis* | *S. aureus* |
|---|---|---|---|
| Alanine | + | + | + |
| Glucose | + | + | — |
| Glucosamine | — | — | + |
| Inorganic phosphate | + | + | + |
| 1 : 4-Anhydroribitol | + | + | + |
| 1 : 4-Anhydroribitol 5-phosphate | + | + | + |
| Ribitol phosphates | + | + | + |
| Ribitol | + | + | + |
| Ribitol glucosaminide | — | — | + |

those described for the wall material, but glycerol and its phosphates were also detected. The absence of glycerol derivatives from cell walls, and the variable amounts present in different teichoic acid preparations from whole cells of *L. arabinosus* and *B. subtilis*, suggest that these products do not represent residues in teichoic acid, but are probably derived from a glycerophosphate polymer which accompanies the acid when obtained from whole cells. The possible significance of this glycerophosphate polymer is discussed on p. 4350.

The formation of ribitol, 1 : 4-anhydroribitol, and their respective phosphates on acid hydrolysis is consistent with the presence in teichoic acid of a polymeric structure, *e.g.*, (I) or its isomers, in which ribitol residues are joined together through phosphodiester linkages. At pH values lower than 4, ribitol monophosphates decompose largely to 1 : 4-anhydroribitol and inorganic phosphate; at the same time equilibration between the isomeric ribitol monophosphates occurs through acid-catalysed migration of the phosphate.[18] Although traces of 1 : 4-anhydroribitol 5-phosphate are formed at these low pH values, no ribitol is liberated. Possible mechanisms for these reactions have been discussed previously.

In a polymer of ribitol phosphate, *e.g.*, (I), acid-catalysed hydrolysis might occur in a more or less random manner. Consequently, the primary hydrolysis products would include ribitol and its mono- and di-phosphate. Ribitol, once formed, would be relatively stable under the conditions of our experiments, but the monophosphates would be largely converted into 1 : 4-anhydroribitol and a trace of its 5-phosphate. Similarly, diphosphates of ribitol would be converted into 1 : 4-anhydroribitol 5-phosphate. The amount of 1 : 4-anhydroribitol 5-phosphate formed by hydrolysis of teichoic acid (estimated visually from paper chromatograms) is considerably greater than would be expected from hydrolysis of ribitol phosphate itself. This, and the presence of free ribitol in hydrolysates, indicates that teichoic acid must contain a chain of ribitol residues linked through phosphodiester groups. It also follows that fission of the secondary phosphate groups can occur in either direction along the polymer chain.



(I)

The hydrolysis of phosphomonoesters at pH 4 takes a different course from that which occurs at low pH values.[20] Hydrolysis of the monoanion occurs smoothly and readily

[20] Desjobert, *Compt. rend.*, 1947, **224**, 575; *Bull. Soc. chim. France*, 1947, **14**, 809; Butcher and Westheimer, *J. Amer. Chem. Soc.*, 1955, **77**, 2420.

with glycerophosphates,[21] ribitol phosphates,[18] and nucleotides [22] to give the respective hydroxy-compounds and inorganic phosphate. Under conditions which convert ribitol phosphates quantitatively into ribitol and inorganic phosphate, the phosphate groups in teichoic acid were almost unaffected. Only a trace of inorganic phosphate was liberated, thus indicating the absence of phosphomonoester groups. Phosphodiesters are relatively stable at pH 4.

Preparations of teichoic acid, whether from whole cells or walls of *L. arabinosus* or *B. subtilis*, yielded alanine and glucose on acid hydrolysis. Further attempts to purify the material by paper chromatography, electrophoresis, or adsorption on charcoal did not alter the relative amounts of these two hydrolysis products, which were present in the ratio 1 : 1. The glucose in the intact compound was non-reducing, readily liberated as glucose by acid hydrolysis, and was unaffected by hot alkali. These properties are consistent with those of a glucoside. The stability of the sugar residues to alkali eliminates the possibility of a linkage between glucose and the remainder of the molecule through a phosphate group. No disaccharides or larger sugar groupings were observed on partial acid hydrolysis of teichoic acid. It is likely then that each glucose unit is joined individually as a glucoside residue to the polymer.

Further information about the nature of the glucoside linkages in teichoic acid comes from its behaviour towards alkali. Although phosphodiesters are normally stable to alkali, they become liable if one or both of the alkyl groups bears a hydroxyl adjacent to the phosphoric ester group. The products of such hydrolyses are usually mixtures of isomeric hydroxyalkyl phosphates.[23] When the acid from *L. arabinosus* was heated with sodium hydroxide solution a mixture of phosphoric esters was obtained. The main components of this mixture were monoesters which, on dephosphorylation with prostate phosphomonoesterase, yielded ribitol derivatives (see Table 2). These were separated into two

TABLE 2. *Products of alkali-hydrolysis, followed by enzymic dephosphorylation, of teichoic acid from different bacteria.*

| | *L. arabinosus* | *B. subtilis* | *S. aureus* |
|---|---|---|---|
| Alanine | + | + | + |
| Inorganic phosphate | + | + | + |
| Ribitol monoglucosides | + | + | — |
| Ribitol diglucoside | + | — | — |
| Ribitol | + | — | — |
| Anhydropentitol | + | + | + |
| Ribitol glucosaminide | — | — | + |
| Residual phosphoric ester | + | — | — |

fractions by paper chromatography in *n*-propyl alcohol–ammonia–water. The faster-moving material gave equimolar amounts of glucose and anhydroribitol on prolonged acid hydrolysis, whereas the slower-moving material gave glucose and anhydroribitol in the molar ratio of 2 : 1. Partial acid hydrolysis of these fractions yielded some ribitol as well as anhydroribitol, and it is concluded that they contain mono- and di-glucosides of ribitol. The faster-moving material was not homogeneous, and at least two, and possibly three, closely related ribitol glucosides were detected. These are presumably isomers in which a glucosyl substituent occupies different positions on the ribitol residue. Further work on the characterisation of these products is in progress.

Alkali-hydrolysates of teichoic acid from *L. arabinosus* contain, in addition to the phosphates of the ribitol glucosides described above, a slower-moving phosphate or mixture of phosphates. This fragment is resistant to both alkali and phosphomonoesterase, but on acid treatment it gives inorganic phosphate, anhydroribitol, and the other products of acid hydrolysis of the ribitol glucoside phosphates. It probably represents a fragment of the

[21] Bailly, *Bull. Soc. chim. France*, 1942, **9**, 340.
[22] Baddiley, Buchanan, and Letters, *J.*, 1958, 1000.
[23] Bailly and Gaumé, *Bull. Soc. chim. France*, 1935, **2**, 354; 1936, **3**, 1396; Baer and Kates, *J. Biol. Chem.*, 1948, **175**, 79; 1950, **185**, 615; Brown and Todd, *J.*, 1952, 52.

teichoic acid where secondary phosphate groups have withstood the action of alkali. This could occur where substituents (presumably glucose units) occupy hydroxyl groups on the ribitol residues adjacent to the phosphate groups.

Neither teichoic acid nor the ribitol glucosides obtained from it were hydrolysed by a commercial β-glucosidase preparation, and it is likely that the glucoside linkages are all α. The pronounced positive rotation, $[\alpha]_D$ + 80·4°, of teichoic acid from *L. arabinosus* is consistent with this view, on the assumption that the main contribution to the rotation will be made by the glucose fraction (25%) of the molecule.

Not all the ribitol residues in teichoic acid from *L. arabinosus* bear one or two glucosyl substituents. Alkali-hydrolysates which have been treated with prostate phosphatase contain appreciable amounts of ribitol. This must have arisen from ribitol residues which did not bear glucosyl substituents.

It is interesting that alkali-hydrolysates always contain a small quantity of an anhydropentitol. Although this is indistinguishable from 1 : 4-anhydroribitol on paper chromatography, it has not been fully identified. The same compound is also formed in very small amounts by the prolonged action of alkali on ribitol phosphates. The mechanism of its formation clearly differs from that which operates under acidic conditions.

Teichoic acids from all sources examined so far yield alanine on acid- or alkali-hydrolysis. The strong positive ninhydrin reaction shown by all preparations suggests that the amino-group in the alanine residue is unsubstituted. Moreover, the great instability of these residues towards alkali suggests that they are joined to the polymer in ester linkage through their carboxyl groups. The reaction of teichoic acid with ammonia confirms this view. Even under the mild conditions of paper chromatography in an ammoniacal solvent the alanine residues were removed completely. The products from these residues were identified by paper chromatography as alanine and its amide. Alanine ethyl ester behaved similarly under comparable conditions. Moreover, the polymer readily yields alanine hydroxamic acid on treatment with hydroxylamine. The alanine ester residues must be attached to a hydroxyl group in either the glucose or ribitol residues in the polymer, but evidence for their exact location is not yet available. We believe that this is the first authentic example of the occurrence of an α-amino-ester in Nature.

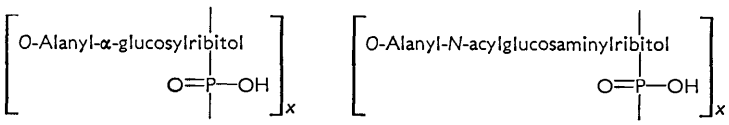
It is of interest that alanine residues bearing free amino-groups have been detected by Brown [24] in cell walls and trichloroacetic acid-extracts of walls. These were described as *N*-terminal groups, presumably of peptide chains. It is probable that most or all of these are alanine ester groups associated with the teichoic acid which would be present in the preparations. Similarly, *O*-acetyl groups have been detected in bacterial walls.[25] These are believed to be associated with sugar residues in the mucopolysaccharide fraction of the walls.[26] It is worth emphasising that the method used in their quantitative determination, *i.e.*, reaction with hydroxylamine followed by addition of a ferric salt, would not normally distinguish between *O*-acetyl and *O*-alanyl groups. This could be misleading in experiments with walls which contain appreciable amounts of teichoic acid.

Preliminary experiments on teichoic acid from *B. subtilis* suggest that the alanine residues are attached directly to the sugar groups. The ribitol residues in this material are destroyed by oxidation with periodate at pH 4·5 during 24 hours. Little or no loss of alanine would occur under these conditions. It follows that an unsubstituted glycol group must be present in each ribitol residue. As the ribitol residues are already substituted in three positions (once by glucose and twice by phosphate), the alanine ester grouping must occur on the sugar residues.

A general structure for teichoic acid, based on the above considerations, is shown in formula (II). This is clearly an over-simplification in the case of the acid from *L. arabinosus*, where some ribitol units have two glucosyl residues, whereas others have none. It is even

[24] Brown, *Biochim. Biophys. Acta*, 1958, **28**, 445.
[25] Abrams, *J. Biol. Chem.*, 1958, **230**, 949.
[26] Brumfitt, Wardlaw, and Park, *Nature*, 1958, **181**, 1783.

possible that the alkali-resistant phosphate obtained from this acid corresponds to ribitol units bearing three glucose residues. On the other hand, teichoic acid from *B. subtilis* is probably more closely represented by formula (II). Alkali hydrolysis, followed by phosphatase treatment, of the acid from this organism gave only one ribitol glucoside, and no ribitol or alkali-resistant phosphates were detected (Table 2).




(II) (III)

Teichoic acid from *S. aureus* differs from the other two described in this paper in that, although it contains alanine ester groups and gives all the degradation products of ribitol phosphates on acid hydrolysis, it does not contain glucose. In this material *N*-acetylglucosamine takes the place of glucose in a relatively simple polymer (III), similar in composition to that from *B. subtilis*. Acid hydrolysates (Table 1) contained glucosamine (identified by the Elson–Morgan test [27] on paper chromatograms and by the slowly developing ninhydrin colour reaction) and a small amount of a ribitol glucosaminide. The identity of glucosamine was confirmed by eluting the purple area of a paper which had been sprayed with ninhydrin solution containing pyridine, and identifying as arabinose the sugar which was formed.[28] The glucosaminide was the only glucosamine-containing product of alkali hydrolysis and phosphatase treatment of the polymer (Table 2). It gave a typical glycoside reaction with the periodate–Schiff reagents for glycols, and a purple colour with ninhydrin. Unlike the original material, it was fairly stable towards acids. On prolonged acid-hydrolysis the products were glucosamine and anhydroribitol.

Acid-hydrolysis of glucosaminides and their *N*-acetyl derivatives has been studied by Foster, Horton, and Stacey,[29] who noted the stability of glucosaminides and concluded that during acid hydrolysis of their *N*-acetyl derivatives two competing reactions occurred. Fission of the glycosidic linkage in the *N*-acetylglucosaminides proceeds readily, but the gradual hydrolysis of the acetyl group in the glycoside gives rise to some free glucosaminide which resists further hydrolysis. These conclusions are in agreement with our findings on the teichoic acid from *S. aureus*. The relative ease with which glucosamine is liberated by acid-hydrolysis suggests the presence of an *N*-acyl substituent. The formation of a small amount of ribitol glucosaminide under acidic conditions is consistent with this view. Under alkaline conditions the acyl group is presumably lost, giving rise to the glucosaminide which would only be hydrolysed slowly by acids. Although we have not identified the acyl substituent, it is probably an acetyl group, since *N*-acetyl-D-glucosamine occurs widely in Nature.

The structure and significance of the glycerophosphate derivative which accompanies teichoic acid when isolated from whole cells is not clear. The high water-solubility and low alcohol-solubility of this material strongly indicate the absence of long-chain fatty ester groups. On acid hydrolysis it gave glycerol, glycerophosphates, and glycerol diphosphates, all identified by paper chromatography and enzymic dephosphorylation to glycerol and inorganic phosphate. Similarly, alkali hydrolysis gave a mixture of glycerol and its mono- and di-phosphate. It follows that this compound is a polymer of glycerophosphate of unknown molecular weight.

It is highly probable that CDP-glycerol is concerned in the biosynthesis of the glycerophosphate polymer, presumably by successive condensation of glycerophosphate residues. The absence of this polymer from the cell walls examined so far, and the failure of CDP-glycerol to accumulate in inhibition studies with Crystal Violet in *S. aureus*, suggest that

[27] Elson and Morgan, *Biochem. J.*, 1933, **27**, 1824.
[28] Stoffyn and Jeanloz, *Arch. Biochem. Biophys.*, 1954, **52**, 373.
[29] Foster, Horton, and Stacey, *J.*, 1957, 81.

the glycerol polymer is not involved in the cell wall. Macromolecular material of uncertain composition, containing glycerophosphate residues, has been isolated from bacteria by Mitchell and Moyle.[30] It is possible that this originated from the protoplast membrane, a thin membrane lying just beneath the cell wall.[31] Recent analyses of such membranes from *M. leisodeikticus* indicate that they contain a glycerophosphate polymer.[32] However, this material is thought to contain long-chain fatty ester groups and it is uncertain what relation it bears to our polymer.

## Experimental

*Isolation of Teichoic Acid from* L. arabinosus *Cells.—L. arabinosus* 17-5 was grown in bottles (10 l.) without aeration on a hydrolysed casein medium, supplemented with yeast extract and trace elements, as described previously.[1] The cells (65 g. wet) were harvested in a Sharples centrifuge and washed three times with 0·8% sodium chloride solution at 0°. They were successively treated in a Waring blendor (30 sec.) with acetone, alcohol, and ether (15 vol.) at room temperature.

The fat-free cells (24 g.) were treated for 1 min. in the blendor with three portions (100 ml. each) of ice-cold 10% trichloroacetic acid. After centrifugation, the combined supernatant solutions were filtered once through " Supercel " and treated with an equal volume of alcohol at 0°. After 12 hr. the material was isolated by centrifugation, reprecipitated from ice-cold 10% trichloroacetic acid (75 ml.) by addition of an equal volume of alcohol, then washed with acetone and ether. The yield was 56 mg. of a white powder containing a little nucleic acid and a high proportion of polyglycerophosphate (Found: P, 8·2; glucose,[33] 22·3; alanine,[34] 11·9%. This represents a molar ratio of glucose : alanine = 1·0 : 1·1).

The cell debris was treated in the blendor (3 min.) with ice-cold 10% trichloroacetic acid (200 ml.), left overnight at 0°, then treated for a further 1 min. in the blendor. The supernant solution, after centrifugation of debris, was filtered once through " Supercel " and mixed with an equal volume of alcohol. After 24 hr. the precipitate (225 mg.) was collected by centrifugation and washed with acetone and ether. Nucleic acid was removed by trituration with water (10 ml.) for several min., followed by clarification in a centrifuge. After 3 days at 0° a further small precipitate was removed from the solution which was next adjusted to pH 6·5 with dilute ammonia. Acetone (1·5 vol.) was added at 0° and after 24 hr. teichoic acid (28 mg.) was collected as a clear resin which solidified to a glass on drying. It contained a little polyglycerophosphate and no nucleic acid (Found: glucose, 25·4%) and had $[\alpha]_D^{19}$ +80·4° (*c* 2·1 in water).

*Isolation of Teichoic Acid from* B. subtilis *Cells.—B. Subtilis* was grown in bottles (10 l.) with aeration on a peptone (2·0%)–sodium chloride (0·5%) medium. Growth was allowed to take place for 48 hr. at 20—22°. The cells (55 g. wet) were harvested in a Sharples centrifuge and washed with 0·8% sodium chloride solution at 0°. They were successively treated in a Waring blendor (30 sec.) with acetone, alcohol, and ether (15 vol.) at room temperature.

More vigorous conditions were required to remove teichoic acid from *B. subtilis* cells than from *L. arabinosus* cells. Fat-free cells (20 g.), treated (3 × 1 min.) in the blendor with 100 ml. portions of ice-cold 10% trichloroacetic acid as described for *L. arabinosus*, yielded no teichoic acid. The residue from this extraction was treated in the blendor (3 min.) with ice-cold 10% trichloroacetic acid (200 ml.), left overnight at 0°, then treated for a further 1 min. in the blendor. The supernatant solution, after centrifugation of debris, was filtered through " Supercel " and mixed with an equal volume of alcohol. After 24 hr. at 0° the precipitate (291 mg). was collected by centrifugation and washed with acetone and ether. Trituration of the precipitate with water (11·0 ml.) dissolved the teichoic acid, and the nucleic acid was removed by centrifugation. After 3 days at 0° a further small precipitate was removed from the solution which was then adjusted to pH 6·5 with dilute aqueous ammonia. Acetone (2 vol.) was added at 0° and after 48 hr. teichoic acid (63 mg.) was collected as a clear resin which solidified

[30] Mitchell and Moyle, *J. Gen. Microbiol.*, 1951, **5**, 981.
[31] McQuillen, *Biochim. Biophys. Acta*, 1955, **17**, 382; Mitchell and Moyle, *J. Gen. Microbiol.*, 1956, **15**, 512.
[32] McQuillen, personal communication.
[33] Park and Johnson, *J. Biol. Chem.*, 1949, **181**, 149.
[34] Meyer, *Biochem. J.*, 1957, **67**, 333.

to a glass on drying. It contained a little polyglycerophosphate and faint traces of nucleic acid.

TABLE 3.

| | $R_F$ in solvent | | | $IO_4^-$–Schiff reaction * | Ninhydrin reaction |
|---|---|---|---|---|---|
| | A | B | C | | |
| Glucose | 0·61 | 0·76 | 0·27 | S, W | — |
| Glucosamine | 0·61 | 0·46 | 0·21 | S, W | + |
| Glycerol | 0·75 | — | 0·49 | R | — |
| Ribitol | 0·65 | 0·83 | 0·30 | R | — |
| Anhydroribitol | 0·75 | 0·88 | 0·47 | S | — |
| Glycerophosphates | 0·33 | — | — | R | — |
| Ribitol 1-phosphate | 0·30 | — | — | R | — |
| Ribitol 2(and 3)-phosphates | 0·33 | — | — | R | — |
| Anhydroribitol 5-phosphate | 0·29 | — | — | S | — |
| Inorganic phosphate | 0·17 | — | — | — | — |
| " Resistant phosphate " | 0·13 | — | — | S | — |
| Ribitol diphosphate | 0·12 | — | — | S, W | — |
| Glycerol diphosphate | 0·12 | — | — | — | — |
| Alanine | 0·63 | 0·67 | 0·30 | — | + |
| Alanine amide | 0·72 | — | — | — | + |
| Ribitol glucosides | 0·55 | — | — | R | — |
| Ribitol diglucoside | 0·40 | — | — | R | — |
| Ribitol glucosaminide | 0·56 | — | — | R | + |

* S = slow reaction, R = rapid reaction, W = weak reaction.

*Isolation of Teichoic Acid from Bacterial Cell Walls.*—50 mg. of a cell-wall preparation (*L. arabinosus* or *S. aureus*) was stirred with 10% trichloroacetic acid (1·5 ml.) overnight at 0°. The cell walls were centrifuged and extracted again with 10% trichloroacetic acid (1 ml.). The combined supernatant solutions were mixed with acetone (2 vol.) and kept at 0° overnight. Teichoic acid was precipitated as a clear resin which solidified to a glass on drying (yield: 5 mg. from *L. arabinosus*; 2·2 mg. from *S. aureus*).

*Acid-hydrolysis of Teichoic Acid.*—Teichoic acid (1·0 mg.) from the above sources and from *B. subtilis* cells was hydrolysed in 2N-hydrochloric acid in a sealed tube for 3 hr. at 100°. After evaporation the samples were examined by paper chromatography in solvent systems *A*, *B*, and *C* (cf. p. 4354). Products were detected by the molybdate reagent for phosphoric esters,[35] the periodate–Schiff reagents for α-glycols,[36] the aniline phthalate reagent for reducing sugars,[37] the ninhydrin reagent for amino-acids, [38] the Elson–Morgan reagents for amino-sugars,[27] and by inspection in ultraviolet light. Products detected are given in Table 1 and $R_F$ values in Table 3.

*Alkali-hydrolysis and Enzymic Dephosphorylation.*—Teichoic acid samples (10 mg.) were heated in N-sodium hydroxide solution (50 μl.) for 3 hr. at 100°. $Na^+$ ions were removed by passing the hydrolysate through a small column of Dowex-50 ($NH_4^+$ form) resin. Ammonia was evaporated from the eluate, its pH was adjusted to 5·5 with acetic acid, and the volume was adjusted to 0·2 ml. A preparation of prostate phosphatase (0·1 ml.) was added and the solution was kept under toluene at 37° for 24 hr. After suitable adjustment of the volume, the solution was chromatographed as a band in solvent system *A*. The products (polyols, glucosides, or glucosaminides) were detected on " marker strips " by using the above spray reagents. They were isolated for further investigation by elution of the appropriate area of the chromatogram. A summary of these results is given in Table 2 and $R_F$ values in Table 3.

*Analysis of Ribitol Glucosides.*—The glucoside spots obtained by the above experiment from *L. arabinosus* were eluted from the chromatogram and separately hydrolysed to glucose and anhydroribitol by heating them at 100° with 2N-hydrochloric acid for 24 hr. After removal of solvent the products were chromatographed in solvent system A and strips containing glucose and anhydroribitol were cut out and eluted. Glucose was estimated as before, and anhydroribitol by quantitative periodate oxidation on a microscale.[39] Control solutions for the estimation were made by eluting strips cut from appropriate areas between tracks on the paper.

[35] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[36] Buchanan, Dekker, and Long, *J.*, 1950, 3162; Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.
[37] Partridge, *Nature*, 1949, **164**, 443.
[38] Consden and Gordon, *ibid.*, 1948, **162**, 180.
[39] Dixon and Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.

The material with the higher $R_F$ value contained glucose and anhydroribitol (originally ribitol) in the ratio of 0·92 : 1·0, and the slower-running material gave a corresponding ratio of 1·84 : 1 0.

*Periodate Oxidation of Teichoic Acid.*—Teichoic acids from whole cells of *L. arabinosus* and *B. subtilis* were used: 0·5 mg. was dissolved in 1% sodium metaperiodate solution (80 µl.) and kept in the dark for 24 hr. The solution was then treated with sulphur dioxide to destroy excess of periodate, and 12N-hydrochloric acid (15 µl.) was added. After hydrolysis for 24 hr. at 100°, followed by removal of solvent, the products were chromatographed in solvent system *A*. A sample (0·5 mg.) of teichoic acid was treated with ammonia solution (*d* 0·88) for 20 min. at room temperature to remove alanine residues. After removal of ammonia, oxidation and hydrolysis were carried out as above. In a control experiment the oxidation step was omitted. The paper was sprayed with the periodate–Schiff reagents, and the anhydroribitol was estimated visually. Periodate oxidation (with or without pre-treatment with ammonia) of teichoic acid from *L. arabinosus* reduced only slightly the amount of anhydroribitol finally formed, *i.e.*, most of the ribitol residues resist periodate oxidation. However, the same treatment of teichoic acid from *B. subtilis* destroyed nearly all the ribitol residues and only a trace of anhydroribitol was finally formed.

It was shown in a separate experiment that no alanine residues are removed from teichoic acid under the conditions of the periodate oxidation.

*Phosphorus Analysis, and Teichoic Acid Content of Bacterial Cell Walls.*—The cell wall preparations studied had the following phosphorus content (Allen's method [40]): *L. arabinosus*, 3·4; *B. subtilis*, 4·2; *S. aureus*, 2·9%. Thus *L. arabinosus* wall is calculated to consist of 49—75% teichoic acid, depending on the proportions of ribitol residues possessing one, or two alanylglucosyl residues; *B. subtilis* and *S. aureus* walls contain 61 and 42% of teichoic acid respectively.

*Formation of Alanine and Alanine Amide from Teichoic Acid.*—Teichoic acid (0·5 mg.) from *L. arabinosus*, *S. aureus*, and *B. subtilis* was dissolved in water (10 µl.) and chromatographed on paper in *n*-butyl alcohol saturated with water. The dried chromatogram was sprayed with the ninhydrin reagent. Teichoic acid remained on the origin and gave a positive reaction. No free alanine was detected. A similar chromatogram was run in the ammoniacal solvent system *A*. The teichoic acid remaining on the origin did not react with ninhydrin and an elongated spot was observed which stretched from the $R_F$ of alanine amide to that of alanine.

Teichoic acid (0·5 mg.) and α-alanine ethyl ester (0·25 mg.) were separately treated with ethyl alcohol–ammonia (*d* 0·88) (1 : 1; 10 µl.) and kept overnight at room temperature. Paper chromatography of the products in solvent system *A* showed that α-alanine ethyl ester had completely decomposed, giving a mixture of alanine and alanine amide, the latter predominating. This behaviour was also observed with teichoic acid.

*Action of Hydroxylamine on Teichoic Acid.*—Teichoic acid (1·0 mg.) from *L. arabinosus* was allowed to react with hydroxylamine under conditions similar to those described by Kaye and Kent,[41] and the products were examined on paper in *n*-butyl alcohol–acetic acid–water (4 : 1 : 5) (organic layer), and *n*-butyl alcohol saturated with water. Spraying of the chromatograms with a solution of ferric chloride in dilute hydrochloric acid showed a reddish spot indistinguishable in $R_F$ from the hydroxamic acid derivative of alanine, produced from alanine ethyl ester by reaction with hydroxylamine. No acethydroxamic acid was detected in the products of reaction of teichoic acid and hydroxylamine, suggesting the absence of *O*-acetyl groups in the intact polymer.

*Confirmation of the Identity of Alanine in Teichoic Acid.*—Teichoic acid (3 mg.) from *L. arabinosus* was hydrolysed in a sealed tube with 2N-hydrochloric acid at 100° for 3 hr. After removal of the solvent the products were run as a band in solvent system A. Only one ninhydrin-positive band was detected. It was cut out and eluted, and the products were examined alongside authentic alanine in solvent systems *B*, *C*, and also phenol–water (9 : 1), *n*-butyl alcohol–acetic acid–water (4 : 1 : 5) (organic layer), *n*-butyl alcohol saturated with water, and methanol–water–10N-hydrochloric acid–pyridine (80 : 17·5 : 2·5 : 10). The chromatograms run in the last-named solvent were dipped in a 0·1% solution of ninhydrin in acetone before heating; the others were sprayed with the usual ninhydrin reagent. In every case only alanine was detected.

*Paper Chromatography.*—Ascending-front chromatography was carried out on Whatman

[40] Allen, *Biochem. J.*, 1940, **34**, 858.
[41] Kaye and Kent, *J.*, 1953, 79.

No. 4 paper which had been washed with 2N-acetic acid, then water. The following solvent systems were used: *A*, *n*-Propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1).[35] *B*, *iso*Propyl alcohol–12N-hydrochloric acid–water (65 : 17 : 18).[42] *C*, *n*-Butyl alcohol–ethyl alcohol–water–ammonia (*d* 0·88) (organic layer) (40 : 10 : 49 : 1).[29]

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[42] Smith and Wyatt, *Biochem. J.*, 1951, **49**, 144.