SUMMARY

1. When [1:2- $^{14}\text{C}_2$]ethanolamine, DL-[3- ^{14}C]-serine, L-[^3H]serine or D-[^3H]serine was administered orally to earthworms, serine ethanolamine phosphodiester and lombricine were found to be radioactively labelled.

2. For each of the precursors the specific radioactivity of serine ethanolamine phosphodiester was greater than that of lombricine in both muscle and gut tissue.

3. The specific radioactivity of serine ethanolamine phosphodiester and lombricine was greater in gut than in muscle.

4. With [1:2- $^{14}\text{C}_2$]ethanolamine most of the radioactivity of muscle lombricine was in the guanidinoethanol portion of the molecule, whereas with DL-[3- ^{14}C]serine most of the radioactivity was in the serine portion.

5. When L-[amidino- ^{14}C]arginine was given, there was negligible radioactivity in serine ethanolamine phosphodiester and considerable radioactivity in lombricine, almost all of which was in the guanidinoethanol portion of the molecule.

6. These findings suggest that serine ethanolamine phosphodiester is the precursor of lombricine in the earthworm and that the amidino group is derived from arginine by the transamidinase-catalysed transfer of the amidino group to serine ethanolamine phosphodiester.

We are indebted to Dr D. I. Magrath and Mrs I. M. Beatty for a sample of synthetic SEP, to Dr R. Norris for advice about the earthworms and to Mr P. Allen for technical assistance.

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Biochem. J. (1960) **76**, 610

Structure of the Ribitol Teichoic Acid from the Walls of *Bacillus subtilis*

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(Received 3 March 1960)

Some micro-organisms contain in their walls a considerable amount of a polymer belonging to the group of phosphoric esters known as teichoic acids (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley, 1959; Armstrong *et al.* 1959*b*). These compounds are polymers of glycerophosphate or ribitol phosphate in which adjacent polyol residues are joined together through phosphodiester linkages. In addition, the glycerol teichoic acids contain alanine in ester linkage with a hydroxyl on the glycerol, whereas ribitol teichoic acids contain both alanine ester linkages and sugars or *N*-acylamino sugars. Hydrolysis of ribitol

teichoic acids with acid, or with alkali followed by phosphatase treatment, suggests that the sugars are attached glycosidically to hydroxyl groups in the ribitol residues.

In the present work the structure of the ribitol teichoic acid from the walls of *Bacillus subtilis* is discussed. A preliminary account of some of these results has been given elsewhere (Armstrong, Baddiley & Buchanan, 1959*a*).

EXPERIMENTAL

Materials. Prostate phosphatase was kindly supplied by Dr G. Schmidt and β -glucosidase by Dr W. J. Whelan. D-Alanine and D-amino acid oxidase were purchased from

* Salters' Student.

L. Light and Co. Ltd., Colnbrook, Bucks. *B. subtilis* was grown from a culture provided by Dr M. R. J. Salton.

Paper chromatography

Paper chromatography was carried out on Whatman no. 1 or no. 4 paper which had been washed with 2*N*-acetic acid and then water. The following solvent systems were used: A, propanol-ammonia (sp.gr. 0.88)-water (6.3:1) (Hanes & Isherwood, 1949); B, butanol-ethanol-water-ammonia (sp.gr. 0.88) (40:10:49:1) (organic phase) (Foster, Horton & Stacey, 1957); C, butanol-pyridine-water (6:4:3) (Jeanes, Wise & Dimler, 1951); D, phenol-water (9:1). The products were detected by E, the improved periodate-Schiff reagent for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956*b*); F, molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949); G, aniline phthalate reagent for reducing sugars (Partridge, 1949); H, ninhydrin reagent for amino acids (Consden & Gordon, 1948).

Isolation of teichoic acid from Bacillus subtilis cells

B. subtilis was grown in bottles (10 l.) with aeration in a peptone (2%)-sodium chloride (0.5%) liquid medium. Growth from an inoculum (250 ml.) of fresh cells was allowed to take place for 48 hr. at 22°. The cells were harvested in a Sharples centrifuge and washed with 0.95% sodium chloride solution at 0°. They were successively treated in a blender (30 sec.) with acetone, ethanol and ether (15 vol. of each) at room temperature.

Short cold extraction. Fat-free cells (36 g.) were extracted three times with 150 ml. portions of ice-cold 10% trichloroacetic acid in a blender for 1 min. After centrifuging, the combined supernatant solutions were filtered through Supercel silica, 2 vol. of ethanol and 2 vol. of acetone were added and the solution was kept for 24 hr. at 0°. The material collected by centrifuging was reprecipitated from ice-cold 10% trichloroacetic acid (100 ml.) by the addition of 2 vol. of ethanol, kept for 18 hr. at 0°, then centrifuged and washed with ethanol and ether. The teichoic acid (200 mg.) contained small amounts of nucleic acid and polysaccharide.

Prolonged cold extraction. The cell debris from the short extraction was treated in a blender (3 min.) with ice-cold 10% trichloroacetic acid (250 ml.), stirred for 18 hr. at 0°, then treated further (1 min.) in the blender. The supernatant solution, after centrifuging of the debris, was filtered through Supercel silica and mixed with 3 vol. of ethanol. After 24 hr. at 0° the material was collected by centrifuging and reprecipitated from 10% trichloroacetic acid (50 ml.) with ethanol (2 vol.). After 18 hr. at 0° the precipitate was removed by centrifuging and dried with acetone and ether. The teichoic acid (1.23 g.) contained 4% of nucleic acid and traces of a glycerophosphate derivative which is probably a glycerol teichoic acid. The addition of a further $\frac{1}{2}$ vol. of ethanol to the supernatant and then allowing it to stand for 24 hr. at 0° yielded a precipitate which was collected by centrifuging and dried with acetone and ether. This teichoic acid (311 mg.) contained 5% of nucleic acid and traces of the glycerophosphate derivative and polysaccharide.

Extraction at 50°. The cell residue from the short cold extraction of fat-free whole cells (15 g.) was stirred with 5% trichloroacetic acid (300 ml.) for 15 min. at 50°. After

centrifuging, the supernatant solution was filtered through Supercel silica and the trichloroacetic acid extracted with 6 $\times$ 150 ml. of ether. The resulting precipitate was removed by centrifuging, the pH of the supernatant was adjusted to 6.3 with ammonia and the volume reduced to 15 ml. by evaporation at 25° under reduced pressure. The solution was cooled and 5 vol. of ethanol were added. After 48 hr. the insoluble material was centrifuged and reprecipitated from 50 ml. of 10% trichloroacetic acid by the addition of 2 vol. of ethanol. After 24 hr. at 0° the precipitate was collected by centrifuging and dried with acetone and ether. The teichoic acid (0.65 g.) contained 5-10% of nucleic acid and glycerol teichoic acid in larger amounts than that present in material obtained from cold extractions.

Isolation of teichoic acid from Bacillus subtilis walls

The organism was grown for 18 hr. at 30° as described previously. The cells (20 g.) were harvested in a Sharples centrifuge, washed with 0.95% sodium chloride solution and water and then suspended in water (175 ml.) at 0°. Portions of the suspension (10 ml.) were mixed with portions (10 ml.) of Ballotini beads and shaken in a Mickle disintegrator for 20 min. at 0°. The beads were removed by filtration through a no. 1 sintered-glass funnel and the filtrate was centrifuged at 15 000 rev./min. for 30 min. The top layer of cell contents, the middle layer of walls and the dense bottom layer of whole cells were separated and the wall fraction was treated in a homogenizer with 0.1*M*-phosphate buffer, pH 7.0. The suspension was centrifuged at 12 000 rev./min. for 20 min., and again the whole cells were removed. This process was repeated 14 times and was followed by ten washes with water and centrifuging at 15 000 rev./min. for 30 min. The preparation was examined under the electron microscope for whole cells and cell contents. The walls (30 mg.) (Found: P, 4.1%) were freeze-dried and samples (2.0 mg.) hydrolysed with 2*N*-hydrochloric acid for 3 hr. at 100°. After removal of hydrochloric acid by evaporation the residues were chromatographed in solvent A. The papers were examined under ultraviolet light and sprayed with reagents E and F (see below).

B. subtilis walls (120 mg.) were stirred with 10% trichloroacetic acid (5 ml.) for 24 hr. at 0°. The walls were centrifuged and extracted twice with portions (5 ml.) of 10% trichloroacetic acid. The supernatant solutions from each extraction were mixed with ethanol (5 vol.) and kept for 24 hr. at 0°. The precipitates were collected by centrifuging and dried with ethanol and ether. The combined precipitates (40 mg.) were dissolved in 10% trichloroacetic acid (2.0 ml.) and fractionally precipitated with ethanol (1.5, 2.5 and 4.5 vol.). The first two precipitates (36 mg.) contained pure teichoic acid. The third contained teichoic acid contaminated with polysaccharide.

The wall residue was suspended in a little water and the trichloroacetic acid was removed by six extractions with ether ($\frac{1}{2}$ vol.). The residue was freeze-dried (74 mg.) (Found: P, 1.83%).

Teichoic acid

Acid hydrolysis. Teichoic acid (2.0 mg.), isolated from walls, was hydrolysed in 2*N*-hydrochloric acid in a sealed tube for 3 hr. at 100°. After evaporation the samples were examined by paper chromatography in solvents A, B, C and D.

Products are given in Table 1 and R_F values in Table 2.

Analysis. Teichoic acid (1.0 mg.), isolated from walls, was hydrolysed in 2N-hydrochloric acid for 9 hr. at 100°. The hydrolysate was neutralized to pH 7.0 with sodium hydroxide and the volume adjusted to 5.0 ml. Phosphorus (Chen, Toribara & Warner, 1956), glucose (Park & Johnson, 1949) and alanine (Rosen, 1957) were determined. The glucose value was corrected for destruction (12%) by acid during hydrolysis. This correction was estimated by heating glucose and alanine together under the conditions of the hydrolysis and determining the amount of glucose destroyed. (Found: ratio P:glucose:alanine, 1.0:0.93:0.46.)

Alkali hydrolysis and enzymic dephosphorylation. Teichoic acid (650 mg.) isolated from whole cells by extraction at 50° (see above) was dissolved in N-sodium hydroxide (7.0 ml.) and the resulting solution heated for 3 hr. at 100°. A slight red-brown precipitate (probably ferric hydroxide) was removed by centrifuging and washed with water. The supernatant and washings were adjusted to 25 ml. with water and passed through a Dowex-50 (NH_4^+ form) (25 ml.) column and the column was washed with water (500 ml.). The eluate was decreased to 25 ml. by evaporation at 30° under reduced pressure. A sample (0.1 ml.) was removed and examined in solvents A and B with reagents E, F, G and H. The results are shown in Table 1.

Table 1. *Hydrolysis products of teichoic acid*

Teichoic acid was hydrolysed for 3 hr. in HCl (2N) or NaOH (N) at 100°. The products were identified by chromatography (see text).

	Acid hydrolysis	Alkali hydrolysis
1:4-Anhydorrbitol	+	-
Ribitol	+	+*
Glucose	+	-
Alanine	+	+
Glucosylribitol	+	+
Ribitol phosphates	+	+*
Glucosylribitol phosphates	+	+
1:4-Anhydorrbitol 5-phosphate	+	-
Inorganic phosphate	+	-

* Traces only.

Table 2. *Paper chromatography of acid hydrolysis products of teichoic acid*

Solvents used were propanol-ammonia (A) and butanol-ethanol-water-ammonia (B).

	R_F A	R_{ribitol} B
Anhydorrbitol	0.75	1.38
Glycerol	0.75	1.47
Ribitol	0.65	1.00
Glucose	0.61	0.65
Alanine	0.63	0.57
Glucosylribitol	0.55	0.55
Ribitol 1-phosphate	0.30	.
Ribitol 2 (and 3)-phosphate	0.33	.
Anhydorrbitol 5-phosphate	0.29	.
Glucosylribitol 1 (and 2)-phosphate	0.28	.
Ribitol diphosphate	0.12	.
Glucosylribitol diphosphate	0.12	.
Glycerol diphosphate	0.12	.

The solution was neutralized to pH 5.5 with acetic acid, 0.4M-ammonium acetate buffer (pH 5.5, 15 ml.) and prostate-phosphatase solution (1.5 ml.) were added and the volume was adjusted to 50 ml. with water. The solution was incubated at 37° under toluene, samples being removed at intervals and analysed for inorganic phosphate and total phosphorus (Fiske & Subbarow, 1925). After 3 days 91% of the phosphorus had been released as inorganic phosphate. A further 0.5 ml. of prostate-phosphatase solution was added and the incubation was continued for a further 17 hr., after which time 99.3% of the phosphorus had been released as inorganic phosphate. The volume was adjusted to 200 ml. with water and the solution was passed quickly through a Dowex-50 (H^+ form) (50 ml.) resin column cooled to 0-5°. The eluate and 250 ml. of column washings were passed directly into a flask containing 30 ml. of Dowex-2 (CO_3^{2-} form) resin. The contents of the flask were poured on to a Dowex-2 (CO_3^{2-} form) (10 ml.) column and the column was washed with water (500 ml.). The eluate was evaporated at 30-35° under reduced pressure to a small volume and then freeze-dried. The dry white solid (386 mg.) was examined in solvents A and B and contained only glycerol, ribitol and glucosylribitol.

The solid (200 mg.) was suspended in hot ethanol and water was added carefully until the solid just dissolved. The solution was kept at room temperature overnight. The rosettes of white needles were filtered off, washed with ethanol and ether, dried and recrystallized from hot aqueous ethanol. The glucosylribitol (157 mg.) had m.p. 137-138°; $[\alpha]_D^{20}$ -28.6° in water (c, 1.0). (Found: C, 41.9; H, 7.6. $\text{C}_{11}\text{H}_{20}\text{O}_{10}$ requires C, 42.0; H, 7.1%). It was examined in solvents A and B and contained no glycerol or ribitol.

Potentiometric titration. Teichoic acid (8.0 mg.) prepared from walls was dissolved in aqueous 2N-ammonia (2.0 ml.) and the solution kept for 5 hr. at room temperature. The excess of ammonia was evaporated, water (5.0 ml.) was added and the solution was passed through a Dowex-50 (H^+ form) (3.0 ml.) resin column to remove alanine, alanine amide and NH_4^+ ions. The column was washed with water (10 ml.) and the eluate and washings were adjusted to 20 ml. with water. The teichoic acid in this solution was titrated against 0.01N-sodium hydroxide, the change of pH being followed on a Pye pH meter. A plot of $\Delta\text{pH}/\Delta V$ against V , where V is the volume of alkali used, gave two peaks, indicating the points of neutralization of the primary and secondary acidic groups. The ratio of primary to secondary groups was 8.9:1.

Periodate oxidation. The alanine residues in teichoic acid (7.0 mg.) from walls were removed by treatment with ammonia as in the previous experiment. The eluate from the Dowex-50 (H^+ form) (3.0 ml.) resin column was neutralized to pH 6.5 with sodium hydroxide, 0.3M-sodium metaperiodate solution (0.4 ml.) was added, the volume was adjusted to 10 ml. with water and the solution was kept at room temperature in the dark. A control experiment was carried out without added periodate. Samples (2.0 ml.) were used to determine the periodate used and the formaldehyde formed (Hough, Powell & Woods, 1956); samples (0.1 ml.) were also used for total phosphorus determination (Chen *et al.* 1956). After 60 hr. 51 μmoles of periodate had been used. 1.96 μmoles of formaldehyde were formed and 17.2 μmoles of phosphorus were present. The ratio of periodate uptake to formaldehyde formed was 26:1; P:formaldehyde, 8.8:1; periodate

uptake: P, 2.97:1. Ratios required for structure (VII) are 28, 9 and 3.1 to 1 respectively.

Action of prostate phosphatase. Teichoic acid (5.2 mg.) from walls was dissolved in water (7.0 ml.). 0.4 M-Ammonium acetate buffer (2.0 ml.), pH 5.5, and prostate phosphatase (0.2 ml.) were added, and the volume was adjusted to 10 ml. with water. The solution was incubated under toluene at 37° and at intervals samples (0.2 ml.) were removed for total phosphorus determination and larger samples (2.0 ml.) for inorganic phosphate determination (Chen *et al.* 1956). After 90 hr. the ratio total P:inorganic P was 27:1. A further quantity (0.1 ml.) of phosphatase was added and incubation continued for 66 hr. The ratio total P:inorganic P decreased to 21:1 (the ratio expected from structure (VII) is 9:1).

Isolation of alanine. Teichoic acid (600 mg.), prepared from whole cells by prolonged cold extraction was hydrolysed with N-sodium hydroxide (6.0 ml.) at 100° for 3 hr. A small amount of ferric hydroxide was removed by centrifuging and washed with water. The supernatant and washings were adjusted to pH 6.2 with acetic acid and a solution of barium hydroxide (350 mg.) in water (2.0 ml.) was added. Ethanol (400 vol.) was added with stirring and the mixture was kept overnight at 0°. The precipitated barium salts (0.72 g.) were collected by centrifuging and washed twice with ethanol (250 ml.) and ether and dried over phosphoric oxide. Chromatography in solvent A (spray reagents E, F and H) indicated that these barium salts contained the normal phosphates produced by alkali hydrolysis of teichoic acid together with a small amount of alanine.

The supernatant and ethanol washings, containing the bulk of the alanine, were diluted with water (65 ml.) and passed through a Dowex-50 (H⁺ form) (35 ml.) resin column. The column was washed with water (500 ml.); no amino compounds were detected in the eluate. The alanine was eluted from the column with 2N-hydrochloric acid, 5 ml. fractions being collected. Appropriate fractions were evaporated to dryness under reduced pressure at 30° and kept over potassium hydroxide. The dry solid was dissolved in water (1 l.), the pH was adjusted to 7 with sodium hydroxide and the solution was passed through a Dowex-2 (OH⁻ form) (50 ml.) resin column. The column was washed with water (1 l.) and the eluate and washings were checked with ninhydrin for the absence of alanine. Alanine was eluted (5 ml. fractions) with N-hydrochloric acid and appropriate fractions were combined and evaporated to dryness under reduced pressure at 30°. Repeated addition of water (0.5 ml.) and evaporation to dryness over potassium hydroxide failed to remove traces of hydrochloric acid. The alanine hydrochloride (89 mg.) was slightly yellow due to impurities from the resin. Chromatography in solvents A and D and butanol-water and spraying with reagent H failed to detect impurities.

Configuration of alanine. The alanine hydrochloride (6.0 mg.) was dissolved in water (2.0 ml.) and samples (0.2 ml.) were diluted to 10 ml. with 0.05 M-pyrophosphate buffer, pH 8.2. The concentration of alanine in these samples was determined colorimetrically with ninhydrin (Rosen, 1957). Standard solutions containing 20 μ moles of D- and L-alanine/ml. were prepared in 0.05 M-pyrophosphate buffer, pH 8.2. Enzymic oxidation was measured in a Warburg apparatus on solutions containing 8 μ moles of amino acid, 5 μ g. of catalase, 20 mg. of D-amino acid oxidase

and buffer to a final volume of 3.2 ml. The centre well contained 20% potassium hydroxide (0.2 ml.) and the reaction was carried out in air at 37°. The alanine from teichoic acid consumed 0.49 mole of oxygen/mole and authentic D-alanine consumed 0.5 mole of oxygen/mole. No oxygen was consumed by authentic L-alanine.

Glucosylribitol

Acid hydrolysis. The compound (1.0 mg. samples) was hydrolysed in 2N-hydrochloric acid for 3, 12 and 24 hr. at 100°. The acid was removed by evaporation and the products were examined by paper chromatography in solvents A, B and C. After 3 hr. the compound was partially hydrolysed to ribitol, anhydribose and glucose; after 12 hr. hydrolysis was complete and the products were ribitol, anhydribose and glucose; after 24 hr. the products were anhydribose and glucose.

Periodate oxidation and borohydride reduction. Glucosylribitol (1.0 mg.) was dissolved in 1% sodium metaperiodate solution (0.4 ml.). It was left for 18 hr. at room temperature, sodium borohydride (2.0 mg.) was added and the solution was kept for a further 24 hr. 2N-Hydrochloric acid (0.2 ml.) was added and the solution was heated for 30 min. at 100°. After evaporation under reduced pressure the product was examined in ethyl acetate-pyridine-water (7:2:1) (Viscontini, Hoch & Karrer, 1955). The only observed product rapidly gave a purple colour with reagent E and had R_F 0.43 (glycerol had R_F 0.43, ethylene glycol had R_F 0.55).

Periodate oxidation. Glucosylribitol (15.8 mg.) was dissolved in water (25 ml.), 0.3 M-sodium metaperiodate (2.0 ml.) was added and the volume was adjusted to 50 ml. with water. A control experiment containing no glucosylribitol was also carried out. The solutions were kept at room temperature in the dark. Samples (5 ml.) were used for the determination of periodate consumed and formic acid produced, and other samples (2 ml.) for formaldehyde determination by the method of Hough *et al.* (1956). The formic acid was titrated with 0.01 N-sodium hydroxide solution to pH 6.25. After 18 hr. glucosylribitol had consumed 3.93 mol.prop. of periodate and produced 0.96 mol.prop. of formaldehyde and 1.0 mol.prop. of formic acid. After 36 hr. 1.2 mol.prop. of formic acid had been produced but the periodate uptake and formaldehyde production were unchanged. To samples (5 ml.) of the glucosylribitol solution and to the control was added ethylene glycol (2 ml.). After 15 min. at room temperature in the dark carbon dioxide-free air was bubbled through, 0.01 N-sodium hydroxide (2 ml.) was added and the samples were kept at room temperature for 1 hr. Water (5.0 ml.) was added and formic acid production was determined by back titration with 0.01 N-oxalic acid. After this treatment 1.9 mol.prop. of formic acid was formed. Further oxidation for 24 hr. at 37° liberated 1.7 mol.prop. of formic acid.

Action of β -glucosidase. Glucosylribitol (1.0 mg.) was dissolved in water (15 μ l.) and β -glucosidase (15 μ l. of a 1% solution) was added. The solution was incubated under toluene for 12 hr. at 30°. The hydrolysate was examined by chromatography in solvents A and C with reagents E and G. Hydrolysis was complete, only glucose and ribitol being detected.

Oxidation and hydrolysis to glyceric acid. Glucosylribitol (32.3 mg.) and α -methyl glucoside (48 mg.) (a molar ratio of

1:2) were separately dissolved in water (10 ml.), 0.3M-sodium metaperiodate (3 ml.) was added to each and the volumes were adjusted to 25 ml. with water. The solutions were kept in the dark at room temperature for 24 hr. and then samples (2 ml.) were removed for determination of periodate consumed (α -methyl glucoside consumed 1.98 mol.prop. of periodate, glucosylribitol consumed 3.93 mol.prop.). The solutions were passed rapidly through Dowex-50 (H^+ form) (10 ml.) resin columns and the resulting acid solutions kept for 24 hr. to hydrolyse formyl esters. Dowex-2 (CO_3^{2-} form) (6 ml.) resin was added to the two solutions and the suspensions were passed through Dowex-2 (CO_3^{2-} form) (5 ml.) columns. The columns were each washed with water (250 ml.) and the neutral eluates were evaporated to 5 ml. under reduced pressure (25–30°). The optical rotations of the solutions are given in Table 3.

The solutions were each adjusted to 35 ml., benzoic acid (146 mg.) and barium hydroxide (198 mg.) were added and the mixtures shaken until the solid dissolved. Bromine (0.3 ml.) was added to the ice-cold solutions in stoppered flasks, which were kept for 60 hr. at room temperature with frequent shaking during the first hour. Air was passed through the solutions to remove excess of bromine, the volumes were decreased to 15 ml. under reduced pressure and the benzoic acid was filtered off and washed with water. The volume of the combined filtrate and washings was decreased to 8 ml., 5N-sulphuric acid (0.9 ml.) was added to each flask and the solutions were heated at 100° for 2 hr. Barium carbonate (0.6 g.) was added to each and after keeping overnight the barium sulphate was filtered off through Supercel silica and washed with water. The filtrate and washings were passed through Dowex-50 (NH_4^+ form) (5.0 ml.) resin columns and the volume was decreased to 5.0 ml. under reduced pressure. The optical rotation of these solutions was measured. To 1 ml. of each solution was added ammonium molybdate (60 mg.) and the optical rotations were measured again (see Table 3).

Chromatography of the oxidation-hydrolysis products in solvent A and detection of products with reagent E confirmed the presence of glyceric acid in both solutions.

Action of β -glucosidase on monophosphates produced by alkali hydrolysis

Teichoic acid (3.0 mg.) was hydrolysed with N-sodium hydroxide at 100° for 3 hr. The hydrolysate was passed through a Dowex-50 (NH_4^+ form) (1.0 ml.) column and chromatographed in solvent A. The monophosphate region was eluted with water (0.2 ml.) and the eluate divided into three parts, which were treated separately as follows: (a) water (45 μ l.) and 0.2M-ammonium acetate buffer, pH 6.0 (45 μ l.), were added; (b) 1% β -glucosidase (45 μ l.)

and buffer (45 μ l.) were added; (c) 1% β -glucosidase (45 μ l.), buffer (45 μ l.) and sodium fluoride to a concentration of 0.012M were added. These solutions were incubated under toluene at 37° for 1 hr. then chromatographed in solvents A and C and the products detected with reagents E, F and G. The β -glucosidase hydrolysed some of the monophosphate giving glucose and an increase in the amounts of ribitol 1- and 2-phosphate. No ribitol 3-phosphate was detected. The fluoride in tube (c) inhibited the action of the phosphomonoesterase known to be present in the β -glucosidase preparation.

Action of phosphomonoesterase on diphosphates produced by alkali hydrolysis

Teichoic acid (2.0 mg.) isolated from whole cells by prolonged cold extraction was hydrolysed with 2N-hydrochloric acid for 3 hr. at 100°. The hydrolysate was evaporated and chromatographed in solvent A and the diphosphate region was eluted. The eluate was evaporated to dryness, the solid was dissolved in 0.4M-ammonium acetate buffer, pH 5.5 (45 μ l.), prostate phosphomonoesterase (45 μ l.) was added and the mixture was incubated under toluene at 37° for 3 days. The hydrolysate was chromatographed in solvent A and the products were detected with reagents E and F. The products were ribitol, glucosylribitol, inorganic phosphate and a faint trace of glycerol.

DISCUSSION

Walls prepared by a modification of Salton & Horne's (1951) method from bacteria which had been ruptured with a Mickle vibrator have been used in this work. Such preparations are free from cell contents and protoplast membrane (personal communication from Dr D. E. Hughes supported by observations in this Laboratory with various organisms). Their purity has been checked by examination with the electron microscope and by the absence of nucleic acid. Acid hydrolysates of walls contained the amino acids and sugars arising from the mucopeptide component (Salton & Ghuyssen, 1957) together with products from the ribitol teichoic acid in this organism described by Armstrong *et al.* (1958). No trace of glycerol or glycerophosphates was detected, and it is concluded that the glycerol teichoic acid present in extracts of whole organism is not located in the wall. This is in agreement with observations (unpublished) on the location of teichoic acids in many other bacteria, although glycerol teichoic acids do occur in the walls of some lactobacilli and staphylococci.

A considerable amount of teichoic acid can be extracted from bacterial walls with cold 10% trichloroacetic acid, thus suggesting that it is associated ionically with the mucopeptide. The approximate amount present in the walls of *B. subtilis* has been determined from the weight of pure teichoic acid recovered from such an extract, allowance being made for that which remained

Table 3. Optical rotations (10 cm. tube) of products during oxidation and hydrolysis of α -methyl glucoside and glucosylribitol

	$\alpha^\circ +$	
	α -Methyl glucoside	Glucosylribitol
Aldehyde from periodate oxidation	0.68	0.12
Glyceric acid	0.06	0.06
Glyceric acid + molybdate	0.28	0.24

For details see text.

(26 %) in the extracted walls. On this basis about 59 % of the weight of the wall is teichoic acid; this value is certainly low because of losses during purification. The assumption is made that the phosphorus content of the extracted walls is a measure of the teichoic acid which has not been removed by trichloroacetic acid. This is justified, since examination of hydrolysates of extracted walls fails to reveal the presence of phosphorus derivatives other than teichoic acid.

No attempt has yet been made to study the relationship between growth conditions and teichoic acid synthesis. However, the amount of teichoic acid which can be extracted from whole bacteria does vary in different batches of organism. The composition of the medium is clearly important since, when *B. subtilis* was grown in the presence of a relatively high concentration (2 %) of glucose, direct extraction of the whole cells yielded no teichoic acid. A considerable amount of a polysaccharide was obtained from the trichloroacetic acid extract of this culture and, as microscopic examination of the organisms suggested the presence of a capsule, it is possible that this polysaccharide was of capsular origin. Polysaccharides from this organism have been described (Sharon, 1957; Sharon & Jeanloz, 1960; Janczura, Perkins & Rogers, 1960).

In earlier work on the ribitol teichoic acid from *B. subtilis* material had been prepared from extracts of whole cells. These preparations invariably contained small amounts of glycerol teichoic acid; consequently, reliable analytical information could not be obtained. Material prepared from walls did not contain glycerol derivatives, but yielded on acid hydrolysis all the products previously ascribed to the ribitol teichoic acid in this organism. No traces of amino acids other than alanine were detected and the only sugar present was glucose. Other hydrolysis products which have been identified before were inorganic phosphate, ribitol, 1:4-anhydribose and its 5-phosphate, and the isomeric monophosphates of ribitol. Ribitol derivatives obtained by hydrolysis are presumably racemic compounds. The relative amounts of the different products varied with the hydrolysis time as discussed previously. Under certain conditions small amounts of hitherto undetected hydrolysis products were obtained. These were incompletely separated from each other on paper chromatograms but had the R_f values expected for diphosphates. On dephosphorylation with prostate phosphatase they yielded a mixture of ribitol and the glucosylribitol described later. Small amounts of glucosylribitol monophosphates were also detected in acid hydrolysates.

The unpurified extract of walls contained in addition to teichoic acid a small amount of a com-

pound which probably contained amino sugars. Although this compound has not been examined fully it resembles in some respects the polysaccharide from *B. subtilis* recently described by Janczura *et al.* (1960) and called by them teichuronic acid.

Analysis of the purified material from walls indicated a molar ratio (phosphorus:glucose) of almost 1:1, but the amount of alanine varied considerably according to the methods employed in extraction and purification. The ratio phosphorus:alanine reported here (1:0.46) is typical but, in view of the remarkable lability of the alanine ester linkages in this and other teichoic acids, it is probable that higher values would be obtained if milder conditions of isolation had been employed. The teichoic acid in *B. subtilis* walls is relatively difficult to extract and considerably higher values have been obtained with teichoic acids from other organisms when mild extraction and purification conditions were used.

The alanine residues in this teichoic acid are linked through their carboxyl groups to hydroxyl groups in the polymer. They are readily released in dilute alkali and react rapidly with ammonia or hydroxylamine. Moreover, the polymer shows an infrared-absorption band at 1751 cm^{-1} in potassium bromide, characteristic of a carboxylic ester. Although the rate of reaction with hydroxylamine has not been determined, the alanine residues in ribitol teichoic acid from *Lactobacillus arabinosus* react with hydroxylamine (Armstrong *et al.* 1959b) at a rate comparable with that for the amino acid-ester linkages in nucleic acid-amino acid compounds concerned in protein synthesis. Experiments with D-amino acid oxidase indicate that the alanine in this teichoic acid, like that from *L. arabinosus* and *Staphylococcus aureus*, has the D-configuration. The location of the alanine ester groups in the polymer is not yet known.

Mild alkali hydrolysis under conditions comparable with those used for the formation of nucleotides from ribonucleic acid had little effect on the phosphodiester linkages in the teichoic acid. However, more vigorous conditions caused complete hydrolysis of phosphodiester linkages, the main products being alanine and a mixture of isomeric phosphomonoesters of glucosylribitol. Although these phosphates were not separated from each other on paper chromatograms their structures were determined by methods described later. Phosphate groups were readily removed from this mixture by prostate phosphatase, the resulting crystalline glucosylribitol being isolated in high overall yield.

Acid hydrolysis of the glucosylribitol yielded glucose, ribitol and anhydribose; the proportion of anhydribose to ribitol increased with

increasing time of hydrolysis. The low negative rotation of the glucosylribitol was consistent with a β -D-glucopyranoside, and this was confirmed by its ready hydrolysis to glucose and ribitol in the presence of a β -glucosidase. This glucoside differs chromatographically (periodate-Schiff reagents) from a glucosylribitol obtained in a similar manner from the ribitol teichoic acid of *L. arabinosus*. The latter is believed to be an α -glucoside, since it has a high positive rotation and is unaffected by a β -glucosidase (unpublished work with Dr A. R. Archibald).

It seemed unlikely that the glucose was attached at the terminal 1 (5)-position of the ribitol since periodate oxidation, followed by reduction of products with sodium borohydride and then acid hydrolysis (Viscontinini *et al.* 1955), gave glycerol but no ethylene glycol. Moreover, if the glucosyl residue is attached to position 3 in the ribitol, the compound should give a yellow colour on paper chromatograms sprayed with the periodate-Schiff reagents for glycols (Baddiley, Buchanan & Carss,

1957a). The glucosylribitol gave the normal purple colour in this test, thus indicating the absence of the glucose at the 3-position.

The structure 4-O-(β -D-glucopyranosyl)-D-ribitol (I) was established for the glucoside by periodate oxidation. It consumed 4 mol.prop. of periodate and gave 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde. It follows that the glucopyranosyl substituent must be at position 2 or 4 on the ribitol residue. The oxidation was carried out with sodium periodate in the absence of buffer, and under these conditions only 1.2 mol.prop. of formic acid was liberated directly. The additional 0.8 mol.prop. of formic acid was released when the solution was made alkaline, and was presumably present as a formic ester in the oxidation mixture. It is concluded that the main course of the oxidation occurs first between carbon atoms 1 and 2 in the ribitol residue to give the 3-O-glucosylerythrose (II) and formaldehyde. Further oxidation of this disaccharide would give a formyl ester (III) and 1 mol.prop. of formic acid. A second mol.prop.

of formic acid would be released under alkaline conditions, the final product being the trialdehyde (IV). The suggested sequence is in agreement with recent views on the course of periodate oxidation of reducing sugars (Warsi & Whelan, 1958; Hough, Taylor, Thomas & Woods, 1958). The preferential oxidation of the acyclic polyol in the first stage of the sequence is supported by Brown, Hall & Letters's (1959) observations on the periodate oxidation of glycerol inositol phosphates. The glycerol residue is readily oxidized under conditions which do not affect the inositol.

Positions 2 and 4 in the ribitol residue can be distinguished from each other by a study of the trialdehyde obtained from the periodate oxidation. 4-*O*-(β -D-Glucopyranosyl)-D-ribitol (I) would give the trialdehyde (IV), whereas 2-*O*-(β -D-glucopyranosyl)-D-ribitol would yield the stereoisomeric compound (V). Both these trialdehydes possess two glyceraldehyde residues; moreover, in both cases the glyceraldehyde residue which arises from carbon atoms 4, 5 and 6 of the original D-glucose will have the D-configuration. The other will have the D-configuration in structure (IV) and the L-configuration in structure (V). It follows that whereas the trialdehyde (IV) would be optically active, that with structure (V) would be a *meso* compound. As all other products of the oxidation are optically inactive, direct measurement of the rotation of the oxidation mixture should be permissible. In fact, this mixture exhibited pronounced optical activity, thus indicating structure (IV) for the trialdehyde. This activity was unaltered after several hours in the presence of acid; these conditions would have effected equilibration of unsymmetrical hemi-acetals of (IV) or (V) which might have arisen during the oxidation.

Further oxidation of the trialdehyde with bromine water, and hydrolysis of the resulting tricarboxylic acid, gave glyceric acid. This acid from structure (IV) would have the D-configuration, whereas from structure (V) it would be racemic. The specific rotation of glyceric acid is considerably enhanced in the presence of molybdate (Isherwood, Chen & Mapson, 1954), and even with the small

quantities of material used in this work a marked positive rotation was observed in this way. It follows that the glyceric acid has the D-configuration and must arise from structure (IV); thus the glucosylribitol is correctly represented by structure (I).

The general behaviour of ribitol teichoic acids, and particularly the nature of their products of acid hydrolysis, indicates that they are polymers in which ribitol residues are joined through phosphodiester linkages. These polymers are analogous to the glycerol teichoic acids, in which it is known that glycerol residues are joined to each other through phosphodiester groups. This is now supported by titration studies on the ribitol teichoic acid from *B. subtilis*, after careful removal of alanine residues with cold dilute ammonia. One primary acidic group is present for each phosphorus atom in the polymer. The low but measurable content of secondary acidic groups suggests the presence of a few phosphomonoester residues, but these are believed to represent an end group terminating the polymer chain.

The alkali-lability of this teichoic acid is explained by a structure in which at least one hydroxyl group is adjacent to each phosphodiester group in the polymer. The mechanism of hydrolysis in alkali would be similar to that proposed for ribonucleic acid (Brown & Todd, 1952; Brown, Magrath, Neilson & Todd, 1956), and must involve the intermediate formation of cyclic phosphates. The somewhat more vigorous treatment required for the hydrolysis of teichoic acid reflects the greater difficulty in forming a cyclic phosphate from an open-chain polyol compared with the sterically easier situation in ribonucleic acid. As the alkali hydrolysis of this teichoic acid gives very largely a mixture of two monophosphates of glucosyl-ribitol it is concluded that hydrolytic fission is unidirectional. It follows that each phosphodiester group in the polymer chain bears only one substituent containing an adjacent hydroxyl group. The structure (VI) satisfies this requirement and is strongly supported by biogenetic consideration. It is reasonable to assume that ribitol teichoic acids

are formed naturally from the bacterial nucleotide cytidine diphosphate ribitol (Baddiley, Buchanan, Carss & Mathias, 1956*a*), the presence of which has been demonstrated in extracts of *B. subtilis* (Clarke, Glover & Mathias, 1959, and unpublished observations in this Laboratory), and that a similar relationship exists between the glycerol teichoic acids and cytidine diphosphate glycerol (Baddiley, Buchanan, Mathias & Sanderson, 1956*c*). Polymer formation through these nucleotides is likely to occur by successive transfer of the polyol phosphate group in the nucleotide. It is known that cytidine diphosphate ribitol is a derivative of D-ribitol 5-phosphate (Baddiley *et al.* 1957*a*), and it follows that one phosphate linkage in ribitol teichoic acids should be at the 5-position in the D-ribitol residues, as shown in (VI).

A sample of teichoic acid from which the alanine residues had been removed was oxidized with periodate. Acid hydrolysis of the oxidized product yielded insignificant amounts of anhydrosorbitol, consequently each ribitol residue in the polymer must contain an unsubstituted α -glycol grouping. From these considerations positions 1 and 5 or 3 and 5 are the only ones available for the phosphodiester linkages in the glucosylribitol residues.

If the phosphodiester linkages are at positions 3 and 5 then each ribitol residue should yield 1 mol.prop. of formaldehyde on oxidation with periodate, whereas a 1:5-phosphodiester would yield formaldehyde only from a glucosylribitol residue at one end of the polymer chain. For every 3 mol.prop. of periodate consumed by the polymer, less than 0.12 mol.prop. of formaldehyde were produced, consequently the teichoic acid must contain only ribitol 1:5-phosphodiester linkages and will have the structure (VII). Although the number of alanine ester residues in this structure is greater than the analysis would indicate, this is intended to represent undegraded teichoic acid.

The course of the alkali hydrolysis of the polymer is readily understood on the basis of structure (VII), which for this purpose can be considered in a simplified form (VIII) still embodying the essential features of the larger molecule. Unidirectional cyclization of the phosphodiester linkages would give the 1:2-cyclic phosphate of glucosylribitol 5-phosphate (IX), the 1:2-cyclic phosphate of glucosylribitol (X) and glucosylribitol (XI). Under the reaction conditions employed the cyclic phosphates would hydrolyse to a mixture of isomeric glucosylribitol diphosphates (XII) and (XIII) and isomeric glucosylribitol phosphates (XIV) and (XV).

The relative amounts of the five products will depend upon the chain length of the polymer. In fact, the main products were the two monophosphates (XIV) and (XV), arising from the glucosylribitol phosphate residues within the chain. A small amount of glucosylribitol (XI) was observed on paper chromatograms of the hydrolysate, and a phosphate-containing spot was detected in the position expected for the mixture of diphosphates (XII) and (XIII); as these products would arise only from the ends of the chain the amounts formed were small. Although the diphosphates have not yet been fully characterized they give glucosylribitol on treatment with phosphatase. The structure of the mixture of monophosphates has been established by hydrolysis with a β -glucosidase. The products were glucose and a mixture of ribitol 1(5)-phosphate and ribitol 2(4)-phosphate. These ribitol phosphates were conveniently identified by paper chromatography, whereby they can be separated from each other and readily distinguished from the isomeric ribitol 3-phosphate (Baddiley, Buchanan & Carss, 1957*b*).

The end groups in structure (VII) are in agreement with the most likely mechanism for the biosynthesis of this teichoic acid. Although the

comprises eight phosphodiester linkages and one phosphomonoester. The presence of a small amount of phosphomonoester groups in the teichoic acid which had had its alanine removed was also demonstrated by the liberation of inorganic phosphate during treatment with prostate phosphomonoesterase. Unfortunately, it was not possible to determine the molecular weight of the polymer in this way, since the liberation of phosphate was very slow and incomplete. The ratio periodate consumed:formaldehyde produced (26:1) and the ratio total phosphorus:formaldehyde produced

(8.8:1) on oxidation of teichoic acid from which alanine has been removed is also consistent with a polymer in which nine glucosylribitol units are joined through phosphodiester linkages, as shown in structure (VII).

At least one of the pneumococci type-specific substances (S. VI) is a ribitol phosphate derivative (Rebers & Heidelberg, 1959*a*). Although the structure of this compound is not yet settled it appears to be a polymer in which a trisaccharide composed of galactose, rhamnose and glucose is attached to the 3-position in the ribitol residues. It is not known whether it is related biochemically to the teichoic acids. Recent work (Rebers & Heidelberg, 1959*b*) suggests that in the type-specific substance the phosphodiester linkages involve a hydroxyl group in the ribitol residues and a hydroxyl at position 2 or 4 of the galactose residues. An analogous structure (XVI) for the teichoic acid from *B. subtilis* is unlikely for several reasons (alanine ester groups have been omitted for convenience).

This is the only alternative to structure (VIII) that would consume 3 mol.prop. of periodate for each repeating polymer unit and give only traces of formaldehyde. It is not easy to understand how such a structure can be formed in any reasonable biogenetic scheme involving cytidine diphosphate ribitol, nor can it yield ribitol diphosphate on acid hydrolysis. Moreover, the absence of glucose 6-phosphate from acid hydrolysates is not readily explained by structure (XVI).

SUMMARY

1. About 60% of the dry weight of *Bacillus subtilis* walls is a ribitol teichoic acid composed of alanine, glucose, ribitol and phosphate. This compound has been separated from the wall mucopeptides by extraction with cold trichloroacetic acid and its structure has been determined.

2. The alanine residues have the D-configuration and are joined to hydroxyl groups in the polymer through their carboxyl groups. These alanine ester linkages are unusually labile.

3. Alkali hydrolysis of the teichoic acid yields a mixture of phosphoric esters of a glucosylribitol. After enzymic removal of phosphate groups a crystalline glucosylribitol was obtained in good yield. The structure 4-O-(β -D-glucopyranosyl)-D-ribitol (I) is established for this compound by periodate oxidation, by the action of β -glucosidase and from a study of the optical activity of oxidation products.

4. Periodate oxidation of the teichoic acid from which alanine residues had been removed indicates that the glucosylribitol residues are joined through

phosphodiester linkages at positions 1 and 5 in the ribitol.

5. Titration studies and other evidence shows that this teichoic acid has the structure (VII), in which nine alanylglucosylribitol 5-phosphate units are joined together. Biogenetic evidence is consistent with this structure.

We thank Mr A. L. Davison and Miss S. Robinson for assistance in the growth of bacteria, Mr E. H. Boulton for assistance in electron microscopy and the Medical Research Council and the Rockefeller Foundation for financial support.

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Biochem. J. (1960) **76**, 621

Quantitative Studies of the Avidity of Naturally Occurring Substances for Trace Metals

4. A POTENTIOMETRIC METHOD FOR HIGHLY INSOLUBLE COMPLEXES, AND ITS APPLICATION TO COMPLEXES OF ADENINE ANION*

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(Received 29 February 1960)

Adenine, in spite of the 1:2-diamine structure involving the 6- and 7-positions, has little tendency to form complexes with the cations of heavy metals, as was shown quantitatively (Albert, 1953). However, by using less acidic solutions it has been found that the anion of adenine has a strong avidity for bivalent cations, which it binds between $N_{(7)}$ and the primary amino group (Harkins & Freiser, 1958).

(I)

In pursuing this observation, we found that the copper complex was so insoluble that it was necessary to work at a greater dilution than has been customary hitherto in potentiometric work. This necessitated refinements of technique and apparatus which it is believed will be generally useful for insoluble complexes.

This work has revealed that, contrary to what

had been supposed, copper binds the adenine anion by almost simultaneous addition of two equivalents, giving the 2:1 complex (I).

METHODS

Materials. Glycine (A.R.) was recrystallized from water and dried at 110° for an hour. Adenine (British Drug Houses Ltd.) was recrystallized from water twice and then found to be chromatographically homogeneous. It was recrystallized once more and dried at 110°. Solutions (5 mM) of these substances were prepared in boiled ion-free water. A part of these solutions was diluted in the titration vessel with ion-free water, added under nitrogen from a grade A burette, to yield the concentrations given in the tables.

Pure copper perchlorate and nickel perchlorate solutions were made by pouring a solution of barium perchlorate on to a column of cation-exchange resin (Amberlite IR 120) previously quantitatively converted into the appropriate metal salt. A barium-free fraction of the eluate was collected and standardized by both a gravimetric and an ion-exchange method. Further dilutions were made with boiled ion-free water.

Apparatus. The titration vessel was a 250 ml. tall beaker sealed into a water-jacket through which water from a thermostatically controlled bath was circulated, thus maintaining the solution in the beaker at 20.0 ± 0.02°. The beaker was closed with a rubber bung carrying the electrodes, a nitrogen inlet, a thermometer and a sealable inlet for the glass capillary of a micrometer syringe. The nitrogen inlet was arranged so that a stream of oxygen-free nitrogen (saturated with water) was directed across the surface of the solution.

The pH was measured to 0.002 pH unit with a Vibron electrometer (model 33B; Electronic Instruments Ltd.), complete with pH attachment, glass electrode (type GG 33)

* Part 3: Albert (1953).