Baddiley, J., Buchanan, J. G. & Carss, B. (1957*a*). *J. chem. Soc.* p. 1869.
Baddiley, J., Buchanan, J. G. & Carss, B. (1957*b*). *J. chem. Soc.* p. 4138.
Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.
Borenfreund, E. & Dische, Z. (1957). *Arch. Biochem. Biophys.* **67**, 239.
Buchanan, J. G. (1951). *Nature, Lond.*, **168**, 1091.
Buchanan, J. G., Dekker, C. A. & Long, A. G. (1950). *J. chem. Soc.* p. 3102.
Buchanan, J. G., Lynch, V. H., Benson, A. A., Bradley, D. F. & Calvin, M. (1953). *J. biol. Chem.* **203**, 935.
Cabib, E. & Leloir, L. F. (1954). *J. biol. Chem.* **206**, 779.
Chambers, R. W. & Moffatt, J. G. (1958). *J. Amer. chem. Soc.* **80**, 3752.
Colowick, S. (1938). *J. biol. Chem.* **124**, 557.
Consden, R. & Stanier, W. M. (1952). *Nature, Lond.*, **169**, 783.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Dixon, J. S. & Lipkin, D. (1954). *Analyt. Chem.* **26**, 1092.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Frahn, J. L. & Mills, J. A. (1959). *Aust. J. Chem.* **12**, 65.
Fried, J. & Titus, E. (1948). *J. Amer. chem. Soc.* **70**, 3615.
Fried, J., Walz, D. E. & Wintersteiner, O. (1946). *J. Amer. chem. Soc.* **68**, 2746.
Ginsburg, V. (1960). *J. biol. Chem.* **235**, 2196.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Heppel, L. A. & Hilmoe, R. J. (1951). *J. biol. Chem.* **188**, 665.
Hershey, A. D., Dixon, J. & Chase, M. (1953). *J. gen. Physiol.* **36**, 777.
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
Kalckar, H. M. & Maxwell, E. S. (1956). *Biochim. biophys. Acta*, **22**, 588.
Kornfeld, S. & Glaser, L. (1960). *Biochim. biophys. Acta*, **42**, 548.
Kowalsky, A. & Koshland, D. E. (1956). *Biochim. biophys. Acta*, **22**, 575.
Kuehl, F. A., Clark, R. L., Bishop, M. N., Flynn, E. H. & Folkers, K. (1949). *J. Amer. chem. Soc.* **71**, 1445.
Kwapinski, J. & Merkel, M. (1957). *Bull. Acad. polon. Sci., Sér. sci. biol.*, **5**, 335.
Manson, L. A. & Lampen, J. O. (1951). *J. biol. Chem.* **191**, 87.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
Maxwell, E. S. (1956). *J. Amer. chem. Soc.* **78**, 1074.
Michelson, A. M. & Todd, A. R. (1956). *J. chem. Soc.* p. 3459.
Moffatt, J. G. & Khorana, H. G. (1958). *J. Amer. chem. Soc.* **80**, 3756.
Okazaki, R. (1960). *Biochim. biophys. Acta*, **44**, 478.
Paladini, A. C. & Leloir, L. F. (1952). *Biochem. J.* **51**, 426.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Pazur, J. H. & Shuey, E. W. (1960). *J. Amer. chem. Soc.* **82**, 5009.
Pontis, H. G. & Blumsom, N. L. (1958). *Biochim. biophys. Acta*, **27**, 618.
Racker, E. (1947). *J. biol. Chem.* **167**, 843.
Reindel, F. & Hoppe, W. (1954). *Chem. Ber.* **87**, 1103.
Staveley, H. E. & Fried, J. (1949). *J. Amer. chem. Soc.* **71**, 135.
Strominger, J. L. & Scott, S. S. (1959). *Biochim. biophys. Acta*, **35**, 552.
Stumpf, P. K. (1947). *J. biol. Chem.* **169**, 367.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Woodward, R. B. (1957). *Festschrift Prof. Dr Arthur Stoll*, p. 524. Basel: Birkhäuser.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

Biochem. J. (1961) **81**, 124

The Ribitol Teichoic Acid from *Lactobacillus arabinosus* Walls: Isolation and Structure of Ribitol Glucosides

BY A. R. ARCHIBALD,* J. BADDILEY AND J. G. BUCHANAN
Department of Chemistry, King's College, University of Durham, Newcastle upon Tyne

(*Received* 10 *February* 1961)

It has been known for some time that considerable amounts of a ribitol teichoic acid occur in the walls of *Lactobacillus arabinosus* 17–5 (Baddiley, Buchanan & Greenberg, 1957; Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan & Carss, 1958*a*; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958*b*). Preliminary studies, carried out largely with the aid of paper chromatography, suggested that this compound is a polymer in which ribitol units are joined together through phosphodiester linkages, and that glucosyl and alanine ester groups are also present (Armstrong *et al.* 1958*b*). It is clear that this teichoic acid resembles the ribitol teichoic acid in the walls of *Bacillus subtilis* (Armstrong, Baddiley & Buchanan, 1960, 1961), but several differences in the detailed structure of the two polymers were observed in the preliminary work. Hydrolysis with acid and alkali

* Frank Schon Fellow.

revealed that, whereas the compound from *B. subtilis* is a regular polymer in which 4-*O*-(β-D-glucopyranosyl)-D-ribitol units are joined through phosphodiester groups, that from *L. arabinosus* contains mono- and di-glucosylribitol units. Preparations from some batches of micro-organisms also contain ribitol units which bear no sugar. Moreover, whereas the glycosidic linkages in the teichoic acid from *B. subtilis* have the β-configuration, those in the *L. arabinosus* polymer probably have the α-configuration.

A more detailed investigation has now been carried out on the ribitol teichoic acid from *L. arabinosus*, and the earlier conclusions have been substantiated. In this paper the isolation and structure of ribitol mono- and di-glucosides from hydrolysates of the teichoic acid is described.

EXPERIMENTAL

Materials. β-Glucosidase was kindly provided by Dr W. J. Whelan; crude yeast hexokinase was a gift from Sigma Chemical Co., St Louis, Mo., U.S.A.; calf intestinal phosphomonoesterase was purchased from Sigma Chemical Co.; DL-glyceric acid, adenosine triphosphate and reduced diphosphopyridine nucleotide were purchased from L. Light and Co. Ltd., Colnbrook, Bucks.; Glucostat reagent was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; prostatic phosphatase was prepared from human prostate glands; *L. arabinosus* 17–5 was grown from a culture supplied by Dr D. E. Hughes.

Methods

Paper chromatography. Paper chromatography was carried out on Whatman no. 1, 4, and 3 mM paper that had been washed with 2N-acetic acid and then water. The following solvent systems were used: *A*, propan-1-ol–ammonia (sp.gr. 0·88)–water (6:3:1) (Hanes & Isherwood, 1949); *B*, butan-1-ol–ethanol–water–ammonia (sp.gr. 0·88) (40:10:49:1) (organic phase) (Hirst, Hough & Jones, 1949); *C*, ethyl acetate–pyridine–water (10:4:3) (Jermyn & Isherwood, 1949).

The products were detected by the periodate–Schiff reagents for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949), aniline phthalate reagent for reducing sugars (Partridge, 1949), silver nitrate reagent for reducing sugars (Trevelyan, Proctor & Harrison, 1950) and ninhydrin reagent for amino acids (Consden & Gordon, 1948).

Paper electrophoresis. Paper electrophoresis was carried out on washed Whatman no. 4 paper in 0·05M-sodium tetraborate at 5v/cm. for 16 hr. The glucosides were located by spraying with 10% acetic acid in butanol, allowing to dry and then spraying with 1% sodium metaperiodate solution containing 5% of acetic acid and developing with the Schiff reagent in the usual manner.

Isolation of teichoic acid from Lactobacillus arabinosus *cells. L. arabinosus* 17–5 was grown in 9 l. batches for 16 hr. at 30° in the following medium: Oxoid Tryptone, 20 g.; Oxoid yeast extract, 10 g.; sodium acetate, 10 g.; glucose, 20 g.; potassium dihydrogen phosphate, 4·5 g.; sodium hydroxide, 1·04 g.; inorganic salts (Barton-Wright, 1946), 5 ml.; distilled water, 100 ml.

The cells (about 80 g. wet wt.) were harvested in a refrigerated Sharples centrifuge and washed with 0·95% sodium chloride solution at 0°. They were treated (30 sec.) in a blender with successive portions of acetone, ethanol and ether (15 vol. of each) at room temperature.

Fat-free cells (160 g.) were extracted by blending for 1 min. with 10% trichloroacetic acid (600 ml.) at 2°. The residue, after removal of the supernatant solution by centrifuging, was extracted twice in a similar manner. The combined extracts were filtered through Celite. To the clear yellow solution (1·5 l.) cold ethanol (2 l.) was added with stirring and the mixture stored at 0° for 24 hr., when the resultant yellow–white precipitate was removed by centrifuging and redissolved in cold 10% trichloroacetic acid (120 ml.). After removal of insoluble material, cold ethanol (150 ml.) was added and, after 24 hr. at 0°, the precipitated teichoic acid was removed by centrifuging and dried with ethanol and ether. Yield, 667 mg. of powder composed largely of ribitol teichoic acid, together with some glycerol teichoic acid and nucleic acid.

The residues from the above extraction were resuspended in 10% trichloroacetic acid (700 ml.) and blended for 1 min. at 0°. The suspension was then stirred at 0° for 24 hr. and again blended for 1 min. before centrifuging. The resulting residue was stirred with 10% trichloroacetic acid (300 ml.) and again centrifuged, the combined solutions then being filtered through Celite and mixed with 1·5 vol. of cold ethanol. After 24 hr. at 0° the precipitated material was collected as before. At this stage the material contained a large amount of nucleic acid, much of which was removed by extraction of the teichoic acid with ice-cold distilled water (100 ml.). The insoluble material (416 mg.) was removed by centrifuging and teichoic acid (1·316 g.) was obtained by precipitation with ethanol. This material contained ribitol teichoic acid and a little glycerol teichoic acid and nucleic acid. The debris, which still contained teichoic acid, was not extracted further.

Isolation of teichoic acid from Lactobacillus arabinosus *walls.* The cells were grown for 16 hr. as described above and, after washing with cold 0·95% sodium chloride, were suspended in cold distilled water (20 g. wet wt. in 100 ml. of water). Walls were prepared as described by Armstrong *et al.* (1960). The yield from a 9 l. culture was 0·8–1·4 g. (Found: P, 2·87–3·3%). No whole cells were detected on examination by electron microscopy. Samples (3 mg.) were hydrolysed for 3 hr. at 100° with 2N-hydrochloric acid. After removal of hydrochloric acid by evaporation over potassium hydroxide, the residues were chromatographed in solvent *A*. The walls (204 mg.) were stirred for 24 hr. in 10% trichloroacetic acid at 2°. After centrifuging the wall residues were suspended in trichloroacetic acid and re-extracted. Ethanol (2 vol.) was added to the combined solutions and the resulting precipitate (42 mg.) collected as before. This material was pure ribitol teichoic acid. Acetone (2 vol.) was added to the supernatant from this precipitation; the resulting precipitate (23 mg.) contained teichoic acid and polysaccharide. The wall residues (125 mg.) were freed from trichloroacetic acid by extraction with ether.

In a similar extraction of walls (412 mg. Found: P, 2·87%), 94 mg. of pure teichoic acid (Found: P, 6·5%),

22·4 mg. of teichoic acid–polysaccharide mixture (Found: P, 5·8%) and 200 mg. of extracted wall residue (Found: P, 0·7%) were obtained. In addition, the supernatant from the acetone precipitation contained 2·4 mg. of phosphorus, which corresponded to partially degraded or low-molecular-weight teichoic acid.

Examination of extracted walls. Extracted walls (3·7 mg.) were hydrolysed in 2N-hydrochloric acid (300 μl.) for 24 hr. at 100°. After evaporation to dryness, the hydrolysate was passed through columns (0·5 ml.) of Dowex 50 (H^+ form) and Dowex 2 (CO_3^{2-} form) resins. The eluate was evaporated to dryness and examined chromatographically (solvent *A*), together with unextracted walls (1·3 mg.) which had been treated similarly. The amount (visual estimation) of anhydroribitol present in the hydrolysate of the extracted walls was less than that present in the unextracted walls containing an equivalent amount of phosphorus.

Extracted walls (46 mg.) were re-extracted by stirring with two successive portions (20 ml.) of 10% trichloroacetic acid at 70°. The wall residue (40 mg.), after removal of trichloroacetic acid by washing with ether, contained 0·36% of phosphorus. No anhydroribitol was detected when this residue (8·9 mg.) was examined as above. Electron microscopy showed that the walls had not disintegrated appreciably. Further extraction at 90° for 1 hr. with three successive portions of 10% trichloroacetic acid left a residue containing 0·04% of phosphorus. After this treatment the walls had disintegrated completely to a granular material. It appears that *L. arabinosus* walls contain small amounts of phosphorus other than that constituting teichoic acid. The nature of this phosphorus is not known.

Teichoic acid

Acid hydrolysis. Teichoic acid (2·0 mg.) isolated from walls 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 solvents *A*, *B*, and *C*. Products are given in Table 1.

Analysis. Teichoic acid (1·5 mg.), isolated from walls, was hydrolysed in 2N-hydrochloric acid (200 μl.) for 4 hr. at 100°. The hydrolysate was neutralized with ammonia and freeze-dried, and the residue was dissolved in water (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 (5%) by acid during hydrolysis (Found: P, 6·44; glucose, 40·3; alanine, 12·1%. Ratio P:glucose:alanine, 1:1·06:0·62). The reducing sugar present in an acid hydrolysate of teichoic acid was shown by assay with the Glucostat reagent to be 98% of D-glucose.

Table 1. *Products obtained by hydrolysis of teichoic acid*

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

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

* Trace.

Action of phosphodiesterase on teichoic acid. Teichoic acid (1·7 mg.), prepared from cell walls, was dissolved in water (3·0 ml.) and mixed with freeze-dried *Crotalus atrox* venom (0·4 mg.) dissolved in 0·1M-glycine buffer, pH 9·0 (1 ml.), and 1 mM-magnesium sulphate (0·3 ml.). The mixture was then incubated at 37° for 24 hr. before being passed through a small column (0·5 ml.) of Dowex 50 (H^+ form) resin and then adjusted to pH 5·5 with aqueous ammonia solution. The solution was freeze-dried, 0·2M-sodium acetate buffer, pH 5·5 (2 ml.), containing prostatic phosphatase (0·1 mg.), was added, and the volume was adjusted to 5 ml. with water. After incubation for 24 hr. at 37°, the ratio inorganic: total phosphate was 0·145:1. After similar incubation with *Crotalus adamanteus* venom, the ratio was 0·093:1. A sample of teichoic acid which was incubated with prostatic phosphatase without previous treatment with snake venom gave after 48 hr. a ratio of 0·10:1. On incubation with a purified calf intestinal phosphomonoesterase, the ratio was 0·145:1.

Alkali hydrolysis and enzymic dephosphorylation. Teichoic acid (980 mg.), isolated from whole cells by extraction at 2°, was dissolved in N-sodium hydroxide solution (20 ml.) and the resulting solution was heated at 100° for 3hr. A red–brown precipitate (probably ferric hydroxide) was removed by centrifuging. The combined supernatant and washings (50 ml.) were passed through a Dowex 50 (NH_4^+) resin column (30 ml.), which was then washed with water (200 ml.). The eluate and washings were concentrated to 10 ml. *in vacuo* or, in a second experiment, by freeze-drying. A sample (0·1 ml.) was removed and examined chromatographically in solvents *A* and *B* (see Table 1).

The pH of the eluate was adjusted to 5·5 with acetic acid, 0·2M-sodium acetate buffer, pH 5·5 (10 ml.), was added, and the solution was adjusted to 50 ml. with water; 4% of the phosphorus present was inorganic. Prostatic phosphatase (1·5 ml.) was added and the mixture was kept at 37°. Samples were removed for analysis after 24 hr. (inorganic P, 69% of total P) and 48 hr. (inorganic P, 70% of total P). A further portion of phosphatase (1·0 ml.) was added and incubation was continued for 24 hr. (inorganic P, 69% of total P). The volume was adjusted to 200 ml. with water and the solution was passed through a column of Dowex 50 (H^+ form) resin (70 ml.) at 2°. The filtrate, together with 250 ml. of column washings, was shaken with Dowex 2 (CO_3^{2-} form) resin (20 ml.), and added to a column of Dowex 2 (CO_3^{2-}) resin (20 ml.), which was then washed with water (400 ml.). The eluate was evaporated under reduced pressure to small volume and freeze-dried. The resulting white solid (288 mg.) was examined chromatographically in solvents *A*, *B* and *C*. Phosphates (520 mg.) were eluted from the Dowex-2 resin with N-sodium hydroxide solution (125 ml.) at 2°. They were isolated by shaking the eluate at 2° with Dowex 50 (H^+) (100 ml.), adding both the supernatant and resin to a column of Dowex 50 (H^+) resin (150 ml.) at 2°, washing with water (1 l.), neutralizing with ammonia and freeze-drying. Those compounds listed in Tables 2 and 3 were detected.

Examination of degradation products

Fractionation of the neutral products. (*a*) On a cellulose column. The neutral fraction (280 mg.) was dissolved in solvent *B* (50 ml.) and applied to a Chromax paper column which had previously been washed with solvent. After washing with three successive portions (10 ml.) of solvent, elution was commenced. Fractions (15 ml.) were collected, and the elution of products was followed qualitatively by paper chromatography and quantitatively by micro-periodate oxidation (Dixon & Lipkin, 1954) on 0·1 ml. samples of eluate after removal of solvent *in vacuo*.

Most of the fractions contained mixtures, the composition of which was determined by descending chromatography on Whatman 3mM paper with solvent *B* (see Table 2).

(*b*) On a charcoal–Celite column. A sample of teichoic acid (500 mg.) was treated with alkali and prostatic phosphomonoesterase as before, and the neutral fraction from this was dissolved in water and passed through a column of equal parts of charcoal and Celite (total weight, 50 g.). The elution of products was followed by paper chromatography, and appropriate fractions were combined and evaporated under reduced pressure (Table 3). Mixtures were separated on Whatman 3mM paper. The 3 glucoside was obtained pure only after repeated chromatographic separation from the 4-glucoside. After this purification all sugar derivatives were adsorbed on short charcoal–Celite columns and eluted with 15% ethanol. The solvent was removed under reduced pressure and the product finally freeze-dried.

Unhydrolysed phosphates. The unhydrolysed phosphates consisted mainly of a monophosphate of diglucosylribitol.

Table 2. *Cellulose separation of products from the action of alkali and phosphatase on teichoic acid*

For details see text.

Fraction no.	Composition
1–59	—
60–97	Anhydroribitol, glycerol
98–145*	Ribitol, 4-glucosylribitol, glucose (trace)
146–155	4-Glucosylribitol
156–226	4-Glucosylribitol, 3-glucosylribitol
227–300†	Mono- and di-glucosylribitol
301–460	Diglucosylribitol

* Also contained a trace of a compound with $R_{ribitol}$ 1·19 (solvent *B*).

† Also contained a trace of a compound with $R_{ribitol}$ 0·29 (solvent *B*).

1:4-*Anhydroribitol.* The eluate from the charcoal column (tubes 151–176) was evaporated to dryness *in vacuo* and the anhydroribitol was purified by sublimation at 145° and 10^{-4} mm. The resulting syrup crystallized after the addition of a seed crystal of DL-anhydroribitol. Yield, about 1·5 mg. of white crystals. The infrared spectrum was identical with that of authentic D-1:4-anhydroribitol. Polarimetric examination in a 1 dm. tube of a solution containing about 0·5 mg. in 1·4 ml. of water gave a rotation of +0·02–0·03°.

Ribitol. Chromatographically pure ribitol crystallized from ethanol as rosettes of white needles (23 mg.), m.p. 99° (Found: C, 39·8; H, 8·2. Calc. for $C_5H_{12}O_5$: C, 39·5; H, 7·9%). The infrared spectrum was identical with that of an authentic sample of ribitol.

Identification of 4-*O*-(α-D-*glucopyranosyl*)-D-*ribitol*

Chromatographically pure 4-glucosylribitol crystallized with difficulty from aqueous ethanol as rosettes of white needles (22 mg.), m.p. 125–126°, $[\alpha]_D^{22}$ +106° in water (*c*, 1·2) (Found: C, 40·0; H, 7·8. $C_{11}H_{22}O_{10},H_2O$ requires C, 39·8; H, 7·2%. Ratio ribitol:glucose, found 1:0·94). It showed a band at 1631 cm.$^{-1}$ in the infrared, characteristic of compounds containing water of crystallization.

Acid hydrolysis. Samples of the glucoside (about 0·5 mg.) were hydrolysed in 2N-hydrochloric acid (100 μl.) for 3 hr. and 24 hr. The hydrolysates were neutralized with ammonia and examined by paper chromatography in solvent *A*. After 3 hr. the glucoside was completely hydrolysed to ribitol, anhydroribitol and glucose; after 24 hr. the products were anhydroribitol and glucose.

Periodate oxidation and borohydride reduction. The glucosylribitol (1·0 mg.) was dissolved in 1% sodium metaperiodate solution (0·4 ml.) and the solution kept at room temperature for 24 hr. Sodium borohydride (2·0 mg.) was then added; after a further 24 hr. 2N-hydrochloric acid (0·2 ml.) was added and the solution was heated at 100° for 30 min. After evaporation under reduced pressure the mixture was examined by ascending chromatography in ethyl acetate–pyridine–water (7:2:1) (Viscontini, Hoch & Karrer, 1955). A single product was observed which rapidly gave a purple colour with the periodate–Schiff reagents and had R_F 0·45 (glycerol had R_F 0·45, erythritol had R_F 0·37 and ethylene glycol had R_F 0·58).

Quantitative periodate oxidation. Glucosylribitol (7·4 mg.) was dissolved in water (10 ml.), 0·04M-sodium metaperiodate (5 ml.) was added, and the volume adjusted to 20 ml. with water. A control experiment containing no glucosylribitol was also performed. Solutions were kept in the

Table 3. *Charcoal chromatography of products from the action of alkali and phosphatase on teichoic acid*

For details see text.

Fraction no.	Eluent	Composition
1–14	Water	—
15–29		Glycerol
30–39		Glycerol, ribitol
40–83		Ribitol
84–130		—
131–150	2% Ethanol	Anhydroribitol (trace)
151–176		Anhydroribitol
177–200		Anhydroribitol (trace)
201–221	3% Ethanol	3- and 4-Glucosylribitol
222–247	4% Ethanol	4-Glucosylribitol
248–320	6% Ethanol	3- and 4-Glucosylribitol, diglucosylribitol
321–390	7·5% Ethanol	Diglucosylribitol
391–411	9·0% Ethanol	Diglucosylribitol (trace)

dark at room temperature and samples were withdrawn at intervals for determination of periodate (4 ml. samples), formic acid (5 ml. samples) and formaldehyde (0·4 ml. samples) (Hough, Powell & Woods, 1956). The formic acid was titrated with 0·01 N-sodium hydroxide solution to pH 6·4. After 24 hr. the glucosylribitol had consumed 4·00 mol.prop. of periodate and 1·02 mol.prop. of formaldehyde had been produced. After 48 hr. 4·10 mol.prop. of periodate had been consumed and 1·06 mol.prop. of formaldehyde and 1·43 mol.prop. of formic acid were produced. On addition of an excess of sodium hydroxide solution and back-titration with 0·01 N-oxalic acid, the value obtained for formic acid was 1·90 mol.prop.

Oxidation and hydrolysis to glyceric acid. The glucosylribitol (1·6 mg.) and α-methyl glucoside (2·0 mg.) were separately dissolved in 0·15 M-sodium metaperiodate (0·5 ml.), and the solutions were kept at room temperature for 48 hr. to ensure complete oxidation. The solutions were passed through columns of Dowex 50 (H^+ form) resin (0·5 ml.) and Dowex 2 (acetate form) resin (0·5 ml.). The columns were washed with water (10 ml.), the eluates were freeze-dried, and acetic acid was removed *in vacuo*. The residues were dissolved in a solution (2 ml.), 100 ml. of which contained 417 mg. of benzoic acid and 565 mg. of barium hydroxide. Bromine (0·02 ml.) was added to the ice-cold solutions which were then shaken in stoppered tubes for 1 hr. and kept for 60 hr. at room temperature. Air was passed through the solutions to remove excess of bromine and the volumes were reduced to 0·8–1·0 ml. by evaporation under reduced pressure. Benzoic acid was removed by centrifuging and washed with a little ice-cold water. The volume of combined filtrate and washings was reduced *in vacuo* to 0·5 ml., 5 N-sulphuric acid (0·07 ml.) was added to each tube, and the solutions were heated at 100° for 2 hr. Barium carbonate (40 mg.) was added to each and the mixtures were shaken at intervals during 24 hr. Insoluble barium salts were removed by centrifuging and washed with water. The supernatant and washings were passed through columns of Dowex 50 (NH_4^+ form) resin (0·5 ml.) and evaporated to dryness *in vacuo*. The glyceric acid was purified by chromatography in solvents *A* and *B*. Paper strips containing glyceric acid, together with corresponding control areas of paper, were cut out and eluted with water or 0·01 N-ammonia solution.

The eluates were diluted to 2·0 ml. with water. Samples were removed for chemical assay of the glyceric acid by chromotropic acid (Bartlett, 1959) or by oxidation with 0·1 mM-sodium metaperiodate (Dixon & Lipkin, 1954). Oxidation was complete in 1 hr., 1 mole of periodate being consumed by each mole of glyceric acid. Samples (0·2–0·4 ml.) were then mixed with 3·5 ml. of a solution composed of 0·01 M-phosphate buffer, pH 7·3 (12·0 ml.), reduced diphosphopyridine nucleotide (about 4·0 mg.), 0·1 M-magnesium sulphate (0·2 ml.), 0·2 M-adenosine triphosphate (0·2 ml.), rabbit-muscle enzyme (10 mg.) and yeast hexokinase (12 mg.). In a control experiment, water (0·2–0·4 ml.) was mixed with 3·5 ml. of the enzyme solution.

Oxidation of the pyridine nucleotide was followed spectrophotometrically at 340 mμ, and under the above conditions was usually complete after 30 min. Oxidation was calculated from the change in extinction at 340 mμ.

The glyceric acid from α-methyl glucoside oxidized 2·0 mol.prop. of pyridine nucleotide, whereas that from the glucosylribitol oxidized 1·9 mol.prop. Authentic D-glyceric acid, prepared by enzymic dephosphorylation of D-glyceric acid 3-phosphate, oxidized 2 mol.prop. of pyridine nucleotide, whereas DL-glyceric acid oxidized 1 mol.prop. of nucleotide at a reduced rate.

Identification of 3-*O*-(α-D-*glucopyranosyl*)*ribitol*

Chromatographically purified 3-*O*-(α-D-glucopyranosyl)-ribitol, from which all 4-glucosylribitol had been removed, was adsorbed on a charcoal–Celite column (300 mg.) which was then washed with water, and the glycoside was eluted with 10% ethanol. The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in water, then freeze-dried to a white powder (1·7 mg.). It had $[\alpha]_D^{20} + 78 \pm 20°$ in water (*c*, 0·284) and a ratio glucose: ribitol of 0·97:1. The amount of glucosylribitol in this solution, as estimated by determination of glucose in an acid hydrolysate, was 1·55 mg.

The solution was quantitatively transferred to a test tube and the volume adjusted to 3·25 ml. with water; the concentration of glucosylribitol in this solution, determined by estimation of the glucose produced on acid hydrolysis, was 0·421 mg./ml. The remainder of the solution was used for quantitative periodate oxidation studies.

Controlled oxidation with sodium metaperiodate. The glucosylribitol solution (1·0 ml.) was mixed with 6 mM-sodium metaperiodate solution (2 ml.) and kept in the dark at room temperature. A control containing no glucosylribitol was also prepared. Samples (0·20 ml.) of each solution were diluted to 10 ml. with water and the periodate content was determined at intervals. Formaldehyde was determined in other samples (0·4 ml.). After 6 hr., 4·1 mol.prop. of periodate were consumed, whereas after 24 hr. 4·8 mol.prop. of periodate were consumed and 2·2 mol.prop. of formaldehyde were formed. After 72 hr., 5·1 mol.prop. of periodate were consumed and 2·3 mol.prop. of formaldehyde were formed.

Oxidation with sodium metaperiodate at pH 8·0. The glucosylribitol solution (0·35 ml.) was mixed with 0·1 M-phosphate buffer, pH 8·0 (1·70 ml.), 0·3 M-sodium metaperiodate (0·27 ml.) and water (1·00 ml.). A control experiment containing no glucosylribitol was also prepared and both solutions were kept in the dark at 37°. Samples (0·4 ml.) were removed for determination of formaldehyde. After 12 hr., 3·12 mol.prop. and after 24 hr. 3·03 mol.prop. of formaldehyde were produced.

Identification of 3:4-*di*-(O-α-D-*glucopyranosyl*)-D-*ribitol*

Diglucosylribitol was precipitated by cooling its solution in aqueous ethanol. It was a deliquescent, microcrystalline, white powder (30 mg.) which changed form at 106° and melted with decomposition at 118–121°. It had $[\alpha]_D^{22} + 137°$ in water (*c*, 1·08) (Found: C, 42·2; H, 7·1. $C_{17}H_{32}O_{15},6H_2O$ requires C, 41·6; H, 6·8%. Glucose:ribitol ratio, found 1·92:1). The hygroscopic nature of the material prevented satisfactory measurement of its infrared spectrum, but a strong absorption at 1639 cm.$^{-1}$, characteristic of hydrated compounds, was observed.

Acid hydrolysis. Diglucosylribitol (about 0·5 mg.) was hydrolysed in 0·1 N-hydrochloric acid (100 μl.) for 90 min. The hydrolysate was neutralized with aqueous ammonia solution and examined chromatographically in solvent *B*.

Unhydrolysed diglucosylribitol (about 50%), glucose, and approximately equal parts of 3- and 4-glycosylribitol were the only compounds detected. In 2N-hydrochloric acid at 100° for 4 hr. the products were glucose, ribitol and anhydroribitol.

Periodate oxidation and borohydride reduction. Acid hydrolysis of the reduced product of periodate oxidation gave two glycols; the principal one had R_F 0·45 and the minor one had R_F 0·39 (glycerol had R_F 0·45 and erythritol had R_F 0·37).

Quantitative periodate oxidation. Diglucosylribitol (4·99 mg.) was dissolved in a little water, 0·02M-sodium metaperiodate solution (5 ml.) was added and the volume was adjusted to 25 ml. with water. The solution, and a control solution, were kept in the dark at room temperature and samples were removed for analysis. After 36 hr., 5·10 mol.prop. of periodate were consumed and 1·80 mol.prop. of formic acid were formed; after 72 hr., 5·21 mol.prop. of periodate were consumed and 1·98 mol.prop. of formic acid and 1·08 mol.prop. of formaldehyde were formed. The oxidation product was stable at pH 6·9, but was unstable above pH 7, giving rise to acidic products.

Partial acid hydrolysis. The diglucoside (10 mg.) was dissolved in 0·1N-hydrochloric acid and heated at 100° for 90 min. The solution was neutralized with ammonia solution and ions were removed by use of a charcoal–Celite column. The resulting monoglucosylribitols were purified by chromatography in solvent *B*, adsorption on charcoal and elution with ethanol as before, and identified as follows:

(*a*) 4-*O*-(α-D-Glucopyranosyl)-D-ribitol. This compound, obtained by hydrolysis of the diglucoside, was indistinguishable on paper chromatography (solvent *B*) and colour reactions from authentic 4-glucosylribitol. On acid hydrolysis, glucose, ribitol and anhydroribitol were the only products detected. The glucose:ribitol ratio was 0·92:1. The pure glucosylribitol (0·8 mg.) was oxidized with periodate, the product was reduced with borohydride then hydrolysed, and fragments were examined as before. Glycerol (R_F 0·45) was the only product detected with the periodate–Schiff reagents. A sample (1·2 mg.) of the glucosylribitol was oxidized with sodium metaperiodate followed by bromine, then hydrolysed and the resulting glyceric acid was examined as before. With α-methyl glucoside as a reference compound, the enzymic procedure showed that 90% of the glyceric acid formed had the D-configuration.

(*b*) 3-*O*-(α-D-Glucopyranosyl)ribitol. This compound from the partial acid hydrolysis gave no reaction with aniline phthalate and had the same chromatographic properties in solvent *B* as had 3-*O*-(α-D-glucopyranosyl)-ribitol. It also gave the characteristic yellow colour with the periodate–Schiff reagents. On acid hydrolysis, glucose, ribitol and anhydroribitol were the only products detected. The glucose:ribitol ratio was 0·97:1. On oxidation with sodium metaperiodate at pH 8·0, 2·80 mol.prop. of formaldehyde were produced.

DISCUSSION

Analysis of this teichoic acid and paper-chromatographic characterization of hydrolysis products were carried out on material which had been prepared from isolated walls as well as from whole cells. The walls were obtained by mechanical rupture of bacteria with a Mickle vibrator followed by differential centrifuging, the technique being adapted from that of Salton & Horne (1951). This method was the most satisfactory one for preparing cell walls from *L. arabinosus* for the present purpose. Much of the teichoic acid could be extracted from walls by treatment with cold 10% trichloroacetic acid at 2° for several hours. Extraction at 37°, or even higher temperatures, was more complete but these vigorous conditions led to the presence of contaminants in the final product. The ease of extraction of this teichoic acid is comparable with that for *Bacillus subtilis* (Armstrong *et al.* 1960), and is consistent with the view that most, or possibly all, of the teichoic acid in bacterial walls is held in the wall structure by ionic linkages.

L. arabinosus walls contain about 3% of phosphorus, 90% of which can be accounted for as ribitol teichoic acid after extraction with trichloroacetic acid solution. The nature of the small amount of unextracted phosphorus is unknown. This organic phosphate is not a teichoic acid. Similar amounts of unextracted organic phosphates have been observed in the walls of other bacteria studied in this laboratory.

Pure teichoic acid was obtained from the extract by precipitation with ethanol, dissolving in water and reprecipitation with ethanol. It contained 6·44% of phosphorus and gave on acid hydrolysis alanine, glucose, inorganic phosphate and the known acid-degradation products of ribitol phosphate (Armstrong *et al.* 1958*b*). The ratio phosphorus:glucose:alanine was 1:1·06:0·62. The amount of alanine depended upon the conditions of preparation but the molar proportion was always less than 1. At present it is assumed that a molar proportion of about 1 occurs in teichoic acid within the wall and that partial loss of alanine takes place during isolation.

A sample of alanine from this teichoic acid was readily oxidized by kidney D-amino acid oxidase (experiment by Dr F. C. Neuhaus) and thus has the D-configuration, a feature common to all teichoic acids examined in this Laboratory. Moreover, the alanine residues react readily at room temperature with dilute ammonia or hydroxylamine, giving alanine amide and hydroxamate respectively. These observations, and the presence of a typical ester carbonyl absorption band at 1751 cm.$^{-1}$ in the infrared, indicate that the alanine residues are joined through their carboxyl groups to hydroxyl groups of either the glucose or ribitol residues. It has been shown that the alanine residues in the ribitol teichoic acid from *B. subtilis* are joined to ribitol and not to glucose (Armstrong *et al.* 1961).

The general properties of this polymer and the nature of the products of its acid hydrolysis are

consistent with a structure in which ribitol residues are joined together through phosphodiester linkages. This similarity to the ribitol teichoic acid from *B. subtilis* walls is in contrast with the nature and number of glucosyl residues attached to ribitol. When the phosphodiester linkages in the *B. subtilis* polymer were hydrolysed by alkali, a mixture of phosphomonesters was produced almost quantitatively. Removal of phosphate from the esters through the action of prostatic phosphatase gave a high yield of 4-*O*-(β-D-glucopyranosyl)-D-ribitol, and it was concluded that the teichoic acid is a polymer in which each glucose residue is attached in a regular manner to each ribitol in the chain. When a similar procedure was applied to the teichoic acid from *L. arabinosus*, a mixture of products was obtained. In order to obtain sufficient of these for adequate characterization, larger-scale experiments were carried out on hydrolysates of teichoic acid prepared from extracts of whole, defatted bacteria. Such preparations were less pure than those from walls, and contained hydrolysis products from the intracellular glycerol teichoic acid that is known to be present in this organism (cf. Baddiley & Davison, 1961).

The main products of hydrolysis were a monoglucosylribitol and a diglucosylribitol, and appreciable amounts of ribitol were also obtained from preparations made from some batches of organisms. In addition, small amounts of 1:4-anhydroribitol and an isomeric monoglucosylribitol were detected in the hydrolysate. The anhydroribitol is probably not a structural unit in the teichoic acid, but could arise during the alkali treatment. A trace of 1:4-anhydroribitol is formed by the prolonged action of hot alkali on ribitol 5-phosphate (unpublished observation by Mr D. A. Applegarth), and it is possible that phosphodiesters in a teichoic acid would yield the anhydro-compound more readily than would the simple phosphomonoester. Occasionally a small amount of an isomeric diglucosylribitol has been detected in hydrolysates, but this is not a regular product.

The hydrolysis products were separated from each other by chromatography on charcoal, followed in some cases by preparative paper chromatography. Anhydroribitol was isolated crystalline by sublimation. It had the same chromatographic mobility in several solvent systems as authentic 1:4-anhydroribitol and its infrared spectrum was identical with that of optically active 1:4-anhydroribitol, which differs from the spectrum of the racemate. Insufficient material was available for accurate measurement of its optical activity, but the solution was slightly dextrorotatory, suggesting that the sample was the D-isomer. Ribitol was isolated crystalline and identified by direct comparison with synthetic material. The predominant isomer of glucosylribitol also crystallized as a monohydrate. It had the same R_F as the glucosylribitol from *B. subtilis* teichoic acid in two solvents and behaved similarly on electrophoresis, but differed slightly in the colour given with the periodate–Schiff reagents. The blue colour given by the β-glucoside from *B. subtilis* closely resembles that from β-methyl glucoside, whereas that shown by the glucoside from *L. arabinosus* possesses a blue–grey component. α-Methyl glucoside gives a marked blue–grey colour in this test.

Acid hydrolysis of the glucosylribitol gave glucose, ribitol and anhydroribitol, whereas under more vigorous conditions equimolar amounts of glucose and anhydroribitol were formed. These were separated from each other by paper chromatography. Anhydroribitol was determined by oxidation with periodate, and glucose by reaction with ferricyanide (Park & Johnson, 1949). The high positive rotation ($[\alpha]_D^{22} + 106°$) of this glucosylribitol suggested that it is an α-glucopyranoside. This is consistent with the observation that it was not hydrolysed with the β-glucosidase preparation which readily hydrolysed the 4-*O*-(β-D-glucopyranosyl)-D-ribitol from *B. subtilis*, and is also consistent with the earlier conclusion that the high rotation of the teichoic acid from *L. arabinosus* is due to the presence of α-glucosyl residues. There is thus a marked difference between the teichoic acid from *L. arabinosus* and that from *B. subtilis* in which all glucose linkages possess the β-configuration.

The position of attachment of glucose to ribitol was established by oxidation with sodium metaperiodate; 4 mol.prop. of periodate were consumed and 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde were produced. For complete liberation of formic acid it was necessary to add an excess of sodium hydroxide. The 4-*O*-(β-D-glucopyranosyl)-D-ribitol from *B. subtilis* teichoic acid behaved similarly, and this is believed to arise from the intermediate formation of a glucosyl-D-erythrose, which then gives a formyl ester of the final oxidation product. The course of the oxidation has been discussed fully (Armstrong *et al.* 1960). It follows from the analysis that the glucose must be attached to a hydroxyl at position 2 or 4 in the ribitol residue (cf. I and II). This conclusion is supported by the observation that the trialdehyde formed by oxidation of the glucoside with periodate, on reduction with borohydride and acid hydrolysis, gave glycerol but not ethylene glycol.

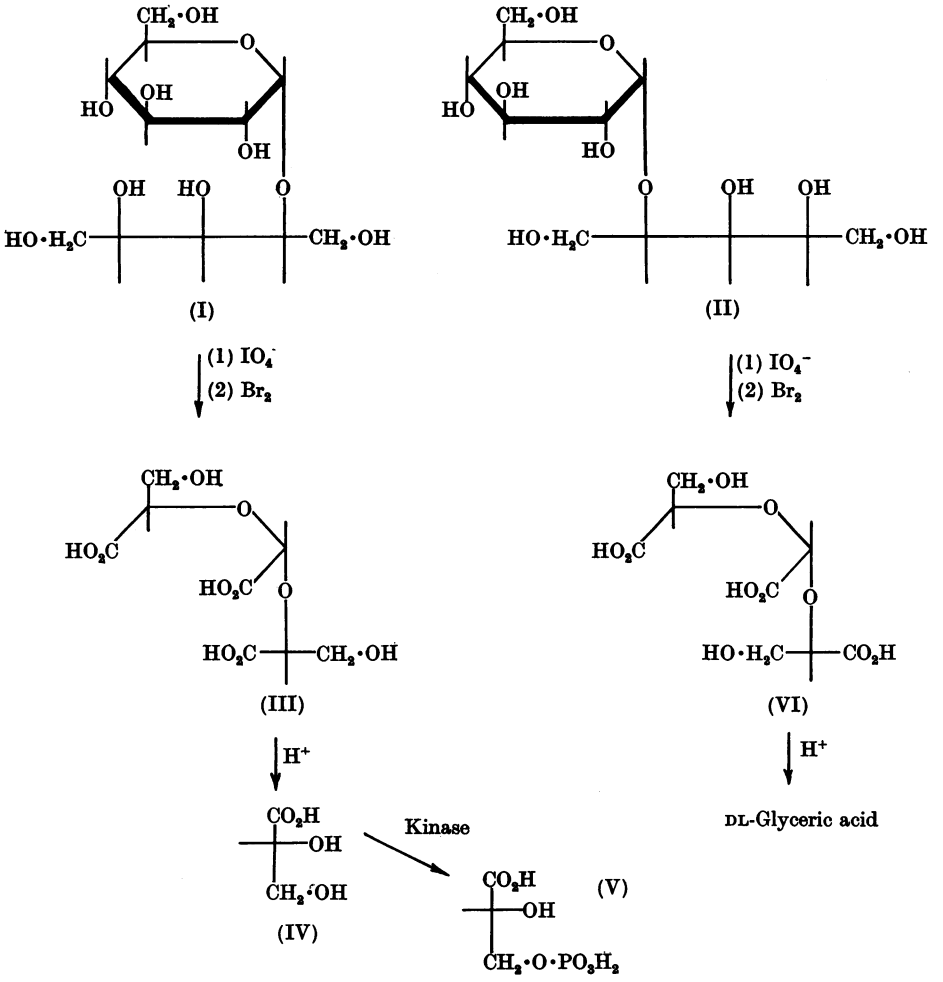
The two possible structures (I and II) for the glucosylribitol can be distinguished from each other by considering the stereochemistry of their oxidation products.

The structure (I), on oxidation with periodate, then with bromine, would give the tricarboxylic

acid (III); this would yield on acid hydrolysis 2 mol.prop. of D-glyceric acid (IX). On the other hand, the alternative structure (II) would give the tricarboxylic acid (VI), which would yield on acid hydrolysis 2 mol.prop. of DL-glyceric acid. In a similar degradation of the glucosylribitol from *B. subtilis* teichoic acid, the configuration of the glyceric acid was determined from its optical rotation in the presence of molybdate, but in the present work an alternative procedure was adopted. The glyceric acid was isolated from the reaction mixture by paper chromatography, eluted from the paper and then determined chemically by oxidation of a sample with sodium metaperiodate. Its configuration was established by phosphorylation with adenosine triphosphate in the presence of crude yeast hexokinase to give D-glyceric acid 3-phosphate (V). This was determined by its ability to oxidize reduced diphosphopyridine nucleotide in the presence of a rabbit-muscle-enzyme preparation. The enzymic method for determining D-glyceric acid 3-phosphate is similar to that used previously for establishing the configuration of the ribitol phosphate residue in cytidine diphosphate ribitol, and is known to be specific for the D-series (Baddiley, Buchanan & Carss, 1957*a*).

When the glucosylribitol from *L. arabinosus* teichoic acid was subjected to this procedure, the resulting glyceric acid, after phosphorylation, oxidized 1·9 mol.prop. of pyridine nucleotide: authentic D-glyceric acid, and the glyceric acid obtained from α-methyl glucoside, oxidized 2 mol.-prop. of pyridine nucleotide; DL-glyceric acid oxidized 1 mol.prop. of nucleotide. Thus, the glyceric acid from the glucosylribitol must have the D-configuration and the glucosylribitol is correctly represented as 4-*O*-(α-D-glucopyranosyl)-D-ribitol (I).

This combined chemical and enzymic method for determining the structure (I) of the glucosylribitol



is conveniently carried out on a small scale (3–4 μmoles of glycoside), and could be applied to the determination of the stereochemical series of many other glycosides. It has been used successfully here for determining the stereochemical series of the glucose in α-methyl D-glucoside and, with minor modification, has been applied in other work on the structure of nucleotides containing D-glucose or D-mannose (Blumsom & Baddiley, 1961). It is likely that this procedure will have considerable general use in the carbohydrate field.

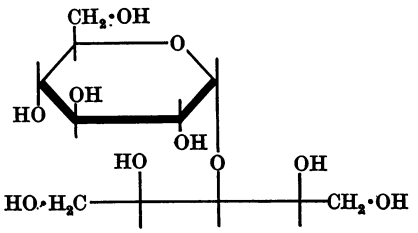
After several chromatographic separations, a pure specimen (1·7 mg.) of an isomeric glucosylribitol was obtained from a teichoic acid hydrolysate. This was non-reducing and on acid hydrolysis gave glucose, ribitol and anhydroribitol. Equimolar amounts of glucose and anhydroribitol were formed on vigorous acid treatment. The optical rotation of the glucosylribitol ($[\alpha]_D^{22} + 78 \pm 20°$) suggests that the glucosyl linkage is α, and this is supported by the stability of this glucoside in the presence of a β-glucosidase.

On oxidation of the isomeric glucosylribitol with sodium metaperiodate in dilute solution in the dark at room temperature, 4–5 mol.prop. of periodate were consumed and 2 mol.prop. of formaldehyde were produced. It follows that the glucose is attached to the hydroxyl at position 3 on the ribitol. Confirmation of the structure 3-*O*-(α-D-glucopyranosyl)ribitol (VII) was obtained by oxidation at pH 8 with a large excess of sodium metaperiodate at 37°. Hough & Perry (1956) have shown that under these conditions compounds that would be expected to be oxidized to derivatives of malondialdehyde are readily susceptible to a further degradation known as 'over-oxidation'. In a glucosylribitol this could only occur if the glucosyl residue is at the 3-position. When the glucoside was oxidized in this way, 3 mol.prop. of formaldehyde was produced, showing that 'over-oxidation' had occurred and therefore that the glucose was attached to the hydroxyl at the 3-position of the ribitol.

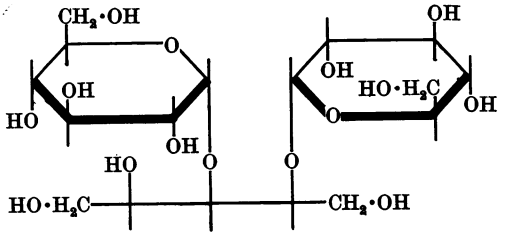
This structure was supported by the characteristic yellow colour given by the compound on paper chromatograms sprayed with the periodate–Schiff reagents. This behaviour has been observed previously with 3-substituted ribitol derivatives (Baddiley, Buchanan & Carss, 1957*b*).

The diglucosylribitol was precipitated from aqueous ethanol as a white deliquescent powder, which was probably microcrystalline. Vigorous acid hydrolysis gave glucose and anhydroribitol in the molar ratio 2:1. The high positive optical rotation indicated that both glucose units are present as α-glucosyl residues; this conclusion was supported by the failure of a β-glucosidase to hydrolyse the glycoside.

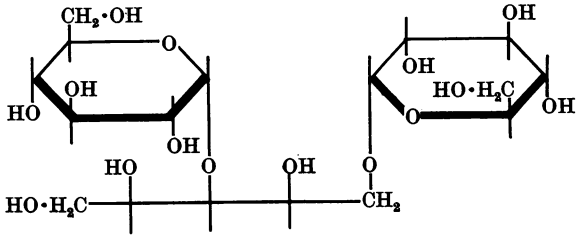
The diglucosylribitol consumed 5 mol.prop. of periodate, giving 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde. Reduction of the oxidation products with borohydride, then acid hydrolysis, gave glycerol and erythritol. Careful acid hydrolysis of the diglucoside gave glucose and two isomeric non-reducing monoglucosides of ribitol. It follows that the two glucose residues are each directly attached to the ribitol and cannot be present in a glucosylglucose unit.



(VII)



(VIII)



(IX)

From the above evidence it follows that the two glucose residues in the diglucoside must occupy positions 3 and either 4 (2) or 5 (1) of the ribitol. The 3:4- and 3:5-diglucosyl-D-ribitol structures are shown in (VIII) and (IX) respectively.

The isomeric structures were distinguished from each other by studies on the monoglucosides obtained by careful acid hydrolysis. The two monoglucosides were separated from each other by paper chromatography, and both gave equimolar amounts of glucose and anhydroribitol on vigorous treatment with acid. One was identified as 3-*O*-(α-D-glucospyranosyl)ribitol (VII) by paper-chromatographic comparison with the 3-glucosylribitol isolated previously. It also gave the characteristic yellow colour with the periodate–Schiff reagents, and on periodate oxidation at pH 8, 'over-oxidation' occurred, 3 mol.prop. of formaldehyde being formed.

The other glucosylribitol from the diglucoside was indistinguishable on paper chromatograms and in colour reactions from 4-*O*-(α-D-glucopyranosyl)-D-ribitol (I). On oxidation with periodate, followed by reduction with borohydride and acid hydrolysis, it gave glycerol but neither erythritol nor ethylene glycol. The 5(1)-glucosylribitol which would be formed by partial hydrolysis of structure (IX) would give ethylene glycol by this procedure; thus, structure (IX) is untenable for the diglucoside. Moreover, the aldehyde obtained by oxidizing the diglucoside with periodate is particularly labile towards alkali. This can be explained readily on the basis of a 3:4 (2) structure as in (VIII).

The structure (VIII) was distinguished from the isomeric 2:3-di-*O*-(α-D-glucopyranosyl)-D-ribitol by stereochemical analysis of the 4(2)-glucosylribitol obtained by partial hydrolysis of the diglucoside. The glyceric acid obtained by oxidizing the glucosylribitol with periodate then bromine followed by hydrolysis had the D-configuration. Consequently, the glucosylribitol must be the 4-glucoside (I), and the diglucosylribitol must be correctly represented as 3:4-di-*O*-(α-D-glucopyranosyl)-D-ribitol (VIII).

Although the glucosylribitol (I) and the diglucosylribitol (VIII) are the principal components of the teichoic acid from *L. arabinosus* walls, their relative amounts and the relative amounts of ribitol and isomeric glycosides vary in different samples of teichoic acid.

SUMMARY

1. It is confirmed that the teichoic acid from the walls of *Lactobacillus arabinosus* is composed of ribitol, phosphate, D-glucose and D-alanine.

2. Hydrolysis in alkali, followed by removal of phosphate from the products with a phosphatase, gave mainly a mono- and a di-glucosylribitol. Small amounts of anhydroribitol and an isomeric monoglucosylribitol are also formed, and occasionally traces of an isomeric diglucosylribitol have been detected. Teichoic acid preparations from some batches of micro-organisms but not others yield appreciable amounts of ribitol.

3. The principal monoglucosylribitol has the structure 4-*O*-(α-D-glucopyranosyl)-D-ribitol (I). This has been established by studies on optical rotation, hydrolysis, periodate oxidation and the stereochemistry of degradation products.

4. A general method is described for determining the stereochemical series of sugars from their glycosides on a microscale. This depends upon oxidation of the glycoside with periodate then bromine, hydrolysing the product with acid, and determining the configuration of the resulting glyceric acid by enzymic phosphorylation then oxidation of reduced diphosphopyridine nucleotide in the presence of a rabbit-muscle preparation.

5. The monoglucosylribitol formed in small amounts is 3-*O*-(α-D-glucopyranosyl)ribitol (VII). The structure of this compound was established by methods similar to those adopted for the isomeric 4-glucoside.

6. The diglucoside is 3:4-di-*O*-(α-D-glucopyranosyl)-D-ribitol (VIII). On careful acid hydrolysis it yields glucose and a mixture of 3- and 4-glucosylribitol identical with those isolated before.

7. The teichoic acid from the walls of *Lactobacillus arabinosus* differs markedly from the other ribitol teichoic acid which has been studied in detail, i.e. that from *Bacillus subtilis*, in that both mono- and di-glucoside residues are present and the glycosidic linkages all have the α-configuration.

We thank the Medical Research Council, the Nuffield Foundation and the Rockefeller Foundation for financial support.

REFERENCES

Armstrong, J. J., Baddiley, J. & Buchanan, J. G. (1960). *Biochem. J.* **76**, 610.

Armstrong, J. J., Baddiley, J. & Buchanan, J. G. (1961). *Biochem. J.* **80**, 254.

Armstrong, J. J., Baddiley, J., Buchanan, J. G. & Carss, B. (1958*a*). *Nature, Lond.*, **181**, 1692.

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. & Greenberg, G. R. (1958*b*). *J. chem. Soc.* p. 4344.

Baddiley, J., Buchanan, J. G. & Carss, B. (1957*a*). *J. chem. Soc.* p. 1869.

Baddiley, J., Buchanan, J. G. & Carss, B. (1957*b*). *J. chem. Soc.* p. 4058.

Baddiley, J., Buchanan, J. G. & Carss, B. (1958). *Biochim. biophys. Acta*, **27**, 220.

Baddiley, J., Buchanan, J. G. & Greenberg, G. R. (1957). *Biochem. J.* **66**, 51P.

Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.

Baddiley, J. & Davison, A. L. (1961). *J. gen. Microbiol.* **24**, 295.
Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 469.
Barton-Wright, E. C. (1946). *Practical Methods for Microbiological Assay of Vitamin Complexes and Essential Amino Acids.* London: Ashe Laboratories.
Blumsom, N. L. & Baddiley, J. (1961). *Biochem. J.* **81**, 114.
Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
Consden, R. & Gordon, A. H. (1948). *Nature, Lond.*, **162**, 180.
Dixon, J. S. & Lipkin, D. (1954). *Analyt. Chem.* **26**, 1092.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 928.
Hough, L. & Perry, M. B. (1956). *Chem. & Ind.* p. 768.
Hough, L., Powell, D. B. & Woods, B. M. (1956). *J. chem. Soc.* p. 4799.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.
Salton, M. J. R. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Viscontini, M., Hoch, D. & Karrer, P. (1955). *Helv. chim. acta*, **38**, 642.

Biochem. J. (1961) **81**, 134

Optical Rotation of Soluble Feather Keratin

BY N. E. DWELTZ*
Department of Physics, University of Madras, Madras 25, India

AND V. MAHADEVAN
Department of Physical Chemistry, University of Madras, Madras 25, India

(*Received* 6 *February* 1961)

A new structure has recently been proposed for feather keratin by Ramachandran & Dweltz (1961), which is based on a collagen-like triple helix and which is in good agreement with its detailed X-ray diffraction pattern. In support of this, they showed that feather keratin and collagen exhibit remarkable resemblances in their infrared-absorption spectra and birefringence, in addition to the nature of oriented crystallization of inorganic salts on the two fibrous proteins and in their behaviour on treatment with chemical reagents. In the present paper details are given of the measurement of the optical rotation of soluble feather keratin, which again is found to be close to that of collagen both in sign and magnitude.

MATERIALS AND METHODS

White goose-feather keratin was used in these experiments. The samples were first thoroughly degreased by washing in a number of changes of benzene, then cleaned by washing in a number of changes of distilled water and subsequently air-dried.

The solution of soluble SH-keratin of feather was prepared according to the method of Woodin (1954, 1956). A weighed amount (about 1 g.) of dry feathers was taken and the extraction made in a 50 ml. mixture of 10 M-urea, 0·1 M-$NaHSO_3$ and 0·05 M-sodium phosphate buffer at a pH of about 8·5 for 48 hr. at 60°. The extract was cooled to room temperature and filtered, and the clear protein solution collected. This was then made up to 50 ml. with the urea mixture and its optical rotation measured accurately at 25° with a Hilger standard polarimeter.

The protein concentration of feather keratin in solution was determined from the non-diffusible N content. For this purpose 4 ml. portions of the urea–bisulphite–protein solution were pipetted into cellophan sacs and dialysed against running tap water for various periods ranging from 1 to 7 days in steps of 1 day each. After the fourth day the estimated N was constant, but decreasing very slowly, probably because a fraction of the protein content was also diffusing out of the cellophan sac. A correction for this was made from the rate of change of concentration observed from the fourth to the seventh day.

The estimation of the N content in each case was carried out as follows: The contents of each dialysis sac was quantitatively transferred after dialysis to a 50 ml. flask and digested with conc. H_2SO_4 (A.R.) for 3 hr., over a microburner. Reaction was completed by adding 1–2 ml. of 30% H_2O_2 so as to remove any faint-yellow colour produced during digestion. The digest was then cooled in an ice bath and neutralized with NaOH (A.R.) added in drops, to a pH of approx. 7, and then made up to 100 ml. The N in this neutralized digest was estimated by nesslerization. The colorimetric measurements were made in a Klett–Summerson photoelectric colorimeter by using a no. 54

* Present address: ATIRA, Ahmedabad-9, India.