- Novoa, W. B., Winer, A. D., Glaid, A. J. & Schwert, G. W. (1959). *J. biol. Chem.* **234**, 1143.
- Plagemann, P. G. W., Gregory, K. F. & Wróblewski, F. (1960). *J. biol. Chem.* **235**, 2288.
- Plagemann, P. G. W., Gregory, K. F. & Wróblewski, F. (1961). *Biochem. Z.* **334**, 37.
- Plummer, D. T., Elliott, B. A., Cooke, K. B. & Wilkinson, J. H. (1963). *Biochem. J.* **87**, 416.
- Plummer, D. T. & Wilkinson, J. H. (1961). *Biochem. J.* **81**, 38 p.
- Quastel, J. H. & Woolridge, W. R. (1938). *Biochem. J.* **22**, 689.
- Rosalki, S. B. & Wilkinson, J. H. (1960). *Nature, Lond.*, **188**, 1110.
- Wróblewski, F. & LaDue, J. S. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 210.

Biochem. J. (1963) **87**, 429

The Intracellular Teichoic Acid from *Staphylococcus aureus* H

By U. L. RAJBHANDARY AND J. BADDILEY

Department of Chemistry, King's College, Newcastle upon Tyne 1

(Received 9 November 1962)

Staphylococcus aureus H contains in its wall a large amount of a ribitol teichoic acid in which each of the eight D-ribitol 5-phosphate units possesses a α - or β -N-acetyl-D-glucosaminyl residue at position 4, and the usual D-alanine ester residues at positions 2 or 3 (Baddiley, Buchanan, Raj-Bhandary & Sanderson, 1962*b*; Baddiley, Buchanan, Martin & RajBhandary, 1962*a*). In earlier work trichloroacetic acid extracts of whole cells of this organism were shown to contain, in addition to the ribitol compound, a small amount of a glycerol phosphate polymer (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). The demonstration that intracellular glycerol teichoic acids occur almost universally in Gram-positive bacteria (Baddiley & Davison, 1961; Davison, 1962) supports the view that the glycerol compound from the staphylococcal extracts is an intracellular teichoic acid.

In view of the serological importance of teichoic acids, several of which are now known to be group antigens (Baddiley & Davison, 1961; Haukenes, Ellwood, Baddiley & Oeding, 1961; Sanderson, Juergens & Strominger, 1961; Morse, 1962; Haukenes, 1962; Wicken, Elliott & Baddiley, 1963), an examination of the structure of the intracellular representative from the *Staphylococcus* was undertaken. Although the ribitol teichoic acid in walls of *S. aureus* is associated with the group antigenic reactions, intracellular teichoic acids from other bacteria are serologically active (Wicken *et al.* 1963); consequently, studies on this intracellular compound were desirable. Moreover, in the two cases examined so far, these so-called intracellular compounds are probably located between the wall and the protoplast membrane (Hay, Wicken & Baddiley, 1963), and thus may be

concerned in transport and similar important metabolic activities.

It is shown here that the compound from *S. aureus* H is a glycerol 1,3-phosphate polymer with D-alanine ester residues and occasional β -gentiobiosyl [β -D-glucopyranosyl-(1 $\rightarrow$ 6)-O-D-glucose] or N-acetylglucosamine residues at position 2 on glycerol. It is not known whether the gentiobiose and glucosamine substituents occur in the same molecule or whether the preparation is a mixture.

EXPERIMENTAL

Materials. *S. aureus* H was grown by Dr A. L. Davison from a culture provided by Dr J. T. Park, calf intestinal phosphatase was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., β -glucosidase (from almond emulsin) from L. Light and Co. Ltd., Colnbrook, Bucks. and Sephadex G-75 from Pharmacia, Uppsala, Sweden.

Analytical methods. Phosphate was determined as described by Chen, Toribara & Warner (1956), alanine as described by Rosen (1957), periodate as described by Dixon & Lipkin (1954), formaldehyde as described by Hough, Powell & Woods (1956) and Hanahan & Olley (1958), and hexoses as described by Dubois, Gilles, Hamilton, Rebers & Smith (1956).

Paper chromatography. Paper chromatography was carried out with Whatman no. 1 or no. 4 paper previously washed with 2N-acetic acid and then water. The following solvent systems were used: A, propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); B, butan-1-ol-ethanol-water-aq. ammonia (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Foster, Horton & Stacey, 1957); C, pyridine-ethyl acetate-water-acetic acid (5:5:3:1, by vol.) (Fischer & Nebel, 1955). Solvent A was used for ascending chromatography on Whatman no. 4, solvent B for descending chromatography on Whatman no. 4 and solvent C for descending chromatography on Whatman no. 1 papers. The products were detected by the

periodate-Schiff reagents for α -glycols (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956), molybdate reagent for phosphate esters (Hanes & Isherwood, 1949), ninhydrin reagent for amino acids and amino sugars (Consden & Gordon, 1948) and alkaline silver nitrate reagent for sugars (Trevelyan, Procter & Harrison, 1950).

The R_F or R_{Glucose} values in Table 1 refer to compounds obtained by degradation of the teichoic acid and to reference compounds. Most of the degradation products were isolated in small amounts by elution from appropriate areas of paper chromatograms, and in subsequent qualitative and quantitative work a corresponding blank area was extracted with water and the extract used for comparison.

Preparation of ribosomal gel fraction. The organism was grown and whole cells were disrupted as described by Baddiley *et al.* (1962b). After centrifuging, the cloudy supernatant was carefully poured from a mobile layer of disintegrated cell walls and centrifuged at 100 000g in a Spinco model L ultracentrifuge for 1 hr. The clear gel that was deposited (about 1.5–2 g. from 30 l. of culture) was suspended in water and then freeze-dried; acid hydrolysis of a sample followed by paper chromatography indicated the presence of protein, nucleic acid and glycerol teichoic acid.

Isolation and purification of glycerol teichoic acid from the gel. The freeze-dried gel (2.4 g.) was stirred with cold 10% trichloroacetic acid solution (50 ml.) at 0–4° for 48 hr. A further amount (25 ml.) of cold 10% trichloroacetic acid solution was added and, after a further 24 hr., insoluble material was removed by centrifuging in the cold at 2000g for 30 min. To the clear supernatant was added 4 vol. of ethanol and, after 12 hr. at 0°, the impure material was collected by centrifuging, washed twice with ethanol and once with ether and then dried (215 mg.). To the supernatant was added 5 vol. (375 ml.) of acetone, but after 12 hr. at 0° a negligible amount of material was precipitated. Further extraction of the gel with cold 10% trichloroacetic acid solution for 96 hr. gave only 7.2 mg. of

crude teichoic acid, and so this procedure was not adopted with later preparations.

The crude preparation (222 mg.) was triturated with cold 10% trichloroacetic acid solution (2.4 ml.) and an insoluble greyish precipitate removed by centrifuging in the cold. To the supernatant was added ethanol (7.2 ml.) and, after 12 hr. at 0°, the precipitated product was centrifuged, washed with ethanol, ether and dried. Further trituration of this teichoic acid with cold 10% trichloroacetic acid solution (1.0 ml.), and repetition of the above procedure, gave a white powder (133 mg.); acid hydrolysis at this state showed that all protein had been removed and the only impurity was RNA and related polynucleotides.

Purification on a Sephadex column. The partly purified material (75 mg.) was dissolved in cold water (1.5 ml.) and some insoluble nucleotides were removed by centrifuging at 15 000g for 1 hr. in the cold. The slightly turbid supernatant was diluted with water (15 ml.), the pH adjusted to 6.5 with dilute aq. ammonia solution, and the solution freeze-dried. The residue was dissolved in water (3.0 ml.) and applied to a column (2 cm. $\times$ 50 cm.) of Sephadex G-75. Substances were eluted with water at an initial flow rate of 0.6 ml./min. and fractions (2.5 ml., 50 drops) were collected automatically. The extinction at 260 m μ and phosphate content were determined on alternate fractions. The elution curve was similar to that observed by Wicken *et al.* (1963) with the glycerol teichoic acid from *Streptococcus* strain 8191. The teichoic acid was separated from much of the RNA but was incompletely separated from smaller oligonucleotides. Appropriate fractions were combined and pure teichoic acid containing very little nucleotide was obtained. The chromatographic procedure was repeated on those fractions that contained both teichoic acid and nucleotides (total yield, 47 mg.).

RESULTS

Studies with the teichoic acid

Analysis. The ratio glucose:phosphorus in purified material was 0.066:1, and was unchanged when determined on a 2N-sulphuric acid hydrolysate of the teichoic acid. The ratio alanine:phosphorus was 0.87:1, determined on a sample that had been kept at room temperature for 5 hr. in 0.02N-sodium hydroxide solution to hydrolyse alanine ester linkages.

Treatment with ammonia. After treatment with aq. 5N-ammonia solution for 1 hr., followed by evaporation and chromatography in solvent A, products were alanine and its amide. The intensity of the spot corresponding to the amide was greater than that corresponding to alanine.

Acid hydrolysis. Teichoic acid was hydrolysed with an excess of 2N-hydrochloric acid at 100° for 3 hr. The hydrolysate was evaporated *in vacuo* over potassium hydroxide and chromatographed in solvent A. The products are given in Table 2. For the detection of sugars the teichoic acid was hydrolysed for either 3 or 24 hr. at 100°; the hydrolysates were passed through columns (0.8 cm. $\times$ 3 cm. for 2 mg. of polymer) of Dowex 3 (OH[−]

Table 1. *Paper chromatography of degradation products from teichoic acid and related compounds*

Solvents and experimental procedures are described in the text.

Solvent	R_F	R_{Glucose}		
		A	B	C
Glycerol	0.79	—	1.52	—
Glycerol 1-phosphate	0.27	—	—	—
Glycerol 2-phosphate	0.33	—	—	—
Glucose	—	—	1.00	1.00
Galactose	—	—	0.90	0.90
Glucosamine	0.60	—	0.81	0.73
Galactosamine	0.60	—	—	0.62
Glycerol diphosphates	0.07	—	—	—
Inorganic phosphate	0.13	—	—	—
Alanine amide	0.70	—	—	—
Alanine	0.60	—	—	—
Gentiobiosylglycerol	0.43	—	—	—
Glucosaminylglycerol	0.62	—	—	—
Cellobiose	—	—	0.56	—
Gentibiose	—	—	0.44	0.65
Laminaribiose	—	—	0.65	—
Sophorose	—	—	0.61	—

form) resin and the eluates were freeze-dried and chromatographed in solvent C; the eluate contained glucose, glucosamine and glycerol (detected by the alkaline silver nitrate reagent). No anhydrosorbitol or ribitol 3-phosphate was detected (periodate-Schiff reagents) by chromatography in solvent B of an acid hydrolysate of 2 mg. of teichoic acid.

Alkaline hydrolysis. This was carried out with *N*-sodium hydroxide solution at 100° for 3 hr. The hydrolysate was passed through a column (0.6 cm. $\times$ 1.5 cm. for 3 mg. of polymer) of Dowex 50 (NH_4^+ form) resin and the eluate evaporated *in vacuo*. Products were examined by chromatography in solvent A and are given in Table 2.

Enzymic hydrolysis of glycerol monophosphates. Glycerol phosphates in an alkaline hydrolysate were identified by chromatography in solvent A, elution and hydrolysis in 0.1M-ammonium carbonate-aq. ammonia buffer, pH 9.5, with the intestinal phosphatase. After incubation for 72 hr. at 37°, buffer was evaporated *in vacuo* and products were identified in solvents A and B. Both glycerol phosphates were completely hydrolysed by the enzyme to glycerol and inorganic phosphate.

Identification of glycerol diphosphates and diglycerol triphosphate. From a similar alkaline hydrolysate of teichoic acid (12 mg.), the glycerol diphosphates and diglycerol triphosphate were together eluted from a paper chromatogram and hydrolysed with the phosphatase under conditions comparable with those described above. The products were inorganic phosphate, glycerol and diglycerol phosphate; the glycerol and diglycerol phosphate reacted rapidly with the periodate-Schiff reagents, giving purple spots on the paper, and the diglycerol phosphate reacted with the molybdate reagent. Diglycerol phosphate was eluted from a chromatogram and hydrolysed with *N*-sodium hydroxide at 100° for 3 hr. After removal of Na^+ ions with a column of Dowex 50 (NH_4^+ form) resin the eluate was freeze-dried. The diester

was completely hydrolysed under these conditions to glycerol and its monophosphates.

Other phosphates formed by alkaline hydrolysis. The small amounts of organic phosphate with R_f 0.17 in solvent A slowly gave a blue spot with the periodate-Schiff reagents. Eluted material was treated with the phosphatase in ammonium carbonate-aq. ammonia buffer as described above. After incubation at 37° for 48 hr. the products were inorganic phosphate and a major product with R_f 0.52 (solvent A), which contained sugar and phosphate as shown by the periodate-Schiff and molybdate reagents respectively; this is probably glucosaminylglycerol glycerol phosphate. A small amount of a compound tentatively identified as gentiobiosylglycerol was also detected.

Periodate oxidation. Alanine was removed from teichoic acid (5 mg.) by treatment with ammonia as described above, followed by passage of the aqueous solution through a column (0.8 cm. $\times$ 3 cm.) of Dowex 50 (H^+ form) resin. The eluate was neutralized with ammonia, evaporated *in vacuo* and the residue dissolved in water (1.1 ml.); 0.8 ml. of the above-mentioned solution was mixed with 1.25 mM-sodium metaperiodate solution (3.2 ml.), a portion was diluted ten times and the change of extinction at 223 $\text{m}\mu$ was measured at intervals. Extrapolation of the curve relating change of extinction with time to zero time (cf. Critchley, Archibald & Baddiley, 1962) gave a ratio phosphorus:periodate consumed by the terminal glycol residue as 13.7:1. Similarly, the formaldehyde produced after 72 hr. gave the ratio phosphorus:formaldehyde as 14.1:1.

Periodate oxidation and acid hydrolysis. Teichoic acid (3.9 mg.) from which alanine had been removed with ammonia was added to 0.3M-sodium metaperiodate solution (0.2 ml.) and the volume adjusted to 5 ml. with water. After 96 hr. in the dark, excess of periodate was destroyed with sulphur dioxide. The solution was aerated and passed through a column of Dowex 50 (H^+ form) resin, and the eluate was evaporated *in vacuo* to a small volume (not to dryness). The residue was hydrolysed with 2*N*-hydrochloric acid at 100° for 3 hr. and the hydrolysate passed through a column of Dowex 3 (OH^- form) resin. The neutral eluate was freeze-dried and chromatographed in solvent C. Although glycerol was present, no glucose or glucosamine was detected with the alkaline silver nitrate reagent.

Periodate oxidation and borohydride reduction. To a solution of the neutral sodium salt of teichoic acid (11 mg. from which alanine had been removed) was added 0.3M-sodium metaperiodate (0.2 ml.) and the volume adjusted to 5 ml. with water. After 96 hr. in the dark, sodium borohydride (10 mg.) was added to a portion (1.3 ml.), and

Table 2. *Products of acid and alkaline hydrolysis of teichoic acid*

Conditions for hydrolysis and methods for the identification of products are described in the text.

	Acid hydrolysis	Alkaline hydrolysis
Glycerol	+	+
Glycerol phosphates	+	+
Glycerol diphosphates	+	+
Diglycerol triphosphate	-	+
Glucose	+	-
Glucosamine	+	-
Alanine	+	+
Anhydrosorbitol	-	+
Gentiobiosylglycerol	-	+
Glucosaminylglycerol	-	+

after a further 18 hr. at room temperature excess of sodium borohydride was destroyed with acetic acid. Sodium ions were removed with a column (0.5 cm. $\times$ 4 cm.) of Dowex 50 (H^+ form) resin, and boric acid was removed by evaporation with methanol in the usual manner. The residue was hydrolysed with 2N-hydrochloric acid at 100° for 5 hr. and the hydrolysate passed through a column of Dowex 3 (OH^- form) resin. The neutral eluate was freeze-dried and chromatographed in solvent B. No tetritol was detected, whereas a comparable experiment on an approximately equivalent amount of cellobiose gave a strong spot corresponding to erythritol.

Action of phosphomonoesterase. Teichoic acid (1.6 mg.) without alanine residues was dissolved in 0.1M-ammonium carbonate-aq. ammonia buffer, pH 9.5 (5 ml.), and treated with the phosphatase (0.15 mg.) at 37° for 21 hr. The ratio inorganic phosphate produced:total phosphorus was 1:35; no more inorganic phosphate was produced by prolonged incubation.

Gentiobiosylglycerol

The alkaline hydrolysate from 12 mg. of teichoic acid was passed through a column (0.8 cm. $\times$ 3.8 cm.) of Dowex 50 (NH_4^+ form) resin and the column was washed with water. The eluate and washings were evaporated *in vacuo* and products separated by chromatography in solvent A. Material with R_f 0.43 was eluted and examined in the following manner.

Acid hydrolysis. A sample was hydrolysed with 2N-hydrochloric acid at 100° for 3 hr. and the hydrolysate was passed through a column of Dowex 3 (OH^- form) resin. The eluate was evaporated *in vacuo* and examined in solvents A and C. Only glucose and glycerol were detected.

Treatment with β -glucosidase. A sample of the aqueous solution of glycoside was hydrolysed with β -glucosidase in the absence of a buffer at 37° for 30 hr., and then evaporated and examined on paper in solvents A and C. The products were glucose and glycerol; no glycosides remained.

Analysis. Gentiobiosylglycerol (from 7 mg. of teichoic acid) was treated with β -glucosidase (0.02 mg.) in water (1 ml.) at 37° for 24 hr. Glycerol was determined as described by Hanahan & Olley (1958) from the amount of formaldehyde produced on oxidation with sodium metaperiodate, and glucose by the method of Dubois *et al.* (1956). The ratio glucose:glycerol was 1.82:1.

Partial acid hydrolysis. Gentiobiosylglycerol (from 10 mg. of teichoic acid) was hydrolysed with 0.33N-sulphuric acid at 100° for 30 min. (Goldstein & Whelan, 1962), and the hydrolysate was passed through a column (0.6 cm. $\times$ 3 cm.) of Dowex 3 (OH^- form) resin. The eluate and water washings

were freeze-dried and examined on paper. In solvent A glycerol was identified, and in solvent B (descending, 48 hr.) the only sugars detected were gentiobiose and a trace of glucose.

Glucosaminylglycerol

The glucosaminide had R_f 0.62 in solvent A and was isolated from a paper chromatogram of an alkaline hydrolysate treated in the manner described above. Part of the eluate was hydrolysed with 2N-hydrochloric acid at 100° for 3 hr., and the rest at 100° for 24 hr. After removal of Cl^- ions on Dowex 3 (OH^- form) resin, the solutions were freeze-dried and examined in solvents A and C. The compound was unchanged after hydrolysis for 3 hr. but was hydrolysed to glucosamine (alkaline silver nitrate reagent, solvent C) and glycerol (periodate-Schiff reagents, solvent A) during 24 hr.

Reaction with nitrous acid. Glucosaminylglycerol (from 10 mg. of teichoic acid) was treated with sodium nitrite (20 mg.) in water (0.5 ml.) and 0.01N-hydrochloric acid (0.05 ml.). The solution was heated at 70° for 3 hr. and passed through a column (0.8 cm. $\times$ 4 cm.) of Dowex 50 (NH_4^+ form) resin. The eluate and water washings were heated at 90° for 10 min. and then freeze-dried; the residue was examined in solvent A. With the periodate-Schiff reagents a strong spot corresponding to glycerol and a very faint spot corresponding to ribitol were obtained; a yellow streak characteristic of chitose was observed that was indistinguishable from that obtained by deamination of glucosamine.

DISCUSSION

The isolation of this intracellular teichoic acid by differential centrifuging of disrupted cells and extraction from the ribosomal fraction follows the general procedure adopted in other cases (Critchley *et al.* 1962; Ellwood, Kelemen & Baddiley, 1963; Wicken *et al.* 1963; Wicken & Baddiley, 1963). Although no ribitol teichoic acid from the walls was detected in the unpurified material, nevertheless complete separation from RNA and oligonucleotides on Sephadex columns was achieved only with considerable difficulty. These considerations, and the smallness of the amount of intracellular teichoic acid in this organism, seriously limited the scale and extent of structural investigations.

From the products of acid hydrolysis (Table 2) it follows that this material conforms to the general structural pattern for intracellular teichoic acids; in particular, the formation of glycerol and its diphosphates establishes the presence of a glycerol phosphate polymer in which the glycerol units are joined through phosphodiester linkages. Alkaline

hydrolysis gave a small amount of a diglycerol triphosphate (I), identified chromatographically and by hydrolysis with a phosphomonoesterase to the alkali-labile diglycerol monophosphate. The formation of the triphosphate establishes the presence in the polymer of glycerol phosphate units joined at the 1,3 positions (Kelemen & Baddiley, 1961; Critchley *et al.* 1962; Ellwood *et al.* 1963).

Other products of acid hydrolysis include alanine, glucose and a small amount of glucosamine. The alanine is joined to the polymer through the usual labile ester linkages, as it was removed readily with ammonia solution at room temperature giving alanine and its amide; no other amino acids were detected. From the ratios alanine:phosphorus (0.87:1) and glucose:phosphorus (0.066:1) it follows that most or all of the alanine is attached to position 2 in glycerol units. The configuration of the amino acid was not determined directly, but it is known that only D-alanine can be isolated from mixtures of glycerol and ribitol teichoic acids extracted from whole cells of this organism (Baddiley *et al.* 1962*b*); it follows that the alanine in the intracellular compound has the expected D configuration.

The sugars produced during acid hydrolysis are attached glycosidically to glycerol residues. Thus hot alkali caused extensive decomposition of the polymer by loss of alanine residues and then hydrolysis of phosphodiester linkages through intermediate glycerol 1,2-cyclic phosphates; in this way all non-terminal glycerol units bearing sugar residues were converted into glycerol glycosides, and only traces of glycoside phosphates from terminal positions were detected. These observations are characteristic of glycerol teichoic acids in which not all glycerol residues bear sugar substituents (Critchley *et al.* 1962; Ellwood *et al.* 1963), and the mechanism of hydrolysis of such structures has been discussed in previous publications. This alkaline lability is in marked contrast with the stability of those teichoic acids in which most or all glycerol residues bear sugar substituents (Wicken & Baddiley, 1963). It is concluded that in the teichoic acid from *S. aureus* sugar substituents occur on a small number of glycerol units that could be more or less randomly distributed. The very small amount of phosphates of glycerol glycosides formed during alkaline hydrolysis indicates that a negligible amount of the sugar occurs on phosphate-terminal glycerol units, and the number of occasions where two glycerol glycoside

residues are adjacent on the chain is also negligible. A small amount of alkali-stable organic phosphate(s) with low R_F is probably a mixture of products, e.g. (II), in which a glycosylglycerol residue is joined through phosphodiester linkage to glycerol 2-phosphate; stable phosphodiester of this type were fully characterized as products of alkaline hydrolysis of the glycerol teichoic acid from *S. albus* walls, and they arise through mechanisms similar to that which causes the production of the alkali-stable diglycerol triphosphate (I) (Ellwood *et al.* 1963).

Paper chromatography of the alkaline hydrolysate revealed a product with the properties of a 2-O-glucosaminylglycerol. It was stable towards mineral acid at 100° during 3 hr., but gave glycerol and glucosamine on prolonged acid hydrolysis. Such stability is typical of glycosides of 2-amino sugars (Foster *et al.* 1957), and has been observed with glucosaminides of ribitol and galactosaminides of glycerol obtained from other teichoic acids. Although insufficient material was available to establish the configuration of the glycosidic linkage, and quantitative periodate oxidation was not possible, the general structure of the compound was established from its behaviour towards nitrous acid and on paper with the periodate-Schiff spray reagents. The reaction with nitrous acid gave glycerol and chitose (2,5-anhydromannose) in a manner analogous to that observed with other polyol aminoglycosides (Baddiley *et al.* 1962*a, b*; Ellwood *et al.* 1963), thus establishing the presence of a glycosidic linkage between glycerol and glucosamine. The glucosaminide reacted slowly and weakly with the periodate-Schiff reagents, giving a blue colour on the paper; it follows that the oxidation produces no formaldehyde, and so the glycosidic linkage must involve position 2 in glycerol. Only a faint trace of ribitol was detected in the products of reaction with nitrous acid, and no anhydriboitol was detected in acid hydrolysates; this indicates the almost complete absence of ribitol teichoic acid from the intracellular material. From the ease with which glucosamine is produced during acid hydrolysis of the teichoic acid it follows that the glucosaminylglycerol residues in the polymer bear N-acyl substituents.

Alkaline hydrolysates also contained a product with R_F 0.43 in solvent A. On acid hydrolysis it gave only glucose and glycerol, and this compound is believed to account for all the glucose in

the teichoic acid. It was readily and completely hydrolysed to glucose and glycerol by a β -glucosidase, and analysis of an enzymic hydrolysate indicated the presence of 2 mol.prop. of glucose to each glycerol molecule; it follows that the compound is a glucosylglucosylglycerol in which both glycosidic linkages possess the β configuration. Partial acid hydrolysis gave glycerol, a little glucose and a much larger amount of a disaccharide identified chromatographically as gentiobiose [β -D-glucopyranosyl-(1 $\rightarrow$ 6)-O-D-glucose]; this disaccharide differed chromatographically from the other reducing disaccharides of glucose containing a β -glycosidic linkage (sophorose, laminaribiose and cellobiose). The high yield of gentiobiose from the partial acid hydrolysis of the glycerol glycoside is consistent with the known stability of 1 $\rightarrow$ 6 linkages in disaccharides (cf. Goldstein & Whelan, 1962), and is in marked contrast with the behaviour of glucose di- and tri-saccharide residues containing 1 $\rightarrow$ 2 linkages in the glycerol teichoic acids from streptococci (Wicken & Baddiley, 1963). The slow development of a blue colour when the gentiobiosylglycerol was treated with the periodate-Schiff reagents on paper chromatograms indicates that sugar is attached to position 2 on the glycerol.

The presence of gentiobiosyl residues in the teichoic acid is consistent with its behaviour towards periodate after removal of alanine. All the glucose and glucosamine was destroyed, and reduction of the oxidized material with sodium borohydride followed by acid hydrolysis gave glycerol but no tetritol. It is concluded that 2-O- β -gentiobiosylglycerol residues occur in the teichoic acid, as shown in (III).

Attempts to determine the chain length of the polymer gave inconclusive results. If it is assumed that the material is homogeneous with respect to sugars and chain length, the ratio glucose:phosphorus indicates a minimum polymer of about 30 glycerol phosphate units, one of which bears a gentiobiosyl residue and presumably about one of which bears a *N*-acylglucosaminyl residue. Alter-

natively, the material could be a mixture of approximately equal amounts of a polymer of 15 units with one gentiobiosyl residue, and one of 15 units with one *N*-acylglucosaminyl residue. Shortage of material prevented a decision about these alternatives. The ratio terminal phosphomonoester:total phosphate determined by the action of phosphomonoesterase was 1:35, whereas in a separate experiment the number of terminal glycol groups determined by periodate oxidation and formaldehyde production was 1 to about 14 phosphorus atoms. Both methods are unreliable if partial loss of terminal phosphate, or hydrolysis of phosphodiester groups, has occurred during purification of the teichoic acid.

SUMMARY

1. The intracellular teichoic acid has been isolated in small amounts from a ribosomal fraction prepared from *Staphylococcus aureus* H. Purification was achieved by chromatography on columns of Sephadex G-75.

2. Chemical and enzymic degradation indicates that it is a glycerol 1,3-phosphate polymer with D-alanine ester residues at position 2 on most of the glycerol units, and a small number of sugar residues attached glycosidically to glycerol.

3. All of the glucose occurs as gentiobiosyl [β -D-glucopyranosyl-(1 $\rightarrow$ 6)-O-D-glucopyranosyl] residues attached in β -glycosidic linkage to position 2 in glycerol. 2-O- β -Gentiobiosylglycerol has been isolated from an alkaline hydrolysate of the teichoic acid, and its structure established by chemical and enzymic degradation.

4. A small amount of *N*-acylglucosamine is attached to glycerol at position 2, as alkaline hydrolysis of the polymer gave a little glucosaminylglycerol. It is not known whether gentiobiosyl and acylglucosaminyl residues occur in a single polymer or whether the preparations contain two or more chemically different teichoic acids.

We thank the Nuffield Foundation, the Medical Research Council and the Department of Scientific and Industrial Research for financial assistance, and Dr W. J. Whelan for samples of sophorose and laminaribiose.

REFERENCES

- Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. & Greenberg, G. R. (1958). *J. chem. Soc.* p. 4344.
- Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.
- Baddiley, J., Buchanan, J. G., Martin, R. O. & Raj-Bhandary, U. L. (1962a). *Biochem. J.* **85**, 49.
- Baddiley, J., Buchanan, J. G., RajBhandary, U. L. & Sanderson, A. R. (1962b). *Biochem. J.* **82**, 439.
- Baddiley, J. & Davison, A. L. (1961). *J. gen. Microbiol.* **24**, 295.

- Buchanan, J. G., Dekker, C. A. & Long, A. G. (1950). *J. chem. Soc.* p. 3102.
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- Consden, R. & Gordon, A. H. (1948). *Nature, Lond.*, **162**, 180.
- Critchley, P., Archibald, A. R. & Baddiley, J. (1962). *Biochem. J.* **85**, 420.
- Davison, A. L. (1962). Ph.D. Thesis: University of Durham.
- Dixon, J. S. & Lipkin, D. (1954). *Analyt. Chem.* **26**, 1092.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
- Ellwood, D. C., Kelemen, M. V. & Baddiley, J. (1963). *Biochem. J.* **86**, 213.
- Fischer, F. G. & Nebel, H. G. (1955). *Hoppe-Seyl. Z.* **302**, 10.
- Foster, A. B., Horton, D. & Stacey, M. (1957). *J. chem. Soc.* p. 81.
- Goldstein, I. J. & Whelan, W. J. (1962). *J. chem. Soc.* p. 170.
- Hanahan, D. J. & Olley, J. N. (1958). *J. biol. Chem.* **231**, 813.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Haukenes, G. (1962). *Immunochemical Studies on Polysaccharide A of Staphylococcus aureus*. Norwegian Universities Press.
- Haukenes, G., Ellwood, D. C., Baddiley, J. & Oeding, P. (1961). *Biochim. biophys. Acta*, **53**, 425.
- Hay, J. B., Wicken, A. J. & Baddiley, J. (1963). *Biochim. biophys. Acta* (in the Press).
- Hough, L., Powell, D. B. & Woods, B. M. (1956). *J. chem. Soc.* p. 4799.
- Kelemen, M. V. & Baddiley, J. (1961). *Biochem. J.* **80**, 246.
- Morse, S. I. (1962). *J. exp. Med.* **116**, 229.
- Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.
- Sanderson, A. R., Juergens, W. G. & Strominger, J. L. (1961). *Biochem. biophys. Res. Commun.* **5**, 472.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Wicken, A. J. & Baddiley, J. (1963). *Biochem. J.* **87**, 54.
- Wicken, A. J., Elliott, S. D. & Baddiley, J. (1963). *J. gen. Microbiol.* **30**, 111.

Biochem. J. (1963) **87**, 435

Condensed Tannins

16. SYNTHESIS OF (–)-FLAVANS FROM (+)-DIHYDROFLAVONOLS*

By D. G. ROUX†

Leather Industries Research Institute, Rhodes University, Grahamstown, South Africa

(Received 18 July 1962)

By means of the reductive conversions of (+)-dihydorobinetin and (+)-fustin [(+)-3',4',5',7-tetrahydroxy- and (+)-3',4',7-trihydroxy-flavanon-3-ol respectively] into their (–)-flavanone and thence into their (+)-flavan-4-ol analogues, Roux & Paulus (1962*b*) were able to correlate the absolute configurations of each of these classes of flavonoid compounds. Similarly, the absolute configurations of the corresponding (–)-flavans may now be predicted by an extension of the same series of interconversions.

From the above and also from previous work on their naturally occurring analogues, the contributions of each of the asymmetric centres to the molecular rotations of natural flavan-3-ols, flavan-3,4-diols, flavanonols and synthetic flavan-4-ols, flavans and flavanones may be assessed. Also, evidence from the conversion of flavan-4-ols into flavans confirms previous notions (Roux & Paulus, 1962*b*) on the contribution of the 4-hydroxyl group to the redness induced in tannins by sunlight.

* Part 15: Drewes & Roux (1963).

† Present address: Chemistry Department, Harvard University, Cambridge 38, Mass., U.S.A.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points were carried out as described by Roux & Maihs (1960*a*). Analysis for C, H, methoxyl and acetyl groups was by K. Jones, Microanalytical Laboratory, C.S.I.R., Pretoria, South Africa. Comparisons of infrared spectra were by Dr P. R. Enslin, National Chemical Research Laboratories, C.S.I.R., Pretoria, South Africa. A 2 dm. tube was used for optical rotations.

Synthesis of (±)-3',4',5',7-tetrahydroxyflavan and derivatives from (±)-dihydorobinetin

Partially racemized dihydorobinetin, $[\alpha]_D +16^\circ$, from the heartwood of *Robinia pseudacacia* (Freudenberg & Hartmann, 1954) was separated into a racemate and into the optically pure (+)-form by chromatographic methods with cellulose as substrate (Roux & Paulus, 1962*b*).

(±)-3',4',5',7-Tetrahydroxyflavan. Racemic dihydorobinetin (500 mg.) was selectively reduced to (±)-robinetin [(±)-3',4',5',7-tetrahydroxyflavanone] (250 mg.), m.p. 276°, with deactivated zinc dust and hydrochloric acid in methanolic solution (Roux & Paulus, 1962*b*). The method of reduction was improved by using AnalaR zinc dust that had been dampened and left in an open beaker at room temperature (20–30°) for 20 days. The zinc set into a hard