The Synthesis of Teichoic Acids

III. GLUCOSYLATION OF POLYGLYCEROPHOSPHATE*

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In the two preceding papers, evidence has been presented for the enzymatic synthesis of polyglycerophosphate (1) and polyribitol phosphate (2) from cytidine diphosphate glycerol and cytidine diphosphate ribitol, respectively. In this paper, we describe the glycosyl transfer from uridine diphosphate glucose to polyglycerophosphate by an enzyme from Bacillus subtilis. Teichoic acids differ in the nature and number of the glycosyl residues attached to the polyol phosphate backbone.

The glycosyl transfer described in this paper gives rise to one of the simplest teichoic acid structures, in which every glycerol residue in the polyglycerophosphate backbone has attached to the hydroxyl at carbon 2 an α-glucopyranosyl residue.

A similar reaction, the transfer of N-acetylglucosamine residues to polyribitol phosphate from UDP-N-acetylglucosamine, has been described by Nathenson and Strominger (4). A second glucosylating system for glycerol teichoic acid has been described in a preliminary communication (5). This system isolated from Bacillus licheniformis is more complex and will be reported in detail later.

EXPERIMENTAL PROCEDURE

B. subtilis NCTC 3610 was grown as described (1). Membranes† were prepared from cells in the logarithmic phase of growth, either by osmotic lysis of protoplasts (1), or by incubating the cells in 0.05 M Tris chloride, 0.01 M MgCl₂, 0.001 M EDTA, pH 8.0, containing 1 mg per ml of lysozyme and 50 μg per ml each of ribonuclease and deoxyribonuclease. After lysis had occurred, as judged by examination under a phase microscope, cell membranes were collected at 32,000 × g for 12 minutes, washed twice with centrifugation with the same buffer, and kept frozen in this buffer at a concentration of approximately 30 mg of dry weight per ml.

Cell walls were prepared by shaking B. subtilis cells in a Nossal shaker and purified by differential centrifugation and digestion with trypsin, ribonuclease, and deoxyribonuclease similar to the method of Cummins and Harris (6). Their purity was checked by examination in a phase microscope. The supernatant fluid, after removal of the cell walls from the cell extract, was centrifuged at 105,000 × g for 60 minutes, and the sedimented particles used to prepare "intracellular" teichoic acid by phenol extraction and Sephadex chromatography (1). Teichoic acid was isolated from the cell wall fraction by two successive extractions with 10% trichloroacetic acid at 3° for 24 hours (7) and was precipitated with 2 volumes of ethanol. After 24 hours at 3°, the precipitate was collected by centrifugation, washed with absolute ethanol, and dried. The dried residue was suspended in 10% trichloroacetic acid, a small insoluble residue was discarded, and the teichoic acid was again precipitated with ethanol.

Degradation of teichoic acid with 60% HF was performed by an unpublished method of Dr. D. Lipkin and Mr. J. W. Abrell, and was kindly made available to us by Dr. D. Lipkin. (For some previous applications of this method, see Lipkin et al. (8, 9).) Samples were dried overnight in polyethylene tubes in a vacuum desicator and incubated with 0.2 ml of 60% HF at 0° for 3 to 5 hours. The sample was cooled to −80°, LiOH sufficient to almost neutralize the HF was added, and the solution was allowed to warm to 0°. The pH was then adjusted to neutrality with lithium carbonate, and insoluble LiF was removed by centrifugation and washed with water. The water eluent was concentrated and put on a column prepared from 1 g of Darco G-60 and 1 g of Celite (10). The column was washed with 40 ml of H₂O and eluted with 10% ethanol.

UDP-glucose was prepared enzymatically (11). It had a specific activity of 118,000 c.p.m. per μmole. α-Glucosidase was prepared from yeast by the method of Lehman and Pratt (12) and was free of β-glucosidase when assayed with salicin as a substrate. β-Glucosidase was obtained from Worthington Biochemical Corporation and refractionated with (NH₄)₂SO₄. It was free of α-glucosidase when assayed with maltose as substrate.

The hydrolysis of glycosylglycerol with α-glucosidase was performed in 0.06 M phosphate buffer, pH 6.8, containing 10⁻³ M mercaptoethanol. Glycosylglycerol was treated with β-glucosidase in 0.05 M acetate buffer, pH 5.0.

Amino groups were determined with 2,4,6-trinitrobenzenesulfonic acid (13). Glucose was determined with glucose 6-phosphate dehydrogenase and hexokinase, glycerol with glycerol dehydrogenase (Worthington Biochemical Corporation), and phosphate by the method of Chen, Toribara, and Warner (14). Sugars were chromatographed in butanol-pyridine-H₂O (6:4:3) (15).

For enzyme assay, incubations were performed as described for the individual experiments and terminated by the addition of an equal volume of pyridine. Aliquots were streaked on strips of Whatman No. 1 paper (4 cm wide) and chromatographed in ethanol-1 M ammonium acetate, pH 3.8 (16). Teichoic acid remains at the origin. The radioactivity was determined in a...
Vanguard paper strip counter with an integrator, and the percentage of the total radioactivity remaining at the origin was determined. The procedure automatically corrects for small errors in spotting and variations in the counter efficiency.

RESULTS

Structure of Teichoic Acid from B. subtilis NCTC 3610—The structure of the ribitol teichoic acid from B. subtilis (strain unspecified) has been examined in detail by Armstrong, Baddiley, and Buchanan (17). Their strain has a glucosylated ribitol teichoic acid in the cell wall and contains as well an “intracellular” glycerol teichoic acid, also heavily glucosylated. It has become clear in the last few years that there is a considerable strain difference in the composition of teichoic acids (see, e.g. (18, 19)), and it is therefore not surprising that the teichoic acid from B. subtilis NCTC 3610 should be different from the acid described by Armstrong et al. (17). The compositions of several preparations of this teichoic acid are illustrated in Table I. The amino group content was very variable and usually less than 0.1 pmole of amino group per pmole of glycerol. No other sugars besides glucose could be identified by chromatography of an acid hydrolysate in butanol-pyridine-water.

The analysis of the wall teichoic acid is consistent with a

<table>
<thead>
<tr>
<th>Source of teichoic acid</th>
<th>Compound analysed</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>4</td>
<td>2.28</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.95</td>
<td>2.28</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>4.0</td>
<td>2.94</td>
<td></td>
</tr>
<tr>
<td>Intracellular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>1.04</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.70</td>
<td>7.45</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>5.70*</td>
<td>7.45</td>
<td></td>
</tr>
</tbody>
</table>

* The excess phosphate in this preparation is due to contamination with oligonucleotides.

Composition of teichoic acid from B. subtilis NCTC 3610

Teichoic acids were prepared as described in “Experimental Procedure.” Analysis for glucose, glycerol, and phosphate were performed after hydrolysis in 1 n HCl for 3 hours at 100°, followed by treatment with Escherichia coli phosphatase. A and B are different preparations of teichoic acid.

Experiment A

Experiment B

Table II

Analysis of glucosylglycerol

Glucosylglycerol was prepared by hydrolysis in 60% HF and purified by chromatography on a Darco-Celite column. A, B, and C are samples prepared from three different teichoic acid preparations.

<table>
<thead>
<tr>
<th>Method of hydrolysis</th>
<th>Product</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl, 1 n, 3 hrs at 100°</td>
<td>Glucose</td>
<td>0.62</td>
<td>6.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>0.48</td>
<td>6.20</td>
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<tr>
<td>α-Glucosidase</td>
<td>Glucose</td>
<td>0.50</td>
<td>0.53</td>
<td>6.60</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>0.53</td>
<td>0.46</td>
<td>5.95</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>Glucose</td>
<td>0.53</td>
<td>0.46</td>
<td>5.95</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>0.53</td>
<td>0.46</td>
<td>5.95</td>
</tr>
</tbody>
</table>

Fig. 1. Alkaline hydrolysis of glucosylpolyglycerophosphate-

UDP-glucose in preincubation | Glycosyl acceptor | Time | Glucosylpolyglycerophosphate formed
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mmoles glucose</td>
<td>hrs</td>
<td>mmoles glucose</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------------</td>
<td>-----</td>
<td>------------------------</td>
</tr>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>+</td>
<td>28</td>
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<td>12.5</td>
</tr>
<tr>
<td>+</td>
<td>56</td>
<td>2</td>
<td>20.0</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>2</td>
<td>35.5</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>35</td>
<td>2</td>
<td>12.6</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table III

Acceptor requirement for synthesis of glucosylpolyglycerophosphate

The enzyme in Experiment A was preincubated with 1 μmole of UDP-glucose, 20 μmoles of Tris-chloride (pH 8.0), 4 μmoles of MgCl₂, and 0.1 μl of enzyme, and was incubated at 37° for 2 hours. The reaction was diluted to 10 ml with 0.05 μ Tris-chloride (pH 8.0), 0.01 μM MgCl₂, and 0.001 μM EDTA, and the enzyme was resuspended by centrifugation at 30,000 × g for 10 minutes. The supernatant fluids were neutralized with KOH and chromatographed in ethanol-1 M ammonium acetate, pH 3.8, and the radioactivity remaining at the origin was determined. Experiment B was performed with the same enzyme preparation, at one third the scale of A, and with intracellular glycerol teichoic acid as glucosyl acceptor. It was assayed as described in “Experimental Procedure.”

Fig. 1. Alkaline hydrolysis of glucosylpolyglycerophosphate-

fully glucosylated glycerol teichoic acid, with one glucose linked to every glycerol unit; alternatively, some of the glycerol units may have oligosaccharides attached to them and some may be unsubstituted. In the former case, the teichoic acid would be expected to be resistant to alkaline hydrolysis (18). In fact,
FIG. 2. Hydrolysis of 14C-glucosylglycerol with α-glucosidase. In A and B, 14C-glucosylglycerol was prepared from UDP-glucose and 14C-polyglycerophosphate. Chromatogram A is a tracing of the radioactivity in a chromatogram of glucosylglycerol before treatment with α-glucosidase. Chromatogram B is the same material after treatment with α-glucosidase. Chromatogram C shows the chromatographic behavior of 14C-glucosylglycerol prepared from 14C-UDP-glucose and unlabeled polyglycerophosphate. Chromatogram D shows the same material after treatment with α-glucosidase followed by oxidation with glucose oxidase to convert glucose to gluconic acid, since glucose and glucosylglycerol do not separate well in this solvent. The Rf value of glucosylglycerol (prepared from cell wall teichoic acid) is 1.1. The counts per minute spotted were approximately 300 in A, C, and D and 600 in B. Solvent was butanol-pyridine-H2O (6:4:3). The standards indicated by the cross-hatched areas are, in order of increasing mobility, gluconic acid, glucose, and glycerol.

no small molecular weight material could be isolated by hydrolysis of the wall teichoic acid in 1 N KOH at 100°C for periods up to 24 hours.

Teichoic acids are resistant to the action of phosphodiesterases. A 60% solution of HF at 0°C will cleave phosphate esters without significant hydrolysis of glycosides. It will cleave monoesters more rapidly than diesters,2 therefore, the expected product from the degradation of fully glucosylated glycerol teichoic acid is glucosylglycerol. Glucosylglycerol could be isolated by treatment of the teichoic acid with HF, followed by chromatography on charcoal Celite columns. It had an Rf value of 1.1 when chromatographed in butanol-pyridine-H2O (6:4:3). Typical analyses are shown in Table II. Since the glucosylglycerol is cleaved quantitatively by α-glucosidase and does not yield formaldehyde on periodate oxidation, the glucose must be linked to carbon 2 of glycerol.

On the small scale in which the hydrolyses was performed, the yield of glucosylglycerol varied between 50 and 75% of theory. Most of the loss was material that adsorbed irreversibly on LiF. Essentially all of the glucose in the supernatant fluid after removal of LiF was recovered as glucosylglycerol after chromatography on Darco G-60. The data cannot, therefore, rule out that a few glycerol units carry no substituents, or that a few glycerol units have disaccharides linked to them.

Table I also shows analyses of the intracellular glycerol teichoic acid. The analyses would be consistent with a mixture of cell wall teichoic acid and free polyglycerophosphate or with a partially glucosylated polymer. The ratio of glucose to glycerol varied in different preparations of intracellular teichoic acid. The former possibility is supported by the observation that the glucosylated fraction of the intracellular teichoic acid is also resistant to alkaline hydrolysis, showing that the glucosyl residues are linked to neighboring glycerol units.

FIG. 3. Effect of time and enzyme concentration on the glucosylation of polyglycerophosphate.3 For the time curve (A) the reaction mixtures contained 10 mmoles of Tris-chloride (pH 8.0), 3 μmoles of MgCl2, 280 mmoles of polyglycerophosphate, 40 μmoles of 14C-UDP-glucose, and 0.03 ml of enzyme, in a final volume of 0.38 ml, and was incubated at 37°C for the times indicated. For the enzyme concentration (B), the reaction mixture was the same with 1 μmole of MgCl2 and a 30-minute incubation period, n, millimicromoles of glucose transferred to polyglycerophosphate.

3 The enzyme preparations used in the experiments shown in Figs. 3 to 7 were preincubated with UDP-glucose to glucosylate acceptor present in the enzyme, and washed twice with 0.05 M Tris-0.01 MgCl2-0.001 M EDTA, pH 8.0. The glucosyl acceptor used in all these experiments was "intracellular" glycerol teichoic acid containing 30% glucosylglycerol; concentrations are expressed in millimicromoles of unsubstituted glycerophosphate units in polyglycerophosphate.
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0.04 0.1 0.3

FIG. 4. The effect of UDP-glucose concentration on the glucosylation of polyglycerophosphate. The reaction mixtures contained 5 μmoles of Tris-chloride (pH 8.0), 1 μmole of MgCl₂, 350 μmoles of polyglycerophosphate, and 0.03 ml of enzyme, in a final volume of 0.26 ml, and were incubated at 37° for 30 minutes. V, millimicromoles of glucose transferred; S, UDP-glucose concentration (M × 10⁻⁵).

0.4 0.8 1.2 1.6

FIG. 5. The effect of polyglycerophosphate concentration on the glucosylation of polyglycerophosphate. The reactions were performed as in Fig. 4 with 30 μmoles of UDP-glucose-¹⁴C. v, millimicromoles of glucose transferred; s, glycerophosphate concentration (in polyglycerophosphate) (M × 10⁻⁵).

Enzymatic Glucosylation of Polyglycerophosphate

When a membrane suspension from *B. subtilis* was incubated with UDP-glucose-¹⁴C and either intracellular teichoic acid from *B. subtilis* (30% glucosylated) or enzymatically synthesized polyglycerophosphate (1), there was a considerable formation of labeled, high molecular weight material. Variable quantities of this material could also be formed in the absence of added acceptor. The system became completely acceptor-dependent if the enzyme was preincubated with UDP-glucose, washed, and then incubated with ¹⁴C-UDP-glucose (Table III). The radioactive polymer formed in the absence of added acceptor precipitated with the protein after addition of perchloric acid (1), the radioactive polymer formed from enzymatically synthesized polyglycerophosphate precipitated about 50% with protein, while the product formed from teichoic acid prepared by trichloroacetic acid extraction fails to precipitate. For this reason, the method used to assay for teichoic acid synthesis in the two previous papers was abandoned in favor of the more cumbersome chromatographic procedure described in "Experimental Procedure."

If unlabeled UDP-glucose was used as a glycosyl donor and ¹⁴C-labeled polyglycerophosphate as glucosyl acceptor, the labeled polyglycerophosphate became alkali-resistant (Fig. 1), as is the material formed from UDP-glucose-¹⁴C and unlabeled polyglycerophosphate.

The product of the reaction was isolated by phenol treatment (1) and after addition of cell wall teichoic acid carrier degraded with 60% HF, and ¹⁴C-glucosylglycerol was isolated by charcoal Celite chromatography. This material had the mobility in butanol-pyridine-water of authentic glucosylglycerol, and after treatment with α-glucosidase was quantitatively converted to ¹⁴C-glucose and ¹⁴C-glycerol (Fig. 2). The yield of ¹⁴C-glucosylglycerol varied from preparation to preparation, but was identical with the yield of glucosylglycerol from the carrier teichoic acid present in the same preparation.

Periodate oxidation of glucosylglycerol ¹⁴C labeled in the

0 5 10 15 20 25 30

FIG. 6. Effect of pH on the glucosylation of polyglycerophosphate. The reaction mixture contained 1 μmole of MgCl₂, 240 μmoles of polyglycerophosphate, 40 μmoles of UDP-glucose-¹⁴C and 10 μmoles of the following buffer: pH 5.5 to 6.5, histidine; pH 6.5 to 7.5, glycine; pH 7.5 to 9.1, Tris-acetate. v, millimicromoles of glucose transferred in 30 minutes.

0 10 20 30 40

FIG. 7. The effect of MgCl₂ on the glucosylation of polyglycerophosphate. The reaction mixtures contained 10 μmoles of Tris-chloride (pH 8.0), 240 μmoles of polyglycerophosphate, 40 μmoles of ¹⁴C-UDP-glucose, 0.03 ml of enzyme, and MgCl₂ as indicated, in a volume of 0.27 ml, and were incubated at 37° for 30 minutes. v, millimicromoles of glucose transferred.
glycerol moiety yielded no 14C-formaldehyde, showing that the glucose is linked to carbon 2 of glycerol. In one experiment, glucosylglycerol (180 c.p.m.) was oxidized with periodate and formaldehyde isolated with carrier as the dimedon derivative, as described in the previous paper. It contained less than 3 c.p.m. The enzymatically glucosylated polymer thus appears to be identical with the glucosylated teichoic acid isolated from the cell wall.

Properties of Enzyme—The proportionality of the assay to time and enzyme concentration is shown in Fig. 3. The $K_m$ for UDP-glucose is $4 \times 10^{-4} M$ (Fig. 4), and for polyglycerophosphate it is $2 \times 10^{-2} M$ in terms of glycerophosphate units, but since the chains have a minimum length of 20 units, the $K_m$ is probably no higher than $1 \times 10^{-4} M$ (Fig. 5). dTDP-glucose-14C (20) is almost as effective as UDP-glucose-14C as a glucosyl donor. The pH optimum is 8.0 (Fig. 6). L-α-Glycerophosphate will not act as a glucosyl acceptor. Vancomycin, novobiocin, and crystal violet do not inhibit the glucosyl transfer reaction.

The enzyme shows an absolute requirement for the addition of a divalent cation. The dependence of the reaction velocity on the MgCl$_2$ concentration is illustrated in Fig. 7. Ca$^{++}$ is effective as well as Mg$^{++}$; Mn$^{++}$ is much less effective than Mg$^{++}$, and when added to a reaction containing optimal Mg$^{++}$ concentration is inhibitory.

**DISCUSSION**

The extensive work of Baddiley (3) and coworkers has provided evidence for a wide variety of glycolylation patterns of teichoic acids. The simplest case has been examined in this paper, where the teichoic acid can be considered as a polymer of 2-glucosylglycerophosphate. It is at present difficult to decide whether glycosylation occurs randomly along the polyglycerophosphate chain, or whether glycosylation occurs by successive glucosylation additions along the chain. The reaction does not show significant reversibility, since we have been unable to detect the formation of UDP-glucose from 14C-glucosylpolyglycerophosphate and UDP.

It will now become of interest to examine the synthesis of more complex teichoic acids in which more than one sugar substituent is present in the teichoic acid. One such case, in which both α- and β-linked N-acetylglucosamine residues are present on ribitol teichoic acid, has been examined by Nathenson and Strominger (4). It is unclear in that case whether the α- and β-linked N-acetylglucosamine residues coexist on the same chain, or whether they form part of different chains (21).

The material isolated as "intracellular teichoic acid" from _B. subtilis_ NCTC 3610 appears to be a mixture of polyglycerophosphate and glucosylated polyglycerophosphate. Polyglycerophosphate has been isolated by McCarty (22) as one of the first teichoic acids isolated from bacteria, but the present situation appears unique in that two different glycerol teichoic acids can be isolated from two different cellular locations.

The "intracellular teichoic acid" may represent a precursor of the wall teichoic acid; however, we have isolated partially glucosylated intracellular glycerol teichoic acid from cells in the stationary phase of growth as well as the logarithmic phase of growth. It is possible that in these cells polyglycerophosphate is not only a precursor of the glucosyl polyglycerophosphate, but also has a specific function.

In a preliminary communication (5), we have previously reported the synthesis of glucosyl polyglycerophosphate by an enzyme from _B. licheniformis_ (ATCC 9945). In that system, the product after alkaline hydrolysis seems to yield mostly α-glucopyranosyl glycerol in which the glucosyl group is linked to one of the primary hydroxyl groups of glycerol. The detailed investigation of that system will be reported in detail at a later date.

**SUMMARY**

The synthesis of glucosylpolyglycerophosphate from uridine diphosphate glucose and polyglycerophosphate has been described. The reaction is catalyzed by a membrane preparation from _Bacillus subtilis_ NCTC 3610, and requires divalent cations for activity.

From _B. subtilis_ NCTC 3610, a teichoic acid associated with the cell wall has been isolated and shown to be a completely glucosylated polyglycerophosphate. The intracellular teichoic fraction in the same organism is a mixture of polyglycerophosphate and glucosylpolyglycerophosphate.

**Acknowledgment**—We are extremely grateful to Professor D. Lipkin for suggesting the use of HF to degrade teichoic acid, and providing details of an unpublished procedure to us. A culture of _Saccharomyces italicus_ Y-5 for the preparation of α-glucosidase was kindly made available by Dr. H. O. Halvorson.

**REFERENCES**
