Teichoic Acid Synthesis in *Bacillus stearothermophilus*

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1. Particulate enzyme preparations obtained from *Bacillus stearothermophilus* B65 by digestion with lysozyme were shown to catalyse teichoic acid synthesis. With CDP-glycerol as sole substrate the preparations synthesized 1,3-poly(glycerol phosphate). It was characterized by alkaline hydrolysis, by glucosylation to the alkali-stable 2-glucosyl-1,3-poly(glycerol phosphate) with excess of UDP-glucose and a *Bacillus subtilis* Marburg enzyme system, by degradation of this latter product with 60% HF and periodate oxidation of the resulting glucosylglycerol. The specificity of the *B. subtilis* system previously reported (Glaser & Burger, 1964), was confirmed in the present work. 2. Pulse-labelling experiments, followed by periodate oxidation of the product and isolation of formaldehyde from the glycerol terminus of the polymer, showed that the *B. stearothermophilus* enzyme system transferred glycerol phosphate units to the glycerol end of the chain. The transfer reaction was irreversible. It was not determined if these poly(glycerol phosphate) chains were synthesized *de novo*, but it was shown that the newly synthesized oligomers were bound to much larger molecules. 3. When the *B. stearothermophilus* enzyme system was supplied with both CDP-glycerol and UDP-glucose, 1-glucosyl-2,3-poly(glycerol phosphate) was synthesized in addition to the 1,3-isomer. The former polymer was characterized by acid and alkaline hydrolysis, degradation with HF and periodate oxidation of the resulting glucosylglycerol, and periodate oxidation of the intact polymer followed by mild acid hydrolysis. This latter procedure removed the glucose substituents without disrupting the poly(glycerol phosphate) chain. 4. The poly(glycerol phosphate) isomers were distinguished by glucosylation with the *B. subtilis* enzymes and alkaline hydrolysis, the 2,3-isomer remaining alkali-labile. The proportion of 2,3-poly(glycerol phosphate) in the product increased with increasing amounts of UDP-glucose in the incubation mixture, but the total glycerol phosphate incorporated into products remained constant. It is suggested that the synthetic pathways of the two poly(glycerol phosphate) species may share a rate-limiting step.

Glycerol teichoic acids occur widely in Gram-positive bacteria and are recognized as important cell constituents. They have been found both as membrane and wall constituents, and the structures of many individual polymers have been established (Archibald *et al.*, 1968a). Nearly all are 1,3-poly(glycerol phosphate), almost invariably substituted with ester-linked D-alanine, and also frequently with glycosyl residues. Two exceptions have been reported: the cell-wall glycerol teichoic acids from *Bacillus stearothermophilus* B65 (Wicken, 1966), and from *Actinomyces antibioticus* (Zaretskaya *et al.*, 1967, 1971) are both 2,3-poly(*sn*-glycerol 3-phosphate) with glycosidic substituents on C-1 of *sn*-glycerol. Wicken (1966) found that most of the glycerol residues in the *B. stearothermophilus* teichoic acid bore α -D-glucosyl residues and that D-alanine was esterified to some of the glucose units, whereas Zaretskaya *et al.* (1971) found O- α -D-galactopyranosyl-(1 $\rightarrow$ 4)- α -D-N-acetyl-galactosamine residues on C-1 of *sn*-glycerol in the

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A. antibioticus polymer. The latter teichoic acid also contained acetyl esters at an unidentified site.

Burger & Glaser (1964) and Glaser & Burger (1964) studied the synthesis of glucosylated 1,3-poly(glycerol phosphate) by cell-free preparations from *Bacillus subtilis* Marburg and found particulate enzymes capable of synthesizing 1,3-poly(glycerol phosphate) from CDP-glycerol, and of glucosylating this polymer with UDP-glucose as donor. Oo (1965) showed that particulate preparations obtained from *B. stearothermophilus* B65 by stirring with glass beads at high speed catalysed the net synthesis of poly(glycerol phosphate) with CDP-glycerol as sole substrate, but found the product to be 1,3-poly(glycerol phosphate) and not the expected 2,3-poly(glycerol phosphate). He characterized the poly(glycerol phosphate) by hydrolysing it with alkali and identifying bis-(glycerol 2-phosphate)-1,1'-(hydrogen phosphate) ('diglycerol triphosphate') as a degradation product. Kelemen & Baddiley (1961) have shown that this product is indicative of a 1,3-poly(glycerol phosphate) and cannot be given by

a 2,3-poly(glycerol phosphate). Oo (1965) found the poly(glycerol phosphate) synthetases from *B. stearothermophilus* and *B. subtilis* to have similar general properties.

Kennedy & Shaw (1968) used periodate oxidation of pulse-labelled polymer to show that the 1,3-poly(glycerol phosphate) synthesized by *B. subtilis* enzymes *in vitro* was extended by addition of glycerol phosphate units to the glycerol terminus of the chain, called 'tailward' elongation by Lipmann (1968). Other bacterial wall polymers investigated are extended in the same way (Hussey *et al.*, 1969; Brooks & Baddiley, 1969). Mechanisms of polymer chain growth have been discussed in general by Robbins *et al.* (1967) and Lipmann (1968). In a poly(glycerol phosphate) one end of the molecule should terminate in a glycerol residue, whereas the other should be a phosphomonoester or a linkage to other cell components. Production of formaldehyde from the terminal glycerol has been used to estimate chain lengths of poly(glycerol phosphate) (Burger & Glaser, 1964; Ghuysen *et al.*, 1965), and phosphomonoesterase is known to remove the terminal phosphate from the other end of the chain (Ellwood *et al.*, 1963; Ghuysen *et al.*, 1965).

The present work was undertaken to investigate further the enzymes of teichoic acid synthesis in *B. stearothermophilus*, and the products synthesized. We show here that 1,3-poly(glycerol phosphate) was synthesized from CDP-glycerol by tailward growth and that 1-glucosyl-2,3-poly(glycerol phosphate) was also synthesized when both CDP-glycerol and UDP-glucose were present.

Experimental

Materials

B. stearothermophilus B65 (Forrester & Wicken, 1966) was grown from a culture supplied by Dr. A. J. Wicken to Dr. K. C. Oo. *B. subtilis* Marburg (A.T.C.C. 6051) was obtained from the American Type Culture Collection, Rockville, Md., U.S.A. NaB³H₄ was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. [1,3-¹⁴C]Glycerol and UDP-[U-¹⁴C]glucose were supplied by Calbiochem, San Diego, Calif., U.S.A., and the International Chemical and Nuclear Corp., Irvine, Calif., U.S.A., respectively. [³²P]P_i was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-[U-¹⁴C]Glucose was diluted with unlabelled UDP-glucose. The UDP-glucose content of the mixture was determined by enzymic assay, and the proportion of radioactivity present as UDP-glucose was determined chromatographically. About 10% of the label was glucose phosphate. [³H]- and [¹⁴C]-Toluene (for standardizing liquid-scintillation counters) were obtained from Packard Instrument

Company Inc., Downers Grove, Ill., U.S.A.; [³H]- and [¹⁴C]-hexadecane (for the same purpose) were from The Radiochemical Centre. Ion-exchange resins were supplied by Bio-Rad Laboratories, Richmond, Calif., U.S.A., Sephadex was from Pharmacia AB, Uppsala, Sweden, and Norit A was from Pfanstiehl Laboratories Inc., Waukegan, Ill., U.S.A. DL-Glycerol 3-phosphate and UDP-glucose were from Sigma Chemical Co., St. Louis, Mo., U.S.A. and other nucleotides were from Calbiochem and Sigma. DL-Glyceraldehyde was purchased from British Drug Houses, Poole, Dorset, U.K., and mannitol ('Micro-Analytical Standard' grade) and 60% (w/w) hydrofluoric acid (technical grade) were from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Glucose 6-phosphate dehydrogenase (suspension), hexokinase (crystal suspension) and glycerol kinase (crystal suspension) were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; UDP-glucose dehydrogenase (type III), glucose oxidase (type II), peroxidase (type I), deoxyribonuclease (purified powder), lysozyme (crystalline freeze-dried), and α -glucosidase (type I) were from Sigma; ribonuclease (crystalline) and *Escherichia coli* alkaline phosphatase (chromatographically purified) were from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; β -glucosidase from almonds (purified powder) was a gift from Dr. R. W. Bailey.

Other chemicals were reagent or analytical grade.

Preparation of substrates

[1-³H]Glycerol was synthesized by NaB³H₄ reduction of DL-glyceraldehyde. Periodate oxidation of this glycerol (see below) released all the ³H as formaldehyde. L-Glycerol 3-phosphate was synthesized from glycerol with ATP and glycerol kinase (Bublitz & Kennedy, 1954) and purified by adsorption on ion-exchange resin (AG 1-X8, HCO₃⁻ form) and elution with 0.05 M-NH₄HCO₃. For glycerol [³²P]-phosphate, [γ -³²P]ATP (Avon, 1961) was used and the product was treated with charcoal. Paper chromatography of glycerol phosphate samples showed that each was free of labelled impurities. CDP-glycerol was synthesized chemically by the general method of Roseman *et al.* (1961), or enzymically with CDP-glycerol pyrophosphorylase (Shaw, 1962). In each case it was purified by chromatography on Whatman 3MM paper in solvents A and B, defined below. CDP-[³H]glycerol, CDP-[¹⁴C]glycerol and [β -³²P]CDP-glycerol were also prepared. Each was shown to be free of labelled impurities and on acid hydrolysis gave glycerol phosphate as the only labelled product. DL-Glycerol 3-phosphate was used in the synthesis of unlabelled CDP-glycerol, and concentrations were calculated as the L-glycerol 3-phosphate isomer.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951), and phosphate by the method of Chen *et al.* (1956). ATP was assayed with hexokinase and glucose 6-phosphate dehydrogenase (Lamprecht & Trautschold, 1963), and UDP-glucose with UDP-glucose dehydrogenase (Mills & Smith, 1963).

Radioactivity measurements. For single radioisotope samples where absolute radioactivities were not required, samples were dried on Whatman no. 1 paper and counted in 10ml of toluene scintillation fluid [toluene, 1 litre; 2,5-diphenyloxazole (PPO), 4g; 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), 50mg] in a Packard model 3214 liquid-scintillation counter. Other samples were mixed with 10ml of dioxan scintillation fluid (Bray, 1960) and counted to at least 5000 counts. ^3H and ^{32}P radioactivities were corrected for radioactive decay. The counting efficiency of representative samples was checked by internal standardization.

Paper chromatography. Whatman no. 1 paper was used except where otherwise stated. Whatman 3MM paper was washed with 2M-acetic acid, and then with water before use. Solvents used (all descending) were: A, ethanol-1M-ammonium acetate, pH 7.5 (5:2, v/v) (Roseman *et al.*, 1961; Burger & Glaser, 1964); B, ethanol-0.5M-ammonium acetate, pH 3.8 (5:2, v/v) (Roseman *et al.*, 1961); C, propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) (Shaw & Baddiley, 1964; Burger & Glaser, 1964; Archibald *et al.*, 1968b); D, ethyl acetate-pyridine-water (7:2:1, by vol.); E, phenol (saturated with water at room temperature); F, butan-1-ol-pyridine-water (6:4:3, by vol.) (Brundish & Baddiley, 1968).

Unlabelled materials were detected by the following methods: periodic acid-benzidine for polyols (Gordon *et al.*, 1956); ferric chloride-sulphosalicylic acid for phosphate esters (Runeckles & Krotkov, 1957); inspection under u.v. light for nucleotides. Radioactive compounds were located with a Packard model 7201 radiochromatogram scanner. When a compound was required for further study the appropriate area was cut out, sewn to a paper wick, and eluted with water.

Identification of labelled compounds from paper chromatograms. In most cases the identity of the compounds (as suggested by their R_f values) was checked by chromatographic identification of the products found after enzymic treatment. The following enzymes and reaction mixtures (normally 0.5ml total) were used: phosphatase, 10 $\mu\text{g}/\text{ml}$ in 2mM-Tris-HCl buffer, pH 8.0; glucose oxidase, 0.2mg/ml in 2mM-sodium acetate buffer, pH 5.5, containing 20 μg of peroxidase/ml and 2 μg of *o*-dianisidine/ml; glycerol kinase, 2 $\mu\text{g}/\text{ml}$ in 2mM- Na_2CO_3 buffer, pH 9.8, containing 1mM- MgCl_2

and 1mM-ATP; α -glucosidase, 0.2mg/ml in 2mM-sodium phosphate buffer, pH 6.8; β -glucosidase, 0.2mg/ml in 10mM-sodium acetate buffer, pH 4.7. Each of the above reactions was found to be essentially complete after 2h at 37°C when tested with 0.25 μmol of appropriate substrate. α - and β -Glucosidases were shown not to hydrolyse cellobiose and methyl α -D-glucopyranoside respectively. Acid and alkaline hydrolysis were also used. After the above treatments, samples were concentrated to 100–50 μl by an air jet directed on to the surface of the liquid, and were spotted directly on to chromatography paper. The overall recovery was about 80% for each such step.

Two specific examples of this technique are described. Material with the mobility of diglycerol triphosphate was treated with phosphatase and gave two products, diglycerol phosphate and glycerol. The latter compound gave glycerol phosphate after treatment with glycerol kinase, and acid hydrolysis of the former gave equal amounts of glycerol and glycerol phosphate. (The glycerol liberated by phosphatase treatment was from glycerol diphosphate, which co-chromatographs with diglycerol triphosphate in the solvent used initially.) Glucosylglycerol phosphate yielded glucosylglycerol after phosphatase treatment, and this product was hydrolysed to glucose and glycerol by acid or α -glucosidase. The glucose gave gluconic acid after treatment with glucose oxidase.

Chemical degradation methods. For acid and alkaline hydrolyses the dried sample was dissolved in 0.2ml of 1M-HCl or 1M-KOH and heated at 100–105°C for 3h in a sealed tube. Acid hydrolysates were dried, and HCl was removed by several additions and evaporations of water. Alkaline hydrolysates were carefully neutralized with HClO_4 , and the supernatant fluid was dried. Hydrofluoric acid was used as described by Glaser & Burger (1964). Glucosylglycerol thus produced was purified by paper chromatography in solvents C, D and E. Acid hydrolysis of each preparation of glucosylglycerol gave glucose and glycerol only. Glucose was characterized by oxidation with glucose oxidase.

Samples were oxidized with periodic acid and formaldehyde was isolated as the dimedone derivative as described by Kennedy & Shaw (1968). Mannitol was added to supply carrier formaldehyde and oxidation proceeded for 4h (glucosylglycerol) or 12h [poly(glycerol phosphate)] at room temperature in the dark. The dimedone derivative was recrystallized to constant specific radioactivity from aq. 50% (v/v) ethanol, dried, weighed and counted for radioactivity. Recovery was usually 85–90%. Sufficient carrier was added to bring all the samples from one experiment to a standard weight so that counting efficiencies would be the same. Radioactivity measurements were corrected for losses of carrier formaldehyde.

Culture of micro-organisms

B. stearothermophilus was grown in medium containing: trypticase (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.), 10g; yeast extract powder (Oxoid Ltd., London E.C.4, U.K.), 2.5g; glucose, 10g [autoclaved separately as a 10% (w/w) soln.]; NaCl, 10g; 0.5M-potassium phosphate buffer, pH 7.4, 100ml (autoclaved separately); salts [4% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04% FeCl_3], 5ml; and water to 1 litre. Cells were grown in 10-litre batches at 55°C in a New Brunswick Microferm laboratory fermenter (stirring rate 400 rev./min, aeration rate 1 litre of air/litre of culture per min) until near the end of exponential-phase growth. A 5% inoculum of late-exponential-phase cells grown in the same medium was used. The culture was cooled by the addition of ice and the cells were harvested with a Sharples continuous-flow centrifuge. Cells were washed twice with 0.1M-Tris-HCl buffer (pH 7.5) and frozen. *B. subtilis* Marburg was grown and harvested in the same way, except that NaCl was omitted from the medium, growth was at 30°C, and the cells were washed with water. Both bacterial cultures were maintained on nutrient agar (Difco Laboratories, Detroit, Mich., U.S.A.) slopes.

Enzyme preparation

Cells of both species were disrupted with lysozyme. Approximately 10g (wet wt.) of cells was suspended in 90ml of 0.1M-Tris-HCl buffer, pH 7.5, containing: magnesium acetate (10mM), lysozyme (60mg) (100mg for *B. subtilis*), ribonuclease (5mg) and deoxyribonuclease (5mg). Lysis was generally found to be almost complete after 2h at 30°C, and intact cells remaining were removed by centrifugation (5000g, 5min). Membranes were collected (20000g, 30min), washed twice with 0.1M-Tris-HCl buffer, pH 7.5, suspended in 0.1M-Tris-HCl buffer, pH 8.0 (about 10mg of protein/ml) and frozen. The supernatant fraction from *B. stearothermophilus* was used as crude CDP-glycerol pyrophosphorylase for CDP-glycerol synthesis.

Enzyme assays

Incubation mixtures contained, in 0.35ml: 20 μmol of Tris-HCl buffer, pH 8.0 (57mM), 10 μmol of MgCl_2 (29mM), 25nmol of substrate (70 μM) and about 1mg of enzyme protein. After incubation (at 30°C unless otherwise stated) the reaction was stopped by addition of HClO_4 (0.25M final concn.) and the mixture was centrifuged (10000g, 10min). This removed all newly synthesized polymeric material from the supernatant. The precipitate was washed three times with 0.6ml of 0.25M- HClO_4 , once with

0.6ml of water, and was dissolved in 0.6ml of aq. 1% (v/v) NH_3 for radioactivity counting in dioxan scintillation fluid. Control incubation mixtures contained enzyme that had been boiled for 5min or, in some cases, to which HClO_4 had been added at zero time.

Product purification

Products were usually isolated by phenol extraction (Burger & Glaser, 1964). The reaction was stopped by the addition of an equal volume of 80% (w/w) phenol. After 40min at 0°C with intermittent stirring, the mixture was centrifuged (10000g, 10min), the aqueous layer was removed, and the phenol layer washed with an equal volume of water. The combined aqueous layers were extracted with an equal volume of chloroform and the chloroform was discarded. The aqueous solution was dialysed for 24h against three changes of 1000vol. of water. No appreciable radioactivity was found in the (discarded) phenol or chloroform layers when *B. stearothermophilus* enzymes were used. *B. subtilis* membranes synthesized some lipid from CDP-glycerol (Oo, 1965). In some experiments material was also chromatographed on Sephadex. In all cases, products were eluted with 0.05M- NH_4HCO_3 , 3ml fractions were collected, and 50 μl samples were counted for radioactivity in dioxan scintillation fluid.

Results

Confirmation of the properties of B. subtilis enzymes

Burger & Glaser (1964) and Glaser & Burger (1964) showed that membrane preparations from *B. subtilis* Marburg synthesized 1,3-poly(glycerol phosphate) from CDP-glycerol, and glucosylated this polymer by using UDP-glucose as donor. These findings were confirmed in the present work with the culture of *B. subtilis* Marburg available in this laboratory. At least 95% of the product obtained after glucosylation of 1,3-poly(glycerol phosphate) was resistant to alkaline hydrolysis when a 10-fold excess of UDP-glucose and the enzyme preparation and assay described above were used.

Structures of the products synthesized by B. stearothermophilus enzymes

Material synthesized from CDP-glycerol and purified by phenol extraction as described above remained at the origin after paper chromatography in solvents A, B and C, and was excluded from Sephadex G-200. Alkaline hydrolysis of material synthesized from CDP-[^3H]glycerol gave the following labelled products: glycerol phosphate (55% of the label recovered), glycerol (22%), glycerol

Table 1. Glucosylation of poly(glycerol phosphate) samples by *B. subtilis* membranes

B. subtilis membrane samples (1.2mg of protein) were incubated in 0.35ml of assay mixture (see the Experimental section) with 0.5 μ mol of UDP-glucose. After 2h at 30°C each reaction mixture was diluted to 2ml with ice-cold 50mM-Tris-HCl buffer, pH8.0. Membranes were recovered by centrifugation (10000g, 10min), washed once with 2ml of the same buffer and resuspended in 0.35ml of assay mixture with UDP-[¹⁴C]glucose (25nmol) and poly([³H]glycerol phosphate) (20nmol of glycerol) synthesized with either *B. stearothermophilus* or *B. subtilis* membranes. After 2h 0.5ml of pyridine was added and reaction mixtures were chromatographed in solvent A. Polymeric material was eluted and counted for radioactivity. Poly(glycerol phosphate) was omitted from tube 3.

Tube	Source of poly(glycerol phosphate)	Glycerol (nmol)	Glucose (nmol)
1	<i>B. stearothermophilus</i>	5.9	3.4
2	<i>B. subtilis</i>	5.5	2.1
3	—	—	0.3

diphosphate (16%), and diglycerol triphosphate (6.8%). When [³²P]CDP-glycerol was used as substrate alkaline hydrolysis gave glycerol phosphate and glycerol diphosphate as the major labelled products. (These compounds were identified by paper chromatography as described in the Experimental section.) The presence of diglycerol triphosphate in alkaline hydrolysates showed that the polymer was 1,3-poly(glycerol phosphate) (Kelemen & Baddiley, 1961).

As an independent method of characterizing this poly(glycerol phosphate), it was treated with UDP-glucose and the enzyme preparation from *B. subtilis*. The *B. stearothermophilus* poly(glycerol phosphate) was at least as good a substrate for the *B. subtilis* glucosylation system as *B. subtilis* poly(glycerol phosphate) (Table 1). The glucosylated product was completely stable to alkali. Degradation of this glucosylated polymer with hydrofluoric acid gave a glucosylglycerol, which was shown to be 2-*O*-D-glucosylglycerol by periodate oxidation, which produced no formaldehyde from the glycerol unless the glucoside was first hydrolysed. As the glucosylated product was completely stable to alkaline hydrolysis it was concluded that the poly(glycerol phosphate) was entirely 1,3-linked. The glucosylated poly(glycerol phosphate) shown in Table 1 was measured by addition of pyridine and by chromatography (Glaser & Burger, 1964), as it had been found that previously isolated poly(glycerol phosphate) no longer co-precipitated completely with protein. Although this procedure gave low recoveries of poly(glycerol phosphate) it was simpler to use than alternative procedures (Glaser & Burger, 1964).

When UDP-glucose and CDP-glycerol were both present in the assay mixture, glucose and glycerol phosphate were both incorporated into material which co-precipitated with protein. This material appeared to be polymeric, as it was non-diffusible on dialysis, was excluded from Sephadex G-200 and remained at the origin after paper chromatography in solvents A, B and C. The polymer could be isolated by phenol extraction in the same way as poly(glycerol phosphate). The ratio of glucose to glycerol was variable, the glucose content generally being 60–90% of the glycerol content. Paper chromatography of acid hydrolysates showed degradation products typical of a glucose-containing teichoic acid. Compounds with the mobilities of glycerol diphosphate, glycerol phosphate, glucose and glycerol were observed. No disaccharides were found after partial acid hydrolysis. Alkaline hydrolysis degraded the polymer to glycerol diphosphate, glucosylglycerol phosphate, glycerol phosphate, glucosylglycerol and glycerol. Glucosylglycerol phosphate accounted for about 70% of the radioactivity from glucosylpoly([³H]glycerol phosphate). No other compounds were observed.

The structure of the glucosylglycerol subunit was determined after degradation of a sample of polymer (synthesized from CDP-[³H]glycerol and UDP-[¹⁴C]glucose) with hydrofluoric acid. The glucoside was purified by paper chromatography in solvents C, D, E and F. No diglucosylglycerol was observed in the last solvent. The glucosylglycerol was recovered unchanged after incubation with β -glucosidase but was hydrolysed by α -glucosidase. Acid hydrolysis of the glucoside before periodate oxidation gave yields of [³H]- and [¹⁴C]-formaldehyde close to those expected (94% and 13% respectively). Approximately half (47%) of the radioactivity from the glycerol was found as formaldehyde after periodate oxidation of the intact glucoside, but no formaldehyde derived from the glucose was detected. This showed it to be 1-*O*- α -D-glucopyranosylglycerol, since in 2-glucosylglycerol the glycerol would have been protected from oxidation, and a glucofuranose unit would have yielded [¹⁴C]formaldehyde. This subunit could be derived from glucosylated 2,3-poly(glycerol phosphate) or from poly(glycerol phosphate glucose). The hydrolysis products found are consistent with the former structure, but would also be given by a mixture of the latter with some 1,3-poly(glycerol phosphate). In any case, the variable glucose:glycerol ratio found is not consistent with poly(glycerol phosphate glucose) as the sole product.

Proof of the polymer structure was obtained from further periodate oxidation studies. Burger & Glaser (1966) used periodate oxidation of intact poly(glycerol phosphate glucose) to show that the glucose and glycerol were linked through a phosphodiester as well as by glucosidic bonds. Oxidation, followed

Fig. 1. Sephadex chromatography of oxidized *B. stearothermophilus* polymer

Purified *B. stearothermophilus* polymer was oxidized with periodate and reduced with borohydride as described in the text. The product was hydrolysed with acid and chromatographed on Sephadex G-75 as described. Specific radioactivities of the substrates used were: CDP-[^3H]glycerol, 35 d.p.m./pmol; UDP-[^{14}C]glucose, 2.2 d.p.m./pmol. The data shown are d.p.m./50 μl of sample counted. ^3H , —; ^{14}C , ----.

by borohydride reduction and acid hydrolysis, gave glycerol phosphate containing C-4, C-5 and C-6 from glucose. This procedure was repeated on a sample of polymer synthesized from CDP-[^3H]glycerol and UDP-[^{14}C]glucose and purified by phenol extraction and chromatography on Sephadex G-200. Glycerol phosphate was purified by paper chromatography and, after phosphatase treatment and addition of carrier glycerol, the glycerol was isolated and counted for radioactivity as glycerol tribenzoate. It contained no ^{14}C . Chromatography of acid hydrolysates showed that the glucose was almost completely oxidized, so linkage of phosphate to C-6 of glucose was excluded. The finding that periodate oxidized the glucose suggested a further approach. Cleavage of the glucose ring and reduction of the resulting dialdehyde would give the extremely acid-labile (Smith & Van Cleve, 1955) acetal of glyceraldehyde (or glyceraldehyde if C-2 was substituted). Hydrolysis of this acetal would remove all glucose carbon atoms from a glucosyl-poly-(glycerol phosphate) without degrading the poly-(glycerol phosphate), but would degrade a poly(glycerol phosphate glucose) to low-molecular-weight fragments. Polymer prepared from CDP-[^3H]glycerol and UDP-[^{14}C]glucose was isolated

by phenol extraction and dissolved in 1 ml of 0.4 M-sodium acetate buffer, pH 4.7, containing 0.06 M-NaIO₄. After 22 h at room temperature in the dark the mixture was chromatographed on Sephadex G-75. Excluded material was dried and reduced with NaBH₄ (5 mg in 1 ml of water) for 30 min at room temperature. Formalin (1 drop) was then added and the reaction mixture was dried. The residue was dissolved in 0.2 ml of 1 M-HCl, incubated for 1 h at 30°C, neutralized with NaOH, and chromatographed on Sephadex G-75. Fig. 1 shows that this procedure removed the glucose carbons from the high-molecular-weight fraction while leaving most of the poly(glycerol phosphate) intact. The excluded material contained 78% of the ^3H and 10% of the ^{14}C eluted. The overall recovery of ^3H was 84%. This reaction sequence shows that the glucose units are not part of the polymer backbone and as the subunit had been found to be 1-glucosylglycerol, the polymer must be a 2,3-poly(glycerol phosphate).

Incubation of *B. stearothermophilus* enzyme with UDP-glucose alone resulted in relatively little incorporation of glucose. Labelled polymeric material could be isolated by phenol extraction and degraded by alkali; glucosylglycerol phosphate, glucosylglycerol and glucose were the major products. The glucosylglycerol had the mobility of 1-glucosylglycerol in solvent D. These results are consistent with some glucosylation of endogenous 2,3-poly-(glycerol phosphate), although the glucose formed on alkaline hydrolysis must have come from a different polymer.

Properties of the enzymes from *B. stearothermophilus*

Preliminary experiments showed that the pH and cation-concentration optima (pH 8.0 and 30 mM for MgCl₂) for poly(glycerol phosphate) synthesis are similar to those found by Oo (1965).

Use of UDP-glucose as sole substrate gave relatively little polymeric material, but glucose incorporation was greatly increased by the simultaneous presence of CDP-glycerol. Prior incubation with CDP-glycerol did not replace this requirement. The presence of UDP-glucose caused only a slight increase in glycerol incorporation (Table 2). These properties were reproducible, and were found with all enzyme preparations. The enzymes which incorporated glycerol phosphate and glucose were stable for months at -20°C.

Table 3 shows that in both the presence and the absence of UDP-glucose, glycerol and phosphate were transferred from CDP-glycerol.

The time-course of incorporation of glycerol plus glucose closely followed that of glycerol alone and was initially rapid (Fig. 2). The sharp decreases in the rates of polymer synthesis after about 10 min are probably due to the exhaustion of substrate, as

CDP-glycerol breaks down under the assay conditions (Shaw, 1962; Glaser, 1965). Experiments in which the enzyme was incubated with substrate, recovered, and incubated with fresh substrate showed that the enzyme remained active.

As the simultaneous presence of CDP-glycerol and UDP-glucose led to the synthesis of 1-glucosyl-2,3-poly(glycerol phosphate), whereas CDP-glycerol alone gave 1,3-poly(glycerol phosphate), the products formed with different proportions of the two substrates were investigated. Table 4 shows that increasing amounts of UDP-glucose in the reaction mixture led to increasing amounts of glucose in the product, without affecting the amount of glycerol incorporated. The proportion of 1,3-poly(glycerol phosphate) in the product was measured by enzymic glucosylation with *B. subtilis* membranes, followed by alkaline hydrolysis. As noted above, when excess of UDP-glucose was used *B. subtilis* membranes would glucosylate 1,3-poly(glycerol phosphate) to

give a product stable to alkali. Table 5 shows that the proportion of 1,3-poly(glycerol phosphate) in the product decreased with increasing UDP-glucose concentration in the reaction mixture. In a separate experiment with CDP-[³H]glycerol and UDP-[¹⁴C]glucose as substrates, material stable to alkali (after enzymic glucosylation with unlabelled UDP-glucose) was shown to be essentially free of [¹⁴C]-glucose (³H:¹⁴C = 500:1).

It was found that 1,3-poly(glycerol phosphate) was not glucosylated with UDP-glucose by *B. stearothermophilus* membranes.

The 2,3-poly(glycerol phosphate) prepared by periodate oxidation and mild acid hydrolysis of glucosyl-2,3-poly(glycerol phosphate) (see Fig. 1) was not glucosylated by either *B. stearothermophilus* or *B. subtilis* membranes.

No butanol-extractable labelled material was detected in reaction mixtures at times up to 20 min.

Direction of poly(glycerol phosphate) chain extension by B. stearothermophilus

The data in Table 6 show that when *B. stearothermophilus* membranes were incubated with CDP-[³H]glycerol a large proportion of the label incorporated was present in the terminal glycerol groups (tube 1), but this proportion was substantially decreased when the same membranes were subsequently incubated with unlabelled substrate (tube 2). As half of the label of each terminal [1,3-³H]glycerol would be recovered as formaldehyde, the two samples contained 30.4 and 20.8% of the labelled glycerol in their glycerol termini respectively, showing that one out of every three labelled glycerol moieties previously susceptible to oxidation had been blocked during the incubation with unlabelled CDP-glycerol. The proportion of labelled formaldehyde produced from the oxidation of the product in tube 1 corresponds to an average chain length of 3.3 glycerol

Table 2. Requirement for the simultaneous presence of CDP-glycerol and UDP-glucose

B. stearothermophilus membranes (0.7 mg of protein/tube) were incubated with 25 nmol of substrate in 0.35 ml of assay mixture for 30 min, sedimented (25000g, 30 min), washed twice with 1 ml of 50 mM-Tris-HCl buffer (pH 8) and resuspended in 0.35 ml of assay mixture with substrate (25 nmol) where indicated. After 30 min reactions were stopped with HClO₄ (see the Experimental section).

Tube	First substrate	Second substrate	Product	
			Glycerol (nmol)	Glucose (nmol)
1	UDP-glucose	—	—	0.08
2	—	UDP-glucose	—	0.04
3	CDP-glycerol	UDP-glucose	3.0	0.07
4	CDP-glycerol + UDP-glucose	—	3.3	1.9

Table 3. Incorporation of glycerol and phosphate from CDP-glycerol

B. stearothermophilus membranes (1.7 mg of protein) were incubated in 0.35 ml with 25 nmol of substrate(s) as indicated. Reactions were stopped with HClO₄ after 30 min. Enzyme in tubes 3, 7 and 8 was boiled.

Tube	Substrate	Products		
		³² P-labelled (nmol)	³ H-labelled (nmol)	¹⁴ C-labelled (nmol)
1	CDP-[³ H]glycerol	—	10.90	—
2	[³² P]CDP-glycerol	11.30	—	—
3	[³² P]CDP-glycerol	0.01	—	—
4	UDP-[¹⁴ C]glucose	—	—	0.20
5	CDP-[³ H]glycerol + UDP-[¹⁴ C]glucose	—	11.40	9.40
6	[³² P]CDP-glycerol + UDP-glucose	10.60	—	—
7	CDP-[³ H]glycerol + UDP-[¹⁴ C]glucose	—	0.01	0.03
8	[³² P]CDP-glycerol + UDP-glucose	0.01	—	—

Fig. 2. Rate of polymer synthesis

B. stearothermophilus membranes (1.7mg of protein) were incubated with 25 nmol of substrate(s) in 0.35 ml of assay mixture. For each point a separate incubation was stopped with HClO_4 at the time indicated, and the product determined as described in the text. ● and ○, substrate CDP-[^3H]glycerol (glycerol incorporated); ■, substrate UDP-[^{14}C]glucose (glucose incorporated); ▲ and △, substrate CDP-[^3H]glycerol + UDP-[^{14}C]glucose (glucose and glycerol incorporated respectively).

Table 4. Glycerol:glucose ratio in the product

B. stearothermophilus membranes (1.7 mg of protein) were incubated in 0.35 ml with 25 nmol of CDP-[^3H]glycerol, and UDP-glucose as indicated. Reactions were stopped with HClO_4 after 30 min.

Tube	UDP-[^{14}C]glucose (nmol)	Products	
		Glycerol (nmol)	Glucose (nmol)
1	—	21.4	—
2	5	21.3	1.9
3	12.5	20.9	6.5
4	25	21.3	14.7
5	50	20.4	26.7

phosphate units. The results from tube 3 show that the enzyme remained active in the second incubation. During this second (longer) incubation the average length of chains synthesized was 4.8 units.

Neither the incubation with unlabelled substrate nor the washing procedure used caused loss of label from the poly(glycerol phosphate) and negligible substrate remained after the washing procedure. The former result suggests that the reaction is irreversible, as CMP [from the non-enzymic breakdown of CDP-glycerol (Shaw, 1962)] would have been present to allow exchange of [^3H]glycerol phosphate with the unlabelled substrate.

Periodate oxidation of poly(glycerol phosphate) was essentially complete in 2 h, and the yield of formaldehyde remained constant thereafter for at least 24 h.

Table 5. Proportion of 1,3-poly(glycerol phosphate) in the product

B. stearothermophilus membranes (1.3 mg of protein) were incubated in 0.35 ml with 25 nmol of CDP-[^3H]glycerol, and UDP-glucose as indicated. After 30 min products were isolated by phenol extraction, glucosylated with *B. subtilis* membranes (1.2 mg of protein) and UDP-glucose (0.5 μmol), and reisolated with phenol. A sample of each was counted for radioactivity, and the remainder was hydrolysed with alkali and chromatographed in solvent A. Radioactive areas were located with a radiochromatogram scanner, peak areas were measured, and the areas corresponding to alkali-stable and alkali-labile compounds were compared.

Tube	UDP-glucose (nmol)	Product (nmol) of glycerol	Area of alkali-stable compound (%)
1	10	3.6	86
2	25	4.1	50
3	50	2.6	43

Table 6. Direction of *B. stearothermophilus* poly(glycerol phosphate) chain extension

B. stearothermophilus membranes (1.25 mg of protein) were incubated in 0.35 ml of reaction mixture (see the Experimental section) with the first substrate shown. After 10 min the reaction mixture was diluted to 2 ml with ice-cold 0.05 M-Tris-HCl buffer, pH 8. Membranes were recovered by centrifugation (15000g, 10 min), washed once with 2 ml of the same buffer, and resuspended in 0.35 ml of reaction mixture with the second substrate. After a further 30 min incubation membranes were again washed as above, and poly(glycerol phosphate) was isolated by phenol extraction. Samples were taken for radioactivity counting and periodate oxidation (see the Experimental section). Treatment with phosphatase (where indicated) was performed immediately before periodate oxidation. 'Product' is the amount of material (as glycerol) in each sample for oxidation. The reaction in tube 1 was stopped (by addition of phenol) at zero time in the second incubation.

Tube	Substrate		Product (nmol)	Phosphatase	% of ^3H as HCHO
	First	Second			
1	CDP-[^3H]glycerol	—	0.63	—	15.2
				+	18.0
2	CDP-[^3H]glycerol	CDP-glycerol	0.61	—	10.4
				+	11.8
3	CDP-glycerol	CDP-[^3H]glycerol	1.61	—	10.4
				+	10.1

This periodate oxidation method was also used to determine the chain length of labelled material synthesized during longer incubations. In a typical experiment *B. stearothermophilus* enzyme was incubated with CDP-[^3H]glycerol for 2 h (see the Experimental section). Products were isolated by phenol extraction and chromatographed on Sephadex G-75. Excluded material was pooled and concentrated, and duplicate samples were oxidized. An average chain length of 4.8 glycerol phosphate units was deduced from the ^3H recovered as formaldehyde (10.5%). This enzyme sample was a different preparation from that used in Table 6 and is clearly less capable of synthesizing long chains. The product was excluded from Sephadex G-75, which is clear evidence that the labelled poly(glycerol phosphate) was bound to a much larger molecule.

Discussion

Enzyme preparations which were capable of synthesizing teichoic acid were presumed to include endogenous glycerol phosphate-containing polymers, and although the use of radioactive substrates ensured that the material synthesized *in vitro* could be distinguished from that already present, the possible presence of the latter caused some problems. Although the specific radioactivities of the substrates were known, these were diluted to undetermined extents, e.g. by glucosylation *in vitro* of endogenous (unlabelled) glycerol in addition to glucosylation of nascent (labelled) poly(glycerol phosphate). This effective dilution of the labelled glycerol would give glucosylglycerol with an apparent excess of glucose. To minimize interference by endogenous materials all methods used depended on the detection of radioactivity in the products that were synthesized.

Both *B. subtilis* and *B. stearothermophilus* enzyme preparations were isolated by the method of Glaser & Burger (1964), and are probably largely cytoplasmic membrane material. As expected, the *B. subtilis* preparation had essentially the same properties as previously reported (Burger & Glaser, 1964; Glaser & Burger, 1964) and was suitable as a test system to identify 1,3-poly(glycerol phosphate). It was shown that 2,3-poly(glycerol phosphate) was not a substrate for glucosylation.

The previous identification (Oo, 1965) of the polymer synthesized by *B. stearothermophilus* enzymes from CDP-glycerol as 1,3-poly(glycerol phosphate) was confirmed, and its identity was further shown by glucosylation to 2-glucosyl-1,3-poly(glycerol phosphate) by *B. subtilis* enzymes.

The product synthesized by *B. stearothermophilus* from CDP-glycerol plus UDP-glucose had properties typical of a glucose-containing teichoic acid, although

the pattern of alkaline hydrolysis products was unusual. Periodate oxidation and enzymic hydrolysis of the glucosylglycerol subunit showed it to be 1-glucosylglycerol. It is known that during degradation of teichoic acids by hydrofluoric acid considerable amounts of glucosylglycerol are lost by adsorption on to lithium fluoride (Glaser & Burger, 1964), and although such compounds as diglucosylglycerol were not detected, small amounts may have been present in the polymer. Repetition of the procedure used by Burger & Glaser (1966) showed that it was not a poly(glycerol phosphate glucose) with 1-6 phosphodiester bonds, and the reaction sequence described established that the glucosyl residues were not part of the polymer backbone, as they could be separated from it. The alkaline hydrolysis products obtained are readily explained by this structure, with glycerol diphosphate coming from some unsubstituted glycerols, and also from the 1,3-poly(glycerol phosphate) present (see Table 5). Although some diglycerol triphosphate should have been formed from this latter polymer, there was presumably insufficient to be detected. This teichoic acid thus has the same structure as that isolated by Wicken (1966) from the walls of the organism. Very little polymeric material was synthesized from UDP-glucose as sole substrate. Products isolated are consistent with some glucosylation of endogenous 2,3-poly(glycerol phosphate), although when this was added it was glucosylated very poorly, if at all.

The *B. stearothermophilus* enzyme system used in the present work appears to have similar properties to other systems capable of synthesizing bacterial-wall polymers. The system is particulate, appears to be derived from the cytoplasmic membrane, and requires a relatively high Mg^{2+} concentration.

Since poly(glycerol phosphate) synthesis was first demonstrated (Burger & Glaser, 1964) it has been assumed that the glycerol phosphate moiety is transferred as a unit in a manner similar to that shown for poly(ribitol phosphate) (Ishimoto & Strominger, 1966). This mechanism has now been directly demonstrated for both polymers studied in the present work by use of [^{32}P]CDP-glycerol.

The decrease in label in the terminal glycerol after incubation with unlabelled substrate indicates that glycerol phosphate units were added at this end of the chain rather than at the phosphate end. If existing chains were extended by addition of units to the phosphate terminus no labelled formaldehyde would be produced by periodate oxidation. Chain synthesis *de novo* in this fashion would give a product yielding labelled formaldehyde from the first glycerol phosphate unit if labelled substrate were present in either incubation, but subsequent addition of unlabelled units would have no effect on the proportion of label recovered as formaldehyde. The data show that this mechanism does not operate.

Yields of formaldehyde after phosphatase treatment were variable, and sometimes less than the yield in the absence of such treatment. This suggests either that chain extension alone was occurring with no initiation of new chains, or that chains were bound through their phosphate termini to some acceptor. Although the phosphatase data are not conclusive it seems likely that the chains synthesized in the present work, whether synthesized *de novo* or not, are linked to some acceptor. Addition of newly synthesized teichoic acid to an acceptor rather than synthesis of free polymer appears to be a general property of cell-free systems (Burger & Glaser, 1964; Glaser, 1964; Burger & Glaser, 1966; Ishimoto & Strominger, 1966; Baddiley *et al.*, 1968). This acceptor would not be a polyprenol phosphate, though such a compound may be an intermediate carrier (Anderson *et al.*, 1972).

The simultaneous synthesis of a poly(glycerol phosphate) and a glucose-containing polymer by the same enzyme preparation is not unusual, but the observation that the two should be interdependent to the extent that the amount of glycerol incorporated remained the same, irrespective of glucose incorporation, was not expected. The requirement that CDP-glycerol and UDP-glucose be present together for glucose to be incorporated is explained by 1,3-poly(glycerol phosphate) not being a substrate for glucosylation, and 2,3-poly(glycerol phosphate) not being synthesized in the absence of UDP-glucose. Synthesis of 2,3-poly(glycerol phosphate) appears to be controlled by the concentration of UDP-glucose, as the amount of glucose incorporated and of 2,3-poly(glycerol phosphate) synthesized both increase with increasing UDP-glucose concentration. The use of the *B. subtilis* glucosylation system may be of more general interest, but as used here the results obtained were not quantitative (use of a chromatogram scanner particularly introduced errors). Glucosyl-2,3-poly(glycerol phosphate) was effectively separated from 1,3-poly(glycerol phosphate) by this glucosylation and hydrolysis procedure however, as shown by the removal of [^{14}C]glucose from the alkali-stable products.

The lack of effect of UDP-glucose on the amount of glycerol incorporated suggests that the two poly(glycerol phosphate) species are not synthesized independently and that they may share a rate-limiting step. Use of the same lipid-bound intermediate by both systems (Anderson *et al.*, 1972) could be such a control point. Our failure to detect such a lipid suggests that, if it exists, it must be present in low concentration. For these two enzyme systems to use the same substrate some degree of spatial organization would be required in the isolated enzyme complex, and mechanical disruption might be sufficient to destroy the coupling between the two systems. The enzyme preparation used in the present work shows some degree of integration, as

glucosyl-2,3-poly(glycerol phosphate) was synthesized from the nucleotide precursors, but exogenous 2,3-poly(glycerol phosphate) was not glucosylated. A similar effect occurs in the glucosylation of poly(ribitol phosphate) (Ishimoto & Strominger, 1966). It is also notable that the preparation first used to demonstrate poly(glycerol phosphate) synthesis by *B. stearothermophilus* was prepared by mechanical disintegration of cells (Oo, 1965), and did not synthesize significant amounts of glucosyl-poly(glycerol phosphate) (K. C. Oo, personal communication). The glucosyl-poly(glycerol phosphate) synthesized has the structure of that extracted from isolated cell walls by Wicken (1966), but as he found no evidence for the presence of 1,3-poly(glycerol phosphate) in the walls it is probable that this latter polymer represents the membrane teichoic acid.

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