# DIRECTION OF POLYGLYCEROLPHOSPHATE CHAIN GROWTH IN BACILLUS SUBTILIS

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Received August 6, 1968

Bray & Robbins (1967) have recently shown that the o-antigen of Salmonella anatum is a polysaccharide which is synthesised by addition of trisaccharide units to the reducing end, with transfer of the growing chain from a lipid carrier to the unit being added. Formation of this antigen thus resembles the synthesis of protein, rather than that of other polysaccharides such as glycogen (Robbins, Bray, Dankert & Wright, 1967). In this communication we show that in Bacillus subtilis ATCC 6051 the synthesis of the teichoic acid, polyglycerolphosphate (Burger & Glaser, 1964), occurs by addition of units to the glycerol terminus.

In bacterial cell walls, teichoic acids are found with the phosphate terminus attached to mucopeptide and the glycerol end free (Ghuysen, Tipper & Strominger, 1965; Hay, Archibald & Baddiley, 1965; Rodgers & Garrett, 1965). Production of formaldehyde by periodate oxidation of the adjacent hydroxyls of the terminal glycerol group has been used to estimate chain length (Burger & Glaser, 1964; Ghuysen et al., 1965). In experiments reported here a cell membrane preparation from B. subtilis was incubated sequentially with the polyglycerol-phosphate precursor CDP-[$^{3}$H] glycerol, and with the unlabelled

substrate. The polyglycerolphosphate formed was isolated and the proportion of label in the terminal glycerol was measured by periodate oxidation and isolation of formaldehyde as the dimedone derivative.

## Experimental

1(3)-[$^3$H] glycerol was prepared by reduction of DL-glyceraldehyde (British Drug Houses Ltd., Poole) with tritiated sodium borohydride (New England Nuclear) and was phosphorylated (Bublitz & Kennedy, 1954) to L-3-glycerolphosphate with crystalline glycerol kinase (Calbiochem). The glycerolphosphate was purified by adsorption on to Dowex-1 (bicarbonate form) and elution with 0.05 M-ammonium bicarbonate. Unlabelled DL-3-glycerolphosphate (Sigma) concentrations were calculated as the L isomer. CDP-glycerol was synthesised chemically (Roseman, Distler, Moffatt & Khorana, 1961), and was purified by paper chromatography on Whatman 3mm paper with ethanol-1.0 M-ammonium acetate pH 7.5 (5:2, v/v) and ethanol-0.5 M-ammonium acetate pH 3.8 (5:2, v/v).

Radioactivity was determined in dioxan scintillation fluid (Bray, 1960).

B. subtilis (ATCC 6051) cells were grown in nutrient broth (Shaw, 1962); at mid-log. phase they were collected by centrifugation, and washed twice with water. Approximately 10 g. (wet weight) of bacteria was suspended in 90 ml. 0.1 M-tris-HCl buffer pH 7.5 containing 100 mg. lysozyme, 5 mg. RNase and 5 mg. DNase (all three enzymes from Sigma); after 2 hr., lysis was found to be almost complete and intact cells were removed by centrifugation (5000 g, 5 min.). The membranes were collected (20,000 g, 30 min.), washed twice with 0.1 M-tris-HCl buffer pH 7.5, suspended in 0.1 M-tris-HCl buffer pH 8.0, and frozen. The protein concentration (Lowry, Rosebrough, Farr & Randall, 1951) was 23 mg./ml.

Incubation mixtures were set up as shown in Table 1. After incubation with the first substrate the contents of tubes 2 & 3 were diluted to 2 ml. with 0.05 M-tris-HCl buffer pH 8.0 at 0° and centrifuged at 10,000 g for 10 min. The membranes were washed once with 2 ml. of the same buffer, centrifuged, and resuspended with the second substrate. The reaction in tube 1 was stopped after the first incubation.

Reactions were stopped by the addition of 0.35 ml. of 80 % (w/w) phenol, and polyglycerolphosphate was isolated by phenol extraction and dialysis (Burger & Glaser, 1964). Samples were taken for counting and for periodate oxidation (Reeves, 1941). The polyglycerolphosphate was oxidised in 12 ml. solution containing 0.2 m-mole mannitol, 1.2 m-mole sodium periodate, and 2.8 m-mole sodium bicarbonate. After 12 hr. at room temperature in the dark, 6 m-mole HCl, 6.4 m-mole sodium arsenite, and 0.2 m-mole sodium acetate were added consecutively in a total volume of 14 ml., and 160 mg. dimedone in 2 ml. ethanol was then added. Next day the dimedone derivative was filtered off, recrystallised from 50 % (v/v) ethanol, dried, weighed, and counted. The amount of radioactivity recovered as formaldehyde (corrected for losses of carrier formaldehyde) is shown in Table 1 as a percentage of the total radioactivity in the sample oxidised.

Control experiments showed that periodate oxidation was complete in 12 hr., and that under the above conditions all of the radioactivity in the [$^3$H] glycerol could be recovered as formaldehyde after periodate oxidation.

## Results and Discussion

The data (Table 1) show that when CDP-[$^3$H] glycerol was incubated with cell membranes (tube 1), label was incorporated and was present in the terminal glycerol groups. As half the label of each terminal glycerol would be recovered as formaldehyde, an average of 1.1 glycerolphosphate units per chain were added during this incubation. Previous incubation with

Table 1

Production of [$^3$H] Formaldehyde from [$^3$H] Polyglycerolphosphate.

| Tube | 1st substrate | 2nd substrate | % label as HCHO |
|---|---|---|---|
| 1 | CDP-[$^3$H] glycerol | -- | 45 |
| 2 | CDP-glycerol | CDP-[$^3$H] glycerol | 40 |
| 3 | CDP-[$^3$H] glycerol | CDP-glycerol | 13 |

Incubation mixtures contained 20 µmole tris-HCl buffer pH 8.0, 10 µmole $MgCl_2$, 25 mµmole substrate, and 50 µl. membrane preparation, final volume 0.35 ml. The first incubation was for 15 min. at 30°, and the second for 30 min. at 30°. Subsequent procedures are described in text.

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unlabelled substrate (tube 2) shows similar results, an average of 1.25 units being added per chain. Incubation with unlabelled substrate after the use of labelled substrate (tube 3) reduces the proportion of label in the terminal glycerol, periodate oxidation of 2 out of every 3 labelled glycerols previously susceptible being blocked. The incubation times were chosen, after trial experiments, to give a high proportion of terminal labelled glycerol.

If existing chains were extended by addition of units to the phosphate terminus, no labelled formaldehyde would be produced by periodate oxidation. De novo synthesis would give a product yielding labelled formaldehyde from the first glycerolphosphate unit if labelled substrate were present in either incubation and subsequent incubation with unlabelled substrate would have no effect on the proportion of label recovered as formaldehyde. Results from Tube 3 exclude this possibility.

Synthesis of polyglycerolphosphate in B. subtilis (ATCC 6051) thus resembles glycogen synthesis, rather than o-antigen synthesis in Salmonella anatum (Robbins et al., 1967).

Acknowledgement This work was assisted by U.S. Public Health Service grant AI-03548.

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