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Biosynthesis of Wall Polymers in *Bacillus subtilis*

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Preparations of membrane plus wall derived from *Bacillus subtilis* W23 were used to study the in vitro synthesis of peptidoglycan and teichoic acid and their linkage to the preexisting cell wall. The teichoic acid synthesis showed an ordered requirement for the incorporation of *N*-acetylglucosamine from uridine 5'-diphosphate (UDP)-*N*-acetylglucosamine followed by addition of glycerol phosphate from cytidine 5'-diphosphate (CDP)-glycerol and finally by addition of ribitol phosphate from CDP-ribitol. UDP-*N*-acetylglucosamine was not only required for the synthesis of the teichoic acid, but *N*-acetylglucosamine residues formed an integral part of the linkage unit attaching polyribitol phosphate to the cell wall. Synthesis of the teichoic acid was exquisitely sensitive to the antibiotic tunicamycin, and this was shown to be due to the inhibition of incorporation of *N*-acetylglucosamine units from UDP-*N*-acetylglucosamine.

It is well established that in many gram-positive bacteria the two major polymers of the wall, peptidoglycan and teichoic acid, are linked covalently. In *Staphylococcus lactis* I3, the teichoic acid, a polymer of *N*-acetylglucosamine-1-phosphate and glycerol, is linked through a phosphodiester bond to the 6-hydroxyl group of a muramic acid residue in the peptidoglycan (4). Chemical evidence also established that in *S. aureus* a trimer of glycerol phosphate acts as a linkage unit between the polyribitol phosphate teichoic acid and the peptidoglycan (8), although the nature of the bond between the peptidoglycan and the linkage unit was not determined.

In vivo experiments with *Bacillus subtilis* (19) and *Diplococcus pneumoniae* (25) have shown that newly synthesized teichoic acid is linked only to peptidoglycan synthesized concomitantly. These observations were largely confirmed in vitro with preparations of membrane plus wall from *B. licheniformis* (31), where the linkage of 80% of the newly synthesized teichoic acid, a 1,3 poly(glycerol phosphate), required the concomitant synthesis of cross-linked peptidoglycan. However, 20% of the teichoic acid was linked to the preexisting wall in the absence of peptidoglycan synthesis. More recently, we have isolated newly synthesized muramic acid phosphate from membrane + wall preparations from *B. licheniformis* in which in vitro synthesis of both teichoic acid and peptidoglycan had occurred (32). Synthesis of the muramic acid phosphate required the simultaneous synthesis of both polymers, and the phosphate moiety was derived from uridine 5'-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc).

Bracha and Glaser (5) used similar enzyme preparations to investigate the linkage of polyribitol phosphate teichoic acid to the peptidoglycan of *S. aureus*. In this organism, synthesis of linked teichoic acid required adenosine 5'-triphosphate, cytidine 5'-diphosphate (CDP)-glycerol and UDP-GlcNAc in addition to CDP-ribitol. Moreover, increased concentrations of UDP-GlcNAc stimulated the incorporation of glycerol phosphate from CDP-glycerol into preexisting wall. Simultaneously, Hancock and Baddiley (14) reported similar results showing that UDP-GlcNAc stimulated the incorporation of glycerol phosphate from CDP-glycerol into polymeric material synthesized by membrane preparations from a number of other gram-positive organisms.

In this communication, we describe a membrane + wall preparation from *B. subtilis* W23, which utilized UDP-GlcNAc, CDP-glycerol, and CDP-ribitol to synthesize polymeric material linked to the preexisting wall. The sequence of addition from these nucleotide precursors in the synthesis of this polymer and some aspects of its chemical composition were investigated. These membrane + wall preparations can also synthesize peptidoglycan and a wall-linked polymer derived from UDP-GlcNAc.

MATERIALS AND METHODS

The organism used, *B. subtilis* B7130, was a mutant defective in the glucosylation of teichoic acid, derived from *B. subtilis* B71 *met glyc glpD* (a strain

of *B. subtilis* W23) (20): Cultures were grown under the conditions described previously for *B. licheniformis* (28) in medium supplemented with methionine (100 μg/ml) and glycerol (0.01%, vol/vol). Organisms were harvested in the mid-exponential phase of growth, and the membrane + wall preparations were isolated as described previously (28), except that the buffer used for washing and final suspension of the enzyme preparation was 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8) containing 60 mM $MgCl_2$ and 1 mM dithiothreitol (TMD-buffer). As used, the membrane + wall preparations contained 10 to 15 mg of protein per ml and 40 to 60 mg (dry weight) of walls per ml.

Materials. UDP-*N*-acetyl[U-^{14}C]glucosamine (300 mCi/mmol), [U-^{14}C]glycerol (46 mCi/mmol), [γ-^{32}P]adenosine 5'-triphosphate (16.25 Ci/mmol), and NaB^3H_4 (870 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, United Kingdom. L-α-Glycerol 3-phosphate radioactively labeled in either the glycerol or phosphate moiety was prepared from [U-^{14}C]glycerol or [γ-^{32}P]adenosine 5'-triphosphate, respectively, and was used for the enzymatic synthesis of CDP-glycerol as previously described (22). The nucleotides were routinely used at specific activities of 2.5 mCi/mmol for ^{14}C and 5 mCi/mmol for ^{32}P. Non-radioactive CDP-glycerol was also prepared by this method. Similarly, CDP-ribitol was synthesized from D-ribitol 5-phosphate and CTP by using a cytoplasmic extract obtained from *B. subtilis* B71 as a source of CDP-ribitol pyrophosphorylase (EC 2.7.7.40). D-Ribitol 5-phosphate was prepared by reduction of D-ribose 5-phosphate with $NaBH_4$. Reduction with NaB^3H_4 yielded D-[^{3}H]ribitol 5-phosphate, which was used for synthesis of CDP-[^{3}H]ribitol at specific activity of 6.28 mCi/mmol. UDP-*N*-acetylmuramyl-LAla-DisoGlu-mA_2pm-DAla-DAla (UDP-MurAc-pentapeptide) radioactively labeled with either diamino[^{3}H]pimelic acid (17.4 mCi/mmol) or [^{14}C]muramic acid (12.4 mCi/mmol) were prepared as previously described (28, 29). Non-radioactive UDP-MurAc-pentapeptide was isolated from *B. licheniformis* after accumulation in a medium lacking Mg^{2+} (11). [^{32}P]UDP-GlcNAc was obtained from cultures of *Micrococcus luteus* grown in 1% peptone (Difco) containing glucose (0.2%), NaCl (0.5%), and ^{32}P-labeled inorganic phosphate and inhibited with vancomycin. The specific activity of the nucleotide obtained ranged from 2.5 to 5.6 mCi/mmol. Tunicamycin, supplied by G. Tamura, University of Tokyo, Tokyo, Japan, was the kind gift of A. H. Rose, University of Bath, Bath, United Kingdom. Other antibiotics were obtained from various commercial sources as previously described.

Determination of polymer synthesis. Biosynthesis of polymeric material was carried out for 30 min at 28°C. The complete reaction mixture contained in a final volume of 200 μl: 0.5 mM UDP-GlcNAc, 0.5 mM CDP-glycerol, 0.31 mM CDP-[^{3}H]ribitol (6.28 mCi/mmol), and membrane + wall preparation (150 μl). The reaction was terminated by the addition of sodium dodecyl sulfate (SDS; 10%, 200 μl), and the SDS-insoluble fraction was isolated as previously described (31). In certain cases, the SDS-soluble material was examined by paper chromatography after removal of the bulk of the SDS by precipitation at 0°C followed by centrifugation. The supernatant was then concentrated by freeze-drying prior to chromatography. In experiments involving the preincubation of the enzyme preparation with certain substrates, excess nucleotides were removed after the first incubation by diluting the incubation mixture (200 μl) with ice-cold TMD buffer (3 ml) and centrifuging (2 min, 17,000 × *g*). After being washed twice more with cold buffer (3 ml), the membrane + wall preparations were resuspended in buffer (100 μl) prior to the second incubation.

Analytical methods. Protein was determined by the method of Lowry et al. (18), with bovine serum albumin as the standard. Amino sugars were determined with a Beckman-Spinco automatic amino acid analyzer after hydrolysis of the samples in 4 M HCl at 100°C for 4 h. Glycerol and phosphorus were determined by published methods (1, 30). Periodate oxidation and the formation of the dimedone complex of the released formaldehyde were carried out as described by Kennedy and Shaw (16). Radioactivity in SDS-insoluble material and in liquid samples was determined in a Packard Tri-Carb 3385 scintillation counter, using toluene-based scintillation fluid [toluene containing 2,5-diphenyloxazole (0.4%), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%), and Biosolv (20%)]. Radioactivity on paper was determined by counting the paper directly in toluene-based scintillation fluid.

Whatman no. 3 paper was used for descending chromatography in the following solvent systems: A, ethanol-1 M ammonium acetate (pH 3.8; 5:2, vol/vol); B, propan-1-ol-NH_3-water (6:3:1, by volume); C, butan-1-ol-pyridine-water (6:4:3, by volume); D, butan-1-ol-acetic acid-water (3:1:1, by volume); E, isobutyric acid-1 M NH_3 (5:3, vol/vol). Electrophoresis on Whatman no. 3 paper was carried out at 70 V/cm in the following buffer systems: F, pyridine-acetic acid-water (pH 6.5; 50:2:948, by volume); G, pyridine-acetic acid-water (pH 3.5; 1:10:989, by volume).

RESULTS

Preliminary experiments with membrane + wall preparations derived from *B. subtilis* B71 synthesized small and variable amounts of both peptidoglycan and teichoic acid. Polymer synthesis was measured by the incorporation of either diamino[^{3}H]pimelic acid or [^{3}H]ribitol into SDS-insoluble material after incubation with the appropriate nucleotide precursors. Resistance of the radioactive labels to extraction by hot SDS has previously been taken as indicating that the newly synthesized material is covalently bound to the preexisting wall (5, 31). Experiments with *B. licheniformis* had shown previously that mutants that are phosphoglucomutase deficient and have reduced levels of autolytic enzymes are generally more efficient

in cell-free synthesis of peptidoglycan than the wild-type organism (28). We therefore examined mutants of *B. subtilis* B71 that were unable to synthesize glucosylated teichoic acid for enhanced activity of polymer synthesis. The following experiments were all carried out with one such mutant, *B. subtilis* B7130, which synthesized increased amounts of both peptidoglycan and teichoic acid and which did not show the variability in yields observed with the parent organism.

Biosynthesis of peptidoglycan. Incubation of the membrane + wall preparations with UDP-GlcNAc and UDP-MurAc-pentapeptide resulted in the synthesis of peptidoglycan (Table 1). Since this synthesis was not inhibited by either benzylpenicillin or cephaloridine (10 μg/ml), we conclude that incorporation of the newly synthesized material occurs by a transglycosylation mechanism rather than by transpeptidation. The incorporation of radioactivity from UDP-MurAc-pentapeptide was, however, dependent on the presence of UDP-GlcNAc. In contrast, when UDP-[^{14}C]GlcNAc was incubated with the membrane + wall preparations, radioactivity was incorporated into the SDS-insoluble material whether or not UDP-MurAc-pentapeptide was present (Table 1). Again this incorporation was insensitive to the presence of beta-lactam antibiotics. Further studies on the nature of the material synthesized from UDP-[^{14}C]GlcNAc are described below.

Biosynthesis of teichoic acid. The incorporation of [^{3}H]ribitol from CDP-[^{3}H]ribitol into SDS-insoluble material required the presence of both UDP-GlcNAc and CDP-glycerol (Table 2). Incorporation increased with time, reaching a plateau after 20 to 30 min of incubation. At this time, polymeric material in the SDS-soluble fraction (i.e., material remaining on the origin after paper chromatography in solvent E) was generally present in a four- to fivefold excess over the SDS-insoluble material. Increasing the concentrations of UDP-GlcNAc in the incubation mixture from 0.1 to 0.5 mM led to a fourfold increase in the amount of [^{3}H]ribitol incorporated into SDS-insoluble material, but the amount of SDS-soluble polymer formed was not investigated.

In contrast to previous findings with *B. licheniformis* (31), synthesis of the SDS-insoluble teichoic acid was independent of the presence of UDP-MurAc-pentapeptide. Under the assay conditions as described in Table 2, the amount of [^{3}H]ribitol incorporated in the presence and absence of UDP-MurAc-pentapeptide (0.5 mM) was 1,491 and 1,467 pmol, respectively. Pretreatment of the enzyme preparations with uridine-5'-monophosphate to remove endogenous membrane-bound intermediates of peptidoglycan biosynthesis by reversal of the phospho-MurAc-pentapeptide translocase (13) was without effect on the subsequent incorporation of radioactivity. Thus, in these enzyme preparations, the concomitant synthesis of peptidoglycan does not appear to be a requirement for the synthesis of SDS-insoluble teichoic acid.

Incubation of membrane + wall preparations with CDP-[^{14}C]glycerol (0.25 mM) alone led to the incorporation of [^{14}C]glycerol (181 pmol/mg of protein per 30 min) into the SDS-insoluble fraction. When UDP-GlcNAc (0.5 mM) was also included in the incubation mixture, the amount of [^{14}C]glycerol increased (345 pmol); the presence or absence of CDP-ribitol had no effect on this incorporation.

TABLE 1. *Biosynthesis of peptidoglycan by membrane + wall preparations from B. subtilis B7130*[a]

Radioactive precursor	Other additions	SDS-insoluble polymer (pmol/mg of protein per 30 min)
UDP-MurAc-[^{3}H-A$_2$pm]pentapeptide	UDP-GlcNAc	231
UDP-MurAc-[^{3}H-A$_2$pm]pentapeptide		21
UDP-MurAc-[^{3}H-A$_2$pm]pentapeptide	UDP-GlcNac + cephaloridine (10 μg/ml)	251
UDP-[^{14}C]GlcNAc	UDP-MurAc-pentapeptide	769
UDP-[^{14}C]GlcNAc		842

[a] The incubation mixture contained in a total volume of 200 μl: 0.5 mM UDP-MurAc-pentapeptide labeled where indicated with diamino[^{3}H]pimelic acid (17.4 mCi/mmol), 0.1 mM UDP-GlcNAc (2.5 mCi/mmol), and membrane + wall preparation (150 μl). After incubation at 28°C for 30 min, the SDS-insoluble fraction was prepared as described in the text.

TABLE 2. *Biosynthesis of teichoic acid by membrane + wall preparations from B. subtilis B7130*[a]

System	SDS-insoluble teichoic acid (pmol of [^{3}H]ribitol/mg of protein per 30 min)
Complete	718
− UDP-GlcNAc	33
− CDP-glycerol	104

[a] The complete system for teichoic acid biosynthesis included in a total volume of 200 μl: 0.1 mM UDP-GlcNAc, 0.05 mM CDP-glycerol, 0.31 mM CDP-[^{3}H]ribitol (6.28 mCi/mmol), and membrane + wall preparation (150 μl). Incubation conditions and isolation of the SDS-insoluble material were as described in the text.

Preincubation of the enzyme preparation at 28°C for 20 min with UDP-GlcNAc (0.5 mM) and CDP-glycerol (0.5 mM), followed by extensive washing to remove nucleotides (see Materials and Methods), reduced the uptake of [^{14}C]glycerol from CDP-[^{14}C]glycerol in a second incubation in the absence of UDP-GlcNAc to 19 pmol. Moreover, similar preincubation (28°C for 20 min) with UDP-GlcNAc and CDP-glycerol completely removed the requirement for these nucleotides for the incorporation of [^{3}H]ribitol such that, in the second incubation, uptake of [^{3}H]ribitol (352 pmol) occurred when CDP-ribitol was the only nucleotide present. This value should be compared with an incorporation of 429 pmol obtained when all three nucleotide precursors were present in the second incubation. Such preincubation experiments were then used to establish the sequence of incorporation of the various components of the teichoic acid. GlcNAc must be incorporated before the glycerol phosphate (Table 3), and, since a short preincubation with UDP-GlcNAc and CDP-glycerol allows addition of ribitol phosphate from CDP-ribitol alone, addition of ribitol phosphate units must be the final stage of the synthesis.

However, in contrast to these observations, on increasing the time of the preincubation with UDP-GlcNAc and CDP-glycerol from 20 to 30 min, no subsequent addition of [^{3}H]ribitol from CDP-[^{3}H]ribitol occurred when UDP-GlcNAc and CDP-glycerol were omitted from the second incubation. This suggested that ribitol phosphate units may be added only to GlcNAc-glycerol units present in the enzyme preparation in some intermediate form and not to GlcNAc-glycerol units already bound to preexisting wall. To test this hypothesis, membrane + wall preparations were preincubated for 30 min with UDP-GlcNAc (0.5 mM) and CDP-[^{14}C]glycerol (0.5 mM), and the SDS-insoluble fraction was prepared from one-half of the material at this stage (fraction A). The remainder was washed extensively prior to a second incubation with UDP-GlcNAc (0.5 mM), CDP-glycerol (0.5 mM), and CDP-[^{3}H]ribitol (0.34 mM) (fraction B). The SDS-insoluble material from both was then subjected to periodate oxidation, and the amount of radioactive formaldehyde released was measured as the dimedone complex (16). One mole of [^{14}C]formaldehyde would arise from oxidation of each terminal [^{14}C]glycerol residue, and 1 mol of [^{3}H]formaldehyde would arise from each terminal ribitol residue. The ratio of radioactive formaldehyde formed to the total radioactivity incorporated gives a measure of the chain length. In fraction A, the total ^{14}C incorporated was 9,725 dpm, of which 1,698 ^{14}C dpm (17.5% of the total) was released as [^{14}C]formaldehyde; in fraction B, 956 dpm of [^{14}C]formaldehyde (16.9%) was obtained from the total 5,673 ^{14}C dpm incorporated. Thus, the number of [^{14}C]glycerol termini was the same in both fractions and corresponded to an average chain length of 1.95 glycerol units in each case. In addition, 2,047 dpm of [^{3}H]formaldehyde was obtained from a total of 36,507 dpm of [^{3}H]ribitol incorporated into fraction B, corresponding to a chain of 18.8 ribitol units in this experiment. Addition of ribitol phosphate units to glycerol termini already present in the wall after the preincubation would lead to a decrease in the number of [^{14}C]glycerol molecules susceptible to periodate oxidation. Since this was not the case, we conclude that ribitol phosphate units must be added to GlcNAc-glycerol units present in the enzyme preparation at the same time as the CDP-ribitol. In experiments using preincubation with UDP-GlcNAc and CDP-glycerol for periods of up to 20 min, followed by omission of these nucleotides in the second incubation, the portion of the carrier containing the GlcNAc and glycerol residues must remain in the membrane + wall preparation during the second incubation. Ribitol phosphate units are then added to this carrier before transfer of the completed polymer to the wall. In the absence of added CDP-ribitol, GlcNAc and glyc-

TABLE 3. *Sequence of addition from nucleotide precursors in teichoic acid biosynthesis*[a]

1st Incubation[b]	2nd Incubation[b]	SDS-insoluble teichoic acid (pmol of [^{3}H]ribitol/mg of protein)
UDP-GlcNAc (0.5)	CDP-glycerol (0.28) CDP-[^{3}H]ribitol (0.31)	1,848
CDP-glycerol (0.55)	UDP-GlcNAc (0.5) CDP-[^{3}H]ribitol (0.31)	281
Water	UDP-GlcNAc (0.5) CDP-glycerol (0.28) CDP-[^{3}H]ribitol (0.31)	1,769

[a] The initial incubation mixture contained in a total volume of 200 μl: membrane + wall preparation (150 μl) and the teichoic acid precursors as shown. After incubation for 20 min at 28°C, the precursors were removed by washing, as described in the text. The washed membrane + wall preparations were then reincubated, together with the precursors shown, for a further 30 min, after which SDS-insoluble material was isolated.

[b] Numbers in parentheses indicate millimolar concentrations.

erol units can be transferred from the carrier to the wall.

Effect of antibiotics on teichoic acid synthesis. Among the antibiotics known to inhibit various stages of peptidoglycan biosynthesis, a number have also been reported to inhibit teichoic acid synthesis. Vancomycin inhibited polyribitol phosphate synthesis by an enzyme preparation from *Lactobacillus plantarum* (12) and the incorporation of glycerol into a water-soluble polymer by membranes of *B. subtilis* W23 (14); however, bacitracin only inhibited teichoic acid synthesis by membrane preparations of *B. licheniformis* if peptidoglycan synthesis was occurring simultaneously (3). In the present investigation, these antibiotics had no significant effect on the incorporation of ribitol into SDS-insoluble material, with vancomycin (1,250 μg/ml) and bacitracin (400 μg/ml) giving less than 10% inhibition. Teichoic acid synthesis had already been shown to be independent of concomitant peptidoglycan synthesis (see above) and, as expected, the beta-lactam antibiotics benzylpenicillin and cephaloridine had no inhibitory effect on the incorporation of [^{3}H]ribitol. In contrast, the synthesis of teichoic acid was extremely sensitive to inhibition by tunicamycin, with greater than 90% inhibition of [^{3}H]ribitol incorporation occurring at an antibiotic concentration of 1 μg/ml (Fig. 1). Preincubation experiments, such as described in the previous section, revealed that only the utilization of UDP-GlcNAc was inhibited. Once the incorporation of GlcNAc had occurred, the synthesis of the complete polymer from CDP-glycerol and CDP-[^{3}H]ribitol was unaffected by concentrations of tunicamycin that inhibited synthesis of the complete polymer by more than 97% (Table 4). When UDP-[U-^{14}C]GlcNAc was incubated with the membrane + wall preparation in the presence of CDP-glycerol and CDP-ribitol, the incorporation of GlcNAc into SDS-insoluble material was also inhibited by tunicamycin but to a lesser extent than the ribitol incorporation (Fig. 1). This result could be explained if GlcNAc is also incorporated into another SDS-insoluble polymer in addition to the teichoic acid, the synthesis of this other product being unaffected by tunicamycin. Tunicamycin-sensitive synthesis of lipid-linked intermediates in teichoic acid synthesis has recently been demonstrated in membranes of *B. subtilis* W23 and *S. aureus* (6, 15). In addition, the membranes of *S. aureus* could synthesize polymeric material from UDP-GlcNAc alone, which was not inhibited by tunicamycin (6).

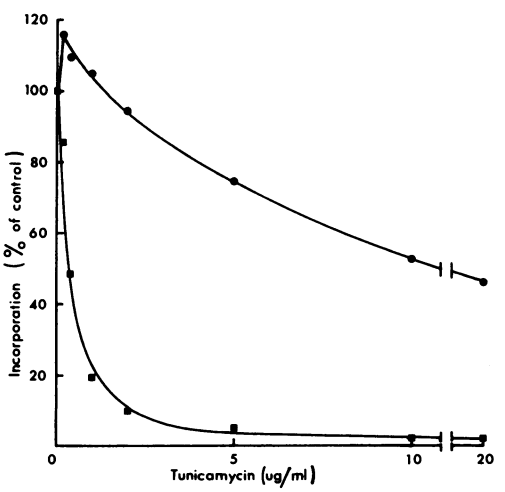

FIG. 1. *Effect of tunicamycin on teichoic acid biosynthesis in B. subtilis B7130. Assays contained UDP-[U-^{14}C]GlcNAc (0.48 mM), CDP-glycerol (0.28 mM), CDP-[^{3}H]ribitol (0.31 mM), and tunicamycin as indicated. Radioactivity in SDS-insoluble material was measured: ■, [^{3}H]ribitol; ●, [^{14}C]GlcNAc.*

Participation of lipid intermediates in teichoic acid synthesis. Tunicamycin is known to inhibit the synthesis of polyisoprenyl *N*-acetylglucosaminyl pyrophosphate intermediates (17, 23, 24) and, since teichoic acid synthesis was exquisitely sensitive to tunicamycin, it seems likely that a polyprenol-linked intermediate may be involved. Attempts to isolate a lipid intermediate in teichoic synthesis were made by incubating the membrane + wall preparation with CDP-[^{3}H]ribitol in the presence of UDP-GlcNAc and CDP-glycerol with one or other of these latter nucleotides labeled with ^{14}C, followed by extraction with 2 volumes of butan-1-ol–6 M pyridinium acetate (pH 4.2; 1:1,

TABLE 4. *Site of tunicamycin inhibition of teichoic acid biosynthesis*[a]

1st Incubation	2nd Incubation	Tunicamycin (20 μg/ml)	SDS-insoluble teichoic acid (pmol of [^{3}H]ribitol/mg of protein)
None	UDP-GlcNAc CDP-glycerol	–	1,331
	CDP-ribitol	+	37
UDP-GlcNAc	CDP-glycerol	–	751
	CDP-ribitol	+	729
UDP-GlcNAc CDP-glycerol	CDP-ribitol	– +	638 661

[a] Substrates and incubation times were as described in footnote *a* of Table 3. Tunicamycin was present during the second incubation where indicated.

vol/vol). This solvent was chosen for the extraction of possible teichoic acid-lipid intermediates since it quantitatively extracts the undecaprenyl lipid intermediates of peptidoglycan synthesis without extracting either the nucleotide precursors or the peptidoglycan (2). Butanol-soluble material containing GlcNAc and glycerol was detected, but no [^{3}H]ribitol was solubilized. A carrier containing GlcNAc and glycerol residues attached to undecaprenol would probably be soluble under these conditions, whereas the further addition of a long chain of ribitol phosphate units would increase the hydrophilic nature of the material and thus render it insoluble in the organic solvent. If UDP-GlcNAc was omitted from the incubation mixture, incorporation of [^{14}C]glycerol was reduced from 664 to 320 pmol/mg of protein per 30 min. Small amounts of the glucosamine-containing acceptor preexisting in the membrane + wall preparation could account for the incorporation of [^{14}C]glycerol under these conditions. Furthermore, formation of butan-1-ol-soluble GlcNAc increased from 646 to 1,617 pmol/mg per 30 min when CDP-glycerol and CDP-ribitol were omitted from the incubation mixture. This could reflect either an increased synthesis of the GlcNAc lipid intermediate or, alternatively, the synthesis of polymeric GlcNAc linked to undecaprenol pyrophosphate as described for membranes of *B. subtilis* 168 (G. E. Bettinger, A. N. Chatterjee, and F. E. Young, Fed. Proc. 34:668, 1973).

Characterization of the newly synthesized teichoic acid. Treatment with 60% HF (−2°C for 16 h) (26) of wall preparations containing teichoic acid synthesized from CDP-[^{3}H]ribitol as the only radioactive precursor released more than 90% of the radioactivity from the walls. Cleavage with HF would be expected to hydrolyze all phosphoester linkages, but the released radioactivity remained at the origin upon chromatography in solvent C. However, after alkaline hydrolysis (1 N KOH, 100°C for 3 h) followed by treatment with alkaline phosphatase, all of the radioactivity had the chromatographic mobility of ribitol.

Similar alkaline hydrolysis of SDS-insoluble material prepared with CDP-[^{14}C]glycerol as the radioactive teichoic acid precursor gave a mixture of glycerol, glycerol monophosphate, and glycerol diphosphate when chromatographed on a column of diethylaminoethyl-cellulose (HCO_3^-) (4). The identity of these various components was further confirmed by chromatography in solvent B with the appropriate marker compounds.

Cleavage with HF of material synthesized from UDP-[^{14}C]GlcNAc as the radioactive teichoic acid precursor also released 90% of the incorporated radioactivity. Chromatography of the released material in solvent C showed that 43.9% of the ^{14}C present had a mobility identical to that of authentic GlcNAc, a further 32.5% appeared as a discrete peak of $R_{GlcNAc} = 0.5$, and the remaining 21.5% was located on the origin of the chromatogram. Acid hydrolysis (4 N HCl, 100°C for 4 h) of these latter two fractions gave glucosamine as the only radioactive component.

Periodate oxidation followed by isolation of the formaldehyde-dimedone complex gave an average chain length of 27.5 units for the newly synthesized polyribitol phosphate. Similar treatment of material prepared from CDP-[^{14}C]glycerol yielded no radioactive formaldehyde. This would be expected if the glycerol phosphate units are part of a linkage unit between the polyribitol phosphate and the preexisting wall. On the assumption that newly synthesized polyribitol phosphate chains are only linked to newly synthesized glycerol phosphate units, a calculation of the average chain length of glycerol phosphate units can be made from an average value of the polyribitol phosphate chain length compared with the total glycerol incorporation. On this basis, an average chain length of 3.4 glycerol phosphate units was obtained.

Since the GlcNAc residues are also involved in the linkage between the preexisting wall and the glycerol phosphate oligomer, no free reducing GlcNAc terminal is available for direct measurement of the chain length. An indirect method of calculation of the chain length similar to that used for the glycerol phosphate oligomer cannot be used for the GlcNAc portion since UDP-[^{14}C]GlcNAc is incorporated into another polymer (see below) in addition to the teichoic acid. SDS-soluble material was prepared by incubation of the enzyme preparation with UDP-[^{14}C]GlcNAc, CDP-glycerol, and CDP-[^{3}H]ribitol. Most of the SDS was removed by precipitation at 0°C, and the water-soluble polymeric material was separated from the remaining detergent and the excess nucleotides by chromatography on a column (1 by 70 cm) of Sephadex G75. Any polymeric material attached to a lipid carrier would have been expected to chromatograph in the detergent fraction. Periodate oxidation of a sample of this material gave an average chain length of 22.5 ribitol phosphate units, a value similar to that found for SDS-insoluble material, suggesting that comparison of SDS-soluble and SDS-insoluble material is valid. An additional sam-

ple of the SDS-soluble material was then reduced with KBH_4 (0.1 M in 0.01 M KOH) at 4°C for 16 h and, after acid hydrolysis (4 N HCl, 100°C for 4 h), the glucosamine and glucosaminol were separated by chromatography on a column of Sephadex G10 equilibrated in borate buffer (9). This gave a chain length of 1.4 GlcNAc units.

Release of the newly synthesized teichoic acid from the preexisting wall. Since treatment with hot 5% SDS does not release the newly synthesized teichoic acid from the preexisting wall and further treatment with trypsin or with butan-1-ol–6 M pyridinium acetate (pH 4.2; 1:1, vol/vol) is without effect, it is assumed that the linkage involves a covalent bond. SDS-insoluble material containing [^{14}C]GlcNAc and [^{3}H]ribitol was synthesized in vitro, and the wall was solubilized completely by digestion with *Streptomyces* muramidase in 0.05 M sodium acetate buffer (pH 5.0; 35°C for 48 h). Analysis showed that more than 90% of the muramic acid residues were then present as free reducing groups. Chromatography of the solubilized material on a column (0.5 by 17 cm) of diethylaminoethyl-cellulose (acetate form) gave two peaks, both of which contained ^{3}H and ^{14}C in a constant ratio. Extraction with 0.1 N NaOH under N_2 at 0°C for 24 h also completely solubilized newly synthesized teichoic acid containing [^{14}C]GlcNAc and [^{3}H]ribitol. The alkaline extract was neutralized on a column (0.5 by 5 cm) of Amberlite IR120 (H^+) with 100% recovery of the radioactivity and then chromatographed on a column (0.5 by 17 cm) of Ecteola-cellulose (Cl^-) eluted with a gradient of 0 to 0.5 M LiCl. A single peak containing both radioactive labels was obtained (Fig. 2).

Mild acid hydrolysis (0.1 N HCl, 100°C for 4 min) also solubilized the newly synthesized teichoic acid, with all of the radioactivity being released from the wall after four successive extractions. This procedure also released more than 97% of the phosphorus present in the cell wall of this organism. Material labeled in any two of the three teichoic acid components was solubilized in this way and, upon electrophoresis in buffers F or G or gel filtration on Sephadex G25, both labels always remained coincident. We therefore conclude that the newly synthesized teichoic acid contains GlcNAc, glycerol phosphate, and ribitol phosphate residues linked together in a single polymer that is covalently bound to the wall.

Polymer synthesized from UDP-GlcNAc. Membrane + wall preparations incorporated radioactivity from UDP-[^{14}C]GlcNAc into SDS-insoluble material in the absence of other nucleotides (Table 1). This radioactivity could subsequently be solubilized by mild acid hydrolysis and then remained at the origin of chromatograms developed with solvents B and C. Further acid hydrolysis (4 N HCl, 100°C for 4 h) of this solubilized material yielded only glucosamine when chromatographed in solvent C. Alkaline hydrolysis (1 N KOH, 100°C for 3 h) of SDS-insoluble material yielded a major product having the electrophoretic behavior of glucosamine phosphate in buffer F. This also yielded glucosamine after acid hydrolysis (4 N HCl, 100°C for 4 h). This material has not been investigated further but may be analogous to the polymer synthesized from UDP-GlcNAc by membranes from *S. aureus* (6).

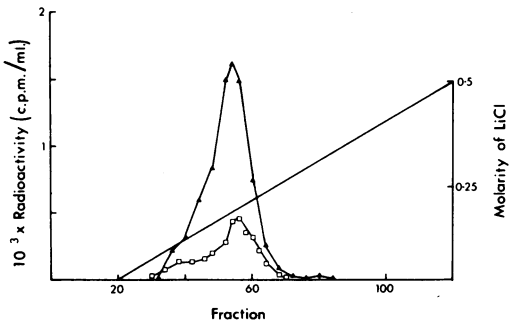

FIG. 2. *Fractionation on Ecteola-cellulose of teichoic acid newly synthesized from UDP-[U-^{14}C]GlcNAc, CDP-glycerol and CDP-[^{3}H]ribitol and solubilized by treatment with dilute alkali. ^{3}H (▲) and ^{14}C (□) radioactivity was measured in 0.2-ml portions of the fractions.*

DISCUSSION

Membrane + wall preparations from *B. subtilis* B7130 were able to synthesize linear peptidoglycan, teichoic acid, and a polymer of GlcNAc, all of which were covalently bound to the preexisting wall. Synthesis of these polymers can occur simultaneously, although each also occurs independently of the others. Teichoic acid synthesis required an ordered incorporation of residues from UDP-GlcNAc, CDP-glycerol, and CDP-ribitol, and all three components were linked together and attached to the preexisting wall. Similar preparations from *S. aureus* (5) required these three nucleotides for teichoic acid synthesis, and toluenized cells of *Micrococcus* sp. 2102 (14) similarly required UDP-GlcNAc for synthesis of the glycerol phosphate linkage unit between teichoic acid and peptidoglycan. However, in neither of these cases was GlcNAc shown to be incorporated into the wall as part of the linkage region of the newly synthesized teichoic acid.

The in vitro synthesis of teichoic acid by *B. subtilis* B7130 was independent of peptidoglycan synthesis, unlike similar preparations from *B. licheniformis* in which teichoic acid synthesis required the concomitant synthesis of cross-linked peptidoglycan (31). In *B. licheniformis*, the two newly synthesized polymers are linked together by a muramic acid phosphate bond, the phosphate arising from a GlcNAc residue in the teichoic acid (32). However, no newly synthesized muramic acid phosphate was found when enzyme preparations from *B. subtilis* B7130 synthesized teichoic acid alone or simultaneously synthesized teichoic acid and linear peptidoglycan. It has also been shown in *B. licheniformis* (27) and *M. luteus* (21) that linear peptidoglycan synthesized in the presence of beta-lactam antibiotics has no associated phosphorus. It therefore seems possible that muramic acid phosphate is only formed when newly synthesized teichoic acid is linked to cross-linked peptidoglycan synthesized concomitantly.

Recently two laboratories reported tunicamycin-sensitive synthesis of lipid-linked intermediates in the biosynthesis of teichoic acid by membranes of *B. subtilis* W23 and *S. aureus* (6, 15). In both cases, UDP-GlcNAc was required for synthesis of the intermediate. Bracha and Glaser (6) did not demonstrate that the intermediate synthesized by membranes from *S. aureus* contained GlcNAc, although their proposed structure shows 1.5 to 2.0 GlcNAc residues per molecule of intermediate, this figure having been calculated from measurements of the excess of glucosamine over muramic acid in the teichoic acid-peptidoglycan complex obtained by digesting cell walls with lysostaphin. In the biosynthetic scheme proposed by Hancock et al. (15), UDP-GlcNAc was required for the addition of the glycerol phosphate trimer to a lipid acceptor, but GlcNAc was not incorporated into the intermediate. The lipid portion of the intermediate was not identified in either case, although both used tunicamycin inhibition to suggest that this was likely to be a polyprenol. Earlier, both Bracha and Glaser (5) and Hancock and Baddiley (14) had simultaneously published similar schemes for the in vitro synthesis of wall-linked teichoic acid in which polyribitol phosphate was transferred from a lipoteichoic acid carrier either (i) to nascent peptidoglycan that already contained glycerol phosphate and GlcNAc residues (5) or (ii) to a glycerol phosphate trimer that had previously been attached to either a growing glycan chain or its polyprenyl-MurAc-pentapeptide-GlcNAc intermediate (14). Neither of these schemes could apply to the wall-linked teichoic acid synthesized in our present experiments since this is clearly independent of any de novo peptidoglycan synthesis. The observations described in this paper lead to the scheme for teichoic acid synthesis shown in Fig. 3. GlcNAc residues are first added to a lipid carrier, which may be a polyprenol since this step is inhibited by tunicamycin; secondly, a glycerol phosphate oligomer is added onto this carrier; and, finally, ribitol phosphate units are added onto the carrier molecule. If ribitol phosphate units were added individually onto the carrier-GlcNAc-glycerol phosphate, the carrier molecules containing only a few ribitol phosphate units would probably have been solubilized by the butan-1-ol–6 M pyridinium acetate; since this was not the case, we conclude that the polyribitol phosphate is

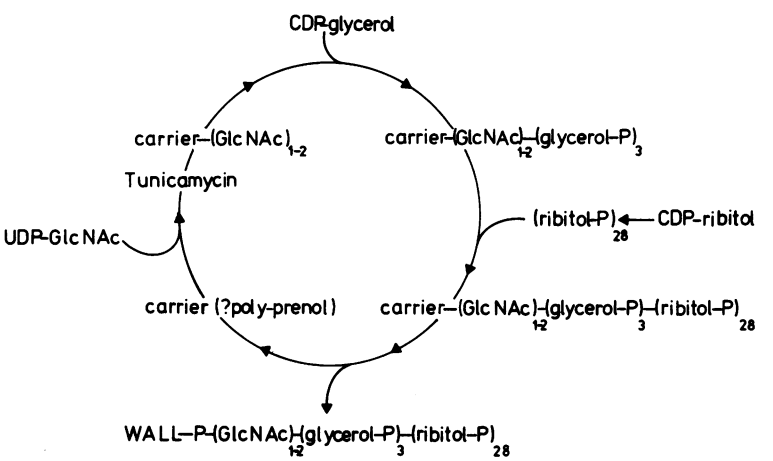

FIG. 3. *Proposed scheme for the biosynthesis of teichoic acid linked to preexisting wall.*

polymerized separately and then added to the carrier-GlcNAc-glycerol phosphate. Such polymerization could occur on lipoteichoic acid carrier as described by Fiedler and Glaser (10). Finally, the completed teichoic acid is covalently linked to the preexisting wall by a bond between a GlcNAc residue in the teichoic acid and an as yet unidentified component of the wall. This scheme is similar to those already published for the synthesis of teichoic acid intermediates (6, 15) but also accounts for covalent linking of the new teichoic acid to preexisting wall in the absence of de novo peptidoglycan synthesis. Confirmation of this or any of the proposed biosynthetic schemes must await isolation of the intermediate at various stages of its assembly and its subsequent utilization for synthesis of linked teichoic acid by cell-free enzyme preparations.

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