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In Vitro System for the Synthesis of Teichoic Acid Linked to Peptidoglycan

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A crude cell wall preparation from *Staphylococcus aureus* H prepared by the method of Mirelman and Sharon (1972) was shown to catalyze the synthesis of polyribitol phosphate linked to the cell wall peptidoglycan. The reaction used cytidine diphosphate (CDP)-ribitol as a substrate and in addition required the presence of CDP-glycerol, uridine diphosphate (UDP)-*N*-acetyl-D-glucosamine, and adenosine triphosphate. Incubation of radioactive CDP-glycerol with the crude cell wall preparation resulted in the transfer of glycerol phosphate residues to the cell wall; this reaction was greatly stimulated by the presence of UDP-*N*-acetylglucosamine. These data suggest that polyribitol phosphate is linked to the cell wall peptidoglycan by an oligomer containing *N*-acetyl-D-glucosamine and glycerol phosphate.

Current knowledge of the synthesis of teichoic acid in *Staphyloccus aureus* H can be summarized by the reactions shown in Fig. 1. Evidence for the synthesis of polyribitol phosphate linked to lipoteichoic acid carrier (LTC) has been presented previously (4, 5), and the transfer of *N*-acetyl-D-glucosamine residues from uridine diphosphate-*N*-acetyl-D-glucosamine (UDP-GlcNAc) to polyribitol phosphate has been demonstrated by Nathenson et al. (17). Reaction III, the transfer of polyribitol phosphate from LTC to peptidoglycan, has remained obscure.

Based on in vivo observations with *Bacillus subtilis* (12) and *Diplococcus pneumoniae* (21) teichoic acid chains are only linked to nascent peptidoglycan but not to preexisting peptidoglycan. D-Alanine also occurs linked to polyribitol phosphate, but the precise mechanism by which it is attached to this polymer is not known (18).

We report in this communication the properties of a crude enzyme system that catalyzes reactions I to III (Fig. 1) and shows an unexpected requirement for the synthesis of an oligomer containing glycerol phosphate and *N*-acetyl-D-glucosamine as a linkage region between peptidoglycan and polyribitol phosphate.

While the work was in progress, Wyke and Ward informed us that they had obtained a system that links polyglycerol phosphate to peptidoglycan from *B. licheniformis* (22). In an accompanying paper, Hancock and Baddiley describe similar observations obtained independently in their laboratory (9).

MATERIALS AND METHODS

S. aureus H was grown in a New Brunswick fermenter in 100 liters of antibiotic medium 3 (Difco). The fermenter was rapidly cooled to 5 C and the cells were harvested by centrifugation in a refrigerated Sharples centrifuge. The cell paste was stored frozen at −20 C. Crude cell wall preparations were prepared as described by Mirelman and Sharon (15).

UDP-*N*-acetylmuramyl-L-ala-D-isoglu-L-Lys-D-ala-D-ala (UDP-muramyl pentapeptide) was prepared from vancomycin-inhibited *S. aureus* H by published procedures (3). Radioactive UDP-muramyl pentapeptide labeled in the muramic acid was prepared either by incubation of *Micrococcus luteus* with radioactive glucose in the presence of vancomycin (13) or by incubation of *S. aureus* H with radioactive *N*-acetyl-D-glucosamine in the presence of vancomycin (R. Bracha, unpublished observations). The specific activity of the nucleotide used in most of the experiments reported here was 9.3 dpm/pmol.

For the preparation of CDP-[^{3}H]ribitol, D-ribose 5-phosphate was reduced with NaB^3H_4 to D-ribitol 5-phosphate, and CDP-[^{3}H]ribitol was prepared from this by the carbodiimide method (7, 19). This nucleotide was used at a specific activity of 20 to 45 dpm/pmol. CDP-[^{14}C]glycerol was prepared similarly from L-α-[^{14}C]glycerol phosphate (New England Nuclear Corp.) and had a specific activity of 22 dpm/pmol (1).

For the assay of polyribitol phosphate synthesis linked to peptidoglycan, the reaction mixture contained: crude cell walls (7 to 8 mg [dry weight]) in 50 mM tris(hydroxymethyl)aminomethane-chloride, pH 7.8, 20 mM $MgCl_2$, 18 mM adenosine triphosphate (ATP), 10 mM spermidine, 75 mM NH_4Cl, 1.0 mM β-mercaptoethanol, and nucleotides as indicated in a final volume of 0.15 ml. In incubation containing UDP-muramyl pentapeptide, 500 nmol of

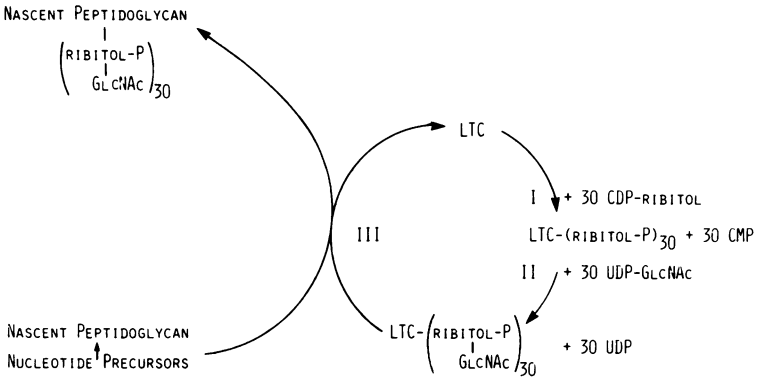

FIG. 1. *Synthesis of cell wall teichoic acid in S. aureus. Abbreviations: LTC, Lipoteichoic acid carrier (4, 5); CMP, cytidine 5'-monophosphate; UDP, uridine 5'-diphosphate. For details see text.*

glycine was also included. After incubation at 25 C for 30 min, the cell walls were sedimented by centrifugation in a Brinkmann microcentrifuge for 2.5 min and washed three times by centrifugation with 1 ml of water. The cell walls were suspended in 1 ml of 1% sodium dodecyl sulfate (SDS) and heated at 100 C for 10 min, and the walls were collected by centrifugation for 10 min. The heating step in SDS was repeated, and the walls were then washed three times with water and once with 1 ml of 0.05 M sodium acetate buffer, pH 6.5. If the cell walls were to be digested with lysozyme, they were then digested with 1 mg of lysozyme in the same buffer for 18 h at 37 C. The residual cell walls were collected by centrifugation for 10 min, and the radioactivity in the supernatant fluid and pellet was determined in a Packard liquid scintillation counter equipped with an absolute activity analyzer using 3a70 (Research Products International) as a counting fluid. Lysostaphin was obtained from Schwarz-Mann.

RESULTS

Incubation of the crude cell wall preparation with CDP-[^{3}H]ribitol in the presence of ATP and UDP-GlcNAc resulted in time-dependent incorporation of radioactivity into an SDS-insoluble product (Fig. 2). The radioactive material after acid hydrolysis (4 N HCl, 12 h at 100 C) showed the chromatographic mobility of anhydroribitol. After cleavage with 60% HF (8) followed by acid hydrolysis, it showed the chromatographic behavior of ribitol when chromatographed on Whatman 3MM paper using butanol-pyridine-water (6:4:3) as a solvent. The radioactive polymer was not released from the cell walls by brief exposure (10 min at 0 C) to 10% trichloroacetic acid, but it could be totally released from the cell wall by digestion with lysostaphin and partially released by digestion with lysozyme (see below) or the *B. subtilis N*-acetylmuramic acid L-alanine amidase (11).

When increasing quantities of UDP-muramyl pentapeptide were added to the incubation mixture, we observed a gradual inhibition of polyribitol phosphate synthesis but an increase (Fig. 3A) in the fraction of the polymer that was released by lysozyme. The inhibition by UDP-muramyl pentapeptide was partially reversed by increasing the level of UDP-GlcNAc in the reaction mixture (Fig. 3B). These data suggest that UDP-muramyl pentapeptide acts as an inhibitor of the reaction by which UDP-GlcNAc stimulates polyribitol phosphate synthesis. This could either be a direct competitive inhibition or both compounds could compete for a common carrier, for example, undecaprenol phosphate.

The cell wall of *S. aureus* is lysozyme resistant due to the presence of *O*-acetyl residues on

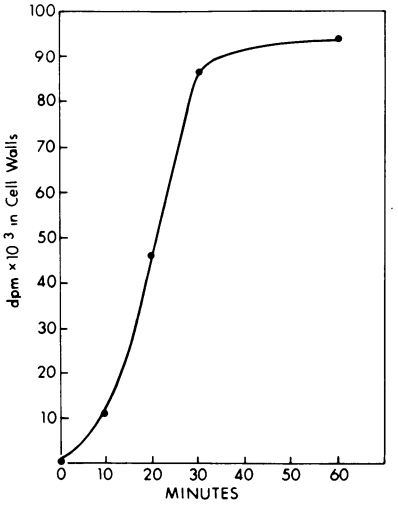

FIG. 2. *Time course of polyribitol phosphate synthesis. Standard assay conditions were used with 10 nmol of CDP-[^{3}H]ribitol and 200 nmol of UDP-GlcNAc.*

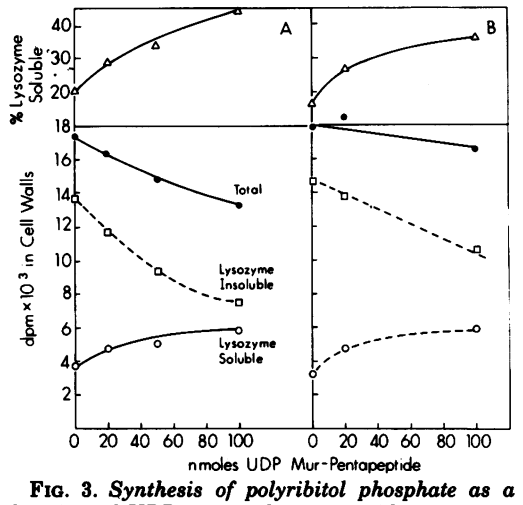

FIG. 3. *Synthesis of polyribitol phosphate as a function of UDP-muramyl pentapeptide concentration. Standard assay conditions were used with 25 nmol of CDP-[³H]ribitol, and in (A) 200 nmol of UDP-GlcNAc and in (B) 1,000 nmol of UDP-GlcNAc. Symbols: (●) Total radioactivity in cell wall; (○) radioactivity solubilized by incubation with lysozyme; (□) insoluble radioactivity after lysozyme digestion; (△) percentage of radioactivity solubilized by lysozyme.*

the muramic acid (6). The peptidoglycan synthesized in vitro was, however, totally degraded by lysozyme or *B. subtilis N*-acetyl muramic acid L-alanine amidase (Table 1), presumably because it lacks *O*-acetyl groups. About 20% of the polyribitol phosphate synthesized enzymatically in the absence of UDP-muramyl pentapeptide was released from the cell wall by lysozyme digestion, but about 40% of the polymer synthesized in the presence of UDP-muramyl pentapeptide was released by lysozyme (Fig. 3, Table 2), suggesting that a fraction of the polyribitol phosphate is attached to newly made peptidoglycan. The data in Fig. 4 also agree with this suggestion. (There was considerable variability in various experiments in the total amount of polymer synthesized per milligram of cell wall. However, the relative rates of polymer synthesis in the presence of various nucleotides were constant from experiment to experiment.) The chromatographic behavior of the enzymatically synthesized polyribitol phosphate that was released from the cell wall with lysozyme was different depending on whether the polymer had been made in the presence or absence of UDP-muramyl pentapeptide, i.e., the presence or absence of peptidoglycan synthesis. The material made in the presence of UDP-muramyl pentapeptide showed a prominent second high-molecular-weight peak that was essentially absent from the polyribitol phosphate synthesized in the absence of peptidoglycan synthesis. The chemical difference between these two polymers is not known, but it is of interest that small quantities of peptidoglycan and glycerol phosphate also eluted in the area of this second peak after lysozyme digestion. The low-molecular-weight peptidoglycan pieces obtained after lysozyme digestion eluted in the included volume (30 ml for the columns used in the experiments in Fig. 4).

Using this crude cell wall system, the incorporation of muramyl pentapeptide into cell wall was inhibited 70% by the addition of penicillin G, but no inhibition of polyribitol phosphate synthesis was observed, suggesting that the newly synthesized polyribitol phosphate is linked to chains that were already linked to the cell wall before the in vitro incubation, but to which additional disaccharide pentapeptide units are added during the enzyme incubation from UDP-muramyl pentapeptide and UDP-GlcNAc (14).

The requirement for UDP-GlcNAc for the linkage of polyribitol phosphate to peptidoglycan was surprising. It seemed unlikely that this was due to reaction II in Fig. 1 since mutants that lack this enzyme (2, 20) incorporate polyribitol phosphate into the cell wall. Equally puzzling was the requirement for ATP in this system, which is not predicted by the reactions in Fig. 1.

The precise chemical structure by which teichoic acid is linked to peptidoglycan is not known. The cell wall of *S. aureus* mutants that lack polyribitol phosphate seem to contain in their cell wall small quantities of glycerol phosphate, although it could not be established with certainty whether these were covalently linked to the peptidoglycan (16). On the assumption that this glycerol phosphate was in the linkage region between polyribitol phosphate and peptidoglycan, we tested the effect of CDP-glycerol addition on polyribitol phosphate synthesis. Since completion of these experiments, J. Bad-

TABLE 1. *Effect of CDP-glycerol on cell wall-linked polyribitol phosphate synthesis*[a]

Expt	Addition (nmol)	dpm in polyribitol phosphate
A	None	6,400
	CDP-glycerol (5)	11,000
	CDP-choline (5)	7,200
	CDP-ethanolamine (5)	6,600
B	None	20,500
	CDP-glycerol (5)	53,000

[a] Standard reaction conditions were used with 25 nmol of CDP-[³H]ribitol, 200 nmol of UDP-GlcNAc, and the indicated quantities of cytidine nucleotide.

TABLE 2. *Digestion of enzymatically synthesized cell wall polymers with lytic enzyme*[a]

Expt	Labeled nucleotide precursors (nmol)	Other additions (nmol)	Total radioactivity in cell wall (dpm)	% Released by lytic enzyme
A	CDP-[^{3}H]ribitol (10)	None	618	20
	CDP-[^{3}H]ribitol (10)	UDP-GlcNAc (1,000)	10,419	19
	CDP-[^{3}H]ribitol (10)	UDP-GlcNAc (1,000) UDP-muramyl pentapeptide (200)	5,337	42
	UDP-[^{3}H]muramyl pentapeptide (50)	UDP-GlcNAc (1,000)	7,550	90
B	CDP-[^{3}H]ribitol	UDP-GlcNAc (200)	3,100	14
	CDP-[^{3}H]ribitol	UDP-GlcNAc (200) UDP-muramyl pentapeptide (60)	4,200	48
	UDP-[^{3}H]muramyl pentapeptide	UDP-GlcNAc (200)	4,500	80

[a] Standard assay conditions were used with additions of nucleotide precursors as indicated. At the end of the reaction the isolated cell walls were digested with lytic enzymes. In A, lysozyme was used; in B, *N*-acetylmuramic acid L-alanine amidase (11) from *B. subtilis* was used to digest the isolated cell walls.

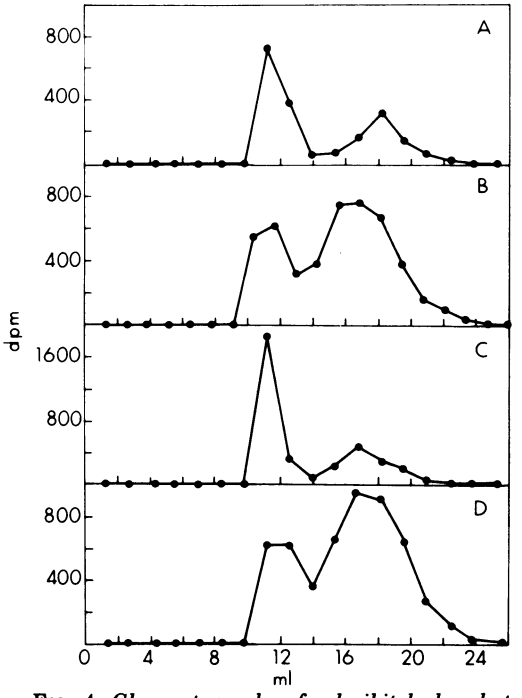

FIG. 4. *Chromatography of polyribitol phosphate on Sephadex G-100. The polymer was released from cell wall by digestion with lysozyme and chromatographed on a column (0.9 by 60 cm) of Sephadex G-100 in 0.1 M tris(hydroxymethyl)aminomethane-chloride, pH 8.0. Fractions of 1.4 ml were collected. (A) The synthetic reaction mixture contained 25 nmol of CDP-[^{3}H]ribitol and 1,000 nmol of UDP-GlcNAc. (B) The reaction mixture contained 25 nmol of CDP-[^{3}H]ribitol, 1,000 nmol of UDP-GlcNAc, and 100 nmol of UDP-muramyl pentapeptide. (C) and (D) are identical to (A) and (B), respectively, but contained in addition 10 nmol of CDP-glycerol. Note that a different scale is used in each experiment.*

diley has informed us that chemical evidence from his laboratory establishes the existence of a linkage region of three or four glycerol phosphate units between polyribitol phosphate and the cell wall peptidoglycan (10).

CDP-glycerol stimulated polyribitol phosphate synthesis, but neither CDP-choline nor CDP-ethanolamine had this effect (Table 1). Addition of excess CDP-ribitol did not substitute for CDP-glycerol.

When CDP-[^{14}C]glycerol was incubated with the cell wall preparations, radioactivity was present in the isolated cell wall and the formation of this product was greatly stimulated by the addition of UDP-GlcNAc but was not affected by the addition of CDP-ribitol or UDP-muramyl pentapeptide (Table 3).

The material incorporated into the cell wall from CDP-[^{14}C]glycerol yielded a mixture of glycerol phosphates after acid hydrolysis which after treatment with *E. coli* alkaline phosphatase showed the chromatographic behavior of glycerol when subjected to descending paper chromatography using butanol-pyridine-water (6:4:3) as the solvent (1). Treatment of the cell walls labeled from [^{14}C]CDP-glycerol with 60% HF (8) resulted in the formation of glycerol as the only radioactive product, suggesting that the glycerol phosphate residues in the cell walls are not substituted.

Maximal stimulation of polyribitol phosphate synthesis by CDP-glycerol was obtained at 2×10^{-5} M CDP-glycerol; increasing concentrations of CDP-ribitol did not substitute for

TABLE 3. *Incorporation of [^{3}H]ribitol and [^{14}C]glycerol into cell walls*[a]

Radioactive precursor (nmol)	Other additions (nmol)	Radioactivity in cell wall (pmol)	% Released by lysozyme
CDP-ribitol (25)	UDP-GlcNAc (1,000) CDP-glycerol (10)	908	13
CDP-ribitol (25)	UDP-GlcNAc (1,000) UDP-muramyl pentapeptide (100)	214	35
CDP-ribitol (25)	UDP-GlcNAc (1,000) UDP-muramyl pentapeptide (100) CDP-glycerol (10)	519	38
CDP-glycerol (10)	None	96.4	2.9
CDP-glycerol (10)	UDP-GlcNAc (1,000)	335	1.0
CDP-glycerol (10)	UDP-GlcNAc (1,000) CDP-ribitol (25)	276	5.2
CDP-glycerol (10)	UDP-GlcNAc (1,000) CDP-ribitol (25) UDP-muramyl pentapeptide (100)	312	14
UDP-muramyl pentapeptide (50)	UDP-GlcNAc (1,000) CDP-glycerol (10) CDP-ribitol (100)	1,747	86

[a] Standard assay conditions were used with concentrations of nucleotide precursors indicated.

CDP-glycerol in this system. At equimolar concentrations neither cytidine triphosphate nor L-α-glycerol phosphate stimulated the reaction, but a mixture of the two was stimulatory, suggesting that CDP-glycerol pyrophosphorylase is present in the crude cell wall preparation and that the synthesis of CDP-glycerol from endogenous precursors in the presence of ATP may account for the synthesis of cell wall-linked polyribitol phosphate in the absence of added CDP-glycerol.

The data in Table 4 show that this prediction is correct. In the absence of ATP, the formation of cell wall-linked polyribitol phosphate was almost totally dependent on the addition of CDP-glycerol, although ATP had an additional stimulatory effect. Also shown in Table 4 is the fact that UDP-D-galactose could not substitute for UDP-GlcNAc in stimulating the synthesis of polyribitol phosphate.

A variety of preincubation experiments have been carried out to establish the sequence of reactions responsible for polyribitol phosphate synthesis. Incubation of the crude cell wall preparation in the absence of ATP resulted in irreversible inactivation of the enzyme. Enzyme activity was retained in the presence of ATP, but uridine or guanosine triphosphate had no effect. Preincubation with ATP and UDP-GlcNAc resulted in a preparation that showed decreased dependence on UDP-GlcNAc for polyribitol phosphate synthesis, but curiously enough preincubation with ATP, UDP-GlcNAc, and CDP-glycerol did not totally abolish the requirement for CDP-glycerol (Table 5).

These crude cell wall preparations also catalyzed the incorporation of *N*-acetylglucosamine from UDP-GlcNAc into SDS-insoluble material, in the absence of other nucleotides, but it is not clear whether these amino sugar residues were added to preexisting polyribitol phosphate or to other cell wall components. In the presence of UDP-muramyl pentapeptide a much larger incorporation of GlcNAc was observed, which was primarily into peptidoglycan.

In all of these experiments we found the formation of large quantities of soluble polymers that were released from the crude cell walls by boiling in SDS. The labeling pattern of this fraction was in every instance identical to that observed in the SDS-insoluble material, but the

TABLE 4. *Effect of CDP-glycerol and ATP on polyribitol phosphate synthesis*[a]

Additions (μmol)	Polyribitol phosphate in cell wall (pmol)
None	63
UDP-GlcNAc (1)	80
ATP (2.7)	113
UDP-GlcNAc (1) + ATP (2.7)	1,072
UDP-Gal (1) + ATP (2.7)	107
CDP-glycerol (0.01)	74
CDP-glycerol (0.01) + UDP-GlcNAc (1)	710
CDP-glycerol (0.01) + UDP-GlcNAc (1) + ATP (2.7)	1,574
CDP-glycerol (0.01) + ATP (1)	104

[a] Standard assay conditions, but ATP was added only to the reaction mixtures indicated. All reaction mixtures contained 25 nmol of CDP-[^{3}H]ribitol.

TABLE 5. *Effect of preincubation on polyribitol synthesis*[a]

Expt	Additions		Ribitol phosphate in cell walls (pmol)
	1st incubation	2nd incubation	
A	ATP		10.2
		UDP-GlcNAc	66
		UDP-GlcNAc, CDP-glycerol	212
		UDP-GlcNAc, ATP	69
	ATP, UDP-GlcNAc		50
		UDP-GlcNAc	63.6
		UDP-GlcNAc, CDP-glycerol	198
		UDP-GlcNAc, ATP	81
	ATP, UDP-GlcNAc, CDP-glycerol		57
		UDP-GlcNAc	103
		UDP-GlcNAc, CDP-glycerol	205
B	ATP		17
		UDP-GlcNAc	56
		CDP-glycerol	11
		UDP-GlcNAc, CDP-glycerol	248
	ATP, UDP-GlcNAc		16
		UDP-GlcNAc	28
		CDP-glycerol	125
		UDP-GlcNAc, CDP-glycerol	193

[a] The crude cell wall preparation was preincubated for 10 min at 25 C with additions indicated in a final volume of 0.15 ml. The reaction mixture was diluted to 1 ml, and the walls were collected by centrifugation and washed again with 1 ml of buffer. The cell walls were then incubated for 15 min with the additions indicated and 15 nmol of ^{3}H-labeled CDP-[^{3}H]ribitol (33 dpm/pmol). Clean cell walls were then isolated by normal procedure. The following quantities of nucleotides were used: UDP-GlcNAc, 200 nmol in the preincubation and 1,000 nmol in the second incubation; CDP-glycerol, 10 nmol; ATP, 2 nmol. Different enzyme preparations were used in experiments A and B.

structure of these polymers has not been further investigated.

DISCUSSION

The crude cell wall fraction that we used in these experiments is able to synthesize all cell wall components and link them together. It has previously been shown by Mirelman and Sharon (15) that this preparation synthesizes new peptidoglycan cross-links, and our data suggest that it synthesizes polyribitol phosphate and links it to peptidoglycan. Only a fraction of the polymers that are synthesized in this system are cross-linked to the preexisting cell wall, and the majority of the polymer made is present as SDS-soluble material.

The observations presented in this paper lead to a revised and more complex scheme for cell wall teichoic acid biosynthesis, which is shown in Fig. 5.

In this scheme polyribitol phosphate is first synthesized linked to LTC, this polymer is then substituted with *N*-acetyl-D-glucosamine residues, and presumably D-alanine residues, and this is then transferred to peptidoglycan that contains attached to it a linkage region containing glycerol phosphate and *N*-acetyl-D-glucosamine residues. It is possible that such a linkage region also exists in cell walls that contain a polyglycerol phosphate teichoic acid, but since both the linkage region and the major chain would be derived from CDP-glycerol, the linkage region would not have been detected in experiments such as those carried out by Wyke and Ward (22).

Several assumptions underlie the scheme in Fig. 5. The most important is that LTC[ribitol phosphate]$_{30}$ is the only product observed when membranes are incubated with CDP-ribitol (5, 6); however, the direct demonstration that the exogenous LTC[ribitol phosphate]$_{30}$ can transfer polyribitol phosphate to the cell wall has not yet been made. Added LTC[ribitol phosphate]$_{30}$ appears not to be used by our preparations of crude cell walls, and pulse chase experiments are inconclusive because of the large amount of soluble polymers made in this system.

ATP plays a complex role in this system; it is not simply required for synthesis of CDP-glycerol or for the formation of peptidoglycan cross-links, but appears to be essential for an early step in the linking of teichoic acid to peptidoglycan. Other nucleotide triphosphates such as uridine or guanosine triphosphate will not substi-

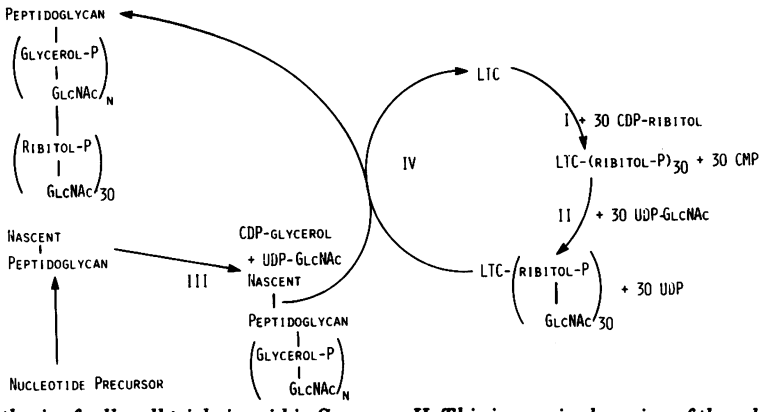

FIG. 5. *Synthesis of cell wall teichoic acid in S. aureus H. This is a revised version of the scheme in Fig. 1 to take into account the new observations presented in this paper. ATP, which also stimulates the reaction, appears to do so at an initial step involving the linkage of GlcNAc residues to peptidoglycan. Abbreviations as in Fig. 1.*

tute for ATP in preincubation experiments such as those shown in Table 5.

Fractionation of this system and elucidation of the individual steps will be necessary in order to clear up these ambiguities.

It is clear from these observations, however, that not only is the cell wall structure of *S. aureus* more complex than was believed heretofore, but also that the cell wall synthesis involves complex regulatory patterns. For example, the ratio of UDP-muramyl pentapeptide and UDP-GlcNAc appears to determine the rate of synthesis of the linkage region in vitro and may well do so in vivo. CDP-glycerol has acquired a new role in cell wall synthesis in organisms that contain polyribitol phosphate in the cell wall.

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