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Teichoic and Teichuronic Acids: Biosynthesis, Assembly, and Location

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INTRODUCTION

The walls of most gram-positive bacteria contain one or more secondary polymers which are covalently linked to the main wall structural component, peptidoglycan. The secondary polymers include the teichoic and teichuronic acids.

The teichoic acids were discovered in 1958 by Baddiley and his colleagues (18, 19) as a result of a search for a role for cytidine 5'-diphosphate (CDP)-glycerol and CDP-ribitol, which were isolated earlier from *Lactobacillus arabinosus*. From these results, and the examination of several other bacteria, the teichoic acids were defined as polymers of glycerol phosphate or ribitol phosphate which were substituted to various extents by ester-linked D-alanine and often also with glycosyl residues. It rapidly became clear, however, that many variations in these basic structures existed in bacteria, and the definition was broadened to include all surface-associated polymers containing glycerol phosphate or ribitol phosphate. Today the term teichoic acid has come to mean any one of a range of polymers possessing "phosphodiester groups, polyols and/or sugar residues, and usually, but not always, D-alanine ester residues" (20). It thus encompasses the wall teichoic acids which are covalently linked to peptidoglycan, certain capsular polymers found particularly in *Streptococcus pneumoniae*, and the membrane-associated lipoteichoic acids.

The first teichuronic acid was isolated from the walls of *Bacillus licheniformis* 6346 and characterized as a polymer containing equal proportions of *N*-acetylgalactosamine and D-glucuronic acid (100). The interrelationships of the acidic polymers of the wall were established when Ellwood and Tempest (53, 54) showed that walls of *B. subtilis* var. *niger* grown in continuous (chemostat) culture with limiting phosphate contained teichuronic rather than teichoic acid. Similar results were obtained with a number of other gram-positive bacilli and cocci. However, when phosphate was resupplied and the organisms were grown under Mg^{2+} limitation, their teichoic acid was again synthesized. Thus, the acidic wall polymers respond to the conditions prevailing in the environment, particularly to the concentration of Mg^{2+} available. These findings led Ellwood and Tempest to conclude that wall teichoic and teichuronic acids are involved in cation assimilation. This function for the teichoic acids was first proposed by Archibald et al. (13) on the basis of their polymeric structure, containing many equally spaced phosphate residues. Subsequently, Heptinstall et al. (89) showed that the presence or absence of wall teichoic acid affected the cells' avidity for Mg^{2+}. It should be noted, however, that even phosphate-limited organisms continue to synthesize lipoteichoic acid (54), and it appears that these membrane-associated polymers are indispensa-

ble for growth. In bacteria such as *Micrococcus luteus* (*lysodeikticus*), *M. flavus* and *M. sodonensis,* which completely lack teichoic acids, the lipoteichoic acid has been replaced by succinylated lipomannan (137, 141, 144). The walls of these organisms contain teichuronic acid, which in the case of *M. luteus,* is a polymer containing equal proportions of D-glucose and D-*N*-acetylmannosaminuronic acid (84, 130, 139). Thus, acidic surface polymers appear to possess a vital role in cellular function, perhaps through the binding of cations to provide the correct ionic environment at the cytoplasmic membrane.

The teichoic acids have been the subject of extensive investigation and have been the subject of several reviews which describe the structure and chemistry of the teichoic acids and also their biosynthesis, function, and immunological properties in detail (9, 10, 20, 21, 111, 113, 179, 180). However, recent advances have been made in our understanding of the biosynthesis of both teichoic acid and certain teichuronic acids and in particular of the mechanisms whereby these polymers become covalently linked to peptidoglycan. I will review these topics and our current understanding of the location of the various membrane and wall polymers. This latter information is largely restricted to teichoic acids. For detailed consideration of the structures and possible functions of the teichoic acids, the reader is directed to the various reviews cited above.

LOCATION OF TEICHOIC ACIDS

Initially, teichoic acids were extracted from both bacteria and their isolated walls by prolonged treatment with cold dilute trichloroacetic acid. However, particularly when whole organisms were examined, the results were confused by the presence in many bacteria of a closely related group of compounds which were also shown to be polymers of poly(glycerol phosphate). These could be extracted from whole bacteria although they were absent from isolated walls (48). These "intracellular" teichoic acids were subsequently shown to be located in the cytoplasmic membrane (158) and for this reason were renamed "membrane teichoic acids." Yet a third change in nomenclature occurred as a consequence of the increased knowledge available about their structure, and they are now recognized as the lipoteichoic acids (111, 113, 178, 179, 180). As this name implies, they consist of a teichoic acid chain, commonly D-alanyl-substituted 1,3-poly(glycerol phosphate), although glycosyl substituents may also be present attached to a glycolipid. The lipid moiety serves to anchor the molecule in the cytoplasmic membrane with the hydrophilic teichoic acid chain presumably extending away from the membrane surface and, in certain organisms, through the wall. This latter situation may occur in all organisms where lipoteichoic acid is present, but has only clearly been demonstrated where a wall teichoic acid is absent. In group D streptococci lipoteichoic acid is the group-specific antigen, and antisera raised against this "membrane component" will agglutinate whole organisms (154, 158). Similar results were obtained with *Lactobacillus fermenti,* although cells of *L. casei* were apparently unaffected by such treatment. However, by application of successive treatments of anti-lipoteichoic acid antiserum and ferritin conjugated to goat anti-rabbit γ-globulin, Van Driel et al. (167) showed that the surfaces of both organisms were labeled (Fig. 1). The differences between the two strains were quantitative rather than qualitative. Thus, as might be expected from the above results, the labeling of *L. fermenti* was more or less confluent, whereas in *L. casei* it was significantly less and patchy. Clearly the extent to which a "membrane component" can act as a surface antigen will be dependent upon several factors, including (i) wall thickness and density in terms of packing of the peptidoglycan, this, in turn, will depend on the length of the glycan chains and the extent of cross-linkage, and (ii) the length of the lipoteichoic acid and its conformation within the wall. A further complicating factor may come from the release of lipoteichoic acids from the membrane and their subsequent reassociation, perhaps through ionic bonds to peptidoglycan, with the outer surface of organisms. Although the extent to which a reassociation of this type could occur remains unclear, the release of large amounts of lipoteichoic acid by several oral streptococci is well documented (101, 117). The two lactobacilli investigated in detail differ markedly in the composition and lysozyme sensitivity of their walls (108, 111), and such differences in wall chemistry might well underlie the differences observed in surface exposure and, hence, penetration of the walls by lipoteichoic acid. Localized changes in wall composition may also explain the patchiness of ferritin labeling observed in *L. casei.* On the basis of their findings Van Driel et al. (167) proposed a model of the wall membrane (envelope) of a gram-positive organism in which the polar poly(glycerol phosphate) chains extend into and, in some cases, through the wall in a highly oriented state. Such an arrangement would provide an additional physical link between the membrane and the wall where the glycolipid terminus of the lipoteichoic acid is held by hydrophobic and possibly ionic bonds in the membrane and by ionic interactions to the

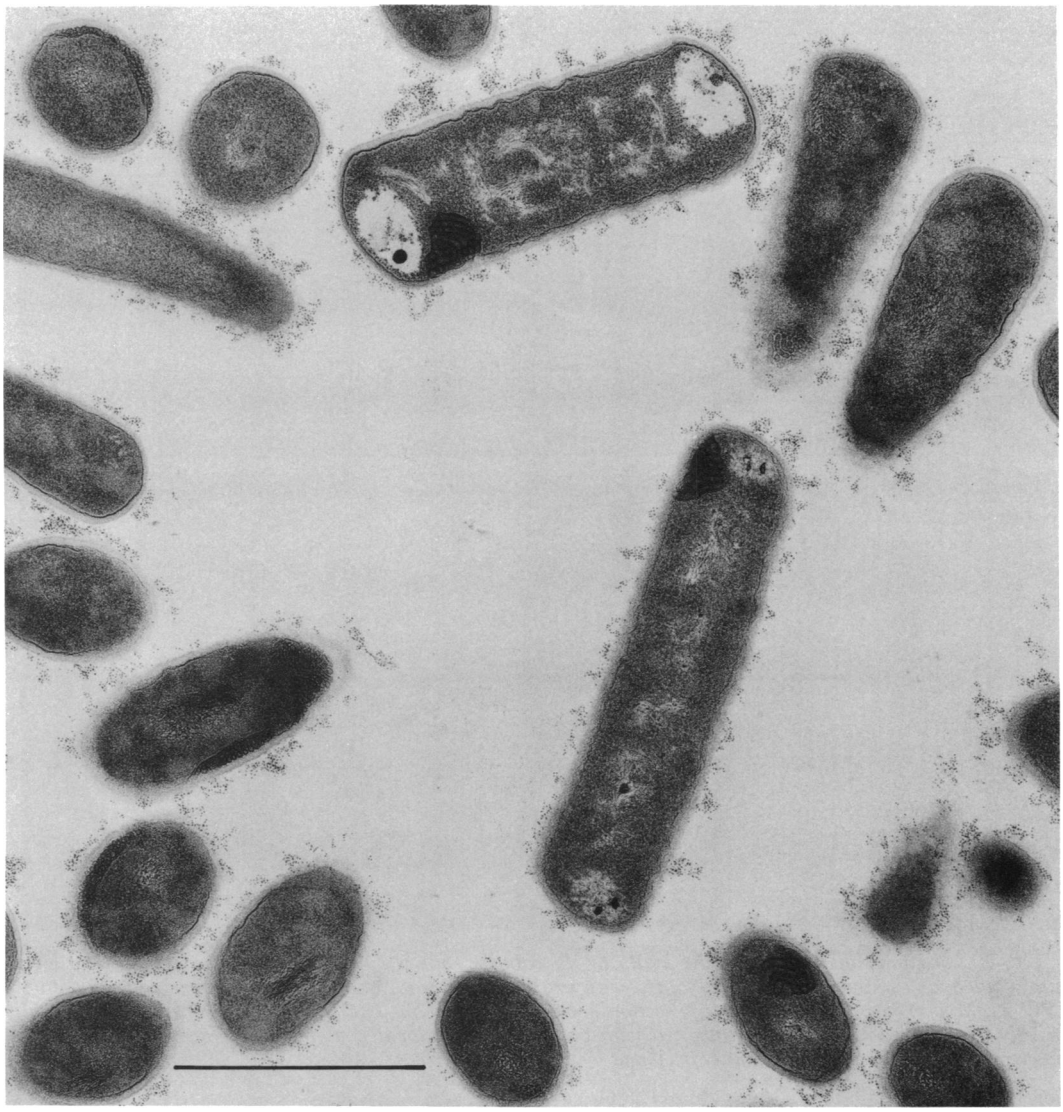

FIG. 1. *Thin sections of L. fermenti treated with antiserum directed against lipoteichoic acid and ferritin-labeled antibody directed against the rabbit gamma globulin. Ferritin conjugate is located on the outside of the wall showing the presence of lipoteichoic acid at the cell surface. Bar, 1 μm. Figure kindly provided by A. J. Wicken and taken with permission from Van Driel et al. (167).*

peptidoglycan. It is assumed that another physical link between the membrane and wall occurs through the growing peptidoglycan chains where the reducing terminus is linked to the undecaprenol lipid carrier held in the membrane. The chain then extends from the biosynthetic site in the membrane and is covalently linked via peptide cross-links to the wall (173). For further consideration of the structure, occurrence, and possible functions of the lipoteichoic acids the reader is directed to four recent reviews (111, 113, 179, 180).

Wall Teichoic Acids

The walls of most gram-positive bacteria appear thick and relatively structureless when seen in section. However, depending upon the nature of the various fixation and poststaining methods used, walls from several species show a triple- or double-layered appearance (Fig. 2). Various authors have concluded that such layering results from a difference in composition of the layers and have speculated that the teichoic and teichuronic acids are restricted to the outer layers

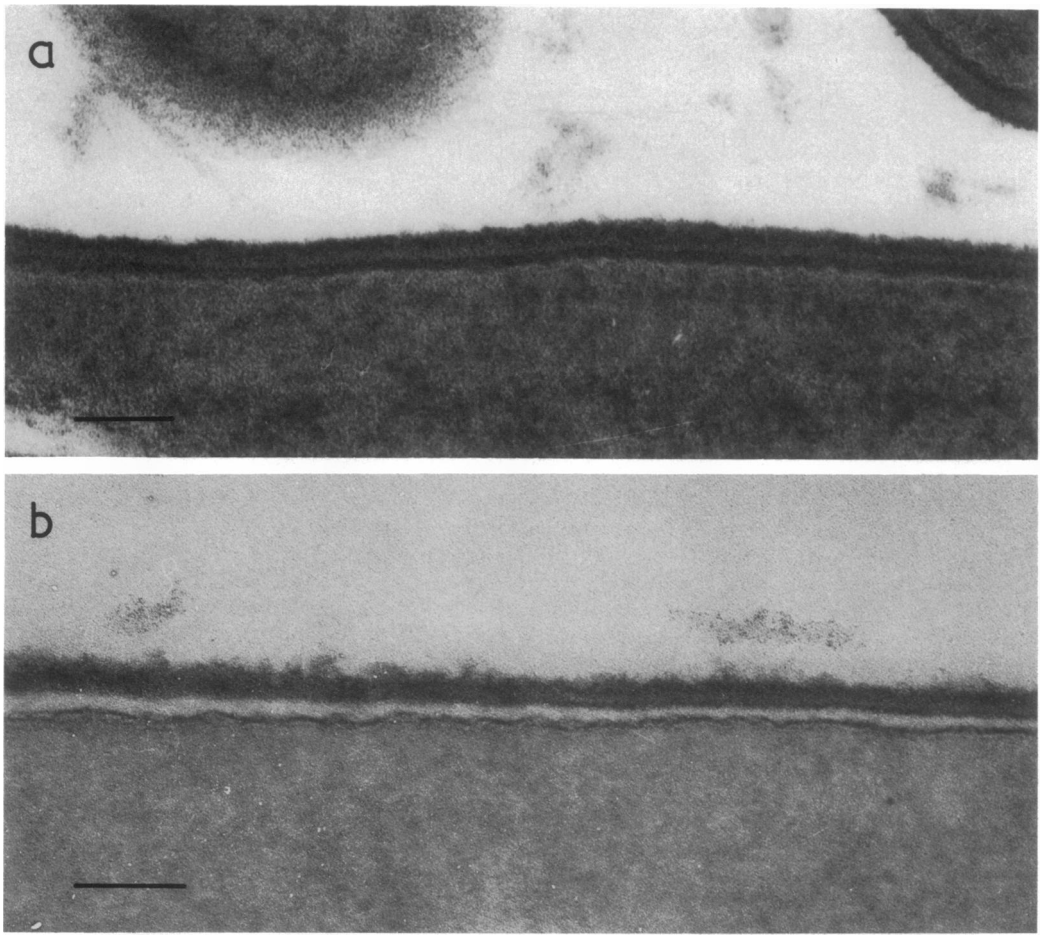

FIG. 2. *Longitudinal sections of the walls (a) B. subtilis showing tribanded structure after fixation in glutaraldehyde-osmium, section poststained with uranium and lead, and (b) B. licheniformis fixed with 1% OsO_4 in the presence of ruthenium red, section not post-stained (36). The bulk of the staining, which results mainly from interaction of ruthenium red with teichoic acid, is located at the outer faces of the wall and membrane. Bars, 1 μm. Figure kindly provided by I. D. J. Burdett.*

(134, 176). On the other hand, Millward and Reaveley (124) concluded that the trilamellar appearance of unstained walls of *Staphylococcus aureus* and *B. licheniformis* resulted from variations in the packing of peptidoglycan rather than in the distribution of secondary polymers. Results in agreement with this conclusion were reported by Garland et al. (68) after studying the effects of various staining and chemical binding treatments in whole cells and walls of *Streptococcus faecalis*. Earlier studies (25, 67), using gold-coupled concanavalin A as a marker for glucosylated teichoic acid, were also interpreted as showing the teichoic acid to be distributed throughout the wall. A layered arrangement of teichoic acid and peptidoglycan was proposed by Nermut (134) after studying walls of *Bacillus megaterium* extracted with hot formamide. This procedure decreased the thickness of the wall by approximately 50%. Walls of *B. megaterium* KM had previously been shown to contain approximately equal amounts of peptidoglycan and a teichoic acid-like polymer (33, 69, 70). On the basis of these results and the knowledge that formamide treatment had been used to isolate pure peptidoglycan from *B. megaterium* (69) and streptococci (112), Nermut assumed that the formamide-insoluble residue was peptidoglycan. However, no chemical investigation of either this residue or the formamide-soluble material was undertaken, and the conclusions of this study must be judged accordingly. Support for a layered arrangement appeared to come from observations on a temperature-sensitive

mutant of *Bacillus subtilis* which at the restrictive temperature had grossly reduced amounts of teichoic acid in its walls (46). Under these conditions, the walls did not show a trilamellar structure, whereas this structure was regained together with wall teichoic acid on transfer to the permissive temperature. On the other hand, extraction of the walls of group A and group C streptococci with various reagents which removed from 20 to 95% of the rhamnose present did not alter the trilamellar appearance, although there were considerable reductions in thickness (169). Similar results were obtained with walls of *S. faecalis* (68, 165) and *L. arabinosus* (13), from which the secondary polymers were extracted with trichloroacetic acid. Clearly, it is not possible to reach firm conclusions about the relationship between the banded appearance of gram-positive walls and the distribution of polymers within them. A simple layered arrangement does, however, seem highly unlikely.

A proportion of the teichoic acid present in walls must be located at the outer surface of the wall where it forms part of the receptor for various bacteriophages and where it can react with specific antibodies and lectins. In these latter cases agglutination of the walls and whole cells often ensues. Reaction particularly with bacteriophages and antibodies has been taken to imply a surface location since it is presumed these cannot penetrate the wall. No description has been published of a bacteriophage which requires teichuronic acid as part of its receptor. Moreover, this polymer appears to be non-immunogenic and in this respect resembles the hyaluronic acid capsules of certain streptococci (94).

The reaction of specific antibody with teichoic acid as a probe for the location of the teichoic acid in walls of bacilli was first studied by Burger (37). Walls of *B. subtilis* 3610 bound only 19% of the total possible antibody, although this value increased greatly when the walls were subjected to brief lysozyme treatment. Surprisingly, whole cells bound only slightly less antibody than did isolated walls, an observation which led Burger to conclude that relatively little teichoic acid was located on the inner surface of the wall. Similar observations were made with *B. subtilis* W23. In contrast, 71% of the possible antigenic sites in *B. licheniformis* 9945A bound antibody, a value which was only marginally increased by lysozyme treatment. This marked difference in the effect of lysozyme, which in *B. subtilis* was thought to loosen the peptidoglycan network to allow access of antibodies to teichoic acid buried in the wall or to alter steric configuration, making them more suitable for antibody binding, may be interpreted to suggest a surface location for teichoic acid in *B. licheniformis*. Alternatively, the differences might be explained by differential access of antibody molecules to teichoic acid situated within the wall. Little information is available concerning the ability of proteins to penetrate walls or steric factors influencing antibody binding. It also seems possible that availability of teichoic acid binding sites could be influenced by the extent to which autolytic enzymes have been able to act during wall preparation. Both *B. licheniformis* and *B. subtilis* are known to possess similar autolysins cleaving the glycan chains and peptide side chains, although strains of *B. licheniformis* are generally considered to be more autolytic (148).

Concanavalin A (ConA) has also been used as a probe for the location of glucosylated poly(glycerol phosphate) teichoic acid in *B. subtilis* 168 (28, 51). Electron microscopy of sectioned bacteria and isolated walls previously treated with ConA revealed a discontinuous "fluffy layer" on their outer surfaces. This layer was absent from untreated or methyl-α-D-glucoside-treated preparations and from a mutant unable to synthesize glucosylated teichoic acid. On the basis of their findings, Birdsell et al. (28) proposed that the polyvalent lectin molecules bind adjacent teichoic acid chains extending from the bacterial surface to produce the fluffy layer. Subsequently, [^{14}C]ConA was used to determine that walls of *B. subtilis* 168 bound only 50 to 56% of the amount bound by autolyzed or lysozyme-treated preparations (51). However, in neither case did the increase in lectin binding parallel the rate of wall hydrolysis. After 10 min of lysozyme treatment, only small changes in turbidity were observed, and the amount of soluble phosphorus released was 15% of the total, whereas the amount of lectin bound increased by 39%. Similar results were obtained upon autolysis of the wall preparations. These results were also interpreted as showing that a loosening of the peptidoglycan network allowed penetration of the lectin to bind with teichoic acid buried within the interior of the wall. Microscopic examination of bacteria treated with fluorescein-labeled ConA revealed that the lectin bound to the whole surface of the organisms, with increased labeling of the septa. Whether this represents a difference in teichoic acid content or simply the presence of a double wall remains unclear. Thus, approximately half of the wall teichoic acid appears to be exposed on the surface of *B. subtilis* 168, with the remainder distributed within the wall. Surprisingly, these studies were unable to demonstrate the presence of any teichoic acid on the inner surface of walls,

although Beveridge and Murray (27) subsequently showed binding of ferittin-conjugated ConA to both outer and inner surfaces of *B. subtilis* 168 walls.

More recently, the insertion of newly synthesized teichoic acid and the subsequent growth of the wall was studied in a series of elegant experiments by Archibald and his colleagues (3, 4, 11, 12, 163). Using chemostat cultures of *B. subtilis* W23 grown under conditions of potassium or phosphate limitation, they used the binding of bacteriophage SP50 as a probe for glucosylated ribitol phosphate teichoic acid (16) (Fig. 3). As described earlier, phosphate limitation results in the replacement of wall teichoic acid by teichuronic acid. Walls from organisms grown under these conditions did not bind the bacteriophage. When these cultures were supplied with phosphate, teichoic acid was again synthesized and incorporated into the wall, where its presence could be measured either as phosphate or glucosylribitol (2). Bacteria harvested from cultures undergoing transition from potassium to phosphate limitation and from cultures undergoing the reverse transition were examined for their ability to bind bacteriophage. Surprisingly, organisms harvested in the early stages after the addition of phosphate and containing only small amounts of newly synthesized teichoic acid were relatively inefficient in phage binding. In contrast, bacteria containing similar small amounts of teichoic acid, but harvested in the late stages of the reverse transition (i.e., potassium to phosphate limitation), had a much greater ability to bind phage. Thus, newly synthesized teichoic acid appeared to be relatively ineffective in promoting phage binding. Subsequent experiments with walls isolated from cultures growing under phosphate limitation, but which had been pulsed with excess phosphate, showed this to be due to the inaccessibility of newly synthesized phage receptor to the phage (4, 12). Electron microscopy of thin sections of walls of bacteria harvested soon after incorporation of teichoic acid commenced, revealed that bacteriophage SP50 was bound only to their inner surfaces (Fig. 3B). Bacteriophage bound maximally to the outer surface only after a lapse of approximately 0.75 to 1 generation, the time necessary for the newly synthesized teichoic acid to reach the cell surface. Walls isolated from organisms harvested after incorporation of teichoic acid had ceased bound the phage almost exclusively to their outer surfaces (Fig. 3C). A small number of phage particles were observed binding to the inner surface of the walls at cell poles. In potassium-limited bacteria transferred to phosphate limitation, binding of SP50 continued until almost all the wall teichoic acid was lost. Eventually, bacteriophage were located only at the poles of cells, suggesting these areas were conserved to a greater extent than were the peripheral walls (3, 4, 11). The findings of Archibald and his colleagues confirm that newly synthesized teichoic acids are synthesized and incorporated into the wall at the wall-membrane interface. With continued synthesis, they pass through the thickness of the wall to appear some time later on the outer surface. Similar conclusions with regard to wall assembly in *B. subtilis* were reached by Pooley (142, 143) in his study of the turnover of pulse-labeled peptidoglycan and by Fan et al. (58) in an electron microscopic study of bacteria that had resumed wall synthesis after being plasmolysed. This being the case, the inability of others to demonstrate increased binding of either antibody or ConA to isolated walls when compared with bacteria is somewhat surprising. In particular, the absence of a fluffy layer on the inner surface of walls treated with ConA suggests marked differences in the organization of the wall polymers on the two wall surfaces.

Although this recent work established that teichoic acids in particular are located throughout the wall, rather than being restricted to one layer, relatively little is yet known concerning the organization of the various polymers within the wall. Earlier work showed that the wall polymers were held in covalent linkage and that prolonged extraction of walls with cold trichloroacetic acid resulted in the solubilization of teichoic acid chains terminating in phosphomonoester residues (14, 17, 23). On the other hand, enzymic hydrolysis of walls released teichoic acid-peptidoglycan and teichuronic acid-peptidoglycan complexes from which phosphomonoester groups were absent. It was concluded that linkage to peptidoglycan involved a terminal phosphodiester bond to a residue in the glycan chain (71, 162). The subsequent isolation of muramic acid phosphate from acid hydrolysates of walls and isolated teichoic acid-glycan complexes from various bacteria gave direct support to this idea (1, 40, 116). Moreover, muramic acid phosphate was also shown to be involved in the linkage of polysaccharides to peptidoglycan in *L. casei* and *L. fermenti* (109, 110) and of teichuronic acid in *B. licheniformis* (91). It was thought that teichoic acids were linked directly by a phosphodiester bond formed between the terminal alditol residue of the teichoic acid chain and a muramyl residue in the glycan. However, at the time Hay et al. (86) argued that such a linkage should not be preferentially hydrolyzed by mild acid or alkali treatment when compared with other phosphodiester linkages in the polymer chain. The recent detailed investigations of

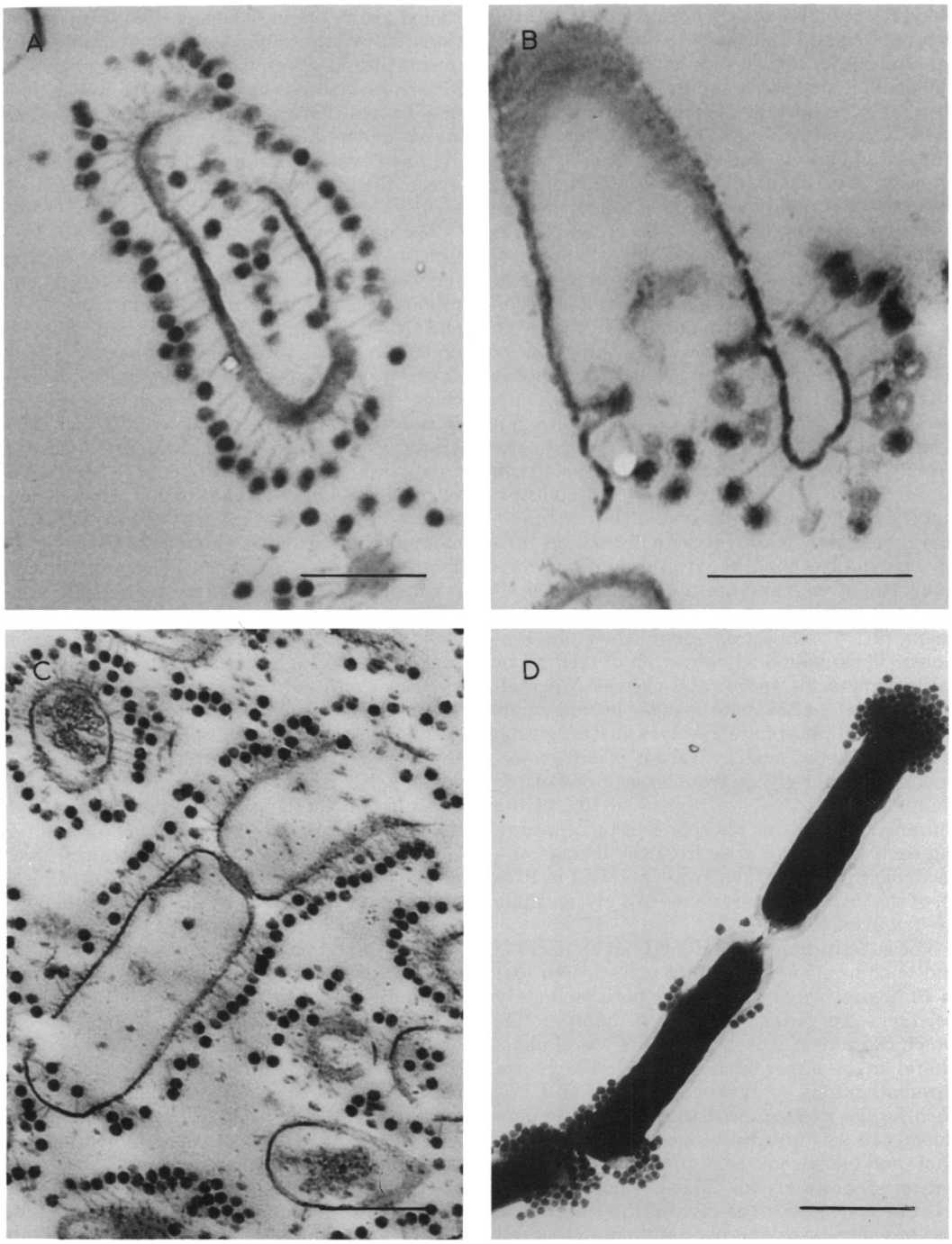

FIG. 3. *Electron micrographs of B. subtilis W23 and isolated walls showing the location of adsorbed bacteriophage SP50 as a marker for glucosylated ribitol teichoic acid. Walls from organisms grown in phosphate-containing medium (A) bind phage particles on both surfaces. After a pulse of phosphate to phosphate-limited organisms the phage particles bind initially to the inner surfaces of the wall (B; organisms harvested 1 to 1.5 h after the pulse) and finally only to the outer surfaces (C; organisms harvested 2.25 to 2.75 h after the pulse). With continued growth (D; organisms harvested 12 to 13 h after the pulse) adsorbed phage particles were only found at cell poles. Bars, 0.5 μm (A, B and C) and 1 μm (D). Figures kindly provided by A. R. Archibald and taken with permission from references 12 and 16.*

Baddiley and his colleagues established that the acid and alkali lability of the linkage resides in an oligomeric linkage unit interposed between the teichoic acid chain and the muramyl residue in the glycan. Details of these chemical studies are in a recent review (47). In brief, the linkage unit of *S. aureus* and various other bacteria showing considerable differences in teichoic acid structure is formed in each case from a residue of *N*-acetylglucosamine (GlcNAc) and three units of glycerol phosphate. The acid lability of the linkage resides in the bond formed as a 1-phosphate of GlcNAc (linked to the 6 position of muramic acid) and the alkali lability in the phosphodiester bond from the 4-position of GlcNAc to the first glycerol phosphate residue of the trimer. A linkage unit also containing GlcNAc and terminating in a 1-phosphate, but lacking glycerol phosphate, is involved in the attachment of teichuronic acid to peptidoglycan in *M. luteus* (85, 149, 161, 177). In contrast, linkage of the teichuronic acid of *B. licheniformis* appears to be direct with formation of a phosphodiester bond between a muramyl residue in the glycan and the reducing terminal *N*-acetylgalactosamine residue of the polymer chain (91; J. B. Ward, unpublished observations). Thus, mild acid extraction of teichoic or teichuronic acids specifically cleaves *N*-acetylhexosamine 1-phosphate bonds, leaving muramic acid 6-phosphate residues in the glycan chains. Stronger acid hydrolysis of either secondary polymer-glycan complexes or of isolated walls appears to release from 5 to 10% of the muramyl residues as phosphate esters. Assuming each of these to arise from the linkage of a secondary polymer, then an average of 1 in 10 to 20 of the disaccharide residues in a glycan chain are involved.

The substitution of peptidoglycan by teichoic and teichuronic acid was first investigated (64) in *B. licheniformis* 6346, where both secondary polymers are present, and in *B. subtilis* 168, which contains glucosylated poly(glycerol phosphate) as the major teichoic acid. Ion exchange chromatography of lysozyme digests of *B. licheniformis* walls showed that 55% of the peptidoglycan was unsubstituted and that the teichoic and teichuronic acids appeared to be present on separate glycan chains. Since lysozyme treatment degrades the glycan, this conclusion was based on lysozyme-resistant material of relatively short average glycan chain length. An apparently similar degree of substitution was found in wall preparations of *B. subtilis* 168, allowed to autolyze under conditions in which the *N*-acetylmuramyl-L-alanine amidase, but not the β-endo-*N*-acetylglucosaminidase, was thought to be active. The autolysates were fractionated by ion-exchange chromatography into unsubstituted glycan (60 to 70% of the total) and a teichoic acid-glycan complex. However, it is now known that the glucosaminidase is in fact active under these conditions, and the results obtained will be influenced by the extent to which the glycan chains have been degraded (170).

The question of secondary polymer substitution in *B. licheniformis* and *B. subtilis* was reinvestigated using walls prepared under conditions known to minimize autolysis (J. B. Ward and N. L. Hemmings, unpublished observations). In these wall preparations the glycan chains are considerably longer, with average chain lengths of 70 to 450 disaccharide units in *B. subtilis* (66). After treatment of the walls with *Myxobacter* sp. *N*-acetylmuramyl-L-alanine amidase (57) to remove peptide side chains, the secondary polymer-glycan complexes were fractionated by ion exchange or ConA affinity chromatography. In both *B. licheniformis* and *B. subtilis* less than 15% of the glycan was unsubstituted. In *B. licheniformis* the teichoic acid-glycan and teichuronic acid-glycan complexes could be separated, thus confirming the earlier suggestion of Hughes et al. (92), whereas in *B. subtilis* ConA affinity chromatography separated glycan chains substituted with glucosylated poly(glycerol phosphate) teichoic acid from those substituted with the minor teichoic acid, poly (glucosyl-*N*-acetylgalactosamine phosphate). These results strongly suggest that the biosynthesis and linkage of teichoic and teichuronic acids to peptidoglycan is an ordered rather than a random process. Since secondary polymers are known to be linked to peptidoglycan synthesized concomitantly (119), it seems possible that the enzymes responsible for the biosynthesis and linkage of both polymers are present in the membrane as specific complexes.

Earlier, hydrolysis of walls of *Staphylococcus lactis* I3 with *Flavobacter* sp. amidase also gave separable glycan and teichoic acid-glycan complexes (15). In this case, 58% of the glycan, average chain length 7 to 8 disaccharide units, was unsubstituted, whereas the remaining 42% of similar average chain length was substituted with one teichoic acid chain per glycan chain. However, both muramic acid and glucosamine reducing terminals were present in the glycan, and the extent to which the wall had been autolysed remains unclear. Two possible arrangements for the teichoic acid-glycan complexes in the wall were considered involving either a radial or a parallel disposition of the glycan chains relative to the surface of the wall. In the latter

structure the thickness of the wall would require multiple layers of glycan, and presumably much of the teichoic acid would be buried within the wall. In view of the observations made subsequently with bacteriophage and lectin binding it seems almost certain that a parallel arrangement or something similar will occur in most, if not all, bacteria. The length of the glycan chains alone precludes a radial arrangement in the walls of the bacilli (170).

BIOSYNTHESIS OF TEICHOIC AND TEICHURONIC ACIDS

In common with other wall polymers, the biosynthetic enzymes for teichoic and teichuronic acids appear to be located in the cytoplasmic membrane, whereas those for the formation of the nucleotide precursors are soluble, i.e., cytoplasmic enzymes. Many of the earlier studies of biosynthesis used particulate enzyme preparations, obtained by differential centrifugation either from osmotically lysed protoplasts prepared by lysozyme digestion of the peptidoglycan or more commonly from sonically disrupted organisms. With the finding that in vitro peptidoglycan synthesis was enhanced in membranes obtained from alumina-ground bacteria (5), this method was generally adopted as the method of choice for studies on wall polymer biosynthesis. It rapidly became apparent, however, that membranes from many gram-positive bacteria did not catalyze the final transpeptidation reaction leading to the formation of cross-linked peptidoglycan. This was only achieved when wall membrane preparations were brought into use (125, 127, 128, 171). It is assumed that these preparations retain some of the spatial relationships existing in the intact organism between the membrane-bound enzymes and the wall. Moreover, the products of biosynthesis can easily be fractionated on the basis of their solubility in hot detergent (sodium dodecyl sulfate is generally used). This procedure distinguishes between newly synthesized polymers which have become covalently linked to the wall and are sodium dodecyl sulfate insoluble and detergent-soluble material which is presumably associated with the membrane. Finally, an additional refinement has been the development of permeabilized cell systems in which the integrity of the cytoplasmic membrane has been destroyed by treatment with organic solvents (toluene is commonly used for gram-positive organisms), and the bacteria have been made permeable to exogenously added nucleotide precursors. This type of preparation was first used to study nucleic acid synthesis in *Escherichia coli* (168) and was subsequently adapted by Fan and his colleagues for the studies of peptidoglycan synthesis in *B. megaterium* (153). Recently, permeabilized bacteria have been widely used to investigate wall biosynthesis in both gram-positive and gram-negative organisms (129, 140). It seems likely that enzyme preparations of this type, which can also be fractionated by detergent treatment, will retain the closest association of the biosynthetic enzymes with the pre-existing wall.

Nucleotide Precursors

The teichoic acids appear to differ from all other wall polymers in that the discovery and characterization, including chemical synthesis, of the nucleotide-linked precursors preceded the discovery and isolation of the polymers themselves. Thus, in the late 1950s Baddiley and his colleagues (for references see reference 21) isolated and characterized CDP-glycerol and CDP-ribitol from *L. arabinosus*. Subsequently, enzymes synthesizing these precursors, CDP-glycerol pyrophosphorylase and CDP-ribitol pyrophosphorylase, were demonstrated in extracts from a number of gram-positive bacteria (155). D-Ribitol 5-phosphate is produced by a reduced nicotinamide adenine dinucleotide-dependent reduction of D-ribulose 5-phosphate (73), and D-glycerol 1-phosphate is assumed to be derived via glycolysis. Other glycosidic substituents are derived from uridine-linked precursors. The activation of D-alanine and the esterification of teichoic acids are described below.

In the case of the teichuronic acids of bacilli the nucleotide precursors are uridine 5'-diphosphate (UDP)-*N*-acetylgalactosamine and UDP-glucuronic acid. Extracts of *B. licheniformis* 9945 (then *B. subtilis*) were shown to contain a UDP-GlcNAc 4'-epimerase (72). UDP-glucuronic acid is derived from UDP-glucose by the action of UDP-glucose dehydrogenase.

The novel precursor of the teichuronic acid of *M. luteus* is UDP-*N*-acetylmannosaminuronic acid (UDP-ManNAcUA) which again is formed from UDP-GlcNAc. The reaction in *E. coli* proceeds in two stages involving the epimerization of UDP-GlcNAc to UDP-*N*-acetylmannosamine (UDP-ManNAc) followed by oxidation at C-6 as follows:

$$\text{UDP-GlcNAc} \rightleftharpoons \text{UDP-ManNAc}$$

$$\text{UDP-ManNAc} \xrightarrow{\text{NAD}^+} \text{UDP-ManNAcUA}$$

The two enzymes were purified from *E. coli*, where ManNAcUA is present in a capsular polysaccharide (98, 103). Dehydrogenase activity was also demonstrated in *M. luteus*, suggesting

that the micrococci utilize a similar mechanism of synthesis.

Biosynthesis of Teichoic Acids

Poly(glycerol or ribitol) phosphate polymers. The initial studies on the biosynthesis of teichoic acids were those of Burger and Glaser (38) using particulate enzyme preparations from sonically disrupted *B. licheniformis* and *B. subtilis*. These experiments established that CDP-glycerol was the only substrate required for poly(glycerol phosphate) polymerase in a reaction which required the presence of either 40 mM Mg^{2+} or 10 mM Ca^{2+} for optimal activity. Degradation studies and end-group analysis of the newly synthesized material were consistent with the product being 1,3-poly(glycerol phosphate). The average chain length of the newly synthesized polymer ranged from 7 to 32 glycerol phosphate units, and ultracentrifugation and chromatographic evidence led the authors to conclude that teichoic acid already present in the enzyme preparation was being used as an acceptor. The presence of acceptor teichoic acid was also noted when the enzyme was prepared from protoplasts of *B. subtilis*, although almost all of the wall teichoic acid had been removed. These observations are particularly interesting in view of the subsequent studies on the role of lipoteichoic acid carrier (LTC) which are described in detail below.

Similar findings were later reported by Kennedy and Shaw (105), although in this case the average chain length of the newly synthesized material was only 1.2 units. Periodate oxidation of the glycerol terminus released 45% of the incorporated radioactivity as formaldehyde. This would not have occurred if the newly incorporated glycerol phosphate had been added at the phosphate terminus where the glycerol moiety would be protected from oxidation. Thus, the direction of synthesis of teichoic acid differs from that found for several other wall (or envelope) components, including peptidoglycan (173), teichuronic acid in *B. licheniformis* (J. B. Ward, unpublished observations), and the O antigen (O side chain) of the lipopolysaccharide of *Salmonella anatum* (146). In each of these polymers newly synthesized units are added at the reducing terminal of the growing chains.

More recently poly(glycerol phosphate) polymerase was solubilized and partially purified from membranes of *B. subtilis* (118). Activity of the purified enzyme was found to be dependent on the presence of a heat-stable acceptor. Both were solubilized by Triton X-100 treatment of the isolated membranes, and separation of the two components was achieved by sucrose density gradient centrifugation or ion exchange chromatography. In the presence of the acceptor, glycerol phosphate units were transferred from CDP-glycerol, and a continuous increase in the average chain length of poly(glycerol phosphate) was obtained. Analysis of the acceptor showed that it contained equimolar amounts of glycerol and phosphate together with much smaller amounts of glucose, glucosamine, and fatty acids. Thus, in composition it resembled the lipoteichoic acids obtained from the membranes of several gram-positive bacteria by phenol extraction. However, attempts to use phenol-extracted material as the acceptor in assays of polymerase activity gave extremely variable results. These findings, possible reasons for which are described in a later section, led directly to the concept of a specific teichoic acid carrier involved in biosynthesis (52, 62). This was subsequently designated LTC. The involvement of LTC in biosynthesis is discussed in more detail below.

Recently, the incorporation of 3,4-dihydroxybutyl-1-phosphonate, an analog of glycerol phosphate, into the walls of *B. subtilis* 168 and *B. subtilis* W23 was reported (107). The organisms differ in their response to treatment with the analog, its effects being bacteriostatic for *B. subtilis* 168 and bactericidal for strain W23. It appears the analog can replace glycerol phosphate residues in phospholipids as well as in poly(glycerol phosphate) teichoic acid in strain 168 and probably in the linkage unit of both organisms. As described below, incorporation of analog residues inhibits the biosynthesis of lipoteichoic acid (49). It remains unclear which of these substitutions is responsible for the observed effects on growth.

Simultaneously with the studies on 1,3-poly(glycerol phosphate) synthesis, similar particulate enzyme preparations from *L. plantarum* (74) and *Staphylococcus aureus* Copenhagen (99) were used to investigate the synthesis of poly(ribitol phosphate) from CDP-ribitol. The polymerase from *S. aureus* was present in a particulate fraction, presumably membranes, sedimenting between 30,000 and 100,000 × *g*. Optimal polymer synthesis required relatively high concentrations (10 to 30 mM) of divalent cations and was inhibited by the addition of cytidine 5′-monophosphate (CMP). The presence of potential acceptors such as isolated walls, peptidoglycan-teichoic acid complexes, or poly(ribitol phosphate) itself were without effect. In contrast, the polymerase of *L. plantarum* remained closely associated with the wall of the organism, and the enzyme preparation used was similar to the wall membrane preparations de-

scribed earlier; preparations substantially free of wall were considerably less active. The polymer synthesized by *L. plantarum* had an average chain length of 7.9 units, although in this case, and in *S. aureus*, the newly synthesized material appeared to be linked to some unidentified acceptor.

Poly(ribitol phosphate) polymerase has now been purified by ion-exchange and gel filtration chromatography of Triton X-100 extracts of membranes from *S. aureus* H (59, 60). The purified enzyme, which showed a requirement for spermidine (or divalent cations), phospholipid, and detergent, was totally dependent for activity on the presence of LTC acting as the acceptor for the newly polymerized units. The carrier was also purified from the same membranes. It could, however, be replaced by similar preparations from a number of other gram-positive bacteria. Unlike the synthesis of poly(glycerol phosphate) described above, the formation of poly(ribitol phosphate) on LTC appeared to be an ordered process. LTC and LTC loaded with poly(ribitol phosphate) were separated by polyacrylamide gel electrophoresis. When the course of polymerization was followed by this method, material of an intermediate size was not found. Thus, polymerization appeared to occur by the addition of ribitol phosphate to single chains rather than the random addition of units to several chains simultaneously, and the length of the poly(ribitol phosphate) chains was limited. The average chain length determined by periodate oxidation and isolation of the radioactive formaldehyde produced was 30 units, a value in reasonably good agreement with that found for teichoic acid isolated from walls of *S. aureus*. The conclusion that teichoic acid chains of a defined length were formed was supported by the finding that preloaded LTC no longer functioned as an acceptor of additional ribitol phosphate units. The mechanism by which the length of the newly synthesized polymer is controlled remains unknown. A possible mechanism envisaged by Fiedler and Glaser (60) is the formation of a hydrophobic complex of polymerase, phospholipid, and LTC in the presence of detergent. As the number of units of ribitol phosphate linked to LTC increases, the newly synthesized polymer becomes increasingly hydrophilic, with the result that chains containing 30 ribitol phosphate residues become dissociated from the complex. In contrast, the poly(glycerol phosphate) polymerase of *B. subtilis* synthesized chains of widely differing lengths (118). Since both enzymes utilized LTC from either organism equally well it suggests that the differences in size of chains polymerized is a function of the polymerases themselves and not of the LTC. The mechanism of attachment of the poly(ribitol phosphate) chain to LTC and the use of the carrier during synthesis in vivo are discussed in detail below.

Glycosylation of poly(glycerol or ribitol) phosphate teichoic acids. Glucosylation of both teichoic acids involves the transfer of glucosyl residues from UDP-glucose to free hydroxyl groups of the polyol residues in the polymer. The particulate enzyme from *B. subtilis* which synthesized poly(glycerol phosphate) also catalyzed this glucosylation reaction (76). Either poly(glycerol phosphate) extracted from walls of the organism or the polymer synthesized in vitro was the substrate for the reaction. The simultaneous synthesis and glucosylation of poly(glycerol phosphate) were not described. More recently, non-glucosylated teichoic acid extracted from the walls of a phage-resistant mutant of *B. subtilis* 168 was used to study the glucosyl transferase of this organism (35). Small amounts of the enzyme were present in the cytoplasm of organisms in the exponential phase of growth, although the majority of the activity was membrane bound. The membrane-bound enzyme was solubilized in an active form by extraction of the membranes with nonionic detergents or several chaotropic reagents. However, no evidence for the participation of a lipid intermediate in the glycosylation reaction was obtained with either form of the enzyme.

Glucosylation of poly(ribitol phosphate) was studied with enzyme preparations from *B. subtilis* W23 (44). The walls of this organism contain both poly(ribitol phosphate) and the glucosylated polymer. The unsubstituted teichoic acid was used as the acceptor for the transferase and, on incubation with UDP-glucose, became fully glucosylated. In addition, teichoic acid present in isolated walls also acted as the acceptor, with approximately 20% of the unsubstituted ribitol residues becoming glucosylated. In each case the linkages synthesized were of the β configuration, in agreement with the known chemistry of the glucosylated teichoic acid. The enzyme preparation used was solubilized from highly fragmented membrane preparations. The observation that poly(ribitol phosphate) teichoic acid present in the walls could become glucosylated strongly suggests the presence in the membrane preparation of two teichoic acid-synthesizing systems, one for each polymer. Thus poly(ribitol phosphate) synthesized by the unsubstituted polymer system is unavailable to the other system (i.e., the glucosylated polymer system). Evidence to support this hypothesis has come from the observation that germinating spores of *B.*

subtilis W23 contain all of the enzymes necessary for synthesis of glucosylated poly(ribitol phosphate), whereas synthesis of the unsubstituted polymer requires the prior synthesis of the necessary enzyme (45).

Particulate enzyme preparations from *S. aureus* also catalyzed the transfer of GlcNAc from UDP-GlcNAc to poly(ribitol phosphate) (133). Initially, teichoic acid of *S. aureus* Copenhagen from which the β-linked GlcNAc substituents had been removed by treatment with β-*N*-acetylglucosaminidase was used as the acceptor. The native teichoic acid contains both α- and β-linked substituents. Later investigations showed, however, that a substantially greater rate of glycosylation was obtained when both CDP-ribitol and UDP-GlcNAc were added simultaneously to the enzyme preparation. Delayed addition of UDP-GlcNAc resulted in a marked decrease in the amount of GlcNAc incorporated. Thus, in *S. aureus* glycosylation of poly(ribitol phosphate) appears to occur more readily with polymer being synthesized concomitantly than does glycosylation of either preformed or exogenously added teichoic acid. These observations suggest that there is a close spatial relationship between the *N*-acetylglucosaminyl-transferase and the ribitol phosphate polymerase in the membrane. Unfortunately, this type of experiment in which teichoic acid synthesis and glucosylation occur simultaneously has not been reported for either of the other two systems described, so that the relative rates of glucosylation are not known.

To extend the earlier observations, the nature of the GlcNAc linkages synthesized by various strains of *S. aureus* was investigated (132). Chemical and immunochemical analyses showed that these teichoic acids contain different proportions of α- and β-linked GlcNAc residues. By using as the acceptor teichoic acids from which β-linked substituents had been removed as described above, it was found that particulate enzyme preparations from the various strains synthesized α and β linkages in a ratio close to that found in their teichoic acids. Thus, *S. aureus* 3258 synthesized linkages mainly of the α configuration, whereas linkages of the β configuration were obtained with strains H and Duncan. *S. aureus* Copenhagen gave results between these two extremes, in agreement with the composition of its teichoic acid.

In both strains of *B. subtilis* and *S. aureus* the glycosylated teichoic acids are recognized as part of the receptor site for various bacteriophages (11). Consequently, there are phage-resistant mutants which can no longer synthesize the glycosylated polymer. Such mutants resistant to bacteriophage φ29 were studied extensively in *B. subtilis* 168 where lesions in both the glucosyl transferase (genetic designation, *gtaA*) and in the ability to synthesize the precursor UDP-glucose (*gtaB* and *gtaC*) were described (186). Similar mutants resistant to phage PBSX have been isolated from *B. subtilis* W23 (11). *S. aureus* mutants resistant to phage 52A were described which lack the *N*-acetylglucosaminyl transferase (41, 88, 156) or were incapable of synthesizing teichoic acid because of their inability to synthesize the linkage unit required for attachment of teichoic acid to peptidoglycan (30). In mutants lacking transferase activity, where the lesion appears only to involve changes in teichoic acid structure, little obvious effect on growth of the organism can be observed.

Recently, changes in the degree of glycosidic substitution of wall teichoic acids were observed by Wicken and his colleagues (A. J. Wicken, personal communication) while investigating the effects of environmental conditions on wall composition. *Lactobacillus plantarum* grown under conditions of glucose limitation showed increased glucosidic substitution of poly(ribitol phosphate) with increasing dilution rate at constant pH or with increasing pH at constant dilution rate, although the amount of teichoic acid present (measured as phosphorus) remained constant. Similar experiments with *Streptococcus mutans* BHT showed that the amount of poly(glycerol phosphate) in the wall again remained constant and that in this case galactosyl substitution was only marginally affected. The reasons underlying these differences in substitution remain unclear. However, the amount of covalently linked wall polymers appeared to be relatively stable when examined under a variety of growth conditions in which the dilution rate and the carbohydrate used as limiting nutrient were changed.

Teichoic acids in which glycosyl residues form part of the polymer chain. Teichoic acids with a polyol phosphate chain require only the presence of the appropriate precursor for synthesis to occur. Although the formation of glycosylated polymers may be enhanced by the simultaneous presence of the precursors of both the polyol phosphate and the glycosyl substituent, the glycosylation of preformed teichoic acid can be demonstrated. In contrast, the biosynthesis of teichoic acids in which the glycosyl residues are part of the polymer chain has an absolute requirement for both precursors. The biosynthesis of these teichoic acids was again first studied by Burger and Glaser (39) by using a particulate enzyme preparation from *B. licheniformis* 9945A. The walls of this organism con-

tain poly(glucosylglycerol phosphate) and poly(galactosylglycerol phosphate) in addition to the poly(glycerol phosphate) described earlier. Synthesis of the glycosylglycerol phosphates occurred when CDP-glycerol and either UDP-glucose or UDP-galactose were present. Unfortunately, the simultaneous synthesis of poly(glycerol phosphate) made interpretation of the results difficult. However, it appeared that polymerization occurred by the sequential addition of monomer units, (i.e., glycerol phosphate and hexose) to some unknown acceptor (X), according to the following reactions:

CDP-glycerol + glucosyl-X →

glycerol-P-glucosyl-X + CMP

UDP-glucose + glycerol-P-glucosyl-X →

glucosyl-glycerol-P-glucosyl-X + UDP

An analogous reaction was proposed for synthesis of the galactose-containing teichoic acid. Preincubation with UDP-glucose did not prevent the subsequent incorporation of galactosyl residues from UDP-galactose. These observations suggest the presence of independent synthesizing systems for the two polymers. The nature of the reactions involved was confirmed by Baddiley and his colleagues (8, 80), who established that the phosphate residue of the glucosyl teichoic acid is derived from CDP-glycerol and not UDP-glucose. Subsequently, Hancock and Baddiley (81) reported the solubilization of an enzyme complex by repeated freeze-thawing of *B. licheniformis* membranes, which synthesized both poly(glycerol phosphate) teichoic acid and the glucose-containing polymer. From the results of these and earlier investigations, they concluded that biosynthesis of teichoic acids required the participation of undecaprenyl phosphate-linked intermediates.

A similar conclusion was reached in other studies of teichoic acid biosynthesis in *S. lactis* I3 (now *Micrococcus* sp. I3) and strain 2102 (now *Micrococcus varians*) (22, 34, 95). Membrane preparations from these organisms catalyzed the synthesis of polymeric materials from glycerol phosphate and GlcNAc 1-phosphate in strain I3 (the repeating unit is shown in Fig. 4a) and from GlcNAc phosphate alone in strain 2102 (Fig. 4b). In each organism biosynthesis of the teichoic acid occurred by the addition of newly synthesized units to the nonreducing terminal of the growing polymer chain (34, 96).

The walls of *B. subtilis* 168 contain a third teichoic acid, poly(glucosyl-*N*-acetylgalactosamine(GalNAc)-phosphate) (157), in addition to poly(glycerol phosphate) and its glucosylated derivative which have been discussed above. The biosynthesis of this third teichoic acid has recently been investigated in a phosphoglucomutase-deficient mutant of *B. subtilis* 168 (87). As a consequence of the enzyme deficiency this strain cannot form UDP-glucose, and the teichoic acid is not made in vivo. However, wall-free preparations supplied with the appropriate precursors will synthesize the polymer in vitro. Formation of the teichoic acid chain occurs by the sequential additon of *N*-acetylgalactosamine phosphate and glucose; evidence for the participation of lipid intermediates was not obtained. Wall membrane preparations also synthesize the polymer and can link a proportion of the newly synthesized material to peptidoglycan. Although the exact nature of this linkage remains unknown, the fact that the polymer can be released from walls by mild alkali treatment suggests the presence of a linkage unit similar to that found in *S. aureus*.

Addition of Ester-Linked D-Alanine Residues

Ester-linked D-alanine residues are common substituents of both wall and membrane teichoic acids, although the mechanism of esterification is only now becoming clear. In 1960 Baddiley and Neuhaus (24) reported that *L. casei, L. arabinosus,* and several other gram-positive bacteria contained a soluble enzyme which required adenosine triphosphate (ATP) for the formation of a D-alanyl-AMP-enzyme complex as follows: D-alanine + ATP + enzyme ⇌ D-alanyl-AMP-enzyme + inorganic pyrophosphate. This complex reacts spontaneously with hydroxylamine to yield D-alanyl hydroxamate. However, attempts to use the enzyme (or complex) to catalyze the esterification of D-alanine residues of teichoic acids or teichoic acid precursors were unsuccessful.

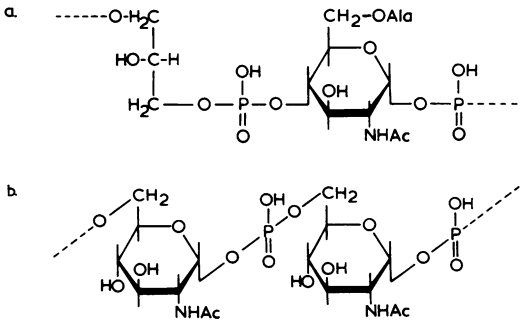

FIG. 4. *Repeating units of the teichoic acids in the walls of (a) Micrococcus sp. I3 (formerly S. lactis I3) and (b) Micrococcus varians (formerly S. lactis 2102).*

Recently, a second membrane-bound enzyme was shown in *L. casei* which catalyzes the transfer of D-alanine into the membrane fraction (115, 136, 145). Similar activities were also present in *S. aureus* H and *Streptococcus pyogenes* (136). This enzyme, D-alanine:membrane acceptor ligase, required ATP, Mg^{2+}, and a supernatant fraction for activity. Again, attempts to link D-alanine residues to exogenously added teichoic acids were unsuccessful. This may reflect a requirement for a precise orientation of the acceptor teichoic acid within the membrane.

The product of the reaction was initially identified by precipitation with antisera specific for poly(glycerol phosphate), and both acid and alkaline hydrolysis, to be D-alanylated 1,3-poly(glycerol phosphate). The fact that *L. casei* does not possess a wall teichoic acid (104) pointed to the acceptor being lipoteichoic acid. This has now been established unequivocally by the isolation and characterization of D-alanyl-lipoteichoic acid synthesized by toluene-treated cells of *L. casei* (43). The supernatant factor required for ligase activity could not be distinguished from the D-alanine-activating enzyme described above. This finding led Neuhaus and his colleagues (136) to conclude that at least two steps are involved in the esterification of the teichoic acid. D-alanine is first activated by the soluble enzyme in the presence of ATP before the amino acid residues are transferred to the polymer chain in a second reaction catalyzed by the ligase as follows: D-alanyl-AMP-enzyme + acceptor → D-alanyl-acceptor + AMP + activating enzyme. It remains unknown whether the addition of D-alanine esters to a particular teichoic acid chain is an ordered process in which all of the residues are attached in sequence or occurs in a more random manner.

A stable L-phase variant of *S. pyogenes* was found to be deficient in ligase activity. The membranes of this organism were shown earlier to contain poly(glycerol phosphate) lacking ester-linked D-alanine residues (160). Subsequent investigation of the enzymes involved in D-alanine addition revealed both the L-phase variant and the parent coccus to be equally efficient in activating D-alanine; the absence of ester-linked D-alanine in the L-phase variant is explained by an absence of ligase activity (42). Whether this lesion is in the ligase itself or in some additional factor involved in transfer requires further investigation.

Although the system described catalyzes the addition of D-alanine esters to lipoteichoic acid, it seems probable that a similar, if not identical, process will function in the formation of these ester linkages in wall teichoic acids.

Biosynthesis of Teichoic Acid Linkage Units

The chemical studies which led to the finding that wall teichoic acids are not linked directly to peptidoglycan, but rather are linked through an oligomeric linkage unit, were reviewed recently (47). Briefly, these studies showed that despite major differences in structure the wall teichoic acids of *B. subtilis* W23, *Micrococcus* sp. I3 and 2102, and *S. aureus* H were all covalently linked to peptidoglycan by a specific linkage unit composed of *N*-acetylglucosamine phosphate and three residues of glycerol phosphate. In each case the linkage unit was found interposed between the teichoic acid chain and the 6-hydroxyl of a muramic acid residue in the glycan.

At the time the first of the chemical studies was being published, Bracha and Glaser (32) reported that biosynthesis and linkage of poly(ribitol phosphate) teichoic acid to peptidoglycan in wall membrane preparations from *S. aureus* H was greatly stimulated by the presence of UDP-GlcNAc and CDP-glycerol. Simultaneously, Hancock and Baddiley (82) described similar results from their study of the synthesis of teichoic acids by membrane preparations of *B. subtilis* W23, *Micrococcus* sp. 2102 (now *M. varians*), and *S. aureus* H. The formation of water-soluble polymers containing additional glycerol residues required the precursors of the teichoic acid and CDP-glycerol. Again, synthesis was stimulated by the additional presence of UDP-GlcNAc. Extraction of the incubation mixtures with organic solvents, particularly butan-1-ol, revealed the presence of lipids containing radioactive GlcNAc and glycerol. Subsequently, the biosynthesis and linkage of poly(ribitol phosphate) teichoic acid to peptidoglycan in wall membrane preparations of *B. subtilis* W23 was shown to require the three nucleotide precursors UDP-GlcNAc, CDP-glycerol, and CDP-ribitol (183). Moreover, by incubating the enzyme preparation with a particular precursor, followed by its removal by washing, and subsequent incubation with the additional precursors, biosynthesis of the teichoic acid was shown to occur by utilizing the nucleotide precursors in the order given above. Lipids containing labeled GlcNAc and glycerol were also demonstrated in this system.

The biosynthesis of the linkage units was first investigated by using membrane preparations from *S. aureus* H (31). Lipids containing radioactive GlcNAc and glycerol were extracted into 70% ethanol. After removal of the solvent, they were converted into water-soluble polymer by further incubation with CDP-ribitol and fresh

membranes. The newly synthesized polymeric material had the electrophoretic mobility of poly(ribitol phosphate) teichoic acid on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In separate experiments, membranes of *B. subtilis* W23 and *S. aureus* H incubated with the three nucleotide precursors UDP-GlcNAc, CDP-glycerol, and CDP-ribitol synthesized two compounds containing poly(ribitol phosphate) (83). These could be extracted from the membrane preparations by treatment with a nonionic detergent and separated by ion-exchange chromatography on diethylaminoethyl-cellulose. One compound was identical to a compound formed by membranes incubated with CDP-ribitol alone. This was assumed to be poly(ribitol phosphate) linked to LTC, the complex known to be synthesized by the acceptor-dependent poly(ribitol phosphate) polymerase purified from Triton X-100 extracts of *S. aureus* H membranes (59, 60). The second compound, synthesized only in the presence of the additional nucleotide precursors, appeared to be teichoic acid attached to the linkage unit. The formation of this teichoic acid-linkage unit complex and the ethanol-soluble lipid described above were sensitive to inhibition by tunicamycin, as was the biosynthesis and linkage of poly(ribitol phosphate) teichoic acid to peptidoglycan in a second strain of *B. subtilis* W23 (83, 183). In membranes of *S. aureus* H and the wall membranes of *B. subtilis* W23, 50% and more than 90% inhibition, respectively, was observed at an antibiotic concentration of 1 μg/ml.

Tunicamycin inhibits the translocation of GlcNAc 1-phosphate from the nucleotide precursor to polyisoprenyl phosphate in both procaryotic and eucaryotic systems. Hence, the inhibition of biosynthesis described above suggested that the linkage unit was also synthesized attached to a similar polyisoprenol carrier, presumably undecaprenol. This being the case, it appeared that formation of the linkage unit occurred by the transfer of glycerol phosphate residues from CDP-glycerol to the nonreducing terminus of a GlcNAc residue attached by a pyrophosphate bond to undecaprenol. This was established by the isolation and detailed characterization of the lipid intermediates from *S. aureus* H and *M. varians* (121, 122, 147). These contained GlcNAc alone (lipid I) and GlcNAc to which were attached one, two, or three residues of glycerol phosphate (lipids II, III, and IV), linked via pyrophosphate to a polyisoprenol. Incorporation of GlcNAc by membranes of *B. subtilis* 168 to form poly(GlcNAc) attached by a pyrophosphate bond to undecaprenol was reported previously (26). However, the relationship of these compounds to linkage units remains unknown.

Little is yet known about the enzymes catalyzing the individual reactions involved in biosynthesis of the linkage unit, although methods to follow the complete process have been described. Initially GlcNAc phosphate is translocated from the nucleotide precursor into the lipid carrier in the following reaction: C_{55}-P + UDP-GlcNAc $\rightleftharpoons$ C_{55}-P-P-GlcNAc + UMP.

The inhibition by tunicamycin of the translocase was mentioned above. In membranes of *B. licheniformis* this occurs at antibiotic concentrations approximately 10-fold lower than those required to inhibit phospho-*N*-acetylmuramyl pentapeptide translocase, the enzyme catalyzing the formation of the first lipid intermediate in peptidoglycan synthesis (135, 172, 174) (50% inhibition requires 0.2 μg of tunicamycin per ml). However, the known ability of bacteria to survive the absence of teichoic acid in their walls suggests that the antibacterial activity of tunicamycin must reside in its ability to inhibit peptidoglycan synthesis despite this being less sensitive to inhibition. Phospho-GlcNAc translocase has not yet been isolated, and nothing is known of the nature of the reaction mechanism. Inactivation of the translocase was reported to occur when *B. subtilis* W23 was transferred from phosphate-containing to phosphate-free medium (79). Under these conditions synthesis of teichoic acid ceases, and the synthesis of teichuronic acid is initiated.

A deficiency in translocase activity was described in *S. aureus* 52A5 (30), which completely lacks ribitol teichoic acid and is resistant to bacteriophage 52A (41), part of whose receptor includes *N*-acetylglycosaminyl ribitol (11). Membranes from the mutant can, however, synthesize poly(ribitol phosphate) attached to LTC. On the other hand, when they are supplied with preformed linkage unit (generally an ethanol extract of *S. aureus* membranes preincubated with UDP-GlcNAc and CDP-glycerol) they will also form poly(ribitol phosphate) attached to the linkage unit. Similar experiments in which ethanol-extractable lipids from membranes incubated with UDP-GlcNAc alone were reincubated with CDP-glycerol, CDP-ribitol, and fresh membranes established that the translocase was the missing enzyme. The two forms of the teichoic acid (i.e., attached to LTC and linkage unit) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

It remains unknown whether the addition of the glycerol phosphate residues to GlcNAc-P-P-polyisoprenol is catalyzed by one or three separate enzymes.

Is Lipoteichoic Acid Carrier Involved in Teichoic Acid Biosynthesis?

The initial studies of Mauck and Glaser (118) on the purification of poly(glycerol phosphate) polymerase which led to the isolation and characterization of LTC as an acceptor for the polymerase were described briefly above. Earlier, Baddiley and his colleages (8, 34, 50, 80, 95, 175) proposed the involvement of polyisoprenol phosphate-linked intermediates in the biosynthesis of a number of teichoic acids. At that time, however, the presence of linkage units remained unknown and with hindsight it seems apparent that most, if not all, of their findings with regard to lipid intermediates can now be explained in terms of the involvement of lipid intermediates in the biosynthesis of linkage units.

Additional evidence for the participation of LTC as a carrier in teichoic acid synthesis came with the isolation and purification of poly(ribitol phosphate) polymerase from membranes of *S. aureus* H (59, 60). As described above, the purified enzyme showed an absolute requirement for LTC which was also purified to apparent homogeneity from Triton X-100 extracts of the same membranes. In composition it resembled the LTC isolated from *B. subtilis*, and in fact the two carriers were interchangeable and could be used for synthesis of either polymer. Lipoteichoic acid obtained from membranes by phenol extraction gave extremely variable results when used as the acceptor; possible reasons for this are discussed below.

The linkage between LTC and poly(ribitol phosphate) was studied by chemical degradation of the isolated complex and enzymic characterization of the products (61). The specific point of attachment was a phosphodiester bond formed between a glycerol residue in the carrier and the terminal ribitol phosphate residue of the teichoic acid chain, the phosphate being derived from CDP-ribitol. This linkage was studied in detail in an investigation of the structural requirements of LTC required for recognition of the carrier by poly(ribitol phosphate) polymerase (64) (Table 1). It was established that the ribitol phosphate units are polymerized on the terminal glycerol residue of the carrier. Consequently, all acceptor activity was lost if the terminal glycerol was either converted to ethylene glycol or completely removed to leave a terminal phosphomonoester. In this second case, acceptor activity was restored by treatment with phosphomonoesterase. On the other hand, both derivatives of LTC and the deacylated carrier were competitive inhibitors of the polymerization reaction on untreated LTC. Thus, the enzyme appeared to recognize other parts of the poly(glycerol phosphate) chain rather than the hydrophobic terminus. These structural requirements were further studied using a series of analogs of LTC prepared by systematically shortening the hydrophilic glycerol phosphate chain (Table 2). This was achieved by treatment of purified LTC with an enzyme preparation from *Aspergillus niger* containing both phosphodiesterase and phosphomonoesterase activities (152). Ability to function as an acceptor in the polymerization reaction required a minimum of four unsubstituted glycerol phosphate residues, although optimum acceptor activity was obtained with a chain length of 20 units. With LTC of longer chain lengths, the terminal glycerol residue is not essential for enzyme recognition, since chemically modified carrier can still interact with and inhibit the enzyme. Whether this is also true for the analog containing four

TABLE 1. *Poly(ribitol phosphate) acceptor activity of lipoteichoic acid and various derivatives*[a]

Lipoteichoic acid or derivative	Acceptor activity (pmol of ribitol polymerized)	
	Expt 1	Expt 2
Lipoteichoic acid (LTC)	925	580
Deacylated	134	
Oxidized and reduced (ethylenediol terminus)	147	
Phosphomonoester terminus	227	
Phosphomonoester after dephosphorylation	531	
Native lipoteichoic acid (contains ester-linked D-alanine)		90
Alanine-free derivative		510
No additions	169	88

[a] Data taken with permission from Fischer et al. (63, 64) where details of the various procedures used and the assay of acceptor activity can be found.

TABLE 2. *Effect of lipoteichoic acid chain length on poly(ribitol phosphate) acceptor activity*[a]

Average chain length of lipoteichoic acid (glycerol phosphate units)	Acceptor activity (pmol of ribitol polymerized)
20.0	845
15.4	724
12.1	701
8.3	673
4.6	383
3.4	109
1.2	55
0	105

[a] Lipoteichoic acid from *Leuconostoc mesenteroides* was systematically shortened by treatment with enzymes from *A. niger*. Data taken with permission from Fischer et al. (64), where further details may be found.

glycerol phosphate units remains unknown. Similarly, the glycolipid (hydrophobic terminus) of the carrier does not appear to be important in enzyme recognition, whereas it is clearly essential for acceptor activity. On the basis of their results Fischer and his colleagues (64) proposed that in vitro the poly(ribitol phosphate) polymerase, in the presence of phospholipid and detergent, exists with LTC in mixed micelles. In this situation the glycolipid (with its hydrophobic terminus) is required to maintain the appropriate orientation of the carrier with respect to the enzyme.

The question then arises whether LTC also functions in vivo as a carrier on which the main teichoic acid chain becomes polymerized. This would occur before the completed chain was transferred to the linkage unit; only then would the teichoic acid become linked to peptidoglycan. After the initial demonstrations of polymerization of LTC in vitro, it was generally assumed (32, 82, 83, 183) that teichoic acid synthesis followed this pathway. In *B. subtilis* W23 the absence of radioactivity derived from CDP-ribitol in butanol extracts of wall membrane preparations was taken as evidence for polymerization occurring before attachment of the teichoic acid to the linkage unit (183). It is now known, however, that addition of the third glycerol phosphate residue to the linkage unit precursor (i.e., formation of lipid IV from lipid III) results in very large increase in the hydrophilicity of the molecule (121). Presumably, the addition of further ribitol phosphate units would only serve to make the intermediate more hydrophilic and perhaps no longer butanol soluble under the conditions used.

The possibility that LTC functions in the polymerization reaction as an analog of the natural acceptor, the linkage unit, was first suggested by Bracha and Glaser (31). At the time, their inability to cleanly separate poly(ribitol phosphate) attached to either LTC or the linkage unit prevented them from deciding whether the LTC-linked polymer was an intermediate in the synthesis of the teichoic acid-linkage unit complex. Baddiley and his colleagues achieved the separation of poly(ribitol phosphate)-linkage unit and poly(ribitol phosphate)-LTC by chromatography on diethylaminoethyl cellulose (83). The finding that membranes of *S. aureus* H and *B. subtilis* W23 incubated under a variety of conditions synthesized both complexes simultaneously led them to conclude that both acceptors were required.

However, recent studies from the laboratories of Fischer and Fiedler (63, 64) appear to provide convincing evidence against a role for LTC in polymer synthesis in vivo. In their structural studies on acceptor recognition by poly(ribitol phosphate) polymerase they found that glycosylation of the glycerol phosphate chain reduced the effectiveness of LTC as an acceptor (64). As a result of these observations a method was developed to isolate LTC from which the naturally occurring ester-alanine substituents had not been removed. These preparations did not function as acceptors for poly(ribitol phosphate) (63).

Since all of the LTC isolated from *S. aureus* and several other gram-positive bacteria appears to be alanylated [in *S. aureus* the degree of substitution of the poly(glycerol phosphate) chain varied from 44 to 73% (65)]. Fischer and his colleagues argued that in vivo polymerization of the teichoic acid must occur on an alternative acceptor, presumably the linkage unit (Fig. 5). They also concluded that LTC acts as an acceptor in vitro because of the extreme alkali lability of the D-alanine ester linkage. Weakly alkaline buffers (pH 7.5 to 8.5) are commonly used for the isolation of both LTC and membranes, and these pH values are sufficient to hydrolyze the ester bonds, resulting in a loss of D-alanine substituents. This would enable LTC, whether membrane bound or purified, to be recognized by the poly(ribitol phosphate) polymerase. The variable acceptor activity of phenol-extracted LTC referred to above may also be explained by different degrees of alanine substitution retained during the isolation process. The remaining ambiguity that the linkage unit contains only three unsubstituted glycerol phosphate residues, whereas the polymerase requires a minimum of four such residues for acceptor activity, could also be explained. Space-filling models of both compounds revealed that both were of the same length with phosphate residues in more or less identical positions (Fig. 6). The GlcNAc phosphate moiety of the linkage unit effectively replaces a glycerol phosphate residue in the LTC analog.

On the other hand, recent studies of McArthur and his colleagues (120) showed that membrane preparations from *S. aureus* H and *M. varians* could synthesize their teichoic acids on LTC and subsequently transfer the polymer to linkage unit. Membrane preparations provided with all necessary precursors (i.e., in *S. aureus* CDP-ribitol, CDP-glycerol, and UDP-GlcNAc), synthesized poly(ribitol phosphate) attached to both LTC and to the linkage unit (83, 120). When membranes were incubated with CDP-ribitol alone, only the LTC-poly(ribitol phosphate) complex was formed, showing that the membranes contained negligible amounts of en-

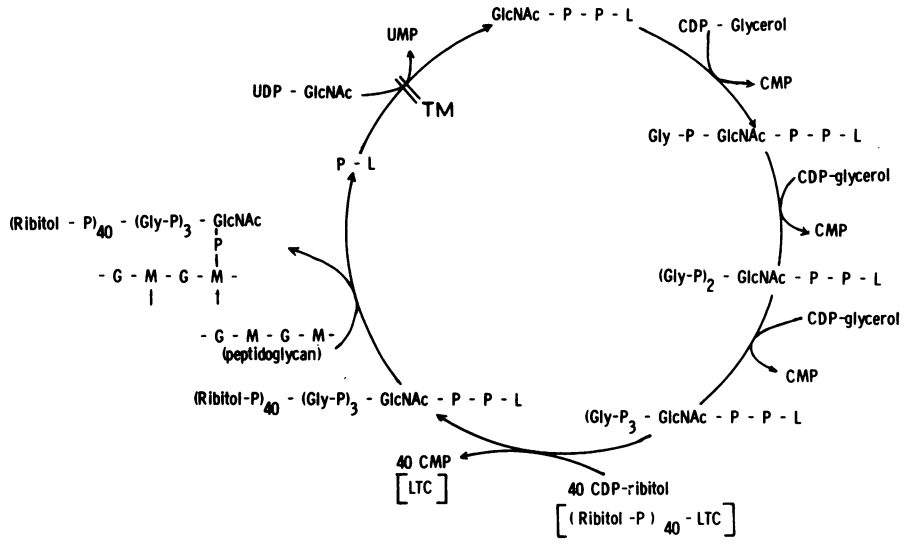

FIG. 5. *Pathway proposed for the biosynthesis of poly(ribitol phosphate) teichoic acid and linkage to peptidoglycan in S. aureus. L is the lipid carrier, presumably undecaprenol. The site of inhibition by tunicamycin (TM) is also shown.*

dogenous linkage unit lipids. However, when membranes were preincubated with CDP-ribitol and the residual substrate was destroyed by phosphodiesterase treatment before the preparation was reincubated with CDP-glycerol and UDP-GlcNAc to allow linkage unit synthesis to occur, poly(ribitol phosphate) was again found attached to both LTC and the linkage unit. Since the main chain of the teichoic acid was initially present only as the LTC complex, transfer from this complex to the newly synthesized linkage unit must have occurred. This was confirmed in experiments when the linkage unit was specifically labeled with [^{14}C]glycerol. Similar results showing transfer of preformed poly-(GlcNAc 1-phosphate) from LTC to newly synthesized linkage unit were also obtained by using membranes from *M. varians*. A lack of specificity in the polymerization or transfer reaction was also shown. Linkage unit lipids containing one, two, or three glycerol phosphate residues were extracted and purified from either *S. aureus* or *M. varians*. Incubation of these lipids with the appropriate precursor and membranes from *S. aureus*, *M. varians*, or *B. subtilis* 3610 resulted in their incorporation into teichoic acid.

Hence, the various polymerases lack specificity in that polymerization can occur in vitro onto incomplete linkage unit lipids. Moreover, with the exception of the small amount of lipid IV present in the extract, these polymerization reactions are utilizing intermediates of less than the minimum length of four unsubstituted glycerol phosphate residues required by the purified poly(ribitol phosphate) polymerase from *S. aureus*.

Thus, in vitro it appears that the main teichoic acid chain can become attached to the linkage unit by two routes. Either polymerization occurs directly on the linkage unit lipid, or alternatively teichoic acid is first polymerized on LTC and then transferred to the linkage unit (Fig. 5). The earlier finding that purified poly(ribitol phosphate) polymerase did not catalyze such a transfer reaction suggests the presence of a specific transferase in the membrane preparation. Whether only one or both of these routes are utilized in vivo still remains open to speculation.

Biosynthesis of Teichuronic Acids

In contrast to the many studies on biosynthesis of teichoic acids, the teichuronic acids have until recently received little attention. An exception has been the ManNAcUA- and glucose-containing polymer in the walls of *M. luteus* (*lysodeikticus*), the biosynthesis of which was investigated in detail by Anderson and Perkins and their colleagues. More recently, the formation of the teichuronic acid of *B. licheniformis* was also studied.

Micrococcus luteus. The teichuronic acid present in the walls of *M. luteus* has the following structure (84, 130, 139):

$$\left[\xrightarrow{4}\text{ManNAcUA}\xrightarrow{\beta\ 1,6}\text{D-glucose}\xrightarrow{1\alpha}\right]_{\sim 40}$$

Linkage to peptidoglycan was reported to occur

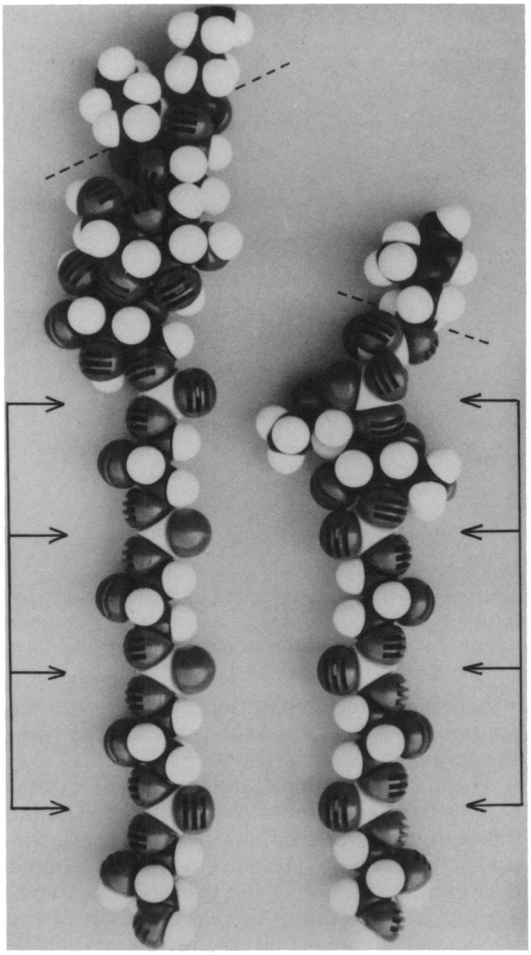

FIG. 6. *Space-filling models of the oligomeric linkage unit of S. aureus (left) and the tetra (glycerol phosphate) derivative of lipoteichoic acid (right). The N-acetylglucosaminyl residue is the α-anomer and substituted by the glycerol phosphate trimer at C-4. Only part of the hydrophobic chains are shown with the hydrophobic-hydrophilic boundary given by the dashed lines. The four phosphate residues required for recognition by poly(ribitol phosphate) polymerase are marked by arrows. Figure kindly provided by W. Fischer and taken with permission from reference 63.*

through a reducing GlcNAc residue and a phosphodiester bond to the 6-hydroxyl of a muramic acid residue in the glycan chain (85). In agreement with this structure early studies on biosynthesis established that formation of polymeric material required the presence of UDP-GlcNAc in addition to the expected precursors UDP-glucose and UDP-ManNAcUA (6, 138). The newly synthesized polymer contained 1 residue of GlcNAc for each 15 to 20 residues of glucose. More recently, it was shown that biosynthesis proceeds in two stages (149, 161). A linkage unit composed of GlcNAc and two residues of ManNAcUA is synthesized first on a polyisoprenol lipid carrier, presumably undecaprenol phosphate (Fig. 7). Supporting evidence has come from the finding that concomitant synthesis of peptidoglycan and lipomannan, biosynthetic processes known to require undecaprenol phosphate, inhibits the synthesis of teichuronic acid. In the second stage the main polysaccharide chain is built up by the transfer of alternating residues of glucose and ManNAcUA (Fig. 7).

The initial reaction in formation of the linkage unit was reported to be the transfer to the lipid carrier of GlcNAc rather than GlcNAc phosphate (149). The product was UDP, and it was this nucleotide that participated in an exchange reaction, rather than UMP. A lipid of this type, *N*-acetylglucosaminyl phosphoryl undecaprenol, is synthesized by membranes of *B. cereus* in a reaction which is insensitive to tunicamycin-like antibiotics (185). However, these authors also reported that they were unable to demonstrate the synthesis of a similar lipid by *M. luteus* membranes. Moreover, the incorporation of GlcNAc into teichuronic acid being synthesized and covalently linked to peptidoglycan by wall membrane preparations of *M. luteus* was sensitive to tunicamycin, complete inhibition being obtained at an antibiotic concentration of 10 μg/ml (140, 177). In other systems, including biosynthesis of teichoic acid linkage units, tunicamycin at low concentrations was shown to inhibit only reactions involving the translocation of GlcNAc 1-phosphate (or analogs such as phospho-*N*-acetylmuramyl pentapeptide) to a polyisoprenoid phosphate carrier (31, 83, 172, 174). Thus, the results obtained by the two groups on the initial reaction in biosynthesis of the linkage unit present something of a paradox. Further work is obviously required to establish whether there is an additional translocase reaction involved in these early stages of biosynthesis.

The two transferases catalyzing the addition of the alternating residues of D-glucose and ManNAcUA to build up the polysaccharide were solubilized by Triton X-100 treatment of *M. luteus* membranes (161). Extraction of the membranes in the presence of 5 to 10 mM Mg^{2+} selectively released the glucosyltransferase. When the Mg^{2+} concentration was then reduced to 1 mM or less the *N*-acetylmannosaminuronyl transferase was also solubilized.

Bacillus licheniformis. The teichuronic acids present in the walls of *B. licheniformis* 6346 grown in batch culture (93) and *B. subtilis* W23 grown under conditions of phosphate limi-

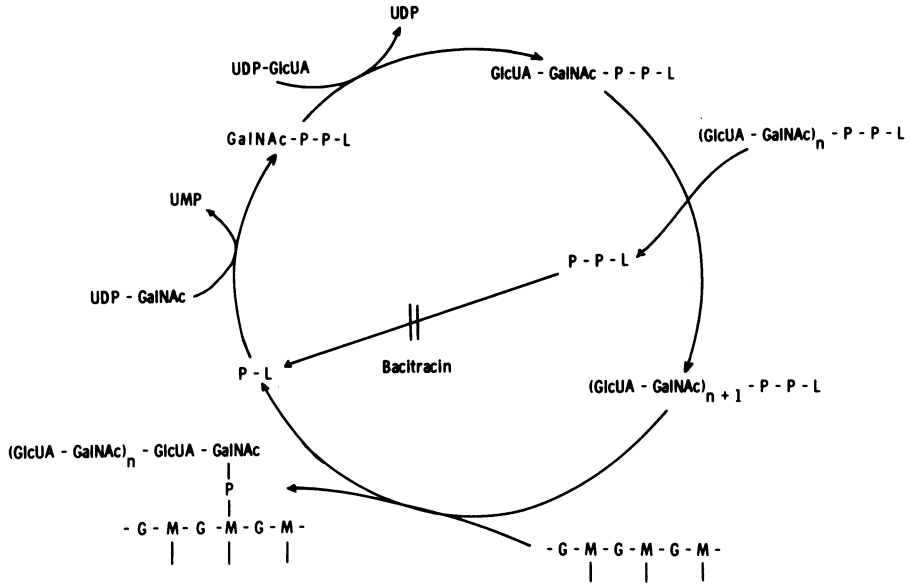

FIG. 7. *Pathway proposed for the biosynthesis of teichuronic acid in M. luteus. L is the lipid carrier, presumably undecaprenol. As discussed in the text the nature of the initial reaction, shown as the formation of GlcNAc-P-L, is not clearly established, and the mechanism of linkage to peptidoglycan remains unknown.*

tation (181) were reported to be linear polysaccharides composed of alternating residues of GalNAc and D-glucuronic acid (D-GlcUA) as follows:

$$\left[\text{4D-GlcUA} \xrightarrow{1,3} \text{GalNAc} \xrightarrow{1}\right]_{23\text{–}25}$$

More recently, the teichuronic acid of *B. licheniformis* 9945 was subjected to detailed chemical analysis and nuclear magnetic resonance spectroscopy (114), and the following structure was proposed:

$$\left[\beta\text{-4D-GlcUA} \xrightarrow{\beta\text{-1,4}} \text{D-GlcUA} \xrightarrow{\beta\text{-1,3}} \text{GalNAc} \xrightarrow{\alpha\text{-1,6}} \text{GalNAc}^{1}\right]_{n}$$

Chemical analysis (91) and the biosynthetic studies described below established that the teichuronic acid of *B. licheniformis* 6346 is linked to a 6-hydroxyl of a muramic acid residue via a phosphodiester bond from the reducing GalNAc residue of the polysaccharide.

The biosynthesis of this teichuronic acid was first investigated by Hughes (90), who demonstrated the synthesis of polymeric material from UDP-GalNAc and UDP-glucuronic acid. More recently, the nature of the reactions involved was studied by using enzyme preparations from a phosphoglucomutase-negative mutant of *B. licheniformis* 94 (174; J. B. Ward, unpublished observations). The absence of this enzyme prevents the formation of teichuronic acid in vivo, although cell-free preparations supplied with the appropriate precursors will synthesize the polysaccharide in vitro.

The initial reaction, catalyzed by a translocase, is the formation of *N*-acetylgalactosamine linked by a pyrophosphate bond to a polyisoprenol lipid carrier. This enzyme is not inhibited by tunicamycin, unlike the translocase catalyzing the initial reaction in the formation of the teichoic acid linkage unit, i.e., involving GlcNAc 1-phosphate. The disaccharide repeating unit of the polysaccharide then appears to be formed by the transfer of glucuronic acid with the release of UDP (Fig. 8). Similar reactions for biosynthesis of the repeating unit of the *B. licheniformis* 9945 polymer would appear to require a translocase followed by the transfer of two residues of D-glucuronic acid and a second *N*-acetylgalactosamine residue. In *B. licheniformis* 94 polymerization of the repeating units occurs by the incorporation of new units at the reducing terminus of the growing chain while this remains attached to the lipid carrier. This direction of chain extension is the same as that found in the biosynthesis of peptidoglycan and the 'O' side chain of lipopolysaccharide (146, 173). The common product of all these polymerizations is polyisoprenol pyrophosphate, which

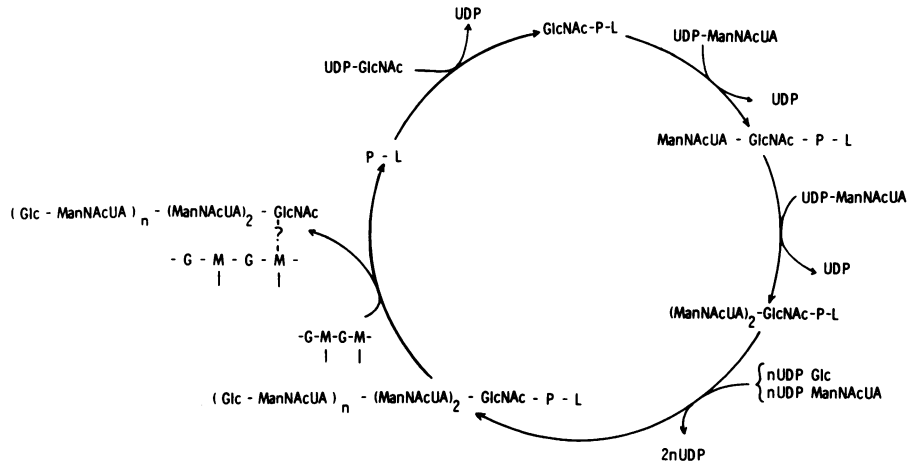

FIG. 8. *Pathway proposed for the biosynthesis and linkage to peptidoglycan of teichuronic acid in B. licheniformis 94. The site of inhibition by bacitracin is shown. L is the lipid carrier, presumably undecaprenol.*

must be converted to the monophosphate before it can be reutilized in biosynthesis. It is this dephosphorylation which is specifically inhibited by bacitracin (159). Thus, the biosynthesis of teichuronic acid is also inhibited by this antibiotic (Fig. 8). Whether a similar series of reactions occurs in *B. licheniformis* 9945, and indeed in other organisms which synthesize teichuronic acids when grown under conditions of phosphate-limitation, remains unknown.

Linkage of Teichoic and Teichuronic Acids to Peptidoglycan

The first evidence on the interrelationships existing between the synthesis of peptidoglycan and secondary wall polymers came from the experiments of Mauck and Glaser (119). This study was based on the earlier observations of Ellwood and Tempest (53, 54), described in the introduction, which showed that the composition of *B. subtilis* walls, with respect to the secondary polymer present, was influenced by the phosphate concentration of the medium.

By transferring the bacilli from phosphate-rich to phosphate-limited medium, and the reverse, both teichoic and teichuronic acids were shown to be linked only to concomitantly synthesized peptidoglycan and not to preexisting peptidoglycan. Subsequent examination of the effects of benzylpenicillin on wall synthesis by *B. licheniformis* (166) and *M. luteus* (126) provided additional evidence. Incubation of both organisms in the presence of the antibiotic resulted in the continued synthesis of soluble uncross-linked peptidoglycan. In neither organism were the secondary polymers linked to the uncross-linked material. Thus, in vivo the linkage of either teichoic or teichuronic acids to peptidoglycan appears to require the concomitant synthesis of cross-links between the newly synthesized peptidoglycan and the preexisting wall.

Similar results were obtained in vitro with wall membrane preparations from the same strain of *B. licheniformis*. Covalently linked peptidoglycan, poly(glycerol phosphate) teichoic acid, and teichuronic acid were synthesized (174, 182). Benzylpenicillin or other β-lactam antibiotics did not affect the overall synthesis of the three polymers, whereas the incorporation of the secondary polymers into the wall (determined as material insoluble in hot detergent) was inhibited by approximately 80%. The concentration of β-lactam antibiotic used inhibited the incorporation of newly synthesized peptidoglycan into the preexisting wall by more than 90% (171). In contrast, wall membranes of *B. subtilis* W23 linked newly synthesized poly(ribitol phosphate) exclusively to preexisting peptidoglycan (183), whereas in similar preparations from *S. aureus* 52A teichoic acid was linked to both preexisting and newly synthesized peptidoglycan (29, 30). Wall membrane preparations of *M. luteus* attached newly synthesized teichuronic acid predominantly to preexisting wall, and the extent of linkage was not significantly stimulated by further peptidoglycan synthesis (177). The absence of attached secondary polymers on soluble peptidoglycan synthesized in vivo was mentioned above. Similar results were obtained with cell-free preparations of *B. licheniformis* (184). More recently, the first demonstration of linkage at this level was shown in *M. varians* (123). Membranes catalyzed the attachment, via a linkage unit, of poly(GlcNAc phosphate) to uncross-linked peptidoglycan. The reasons underlying these observations remain unclear, but

they presumably reflect differences in the specificity of the enzymes catalyzing the linkage of the two polymers.

In the absence of synthesis of the main chain, teichoic acid linkage units may become attached to peptidoglycan. However, in wall membrane preparations of *B. subtilis* W23, at least, there is no evidence which suggests that polymerization of poly(ribitol phosphate) can occur on these transferred units (183).

As mentioned above, the linkage of all secondary polymers is thought to occur through a phosphodiester bond formed between the polymer (or linkage unit) and a 6-hydroxyl of a muramic acid residue in the glycan. Newly synthesized muramic acid phosphate was isolated from wall membrane preparations of *B. licheniformis* in which synthesis of peptidoglycan and either teichoic or teichuronic acid occurred (184, J. B. Ward, unpublished observations). In both cases formation of muramic acid phosphate required the concomitant synthesis of the secondary polymer and peptidoglycan. The phosphate residue which forms the phosphodiester bond in the wall was derived biosynthetically from UDP-GlcNAc in the attachment of teichoic acid (184) and from UDP-*N*-acetylgalactosamine in teichuronic acid. This observation provided the first evidence for the involvement of a linkage unit in the attachment of a poly(glycerol phosphate) teichoic acid. More recently, confirmation came from chemical studies on the linkage of glucosylated poly(glycerol phosphate) teichoic acid in *B. subtilis* (47). The mechanism of linkage of teichuronic acid differs in that no specific linkage unit is involved; the phosphodiester bond is formed directly between the reducing terminus of the polymer and the glycan. Thus, *B. licheniformis* and perhaps other organisms have evolved quite distinct mechanisms for the biosynthesis and linkage of the major secondary polymers present in their walls.

Attempts to determine the biosynthetic origin of muramic 6-phosphate in wall membrane preparations of *B. subtilis* W23 synthesizing poly(ribitol phosphate) teichoic acid and *M. luteus* synthesizing teichuronic acid were not successful (177, 183). However, in *M. luteus* the kinetics of release of free reducing groups of GlcNAc and the removal of phosphate presumably from muramic 6-phosphate in peptidoglycan did not agree (see Fig. 1 in reference 85). Whether these results indicate an additional residue(s) interposed between the GlcNAc and muramyl phosphate or simply reflect the inability of alkaline phosphatase to hydrolyze the phosphomonoester remains unclear. The former situation could explain the differences observed in the biosynthesis of the linkage unit with respect to transfer of GlcNAc and tunicamycin sensitivity. In this context it is perhaps worth mentioning that glucosamine 6-phosphate was isolated from hydrolysates of the walls of *M. luteus* (102, 131). However, any suggestion that such residues are involved in the linkage of the teichuronic acid is purely speculative.

CONTROL OF TEICHOIC AND TEICHURONIC ACID BIOSYNTHESIS

Before discussing the specific control of teichoic and teichuronic acid biosynthesis it seems pertinent to consider certain aspects of the control of wall biosynthesis as a whole. The finding that biosynthesis of both teichoic acid linkage unit and teichuronic acid, in addition to peptidoglycan, requires the participation of polyisoprenoid lipid intermediates shows that the cell has a single point of control over the formation of these wall polymers. In their detailed studies of the lipid intermediates involved in peptidoglycan synthesis, Strominger and his colleagues described enzymes capable of phosphorylating undecaprenol and dephosphorylating undecaprenol phosphate (for review, see reference 164). They argued that the combined action of the kinase and phosphatase would provide a mechanism for regulation of cellular levels of the lipid carrier and hence peptidoglycan biosynthesis. This regulatory system may also be required to enable the organism to establish priority for the synthesis of a particular polymer. Evidence for the control of peptidoglycan and teichoic acid biosynthesis through the availability of the lipid carrier came from experiments with *S. lactis* I3 (175), *B. licheniformis* 9945, and *B. subtilis* (8). Membrane preparations were used which synthesized both polymers. Addition of the nucleotide precursors of teichoic acid to membranes of the bacilli synthesizing peptidoglycan resulted in the initiation of teichoic acid synthesis and inhibition of peptidoglycan formation. In the reverse experiment, the addition of peptidoglycan precursors to membranes synthesizing teichoic acid inhibited synthesis of teichoic acid. Maximal inhibition was obtained when optimal conditions for synthesis of the competing polymer were used. Further evidence came from a study of the effects of bacitracin on the same membrane systems. The antibiotic, which inhibits peptidoglycan synthesis but not teichoic acid biosynthesis, causes an accumulation of the lipid carrier as undecaprenyl pyrophosphate with a consequent depletion of undecaprenyl phosphate. Bacitracin specifically inhibits the dephosphorylation of undecaprenyl pyrophosphate. It does not inhibit teichoic acid biosyn-

thesis in vitro, since undecaprenyl phosphate rather than pyrophosphate is the reaction product. However, the addition of bacitracin and peptidoglycan precursors to membrane preparations synthesizing teichoic acid resulted in increased inhibition of teichoic acid formation when compared with preparations from which the antibiotic was omitted. Similar results were obtained using membrane preparations of *S. lactis* I3 [*Micrococcus* sp. I3 (175)]. The authors (8, 175) interpreted their findings as showing the interdependence of the peptidoglycan and teichoic acid synthesizing systems due to the use of undecaprenyl phosphate, as the common lipid carrier. It was also suggested that the lipid carrier molecules remained associated with a particular enzyme complex to allow synthesis of a complete polymer molecule before becoming dissociated and thus available for use by another enzyme system. In this way the formation of partially polymerized molecules would be prevented.

A similar "compartmentalization" of the lipid carrier between the enzymes synthesizing peptidoglycan and lipopolysaccharide O side chain of *Salmonella typhimurium* was described previously (151). Membranes prepared from a mutant strain in which the accumulation of incomplete lipopolysaccharide units was induced were used to assay the initial exchange reactions involved in peptidoglycan and polysaccharide biosynthesis. Phospho-*N*-acetylmuramylpentapeptide translocase was present with an activity almost identical to that found in membranes from organisms in which no accumulation occurred, but the activity of galactose 1-phosphate translocase was markedly reduced (87% inhibition). Thus, in vivo the two enzymes appear to interact with different pools of the lipid carrier. This conclusion is based on the assumption that both enzymes utilize undecaprenyl phosphate as the carrier and that this phosphate is required for the translocase exchange reaction. However, when taken together with the observations made on peptidoglycan and teichoic acid biosynthesis with *S. lactis, B. licheniformis,* and *B. subtilis,* these studies provide strong evidence for a control mechanism for wall polymer biosynthesis based on the availability of the lipid carrier.

Investigations of the specific control of teichoic and teichuronic acid biosynthesis have almost all been based on the changeover in secondary wall polymers which occurs as a result of environmental changes.

The transfer of either bacilli or staphylococci from growth in phosphate-rich medium to conditions of phosphate limitation results in the rapid cessation of wall teichoic acid biosynthesis and its replacement by teichuronic acid. This changeover occurs at much faster rates than can be accounted for by a simple dilution of the preexisting wall, and wall turnover is now recognized to play an important part in this process. Autolytic enzymes in both groups of organisms hydrolyze peptidoglycan to release the degraded peptidoglycan and secondary polymers into the medium (148). Turnover in *B. subtilis* 168 growing under steady-state conditions was shown to release as much as 50% of the wall in each generation (142).

Initially, control of teichoic and teichuronic acid biosynthesis was suggested to occur at the level of the nucleotide precursors. Ellwood and Tempest (54) postulated that the teichoic acid precursors CDP-glycerol and CDP-ribitol might repress enzymes required for teichuronic acid biosynthesis. In organisms transferred from phosphate-rich to phosphate-limited conditions, formation of teichoic acid precursors would cease, and as their intracellular concentration fell, the enzymes of teichuronic acid biosynthesis would become derepressed. Earlier, Glaser (75) described CDP-glycerol pyrophosphatase in *B. subtilis* and suggested that this enzyme could control intracellular concentrations of CDP-glycerol when the rate of teichoic acid biosynthesis was decreased.

The repression of enzymes required for the synthesis of both teichoic and teichuronic acid was described in *B. subtilis* 168. Rosenberger (150) studied CDP-glycerol pyrophosphorylase and UDP-glucose dehydrogenase, enzymes directly involved in the synthesis of the two secondary polymers. Organisms grown with limited phosphate contained less than 5% of the pyrophosphorylase activity found in cells from phosphate-rich medium (1.6 and 42.5 nmol of product synthesized mg of protein^{-1} min^{-1}, respectively), whereas amounts of UDP-glucose dehydrogenase varied inversely. No evidence was obtained which supported the suggestion that nucleotide precursors of one polymer inhibited enzymes required for the biosynthesis of the other. However, if protein synthesis were inhibited when *B. subtilis* was transferred from phosphate-rich to phosphate-starved conditions then synthesis of teichoic acid would continue. In the reverse experiment (i.e., addition of phosphate to phosphate-starved cultures), synthesis of teichuronic acid continued. Thus, phosphate concentrations as such did not appear to control formation of the alternative secondary polymer; rather, the synthesis of new protein(s) appeared to be required for teichoic (or teichuronic) acid synthesis to be inactivated. More recently, Glaser and Loewy (78) demonstrated that addi-

tion of inorganic phosphate to batch-grown cultures of *B. subtilis* in the early stages of phosphate-starvation leads to the rapid recommencement of teichoic acid synthesis even when protein synthesis is inhibited. At later times, however, reversal of the inhibition of teichoic acid synthesis by phosphate requires new protein synthesis. During phosphate starvation, CDP-glycerol pyrophosphorylase activity decreased slowly, but this change was clearly insufficient to account for the inhibition of poly(glycerol phosphate) synthesis observed. Glaser and Loewy (78) concluded that control of teichoic acid synthesis occurs in a two-step process. The first of these results in a reversible inhibition, and the second results in an irreversible inactivation of the synthesizing system as a consequence of the induction, under conditions of phosphate limitation, of a protein or proteins which inactivates one or more of the biosynthetic enzymes. Subsequently (79), they showed that incubation of *B. subtilis* W23 without phosphate resulted in the inactivation of GlcNAc 1-phosphate translocase, the first membrane-bound enzyme specifically involved in teichoic acid biosynthesis. However, other biosynthetic enzymes were not assayed. Thus, in this organism control of synthesis may reside in the formation of the linkage unit rather than in polymerization of the main chain of the teichoic acid, but this remains to be established.

Baddiley and his colleagues (7, 97) studied the control of teichoic acid synthesis in *B. licheniformis* 9945, which contains both teichoic and teichuronic acids in its walls when grown in batch culture. When chemostat cultures were changed from glucose limitation (phosphate-rich conditions) to phosphate limitation, a rapid decrease in the activity of CDP-glycerol pyrophosphorylase and poly(glucosylglycerol phosphate) polymerase began some 3 h after the medium change (97). Loss of both enzymes occurred at rates much faster than the theoretical dilution rate expected if cessation of protein synthesis alone were responsible. A similar decrease was observed when batch cultures entered stationary phase, and this happened even in the presence of excess phosphate, and when protein synthesis was inhibited (see Fig. 4 in reference 97). In the chemostat cultures teichoic acid synthesis ceased 5 h after the medium change, when the activity of the pyrophosphorylase had reached very low levels but when significant polymerase activity was detected. Thus, in *B. licheniformis* CDP-glycerol pyrophosphorylase appears to be a particularly labile enzyme. Activity is lost even without phosphate limitation, and this loss does not require protein synthesis. In the reverse experiment, transfer of phosphate-limited bacilli to phosphate-rich conditions resulted in a large and immediate increase in pyrophosphorylase activity. This did not occur when protein synthesis was inhibited. Clearly, a potential control mechanism over the biosynthesis of teichoic acid in *B. licheniformis* could reside in the relative instability of CDP-glycerol pyrophosphorylase. Intracellular phosphate concentrations may in some way influence this control. Unfortunately, the effects of phosphate limitation on the GlcNAc phosphate translocase (and hence linkage unit biosynthesis) do not appear to have been investigated in this organism. Therefore, it remains unknown whether *B. subtilis* and *B. licheniformis* evolved different control mechanisms over biosynthesis of teichoic acid.

BIOSYNTHESIS OF LIPOTEICHOIC ACIDS

In contrast to the detailed information available describing the biosynthesis of wall teichoic acids, the lipoteichoic acids have received relatively little attention. Initial studies on the biosynthesis of lipoteichoic acid were performed as pulse-chase experiments using *Streptococcus sanguis* and *Staphylococcus aureus* H (55, 77). Organisms in exponential growth were incubated with radioactive glycerol for 10 and 2 min, respectively. An excess of nonradioactive glycerol was then added, and the distribution of the radioactive label was followed during chase periods of 60 and 45 min. During this time a turnover of 68 to 87% of the radioactivity initially isolated from the cells as phosphatidylglycerol was observed. This was accompanied by an increase in radioactive material having the characteristics of lipoteichoic acid. In each organism this polymer was identified by polyacrylamide gel electrophoresis, and in *S. aureus* H 50% of the isolated material was able to act as the acceptor of poly(ribitol phosphate). Glaser and Lindsay (77) suggested that the remaining 50% was already blocked with poly(ribitol phosphate). However, the recent observations of Fischer et al. (63) on the effects of alanine substitution on acceptor activity present an alternative explanation. It seems more likely that the 50% acceptor activity represents the extent to which the ester-alanine substituents were lost during the extraction and purification of the lipoteichoic acid. Recently, 3,4-dihydroxybutyl 1-phosphonate, an analog of glycerol 3-phosphate, was reported to inhibit phosphatidylglycerol synthesis and hence the biosynthesis of lipoteichoic acid (49).

In vitro synthesis of both poly(glycerol phosphate) and lipoteichoic acid from phosphatidyl-

glycerol by enzyme preparations from *S. sanguis* was reported (56, 116a). Synthesis of lipoteichoic acid required the additional presence of UDP-glucose. The products were fractionated into saline-soluble polymers which contained no fatty acids and detergent soluble polymers which did (116a). Radioactivity from [^{14}C]acetate-labeled phosphatidylglycerol was incorporated into lipoteichoic acid, suggesting that the phospholipid may be the precursor of acyl residues in the glycolipid terminus in addition to the glycerol phosphate chain. The relationships of the poly(glycerol phosphate) synthesized from phosphatidylglycerol alone and the saline-soluble polymers lacking fatty acid substituents remains unclear. One possible explanation is that they represent deacylated lipoteichoic acids. Kessler and Shockman (106) recently reported the enzymic deacylation of lipoteichoic acid by a membrane-bound enzyme from protoplasts of *S. faecium*. A similar enzyme present in *S. sanguis* might well explain the results obtained.

Lipoteichoic acid is synthesized by membrane preparations of *S. faecium* (*S. faecalis*) from phosphatidylglycerol and phosphatidyl-kojibiosyl diacylglycerol (66a). Polymer synthesis was stimulated by mercaptoethanol and Triton X-100, whereas Mg^{2+} was without effect. Incorporation of the phosphoglycolipid into polymer initially gave a product soluble in chloroform-methanol-water, although with the addition of further glycerol phosphate units, the newly synthesized material became water soluble. The product was characterized as unsubstituted lipoteichoic acid lacking the D-alanyl and kojibiosyl substituents found in vivo.

D-Alanyl lipophilic compounds were also synthesized by toluene-treated cells and membranes of *L. casei* which were incubated with D-alanine, the D-alanine activating enzyme, D-alanine membrane acceptor ligase, and ATP (32a). The products were characterized as D-alanyl-lipoteichoic acid with short chains of poly(glycerol phosphate). These short chains were also soluble in chloroform-methanol, but they partitioned into chloroform when the system was made biphasic. Again, as further glycerol phosphate units were added their solubility changed so that they partitioned into the aqueous phase. This was accomplished in toluene-treated cells by treatment with excess phosphate. It was concluded that the phosphate stimulated the synthesis of phosphatidylglycerol which then donated glycerol phosphate units to the D-alanyl lipophilic compounds resulting in chain extension. In addition, glycerol phosphate units appeared to be transferred to glycolipid to generate new lipophilic compounds. From this evidence it would appear that the compounds from *L. casei* are earlier intermediates in the assembly process than those obtained from *S. faecium*. Emdur and Chiu (56) also isolated chloroform-methanol-soluble lipoteichoic acid from *S. sanguis* which may be analogous to one or both of these D-alanyl-lipoteichoic acid intermediates.

As described in the Introduction, the basic structures of the main chain of poly(glycerol phosphate) wall teichoic acids and lipoteichoic acids differ only in that the glycerol phosphate residues are of the opposite sterochemical series. It was reported previously (54) that lipoteichoic acids continued to be synthesized in *B. subtilis* grown under phosphate limitation, a situation which quickly leads to the cessation of wall teichoic acid synthesis and its replacement by teichuronic acid. The investigations described above appeared to provide an explanation for this apparent paradox by establishing that the mechanisms of synthesis of the two polymers are quite different. However, *B. licheniformis* grown under phosphate limitation was found to contain only 10% of the lipoteichoic acid present in magnesium-limited cells. (D. Button, M. K. Choudry, N. L. Hemmings, Proceedings of the Society for General Microbiology, **2**:45, 1975). Moreover, phosphate-limited *B. subtilis* cells contain only 10% of their phospholipid as phosphatidylglycerol, whereas this is increased to 54% in magnesium-limited organisms (124a). Thus, in contrast to the earlier observation, phosphate limitation appears to inhibit both phosphatidylglycerol and lipoteichoic acid biosynthesis. Presumably, the residual amounts of the lipoteichoic acid synthesized under these conditions are sufficient to meet any requirements the organism might have. However, the effect of phosphate limitation on lipoteichoic acid and phospholipid biosynthesis clearly deserves further investigation.

CONCLUDING REMARKS

Considerable advances have been made in recent years in our understanding of the biosynthesis and assembly of secondary wall polymers. In particular information is now available which describes the mechanism whereby wall teichoic acids become covalently linked to peptidoglycan. Largely as a result of the detailed chemical and biosynthetic studies of Baddiley and Glaser and their colleagues, the presence of specific linkage units interposed between the main teichoic acid chain and muramyl residues in the glycan of peptidoglycan was established. Moreover, the similarity in structure of the various linkage units so far examined suggests that organisms possessing wall teichoic acids of widely differing

structure developed almost identical mechanisms for linkage of the two polymers.

The early investigations of Baddiley et al. suggested the involvement of polyisoprenyl-linked intermediates in the biosynthesis of several teichoic acids. However, these observations, which with hindsight were apparently related to the formation of linkage units rather than the teichoic acid itself, were thrown into doubt with the finding that lipid intermediates were not involved in the polymerization reactions catalyzed by both poly(glycerol phosphate) and poly(ribitol phosphate) polymerases. Rather, these purified enzymes were dependent for their activity on an acceptor identified as LTC. The chemical characterization of linkage units initially in isolated walls of *S. aureus* and the more or less simultaneous demonstration of their biosynthesis in a lipid-linked form, appeared to complicate the process of teichoic acid biosynthesis even further. To utilize the two carriers, it was concluded that the biosynthesis and linkage to peptidoglycan of wall teichoic acids was a three-step process. Thus, the main teichoic acid chain was thought to be polymerized on LTC before the completed unit was transferred on to a linkage unit. Only then would the completed polymer become covalently linked through a phosphodiester bond to a muramyl residue in the peptidoglycan. However, the finding that poly(ribitol phosphate) was polymerized directly on linkage unit lipids (29, 31, 74) together with the results of Fischer et al. (63, 64) appeared to eliminate LTC from any role in the biosynthesis of teichoic acid by *S. aureus*. This would simplify the mechanism of polymer assembly to one in which single polyol-phosphate units are added sequentially to the nonreducing terminus of first the linkage unit and subsequently the growing polymer chain (Fig. 5). The recent finding (120) that membrane preparations of both *S. aureus* and *M. varians* catalyze the transfer of preformed teichoic acid chains onto newly synthesized linkage unit lipids must reopen this quesiton. Clearly, in *S. aureus* in vitro teichoic acid biosynthesis appears to occur by either of these two mechanisms; whether both operate in vivo remains unknown, and this area still requires further investigation.

On the other hand these major developments in our understanding of the biosynthesis and linkage of teichoic acids do not provide an answer to the problems associated with the vectorial aspects of assembly. Peptidoglycan, teichoic acid, and teichuronic acid are all synthesized by membrane-bound enzymes from intracellular nucleotide precursors, and in each case the final polymeric product is located outside the membrane. Lipid intermediates are now known to participate in the biosynthetic reactions, and these may well be involved in the translocation of newly synthesized polymer units across the cytoplasmic membrane. However, whereas polymerization of peptidoglycan and teichuronic acid occurs by the addition of newly synthesized units from the lipid intermediate to the reducing terminal of the growing polymer chain, a different situation exists for teichoic acids. Here, the additional units are polymerized by a direct transfer from the cytoplasmic nucleotide to the nonreducing terminus of the growing chain. The proposed mechanism of polymerization of peptidoglycan and teichuronic acid would appear to allow the biosynthetic enzymes to remain in the membrane. Thus, polymerization would occur at the wall-membrane interface, with the lipid intermediates being directly involved in transmembrane movement of the newly synthesized units. Once the teichuronic acid had reached an appropriate length then linkage to peptidoglycan would occur. It should be noted however that recent physical studies of the mobility in membranes of the peptidoglycan lipid intermediates suggests that any transmembrane movement would be exceedingly slow. Clearly, the above description presents an oversimplification of the mechanism of synthesis of peptidoglycan and teichuronic acid. It does however serve to highlight the problems involved in the assembly and linkage of teichoic acid. By adopting a process where access to cytoplasmic precursors is required for synthesis of the teichoic acid chain the bacterium appears to have developed a mechanism of synthesis whereby the completed polymer, rather than newly synthesized repeating units, must be translocated across the cytoplasmic membrane. An alternative mechanism would require the transmembrane transport of the nucleotide precursors themselves allowing polymerization to occur at the wall-membrane interface rather than at the membrane-cytoplasm interface.

This latter possibility is supported by recent experiments of Baddiley and his colleagues (J. Baddiley, personal communication) which showed that protoplasts of *B. subtilis* W23 supplied with the appropriate nucleotide precursors will efficiently synthesize teichoic acid-linkage unit complexes. Moreover, biosynthesis of the complexes was inhibited by mild trypsin treatment, and all attempts to demonstrate penetration of the precursors through the membrane were unsuccessful. Thus, polymerization appeared to be occurring at the membrane surface. These observations clearly represent an exciting development in our understanding of the process

of wall polymer assembly and will undoubtedly serve to stimulate further research in this area.

It will also be important to investigate further the controls exercised by the organism over the synthesis of teichoic and teichuronic acids and in particular to establish in other organisms the effects of environmental changes on the enzymes involved in the biosynthesis of both teichoic acids and their linkage units. Related to this and to the problem of wall assembly as a whole is the relationship of peptidoglycan biosynthesis and structure to the biosynthesis and linkage of secondary polymers in general. The technical problems involved in this area of research present difficulties, particularly in terms of the control of changes in environmental conditions. However, the results obtained from such investigations would undoubtedly further our knowledge of wall assembly in gram-positive bacteria and perhaps contribute to a better understanding of related processes in other organisms.

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