Chapter II

*Bacterial Cell Walls**

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1. Biology of bacterial cell walls[1-8]

The bacterial cell wall is a rigid water-insoluble envelope surrounding the cytoplasmic membrane. This supporting structure, of which the thickness ranges from 150 to 350 Å, is essential for maintaining the cell alive in ordinary environmental conditions which most often are characterized by hypotonicity. If the cell wall is solubilized *in situ* with the help of appropriate enzymes or if the ability of growing bacteria to synthesize new cell walls is blocked through the action of selectively toxic agents such as penicillins, the bacteria burst owing to the high internal osmotic pressure. They can be protected by adding to the medium an adequate concentration of a solute, such as sucrose, to which the cell is impermeable. In this latter case, the bacteria are transformed into spherical bodies characterized by extreme osmotic fragility. These spherical bodies called protoplasts[9] or spheroplasts, can perform almost all of the biochemical activities of the bacteria from which they originate. In a few cases the organisms will continue to grow and divide as spheroplasts; in most cases, however, cell division does not occur. When

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the formation of spherical forms has been induced by an inhibitor of cell wall synthesis, the culture may revert to the normal form if the inhibitor is worked out; such reversion has not been observed when the formation of spherical forms has been induced by bacteriolytic enzymes.

The modern study of the bacterial cell walls started when, in the 1950's, Salton[10] succeeded in isolating and purifying intact cell walls from various bacteria. The study of the building blocks which compose their rigid matrix rapidly led to the very important discovery of a new heteropolymer not found elsewhere in nature. While walls of higher protists consist of polysaccharides like α-cellulose, hemicellulose, glucan, mannan or chitin, the rigid portion of the cell walls, ubiquitous in the bacterial world, is a peptidoglycan complex, also called glycopeptide or mucopeptide or glycosaminopeptide or murein. It is composed of two different *N*-acetylhexosamines and of a few different amino acids. The two *N*-acetylhexosamines are *N*-acetyl-D-glucosamine and its 3-*O*-D-lactic acid ether, called *N*-acetylmuramic acid. This latter amino sugar, found only in bacteria, was first discovered by Strange and Powell[11] in 1954 and its structure was further elucidated by synthesis in 1959 by Strange and Kent[12]. The amino acids are alanine, glutamic acid and a dibasic amino acid, most often lysine or α,α′-diaminopimelic acid (DAP). DAP is another compound found only in bacteria and a few blue-green algae. It was discovered, isolated and characterized by Work[13,14] (1949–1951). Another striking feature of the cell wall is the occurrence in it of amino acids with the D-configuration, this also being an unusual feature of bacterial cell walls. Since the discovery in 1955 by Snell and his colleagues of D-alanine as a component of bacterial cell walls[15,16], the list of the "non-natural" amino acids present in this structure has been extended. Glutamic acid is usually in the D-form and all three DAP isomers, LL-, DD- or meso-, have been encountered. Lysine however is, as far is known, always in the L-form. Soon, it was observed that the relative proportion of these constituents was in many bacterial cell walls grossly equal to *N*-acetylglucosamine 1, *N*-acetylmuramic acid 1, L-ala 1, D-ala 1, D-glu 1 and either L-lys or DAP 1. This led to the hypothesis that the peptidoglycan complex resulted from the polymerization of disaccharide–peptide subunits which obviously possessed many function groups for peptide cross-linking.

It was also early observed that bacterial cell walls are never pure peptidoglycan. In most Gram-negative bacteria, the non-peptidoglycan components represent as much as 90% of the weight of the cell walls and are protein–polysaccharide–lipid (PPL) complexes[17,18] in layers. The direct experimental evidence of multiple layers in this type of cell walls was brought about by several techniques. Rod-shaped Gram-negative bacteria, when growing in a sucrose–penicillin solution, are transformed into spherical bodies[19], called spheroplasts, which still have "weakened" cell walls as shown by electron microscopy. If the osmotic pressure of the external medium is then progressively reduced, true protoplasts first emerge from within the spheroplasts and finally burst when an extreme dilution is reached, leaving in the medium "ghosts" composed of the non-glycopeptide part of the cell walls[2–20]. Similar observations were made by selectively dissolving the rigid cell wall peptidoglycan in Gram-negative cells by enzymatic treatment[21]. Conversely the non-peptidoglycan components have been peeled off from isolated cell walls of Gram-negative bacteria with the help of detergents and phenol, leaving an insoluble residue (about 10% of the weight of the cell wall) still having the characteristic shape and the rigidity of the original structure[22,23].

In Gram-positive bacteria, the non-peptidoglycan components represent 10–50% of the weight of the cell walls and for example, may be proteins (in *Streptococcus*), polymers of polyol phosphate (teichoic acids in various strains of *Staphylococcus*, *Bacillus* and *Lactobacillus*), polymers of *N*-acetylgalactosamine and glucuronic acid (teichuronic acid in *Bacillus subtilis*), polymers of L-rhamnose and *N*-acetyl-D-glucosamine (polysaccharide C in *Streptococcus pyogenes*). None of these polymers has been isolated as a distinct anatomical structure and all of them probably are bound through covalent linkages to the peptidoglycan. As a consequence, when the integrity of this latter complex is lost, the non-peptidoglycan components are also solubilized. If such a selective treatment is carried out on living Gram-positive cells in hypertonic media, true protoplasts are obtained.

The non-peptidoglycan components of bacterial cell walls may appear as useless accessories. Actually, owing to their external location on the surface of the cells, they are the sites of important biological activities. For example, they are antigens or at least they contain the chemical determinants responsible for the antigenic specificity of the bacteria. They contain the specific receptors which mediate phage fixation. Some are powerful endotoxins, while others seem to be implicated in the virulence of bacteria.

Bacterial cell wall biochemistry has rapidly developed along different lines during the past fifteen years and reviews[1–8] have periodically appeared which more specially emphasized one or another aspect of the research. Salton's book *The Bacterial Cell Wall* published in 1964 was an exhaustive

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record of the field[1]. Very recent progress, however, has considerably enlarged our understanding of two major areas, *viz.* the detailed macromolecular structure of, and the biosynthesis pathway to, the complex peptidoglycan, and have provided evidence for the precise mechanism of action of a number of bacteriolytic enzymes and of several antibiotics. These research areas have now reached the stage where biological phenomena begin to be understood at a molecular level.

2. Structure of the bacterial cell wall peptidoglycans

(*a*) *Structure of the peptidoglycan in cell walls of Staphylococcus aureus, strain Copenhagen and of Micrococcus roseus, strain R 27*

Basically, the peptidoglycan is built up of polysaccharide chains cross-linked through peptides, as schematically represented in Fig. 1. The glycan portion is made up of alternating β-1,4-linked units of *N*-acetylglucosamine and *N*-acetylmuramic acid arranged in linear chains. Virtually all of the

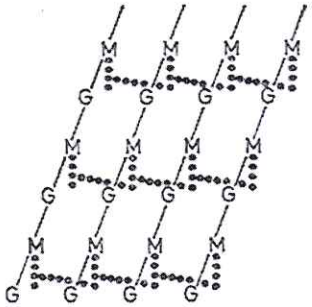

Fig. 1. Schematic representation of a peptidoglycan sheet in cell walls of *Staphylococcus aureus* Copenhagen and *Micrococcus roseus* R 27. The polysaccharide chains consist of alternating β-1,4-linked residues of *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M). The vertical dots from M represent the tetrapeptide subunits, L-ala–γ-D-glu(α-$CONH_2$)-L-lys-D-ala. The horizontal dots represent cross-linking peptide bridges: pentaglycine in *S. aureus*: tri-L-alanyl-L-threonyl in *M. roseus*.

carboxyl groups of the *N*-acetylmuramic acid residues are involved in amide linkages to terminal L-alanine residues of the peptide moiety. This peptide portion is itself composed of peptide subunits, L-alanyl–γ-D-isoglutaminyl–L-lysyl–D-alanine cross-linked by peptide bridges which extend from the

carboxyl group of the terminal D-alanine of one peptide subunit to the ε-amino group of the lysine residue in another peptide subunit. This peptide subunit, or its analog in which L-lysine is replaced by diaminopimelic acid, is probably common to all bacterial cell wall peptidoglycans. However, the chemical composition and the length of the peptide bridges vary according to the bacterial species: pentaglycine bridges occur in cell walls of *S. aureus* Copenhagen and L-ala–L-ala–L-ala–L-thr bridges in cell walls of *M. roseus* R27. This type of structure results in a tight network which, in fact, is an enormous macromolecule encompassing the entire bacterium. It is probably the largest macromolecule in the cell. For obvious reasons, partial acid or base hydrolysis which has been a valuable technique in determining the structure of other natural polymers, was of almost no help in the present case. Fortunately, enzymes which attack specific linkages in the glycan and in the peptide portion of the cell walls have become available within the past few years[8]. They provide the specific tools necessary for study of cell wall structure. With their help, many different peptidoglycan fragments have been obtained from *S. aureus* and *M. roseus* cell walls.

(*i*) *The polysaccharide moiety*

The *Chalaropsis* B enzyme[24,25], the *Streptomyces* 32 enzyme[26,27], and the *Streptomyces* F_1 enzyme[28,29], are all lytic endo-*N*-acetylmuramidases active on *S. aureus* cell walls. They have been used to hydrolyse all of the glycosidic linkages of *N*-acetylmuramic acid to *N*-acetylglucosamine and consequently to *quantitatively* split the polysaccharide chains into disaccharide units (Fig. 2, hydrolysis at linkage A). These disaccharides still linked to the peptide moiety, were liberated as free disaccharides by the action of the *Streptomyces N*-acetylmuramyl-L-alanine amidase[26–30] (Fig. 2, hydrolysis at linkage C). Two types of disaccharides (Fig. 3) were isolated with a yield close to theoretical: disaccharide I is *N*-acetylglucosaminyl–β-1,4-*N*-acetylmuramic acid and disaccharide II is *N*-acetylglucosaminyl–β-1,4-*N*-6-*O*-diacetylmuramic acid[31,32].

The endo-*N*-acetylglucosaminidase of lysostaphin[33,34], which catalyzes hydrolysis at linkage B (Fig. 2), acting in conjunction with a *N*-acetylmuramyl–L-alanine amidase and an endopeptidase also present in the same enzyme preparation, liberated from *S. aureus* cell walls, also in excellent yield, disaccharide units to which the following structures were assigned[35] (Fig. 3): disaccharide III: *N*-acetylmuramyl–β-1,4-*N*-acetylglucosamine and disaccharide IV: *N*,6-*O*-diacetylmuramyl–β-1,4-*N*-acetylglucosamine.

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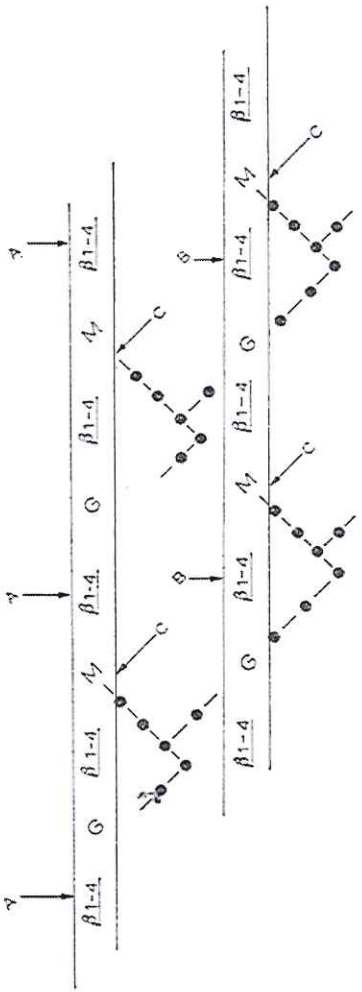

Fig. 2. Degradation of the cell wall peptidoglycans of *Staphylococcus aureus* Copenhagen into disaccharide G–M and disaccharide M–G. For meaning of M, G, and vertical and horizontal dots, see Fig. 1. (A) site of action of endo-*N*-acetylmuramidase, (B) site of action of endo-*N*-acetylglucosaminidase, (C) site of action of *N*-acetylmuramyl-L-alanine amidase. Hydrolyses at A and C yield disaccharide I and disaccharide II. Hydrolyses at B and C yield disaccharide III and IV.

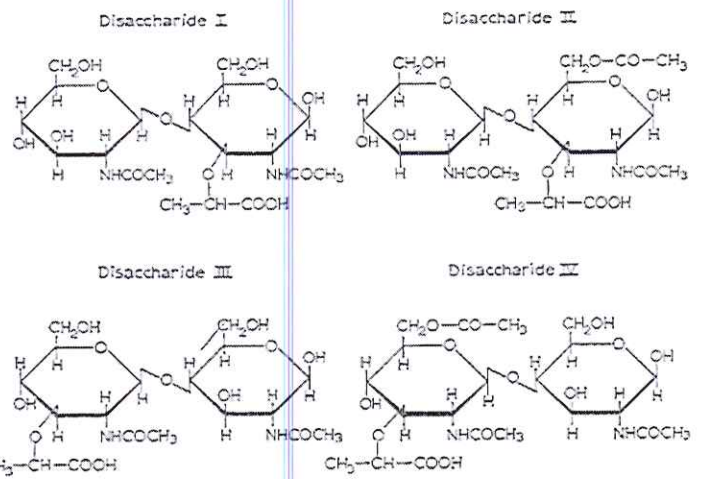

Fig. 3. Structure of disaccharides isolated from the peptidoglycan of *Staphylococcus aureus* Copenhagen. Disaccharide I: *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid. Disaccharide II: *N*-acetylglucosaminyl-β-1,4-*N*,6-*O*-diacetylmuramic acid. Disaccharide III: *N*-acetylmuramyl-β-1,4-*N*-acetylglucosamine. Disaccharide IV: *N*,6-*O*-diacetylmuramyl-β-,1,4-*N*-acetylglucosamine. Disaccharide I has also been obtained from the peptidoglycan of both *M. roseus* and *M. lysodeikticus* and Disaccharide III from *M. lysodeikticus*.

From the foregoing, it follows that a linear sequence of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid, both in the pyranose ring form, can be drawn for the whole glycan portion of the *S. aureus* cell wall peptidoglycan. About 60% of the *N*-acetylmuramic acid are substituted on C-6 by labile *O*-acetyl groups. Whether these substituents are distributed along the chains, in a random or in a well defined order, is however, still unknown.

Intact polysaccharide chains were isolated from *S. aureus* cell walls[36] through the action of a *Myxobacterium* enzyme complex[37,38] which contains an *N*-acetylmuramyl-L-alanine amidase and an endopeptidase, but which is devoid of glycosidase. After fractionation, one portion of the polysaccharide was isolated associated with teichoic acid, *i.e.*, the non-peptidoglycan component of the cell walls, and the rest was obtained with a low content of peptide. Periodate oxidations carried out on the borohydride-reduced carbohydrate indicated an average chain length of about 12 disaccharide units. The intact polysaccharide fraction seems, however, to

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be highly polydisperse. It probably contains constituents composed of 4 to 100 hexosamine residues.

The glycan portion of the *M. roseus* peptidoglycan has not yet been thoroughly studied. However, a structure identical to that found in *S. aureus* can be assigned to it, except that in *M. roseus* the glycan has no *O*-acetyl substituents. Indeed, the glycan moiety of *M. roseus* has also been quantitatively degraded[29] into free disaccharide I with the help of the *Streptomyces* F_1 endo-*N*-acetylmuramidase and of the *Streptomyces* *N*-acetylmuramyl-L-alanine amidase (Fig. 2, hydrolysis at linkage A and linkage C). Study of this disaccharide has provided direct evidence for the alternation of the two acetamido sugars, the β-1,4-linkage of acetylglucosamine to acetylmuramic acid and the pyranose ring form of all of the *N*-acetylglucosamine residues. After digestion of the cell walls with the *Myxobacterium* enzyme complex[39], intact polysaccharide has also been isolated, associated in this case with an as yet unidentified non-peptidoglycan polymer.

The glycan portion of *S. aureus* and *M. roseus* cell wall peptidoglycan can therefore be visualized as a substituted chitin: *i.e.* a polymer of β-1,4-linked *N*-acetylglucosamine residues in which every other sugar is substituted by a 3-*O*-lactyl group. In contrast to chitin, the carbohydrate of the cell wall peptidoglycan is soluble, and thus is, by itself, devoid of any rigidity.

(ii) The peptide moiety

The structure of the peptide moiety was elucidated by analyzing the products of sequential enzymatic degration of the cell walls[39] (Fig. 4). The cell walls were first solubilized by the *Streptomyces* SA endopeptidase which hydrolyses D-alanyl–glycyl linkages in *S. aureus* and D-alanyl–L-alanyl linkages in *M. roseus*, *i.e.* at the amino terminus of the peptide bridges and at the carboxyl terminus of the tetrapeptide subunits (Fig. 4, hydrolysis at linkage 1). The opened bridges were then degraded with the aid of the *Streptomyces* aminopeptidase until all of the lysine residues of the cell walls had their ε-amino groups free (Fig. 4, hydrolysis at 2). At the end of this degradation, the aminopeptidase had liberated, per lysine residue, 5 glycines from *S. aureus* and *seriatim*, three L-alanines and one L-threonine from *M. roseus*. At this stage, the postulated tetrapeptide units Ala–Glu–Lys–Ala had been disconnected from each other but were still attached to an intact glycan polymer. This glycan was then completely degraded into disaccharide–peptide units by means of the *Streptomyces* F_1 endo-*N*-acetylmuramidase (Fig. 4, hydrolysis at linkage 3).

The sequence L-ala–γ-D-glu(α-$CONH_2$)–L-lys–D-ala for the majority of the tetrapeptide units of the *S. aureus* disaccharide–peptide was next established. Treatment of the disaccharide-peptide with the *N*-acetylmuramyl-

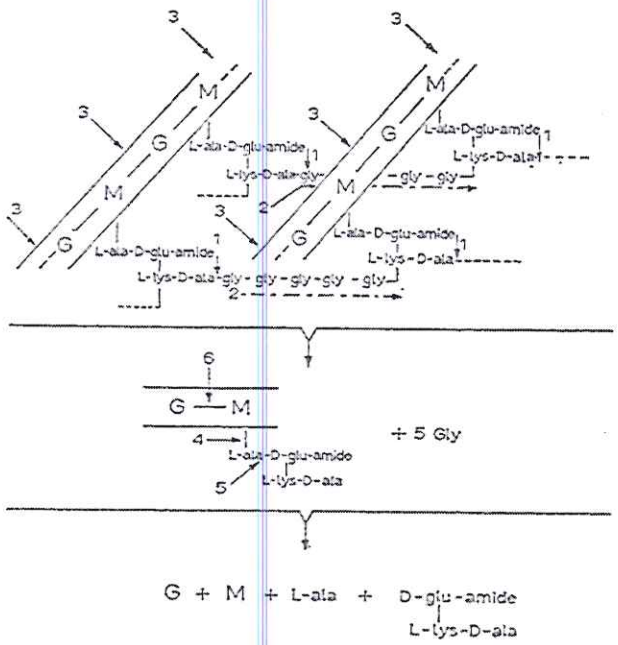

Fig. 4. Sequential degradation of peptidoglycans of *Staphylococcus aureus* Copenhagen and *M. roseus* R 27. (A) Preparation of the disaccharide–peptide subunit. (1) Site of action of *Streptomyces* SA endopeptidase. (2) Degradation of the uncross-linked peptide bridges with *Streptomyces* aminopeptidase. (3) Site of action of *Streptomyces* F_1 endo-*N*-acetylmuramidase. (B) Further degradation of the isolated disaccharide–peptide. (4) Site of action of *Streptomyces* *N*-acetylmuramyl–L-alanine amidase. (5) Site of action of *Streptomyces* aminopeptidase. (6) Site of action of exo-β-*N*-acetylglucosaminidase (from pig epidymis).

L-alanine amidase liberated, per lysine, one disaccharide unit and unmasked one *N*-terminal L-alanine (Fig. 4, hydrolysis at linkage 4). Finally, a second treatment with the *Streptomyces* aminopeptidase liberated, per lysine, one L-alanine residue and unmasked one *N*-terminal D-glutamic acid (Fig. 4, hydrolysis on linkage 5). The tripeptide Glu–Lys–Ala left intact at the end of this sequential degradation was isolated from the final mixture in the form of a di-dinitrophenyl derivative. When subjected to chromatography in var-

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ious solvents with internal markers[40], the natural di-DNP–tripeptide was undistinguishable from the di-DNP derivatives of the synthetic tripeptides γ-L or D-glu(α-$CONH_2$)–L-lys–D-ala but was readily separated from the di-DNP derivative of the same L-glutamyl tripeptide previously deamidated through the action of the *Streptomyces* aminopeptidase. All of the lysine residues in the tetrapeptide had their ε-amino groups free, and D-alanine was *C*-terminal. The presence of ammonia on the α-carboxyl group of glutamic acid had previously been demonstrated by Edman degradation[41], carried out on the peptide moiety of *S. aureus* cell walls. This amidation is compatible with the electrophoretic properties[40] of the disaccharide–peptide which is neutral at pH 5.5 and of the tetrapeptide and the tripeptide respectively obtained in the sequential degradation, which are basic at pH 5.5. Glutamic acid in all cell wall peptidoglycans is known to be in the D-form. *Streptomyces* or leucine aminopeptidases do not deamidate the peptide isolated from the disaccharide–peptide, thus providing further evidence for the D-configuration of the isoglutaminyl residue.

The tetrapeptide from cell walls of *M. roseus* has the same structure. In addition, the tripeptide, L-ala–γ-D-glu(α-$CONH_2$)–L-lys occurs in the cell wall of this organism to the extent of about 25% of the total peptide subunits. The tripeptide must exist in the cell wall peptidoglycan at the COOH-terminus of the polypeptide since it lacks the terminal D-ala residue which serves as anchor point for the *N*-terminal end of the tri-L-ala–L-thr bridges. Similar tripeptide subunits (with DAP instead of lysine) have also been observed in *Corynebacterium diphtheriae*[42].

Molecular weight measurements and peptide end-groups determinations were carried out on endo-*N*-acetylmuramidase-degraded peptidoglycans (in which disaccharide units are interlinked by the intact peptide moiety). The product obtained from *S. aureus* cell walls[43] has a sedimentation constant $s^{\circ}_{20}=1.12$, a diffusion constant $d_{20}=9\cdot10^{-7}$ cm²/sec and a partial specific volume $v=0.67$. These data yield a weight average molecular weight of 9200. Since a disaccharide–tetrapeptide unit with a pentaglycine chain attached to it, has a molecular weight of about 1300, an average degree of polymerization of about 7 can be assigned to the peptide moiety. An estimation of the number average molecular weight is obtained by determining the ε-amino groups of lysine, after completely degrading the native uncross-linked peptide bridges with the aminopeptidase. After such treatment about 150 and 250 respectively ε-amino groups of lysine per 1000 total lysine residues were detected in the soluble glycopeptides of *S. aureus*[43] and *M. roseus*[39], indicating an average degree of polymerization of 6 and 4. It is thus clear that the peptide-linked polymers in the peptidoglycans are relatively small in size and soluble. Like the glycans, the polypeptides cannot by themselves impart rigidity or insolubility to the bacterial cell walls.

(*iii*) *The peptidoglycan*

The insolubility and mechanical strength of the bacterial cell wall peptidoglycan is thus a property of the network as a whole. Both peptide and glycan are essential and they complement each other. Cleavage of either glycosidic or peptide bonds results in solubilization of the whole network.

The cell wall peptidoglycan as represented in Fig. 1, is a two-dimensional network. In building a three-dimensional structure, it would be necessary to provide not only for "horizontal" cross-bridges, but also for "vertical" peptide cross-links between superposed layers. *A priori*, these two kinds of bridges might be different chemically. The mechanisms of action of certain lytic endopeptidases suggest the existence in the cell wall peptidoglycans of *S. aureus* and *M. roseus* of two types of peptide bridges. However, the study of the three-dimensional structure of the cell wall peptidoglycan remains a completely unexplored area of research.

(*b*) *Variations in bacterial cell wall peptidoglycans*

Many variations occur in the cell wall peptidoglycans from bacteria of different origin. It has already been mentioned that instead of lysine, one or more of the LL-, DD- or meso-isomers of diaminopimelic acid occur in the peptide subunit. In fact DAP is the most widely encountered dibasic amino acid. It is found virtually in all Gram-negative bacteria and in a number of strains of Gram-positive bacteria such as: *Lactobacillus*, *Bacillus*, *Propionibacterium*, *Listeria*, *Clostridium* and *Corynebacterium* sp. L-Lysine is only found in certain Gram-positive species such as *Staphylococcus*, *Streptococcus*, *Pneumococcus*, some *Arthrobacter*, *Sarcina*, *Sporosarcina* and *Micrococcus* sp. A few bacterial cell walls, contain no lysine or DAP. Instead, L-2,4-diaminobutyric acid[44], L- and possibly D-ornithine[44–46], hydroxylysine[47] or 2,6-diamino-3-hydroxypimelic acid[48] occur in the peptidoglycan. Although no direct evidence has been forthcoming in many cases, it is assumed that all these dibasic amino acids are involved in peptide cross-links.

The nature and the length of the peptide bridges which cross-link peptide

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subunits also vary according to the bacteria species: pentaglycine in *S. aureus* Copenhagen[39], tri-L-ala–L-thr in *M. roseus* R 27[39], tri-L-ala in another strain of *M. roseus*[39] di- or tri-L-ala in *Streptococcus pyogenes* group A (ref. 40), diglycine in *Micrococcus radiodurans* (unpublished), and L-alanine in *Arthrobacter crystallopoietes*[49]. The foregoing L-lysine or L-ornithine (in the case of *M. radiodurans*) containing cell walls are sensitive to the *Streptomyces* SA endopeptidase[39] and the opened bridges can then be degraded with the *Streptomyces* aminopeptidase. Cross-linkage in which no additional amino acid is involved can occur in the form of a direct D-alanyl–N^{ε}-lysine bond. The *Streptomyces* SA endopeptidase is not active on this type of linkage. Another *Streptomyces* endopeptidase, the ML endopeptidase, has been isolated which selectively hydrolyzes this peptide bond in cell walls of *Micrococcus lysodeikticus* and *Sarcina lutea*[39]. ML endopeptidase also digests cell walls of *Micrococcus flavus* and *Micrococcus citreus*. The *Streptomyces* SA and ML endopeptidases do not act upon any of the numerous DAP containing cell walls which have been examined, including these in which DAP is in the LL-form, such as in *Propionibacterium petersonii*, *P. rubrum* and in the atypical *Corynebacterium anaerobium*[39]. The *Streptomyces* MR endopeptidase has still a more restricted activity spectrum since it is only active on strains of *Micrococcus roseus*[39].

In the DAP group of cell walls, no cross-linking peptide bridge exists so far is known. A direct linkage from D-alanine to DAP occurs in cell walls of *Escherichia coli*[5] and in *Corynebacterium diphtheriae*[50]. This type of peptide cross-link has been demonstrated with the help of two specific endopeptidases, one found in the autolytic system of *E. coli*[51] and the other, the L_3 enzyme, excreted by a strain of *Streptomyces*[52]. The stereochemical configuration of the *N*-terminal group of the meso-DAP residue engaged in the peptide bond with the D-alanine is not yet known.

Variations also occur in the degree of peptide cross-linking. In the case of *S. aureus* and possibly *M. roseus* peptidoglycans (Fig. 1), peptide-linked oligomers at least as large as octomers occur. Other cell walls have a low degree of peptide cross-linking. From extensive studies carried out on *E. coli* peptidoglycan[5], it can be concluded that all disaccharide units in the glycan are substituted by peptide subunits of the composition L-ala–D-glu–meso-DAP–D-ala but that only about 50% of them are joined through D-ala–DAP linkages. The only peptide-linked oligomers in the cell wall of this organism are dimers. Similar results have been obtained for *C. diphtheriae*[42]. Another example of very low degree of peptide cross-linking is given by cell walls of *M. lysodeikticus* in which 75% of the lysine residues have free ε-amino groups[39].

Isoglutamine occurs in cell walls of *S. aureus* Copenhagen[40,41], of *M. roseus* R 27 (ref. 41) and also of *Streptococcus pyogenes*[41] and *C. diphtheriae*[40,42]. Similarly, one of the carboxyl groups of DAP is, in some strains of *Corynebacterium*, substituted by an amide[42]. The masking in the form of a carboxamide of the carboxyl groups of Glu or DAP not used for cross-linking is not, however, a general property of bacterial cell walls. Glycine, for example, substitutes the α-carboxyl group of glutamic acid in cell walls of *Micrococcus lysodeikticus*[41], and in *E. coli* both the α-carboxyl of glutamic acid and one of the carboxyl groups of DAP are unsubstituted.

The structure of the glycan portion has only been extensively elucidated in cell walls of *S. aureus* Copenhagen, *M. roseus* R 27 (see above), *M. lysodeikticus*[53] and *E. coli*[5]. In the latter case, β-1,6-linkages have been proposed for the *N*-acetylmuramyl-*N*-acetylglucosamine linkages, but this assignment should be reinvestigated. Indeed, in early work, β-1,6-linkages had also been proposed for *M. lysodeikticus*[54,55] and *S. aureus*[31] cell walls but it was later shown that this conclusion was based on an erroneous assumption[56,32,53]. Variations might also occur in the structure of the glycan. For example, part of the glycosidic linkages in the *Streptococcus pyogenes* peptidoglycan might be of the 1,3-type[57].

Most of the glycosidases and particularly the endo-*N*-acetylmuramidase egg-white lysozyme, have limited activity spectra. In many cases, it has been shown that the non-peptidoglycan components of the cell walls such as the lipopolysaccharides in Gram-negative bacteria or teichoic acid in *S. aureus*, prevent the enzymes from reaching the sensitive linkages in the peptidoglycan. Much simpler substituents on the peptidoglycan, such as *O*-acetyl groups in some strains of *S. aureus* and *M. lysodeikticus*, can also depress or even abolish the affinity for lysozyme. *Streptomyces* F_1 endo-*N*-acetylmuramidase, however, lyses bacteria or digests the corresponding cell walls of virtually all Gram-positive bacteria[29]. This enzyme seems to hydrolyse preferentially those acetylmuramyl linkages substituted by peptides. Cell walls were prepared from about 30 different strains of the following species: *Staphylococcus*, *Micrococcus*, *Sarcina*, *Bacillus*, *Lactobacillus*, *Propionibacterium*, *Bifidobacterium*, *Streptococcus*, *Listeria*, *Clostridium* and *Corynebacterium*. They all undergo complete solubilization when incubated with the F_1 enzyme. Kinetics of the digestion of these cell walls and analyses of

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the digested products presented striking differences. A complete solubilization of cell walls of *S. aureus* Copenhagen or of *M. roseus* R 27 requires the hydrolysis of all of the glycosidic linkages of *N*-acetylmuramic acid. The number of disaccharide units (still cross-linked through peptides) appearing at the end of the degradation is equivalent to the number of lysine residues of the cell walls, *i.e.* to the number of repeating subunits in the peptidoglycan. In all other cell walls examined, only part of the glycosidic linkages of *N*-acetylmuramic acid or even a very small number of them as in *M. lysodeikticus*, are susceptible to hydrolysis by F_1 enzyme. Yet the incomplete splitting of the polysaccharide chains is sufficient to induce cell wall solubilization. The glycan portion of one cell wall of this group, that of *M. lysodeikticus*[53], has been shown to have a structure identical to that of *M. roseus* or of *S. aureus* (if deprived of *O*-acetyl group). In *M. lysodeikticus*, however, at least 50% of the *N*-acetylmuramic acid residues are not substituted by peptide[53,29]. Since only part of the *N*-acetylmuramic acid residues serve as branching points for cross-links, it is conceivable that the whole peptidoglycan network can be solubilized through the hydrolysis of only a small number of glycosidic bonds. It thus appears that the frequency and the location of the peptide substituents along the polysaccharide chains is another frequent variation among bacterial cell walls.

Tight peptidoglycan networks exclusively built up of cross-linked disaccharide-peptide units as found in cell walls of *S. aureus* and *M. roseus* (Fig. 1) would rather be exceptional. In many cell walls, loose peptidoglycan networks occur, characterized by a low degree of cross-linking between the polysaccharide chains. However, despite all these chemical and structural variations, the integrity of both the polysaccharide chains and of the cross-links between the peptides are, in all cases, essential for the rigidity of the bacterial cell walls.

3. Biosynthesis of the bacterial cell wall peptidoglycans

There are three stages in the biosynthesis of the peptidoglycan of bacterial cell walls. These will be discussed in turn. They are: (*a*) The biosynthesis of the precursors of the peptidoglycan, (*b*) The utilization of these precursors to form linear peptidoglycan strands, and (*c*) The cross-linking of the linear peptidoglycan strands to form a two- or three-dimensional network. Antibiotics are known which are specific inhibitors of each stage of cell wall synthesis. D-Cycloserine and *O*-carbamyl D-serine interfere with the biosynthesis of the cell wall precursors. Vancomycin, ristocetin and bacitracin interfere with the formation of the linear peptidoglycan strands. Penicillins and cephalosporins interfere with the cross-linking of the strands. Studies of the mechanism of action of these antibacterial substances are intimately associated with studies of the biosynthesis of bacterial cell walls and indeed have been a major impetus to these studies. It is, therefore, necessary also to discuss these mechanisms in connection with a discussion of cell wall synthesis.

(*a*) *Biosynthesis of the peptidoglycan precursors*

In 1949, at the same time Leloir and his collaborators isolated uridine diphosphoglucose from yeast[58], Park and Johnson[59] isolated a material containing uracil and phosphate which accumulated in large amounts in cells of *Staphylococcus aureus* treated with penicillin. It was later shown that this material could be separated into 3 uridine diphosphate derivatives containing an unknown acetamido sugar[60]. One of these compounds contained no additional component, another contained one residue of L-alanine and a third contained a peptide composed of DL-alanine, D-glutamic acid and L-lysine in a molar ratio of 3:1:1. Almost ten years were required for the complete elucidation of the structure of these nucleotides. An unknown acetamido sugar had also been found in a peptide which is solubilized during germination of spores. This sugar was eventually crystallized from spore material and shown both by degradation and by chemical synthesis to be a 3-*O*-D-lactic acid ether of *N*-acetyl-D-glucosamine, namely *N*-acetylmuramic acid[11,12]. A variety of experimental data established that the sequence of the peptide was L-alanyl–D-γ-glutamyl–L-lysyl–D-alanyl–D-alanine[61–63]. In this peptide the α-carboxyl group of glutamic acid and the ε-amino group of lysine are free. Leloir and his collaborators also isolated from yeast uridine diphospho-acetylglucosamine, a second compound of importance in the biosynthesis of bacterial cell walls[64]. Later uridine nucleotides similar to those found in *S. aureus* were isolated from *Escherichia coli*. However, these latter compounds contained meso-diaminopimelic acid instead of L-lysine in the peptide sequence[4].

Uridine nucleotide accumulation in *S. aureus* can be induced by means other than penicillin[4]. Treatment with D-cycloserine induces accumulation of UDP–acetylmuramyl–L-ala–D-glu–L-lys. Lysine deprivation leads to accumulation of UDP–acetylmuramyl–L-ala–D-glu. Finally, treatment with

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gentian violet leads to accumulation of UDP–acetylglucosamine, UDP–acetylglucosamine pyruvate enol ether, and UDP–acetylmuramic acid. During the course of isolation of these compounds it was apparent that they represented a biosynthetic sequence. With the isolated compounds available as substrates it was possible to establish the reaction sequence[66-71] shown in Fig. 5. The first two reactions are phosphorylations of UMP and

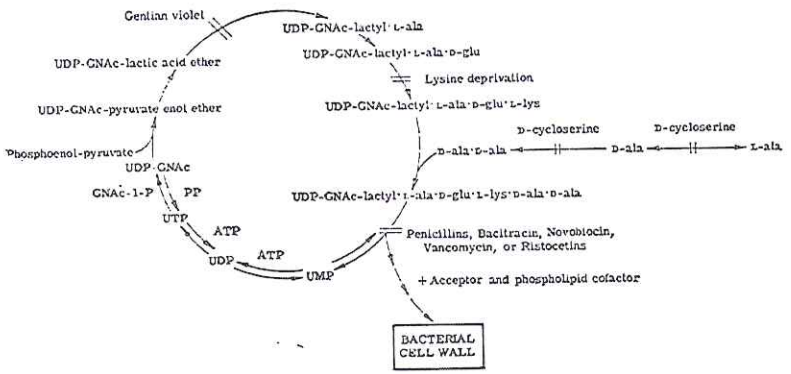

Fig. 5. Reaction cycle which leads to the synthesis of uridine diphosphate acetylmuramyl–pentapeptide, a bacterial cell wall precursor. Points of inhibition by antibiotics and other substances are indicated.

UDP by ATP to yield UTP. UTP then reacts with α-D-*N*-acetylglucosamine 1-phosphate to yield UDP–acetylglucosamine and inorganic pyrophosphate[66]. This reaction is catalyzed by an enzyme termed UDP–acetylglucosamine pyrophosphorylase, and is analogous to the reactions which lead to the synthesis of NAD, UDP–glucose and many other compounds of this type.

A three-carbon fragment can be transferred to UDP–acetylglucosamine from 2-phosphoenol pyruvate, the glycolytic intermediate[67]. The products are the pyruvate enol ether of UDP–acetylglucosamine and inorganic phosphate. The following step, the reduction of the UDP–*N*-acetylglucosamine pyruvate enol ether to UDP–*N*-acetylmuramic acid, was detected in extracts of *S. aureus*, but never carefully studied[4]. It should not be regarded as having been established.

The amino acids are added step-wise to UDP–acetylmuramic acid, each

step being catalyzed by an enzyme which is highly specific for both its uridine nucleotide and amino acid substrates[68-71]. Thus, the sequence of the peptide is determined entirely by the sequence of the individual enzymes which catalyze these amino acid adding reactions, and no evidence for participation of a polynucleotide template in this process has ever been found. In the first three steps L-alanine, D-glutamic acid and L-lysine are added sequentially to the uridine nucleotide, UDP–acetylmuramic acid. Each of these reactions is catalyzed by a separate enzyme which requires ATP and a divalent cation, either Mg^{2+} or Mn^{2+}. For addition of the last two amino acids first D-alanine is utilized to make the dipeptide, D-alanyl–D-alanine and then the dipeptide is added to the uridine nucleotide containing the tripeptide to form the final pentapeptide-containing product. Once again these reactions are catalyzed by separate enzymes which require ATP and a divalent cation. These enzymes have been found in a large number of bacteria and the following have been purified and studied: The alanyl–alanine synthetase of *Streptococcus faecalis*[72,73], the alanyl–alanine adding enzyme from *E. coli*[74], and the L-lysine and D-glutamic acid adding enzymes from *S. aureus*[70,71]. A meso-diaminopimelic acid adding enzyme of *Corynebacterium xerosis*, which catalyzes a reaction analogous to that catalyzed by the L-lysine adding enzyme, has also been purified and studied[75,76]. In all of these cases it has been shown that the adenine nucleotide reaction products are ADP and inorganic phosphate. Particularly noteworthy in the specificity of these enzymes for their amino acid substrates are the strict specificities of the L-lysine adding enzyme for L-lysine and the meso-diaminopimelic acid adding enzyme for meso-diaminopimelic acid. One, but not both, of these enzymes is present in bacterial species and, therefore, an important feature of the specificity of the peptide sequence, *i.e.* the nature of the dibasic amino acid, is strictly determined by the specificity of a single enzyme which is able to utilize either L-lysine or meso-diaminopimelic acid as its substrate. Presumably the same specificity features operate in cases where other dibasic amino acids occur in the peptidoglycan, such as L-ornithine or L-diaminobutyric acid, but these have not yet been investigated. A requirement of the alanyl–alanine synthetase of *S. aureus* for a still unidentified heat stable cofactor has been noted but no further information has been obtained[77].

A number of other enzymes are required for the synthesis of UDP–acetylmuramyl–pentapeptide. These are the enzymes which lead to the synthesis of D-alanine, D-glutamic acid, or of *N*-acetylglucosamine 1-phosphate. D-Alanine is formed from L-alanine in a reaction catalyzed by alanine

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racemase (EC 5.1.1.1)[78]. This enzyme is a pyridoxal phosphate containing protein which catalyzes the reversible interconversion of the two optical antipodes. There are two mechanisms for formation of D-glutamic acid. One of these is the reversible interconversion of L-glutamic acid and D-glutamic acid catalyzed by glutamic acid racemase (EC 5.1.1.3)[79]. The other mechanism is the stereospecific transamination of α-ketoglutarate by D-alanine to yield D-glutamic acid and pyruvate[80,81]. The formation of acetylglucosamine 1-phosphate by glycolytic enzymes requires three steps: the amidation of fructose 6-phosphate by glutamine to yield glucosamine 6-phosphate, the acetylation of the latter with acetyl-CoA to yield acetylglucosamine 6-phosphate, and finally, the formation of acetylglucosamine 1-phosphate catalyzed by phosphoacetylglucosamine mutase (EC 2.7.5.2)[82].

D-Cycloserine and *O*-carbamyl-D-serine are specific inhibitors in the metabolic pathway which leads to the synthesis of these nucleotides[83–85]. Although both of these antibiotics induce accumulation of UDP–acetylmuramyl–L-ala–D-glu–L-lys, neither of them inhibits the addition of D-ala–D-ala to the tripeptide-containing nucleotide. Instead they are both inhibitors of the formation of D-alanyl–D-alanine. D-Cycloserine is a true competitive inhibitor of both alanine racemase and D-alanyl–D-alanine synthetase (EC 6.3.2.4.)[3,86]. Extensive kinetic studies have been carried out on the inhibition of D-alanyl–D-alanine synthetase and it has been shown that this enzyme contains two sites for binding of alanine, termed the donor and acceptor sites[73]. The antibiotic inhibits this enzyme mainly by competing at the donor site. The Michaelis constants for D-alanine for both alanine racemase and for the donor site of alanyl–alanine synthetase are about $5 \cdot 10^{-3}$ *M*. On the other hand, the K_i for the antibiotic is 100 times smaller, of the order of $5 \cdot 10^{-5}$ *M* in each case. The antibiotic is thus bound by the inhibited enzymes about 100 times more effectively than they bind their natural substrate. The molecular basis for the competition lies in the striking structural similarity of antibiotic and substrate (Fig. 6). It is believed that the greater affinity of D-cycloserine for the inhibited enzymes may be due to the fact the D-cycloserine is held in the conformation preferred by the substrate binding sites of these enzymes as a consequence of the formation of the 5-membered ring in the antibiotic[4,87]. The presence of this ring restricts rotation at the carbon–carbon bonds and thereby restricts the possible conformations of the antibiotic as compared to the substrate which can have many possible conformations. L-Cycloserine is not an inhibitor of alanine racemase despite the fact that L-alanine is one of its substrates. This failure of L-cycloserine

to inhibit may be due to the inability of this synthetic substance to assume the conformation required on the substrate binding site[87].

O-Carbamyl-D-cycloserine is a competitive inhibitor of alanine racemase but it does not inhibit D-alanyl–D-alanine synthetase[85]. The acquisition of resistance to these antibiotics is a stepwise phenomenon; that is, increasing levels of resistance are obtained by successive isolations of resistant mutants[88].

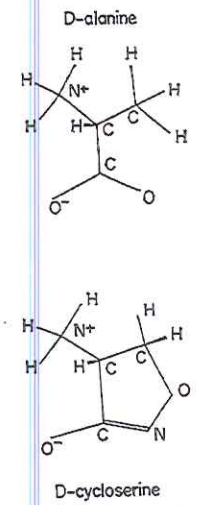

Fig. 6. Structures of the substrate, D-alanine, and the antibiotic, D-cycloserine. The antibiotic is a competitive inhibitor of the utilization of this substrate for bacterial cell wall synthesis.

In the case of D-cycloserine the acquisition of the resistant state is associated with increased levels of both alanine racemase and alanyl–alanine synthetase[89]. On the other hand, the acquisition of resistance to *O*-carbamyl-D-serine is associated with increased level of alanine racemase only.

(*b*) *Utilization of uridine nucleotide precursors to form linear peptidoglycans*

In 1964 two systems were described for the simultaneous utilization of UDP–acetylmuramyl–pentapeptide and UDP–acetylglucosamine for the synthesis of products which in one case was defined as a chromatographically immobile product[90] and in the other case as an acid-precipitable product[91]. The formation of the chromatographically immobile product could be ob-

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tained only when incubations were carried out on a filter paper support. The two enzymes were prepared in different ways. In both cases they were the particulate fraction obtained from cell disintegrates by high speed centrifugation after preliminary low speed centrifugation to remove inactive materials. Subsequent events have shown that enzymes obtained after disintegration of cells by sonic oscillation and employed in incubations on filter paper[90], catalyze all the reactions in a complex sequence which leads to the synthesis of a linear glycopeptide. It was subsequently found that enzymes prepared after alumina grinding of cells catalyze the entire sequence without the necessity of a filter paper support[92,93]. On the other hand, preparations obtained after disintegration with glass beads[91] apparently catalyze predominantly only the initial stages of this reaction sequence[94]. Thus, the manner of preparation of the enzyme is of great importance in the study of these reactions.

In the study of the overall reactions several features of product identification were especially noteworthy. The peptidoglycan reaction product contained both of the D-alanine residues present in the uridine nucleotide precursor[93], *i.e.* it contained pentapeptide substituents on the acetylmuramic acid rather than the tetrapeptide substituents which are found in the completed peptidoglycan. Analysis of the uridine nucleotide products of the reaction indicated that UDP was formed from UDP–acetylglucosamine as expected for such a transglycosylation reaction[92], and as had been demonstrated previously for many other transglycosylations of this type. However, no UDP was formed from UDP–acetylmuramyl–pentapeptide, and instead it could be shown in two organisms that UMP and inorganic phosphate were the primary products of the transglycosylation reaction involving this uridine nucleotide[90,92]. This unexpected finding then led to the elucidation of the complex reaction sequence for formation of a linear peptidoglycan, as illustrated in Fig. 7. The initial acceptor is a membrane-bound phospholipid and phosphoacetylmuramyl–pentapeptide is transferred to it from UDP–acetylmuramyl–pentapeptide with formation of UMP. This initial attachment is reversible[95]. Acetylmuramyl–pentapeptide is attached to the phospholipid through a pyrophosphate bridge, one phosphate of which is present in the phospholipid[96,97].

After attachment of the phosphoacetylmuramyl–pentapeptide to the phospholipid acceptor, acetylglucosamine is transferred from UDP–acetylglucosamine to form disaccharide–pentapeptide phospholipid[92]. The disaccharide moiety is then transferred to some endogenous acceptor (which

has not been identified but is presumed to be an incomplete glycopeptide chain). Phospholipid pyrophosphate is a product of this reaction and is then dephosphorylated to yield the original phospholipid phosphate carrier. By repeated cycles of this kind a linear peptidoglycan chain can be built up.

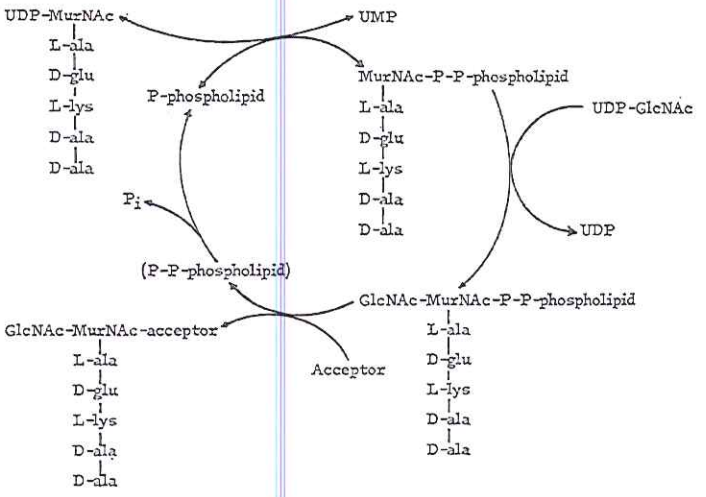

Fig. 7. The phospholipid cycle in the biosynthesis of the peptidoglycan of the cell walls of *Staphylococcus aureus* and *Micrococcus lysodeikticus*. A portion of the cycle which is common to both organisms is shown.

From examination of this reaction sequence, the origins of the UDP, UMP, and inorganic phosphate formed in the overall reaction are obvious. The attachment of the sugar fragments to the phospholipid carrier may serve as a means of transporting the sugar fragments from their intracellular site of synthesis to their extracellular site of utilization. The cell wall lies outside of the cell membrane, the permeability barrier, and is in this sense an extracellular product.

This reaction cycle which operates *in vitro* is, however, a simplified form of the cycle which operates *in vivo*. Modification of the disaccharide–pentapeptide moiety occurs while it is attached to the lipid intermediate. In *S. aureus* this modification includes amidation of the α-carboxyl group of glutamic acid and addition of a pentaglycine chain attached to the ε-amino group of lysine (Fig. 8). The amidation of the α-carboxyl group of glutamic acid requires ammonium ions and ATP[98,99]. Demonstration of this reaction

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requires careful preparation of ammonia-free substrates. The donor of glycine for the formation of the pentaglycine chain is glycyl–sRNA[91,93]. The participation of sRNA in this reaction was the first demonstrated example of the role of sRNA in any process other than protein synthesis.

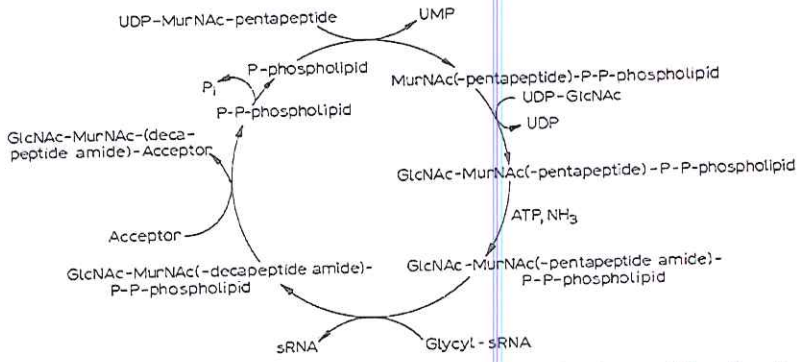

Fig. 8. The phospholipid cycle in the biosynthesis of the peptidoglycan of the cell wall of *Staphylococcus aureus*. In addition to the common part of the cycle shown in Fig. 7, this reaction mechanism includes reactions for modification of the lipid by amidation of the α-carboxyl group of glutamic acid and addition of an open pentaglycine chain to the ε-amino group of lysine in the pentapeptide.

Among the many questions still to be answered about this system is the question of whether the sRNA molecule which participates in cell wall synthesis is the same sRNA molecule which carries glycine for incorporation into protein. It is conceivable that a specific sRNA molecule is present for activation of glycine for cell wall synthesis, but the answer to this question will require much further purification of the components of this system. The activation of glycine in this manner is not a phenomenon which is restricted to *S. aureus*. It has also been demonstrated that threonine is activated as threonyl–sRNA by enzyme preparations obtained from *M. roseus* for incorporation as the first amino acid in the bridge[100]. Thus, it is possible that activation of amino acids as sRNA derivatives is a general feature of cross-peptide bridge synthesis in bacteria.

In *M. lysodeikticus* a slightly different reaction sequence occurs (Fig. 9). In this organism a single glycine residue is added to the α-carboxyl group of glutamic acid[98,101,102]. The glycine incorporated has a free carboxyl group. Although it has not yet been possible to demonstrate that ADP and inorganic phosphate are the adenine nucleotide reaction products in the forward

direction due to contamination of the enzyme preparation by ATPases, it has been shown that in the reverse direction the arsenolysis of the glycine containing product specifically requires ADP[102]. This provides presumptive evidence that ATP is split to ADP and inorganic phosphate in the reaction.

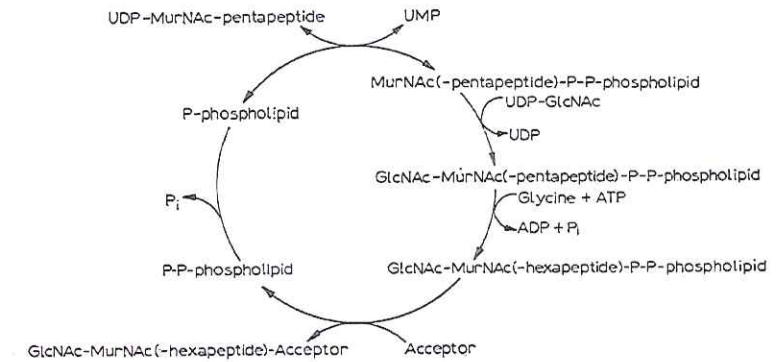

Fig. 9. The phospholipid cycle in the biosynthesis of the peptidoglycan of the cell wall of *Micrococcus lysodeikticus*. In addition to the common part of the cycle shown in Fig. 7, this reaction cycle includes a reaction in which the lipid is modified by addition of glycine to the α-carboxyl group of glutamic acid.

So far as is known no further modification of the disaccharide hexapeptide is required before its utilization for formation of the linear glycopeptide strands.

The phospholipid intermediates were originally detected as radioactive compounds which ran near the solvent front in paper chromatograms of incubation mixtures. They have subsequently been isolated by chromatography on columns of DEAE-cellulose and analysis of one of the lipid intermediates from both *M. lysodeikticus* and *S. aureus* indicate that they are both complex glycerophosphatides related to the cardiolipins[103]. Small differences in the analyses of the intermediate from the two sources as well as some apparent differences in stability of the two compounds have been apparent. At the present time no definite conclusions can be made as to whether the two lipid intermediates are identical or not. This question requires very careful investigation. The particulate enzyme preparation which catalyzes these reactions is believed to be derived from cell membrane. It contains both the phospholipid acceptor and the enzymes which catalyze these reactions. The lipid and protein components of this enzyme have been

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separated by extraction with chloroform–methanol at low temperatures[98]. They could be reconstituted in the presence of low concentrations of deoxycholate.

In fact, a wide variety of phospholipid intermediates in glycopeptide synthesis can be isolated differing in the precise nature of the substituents on the sugar fragment. Many of them have been isolated and shown to be utilized as substrates in the absence of added uridine nucleotides, thus proving their role as true intermediates in the reaction cycle[98].

Three antibiotics, vancomycin, ristocetin and bacitracin, interfere in this reaction cycle. At low concentrations the only reaction inhibited is the utilization of the phospholipid intermediate for peptidoglycan synthesis, the last reaction in the cycles shown in Figs. 7–9. The enzyme which catalyzes this last reaction and is inhibited by these substances is termed peptidoglycan synthetase. At low concentrations (20 μg/ml) of ristocetin or vancomycin these substances inhibit peptidoglycan synthetase but do not interfere with the synthesis of the lipid intermediate[90,92]. At much higher concentrations (100 μg/ml or greater) inhibition of some of the reactions which lead to the synthesis of lipid intermediates can also be demonstrated[90,92,95,97]. The precise manner in which these substances interfere with peptidoglycan synthetase is presently unknown. The effects of bacitracin are more complicated than those of vancomycin and ristocetin, and, although it appears to interfere at some point in this reaction mechanism, much more information about it is required.

(c) *Cross-linking of peptidoglycan strands*

After completion of the structural studies described above, it seemed obvious that in some manner terminal D-alanine residues of UDP–acetylmuramyl–pentapeptide which were retained in the formation of the linear peptidoglycan strands were lost in some manner in the final formation of the cross-linked peptidoglycan. In all organisms examined the peptide bridge was attached at its *N*-terminal end to the carboxyl group of the subterminal D-alanine of the pentapeptide. Thus, it seemed possible that this bridge-forming reaction was a transpeptidation in which the bond energy of the terminal peptide bond in the pentapeptide was utilized for the formation of the cross-link, with the release of the terminal D-alanine residue. Efforts to demonstrate this transpeptidation in the two organisms which had been extensively studied from a biosynthetic standpoint, namely *S. aureus* and *M. lysodeikticus*, were completely unsuccessful. The reason why this activity is lost during preparation of the particulate enzyme system remains obscure. However, for other reasons, attention was directed to the synthesis of the peptidoglycan in the cell wall of *E. coli*. It was immediately apparent that the *E. coli* system catalyzed not only the formation of the linear glycopeptide strands described above but also the cross-linking of these strands[104]. For these experiments labeled uridine nucleotides containing DAP rather than lysine were employed. Initial reactions were similar or identical to those which occur in *S. aureus* and *M. lysodeikticus* except for the substitution of DAP for lysine in the various intermediates, as in Fig. 7. These enzyme preparations also catalyzed the cross-linking reaction (Fig. 10). In this reaction the terminal D-alanine of

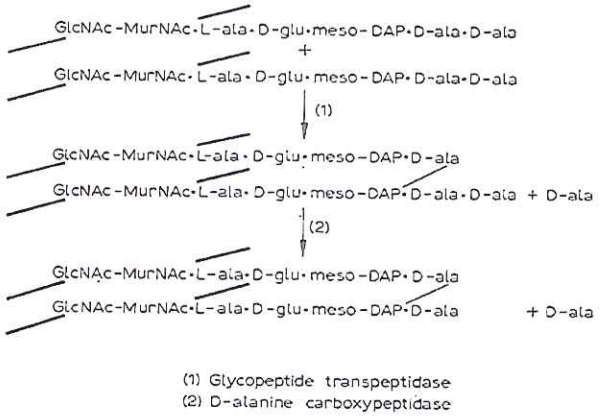

Fig. 10. Formation of peptide-linked dimers in the peptidoglycan of the cell wall of *Escherichia coli* catalyzed by (1) peptidoglycan transpeptidase and (2) D-alanine carboxypeptidase.

the pentapeptide is released. No external energy source, such as ATP, is required. Presumably this cross-linking reaction occurs outside of the cell membrane at the site of the cell wall and may have been evolved by bacterial cells as a means of catalyzing an important synthetic reaction at a site where no ATP is available. In addition to the transpeptidation the enzyme preparations from *E. coli* contain a D-alanine carboxypeptidase which removes a D-alanine residue from the second strand involved in the cross-linking (Fig. 10). As indicated above the cell wall of the peptidoglycan of *E. coli* does not contain peptide-linked oligomers larger than the dimer. Apparently the D-

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alanine carboxypeptidase provides the means by which the size of the peptide oligomers is controlled, since removal of the D-alanine residue in this manner prevents further transpeptidation and further enlargement of the polypeptide network.

Penicillins and cephalosporins are specific inhibitors of this cross-linking reaction. Initially it was shown in experiments with whole cells by a number of investigators[105-111] that in the presence of penicillin uncross-linked cell walls were formed. Of particular interest is the original demonstration by Martin[105,106] that cells of *Proteus mirabilis* in the presence of penicillin continued to grow, but as spheres rather than rods. The sphere form contained a normal cell wall quantitatively but the wall which was present appeared to be uncross-linked. The reason why this particular bacterial cell will continue to grow and divide with an uncross-linked wall, while other bacterial cells are unable to grow in this manner, remains obscure.

It was further hypothesized that penicillin is an analog of the D-alanyl–D-alanine at the end of the linear peptidoglycan strand[108,109]. Molecular models revealed a striking similarity in structure between penicillins or cephalosporins and the end of this strand (Fig. 11). In particular it should be pointed out that the highly reactive CO–N bond in the β-lactam ring of penicillin is the analog of the peptide bond in D-alanyl–D-alanine which is involved in the transpeptidation. A reaction mechanism for the transpeptidation (Fig. 12) was proposed and it was suggested that penicillin acylates the transpeptidase through the β-lactam ring at the active site involved in the transpeptidation.

When the cell-free preparation which catalyzed transpeptidation was obtained from *E. coli*[104], it was possible to demonstrate directly that this enzyme was irreversibly inactivated by low concentration of penicillin G, other penicillins and cephalosporins. The sensitivity of these enzymes to various penicillins and the sensitivity of the growth of *E. coli* cells to penicillins is summarized in Table I. It may be noted that with one exception the sensitivity of growth of the cells to penicillins was paralleled by the sensitivity of peptidoglycan transpeptidase to these substances. The important exception is penicillin G, where it may be noted that the enzyme was 10 times more sensitive to this substance than were the cells. These data appear to indicate that penetration of the antibiotic to the site of the sensitive enzyme may be an important factor in determining antibiotic sensitivity. Failure of penetration could explain the relative insensitivity of most Gram-negative bacteria to some penicillin preparations. However, another factor appears to operate variations in the nature of the transpeptidases themselves. For example, the

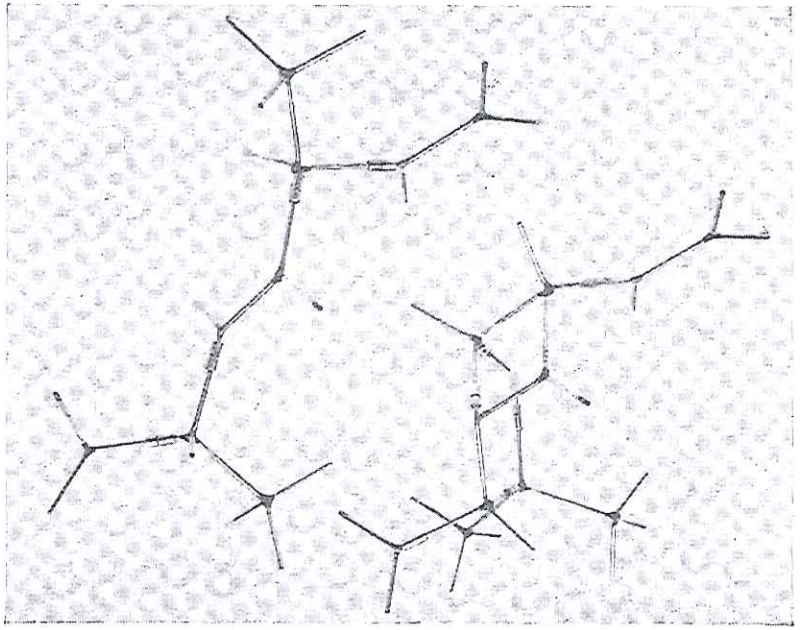

Fig. 11. The molecular structure of a penicillin (left) and of the D-alanyl–D-alanine end of a peptide subunit in the peptidoglycan (right). Dreiding stereomodels are shown. The correspondence in position of the CO–N bond in the β-lactam ring of penicillin and the peptide bond in D-alanyl–D-alanine is particularly noteworthy.

transpeptidase of *E. coli* is virtually insensitive to methicillin, but it is well known that cells of *S. aureus* are very sensitive to this antibiotic. It may be presumed that the transpeptidase in *S. aureus*, when it can be demonstrated, will prove to be equally sensitive.

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The D-alanine carboxypeptidase present in *E. coli* was even more sensitive to penicillins than was the transpeptidase. Its inhibition cannot be a lethal reaction since it was inhibited at concentrations far below the growth inhibitory concentrations for penicillins. Also shown in Table I is the fact that the peptidoglycan synthetase in *E. coli* is far more sensitive to ristocetin, vancomycin and bacitracin than are the cells. Once again it appears that the cells contain the sensitive component but the antibiotics must have failed to penetrate to its site within the cell.

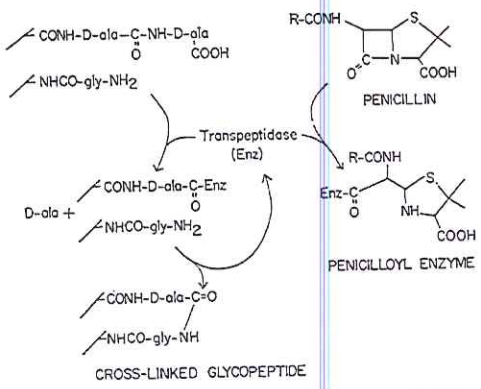

Fig. 12. The proposed mechanism of transpeptidation and its inhibition by penicillins.

TABLE I

COMPARISON OF THE ANTIBIOTIC SENSITIVITY OF CELLS OF *Escherichia coli* AND OF SEVERAL ENZYMES OBTAINED IN CELL-FREE PREPARATIONS

Antibiotic	*Concentrations required for 50% inhibition (μg/ml)*			
	Growth	*Glycopeptide synthetase*	*Glycopeptide transpeptidase*	*D-Alanine carboxypeptidase*
Ampicillin	3	—	3	0.04
Penicillin G	30	—	3	0.02
Cephalothin	50	—	50	1
Methicillin	1000	—	1000	1
Ristocetin	1000	3	—	—
Vancomycin	100	10	—	—
Bacitracin	1000	40	—	—

—, Not inhibited

4. Non-peptidoglycan components of cell walls of Gram-positive bacteria

The bacterial cell wall is defined operationally as that component of the bacterial cell which remains easily sedimentable after mechanical rupture of the cellular "envelope". Thus the nature of its components depends to some extent on the preparation procedure, such as the vigor and length of application of the disruptive force and the extent of subsequent washing. In particular, enzyme digestion with nucleases and trypsin (EC 3.4.4.4) is often used in order to remove cytoplasmic and membrane components from cell walls, and may concomitantly remove true wall substituents. In general, materials that can be removed by washing at neutral pH in the absence of detergents are not regarded as cell wall components even though they may exist outside the plasma membrane. This category includes capsular materials and also applies to enzymes like invertase (EC 3.2.1.26) in yeast and *Neurospora* and the so-called "intracellular" glycerol teichoic acid of streptococci, all of which are located between the plasma membrane and the wall proper, and are released in soluble form on disruption of the cells.

A chemical definition of Gram-positive cell walls can only be attempted in those cases which have been most extensively studied. It would describe the walls as an insoluble polymeric complex of the recognized cell wall components in their characteristic ratios. Thus, in the case of *S. aureus* strain Copenhagen, a cell wall unit consisting of one unit of the peptidoglycan with one unit of the teichoic acid has a molecular weight of 2080, so that 1 mg of a cell wall containing nothing but these components, in a 1:1 ratio, should contain 0.48 μmole of each unit. Analyses of several preparations yield analyses varying between 0.45 and 0.47 μmole/mg, but these were all trypsin-treated preparations. Non-trypsin-treated preparations of *S. aureus* cell walls will autolyze completely, and presumably also carry Jensen's antigen A, a trypsin-positive polypeptide antigen (see below). Many cell wall preparations which have not been "killed" by heat or detergent treatment will autolyze, and so contain lytic enzymes, which are part of the cell wall according to the operational definition. Other enzymes, such as those involved in the terminal reactions of cell wall biosynthesis, may be so integrated with their product that they also occur in cell wall preparations, however well they are washed. One example may be a uridylic acid phosphatase which occurred in a preparation of *Staphylococcus aureus* cell walls[113]. Presumably such cell wall components are removed by trypsin treatment. Treatment of *S. aureus* cell walls with an impure pancreatic DNAase preparation[114]

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results in the solubilization of a peptide agglutinogen (see below). As a result of the frequent use of trypsin in cell wall preparations, the bulk of available information on non-glycopeptide cell wall components is concerned with carbohydrate polymers, and a review of this information will comprise the major part of this section. The structure of the "intracellular" glycerol teichoic acids will be briefly discussed, because of their close structural relationship to cell wall teichoic acids.

The influence of growth conditions and phase of growth on the structure of isolated cell walls has been little studied, but may be profound, especially on the ratio of peptidoglycan to non-peptidoglycan constituents. The rate of cell wall synthesis in *B. megaterium* seems to vary less with medium composition than does the rate of cytoplasmic synthesis, with the result that faster growing cells have less cell wall[115]. The cell wall compositions were not extensively studied. Deprivation of *Streptococcus faecalis* of a non-cell wall amino acid required for growth results in a thickening of the walls without cell division[116]. D-Serine is incorporated into the cell wall peptidoglycan of *M. lysodeikticus* when it is added to the medium, and glycine incorporation is decreased[117]. When sub-optimal amounts of lysine are present, hydroxylysine is incorporated into the peptidoglycan in place of lysine by *Streptococcus faecalis* and *Leuconostoc mesenteroides*[118]. *Bacillus stearothermophilus* and *Bacillus coagulans* grow faster at 55° than at 37° and at 55° produce cell walls with a decrease in both teichoic acid content and mechanical fragility[119]. *Bacillus subtilis* (168 I^-C^+) cell walls were found to vary in galactosamine content during log phase and early stationary phase growth in normal medium, and to have very low galactosamine contents when grown in supplemental media[120]. Teichoic acid itself was not measured in these studies. Galactosamine in these walls fractionates with the teichoic acid after autolysis, but it is not known if it is an integral part of this cell wall component[121].

Finally, cell wall preparations can be examined in the electron microscope and are seen as preparations of collapsed sacs whose shape derives from that of the parent cell, and which should be visibly free of other kinds of fragments such as membranous residues. This gives some evidence of the purity of wall preparations. While electron microscopy has given much data on the spatial interrelationships of the various components of Gram-negative cell walls, Gram-positive cell walls in general reveal no interpretable fine structure.

Knowledge of this field has recently been detailed in an excellent review[112] and this chapter follows a similar pattern with the addition of more recent information. The reader is referred to this review for references to older work which are not included here.

(*a*) *Protein antigens*

The most extensive information available on protein antigens of bacterial cell walls comes from studies of group A *Streptococci*, especially of the M protein. Most human infections are due to group A *Streptococci*, which are defined on the basis of a common carbohydrate antigen. Immunity and protection are not related to this antigen.

(*i*) *The M protein*

The M proteins of group A *Streptococci*[122], together with hyaluronic acid capsules, determine the virulence of these organisms. They have in common the property of greatly retarding phagocytosis of the organisms, thus protecting them from destruction in the early phase of the infectious process. The M protein is also the type-specific antigen that differentiates the diverse groups of *Streptococci* in group A. M protein can only be removed from the cell wall by mild acid hydrolysis or treatment of the walls with enzymes[123], suggesting a covalent linkage to the peptidoglycan. Protoplasts of group A *Streptococci*, growing in dilute agar medium after removal of the cell wall with lytic enzymes, release an antigen into the medium which is indistinguishable from M protein[124]. M proteins are completely destroyed by proteolytic enzymes without effect on cell wall integrity or cell viability, which suggests that they are localized at the outside of the cell wall. In growing cells they are available for reaction with fluorescent antibody, and after treatment with trypsin, newly synthesized M protein is demonstrable by this technique only in areas of new cell wall production, indicating a chemical integration of cell wall and antigen synthesis[125]. Analysis of the products of digestion of isolated cell walls with lytic enzymes[122] showed the solubilized M protein to be antigenic, resistant to short-term hydrolysis at pH 2 and 100°, alcohol-soluble, susceptible to proteolytic enzymes, and to have a p*I* of about 5.3. M proteins extracted from isolated cell walls by mild acid hydrolysis were purified by chromatography on CM cellulose and shown to contain about 3% pentose, 1.4–3.2% phosphorus but no detectable amino sugars[126].

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(*ii*) *Other protein antigens of group A streptococcal cell walls*

Lancefield identified two antigens, both distinct from M protein, in cells of group A *Streptococci*, and showed them to occur in the isolated cell walls[112]. They were designated T and R antigens, and shown to be proteins, though they are much more resistant to proteolytic digestion than M protein. They lack antiphagocytic activity and play no demonstrable role in virulence.

A single T or R antigen may occur in strains of several different types characterized by different M proteins, and different strains of a single M type may have different R antigens. Other protein antigens may exist forming further dimensions for variation within a given M type. Kaplan[127,128] has identified an antigen occurring only in certain members of group A strains type 5 and 19, which cross-reacts with antigens in human and rabbit myocardium. The antigen is a protein component of the cell walls that can be extracted by mild acid hydrolysis. The presence of circulating antibody specific for this antigen has been found in the sera of a majority of patients who had a streptococcal infection with associated subsequent scarlet fever, rheumatic heart disease or glomerulonephrosis. The antigen was also found in patients with none of these complications. The heart-related antigen is separable from M antigen.

(*iii*) *Proteins in other cell walls*

Bacillus licheniformis strain 6346 contains a protein which makes up 7–10% of the cell wall weight and which is not solubilized by trypsin, pepsin or lysozyme treatment of the isolated cell walls, although the latter enzyme solubilizes the rest of the cell wall[149,130]. Cell walls of *Corynebacterium diphtheriae* contain a protein antigen that is probably type-specific, since antisera agglutinate only those cell walls prepared from organisms of the immunizing strain[131]. *Staphylococcus aureus* cell walls which have not been digested with protease contain 30% by weight of protein, and this is solubilized on digestion of the cell walls with "Dornavac", a commercial preparation of beef pancreas DNAase[114]. The extract contained at least two protein antigens, and one of them was purified and shown to be the principal staphylococcal surface component responsible for agglutination. It is common to all the recognized pathologic serotypes, and is identical to Jensen's "antigen A" (*cf.* ref. 132). It is a protein with a molecular weight of about 13 000 and a p*I* between 7.4 and 8.6. The nature of the bonds hydrolyzed by "Dornavac" in its release are unknown.

(*b*) *Polysaccharide components*

The studies of Cummins and Harris[133] have shown that isolated cell walls of most Gram-positive bacterial species contain carbohydrate components other than glucosamine and muramic acid, and that these components are often a species-specific characteristic. Although it is probable that these carbohydrates are components of polysaccharide antigens, this has only been demonstrated in a few species, principally *Streptococci*, *Pneumococci*, and *Lactobacilli*. Even in these instances, while the isolated cell walls can elicit the production of specific antibodies, the isolated polysaccharides are only antibody-precipitating haptens.

(i) Streptococcal cell wall polysaccharides

Hemolytic *Streptococci* were originally classified by Lancefield[134] into groups on the basis of specific precipitating haptens extracted from the cells by boiling at pH 2. These were later identified as the cell wall polysaccharide in the more common groups, but as the "intracellular" glycerol teichoic acid in group D (see below). The cell wall carbohydrates appear to be the type-specific antigens within group D[135], and probably also in group N[136].

The polysaccharides can also be extracted by autoclaving in 0.9% saline and by treatment with anhydrous formamide at 170°. After lysis of isolated cell walls with enzymes which degrade the peptidoglycan, the native polysaccharides isolated contain peptidoglycan components from which they cannot be separated by physical means, indicating a covalent linkage between the two. The products obtained by all these procedures have similar serological activity. Since the high-molecular weight polysaccharides isolated by the extraction procedures listed above are virtually devoid of peptidoglycan components, the extraction procedures must involve hydrolysis either of linkages to peptidoglycan or within a terminally linked polymer. Linkage of the reducing terminus of the polysaccharide to the peptidoglycan *via* a phosphodiester has been demonstrated in *Lactobacillus casei*[165] and covalent linkage of ribitol teichoic acid to peptidoglycan has also been demonstrated in *S. aureus*[137,138], *B. subtilis*[121] and *Arthrobacter crystallopoietes*[141]. Periodate degradation studies demonstrate that this linkage cannot be multiple[139,140], and it may be that linkage of antigenic, non-peptidoglycan components to the peptidoglycan by covalent bonds at one end of the antigen is a general feature of Gram-positive cell walls. Evidence is included in the sections dealing with various specific cell wall components. Biosyn-

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thetically, the peptidoglycan might be the initial acceptor for polymerization of the antigenic components, or the linkage might occur after transport of preformed polymers through the cytoplasmic membrane.

The cell wall polysaccharide of group A *Streptococci*[112] contains only L-rhamnose (2 parts) and *N*-acetyl-D-glucosamine (1 part). It appears to be a highly branched polysaccharide with non-reducing terminal residues of β-linked *N*-acetylglucosamine, since treatment of the polysaccharide with an exo-β-*N*-acetylglucosaminidase releases 75% of the glucosamine. Concomitantly, the group A serological activity is destroyed, demonstrating that these end groups are the primary determinants of this activity. The remaining polysaccharide now has unmasked determinants of A-variant specificity and can be completely hydrolyzed by V enzyme[142] which is without action on the A and C polysaccharides. A-variant strains are mutants which retain the M-serotype of the group A parent organism, but which have lost group A antigenic activity, along with most of the glucosamine of the cell wall polysaccharide.

The A-variant polysaccharide is hydrolyzed by the V enzyme to products consisting mostly of dialyzable rhamnose oligosaccharides. The products are inhibitors of the precipitation of A-variant polysaccharide by specific antibody, with potency increasing with their chain length, while free L-rhamnose has no effect, demonstrating that A-variant specificity resides in multiple L-rhamnosyl residues. *O*-Acetyl residues detected in the enzymatic products may also be important in determining serological specificity. Serological[143] and chemical evidence suggests that a majority of the rhamnosyl–rhamnose linkages are 1,3. Different preparations of A polysaccharide have different degrees of terminal *N*-acetylglucosaminyl substitution on the rhamnosyl side-chains, as indicated by varying degrees of cross-reactivity with A-variant antisera, and the polysaccharide isolated from a mutant designated A-intermediate reacts well with both A and A-variant antisera, and carries terminal *N*-acetylglucosamine residues on approximately half its side-chains. This may indicate varying degrees of deficiency in the mechanism by which the terminal residues are attached to the A-variant polysaccharide.

Group C specificity resides in non-reducing terminal residues of *N*-acetyl-D-galactosamine, probably α-linked to a very similar branched rhamnosyl polysaccharide[112]. Polysaccharides isolated from C-variant mutants have lost most of their galactosamine and are similar serologically and chemically to A-variant polysaccharide, and C-intermediate mutants have a polysaccharide which reacts with both C- and A-variant antisera and which has a reduced galactosamine content. Group C polysaccharide is probably the receptor for phage specific for this group.

The formamide-extracted cell wall polysaccharide of group G also carries the group specificity and contains rhamnose (41%), *N*-acetylgalactosamine (20%), galactose (26%) and traces of peptidoglycan components[144]. Inhibition of its precipitation by antiserum to group G is strongly inhibited by free L-rhamnose (in contrast to non-inhibition of A-variant precipitation). The low cross-reactivity with anti-A-variant sera which this polysaccharide possesses is completely destroyed by the V enzyme, with removal of rhamnosyl oligosaccharides, and without affecting group G activity. This polysaccharide may, therefore, have a rhamnose polysaccharide core similar branched to that found in groups A and C.

Formamide extraction of trypsinized cell walls of a group B *Streptococcus* gave a mixture of polysaccharides from which the B-specific component was separated by fractional precipitation[145]. It contains L-rhamnose (50%), glucosamine (12%) and galactose (9%) and cross-reacts with anti-G serum. Similarly, G polysaccharide cross-reacts with anti-B serum, and L-rhamnose inhibits strongly both these cross-reactions and the homologous B–anti-B precipitation. This suggests that terminal non-reducing residues of L-rhamnose are major determinants of serological specificity in the polysaccharide antigens of both groups B and G. The group specific polysaccharide antigen of group F *Streptococci*[146] contains L-rhamnose, glucose and *N*-acetylgalactosamine. Inhibition of its precipitation by anti-F sera with cellobiose, which is much more effective than glucose, indicates that glycosidically linked β-glucosyl residues are probably important in the serological determinant group. Partial acid hydrolysis gave a disaccharide, 3-*O*-β-D-glucopyranosyl-*N*-acetyl-D-galactosamine which was a 300 times more effective inhibitor than cellobiose, and a tetrasaccharide dimer of this disaccharide was even more effective, and is believed to be a determinant group of the group F polysaccharide.

Information on other streptococcal cell wall polysaccharide antigens is confined to the identification of monosaccharide components. The type-specific antigens of group D contain similar constituents[135] (L-rhamnose, glucose, galactose, galactosamine) to the group antigens described above. Components of the cell walls of 200 streptococcal strains have been listed[147] in 26 different patterns of carbohydrate components. One strain was unique in containing 6-deoxytalose and two strains contained unknown amino sugars.

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(*ii*) *Pneumococcal C-polysaccharide*

This polysaccharide is an antigen common to all *Pneumococci* and distinct from the type-specific capsular antigens. It is solubilized on autolysis, which occurs readily in this organism. However, cell walls have been isolated with minimal autolysis and found to contain C-polysaccharide[112]. Hydrolysates of the C-polysaccharide after autolysis[148] contained considerable amounts of amino acids typical of peptidoglycan (lysine, glutamic acid, D- and L-alanine, serine and glycine in the ratios 1.0:1.2:1.1:1.1:0.3:0.1 respectively) and even larger amounts of muramic acid and glucosamine. They also contained small amounts of muramic acid phosphate, some glucose, and large amounts of galactosamine 6-phosphate (35% of the total weight), ammonia and acetate. Only 80% of the weight was accounted for by these components, and the high yield of ammonia suggests the presence of additional nitrogenous compounds which break down during hydrolysis. Galactosamine 6-phosphate is probably the determinant of serological specificity. The pneumococcal C-polysaccharide isolated from a different strain[149] was found to contain DAP and a uridine nucleotide.

(*iii*) *Corynebacterium diphtheriae*

Cell walls of this organism contain arabinose, mannose, and galactose. A short-chain length polysaccharide containing these components has been obtained by various extraction procedures, all of them probably hydrolytic. Lysis of delipidated cell walls of this organism by a *Myxobacterium* enzyme[150] results in the production of soluble polysaccharide which is excluded from Sephadex G-75 and so is of high molecular weight, and which is associated with a portion of the glycan derived from the peptidoglycan. Periodate oxidation of this material rapidly destroys the galactose residues, as has been observed with other preparations. The original cell walls contain lipid and a strain-specific protein antigen and are agglutinated only by specific antisera. Protein-free cell walls, however, cross react with antisera to other *C. diphtheriae* strains, indicating that the polysaccharide antigenic determinants may be common to several strains. For instance, antisera to *C. diphtheriae* strain *mitis* agglutinated protein-free cell walls of *intermedius* and *gravis* strains[131]. *C. xerosis* cell walls contain arabinose, galactose and mannose. *C. renale* cell walls contain glucose and galactosamine in addition; *C. hofmannii* cell walls contain arabinose, galactose, glucose and galactosamine and *C. ovis* cell walls contain arabinose, galactose, glucose and mannose.

(*iv*) *Bacillus anthracis*

Polysaccharide obtained from lysis of anthrax bacilli with $(NH_4)_2CO_3$ contained galactose (38–43%) and glucosamine (38–43%), together with 3% of peptidoglycan constituents (glutamate, aspartate, alanine, glycine, DAP and muramic acid)[151,152]. The material was homogeneous in electrophoresis and ultracentrifugation[153]. This polysaccharide is, therefore, almost certainly a cell wall component, although it has also been isolated from culture filtrates of relatively young organisms[154]. Serological cross-reactivity with Type XIV pneumococcal antiserum indicates the common presence of terminal galactose residues, and of other undefined antigenic determinants[155].

(*v*) *Bacillus megaterium*

Strain M has intracellular and cell wall teichoic acid together with a polyglutamate capsule and polysaccharide which may be both a cell wall and a capsular component[156]. Polysaccharide which can be solubilized by extraction with boiling water contains galactosamine, glucosamine and an unknown amino sugar[157]. Material containing peptidoglycan components as well as galactosamine and phosphate was prepared from non-encapsulated cells or from encapsulated cells previously extracted with boiling water by partial lysozyme digestion. The phosphate is probably derived from teichoic acid. All three preparations were immunologically active and precipitated only with homologous antisera.

Bacillus megaterium KM cell walls contain a phosphomucopolysaccharide which, when solubilized by treatment of the walls with endoacetylmuramidases, contains peptidoglycan components which are not separated from the bulk of the peptidoglycan fragments by the electrophoresis used to fractionate the polysaccharide (on the basis of its negative charge)[158]. These components are removed by further treatment with an enzyme preparation containing *N*-acetylmuramyl–L-alanine amidase. The polymer contains phosphate, glucose and *N*-acetylglucosamine in the ratio 1:2:1.3 and makes up 40% of the cell walls. It has no phosphomonoester end groups, but these are liberated on alkaline hydrolysis to the extent of 80% of the total phosphate content. Subsequent paper chromatography separates glycerol phosphate from an immobile polymer containing the sugars and 10% of the original phosphate. The original polymer therefore probably consisted of a polysaccharide of glucosyl and *N*-acetyl-glucosaminyl residues linked to unsubstituted polyglycerolphosphate chains.

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(*vi*) *Bacillus licheniformis*

The cell walls of a *Bacillus licheniformis* strain (originally designated *Bacillus subtilis*) (N.C.T.C. 6346) were found to contain teichoic acid, protein (see above) and teichuronic acid[129,130]. Teichuronic acid is a high molecular weight polysaccharide whose extraction from isolated trypsin-digested cell walls by 5% trichloroacetic acid at room temperature is considerably more difficult than the extraction of teichoic acid, which was subsequently shown to be covalently linked to the peptidoglycan in this organism[130]. Its homogeneity was demonstrated by fractionation on DEAE cellulose. It contains glucuronic acid and *N*-acetylgalactosamine, probably with α-linkages as indicated by specific rotation and infrared spectroscopy data. This polymer has already been discussed by Jeanloz in volume 5 of this series, p. 275.

(*vii*) *Micrococcus lysodeikticus*

The cell walls contain a polymer of equal parts of glucose and 2-acetamido-2-deoxy-mannuronic acid in unknown linkage[159]. It is extracted very slowly from the cell walls by 5% TCA at 35°, and can then be fractionated from peptidoglycan components by paper electrophoresis, by virtue of its negative charge at pH 4.7.

(*viii*) *Lactobacillus casei*

Sharpe[160] classified lactobacilli into six major serological groups on the basis of antigenicity of hot dilute acid extracts of intact organisms. The groups B and C antigens have been shown to be polysaccharide components of the corresponding cell walls; the groups D and E antigens are the cell wall teichoic acids, and the group A and possibly the group F antigens are the intracellular teichoic acids (see below). Strains of *L. casei* var. *casei* belong to both groups B and C[161]. All group B strains contained L-rhamnose as a major cell wall constituent together with lesser and variable amounts of glucose and galactose. Only *L. casei* var. *rhamnosus* strains among group C strains contained significant amounts of cell wall rhamnose, while group C strains all contained glucose and galactose as major cell wall components. The cell walls from one strain of *L. casei* var. *rhamnosus* also contained mannose. Cell walls were lysed with an enzyme preparation from *Streptomyces albus*, and after removal of insoluble material, fractionated into material insoluble (fraction I) and soluble (fraction II) in 70% saturated ammonium sulfate[162]. All fractions contained considerable amounts of peptidoglycan components. The rhamnose to glucose ratios in the fractions from

L. casei var. *casei* cell walls indicated little fractionation by this technique, most of the carbohydrate being in fraction II and containing 39–48% rhamnose in 3 group B strains and 1.7% rhamnose, 27.5% glucose and 23% galactose in the fraction from a group C strain. Almost all of the rhamnose from *L. casei* var. *rhamnosus* cell walls was found in fraction I which contained most of the carbohydrate, and the glucose rich polysaccharide in fraction II was found to have the group C specificity. It also resembled the fraction II polysaccharide from the group C *L. casei* var. *casei* cell walls in chemical composition.

Precipitation of group B polysaccharides by group B antisera was significantly inhibited by L-rhamnose, and glucose was the most effective inhibitor of precipitation of group C polysaccharides by group C antisera. β-D-Glucosides were inhibitory, the most effective derivative being gentiobiose. It is suggested that the fractions I and II from *L. casei* var. *rhamnosus* cell walls represent two distinct antigens, one of which carries the group C serological specificity and the other being rich in rhamnose and probably the type-specific antigen for *L. casei* var. *rhamnosus*. Fraction I from these walls reacted only with homologous antisera, but again β-D-glucosides were the most effective inhibitors of precipitation. An encapsulated strain of *L. casei* var. *rhamnosus* was found to contain none of the fraction I polysaccharide in the cell wall, while it formed the main component of the capsule[163]. This capsule then seems to result from non-attachment of this polysaccharide to the peptidoglycan.

Partial hydrolysis of fraction II from group C *L. casei* cell walls yielded oligosaccharides, the most effective of which in inhibiting the group C precipitation reaction[164] being Glu–β→6–Gal–β→6–GNAc. Fraction II from the *L. casei* var. *casei* group C strain was further purified by DEAE chromatography, and still found to contain peptidoglycan components and phosphate. It was shown that the polysaccharide was probably linked by a phosphodiester joining the reducing group of the terminal, 3-substituted *N*-acetylgalactosamine residue to an unidentified group on the peptidoglycan[165]. Very mild acid hydrolysis liberated the intact polysaccharide from the cell walls, with a molecular weight of about 10 000, leaving the insoluble peptidoglycan[166], which became lysozyme-sensitive. This is the only case where positive identification of the linkage between antigen and peptidoglycan has been obtained.

Lactobacillus bifidus var. *pennsylvanicus*, a strain with ornithine rather than lysine in the peptidoglycan, and requiring β-*N*-acetylglucosaminide

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derivatives for growth, has been shown to contain rhamnose, glucose, galactose and phosphate in its cell walls[167] in the ratio of 3.9:1.7:1.0:0.8. The walls also contain glycerol and the presence of a glycerol teichoic acid is suspected.

(c) *Teichoic acids*

Teichoic acids were first described as phosphodiester-linked polymers of glycerol or ribitol carrying glycosidically-linked sugars and ester-linked D-alanine, found in cell walls of Gram-positive bacteria (see recent reviews by Baddiley[168,169]). The term now includes the intracellular teichoic acids, which are glycerophosphate polymers probably occurring in most Gram-positive bacteria and which are solubilized during cell wall preparation, but which are usually precipitated with the 100 000 *g* ribosomal pellet. The glucosyl residues in the intracellular teichoic acid from a group D *Streptococcal* strain are substituted by a few ester-linked L-lysine residues[170]. A teichoic acid-like polymer has been shown to occur in *Escherichia coli*[171]. The polymer from cell walls of a strain of *Staphylococcus lactis* in which *N*-acetylglucosamine 1-phosphate is an integral part of the polymer chain[172] is also called a teichoic acid. However, the related type-specific pneumococcal capsular substances, many of which contain ribitol or glycerol and phosphate, are not called teichoic acids. Two of these substances, S6 and S34 contain phosphodiester-linked repeating units[173,174] of oligosaccharides, glycosidically-linked to ribitol. Structures of this type are precluded for the teichoic acids by examination of the products of acid and alkaline hydrolysis, with the possible exception of the cell wall teichoic acid of a strain of *Lactobacillus plantarum* (J. Baddiley, quoted in ref. 191).

The following is a summary of teichoic acid structures, mostly determined on trichloroacetic acid (TCA) extracts of walls or of materials obtained from the 100 000 *g* sediment of cell extracts. Ribitol is numbered as if derived from D-ribose, and glycerol as if derived from D-glyceraldehyde.

(i) *Staphylococcus aureus (wall)*

The teichoic acid isolated from walls of strain H by TCA extraction and precipitation is a polymer of 1,5-phosphodiester-linked ribitol units, each of which carries an *N*-acetylglucosaminyl residue on C-4, and about half of which are esterified on C-2 or C-3 by D-alanine. The majority of the glycosidic linkages are β, and there is one phosphomonoester group and one formaldehydogenic group per 8 phosphates[175,176].

The teichoic acid similarly isolated from strain Copenhagen[177] has a similar structure with a longer chain length of 12–16 units and 15% of α-linked *N*-acetylglucosamine residues. It was demonstrated that major agglutinins formed in rabbits against bacteria of this strain were directed against these groupings. Similarly it was demonstrated that β-linked *N*-acetylglucosamine residues on the wall teichoic acids of strains H and NYH-6 were major determinants of agglutinins for these walls (for references see ref. 178). It was subsequently demonstrated[178] that the teichoic acids from strain Copenhagen could be fractionated by immune precipitation into separate polymers carrying either α- or β-*N*-acetylglucosamine determinants, and that the proportions of these polymers in strains Copenhagen and NYH-6 were 1:6 and about 1:1 respectively. Strains Duncan and 3528 wall teichoic acids have virtually all β- and α-acetylglucosaminyl residues respectively, corresponding to the immunological determinants in these strains[179].

All of 16 *Staphylococcal* strains examined had an intracellular glycerol teichoic acid[180]. All the *aureus* strains had an *N*-acetylglucosaminyl ribitol wall teichoic acid and all the *epidermidis (saprophyticus, albus)* strains had a glucosyl–glycerol wall teichoic acid. It had previously been demonstrated[181] that the glucosylglycerolphosphate polymer extracted from walls of a strain of *S. epidermidis (albus)* by heating at 100° at pH 2, is the group-specific antigen. Hapten inhibition studies indicated that α-D-glucosyl residues are involved in the serological specificity. The material contained 8% of peptidoglycan components and had a phosphate to glucose ratio of 1.4. A full discussion of the relationships between teichoic acids and group antigens will be found in the review by McCarty and Morse[112]. *Staphylococcus lactis* strains were found to be heterogeneous[180], some having no wall teichoic acid and others having glycerol teichoic acid containing either galactosamine or glucosamine. The teichoic acid from one strain designated as *S. lactis*, but which was probably *Gaffkya tetragena*, was extensively studied[172] and found to have an unusual structure. The *N*-acetylglucosamine residues are phosphodiester-linked at carbon-1 to carbon-1 of glycerol, which in turn is phosphodiester-linked at positions 2 or 3, probably to position 3 of the next *N*-acetylglucosamine residue. The polymer also contains ester-linked D-alanine, and one in 25 of the phosphate groups are released by intestinal phosphomonoesterase. A similar type of polymer probably occurs in the walls of Group N streptococci[173,136].

The glycerol teichoic acid isolated by TCA extraction and alcohol precipitation from the cell walls of a strain designated *Staphylococcus albus*[182]

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was found to be 1,3-linked with *N*-acetylgalactosamine residues on C-2 of about one-third of the glycerol residues, the remainder being esterified by D-alanine. The chain length was 18 residues. Most of the glycosidic linkages were α, but 20% may be β.

A soluble, covalently-linked complex of teichoic acid and peptidoglycan has been isolated from walls of strain Copenhagen[137], and its structure extensively studied[138]. It contained no phosphomonoester groups and its formaldehydogenic end-groups (moles of formaldehyde produced on periodate oxidation of ester alanine-free material, per moles of total phosphate) corresponded to a chain length of 26–30 units. Ultracentrifugation data yielded a weight-average chain length of about 40 units. A similar complex containing only glycan components of the peptidoglycan has been isolated[141], demonstrating the probable site of attachment to the peptidoglycan. It has been suggested on the basis of the reaction of cell walls with hydrazine derivatives[183], that this linkage involves a phosphoramidate on the amino group of a hexosamine at the reducing terminus of the glycan.

The intracellular teichoic acid of strain H was isolated by extraction of the 100 000 *g* pellet fraction with 5% TCA, and shown to be a 1,3-linked polyglycerophosphate polymer, esterified by D-alanine on most of the glycerol C-2 positions, but also carrying a small number of β-linked gentiobiosyl and *N*-acetylglucosaminyl residues at this same position[184].

The majority of structural studies have been performed on TCA extracts from which the teichoic acid has been isolated by precipitation with acetone or ethanol. The extraction procedure hydrolyses either the linkage to the peptidoglycan or linkages within the teichoic acid itself. Complete hydrolysis of teichoic acid to monophosphates under the conditions of extraction has been demonstrated[138]. However, it is probable that the majority of the precipitated materials studied have been cleaved at some specially acid-labile phosphodiester-linkage[7,65,186]. These could be within the teichoic acid chain, or at the linkage to peptidoglycan. The ribitol teichoic acids so isolated carry phosphomonoester groups equal in number to the moles of formaldehyde produced on periodate oxidation after treatment with ammonia to remove ester-linked alanine. Since every ribitol carries a glycosidic substituent on C-4, the formaldehydogenic groups must be unsubstituted C-1 to C-2 glycols. If every molecule is identical, then each has a single phosphomonoester group on C-5. This would not be the expected product from random cleavage of the chain, since acid hydrolysis of a phosphodiester having a neighboring free hydroxyl group (*i.e.* on C-2 of ribitol) occurs by

a mechanism involving cyclic phosphate formation, which should yield ribitol 1- or 2-phosphate. After alkaline treatment of strain *Oxford* cell walls, it has been shown that 15% of the total phosphate remains unextractable by 0.005 *M* periodate[140]. Significantly, the formaldehyde production from these cell walls under these conditions gave a chain length of 30, very similar to that reported for the soluble complex with peptidoglycan[138], while oxidation under more vigorous conditions gave a chain length of 11, corresponding more closely to the results on TCA-extracted materials.

(ii) Bacillus subtilis (wall)

Teichoic acid, prepared by precipitation from TCA extracts of isolated walls[187], consists of ribitol units joined by 1,5-phosphodiester links, carrying a β-D-glucosyl residue on each C-4 and esterified by D-alanine on C-2 or C-3. The ease with which the alanine in these compounds is eliminated in alkali is probably due to the effect of the neighboring phosphate or hydroxyl groups[188], and the rate of elimination may be useful as a diagnostic test for this type of grouping. It is absent in the group D *Streptococcal* intracellular glycerol teichoic acid and in the teichoic acid from *Bacillus stearothermophilus* walls (see below), where the D-alanine is esterified to the glucosyl residues, and is much more resistant to alkaline hydrolysis. The *Bacillus subtilis* teichoic acid had one phosphomonoester group and one formaldehydogenic group per 9 total phosphates.

After complete autolysis, 80% of the total phosphate in cell walls of *Bacillus subtilis* 168 (I^-C^+) was found to be non-dialyzable and separable by DEAE chromatography from the bulk of the peptidoglycan components[121]. The isolated teichoic acid was a complex containing all of the galactosamine (0.15 mole per mole of phosphate) in the original cell wall together with a small amount of peptidoglycan components. It contained no detectable phosphomonoester groups. The TCA-extracted material derived from these same walls had one phosphomonoester group per 9 phosphates and contained nothing but ribitol, phosphate, glucose and alanine.

(iii) Bacillus licheniformis NCTC 6346

Both teichuronic acid and teichoic acid are extracted by TCA from walls of this strain[129,130]. The extract contains 95% of the total phosphate and all of the uronic acid, but while none of the uronic acid is dialyzable, all but 33% of the phosphate is dialyzable. The non-dialyzable teichoic acid is separable from the teichuronic acid by DEAE chromatography, and closely

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resembles the *B. subtilis* teichoic acid described above. It contains glucose, phosphate and ribitol in approximately equimolar amounts and also contains alanine. 10% of its phosphate is liberated as inorganic phosphate by wheat-germ acid phosphatase. However, the intact walls and the whole TCA extract contain only one glucose for four phosphates, and the dialyzable part of the extract presumably contains short-chain length ribitol phosphate oligomers with a very low degree of glucosylation. Such fragments might not be alcohol precipitated in the usual preparation procedure. Lysozyme completely solubilized everything but the protein in these cell walls, and the digest was fractionated into three components, L_1, L_2, and L_3 by DEAE chromatography. L_1 contained only peptidoglycan components (54% of total), L_2 contained all the teichuronic acid, 33% of the peptidoglycan and part of the teichoic acid. The peptidoglycan is probably bound to the teichoic acid fraction, and the two are separable from each other and teichuronic acid only after mild acid treatment. This fraction of the teichoic acid then appears to be identical to the non-dialyzable TCA-extracted teichoic acid. Fraction L_3 contains another teichoic acid–peptidoglycan complex with a phosphate to glucose ratio of 6, and presumably is the fraction giving rise to dialyzable fragments on TCA-extraction. Thus these cell walls contain two separable fractions of peptidoglycan-linked teichoic acid. It is not known if the teichuronic acid is also linked to peptidoglycan or teichoic acid.

Another strain of *Bacillus licheniformis* contains a 1,3-linked glycerol teichoic acid[189,190]. Material extracted from membrane particles with 80% phenol had one glucose per seven phosphates and a chain length of 24–30 residues and contained small amounts of muramic acid, glucosamine and galactosamine.

(*iv*) *Bacillus coagulans and Bacillus stearothermophilus*

The proportion of teichoic acids in the cell walls of two thermophilic bacteria was found to decrease at higher temperatures, which favored increased growth rates[119]. The *Bacillus coagulans* teichoic acid was a 1,3-linked polyglycerophosphate containing glucose and galactose but no alanine. The TCA-extracted *Bacillus stearothermophilus* teichoic acid was found to be a 2,3-linked polyglycerophosphate polymer of chain length 18–19, with α-D-glucosyl residues on C-1 of 14–15 of the glycerol residues[191]. About seven ester-linked D-alanines were present per polymer, and their unusual resistance to alkaline hydrolysis combined with the result of periodate oxidation studies indicated them to be on the 6 position of glucosyl residues. A very few glucosyl residues carried a phosphomonoester group at position 3. A similar esterification of glucosyl residues by D-alanine is found in the intracellular glycerol teichoic acid of group D *Streptococci*. On autolysis, the *Bacillus stearothermophilus* teichoic acid was solubilized as a complex with peptidoglycan which could not be fractionated by DEAE cellulose chromatography[191].

(*v*) *Streptococci*

The intracellular teichoic acid of group D *Streptococci* is the group-specific antigen[192] and that in strain 8191 has been shown to be located between the membrane and the cell wall proper[193], possibly in close association with the membrane[194]. The same is true of the intracellular teichoic acid of a strain of *Bacillus megaterium*[193]. The teichoic acids from *Streptococci* strain 39 and 8191 were shown to be 1,3-linked glycosylglycerolphosphate polymers, the latter having a chain length of 20 residues. The glycosyl residues are kojibiose (2-*O*-α-D-glucosyl–D-glucose) and kojitriose respectively, and occur on nearly all the glycerol C-2 hydroxyls. The glucose residues are esterified by D-alanine in both polymers and by a smaller amount of L-lysine in the 8191 polymer. This strain has a ribitol teichoic acid in its cell wall.

(*vi*) *Lactobacilli*

Like *Staphylococci*, all *Lactobacilli* studied have intracellular teichoic acid, and this polymer is the group-specific antigen in group A, and probably also in group F[195]. These polymers have been studied in two strains. *Lactobacillus casei* A.T.C.C. 7469 for which, as in all group B and C strains, the group-specific antigen is a cell wall polysaccharide (see above), contains an intracellular, 1,3-linked glycerolphosphate polymer, probably devoid of glycosyl residues and esterified by D-alanine on most of the glycerol C-2 hydroxyls[196]. There is no cell-wall teichoic acid in groups B.C.F. and G strains. Two group A strains were shown to have a cell wall glycerol teichoic acid, probably without glycosidic substituents, which may account for their lack of serological activity[195]. Four group E strains were found to have an intracellular glycerol teichoic acid, and a glucosylglycerolphosphate polymer in their cell wall[195]. One of these also contained galactose. The cell wall teichoic acids of groups D and E are the group-specific antigens.

Lactobacillus arabinosis 17-5 (group D) has an intracellular 1,3-linked glycerolphosphate polymer of chain length about 18 residues, with α-D-

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glucopyranosyl residues on the C-2 hydroxyl of two of the glycerols, most of the remaining glycerols being esterified on position 2 by D-alanine[197]. The serological activity of this polymer was not reported. The group-specific antigen in the cell wall is a 1,5-linked glucosyl–ribitol phosphate polymer. The teichoic acid isolated after TCA extraction and ethanol precipitation[198] carries α-D-glucosyl residues on either C-4 or on both C-3 and C-4 of each ribitol residue, and ester-linked D-alanine is present on at least 60% of the ribitol residues.

The teichoic acid isolated from cell walls of *Lactobacillus buchneri* NCIB 8000 (Group E) by TCA extraction and alcohol precipitation[199] is a 1,3-linked glycerolphosphate polymer with a chain length of about 14 residues. Four of the glycerol residues carry 2-*O*-α-D-glucosyl residues, and these are problaby randomly distributed. About half the glycerol residues are esterified on C-2 by D-alanine. No cross-reactions were observed between this teichoic acid, *Staphylococcus epidermidis* wall teichoic acid, or group A *Streptococcal* intracellular teichoic acid, all of which carry D-glucosyl residues.

(*d*) *Lipids*

The occurrence and nature of lipids in the cell walls of Gram-positive bacteria have been well studied only in *Mycobacteria* and *Corynebacteria*. Both the myoinositol–oligomannoside–phospholipids in the former[200] and the structures of other components, mycolic acid diesters of trehalose and the complex peptidoglycan–polysaccharide–lipid material called Wax D, have been recently reviewed[201]. The occurrence of muramic acid in Wax D preparations has been recently reported[202], and a comprehensive review of bacterial glycolipids will be found in *Annual Review of Biochemistry* for 1965[203]. The occurrence of glycolipids in other Gram-positive organisms has been described[204].

(*e*) *Enzymatic synthesis of the non-peptidoglycan components*

The teichoic acids are the only non-peptidoglycan components of Gram-positive bacterial cell walls whose enzymatic synthesis has been extensively studied. CDP–glycerol is the donor of phosphoglycerol units for the synthesis of polyglycerol phosphate and CDP–ribitol is the donor of phosphoribitol for the synthesis of polyribitol phosphate. Glycosylation of these polymers occurs by a mechanism in which the glycosyl donors are uridine diphosphate sugar compounds. In many cases the glycosyl residues which are introduced in this way are the determinants of immunological specificity (see below). These glycosylating enzymes are, therefore, responsible for the synthesis of the important immunological determinants. For example, in the case of *Staphylococcus aureus*, either α-or β-acetylglucosaminyl residues may be substituted on ribitol. Two different immunological specificities result and these specificities are determined by two enzymes which catalyze the synthesis of the glycoside in either the α- or the β-configuration. Nothing is presently known about the mechanism by which the D-alanine ester is incorporated into the teichoic acid. Biosynthesis of the teichoic acid will not be extensively discussed in this section and readers are referred to recent reports for more detailed information[179,205–214].

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