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THE SYNTHESIS, ASSEMBLY AND TURNOVER OF CELL SURFACE COMPONENTS

Edited by

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Biosynthesis and assembly of bacterial cell walls

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Jean-Marie GHUYSEN

1. Introduction

The distinction between eukaryotic and prokaryotic cells rests upon a limited number of major features (Salton, 1974): the organization of the nucleus, the types of ribosomes, the respiratory and photosynthetic equipment, the absence of nuclear membrane and endoplasmic reticulum in bacteria, their inability (except the mycoplasma) to synthesize sterols, and finally, the occurrence of a whole class of unique heteropolymers in the latter's cell walls and envelope layers.

Electron microscopy has proven to be an invaluable tool for investigating the architecture of the bacterial surface components and for showing the degree of purity of the various preparations of organelles or layers. It has also been an essential technique in understanding the distinctions between the two broad groups of bacteria that are separated by the gram-stain reaction, the gram-positives and the gram-negatives.

Bacteria are surrounded by a delicate plasma membrane that contains or has fixed on it many enzymes and biologically active compounds. Because of the large concentration gradient between the inside and the outside of the plasma membrane in bacteria, water has a strong tendency to flow inward. To preserve the plasma membrane against osmotic disruption, a cell envelope of high tensile strength outside the plasma membrane has evolved. The cell envelopes in gram-positive bacteria differ markedly in structure from those in gram-negative bacteria. Both types, however, perform the same essential function, to keep the cell alive under ordinary hypotonic environmental conditions.

Thin sections of gram-positive bacteria (Fig. 1) usually reveal (1) a well-defined, seemingly homogeneous, thick (15–30 nm) and rigid outer cell wall; (2) an underlying and cell-limiting plasma membrane; and (3) membraneous mesosomes that appear to be internal invaginations of the plasma membrane (Ellar et al., 1967). The wall represents about 15 to 30% of the dry weight of the cell. It is the cell supporting structure because of the presence of a rigid, water-

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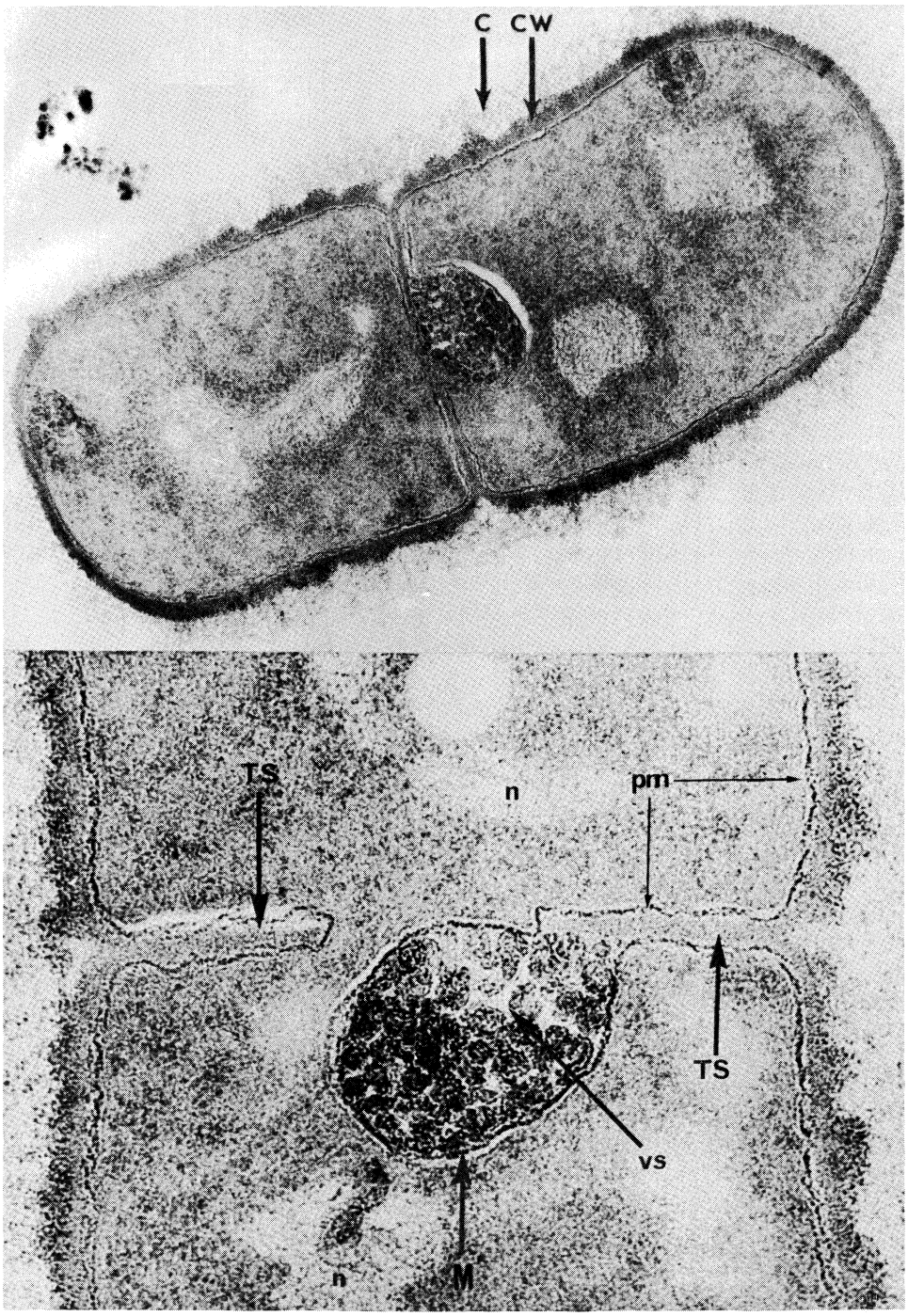


Fig. 1. Electron micrograph of a section of *Bacillus megaterium* ATCC 19213, taken from a synchronously dividing population at 3 hours. CW, cell wall; c, surrounding capsular material; TS, nascent transverse septum; pm, plasma membrane; M, mesosomes; vs, mesosomal vesicles; n, nuclear material. (Ellar et al., 1967.) (Reprinted by courtesy of the American Society for Microbiology.) 32,000 ×: upper part; 64,000: lower part.

insoluble polymer, the peptidoglycan (also called mucopeptide, glycopeptide, or murein).

Thin sections of gram-negative bacteria (Fig. 2) reveal a more complex layering in the cell envelope (Freer and Salton. 1971). The electron-dense peptidoglycan-containing layer is considerably thinner (1–2 nm) than that in the corresponding wall of the gram-positive bacteria, and it occurs sandwiched between the inner plasma membrane and an additional outer membrane that is similar in appearance to the former. Mesosomes are also present but their development is often less conspicuous than in the gram-positive bacteria. In electron micrographs the intermediate, rigid peptidoglycan-containing layer can be seen well separated from the plasma membrane by an electron-transparent zone. Depending on the bacterial species and the preparation techniques used, the peptidoglycan-containing layer may be seen either as a layer that is well separated from the outer membrane by another electron-transparent zone or is firmly connected to its inner segment and is not therefore discernible (unless the cells are submitted to heat or trypsin treatment). The profile of the outer membrane also varies (Freer and Salton, 1971). In some species, it exhibits a typical convoluted, wavy appearance. The structures that include both the peptidoglycan layer and those external to it are generally referred to as the wall of the gram-negative bacteria.

The freeze-fracture technique has amply confirmed the results obtained by thin-section studies. It has also provided valuable additional information by allowing intimate membrane and wall details to be revealed in a state believed to be closer to the "native" one. Instead of showing cross-sectional profiles, this technique shows the topography of outer and inner surfaces of walls and membranes (after etching) or the internal cleavage faces of these structures. Mesosomes are only rarely seen in freeze fractures of unfixed cells, whereas they are seen in high frequency in freeze fractures after fixation with glutaraldehyde (Higgins et al., 1976). In fact, a quantitative agreement was obtained between the increasing numbers of mesosomes seen in central cell fractures and the degree to which these cells had been cross-linked by glutaraldehyde. Moreover, it was observed that with increasing fixation times, the mesosome was progressively displaced from the periphery of the cell, and in the process a "tail" of membrane was created between the septal membrane and the displaced mesosome body. A mechanism has been proposed whereby fixation renders mesosomes observable in freeze fractures. At present, morphology, location, number per cell, and function of mesosomes are still being debated (section 2.2.2.)

This chapter will concentrate on selected aspects of some of the unique structures that are located outside the plasma membrane of the gram-positive and gram-negative bacteria. The goal is to describe the multiplicity of functions they perform in molecular structures and processes of synthesis and assembly. Factors that govern cell surface enlargement, division sites, and changes in shape during the division cycle will not be discussed in detail. These complex factors, which must be integrated with the other cellular processes, are discussed by Daneo-Moore and Shockman in this volume. Capsules that may occur outside

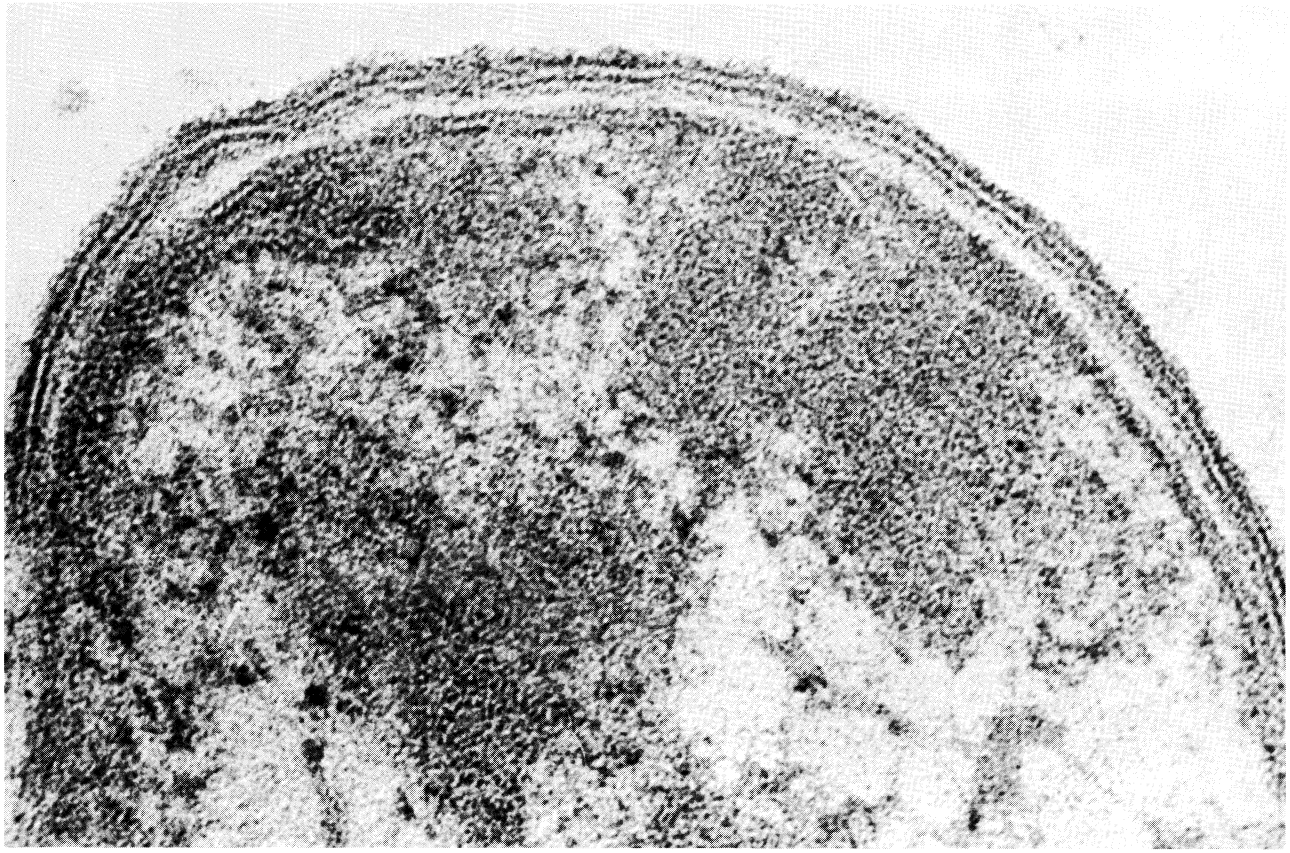


Fig. 2. Electron micrograph of a section of *Proteus vulgaris* P18 heated (5 min at 80°C) showing the complex multilayered structure of the cell envelope. The peptidoglycan is located in the intermediate, electron-dense layer that is sandwiched between the two membrane units (the plasma membrane and the outer membrane, respectively) 240,000 ×

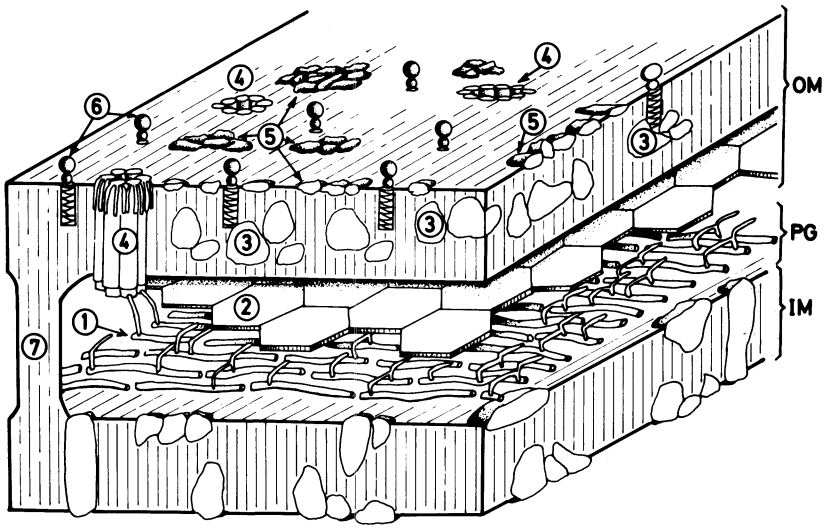


Fig. 4. Schematic representation of the cell envelope of a gram-negative bacterium. IM, inner (cytoplasmic) membrane; PG, mono-layered peptidoglycan; OM = outer membrane; ①, glycan strands of the peptidoglycan monolayer; ②, hexagonally-packed protein that may be closely associated with (but not covalently linked to) the peptidoglycan (Rosenbush, 1974); ④, lipoprotein covalently linked to peptide units of the peptidoglycan. This lipoprotein occurs not only in covalent linkage with the peptidoglycan but also in free form. Bound and free lipoproteins are represented as if they formed cylindrical channels providing the outer membrane with diffusion pores (Inouye, 1974). However, this type of arrangement for the lipoprotein molecules was not supported by more recent experiments (Nakae, 1976). In addition to the lipoprotein and phospholipids (not shown), the outer membrane contains a set of major ③ and minor ⑤ proteins and lipopolysaccharide ⑥. Inner and outer membranes are connected to each other by adhesion sites ⑦ (Bayer, 1968.) (Drawing courtesy of Dr. J. Dusart, University of Liège.)

lowed by differential centrifugation (and sometimes by treatments with proteases and nucleases). Walls isolated from cocci are spherical, whereas walls isolated from bacilli are cylindrical (Salton, 1964) (Fig. 5). Polymers other than peptidoglycan also occur in the walls. Depending on the bacterial species, the wall-associated polymers exhibit extreme variations in chemical nature and in structural complexity. All gram-positive bacteria, however, contain in their walls anionic polymers that are covalently attached to the peptidoglycan. These polymers (teichoic and teichuronic acids; see section 2.3.) are water-soluble. They do not contribute to the rigidity and insolubility of the walls, and can be selectively removed by various means (dilute acid, dilute alkali, hot formamide, dilute periodate in the cold) without affecting the shape and mechanical properties of the residual peptidoglycan.

Crude cell envelopes of gram-negative bacteria can also be obtained by mechanical disruption of the cells and differential centrifugation (Fig. 6a). The protein composition of the isolated cell envelopes is exceedingly complex; about 150 different proteins have been visualized in those of *Escherichia coli* and *Sal-*

the walls in some representatives of both gram-positive and gram-negative bscteria are also beyond the scope of this chapter.

In an attempt to help orient the reader, diagrammatic representations of the location of the various components and layers of the bacterial cell envelopes in both gram-positive and gram-negative bacteria are presented in Figs. 3 and 4. These drawings, however, are purely illustrative and should not be taken literally.

2. *Structure and assembly of bacterial walls*

2.1. The peptidoglycan in gram-positive and gram-negative bacteria

2.1.1. Isolation

Walls of gram-positive bacteria can be readily obtained reasonably free of cytoplasm and membranous contaminants by mechanical disruption of the cells fol-

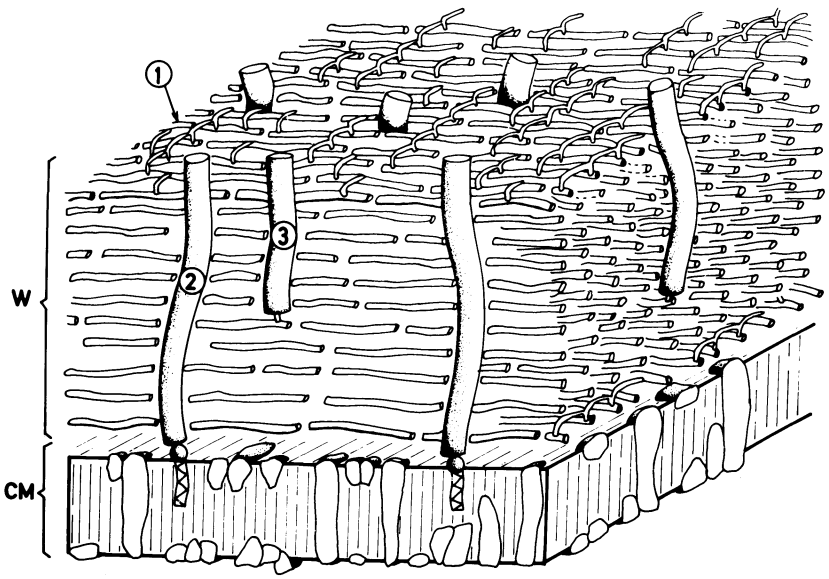


Fig. 3. Schematic representation of the cell envelope of a gram-positive bacterium. CM, cytoplasmic membrane; W, wall. The wall essentially consists of a thick, multilayered peptidoglycan structure (1) and of anionic polysaccharides (2) and (3). Wall teichoic acids (3) are covalently linked to glycan strands of the peptidoglycan. Lipoteichoic acids (2) are anchored in the cytoplasmic membrane. Depending on growth conditions, the wall teichoic acids can be replaced by other anionic polysaccharides (teichuronic acids) that are also linked to the peptidoglycan. Lipoteichoic acids are permanent constituents of the cell envelope, and are not dependent on growth conditions. (Drawing courtesy of Dr. J. Dusart, University of Liège.)

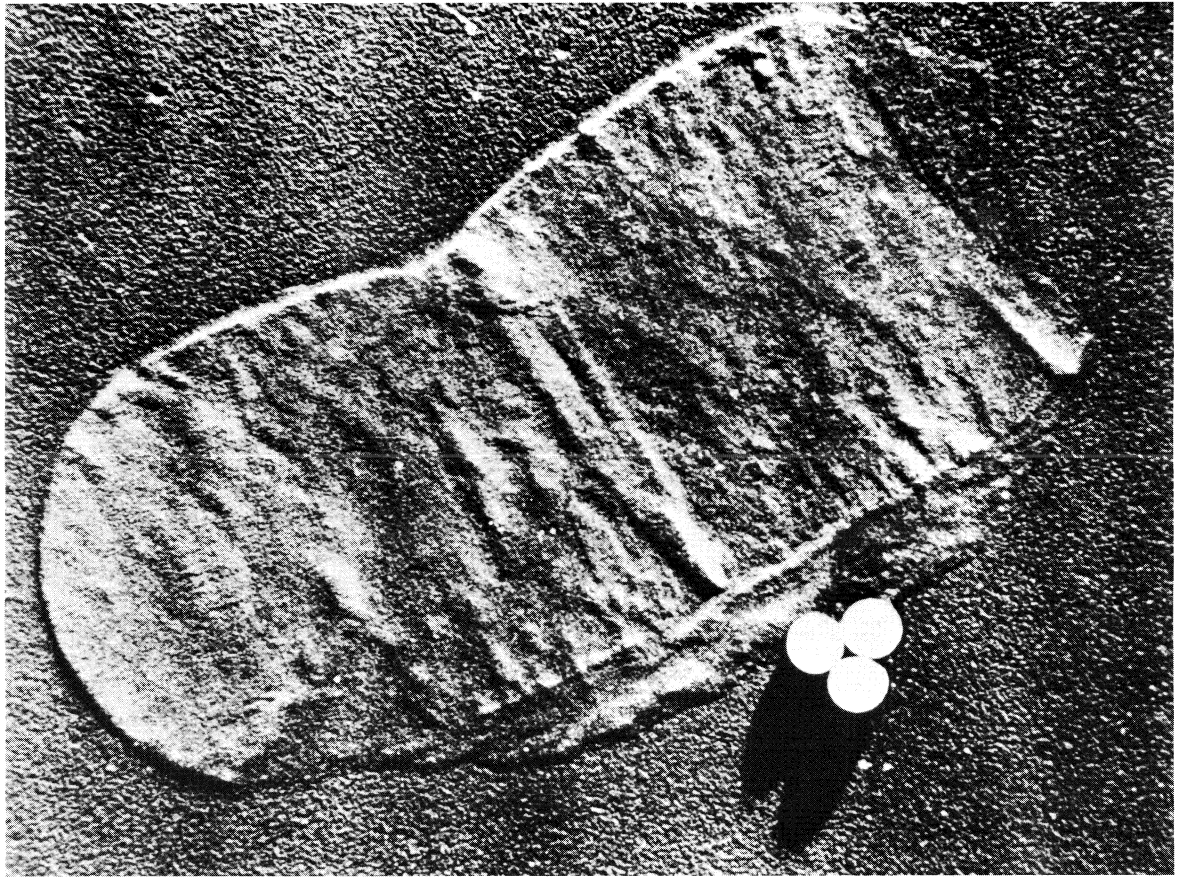
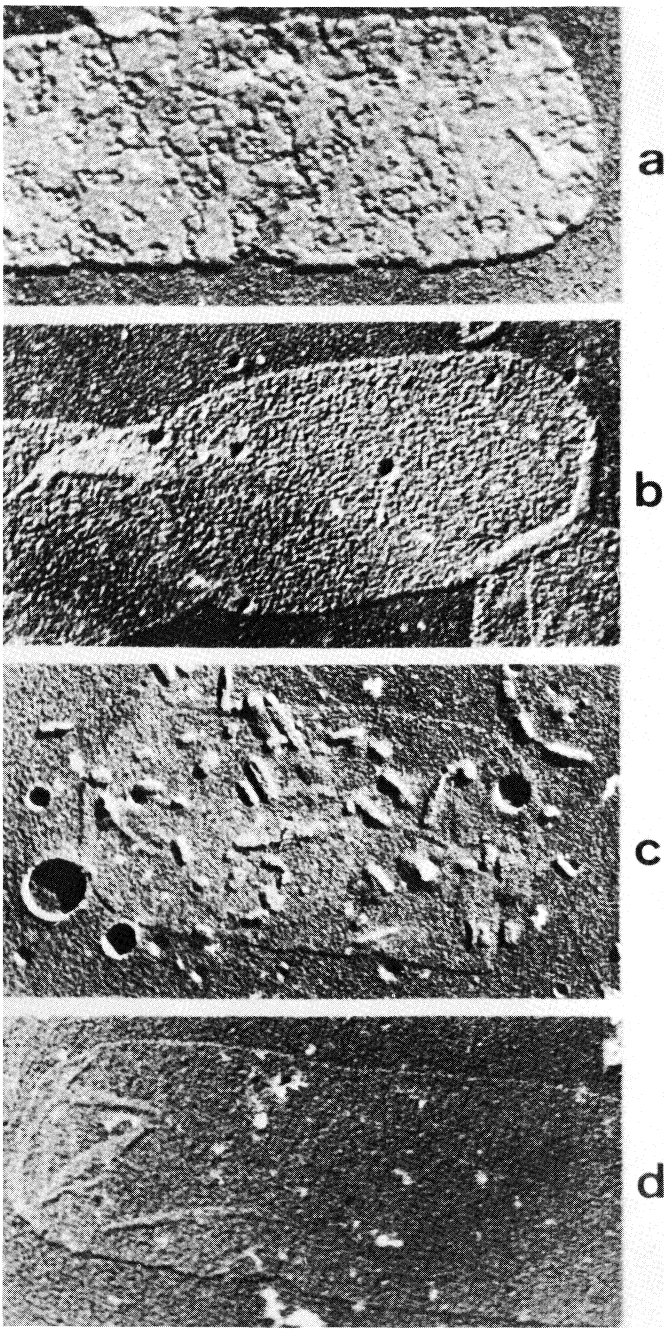


Fig. 5. Isolated cell wall of *Bacillus megaterium.* No well-defined fine structure is detectable but wall has a "fibrous" appearance. Latex spheres are 250 nm. (Salton and Williams, 1954.) (Reprinted courtesy of Elsevier/North-Holland, Amsterdam.) 32,000 ×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monella typhimurium (Ames and Nikaido, 1976). Extraction of the isolated envelopes, usually with 2% sodium dodecylsulfate (SDS) at 100°C, yields a clear solution from which a small sediment can be recovered by high speed centrifugation (Fig. 6b). Further treatment of the pellet with trypsin (or pepsin) removes lipoprotein molecules (Fig. 6c) (see section 2.5.) which, like the teichoic and teichuronic acids in the walls of gram-positives, are covalently attached to the peptidoglycan of gram-negative bacteria. The residual material is reasonably well purified peptidoglycan material (Martin and Frank, 1962). After shadowing it appears in the electron microscope as a thin, translucent, collapsed cylindrical structure similar in shape to that of the original bacterium (Fig. 6c).

2.1.2. Primary structure

In marked contrast to the eukaryotic cell-supporting exostructures that consist of α-cellulose, hemicellulose, glucan, mannan, or chitin, bacterial peptidoglycans are composed of glycan chains cross-linked by peptide chains (Fig. 7). These peptides contain both D- and L-amino acids. A complete bibliography on this topic may be found in specialized monographs and reviews (Salton, 1964; Weidel and Pelzer, 1964; Martin, 1966; Ghuysen, 1968; Tipper, 1970; Schleifer and Kandler, 1972; Ghuysen and Shockman, 1973; Rogers, 1974).

2.1.2.1. The glycan chains. The glycan moiety of all peptidoglycans examined consists of linear strands of alternate residues of 2-N-acetylamino-2-deoxy-D-glucose (N-acetylglucosamine) and 2-N-acetylamino-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucose (N-acetylmuramic acid). On the basis of extensive chemical investigation carried out in one or two cases (Tipper et al. 1965; Sharon et al., 1966; Jeanloz, 1967) and, more generally, on the basis of the lytic activity of enzymes that have a specificity for 1-4,β bonds or act as endo-β-N-acetylhexosaminidases (Ghuysen, 1968), it is believed that the glycan linkages are uniform in all bacteria and the pyranoside residues of N-acetylglucosamine and N-acetylmuramic acid are linked together by 1-4,β bonds. It is thus a chitin-like structure in which each alternative N-acetylglucosamine residue contains a D-lactyl group ether linked to C-3 (Fig. 8).

Variations occur in the glycan strands, but they do not appear to alter their three-dimensional organization. The C-6 hydroxyl function of N-acetylmuramic acid residues are often substituted by acetyl or phosphodiester groups (Ghuysen, 1968). In at least several species of *Nocardia* and *Mycobacteria*, N-acetylmuramic acid is replaced by N-glycolylmuramic acid (Azuma et al.,

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Fig. 6. The cell envelope of *Escherichia coli* B. (a) The isolated wall. (b) The isolated rigid layer, i.e., the peptidoglycan-lipoprotein complex. (c) Same as (b) after treatment with pepsin and before washing. Particles of detached and aggregated lipoprotein still adhere to the sacculus. (d) Peptidoglycan sacculus after treatment of the rigid layer with pepsin and washing. (d) A pure monolayered peptidoglycan or murein layer. (Martin and Frank, 1962.) (Reprinted courtesy of Verlag Zeitschrift für Naturforschung, Tübingen.) 41,600 ×

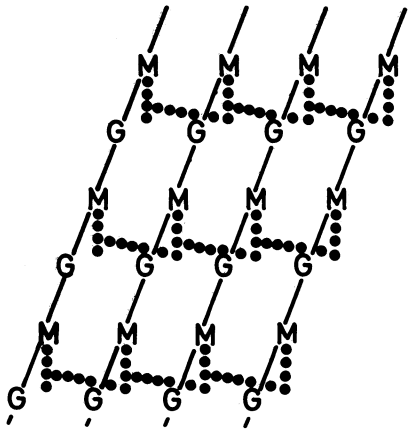


Fig. 7. Schematic representation of a wall peptidoglycan. Glycan chains are composed of N-acetylglucosamine (G) and N-acetylmuramic acid (M). Vertical dots from M represent the amino acid residues of the tetrapeptide subunits. Horizontal dots represent the peptide cross-linking bridges. Five bridging amino acids are shown, corresponding to the peptide bridges of *Staphylococcus aureus* presented in Fig. 11.

1970; Guinand et al., 1970). In spore peptidoglycans, a portion of the muramic acid is replaced by the lactam derivative (Warth and Strominger, 1969). Finally, in a few instances, small amounts of mannomuramic acid were reported to occur along with the glucose derivative (Hoshino et al., 1972). A survey including more than 40 species of gram-positive and gram-negative bacteria, however, failed to reveal the occurrence of anything except glucosamine and glucomuramic acid (Wheat and Ghuysen, 1971).

2.1.2.2. The peptide units (Ghuysen, 1968; Schleifer and Kandler, 1972). In most peptidoglycans that have been examined, every D-lactyl group of the N-acetylmuramic acid is peptide-substituted. In a few species such as *Micrococcus lysodeikticus* (and related *Micrococcaceae*), about 30 to 40% of the total N-acetylmuramic acid is unsubstituted and oligosaccharides as long as octasaccharides with no peptide attached have been identified. All glycans have short tetrapeptide units L-alanyl-D-glutamyl $\ulcorner$L-R_3-D-alanine (Fig. 9) (and sometimes tripeptide units L-alanyl-D-glutamyl $\ulcorner$L-R_3) linked to their muramyl carboxyl groups. L-alanine at the N-terminus can be replaced by L-serine or glycine. Thus, except for the occasional appearance of glycine at this position, the backbone of all tetrapeptides exhibits an alternating LDLD sequence. The peptide linkages are α except the bond between D-glutamic acid and the L-R_3 residue which is in a γ linkage. The α-carboxyl group of D-glutamic acid can be either free, amidated, substituted by a C-terminal glycine or by a glycine amide. Threo-3-hydroxylglutamic acid may occur instead of glutamic acid. Finally, the L-R_3 residue may be a neutral amino acid (e.g., L-alanine, L-homoserine), a dicarboxylic amino acid (e.g., L-glutamic acid) or a diamino acid (e.g., L-diaminobutyric acid, L-ornithine, L-lysine, LL-diaminopimelic acid or *meso*-diaminopimelic acid).

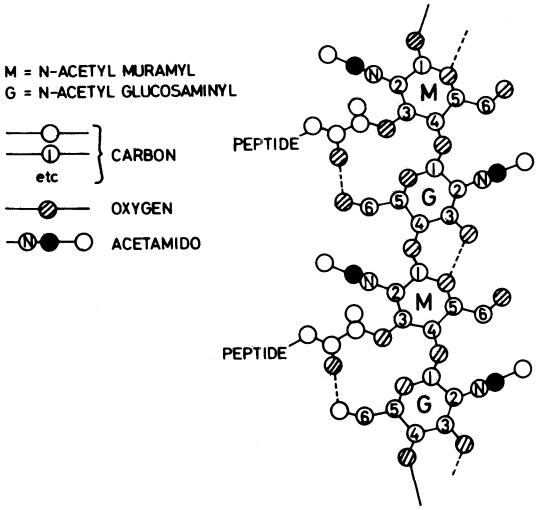


Fig. 8. Tetrasaccharide segment of a glycan strand with the chitin-like configuration. The dashed lines represent hydrogen bonds. G, N-acetylglucosamine; M, N-acetylmuramic acid (Tipper, 1970.) (Reprinted courtesy of Iowa State University Press.)

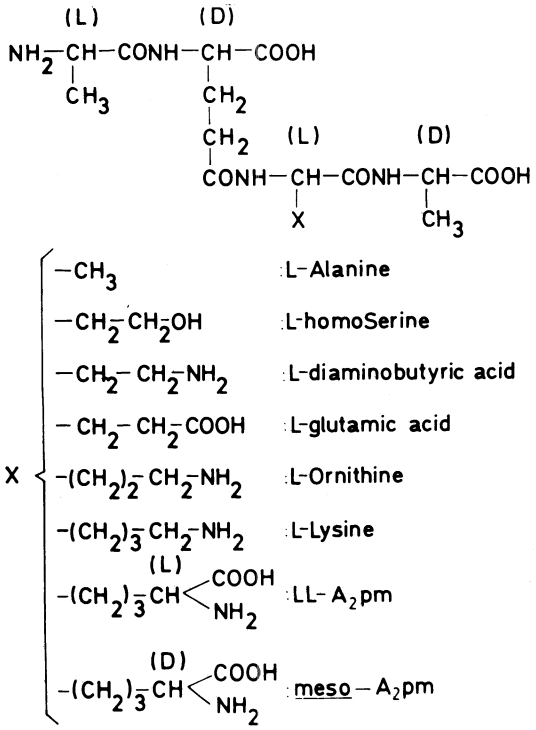


Fig. 9. General structure of tetrapeptide L-alanyl-γ-D-glutamyl-L-R_3-D-alanine subunits. Side chains of amino acids known to occur in the L-R_3 position are shown. A_2 pm, diaminopimelic acid. (Ghuysen, 1968.)

When *meso*-diaminopimelic acid occurs, both its amino group linked to D-glutamic acid and its carboxyl group linked to D-alanine are located on the same L-center. Finally, the carboxyl group on the D-carbon of *meso*-diaminopimelic acid may be either free or amidated.

2.1.2.3. The interpeptide bridges (Ghuysen, 1968; Schleifer and Kandler, 1972) Peptide units substituting adjacent glycan chains are covalently linked together by means of "bridges." Based on the composition and location of these interpeptide bridges, peptidoglycans have been classified into four main types. The bridge between two peptide units always extends between the C-terminal D-alanine residue of one tetrapeptide and either the ω-amino group of the L-R_3 diamino acid (types I, II and III) or the α-carboxyl group of D-glutamic acid (type IV) of another (tri or tetra) peptide unit. Tripeptides L-alanyl-D-glutamyl-L-R_3, which lack the D-alanine residue, are thus necessarily either uncross-linked or located at the C-terminus of a peptide oligomer.

In type I, the bridging consists of a direct N^{ω} (D-alanyl)-L-R_3 peptide bond (Fig. 10). In type II, the bridging is mediated via a single additional amino acid residue (glycine, L-amino acid or D-amino acid) or an intervening short peptide containing up to five amino acid residues. Variations are almost endless (Fig. 11). In type III, the bridging is composed of one or several peptides each having the same amino sequence as the peptide unit attached to muramic acid (Fig. 12). This type of bridging is found in *M. lysodeikticus* where a high proportion of the N-acetylmuramic acid residues in the glycan strands are not peptide substituted as if, at a certain stage of biosynthesis, some peptide units had moved from their muramyl residues into a bridging position. Finally, in type IV, the bridge extends between two carboxyl groups belonging to D-alanine and D-glutamic acid, respectively (Fig. 13). Thus it necessarily involves either a diamino acid residue (which often also has a D configuration) or a diamino acid-containing short peptide.

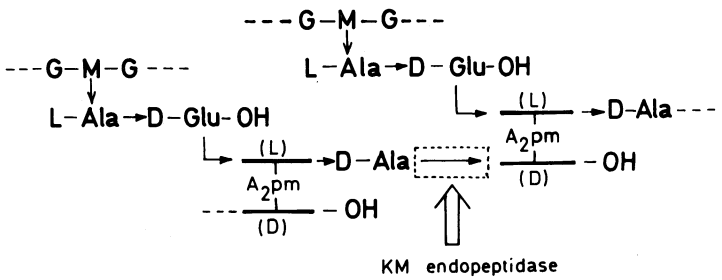


Fig. 10. Peptidoglycan of chemotype I with *meso*-diaminopimelic acid in the L-R_3 position. This structure occurs in the wall of *E. coli*, of probably all other gram-negative bacteria, and of many bacilli. The interpeptide linkage is a D-alanyl-(D)-*meso*-diaminopimelic acid linkage. In *E. coli*, the carboxyl groups are not amidated. The arrow indicates the site of action of the lytic KM endopeptidase from *Streptomyces* strain *albus* G. In some *Bacillaceae*, the α-carboxyl group of D-glutamic acid and/or the carboxyl group of diaminopimelic acid that is not in a peptide bond, are amidated. G, N-acetylglucosamine; M, N-acetylmuramic acid. (Ghuysen, 1968.)

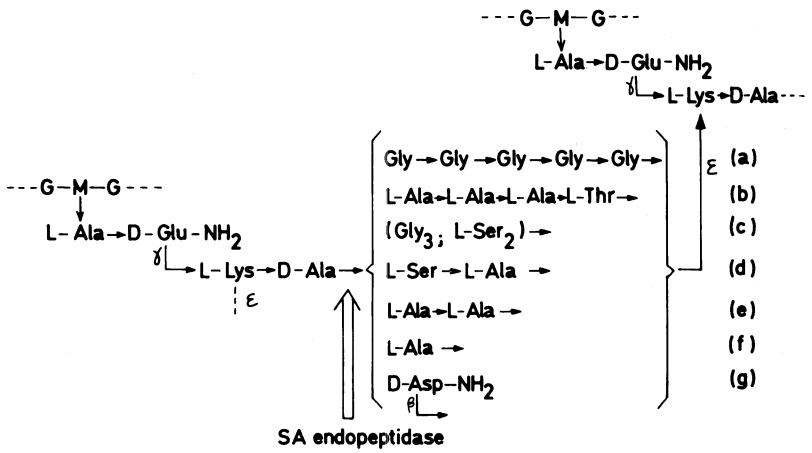


Fig. 11. Peptidoglycans of chemotype II that occur in the walls of (a) *Staphylococcus aureus* Copenhagen; (b) *Micrococcus roseus* R27; (c) *Staphylococcus epidermidis* Texas 26; (d) *Lactobacillus viridescens;* (e) *Streptococcus pyogenes* Group A, type 14; (f) *Arthrobacter crystallopoietes;* (g) *Streptococcus faecalis* (faecium) ATCC 9790; and *Lactobacillus casei* RO94. Arrow indicates the site of action of the lytic SA endopeptidase from *Streptomyces albus* G upon walls (a), (b), (e), and (g). This enzyme has not been tested upon walls (c), (d) and (f). G, N-acetylglucosamine; M, acetylmuramic acid. (Ghuysen, 1968.)

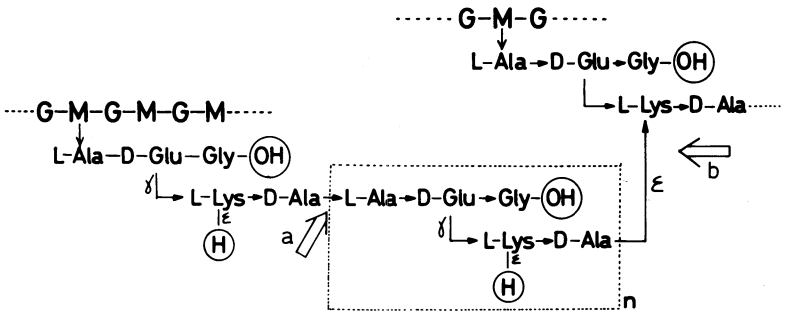


Fig. 12. Peptidoglycan of chemotype III that occurs in the wall of *Micrococcus lysodeikticus.* The site of action of *Myxobacter* ALI enzyme is indicated by arrow a and that of the ML endopeptidase from *Streptomyces albus* G by arrow b. Also shown are the unsubstituted N-acetylmuramic acid residues. G, N-acetylglucosamine, M, N-acetylmuramic acid. (Ghuysen, 1968.)

Recent experiments have shown, in addition to types I to IV, the existence of "atypical" interpeptide bridges extending between the R_3 residue of one peptide to the same R_3 residue of another. To date such atypical bridges have only been found in Mycobacteria. In these organisms cross-linking is mediated through both classical D-alanyl-(D)-*meso*-diaminopimelic acid (Fig. 10) and atypical *meso*-diaminopimelyl-*meso*-diaminopimelic acid linkages occurring in a ratio of about 2:1 (Wietzerbin et al., 1974). The stereochemistry of this atypical bridging is unknown and its physiological significance is still being debated.

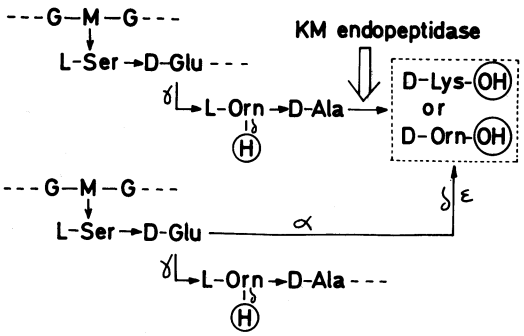


Fig. 13, Peptidoglycan of chemotype IV that occurs in the wall of *Butyribacterium rettgeri*. Arrow indicates the site of action of the KM endopeptidase from *Streptomyces albus* G. G, N-acetylglucosamine; M, N-acetylmuramic acid. (Ghuysen, 1968.)

2.1.3. Evolutionary trends in peptidoglycan structures (Schleifer and Kandler, 1972)

The most frequent type of peptidoglycan is that of type I with *meso*-diaminopimelic acid at the L-R_3 position in the peptide unit. Bridging is direct and is mediated via a D-alanyl-(D)-*meso*-diaminopimelic acid bond (Fig. 10). It occurs in the gram-positive bacilli, probably in all gram-negative bacteria, and is also found in highly evoluted prokaryotes such as myxobacteria and blue-green algae. The enormous diversification and complexity of primary structures briefly described above (Figs. 9–13) have only been found in gram-positive bacteria. As Schleifer and Kandler (1972) pointed out, the fact that at present so many different peptidoglycan structures are still conserved by gram-positive bacteria clearly shows that such a diversification was not connected with an appreciable selective advantage or disadvantage. If one accepts the idea that phylogenetic advancement is expressed by simplification and loss of variability (fixation), the complex peptidoglycans of the gram-positive bacteria may represent a primitive stage. Following this view, the directly cross-linked *meso*-diaminopimelic acid-containing peptidoglycan is not only the most successful but would also be the most advanced.

In all gram-positive bacteria shape maintenance and physical protection of the plasma mebrane are almost exclusively, if not entirely, undertaken by the vast amount of multilayered peptidoglycan that is present in their thick wall. Some degree of expansion and contraction has been observed and actually measured based on the impermeability of peptidoglycan to dextran molecules (Marquis, 1968; Ou and Marquis, 1970, 1972). Since these changes, induced by variations of the ionic strength of the medium, are especially sensitive to pH, it has been assumed that they are caused primarily by electrostatic forces within the wall.

In contrast to the gram-positive bacteria (including the bacilli that conserve the multilayered peptidoglycan), all the gram-negative bacteria have developed a peptidoglycan structure that is considered to be a monolayer. This highest level

of simplification is such that the supporting function of the peptidoglycan is barely maintained. The outer membrane (linked to the underlying peptidoglycan via a covalent attachment between lipoprotein molecules and the peptidoglycan; see section 2.5.) not only confers the advantage of an additional permeability barrier on the gram-negative bacteria but also reinforces the mechanical strength of the whole cell envelope and further protects the inner plasma membrane against osmotic hazards.

The relative fragility of the *E. coli* K12 peptidoglycan is well demonstrated by the effects of guanidine hydrochloride (Leduc and Van Heijenoort, 1975). The presence of a 6 M concentration of this reagent in the cold causes a suspension of this peptidoglycan to undergo complete clarification. Upon dilution or dialysis of the resulting solution peptidoglycan precipitates; but as shown by electron microscopy, it is recovered as distorted sacculi lacking the initial rod shape. Hence, this fragile peptidoglycan may be converted into a soluble form without breaking covalent linkages. Hydrogen bonding and perhaps electrostatic interactions must contribute significantly to the nondenatured form of this polymer.

The effects of chelating agents on gram-negative cells are also revealing in this respect. When suspended in sucrose solution in the presence of chelating agents such as ethylenediaminetetraacetate (EDTA), exponentially growing cells of *E. coli* (and of other gram-negative bacteria) undergo lysis upon subsequent dilution of sucrose but remain rod-shaped. As shown by Eagon and Carson (1965), Leive (1965a,b), Gray and Wilkinson (1965a,b), Asbell and Eagon (1966), Leive et al. (1968), Roberts et al. (1970), Gilleland et al. (1973), and Leive (1974), the only damage caused by EDTA is removal of divalent cations, which affects the stability of the outer membrane and induces the solubilization of part of its components (lipopolysaccharide together with minor amounts of phospholipids and proteins; see section 2.7). Since the ghosts produced by EDTA and osmotic shock are rod-shaped, it follows that the shape-maintaining properties of the rigid layer are not impaired. The observed lysis, however, suggests that under conditions where the outer membrane has lost its integrity, the very thin peptidoglycan monolayer might not possess enough tensile strength in various areas to resist the high internal osmotic pressure. A thin peptidoglycan monolayer containing areas of low physical strength might also explain the flexibility of some gram-negative bacteria such as the gliding bacteria and *Myxobacteriales* (Verma and Martin, 1967; White et al., 1968). Experimental evidence also supports the view that the peptidoglycan monolayer in gram-negative bacteria is discontinuous in certain areas. These areas would correspond to the adhesion sites that occur between the plasma and the outer membrane (section 2.10.).

Evidence also exists showing that the outer membrane actually contributes to the shape of the wall. Treatment of *E. coli* cells with lysozyme in the absence of EDTA but in the presence of 0.5 M sucrose does not alter their morphology (Birdsell and Cotta-Robles, 1967). However, peptidoglycan is degraded, and dilution in water causes the formation of spherical structures.

Rod-shaped "ghosts" have been prepared from *E. coli* and a number of other

bacteria (section 2.8.). Essentially these ghosts consist of a unit membrane derived from the outer membrane. They lack peptidoglycan and have lost virtually all intracellular material. Hence, the outer membrane can maintain its rod shape.

2.1.4. Size

The concept suggested by Weidel and Pelzer (1964) that the peptidoglycan is a bag-shaped macromolecule which completely surrounds the cell is still valid, at least in essence. Studies of primary structure have largely confirmed the netlike arrangement of this polymer. The initial idea, however, might lead to the false impression of extremely long glycan strands interconnected by means of a single, enormous, branched peptide moiety. This is not true, as many terminal groups are present in both the glycan and the peptide moieties.

Average chain lengths for several peptidoglycans have been reported to be of the order of only 10 to 50 disaccharides (Krulwich et al., 1967a, b; Tipper et al., 1967; Kolenbrander and Ensign, 1968; Tipper, 1969). The largest glycan synthesized by a (autolytic-deficient) mutant of *Bacillus licheniformis* contains about 100 to 150 disaccharide units (Ward, 1973; Rogers, 1974). As discussed by Rogers (1974), such a 150-disaccharide unit glycan strand has a length of about 150 nm. Since the length of a bacillus is approximately 1,500 to 3,000 nm, and its circumference is about 2,000 nm, ten or more glycan chains could be stretched in either direction.

The proportion of peptide units that are cross-linked varies from species to species but is invariably rather low (Ghuysen, 1968). The most highly cross-linked peptide is that of *Staphylococcus aureus,* and at least one estimate indicates that the average size does not exceed ten peptide units. The other extreme includes many peptidoglycans of type I (from *E. coli, Proteus vulgaris* and probably most gram-negative bacteria), where approximately equimolar amounts of uncross-linked peptide monomer and cross-linked peptide dimer exist. In spite of the small size of both glycan and peptide moieties, a continuous net of the type shown in Fig. 14 can be built. Such a sheet still may consist of a single macromolecule covering the entire surface of the bacterium. Points of weak mechanical resistance are easily visualized.

2.1.5. Three-dimensional structure

Three-dimensional atomic models of peptidoglycans have been proposed by Tipper (1970), Kelemen and Rogers (1971), Higgins and Shockman (1971), Formanek et al. (1974, 1976) and Oldmixon et al. (1974), respectively. In all of these models the glycan chains are assumed to have the structure of chitin. An α-helical conformation of the peptide units is judged impossible for the obvious reason that the glutamyl bond is γ, and therefore all the carbon atoms of this residue are in the chain. Moreover, in all models proposed, efforts are made to stabilize the peptides by as many hydrogen bonds as possible (like all other known peptide and protein structures) which implies a fairly extended, rather

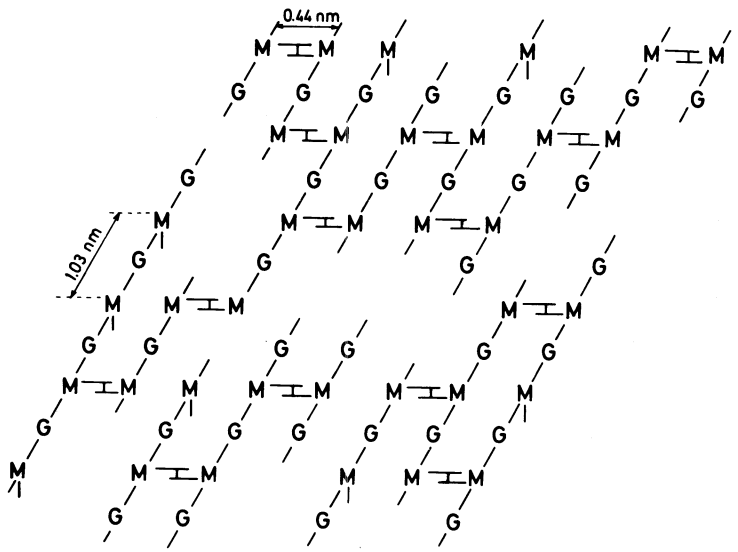


Fig. 14. Schematic representation of the peptidoglycan monolayer of *E. coli.* All of the N-acetylmuramic acid residues (M) are substituted, either by uncross-linked peptide monomers or by cross-linking peptide dimers. Peptide oligomers larger than dimers have not been observed. G, N-acetylglucosamine; M, N-acetylmuramic acid. Note that a continuous net of this type can be built even if both the glycans and the peptides are short. The periodicity of the structure is assumed to be about 1.00 and 0.44 nm (see text).

flat conformation. As pointed out by Formanek et al. (1974) for a structure of β-1,4 linked glucose residues, an X-ray reflection corresponding to a periodicity near 1.0 nm can only occur if the sugar residues form a twofold screw axis (Fig. 8). This is actually the case in cellulose and chitin. Moreover, the carbohydrate chains of chitin are packed in a parallel array side by side, with a periodicity of 0.476 nm perpendicular to their long direction. This packing is energetically favored because of the formation of hydrogen bonds between the N-acetyl groups of adjacent carbohydrate chains. Interestingly, X-ray diffraction of dried foils of peptidoglycans from both the gram-negative *Spirillum serpens* and the gram-positive *Lactobacillus plantarum* showed Debye-Scherrer rings indicating periodicity of about 1.00 and 0.44 nm (Fig. 14) (Formanek et al. 1974). In addition, a comparison of the infrared spectra of chitin and peptidoglycan showed that the amide I and amide II bonds have the same frequencies (Formanek et al., 1974, 1976). These experimental data strongly suggest that the structure of the glycan chains in the peptidoglycans may well be similar to that of chitin (and cellulose). In order to fit into a periodic structure of the glycan moiety of 0.44 and about 1 nm, the peptide units must be very flat. Therefore the peptide units were assigned the hypothetical 2.2_7 helix conformation. In this representation, the peptide units form two hydrogen bonds with the sugar residues. Similar models were proposed by Oldmixon et al. (1974) that take into

account the ability of walls from gram-positive bacteria to change in volume with ionic conditions (Marquis, 1968).

An interesting conclusion of these studies is that a peptidoglycan monolayer appears to be a highly asymmetric structure. Indeed, on the basis of a chitin-like conformation, the twofold screw axis in the glycan chains of the peptidoglycan necessarily directs all the peptide chains on the same side of the glycan to stack, whereas the O–6 positions of the N-acetylmuramic acid residues are exposed on the other side of the structure sterically unhindered, and therefore are readily available for substitution with acetyl and phosphodiester groups (Fig. 8).

Despite these recent advances, more work remains to be done before a clear representation of the three-dimensional structure of the peptidoglycans can be obtained. In particular, as pointed out by Braun and Wolff (1975), a very dense crystalline, chitin-like arrangement in the *E. coli* rigid monolayer can only exist in certain areas that must be interrupted by less densily packed and less ordered structures to account for the permeability of the peptidoglycan layer, the number of structural repeating units per cell surface area, and the occurrence of the adhesion sites extending between the plasma and the outer membranes. A continuous packing density of the chitin-like arrangement, as proposed by Formanek et al., (1974) would not allow water to pass through and would require that only 30% of the cell surface be covered by peptidoglycan (Braun, 1975).

2.1.6. Peptidoglycan-degrading enzymes (Ghuysen, 1968)

Peptidoglycans can be solubilized by enzymes that hydrolyze bonds either in the glycan strands (endo-N-acetylmuramidases and endo-N-acetylglucosaminidases), in the peptide moiety (endopeptidases hydrolyzing peptide bonds in the interior of the peptide bridges, or those bonds that involve the C-terminal D-alanine residue of the peptide units; see Figs. 10–13), or at the junction between the glycan strands and the peptide units (N-acetylmuramyl-L-alanine amidase). Peptidoglycan solubilization causes cell lysis. Lysis can occur either from within (by autolysins; see below) or externally, when the enzymes are added to cell suspension. Lytic enzymes have been used as the method of choice for the elucidation of the primary structures of all peptidoglycan types. This procedure requires several sequential steps, each involving the use of one specific enzyme so that the original complex heteropolymer can be progressively degraded into small fragments in a controlled manner.

2.1.7. Lysis from within

Bacteria possess enzymes called autolysins, that are capable of hydrolyzing their own peptidoglycan. When such enzymes are permitted to act, cells lose their osmotic protection and autolyze. Autolysins have been found to be localized in the cytoplasm, associated with the membrane, concentrated in the periplasmic region, firmly fixed on the wall, or even excreted in the growth medium. Some are specifically localized in the region of the dividing septum. The first evidence for such localization was suggested by Mitchell and Moyle (1957), who made the intriguing observation that *Staphylococcus* spontaneously becomes osmotically

fragile when incubated at 25°C in 1.2 M sucrose, and on further dilution of the sucrose eventually gives rise to hemispheric wall fragments.

The specificities of the autolysins found in various species correspond to those described above: N-acetyl-muramidase, N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase, and endopeptidase. The nature and complexity of the autolysin system, however, vary largely among different species. In *S. faecalis* 9790 (Shockman et al., 1967) and *L. acidophilus* strain 63 AM Gasser (Coyette and Ghuysen, 1970b), the only detectable activity is an endo-N-acetylmuramidase. Yet the localization and mode of action of the enzymes seem not to be the same in the two organisms (Higgins et al., 1973). In *D. pneumoniae* (Mosser and Tomasz, 1970; Howard and Gooder, 1975), various bacilli (Brown and Young, 1970), and in *Clostridia* (Tinelli, 1968; Takumi et al., 1971), the major, if not the only activity appears to be an N-acetylmuramyl-L-alanine amidase. In marked contrast, *E. coli* possesses at least six different hydrolases capable of hydrolyzing linkages found in the peptidoglycan. They include N-acetylglucosaminidase, N-acetylhexosaminidase, amidase and cross-bridge splitting peptidases (Weidel and Pelzer, 1964). One of these autolysins was recently discovered in *E. coli* and exhibits a novel and interesting specificity (Taylor et al., 1975; Höltje et al., 1975). In an in vitro system this enzyme has the unique property of degrading the *E. coli* rigid layer peptidoglycan into disaccharide fragments that lack reducing ends and contain muramic acid as an internal (anhydro) structure, apparently 1→6 linked (Fig. 15). Such an in vitro conversion of the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine into an internal 1→6 anhydro N-acetylmuramyl (hemiacetal) bond is an intramolecular transglycosylation reaction. Therefore this enzyme behaves as a peptidoglycan: peptidoglycan-6-muramyl transferase. A possible role in remodeling the peptidoglycan sacculus during the life cycle of the bacterium can be assigned to this enzyme. Indeed, rupture of a glycan strand could be followed by reattachment of the terminal anhydro N-acetylmuramic acid thus formed to the nonreducing terminal N-acetylglucosamine residue of another glycan fragment, thus conserving the energy of the original sensitive glycosidic bond. As noted by Höltje et al. (1975), however, the in vitro reaction products could be artifacts due to a misdirected intramolecular transfer reaction, whereas the enzyme could serve other functions in vivo. Side reactions similar to that occurring in vitro might also occur to a minor extent in vivo, which would explain the small amounts of nonreducing peptidoglycan fragments that can be detected in lysozyme digests of *E. coli* peptidoglycan (Primosigh et al., 1961).

In view of the ubiquity and multiplicity of autolysins, it could be argued that the many terminal groups found in all peptidoglycans as they are isolated (section 2.1.4.) are artifacts caused by the uncontrolled action of these enzymes during isolation. If special care is not taken to prevent autolysin action, terminal groups can be "artificially" created, and at the limit no insoluble peptidoglycan or wall can be isolated at all. It is striking, however, that the sizes of both the glycan and peptide moieties do not vary to any great extent, among peptidoglycans isolated from cells of which the autolytic systems are markedly different.

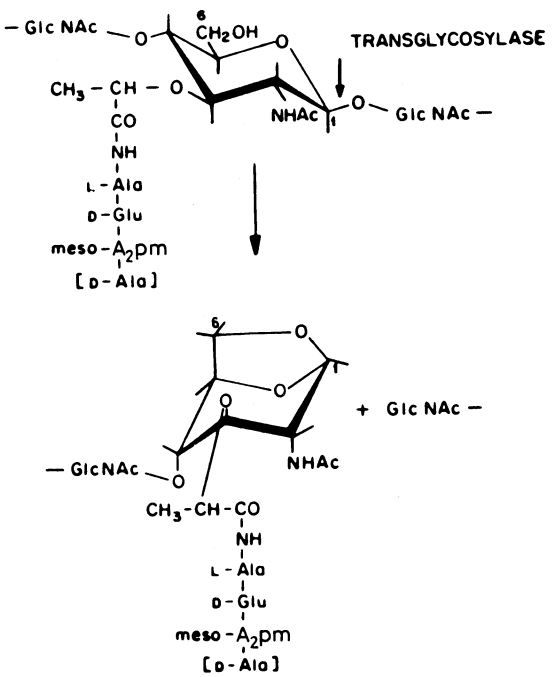


Fig. 15. Conversion of the muramic acid moiety from the $^4C_1$ into the $^1C_4$ conformation upon transglycosylase action by one of the autolysins of *Escherichia coli.* (Höltje et al., 1975.) (Reprinted courtesy of the American Society for Microbiology.)

Thus, the peptide moiety of the peptidoglycan of *L. acidophilus* is essentially a mixture of monomer, dimer, and trimer (Coyette and Ghuysen, 1970a) although the only autolysin actively detected in the cells is an endo-N-acetylmuramidase. Short glycan strands occur in the peptidoglycans of various bacilli (Rogers, 1974), while the main autolysin detected is an N-acetylmuramyl-L-alanine amidase (although hexosaminidase activity has also been detected; Brown and Young, 1970; Fan and Beckam, 1973). *E. coli* has a full complement of autolysins of various types, but its peptidoglycan is not very different in size from those of the same type isolated from bacilli. Finally, except for *M. lysodeikticus,* where a high proportion of the N-acetylmuramic acid is unsubstituted (Leyh-Bouille et al., 1966; Ghuysen, 1968), in all other bacteria the N-acetylmuramic acid is fully substituted (even in those where very active amidases are present in the membranes and walls). From these observations, it seems clear that many of the terminal groups in the peptidoglycan are not artifacts but rather reflect important properties of the peptidoglycan biosynthetic machinery and secondary modifications that occur following wall assembly.

The potentially dangerous autolysins probably play an important role in various cellular events such as remodeling the cell shape throughout the division cycle, cell separation, wall turnover (when it occurs), sporulation, the ability of

cells to become competent for transformation, and finally, the excretion of toxins and exoenzymes. Actually, peptidoglycan hydrolases of any specificity could function effectively for all these processes. It has also been proposed that autolysins are actively involved in bacterial growth itself (Higgins and Shockman, 1971). The relationships of peptidoglycan hydrolases to surface growth and cell division are discussed in chapter 9 by Daneo-Moore and Shockman.

2.2. *The bacterial membrane systems in gram-positive and gram-negative bacteria*

2.2.1. Isolation

Enzymic degradation of the wall peptidoglycan normally causes cell lysis. However, lysis can be prevented for a limited period if the external medium contains a solute to which the cell is impermeable (sucrose) at a concentration that approximately balances the high osmotic pressure of the cell. Under these conditions, bacteria undergo transformation into osmotically fragile bodies. These bodies are either wall-less bacteria (i.e., protoplasts from gram-positives) or bacteria with some defect in their peptidoglycan component (i.e., spheroplasts from gram-negatives).

Plasma membranes of gram-positive bacteria are isolated by using any enzymes that selectively degrade the peptidoglycan and digest the whole wall structure. From the lysate thus obtained, plasma membranes free of insoluble wall residue and of cytoplasmic contaminants can be isolated. Alternatively, the procedure may include protoplast formation followed by osmotic shock.

The isolation of the mesosome intrusions is a more difficult task that has been successfully accomplished with a limited number of gram-positive bacteria (Salton, 1971). Essentially, the procedure is based on controlled and careful extrusion of the mesosome content during protoplasting of the cells, followed by careful differential centrifugation. Fig. 16 shows how the mesosome pocket opens and liberates its membranous content when a gram-positive bacterium is transformed into protoplast in a hypertonic medium (which causes plasmolysis) (Ryter, 1974).

Various methods have been proposed for the physical separation of the outer membrane of the gram-negative bacteria from the plasma membrane and for the selective isolation of both structures (Miura and Mizushima, 1968a,b; Schnaitman, 1970a,b; Osborn et al., 1972a,b). Most methods include the preparation of the total (inner plus outer) membrane fraction through the lysis of spheroplasts obtained by the action of lysozyme and EDTA, although this technique may be damaging to the outer membrane. Extensive loss of its lipopolysaccharide molecules may occur, probably due to the use of EDTA. Recently, Osborn's group showed that loss of lipopolysaccharide from the outer membrane of *Salmonella typhimurium* can be prevented by using an EDTA concentration just sufficient to prevent the aggregation of membranes (Osborn et al., 1972a,b). The two membranes can then be separated from each other by isopyc-

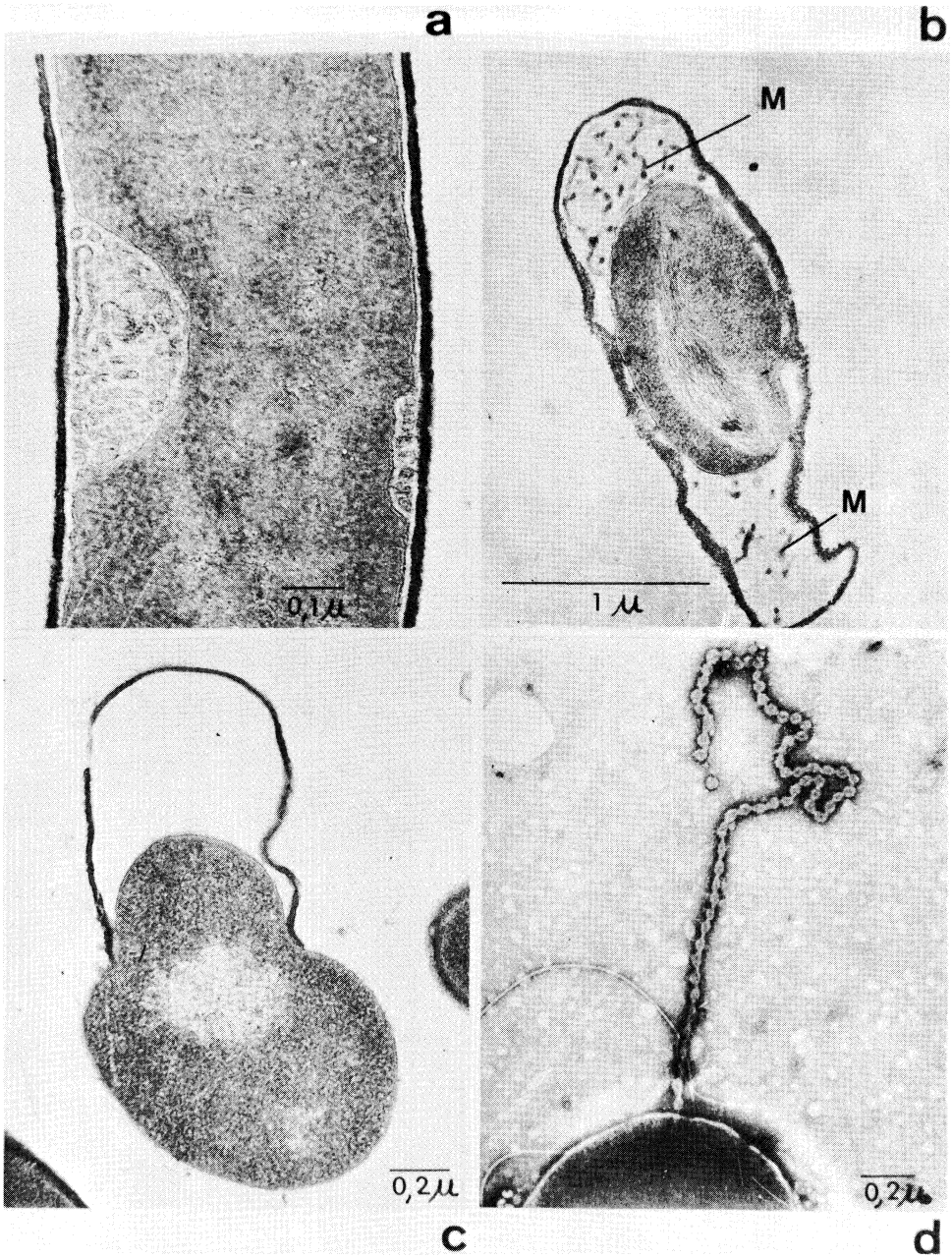


Fig. 16. Protoplast formation and mesosome extrusion (Ryter, 1974). (a) Beginning of plasmolysis. The mesosomic pocket opens. (b) End of plasmolysis. The mesosomic tubules (M) are at the poles of the bacterium between the wall and the plasma membrane. (c) Emerging of the protoplast. (d) Mesosomic tubule still attached to the protoplast. (Reprinted courtesy of Ediscience/McGraw-Hill. Paris.)

nic sucrose density gradient centrifugation of the total membrane fraction. The outer membrane bands at a buoyant density of about 1.22 g/cc, whereas the plasma membrane bands at densities ranging from 1.14 to 1.12 g/cc. The same technique, slightly modified if necessary, can be applied to other gram-negative bacteria. Fig. 17 shows the negatively stained outer and plasma membranes isolated from *E. coli* K12 (Pollock et al., 1974).

Finally, mention should be made of the antibiotic EM49, an octapeptide containing a C:10 or C:11 β-hydroxy fatty acid with no threonine residues, which has the property shared by no other antibiotic, to disrupt the *E. coli* outer-membrane structure and to release from the cells membrane fragments having a density identical to that of the isolated outer membrane (Rosenthal et al., 1976).

2.2.2. Functions

The physiological functions fulfilled by the plasma-mesosome membranes involving enzyme activities are numerous and important (Salton, 1971). Membranous organelles such as nuclear membrane, mitochondria, Golgi, and endoplasmic reticulum do not exist in bacteria. The functions associated with these organelles in the eukaryotic cells are packaged in the bacterial multifunctional plasma-mesosome membrane system. At present, a distribution of specific enzymes between plasma membranes and mesosomes has not been established except for the adenosinetriphosphatase (ATPase) connected with oxidative phosphorylation and for the mannosyl-1-phosphorylundecaprenol synthetase involved in mannan synthesis (section 3.2.), which were shown to occur only in the plasma membrane and to be absent from the mesosomes. Concerning all other enzyme activities, including those involved in the biosynthesis of the exocellular cell envelope structures, differences in activity levels of certain enzymes between plasma membrane and mesosomes were reported. The significance of these data, however, has not yet been assessed.

In contrast to the plasma membrane, very few physiological functions involving enzyme activities seem to be performed by the outer membrane of the gram-negative bacteria (Salton, 1971). Enzyme activities associated with terminal electron transport and with the active transport of solutes, which are classic plasma membrane functions, are entirely lacking in the outer membrane. However, phospholipase activities tentatively identified as a mixture of phospholipase A and lysophospholipase, were found associated primarily with the outer membrane of *S. typhimurium* (Osborn et al., 1972a,b) but the role they may play is unclear. UDP-sugar hydrolase, ribonuclease I and endonuclease I activities were found in both plasma and outer membranes (Osborn et al., 1972a,b). Finally, some peptidoglycan hydrolases of unidentified specificity also appear to be confined to the outer membrane (Hakenbeck et al., 1974). Their role remains obscure.

The outer membrane, essentially a phospholipid-lipopolysaccharide-protein structure stabilized by Mg^{2+} cations, contributes to the mechanical stability of the gram-negative bacteria cell envelope and provides them with an additional permeability barrier (Leive, 1974). This barrier function is, at least in part,

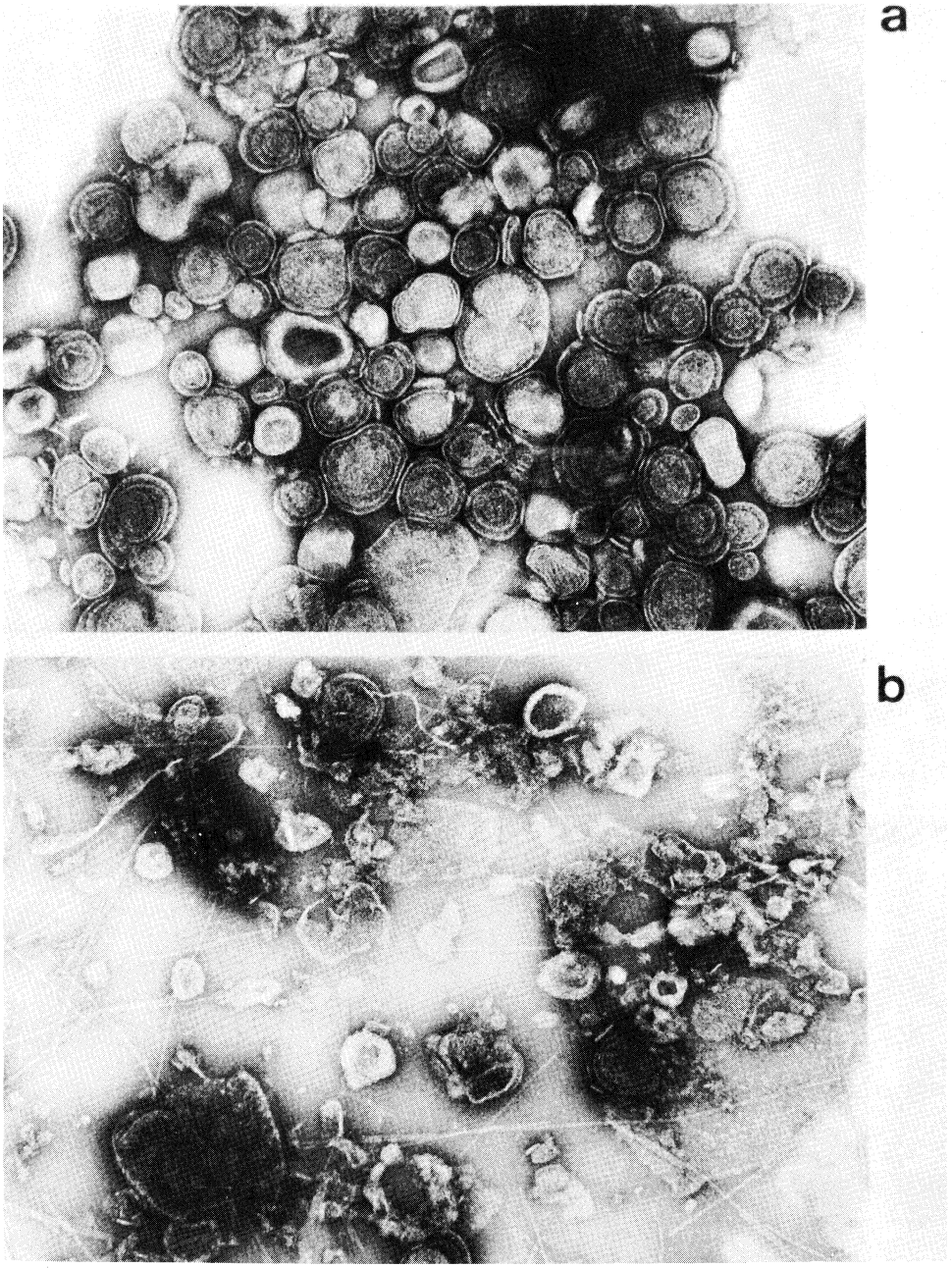


Fig. 17. (Top) Electron micrograph of the outer membrane of *E. coli* K12, mutant strain 44. Note the spherical shape of the membranes containing concentric structures typical of isolated lipopolysaccharide. Bar, 0.5 μm. (Bottom) Electron micrograph of the inner membrane of *E. coli* K 12 mutant strain 44. The plasma membrane fragments are covered with small particles. Pili also occur in the preparation. Bar, 0.5 μm. (Pollock et al., 1974). (Reprinted courtesy of the Federation of European Biochemical Societies.)

related to the lipopolysaccharide component. Treatment with EDTA (Leive, 1965a,b; Voll and Leive, 1970) and mutational alterations of the lipopolysaccharide structure (Tamaki et al., 1971; White et al., 1971) cause a marked increased permeability of the outer membrane to antibiotics, detergents, and other agents. As stressed by Haller et al. (1975), the term membrane should be used as a morphological designation, since it is only meant to describe the unit membrane profile revealed by electron microscopy.

2.2.3. General composition of the outer membrane of the gram-negative bacteria

In *S. typhimurium, E. coli,* and other gram-negative bacteria (Osborn et al., 1972a,b) the phospholipid composition of the outer membrane is qualitatively similar to that of the plasma membrane: phosphatidylethanolamine and phosphatidylglycerol are the major constituents and small amounts of cardiolipin are also present. Quantitatively, the composition of the two membranes differs markedly. The ratio of phosphatidylglycerol to phosphatidylethanolamine and that of cardiolipin to phosphatidylethanolamine in the outer membrane are reduced to about 50% and 25%, respectively, of those observed in the plasma membrane. Moreover, the outer membrane has an appreciably low ratio of total phospholipid to protein as compared to those of the plasma membrane and other biological membranes. The outer membrane is distinguished by a highly characteristic pattern of protein bands in SDS-polyacrylamide gel elctrophoresis (Schnaitman, 1970a,b; 1971a,b) and by the occurrence in it of several unique components (lipoprotein and lipopolysaccharide; see sections 2.5. and 2.7.). One could assume that the phospholipids occur primarily in a bilayer structure and that the proteins interact with both the polar headgroups and the internal hydrocarbon chains of the lipid layer. This classic view, however, does not explain the many unusual features and properties of the outer membrane. These properties are such that the fluid mosaic model of Singer and Nicolson (1972), in essence a fluid lipid matrix in which the proteins are randomly disposed without forming a long-range ordered system, does not apply to the outer membrane (see section 2.8.).

2.3. Wall and membrane teichoic acids in gram-positive bacteria

The term teichoic acid was first introduced by Baddiley to designate polymers of either ribitol phosphate or glycerol phosphate in which the repeating units were joined together through phosphodiester linkages (Armstrong et al., 1958). Typical ribitol and glycerol teichoic acids are shown in Fig. 18. This term is intended to include all polymers containing glycerol phosphate or ribitol phosphate residues, associated with the membrane, the wall, or the capsule (Baddiley, 1972). Teichoic acids have been found to occur in gram-positive bacteria and are apparently absent from gram-negative bacteria. They are very effective antigens and have often been identified as group- or type-specific substances.

Wall teichoic acids are either glycerol or ribitol phosphate polymers. They are covalently linked to the peptidoglycan. Membrane-associated teichoic acids are

always glycerol phosphate polymers. They are covalently linked to a glycolipid that is part of the plasma membrane, and thus they are also called lipoteichoic acids. Both wall and membrane teichoic acids often occur in the same organism.

2.3.1. Isolation (Coley et al., 1975a)

Wall teichoic acids are usually extracted from purified walls. As they are isolated, however, walls may contain substantial amounts of membrane fragments and lipoteichoic acid. Thus special attention must be paid to the removal of membrane contamination. This can be achieved by extraction with 40 to 80% aqueous phenol. Wall teichoic acid can then be extracted by cold dilute trichloracetic acid, followed by precipitation with organic solvents. Extraction periods of 1 to 2 days or more at 4°C are necessary. In this procedure solubilization is caused by hydrolysis of some of the phosphodiester linkages. Obviously, the compounds thus obtained are of no value for the study of chain length. "Native" teichoic acids can be isolated by selective enzymic degradation of the wall peptidoglycan (section 2.3.2.3.).

Lipoteichoic acids are usually isolated by extraction of whole cells with 40 to 80% aqueous phenol, followed by chromatography on Sepharose 6B. Attention should be paid to the fact that the glycolipid moiety of the lipoteichoic acid is in hydrophobic interaction with the lipids of the plasma membrane. Substantial loss of lipoteichoic acid from the membrane can result from washing with water (Shockman and Slade, 1964). Mg^{2+} ions play a role in maintaining the association. Protoplasts made in the absence of Mg^{2+} ions lack lipoteichoic acid (van Driel et al., 1973). Finally, release of lipoteichoic acids from the membrane through the walls has been observed in several species (Joseph and Shockman, 1975; Markham et al., 1975).

2.3.2. Wall teichoic acids

Wall teichoic acids may exhibit the full range of glycerol phosphate-and ribitol phosphate-containing structures.

2.3.2.1. Structure. One of the first ribitol teichoic acids that was submitted to thorough structural investigation was found in the walls of a strain of *Staphylococcus aureus* (Baddiley et al., 1962a,b; Ghuysen et al., 1964). Its basic structure is shown in Fig. 18. In all strains examined the phosphodiester linkages are between positions 1 and 5 on ribitol, and N-acetylglucosaminyl residue is present at the D-4 position of each ribitol residue. The glycosidic linkages may be α or β, but both types of linkage are present in the majority of strains. Most of the ribitol residues have a D-alanine ester residue that occupies position D-2. Similar wall ribitol teichoic acids occur in many bacilli and lactobacilli. The sugar substitutes are β-glucopyranosyl in a strain of *B. subtilis* (Armstrong et al., 1961) and are α-glucopyranosyl in *Lactobacillus arabinosis* 17-5 (Archibald et al., 1961).

More complex wall ribitol teichoic acids occur in other species. For example, in *Diplococcus pneumoniae* strain R36A, the wall teichoic acid is not completely characterized but is known to contain choline in addition to glucose, ribitol,

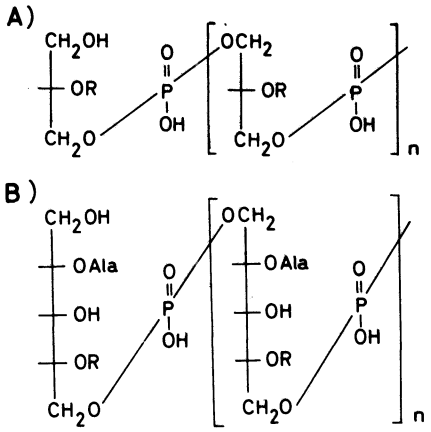


Fig. 18. (a) Typical glycerol teichoic acid (R = H, glycosyl or D-alanyl). (b) Typical ribitol teichoic acid (R = glycosyl; Ala-D-alanyl).

phosphorus, galactosamine, and 2,4,6-trideoxy-2, 4-diaminohexose. This latter teichoic acid has been identified as the serologically somatic antigen known as "C-substance" (Brundish and Baddiley, 1968; Mosser and Tomasz, 1970; Watson and Baddiley, 1974). Wall teichoic acids in which the sugar residues form a part of the main polymer chain are also known. Polymers of glucosylglycerol phosphate (Fig. 19) and galactosylglycerol phosphate occur together as separate molecules in the walls of *Bacillus licheniformis* ATCC 9945 (Burger and Glaser, 1966). Similarly, walls of *Lactobacillus plantarum* N1RD C106 contain a mixture of one polymer of glucosylglycerol phosphate and two polymers of isomeric diglucosylglycerol phosphates (Fig. 20) (Adams et al., 1969). Walls of various bacteria possess teichoic acids which contain sugar-1-phosphate residues. The first example known was *Staphylococcus lactis* 13; glycerolphosphate is attached to the hydroxyl at C-4 on N-acetylglucosamine-1-phosphate, whereas D-alanine is fixed on the hydroxyl at C-6 (Fig. 21) (Archibald et al., 1971). Finally, in *Lactobacillus acidophilus,* the teichoic acid is probably a mixture of (α or β)-1,6-linked polyglucose polymers with monomeric α-glycerol phosphate side chains attached to them on the C_2 or C_4 position (Coyette and Ghuysen, 1970a).

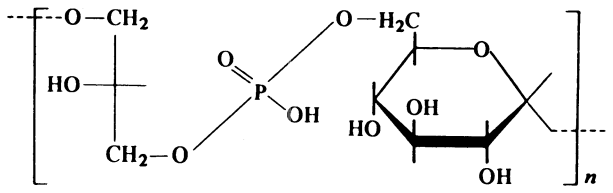


Fig. 19. Glucosylglycerol phosphate teichoic acid from *Bacillus licheniformis* ATCC 9945. (Burger and Glaser, 1966).

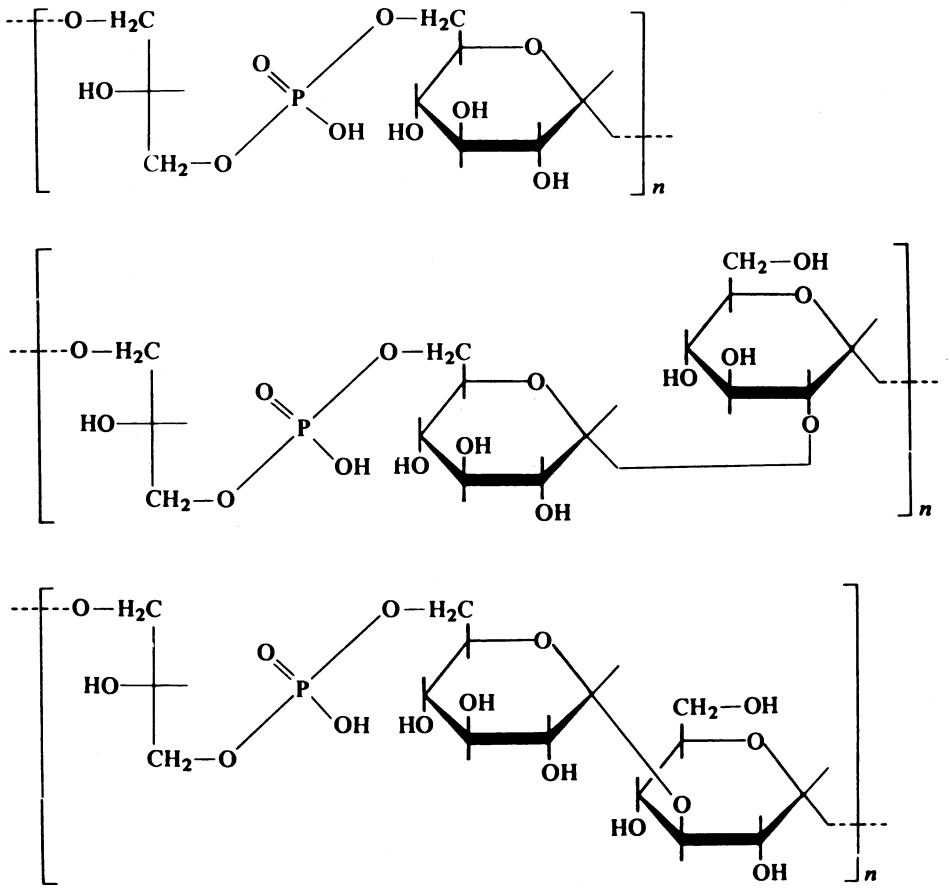


Fig. 20. Mixture of teichoic acids in walls of *Lactobacillus plantarum* NIRD C106. (Adams et al., 1969.)

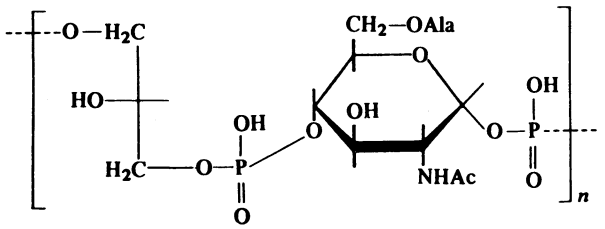


Fig. 21. Teichoic acid containing sugar 1-phosphate linkages from the walls of *Staphylococcus lactis* 13. (Archibald et al., 1971.)

2.3.2.2. Occurrence. The few examples given above illustrate the extreme variability in structure exhibited by wall teichoic acids. Other anionic polymers that lack polyol phosphate, and thus are not strictly teichoic acids, may also occur in the walls, such as polymers containing N-acetylglucosamine 1-phosphate (Archibald and Stafford, 1972) and the so-called teichuronic acids that are acidic polymers containing hexuronic acids. In the walls of *B. subtilis,* the teichuronic acid is a polymer of N-acetylgalactosamine and glucuronic acid (Janczura et al, 1961), and that of *M. lysodeikticus* is a polymer of glucose and 2-acetamido-2-deoxymannuronic acid (Perkins, 1963). An almost endless variety of acidic polysaccharides and teichoic acids constitute the nonpeptidoglycan portion of virtually all the walls of gram-positive bacteria. Growth conditions (especially phosphate and magnesium limitations) deeply influence the composition of the wall in altering the relative proportion of its teichoic acids and anionic polysaccharides (Ellwood and Tempest, 1969). This mobility occurs despite the fact that both of these polymers are covalently linked to the peptidoglycan, and thus implies the presence of an active system for turnover.

2.3.2.3. Linkage with peptidoglycan. Little information is available to date on the exact nature of the bond by which the teichoic acids and acidic polysaccharides are fixed to the walls. Clearly, however, teichoic acids are covalently attached to the glycan moiety of the peptidoglycan. This was proved for the first time by selectively degrading the peptidoglycan of the walls of *S. aureus* Copenhagen with the help of specific enzymes and by isolating the ribitol teichoic acid (Ghuysen et al., 1964). The polymer thus purified had short peptidoglycan fragments still attached to it. By using this technique, an average chain length of about 30 to 40 ribitol phosphate units was estimated.

The fact that muramic acid 6-phosphate occurs in various walls containing teichoic acids (Liu and Gotschlich, 1963; Munoz et al., 1967) strongly suggests that the attachment between wall teichoic acid and peptidoglycan involves a bond between a terminal phosphate group of teichoic acid and C-6 of muramic acid. Good evidence has also been obtained that wall polysaccharides are attached to peptidoglycan through a sugar-1-phosphate linkage between the reducing terminal sugar of the polysaccharide and a N-acetylmuramic acid residue in the peptidoglycan (Knox and Hall, 1965; Knox and Holwood, 1968; Hughes, 1970). So far, however, direct evidence for the nature of the linkage between wall teichoic acid and peptidoglycan has been obtained in only two cases. In *Staphylococcus lactis* 13, the wall glycerol teichoic acid has its terminal glycerol phosphate residue fixed to muramic acid (Button et al., 1966) (Fig. 22). Nearly 40% of the glycan of the peptidoglycan has teichoic acid attached to it, and in that fraction of the wall that contains the two polymers linked to each other each glycan chain (9 disaccharide units) is substituted by only one teichoic acid chain (24 repeating units) (Baddiley, 1972). In the bacteriophage resistant mutant of *S. aureus* H, the wall ribitol teichoic acid (40 repeating units) is also fixed to muramic acid, but in this case the link between the two polymers is mediated via a short oligomer containing 3 or 4 glycerol phosphate residues (Fig. 22) (Heckels

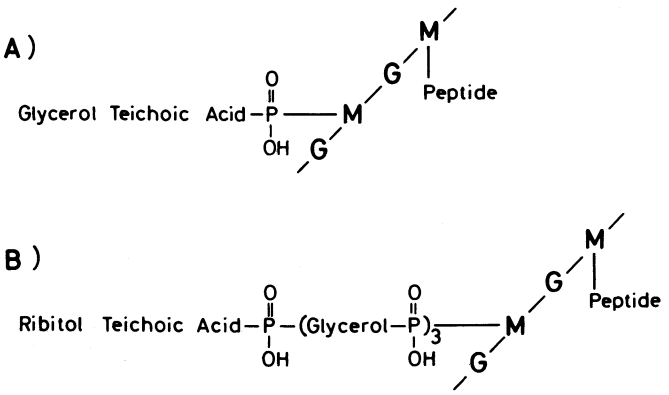


Fig. 22. Wall teichoic acids in *S. lactis* 13 (a) and in *S. aureus* H (b). The attachment to the peptidoglycan is probably on C_6 of N-acetylmuramic acid. G, N-acetylglucosamine; M, N-acetylmuramic acid.

et al., 1975; Coley et al., 1975b, 1976; Hancock and Baddiley, 1976) and perhaps N-acetyl-D-glucosamine (Bracha and Glaser, 1976). It is not known whether this type of link occurs with all ribitol teichoic acids. It is striking, however, that even after trypsin treatment and aqueous phenol extraction small amounts of glycerol phosphate are found in the walls of various bacteria that contain ribitol teichoic acids (Archibald and Stafford, 1972). This observation suggests that an intervening short oligomer of 3 to 4 glycerol phosphate units between peptidoglycan and ribitol teichoic acid may usually occur. Such a structural feature would be consistent with the finding that, at least in some bacteria, the glycerol lipoteichoic acid carrier is involved in the biosynthesis of the wall ribitol teichoic acid (Fiedler and Glaser, 1974; also see section 3.2.).

2.3.2.4. Arrangement. Peptidoglycan, together with the teichoic acids and the acidic polysaccharides covalently attached to it, should be considered as components of the same macromolecule and not as separate polymers (Rogers, 1974). The question arises, however, whether the attachment sites for the nonpeptidoglycan polymers are distributed randomly or nonrandomly within the wall. A nonrandom distribution could give rise to distinct wall areas containing unsubstituted and substituted peptidoglycan, respectively. This problem has not been solved. Clearly at least a portion of the teichoic acid must be localized close enough to the surface for reactions to occur between whole organisms and teichoic acid—specific antibodies (Burger, 1966; Knox and Wicken, 1971, 1973), plant lectins (Birdsell et al., 1975) and bacteriophages (Young, 1967; Coyette and Ghuysen, 1968; Chatterjee, 1969; Doyle et al., 1973; Archibald, 1976). For example, concanavalin A, which interacts specifically and reversibly with the polyglucosyl glycerolphosphate teichoic acid of *B. subtilis* 168 walls, has been used as a probe to study the organization of the surface teichoic acid in this organism (Birdsell et al., 1975). Treatment of whole cells and walls with Con A caused the appearance of a discontinuous, irregular fluffy layer (25–60 nm thick) on the

outer profile of the wall. On this basis, the portion of teichoic acid exposed at the cell surface was proposed to be oriented perpendicularly to the long axis of the cell.

2.3.3. Membrane lipoteichoic acids

Membrane lipoteichoic acids are almost always of the classical glycerolphosphate polymer type. They are almost universally present in the gram-positive bacteria, and this presence does not depend on growth conditions as do wall-associated teichoic acids.

2.3.3.1. Structure. Lipoteichoic acids are amphipathic molecules, each having a long, polar glycerolphosphate chain linked to a small hydrophobic lipid portion. The polar portion consists of 1→3 phosphodiester-linked chains of 25 to 35 glycerolphosphate residues variously substituted with glycosyl and D-alanine ester groups (Fig. 18). The lipid moiety is a glycolipid that is probably identical to the free glycolipid of the plasma membrane (Button and Hemmings, 1976). In *S. aureus* H, the lipoteichoic acid (Fig. 23) contains one molecule of gentiobiosylglycerol for every 30 glycerol phosphate units. The polyglycerolphosphate chain is linked at its phosphate terminal end to the hydroxyl group at position 6 of the terminal glucose moiety of diacylgentiobiosylglycerol (Duckworth et al., 1975). In *S. faecalis,* this glycolipid has been characterized as a phosphatidylkojibiosyl diglyceride (Toon et al., 1972; Ganfield and Pieringer, 1975). The two portions of the lipoteichoic acids are linked by a phosphodiester bond that involves a sugar hydroxyl group of the glycolipid and the terminal glycerolphosphate residue of the teichoic acid chain. The partial structures of the lipoteichoic acids in other *Streptococci* and in various *Lactobacilli* (Knox and Wicken, 1973; Wicken and Knox, 1975) are also known. Deacylation removes the fatty acyl and D-alanine ester groups and yields glycerolphosphate chains still attached to the glycerol glycoside of the original polymer. The phosphodiester link between the glycerolphosphate chain and the glycolipid is acid-labile. Treatment with cold trichloracetic acid yields teichoic acid devoid of glycolipid.

Not all gram-positive bacteria possess conventional lipoteichoic acids. In *M. lysodeikticus, sodonensis,* and *flavus,* the presence of lipoteichoic acids has been

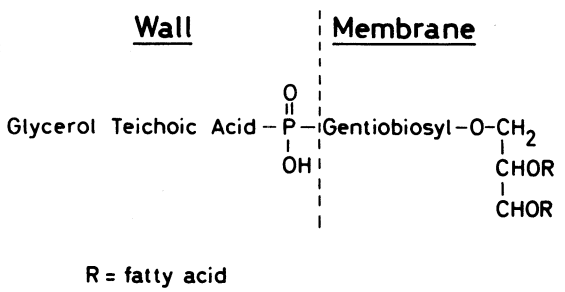


Fig. 23. Lipoteichoic acid in *Staphylococcus aureus* H.

precluded, but there are membrane-associated lipomannans that have the same properties as lipoteichoic acids (Powell et al., 1974, 1975; Schmit et al., 1974; Owen and Salton, 1975a,b,c). Lipomannans have mannose as the only sugar component and also contain glycerol, ester-linked fatty acids, and succinic acid substituents. In *M. Lysodeikticus,* the mannan has a chain of about 60 hexose units linked at its reducing end to a glycerol residue which itself bears 2 fatty acid esters (Powell et al., 1975). This hydrophobic terminal is similar to the linkage of the polyglycerolphosphate chain to lipid in conventional lipoteichoic acids and ensures the anchorage of the mannan to the plasma membrane. Lipomannans occur in both plasma and mesosomal membranes (Owen and Salton, 1975c).

D. pneumoniae is another exception. In this organism, the so-called pneumococcal Forssman antigen can probably be regarded as a lipoteichoic acidlike component. It is localized in the plasma membrane and contains bound lipids and choline (Goebel et al., 1943; Brundish and Baddiley, 1968; Briles and Tomasz, 1973; Höltje and Tomasz, 1975a,b).

2.3.3.2. Lipoteichoic acids as surface components. Despite their inner cellular localization, reaction of whole organisms with antibodies specific for lipoteichoic acids has provided evidence for the exposure of lipoteichoic acids at the cell surface (Wicken and Knox, 1975). Thin sections of *Lactobacillus plantarum* treated with rabbit IgG specific for the glycerolphosphate sequence of the lipoteichoic acid and then with ferritin-labeled goat antibodies to rabbit IgG showed the label extending from the outer surface of the plasma membrane throughout the wall and, in some cases, outside the outer boundary of the cell. Mesosomes were also labeled by the indirect ferritin antibody technique, indicating that both types of membrane carry lipoteichoic acid. However, variations may occur. Thus *L. fermenti,* but not *L. casei,* was agglutinated by antisera to lipoteichoic acid. By use of the same ferritin technique as described above, *L. casei* showed some surface adsorption of the antibody, but this was irregular and less significant than the confluent labeling seen with *L. fermenti* (Knox and Wicken, 1973; van Driel et al., 1973). On the basis of these observations and others, it has been proposed that the long, polar glycerolphosphate chains of lipoteichoic acids probably extend through the network of the wall until, in at least some cases, they come near enough to the outer surface to act as surface antigens. Surprisingly, extracellular lipoteichoic acids have been found in cultures of *S. faecalis* 9790, *Streptococcus mutans* FA-1 and in a variety of other bacterial species (Joseph and Shockman, 1975; Markham et al., 1975). They occur in both the deacylated and micellar (and presumably acylated) forms. Extracellular lipoteichoic acids do not seem to be products of cellular lysis, and the role of excretion of large amounts of such energy-rich polymers remains to be determined. Recent observations, however, suggest they may play a role in regulating the activity of the cell autolysins (section 3.1.6.).

2.3.4. Functions of wall and membrane teichoic acids

Because of the widespread distribution of wall and membrane teichoic acids in gram-positive bacteria, it has long been assumed that these polymers confer

advantages on these bacteria, or that they fulfill one or several important functions.

When *B. subtilis* (and other bacteria) are grown under conditions of phosphate limitation, teichoic acid is no longer present in the wall. The preexisting teichoic acid is removed by wall turnover and is replaced by teichuronic acid (Ellwood and Tempest, 1969). Conversely, when *B. subtilis* is grown under various limitations in the presence of excess phosphorus, the walls always contain teichoic acid but no teichuronic acid. The amount of teichoic acid formed, however, varies depending on the culture limitations. Among the limitations tested, the one that yields the largest amount of wall teichoic acid is magnesium starvation (Ellwood, 1975). The substitutions between teichoic and teichuronic acid are phenotypic responses. They occur rapidly as pointed out by Rogers (1970), "providing either one or another of the groups of negatively charged polymers is present on its surface, the microorganism seems content". Significantly, even under conditions of phosphate limitation when no wall teichoic acid is formed, the membrane lipoteichoic acid is still produced. Thus it seems that cell must possess membrane lipoteichoic acid but that "all that is required in the wall is one or more acidic polymers" (Rogers, 1970).

The most gram-negative bacteria do not possess teichoic acids. Glycerol teichoic acids, however, have been isolated from some strains of *Butyrivibrio fibrisolvens* (Sharpe et al., 1975). Moreover, gram-negative bacteria have in their outer membrane a related class of macromolecules, the lipopolysaccharides, which include in their structures, phosphodiester groups, sugar residues, and basic centers (section 2.7.). These may serve a purpose similar to that of teichoic acids. This idea is further supported by findings that phosphate esters may be constituents of the O-side chain of the lipopolysaccharide in some gram-negative bacteria. Glycerolphosphate was found in one strain of *E. coli* (Jann et al., 1970) and ribitol phosphate occurs in *Proteus mirabilis* (Gmeiner, 1975, 1977; Gmeiner and Martin, 1976) as a side branch of the sugar polymer. Moreover, uronic acids are also frequently found in lipopolysaccharides of *P. mirabilis* as if this organism could satisfy its need for anionic groups in the O-side chain either by phosphodiester groups or by uronic acids. This situation is reminiscent of that in gram-positive bacteria, where a reversible shift from teichoic acid to teichuronic acid, and vice versa, occurs depending on growth conditions.

2.3.4.1. Magnesium uptake. Teichoic acids and other negatively charged polymers bind divalent cations. Similarly, the succinic acid substituents on lipomannan determine its charged properties and also enable this polymer to bind divalent cations efficiently. Baddiley and his colleagues have suggested that the two regions of anionic polymers (i.e., the wall and the plasma membrane, respectively) would function as an integrated cation-exchange system between the exterior of the cell and the plasma membrane, ensuring to the latter the high concentration of Mg^{2+} required for stability and many enzymic functions (Heptinstall et al., 1970; Hughes et al., 1971; Baddiley, 1972). This view is not only consistent with the cation-binding properties of these polymers but, as stressed by Baddiley (1972), would also explain their structural diversities since a variety of phos-

phate, hydroxyl, and amino groups could effectively regulate cation binding. Cells could control this activity in part by controlling the amount of ester-bond alanine in the teichoic acids.

The interaction between Mg^{2+} ions and wall teichoic acids has been studied by equilibrium dialysis (Lambert et al., 1975). On the basis of binding data, the apparent association constants and the number of Mg^{2+} binding sites on the teichoic acid molecules were shown to vary depending on the pH, ionic strength, and the presence of other divalent cations. Thus in 10 mM NaCl aqueous solution at pH 5.0, one Mg^{2+} ion is bound for every two phosphate groups in the wall teichoic acid of *Lactobacillus bulchneri* with a $K_{assoc.}$ apparent value of $2.7 \times 10^3\ M^{-1}$. Moreover, the number of Mg^{2+} binding sites was shown to be reduced by the presence of alanine ester substituents. These properties and apparent association constant values presumably reflect an optimum balance between two antagonistic requirements. The first requirement is that wall teichoic acid must possess sufficient affinity and selectivity for Mg^{2+} ions to be able to scavenge them from an excess of competing ions. The second is that Mg^{2+} ions must not be too tightly bound in order to be transferred to the plasma membrane. On the basis of equilibrium dialysis experiments, the Mg^{2+} binding properties of walls of *B. subtilis* W13 containing teichoic acid were compared with those of walls of the same organism containing teichuronic acid (Heckels et al., 1977). Both walls had similar properties and their affinity was greater than that displayed by the isolated polymers in solution.

The relative affinities of various cations for anionic sites in isolated walls were also assessed by a technique involving displacement of one cation by another (Marquis, R.E., personal communication). The affinity series was $H^+ >> La^{3+} >> Sr^{2+} > Ca^{2+} > Mg^{2+} >> K^+ > Na^+ > Li^+$. The total amounts of magnesium that could be displaced with Na^+ or H^+ from the magnesium forms of isolated walls varied from 73μmole/g, dry weight, for walls of the teichoic acid deficient *S. aureus* 52A5 to about 520 μmole/g for walls of *B. megaterium* KM. These studies have also shown that interaction of the walls with cations is a complex phenomenon. Thus, for cells grown in usual laboratory media that often contain an excess of monovalent versus divalent cations, there is a mix of small cationic counterions in the wall and monovalent cations may predominate even if the wall has a higher affinity for diavalent cations.

Mutants of bacilli have been used to show that the presence of the proper amounts of negatively charged polymers attached to peptidoglycan in gram-positive bacteria is important (Rogers, 1975, 1976; Rogers et al., 1971, 1974, 1976). Three classes of mutants of *B. subtilis* that have phenotypes with reduced amounts of these polymers in their walls are of particular interest since they do not grow as rods but as deformed cocci. These mutants are the following: (1) the *Rod* A mutant, when grown at 45°C, has a wall containing only about 20% of the amount of teichoic acid present when grown as rods at 30°C (Rogers et al., 1974); (2) the phosphoglucomutase negative mutants, when grown under phosphate limitation, have walls consisting almost entirely of peptidoglycan (Forsberg et al., 1973); and (3) the CDP-glycerol pyrophosphorylase mutants also have markedly

reduced amounts of teichoic acids in the walls (Rogers, 1975, 1976). Surprisingly, at first sight, *Rod* B mutants of *B. subtilis* under restrictive conditions have a deformed coccal morphology but virtually normal amounts of wall teichoic acid (Rogers et al., 1971). However, these mutants are grossly deficient in their capability to use Mg^{2+} ions, probably due to a deficient transport system. Thus, it seems likely that the *Rod* type mutants as a group are disturbed in the supply and/or movement of the Mg^{2+} ions, this disturbance being related either to the absence of wall teichoic acids in proper amounts or to a deficient transport system for this cation.

Manipulations of the Mg^{2+} concentration and of the nature and concentration of halogen anions can alter the morphology of at least some of these mutants (Rogers, 1975, 1976). Thus if 10 mM, instead of 1 mM Mg^{2+}, is added to the growth medium the CDP-glycerol pyrophosphorylase mutants change from deformed cocci to rods. Rapid growth of *Rod* B mutant as deformed cocci can be obtained with 10mM Mg^{2+} and 20 mM Cl^-. But if Cl^- is substituted by 15 mM Br^- or 5 – 10 mM I^-, the morphology is that of rods. Moreover, by using a constant concentration of the latter two halogen ions, the morphology can be precisely controlled by the Mg^{2+} concentration. Thus, in some bacteria at least, Mg^{2+} uptake seems to be an important factor related to cell morphology. A further discussion of the effects of changes in the anionic polymer content of walls can be found in the chapter by Daneo-Moore and Shockman in this volume.

2.3.4.2. Other functions. In addition to the suggested role of the negatively charged polymers in wall and membrane as the suppliers of Mg^{2+} ions to bacterial cells, other important functions are fulfilled by both wall teichoic acids and lipoteichoic acids that are related to the autolytic activity of the cells. These functions are discussed in section 3.1.6. Moreover, lipoteichoic acids were shown to serve, at least in some bacteria, as assembly sites for the wall teichoic acids (and perhaps for the assembly of the peptidoglycan). This role is discussed in section 3.2.2.5.).

2.4. Wall proteins in gram-positive bacteria

Walls of most gram-positive bacteria contain some protein. Little is known about their association with the other wall polymers. In some cases, protein and peptidoglycan are covalently bound to one another. Thus, protein A in the walls of *Staphylococcus aureus* is linked to an amino group of the peptide moiety of the peptidoglycan (Sjöquist et al., 1972; Movitz, 1976; Sjödahl, 1977; Lindmark et al., 1977). In other cases, proteins cover the outer surface of the cell from which they can be removed by trypsin. Thus the M protein antigen of group A streptococci is labile to proteolytic enzymes and can be digested without affecting the viability of the cells or the insolubility and electron microscopic appearance of isolated walls (Fox, 1974). Regular surface patterns due to outer protein-containing layers have also been described; a well-characterized one is the so-

called T-layer from *Bacillus brevis* (Brinton et al., 1969; Aebi et al., 1973; Howard and Tipper, 1973). T designates the tetragonal symmetry of the surface layer. It is located on the exterior surface of the cell and consists of a single polypeptide chain (molecular weight: 140,000) and is involved in phage binding. It is not linked covalently to the peptidoglycan since it can be released with 2 M guanidine hydrochloride and is cleaved by proteolytic enzymes. The purified protein can reassociate from a subunit state to give rise to planar T-layer sheets. Pronase-treated protein (molecular weight: 125,000) can also reassociate to give rise to hollow cylinders with the diameter of the cells. The ultrastructure of planar and cylindrical reassociates has been studied. *Bacillus polymixa* also possesses a patterned surface layer, containing mainly protein and carbohydrates, which is noncovalently associated with the underlying peptidoglycan structure (Baddiley, 1964; Goundry et al., 1967).

The wall of *Bacillus subtilis* 168, strain Cbl-1, exhibits several unusual features (Leduc et al., 1973). The most striking is the presence of a high molecular weight protein, instead of wall teichoic acid, that accounts for over 50% of the wall material. To our knowledge, this strain appears to be the only one known that contains such a "novel" wall protein. This protein is not covalently linked to the peptidoglycan and can be readily removed by treating the walls with 6M LiCl. It consists of two noncovalently associated chains. Treatment of walls with SDS yields a peptidoglycan that exhibits the classical rod-shaped morphology. Very thin homogeneous ghosts are seen that are reminiscent of the rigid layer of *E. coli*. Like this latter structure, the isolated peptidoglycan layer of the *B. subtilis* Cbl-1 is especially fragile; it can be dissolved with 6M guanidine hydrochloride (Leduc and Van Heijenoort, 1975). The isolated peptidoglycan fraction contains galactosamine and an excess of glucosamine, suggesting that a polysaccharide containing these two hexosamines is bound to the peptidoglycan. The mutant fails to grow on a minimum glucose medium and exhibits a long lag before growth on complex media. The occurrence of lipoteichoic acid in the mutant was not investigated. The role played by the novel wall protein in the physiology of the Cbl-1 strain is unknown. Whether or not this protein may serve some of the same functions as the wall teichoic acids or acidic polysaccharides remains unresolved.

2.5. The wall lipoprotein of the gram-negative enteric bacteria

2.5.1. Localization

The peptidoglycan in *E. coli* (and in other enteric bacteria) has covalently attached lipoprotein, a situation reminiscent of the peptidoglycan-anionic polymer complexes found in the walls of gram-positive bacteria. Lipoprotein, however, occurs not only in covalent linkage with the peptidoglycan but also in one free form. Both covalently bound and free lipoproteins are part of the outer membrane, where they represent one of the major proteins. In *E. coli*, the free form is

present in twice the amount of that in the peptidoglycan-linked form (Braun and Sieglin, 1970; Braun and Wolff, 1970; Inouye et al., 1972; Hirashima et al., 1973b). The total number of lipoprotein molecules in cells growing exponentially in a rich medium is about 750,000 per cell, and approximately 250,000 are attached to the peptidoglycan. The number of disaccharide peptide units required to form a monomolecular layer of peptidoglycan in one cell has been estimated to be about 3,500,000. Thus one lipoprotein molecule is statistically linked to every 10th to 12th disaccharide peptide unit (Braun et al., 1974a). Because of the covalent association between lipoprotein and the peptide moiety of the peptidoglycan, one may assume that the side of the glycan stack of the peptidoglycan monolayer that bears the peptide side chains is oriented toward the outer membrane of the cell.

2.5.2. Isolation

The peptidoglycan-lipoprotein complex (or murein-lipoprotein complex) is usually obtained as the residue left after extraction of the isolated cell envelopes with 2 to 4% SDS at 100°C. This complex has been also called the rigid layer, or sacculus. Trypsin selectively cleaves bonds at the junction between the lipoprotein and the peptidoglycan so that the two components can then be separated from each other. As mentioned earlier, the lipoprotein-free peptidoglycan exhibits fragile, rodlike structures similar in shape to that of the intact bacterial cell (Fig. 6).

When isolated *E. coli* cell envelopes are submitted to SDS polyacrylamide gel electrophoresis, only the free form of the lipoprotein migrates into the gel to a position corresponding to a molecular weight of about 7,500 (Inouye et al., 1974). Because of its attachment to peptidoglycan, the bound lipoprotein remains on the top of the gel. If, prior to electrophoresis, the cell envelope is treated with an endo-N-acetylmuramidase (such as lysozyme) that degrades the peptidoglycan into fragments the bound lipoprotein can then penetrate the gel. Since it has an attached peptidoglycan fragment, this substituted lipoprotein migrates to a position corresponding to a higher molecular weight than that of the free protein. If the isolated peptidoglycan-lipoprotein complex is treated with trypsin prior to electrophoresis, then all the lipoprotein migrates as the free form. After double labeling of the total proteins of the cell envelope with L-[^{3}H]arginine and L-[^{14}C]histidine, the lipoprotein (which lacks histidine) is readily characterized as the one that bears only the tritium label. By using these techniques, the quantitative relationship of the two forms of lipoprotein was investigated (Inouye et al., 1974). Recently, a procedure has been described (Hindennach and Henning, 1975) that allows the isolation of the free lipoprotein (and the other major proteins) of the outer membrane of *E. coli*. The method involves differential extraction of cell envelopes with ionic and nonionic detergents with and without Mg^{2+}, and the proteins are finally separated from each other by molecular sieve chromatography in the presence of SDS (yield: 30 mg of pure lipoprotein from 200 g cell paste).

2.5.3. Primary structure

The lipoprotein of *E. coli* contains 58 amino acids (molecular weight: 7,800) and lacks several amino acids including histidine. The amino acid sequence has been determined by Braun and coworkers (Braun and Rehn, 1969; Braun and Sieglin, 1970; Braun and Wolff, 1970; Braun et al., 1970; Braun and Bosh, 1972a,b; Braun 1973; Hantke and Braun, 1973; Braun and Hantke, 1974; Braun et al., 1974a,b; Braun, 1975; Braun and Wolff, 1975). Fig. 24, in which the attachment site to the peptidoglycan is shown, has been drawn in a way that emphasizes the repetitive design of the molecule. Essentially, tripeptides extend at each end of a middle repetitive section and the lipid and the peptidoglycan are attached there.

The lipid is at the N-terminal end of a Cys-Ser-Ser sequence. A fatty acid is bound as an amide to the N-terminal α-amino group of cysteine, and other fatty acids are bound as esters to the hydroxyl groups of S-glyceryl cysteine. The fatty acids bound as amides are mainly palmitic acid (65%), palmitoleic acid (11%) and *cis*-vaccinic acid (11%). The fatty acids bound as esters are mainly palmitic acid (45%), palmitoleic acid (11%), *cis*-vaccinic acid (24%), cyclopropylenehexadecanoic acid (12%) and cyclopropyleneoctadecanoic acid (8%).

The first 15 amino acid residues of the middle section are duplicated almost identically and the next stretch of 7 amino acid residues is almost identically quadruplicated. This amino acid sequence suggests the possible evolution of the molecule from a gene that coded originally for 15 amino acids, which was duplicated and only the C-terminal half was then added four times. The dashes in Fig. 24 represent deletions that may have occurred during evolution.

The attachment site to the peptidoglycan at the C-terminal of the lipoprotein is a Tyr-Arg-Lys sequence and the lipoprotein is fixed by the ϵ-amino group of its C-terminal lysine residue to the carboxyl group at the L-center of *meso*-diaminopimelic acid of the peptidoglycan (i.e., where a D-alanine residue occurs in a conventional tetrapeptide unit) (Fig. 24). The tyrosine, arginine, and lysine residues that accumulate at this attachment site fit especially well into the active center of trypsin. The Arg-Lys bond is preferentially cleaved and the lipoprotein is detached from the peptidoglycan. The released lipoprotein lacks its C-terminal lysine, and this lysine residue is found at the C-terminal end of one of 10th to 12th tetrapeptide L-Ala-D-Glu ⌐(L)-*meso*-A_2pm-(L)-L-Lys units in the peptidoglycan.

These structural studies were carried out on the peptidoglycan-bound lipoprotein. The protein part of the free protein obtained by the procedure of Hindennach and Henning (1975) does not differ substantially from that of the bound form.

2.5.4. Conformation

Circular dichroism of the lipoprotein in aqueous environment reveals an α-helical content of about 80% (Braun, 1975). This high degree of ordered structure explains why the peptide bonds involving arginine or lysine, which are localized in the helical portion of the molecule, are fairly resistant to trypsin action. Another remarkable property of the molecule is that, starting with its

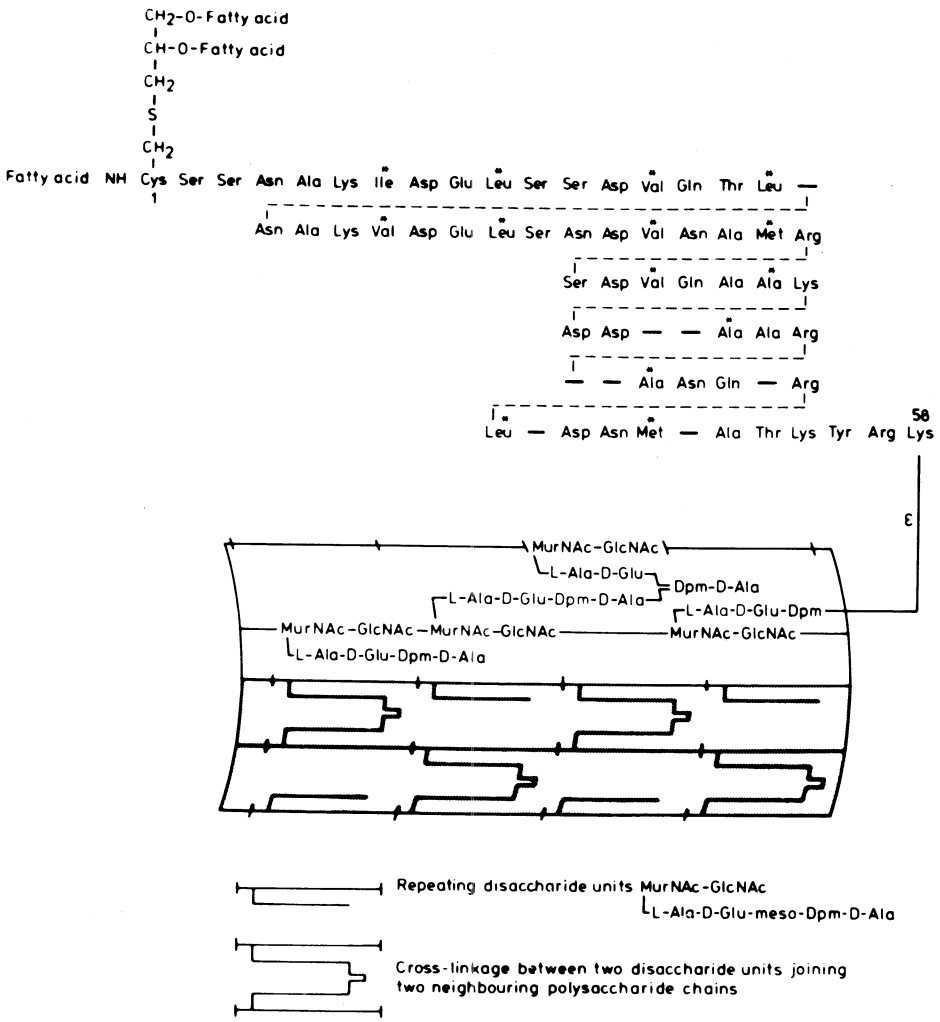


Fig. 24. Structure of the peptidoglycan-lipoprotein complex (rigid layer) of the outer membrane of *E. coli*. The cylindrical section indicates a part of the peptidoglycan with the shape of the rodlike *E. coli* cell. It is not known how the glycan chains span the cell relative to the long axis of the cylinder. In this model they are arbitrarily drawn parallel. The peptidoglycan is composed of roughly 10^6 repeating units, to which approximately 10^5 lipoprotein molecules are covalently bound. The lipoprotein replaces D-alanine on the diaminopimelate residue. Dashes represent hypothetical deletions of amino acids that may have occurred during evolution (see text). Stars indicate the hydrophobic amino acids at every 3.5th position. (Braun, 1975.) (Reprinted courtesy of Elsevier/North-Holland, Amsterdam.)

repetitive segment, every fourth and third amino acid residue, respectively, is hydrophobic. Since a helical turn contains 3.6 residues, all the hydrophobic residues would be localized on one face of the helical rod. Fig. 25 emphasizes the peculiar distribution of the amino acid residues with all the hydrophobic side

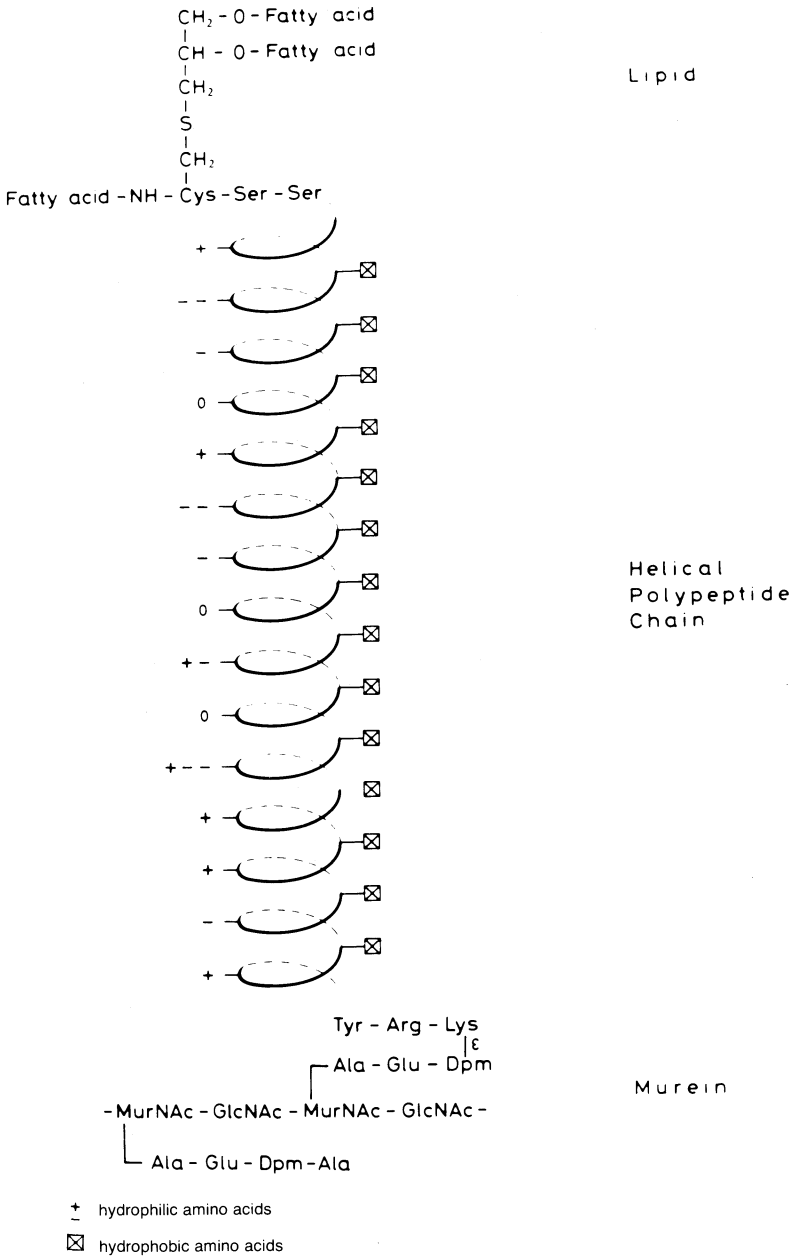


Fig. 25. Tentative model of the lipoprotein drawn as a single helical rod with the lipid and two peptidoglycan repeating units attached. ±, Hydrophilic amino acids. ×, Hydrophobic amino acids. In aqueous solution, intermolecular interactions along the hydrophobic face must occur, leading to aggregation of molecules which, in fact, is observed. (Braun, 1975.) (Reprinted courtesy of Elsevier/North-Holland, Amsterdam.)

chains on one face of the α-helix and all the hydrophilic side chains on the other.

In a search for an experimental approach to the possible conformations of the lipoprotein, Braun (1975) and Braun et al. (1976a) used the rules that Chou and Fasman (1974) derived from 15 proteins of known conformation, which allow one to predict helix, β, and coiled regions of proteins with known sequence with up to 80% accuracy. The model derived from such a calculation is shown in Fig. 26. The simple helical rod initially proposed on the basis of the amino acid sequence and circular dichroism measurements, is now broken by a β-loop occurring approximately at the middle of the amino acid sequence. Amino acid residues 1 to 4 form a β-loop, 5 to 24 an α-helix (symbolized by a cylinder), 25 to 29 a β-loop, 30 to 47 an α-helix (also symbolized by a cylinder), 48 to 51 a β-loop, 52 to 56 a β-sheet, and residues 57 and 58 a coil. One interesting feature of the model is that both the attachment sites of the lipid and the peptidoglycan protrude from the helical part of the molecule. They may serve as recognition sites for the enzyme system that are responsible for the transfers of the lipid to the polypeptide chain and for the attachment of the lipoprotein to the peptidoglycan (Braun, 1975). The secondary structure of the lipoprotein was also examined by Green and Flanagan (1976) using the method of Lim (1974a,b). The two methods of Chou and Fasman, and of Lim, respectively, agreed moderately well.

2.5.5. Function

One possible function of the bound lipoprotein is to connect the outer membrane with the peptidoglycan layer. Thus, it probably plays an important role in the construction and stabilization of the cell envelope of the *Enterobacteriaceae*. It is noteworthy that *E. coli* mutants lacking different major proteins in the outer membrane or even all of them except the lipoprotein (section 2.8.2.) have been found, suggesting that the lipoprotein may well be an essential structural component of the outer membrane. Lipoprotein is a normal wall constituent of all *Enterobacteriaceae*. Contrary to a previous report that lipoprotein was lacking in at least one enteric bacterium, *Proteus mirabilis*, lipoprotein has been shown to occur in covalent linkage to peptidoglycan in this organism, at least in the stationary phase of growth (Gruss et al., 1975). This lipoprotein, however, differs from that of *E. coli* because the *P. mirabilis* lipoprotein has a lower molecular weight (5,500) and has glycine and phenylalanine as specific components that are absent in lipoproteins of other enteric bacteria. The lipoprotein substitutes every 15th to 20th peptidoglycan unit; treatment with trypsin leaves lysine as the only lipoprotein amino acid attached to the peptidoglycan.

The fact that, at least in *E. coli*, there is twice as much free compared to bound lipoprotein suggests another function for the lipoprotein. By assuming that the lipoprotein molecule is a continuous, single helical rod (Fig. 25) and based on theoretical considerations, Inouye (1974, 1975) proposed an assembly model for the bound and free lipoprotein molecules in the form of cylindrical channels that might provide the outer membrane with passive diffusion pores. In this model, the lipoprotein α-helices in the outer membrane are arranged so that the

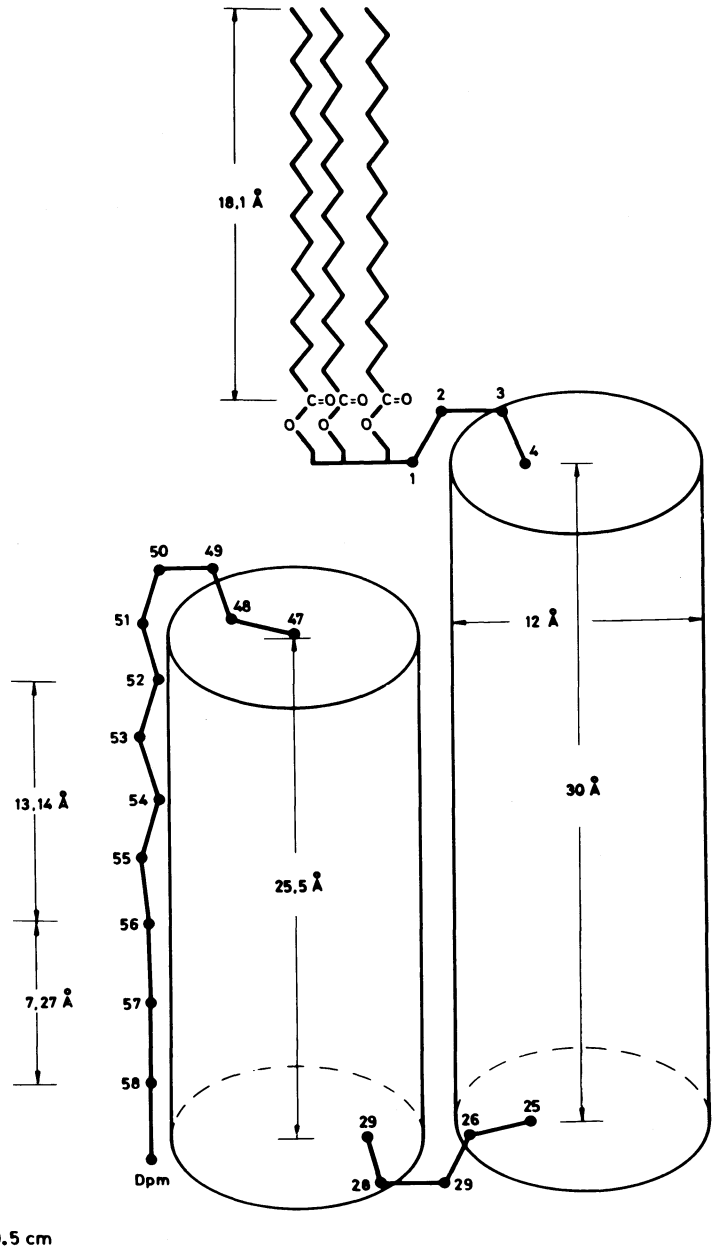


Fig. 26. Three-dimensional structure of the lipoprotein as deduced from the circular dichroism measurements and the amino acid sequence, applying the Chou-Fasman rules. Amino acid residues 1–4 form a β-loop, 5-24 an α-helix with the possible exception of 13-17, which also could be arranged in a β-sheet, 25-29 clearly form a β-loop, 30-47 an α-helix, 48-51 a β-loop, 52-56 a β-sheet and residues 57 and 58 a coil. The arrangement of the amino acid residues in the helical portions is only symbolized by the 2 cylinders. The length of the lipoprotein (48 Å) would span just half the thickness of the outer membrane, suggesting that the lipid portion of the lipoprotein immerses into the inner layer of the lipid bilayer of the outer membrane. (Braun, 1975.) (Reprinted courtesy of Elsevier/North-Holland, Amsterdam.)

hydrophobic residues face outward from the assembly whereas the hydrophilic residues are localized inside. In particular, six α-helical coils may form a superhelix or a coiled-coil with the hydrophobic residues of each of the six α-helices pointed toward the outer surface (structure 4, Fig. 4). This arrangement makes a number of ionic interactions possible between adjacent molecules, a situation that enhances stabilization of the entire assembly. Moreover, according to the model, the whole assembly structure (height: 76 Å) would penetrate through the outer membrane (75 Å thick), providing additional hydrophobic interactions between the outer surface of the assembly and the lipid bilayer of the membrane. Further stabilization of the arrangement could also be achieved by assuming that the three hydrocarbon chains at the amino terminal ends of the lipoproteins are flipped back over the helix and inserted into the bilayer structure (Fig. 4), whereas at the carboxyl terminal end of the assembly, two out of the six lipoprotein molecules would be covalently linked to the peptidoglycan layer, thus preventing the assembly from moving freely in the lipid bilayer (Fig. 4). The channel thus created through the outer membrane would have a diameter of 12.5 Å. On a basis of a twelve (instead of six) lipoprotein assemblies, the diameter would be 35.8 Å. Accordingly, the total number of channels per cell would change from 1.25×10^5 to 0.63×10^5, and the area occupied by these channels would change from 35 to 46% of the total cell surface. Recently, the lipoprotein was obtained in the form of paracrystals by adding acetone to a solution of purified lipoprotein made in Triton X-100 or SDS and in the presence of about 0.01 M $MgSO_4$ (De Martini et al., 1976, Inouye et al., 1976). When examined by the electron microscope after negative staining, these paracrystals showed considerable ultrastructure and the observed patterns were found to be compatible with the tubular superstructure proposed for the lipoprotein assembly in the outer membrane.

If one accepts the idea that one function of the lipoprotein is to provide the outer membrane with passive diffusion pores, then the accessibility of these channels should be controlled, at least in part, by the polysaccharide chains of the lipopolysaccharides (also localized on the outer surface of the outer membrane; see section 2.7.). Inouye suggested that these polysaccharides might cover the entrance of the channels, preventing some molecules from passing through them. Such an arrangement would explain the known effect of EDTA on the permeability barrier of the cell (Leive, 1965a,b; Vol and Leive, 1970). By removing part of the lipopolysaccharide, EDTA would readily expose the entrance of the channels to the outside of the cell. Immunological studies do not contradict this view. Both free and bound forms of lipoprotein are available to lipoprotein-directed antibodies only in those mutants that have incomplete lipopolysaccharides and hence a defective cell surface structure (Braun et al., 1974a). In wild strains, where complete lipopolysaccharides occur, lipoprotein does not function as an antigen and is probably shielded by other surface structures (Braun, 1975; Braun et al., 1976b).

Although Inouye's model is very attractive in many respects, at the present time it should not be taken literally. In particular, if the conformation for the

lipoprotein most recently proposed by Braun (Fig. 26) is that occurring in the membrane, then the length of the lipoprotein when broken by a β-loop approximately at the middle of it, is about 48 Å. Thus the lipoprotein would span half of the outer membrane thickness and the lipid moiety of the lipoprotein would be immersed in the inner portion of the outer membrane's lipid bilayer. Exactly how the free and bound forms of lipoprotein are arranged in the outer membrane remain matters for speculation (see also section 2.9).

2.6. Possible occurrence of a patterned inner protein layer in the walls of gram-negative enteric bacteria

In the 1960s, a periodic structure was observed in *E. coli* that apparently was buried deep in the cell envelope because extractions with phenol or detergents were necessary to reveal it (Weidel et al., 1960; Bayer and Anderson, 1965; Boy de la Tour et al., 1965). Subsequently, the occurrence of a regular array was confirmed by using other preparative procedures (de Petris, 1965, 1967; Fischman and Weinbaum, 1967; Bayer and Remsen, 1970; Nanninga, 1970) but the component(s) responsible for the observed pattern could not be identified. Recently, by using a differential heat extraction procedure in SDS, Rosenbush (1974) isolated a protein that might be responsible for the occurrence of this periodic pattern.

2.6.1. Isolation

Essentially, the procedure involves treatment of the cell envelopes with a large excess of SDS at 60°C. Variations in temperature between 30 and 70°C do not affect the procedure, but higher temperatures must be avoided. A turbid solution is obtained and, after sedimentation, the washed pellet consists of classic rod-shaped structures. When compared to the rigid layer obtained after extraction at 100°C in the detergent (i.e., the peptidoglycan-lipoprotein complex studied in the previous section), the rod-shaped structures obtained at lower temperature are less translucent and their surfaces show a regular pattern (Fig. 27a). Further extraction in SDS at 100°C for 5 minutes eliminates this regular array, and the released protein that was apparently responsible for it can be purified from the supernatant fraction and subsequently freed of the detergent.

2.6.2. Properties

The protein-lipoprotein-peptidoglycan complex (obtained after extraction at 60°C with SDS) is composed of about 65% envelope protein, the remaining mass being accounted for almost exclusively by the lipoprotein-peptidoglycan complex. In this bound form the protein is resistant to prolonged incubations with trypsin, in marked contrast to the lipoprotein. It does not bind SDS tightly even after prolonged exposure to a great excess at 60°C, and is insoluble in 5 M guanidine hydrochloride. There are 1.1×10^5 molecules of this polypeptide per cell. Diffraction pattern and electron micrograph measurements (Steven et al.,

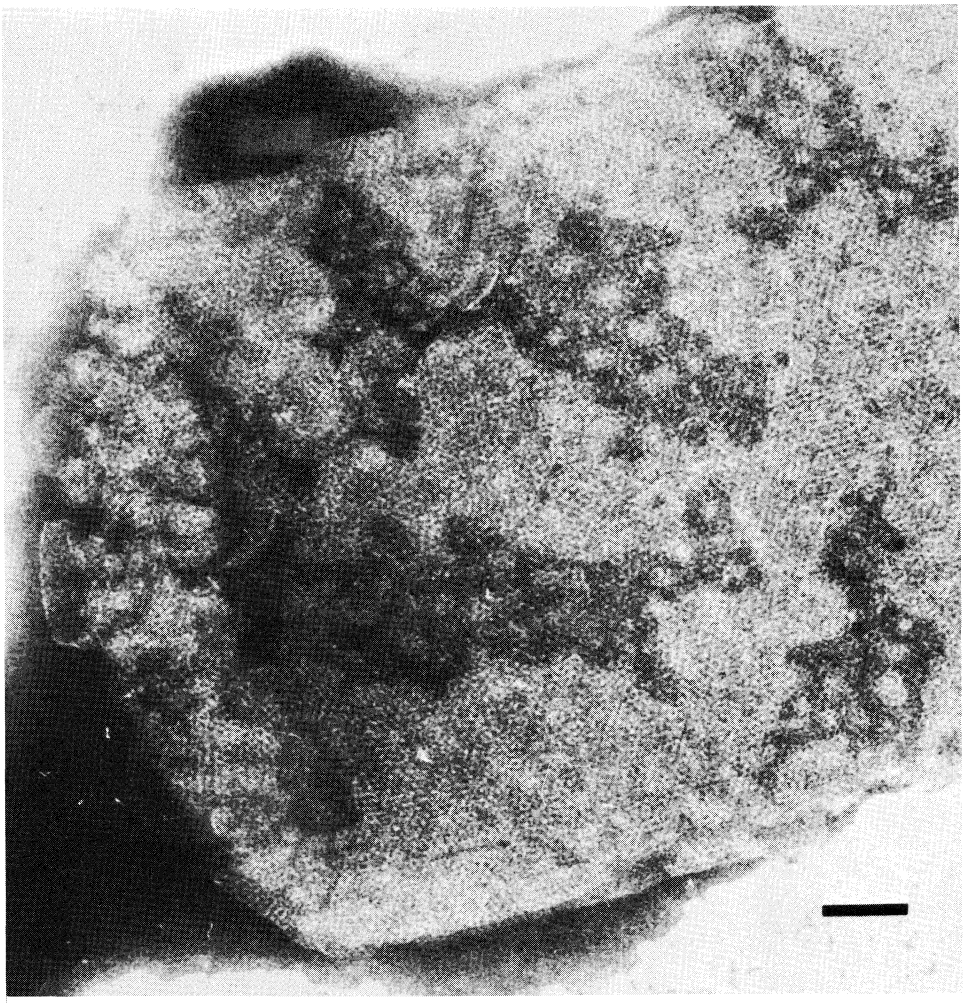
1977) revealed that they are arranged in a lattice structure with hexagonal symmetry and a periodicity of 7.5 nm (Fig. 27a). Electron microscopic evidence, the known asymmetry of the peptidoglycan layer itself, and the fact that antibodies specific for the solubilized protein selectively agglutinate uninverted peptidoglycan-envelope protein complexes strongly suggest that the protein occurs solely on the outer face of the peptidoglycan. On the basis of 1.1×10^5 molecules per cell, a 56 nm^2 value for the area of each unit, and an average value of 3 μm^2 for the surface area of the peptidoglycan layer, two subunits per morphological unit has been estimated. The lattice structure appears to be due to the protein. Treatment with SDS at 100°C dissociates the protein from the complex and leaves the latter devoid of the characteristic array. Moreover, the released protein constitutes more than 90% of the components dissociated by boiling in the detergent. Clearly, the protein is not covalently linked to the peptidoglycan (in contrast to the lipoprotein), but the nature of the interactions between the protein and the peptidoglycan-lipoprotein network as well as the neighboring protein subunits is unknown.

The dissociated detergent-free protein has been found homogeneous on the basis of several criteria. It has a molecular weight of 36,500 and exhibits moderate hydrophobicity. Circular dichroism and infrared spectroscopy indicated that a large fraction of the protein exists as β-structure. In contrast to the bound protein, the free protein binds SDS in amounts equivalent to those found with most polypeptides (Helenius and Simons, 1975), and is denatured by guanidinium ions to a random coil conformation. The same protein in an amorphous state has also been obtained by Hindennach and Henning (1975) by using the procedure described earlier (section 2.5.2). The yield is about 120 mg protein from 200 g cell paste. Henning calls this protein I.

2.6.3. Occurrence and possible function

Examination of 10 strains of *E. coli* revealed strain-specific variations in the binding properties of the protein to the complex (e.g., as a function of the temperature) rather than variations in the amount of protein per cell (Rosenbush, 1974). Proteins corresponding to that described here apparently occur in various gram-negative organisms other than *E. coli*. *Spirillum serpens* (Murray, 1963) is remarkable because it possesses both an inner regular structure comparable to that of *E. coli* and a protein surface lattice. This lattice is probably analogous to those found in various gram-positive bacteria as, for example, the T-layer of *Bacillus brevis* (section 2.4.). The surface lattices are very different from the inner ones. As previously mentioned, the surface lattices are localized on the exterior surface of the cell, released by 2 M guanidine hydrochloride, cleaved by proteolytic enzymes, and during renaturation they reassemble into highly organized structures.

The peptidoglycan is generally accepted as the main shape-maintaining structure in bacteria. However, it probably does not contain shape-determining information. For this function, one could assume that a protein matrix in the form



(a)

Fig. 27. Wall peptidoglycan in *Escherichia coli* with its associated envelope protein: (a) Prepared by SDS treatment at 60°C according to the procedure of Rosenbush (1974). Electron microscopy reveals particles covered with a regular pattern. (See text for details.) Bar, 100 nm. Particles stained negatively with potassium phosphotungstate. (Reprinted from Rosenbush (1974) by courtesy of the Journal of Biological Chemistry). (b) Prepared by SDS treatment at room temperature by Dr. M. Bayer (unpublished data). Unfixed cells from a growing culture were quickly frozen and cut in frozen state with the knife of an ultramicrotome. Sections containing large segments of the cells were picked up on an electron microscope grid, treated with 0.5% SDS at room temperature for 5 min, briefly washed and negatively stained. In the process, more than 50% of the dry mass of the wall disappeared. When treated with lysozyme (which degrades the peptidoglycan), the residual structure shown in the figure disintegrates into small spherical units some of which can also be seen in the background. These spherical elements are destroyed by both trypsin and pronase. Note the gap in the tight protein cover. Crystallinity could not be detected in the layer shown in the figure even by employing optical diffraction. (Unpublished figure and data courtesy of Dr. M. Bayer, Institute for Cancer Research, Philadelphia, Pa.)

Fig. 27. Part (b).

of a two-dimensional lattice would be the most likely candidate (Henning and Schwartz, 1973). Since surface lattices are located on the outer surface of the cell, and since their absence does not noticeably affect the bacteria, it is not possible to correlate these structures with such a function. The periodic structure that seems to be deeply buried in the envelope of the gram-negative bacteria in close contact with the peptidoglycan structure may have played such a role. However, *E. coli* mutants that lack protein I (and other major proteins except the lipoprotein) in the outer membrane exist and do not show any gross defect (Henning and Haller, 1975). Therefore it is also not possible to assign a shape-determinig function to Rosenbush's protein. At present, the functional significance of this protein in cellular processes is unknown.

Finally, the occurrence of a deep, regular, or crystalline structure in the walls of gram-negative bacteria should not be regarded as being established beyond a doubt. Thus for example, examination of sections of *E. coli* cell wall like that shown in Fig. 27b (in which case treatment with SDS was carried out at room temperature on the electron microscopic grid; see figure legend) failed to reveal any crystallinity in the layer even after employment of optical diffraction (M. Bayer; personal communication).

2.7. The wall lipopolysaccharide of gram-negative enteric bacteria

The lipopolysaccharides, major somatic antigens (O-antigens) of enteric bacteria, are very powerful toxic agents (endotoxins). Their chemical structure, physical structure, synthesis, and genetic control have been studied extensively and numerous reviews have appeared on these topics (Nikaido, 1968, 1970, 1973; Luderitz et al., 1966, 1968, 1971, 1973, 1974; Osborn, 1969, 1971; Osborn et al., 1971, 1974; Rothfield and Romeo, 1971; Mäkelä and Stocker, 1969).

2.7.1. Isolation

Although the lipopolysaccharides are localized exclusively in the outer membrane, the most common procedure used for their isolation is phenol-water extraction of the intact cells (45% aqueous phenol at 65–68°C). Cooling the extracts causes partitioning of the extracted substances between phenol layer and aqueous layer. Usually the lipopolysaccharide occurs in the aqueous layer in the form of large aggregates. It can be recovered and purified by ultracentrifugation.

Lipopolysaccharides produced by certain mutants have lost part or most of their hydrophilic polysaccharide moiety. They are hydrophobic. In these cases an extraction method with phenol-petroleum ether-chloroform has been developed that generally gives rise to homogeneous preparations (Galanos et al., 1969).

The lipopolysaccharides contain many ionic groups and thus they usually carry a mixture of various cations (including alkali, alkaline earths, and heavy metal ions) and amines such as spermine, spermidine, and putrescine. Further

purification can be achieved by electrodialysis. After such treatment, the lipopolysaccharides are acidic and water-insoluble. They can then be solubilized in a controlled manner by using any base.

2.7.2. Structure

Lipopolysaccharides are complexes of polysaccharide chains covalently linked to a unique glucosamine-containing lipid known as lipid A. An important feature of these molecules is the occurrence of three different regions (Fig. 28). Typically, the polysaccharide moiety is composed of two distinct parts, the so-called superficial O-antigen chains (region I) and the "core" (region II), which is characterized by two unique sugars in it, L-glycero-D-mannoheptose and 2-keto-3-deoxyoctonate or KDO (Fig. 29). Region II is, in turn, linked to lipid A or region III (Fig. 28). Detailed studies have been performed mainly on lipopolysaccharides of *Salmonella, E. coli* and *Shigella flexneri.* The following description is related to the lipopolysaccharide of *S. typhimurium* which is especially well known (Fig. 28) (Luderitz et al., 1973, 1974; Hämmerling et al., 1973).

Structural studies were made possible because of the isolation of various types of mutants that were defective at various stages of the lipopolysaccharide synthesis (Nikaido, 1968; Mäkelä and Stocker, 1969; Stocker and Mäkelä, 1971). However, the innermost part of the molecule, the KDO-lipid A portion, seems to be indispensable for the survival of the bacteria. So far, mutants defective in the

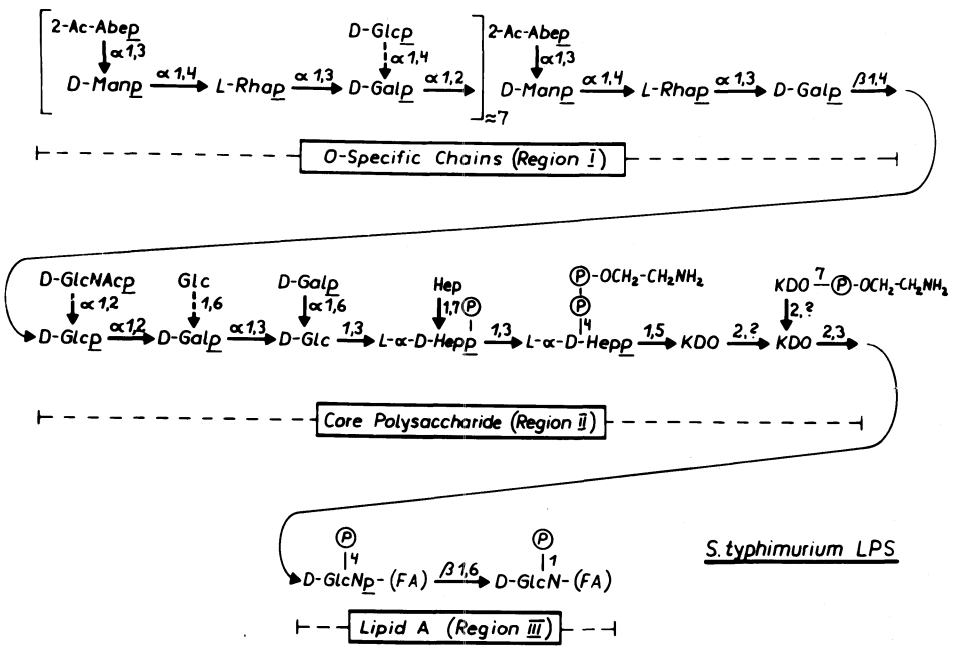


Fig. 28. Chemical structure of the lipopolysaccharide of *Salmonella typhimurium* S form. (Lüderitz et al., 1974). (Reprinted courtesy of Avicenum, Czechoslovak Medical Press, Prague.)

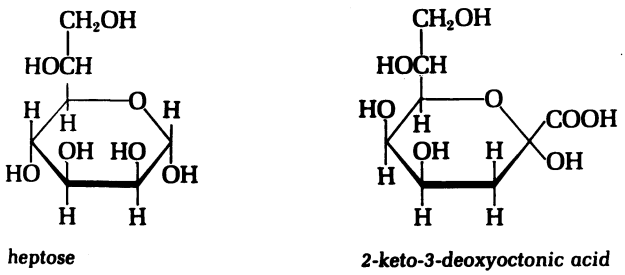


Fig. 29. Structures of the C_7 and C_8 constituents of the lipopolysaccharide core region.

synthesis of this portion (except conditional mutants) have never been isolated (Osborn et al., 1974). The O-side chain and the greatest part of the polysaccharide core are not indispensable. Many different mutants lacking these components to various extents are known. Wild-type organisms usually produce complete lipopolysaccharides with O-side chains. They form smooth colonies on solid media (S strains) and they react with anti-O-antibodies. Mutants without O-side chains grow as rough-surface colonies on solid media (R strains). They do not react with anti-O-antibodies. The smooth appearance of the S colonies is due presumably to the hydrophilic and water-retaining properties of the O-side chains which cover the surface of the cells. In addition to the S and R types, SR mutants are known that can be regarded as intermediates between the S and the R mutants. These SR mutants are defective in the polymerization of O-repeating units, so their lipopolysaccharides have very short unpolymerized O-side chain units (Naide et al., 1965; Mäkelä, 1966; Nikaido et al., 1966; Yuasa et al., 1970).

Lipid A (region III) at one end of the molecule is an unusual phospholipid because it contains no glycerol (Fig. 30) (Luderitz et al., 1974). Its backbone is a disaccharide of D-glucosamine (GlcN-β-1,6-GlcN) with the specific 3-D-hydroxymyristic acid in an amide linkage to the amino groups. The glucosamine disaccharide is substituted at position 3′ with the KDO trisaccharide of the core (region II) and at position 4′ and 1 with phosphate residues. These latter residues may be used to link 3 to 4 individual lipid A molecules through pyrophosphate bridges. The remaining hydroxyl groups of glucosamine (positions 6′, 3 and 4) are esterified by long-chain saturated fatty acids (lauric, palmitic, and 3-D-myristoxymyristic acid). Mild acid hydrolysis—for example, autolysis at 100°C of the acidic lipolysaccharide in water (pH 3–4)—cleaves the ketosidic linkage between KDO and lipid A and releases the polysaccharide-free lipid A as a water-insoluble compound. Neutralization produces a viscous and opalescent solution (Lüderitz et al., 1974). Lipid A is immunogenic, when administered in a suitable form, that is, incorporated into liposomes (Galanos et al., 1971) or exposed on bacterial cells (Lüderitz et al., 1973). Lipid A is also the endotoxic center of the lipopolysaccharides. This has been demonstrated from isolated lipid A solubilized by complexing it with proteins and other solubilizing carriers (Lüderitz et al., 1973).

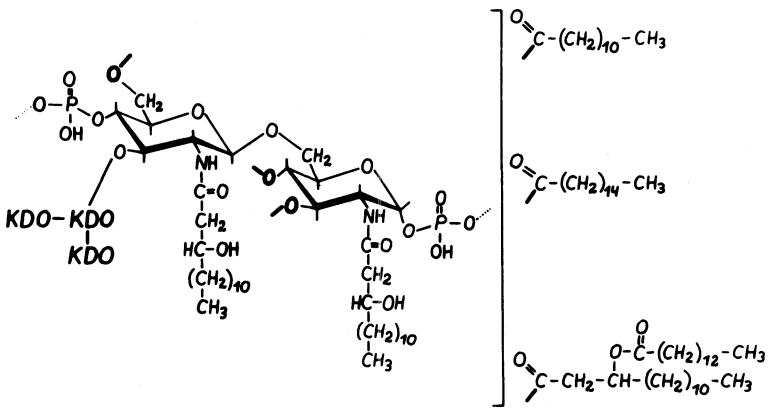


Fig. 30. Chemical structure of a *Salmonella* lipid A unit with an attached KDO trisaccharide. The three acyl residues shown are linked in an unknown distribution to the hydroxyl groups of the glucosamine residues. (Lüderitz et al., 1974). (Reprinted courtesy of Avicenum, Czechoslovak Medical Press, Prague.)

The O-specific polysaccharide (region I) at the other end of the molecule contains (in *S. typhimurium*) five sugar constituents (abequose, mannose, rhamnose, galactose, and glucose) that form the repeating, branched pentasaccharide units of one chain. The great variety of lipopolysaccharides that occur in the various strains, species, and bacterial groups is determined by the nature and linkages of the sugar constituents of region I. Often the chains are composed of repeating oligosaccharide units, as in *S. typhimurium*. These O-specific chains determine the serological specificity of the lipopolysaccharides and the species that contain them.

The core (region II) extends between the O-specific chains and lipid A. Heptose and KDO accumulate in its inner portion. Both residues occur as branched trisaccharides that are substituted by phosphate, phosphorylethanolamine, and pyrophosphorylethanolamine residues, causing an accumulation of charged groups in that part of the molecule. The outer portion of the core is more conventional and consists of a branched polysaccharide of glucose, galactose, and N-acetylglucosamine. Region II carries antigenic determinants that are hardly detectable in complete lipopolysaccharides but become immunologically active in mutants with defective lipopolysaccharides. The structure of region II is invariant within the various *Salmonella* serotypes. The core structures in other *Enterobacteriaceae,* however, may be different. Various distinct groups of *E. coli* with different core structures have been isolated by serological methods. Finally, in gram-negative bacteria other than the *Enterobacteriaceae,* the core structures may lack heptose or KDO, or even both (Jann et al., 1973). Core-defective R mutants in *Salmonella* are known (Fig. 31). The terminal sugar units are the main determinants responsible for the serological specificity of the corresponding lipopolysaccharides. All the R lipopolysaccharides, even those with very pro-

found defects such as the Re mutants, which lack heptose and contain only KDO and lipid A, are potent endotoxins—a finding consistent with the idea that lipid A is the endotoxic center of all lipopolysaccharides (Lüderitz et al., 1973).

2.7.3. Localization and arrangement

The lipopolysaccharides are asymmetrically distributed in the outer membrane with preferential localization on its outer face (Mühlradt and Golecki, 1975). Their surface density is relatively constant ($0.7–1.0 \times 10^5$ molecules per μm^2) and only about 25% of the cell surface is occupied by the lipid portion of the lipopolysaccharides (Mühlradt et al., 1974).

The lipopolysaccharides exhibit surface mobility. Lateral diffusion is low with a constant of about 3×10^{-13} cm^2/s (Mühlradt et al., 1974). This value is orders of magnitude lower than the diffusion constant values of phospholipids in most biological membranes ($D \approx 5 \times 10^{-9}$ cm^2/s) (Lee et al., 1973) and of animal cell-surface antigens ($D \approx 5 \times 10^{-11}$ cm^2/s) (Frye and Edidin, 1970). Transmembrane movement, flip-flop transition, can also occur but only in an area where the peptidoglycan layer is defective and the temperature is sufficiently high (25–37°C) to allow it (Mühlradt and Golecki, 1975). For example, by using ferritin-conjugated antibodies directed against the polysaccharide moiety of the lipopolysaccharide, the walls of *S. typhimurium* from which the peptidoglycan had been removed by lysozyme digestion at 0°C were shown to carry the label only on the outer face of the membrane. When removal of the peptidoglycan was performed at physiological temperatures (25–37°C), lipopolysaccharide was localized on both membrane faces.

Divalent cations are essential for integration of the lipopolysaccharide molecules into the outer membrane. The outer membrane is able to withstand treatment with nonionic detergent (Triton X-100) if enough Mg^{2+} ions are present (De Pamphilis and Adler, 1971; Schnaitman, 1971b), but treatment with EDTA in the absence of detergent causes the selective release of large amounts of lipopolysaccharide (Leive, 1965a; Leive et al., 1968). About 50% of the total lipopolysaccharide molecules are freed and the released material has an overall composition of 85 to 90% lipopolysaccharide, 5 to 10% protein and 5% phospholipid. Clearly, such a release is not a peeling apart of the outer membrane. Newly synthesized lipopolysaccharide molecules are essentially nonreleasable. Subsequently they become evenly distributed between releasable and nonreleasable fractions. Actually, the two fractions are in constant equilibrium (Levy and Leive, 1968). These phenomena, however, are not yet understood.

The lipopolysaccharide molecules play an important role in the permeability barrier of the outer membrane (section 2.9.). They exert a masking effect on various proteins localized on the outer cell surfaces (section 2.8.3.); yet agents such as colicin, phages, and antibodies directed against components other than the lipopolysaccharide can bind to and affect the bacterial cell surface. These observations suggest that the lipopolysaccharide molecules are not evenly distributed on the exterior of the cell. In this context, one may mention that according

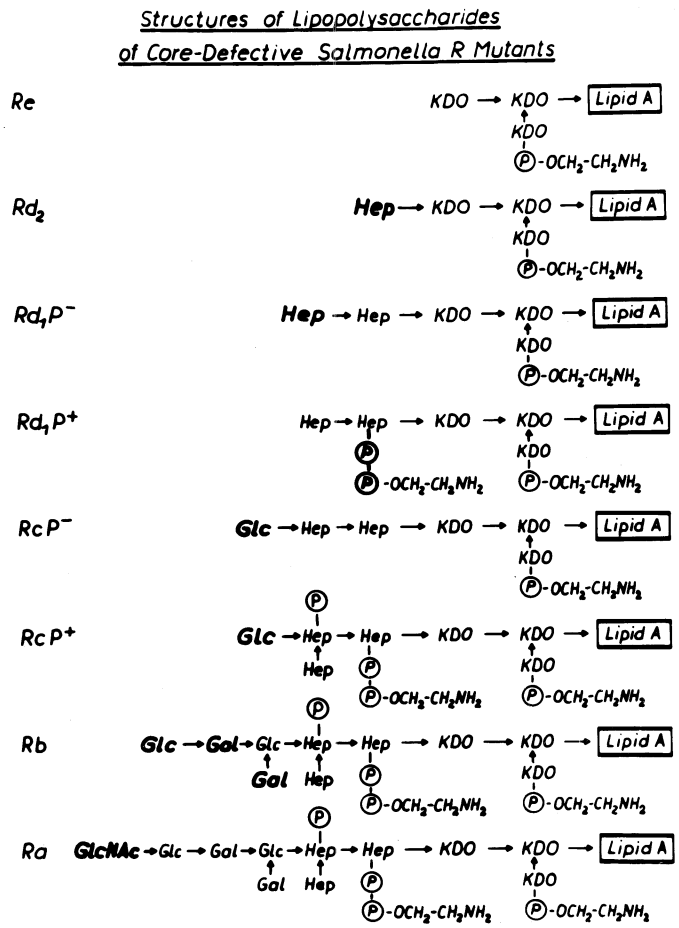


Fig. 31. Chemical structure of the defective lipopolysaccharides from R mutants of chemotype Ra through Re. (Lüderitz et al., 1974). (Reprinted courtesy of Avicenum, Czechoslovak Medical Press, Prague.)

to recent reports, lipopolysaccharide in its native state might occur in close association with protein. In at least one case (*E. coli* 0111-B$_4$) where mild detergent extraction was used, the lipopolysaccharide was quantitatively recovered as an apparent covalently linked polysaccharide-protein complex (Wu and Heath, 1973). It has also been hypothesized (Verkleij et al., 1977) that the many particles seen on the outer fracture face of the outer membrane by freeze fracturing electron microscopy of *E. coli* K12, would consist of lipopolysaccharide aggregates stabilized by divalent cations and complexed with protein and/or phospholipid.

The lipopolysaccharide molecules also exert a masking effect on the phospholipid head groups present in the outer membrane. Whole cells of *S.*

typhimurium were treated with phospholipase C (which should hydrolyze the phosphatidylethanolamine head groups) or with CNBr-activated dextran (which should undergo coupling with these groups). Results did not indicate the presence of any accessible head groups on the outer surface of strains producing lipopolysaccharides of S or R_c type. In contrast, with strains that produce less complete R_d or R_e lipopolysaccharides and reduced amounts of proteins, both methods showed the presence of exposed phosphatidylethanolamine head groups (Kamio and Nikaido, 1976). Resistance of phospholipids in whole cells of wild type of *E. coli* K12 to exogenous phospholipases was also attributed by van Alphen et al. (1977b) to shielding by some other outer membrane components. Proteins b and d, the heptose-bound glucose of lipopolysaccharide and divalent cations would be involved in the phenomenon.

2.7.4. Functions

Available data is limited: (1) Clearly, lipopolysaccharide is an important constituent of the outer permeability barrier of the cell. (2) The presence of at least the glycolipid found in the Re-mutants (Fig. 31) seems to be essential for the survival of the cell, but the function of this apparently indispensable component is not clear. It has been hypothesized that at least this portion of the lipopolysaccharide is needed for the proper assembly of the outer membrane. (3) Wild strains, as they are isolated, are always smooth and contain lipopolysaccharides with O-side chains. When kept as pure cultures, R mutants appear and sometimes overgrow the parent organism. The well-known *E. coli* K12, B, and C strains, for example, are rough strains. O-side chains enable pathogenic organisms to escape phagocytosis in host animals that do not possess the proper antibody. Complete lipopolysaccharides may play a similar role in nature, such as preventing phagocytosis by protozoa. (4) Finally, the lipopolysaccharides exhibit overall structural features which resemble those of the teichoic acids to some extent (see section 2.3.4.). These two types of polymers, though especially devised for gram-positives and gram-negatives, respectively, might, at least in part, be functionally related.

2.8. Protein arrangement in the outer membrane of Escherichia coli *and other gram-negative bacteria*

2.8.1. The membrane ghost

When treated with lysozyme (which destroys the peptidoglycan), *E. coli* cells plasmolyzed in sucrose become osmotically fragile. Nevertheless they retain their rod shape, and it is only upon sucrose dilution that the cells round up into spheres (Birdsell and Cota-Robles, 1967). Sphere formation is due to osmotic pressure and/or surface tension phenomena. These phenomena can be avoided by disrupting the membrane or by making it leaky. Under these conditions, the ghosts obtained remain rod-shaped upon sucrose dilution (Henning et al., 1973a). Rod-shaped ghosts can be obtained by treating sucrose-plasmolyzed *E.*

coli cells with 1% Triton X-100 or Brij 18 to disrupt the plasma membrane. Subsequent steps usually include treatments with urea, trypsin, $MgSO_4$, and lysozyme (Henning et al., 1973a).

Rod-shaped ghosts (Fig. 32) consist almost exclusively of an outer membrane unit (and sometimes of inner membranous material; this material, however, does not contribute to shape maintenance). Peptidoglycan is lacking (Henning et al., 1973a; Haller and Henning, 1974; Schweizer et al., 1975). The phospholipid (20–30%) of the ghosts is almost entirely phosphatidylethanolamine. Very little phosphatidylglycerol and only traces of cardiolipin are present.

The rod shape of the ghosts is resistant to pronase, trypsin, chymotrypsin, EDTA, Triton X-100 and Brij 58; however, SDS (1%) causes complete solubilization. Ghosts delipidated by chloroform methanol extraction retain their rod shape (Henning et al., 1973b). They are distorted and exhibit few local breaks but are otherwise indistinguishable from normal ghosts. They contain about 70% protein and a substantial but imprecisely known amount of lipopolysaccharide (25–30%). The proteins have been classified into major and minor proteins (for a recent review, see Henning, 1975).

2.8.2. The major ghost proteins

Trypsinized ghosts from *E. coli* contain four major proteins which, on the basis of SDS polyacrylamide gel electrophoresis, exhibit the following molecular weights (Henning et al., 1973a,b; Garten and Henning, 1974): protein I (mol. wt. ≈ 38,000), protein II (mol. wt. ≈ 28,000), protein III (mol. wt. ≈ 17,000) and protein IV (mol wt. ≈ 7,000–10,000). Protein I is Rosenbush's protein (section 2.6), but protein I consists of two main components (Ia and Ib), both of which contain six separable isoelectric species (Schmitges and Henning, 1976). These components are almost identical in their primary structure, and represent essentially the same polypeptide. Protein II, probably an homogeneous polypeptide, is derived from a protein II* (mol wt. about 33,000) by the action of trypsin, which is usually used during preparation of the ghosts. Evidence for the structural gene of protein II* has been obtained (Henning et al., 1976; Datta et al., 1976). Protein III occurs in small amounts that vary from preparation to preparation. Protein IV is Braun's lipoprotein (section 2.5.). The molar ratio of polypeptides I, II*, and IV in nontrypsinized ghosts about 1:1:8–10 (Henning et al., 1973b). Henning's nomenclature is used for the major proteins. Protein I is probably identical with protein I of Schnaitman (1974), with protein A_1 of Bragg and Hou (1972), with protein B of Koplow and Goldfine (1974), and with the Salmonella 35K protein of Ames (1974). Protein II is probably identical with protein B* of Reithmeier and Bragg (1974). Protein II* is probably identical with protein d of Lugtenberg et al. (1975), with protein 3a of Schnaitman (1974) and with protein B of Reithmeier and Bragg (1974).

As previously mentioned (section 2.5 and 2.6), preparative isolation of all major membrane proteins has been accomplished (Hindennach and Henning, 1975). The yields (per 200 g cell paste) were ≈ 120 mg protein I, ≈ 110 mg protein II*, ≈

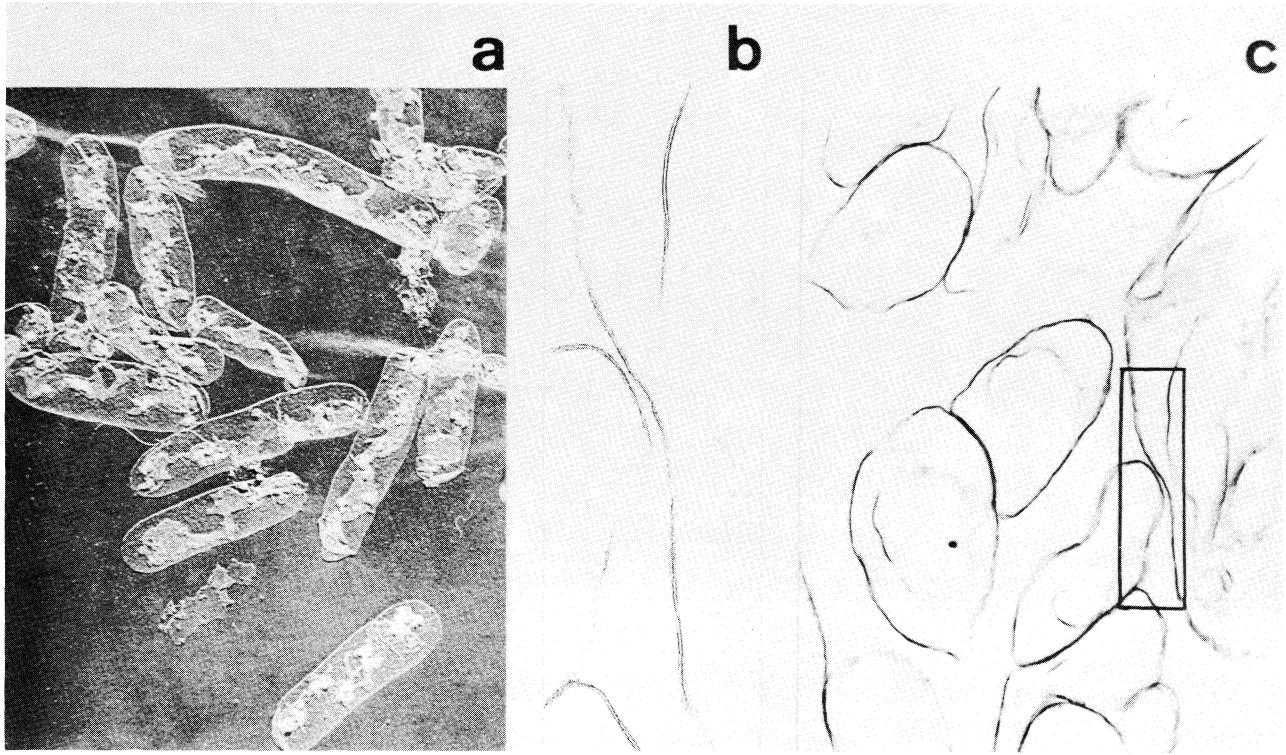


Fig. 32. Shadowed and sectioned ghosts of *Escherichia coli* K12. Insert b is a 2.5-fold further magnification of c. Each bar represents 1 μm. (Henning et al., 1973a.) (Reprinted courtesy of the Federation of European Biochemical Societies.)

50 mg protein III, and 30 mg protein IV. A comparison of all major proteins can be found in recent papers by Garten et al. (1975a,b).

Cross-linking of nontrypsinized ghosts with bifunctional diimidoester cross-linking reagents, with spanning distances from about 0.3 nm to 1.8 nm, produces a macromolecule that shows the size and shape of the cell and resists boiling SDS (Haller and Henning, 1974). Before treatment with SDS, these sacs contain 60 to 70% protein. After treatment, they consist of virtually pure protein. Ammonolysis of the cross-linked sacs shows that the four major proteins (I to IV) found in normally prepared ghosts have been cross-linked. Since reagents spanning only about 0.3 nm can stabilize the ghosts into covalently closed structures, it is clear that these proteins are very densely packed. Ghost membranes do not fit the fluid mosaic membrane structure model of Singer and Nicolson (1972) but rather constitute an extreme case of the types of models discussed by Capaldi and Green (1972). The fact that protein III always occurs in small and variable amounts and that the molar ratio of polypeptides I, II*, and IV is 1:1:8–10 suggests that cross-linking requires repeating I-II* sequences. Protein II* involvement in cross-linking poses an interesting problem because trypsinized ghosts cannot be cross-linked with the diimidoesters (but they can be cross-linked with glutaraldehyde) (Haller and Henning, 1974). This may be due to the removal of about 30% of the amino acid residues of protein II* by trypsin action, causing its transformation into protein II.

Because of the noncovalent, long-range ordered structure exhibited by the ghost membrane, the outer membrane might play a role in shape determination and the major membrane proteins might belong to a self-assembly system participating in or responsible for cellular shape. However, this is not the case (Henning and Haller, 1975). Rod-shaped mutants that lack one or another major protein, or all of them but lipoprotein IV, did not show any apparent gross fundamental defects when isolated. In the mutants where all major proteins (except protein IV) were lacking, no detectable increase occurred in any other single protein species and the integrity of the outer membrane seemed to be perfectly maintained. It is not known whether the space thus made available has been "filled up" with phospholipid or other components. Whatever the situation, it seems obvious that an assembly of the major proteins is not required for the generation or for the shape maintenance of the outer membrane.

Ghosts morphologically identical to those of *E. coli* were prepared from *Serratia marcescens, Proteus mirabilis, Salmonella typhimurium, Spirillum serpens* and *Pseudomonas aeruginosa* (Henning et al., 1973a,b). Sometimes the procedure had to be modified slightly; that used for *S. serpens* (Schweizer et al., 1975) includes treatment with 2 M guanidine hydrochloride to remove the protein cell surface lattice. Except for *Pseudomonas aeruginosa,* the band patterns in SDS gels are identical (*Salmonella*) or at least similar to that of *E. coli.* The ghosts membranes of *S. serpens* contain three major proteins (I:mol. wt., 40,000, II:mol. wt., 26,000, and III:mol. wt., 21,000). In contrast to *E. coli,* all the proteases used cause desintegration of the *S. serpens* ghosts (Schweizer et al., 1975).

2.8.3. The minor ghost proteins (Haller et al., 1975)

One step in the preparation of the *E. coli* ghosts is trypsin treatment, which removes a heterogeneous set of proteins and transforms protein II* into protein II. It has been found that most of these proteins in nontrypsinized ghosts can be covalently linked to each other by oxidation with $CuSO_4$-O-phenanthroline or ferricyanide-ferrocene. The resulting rod-shaped "oxidation containers" apparently held together by disulfide bridges are virtually pure protein. They retain the size and shape of the cell even when treated with hot SDS. When reduced, they are soluble in SDS and contain a set of about 30 different polypeptide chains. Remarkably, the four major proteins (I, II, III, and IV) are not among the "oxidation proteins."

The oxidation proteins are mainly, if not exclusively, localized on the outer surface of the ghosts, and the same localization and arrangement was found in cells. The trypsin-sensitive portion of protein II* is also exposed at the outer surface of the membrane. Protein II* was found together with lipopolysaccharide to stoichiometrically inhibit the F-pilus mediated conjugation. All data available indicate that protein II* acts as a receptor in conjugation (Schweizer and Henning, 1977; van Alphen et al., 1977a). Asymmetry of membrane architecture, especially with regard to localization of proteins and phospholipids, exists in various systems (Blaurock and Stoeckenius, 1971; Fukui et al., 1971; Bretscher, 1972; Zwaal et al., 1973). The outer membrane of *E. coli* (and other gram-negative bacteria) may be one of the most extreme cases of asymmetric protein distribution. At present, however, a model cannot be drawn for the outer membrane since the arrangements of phospholipid and lipopolysaccharide are unknown. Interestingly, it has been shown with *Salmonella typhimurium* that native cells of deep rough mutants, but not the wild-type cells, can be oxidized with ferrocene, suggesting that in the wild type the lipopolysaccharides protect the external face of the outer membrane from the required lipophilic oxidant (Haller et al., 1975).

2.8.4. Outer membrane proteins involved in DNA replication and cell morphogenesis

Recent findings have shed new light on the involvement of some of the outer membrane proteins in DNA synthesis, cell elongation, and septation. Thus, protein G (mol. wt., 15,000) appears to be a structural protein that is specific for cell elongation (James, 1975); protein D (mol. wt. 80,000) appears to be synthesized just before initiation of a round of DNA synthesis (Gudas et al, 1976); nalidixic acid, which inhibits DNA synthesis, causes the inhibition of the envelope protein D and stimulates the synthesis of a protein X (Gudas and Pardee, 1976; Gudas et al., 1976); another protein (mol. wt., 76,000) is only synthesized during a brief period near the time of bacterial division (Churchward and Holland, 1976). R plasmid RM98 apparently mediates an increase of this 76,000 dalton protein in the outer membrane (and the specific loss of a 36,000 dalton protein (Iyer et al., 1976). The relationships of outer membrane proteins and cell cycle are discussed in this volume by Daneo-Moore and Shockman.

2.9. The outer membrane as a permeability barrier

The outer membrane behaves as a fine molecular sieving barrier on the surface of the gram-negative bacteria with an exclusion limit (for uncharged oligosaccharides) of about 650 to 900 molecular weight (Nikaido, 1973, 1976; Nakae and Nikaido, 1976; Dacad and Nikaido, 1976). This limit is much smaller than that of the multilayered peptidoglycan-containing walls of gram-positive bacteria. An upper limit of 100,000 has been estimated in *B. megaterium* (Scherrer and Gerhardt, 1971). According to more recent estimates, the apparent exclusion threshold of the walls of *B. subtilis* and *B. licheniformis* would correspond to molecules with a diffusion radius of 2.5 nm (i.e., molecular weight ca. 70,000). Isolated, resealed outer membrane vesicles can be penetrated by saccharides with molecular weights higher than 1,000 (Nakae and Nikaido, 1976). This penetration seems to occur through "cracks" that are different from the physiological "pores" and are probably due to incorrect resealing. Such resealing may be a reflection of the high degree of protein-to-protein interaction that characterizes the outer membrane (section 2.8.). Since phospholipid bilayers are essentially impermeable to sugars larger than pentoses, and since mixed bilayers of both phospholipid and lipopolysaccharide have similar permeability properties, proteins are the best candidates responsible for outer membrane permeability. Reconstruction of sucrose-permeable vesicles from lipopolysaccharide, phospholipids, and proteins of the outer membrane may lead to the final identification of the permeability-conferring protein (Nakae and Nikaido, 1976). A protein complex participating in selective membrane permeability has been isolated. The active fractions contained three major protein species and the Braun lipoprotein was not one of them (Nakae, 1976). Peptidoglycan-associated outer membrane proteins were also isolated and compared. They would form, or be part of hydrophilic channels through the outer membrane (Lugtenberg et al., 1977). One of them, protein c, acts as a specific phage receptor in *E. coli* (Verhoef et al., 1977). A gene *meo* A, resonsible for the lack of this protein in some *coli* strains was mapped at 48 min on the linkage map (Verhoef et al., 1977).

Whereas hydrophobic substances mainly penetrate by dissolving into the hydrocarbon interior of the outer membrane (Nikaido, 1976), hydrophilic molecules that are too large to cross the pores must also pass through the outer membrane to support cell growth. Thus, other permeation mechanisms must exist. Recent studies on transport of Fe^{3+} ions have revealed complex functional interplay between specific receptor sites on the outer membrane, the active transport systems of the plasma membrane (Kaback, 1973), and specific binding proteins in the periplasmic space (i.e., the region between the outer and plasma membranes) (Rosen and Heppel, 1973).

E. coli K12 has a low affinity system for iron uptake that satisfies the cell requirement under conditions where a high concentration of Fe^{3+} ions is present in the medium (Frost and Rosenberg, 1973). It has also three high affinity systems through which Fe^{3+} ions are taken up either as complexes with citrate (Frost and Rosenberg, 1973; Hancock et al., 1976), enterochelin (Langman et al., 1972), or ferrichrome (Pollack et al., 1970; Luckey et al., 1972). The protein

specified by the *ton* A gene (at 3 min on the linkage map) is involved in the ferrichrome-dependent iron uptake. This protein has been isolated by Braun and coworkers (Braun and Wolff, 1973; Braun et al., 1973; Braun and Hantke, 1974; Braun et al., 1974b; Hantke and Braun, 1975a,b). It consists of a single polypeptide (mol. wt., 85,000 daltons), and is localized in the outer membrane, where it also serves as receptor for phages T_5, T_1 and $\phi 80$ and for colicin M; whereas T_5 binds irreversibly to the protein and releases its DNA, productive infection by T_1 and $\phi 80$ and effective peptidoglycan degradation (and spheroplast formation) by colicin M require in addition, another function, the *ton* B function. This function has not yet been identified biochemically but it may be a "binding protein" for the three types of iron complexes, serving as a shuttle between the outer membrane and the plasma membrane (Boos, 1974). Finally a *feu* function (specified by a gene at 60 min), also localized in the outer membrane, is involved in the enterochehin-dependent iron uptake (Hantke and Braun, 1975a). It might be regarded as an analog of the *ton* A protein for the ferrichrome transport. Thus it appears that the transport of the large iron complexes (molecular weight higher than 700) through the outer membrane depends on the *feu* and the *ton* A functions (specific receptor sites) together with the *ton* B function (periplasmic binding protein).

Other receptor-dependent high affinity transport systems also exist in the outer membrane of *E. coli* such as the receptor protein for vitamin B_{12}, which is also utilized by the E colicins and by phage BF_{23} (Di Masi et al., 1973) and the maltose receptor system which is also utilized by phage λ (Schwartz, 1975, 1976; Schwartz and Le Minor, 1975; Schwartz et al., 1976; Szmelcman and Hofnung, 1975; Szmelcman et al., 1976; Ryter et al., 1975). Both vitamin B_{12} and maltose systems depend on additional periplasmic "binding proteins." These findings suggest that many, if not all, of the receptor proteins evolved as high-affinity binding components of the outer membrane. Initially devised to take up substrates present in the cell environment, these proteins were alternatively utilized by toxic agents such as phages and colicins in order to enter the bacteria.

Because of the presence of the outer membrane, many enzymes that are typically extracellular in gram-positive bacteria are "periplasmic" in the gram-negative bacteria (Heppel, 1967). The retention of these enzymes also poses an interesting problem because it may involve a complex mechanism. Indeed, mutants are known that are leaky (i.e., able to degrade substrates in the medium by an enzyme that is normally periplasmic) for only one or two periplasmic enzymes but not for all of them (Lopes et al., 1972). The molecular basis of this selective mechanism is not understood. At least one of these "leaky" mutants is known to possess an altered outer membrane protein (Lopes et al., 1972).

2.10. Adhesion zones between outer and plasma membranes

Sections of plasmolyzed *E. coli, Salmonella,* and other gram-negative bacteria reveal that not all the plasma membrane is retracted from the more rigid outer

membrane, but that both the outer and plasma membranes remain attached to each other at many distinct areas (Cota-Robles, 1963; Bayer, 1968, 1974, 1975). There are about 200 to 400 such adhesion zones per growing cell, each of them measuring approximately 25 to 50 nm in cross-sections (Fig. 33). Following deplasmolysis both membranes return to their normal positions more or less in contact with each other. Adhesion zones are not seen in stationary cells. They may play a part in cell growth and the question then arises of what function(s) could be ascribed to them.

2.10.1. Export sites of lipopolysaccharides

Bayer (1974), Mühlradt et al. (1973), and Kulpa and Leive (1976) have shown independently that these adhesion sites are physical channels through which the lipopolysaccharides are translocated across the peptidoglycan layer to the outer membrane once they have been synthesized at the plasma membrane (see section 3.4.4.).

2.10.2. Phage adsorption

Phage adsorption is not a "function." However, most cell surface structures of gram-negative bacteria are utilized by phages in order to inject their nucleic acid inside the bacterium. This is true for the following: (1) the F-pili that provide the adsorption sites for two classes of pili-specific phages, the filamentous DNA phages that adsorb to the tip and the icosahedral RNA phages that adsorb along the length of the pili (Curtis et al., 1969), (2) the flagellae on which phages such as PBS1 (Raimondo et al., 1968) and X*l* (Meynell and Aufreiter, 1969) are initially attached, and (3) the outer membrane, which shows a wide spectrum of phage receptors assimilated with lipopolysaccharides and proteins (Bayer, 1974). Regardless of the strain used, localization of the phage receptors by the plasmolysis technique reveals that at low multiplicities of infection (maximum 100) phages are positioned preferentially over the adhesion zones. These phages are frequently seen with empty heads suggesting that release of the viral mucleic acid also occurs at these zones. Finally many, if not all, of the adhesion sites are able to adsorb all phages tested. Since a limited number of adhesion sites are available and many different phages can be adsorbed on these sites, each site appears to have more than one type of receptor.

2.11. Conclusions

Recently the gap that existed between the different conceptual approaches to bacterial wall structure, those of the electron microscopists, biochemists and physiologists, began to narrow and a unified molecular structural view emerged.

Walls exist in both gram-positive and gram-negative bacteria to preserve the plasma membrane against osmotic disruption. Gram-positive bacteria probably represent a primitive stage of evolution. They have solved the problem by synthesizing a vast amount of solid, multilayered peptidoglycan which, depending on

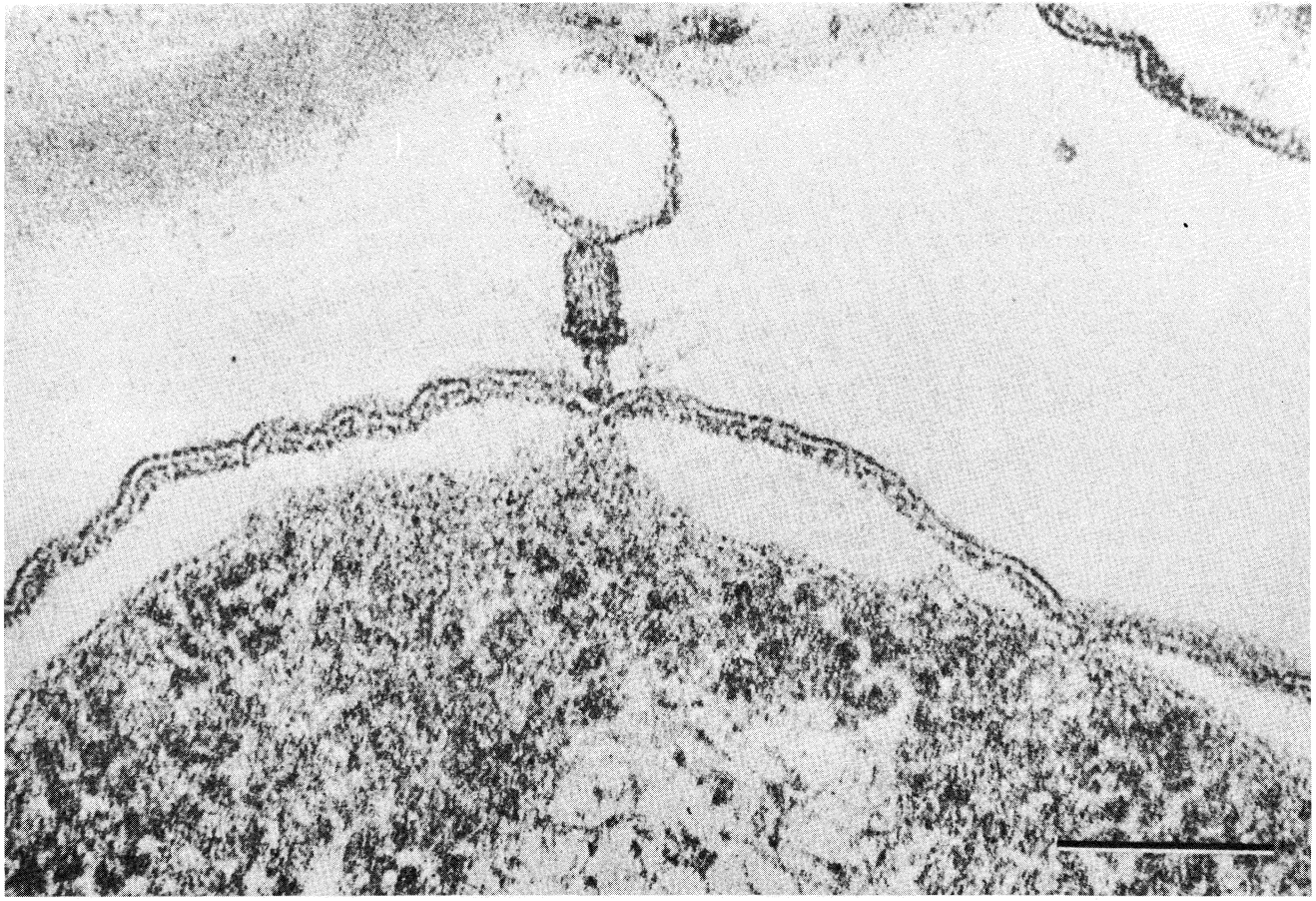


Fig. 33. High-resolution micrograph of ultrathin section of plasmolyzed *E. coli* B cell with an adsorbed bacteriophage T_2. (Bayer, 1975.) (Reprinted courtesy of Plenum Press, New York.) 288,000 ×

the species, exhibits many different structures. The thick walls of the gram-positive bacteria are highly permeable and do not pose any serious problems to the penetration of substances necessary for cell growth. Highly specialized acidic polymers such as teichoic acids, teichuronic acids, and anionic polysaccharides are embedded in the solid peptidoglycan matrix. They are covalently attached to the peptidoglycan or anchored in the underlying plasma membrane. At least a portion of them is localized very close to or over the cell surface. These non-peptidoglycan components seem to be involved in magnesium uptake, possibly functioning as a highly selective cation-exchange system between the exterior of the cell and the plasma membrane. Other important metabolic roles have also been assigned to these polymers, especially in regulating the autolytic activities of the cell.

Gram-negative bacteria have reached a much more advanced stage. They adopted the same simple structure for their peptidoglycan which, in addition, evolved as a monolayer. This simplification was due to the development of an outer membrane which, in addition, gave these bacteria the appreciable advantage of having an additional fine, molecular sieving barrier on their surface.

Both the peptidoglycan and the outer membrane contribute to the mechanical strength of the cell envelope. The plasma membrane, the peptidoglycan layer, and the outer membrane are closely associated. The peptidoglycan and the outer membrane are covalently attached to each other through part of the lipoprotein molecules. The plasma membrane and the outer membrane are also connected to each other through adhesion zones that extend through the peptidoglycan layer and are sites of export for at least some of the specific constituents (lipopolysaccharides) of the outer membrane. The plasma membrane and the outer membrane are also connected to each other through adhesion zones that extend through the peptidoglycan layer and are sites of export for at least some of the specific constituents (lipopolysaccharides) of the outer membrane. Many molecules required for cell growth cannot cross the pores of the outer membrane; consequently, specialized permeation mechanisms evolved in which "periplasmic" binding proteins serve as shuttles between specific receptor sites in the outer membrane and active transport systems in the plasma membrane. The outer membrane is a unique structure. Not only does it contain components that are not found elsewhere in nature but it also exhibits a noncovalent long-range ordered structure which does not fit the classical fluid mosaic model of membrane structure and may represent the most extreme case of asymmetric protein distribution ever encountered.

Finally, teichoic acids and anionic polysaccharides in gram-positive bacteria, lipopolysaccharides and proteins in the gram-negative bacteria—in fact virtually all the specialized components of the bacterial envelope that are distributed on the exterior of the cells and were devised to fulfill important physiological functions—are also utilized by toxic agents such as phages and/or colicins to enter the cell.

3. Biosynthesis and mode of assembly of bacterial walls

Bacterial walls are exocellular structures, and wall biosynthesis is therefore a three-stage process, each stage occurring at a different cell site: in the cytoplasm, on the membrane, and within the wall. Biosynthesis will be described for the four following main wall components: peptidoglycan, teichoic acid, lipoprotein, and lipopolysaccharide. The wall polymers are assembled from cytoplasmic precursors on specific "centers" that are functionally specialized portions of the plasma membrane. Each of these assembly centers contains (1) an "acceptor," or "carrier" to which the various units of a given wall polymer are transferred from the proper cytoplasmic precursors; (2) a series of transferases that function in a specific sequence so that the transfer reactions are catalyzed in the right order; and (3) phospholipids, of defined structure, which may act as ligands or affectors for some enzymes of the assembly centers. Normal wall or cell envelope synthesis requires an intimate functional integration of the various assembly centers involved.

3.1. Biosynthesis of peptidoglycan

3.1.1. The strategy

Contributions from the research groups of Strominger, Park, and Neuhaus, respectively, were essential in unraveling the processes involved in peptidoglycan synthesis (Strominger, 1970). In essence, the peptidoglycan precursors made on a uridylic acid cytoplasmic carrier (stage 1) are transferred from uridylic acid to the membrane assembly centers (stage 2), and from these to the expanding wall peptidoglycan where attachment occurs (stage 3). At some point during stage 3 peptidoglycan becomes insoluble.

Reactions of stage 1 lead to the synthesis of two nucleotide precursors: UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-L-alanine-D-glutamyl-L-R_3-D-alanyl-D-alanine (Fig. 34). The peptide moiety of the latter is not a tetrapeptide as usually found in the completed wall peptidoglycans, but a pentapeptide that ends in a D-Ala-D-Ala sequence. Reactions of stage 1 are catalyzed by soluble, cytoplasmic enzymes.

Reactions of stage 2 ensure the assembly of the activated precursors and their transfer through the plasma membrane. The membrane carrier of the assembly centers involved in peptidoglycan synthesis is a C_{55}-polyisoprenoid alcohol phosphate (undecaprenyl phosphate). At the end of the process the uridylic acid carriers are regenerated into the cytoplasm pool. Reactions of both stage 1 and stage 2 are linked. They function as two integrated cycles: the nucleotide cycle and the lipid cycle, respectively.

Reactions of stage 3 occur on the outer face of the plasma membrane. They are also catalyzed by membrane-bound enzymes, and result in the insolubilization of the newly synthesized soluble peptidoglycan by incorporation into the preexisting wall peptidoglycan. One of the reactions involved is a transpeptida-

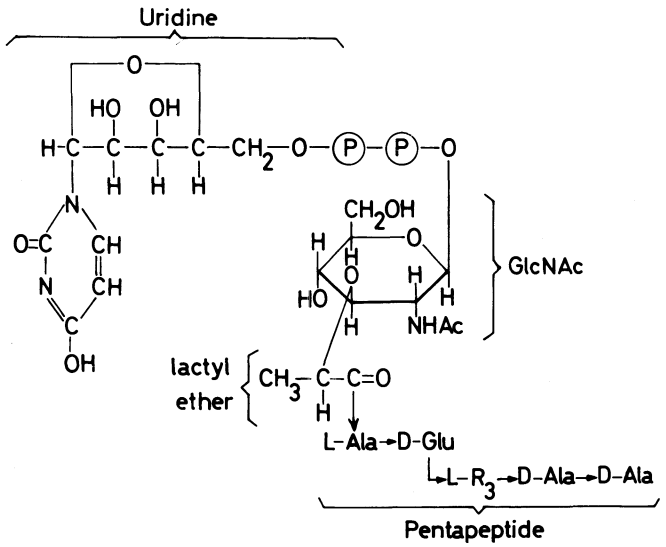


Fig. 34. The completed nucleotide precursor UDP-N-acetylmuramylpentapeptide.

tion. It causes peptide cross-linking between "new" polymers as well as between the "new" and the "old" polymers with concomitant release of one of the C-terminal D-alanine residues originally present in the nucleotide precursor. The released D-alanine can be reutilized by the cell either for peptidoglycan synthesis or for other processes by means of a specific transport system. In *E. coli* the transport for D-alanine, L-alanine, and glycine has been resolved. It is mediated by at least two enzymes. The system for D-alanine and glycine are related and differ from that of L-alanine (Neuhaus et al., 1972).

Figure 35 shows the main reactions involved in the synthesis of a type I peptidoglycan where additional amino acid residues do not occur in the interpeptide bridging.

In all peptidoglycans other than type I, the interpeptide bridges are not direct but consist of one or several additional amino acid residues (section 2.1.2.3.). The incorporation of those bridging residues onto the pentapeptide units L-Ala-D-Glu-L-R_3-D-Ala-D-Ala takes place during stage 1 or, usually during stage 2. Mechanisms also exist that are responsible for the variations encountered in both the glycan and the peptide moieties of the peptidoglycans of various bacterial species. These variations are of minor importance in terms of the general structure of the polymer (section 2.1.2.). In most cases the mechanisms involved are poorly understood or unknown. It is known, however, that in *S. aureus,* conversion of D-glutamic acid into D-isoglutamine (Fig. 11) is accomplished by amidation in the presence of ATP and ammonium ions at the level of the lipid cycle (Siewert and Strominger, 1968). Similarly, in *M. lysodeikticus,* substitution of the same α-carboxyl group of D-glutamic acid by a glycine residue (Fig. 12) is carried out in the presence of ATP at the lipid cycle level (Katz et al., 1967).

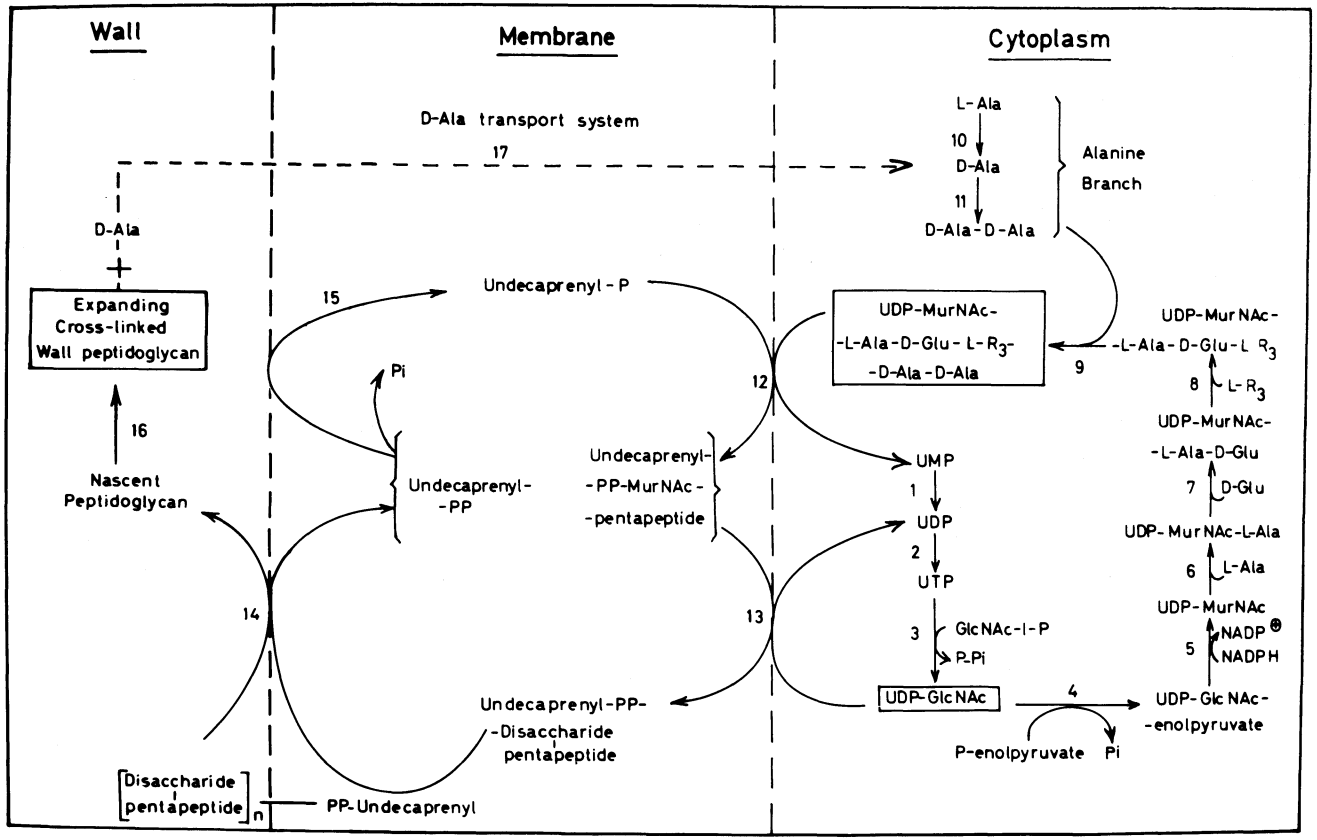


Fig. 35. Schematic representation of the biosynthesis of a peptidoglycan of type I. The three stages—cytoplasmic, membrane-bound, and wall-bound—are separated by the dashed vertical lines. GlcNAc = N-acetylglucosamine; MurNAc = N-acetylmuramic acid; L-R_3 = for example, *meso*-diaminopimelic acid. For peptidoglycans of types II, III and IV, interpeptide chains are incorporated at the cytoplasmic level or, more often, at the lipid cycle (see text). Structure of the undecaprenyl-PP is given in Fig. 36.

3.1.2. The membrane-bound lipid carrier

The lipid carrier of the peptidoglycan assembly centers in *S. aureus* has been isolated to a very high degree of purity, allowing its structure to be ascertained by mass spectrometry (Higashi et al., 1967, 1970b). It is a C_{55}-isoprenoid alcohol containing 11 isoprene units, with the chain ending in an alcoholic function (Fig. 36). In order to be functional, the carrier must be phosphorylated. In *S. aureus,* phosphorylation is achieved by a membrane-bound isoprenyl alcohol phosphokinase in the presence of ATP (Higashi et al., 1970a; Higashi and Strominger, 1970). The active enzyme is composed of a protein fraction and a phospholipid (phosphatidylglycerol). The enzyme-lipid complex is soluble and stable in several organic solvents. The protein fraction is inactive and insoluble both in water and in organic solvents. Its activity and solubility in organic solvents can be restored by addition of the phospholipid fraction. In other organisms, phosphorylation of the lipid carrier is achieved by a different route. In *Salmonella newington,* a particulate enzyme catalyzes the synthesis of C_{55}-lipid pyrophosphate from farnesyl pyrophosphate and isopentenyl pyrophosphate which, in turn, is derived from mevalonate (Christenson et al., 1969; Rothfield and Romeo, 1971). The C_{55}-lipid pyrophosphate is then dephosphorylated into inorganic phosphate and active C_{55}-isoprenoid alcohol phosphate carrier. Bacitracin prevents the action of the membrane-bound pyrophosphatase.

Lipid intermediates are key compounds in the biosynthesis of complex polysaccharides in prokaryotes and in eukaryotes. They have been well characterized in the biosynthesis of the peptidoglycans and the O-antigen lipopolysaccharides (section 3.4.). Their participation has also been demonstrated in the synthesis of a capsular polysaccharide (Troy et al., 1971), the membrane-bound lipomannan of *M. lysodeikticus* (section 3.2.3.), and mannolipids in *Mycobacteria* (Takayama and Goldman, 1970). These lipid intermediates generally contain a pyrophosphate or sometimes a monophosphate linkage between carbohydrate and lipid moieties, and in bacteria the latter is the polyisoprenoid alcohol with 55 carbon atoms. Similar structures are also involved in the synthesis of the yeast mannan (Tanner et al., 1971; Sentandreu and Lampen, 1971) and of mammalian glycolipids and glycoproteins (Oliver et al., 1975; also see the chapter by Cook in this volume). Mammalian glycolipids consist of a moiety with the properties of dolichol (or a similar compound), linked through a phosphate group to a carbohydrate residue. Dolichol is a mixture of very long-chain polyprenols

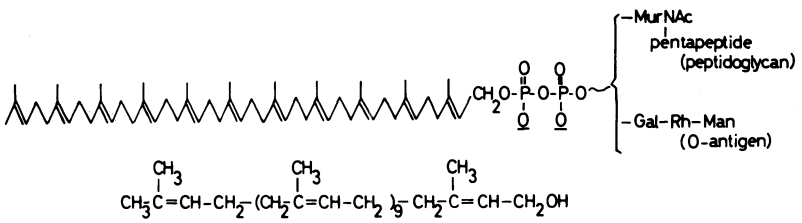


Fig. 36. The C_{55}-isoprenoid alcohol charged with either peptidoglycan or O-antigen precursors (see section 3.4.3.) *via* a pyrophosphate bridge.

(C_{80}–C_{105}) that are widely distributed in animal cells (Butterworth and Hemming, 1968).

3.1.3. Stage 1. The nucleotide cycle (Figure 35)

3.1.3.1. Synthesis of the precursor UDP-N-acetylglucosamine. UTP, produced by phosphorylation of UMP and UDP by ATP (reactions 1 and 2), reacts with α-N-acetylglucosamine-1-phosphate to yield inorganic pyrophosphate and the nucleotide precursor UDP-N-acetylglucosamine (reaction 3). The reaction is catalyzed by UDP-N-acetylglucosamine pyrophosphorylase and is analogous to reactions leading to UDP-glucose and other compounds of this type.

3.1.3.2. Synthesis of the precursor UDP-N-acetylmuramylpentapeptide. The next two reactions of the cycle consist of the transfer of a three-carbon unit from 2-phosphoenolpyruvate with formation of the pyruvate enol ether of UDP-N-acetyl-D-glucosamine (reaction 4), followed by reduction into UDP-N-acetylmuramic acid (reaction 5). The reactions are catalyzed by a transferase and a UDP-N-acetylglucosaminepyruvate reductase, respectively (Gunetileke and Anwar, 1968). The transferase is the specific target of the antibiotic phosphonomycin. The mode of action of phosphonomycin at the molecular level is known (Kahan et al., 1974).

Completion of the UDP-N-acetylmuramylpentapeptide is carried out by stepwise addition of L-alanine (reaction 6), D-glutamic acid (reaction 7), the L-R_3 residue (reaction 8) and, finally, a preformed D-alanyl-D-alanine dipeptide (reaction 9). Each step is catalyzed by a separate enzyme requiring ATP and either Mg^{2+} or Mn^{2+}. The synthesis is not directed by a nucleic acid template, and the constancy of peptidoglycan composition depends on the specificity of the relevant enzymes for their substrates (Strominger, 1970).

The synthesis of D-Ala-D-Ala (reactions 10 and 11) and its addition to the nucleotide tripeptide UDP-N-acetylmuramyl-L-Ala-D-Glu⌋L-R_3 (reaction 9) are carried out by three enzymes: (1) alanine racemase; (2) D-Ala:D-Ala ligase (ADP) or D-Ala-D-Ala synthetase; and (3) UDP-N-acetylmuramyl-L-Ala-D-Glu⌋L-R_3; D-Ala D-Ala ligase (ADP) or D-Ala-D-Ala adding enzyme (Neuhaus et al., 1972). Several D-alanine antagonists are known, and among them D-cycloserine is of great importance. It behaves as a competitive inhibitor of both the racemase (with either D-Ala or L-Ala as substrate) and the synthetase, with K_i values considerably smaller than the K_m values for the substrates, D-cycloserine does not inhibit the D-Ala-DAla-adding enzyme.

In *E. coli* and *B. subtilis,* the alanine racemase is inhibited by both D- and L-cycloserine (Neuhaus et al., 1972; Johnston et al., 1966), whereas in *S. aureus* (Roze and Strominger, 1966) and *S. faecalis* (Lynch and Neuhaus, 1966; Wood and Gunsalus, 1951), this enzyme is inhibited by D- but not L-cycloserine. It has been suggested that some racemases have a single site for both D- and L-alanine and that others have two distinct binding sites. Racemases may also occur in two forms, one of which would bind L-alanine and the other D-alanine (Johnston and Diven, 1969).

The synthetase

$$(2\ \text{D-Ala} + \text{ATP} \xrightarrow[K^+]{Mg^{2+}} \text{D-Ala-D-Ala} + \text{ADP} + \text{Pi})$$

is inhibited by D-cycloserine, D-Ala-D-Ala and analogs of D-Ala-D-Ala (Neuhaus et al., 1972). Inhibition is specific for dipeptides. Additions to the N-terminal residue decrease the inhibitory activity (e.g., D-norvalyl-D-alanine is not an inhibitor), whereas additions to the C-terminal residue sometimes enhance the effectiveness of the peptides as inhibitors (e.g., D-alanyl-D-norvaline is a better inhibitor than D-Ala-D-Ala). Multiple binding sites on the enzyme exist, and it is believed that the function of these sites is to control the rate of dipeptide formation.

The specificity pattern of the D-Ala-D-Ala-adding enzyme for the addition of D-Ala-D-Ala analogs has also been studied. Remarkably, the substrate profile of this enzyme complements that of the D-Ala-D-Ala synthetase (Neuhaus and Struve, 1965). For example, the synthetase can incorporate D-norvaline (and other D-amino acids) in the C-terminal end of a dipeptide but not in the N-terminal end. The adding enzyme, in turn, binds and is inhibited by D-norvalyl-D-alanine but not by D-alanyl-D-norvaline. This combination of specificities also contributes to the relative accuracy of synthesis of the complete nucleotide precursor. Further, it accounts for the growth-inhibitory action exerted by some D-amino acids. Modification of peptidoglycan structure as a result of growth in unbalanced media has been investigated by Schleifer (Schleifer, 1975; Schleifer et al., 1976).

3.1.3.3. Incorporation of interpeptide chains (not shown in Figure 35). In *Lactobacillus viridescens,* the interpeptide chain consists of one L-alanine residue. L-alanine is incorporated to the ϵ-amino group of L-lysine of the nucleotide UDP-N-acetylmuramylpentapeptide by transfer from L-alanyl-tRNA (Plapp and Strominger, 1970). The reaction is catalyzed by a soluble enzyme of poor specificity that is able to transfer not only L-alanine but also, although less efficiently, L-serine, L-cysteine, and probably glycine from the corresponding tRNAs. *L. viridescens* is unusual. In all other bacteria examined (section 3.1.4.3.), incorporation of the interpeptide bridges occurs at the lipid intermediate level (Strominger, 1970).

3.1.3.4. Regulation. Osmotically fragile temperature-sensitive mutants of *E. coli* K12 impaired at the level of UDP-N-acetylglucosamine enolpyruvate reductase (proposed symbol : MurB), L-alanine-adding enzyme (MurC), *meso*-diaminopimelic acid-adding enzyme (MurE) or D-Ala-D-Ala adding enzyme (MurF), respectively, were isolated (Matsuzawa et al., 1969; Lutgenberg, 1971; Lutgenberg and de Haan, 1971; Wijsman, 1972). Mutants impaired at the level of UDP-N-acetylglucosamine-2-phosphoenolpyruvate transferase (MurA) and of the D-Glu-adding enzyme (MurD) have not yet been obtained. The MurC, E and F genes are localized extremely close to each other (at 1–1.5 min of the chromosome map). They might form or be part of an operon. The MurB gene is

located at 77 minutes. Mutants with impaired L-Ala racemase (alr, at 3 min) and D-Ala-D-Ala ligase (ddl, at 17 min) activities have also been obtained.

D-cycloserine causes an enormous accumulation of UDP-N-acetylmuramyl-tripeptide in *E. coli* K12, but penicillin, which inhibits one stage 3 reaction of biosynthesis (section 3.1.5.), fails to cause the accumulation of UDP-N-acetylmuramylpentapeptide. Similarly, mutants altered in one of the membrane-bound enzymes that could accumulate this nucleotide apparently do not exist. These observations suggest that in *E. coli* K12 UDP-N-acetylmuramylpentapeptide may regulate its own biosynthesis by feedback inhibition (Lutgenberg, 1971). This phenomenon is certainly not frequent. *S. aureus* lacks this type of regulation, since penicillin causes a large accumulation of nucleotides. This accumulation was one of the key observations leading to our current knowledge of penicillin's mode of action (Wise and Park, 1965; Tipper and Strominger, 1965).

The amount of D-Ala-D-Ala available for condensation with UDP-N-acetylmuramyl-L-Ala-D-Glu⌐L-R_3 is submitted to complex regulation. In addition to the occurrence of several product-binding sites on the synthetase the phenomenon also includes (1) regulation of the size of the intracellular pools of D- and L-alanine (Wargel et al., 1971); (2) competition for available D-alanine by other reactions that utilize it (e.g., teichoic acid synthesis); (3) D-Ala is a specific inducer of L-alanine dehydrogenase (in *B. subtilis*) and hence limits the amount of L-alanine available to the racemase (Berberich et al., 1968). In other bacteria (*Pseudomonas aeruginosa*), D-Ala induces a D-amino acid dehydrogenase which limits the level of D-Ala (Marshall and Sokatch, 1968); (4) in *E. coli,* the synthesis of the racemase is repressed by high concentrations of alanine (Neuhaus et al., 1972); and (5) the kinetic properties of the racemases in both *E. coli* and *S. faecalis* are such that the intracellular L-Ala pool must be larger than the D-Ala pool for the reaction to proceed in the L→D direction with the required velocity (Neuhaus et al., 1972).

3.1.4. Stage 2. The lipid cycle in the assembly centers of the plasma membrane (Figure 35)

The first two reactions consist of translocating the peptidoglycan precursors from the hydrophilic environment of the cytoplasm to the hydrophobic environment of the membrane. This interchange of carriers leads to the synthesis of disaccharide-peptide β-1,4-N-acetylglucosaminyl-N-acetylmuramylpeptide units, a mechanism that ultimately is responsible for the alternating sequence of N-acetylglucosamine and N-acetylmuramic acid in the glycan strands of the completed wall peptidoglycan (Neuhaus, 1971).

3.1.4.1. Transphosphorylation (Neuhaus, 1971). First, phospho-N-acetylmuramylpentapeptide is translocated from UDP-N-acetylmuramyl-pentapeptide to the membrane undecaprenyl phosphate carrier (reaction 12). Undecaprenyl-PP-N-acetylmuramylpentapeptide is formed and UMP is generated. The translocase has been solubilized and partially purified (Heydanek and Neuhaus, 1969). The transphosphorylation reaction proceeds with-

out loss of energy: UDP-MurNAc (pentapeptide) + undecaprenyl-P $\rightleftharpoons$ undecaprenyl-PP-MurNAc (pentapeptide) + UMP. The equilibrium may be reached by either route and the K_{eq} value is about 0.25. Forward and reverse reactions require Mg^{2+}. Potassium ions stimulate the forward reaction and UMP inhibits it. An enzyme-P-N-acetylmuramyl (pentapeptide) complex is transitorily formed (with release of UMP; exchange reaction), and then P-N-acetylmuramyl (pentapeptide) is transferred from the enzyme to the undecaprenyl phosphate carrier (transfer reaction). The translocase has a high specificity for the uracyl moiety. Fluorouracil causes great accumulation of FUDP-N-acetylmuramyl-pentapeptide. A C-terminal D-Ala-D-Ala sequence is also an essential feature of the substrate. The specificity profiles of both exchange and transfer reactions complement those of the earlier reactions in the synthesis.

3.1.4.2. Transglycosylation (Chatterjee and Park, 1964; Meadow et al. 1964; Struve and Neuhaus, 1965). N-acetylglucosamine is, in its turn, translocated from UDP-N-acetylglucosamine to undecaprenyl-PP-N-acetylmuramylpentapeptide (reaction 13). Undecaprenyl-PP-disaccharidepentapeptide is formed and UDP is generated. This transglycosylation differs from the preceding reaction in that only N-acetylglucosamine and not the terminal phosphate of UDP is transferred to the lipid intermediate. Tunicamycin prevents formation of disaccharide lipid intermediate by blocking the transfer of N-acetylglucosamine onto undecaprenyl muramylpentapeptide pyrophosphate (Bettinger and Young, 1975).

3.1.4.3. Incorporation of interpeptide chains (not shown in Figure 35). With most type II peptidoglycans (section 2.1.2.3.), incorporation of interpeptide chains occurs within the peptidoglycan assembly centers by extension of the length of the side chain of the L-R_3 residue of the lipid intermediate through substitution of the ω-amino group by one or several amino acid residues. These transfers are catalyzed by particulate enzymes and differ completely from mRNA-coded protein synthesis on ribosomes.

tRNAs participate in the incorporation of glycine and L-amino acid residues. Thus for example, in *S. aureus,* five glycine residues from glycyl-tRNA are sequentially added to the ϵ-amino group of L-lysine on the undecaprenyl-PP-disaccharide-peptide intermediate (Matsuhashi et al., 1967; Kamiryo and Matsuhashi, 1969; Thorndike and Park, 1969). Four species of tRNA-Gly exist in *S. aureus* and support glycine incorporation in the peptidoglycan. Three of them participate in template-directed polypeptide synthesis. The fourth one is apparently peptidoglycan specific; it may be a unique gene product. A similar mechanism occurs in *Arthrobacter crystallopoietes* for the incorporation of one L-alanine residue (from L-alanyl-tRNA) (Roberts et al., 1968) and in *Staphylococcus epidermidis* for the incorporation of 3 glycine and 2 L-serine residues (from glycyl-tRNA and L-seryl-tRNA) (Petit et al., 1968).

Contrary to the aforementioned examples, the interpeptide bridges in *S. faecalis* and *L. casei* consist of a single iso-asparaginyl residue that has the D-configuration. In these cases tRNAs do not participate in the incorporation

(Staudenbauer and Strominger, 1972; Staudenbauer et al., 1972). D-aspartic acid is activated as β-D-aspartyl-phosphate by a membrane-bound enzyme and is then transferred to the ε-amino group of L-lysine of the lipid intermediate. Finally, amidation of the α-carboxyl group is achieved in the presence of NH_3 and ATP.

3.1.4.4. Transfer of disaccharide peptide (Figure 35). The transfer of the disaccharide peptide units from the undecaprenyl-PP-disaccharide-peptide to an appropriate acceptor generates undecaprenyl pyrophosphate (reaction 14) which, in turn, is dephosphorylated by a membrane-bound pyrophosphatase (reaction 15). This latter reaction yields inorganic phosphate and the initial C_{55}-isoprenoid alcohol phosphate carrier which can begin a new cycle. As previously mentioned, bacitracin is an inhibitor of the pyrophosphatase (Siewert and Strominger, 1967; Siewert, 1969; Storm, 1974). Transfer of the disaccharide peptide from the lipid carrier and wall peptidoglycan expansion involves two reactions: one insures elongation of the glycan chains by transglycosylation and the other insures peptide cross-linking by transpeptidation. These reactions are part of stage 3 of biosynthesis and are discussed in the following section.

3.1.5. Stage 3. Wall peptidoglycan expansion

The first attempts to study the mechanism of wall peptidoglycan expansion were made by Mirelman, Sharon, and coworkers (Mirelman and Sharon, 1972; Mirelman et al., 1972). *S. aureus* and *M. lysodeikticus* were used as models for these studies (section 3.1.5.3.). Since additional amino acid residues occur in the interpeptide bridges in these bacteria (Figs. 11 and 12), the processes involve a complex series of steps. Since bacilli that have a type I peptigolycan with a direct cross-linkage between the peptide units (Fig. 10) are simpler models, studies with these bacteria will be described first.

3.1.5.1. Synthesis of nascent peptidoglycan in bacilli. The elegant studies of Ward and Perkins (1973, 1974) on the biosynthesis of peptidoglycan by cell-free membrane preparations from a poorly lytic mutant of *Bacillus licheniformis* have shown that chains consisting of multiple disaccharide peptide units grow by addition of the new disaccharide peptide units at the reducing terminal of the lengthening chain. In the process the reducing terminal end (i.e., N-acetylmuramic acid) of the growing chain is transferred from its link with the membrane to the nonreducing terminal residue (i.e., N-acetylglucosamine) of the new disaccharide peptide unit which is itself linked to the membrane. In their original papers Ward and Perkins interpreted their observations as polymerization of nascent glycan chains on the undecaprenyl phosphate carrier (Fig. 37), but the nature of the acceptor was not characterized. Regardless of the exact mechanism, the nascent peptidoglycan thus formed emerges on the exterior of the plasma membrane, which has actually been seen using isolated protoplasts of *B. megaterium* (Fritz-James, 1974) and of *B. licheniformis* (Elliott et al., 1975a,b).

The synthesis of an uncross-linked, linear peptidoglycan by purified plasma

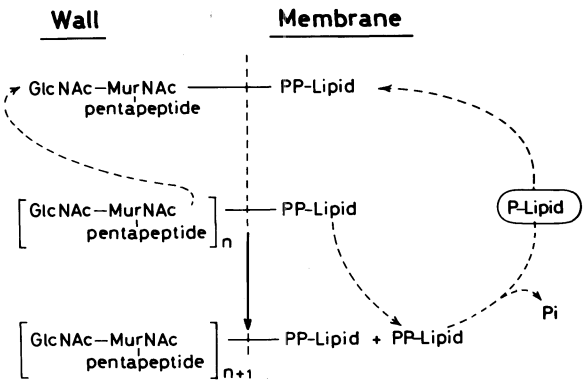


Fig. 37. Synthesis of the nascent peptidoglycan on the undecaprenyl phosphate carrier. Elongation of the glycan chain by transglycosylation on the lipid intermediate. The reaction shows the addition of a new disaccharide peptide unit.

membranes depends strictly on the presence of exogenously added UDP-N-acetylglucosamine and UDP-N-acetylmuramylpentapeptide precursors. It is entirely prevented by the presence of lysozyme (which degrades the polymer as it is being formed) but is unaffected by penicillin.

3.1.5.2. Insolubilization of nascent peptidoglycan by attachment to preexisting wall peptidoglycan and by autopolymerization in bacilli. The attachment of the nascent peptidoglycan to the preexisting wall peptidoglycan in the same poorly lytic mutant of *B. licheniformis* was studied using a cell-free, wall plus membrane preparation (Ward, 1974). Attachment is achieved by transpeptidation (Fig. 38). In this reaction, the penultimate C-terminal D-alanine of a pentapeptide unit acting as donor is transferred to the proper N-terminal group of another peptide unit acting as acceptor. Interpeptide bonds are formed in the absence of exogenous energy and equivalent amounts of D-alanine residues are liberated from the donor peptides (Tipper and Strominger, 1968). The reaction is catalyzed by a membrane-bound transpeptidase and is inhibited by the β-lactam antibiotics (penicillins and cephalosporins) (Wise and Park, 1965; Tipper and Strominger, 1965).

The direction of the transpeptidation reaction with the wall plus membrane preparation from *B. licheniformis* was established by using a nucleotide precursor UDP-N-acetylmuramylpentapeptide in which the free amino group of *meso*-diaminopimelic acid (i.e., the potential acceptor group for transpeptidation) had been blocked by an [^{14}C]acetyl group (Ward and Perkins, 1974). Incorporation of the resulting acetylated nascent peptidoglycan in the wall material occurred, demonstrating that the nascent peptidoglycan must act as donor (through its C-terminal D-Ala-D-Ala sequences) and that the preexisting peptidoglycan must act as acceptor (through the amino group located on the D-center of *meso*-diaminopimelic acid). In vivo studies confirmed the overall process, that attach-

ment of the nascent peptidoglycan is mainly performed by transpeptidation whereas addition of newly synthesized material to the glycan strands of the preexisting wall peptidoglycan by transglycosylation could be at best a minor pathway of peptidoglycan synthesis in *B. licheniformis*.

The system involved in peptide crosslinking contains more than one single enzyme and consists of at least two antagonistic activities. In addition to the transpeptidase activity which catalyses peptide bound formation, bacteria also contain a DD-carboxypeptidase activity which simply hydrolyses the C-terminal D-Ala-D-Ala sequences of pentapeptide units without performing any transfer reaction. As the transpeptidase is sensitive to penicillin, so too is the DD-carboxypeptidase activity. The DD-carboxypeptidase activity is probably there to limit the number of pentapeptide units made available to the transpeptidase activity and therefore, to control the extent of peptide crosslinking. The specific inhibition of the DD-carboxypeptidase activity in *B. subtilis* and *B. stearothermophilus* does not cause detectable damage to the cell (Blumberg and Strominger, 1971; Yocum et al., 1974), suggesting that the transpeptidase is the enzyme that is physiologically important.

Cells incubated in the presence of β-lactam antibiotics released uncross-linked peptidoglycan material in the medium (Tynecka and Ward, 1975). A similar observation was made with *Brevibacterium divaricatum* (Keglevic et al., 1975). Again, these observations showed that glycan elongation on the plasma membrane by transglycosylation was insensitive to these antibiotics, whereas attachment of the nascent peptidoglycan to the preexisting wall peptidoglycan was inhibited under these conditions.

Quantitation of the nascent peptidoglycan attachment by the wall plus membrane preparation of *B. licheniformis* showed that the acetylated nascent peptidoglycan was utilized less efficiently than the natural compound, only about 23% as much being incorporated under identical conditions. This reduced degree of cross-linking could suggest that with the natural precursor not only attachment to the "old" wall peptidoglycan occurred but many crosslinks were also formed between the new chains themselves (Fig. 38), a process that was precluded by the use of the acetylated precursor. This assumption is probably correct, as shown by the remarkable study by Rogers and coworkers (Elliott et al., 1975a,b) on the reversion of protoplasts of *B. licheniformis* (MH-1 and 6346His$^-$) to normally rod-shaped bacilli. In liquid suspension growing protoplasts excrete the soluble, nascent peptidoglycan they produce. Obviously, at some stage of the extension, detachment from the carrier must occur. The mechanism of this reaction is not known. When incubated on the surface of a medium containing 2.5% agar, however, protoplasts of *B. licheniformis* can reverse successfully to normal bacilli. Hence insolubilization of the nascent peptidoglycan by peptide cross-linking (and incorporation of the other wall polymers synthesized simultaneously) can be achieved by cells that completely lack preexisting walls at the start of the process. The operation, however, is difficult and immobilization of the protoplasts by attachment to the agar in a medium of low fluidity is necessary.

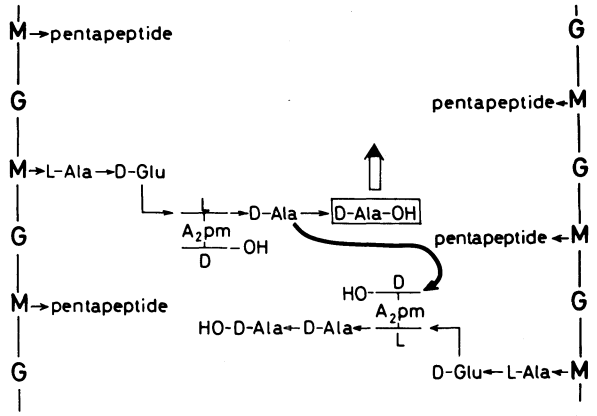
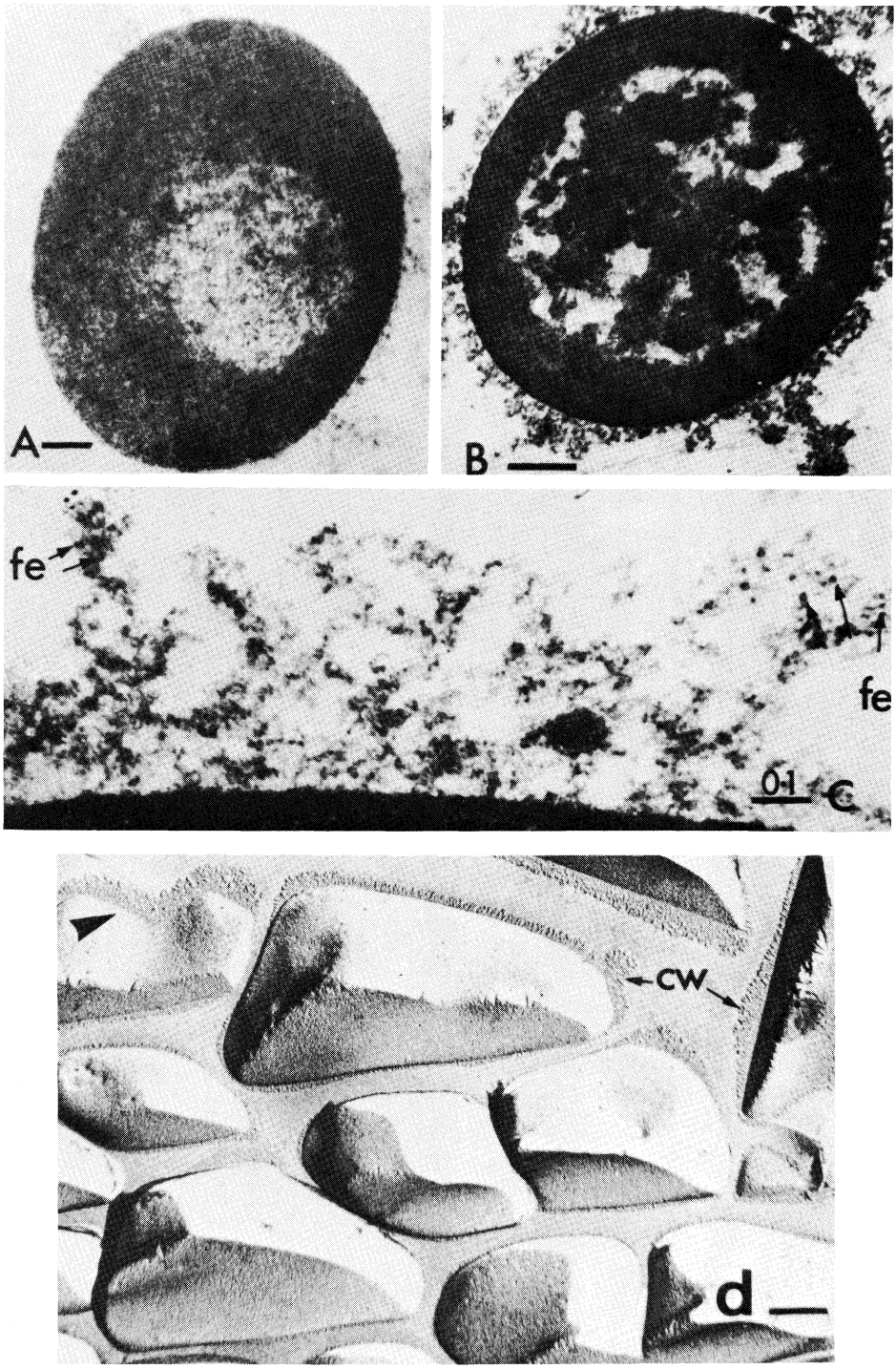


Fig. 38. Formation of a cross-linkage by transpeptidation between two nascent strands of peptidoglycan (adapted from Perkins et al., 1975). In the process, one pentapeptide serves as peptide donor (through the C-terminal D-Ala-D-Ala sequence) and another pentapeptide serves as peptide acceptor (through the N-terminal group of the L-R_3 residue; here a *meso*-diaminopimelic acid residue). Attachment of the nascent peptidoglycan to the preexisting wall peptidoglycan is also catalyzed by transpeptidation. (See text for details.)

In the experiments reported here (Elliott et al., 1975a,b) protoplasts were incubated on the surface of a medium containing 2.5% agar, N-acetyl [^{14}C]glucosamine to measure peptidoglycan synthesis, [2-^{3}H]glycerol to measure teichoic acid synthesis, and [^{3}H]tryptophan to measure protein synthesis. Early stages of peptidoglycan synthesis were visualized by use of ferritin-conjugated antibody. Cells still attached to the agar were also studied by freeze-etching, and cells scraped off the agar were used for biochemical work. A study of the sequence of events occurring during reversion of protoplasts to bacilli showed that at early stages the glycan chains were very short and the peptides poorly cross-linked. Reaction with ferritin-labeled peptidoglycan antibody showed patches of long, thin, flexible fibrils emerging from large, misshapen membranous bodies. Subsequently, the surface became covered increasingly with fibrils emerging from an increasing number of points, and after several hours a layer of loosely organized material was seen surrounding the cells using osmium fixatives or freeze-etching (Fig. 39). At this stage, however, the cells still rounded up when removed from the agar and suspended in isoosmolal solutions. As reversion proceeded, cross-linking reached its maximal value before the glycan chains reached their normal, final average length. After complete reversion, the wall material was rod-shaped and identical to that of the original bacilli. It contained peptidoglycan, teichoic acid, and teichuronic acid in about the same proportions. During the process, soluble peptidoglycan was found in the reversion medium in amounts that decreased as the reversion proceeded. Soluble products, however, were not formed by reverting protoplasts of an autolysin-deficient mutant.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Reversion of fused protoplasts to the bacillary form has opened a new tool in bacterial genetics. While transformation, conjugation, transduction, sexduction and transfection are unidirectional processes transferring DNA, and only DNA, from a donor into a recipient bacterium, fusion occurring in mixed populations of protoplasts derived from two parental strains which are both nutritionally-complementing and polyauxotrophic, followed by wall regeneration can lead to the formation of prototrophic bacteria. Recently such a process was applied successfully to *B. subtilis* by Schaeffer et al. (1976) and to *B. megaterium* by Fodor and Alföldi (1976).

B. megaterium expands its wall peptidoglycan in a way similar, if not identical, to that described for *B. licheniformis* (Reynolds, 1971; Schrader et al., 1974; Schrader and Fan, 1974; Taku et al., 1975; Fuchs-Cleveland and Gilvary, 1976). Isolated membranes catalyzed glycan chain elongation and also partial attachment of these nascent chains to each other by transpeptidation. Partially cross-linked peptidoglycan not attached to preexisting wall peptidoglycan and exhibiting an average molecular weight greater than 6×10^6 was shown to be formed (Schrader et al., 1974). Since peptidoglycans of smaller molecular weights were obtained in the presence of penicillin, transpeptidation was thus partially responsible for the large size of the product.

Toluenized cells of *B. megaterium,* where wall and membrane remain in close contact, catalyzed both peptidoglycan synthesis and its attachment to preexisting wall peptidoglycan by cross-linking (Schrader and Fan, 1974). Toluene treatment seems to preserve remarkably the cross-linking machinery of the plasma membrane. Extraction with LiCl of the toluene-treated cells resulted in a greatly diminished capability of peptidoglycan synthesis. Recovery of the original activity could be obtained by readding the LiCl solubilized proteins to the deficient cells (Taku et al., 1975). One of these protein factors, a glycoprotein with a molecular weight of about 42,000 to 52,000 was purified 124-fold (Taku and Fan, 1976a,b). At present, the nature and exact physiological role of these protein factors in peptidoglycan synthesis are still unknown.

In vivo studies of the peptide cross-linking reaction in *B. megaterium* and its sensitivity to penicillin were performed by Fordham and Gilvarg (1974). One interesting result showed that in the absence of penicillin the reaction continued for many minutes after incorporation of precursor subunits into peptidoglycan.

3.1.5.3. Wall peptidoglycan expansion gram-positive cocci. Synthesis of a nascent

Fig. 39. Reversion of protoplasts of *Bacillus licheniformis.* Sections of reverting protoplasts after 6 h of incubation: (a) Treated first with unconjugated antibody against peptidoglycan followed by reaction with ferritin-conjugated antibody, showing absence of ferritin particles. (b) Low magnification view of section after direct reaction with ferritin-labeled antibody. (c) Appearance examined at higher magnification (fe, ferritin particles). (a,b and c) Cells removed from the agar and suspended in liquid medium. Bars represent 0.5 μm unless otherwise indicated. (d) Freeze-etched cells examined in situ, showing the shape and the fringe of variable length around the cells (CW). Arrow shows the direction of shadowing of replicas. The bar represents 0.5 μm. (Elliott et al., 1975.) (Reprinted courtesy of the American Society for Microbiology.)

peptidoglycan and its attachment to the "old" wall peptidoglycan by transpeptidation are also essential features of peptidoglycan expansion in gram-positive cocci. Crude wall preparations of *S. aureus* containing strongly associated membrane fragments were utilized both as enzyme sources and as acceptors for peptidoglycan synthesis (Mirelman and Sharon, 1972). These preparations also contain tRNA and glycyl-tRNA synthetase, so that they incorporate glycine into the preexisting wall peptidoglycan. Both cross-linking of the newly synthesized peptidoglycan by transpeptidation and glycine incorporation in the wall fraction were strongly inhibited by penicillin.

Autoplasts of *Streptococcus faecalis* 9790 produced by the action of the native autolytic N-acetylmuramidase were grown in the presence of heat-inactivated cell walls (to bind autolytic enzyme and thus reduce the level of peptidoglycan hydrolysis) and of tetracycline (to inhibit further autolysin synthesis) (Rosenthal and Shockman, 1975). Autoplasts secreted soluble, infrequently peptide cross-linked glycan chains. Transfer of this material to the exogenously added walls did not occur, suggesting that a close connection between wall and membrane is necessary for the wall to act as acceptor in transpeptidation. The transpeptidation reaction was studied with whole cells continuously labeled during exponential growth. The pattern of distribution of monomers, dimers, and trimers suggested that cross-linking between peptides was not a random condensation process but proceeded by a monomer addition mechanism (Dezélée and Shockman, 1975; Oldmixon et al., 1976).

In some cocci, transpeptidation is not the only mechanism of precursor incorporation. Thus in *M. lysodeikticus,* some incorporation of precursors in the wall peptidoglycan still occurs in the presence of penicillin at concentrations that completely block transpeptidation. The amounts of precursor attached under these conditions is about 30% of that incorporated in the absence of penicillin. Presumably, this penicillin-insensitive incorporation results from elongation of the preexisting wall glycan strands by transglycosylation (Fig. 40). With *M. lysodeikticus,* additional complications also arise because of its complex interpeptide bridges (Fig. 12). The linear uncross-linked glycan strands secreted by this bacterium (Mirelman et al., 1972; 1974a,b,c; 1975; Mirelman and Bracha, 1974) in the presence of penicillin contain about 150 disaccharide units. About half of these strands are substituted by the hexapeptide L-Ala-D-Glu(Gly) ┌ L-Lys-D-Ala-D-Ala, whereas the others have their muramic acid residues with free carboxyl group. Free hexapeptide is also secreted in the medium and occurs in amounts equivalent to that of the unsubstituted muramic acid residues. The secretion of this free peptide must be the result of a penicillin-insensitive amidase acting on the nascent peptidoglycan. The latter enzyme is presumably involved in the translocation of some peptide units from their muramyl residues into a bridging position. Incorporation of the liberated peptide units in that position requires formation of D-Ala-L-Ala linkages. Evidence has been obtained that this link is probably also made by a transpeptidation reaction in which the C-terminal D-alanine of one hexapeptide that is attached to the glycan is transferred to the N-terminal L-alanine of a free hexapeptide. Amidase action and

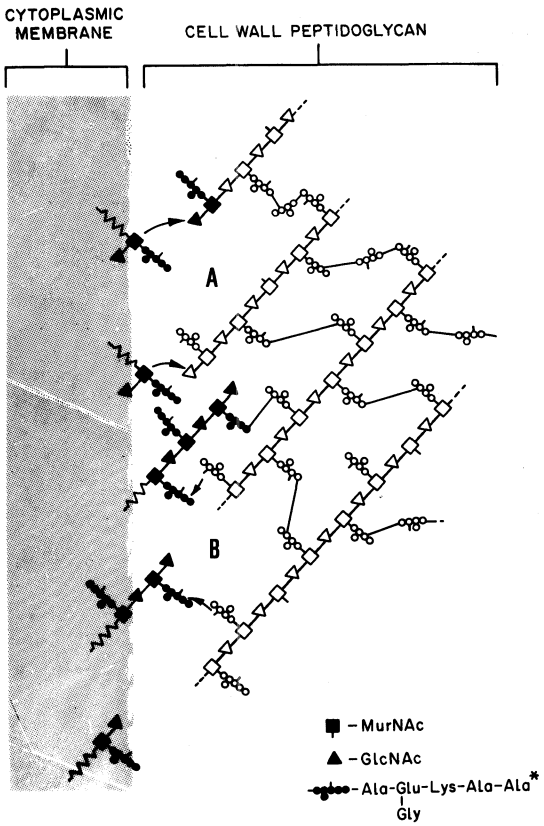

Fig. 40. Model of growth of *M. lysodeikticus* wall peptidoglycan. Newly synthesized strands are attached to preexisting wall peptidoglycan by two mechanisms. The main one is transpeptidation to an amino group on a preexisting peptide side chain, with concomitant release of terminal D-alanine from the newly synthesized peptidoglycan chain (see B on Fig.). The second mechanism is the attachment by transglycosylation of an oligosaccharide-peptide intermediate to a nonreducing end of a preexisting glycan chain (see A on Fig.). Symbols representing preexisting glycan strands are: GlcNAC-MurNAc, △—□; and the peptide

Ala-Glu-Lys-Ala-Ala, oo$\overset{o}{o}$oo.
Gly

Newly synthesized peptidoglycan is in black. The strands incorporated either singly or in polymerized form are depicted as lipid bound, although it is possible that the lipid moiety is removed before incorporation. (Mirelman et al., 1974c.) (Reprinted courtesy of the New York Academy of Sciences.)

D-Ala-L-Ala peptide bond formation must occur in strict coordination. By repetition of the process, interpeptide bridges of increasing size can be achieved. The penicillin susceptibility of the formation of D-Ala-L-Ala crosslinkages is about 50-fold less than that of the N^{ϵ}-(D-Ala)-L-Lys linkages. Fig. 41 shows the proposed sequence for the release of hexapeptide units (through amidase action at sites 2, 4, 6, 8), the formation of a cross-linking peptide chain composed of four

pentapeptide units (through D-Ala-L-Ala formation at sites 9, 7, 5, 3), and the closure of the cross-linking bridge (through N^{ϵ}-(D-Ala)-L-Lys formation at site 1) (Ghuysen, 1968). Two transpeptidases exhibiting different penicillin susceptibility may exist. Alternatively, only one transpeptidase might be responsible for both peptide cross-linking reactions and the variations in penicillin susceptibility might be ascribed to differences in location of the reactions in the cell.

The direction of transpeptidation in *Gaffkya homari* (Hammes, 1976; Hammes and Kandler, 1976) was shown to proceed in such a way that the preexisting wall peptidoglycan functions as carbonyl donor and the newly synthesized peptidoglycan strands function as amino acceptors. This situation is entirely different from that described above for *B. licheniformis* (see section 3.1.5.2.). The mechanism is especially complex. Indeed, these peptide units of the nascent peptidoglycan function as carbonyl donor and the newly synthesized peptidoglycan strands function as amino acceptors. This situation is entirely different units must remain present in this nascent peptidoglycan in such a way that through the active transpeptidation of the tetrapeptide units, these pentapeptide units are passively incorporated into the wall peptidoglycan where they can serve as donor units for further expansion of the polymer. In vivo, the newly synthesized peptidoglycan strands which undergo attachment probably contain equimolar amounts of pentapeptide and tetrapeptide units. Such a ratio would allow a maximum degree of crosslinking of 50% and oligomers larger than dimers could not be formed. In fact, such structural features are those found in the wall peptidoglycan of *G. homari*. Whereas the specific inhibition of the DD-carboxypeptidase activity in various bacilli by penicillin does not cause detectable damage to the cell (see section 3.1.5.2), the specific inhibition of the DD-

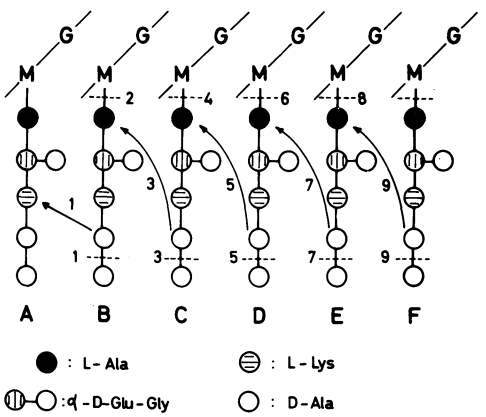

Fig. 41. Proposed biosynthetic sequence of the modification of the peptidoglycan in *Micrococcus lysodeikticus*. Reactions 2, 4, 6 and 8 are the result of hydrolysis by an N-acetylmuramyl-L-alanine amidase. Reactions 1, 3, 5, 7 and 9 are transpeptidations. Reaction 1 leads to formation of N^{ϵ}-(D-Ala)-L-Lys linkage. Reactions 3, 5, 7 and 9 lead to formation of L-Ala-D-Ala linkages. (Ghuysen et al., 1968.) (Reprinted courtesy of the American Chemical Society.)

carboxypeptidase activity in *G. homari* prevents peptidoglycan incorporation (Hammes, 1976). Hence, an unified view on the nature of the enzyme on which penicillin binding causes cessation of cell growth (and cell lysis) cannot be proposed. The concept of a penicillin "killing site" may well escape a precise definition.

3.1.5.4. Effect of the microenvironment of the assembly centers of peptidoglycan synthesis. Virtually nothing is known about the functioning of the enzymes in the peptidoglycan assembly centers. Mention should be made, however, of recent experiments by Neuhaus and coworkers who introduced a new and promising methodology for studying the effects of the microenvironment on, and the dynamics of, the processes that lead to the synthesis of the nascent peptidoglycan. The synthesizing system of *Gaffkya homari* (Hammes and Neuhaus, 1974a,b) was utilized to synthesize a spin-labeled nascent peptidoglycan from UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-(TEMPO-ϵN)-pentapeptide (Johnston et al., 1975; Johnston and Neuhaus, 1975). Spin labels are sensitive probes to study the microenvironments of intermediates in membrane-catalyzed reactions, since the electron-spin resonance spectrum of the spin label is a function of the motion that the probe experiences and of the polarity of the medium surrounding the probe. In the above system 188 pmoles of lysozyme-sensitive polymer were synthesized in 15 minutes. The spectrum of the spin-labeled lipid intermediate indicated that it was weakly immobilized relative to the spin-labeled nucleotide. The spectrum of the spin-labeled nascent peptidoglycan had a line-broadening characteristic of nitroxide-nitroxide interactions due to the high concentration of spin labels. Incubation with lysozyme resulted in marked sharpening of the spectrum. Both spin-labeled lipid intermediate and spin-labeled peptidoglycan were found to be accessible to vancomycin and ristocetin. The mechanism of action of these two antibiotics is related to their ability to complex with the C-terminal D-Ala-D-Ala moieties that are present at various phases of peptidoglycan synthesis (Perkins and Nieto, 1974).

3.1.5.5. Peptidoglycan expanson in E. coli. In their studies with *E. coli* W7 and W945T3282, Braun and coworkers (Braun and Bosch, 1973; Braun et al., 1974a; Braun and Wolff, 1975) observed that the only detectable radioactive peptidoglycan in the plasma membrane that could be chased from the plasma membrane into the rigid layer was the disaccharide peptide unit linked to the lipid carrier. The technique failed to reveal any formation of glycan chains before transfer from the lipid. Thus, peptidoglycan extension in *E. coli* might proceed by direct insertion of newly synthesized disaccharide-peptide monomer units. The results obtained also indicated that the rigid layer was not enlarged at growing points distributed at random throughout the rigid layer. Instead, during a 1-minute pulse, the diaminopimelate label was shown not to be incorporated in those regions where lipoprotein was attached to peptidoglycan; areas comprising 30% of the peptidoglycan remained largely preserved from growth. Prevention of peptidoglycan unit insertion at lipoprotein attachment sites might

be due to steric reasons since, for example, lysozyme is also sterically hindered by lipoprotein and leaves about three peptidoglycan units attached to each lipoprotein molecule. In this process, attachment of lipoprotein to the newly synthesized peptidoglycan portions would occur subsequently.

Assuming that the above model is correct, acceptor sites in the preexisting glycan chains (i.e., nonreducing N-acetylglucosamine) could be created by any type of endo-N-acetylmuramidase autolysin. Following insertion or concomitant with it, closure of bridges between peptide units belonging to adjacent strands would be achieved by transpeptidation. Virtually nothing is known about the transglycosylation reaction; however, endo-N-acetylmuramidases exhibiting potential biosynthetic activities are known. Hen egg white lysozyme, for example, is able to catalyze transglycosylation reactions (Chipman et al., 1968; Pollock and Sharon, 1970). The same endo-N-acetylmuramidase, occurring perhaps in two forms, might catalyze both creation of acceptor sites in the glycan strands by hydrolysis and insertion of new units by transglycosylation. The recently discovered peptidoglycan:peptidoglycan-6-muramyl transferase (section 2.1.7.) might also fulfill such a function.

A different view is expressed by Mirelman, Yashow-Gan, and Schwarz (1976) who showed experimentally that the mechanism of wall peptidoglycan expansion in *E. coli* (a thermosensitive division mutant PAT84) may be very similar to that in bacilli. *E. coli* PAT84, made permeable to nucleotide precursors by ether treatment, catalyzes peptidoglycan synthesis and its attachment to the preexisting wall peptidoglycan. Synthesis during a bacterial division cycle was investigated by initiating synchronous cell division with a shift down from restrictive (42°C) to a permissive (30°C) growth temperature. An abrupt increase in cell number occurred 20 to 30 minutes after the temperature shift down indicating that in these cells a synchronous triggering of septum and polar cap formation had occurred. At various times after the temperature shift down cells were collected, treated with ether, and utilized as representatives of the various stages during cell division. Nonseptated filaments formed at the restrictive temperature were similarly investigated. In these experiments newly synthesized, mostly peptide uncross-linked macromolecular peptidoglycan was estimated to be insoluble in trichloracetic acid but soluble in hot sodiumdodecylsulfate. Only the peptidoglycan that became covalently linked to preexisting wall peptidoglycan (i.e., the sacculus in Fig. 42) was insoluble in both reagents (Fig. 42).

Of interest was the finding that the amount of pentapeptide units in the SDS-soluble peptidoglycan intermediate was under the control of a DD-carboxypeptidase activity. Release of D-alanine residues (by both transpeptidase and DD-carboxypeptidase), covalent attachment of new peptidoglycan and formation of peptide cross-linkages were all completely inhibited by high concentrations of ampicillin (50 μg/ml). Low dose levels of ampicillin (0.5 μg/ml), however, specifically inhibited the DD-carboxypeptidase activity of cells grown at permissive temperature without affecting transpeptidation. Peptidoglycan incorporation in the preexisting wall was increased and septum formation was inhibited. Similarly, the DD-carboxypeptidase activity of nonseptated filaments

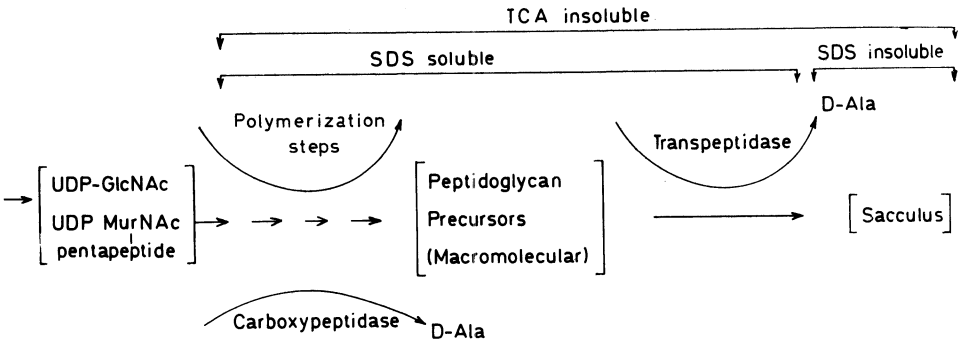

Fig. 42. Schematic model of principal reactions which participate in the synthesis and attachment of newly synthesized peptidoglycan strands to preexisting ones in the *E. coli* cell wall. The SDS-insoluble material accounts only for newly synthesized peptidoglycan bound covalently to the preexisting cell wall. The macromolecular peptidoglycan intermediates remain in the cell upon treatment with TCA, and are predominantly uncross-linked at its peptide side-chains (Mirelman et al., 1976.) (Reprinted courtesy of the American Chemical Society.)

grown at restrictive temperature was lower than that of the transpeptidase. Very little SDS-soluble peptidoglycan could be detected in these filaments and the extent of cross-linkage was increased when compared to that of cells grown at permissive temperature. Thus an increased ratio of transpeptidase to DD-carboxypeptidase, induced either by low ampicillin concentration at permissive temperature or by a shift up to nonpermissive temperature, appeared to result in an increased amount of substrate donor available for transpeptidation. Consequently, an increased rate of peptidoglycan incorporation in the rigid layer and a greater extent of peptide cross-linking were obtained. Also, under conditions where the extent of transpeptidation was not controlled by DD-carboxypeptidase activity, triggering of formation of new septa was prevented. Regardless of the exact situation, it is clear that a balance of the relative activities of transpeptidase and DD-carboxypeptidase is important for cell division and morphology. On the basis of these observations, and more recently, of the effects caused by cephalexin and nalidixic acid on peptidoglycan synthesis at the permissive temperature (Mirelman et al., 1977), the attractive working hypothesis was made that in *E. coli,* the nascent peptidoglycan would act as carboxyl donor for transpeptidation reactions with two types of acceptors. Reaction with the preexisting wall peptidoglycan would lead to wall elongation, whereas reaction with a modified nascent peptidoglycan previously deprived of donor sites by DD-carboxypeptidase action and accumulated at the equator of the cell (i.e., where it is synthesized) would lead to septum formation. The specific inhibition of the DD-carboxypeptidase responsible for the destruction of the donor sites of the nascent peptidoglycan (and for its accumulation) and/or the specific inhibition of the transpeptidase which uses this modified nascent polymer as amino acceptor would therefore prevent cell septation and would cause cell filamentation.

Recently, the work of Spratt and Pardee (1975) and Spratt (1975, 1977) has

shed new light on the subtle processes that control cell wall growth and septation in *E. coli.* These authors have shown that a set of membrane-bound proteins in this organism are apparently involved specifically in cell division, cell elongation, and cell shape. [^{14}C]benzylpenicillin and [^{14}C]penicillin FL-1060 were bound to the plasma membrane selectively solubilized with Sarkosyl NL-913. Penicillin FL-1060, the amidino-penicillanic acid (Lund and Tybring, 1972), differs from other β-lactam antibiotics in that penicillin FL-1060 at low concentration produces ovoid shaped gram-negative cells without inhibiting cell division or causing lysis. Penicillin FL-1060 is known to interfere with an event that starts at the beginning of the C period of the cell division cycle in *E. coli* and is responsible for normal cell elongation (James et al., 1975). Six main penicillin-binding proteins (numbered 1 to 6 on the basis of decreasing molecular weights) and a series of other minor binding proteins were separated on sodium dodecylsulfate polyacrylamide slab gels and detected by fluorography. Evidence that protein n°2 (molecular weight 66,000) was involved in rod-shape maintenance was strong. Indeed [^{14}C]penicillin FL-1060, which specifically causes ovoid cells, bound exclusively to protein n°2. Moreover, a mutant (B-6) was isolated that grew as round cells and failed to bind β-lactam antibiotics to protein n°2. About 10 copies of protein n°2 exist per cell. Weaker evidence indicated the probable involvement of protein n°3 (mol. wt., 60,000) in cell division and of protein n°1 (mol. wt. 91,000) in cell elongation. Bulge formation, typical of the early stages of penicillin intoxication, might result from inhibition of proteins 2 and 3 since the β-lactam antibiotics that caused bulges in the strain used also showed higher affinities for protein 2 and 3 than for protein 1. Finally, since the peptidoglycan produced under conditions where either protein n°2 was inhibited (by ovoid cells) or protein n°3 was inhibited (by filaments) was apparently not defective, and since inhibition of protein n°1 stopped cell elongation, protein n°1 was proposed as the "main" transpeptidase responsible for the attachment to the wall of the newly synthesized peptidoglycan. The significance of this work, however, remains incomplete as long as the possible correlation between those multiple penicillin-binding proteins and the known multiple penicillin-sensitive enzymes remains unknown.

Finally, Goodell and Schwarz (1975) have shown that in addition to the protein 2 of Spratt and Pardee the peptidoglycan itself may play a role in cell morphogenesis. Spherical *E. coli* cells retaining an apparently mechanically intact but spherical peptidoglycan were produced by mutation and by treatment with penicillin FL-1060. Under proper conditions these spherical cells were seen to reshape themselves into rods. During this process the areas of the cell envelope that had been the ends of the original rods became the ends of the newly formed rods. In contrast, osmotically sensitive spheroplasts lacking the rigid layer were unable to revert to rods although they were able to synthesize a new but spherical rigid layer. Loss of the original rigid layer in *E. coli* might thus be paralleled by loss of the ability to revert to a rod, that is, to retain cell polarity.

3.1.5.6. The peptide cross-linking enzyme system Transpeptidation is a key reaction in peptidoglycan expansion. The complexity of the enzyme system involved in

the peptide cross-linking reaction varies greatly depending on the bacteria (Ghuysen, 1977a,b). The most simple systems appear to occur in *Streptomyces*, gram-positive eubacteria that form a characteristic mycelium. The enzyme system seems to consist, of one membrane-bound enzyme in *Streptomyces* strain *rimosus* (a transpeptidase that exerts a low DD-carboxypeptidase activity) and two membrane-bound enzymes in *Streptomyces* strains K15 and R61 (a transpeptidase that exerts a low DD-carboxypeptidase activity, and a DD-carboxypeptidase that shows a low transpeptidase activity). Strains R61 and K15 also possess two other DD-carboxypeptidases performing a low transpeptidase activity; one of them can be released from the cells during protoplast formation by lysozyme treatment and the other is excreted in the culture medium during growth. Membrane-bound, lysozyme-releasable and exocellular DD-carboxypeptidases are immunologically related to each other (Ghuysen, 1977b). The membrane-bound peptide cross-linking enzyme system in *E. coli* is exceedingly complex (Pollock et al., 1974; Nguyen-Distèche et al., 1974a,b; Mirelman et al., 1976; Ghuysen, 1977a). Fractionation of the extracted complex suggested the occurrence of (1) a transpeptidase whose main function is to catalyze transpeptidation, that is, dimerization between two peptide units. An in vitro specific test for the transpeptidase has been devised. It consists of a mixture containing a low concentration of radioactive pentapeptide L-Ala-D-Glu$_\Gamma$-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]Ala-D-[^{14}C]Ala (acting mainly as donor) and a high concentration of amidated tetrapeptide L-Ala-D-Glu(amide)$_\Gamma$-(L)-*meso*-diaminopimethyl-(L)-D-Ala (acting exclusively as acceptor). D-[^{14}C]Ala is liberated and the monoamidated octapeptide dimer is formed (Fig. 43); (2) a DD-carboxypeptidase whose main function is to hydrolyze the C-terminal D-Ala-D-Ala peptide bond of pentapeptide units (whether they occur free, as disaccharide peptide, in the form of the nucleotide precursor, or at the C-terminal end of a peptidoglycan polymer) without concomitant transpeptidation; and (3) an endopeptidase whose main function is to hydrolyze the dimers formed by transpeptidation into monomers. Since the interpeptide bond D-Ala-(D)-*meso*-diaminopimelic acid made by transpeptidation extends between two D-centers and is in α-position to a free carboxyl group (Fig. 43), the endopeptidase that hydrolyzes it has the specificity of a DD-carboxypeptidase. Although they evolved to perform one of these specific activities with high efficiency, each of these enzymes seems also to be able to perform the other activities with low efficiency. The situation becomes more complicated since this multienzyme (transpeptidase-DD-carboxypeptidase-endopeptidase) complex probably occurs in two distinct forms, one of them sensitive to very low dose levels and the other to higher dose levels of ampicillin.

As discussed earlier (section 3.1.5.3.), the peptide cross-linking enzyme system in *M. lysodeikticus* is also exceedingly complex since its functioning probably implies the involvement of two transpeptidases and a N-acetylmuramyl-L-alanine amidase. In addition, *M. lysodeikticus* contains a periplasmic DD-carboxypeptidase activity of undetermined physiological function (Linder and Salton, 1975).

Bacteria may contain LD-carboxypeptidase-transpeptidase enzyme systems.

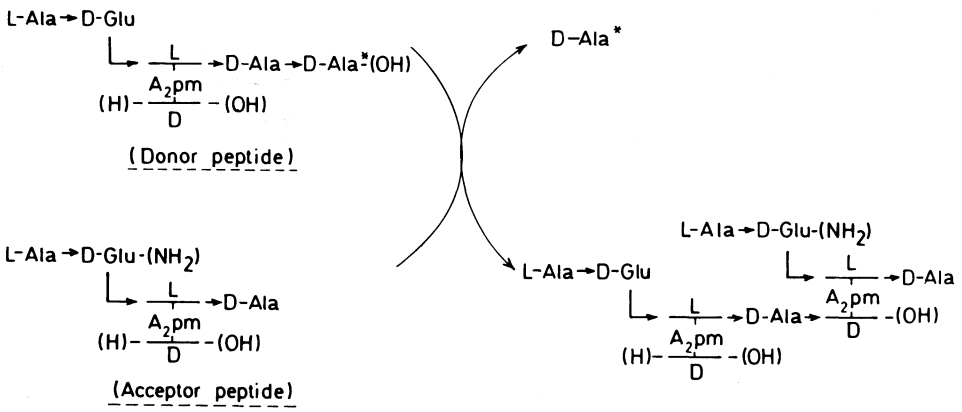

Fig. 43. Mono-amidated octapeptide dimer formed *in vitro* by the *E. coli* peptide cross-linking enzyme system from pentapeptide L-Ala-D-Glu ⌐(L)-*meso*-diaminopimelyl-(L)-D-Ala-D-Ala and amidated tetrapeptide L-Ala-D-Glu (amide) ⌐(L)-*meso*-diaminopimelyl-(L)-D-Ala.

These systems liberate the C-terminal D-alanine residues from tetrapeptides L-Ala-D-Glu ⌐L-R_3-D-Ala (that are formed by action of a DD-carboxypeptidase) either without concomitant transpeptidation (LD-carboxypeptidase) or with concomitant transfer of the tripeptide L-Ala-D-Glu ⌐L-R_3 to a proper amino group acceptor ("atypical" transpeptidase). *E. coli* possesses an LD-carboxypeptidase which is periplasmic (Strominger, 1970). Its activity is not constant throughout the division cycle (in cells of D11/lac $^+$pro$^+$), detectable activity being highest at the time of division by a factor of three (Beck and Park, 1976). The corresponding atypical transpeptidase has not been detected but its involvement in the covalent attachment of the lipoprotein to the peptidoglycan has been postulated (section 3.3.). *Streptococcus faecalis* possesses a membrane-bound "atypical" transpeptidase that is inhibited by various β-lactam antibiotics (Coyette et al., 1974). Its physiological role is unknown. Such "atypical" transpeptidases might catalyze the synthesis of "atypical" L-R_3L-R_3 interpeptide bridges described earlier (section 2.1.2.3.).

Multiple penicillin binding sites exist in both gram-negative and gram-positive bacteria. These binding sites occur in the plasma membrane and, to all appearances, they are distinct proteins. The fluorography technique has revealed the presence of at least 6 penicillin binding sites in *E. coli* (see section 3.1.5.5.), 5 in *S. typhimurium* (Shepherd et al., 1977), in *B. subtilis* and *B. megaterium*, 4 in *B. stearothermophilus* and 3 in *B. cereus* (Blumberg and Strominger, 1974). In most cases, the physiological role and the possible enzymatic function of these binding sites remain to be discovered. However, proteins 5 and 6 in *E. coli* were shown to be DD-carboxypeptidases-transpeptidases (Spratt and Strominger, 1976). Similarly, proteins 1, 4 and 5 in *Salmonella typhimurium* were shown to be DD-carboxypeptidases-transpeptidases, protein 4 having the greatest capacity for carrying out transpeptidation *in vitro* (Shepherd et al., 1977). It has also been suggested that in *B. subtilis*, the penicillin binding component n°2 would be the most likely target for killing by penicillins (Buchanan and Strominger, 1976).

Extraction and characterization of a membrane-bound transpeptidase pose technical problems because experiments of this type cannot be attempted unless the transpeptidase activity can be directly and specifically estimated with the help of a proper system of donor and acceptor peptides. The nucleotide precursors UDP-N-acetylglucosamine and UDP-N-acetylmuramylpentapeptide are not substrates of the transpeptidase. Nascent peptidoglycans are poorly characterized, both chemically and physically, and preclude precise kinetic studies. Defined substrate systems that allowed these enzymes to be estimated independently of the preceding biosynthetic reactions have been developed and their use has allowed several transpeptidases to be isolated and studied (Ghuysen, 1977a,b). Several strains of *Streptomyces* were shown to excrete enzymes during growth that appeared to be soluble forms of the membrane-bound transpeptidases. Some of these extracellular enzymes were purified to protein homogeneity. Each extracellular enzyme was shown to be capable of performing both DD-carboxypeptidase and transpeptidase activities, and the ratio between these activities could be modified by changing the environmental conditions (e.g., pH, polarity of the incubation medium, ionic strength, acceptor and donor concentrations) (Frère et al., 1973; Ghuysen et al., 1973). Membrane-bound DD-carboxypeptidases-transpeptidases were also solubilized and partially purified. Both membrane-bound and exocellular enzymes were used as models to study the transpeptidation reaction and the interaction with penicillin (Ghuysen, 1977a,b). β-Lactam antibiotics are actually substrates of these enzymes. Enzyme-antibiotic complexes are formed that subsequently undergo spontaneous breakdown, and the half-lives of these complexes vary from minutes to days depending on the enzyme and the antibiotic, so that the velocity with which the antibiotic is degraded during the interaction may vary greatly. By using the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* strain R61, benzylpenicillin was shown to be fragmented into phenylacetylglycine (Frère et al., 1975) and N-formyl-D-penicillamine (Frère et al., 1976).

3.1.6. Regulation of the autolysins

Wall growth and the changes that occur in the shape of the peptidoglycan network during the life cycle are complex phenomena. They are discussed by Daneo-Moore and Shockman elsewhere in this volume. The cell autolysins are actively involved in these processes. Obviously, the peptidoglycan hydrolases must be prevented from uncontrolled action; a "barrier" exists between these enzymes and their substrate.

3.1.6.1. The "barrier" between autolysins and peptidoglycan in E. coli. *E. coli* possesses a set of different types of membrane-bound peptidoglycan hydrolases despite which peptidoglycan breakdown will not occur if intact cells from either the exponential or stationary phases are maintained in buffer at 37°C (Hartmann et al., 1974). The characteristics of the barrier are unknown but its maintenance clearly depends on the integrity of the cell envelope. Breaking the barrier, and triggering hydrolase activity, can be achieved in many different ways, as follows:

(1) mechanical opening of the cells; (2) changing osmotic pressure (e.g., treatment of the cells with 1 M NaCl at 0°C or with 20% sucrose); (3) removal of divalent cations by EDTA; (4) interaction with penicillin (for further details on the effects of penicillin on cell surface growth and division, see Daneo-Moore and Shockman, chapter 9 in this volume) and (5) treatment of the cells with 5% trichloracetic acid. The latter treatment causes optimal activation of the peptidoglycan hydrolase complex, and this shows that the membrane-bound hydrolases in their native environment are well protected against otherwise efficient denaturing agents. Despite the presence of this barrier, localized hydrolase action appears to be triggered at a given stage of the cell life cycle. Presumably "enzyme action would be permitted for a defined time period in a specific cell area by controlled local breakdown and reestablishment of the barrier" (Hartmann et al., 1974). Experimentally, peptidoglycan hydrolase activities in synchronized cultures of *E. coli* B/r were shown to increase discontinuously during the cell cycle with a maximal activity occurring at the same time that the rate of wall synthesis was maximal (Hakenbeck and Messer, 1977). The transpeptidase-DD-carboxypeptidase-endopeptidase system in *E. coli* also seems to be controlled by some "barrier." Thus, the lower DD-carboxypeptidase activity exhibited by the ether-treated filaments of *E. coli* (obtained at the nonpermissive temperature; section 3.1.5.5.) is not due to temperature sensitivity of the enzyme. Mechanical disruption of the filaments by sonication causes a great increase in DD-carboxypeptidase activity, suggesting that this enzyme is partially inactive in its natural environment in the intact filament (Mirelman et al., 1975).

3.1.6.2. The "barrier" between autolysins and peptidoglycan in gram-positive bacteria. The barrier between autolysins and peptidoglycan in gram-positive bacteria can be partially described in biochemical terms. In *S. faecalis,* the occurrence of two interconvertible forms of the autolytic enzyme, one active and the other latent, provides a possible mechanism for the mode of control of the wall autolysin (Higgins and Shockman, 1971; Joseph and Shockman, 1976). Various defined wall components are also involved in this control; they include the lipoteichoic acids, the wall teichoic acids, the wall teichuronic acids and specialized autolysin modifiers.

Membrane lipoteichoic acids isolated from several bacterial species (with common backbone of polyglycerol phosphate, but differing in sugar substituents and in glycolipids) and the pneumococcal Forssman antigen (section 2.3.3.) have been examined for effects on various wall autolysins (endo-N-acetylmuramidases of *S. faecalis* and *L. acidophilus*; N-acetylmuramyl-L-alanine amidases of *B. subtilis* and *D. pneumoniae*) (Cleveland et al., 1975). The conventional lipoteichoic acids inhibit both N-acetylmuramidases and the *B. subtilis* amidase at relatively low concentrations, but fail to inhibit the pneumococcal amidase. Conversely, the pneumococcal Forssman antigen inhibits the pneumococcal amidase but has no effect on the three other autolysins. Deacylation of the lipoteichoic acids results in loss of lytic inhibitory activity on the sensitive autolysins, and similarly the pneumococcal choline-containing wall

teichoic acid is 700-fold less active with the pneumococcal amidase than the Forssman antigen. Further work is required to unravel the specificities of the observed effects, but the occurrence of the lipoteichoic acids in both acylated and deacylated forms implicates a role for these polymers in the in vivo regulation of autolytic activity. It should also be mentioned that autolysis of intact cells of *S. faecalis* was found to be inhibited to a greater extent by phospholipids than by lipoteichoic acid (Cleveland et al., 1976). This observation suggests a possible difference in the accessibility of native autolysin to these various substances.

In vivo, the specificity of at least some autolysins can be modulated by specific modifiers. Thus, both the N-acetylmuramyl-L-alanine amidase autolysin and a specific modifier protein were purified from *B. subtilis* ATCC 6051 (Herbold and Glaser, 1975a,b). The modifier protein combines stoichiometrically with the enzyme and stimulates its activity threefold. The major effect of the modifier, however, is not to alter the level of enzyme activity but rather to change the pattern of cleavage from a random one, when only the enzyme hydrolyzes the wall, to a more sequential, specific pattern in the presence of the modifier protein.

Wall teichoic acids have been assigned a role as "allosteric" (or "allotopic") ligands for wall autolytic enzymes (Tomasz and Westphal, 1971; Herbold and Glaser, 1975a,b). These ligands appear to be responsible for the tight binding of at least some autolysins to their wall substrate. One revealing example of this type of interaction is found in *D. pneumoniae*. Walls from pneumococci grown on choline-containing medium have a choline-containing teichoic acid and are substrates of the N-acetylmuramyl-L-alanine amidase autolysin. Removal of the wall teichoic acid by treatment with periodate or with formamide renders the walls resistant to the amidase (Mosser and Tomasz, 1970). Moreover, pneumococci grown on the choline analog, ethanolamine, incorporate ethanolamine in the teichoic acid where it replaces choline, resulting in synthesized walls that are resistant to the homologous autolysin (Höltje and Tomasz, 1975b). In vitro methylation of these autolysin-resistant, ethanolamine-containing walls under conditions where virtually all the ethanolamine residues are converted to choline results in recovery of amidase sensitivity. Clearly, the autolysin-catalyzed hydrolysis of the amide bonds on the peptidoglycan requires interaction between the enzyme and the choline residues of the wall teichoic acid. The wall choline-containing teichoic acid activates the amidase, whereas the membrane choline-containing lipoteichoic acid inhibits it. Thus, regulation of the enzyme activity appears to depend on the relative amount, activity, or localization of these two ligands.

Another example of the ligand properties of wall teichoic acids toward autolysins is *B. subtilis* 6051. In this bacterium the teichoic acid is a polyglycerolphosphate polymer containing α-linked glucosyl residues, and teichoic acid is also required for the action of the modifier protein on the amidase autolysin. Herbold and Glaser (1975a,b) have suggested that the apparent absence of wall hydrolytic enzymes in teichoic acid-negative mutants may not be due to the lack of enzyme synthesis by these cells, but may instead reflect the fact that in the

absence of teichoic acid the enzymes are not bound tightly to the wall and are probably lost from the cell *in vivo* and during cell breakage and wall isolation.

The studies mentioned above led to an interesting observation concerning the mode of action of penicillin (and other antibiotics inhibiting peptidoglycan synthesis) at the cellular level. Working with a mutant pneumococcus defective in autolysin, Tomasz and Waks (1975) noted: (1) growth of the mutant was inhibited by penicillin at the same concentration as the one that induced lysis of the wild type; (2) exogenous, wild-type autolysin alone had no effect on the growth of the mutant but caused its lysis if penicillin (at the minimum growth inhibiting concentration) was added simultaneously to the medium; (3) penicillin and other inhibitors of peptidoglycan synthesis caused the escape into the medium of a trichloracetic acid-precipitable, choline-containing polymer; and (4) growth inhibition of the mutant, its sensitization to exogenous penicillin, and release of the choline-containing polymer all showed the same dose response as that of the penicillin-induced lysis of the wild type. One probable interpretation of these results is that inhibition of peptidoglycan synthesis destabilizes the complex between the autolysin and its inhibitor (lipoteichoic acid) and triggers autolysin activity.

Finally in *B. licheniformis*, it seems that the wall teichuronic acid (and not the wall teichoic acid) controls lytic activity. Cells grown under certain conditions, contain both polymers in their walls. Removal of teichoic acid from isolated walls had no effect on autolysin sensitivity, whereas removal of teichuronic acid made the walls resistant to autolysin. Moreover a novobiocin-resistant mutant lacking teichuronic acid was isolated (Robson and Baddiley, 1977). It was defective in its cell morphology, the isolated walls had an increased resistance to autolysin and did not possess such autolysin. It was concluded that teichuronic acid was necessary for both the binding of the autolysin to the wall and for the bound enzyme to hydrolyse the peptidoglycan. It was also suggested that small variations in the localization of teichuronic acid in the wall might result in different rates of autolysis in specific regions and that cell separation might be achieved by discontinuities in the distribution of this polymer across the septal wall (Robson and Baddiley, 1977).

3.2. Biosynthesis of teichoic acids and other anionic polysaccharides

3.2.1. Synthesis of lipoteichoic acids

Little is known about the synthesis of lipoteichoic acids. Attempts to demonstrate lipoteichoic acid formation from CDP-glycerol in a variety of microorganisms failed. Studies with *S. aureus* H (Glaser and Lindsay, 1974) suggested that phosphatidylglycerol was a precursor of lipoteichoic acid, not only of the hydrophobic end of the molecule but also of at least a portion of the polyglycerol phosphate chain. Data were also consistent with the assumption that only the glycerol-phosphate moiety of phosphatidylglycerol was used in the synthesis of lipoteichoic acid. Work on the biosynthesis of the phosphatidylkojibiosyl dig-

lyceride, the phosphoglycolipid moiety of the lipoteichoic acid in *S. faecalis,* is in progress (Pieringer and Ganfield, 1975).

Attempts to demonstrate the incorporation of D-alanine into isolated lipid- and D-alanine-free teichoic acids were unsuccessful. Thus, it was thought that D-alanine incorporation might occur only when the polymer was associated with the membrane, perhaps in a particular conformation or environment. Such a possibility was supported by other observations showing that isolated teichoic acid has lost properties that it performs when it is integrated in the cell envelope. For example, bacteriophages adsorb to teichoic acid bound with the wall but not to isolated teichoic acid as if adsorption requires a given conformation of the receptor or a special orientation of the sugar residues that are imparted by the binding of teichoic acid to the cell envelope (Young, 1967; Coyette and Ghuysen, 1968). Incorporation of D-alanine into membranes was therefore investigated (Reusch and Neuhaus, 1971). *Lactobacillus casei* was chosen for these studies because its membrane contains a simple lipoteichoic acid consisting of a linear polyglycerol phosphate with D-alanine esterified at position 2 of the glycerol moiety (Kelemen and Baddiley, 1961). In the presence of Mg^{2+} ions and ATP, D-alanine incorporation appeared to proceed through a two-step reaction involving two cytoplasmic, soluble enzymes, a D-alanine activating enzyme (E_1) and a D-alanine:membrane acceptor ligase (E_2) (Linzer and Neuhaus, 1973; Neuhaus et al., 1974):

$$\text{D-Ala} + E_1 + \text{ATP} \rightleftharpoons E_1 \cdot \text{AMP-D-Ala} + \text{PP}$$

$$E_1 \cdot \text{AMP-D-Ala} + \text{MEMBRANE ACCEPTOR} \xrightarrow{E_2} E_1 + \text{AMP} + \text{D-Ala-MEMBRANE ACCEPTOR}$$

The acceptor has not been characterized. It may be the lipoteichoic acid itself. The system is exceedingly complex as has been revealed particularly by a study of the membrane glycerol teichoic acid of a stabilized L-form of *Streptococcus pyogenes* (Chevion et al., 1974). The glucose-containing teichoic acid from this L-form lacks D-alanyl esters and is shorter in length than that of the parental coccus (13 *versus* 25 units) (Slabyi and Panos, 1973). Like the parental coccus, the stabilized L-form contains both D-alanine activating and ligase enzymes and the L-form enzymes successfully catalyze D-alanine incorporation into the membranes of the parental coccus. No incorporation was observed, however, when the L-form membranes were used as substrates, demonstrating that the L-form membranes lack functioning D-alanine acceptor activity. The exact reason for this situation is unclear. It may be due to an alteration of the lipoteichoic acid acceptor (which appears to be shorter than that of the parental strain), or to a change in its conformation, the absence of one enzyme or several enzymes associated with the membrane or, finally, to changes in the complex lipid and fatty acids of the L-form membrane.

3.2.2. Synthesis of wall teichoic acids

Wall teichoic acids are synthesized through a three-stage process which, in essence, is similar to that described for peptidoglycan synthesis.

3.2.2.1. The nucleotide precursors. CDP-glycerol is the precursor of the wall glycerol teichoic acids and CDP-ribitol is the precursor of the wall ribitol teichoic acids (Fig. 44). CDP-glycerol is synthesized from D-glycerol-1-phosphate (which arises from glycolysis) and CTP. The reaction is catalyzed by a CDP-glycerol pyrophosphorylase: D-glycerol-1-P + CTP $\rightleftharpoons$ CDP-glycerol + PP. Similarly, CDP-ribitol is synthesized from D-ribitol-5-phosphate and CTP by a CDP-ribitol pyrophosphorylase. D-ribitol-5-phosphate is formed by enzymic reduction of D-ribulose-5-phosphate by NADH. CDP-glycerol and CDP-ribitol are known to occur in gram-positive bacteria and were actually characterized by Baddiley and his colleagues before the polymers for which they serve as biosynthetic precursors were discovered (review, Baddiley, 1972).

An interpendence of the peptidoglycan and teichoic acid synthesizing systems exists at the level of the relevant nucleotides. Such an early reciprocal control is suggested by the fact that a precursor of one wall polymer can interact with an enzyme involved in the synthesis of the second wall polymer. The UDP-N-acetylglucosamine pyrophosphorylase and the phosphoenolpyruvate UDP-N-acetylglucosamine enolpyruvyl transferase involved in peptidoglycan synthesis and the CDP-glycerol pyrophosphorylase involved in teichoic acid synthesis appear to be possible control points in the synthesis of the wall of *B. licheniformis* (Anderson et al., 1972, 1973) (Fig. 45). UDP-N-acetylmuramylpentapeptide inhibits all three enzymes, influencing both peptidoglycan and teichoic acid syntheses. CDP-glycerol can affect peptidoglycan synthesis by inhibiting UDP-N-acetylglucosamine pyrophosphorylase. The only stimulating effects observed are

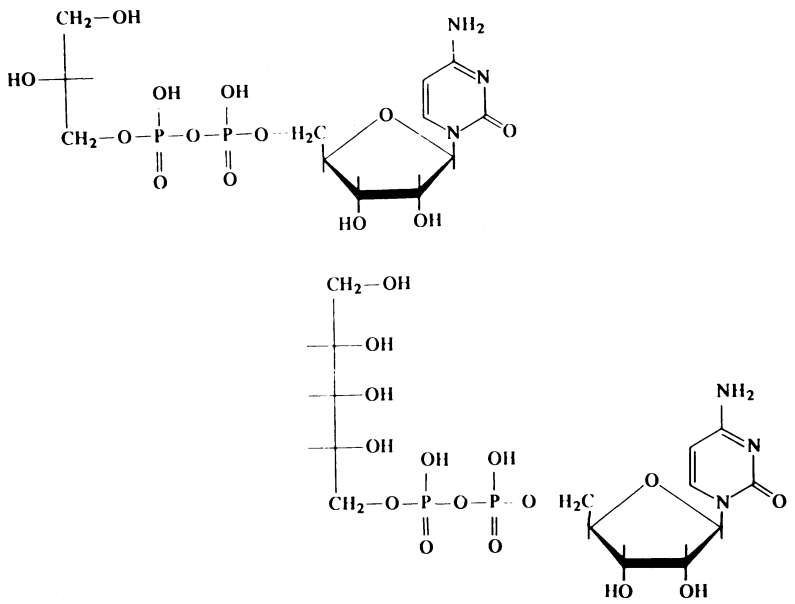

Fig. 44. Cytidine diphosphate glycerol and cytidine diphosphate ribitol.

on CDP-glycerol pyrophosphorylase by low concentrations of UDP-N-acetylglucosamine and UDP-N-acetylmuramylpentapeptide. Finally, the pyrophosphorylases investigated are inhibited by their reaction products. The relative importance of these possible regulatory processes, remains to be determined.

3.2.2.2. Polymerization and transglycosylation reaction. The synthesis of those teichoic acids where the backbone is a simple polymer of polyglycerol- or polyribitol phosphate units (Fig. 18) proceeds through sequential transfers of polyol phosphate residues from CDP-glycerol or CDP-ribitol to a membrane lipid carrier. These reactions are transphosphorylations where CMP residues are released. Incorporation of the side-chain sugars onto the polyolphosphate backbone is performed by transfer of glycosyl residues from UDP-glucose or UDP-N-acetylglucosamine to the free hydroxyl groups of the polyol. These reactions are transglycosylations; UDP residues are released. Transphosphorylation and transglycosylation reactions can occur concomitantly, and bivalent cations are required for optimal synthesis. The first successful syntheses of this type were carried out with particulate preparations from various bacilli and staphylococci (Glaser, 1964). Preformed poly(glycerol or ribitol) phosphate polymers can be used as substrates by the transglycosylases. For example, glucosyl transfer from UDP-glucose to polyribitol phosphate backbone was achieved with a particulate enzyme preparation from *B. subtilis* W23. All the linkages formed had the β-configuration (Chin et al., 1966). Similarly, insertion of both α- and β-N-acetylglucosamine residues on a polyribitol phosphate backbone from

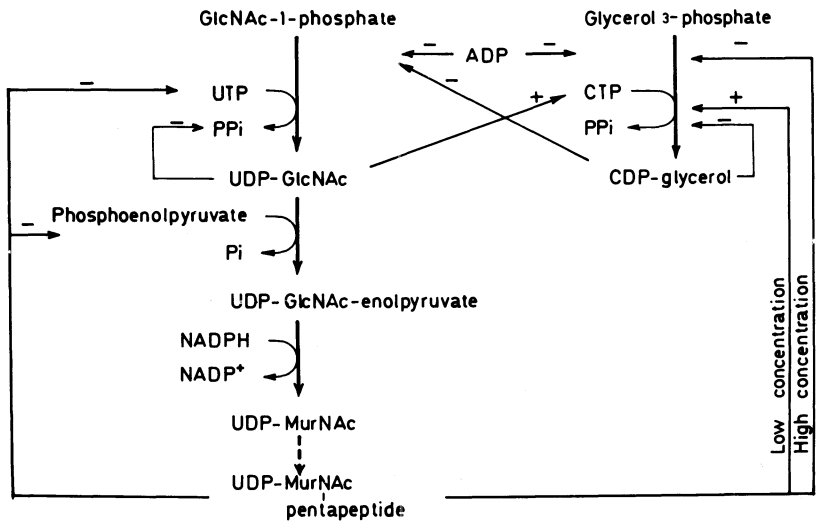

Fig. 45. Interrelations in the synthesis of UDP-GlcNAc, UDP-MurNAc-pentapeptide and CDP-glycerol. Stimulatory effects are indicated as positive, inhibitory effects as negative. A_2pm, diaminopimelate. (Anderson et al., 1973.) (Reprinted courtesy of the Biochemical Society.)

UDP-N-acetylglucosamine was achieved by enzyme preparations from *S. aureus* (Nathenson and Strominger, 1966).

The synthesis of those teichoic acids where the sugar residues are part of the polymeric chain (Figs. 19 to 21) is more complex. For synthesis of the teichoic acid of *S. lactis* 13 (Fig. 21), both CDP-glycerol and UDP-N-acetylglucosamine must be incubated simultaneously with the cell-free enzyme preparation. UDP-N-acetylglucosamine donates an N-acetylglucosamine-1-phosphate residue to a membrane lipid carrier and the intermediate thus formed accepts a glycerol phosphate residue from CDP-glycerol. Both reactions are transphosphorylations and UMP and CMP are released. The product formed possesses the complete repeating unit of the polymer. In the process, the glycerol phosphate moiety is provided by CDP-glycerol and the N-acetylglucosamine-1-phosphate moiety is provided by UDP-N-acetylglucosamine (Fig. 46). For synthesis of the teichoic acid of *B. licheniformis* ATCC-9945 (Fig. 19), glucose incorporation proceeds by transglycosylation from UDP-glucose to a membrane lipid carrier with release of UDP, and glycerol phosphate incorporation proceeds by transphosphorylation from CDP-glycerol with release of CMP. As a result, the intermediate contains glucose and glycerol phosphate in the form of the repeating unit of the polymer (review, Baddiley, 1972.)

3.2.2.3. The membrane carrier. The polyglycerol phosphate and polyribitol phosphate polymers being synthesized are linked to an endogenous acceptor. The polyglycerol phosphate polymerase (from *B. subtilis*) and the polyribitol phosphate polymerase (from *S. aureus*) involved in these polymerizations were extracted from the relevant bacterial membranes and purified to the stage where they depended almost entirely on the addition of a heat-stable component for activity (Mauck and Glaser, 1972, 1973; Fiedler and Glaser, 1974; Fiedler et al., 1974). This component was also isolated. It was not an activator of the reaction, but behaved as an acceptor for the growing polymer. With the *S. aureus* system

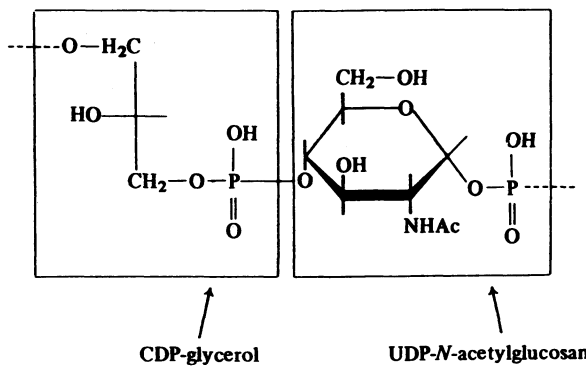

Fig. 46. Transfer of residues from nucleotide precursors to teichoic acid from *Staphylococcus lactis* 13. (Baddiley, 1972.)

the length of the complete polyribitol phosphate formed on this acceptor was about 35 units, in fair agreement with a chain length of 40 to 50 units for the teichoic acid in the completed wall (Ghuysen et al., 1964). In addition to the acceptor the *S. aureus* polyribitol phosphate polymerase also required a phospholipid for full activity. Cardiolipin was found to be a very effective activator, but a variety of other phospholipids (e.g., phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine) could substitute for cardiolipin with diminished efficiency (Fiedler et al., 1974).

Transfer of glycerol phosphate and ribitol phosphate units to the acceptor is a direct process that does not involve an additional intermediate carrier. Both *S. aureus* and *B. subtilis* polymerases did not appear to require any addition of undecaprenol phosphate (i.e., the lipid carrier involved in peptidoglycan synthesis) and formation of undecaprenol pyrophosphate glycerol or ribitol could not be detected in these systems. The carrier was subsequently identified as a (polyglycerol) lipoteichoic acid. Hence, the nascent teichoic acid that ultimately undergoes insertion in the wall peptidoglycan grows on a preformed lipoteichoic acid; lipoteichoic acid serves as the assembly center for the wall teichoic acid. The growing site for the future wall teichoic acid on the lipoteichoic acid is probably a glycerol residue located at the hydrophobic end of the carrier where all the D-glucose and fatty acids accumulate (Fiedler and Glaser, 1974). Thus the hydrophobic end of the lipoteichoic acid carrier would serve not only to anchor the carrier into the membrane but would also be the active site of the molecule. Such a model could explain why a polyglycerol phosphate polymer that lacks the hydrophobic end of the lipoteichoic acid cannot substitute for the carrier in the teichoic acid polymerase reaction.

Lipoteichoic acid carriers do not exhibit a high specificity with respect to the type of wall teichoic acid for which they serve as carriers. Lipoteichoic acid carriers that are active with the polyribitol phosphate polymerase of *S. aureus* have been extracted from various gram-positive bacteria. Similarly, lipoteichoic acid isolated from *S. aureus* also acts as a carrier for the polyglycerol phosphate polymerase of *B. subtilis*.

Whether the lipoteichoic acid that acts as carrier for wall teichoic acid synthesis is distinct or not from the bulk of lipoteichoic acid which occurs in the plasma membrane of the gram-positive bacteria is not definitely established. Thus, the purified lipoteichoic acid carrier of *S. aureus* H was considered by Fiedler and Glaser (1974) to be an acylated polyglycerol phosphate with a chain length of 12 to 14 units and one glucose molecule per chain. Duckworth et al. (1975), on the other hand, assigned to the lipoteichoic acid isolated from the same organism a chain of 28 to 30 units linked to the terminal glucose moiety of diacetylgentiobiosylglycerol. The proportions of glycerol, phosphorus, glucose and fatty acids reported by Fiedler and Glaser, and by Duckworth and associates were virtually identical. The difference between the reported properties of the two preparations might be resolved on the basis that the lipoteichoic acid carrier actually contains two molecules of glucose per chain, and the published evidence does not exclude this possibility.

Finally, the high levels of D-alanine ester residues in wall teichoic acid also pose the question of D-alanine incorporation in this polymer. Presumably, such incorporation might occur when the growing wall teichoic acid is linked to its lipoteichoic acid carrier. The exact mechanism involved is unknown.

3.2.2.4. The direction of chain extension. The synthesis of the wall polyglycerol phosphate chains in *B. subtilis* was studied by pulse-labeling techniques (Kennedy and Shaw, 1968). The individual units of glycerol phosphate were shown to be transported from the precursor CDP-glycerol so that the newly added glycerol on the carrier was susceptible to oxidation with periodate, yielding formaldehyde. The most recently introduced glycerol phosphate unit was at the glycol end of the growing chain (Fig. 47). Thus, chain extension in teichoic acid synthesis, at least in *B. subtilis,* is similar to glycan chain extension in glycogen synthesis where the glucose units are directly transferred from nucleotide diphosphate sugar to the nonreducing end of the chain. These processes are entirely different from those of peptidoglycan synthesis (section 3.1.5.) and O-antigen synthesis (section 3.4.) that occur through undecaprenol phosphate intermediates where chain growth proceeds by extension from the reducing end of the oligosaccharide. The latter processes are comparable to the synthesis of peptide chains in proteins that occurs at the carboxyl end by transfer of the growing oligopeptide from a peptidyl tRNA to an aminoacyl-RNA.

3.2.2.5. Attachment of wall teichoic acid to peptidoglycan. When the growing (wall) teichoic acid linked to its lipoteichoic acid carrier has reached the correct length, the enzyme reaction stops and the polymer is translocated to the proper N-acetylmuramic acid residue of the peptidoglycan chain (section 2.3.2.3.). In the process, a chain of 3 to 4 glycerolphosphate residues is inserted as the link between the wall teichoic acid and the 6-position of an N-acetylmuramic acid residue of the peptidoglycan (Heckels et al., 1975; Coley et al., 1976).

With cell-free membrane preparations, teichoic acid synthesis leads to products that are not attached to peptidoglycan. Thus the question arose whether the teichoic acid in vivo is inserted to a preexisting wall peptidoglycan or to a concomitantly synthesized peptidoglycan. On the basis of elegant double-label experiments, Mauck and Glaser (1972) showed that in *B. subtilis* cells newly synthesized teichoic acid (and teichuronic acid) were linked only to peptidoglycan which had been synthesized simultaneously.

More recent experiments by Wyke and Ward (1975) performed with a cell-free membrane plus wall preparation of *B. licheniformis* largely confirmed these

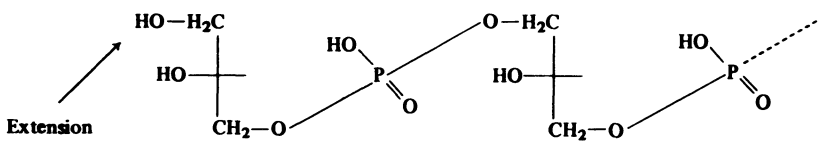

Fig. 47. Growth of a poly(glycerol phosphate) (Baddiley, 1972.)

results. Such membrane plus wall preparations synthesized teichoic acid from CDP-ribitol as well as peptidoglycan from UDP-N-acetylglucosamine and UDP-N-acetyl-muramylpentapeptide. The following observations were made: (1) Addition of penicillin to the incubation mixtures at concentrations that completely inhibited attachment of the nascent peptidoglycan to the preexisting wall peptidoglycan by transpeptidation had no effect on the synthesis of the nascent teichoic acid but caused an 81% decrease in the amount of teichoic acid linked covalently to the wall. (2) The same result was obtained when teichoic acid synthesis was carried out in the absence of added peptidoglycan precursors in the reaction mixtures. Since, as shown by Tynecka and Ward (1975), the nascent peptidoglycan synthesized by *B. licheniformis* under conditions where transpeptidation is inhibited lacks wall-associated teichoic acid, it follows that, at least with this cell-free system, the incorporation of the majority of the newly synthesized teichoic acid to the wall peptidoglycan requires the concomitant synthesis of both teichoic acid and cross-linked peptidoglycan. These observations suggest that the nascent teichoic acid made on the lipoteichoic acid assembly center could not undergo attachment to the nascent peptidoglycan made on the undecaprenol phosphate assembly center unless the latter polymer would exhibit the proper orientation or correct alignment that would be imparted by its concomitant fixation on the "old" wall peptidoglycan by transpeptidation (Fig. 48). Obviously, perfect coordination among all these events would be essential.

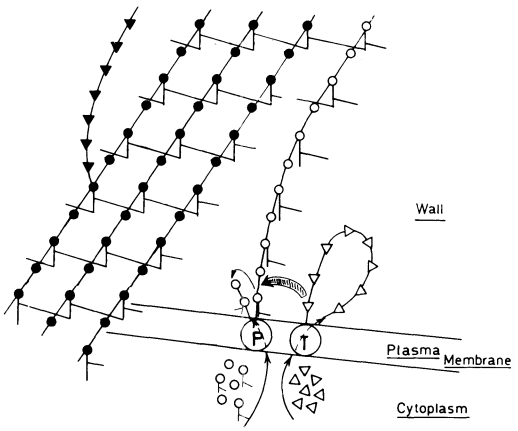

Fig. 48. Model of wall assembling in Gram-positive bacteria. In black: preexisting wall crosslinked peptidoglycan-teichoic acid complex. ●—● = peptidoglycan; ▲—▲ = teichoic acid. In white: nascent peptidoglycan (○—○) and nascent teichoic acid (△—△). ○ and ○: peptidoglycan precursors. △: teichoic acid precursors. Ⓟ: undecaprenol phosphate-containing assembly center for peptidoglycan synthesis. Elongation proceeds at the reducing terminal of the growing chain. Ⓣ: lipoteichoic acid-containing assembly center for wall teichoic acid synthesis. Elongation proceeds at the nonreducing terminal of the growing chain. The model shows the attachment of the nascent teichoic acid on a N-acetylmuramic acid residue (arrow) of the simultaneously synthesized peptidoglycan, which itself undergoes attachment to the preexisting wall peptidoglycan by peptide cross-linking. Attachment of nascent ribitol teichoic acid to nascent peptidoglycan involves an intervening 3-glycerol phosphate residues chain unit. A possible mechanism for the insertion of this link is shown in Fig. 49.

The integration and reciprocal control of peptidoglycan and wall teichoic acid syntheses at the level of the corresponding assembly centers is a major problem that remains to be solved. Recent experiments by Bracha and Glaser (1976) with *S. aureus* H, and by Hancock and Baddiley (1976) with the same and other organisms, have shed new light on the processes involved. Fig. 49 shows a possible mechanism (Hancock and Baddiley, 1976) for synthesis of the 3-glycerol phosphate residue-containing chain that links wall teichoic acid to peptidoglycan and for incorporation of teichoic acid into wall material. Peptidoglycan synthesis on the undecaprenyl phosphate carrier and wall teichoic acid synthesis (here, a polyribitol phosphate polymer) on the lipoteichoic acid carrier are represented in the upper part and the lower part, respectively, of Fig. 49. It is suggested that the acceptor for the 3-glycerol phosphate residue-containing linkage unit is the undecaprenyl-PP-disaccharide peptide intermediate. Subsequently, the completed wall teichoic acid is transferred from the lipoteichoic acid carrier and the complete unit thus formed is incorporated into a growing peptidoglycan chain and then into the wall. At present, a choice cannot be made between addition of the 3-glycerol phosphate residues linkage unit to peptidoglycan at the lipid intermediate stage or to the growing glycan chain. The mechanism proposed by Bracha and Glaser (1976) differs from that represented in Fig. 49: the oligomer which links the polyribitol phosphate to the wall peptidoglycan would contain not only glycerol phosphate but also N-acetyl-D-glucosamine.

Finally, the dynamics of the wall teichoic acid synthesis in *B. subtilis* has been studied by Archibald (1976). On the basis of the development of bacteriophage-binding properties as a result of the pulsed incorporation of teichoic acid, the newly synthesized receptors appeared to be incorporated at the inner surface of the wall and became exposed at the outer surface during subsequent growth.

3.2.3. Synthesis of acidic polysaccharides other than teichoic acids

The synthesis of most of these wall polyanions has not been studied extensively, but from what is known the process appears to resemble that described for wall teichoic acids. During logarithmic growth in phosphate-rich medium *B. subtilis* synthesizes wall teichoic acid. Under conditions of phosphate limitation, teichoic acid synthesis stops and teichuronic acid synthesis is activated. Wall insertion of teichuronic acid induced by phosphate deprivation was found to proceed exactly as wall insertion of teichoic acid in a rich medium; teichuronic acid was linked only to peptidoglycan strands synthesized at the same time as teichuronic acid was produced (Mauck and Glaser, 1972). The assembly center for teichuronic acid synthesis might be the lipoteichoic acid carrier involved in wall teichoic acid synthesis, although this has not yet been proven.

Another interesting example is that of the D-glucose and N-acetyl-D-mannosaminuronic acid-containing polymer that occurs in the walls of *M. lysodeikticus* (section 2.3.2.1.). A particulate enzyme fraction isolated from this organism was shown to synthesize this polymer from the nucleotides UDP-

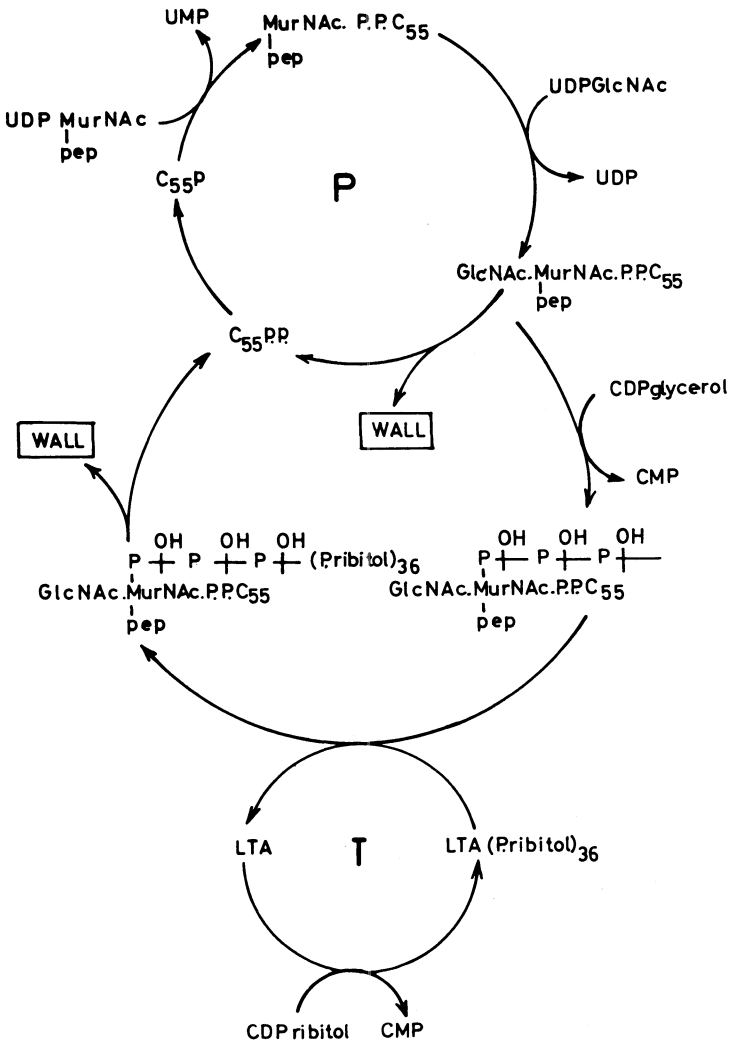

Fig. 49. Possible mechanism for the synthesis of the 3-glycerol phosphate residues linkage unit that links wall ribitol teichoic acid to peptidoglycan and for the incorporation of teichoic acid into wall material (Hancock and Baddiley, 1976.)
Ⓟ: undecaprenol phosphate-containing assembly center for peptidoglycan synthesis.
Ⓣ: lipoteichoic acid-containing assembly center for wall teichoic acid synthesis.
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glucose, UDP-N-acetylhexosaminuronic acid, and UDP-N-acetylglucosamine (Page and Anderson, 1972; Anderson et al., 1972). Glucose and N-acetylhexosaminuronic acid were incorporated in approximately equimolar amounts, and N-acetylglucosamine was incorporated only to the extent of one

residue or less for each 15 glucose residues. Wall polysaccharide contains the two former sugars in about equal amounts. Interestingly, the polysaccharide-synthesizing system required the simultaneous presence of a high molecular weight, heat-stable factor. Although the data suggested that this factor might play an acceptor role, its nature is still unknown. At present, it is also not known whether the membrane-associated succinylated lipomannan might play a role in the synthesis of this wall polysaccharide analogous to that of the lipoteichoic acid carrier in wall teichoic acid synthesis.

Synthesis of the membrane-bound succinylated mannan in *M. lysodeikticus* was elucidated by the important studies of Lennarz and coworkers who established that the carrier lipid mannosyl-1-phosphorylundecaprenol is an obligatory intermediate (Scher et al., 1968; Lahav et al., 1969; Scher and Lennarz, 1969; Lennarz and Scher, 1972). Synthesis involves transfer of mannosyl groups from GDP-mannose to the carrier lipid and is followed by subsequent transfer of hexose from mannosyl-1-phosphorylundecaprenol to mannan acceptor, the majority of the mannose units thus incorporated being located on the nonreducing termini of mannan (Scher and Lennarz, 1969). Thus, both peptidoglycan and mannan in this organism are synthesized on the same undecaprenol phosphate-containing assembly centers. Mannan has been localized in the plasma membrane and in the mesosomal membranes of *M. lysodeikticus* (Owen and Freer, 1972). It was therefore tempting to explore the possibility that its synthesis might be localized in the mesosomes, providing a unique enzymatic function for these structures. Owen and Salton (1975b,c) found that unlike the plasma membrane isolated mesosome vesicles could not catalyze the transfer of mannose from GDP-mannose into mannan because of the inability of the vesicles to synthesize the intermediate mannosyl-1-phosphorylundecaprenol. This situation was not attributed to the absence of undecaprenol phosphate in the mesosome vesicles but rather to the absence of the enzyme mannosyl-1-phosphorylundecaprenol synthetase in these organelles. Since the mannan located on both mesosomal and plasma membranes can accept mannosyl units, it was suggested that the juxtaposition of mesosomal vesicles and mesosomal sacculus (i.e., the region of the plasma membrane that surrounds the mesosomal vesicles) in vivo might allow the mannan located on the mesosomal vesicles to accept mannosyl units either from the carrier lipid of the sacculus membrane or by direct carrier lipid exchange between the two membrane systems (Owen and Salton, 1975c). Thus it is significant that newly synthesized bactoprenol has been shown to be localized mainly in the septal region of *Lactobacillus* cells (Thorne et al., 1974) that have just undergone division, and that in many bacteria mesosomes are closely associated with the growing cross wall.

3.3. Biosynthesis of the lipoprotein of the outer membrane of E. coli

Biosynthesis of the outer membrane lipoprotein is a sequential process. The free

form is synthesized first and the newly synthesized free form is effectively incorporated into the outer membrane without accumulating in the inner membrane (Inouye et al., 1972, 1974; Lee and Inouye, 1974). Part of the free form is then converted as the peptidoglycan-bound form. A dynamic equilibrium may exist between the two forms (Inouye et al., 1972).

3.3.1. Synthesis of the free form

In vivo biosynthesis of the free lipoprotein presents several striking features: (1) the extraordinarily long half-life of the relevant mRNA (Hirashima and Inouye, 1973; Hirashima et al., 1973a); (2) the extremely strong resistance of the synthesizing enzyme system to puromycin (Hirashima et al., 1973a); and (3) the exclusive synthesis of lipoprotein in cells deprived of histidine (Hirashima et al., 1973a). A cell-free system was devised so that the lipoprotein synthesis was directed by the purified relevant mRNA. (Hirashima et al., 1974). The translation product was characterized as the protein moiety of the lipoprotein by immunoprecipitation and peptide mapping. Using slab-gel electrophoresis the mRNA purified from exponentially growing cells gave rise to two closely adjacent bands migrating similar to 7s RNA. Band 1 consisted of about 250 and band 2 of about 230 nucleotides. Whether or not both of these RNAs have the lipoprotein-specific mRNA activity is not known. Nevertheless, since lipoprotein contains 58 amino acid residues, at least 180 nucleotides are required for its synthesis (i.e., 3×58; + 1 for initiation codon; + 1 for termination codon) and thus the mRNA should contain 50 to 70 untranslated nucleotides. These nucleotides might be used for the ribosome recognition site at the 5′ end of the mRNA and for the termination signal at the 3′ end. Whether the amino-terminal end of the protein is modified in the cell-free system is also unknown. When radioactive palmitic acid was added to the incubation mixture, its incorporation into product could not be detected. Since in a cell-free protein synthesizing system the initiator amino acid, N-formyl-methionine, is known to remain at the N-terminal of the product, perhaps N-formyl-methionine should first be removed from the synthesized protein portion of lipoprotein to allow palmitic acid to undergo attachment. Recent results have suggested that the synthesis of the apoprotein portion of the lipoprotein proceeds independently of the attachment of diglyceride to the SH group of the N-terminal cysteine (Jung-Ching Lin and Wu, 1976) and that the protein is transferred to the outer membrane and linked to the peptidoglycan (see section 3.3.2) at least in the absence of glyceride-linked portion of the lipoprotein lipids (Suzuki et al., 1976). With the cell-free system, mRNA and ribosomes were required for lipoprotein synthesis. Since the mRNA was relatively small, the size of the polyribosomes involved in the synthesis was also expected to be small. This prediction proved to be right. Fractionation of the polyribosomes showed that lipoprotein synthesis was carried out on relatively small polyribosomes, possibly tri- or tetra-ribosomes (Hirashima and Inouye, 1975).

Many intriguing questions remain: (1) Are the polyribosomes membrane-

bound? It is well established that in eukaryotic cells, secreted proteins are synthesized on membrane-bound polysomes. Randall and Hardy (1977) showed that the major products of protein synthesis in vitro by membrane-bound polysomes from *E. coli* included proteins of the outer membrane and the maltose-binding protein, a typical secreted periplasmic protein. On the contrary, the major product synthesized in vitro by free polysomes of *E. coli* was characterized as the ribosomal elongation factor Tu, a cytoplasmic protein for which a peripheral location at the inner surface of the plasma membrane has been postulated (Jacobson and Rosenbusch, 1976a,b; Jacobson et al., 1976). Randall and Hardy (1977) also found that the activity of membrane-bound polysomes in vitro was more resistant to puromycin than was the activity of free polysomes and in addition, the mRNA associated with membrane-bound polysomes was more stable than the bulk of cellular mRNA. (2) Which structural feature of the lipoprotein is responsible for the extreme distribution coefficient that the lipoprotein exhibits in favor of the outer membrane? (3) What is the molecular basis for the extreme resistance of the lipoprotein synthesizing system toward puromycin? In the presence of 800 μg/ml of puromycin, lipoprotein synthesis was inhibited by only 30% (Hirashima et al., 1973a). One could hypothesize that because of some sort of compartmentalization membrane-bound ribosomes would behave differently or would be less accessible to puromycin. (4) What is the molecular basis for the very long half-life of the lipoprotein mRNA? The half-life for this mRNA is 11.5 minutes, a value that can be compared with 2 minutes for the cytoplasmic proteins mRNAs and 5.5 minutes for the envelope proteins mRNAs. Obviously, this small lipoprotein mRNA is worth sequencing.

3.3.2. Attachment of the free form to the peptidoglycan

The following mechanism is hypothetical. Attachment of lipoprotein to the underlying peptidoglycan might proceed by a transpeptidation reaction in which the free lipoprotein molecules would act as the acceptors and tetrapeptide units L-Ala-D-Glu┌*meso*-A_2pm-D-Ala of the peptidoglycan would act as the donors. The reaction would be catalyzed by an LD-carboxypeptidase-transpeptidase system as shown in Fig. 50. Tripeptide units L-Ala-D-Glu┌*meso*-A_2pm would be the products of the hydrolysis pathway of the reaction (such tripeptide units are actually present in the completed wall peptidoglycan), and the ratio between the tripeptide units and peptidoglycan-bound lipoproteins might depend on the availability of water to the enzyme complex. The tetrapeptide units assumed to be involved as donors in the reaction are themselves products of the hydrolysis pathway of the membrane-bound DD-carboxypeptidase-transpeptidase system (section 3.1.5.6.).

The attachment process in *E. coli* is unaffected by benzylpenicillin and penicillin FL-1060. Penicillin FL-1060 has stimulated interest. Under the influence of this antibiotic peptidoglycan is at most 50% inhibited, as if the inhibition of an enzymatic step of peptidoglycan synthesis could be partially bypassed by a second enzyme, less efficient but resistant to penicillin FL-1060. The rate of lipo-

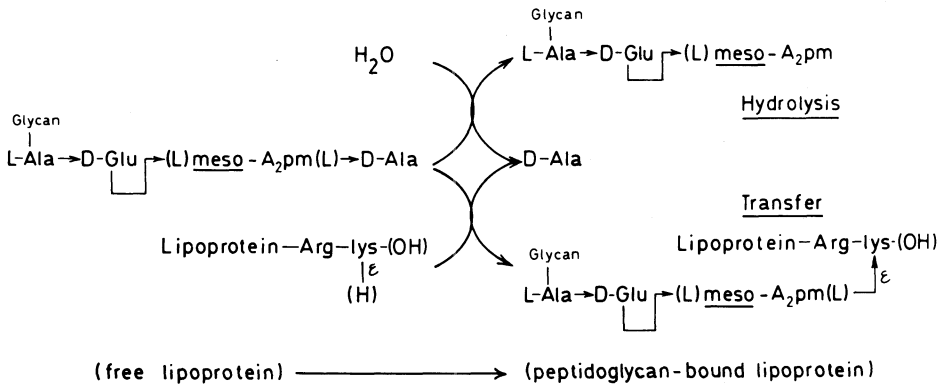

Fig. 50. Possible mechanism for the enzymatic attachment of lipoprotein to peptidoglycan.

protein attachment is far less affected, and therefore increasing amounts of lipoprotein become attached with time. When the stationary growth phase has been reached, the lipoprotein content of the peptidoglycan has doubled (Braun and Wolff, 1975).

Recent studies by James and Gudas (1976) have shown that the incorporation of free lipoprotein into the outer membrane of *E. coli* is a cell cycle-specific process with a maximal rate of incorporation occurring at the time of septation. Since no corresponding increase in the rate of incorporation of bound lipoprotein during the cell cycle was observed, an appreciable decrease in the ratio of bound to free lipoprotein must occur shortly before septation. This observed phenomenon may be relevant to the separation of outer membrane and peptidoglycan layer observed at an early stage in septation. Burdett and Murray (1975) have shown that the ingrowing septum consists of a fold of plasma membrane plus peptidoglycan, a double structure composed of two opposed lamellae separated by an electron-transparent gap. Clearly, the outer membrane is absent and only enters the septum during cell separation.

The lipoprotein attachment process presents other intriguing peculiarities. *E. coli* cells were pulse-labeled for 5 minutes with [^{3}H]arginine and then chased with nonradioactive arginine for several cell-doubling times. After one cell-doubling time, 40% of the newly synthesized lipoprotein was attached to peptidoglycan, and this percentage remained constant following 2 and 3 cell-doubling times (Braun and Wolff, 1975). These observations are not understood. They could suggest that there is a reversible conversion of the free and bound forms of the lipoprotein and that the newly synthesized lipoprotein mixes with a large pool of the free form. Alternatively, 40% of the newly synthesized free form may become irreversibly attached to the peptidoglycan in one doubling time, whereas the rest would undergo compartmentalization in some areas of the cell envelope where it would no longer become attached to peptidoglycan

(Braun, 1975). As mentioned earlier the peptidoglycan units newly inserted into the preexisting wall peptidoglycan appear very slowly at lipoprotein attachment sites (section 3.1.5.6.). Peptidoglycan extension is confined to areas without attached lipoprotein. The enzyme system responsible for peptidoglycan extension may be hindered sterically at these attachment sites. Regardless of the precise situation, it seems clear that the redistribution of the bound lipoproteins over the peptidoglycan sacculus must result from the incorporation of lipoprotein molecules into the newly synthesized peptidoglycan region (Braun and Wolff, 1975).

3.4. Lipopolysaccharide biosynthesis

Biosynthesis of lipopolysaccharide is peculiar because the core oligosaccharide is made by stepwise extension of lipid A whereas the O-side chains are independently synthesized. Joining of the two preformed macromolecules then occurs and the resulting complete structure that has not yet left the plasma membrane is translocated to the outer membrane. The enzymes that perform the O-side chain syntheses are localized exclusively in the plasma membrane (Osborn et al., 1972a,b). Pulse-chase experiments with radioactive mannose (a specific precursor of the O-antigen) also showed that about 70% of the mannose incorporated into the O-antigen chains during a 1-minute pulse with [^{14}C]mannose was in the plasma membrane and that this pulse-labeled lipopolysaccharide was completely chased in the outer membrane during a subsequent 2-minute chase with nonradioactive mannose (Osborn et al., 1974).

The core-synthesizing enzymes are not firmly bound to the plasma membrane. They are released in soluble form during membrane preparation and thus cannot be used as markers for the synthesis site. Pulse-chase experiments, however, demonstrated that the plasma membrane is also the place where core assembly proceeds (Osborn et al., 1974).

Mutants have been extremely useful for unraveling the structure of lipopolysaccharides (section 2.7.2.). They are also extremely useful in devising cell-free systems for the study of biosynthesis. While it would be impossible to show in vitro transfers of lipopolysaccharide constituents in wild-type cells because of lack of empty sites for incorporation, the cell envelope fractions of mutants containing all its lipopolysaccharide in an incomplete form are ideal acceptor substrates for incorporation experiments. Only the principles of the biosynthesis pathways are presented here. Details and a complete reference list can be found in an excellent review by Nikaido (1973).

3.4.1. The sugar nucleotide precursors

The sugar nucleotide precursors are synthesized by soluble, cytoplasmic enzymes. The pathways for the synthesis of the various sugar nucleotide precur-

sors in *S. typhimurium* are known (Nikaido, 1973). In particular, the nucleotide precursor for KDO incorporation is CMP-KDO (Heath et al., 1966). KDO arises from arabinose through the action of a KDO-8-P synthetase according to the reaction D-arabinose-PP + phosphoenolpyruvate → KDO-8-P + P (Rick and Osborn, 1972). The nucleotide diphosphate derivative of L-glycero-D-mannoheptose has not yet been isolated, but because a transketolase is needed for heptose synthesis it is thought that sedoheptulose-7-phosphate is an obligatory precursor (Eidels and Osborn, 1971). Radioactive sedoheptulose-7-phosphate was shown to label specifically the L-glycero-D-mannoheptose residues of the lipopolysaccharide of a transketolase-negative mutant of *S. typhimurium* (Eidels and Osborn, 1974). Synthesis of the nucleotide of L-glycero-D-mannoheptose would proceed through the following pathway:

$$\text{SODOHEPTULOSE}-7-\text{P} \xrightleftharpoons{\textit{isomerase}} \text{D}-\text{GLYCERO-MANNOHEPTOSE}-7-\text{P} \xrightleftharpoons{\textit{mutase}}$$

$$\text{D-GLYCERO-D-MANNOHEPTOSE}-1-\text{P} \xrightleftharpoons{\textit{NDP-heptose synthetase + NTP}}$$

$$\text{NDP-D-GLYCERO-D-MANNOHEPTOSE} + \text{PP} \xrightleftharpoons{\textit{epimerase}}$$

$$\text{NDP-L-GLYCEROL-D-MANNOHEPTOSE}$$

(NTP = NUCLEOTIDE TRIPHOSPHATE; NDP = NUCLEOTIDE DIPHOSPHATE).

3.4.2. Synthesis of the core-lipid A moiety

Very little is known about the synthesis mechanism of lipid A (for the structure of lipid A, see Fig. 30). Presumably, specific transferases move each fatty acid to the correct location on the glucosamine disaccharide skeleton. Since 3-hydroxytetradecanoic acid is a specific component of lipid A, its transfer to lipid A preparations were attempted but were unsuccessful. 3-Hydroxytetradecanoic acid, could, however, be transferred from its acyl carrier protein derivative to lysophosphatidylethanolamine, but no evidence was obtained that the resulting product would transfer the fatty acid to lipid A (Taylor and Heath, 1969).

The synthesis of the heptose and KDO containing parts, the inner part of the core lipopolysaccharide (Fig. 28), is also obscure. The first step is probably the transfer on lipid A of a KDO residue from CMP-KDO (Heath et al., 1966). A glucosamine oligosaccharide in which the amino groups were substituted by 3-hydroxytetradecanoic acid residues was shown to be a very good acceptor. This observation might suggest that KDO is transferred before the glucosamine residues are O-acylated. The mechanisms of addition of the two remaining KDO residues and of the heptose moieties are unknown. Phosphorylation is catalyzed at the expense of ATP by an enzyme that can be released by washing EDTA-

lysozyme treated cells (Mühlradt et al., 1968; Mühlradt, 1971). Based on the acceptor efficiency of various defective lipopolysaccharides the transfer of the first glucose residue of the outer core might precede phosphorylation of the inner core.

The synthesis of the lipopolysaccharide outer core (Fig. 28) has elicited much interest (Romeo et al., 1970; Hinckley et al., 1972; Müller et al., 1972). It proceeds through a series of conventional glycosyl transferase reactions, each residue being transferred from its proper nucleotide precursor directly to the nonreducing terminal of the growing polymer. Concomitantly, the corresponding nucleotide diphosphate is released. The involvement of undecaprenylphosphate or of other intermediate carriers between the sugar nucleotides and the outer core of the lipopolysaccharide has been excluded. Purified defective lipopolysaccharides (from R_C mutants; see Fig. 31) were completely inactive as acceptors, but activity could be restored by readding the phospholipids extracted from the cell envelope. Actually, the (incomplete) lipopolysaccharide-phospholipid complex but not the (incomplete) lipopolysaccharide alone is the acceptor for the glycosyl transfer reactions (in the presence of Mg^{2+}). Ternary lipopolysaccharide-phospholipid-transferase complexes were isolated by isopyknic sucrose gradient centrifugation and were shown to interact with the relevant nucleotide precursors with transfer of the glycosyl residues. Several transferases were isolated and purified to homogeneity (mol. wt., approximately 20,000). The galactosyl transferase contains glucosamine, perhaps neutral sugars, and a lipid of unknown structure that is probably responsible for the tendency of the transferase to aggregate in an aqueous environment. The efficiency of various phospholipids in transferase reactions has been studied. Phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid, are active, and the fatty acid composition also exerts a strong influence.

A model for the assembly of the outer core has been proposed by Rothfield and Romeo (1971). Lipid A is first synthesized and anchored in the plasma membrane by insertion of its fatty acid chains into the membrane phospholipids. Lipid A with the added inner core has surface mobility. When it encounters a transferase a specific interaction occurs, resulting in the formation of a ternary complex. If the proper sugar nucleotide is unavailable, the complex is blocked (complexes of this type are released from the cells by EDTA treatment). If the proper sugar nucleotide is available, the transfer reaction occurs, the complex dissociates, and the released lipopolysaccharide is then ready to bind with the transferase thus catalyzing the next step in the sequence (Fig. 51). In this model, the incomplete lipopolysaccharide is assumed to move from one transferase to the next. Consequently, the enzymes must be aligned in correct order. It may be that the transferases interact specifically with each other or that the multienzyme system contains a structural protein to which the transferases are attached in the required sequence.

3.4.3. Synthesis of the O-side chains

The O-side chains are assembled independently of the other portions of the

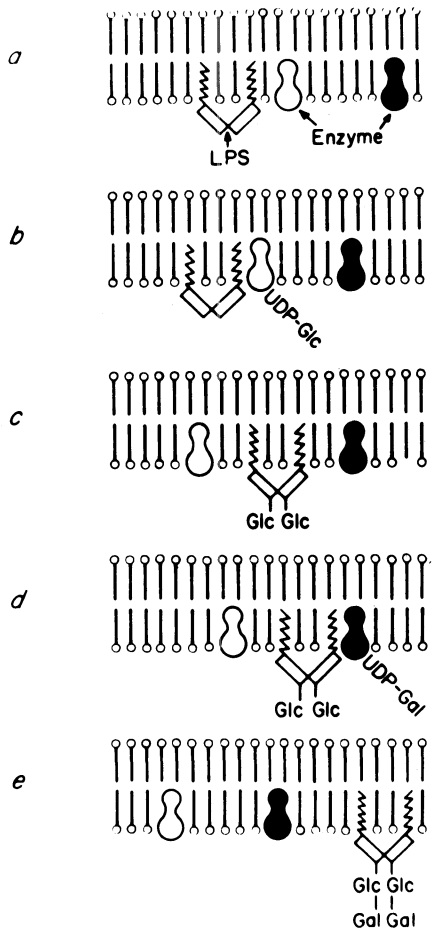

Fig. 51. Mechanism of core oligosaccharide extension on the surface of the membrane. The sequence of events beginning at (a) and ending at (e) are shown, which results in the successive transfer of glucose and galactose residues. It is based on the proposal of Rothfield and Romeo (1971). In essence, incomplete lipopolysaccharide molecules are supposed to move along the phospholipid bilayer membrane, and to accept sugar residues when they encounter transferase enzymes which are half buried in the bilayer. Possibly, transferase enzymes are arranged in the correct order through the formation of a protein aggregate within the membrane; this possibility is not shown here. (Nikaido, 1973.) (Reprinted courtesy of Marcel Dekker, New York.)

lipopolysaccharide. Their synthesis involves a membrane-bound multienzyme system and the same undecaprenyl-phosphate lipid carrier (also called glycosyl carrier lipid or P-GCL) as that involved in peptidoglycan synthesis (Bray and Robbins, 1967; Robbins and Wright, 1971; Lennarz and Scher, 1972; Osborn, 1971). The reaction sequence is analogous to that leading to the formation of the nascent peptidoglycan (section 3.1.4.). The first reaction is a transphosphory-

lation. Galactose 1-phosphate is transferred from UDP-galactose to the undecaprenyl-phosphate carrier with formation of galactose-PP-undecaprenyl and release of UMP. The next sugar residues are then added sequentially by a series of transglycosylation reactions (with, in each case, the release of the corresponding nucleotide diphsophate) until the O-side chain repeating unit is completed. Through a series of lipid cycles, where each newly synthesized repeating unit linked to the lipid accepts the growing chain made during the preceding runs, a long-chain polymeric intermediate is synthesized while remaining linked at its reducing terminal to the lipid carrier (see Fig. 52, and compare with Fig. 37 for synthesis of the nascent peptidoglycan). After each transfer, the PP-lipid carrier is liberated and the active monoester coenzyme is regenerated by a pyrophosphatase. Like peptidoglycan synthesis, O-side chain synthesis is sensitive to bacitracin.

3.4.4. Assembly of the complete lipopolysaccharide and its transfer to the outer membrane

Transfer of the polymerized O-side chains to the lipopolysaccharide core is catalyzed by an O-antigen: lipopolysaccharide ligase (Cynkin and Osborn, 1968). The mechanism is far from being understood and probably involves a series of reactions. Sonicated cell envelope fraction from *S. typhimurium* can transfer in vitro incomplete repeating units (Nikaido, 1973). This situation never occurs in intact cells, suggesting that control mechanisms exist that preclude premature transfers.

After the joining of the two preformed macromolecules, the resulting complete lipopolysaccharide molecules are translocated into the outer membrane through the adhesion sites described in section 2.10. Once they have emerged from these sites, the lipopolysaccharides are distributed over the entire surface of the cell with preferential localization on the outside face of the outer membrane (Kulpa and Leive, 1972, 1976; Bayer, 1974, 1975; Mühlradt et al., 1973, 1974; Mühlradt, 1976). In his study, Bayer made use of a phenomenon known as "lysogenic conversion" where the O-antigen composition of the cell can be rapidly changed as a result of infection with a given bacteriophage. As a consequence of infection with phage $\epsilon 15$, the original O-antigen 10 of *Salmonella anatum* is rapidly diluted by increasing amounts of O-antigen 15. This conversion provides a natural marker for newly synthesized O-antigens either by using immunologic techniques or because the new antigen serves as a receptor for phage $\epsilon 34$ but not for phage $\epsilon 15$ (i.e., the converting phage deprives itself of new adsorption sites while it induces the synthesis of receptor for $\epsilon 34$). Mühlradt and his colleagues used a *Salmonella typhimurium* strain lacking UDP-galactose-4-epimerase. This mutant bears incomplete lipopolysaccharide, and produces the wild-type lipopolysaccharide only after addition of galactose to the medium. After this addition, the preexisting incomplete lipopolysaccharide molecules remain unfinished and only the newly synthesized molecules carry the

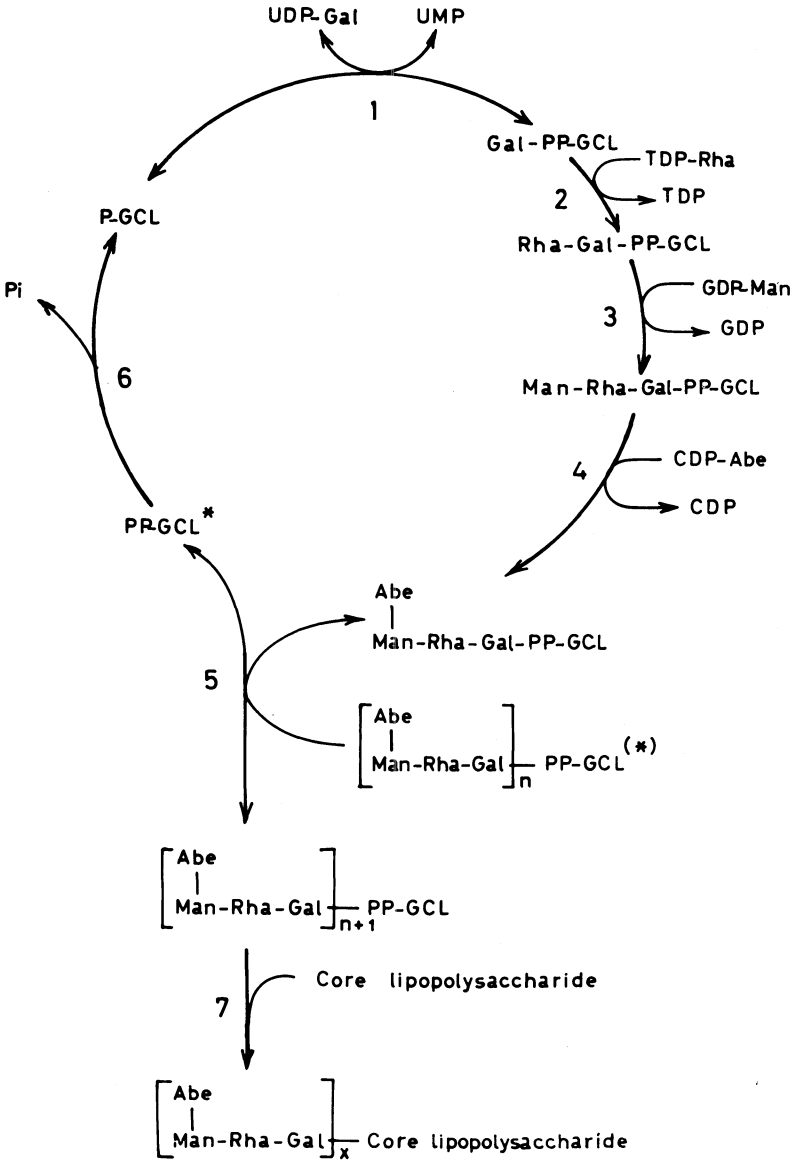

Fig. 52. Pathway of biosynthesis of O-antigen in *S. typhimurium* (reactions 1–6) and transfer of the completed O-side chain to the preformed core lipopolysaccharide (reaction 7). Note that the growing chain made during the preceding runs

Abe

$[\text{Man-Rha-Gall}]_n$ is transferred from the lipid carrier to the nonreducing end of the newly synthesized repeating unit linked to the lipid.

wild-type specificity, providing a means for their specific localization by using ferritin-conjugated antibodies. Finally, Kulpa and Leive used a mutant of *E. coli* that also lacks UDP-galactose-4-epimerase, and thus produces a lipopolysaccharide with less carbohydrate than the parent unless galactose is added exogenously. Because outer membrane fragments containing carbohydrate-rich lipopolysaccharide are denser than fragments containing carbohydrate-poor lipopolysaccharide, the "insertion" points through which newly synthesized lipopolysaccharide enters the outer membrane can be isolated by isopyknic centrifugation. This technique should make it possible to determine how these areas differ in composition and function from the remainder of the outer membrane.

The three methods used, each with very different limitations, have supported the same conclusions, at least on a qualitative level. In *Salmonella typhimurium,* newly synthesized lipopolysaccharides were seen to appear in about 200 patches on the exterior of each cell 30 seconds after addition of galactose. These patches always occurred over the adhesion sites, and after 2 to 3 minutes of growth the labeled molecules were seen evenly spread over the entire cell surface. In both *S. anatum* and *E. coli* the process was different in that export occurred at a limited number of sites (10–50 per cell) and complete spreading over the cell surface took 10 to 20 minutes.

It should be emphasized that the translocation and integration processes undergone by the lipopolysaccharide and the lipoprotein may also be used for all the other constituent elements of the outer membrane. The outer membrane is assembled from components that are synthesized elsewhere. For example, the outer membrane phospholipids are also synthesized in the plasma membrane and are secondarily translocated to the outer membrane (White et al., 1971). Translocation of pulse-labeled phosphatidylethanolamine from the plasma to the outer membrane is essentially complete within about 7 minutes, and the extent of translocation corresponds to the theoretical value from the known distribution of the phospholipids between the two membranes (section 2.2.3.). At present, the molecular bases for these unidirectional translocations into the outer membrane are not understood. Obviously they represent one of the major areas for future research.

4. Conclusions

An attempt has been made in this chapter to assess the present state of progress in the field of assembly and synthesis of bacterial walls. Thus, it might be appropriate to try to predict how research in this area might develop within the next decade or so. There are number of questions to be answered.

Undoubtedly, the exact functioning, reciprocal control, and coordination of the multienzyme assembly centers for the various wall polymers in the microenvironment of the plasma membrane, the exact functioning, regulation, and triggering of the autolytic system in a specific cell area for a defined time period during the cell life cycle, and the integration of all these activities in terms of cell shape expression and determination are essential topics that will continue to stimulate increasing interest.

The operating units in the various wall assembly centers of the plasma membrane are obviously enzymes. Some of these enzymes are known to be the targets of important antibiotics. Thus, β-lactam antibiotics prevent the functioning of the DD-carboxypeptidase-transpeptidase enzyme systems. Other enzymes may be potential targets for new antimicrobial agents. At present, however, none of these membrane-bound enzymes has been fully characterized or even purified to strict homogeneity. Studies of these fascinating enzymes with respect to amino acid sequence, conformation (both in their natural environment and outside), distribution of hydrophobic and hydrophilic portions, mechanisms of the reactions catalyzed, structure and conformation of active centers, requirement for phospholipid, regulation, and so on leave unanswered questions. In light of our present knowledge, future advances must lead to substantial and gratifying developments and, perhaps, to entirely unexpected discoveries.

All the wall components of the gram-negative bacteria, except the peptidoglycan, are part of a membrane unit which is the outermost structure of the cell. The unidirectional translocation of these components from the plasma membrane where they are made to the exterior of the cell where they are eventually assembled and form a relatively fluid and extremely asymmetric structure, is obviously the expression of a very high distribution coefficient that these components exhibit in favor of the outer membrane. The molecular basis of this astonishing behavior is entirely unknown. Is this a purely physical phenomenon? Do carriers serve as shuttles between the two membranes involved in the process? What is the exact nature of the mixed or fused regions that form the adhesion sites between the plasma and the outer membranes. Do these sites, which appear to be export sites for the lipopolysaccharide, fulfill the same function for the other components? Satisfying answers to any of these questions, and others, cannot be ventured at present.

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