2

Functions of Subcellular Structures

2.1. BACTERIAL WALLS AND MEMBRANES

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STUDIES of the structure and biosynthesis of bacterial walls are intimately associated with the mechanism of action of several antibacterial agents.

2.1.1. *Bacterial Walls*

The cell envelope of gram-positive bacteria is composed of two separate and distinct structures: a thick (200–800 Å) external layer constituting the outer wall, and an alternating electron-dense electron-transparent layer representing the inner plasma membrane. Although isolated walls viewed under the electron microscope generally appear as empty and rather amorphous bags retaining the size and shape of the original cell, their complex multilayered structure has recently been revealed with the aid of improved sectioning and staining techniques. One or more outermost layers are interlinked in a specific and organized manner and contain the chemical determinants responsible for the immunological and phage fixation properties of the cells. These are bound to a deep rigid layer, about 100 Å thick, which accounts for about 50% of the weight of the isolated wall. The rigid layer of the gram-negative bacteria is much thinner (20–30 Å) and represents as little as 5 to 10% of the cell envelope by weight. It is sandwiched between the plasma membrane and an outer highly complex multiple-track layer of lipoproteins and lipopolysaccharides, i.e. the O-antigens or the bacterial endotoxins.

The rigid layer of the wall is found in all procaryotic cells (with the exception, however, of the halobacteria), where it functions as an insoluble, supporting structure allowing the bacteria to live under hypotonic environmental conditions. This layer is visualized as a network of glycan strands interlinked by means of peptide chains. It is thus a peptidoglycan polymer. (The terms mucopeptide, glycopeptide and murein used by some authors are synonymous with peptidoglycan.)

Although the wall peptidoglycan is an immense macromolecule completely surrounding the cell, both the glycan and the peptide moieties are small, water-soluble oligomers; rigidity and insolubility hence are properties of the intact polymer. A loss of integrity resulting from the breakdown of either the glycan or the peptide component brings about the solubilization of the entire polymer. During cell expansion and division, the safe enlargement of the net results from a strict coordination between the creation of additional receptor sites by hydrolysis and the insertion of newly synthesized building blocks into the gaps thus formed. Such

coordination in the cell machinery is a hallmark of well-balanced growth. Under normal conditions, and despite the fact that the wall is one of the most dynamic structures of the whole cell, the mechanical strength of its peptidoglycan network is never impaired.

2.1.2. *Structure of the Bacterial Wall Peptidoglycan*

In all bacterial peptidoglycans, the glycan chains consist of alternating β-1,4 linked 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine; GlNAc) and 2-acetamido-2-deoxy-3-O-(D-1-carboxyethyl)-D-glucose (N-acetylmuramic acid; MurNAc) pyranoside residues, i.e. a chitin-like structure with the exception that every other sugar is substituted by a 3-O-D-lactyl group (Fig. 1). The only possible variations encountered to date are the presence of O-acetyl groups on C6 of some of the N-acetylmuramic acid residues or the replacement of N-acetylmuramic acid by N-glycolylmuramic acid (in *Mycobacterium* sp. and related bacteria).

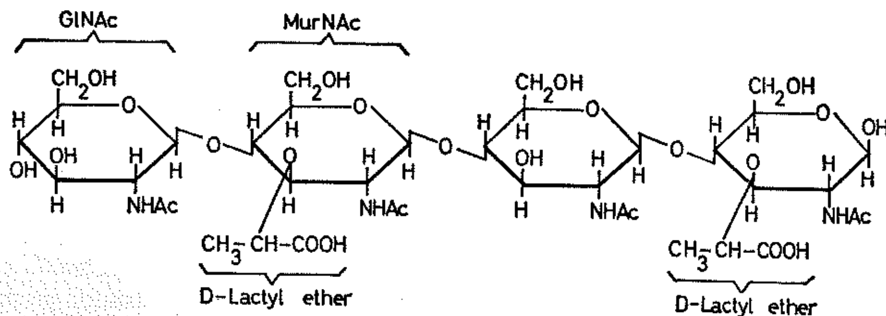

FIG. 1. A portion of a glycan strand. In the peptidoglycan network, the COOH of the D-lactyl groups are peptide-substituted.

The peptide moieties of the wall peptidoglycans are essentially composed of *tetrapeptide subunits*, which substitute through their N-termini the D-lactic acid groups of the glycans, and *peptide bridges*, which cross-link the tetrapeptide subunits so that adjacent glycan chains are paired (Figs. 2 and 3). The tightness of the net thus depends upon the length of the glycan strands, the frequency with which the glycan strands are peptide-substituted, and the frequency with which the peptide subunits are cross-linked. Depending upon the bacteria, the chain length of the glycan strands averages from 20 to 140 hexosamine residues, the percentage of peptide-substituted N-acetylmuramic acid residues in the glycan chains varies from 50 to 100, and the average size of the peptide moieties is between two and ten cross-linked peptide subunits. Evidently, many terminal groups are present in both the glycan and the peptide parts of the net. They reflect, at least in part, the dynamics of bacterial growth.

The consistency of structure seen in the glycan moiety is not reflected in the peptide substituents. The tetrapeptide subunits have the general sequence R_1-γ-D-glutamyl-R_3-D-alanine. The R_1 residue, that is, the one which substitutes the glycan chains, is usually L-alanine but can be L-serine or glycine. D-glutamic acid

----M–G–M–G----= Glycan Strand ; L-Ala→D-Glu→NH_2 γL→L-Lys→D-Ala = Peptide subunit ; $(Gly)_5$ = Peptide Bridge

FIG. 2. Monolayer representation of a portion of the wall peptidoglycan in *Staphylococcus aureus*. M = MurNAc, G = GlNAc. The arrows indicate the CO → NH direction of the linkage. Usual α-peptide linkages are represented by horizontal arrows. The peptidoglycan in gram-negative bacteria most likely occurs as a two-dimensional monolayer network (20–30 Å thick). The peptidoglycan in gram-positive bacteria (about 100 Å thick) is probably a three-dimensional multilayered network. One may visualize that some of the peptide bridges are used to interconnect several superimposed peptidoglycan sheets.

is always linked through its γ-carboxyl group to the third amino acid in the sequence. The third residue is sometimes a neutral amino acid such as homoserine; most often, however, it is a diamino acid such as L-lysine (Fig. 2), L-ornithine, LL-diaminopimelic acid or *meso*-diaminopimelic acid (Fig. 3). L-lysine and L-ornithine are linked to D-glutamic acid through their α-amino group so that the

FIG. 3. The peptide subunit and bridging in *Escherichia coli*.

ε- and δ-amino groups, respectively, are free. Both the amino and carboxyl groups of diaminopimelic acid, linked respectively to D-glutamic acid and D-alanine, are located on the same asymmetrical carbon which, in the case of *meso*-diaminopimelic acid, is the L-carbon (Fig. 3). Also depending upon the bacterial species, the α-carboxyl group of glutamic acid can be either free or amidated (the second amino acid in the sequence then being a D-isoglutamine residue) (Fig. 2), or substituted by an additional amino acid such as glycine. Similarly, the carboxyl group of the diaminopimelic acid residue not engaged in peptide bonding is either free or amidated.

The cross-linking between the tetrapeptide subunits always involves the carboxyl group of the terminal D-alanine residue of one peptide subunit and, usually but not always, the free amino group of the diamino acid of another peptide subunit. The peptide bridges may consist of direct peptide bonds (Fig. 3) such as D-alanyl-(D)-*meso*-diaminopimelic acid linkages (gram-negative bacteria; some gram-positive *Bacillaceae*), i.e. a bond extending from the amino group located on the asymmetrical D-carbon of *meso*-diaminopimelic acid of one peptide subunit to the C-terminal D-alanine residue of another. Peptide bridging between peptide subunits may also be mediated via a single additional amino acid (a D-isoasparagine residue in many *Lactobacillaceae*) or via an intervening peptide chain such as a pentaglycine (in *Staphylococcus aureus*) (Fig. 2), an L-alanyl-L-alanyl-L-alanyl-L-threonine sequence (in *Micrococcus roseus*), or one or several peptides each having the same amino-acid sequence as the peptide subunit (as in *Micrococcus lysodeikticus*). Finally, in a few bacteria such as some plant pathogenic *Corynebacteria* and *Butyribacterium rettgeri*, the peptide bridges extend from the α-carboxyl group of D-glutamic acid of one peptide subunit again to the C-terminal D-alanine of another subunit. The latter type of cross-linking necessarily involves a diamino-acid residue. D-ornithine and D-lysine were found to be involved in this type of bridging.

2.1.3. *Biological Effects of Isolated Wall Peptidoglycan*

Peptidoglycans chemically isolated by means of hot formamide from the walls of streptococci, of *Gaffkya tetragena* and of some other gram-positive cocci exert general and/or dermal endotoxin-like effects such as a rise in temperature, a fall in the number of circulating granulocytes in the blood or the growth of hard nodular lesions in rabbit dermis. 5-hydroxytryptophan may be involved in the pyrogenic response [10] and the importance of a toxic peptidoglycan in virulence has been postulated [11].

2.1.4. *Biosynthesis of Bacterial Wall Peptidoglycan*

In spite of extremely wide variations in structural details, all the bacterial peptidoglycans are probably synthesized by mechanisms which are consistent throughout the bacterial world. A multiple-stage synthesis has been proposed which takes place first inside the cell, next on the plasma membrane and finally within the growing wall [2, 3, 4].

Stage 1. The two nucleotide precursors, uridine-5′-pyrophosphoryl-N-acetylglucosamine (UDP-GlNac) and uridine-5′-pyrophosphoryl-N-acetylmuramic acid-pentapeptide (UDP-MurNAc-R_1-γ-D-Glu-R_3-D-Ala-D-Ala) (Fig. 4), are synthesized by means of soluble enzymes. UDP-MurNAc originates from UDP-GlNAc. A three-carbon fragment is transferred from 2-phosphoenol pyruvate (the glycolytic intermediate) to UDP-GlNAc and the resulting UDP-GlNAc-pyruvate enol ether is then reduced to UDP-MurNAc. The first three amino acids of the pentapeptide substituent are then added sequentially in the presence of ATP and either Mg^{++} or Mn^{++}, each step being catalysed by a specific enzyme. The last D-alanyl-D-alanine sequence, however, is added *en bloc* to the incomplete precursor UDP-MurNAc-R_1-γ-D-Glu-R_3, thus giving rise to the complete precursor UDP-MurNAc-R_1-γ-D-Glu-R_3-D-Ala-D-Ala.

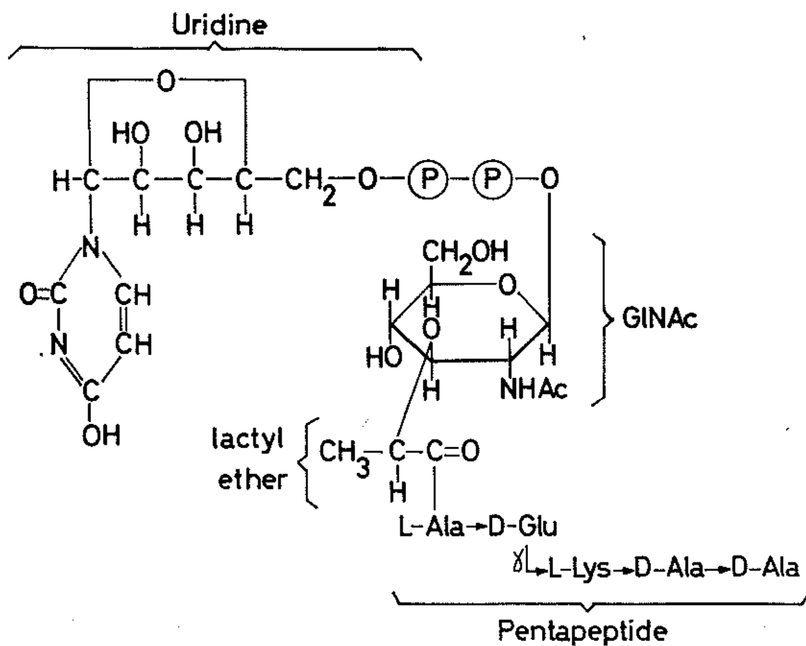

FIG. 4. The nucleotide precursors UDP-GlNAc and UDP-MurNAc-pentapeptide in *Staphylococcus aureus.* Note that D-glutamic acid is linked to the α-amino group of L-lysine through its γ-carboxyl group and that the α-carboxyl group of D-glutamic acid is free. The same precursors are found in *Escherichia coli* except that *meso*-diaminopimelic acid replaces L-lysine.

Stage 2. This stage comprises the assembly of the two nucleotide precursors into disaccharide pentapeptide units, β-1,4-GlNAc-MurNAc-pentapeptide, and their transport via an intermediate carrier to a final acceptor, i.e. the growing wall peptidoglycan (Fig. 5) Several particulate enzymes and a C_{55} polyisoprenoid alcohol phosphate, most probably located on the cytoplasmic membrane, are involved in a complex cyclic reaction that may be visualized as a sequence of three transfer reactions. First, the MurNAc (pentapeptide)-monophosphate residue

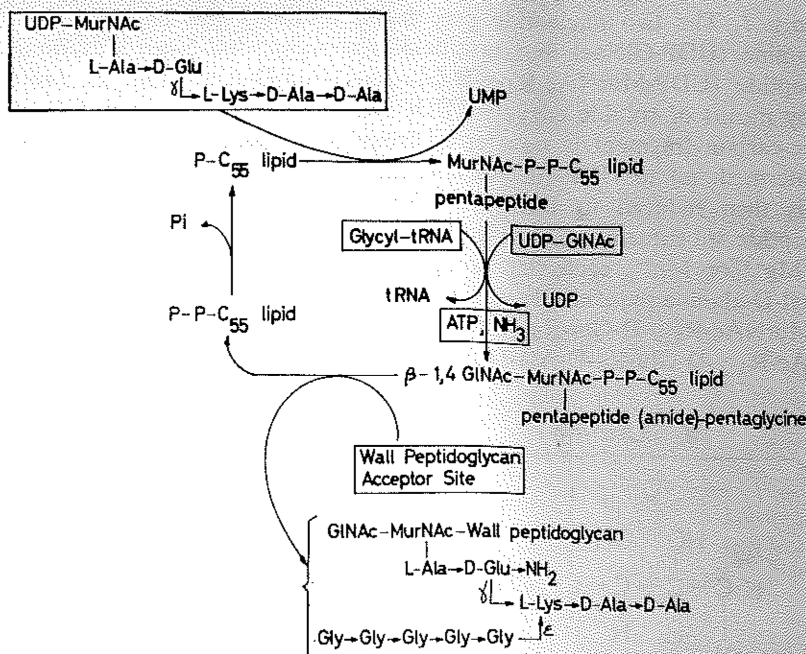

FIG. 5. The lipid cycle in *Staphylococcus aureus* with formation of uncross-linked peptidoglycan strands. In *Escherichia coli* the same lipid cycle occurs with the exception that D-glutamic acid is not amidated and that no additional amino acids are incorporated into the pentapeptide moiety.

is transferred from UDP-MurNAc pentapeptide to the P-C_{55} lipid, resulting in the formation of UMP and in the attachment of MurNAc-pentapeptide to the lipid by means of a pyrophosphate bridge (MurNAc(pentapeptide)-P-P-C_{55}). Secondly, GlNAc is transferred by transglycosylation from UDP-GlNAc with the liberation of UDP and the formation of β-1,4-GlNAc-MurNAc(pentapeptide)-P-P-C_{55} lipid. Thirdly, the disaccharide pentapeptide is transferred to the wall receptor sites (i.e. non-reducing N-acetylglucosamine termini). Concomitant with this, the liberated C_{55} lipid pyrophosphate is dephosphorylated with formation of inorganic phosphate, following which the P-C_{55} carrier can then begin a new cycle. This cyclic reaction is evidently the mechanism by which the disaccharide peptide units are transported through the plasma membrane from the intracellular sites of synthesis to the extracellular sites of incorporation.

Stage 3. In many cases, the pentapeptide moiety cannot be utilized to extend the wall peptidoglycan network without prior chemical modification. Depending upon the bacteria, these modifications may consist of the amidation of some carboxyl groups (in the presence of ATP and NH_3) (Fig. 5), the substitution of the α-carboxyl group of D-glutamic acid by one additional amino acid, or the incorporation of those amino-acid residues which in the completed peptidoglycan will function as "specialized" peptide cross-linking bridges. In *S. aureus*, for example,

pentaglycine is added to the α-amino group of L-lysine (the third amino acid in the pentapeptide sequence) while the disaccharide pentapeptide is bound to the lipid carrier (Fig. 5). The glycyl donor for the incorporation is glycyl-tRNA. Ribosomes, however, are not involved in this process, which thus differs from a "normal" protein synthesis. Again, such a mechanism is not ubiquitous. An amino-acyl-tRNA is not always required for the bridge incorporation in the pentapeptide subunit. The initial acceptor for the tRNA-dependent incorporation likewise may not be the lipid intermediate but the soluble nucleotide precursor itself.

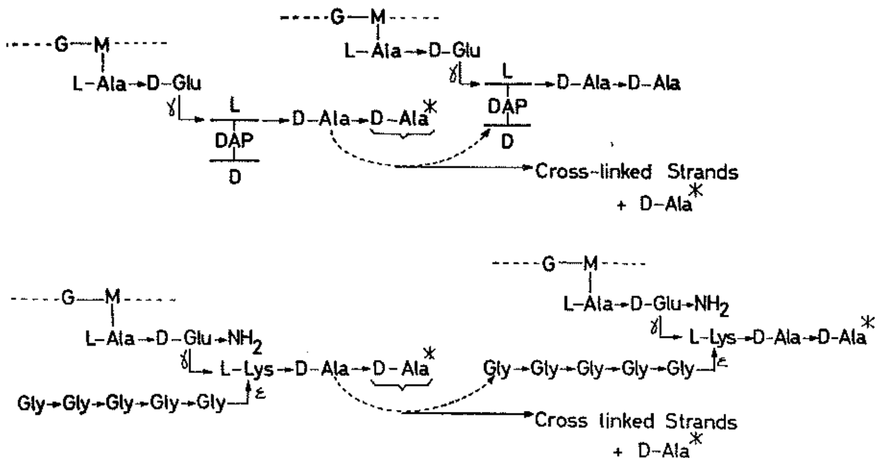

FIG. 6. The transpeptidation reaction with formation of cross-linked peptidoglycan strands and the transformation of pantapeptide units into tetrapeptides. Top: in *Escherichia coli* (cf. Fig. 3). Bottom: in *Staphylococcus aureus* (cf. Fig. 2).

Stage 4. The insertion of newly synthesized and, where necessary, suitably modified disaccharide peptide units into the growing peptidoglycan must be followed by the closure of the peptide bridges if the process is to yield a rigid, insoluble two- or three-dimensional network. This last reaction occurs outside the plasma membrane, that is at a site where ATP is not available. It has been proposed that the bridge closure between two peptide subunits results from a transpeptidation (i.e. a reaction not requiring any exogenous energy source) in which the bond energy of the terminal D-alanyl-D-alanine dipeptide of one peptide unit is utilized to transfer the carboxyl group of the penultimate D-alanine residue to the amino group acceptor of a second peptide unit, with concomitant release of the terminal D-alanine (Fig. 6). The nature of the amino-acceptor varies, of course, with the bacteria. The end product, however, is always an insoluble network of glycan chains substituted by cross-linked tetrapeptide subunits (R_1-γ-D-Glu-R_3-D-Ala), and the C-terminal D-alanine residues of these tetrapeptides are always involved in the bridging. A direct demonstration of this transpeptidation reaction has been achieved in one case, that of *Escherichia coli.* The hypothesis that this reaction is in fact a general bacterial mechanism rests upon indirect but convincing evidence.

2.1.5. *Antibiotics that interfere with the Biosynthesis of Bacterial Wall Peptidoglycan*

In the presence of certain antibiotics, the processes involved in bacterial growth continue to take place with the exception that the cells can no longer synthesize a normal rigid wall peptidoglycan. As a result, the cells are unable to withstand the high internal pressure and die of osmotic disruption.

D-cycloserine is a competitive inhibitor of both the alanine racemase which catalyses the reversible interconversion of the two optical antipodes (L-alanine → D-alanine) and of the D-alanyl-D-alanine synthetase. Consequently, D-cycloserine blocs the synthesis of the muramyl nucleotide precursor (Fig. 4) at an early stage and induces the accumulation of the incomplete precursor UDP-MurNAc-X_1-γ-D-Glu-X_3 which lacks the essential terminal D-alanyl-D-alanine dipeptide. It is believed that the molecular basis for the D-cycloserine inhibition lies in the structural analogy between the antibiotic and D-alanine (Fig. 7)[2].

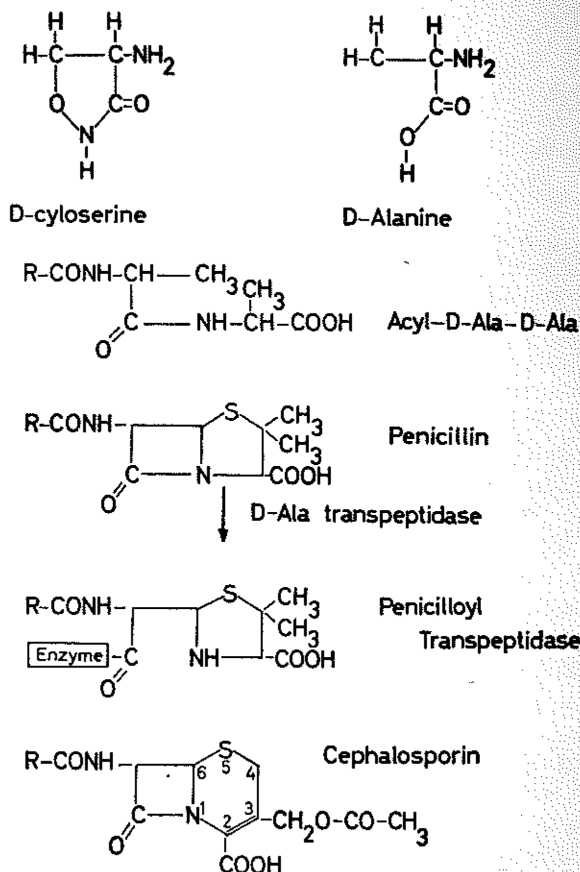

FIG. 7. Molecular basis for antibiotic actions of D-cycloserine and penicillin.

O-carbamyl-D-serine competitively inhibits the alanine racemase but does not inhibit the D-alanyl-D-alanine synthetase. In the presence of O-carbamyl-D-serine, there is accumulation of the same incomplete nucleotide precursor as in the previous case.

Vancomycin is known to combine readily with the isolated complete precursor UDP-MurNAc-X_1-γ-D-Glu-X_3-D-Ala-D-Ala whose specific combining site is the C-terminal D-alanyl-D-alanine sequence [5]. The attachment of vancomycin to the nucleotide precursor does not prevent transphosphorylation to the P-C_{55} lipid carrier (and the formation of UMP. See stage 2 of the biosynthesis, Fig. 5). However, the vancomycin-nucleotide precursor-lipid complex no longer constitutes a substrate for the transglycosylation enzymes and hence cannot be used for further syntheses. The antibiotic's chemical structure is largely unknown. It contains phenols, chlorophenols, aspartic acid, N-methylleucine and glucose, forming a complex with the isolated UDP-MurNAc-pentapeptide precursor when equimolar proportions of peptide and antibiotic (calculated on the basis of one residue each of glucose, aspartic acid and N-methylleucine) are present.

Ristocetin exerts its effects by a mechanism similar to that of vancomycin. Its chemistry is likewise not known. Like vancomycin, it is an amphoteric substance containing amino and phenolic groups and sugar residues (glucose, mannose, arabinose and rhamnose).

Bacitracin, a polypeptide antibiotic, interferes like vancomycin and ristocetin with the lipid cycle (Fig. 5), preventing the nucleotide precursors from being used to form linear uncross-linked peptidoglycan strands. Bacitracin's mechanism of action, however, is entirely different [6]. It specifically inhibits the enzymatic dephosphorylation of the pyrophosphate C_{55} polyisoprenoid alcohol which appears as the end product of the cycle reaction. As a result, the P-C_{55} lipid carrier fails to be regenerated and is thus prevented from re-entering the cycle. The molecular basis of this inhibition has not been elucidated.

Penicillins and *Cephalosporins* are specific inhibitors of the peptide bridge closure reaction (Fig. 6), so that an uncross-linked wall peptidoglycan deprived of mechanical strength is formed [2, 7, 8]. It has been proposed that penicillin G [(6-phenylacetamido) penicillanic acid, i.e. essentially an acetylated cyclic dipeptide of L-cysteine and D-valine] as well as other penicillins and cephalosporins function as analogues of the D-alanyl-D-alanine dipeptide sequence at the C terminus of the pentapeptide units (Fig. 7) [2]. The D-alanine transpeptidase that is presumed to be capable of recognizing the acyl-D-alanyl-D-alanine configuration would accept penicillin as an analogue. This would irreversibly inhibit the enzyme via acylation through the highly reactive CO–N bond in the lactam ring with the formation of a penicilloyl-transpeptidase complex.

Novobiocin, an antibiotic consisting of a substituted phenol, a substituted coumarine and noviose (the carbamate ester of a derivative of L-rhamnose), is known to induce the accumulation of uridine nucleotide precursors in bacteria when used at dose levels near the growth inhibitory concentration. However, novo-

biocin also inhibits protein and nucleic acid synthesis. It may well be that the inhibition of peptidoglycan synthesis and other effects exerted by this antibiotic result from another as yet undiscovered primary metabolic lesion [9].

2.1.6. *Conclusion*

The primary structure and biosynthesis of the bacterial wall peptidoglycans have been elucidated and are now understood at a molecular level. These studies have provided evidence for the exact mechanism of action of several highly selective antibacterial compounds. A striking feature is the importance of the D-alanyl-D-alanine sequence as a target for a number of these antibiotics. D-cycloserine and O-carbamyl-D-serine inhibit the synthesis of the dipeptide, with the result that the wall peptidoglycan nucleotide precursor remains incomplete. Vancomycin and ristocetin attach themselves to the complete nucleotide precursor due to the presence of the D-alanyl-D-alanine sequence and make it unavailable for further syntheses by membrane-bound enzymes. Finally, in the presence of penicillin and related antibiotics, the D-alanyl transpeptidase would recognize the acyl-D-alanyl-D-alanine configuration in the antibiotic and, by accepting it as an analogue, would be irreversibly denatured.

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