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# Bacterial Peptidoglycans in Relation to the Membrane and the Mechanism of Action of Penicillin

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This paper is dedicated to Professor Maurice Welsch on his 60th birthday in acknowledgement of his pioneering work* on the bacteriolytic enzymes from *Streptomyces* sp. and of his continuing stimulating support to the research group of the University of Liège.

## INTRODUCTION

The framework of the bacterial envelope is a peptidoglycan network (the terms mucopeptide, glycopeptide or murein, used by some authors, are all synonymous with peptidoglycan) composed of glycan strands that are interconnected through peptide chains (Fig. 1). Study of the topology of the layers within the bacterial envelope[1] has revealed that the peptidoglycan is sandwiched between the underlying plasma membrane and one or several superimposed outermost layers. In Gram-positive bacteria, at least, the peptidoglycan contributes to the cell-surface properties by providing the chemical groups to which the superficial polymers are covalently bound. In *Staphylococcus aureus*, for example, the N-acetylglucosamine residues of the teichoic acid are known to be essential for phage fixation but, in order to be operative, these groups must possess a definite orientation that is imparted by the binding of the teichoic acid to the supporting peptidoglycan[2]. The main function of the peptidoglycan, however, is the preservation of the integrity of the cell within its fragile cytoplasmic membrane which contains high osmotic pressure. In this respect, the obvious advantage of a net-like structure (Fig. 1) is that its mechanical strength, or perhaps better its elastic restraining structure[3], is not impaired by the hydrolysis of a limited number of linkages. The safe enlargement of the net during cell expansion and division can thus result from a strict coordination between the creation in the peptidoglycan of new receptor sites by hydrolysis and the insertion of newly synthes-

* Welsch M.: Rev. Belge Pathol. Méd. Exp. **28,** Suppl. 2, 1 (1947); Welsch M.: Pathol. Microbiol. **29,** 571 (1966).

ized building blocks into the gaps thus formed. In this process, the membrane plays roles of prime importance. It provides an important part of the biosynthetic machinery *(see below)* and at the same time, it may be involved in the protection of this machinery, by means of specialized enzymes, against the deleterious effects of some chemicals, such as penicillins, that can occur in the cell environment[4]. As the title suggests, this paper will be devoted to some properties of both the peptidoglycan and the membrane. Our intention is to rationalize these properties in terms of the functions played by each of these layers within the complex bacterial envelope.

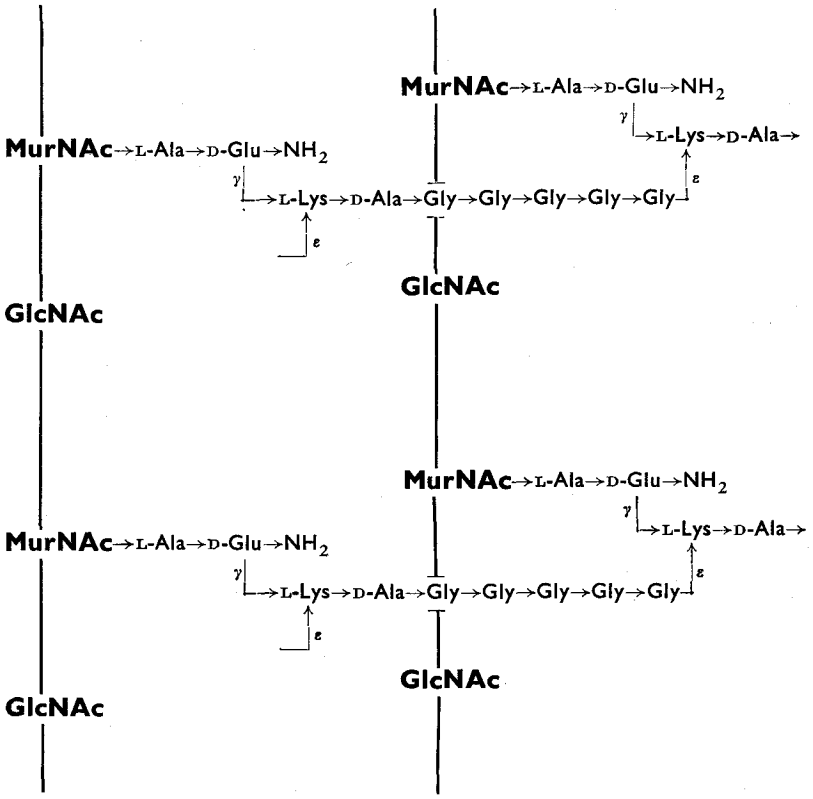


Fig. 1. The peptidoglycan net in *S. aureus* strain Copenhagen. The peptidoglycans are always composed of:

1) *glycan chains* consisting of β-1,4-linked N-acetylglucosamine (**GlcNAc**) and N-acetylmuramic acid (**MurNAc**) (*see* Fig. 2);

2) *tetrapeptide units* (in *S. aureus* L-alanyl-γ-D-*iso*glutaminyl-L-lysyl-D-alanine);

3) *peptide bridges* (in *S. aureus* pentaglycines).

The arrows indicate the CO → NH direction of linkage. α-Peptide linkages are represented by horizontal arrows.

## PRIMARY STRUCTURES OF THE PEPTIDOGLYCANS

The structural features of the bacterial peptidoglycans have been recently reviewed[5]. The main purpose of this section is to emphasize the remarkable consistency of structure exhibited by the peptidoglycans both in the glycan and in the peptide moieties.

### THE GLYCAN MOIETY

The glycan moiety is essentially a chitin-like structure formed by linear strands of $\beta$-1,4-linked N-acetylglucosamine pyranoside residues except that every other sugar is substituted on $C_3$ by a lactyl group which has the D-configuration (*i.e.* an alternating sequence of N-acetylglucosamine and N-acetylmuramic acid; Fig. 2). For a long time the $\beta$-anomery of the linkage between N-acetylmuramic acid and N-acetylglucosamine rested upon the assumption that the *endo*-N-acetylmuramidases which hydrolyze these linkages within the glycan, had a strict $\beta$-specificity. Recently[6], the $\beta$-anomery has been directly demonstrated, in *S. aureus* at least, by NMR spectroscopy of the N-acetylmuramyl-N-acetylglucosamine disaccharide isolated after ap-

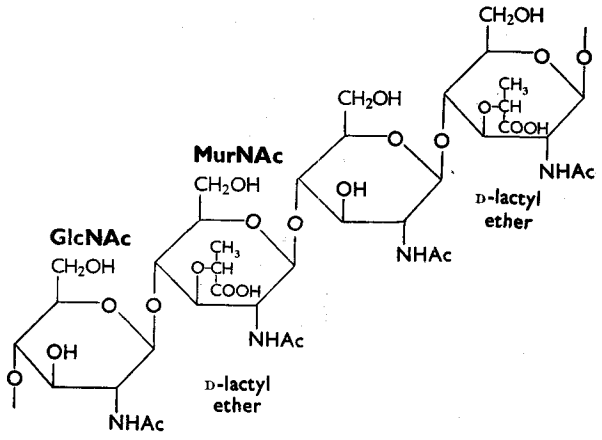


Fig. 2. A portion of a glycan strand. In the intact peptidoglycans, the tetrapeptide units substitute through their N-termini (Fig. 3), the D-lactyl groups of the glycan strands.

propriate enzymatic degradation of the walls. Glucosamine, after acid hydrolysis, has been routinely identified not only by chromatography retention time but also by means of the specific D-glucosamine-6-phosphate N-acetyltransferase. Moreover, a recent survey including a large number of Gram-negative and Gram-positive bacteria from a wide variety of taxonomic groups has revealed that only *gluco*-muramic acid occurs naturally[7,8]. No *galacto*-muramic acid could be identified. It thus appears

that the linear arrangement of β-1,4-linked 2-deoxy-D-glucose derivatives confers to the glycan backbone a conformation which is probably essential for the function of the polymer so that any mutation which would alter this conformation would probably be lethal (*galacto*-muramic acid could readily result, in principle, from the action of a 4′-epimerase upon the nucleotide precursor UDP—*gluco*-muramic acid). Alterations on $C_2$, however, that do not modify the basic conformation of the polymer may possibly occur. The presence of very small amounts of *manno*-muramic acid in walls of *Micrococcus lysodeikticus* has been briefly reported[9].

Alterations in the glycan moiety may affect the nature of the substituent on the $C_2$-amino group or they may consist of the presence of substituents on the $C_6$-hydroxyl group of muramic acid. In many Gram-positive bacteria phosphodiester bridges extending from the $C_6$ of some muramic acids are involved in the binding of the outermost polymers to the wall peptidoglycan matrix[5]. In *S. aureus*, in some strains of *M. lysodeikticus* and in *Lactobacillus acidophilus*[10], 60 to 70% of the muramic acid residues occur as N,6-O-diacetylmuramic acid. In *Mycobacteria* and *Nocardia*, N-acetylmuramic acid is replaced by N-glycolylmuramic acid[11]. In the spore peptidoglycan of *Bacilli*, about half of the muramic acid has been reported to occur in the form of a lactam derivative[12], a compound which must be under the control of a "sporulation-specific" gene since it is unique to sporulation.

Unlike chitin or cellulose, the bacterial glycan is a soluble polymer composed of chains of short length, which is unsuitable for wall function unless its strands are interlinked through peptide chains. Insolubility and mechanical strength are properties solely of the intact peptidoglycan and a loss of integrity resulting from the breakdown of either the glycan or the peptide moieties brings about the solubilization of the whole complex.

## THE PEPTIDE MOIETY

The consistency of structure found in the glycan is reflected in the peptide moiety although at first sight this unity is hidden by a wide variation in structural details.

1) The tetrapeptide units which substitute through their N-termini the D-lactic acid of the muramic acid residues in the glycan or at least of some of them, have always the general sequence L-alanyl (or sometimes L-seryl or glycyl)—γ-D-glutamyl——L-$R_3$—D-alanine (Fig. 3). The glutamyl linkage is always γ. The other linkages are always α. Depending upon the bacterial species, the L-$R_3$ residue may be L-homoserine, or a diamino acid such as L-diaminobutyric acid, L-ornithine, L-lysine, LL-diaminopimelic acid or *meso*-diaminopimelic acid. In the latter case, both the amino group linked to D-glutamic acid and the carboxyl group linked to D-alanine are located on the same asymmetric carbon, that which has the L-configuration[13].

2) The cross-linking between two tetrapeptides of adjacent glycan chains always involves the C-terminal D-alanine residue of one tetrapeptide and either the amino

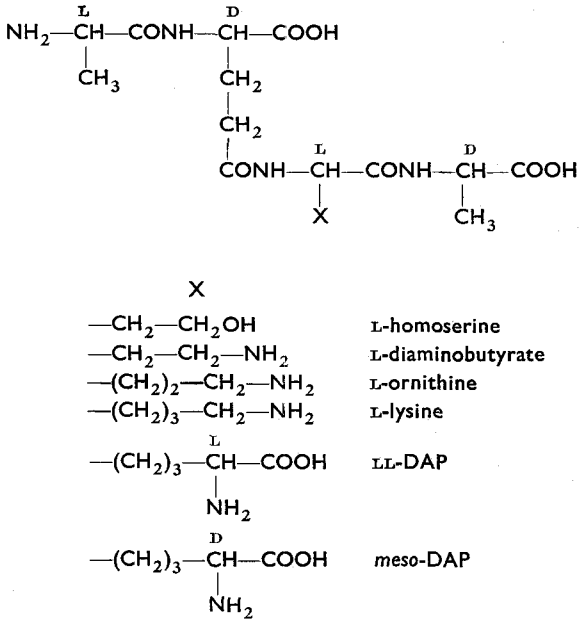


Fig. 3. Structure of the tetrapeptide units. The main variations reside in the structure of the side chain of the L-$R_3$ residue. Sometimes the N-terminal amino acid is L-serine or glycine (*see* Fig. 7).

group at the end of the side chain of the L-$R_3$-diamino acid (chemotypes I, II and III; Figs 4—6) or the α-carboxyl group of D-glutamic acid (chemotype IV; Fig. 7), of another tetrapeptide. In chemotype I, the bridge consists of a direct $N^{\omega}$-(D-alanyl—L-$R_3$) linkage. In chemotype II, one additional amino acid or an intervening short peptide extends between the two tetrapeptides. In chemotype III, a variation of chemotype II, the bridge is made up from one or several peptides each having the same amino acid sequence as the peptide subunit. In chemotype IV, one additional

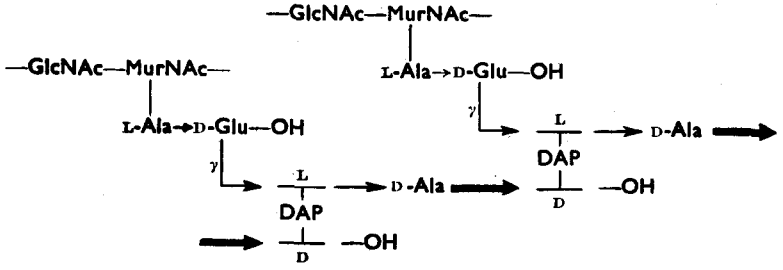


Fig. 4. Peptidoglycan of chemotype I (*E. coli*). Note that the cross-linking bridge is a direct D-alanyl-(D)-*meso*-diaminopimelic acid linkage in a C-terminal position (*heavy arrows*). In other bacteria (*i.e. Bacilli*) the COOH groups not engaged in peptide bonding, or some of them, are amidated.

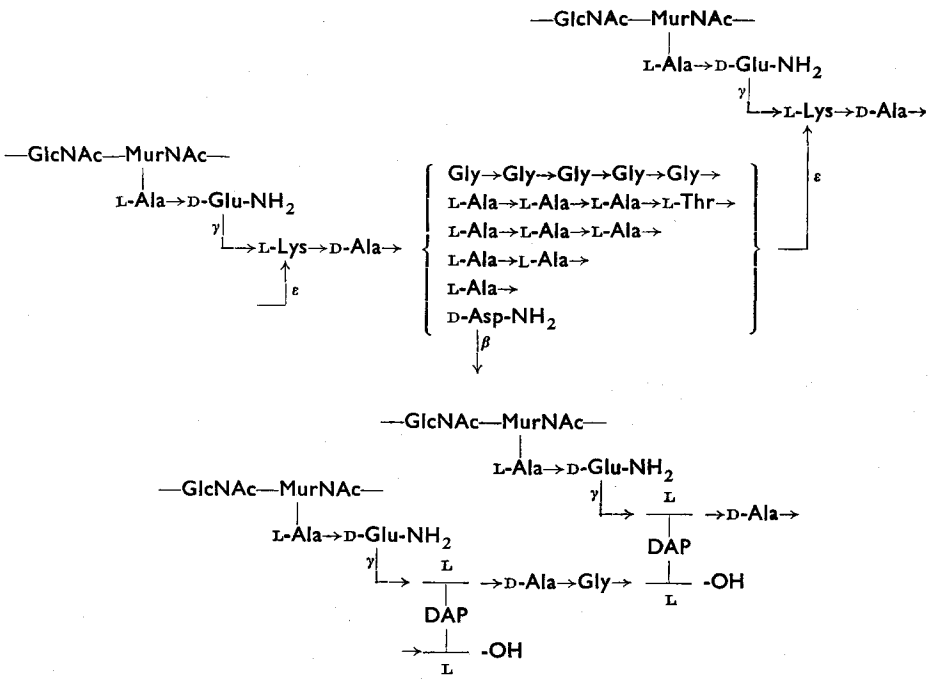


Fig. 5. Peptidoglycan of chemotype II. *Upper* part, *see* Ref.[5]. The pentaglycine bridge is the one found in *S. aureus* (Fig. 1). *Lower* part, peptidoglycan in *Streptomyces* sp.[14].

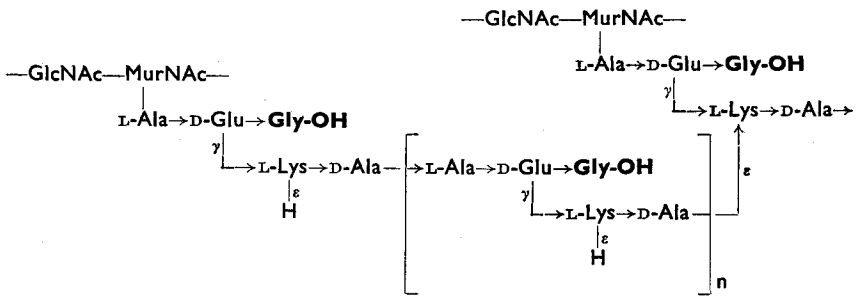


Fig. 6. Peptidoglycan of chemotype III (*M. lysodeikticus* and related *Micrococcaceae*[5,15]). Note that the α-carboxyl group of D-glutamic acid is substituted by a glycine residue (*heavy type*).

diamino acid such as D-diaminobutyric acid, D-ornithine or D-lysine or a short peptide containing a diamino acid, extends from the C-terminal D-alanine to the α-carboxyl group of D-glutamic acid.

3) Depending upon the bacterial species, the α-carboxyl group of D-glutamic acid when not engaged in peptide cross-linking, can be either free or amidated or substituted by an additional amino acid such as glycine. Similarly, the carboxyl groups of the diaminopimelic acid residues which are not engaged in peptide bonding are either free or amidated.

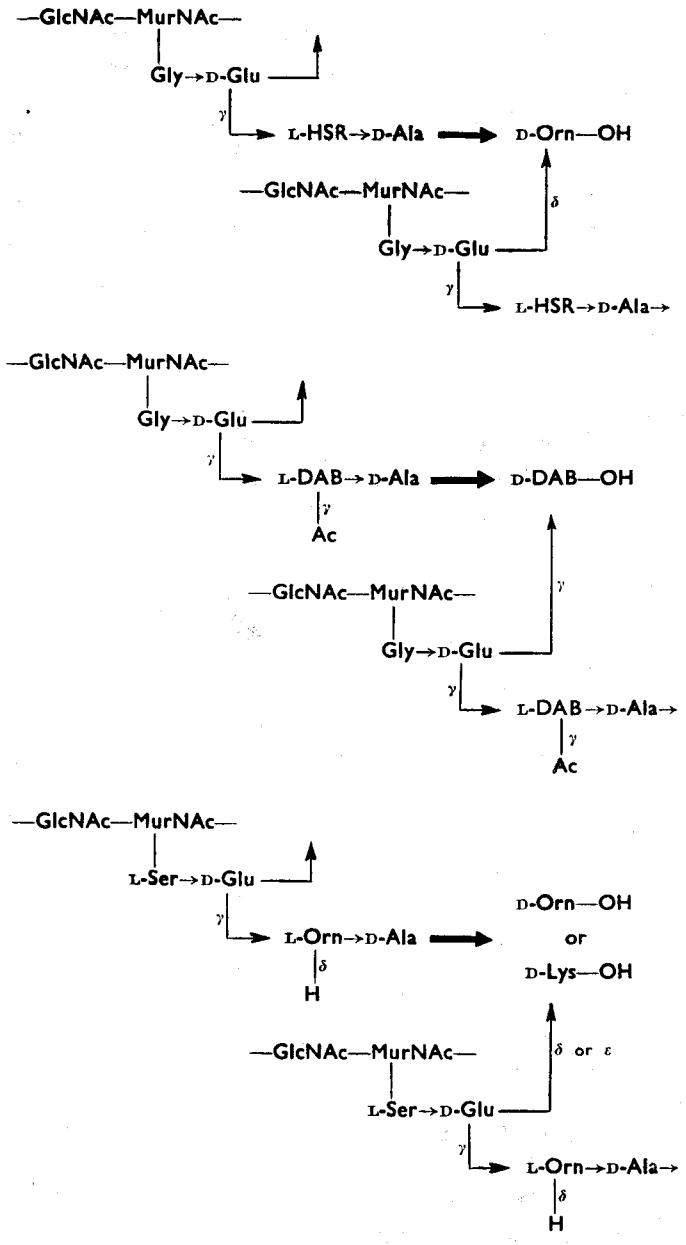


Fig. 7. Peptidoglycan of chemotype IV. From *top* to *bottom: Corynebacterium poinsettiae*[16], *Corynebacterium insidiosum*[17] and *Butyribacterium rettgeri*[18]. Note that the cross-linking bridges are mediated *via* D-alanyl-D-linkages in C-terminal position (*heavy arrows*).

## THE NET

The tightness of the peptidoglycan net depends upon the length of the glycan strands, the frequency with which the glycan strands are substituted and the frequency with which the tetrapeptides 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 muramic acid varies from 50 to 100 and the average size of the peptide moieties is between 2 and 10 cross-linked peptide subunits. Evidently, many terminal groups are present in both the glycan and the peptide moieties of the net. They reflect, at least in part, the dynamics of bacterial growth *(see below)*.

No study has yet been made of the secondary structures of the peptidoglycans. Recently, however, models of each of the four chemotypes described above have been constructed[19]. They suggest that extensive hydrogen bonding giving rise to a regular net-like arrangement is possible only if the polysaccharide chains with the preferred conformation of chitin are run head to tail and if the peptides are extended in the pleated sheet or $\beta$-configuration. Of particular interest is the observation that these structures require the D-glutamic acid residue $\gamma$-linked to the next amino acid in the sequence of the tetrapeptides.

## BIOSYNTHESIS OF THE PEPTIDOGLYCANS

Many important steps of the biosynthesis of the wall peptidoglycan[20] are carried out on or in the close vicinity of the plasma membrane from the two following presynthesized nucleotide precursors: uridine-5'-pyrophosphoryl—N-acetylglucosamine (UDP-GlcNAc) and uridine-5'-pyrophosphoryl—N-acetylmuramyl—L-alanyl (or L-seryl, or glycyl)—$\gamma$-D-glutamyl—L-$R_3$—D-alanyl—D-alanine (UDP-MurNAc-pentapeptide). Note that the peptide moiety of the precursor differs from the tetrapeptide unit in the completed peptidoglycan (Fig. 3) in that the precursor ends in a D-alanyl——D-alanine sequence. The machinery involved is complex and has to carry out: 1) The assembly of the activated precursors into $\beta$-1,4-N-acetylglucosaminyl—N-acetylmuramyl peptide units and, when necessary, appropriate alterations of the peptide moiety *(see below)*; 2) The creation of receptor sites in the growing wall peptidoglycan; 3) The transport *via* an intermediate carrier, of the disaccharide-peptide units from the inner surface of the membrane to the receptor sites on the outside of the membrane; 4) The polymerization of the newly inserted peptidoglycan material into an insoluble net-like structure. The carrier involved in the process has been identified as a $C_{55}$-polyisoprenoid alcohol phosphate. This lipid is clearly relevant to problems of orientation and transport. It is not unique to wall-peptidoglycan synthesis[21].

## SYNTHESIS AND TRANSPORT OF DISACCHARIDE-PEPTIDE UNITS

The sequence of the reactions can be visualized as follows[20] (Fig. 8). 1) The MurNAc (pentapeptide)-monophosphate residue is transferred from UDP-MurNAc pentapeptide to the *P*-$C_{55}$ lipid, resulting in the formation of UMP and of MurNAc-

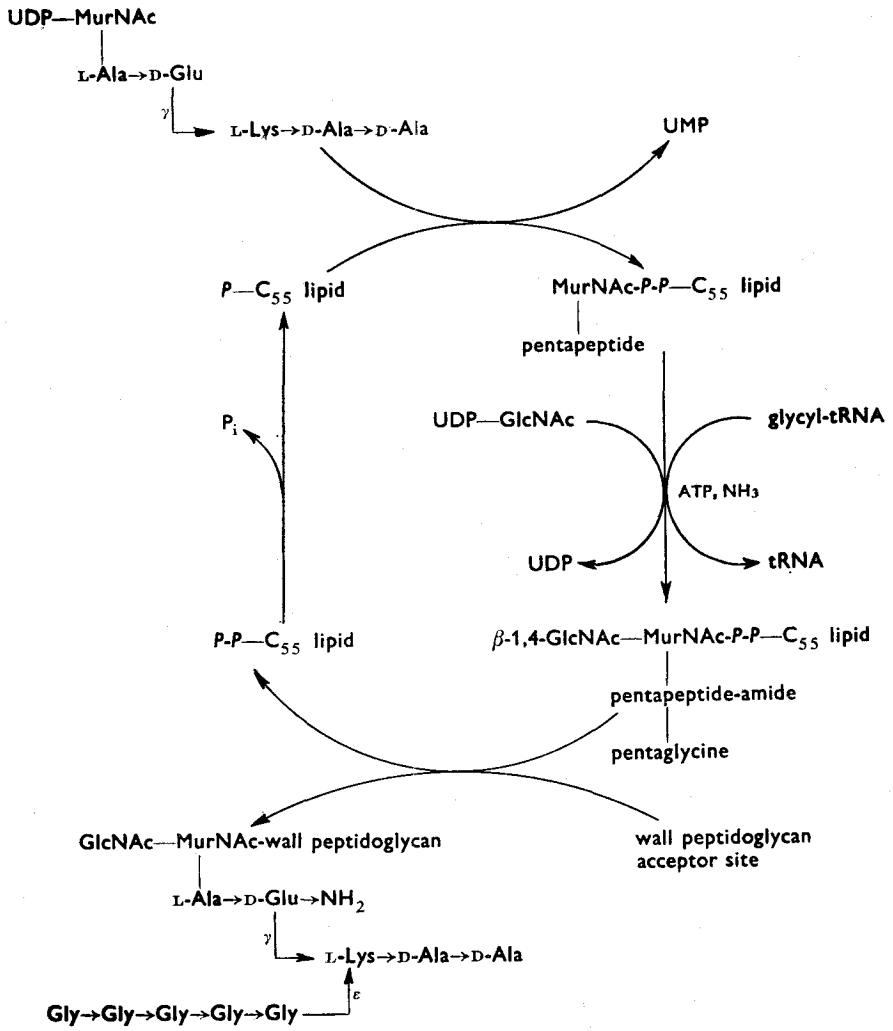


Fig. 8. The lipid cycle in *S. aureus*[20] with formation of disaccharide-peptide units followed by their transport through the plasma membrane and their insertion into the growing wall peptidoglycan (*compare* with Figs 1 and 5). In *E. coli* the same lipid cycle occurs with the exception that D-glutamic acid is not amidated and that no additional amino acid is incorporated into the pentapeptide moiety. Note that at the end of the cyclic reaction, the incorporated disaccharide-peptide units are uncross-linked.

(pentapeptide)-*P*-*P*-$C_{55}$ lipid; 2) GlcNAc is transferred by transglycosylation from UDP-GlcNAc with the liberation of UDP and the formation of $\beta$-1,4-GlcNAc-MurNAc(pentapeptide)-*P*-*P*-$C_{55}$ lipid; 3) Often, but not always, the pentapeptides undergo modifications such as the amidation of some carboxyl groups or, more important, such as the incorporation on the side chain of the $R_3$-residue (chemotypes II and III, Figs 5 and 6) or on the $\alpha$-carboxyl group of glutamic acid (chemotype IV, Fig. 7) of those amino-acid residues which in the completed peptidoglycan will function as "specialized" peptide cross-linking bridges. Sometimes, but not always, this incorporation is tRNA-dependent and the acceptor is the disaccharide-pentapeptide-lipid intermediate; 4) The disaccharide pentapeptide (that has been modified, if necessary) is finally transferred from the lipid intermediate to the wall-peptidoglycan receptor sites. The liberated *P*-*P*-$C_{55}$ lipid is dephosphorylated so that the *P*-$C_{55}$ carrier can begin in a new cycle. Evidently this cyclic reaction through which pre-synthesized disaccharide-peptide units are transported through the plasma membrane to the extracellular sites of incorporation, is responsible for the alternating sequence of N-acetylglucosamine and N-acetylmuramic acid residues found in the completed wall-glycan strands.

## THE WALL-PEPTIDOGLYCAN RECEPTOR SITES

From the above proposed mechanism of disaccharide-peptide transport, the appropriate receptor sites in the expanding peptidoglycan should be nonreducing N-acetylglucosamine termini, *i.e.* termini that result from the action of autolytic *endo*-N-acetylmuramidases which specifically hydrolyze $\beta$-1,4-N-acetylmuramyl-N--acetylglucosamine linkages in *endo* position in the glycan strands. Actually, *endo*--N-acetylmuramidases have been identified in many autolytic systems and shown to be involved in wall biosynthesis. In *Streptococcus faecalis*[22-24], the site of action of the *endo*-N-acetylmuramidase autolysin has been localized exactly at the septum formation. Moreover, it has been proved that the wall-peptidoglycan material first to be dissolved through its action is the most newly synthesized part of the peptidoglycan so that the cell equator appears to be the region of new wall synthesis and the region where active autolysin is localized. In *E. coli*[25], inhibition of wall synthesis by penicillin was shown to induce bulge formation on the cell envelope exactly at the sites where normally new cell wall is formed in the process of cell division. The interpretation was that more bonds in the wall peptidoglycan were locally hydrolyzed than could be closed by the inhibited biosynthetic apparatus. With *L. acidophilus* strain 63 AM Gasser[26], the autolytic activity of the cell population progressively disappears during the stationary phase. The walls isolated from both log-phase cells and stationary-phase cells, however, undergo rapid solubilization through the action of a powerful *endo*-N-acetylmuramidase. It is possible that during log phase the enzyme, or at least some of it, is not in the wall, but can be transported to it and

that in the transition to stationary phase this ability is rapidly lost. It thus appears that simultaneously with the ability to divide, the cells lose the capability of using the muramidase autolysin which, somehow, is prevented from reaching the wall-peptidoglycan substrate. It may be hypothesized that "*the signal which releases the* endo-*N-acetylmuramidase autolysin is transmitted* via *the plasma membrane to which the replicating DNA and the autolysin would be structurally bound*"[25].

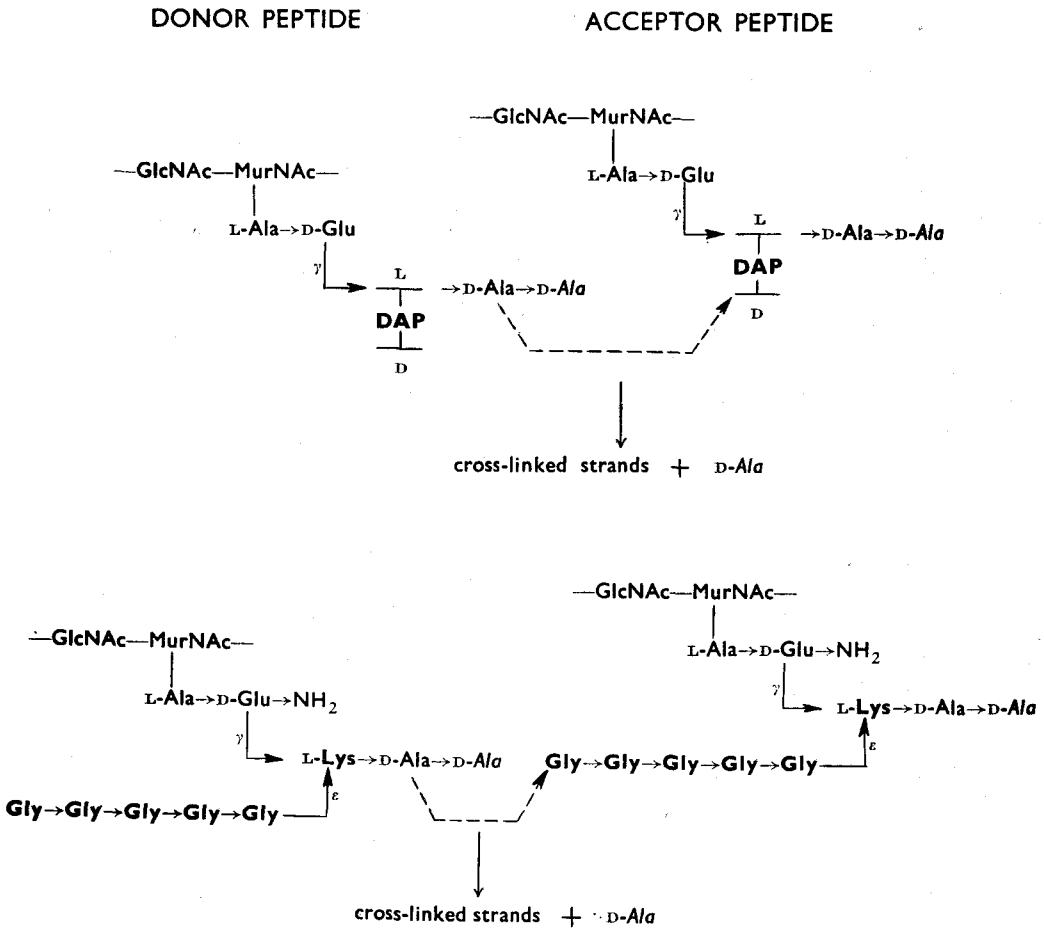


Fig. 9. The transpeptidation reaction with formation of cross-linked peptidoglycan strands and the transformation of the pentapeptide units into tetrapeptides. *Upper* part: transpeptidation in *E. coli*. Note that the residue at the $R_3$ position is *meso*-diaminopimelic acid (*heavy type*), *i.e.* that found in the nucleotide precursor. *Lower* part: transpeptidation in *S. aureus*. Note that the residue at the $R_3$ position is $N^{\varepsilon}$-pentaglycyl-L-lysine (*heavy type*).

## POLYMERIZATION OF THE NASCENT PEPTIDOGLYCAN

The insertion into the wall peptidoglycan of newly synthesized disaccharide-peptide units, whatsoever its precise mechanism, must be followed by the closure of the peptide bridges if the process is to yield an insoluble network. This last reaction which occurs at sites where ATP is not available, is believed to be introduced by transpeptidation[20]. The mechanism of this reaction is such that the penultimate C-terminal D-alanine residue of a *donor* peptide is transferred to the amino group of an *acceptor* peptide (Fig. 9). Interpeptide bonds are formed and equivalent amounts of D-alanine residues are released from the donor peptides.

## REGULATION OF THE SIZE OF THE PEPTIDE MOIETY

Three mechanisms, working alone or in conjunction, may be involved in the regulation of the size of the peptide moiety of the wall peptidoglycans: the efficiency of the transpeptidation reaction, the control of the number of peptides that undergo transpeptidation, the hydrolysis of the peptide cross-linkages in the completed wall peptidoglycan.

1) The transpeptidation reaction does not involve large changes in free energy and probably never reaches completion. The efficiency of the transpeptidation in a given organism should thus be reflected, at least in principle, by the extent of peptide cross-linking in the completed wall. Such relationship is valid only for those bacteria such as *S. aureus* and *L. acidophilus* 63 AM Gasser (Fig. 10) in which the residual not cross-linked C-termini of the wall-peptide moieties have retained the D-alanyl—D-alanine sequence found in the nucleotide precursor, thus demonstrating that peptide hydrolases are not involved in the regulation of the size of the peptides. The extent of peptide cross-linking indicates that the transpeptidation reaction is most efficient in *S. aureus* (80%)[27] whereas it is poorly efficient in *L. acidophilus* (30%)[10].

2) In contrast to *S. aureus* and *L. acidophilus*, the C-termini of the peptides in the wall peptidoglycans of most bacteria have not retained the D-alanyl—D-alanine sequence of the nucleotide precursors. This structural feature indicates, of course, the active presence in these bacteria of D-alanyl—D-alanine carboxypeptidases. Such enzymes were actually isolated[20,28,29] and were shown to hydrolyze specifically the C-terminal D-alanyl—D-alanine linkage in nucleotide precursors UDP-MurNAc-pentapeptide, in $\beta$-1,4-N-acetylglucosaminyl—N-acetylmuramyl—pentapeptide units and in a number of related peptides. Evidently, the size of the peptide moiety in these bacteria does not depend solely upon the efficiency of the transpeptidation reaction. It also depends upon the number of peptide units ending in D-alanyl—D-alanine that are allowed to undergo transpeptidation.

3) Finally, the interpeptide bonds in some wall peptidoglycans are mediated through D-alanyl—D-linkages in C-terminal position (Fig. 4 and 7) which, because of this peculiarity, are susceptible to be hydrolyzed by the DD-carboxypeptidases[28,29]. It thus follows that the extent of peptide cross-linking in these bacteria may also be controlled through the "*endo*peptidase" activity of the DD-carboxypeptidases upon the completed wall peptidoglycan.

If the biosynthesis of the bacterial peptidoglycan is understood at the molecular level, our concept of the mode of growth, replication and regulation of the wall at the cellular level is still largely obscure. Further progress depends upon a better

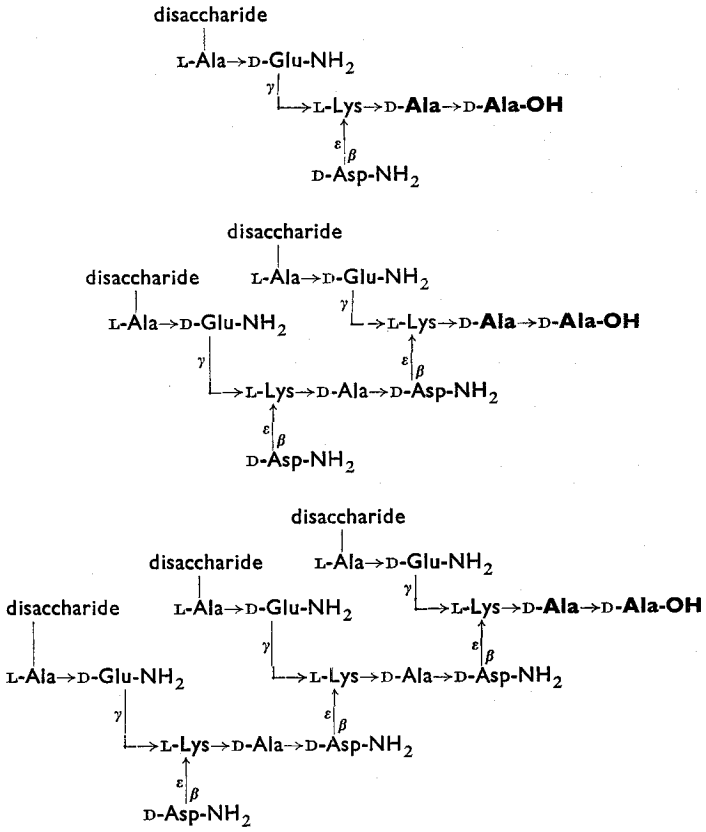


Fig. 10. The peptide moiety in *L. acidophilus* 63 AM Gasser. Disaccharide-peptide monomer (*above*), bis-disaccharide-peptide dimer (*middle*) and tris-disaccharide-peptide trimer (*below*) were isolated after solubilization of the walls (from log-phase cells) through the action of the *endo*-N-acetylmuramidase autolysin. In log-phase walls, 10% of the peptide subunits occur as monomers, 37% as dimers and 30% as trimers. Note that the C-termini of the peptides have retained the D-alanyl-D-alanine sequence found in the nucleotide precursor (*heavy type*). disaccharide = $\beta$-1,4-GlcNAc—MurNAc; some of the N-acetylmuramic acid have an additional acetyl group on $C_6$.

understanding of the precise architecture of the membrane and of the "vectorial" enzymology that is involved in membrane transport. It also depends upon technical improvements in the isolation of the relevant "particulate" enzymes from their sites of attachment in the membrane. Progress in this direction has been reported[21].

## MECHANISM OF ACTION OF PENICILLIN*

Penicillin when added at sublethal-dose levels to growing Gram-positive bacteria, was shown to reduce the extent of wall peptide cross-linking, *i.e.*, it decreased the efficiency of the transpeptidation reaction[30]. This conclusion is further supported by the demonstration that penicillin was able to block the transpeptidase activity of a particulate cell-free system prepared from *E. coli*[20]. Among the numerous enzymes involved in peptidoglycan synthesis, the transpeptidase is thus the target of the penicillin molecule. Two mechanisms, *i.e.* penicilloylation and/or competitive inhibition, possibly working in conjunction, may be invoked to explain the molecular basis of penicillin action.

### THE PENICILLOYLATION HYPOTHESIS

Analyses of the effect of penicillin on the *E. coli* cell-free system indicated that the transpeptidase was inactivated, rather than inhibited, *via* acylation through the highly reactive CO-N bond in the lactam ring of the penicillin molecule, with the formation of an inactive penicilloyl—protein complex. More recently, a particulate D-alanyl—D-alanine carboxypeptidase from *B. subtilis* (*i.e.* the "uncoupled" transpeptidase; *see below*) was also shown to be inactivated by penicillin in a similar fashion[20]. Further treatment of the penicillin-inhibited DD-carboxypeptidase with hydroxylamine or ethylmercaptan reversed the binding. Penicilloylhydroxamate or ethylthiopenicilloate were formed and, concomitantly, the carboxypeptidase activity was restored[20]. It is not known, however, whether penicilloylation occurs on the enzyme itself or on membrane sites localized in its vicinity. The acylating activity of penicillin is not specific for the membrane-bound transpeptidase or the particulate DD-carboxypeptidase. It is also responsible for the formation of penicilloyl-allergen through acylation of serum protein. Finally, 6-aminopenicillanic acid has no antibacterial activity although its $\beta$-lactam ring is intact. It thus appears probable that acylation is at least not the essential part of the mechanism of penicillin action.

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* For more recent views of the mechanism of action of penicillin from these laboratories, *see* Refs[35,36].

## THE STRUCTURAL ANALOGY HYPOTHESIS

Further progress in the elucidation of the exact mechanism of action of penicillin would require the isolation from the membrane of the relevant transpeptidase protein followed by its purification and characterization. The problem is made difficult because it is not known how much of the *in situ* structural integrity must be retained by both the enzyme and the substrate in order to be operative *in vitro*. Another approach[28,29,31] has been recently undertaken which rested upon the observations that DD-carboxypeptidases are spontaneously excreted in the culture media by strains of *Streptomyces* and that there is a remarkable similarity between the substrate requirements of these DD-carboxypeptidases and those of the transpeptidase involved in the peptidoglycan-bridge closure reaction. Studies carried out with small, well defined peptides demonstrated that there is not only a strict requirement for the C-terminal residue of the peptides to have a D-configuration and for the penultimate C-terminal residue to be solely D-alanine, but also that the length, the structure and the polarity of the side-chain of the L-amino acid preceding the C-terminal D-alanyl—D-sequence (*i.e.* the $R_3$-side-chain) are of prime importance for the enzyme activity (Tables I and II). From the scanty information so far available[20,33], the same observation can be made with the DD-carboxypeptidase solubilized from *E. coli* by physical disruption of the cells. Though the structural features of the $R_3$-side-chains that fulfill the requirements of the DD-carboxypeptidases vary according to the bacteria (Table I and footnote), it appears that those structures occurring in the wall peptides before cross-linking, as deduced from the structures of the final peptidoglycans, are compatible with the observed specificity of the DD-carboxypeptidases. In other words, peptide cross-linking does not occur unless the $R_3$-side-chain is such that the C-terminal D-alanine can be removed by the DD-carboxypeptidases. This conclusion gave rise to the idea[29] that the DD-carboxypeptidases are, in fact, the membrane-bound transpeptidases that have undergone solubilization (*i.e.* uncoupled transpeptidases) and that the difference in effective function of the enzyme could be, at least partially, a question of the availability of water. In an aqueous environment, attack of the peptide—enzyme complex, after elimination of the terminal D-alanine, by $OH^-$ would lead to simple hydrolysis. In the hydrophobic environment of the membrane, attack by a recognizable $-NH_2$ would lead to transpeptidation:

$$\text{R—D-Ala—D-Ala} + \text{enzyme } (E) \rightarrow \text{R—D-Ala—D-Ala—}E$$

$$\text{R—D-Ala—D-Ala—}E \rightarrow \text{R—D-Ala—}E + \text{D-Ala}$$

$$\text{R—D-Ala—}E + OH^- \rightarrow \text{R—D-AlaOH} + E \text{ (carboxypeptidase activity)}$$

$$\text{R—D-Ala—}E + \text{R'-NH}_2 \rightarrow \text{R—D-Ala—NH-R'} + E \text{ (transpeptidase activity)}$$

TABLE I

Efficiency of Hydrolysis by the DD-Carboxypeptidases from *S. albus* G and from *Streptomyces* R 61[a]

| Substrates[b] | *S. albus* G[29,31,c] | | | *Streptomyces* R 61[32,d] | | |
|---|---|---|---|---|---|---|
| | $K_m$ | $V$ | $E$ | $K_m$ | $V$ | $E$ |
| Ac → L-Ala → γ-D-Glu → L-Lys → D-Ala → D-Ala (Ac on ε-Lys) | 1.80 | 100 | 56 | 10 | 215 | 21 |
| Ac → L-Lys(Ac) → D-Ala → D-Ala | 0.33 | 100 | 300 | 12 | 890 | 72 |
| Ac → L-Lys(Ac) → D-Ala → D-Lys (H on ε-Lys) | 0.80 | 85 | 106 | 13 | 90 | 7 |
| Ac → L-Lys(Ac) → D-Ala → D-Leu | 0.33 | 33 | 100 | 10 | 50 | 5 |
| Ac → L-Lys(Ac) → D-Ala → Gly | 2.50 | 60 | 24 | 36 | 200 | 6 |
| Ac → L-Lys(Ac) → D-Ala → L-Ala[e] | no hydrolysis | | | virtually no hydrolysis | | |
| Gly → D-Ala | 15.0 | 107 | 7 | 15.5 | 1.7 | 0.1 |
| Ac → L-Lys(Ac) → D-Leu → D-Ala[e] | no hydrolysis | | | 10 | 10 | 1 |
| Ac → L-Lys(Ac) → L-Ala → D-Ala[e] | no hydrolysis | | | virtually no hydrolysis | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ac → L-Lys → D-Ala → D-Ala<br>↑ε<br>H | 6.0 | 20 | 3 | 15 | 4 | 0.3 |
| UDP-MurNAc → L-Ala → γ-D-Glu → (L)-*meso*-DAP-(L) → D-Ala → D-Ala | 0.4 | 10 | 25 | 11 | 3 | 0.3 |
| $N^{\alpha}$-(GlcNAc-MurNAc → L-Ala → γ-D-Gln) → Lys → D-Ala → D-Ala<br>H-(Gly)$_5$—↑ε | 0.28 | 9 | 32 | 14 | 800 | 57 |

[a] *Upper* and *middle* parts: influence exerted by the C-terminal dipeptide sequence; *lower* part: influence exerted by the preceding residue at the $R_3$-position. $K_m$ values are expressed in mM; $V$ values in μmol per mg of enzyme per h; efficiency ($E$) in $V/K_m$. These values were obtained at 37 °C in 0.01 M Tris-HCl buffer pH 7.5 (R 61 enzyme) or in 0.02 M Tris-HCl buffer pH 7.5 supplemented with 2 mM-$MgCl_2$ (*S. albus* G enzyme).

[b] DAP = diaminopimelic acid, GlcNAc-MurNAc = β-1,4-N-acetyl-glucosaminyl-N-acetylmuramic acid.

[c] Penicillin-resistant.

[d] Penicillin-sensitive.

[e] These peptides are not inhibitors.

*Footnote to Table I.*

1) Note that in *Streptomyces* sp., the amino group involved in transpeptidation is a glycyl residue which substitutes the LL-diaminopimelic acid at the $R_3$ position (Fig. 5, *bottom*). In *E. coli*, this amino group is that located on the D-carbon of *meso*-diaminopimelic acid (Fig. 9, *upper part*).

2) The nature of the residue at the $N^{\alpha}$-terminus of the LDD-tripeptides has no great influence on the activity of the *Streptomyces* enzymes.

3) Both *Streptomyces* enzymes have a strict requirement for a C-terminal D-alanyl—D-linkage. The penicillin-sensitive R 61 enzyme has a more strict requirement for a D-alanine at the C-terminal position.

4) With the penicillin-sensitive *Streptomyces* R 61 enzyme, there is a strict requirement for an amino group on the L-residue (here an $N^{\varepsilon}$-L-lysine group) to be either acylated (here an acetyl group) or transformed into an α-amino group by its substitution by a pentaglycine. With the penicillin-resistant *S. albus* G enzyme, the transformation of the ε-amino group of L-lysine to an α-amino group by introduction of a carboxyl group in α-position (*i.e.* the replacement of L-lysine by diaminopimelic acid) is sufficient to induce a high efficiency of hydrolysis.

5) By comparison with the penicillin-resistant *S. albus* G enzyme, the penicillin-sensitive R 61 enzyme exhibits more strict substrate requirements, has a mediocre fit with all the substrates that were used (high $K_m$ values) but has better groups to promote the hydrolysis of at least some of them (high $V$ values).

6) Note that no activity at all of the *E. coli* carboxypeptidase was demonstrable when UDP-MurNAc-pentapeptide containing L-lysine rather than *meso*-diaminopimelic acid was used[33].

TABLE II

Influence of the Length of the Side-Chain of the Residue that Precedes C-Terminal D-Alanyl—D-alanine on Carboxypeptidase Activity[31,32]

| Substrate | Specific activity[a] of enzyme from | |
|---|---|---|
| | *S. albus* G | *Streptomyces* R 61 |
| Ac → L-Ala ⟶ D-Ala → D-Ala | 0.3 | 0.65 |
| Ac → L-DAB[b] → D-Ala → D-Ala<br>↑γ<br>Ac | 22 | 4 |
| Ac → L-Orn ⟶ D-Ala → D-Ala<br>↑δ<br>Ac | 22 | 21 |
| Ac → L-Lys ⟶ D-Ala → D-Ala<br>↑ε<br>Ac | 40 | 47 |

[a] Expressed in microequivalent of D-Ala → D-Ala linkage hydrolyzed per mg of enzyme per h. Conditions of incubation: 15 nmol of peptide were incubated at 37 °C in the presence of enzyme in a final volume of 35 µl of 0.01 M Tris-HCl buffer pH 7.5 (enzyme from *Streptomyces* R 61) or of 0.02 M Tris-HCl buffer pH 7.5 supplemented with 2 mM-$MgCl_2$ (enzyme from *S. albus* G).

[b] Diaminobutyric acid.

The postulated identity between the DD-carboxypeptidases and the transpeptidases is further supported by the fact that the DD-carboxypeptidase solubilized from *E. coli*[20] is sensitive to penicillin and so, too, is the transpeptidase of this organism. Similarly, the DD-carboxypeptidase excreted by the penicillin-sensitive *Streptomyces* strain R 61 is, also, very sensitive to penicillin[32]. In marked contrast to both the particulate transpeptidase from *E. coli* and the particulate DD-carboxypeptidase from *B. subtilis* which are inactivated by penicillin apparently through acylation, both soluble DD-carboxypeptidases from *E. coli* and from *Streptomyces* R 61 are inhibited by penicillin G in a true competitive fashion ($K_i = 1.6 \times 10^{-8}$ M and $7.5 \times 10^{-8}$ M, respectively), demonstrating that penicillin G resembles the substrate sufficiently so as to be bound in its stead on the active site. A structural analogy between penicillin and the peptides involved in transpeptidation is thus likely to be relevant to the antibacterial action of penicillin. Both parts of the penicillin molecule, *i.e.* the acyl group and the 6-aminopenicillanic acid moiety, must be involved in the analogy. Indeed, it has been shown[33] that penicilloic acid and 6-aminopenicillanic acid are also competitive for the substrate of the *E. coli* DD-carboxypeptidase but only at concentrations which are 100-fold higher than the concentrations required of penicillin G. Actually, one edge of the penicillin molecule, *i.e.* the 6-aminopeni-

cillanic acid part of it, has a conformation that resembles one of the conformations of the C-terminal D-alanyl—D-alanine backbone[34], *i.e.* the *donor* site in the transpeptidation. A structural analogy with the *acceptor* site involved in transpeptidation, however, is not apparent in the constructed models of the penicillin molecule.

The penicillin-resistant *Streptomyces albus* strain G secretes a DD-carboxypeptidase which is not inhibited by penicillin. The enzyme was isolated and studied[28,29,31]. The $K_m$ and $V$ values were found to be quantitatively different from those exhibited by the R 61 penicillin-sensitive enzyme[32] (Table I and Footnote). Again, however, the values demonstrated that both the C-terminal D-alanyl—D-sequence and the side chain of the preceding L-amino acid in the peptides are essential for the enzyme activity. The isolation of such an enzyme which hydrolyses peptides ending in D-alanyl—D-alanine, but which is not inhibited by penicillin, seems to be at variance with the structural analogy hypothesis or, at least, it proves that this analogy is not universal among bacteria. One explanation is that both the penicillin-sensitive and penicillin-resistant "DD-carboxypeptidase—transpeptidase" systems would recognize one specific configuration in the substrates and that penicillin would be structurally analogous only to the configuration recognized by the penicillin-sensitive enzyme. The molecular basis for such a penicillin resistance that does not involve the enzymatic degradation of the antibiotic, must reside in a peculiar structure of the enzyme that, essentially, does not modify its catalytic activities but prevents it from being attacked by the penicillin molecule*. Evidently, this resistance must result from one or several mutations in the corresponding structural gene. Hence penicillin-sensitive and penicillin-resistant carboxypeptidases could be used as markers in a genetic approach to the exact molecular basis of the antibacterial action of penicillin. Similarly, the comparative biochemistry of isolated and purified DD-carboxypeptidases that differ from each other by their capabilities of recognizing penicillin, should also contribute to the solution of this fascinating problem.

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* The penicillin-sensitive DD-carboxypeptidase from *Streptomyces* R 61 has a poorer fit (higher $K_m$) than the penicillin-resistant enzyme from *S. albus* G (Table I). Hence, at least in this case, all the difference could be explained as a narrowing of the type of molecules accepted by the active site of the latter so that it will no longer accept penicillin. This explanation, however, may not be universal since the DD-carboxypeptidase from *E. coli* has an excellent fit[33] (low $K_m$, Table I) for its substrate and, at the same time, it is competitively inhibited by very low concentrations of penicillins.

** A more complete list of references can be found in the Refs[5,20].

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