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THE PENICILLIN TARGETS IN BACTERIA

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INTRODUCTION

Penicillin as a lytic agent

Bacteria possess enzymes which are able to hydrolyse their own wall. These enzymes are called autolysins. When they are permitted to act, the cells lose their osmotic protection and lyse. The active presence of these autolysins is usually observed under conditions which involve the inhibition of the continued synthesis of a particular polymer which constitutes the rigid matrix of the bacterial cell wall. This polymer is called peptidoglycan, glycopeptide or murein. Cellular autolysis may be caused by the deprivation of nutritionally required peptidoglycan precursors or by the addition of inhibitors of peptidoglycan synthesis in the growth medium. Penicillin (*i.e.* the various penicillins and cephalosporins) is one of the most specific and potent inhibitors of peptidoglycan synthesis. Penicillin action, however, does not in itself result in cell lysis. The active participation of the autolysins is required as shown, for example, by the absence of penicillin-induced lysis in an autolytic defective mutant of *Diplococcus pneumoniae* [Tomasz *et al.* (1970)].

Structure of the wall peptidoglycan

The wall peptidoglycan is a net-like macromolecule which completely surrounds the plasma membrane and provides the cell with a supporting structure of

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high tensile strength. The glycan moiety of the peptidoglycan consists of linear strands of alternating β-1,4 linked pyranoside N-acetylglucosamine and N-acetylmuramic acid residues. The carboxyl groups of the N-acetylmuramic acid residues, or at least some of them, are substituted by tetrapeptide units L-alanyl-γ-D-glutamyl-L-R_3-D-alanine. Often, but not always, the L-R_3 residue is a diamino acid such as L-ornithine, L-lysine, *LL*- or *meso*-diaminopimelic acid. The peptide units belonging to adjacent glycan strands are, in turn, crosslinked through *specialized* bridges. In many bacteria, these bridges extend from the C-terminal D-alanine of one peptide to the ω-amino group of the diamino acid at the L-R_3 position of another peptide. The bridges may consist of direct N^{ω}-(D-alanyl)-L-R_3 amide bonds or may be mediated *via* a single additional amino acid residue or *via* an intervening peptide. [For more details, see Ghuysen (1968)].

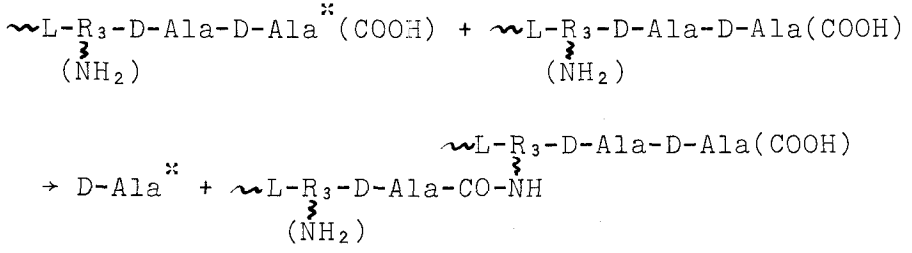
Table 1
Examples of bridges between two peptide units

Organism	Interpeptide bridges
Escherichia coli	L-Ala-D-Glu L-Ala-D-Glu L DAP-D-Ala~~ L ~~DAP-D-Ala-DAP D D
Bacilli	
Streptomyces R39	
Streptomyces R61, K11, *albus* G	L DAP-D-Ala~~ L ~~DAP-D-Ala-Gly-DAP L
Staphylococcus aureus Copenhagen	L-Lys-D-Ala~~ ε L-Lys-D-Ala-(Gly)5 ε

DAP = diaminopimelic acid. The α-carboxyl group of D-Glu (in *Streptomyces* R39 and in some *Bacilli*) and the carboxyl group located on the D center of *meso*-DAP (in some *Bacilli*) are substituted by amide groups.

Biosynthesis of the wall peptidoglycan

Important steps of the biosynthesis of the wall peptidoglycan are carried out on the plasma membrane from the two following cytoplasmic precursors : uridine-5'-pyrophosphoryl-(UDP)-N-acetylglucosamine and UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-R_3-D-alanyl-D-alanine. Note that in the latter precursor, the peptide ends in a C-terminal D-alanyl-D-alanine sequence. The biosynthesis proceeds through the coordinated action of several membrane-bound enzymes and involves a C_{55} polyisoprenoid alcohol phosphate carrier. [For more details, see Strominger (1970)]. This multi-enzyme complex essentially catalyses 1) the formation of β-1,4-N-acetylglucosaminyl-N-acetylmuramyl-pentapeptide units; 2) the substitution, when required, of the L-R_3 residues of the peptides by those amino acids which will serve as peptide bridges in the completed wall peptidoglycan; 3) the transfer of the disaccharide-peptide units to the exocellular sites of incorporation in the expanding wall peptidoglycan; 4) the closure of the bridges between the peptide units. This reaction, which brings about the insolubilization of the peptidoglycan network, occurs by transpeptidation and does not require any input of exogenous energy. In this reaction, the penultimate C-terminal D-alanine residue of a peptide donor is transferred to the ω-amino group at the L-R_3 position of a peptide acceptor of the same composition. Interpeptide bonds are formed and equivalent amounts of D-alanine residues are released from the peptide donors.



Examples of completed crosslinking bridges are given in Table 1.

ENZYME ACTIVITIES THAT ARE SUSCEPTIBLE TO PENICILLIN

Transpeptidase activity

Penicillin specifically abolishes or reduces the efficiency of the membrane-bound transpeptidase that catalyses the peptide crosslinking. Hence, the membrane-bound transpeptidase is thought to be the killing target of penicillin. The mechanism of peptide crosslinking and of its inhibition by penicillin was first revealed by Wise and Park (1965) and Tipper and Strominger (1965) by a study of the effects of benzylpenicillin on *S. aureus*. Benzylpenicillin, when added at sublethal doses to growing cells, decreased the extent of peptide crosslinking. Walls isolated from cells grown in the presence of low concentrations of the antibiotic contained higher amounts of uncrosslinked peptide units than walls isolated from cells grown in the absence of penicillin. The peptide units which had not undergone transpeptidation because of the presence of benzylpenicillin, retained the C-terminal D-alanyl-D-alanine sequence of the peptidoglycan precursors. In 1966, cell-free particulate multi-enzyme preparations were obtained from *E. coli* and *Salmonella*, which catalysed the *in vitro* utilization of the nucleotide precursors UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide for the entire sequence of peptidoglycan synthesis including the peptide crosslinking reaction [Izaki *et al.* (1966), (1968); Araki *et al.* (1966 a,b)]. The transpeptidase activity was suppressed by low concentrations of penicillin and could not be restored by washing or treatment with β-lactamase. It took several more years before cell-free systems able to carry out a similar sequence of reactions could be obtained from Gram-positive bacteria. Wickus and Strominger (1972) prepared from cells of *Bacillus megaterium* a particulate fraction which in addition to peptidoglycan synthesis and peptide crosslinking, catalysed the incorporation of *meso-* or *DD-* (but not *LL-*) diaminopimelic acid at the C-terminus of a disaccharide-pentapeptide peptidoglycan where it replaced the D-alanine residue found in the nucleotide precursor. Both peptide crosslinking and diaminopimelic acid incorporation were sensitive to low concentrations of cloxacillin. However,

inhibition of the former reaction was virtually fully reversible whereas inhibition of the latter was only 50-60 % reversible, suggesting the possible occurrence of two transpeptidase activities in *B. megaterium*. A crude wall fraction containing strongly associated membrane components was prepared from *S. aureus* by Mirelman and Sharon (1972). When exposed to UDP-N-acetylmuramyl-[^{14}C]-pentapeptide together with glycine and UDP-N-acetylglucosamine, this preparation catalysed the release of some D-[^{14}C]-alanine as well as peptidoglycan synthesis. Both reactions were dependent upon the presence of UDP-N-acetylglucosamine. Benzylpenicillin inhibited the release of D-[^{14}C]-alanine. Altogether these properties indicated that the newly synthesized peptidoglycan was probably partially crosslinked but crosslinking was not directly demonstrated. The system appeared to be very delicate in nature. It lost its activity by treatment with pronase and detergents and by sonication. A crude *cell envelope* fraction was also prepared by Mirelman *et al.* (1972) from *Micrococcus lysodeikticus* which similarly appeared to be effective in peptidoglycan synthesis and peptide crosslinking.

These studies have the following obvious limitations. The assays, as they were devised, do not allow measurement of the transpeptidation reaction *per se*, *i.e.* independently of the preceding biosynthetic sequential reactions. Moreover, unfractionated *particulate* preparations were always used. At present, a transpeptidase has not yet been isolated from bacterial membranes, purified and characterized. For a long time, the problem was made difficult because it was not known how much of the *in vivo* structural integrity must be retained by both the enzyme and the substrate for the system to be operative *in vitro*. As pointed out by Reynolds (1971), "in a membrane-bound multi-enzyme biosynthetic pathway, the arrangement of the enzymes with respect to one another is likely to be all important in determining the overall activity of the complex which is being studied *in vitro*". In agreement with this view, membranes prepared from *bacilli* were found to be relatively inactive in peptidoglycan synthesis unless the protoplasts from which they were derived were first *reconditioned* by incubation in a growth medium [Reynolds (1971)]. Reconditioning was thought to

correct the damage caused by the method used for the preparation of the protoplasts. In spite of these difficulties, a new approach to the problem was opened which led to the isolation of proteins performing transpeptidase activities with well defined systems of peptide donors and acceptors (*vide infra*).

Carboxypeptidase activity

In *S. aureus* and in *L. acidophilus*, the peptides at the uncrosslinked C-termini of the wall peptidoglycans retain the D-alanyl-D-alanine sequence found in the UDP-N-acetylmuramyl-pentapeptide precursor. These two organisms are exceptional in this respect [Ghuysen (1968)]. In most bacteria, one of these D-alanine residues or even both of them are removed by carboxypeptidases. A DD-carboxypeptidase (or carboxypeptidase I) hydrolyzes the C-terminal D-alanyl-D-alanine peptide bond. An LD-carboxypeptidase (or carboxypeptidase II) hydrolyzes the C-terminal L-R_3-D-alanine peptide bond exposed by prior action of the DD-carboxypeptidase [Izaki and Strominger (1968)]. These enzymes probably play an important role in peptidoglycan synthesis by limiting the number of donor peptides available for transpeptidation and by preventing further propagation of the crosslinking system.

Table 2
DD-carboxypeptidase activities

(1) UDP-L-Ala-γ-D-Glu-L-R_3-D-Ala$\downarrow$D-Ala $\xrightarrow{\text{Enzyme}}$ D-Ala + UDP-L-Ala-γ-D-Glu-L-R -D-Ala.

(2) ~~L-R_3-D-Ala$\downarrow$D-Ala
~~D-Ala-(Bridge)┘ $\xrightarrow{\text{Enzyme}}$
D-Ala + ~~L-R_3-D-Ala
~~D-Ala-(Bridge)┘

(1) action on nucleotide precursor; (2) action on a crosslinked peptide dimer.

Many *Streptomyces* excrete a DD-carboxypeptidase activity into the external medium during growth. In all the other cases so far studied, the activity is solely membrane-bound. It could be released by sonication [*E. coli*; Izaki and Strominger (1968), Bogdanovsky *et al.* (1969)], by treatment with Brij 36 T [*E. coli*; J. Pollock and M. Distèche (unpublished)], with Nonidet [*B. subtilis*; Blumberg and Strominger (1972)], with butan-1-ol [*B. stearothermophilus*; Barnett (1973)], or with urea [*Streptomyces* R61 (unpublished)]. The easiness of the assay devised to estimate the DD-carboxypeptidase activity, *i.e.* the hydrolysis of the C-terminal D-alanyl-D-alanine peptide bond, greatly facilitated the isolation and the purification of these enzymes. At present, the exocellular DD-carboxypeptidases from *Streptomyces albus* G [Ghuysen *et al.* (1970), Leyh-Bouille *et al.* (1970 b,c)], R61 [Leyh-Bouille *et al.* (1971), Frère *et al.* (1973 a)], K11 and R39 [Leyh-Bouille *et al.* (1972)], and the membrane-bound enzyme from *B. subtilis* [Wickus and Strominger (1972)] were obtained in a very highly purified state.

Many DD-carboxypeptidases but not all of them were sensitive to penicillin. Their responses to these antibiotics differed greatly with the various bacterial species. Some enzymes were irreversibly inactivated (*i.e.* activation was not restored by treatment with β-lactamase; *B. megaterium* and *B. subtilis* enzymes). Others were reversibly inhibited. Kinetics of inhibition were competitive (solubilized enzyme obtained by sonication of *E. coli*; exocellular enzymes from *Streptomyces* R61 and K11), hyperbolic competitive (membrane-bound enzyme of *B. stearothermophilus*) or noncompetitive (exocellular enzyme from *Streptomyces* R39). The exocellular DD-carboxypeptidase from *Streptomyces albus* G was not inhibited by β-lactam antibiotics [Leyh-Bouille *et al.* (1970 c)] whereas among the DD-carboxypeptidase activities of *B. megaterium* there was one which was stimulated in the presence of benzylpenicillin [Wickus and Strominger (1972)].

The LD-carboxypeptidase of *E. coli* was separated from the DD-carboxypeptidase [Izaki and Strominger (1968)] and was not inhibited by penicillins and cephalosporins at about 200 µg/mℓ .

IRREVERSIBLE PENICILLIN BINDING SITES IN BACTERIAL CELLS

It has been known for almost 20 years that penicillin is fixed by bacteria and that a correlation exists between the sensitivity of the bacteria and the amount of penicillin which is irreversibly attached to them. Irreversibly fixed penicillin is the amount of penicillin which cannot be released from the cells by washing in neutral or acidic buffers, by incubation with β-lactamase, by a combination of the two former treatments, or (when fixation has been carried out with radioactive penicillin) by a large excess of non radioactive penicillin. Dilute alkali, hydroxylamine and ethanethiol release at least part of the fixed penicillin as penicilloic acid, penicilloyl hydroxamate or an α-ethylthiol derivative of penicilloic acid, suggesting that penicillin or part of it might bind covalently to the cells. The nature of the postulated covalent linkage between penicillin and the sites in the cells (assuming it really occurs) is unknown. Relatively small amounts of penicillin are irreversibly fixed to the bacteria. Estimates vary from 200-1000 [Gale *et al.* (1972)] to 1000-8000 [Suginaka *et al.* (1972)] molecules per cell.

Irreversible binding of penicillin to S. aureus

Edwards and Park (1969) showed that the irreversible binding of various penicillins and cephalosporins to *S. aureus* correlated well with the growth inhibitory concentrations of these antibiotics. This correlation was true for antibiotics with growth inhibitory concentration values (MIC) ranging from 0.06 to 150 µg/mℓ . Moreover, the observed competition of the penicillins and cephalosporins against [^{14}C]-benzylpenicillin demonstrated that all these antibiotics were competing for the same cellular sites. In a more recent work, however, Park *et al.* (1971) examined a series of β-lactamase-negative, benzylpenicillin-resistant mutants of *S. aureus*. MIC values, in µg/mℓ, were 0.06 for the parent and ranged from 1.8 to 5,000 for the mutants. All strains responded similarly to the antibiotic. "A highly resistant mutant, *e.g.* one which required 1,000 times as much penicillin as the parent for its growth to be prevented,

required 1,000 times as much penicillin to show a comparable effect" ... on peptidoglycan synthesis and on the accumulation of nucleotide precursors. Surprisingly, however, the amount of penicillin irreversibly fixed to the penicillin-resistant mutants was somewhat less than the amount bound by the penicillin-sensitive parent and roughly the same amount of penicillin was bound to the various mutants. Highly resistant strains bound almost as much penicillin as the parent even though their resistance to benzylpenicillin was up to 160,000 times greater. Hence, this great increase in resistance could not be accounted for by a change in the number and/or the reactivity of the binding sites as measured by the irreversible binding of penicillin to the cells. Clearly, the sites in the cells to which penicillin is irreversibly attached are important for antibiotic action but they, or at least the great majority of them, appear not to be the killing target of penicillin. As already pointed out by Maas and Johnson in 1949, "it is entirely possible that the observed uptake is due to a mechanism unconnected with the antibiotic activity of penicillin". This remark was especially pertinent since penicillin is a very active chemical agent. Penicillin combines with ε-amino groups in poly-lysine [Schneider and de Weck (1965)] and in proteins [Batchelor *et al.* (1965)] forming penicilloyl compounds. Penicillin also interacts with guanosine and breaks the strong guanosine-cytidine pairing by forming a binary complex of penicillin-guanosine [Kan *et al.* (1972)].

"Sensitive" vs *"irrelevant" penicillin binding sites in* S. aureus

Other puzzling observations which have also been known for many years are that penicillin does not affect bacteria unless they are growing and that cells saturated with penicillin, when reincubated in fresh medium, are able to grow. In other words, the irreversibly attached penicillin would not be antibiotically active. Cooper (1956) suggested that when reexposed to a fresh, penicillin-free medium, the bacteria would be able to synthesise rapidly new wall assembly sites (transpeptidases !) which would allow growth in spite of a full loading of the old ones with the antibiotic.

Rogers (1967) showed that cells of *S. aureus* previously saturated with benzylpenicillin in buffers, synthesised peptidoglycan when reincubated in a fresh medium in the presence of chloramphenicol, *i.e.* under conditions allowing peptidoglycan synthesis but where no more protein could be formed and growth did not occur. In this system, peptidoglycan synthesis is topographically atypical and leads to the formation of a thickened abnormal wall [Higgins and Shockman (1971)]. Nevertheless, peptidoglycan proceeded at a normal rate for at least 40 min during which time at least 90 % of the fixed benzylpenicillin (*i.e.* about 170 molecules per cell) remained firmly cell-bound. Hence, the great majority of the irreversible sites for penicillin were *irrelevant* as far as peptidoglycan synthesis was concerned and the fixation of most of the benzylpenicillin was interpreted as a possible detoxication mechanism. During the recovery, the progress of peptidoglycan synthesis was paralleled by the exposure of a limited number of new fixation sites for benzylpenicillin (30-40 per cell). After recovery, the cells were about two orders of magnitude more sensitive to exogenous benzylpenicillin and the combination of the highly sensitive sites by benzylpenicillin approximately matched the degree of inhibition of peptidoglycan synthesis. Hence these newly exposed (in the absence of protein synthesis), highly sensitive sites for penicillin were directly related to peptidoglycan synthesis. It is possible that they were already present in the untreated cells but escaped combination with penicillin during the initial treatment because the available benzylpenicillin molecules mainly reacted with the *irrelevant* fixation sites. It is also possible that both *sensitive* and *irrelevant* sites were first saturated but that combination of the *sensitive* ones with benzylpenicillin was somehow reversed during the period of recovery. Other work, subsequent to that of Rogers (1967), revealed that the problem of the relevance of penicillin fixation to inhibition of peptidoglycan synthesis was still much more complex than at first expected. It was shown by Park *et al.* (1971) that exposure of cells of *S. aureus* to benzylpenicillin for 15-30 min under conditions allowing growth caused a very severe inhibition of subsequent peptidoglycan synthesis

(in the absence of antibiotic). Moreover, the ability of the cells to synthesise peptidoglycan when reincubated in a fresh medium, was completely lost if this medium contained an inhibitor of protein synthesis (tetracycline). Hence with cells in growth medium, the inactivation by penicillin was more permanent, as if the killing target of penicillin was made more available and/or susceptible, and recovery appeared to require the synthesis of new peptidoglycan assembly proteins (transpeptidases ?). It was also shown by Tipper and Strominger (1968) that the uncrosslinked peptide units incorporated in the wall during growth in the presence of low concentrations of benzylpenicillin could not subsequently be crosslinked when penicillin was removed and growth allowed to proceed again. One possible explanation was that during the abnormal wall synthesis where a certain number of crosslinked and uncrosslinked units were introduced into the expanding peptidoglycan, the uncrosslinked units became spatially removed from that region of the membrane where peptide crosslinking would occur.
All these experiments pose the problem of the localization and topography of wall growth and peptidoglycan assembly. Many facts [for details, see Higgins and Shockman (1971)] point to the septum region of both Gram-positive and Gram-negative bacteria as one important membrane site concerned with peptidoglycan synthesis and transpeptidation. In the model proposed for the coccus *Streptococcus faecalis*, the peptidoglycan would be synthesised and assembled at or near the nascent septum from which it would find its way into peripheral wall, cross wall and thickened wall. Autolysins would play an essential role in controlling the rates of these processes. With such a model in mind, one could propose that those peptidoglycan assembly sites on the membrane which as a result of growth would no longer be located in the septum region would become inactive (*i.e.* irrelevant !) in peptidoglycan synthesis [Park *et al.* (1971)].

Irreversible binding to Gram-negative bacteria

Increased complexity could be expected with Gram-negative bacteria because of the occurrence of an additional outer membrane containing lipopoly-

saccharides and lipoproteins [Osborn (1969)]. Nevertheless, Schmid and Plapp (1972) showed that the irreversible binding of benzylpenicillin to the β-lactamase-negative *Proteus mirabilis* D52 followed saturation type kinetics and that saturation corresponded to a decrease of about 90 % of the number of viable cells. Time course of binding, however, was atypical in that some part of the large amount of benzylpenicillin fixed during the initial phase was subsequently released in an unaltered form. Very little inhibition of the binding of benzylpenicillin by 6-aminopenicillanic acid was observed whereas 6-aminopenicillanic acid induced spheroplast formation at only a 25 times higher concentration than benzylpenicillin. Hydroxylamine removed up to 50 % of the radioactivity eventually bound after reaction with [^{14}C]-benzylpenicillin, in the form of a product which was not identified except that it was not the expected benzylpenicilloyl-hydroxamate. Phenacetylglycine, a compound in which the amino group of glycine is substituted by the same residue as the amino group of 6-aminopenicillanic acid in benzylpenicillin, was effective in decreasing the amount of benzylpenicillin fixed to the cells to about 50 % of its normal value. But phenacetylglycine did not antagonize the antibiotic action of benzylpenicillin, thus suggesting that those sites for which benzylpenicillin and phenacetylglycine compete, are not related to the killing by the antibiotic. The significance of all these observations remains unclear.

Isolation of irreversible penicillin binding sites

Several techniques were employed by Suginaka *et al.* (1972) to isolate the irreversible penicillin-binding sites of *S. aureus*, *B. subtilis*, *B. cereus* and *E. coli*. Starting materials were particulate preparations obtained after grinding the cells with alumina. Binding was in the same range as that recorded with whole cells. Nonionic detergents and chaotropic agents were effective means for the solubilization of the binding components. A combination of 1 % Nonidet P-40 and 9 M urea was selected and the separation of the solubilized [^{14}C]-benzylpenicillin-binding components was carried out by chromatography on DEAE cellulose, by electrofocusing

in polyacrylamide gels. A variety of major and minor penicillin-binding components were shown to exist in each of these organisms. Both *Bacilli* contained two main components, whereas *S. aureus* and *E. coli* appeared to have only a single major one. As previously found by Edwards and Park (1969), in *S. aureus* the number of binding sites available to benzylpenicillin and cephalothin was nearly the same. In marked contrast, in *B. subtilis* many more of the penicillin binding sites were saturated during prior treatment with benzylpenicillin than with cephalothin. When membrane particles of *B. subtilis* first treated with unlabelled cephalothin and then with radioactive benzylpenicillin were analysed, it was observed that one of the major penicillin-binding components had reacted with radioactive benzylpenicillin. This finding suggested that in *B. subtilis*, one of the two major components which reacted with benzylpenicillin did not react readily with cephalothin.

The use of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate permitted more thorough analyses of the penicillin-binding components of membranes of *B. subtilis* [Blumberg and Strominger (1972)]. This technique revealed the existence of 5 distinct (I to V) components. Component V, a DD-carboxypeptidase, was predominant and after its isolation by affinity chromatography on 6-aminopenicillanic acid-substituted Sepharose, it was found to be at least three orders of magnitude less susceptible to cephalothin than were the other compounds. A technique was then devised which made use of this property and allowed the isolation and the purification of component V (99 % purity) with an absolute yield of 79 % [Blumberg and Strominger (1972)]. The *solubilized* membranes (using 1.0 M NaCl ; 2 % Nonidet P-40 and 4 mM 2-mercaptoethanol) were first treated with cephalothin under conditions which allowed saturation of component I-IV but not of component V. Component V was then isolated by affinity chromatography. It was identified as the DD-carboxypeptidase. In this process of purification, the DD-carboxypeptidase underwent irreversible attachement to the 6-aminopenicillanic acid-Sepharose matrix from which it was released in an active form by hydroxylamine. The *B. subtilis* DD-carboxypeptidase represents 1 % of the membrane

proteins. It is the first membrane-bound DD-carboxypeptidase that has been isolated and purified to protein homogeneity.

THE TRANSPEPTIDASE-DD-CARBOXYPEPTIDASE SYSTEM

The techniques described in the above section for the resolution of membrane components to which penicillin is irreversibly attached may also be a point of departure for an attack on the problem of the isolation and identity of the killing target of penicillin, *i.e.* the transpeptidase. There are, however, several limitations to this approach. Like the DD-carboxypeptidases not all transpeptidases irreversibly fix penicillin. A procedure that would release a transpeptidase in an active form from its covalent association with penicillin has not yet been devised. As already pointed out, the transpeptidase which is part of a membrane-bound multienzyme system may require, in order to be operative, the exact substrate which is produced by the preceding reaction of the sequence pathway, *i.e.* some imprecisely identified *nascent peptidoglycan*. Recent work, however, showed that at least some transpeptidases can operate *in vitro* with well defined substrates.

The membrane-bound transpeptidase system in Streptomyces *R61*

Membranes were prepared from *Streptomyces* R61 which catalysed the transfer of N^{α},N^{ε}-diacetyl-L-lysyl-D-alanine from the tripeptide N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine to the dipeptide glycyl-glycine, yielding free D-alanine and the tetrapeptide N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-glycyl-glycine [Dusart *et al.* (1973)]. Liberation of D-alanine from the tripeptide donor was dependent on the presence of the acceptor glycyl-glycine, *i.e.* the membrane preparation did not exhibit any carboxypeptidase activity. In this system, N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine is an analogue of the L-R_3-D-alanyl-D-alanine donor site and glycyl-glycine is an analogue of the glycyl-LL-diaminopimelyl acceptor site involved in transpeptidation *in vivo*. Indeed, both the crosslinking made by transpeptidation in *Streptomyces* R61 [Leyh-Bouille *et al.* (1970 a); Table I]

and the crosslinking made by transpeptidation when the membrane-bound enzyme was exposed to the above synthetic system, resulted in the synthesis of the same D-alanyl-glycyl linkage in an endo-position. Furthermore, there was a good correlation between the *in vivo* susceptibility of the *Streptomyces* R61 toward a series of penicillins and cephalosporins and the *in vitro* susceptibility of the membrane-bound transpeptidase toward the same antibiotics [Dusart *et al.* (1973)]. Under the growth conditions used in these studies, β-lactamase activity was not involved in the *in vivo* susceptibility to β-lactam antibiotics [Dusart *et al.* (1973), Johnson *et al.* (1973)]. Altogether, these observations strongly suggested that in *Streptomyces* R61, as revealed by using these tripeptide donor and dipeptide acceptor as substrates for transpeptidation, the membrane-bound transpeptidase was the killing target of penicillin.

Chaotropic agents (2 M urea) solubilized at least part of the membrane-bound transpeptidase activity. However, when exposed to the tripeptide donor alone, the solubilized preparation hydrolyzed it into D-alanine and dipeptide N^{α},N^{ε}-diacetyl-L-lysyl-D-alanine (unpublished experiments), *i.e.* it behaved as a DD-carboxypeptidase as well as a transpeptidase. It can be argued that the only difference between the two activities is that in transpeptidation the enzyme catalyses a reaction with a free amino group to complete peptide crosslinking, whereas in hydrolysis the enzyme catalyses a reaction with water.

Ac_2-L-lys-D-Ala-D-Ala + Gly-Gly → D-Ala + Ac_2-L-lys-D-Ala-Gly-Gly.

Ac_2-L-lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2-L-lys-D-Ala.

The hydrophobic environment of the membrane would favor transpeptidation whereas water is accessible to the *solubilized* enzyme.

The exocellular transpeptidase-DD-carboxypeptidase of Streptomyces *R61*

The exocellular DD-carboxypeptidases that is excreted by *Streptomyces* R61 during growth [Leyh-Bouille *et al.* (1971)] was found to function either as

carboxypeptidase or transpeptidase depending upon the availability of nucleophiles (H_2O or NH_2R) [Pollock *et al.* (1972), Perkins *et al.* (1973), Frère *et al.* (1973 b)]. This transpeptidase-DD-carboxypeptidase was purified to protein homogeneity (95 % purity). It consists of a single polypeptide chain with a molecular weight of 38,000 . The acidic residues (20 %) outnumber the basic ones (5 %). About 50 % of the residues are non polar. Several physical and chemical properties were studied by circular dichroism and UV absorption and fluorescence [Frère *et al.* (1973 a), Nieto *et al.* (1973 a)].

Irrespective of the penicillins and cephalosporins used, inhibition of the transpeptidase activity of the exocellular enzyme always occurred at those antibiotic concentrations that inhibited the carboxypeptidase activity [Dusart *et al.* (1973)]. Qualitatively, its susceptibility to penicillins and its specificity profile for various acceptors resembled those of the membrane-bound transpeptidase, but quantitative differences were observed between the two enzymes. In spite of these differences, it can be argued that the exocellular enzyme is probably a soluble form of the membrane-bound enzyme. Indeed, the enzyme conformation in the hydrophobic environment of the membrane must be different from that in an aqueous solvent and a change in orientation of one or more amino acid residues in or near the catalytic centre(s) is likely to alter the specificity profile and susceptibility to antibiotics. In any event, the exocellular R61 transpeptidase-DD-carboxypeptidase was the first model to allow a study of the transpeptidation reaction and of its inhibition by penicillin at the molecular level.

Transfer and hydrolysis reactions catalyzed by the exocellular R61 transpeptidase-DD-carboxypeptidase

In carboxypeptidase assays, the enzyme showed a considerable specificity for peptides ending in a tripeptide sequence L-R_3-D-Ala-D(OH) and for the occurrence of a long aliphatic side-chain containing at least 4 (CH_2) groups on the L-R_3 residue. The C-terminal D residue was preferentially D-alanine [Leyh-Bouille *et al.* (1971)]. In transpeptidation assays (in which N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine was used as donor), the enzyme utilised with varying

efficiency a wide range of compounds such as glycine, D-alanine (but not L-alanine), peptides with N-terminal glycine or D-alanine, aminohexuronic acids, 6-aminopenicillanic acid and D-cycloserine [Perkins *et al.* (1973)].

The proportion of the enzyme activity that could be channelled into the transpeptidation and the hydrolysis pathways greatly depended upon the environmental conditions [Frère *et al.* (1973 b)]. Transpeptidation was favored both by increasing the pH and by decreasing the water content of the reaction mixtures. Kinetics suggested that the reactions proceeded through an ordered mechanism in which the acceptor molecule binds first to the enzyme. Acceptors were shown to behave as non competitive inhibitors of the hydrolysis pathway and some of them, when used at high concentrations, also inhibited the transpeptidation reaction, thus suggesting the occurrence on the enzyme of additional inhibitory binding sites [Frère *et al.* (1973 b)].

The activity of the enzyme (as measured in carboxypeptidase assays) could be inhibited by peptides that were analogues of peptide donors [Nieto *et al.* (1973 b)]. These studies showed that the enzyme binds peptide donors that have a *cis* configuration in the C-terminal amide linkage and suggested a possible sequence of events leading to hydrolysis. According to the model, the enzyme would select the small proportion of *cis* isomer, hence displacing the configuration equilibrium. The free enthalpy of binding would be mainly provided by the interaction of the two C-terminal residues whereas the side-chain of the L-R_3 residue would induce a conformational change in the enzyme which, in turn, would result in the previously *cis*-amide linkage adopting a configuration intermediate between *cis* and *trans* thus losing all double-bond character. The conformational change in the enzyme together with the interactions between enzyme groups and substrate would have to supply an energy of about 30-40 kJ . The active intermediate in the previous step would then break down into an acyl-peptide and the C-terminal amino acid. According to this model, distortion of the amide bond only occurs once the substrate is bound to the enzyme and the change in the enzyme only occurs in the presence of bound substrate.

The exocellular R61 transpeptidase-DD-carboxypeptidase as a model for penicillin action

Inhibition of the R61 enzyme by penicillin was completely reversible. Large concentrations of β-mercaptoethanol neither impaired the enzyme activity nor prevented its inhibition by benzylpenicillin [Nieto *et al.* (1973 a)]. Kinetically, the inhibition of the carboxypeptidase activity by benzylpenicillin and cephalothin was competitive (Ki = 7.5×10^{-8} M and 1.06×10^{-6} M, respectively) [Leyh-Bouille *et al.* (1971)]. The inhibition of the transpeptidase activity by penicillin V was also competitive with respect to the tripeptide donor (N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine) but was noncompetitive with respect to the acceptor (*meso*-diaminopimelic acid was used in this case) [unpublished results]. Binding of benzylpenicillin extensively affected the near UV circular dichroism of the enzyme and caused quenching of its fluorescence [Nieto *et al.* (1973 a)]. The association constant of the interaction was very high and comparable with the inhibition constant determined kinetically. Binding was comparatively slow and also occurred under conditions where the protein had been made enzymically inactive. Good acceptor substrates for transpeptidation had no effect on the interaction between the enzyme and benzylpenicillin. Donor substrates and donor-analogues that were competitive inhibitors effectively decreased the affinity of benzylpenicillin for the enzyme and, in this respect, behaved like general anions. However, when used at concentrations equivalent to 5 times their Km or Ki values, respectively, these peptide donors and competitive inhibitors had a very small effect on penicillin binding to the enzyme. This latter observation was in apparent conflict with the fact that kinetically, benzylpenicillin was a competitive inhibitor for peptide donors in carboxypeptidase assays. This type of kinetics, however, does not necessarily indicate that inhibitor and substrate compete for the same catalytically active site on the free enzyme. Altogether, these experiments suggested a *conformational response* model for penicillin action on the R61 enzyme in which the antibiotic binds to a specific site not directly concerned in catalysis and independent of the binding sites for the substrates [Leyh-Bouille *et al.* (1970 c)].

Interaction of benzylpenicillin produced a conformational change in the enzyme and the liberation of a Gibb's free energy of some 45 kJ (at 25°C) [Nieto *et al.* (1973 a)]. Hence, the enzymic splitting of the amide bond of peptide donors which would require a supply of energy of about 35 kJ in the absence of benzylpenicillin (see above), might require a supply of energy of about 80 (*i.e.* 35 + 45) kJ in the presence of the antibiotic. It can be argued that the enzyme would then be unable to surmount such an energy barrier. In other words, it was proposed that penicillin inhibits the enzyme because penicillin *freezes* the enzyme in a conformation that prevents activity [Nieto *et al.* (1973 a)].

The exocellular R39 transpeptidase-DD-carboxypeptidase

A membrane preparation capable of performing transpeptidations has not yet been obtained from *Streptomyces* R39 but the exocellular DD-carboxypeptidase-transpeptidase that is excreted by this organism [Leyh-Bouille *et al.* (1972)] was isolated to protein homogeneity. It consisted of a single polypeptide chain exhibiting a molecular weight of about 55,000 (unpublished results). Its substrate requirements for peptide donors also showed a considerable specificity for a C-terminal L-R_3-D-Ala-D(OH) sequence. Km and Vmax values, however, were different from those of the R61 enzyme. Moreover, the occurrence of ionized groups at the end of the aliphatic side-chain of the L-R_3 residue differently influenced the activity of the two enzymes [Leyh-Bouille *et al.* (1972)]. The enhancement of the transpeptidase activity of the R39 enzyme relative to its carboxypeptidase activity by high pH , by high concentrations of phosphate and by increasing the hydrophobic character of the reaction mixtures also pointed to the influence that the environment exerts on the functioning of the enzyme [Ghuysen *et al.* (1973 a)]. Penicillins and cephalosporins inhibited the transpeptidase activity at those concentrations which inhibited the carboxypeptidase activity but the susceptibility of the R39 enzyme toward these antibiotics was different from that of the R61 enzyme [Dusart *et al.* (1973)].

The most striking peculiarity of the R39 enzyme was its inability to catalyse transpeptidation reactions when peptides, instead of amino acids, were tested as possible acceptors [Pollock *et al.* (1972), Perkins *et al.* (1973)], *i.e.* the R39 enzyme was unable to catalyse the synthesis of peptide bonds in an endo-position. In fact, this property exactly reflected an important peculiarity of the wall peptidoglycan of *Streptomyces* R39. Contrary to what was observed in *Streptomyces* R61 [Leyh-Bouille *et al.* (1970 a, 1971)], peptide crosslinking in *Streptomyces* R39 is made *via* a D-alanyl-(D)-*meso*-diaminopimelic acid linkage in α position to a free carboxyl group (Table 1). This remarkable correlation strongly suggested that the exocellular enzyme was also a soluble form of the enzyme that catalyses peptide crosslinking *in vivo* [Ghuysen *et al.* (1973 a)].

The substrate requirements of the R39 enzyme for acceptors were studied with peptides which were either similar or identical to the natural peptide [Ghuysen *et al.* (1973 a)]. N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine was used as peptide donor. The natural peptide was L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine and the amino group located on the D carbon of *meso*-diaminopimelic acid in α position to the free carboxyl group was the one which was utilized as acceptor for transpeptidation. These studies pointed to the existence of multiple substrate sites on the enzyme and of mechanisms that controlled its hydrolyzing and synthesizing activities. In this respect, the amide substituent on the α-carboxyl group of D-glutamic acid appeared to play a role of prime importance. Control mechanisms also included inhibition by excess acceptor and by transpeptidation product, and susceptibility of the transpeptidation product to hydrolysis through the carboxypeptidase activity of the enzyme.

The R39 enzyme was not inhibited by any of the analogues of the peptide donor that inhibited the R61 enzyme [Nieto *et al.* (1973 b)]. Moreover, kinetically the inhibition of the carboxypeptidase activity of the R39 enzyme by benzylpenicillin was non-competitive and increasing antibiotic concentrations caused disproportionate decreases in the catalytic rate [Leyh-Bouille *et al.* (1972)]. Clearly, a unified

view on the mechanism of action of penicillin cannot yet be proposed.

The albus *G DD-carboxypeptidase*

The exocellular DD-carboxypeptidase excreted by *Streptomyces albus* G [Ghuysen *et al.* (1970), Leyh-Bouille *et al.* (1970 b,c)], exhibited a specificity profile for peptide donors that very closely resembled that of the R39 enzyme. The *albus* G enzyme, however, was unable to perform transpeptidations with any of the compounds, including free amino acids, which were tested as possible acceptors [Pollock *et al.* (1972), Perkins *et al.* (1973)]. Furthermore, the *albus* G enzyme was not inhibited by β-lactam antibiotics [Leyh-Bouille *et al.* (1970 c)]. The most likely explanations are that either the enzyme has lost both the penicillin binding site and the acceptor binding site or these sites on the enzyme have been modified so that penicillin and acceptors would have no effect on binding of peptide donors and the hydrolytic activity of the enzyme. A similar situation was found by Barnett (1973) with the membrane-bound DD-carboxypeptidase of *B. stearothermophilus*. Conditions which favoured stability of the catalytic site of the enzyme led to loss of susceptibility to benzylpenicillin whereas under conditions in which susceptibility to benzylpenicillin was retained, catalytic activity was readily lost. These observations were also taken as evidence that substrate and penicillin bind to separate sites on the enzyme.

Conclusions

The *DD-carboxypeptidase-transpeptidase* system in bacteria may consist of a single protein. Depending upon its localization (within the membrane, on the membrane, in the periplasmic region or in the external medium), *i.e.* depending upon the availability of water, the protein could function in an anabolic sense as a transpeptidase or in a catabolic sense as a carboxypeptidase. Depending upon its precise microenvironment within the membrane, the protein could undergo conformational changes affecting its substrate requirements and/or susceptibility to penicillins and cephalosporins. The studies carried out with the R61 and R39 enzymes gave direct

experimental supports to these ideas. In fact, such a situation is equivalent to that where more than one DD-carboxypeptidase-transpeptidase would occur in the cell, each of them being involved in different growth cell processes. It is a well known fact that cell elongation and cell division in *E. coli* [Schwarz *et al.* (1969)] and in *Proteus vulgaris* [Fleck and Mock (1972)] are two distinct targets of penicillin in that they exhibit large differences in their susceptibility to these antibiotics. In addition to the DD-carboxypeptidase(s)-transpeptidase(s) protein(s), there may exist one or several carboxypeptidases that would solely function as hydrolyzing enzymes. The *Streptomyces albus* G enzyme is probably such an uncoupled DD-carboxypeptidase.

At present, no unified view can be proposed for the mechanism of action of penicillin except that, from an integration of all the results so far accumulated, penicillin appears to act as an allosteric modifier of the bacterial transpeptidases-DD-carboxypeptidase systems [Nieto *et al.* (1973 a), Ghuysen *et al.* (1973 b)].

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