

The Concept of the Penicillin Target from 1965 until Today

The Thirteenth Marjory Stephenson Memorial Lecture

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The Biochemical Laboratory at Cambridge is the place where Marjory Stephenson established microbiology as a biochemical science and demonstrated that bacteria were useful as experimental material quite apart from their disease-producing action. It is also the place where in the early 1950s Professor Milton R. J. Salton, at that time a graduate student of Professor Ernest Gale, initiated biochemical investigations of bacterial cell walls. Marjory Stephenson died too early (1948) to witness this remarkable event but one can guess the wonder she would have expressed for the enzymes involved in the synthesis of the wall peptidoglycan, a structure which contains muramic acid and several D-amino acids and is unique to the bacterial world. Perhaps she would have found these enzymes even more extraordinary than those concerned with the utilization of molecular hydrogen and with the oxidation and reduction of inorganic substrates, about which she wrote in 1939 'all the enzymes found in the animal will ultimately turn up in some bacterium or other whilst these found only in bacteria will be far more numerous' (Stephenson, 1939). A few years later, appraising the progress made in bacterial metabolism, Marjory Stephenson, who was always fascinated by the life of the organisms, noted with enthusiasm: 'information is now being rapidly gained on the course of the biochemical process leading to cell synthesis . . . We seem, in fact, to be witnessing a transition from katabolic to anabolic studies, made possible only by the use of the microbe as experimental material' (Stephenson, 1949). Among the new sources responsible for this fresh stream of knowledge were studies of the intracellular changes resulting from chemotherapeutic agents, antibiotics and other cell poisons. We all know how crucial for the bacterial cell are the various steps by which the wall peptidoglycan precursors are synthesized in the cytoplasm and are assembled into a rigid, net-like polymer outside the plasma membrane. We also know how essential for the unravelling of the mechanisms involved was the use of D-cycloserine, bacitracin and penicillin.

The decision of the Society to pay tribute to Marjory Stephenson by organizing a lecture on the biosynthesis of the bacterial cell wall peptidoglycan and on the mode of action of penicillin, therefore seems to be most appropriate. However, as I stand before you on this occasion, I do so with mixed feelings. I am deeply honoured by the invitation but I am also convinced that friends who have much contributed to these problems, deserve this honour more than I do and would fulfil the duty in a more elegant way. Moreover, I have been fortunate in having extremely able colleagues: Mélina Leyh-Bouille and Jacques Coyette who were with me from the start of our work; Jean-Marie Frère, Jean Dusart and Martine Nguyen-Distèche who later joined us with fresh ideas; Harold R. Perkins now in Liverpool, Manuel Nieto now in Madrid, Peter E. Reynolds from Cambridge, and Jerry J. Pollock now in Stony Brook all played important, sometimes decisive roles in the work. I am sad that we cannot share this moment together.

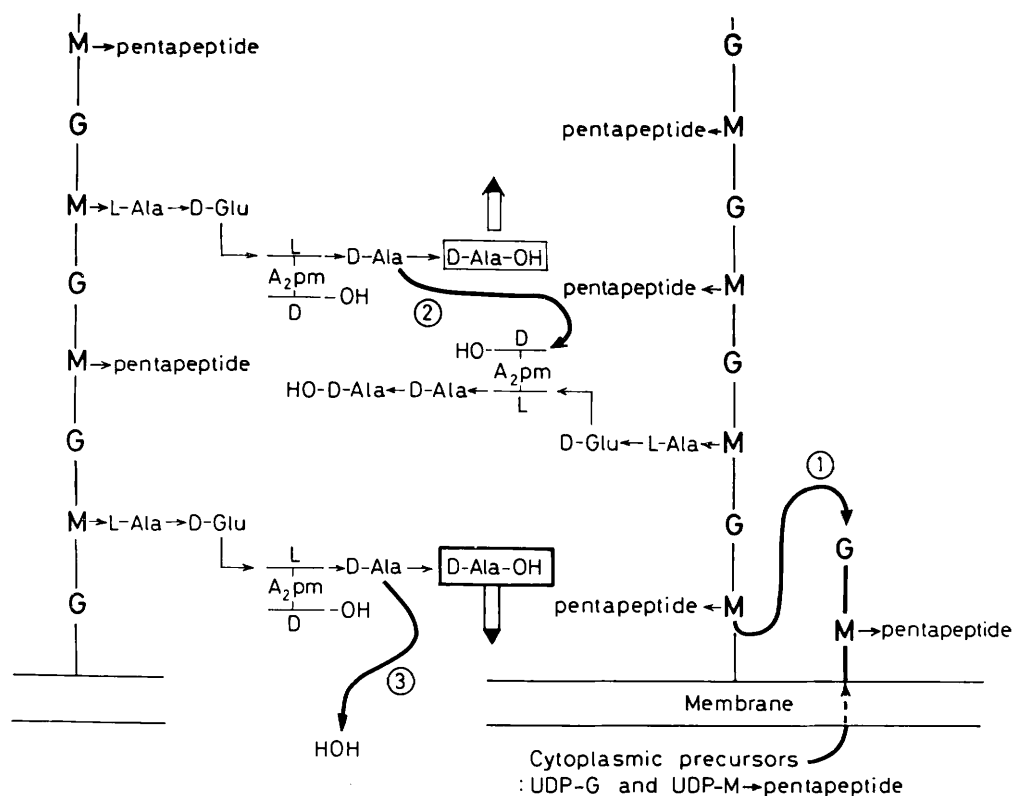
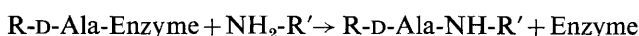
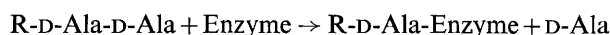


Fig. 1. Wall peptidoglycan synthesis. (1) Elongation of a nascent peptidoglycan strand on the plasma membrane. (2) Transpeptidation between two nascent peptidoglycan strands leading to crosslinking and release of D-Ala. (3) Hydrolysis by DD-carboxypeptidase action leading to release of D-Ala without formation of a peptide bond. G, N-Acetylglucosamine; M, N-acetylmuramic acid.

Penicillin: the ideal chemotherapeutic agent?

In 1965, in the same issue of the *Proceedings of the National Academy of Sciences*, Wise & Park and Tipper & Strominger proposed, independently, that penicillin blocked the biosynthesis of the bacterial wall peptidoglycan by preventing the closure of the peptide bridges between adjacent strands. The reaction inhibited was a transpeptidation as a result of which, under normal conditions of growth, the carboxyl group of the penultimate D-alanine residue of the pentapeptide donor ending in D-Ala-D-Ala was transferred to the free ω -amino group of another peptide acceptor (Fig. 1) with the result that new peptide bonds were formed, equivalent amounts of D-Ala were released from the peptide donors and the newly made peptidoglycan became insoluble. Tipper & Strominger suggested a possible mechanism for the transpeptidation reaction and for its inhibition by penicillin. In the absence of the antibiotic, the C-terminal D-Ala-D-Ala of a pentapeptide donor would first react with the transpeptidase to form an acylenzyme intermediate with the release of D-Ala and, subsequently, the amino end of the peptide acceptor would react with this intermediate to form the cross-bridge. The proposed model was a Ping-Pong Bi Bi mechanism:



Penicillin, assumed to be a close structural analogue of the C-terminal D-Ala-D-Ala, would react with the donor binding site on the transpeptidase, yielding an irreversible, inactive

penicilloyl-enzyme complex. Penicillin had a high selective toxicity because it was postulated to act on one enzyme which occurred solely in bacteria and because the initial driving force for the interaction was due to the presence in the enzyme of a penicillin-binding site which normally recognized a C-terminal D-Ala-D-Ala sequence, a structural feature completely missing in eukaryotic cells. Penicillin was very efficient because of the irreversible nature of the bond formed between the drug and the enzyme. Of the antimicrobial agents known, penicillin was the one which best fulfilled the canons of chemotherapy (at least if one does not take into account the allergic reactions associated with it and its destruction by penicillinase action). The hypothesis remains, but investigations carried out along different lines in various laboratories in the last 12 years do not substantiate it in all its details and the precise molecular mechanism by which β -lactams exert their lethal effect remains elusive.

The multiple enzymes concerned with the wall peptidoglycan crosslinking

As early as 1966, it was known that the system involved in peptide crosslinking contained more than one single enzyme and that it consisted of at least two antagonistic activities (Araki *et al.*, 1966; Izaki, Matsushashi & Strominger, 1966). In addition to the transpeptidase activity which catalyses peptide bond formation, bacteria also contain a DD-carboxypeptidase activity which simply hydrolyses the C-terminal D-Ala-D-Ala sequences of pentapeptide units without performing any transfer reaction (Fig. 1). As the transpeptidase is sensitive to penicillin, so too is the DD-carboxypeptidase activity. The idea immediately brought forward was that the DD-carboxypeptidase activity was there to limit the number of pentapeptide units made available to the transpeptidase activity and, therefore, to control the extent of peptidoglycan crosslinking. It was also suggested that the DD-carboxypeptidase might be an uncoupled transpeptidase. Roughly the idea was correct. Yet, it was far from expressing the real complexity of the problem.

The concept of a 'nascent peptidoglycan', which was rather vague in 1965, was rigorously substantiated thanks to the elegant studies of Ward & Perkins (1973). In *Bacillus licheniformis*, chains consisting of multiple disaccharide-peptide units were shown to grow by addition of the new units at the reducing terminus of the lengthening chain. In the process, the reducing terminal *N*-acetylmuramic acid of the growing chain is transferred from its link with the membrane to the non-reducing *N*-acetylglucosamine terminus of the new disaccharide-peptide unit which is itself linked to the membrane (Fig. 1). The membrane acceptor on which the new disaccharide-peptide units are made is the undecaprenyl phosphate carrier. However, the membrane acceptor on which polymerization of the nascent peptidoglycan chains occurs has not yet been definitely characterized.

When growing in liquid suspensions, protoplasts excrete the soluble, nascent peptidoglycan they make and new walls cannot be formed. However, when incubated on the surface of an agar medium of low fluidity, protoplasts of *B. licheniformis* were shown to be able to revert successfully to normal rod-shaped bacilli (Elliott *et al.*, 1975*a, b*). Hence, insolubilization of the peptidoglycan by peptide crosslinking between nascent strands can be carried out by cells which at the onset of the process completely lack pre-existing walls (Fig. 1). The same process probably takes place in normal, wall-containing cells. In this case, however, insolubilization of the nascent peptidoglycan is mainly achieved by attachment to the pre-existing wall peptidoglycan. Attachment of part of the precursor can be carried out by transglycosylation at the level of the glycan strands, a reaction which is not sensitive to penicillin (Mirelman, Bracha & Sharon, 1972, 1974), but transpeptidation at the level of the peptide units appears to be the major mechanism involved. Two alternatives may exist: the nascent peptidoglycan may act as carboxyl donor through its C-terminal D-Ala-D-Ala sequences while the pre-existing wall peptidoglycan would act as amino acceptor; conversely, D-Ala-D-Ala sequences in the pre-existing wall peptidoglycan may function as donors, and amino groups of the newly synthesized peptidoglycan strands would

function as acceptors. In fact, the transpeptidation reaction can proceed in either direction. The first mechanism occurs in *B. licheniformis* (where it may prevail; Ward & Perkins, 1974) (Fig. 2) and the second in *Gaffkya homari* (Hammes, 1976; Hammes & Kandler, 1976) (Fig. 3). This latter mechanism is especially complex. Indeed, those peptide units of the nascent peptidoglycan which serve as acceptor groups for transpeptidation are tetrapeptides resulting from prior DD-carboxypeptidase action. However, pentapeptide units must remain present in this nascent peptidoglycan in such a way that through the active transpeptidation of the tetrapeptide units, these pentapeptide units are passively incorporated into the wall peptidoglycan where they can serve as donor units for further expansion of the polymer. *In vivo*, the newly synthesized peptidoglycan strands which undergo attachment probably contain equimolar amounts of pentapeptide and tetrapeptide units. Such a ratio would allow a maximum degree of crosslinking of 50 % and oligomers larger than dimers could not be formed. In fact, such structural features are those found in the wall peptidoglycan of *G. homari*.

In parallel to the different mechanisms of wall peptidoglycan expansion, the specific inhibition of the DD-carboxypeptidase activity in *B. subtilis* and *B. stearothermophilus* does not cause detectable damage to the cell (Blumberg & Strominger, 1971; Yocum, Blumberg & Strominger, 1974), suggesting that the transpeptidase is the enzyme that is physiologically important. On the contrary, the specific inhibition of the DD-carboxypeptidase activity in *G. homari* is sufficient to prevent peptidoglycan incorporation and to cause cessation of cell growth (Hammes, 1976). Hence, a unified view on the nature of the enzyme on which penicillin binding causes cessation of cell growth (and cell lysis) cannot be proposed. The concept of a penicillin 'killing site' may well escape a precise definition.

In the Gram-negative bacteria and in various Gram-positive bacilli, the interpeptide bond made by transpeptidation is a D-Ala-(D)-*meso*-diaminopimelic acid linkage in α position to a free carboxyl group (Fig. 1), a structural feature which fits well the substrate requirements for DD-carboxypeptidase action. Hence the extent of peptidoglycan crosslinking is susceptible to regulation not only by DD-carboxypeptidase action on the pentapeptide units before incorporation of the nascent peptidoglycan but also by DD-carboxypeptidase action on the interpeptide bonds in the completed peptidoglycan. DD-Carboxypeptidases which perform such endopeptidase activity exist; they are also sensitive to penicillin (Bogdanovsky, Bricas & Dezélee, 1969).

Septum formation in *Escherichia coli* has received much attention. A bold and attractive working hypothesis made by Mirelman, Yashow-Gan & Schwarz (1976) is that the nascent peptidoglycan would act as carboxyl donor for transpeptidation reactions with two types of acceptors. Reaction with the pre-existing wall peptidoglycan would lead to wall elongation, whereas reaction with a modified nascent peptidoglycan previously deprived of donor sites by DD-carboxypeptidase action and accumulated at the equator of the cell (i.e. where it is synthesized) would lead to septum formation. The specific inhibition of the DD-carboxypeptidase responsible for the destruction of the donor sites of the nascent peptidoglycan (and for its accumulation) and/or the specific inhibition of the transpeptidase which uses this modified nascent polymer as amino acceptor would therefore prevent cell septation and would cause cell filamentation, a morphological damage which can be induced in *E. coli* cells by low levels of ampicillin. In fact, fractionation of the membrane-bound peptide cross-linking enzyme system of *E. coli* has shown that the DD-carboxypeptidase-transpeptidase-endopeptidase activities occur in two distinct forms, one of them being sensitive to low levels of ampicillin (and perhaps involved in cell septation) and the other being sensitive to much higher levels of ampicillin (and perhaps involved in cell elongation) (Pollock *et al.*, 1974; Nguyen-Distèche *et al.*, 1974a, b).

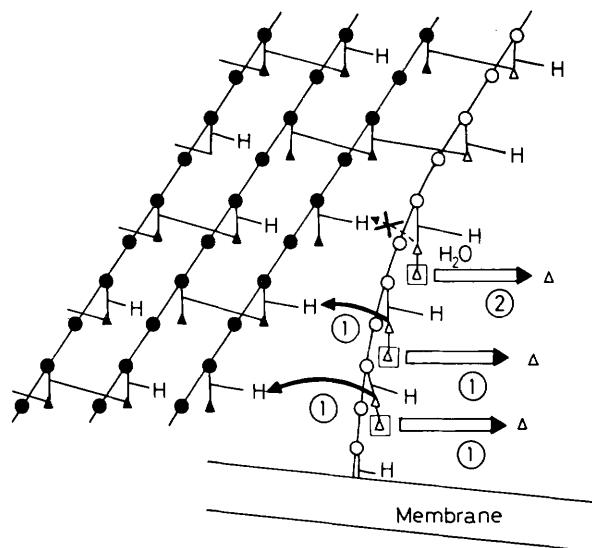


Fig. 2. Attachment of a nascent peptidoglycan strand (open symbols) to the pre-existing wall peptidoglycan (black symbols) in *Bacillus licheniformis*. The nascent peptidoglycan acts as carboxyl donor (\triangle = D-Ala-D-Ala) and the pre-existing wall peptidoglycan acts as amino acceptor ($-H$). (1) Transpeptidase action; (2) DD-carboxypeptidase action. Prior DD-carboxypeptidase action on the nascent peptidoglycan prevents further transpeptidation.

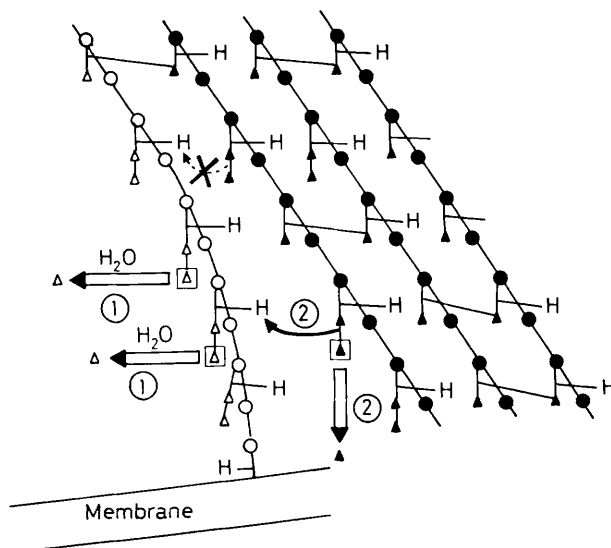


Fig. 3. Attachment of a nascent peptidoglycan strand (open symbols) to the pre-existing wall peptidoglycan (black symbols) in *Gaffkya homari*. The pre-existing wall peptidoglycan acts as carboxyl donor (\triangle = D-Ala-D-Ala) and tetrapeptide units of the nascent peptidoglycan act as amino acceptors ($-H$). (1) DD-Carboxypeptidase action; (2) transpeptidase action. Prior DD-carboxypeptidase action on the nascent peptidoglycan is required for further transpeptidation.

The penicillin-binding components

Tremendous efforts are being made in order to study the penicillin-binding components from penicillin-sensitive cells on the assumption that penicillin-sensitive enzymes and penicillin-binding proteins are synonymous. These studies, initiated in Professor Strominger's laboratory, yielded important results (for a review, see Blumberg & Strominger, 1974). In agreement with the known complexity of the peptidoglycan crosslinking enzyme system, multiple penicillin-binding sites exist in both Gram-positive and Gram-negative bacteria. These binding sites occur in the plasma membrane and, to all appearances, they are distinct proteins. They can be detected by dodecylsulphate-polyacrylamide slab gel electrophoresis of membranes to which radioactive penicillin has been bound; they are numbered in the order of decreasing molecular weight. This technique has revealed the presence of at least six penicillin-binding proteins in *E. coli*, five in *B. subtilis* and *B. megaterium*, four in *B. stearothermophilus* and three in *B. cereus*.

Two complementary approaches have been taken. One, which has been more especially applied to *E. coli*, is to try to relate one or several of the penicillin-binding components to the various morphological responses that β -lactams can elicit in this organism: inhibition of cell septation and cell division; bulge formation; formation of osmotically-stable ovoid cells; cessation of cell growth and cell lysis (Spratt, 1975, 1977). The six main penicillin-binding proteins in *E. coli* are minor components of the plasma membrane. If one assumes that one penicillin-binding site occurs per molecule, then one cell contains about 230 molecules of protein 1 (mol. wt 91000), 20 of protein 2 (mol. wt 66000), 50 of protein 3 (mol. wt 60000), 110 of protein 4 (mol. wt 49000), 1800 of protein 5 (mol. wt. 42000) and 570 of protein 6 (mol. wt 40000). Proteins 4, 5, and 6 are probably not involved in the effects on cell morphology produced by β -lactams. Protein 2 is required for the growth of *E. coli* as a rod-shaped cell and is the protein on which the amidinopenicillanic acids act to produce ovoid cells. Binding of β -lactams to protein 3 seems to cause inhibition of cell division and filamentation. Binding to both proteins 2 and 3 seems to cause bulge formation. Protein 1 would then be the site at which β -lactams bind to inhibit cell elongation, to cause cessation of cell growth and to induce cell lysis, i.e. protein 1 would be the 'main' transpeptidase. Protein 1, however, may be two proteins, a situation which makes the problem more complex. Furthermore, in *Salmonella typhimurium*, protein 4, and not protein 1, appears to have the greatest capacity for carrying out transpeptidation *in vitro* (Shepherd, Chase & Reynolds, 1977). Finally, inhibition of cell elongation and cessation of growth may be caused by the inhibition of a penicillin-sensitive enzyme which cannot be detected as a penicillin-binding protein. Thus, for example, the penicillin-sensitive DD-carboxypeptidase 1B of *E. coli* apparently cannot be detected by this technique (Tamura, Imae & Strominger, 1976). Hence, the penicillin-binding components detected by autoradiography of the slab gels do not necessarily reflect the complexity of the peptidoglycan crosslinking enzyme complex. Conversely, the efficiency of the binding largely depends upon the experimental conditions (antibiotic concentration, time of exposure etc.) and, under certain circumstances, penicillin may react non-specifically with proteins – in the sense that they are not members of the enzyme complex – which consequently are revealed as penicillin-binding components.

A second approach is to isolate and characterize the penicillin-binding components (Blumberg & Strominger, 1974). Protein 5 of *B. subtilis* is a DD-carboxypeptidase. It was isolated by affinity chromatography on 6-aminopenicillanic acid-substituted Sepharose of solubilized membranes previously treated with cephalothin under conditions which allowed saturation of compounds 1 to 4 but not of compound 5. Protein 3 of *B. stearothermophilus* (Yocum *et al.*, 1974) and proteins 5 and 6 of *E. coli* are also DD-carboxypeptidases (Spratt & Strominger, 1976).

Obviously, the full significance of the penicillin-binding sites cannot be understood as

long as the relationship between them and the multiple penicillin-sensitive enzymes is not clearly established. One of the difficulties of the problem is the following. DD-Carboxypeptidase activity can be easily estimated by measuring the C-terminal D-Ala residue released from the relevant UDP-*N*-acetylmuramyl-pentapeptide precursors. Parallel to this, at least until recently, the penicillin-binding sites to which an enzyme activity could be assigned were DD-carboxypeptidases. The test for transpeptidase activity as it is performed in many laboratories makes use of various particular cell-free systems containing both the pre-formed wall peptidoglycan and all the enzymes involved in the synthetic pathway beyond the level of the nucleotide precursors. In the presence of the exogenously added nucleotides, the reaction proceeds through the complex series of steps and culminates in peptidoglycan crosslinking. Of course, such an assay is of no value for testing a transpeptidase separated from the bacterial envelope and disconnected from the other enzymes which precede the transpeptidation reaction. The difficulty, however, can be overcome.

*Exocellular enzymes from Streptomyces R61 and Actinomadura R39 as models
for the study of the peptidoglycan crosslinking enzyme system*

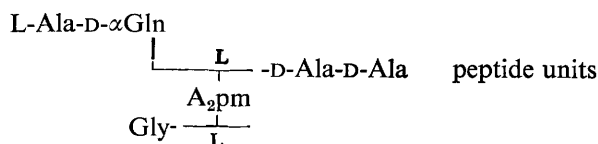
When a natural phenomenon is complex, it is useful to set up an artificial, well-defined model which mimics it as closely as possible. Such a model could be devised using enzymes from some actinomycetes, in particular *Streptomyces* R61 and *Actinomadura* R39. During growth these strains excrete exocellular enzymes able to catalyse reactions which resemble those catalysed *in vivo* by the corresponding crosslinking enzyme systems. Each of these enzymes consists of one single polypeptide chain with a molecular weight of 38000 for the R61 enzyme and 53000 for the R39 enzyme. Many of their physicochemical and enzymic properties are described in two recent reviews where details and a complete list of references can be found (Ghuysen, 1977; Frère *et al.*, 1976*b*). Each of these enzymes performs three activities: a DD-carboxypeptidase activity, a transpeptidase activity and a penicillin-degrading activity. What, in summary, did we learn from them?

DD-Carboxypeptidase-transpeptidase activity

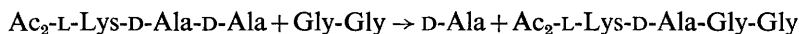
(1) Both enzymes catalyse hydrolysis reactions; they are DD-carboxypeptidases. The C-terminal sequence giving the highest activity is D-Ala-D-Ala but the preceding L-amino acid residue also has a considerable effect on enzyme activity. Although differences exist between the two enzymes with regard to the influences of charged groups at the end of the side-chain of this L-amino acid residue, the synthetic tripeptide Ac₂-L-Lys-D-Ala-D-Ala is an excellent substrate for each of them. The standard reaction is



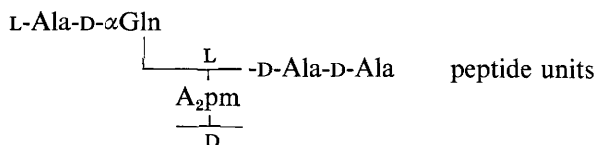
(2) Both enzymes are also transpeptidases. They catalyse transpeptidation reactions between the above tripeptide acting as donor and certain peptides acting as acceptors. In order to function as acceptor, the amino group must belong to a structure resembling that found in the natural peptide acceptor involved in the *in vivo* transpeptidation. The transpeptidation reaction in *Streptomyces* R61 occurs between



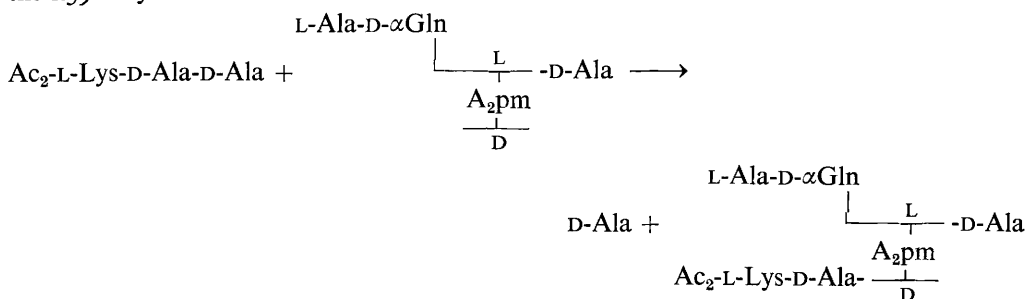
where the acceptor group is the N-terminal glycine residue. Of the peptides that function as acceptors for the R61 enzyme, the most effective are those with an N-terminal glycine, and Gly-Gly is one of the best. The standard reaction catalysed by the R61 enzyme is



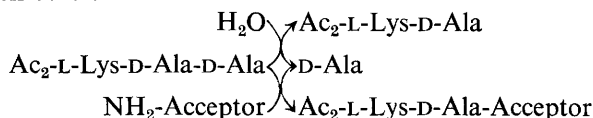
The transpeptidation reaction in *Actinomadura* R39 occurs between



where the acceptor is the amino group located on a D-carbon (of *meso*-diaminopimelic acid) in α position to a free carboxyl group. The basic requirement of the R39 enzyme for an acceptor is precisely such a structural feature. Hence, the R39 enzyme is unable to catalyse transpeptidation reactions leading to the synthesis of peptide bonds in an endo-position and therefore it differs strikingly from the R61 enzyme. The standard reaction catalysed by the R39 enzyme is



(3) Whenever the enzyme performs transpeptidation, concomitant hydrolysis of the peptide donor also occurs. D-Ala is thus released by two pathways: by hydrolysis (DD-carboxypeptidase activity) and by transpeptidation (transpeptidase activity), the two processes competing with each other.

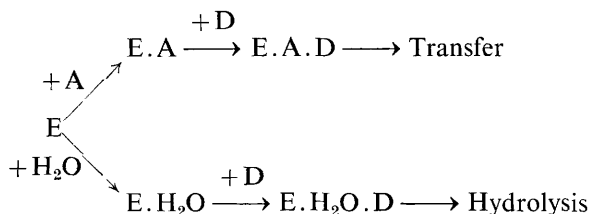


In aqueous media, at neutral pH and at low acceptor concentrations, both enzymes act mainly as DD-carboxypeptidases; their transfer activities are low. However, the proportions of the catalytic activity that can be channelled into the hydrolysis and transpeptidation pathways, respectively, greatly depend upon the environmental conditions. Replacement of part of the water of the reaction mixture by solvents of low polarity preferentially decreases the DD-carboxypeptidase activity so that transpeptidation may largely supersede hydrolysis. Transpeptidation is also favoured by raising the pH value of the reaction mixture. The DD-carboxypeptidase activity of the R39 enzyme is little affected by the ionic strength but its transpeptidase activity is specifically enhanced at high ionic strength. The R61 enzyme, however, has maximal hydrolytic and transfer activities at low ionic strength. Finally, peptide acceptors behave as non-competitive inhibitors of the hydrolysis pathway and therefore high acceptor concentrations favour transpeptidation.

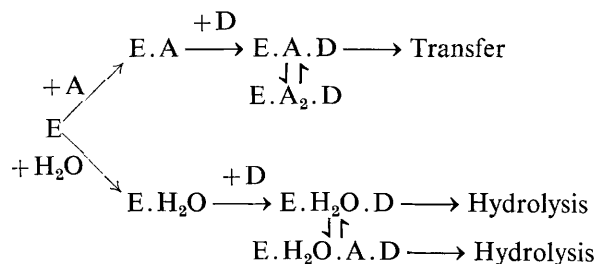
(4) With the R39 enzyme and at low donor concentrations, hydrolysis is inhibited by increasing concentrations of acceptor to a greater extent than transpeptidation is increased. The acceptor is an inhibitor of the total enzyme activity. However, at high peptide donor concentrations when the donor site of the enzyme is saturated, the increase in the rate of the transfer reaction and the decrease in the rate of hydrolysis caused by increasing concentrations of acceptor (at least within certain limits) are commensurate. The enzyme activity that is diverted from the hydrolysis pathway because of the presence of the acceptor is channelled entirely into the transfer pathway. Under these conditions, the acceptor is an inhibitor of

hydrolysis but not of the total enzyme activity which remains constant. Finally, another interesting feature of the system is that when complex peptide acceptors are used, not only hydrolysis but transpeptidation itself may be inhibited by excess acceptor.

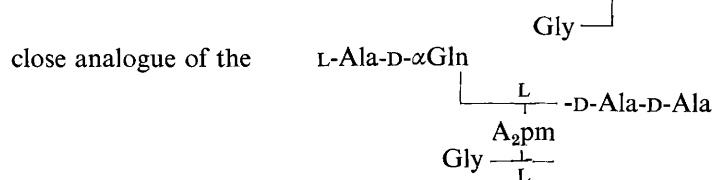
(5) The effects that the concentrations of the donor and the acceptor, respectively, exert on the rates of transfer and hydrolysis reactions concomitantly catalysed by the R61 enzyme suggest that these reactions proceed through an ordered mechanism in which the acceptor binds first to the enzyme (A, acceptor; D, donor; E, enzyme):



The results obtained are not compatible with a mechanism where the donor binds first to the enzyme as in the Ping-Pong Bi Bi mechanism suggested by Tipper & Strominger (1965). For complex peptide acceptors which at high concentrations inhibit the transpeptidation pathway, an additional acceptor binding site would be present on the enzyme leading to the formation of an (enzyme-acceptor₂-donor) complex which would be non-productive for transpeptidation, and to the formation of an (enzyme-H₂O-acceptor-donor) complex remaining productive for hydrolysis. The above scheme would then be modified:

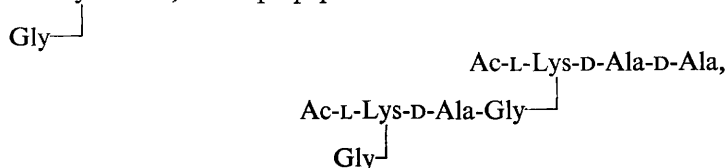


(6) Both R61 and R39 enzymes can catalyse transpeptidation reactions in which the same peptide serves as donor and acceptor, thus leading to the formation of peptide dimers and, sometimes, peptide trimers. The tetrapeptide Ac-L-Lys-D-Ala-D-Ala can be regarded as a

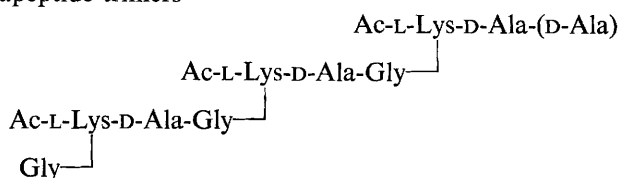


peptide moiety of the peptidoglycan precursor in *Streptomyces* R61. Ac-L-Lys-D-Ala-D-Ala

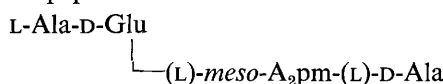
is utilized by the R61 enzyme in media of low polarity with the result that *N*⁶-(D-Ala-Gly)-L-Lys bonds are formed. The reaction products are D-Ala, the tripeptide monomer Ac-L-Lys-D-Ala, the heptapeptide dimer



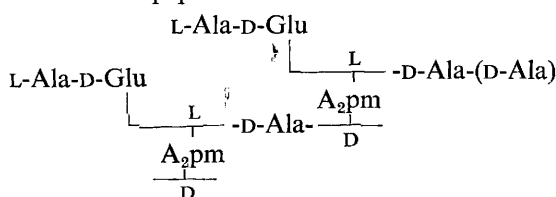
the hexapeptide dimer (i.e. the former dimer lacking the C-terminal D-Ala) and a mixture of nona- and decapeptide trimers



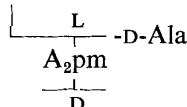
Similarly, in media of high ionic strength, the R39 enzyme utilizes the pentapeptide L-Ala-D-Glu to form free D-Ala, the tetrapeptide



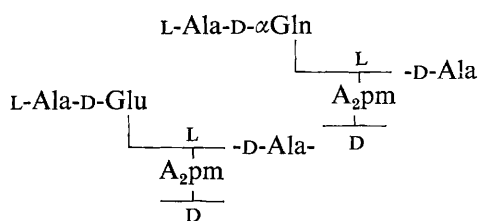
and a mixture of octa- and nonapeptide dimers



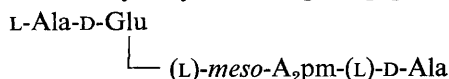
Increased yields of peptide dimer are obtained by providing the R39 enzyme with the above pentapeptide at a concentration (300 μM) which saturates the peptide donor site of the enzyme but is too low to induce its own dimer formation, and a high concentration (60 mM) of the amidated tetrapeptide L-Ala-D- α Gln



(which lacks the C-terminal D-Ala-D-Ala sequence and thus functions only as acceptor). Under these conditions, the rate of formation of the mono-amidated peptide dimer



is twofold higher than the rate of hydrolysis of the pentapeptide into



Note that the above peptide dimers made by the action of the R39 enzyme are either identical or extremely similar to those present in the Gram-negative bacteria, in many bacilli and in *Actinomadura* R39 itself.

Penicillin-degrading activity

(1) Like various β -lactamases, acylases and esterases, both the R61 and R39⁺ enzymes degrade β -lactams. The reaction, however, is peculiar in two respects. One peculiarity

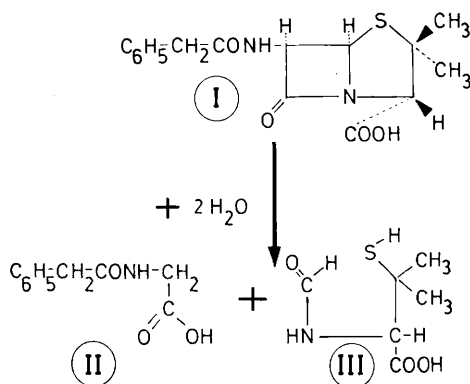
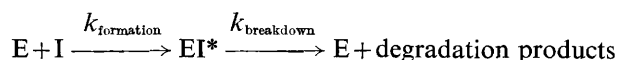


Fig. 4. Enzymic fragmentation of benzylpenicillin (I) into phenylacetylglycine (II) and *N*-formyl-D-penicillamine (III).

During prolonged incubation, the released *N*-formyl-D-penicillamine undergoes spontaneous dimerization into di-(*N*-formyl-D-penicillamine) disulphide. The mechanism of the catalysis remains to be discovered. In particular, one should mention that fragmentation of benzylpenicillin by the DD-carboxypeptidase of *B. stearothermophilus* also yields phenylacetylglycine (Hammarström & Strominger, 1975). 5,5-Dimethyl- Δ^2 -thiazoline carboxylic acid would be the other fragment (Hammarström & Strominger, 1976). However, the fragment arising from the thiazolidine moiety of penicillin was almost certainly mischaracterized since under the conditions prevailing during the reaction (55 min; 55 °C; pH 6.5), 5,5-dimethyl- Δ^2 -thiazoline carboxylic acid has a half-life of 130 s and is transformed into *N*-formyl-D-penicillamine (J. M. Frère, unpublished data). Under these conditions, the mechanism proposed for the enzymic fragmentation of penicillin on the assumption that 5,5-dimethyl- Δ^2 -thiazoline carboxylic acid was one of the primary products released remains entirely hypothetical.

concerns the nature of the degradation products. Benzylpenicillin is degraded into phenylacetylglycine and *N*-formyl-D-penicillamine with the formyl substituent arising from C₅ of benzylpenicillin (Fig. 4). When phenoxyethylpenicillin is used instead of benzylpenicillin, phenoxyacetylglycine and *N*-formyl-D-penicillamine are produced. Hence the nature of the acyl substituent does not modify the pathway of the reaction. The overall reaction involves the addition of two H₂O molecules and results in the hydrolysis of the amide bond and in the rupture of both C₅-C₆ and C₅-S linkages. Enzyme reactivation and fragmentation of the penicillin molecule are concomitant events. However, *N*-formyl-D-penicillamine may not be the initial fragment arising from the thiazolidine moiety of the penicillin molecule but the nature of the possible intermediate remains to be determined. The products resulting from the interaction with cephalosporins have not yet been established. It is certain, however, that these antibiotics are also irreversibly destroyed during the reaction. Another peculiarity of the reaction is that it is a slow or a very slow process. Depending upon the enzymes and the β -lactams, the k_{cat} values for the reactions range from 0.28×10^{-6} to $1.4 \times 10^{-4} \text{ s}^{-1}$ (Table 1). For the purposes of comparison, the k_{cat} values for the hydrolysis of Ac₂-L-Lys-D-Ala-D-Ala into D-Ala and Ac₂-L-Lys-D-Ala by the R61 and R39 enzymes are about 55 and 17.5 s⁻¹, respectively.

(2) The main feature of the reaction is that it proceeds through (i) the formation of a rather stable, stoichiometric enzyme- β -lactam complex EI* in which the enzyme (E) is inactivated and the β -lactam (I, for inhibitor) is chemically modified, and (ii) the subsequent breakdown of complex EI* with regeneration of the enzyme and release of the antibiotic as biologically inactive metabolites



Formation of complex EI* is characterized by a second order rate constant ($k_{\text{formation}}$ in M⁻¹ s⁻¹), and breakdown by a first order rate constant ($k_{\text{breakdown}}$ in s⁻¹). In some cases,

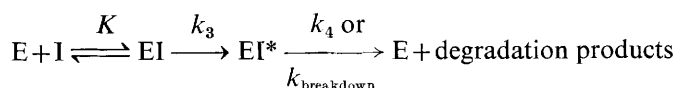
Table 1. Interaction between β -lactams and the exocellular *Streptomyces* R61 and *Actinomadura* R39 enzymes†

Interaction β -lactam-enzyme	$k_{\text{formation}}$ ($\text{M}^{-1} \text{s}^{-1}$) or k_3/K	K (M)	k_3 (s^{-1})	$10^5 \times$ $k_{\text{breakdown}}$ or k_4 at 37°C (s^{-1})	Half-life of complex EI* at 37°C (min)	K_i (nM)	$10^8 \times$ ID_{50} at 37°C (M)
Benzylpenicillin-R39	> 90000 (20 $^\circ \text{C}$)			0.28	4100	0.01	(5)‡
Ampicillin-R39	74000 (20 $^\circ \text{C}$)			0.44	2600	0.06	(3)‡
Cephaloglycine-R39	74000 (20 $^\circ \text{C}$)			0.08	14000	0.03	(6)‡
Cephalosporin C-R39	66000 (20 $^\circ \text{C}$)	0.19	12.5	0.028	40000	0.004	(5)‡
Benzylpenicillin-R61	13700 (25 $^\circ \text{C}$)	13	180	14	80	10	14
Carbenicillin-R39	2900 (20 $^\circ \text{C}$)			0.54	2125	1	200
Cephalosporin C-R61	1150 (37 $^\circ \text{C}$)			0.1	11200	1	45
Carbenicillin-R61	820 (37 $^\circ \text{C}$)	0.11	0.09	14	80	170	800
Ampicillin-R61	107 (37 $^\circ \text{C}$)	7.2	0.77	14	80	1300	2600
Cephaloglycine-R61	22 (37 $^\circ \text{C}$)	0.4	0.009	0.3	3700	150	6900

† For further details and data, see Ghuyssen (1977).

‡ In these cases, the k_3/K ratio values are so high and the k_4 values are so low that the determinations are probably equivalent to a simple titration of the enzyme used in the experiments.

complex formation could be analysed by fast kinetics techniques. The simplest model which best explains the data is



Enzyme and β -lactam first react to form a complex EI (characterized by a dissociation constant K , in M) which, in turn, isomerizes into complex EI*. The isomerization step is characterized by a first order rate constant k_3 (in s^{-1}). Of course, k_4 and $k_{\text{breakdown}}$ are synonymous and the k_3/K ratio (in $\text{M}^{-1} \text{s}^{-1}$) is equal to $k_{\text{formation}}$. Table 1 gives the $k_{\text{formation}}$ (with, in some cases, the individual K and k_3 values) and the $k_{\text{breakdown}}$ values for various enzyme- β -lactam systems. The $k_{\text{breakdown}}$ values have been converted into the half-lives of the complexes EI*: these range for 80 to 40000 min. The degradation of the complexes EI* is thus the rate-limiting step and the $k_{\text{breakdown}}$ values are equivalent to the k_{cat} values for the overall reaction: antibiotic \xrightarrow{E} degradation products.

(3) In the strict sense, one could claim that β -lactams are substrates of the R6I and R39 enzymes. However, by immobilizing the enzymes in the form of inactive complexes EI*, at least for some time, they also behave as inhibitors. The lower the K value, the higher the k_3 value (i.e. the higher the $k_{\text{formation}}$) and the higher the k_4 value, the better is the antibiotic as a substrate. Conversely, the lower the K value, the higher the k_3 value (i.e. the higher the $k_{\text{formation}}$) but the lower the k_4 value, the more active is the antibiotic as an inhibitor. At a low $[I]$ value such that $[I] < K$, the important parameter is the $k_{\text{formation}}$ or k_3/K value since the rate of formation of complex EI* directly depends on it. At a very low $[I]$ value, the k_4 value is also very important since, at the steady state, the smaller its value, the higher is the proportion of the total enzyme that is immobilized in the form of complex EI*. The effectiveness of a β -lactam as an inhibitor can be expressed by the K_i value,

$$K_i = \frac{[E][I]}{[EI] + [EI^*]} = \frac{k_4 K}{k_3 + k_4}$$

which is equivalent to the Henri-Michaelis constant for the reaction β -lactam \rightarrow degradation products. If, as is the case with the exocellular enzymes, $k_4 \ll k_3$, then

$$K_i = \frac{k_4 K}{k_3} = \frac{k_{\text{breakdown}}}{k_{\text{formation}}}$$

Obviously, the lower the K_i value, the more active is the β -lactam as an inhibitor. The effectiveness of the inhibitor, however, is expressed more accurately by

$$\frac{[E]_{\text{steady state}}}{E_0} = 1 + \frac{[I]}{K} + \frac{k_3}{K} \cdot \frac{[I]}{k_4}$$

which gives the fraction of enzyme which remains functional at the steady state. Thus for $[I] \gg k_4 K/k_3$ and at the steady state, all the enzyme is immobilized in the form of complex EI* irrespective of the absolute k_4 value. At $[I] = k_4 K/k_3$ if both k_4 and k_3 values are very small, then the formation of complex EI* may be so slow that within the duration of the experiment, it may not occur at all, i.e. the enzyme remains active.

(4) In no case is the recognition of the antibiotic by the enzyme, as expressed by the K value, exceedingly good (Table 1) but there is a 20000-fold variation between the highest and the lowest k_3 values. Values of 180 and 13 s^{-1} for the k_3 constant are similar to the k_{cat} value of a true enzymic reaction. Although slow, breakdown of the complexes EI* is enzymic at least in the sense that active groups in the enzyme and/or a proper configuration of the protein are involved in the process. Thus boiling of the complex EI* made between

Table 2. *Relation between the rates of formation of complexes EI* with the exocellular Streptomyces R61 and Actinomadura R39 enzymes and the corresponding ID₅₀ values*

Antibiotic	a	b	a/b
	$\frac{k_{\text{formation}}(\text{R39})}{k_{\text{formation}}(\text{R61})}$	$\frac{\text{ID}_{50}(\text{R61})}{\text{ID}_{50}(\text{R39})}$	
Benzylpenicillin	7.3	2.8	2.6
Carbenicillin	3.6	4	0.9
Cephalosporin C	31	9	3.4
Ampicillin	626	867	0.7
Cephaloglycine	3523	1150	3.1

benzylpenicillin and the R61 enzyme stabilizes it (and makes it sensitive to pronase and thermolysine). Serine is the residue involved in penicillin binding to the R61 enzyme (Frère *et al.*, 1976*a*). Another example is given by the R39 enzyme. Breakdown of the complex formed between this latter enzyme and benzylpenicillin yields phenylacetyl glycine (and probably *N*-formyl-D-penicillamine) when breakdown occurs in a medium of high ionic strength under which conditions the enzyme is reactivated. At low ionic strength (where the R39 enzyme is not stable), enzyme reactivation does not occur and breakdown of the complex yields benzylpenicilloic acid. Hence, under certain conditions, the enzyme behaves although with a low efficiency, as a classical penicillinase.

(5) It had been known for several years that the R39 enzyme was more sensitive to β -lactams than the R61 enzyme. Table 1 gives the antibiotic concentrations required to inhibit enzyme activity by 50 % (ID₅₀ values). With all the β -lactams tested, the $k_{\text{formation}}$ values are always higher with the R39 enzyme than with the R61 enzyme, and the $k_{\text{breakdown}}$ values are always lower with the R39 enzyme than with the R61 enzyme. Necessarily, the R61 enzyme is more resistant than the R39 enzyme. The $k_{\text{formation}}$ value, however, is the important parameter (at least under the conditions where the ID₅₀ values were determined) as shown by the fact that for each antibiotic, the ratio of $k_{\text{formation}}$ (for the R39 enzyme) to $k_{\text{formation}}$ (for the R61 enzyme) is very similar to the ratio of ID₅₀ (for the R61 enzyme) to ID₅₀ (for the R39 enzyme) (Table 2).

(6) The differences between the R61 and R39 enzymes with respect to their action on the β -lactams leads to a new concept of penicillin resistance. They show that alterations in the structure and/or the conformation of the enzymes that result in increased $k_{\text{breakdown}}$ values (without altering the $k_{\text{formation}}$ value) or in decreased $k_{\text{formation}}$ values (without altering the $k_{\text{breakdown}}$ value) lead to 'intrinsic resistance'. The comparison between the R61 and R39 enzymes also provides an explanation for the large differences in penicillin sensitivity exhibited by the enzymes of the peptide crosslinking system.

(7) The concomitant interaction between enzyme, peptide donor Ac₂-L-Lys-D-Ala-D-Ala and β -lactam was studied both on the basis of the effect of the β -lactam on the rate of hydrolysis of the peptide donor and on the basis of the effect of the peptide donor on the rate of formation of complex EI*. Kinetically, the interaction was competitive but the conclusions were that it was not possible to decide whether or not binding of peptide donor and penicillin to the enzymes was mutually exclusive, and that a structural analogy between penicillin and peptide donor could not be justified on the basis of such experiments. It is clear that binding of penicillin to its site on the R61 and R39 enzymes with formation of the complexes EI* induces conformational changes in the enzymes. These changes might be sufficiently pronounced to freeze them in conformations that prevent both DD-carboxypeptidase and transpeptidase activities. Other techniques are being used in order to solve this important problem.

*The cell-bound peptidoglycan crosslinking enzyme system
in Streptomyces R61*

The exocellular R61 enzyme is 'lost' in the culture medium. The question therefore arises whether the model derived from the exocellular enzymes for the various reactions concerned with peptide donor, peptide acceptor and β -lactam applies to the physiological, cell-bound peptidoglycan crosslinking enzyme system.

The composition and functioning of the crosslinking enzyme system in *Streptomyces* are not yet fully understood. Recent progress has been made and I am indebted to Drs Mélina Leyh-Bouille, Martine Nguyen-Distèche and J. Dusart, and to Dr P. E. Reynolds from the University of Cambridge, for results that have not yet been published elsewhere. What do we know of the system?

(1) The tripeptide Ac₂-L-Lys-D-Ala-D-Ala is both hydrolysed and utilized in a transfer reaction with Gly-Gly by at least two types of cell-bound enzyme activities. The two activities are located in distinct parts of the cell envelope and are antagonists in the sense that one of them acts mainly as a DD-carboxypeptidase and is highly sensitive to penicillin whereas the other acts mainly as a transpeptidase and has a low sensitivity to penicillin.

(2) The enzyme which acts mainly as a DD-carboxypeptidase (with a low transpeptidase activity, at least in water, at neutral pH and at low acceptor concentrations) and has a high sensitivity to penicillin is released from the mycelium during transformation into protoplasts in sucrose medium (unpublished data). This lysozyme-releasable enzyme is very similar, perhaps identical, to the exocellular enzyme: the proportion of total activity that is channelled into hydrolysis and transfer depends upon the environmental conditions; the enzyme utilizes the tetrapeptide Ac-L-Lys-D-Ala-D-Ala with formation of peptide dimer;

Gly—

when treated with [¹⁴C]benzylpenicillin and submitted to SDS slab gel electrophoresis, it migrates exactly as the exocellular enzyme; finally, its activities are inhibited by antibodies prepared against the exocellular enzyme. Its exact localization in the cell is not known, but the comparison between the levels of exocellular and lysozyme-releasable enzymes among various streptomycetes suggests that a correlation exists between the amount of enzyme that is excreted in the medium and the amount of lysozyme-releasable enzyme present in the mycelium. *Streptomyces rimosus*, for example, contains extremely low levels of lysozyme-releasable enzyme and excretes only traces of exocellular enzyme.

(3) The enzyme which acts mainly as a transpeptidase (with a low DD-carboxypeptidase activity) and has a low sensitivity to penicillin is firmly associated with the plasma membrane. Gly-Gly, various peptides possessing an N-terminal glycine residue and other amino compounds are utilized by the isolated membranes for transpeptidation reactions with Ac₂-L-Lys-D-Ala-D-Ala as donor. Qualitatively, the specificity profile of the membrane-bound enzyme fits well the requirements for amino acceptor exhibited by the exocellular enzyme (Dusart *et al.*, 1973). There is an exception, however: Ac-L-Lys-D-Ala-D-Ala is

Gly—

not utilized as substrate by the isolated membranes (unpublished data). The kinetics of the membrane-catalysed transpeptidation between Ac₂-L-Lys-D-Ala-D-Ala and Gly-Gly fit the general initial rate equation for an enzyme-catalysed bimolecular reaction

(S₁ + S₂ \rightleftharpoons S₁' + S₂'):

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_2}{[S_1]} + \left(\Phi_1 + \frac{\Phi_{12}}{[S_2]} \right) \frac{1}{[S_1]}$$

where Φ_0 is equal to the reciprocal of the maximal rate, and Φ_1 and Φ_2 are related to the Michaelis constants by $\Phi_1/\Phi_0 = K_{m_1}$ and $\Phi_2/\Phi_0 = K_{m_2}$ (unpublished data). One astonishing property of the isolated membranes is their ability to perform transpeptidation reactions

Table 3. *Interaction between β -lactams and the membrane-bound transpeptidase of Streptomyces R61*

Antibiotic	$10^6 \times$		Half-life of complex EI*	K_i †	Excl. D_{50} ‡	LD_{50} ‡§	ID_{50} ‡
	$k_{\text{formation}}$ at 37 °C† ($M^{-1} s^{-1}$)	$k_{\text{breakdown}}$ at 37 °C† (s^{-1})					
Ampicillin	400	330	3.5	10	9	5	8
Phenoxymethylpenicillin	140	28	41	2	4	5	18
Benzylpenicillin	50	11	104	2.2	3	5	2
Carbenicillin	15	7.3	160	5	30	33	34
Cloxacillin	13	12.3	94	10	45	50	39
Cephalosporin C	5	330	3.5	660	220	112	540

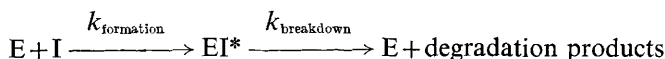
† Unpublished data.

‡ From Marquet *et al.* (1974); half-life, Excl. D_{50} , LD_{50} and ID_{50} values for β -lactams other than those listed above can be found in Marquet *et al.* (1974).

§ Concentration which gives 50 % inhibition of the ability of conidia to form single cell colonies.

in a solid, frozen state. A temperature as low as -30 °C is required to prevent the enzyme from catalysing the reaction, suggesting that the enzyme functions in a lipid environment that remains remarkably fluid at low temperature (Dusart *et al.*, 1975).

(4) The isolated membranes fix in a rather stable manner about 25 pmol [^{14}C]benzylpenicillin per mg protein, under which conditions the transpeptidase activity is totally inhibited (Marquet *et al.*, 1974). The interaction between the membrane transpeptidase and β -lactams fits the general scheme (Marquet *et al.*, 1974)



During breakdown, the transpeptidase is reactivated and recovers its initial ability to react with penicillin. With [^{14}C]benzylpenicillin, release of the radioactivity and enzyme recovery proceed with the same $k_{\text{breakdown}}$ value of $1.1 \times 10^{-4} s^{-1}$ (indicating a half-life value of 104 min) (Marquet *et al.*, 1974). A major part of the released radioactivity occurs in the form of [^{14}C]benzylpenicilloic acid (about 75 %) and a minor part of it in the form of another metabolite X (about 25 %; unpublished data). Hence, it seems safe to conclude that (i) the transpeptidase in the membrane reacts with β -lactams according to the model proposed for the exocellular enzyme, (ii) it functions as a penicillinase with a low k_{cat} value and (iii) at least most of the penicillin-binding sites on the membrane are the transpeptidase molecules.

(5) Table 3 gives the $k_{\text{formation}}$ and $k_{\text{breakdown}}$ values for the interaction between β -lactams and the membrane-bound transpeptidase (unpublished data). These determinations were possible because the isolated membranes of *Streptomyces* R61 do not possess any 'classical' penicillinase activity. Penicillinase activity would degrade penicillin into penicilloic acid at a much faster rate than transpeptidase activity is regenerated through breakdown of complex EI*. In fact, membrane-bound and exocellular penicillinase activities occur in many streptomycetes other than strain R61. On the basis of the K_i values of Tables 1 and 3, the membrane-bound transpeptidase is less sensitive to β -lactams than the exocellular enzyme. Thus the K_i values for benzylpenicillin are 10 nM with the exocellular enzyme and 2.2 μM with the membrane-bound enzyme. With all the β -lactams tested, except for ampicillin, the $k_{\text{formation}}$ is always higher with the exocellular enzyme than with the membrane-bound enzyme (Tables 1 and 3).

(6) The data of Table 3 also show that the effectiveness of β -lactams is very similar with regard to their ability to inhibit the membrane-bound transpeptidase (K_i and ID_{50} values), to bind to isolated membranes (as shown by competition; Excl. D_{50} values) and to inhibit the formation of single cell colonies from conidia on agar nutrient plates (LD_{50} values). Hence one (or the) enzyme which has to be inactivated in order to prevent the early develop-

ment of the mycelium from the spores appears to be the membrane-bound transpeptidase. It may be that the lysozyme-releasable enzyme is not present at that early stage of cell growth. If present, then, because of its high penicillin sensitivity, it may simply immobilize a certain amount of β -lactam (detoxication effect). The present results, however, do not exclude the possibility that the lysozyme-releasable enzyme is, in turn, physiologically important at later stages of the mycelial growth. This question is currently under investigation.

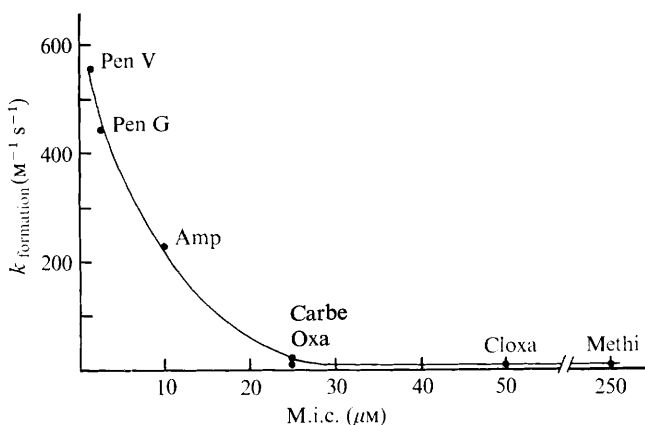
(7) Antibodies specific for the R61 exocellular enzyme do not bind to the isolated membranes and have no effects on its transpeptidase activity (unpublished data). The enzyme might be buried too deeply within the lipid phase of the membrane to be accessible. However, analysis on SDS slab gels of the membranes previously charged with radioactive benzylpenicillin reveals two to three penicillin-binding components of molecular weight of about 20000 to 25000, in addition to another penicillin-binding component of molecular weight of about 50000 (unpublished data). These components have molecular weights that are either considerably lower or higher than the molecular weights of the lysozyme-releasable and exocellular enzymes. Extraction of the membranes (or of the washed mycelium) with the cationic detergent *N*-cetyl-*N,N,N*-trimethylammonium bromide, followed by filtration on Sephadex G-100, yields a soluble preparation (Dusart *et al.*, 1975) enriched in enzyme activity. The enzyme thus solubilized remains a transpeptidase with low DD-carboxypeptidase activity but its properties are somewhat altered with the results that (i) it has a better fit for the two synthetic substrates $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ and Gly-Gly (as revealed by the Φ_D and Φ_A values), (ii) both the $k_{\text{formation}}$ and $k_{\text{breakdown}}$ values for the interactions with β -lactams are modified and (iii) [^{14}C]benzylpenicillin is now fragmented with formation of [^{14}C]phenylacetylglycine as observed with the exocellular enzyme (unpublished data).

(8) The relationship between the membrane-bound transpeptidase and both the lysozyme-releasable and exocellular enzymes in *Streptomyces* R61 is still rather mysterious. However, an interesting observation was made with *Streptomyces* K15 (unpublished data). Like strain R61, *Streptomyces* K15 excretes an exocellular DD-carboxypeptidase, contains a lysozyme-releasable DD-carboxypeptidase and has a transpeptidase fixed on its membrane. The two former enzymes – but not the membrane-bound transpeptidase – are sensitive to the anti-exocellular R61 enzyme antibodies. *Streptomyces* K15, in addition, possesses on its membrane a DD-carboxypeptidase able to perform low transpeptidase activity. This membrane-bound DD-carboxypeptidase which is hardly detectable in *Streptomyces* R61, has, in *Streptomyces* K15, the following properties: (i) it is inhibited by the anti-exocellular R61 enzyme antibodies; (ii) after extraction with the cationic detergent and on the basis of its elution pattern on Sephadex G-100, it has a molecular weight higher than that of the membrane-bound transpeptidase; and (iii) it enables the isolated membranes to react with $\text{Ac-L-Lys-D-Ala-D-Ala}$. It is therefore tempting to postulate that in *Streptomyces* K15, the

Gly—
membrane-bound DD-carboxypeptidase is at the branching point of two distinct pathways: one leading via the lysozyme-releasable enzyme to enzyme excretion, and the other leading somehow to a membrane-bound transpeptidase in the form of iso-enzymes responsible for the presence of multiple penicillin-binding sites in the membrane. One may also postulate that the same situation occurs in all streptomycetes but that, depending upon the strain and/or the stage during cell growth, great variations in the amounts of enzymes present may occur with the result that some of them may be beyond the limits of detection. The membrane-bound transpeptidase, however, is always detectable.

Table 4. Interaction between β -lactams and the membrane-bound DD-carboxypeptidase of *Streptococcus faecalis*†

Antibiotic	$k_{\text{formation}}$ at 37 °C (M ⁻¹ s ⁻¹)	$10^5 \times$ $k_{\text{breakdown}}$ at 37 °C (s ⁻¹)	Half-life of complex EI* at 37 °C (min)	K_i (μ M)	Growth inhibition in m.i.c. (μ M)
Phenoxymethylpenicillin	560	10	130	0.18	1
Benzylpenicillin	445	4.4	263	0.10	2
Ampicillin	230	1.5	820	0.07	10
Carbenicillin	19	3.7	310	2	25
Oxacillin	4.5	24	50	60	25
Cloxacillin	0.75	6	205	80	50
Methicillin	1.85	6.5	183	35	250

† From Coyette *et al.* (1977b).Fig. 5. Relationship between the m.i.c. values for *Streptococcus faecalis* and the second order $k_{\text{formation}}$ values for the formation of the membrane-bound DD-carboxypeptidase- β -lactam complexes. Pen V, phenoxymethylpenicillin; Pen G, benzylpenicillin; Amp, ampicillin; Oxa, oxacillin; Carbe, carbenicillin; Cloxa, cloxacillin; Methi, methicillin.*The membrane-bound DD-carboxypeptidase in Streptococcus faecalis*
ATCC9790 as the target of β -lactams

Bacteria, more conventional as sources of biochemical material than streptomycetes, were also investigated. *Streptococcus faecalis* provides another example where a relationship exists between the effects of β -lactams on bacterial growth and their activity *in vitro* upon one defined cell-bound enzyme. In the present case, the enzyme is a membrane-bound DD-carboxypeptidase active on Ac₂-L-Lys-D-Ala-D-Ala and able to catalyse exchange reactions with simple amino compounds such as D-Ala, Gly and Gly-Gly (Coyette *et al.*, 1974, 1977a, b). The isolated membranes of *Streptococcus faecalis* contain six penicillin-binding proteins which altogether fix about 100 pmol benzylpenicillin per mg protein (R. Fontana, unpublished data). The DD-carboxypeptidase is protein 6 and it represents about one-third of all the binding sites. The $k_{\text{formation}}$ and $k_{\text{breakdown}}$ values for the interaction between the membrane-bound DD-carboxypeptidase and β -lactams are given in Table 4. Interaction with benzylpenicillin yields phenylacetylglutamine and *N*-formyl-D-penicillamine. Fragmentation is prevented by heating the membrane-penicillin complex at 100 °C. All the complexes tested have half-lives which are equal to or longer than the generation time of the organism (30 min). Finally, the relative effectiveness of the β -lactams *in vivo* rests

almost entirely upon the $k_{\text{formation}}$ values: as shown by the data of Table 4 and Fig. 5, the higher the $k_{\text{formation}}$ value the lower is the minimum inhibitory concentration (m.i.c.) of the corresponding β -lactam. Thus, in *Streptococcus faecalis*, normal functioning of the membrane-bound DD-carboxypeptidase enzyme may be important if not necessary for cell growth.

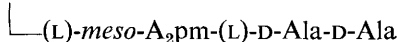
The DD-carboxypeptidase of the unstable spheroplast of Proteus mirabilis

Mention should be made of enzymes which may well form ternary complexes with peptide donor and β -lactam. One of them is the membrane-bound DD-carboxypeptidase isolated from spheroplasts of the unstable L-form of *Proteus mirabilis* strain 19 (Martin, Maskos & Burger, 1975; Martin, Schilf & Maskos, 1976; Schilf *et al.*, 1977). Its interaction with benzylpenicillin is characterized by a $k_{\text{formation}}$ of about $4000 \text{ M}^{-1} \text{ s}^{-1}$ (W. Schilf & J.-M. Frère, unpublished data) and a $k_{\text{breakdown}}$ of about $3.3 \times 10^{-3} \text{ s}^{-1}$, i.e. a half-life of about 3 min (at 37°C). Benzylpenicilloic acid is released. Of course, such a rapid breakdown confers a high penicillin resistance to the enzyme. The *Proteus* bacterial form possesses the equivalent of the L-form DD-carboxypeptidase and, in addition, a second DD-carboxypeptidase characterized by a much higher penicillin sensitivity and a half-life of several hours for the complex EI* with benzylpenicillin (H. H. Martin, personal communication). Protein 5 of *E. coli* (Spratt, 1977) and protein 5 of *Salmonella typhimurium* (Shepherd *et al.*, 1977) are other examples of enzymes forming complexes EI* with benzylpenicillin which have very short half-lives (5 min).

Another interesting feature of the *Proteus* L-form enzyme is that, kinetically, it is inhibited by penicillin in a non-competitive manner. Competitive kinetics are never persuasive because a ternary complex may be formed only at higher concentrations of either the substrate or penicillin than those which are, or can be, used. Conversely, non-competitive kinetics indicate with some weight that binding of donor substrate and binding of penicillin occur on at least partially independent sites on the enzyme.

Conclusions

The use of artificial substrates in uncoupled transpeptidation assays can probably be extended to all bacteria. At present, its application has been limited, presumably because it is difficult to design a proper system of donor and acceptor. However, dimerization of the pentapeptide L-Ala-D-Glu is achieved not only by the



exocellular enzyme of *Actinomadura* R39, but also by various membrane-bound transpeptidases, such as those of *E. coli* (Nguyen-Distèche *et al.*, 1974*a, b*) and *Salmonella typhimurium* (Shepherd *et al.*, 1977). In fact, the penicillin-binding proteins 1, 4 and 5 of *Salmonella typhimurium* are DD-carboxypeptidases-transpeptidases able to catalyse dimer formation (Shepherd *et al.*, 1977). That the same reaction can be catalysed by enzymes originating from organisms taxonomically very different is not surprising when the wall peptidoglycans in these organisms have the same primary structure.

There are reports in the literature where enzymes are designated as transpeptidases on the basis of their ability to replace the C-terminal D-Ala residue of a peptide donor by Gly or a simple D-amino acid residue. Such an exchange reaction does not prove the occurrence in the enzyme of an 'acceptor site' concerned with peptide dimerization. This lack of accuracy in the terminology is unfortunate since it adds confusion to the picture.

The present review has been necessarily limited to selected topics. However, from an integration of these and other studies, it has become certain that the mode of action initially proposed for penicillin in order to explain its effect on the exocellular R61 and R39 enzymes is general. Quantitatively, the constants involved vary considerably depending upon the enzymes and their bacterial origin and, qualitatively, there is more than one possible pathway for the enzymic degradation or fragmentation of the antibiotic molecule.

The concept of chemotherapy, which stems back to the discovery of Prontosil in 1935, was firmly established by the first cures achieved by penicillin in 1941. A second penicillin era, which we are still living in, started in 1957 when it was learnt that semi-synthetic penicillins with increased penicillinase resistance could be made by adding side-chains to the 6-amino-penicillanic acid nucleus. This revolution was 'to open the possibility of chemotherapy being able to adapt itself by producing new products to meet the adaptations nature produces in the micro-organisms' (Wilson, 1976). One day, the peptidoglycan crosslinking enzyme system may be so precisely known that specific inhibitors will be built on a rational basis and used as new antibacterial agents. It is difficult to predict when biochemists will open this third revolution. It is certain that the problem is being fiercely attacked from several quarters.

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