ACKNOWLEDGMENTS

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DISCUSSION

STEWART — What is the activity on ileum of your all-D-retro-[-Ala^7]-AII compared to AII ? I believe its activity would be similar to that of all-D-retro-bradykinin

PAIVA — Our retroenantiomers have been assayed by direct comparison with their respective parent peptides. I do not think that you should refer their activity to that of AII, while all-D-retro bradykinin should be compared with bradykinin.

PENICILLIN-TRANSPEPTIDASE INTERACTION

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1. INTRODUCTION

Peptidoglycan is a giant macromolecule in the form of a three-dimensional network which surrounds completely the bacterial cell and is responsible for the shape and the rigidity of the bacterial wall. It consists of parallel saccharidic chains that are cross-linked by short peptide bridges. The saccharidic chains are copolymers of alternating N-acetylglucosamine and N-acetylmuramic acid residues and the D-lactyl substituents of these latter residues serve as attachment sites for the peptides (Fig. 1).

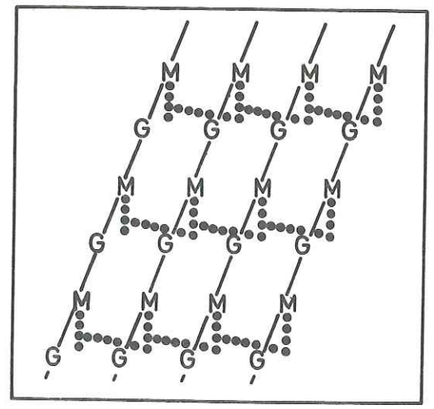


Figure 1: General structure of the bacterial wall peptidoglycans. G = N-acetylglucosamine; M = N-acetylmuramic acid. The dots represent amino-acid residues (from [1]).

Peptidoglycan, which is an exocellular structure, is too big to be exported as such through the membrane. It is assembled on the exterior of the plasma membrane, i.e. in a region where the usual cellular sources of energy (e.g. ATP) are absent. In order to overcome this thermodynamical problem, the bacterial cell exports through the membrane preformed disaccharide-peptide units that are linked to a membrane lipid carrier through an energy-rich phosphoester bond (Fig. 2).

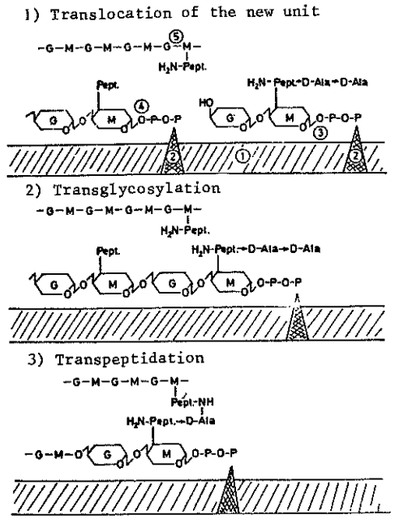


Figure 2: Final steps in the biosynthesis of peptidoglycan. (1) : cytoplasmic membrane; (2) : C_{55} isoprenoid lipid carrier; (3) : new disaccharide peptide unit; (4) : growing saccharide chain; (5) : preexisting chain.

The energy of this phosphoester bond is then utilized for the synthesis of a new saccharide bond. A different mechanism is involved in the synthesis of the peptide cross-bridges. When compared to the terminated peptidoglycan, the disaccharide-peptide unit precursor that is translocated through the membrane contains one additional C-terminal D-alanine residue. In most bacteria, the structure of the peptide moiety of the

precursor is L-Ala-D-Glu-L-R_3-D-Ala-D-Ala. Depending upon the bacterial species, the residue L-R_3 may be a diamino-acid (such as lysine) or a diamino-diacid (LL or meso-diaminopimelic acid) and its ω amino group may be either free or substituted by one additional amino-acid residue or a short peptide (for a complete review on the structure of peptidoglycan, see [1]). The closure of the peptide bridge between two peptide unit precursors is made by transpeptidation, a reaction that does not require any exogenous energy. The mechanism is such that the C = O of the penultimate D-Ala residue of one peptide (the donor peptide) is tranferred on the free ω amino group at the L-R_3 position of another peptide (the acceptor peptide); concomitantly, the C-terminal D-alanine residue of the donor peptide is released. Figure 3 shows the transpeptidation reaction as it occurs in *Streptomyces* sp. Various strains of *Streptomyces* have been used in our studies.

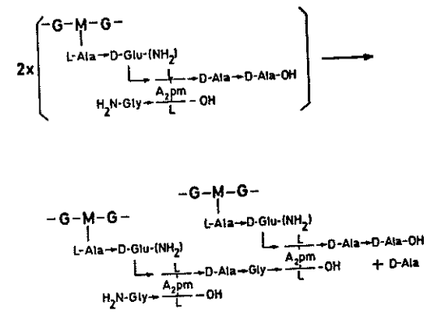


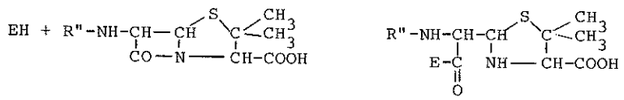
Figure 3: Transpeptidation reaction in Streptomyces R61.

In 1965, penicillins were shown to inhibit the transpeptidation reaction by acting on a membrane-bound transpeptidase. At that time, Tipper and Strominger [2] propose an elegant hypothesis according to which penicillins would be structural analogues of the backbone of the D-Ala-D-Ala moiety of the donor peptide. In the transpeptidation reaction, the enzyme (EH) and the donor peptide would first react to form an acyl-enzyme intermediate which, in turn, would react with the acceptor peptide.

EH + R-D-Ala-D-Ala → D-Ala + R-D-Ala-E

R-D-Ala-E + R'-NH_2 → EH + R-D-Ala-NH-R'.

Because of their analogy with the donor peptide, penicillins would also react with the enzyme. However, the acyl-enzyme intermediates thus formed would be stable leading to the irreversible inactivation of the enzyme.



This hypothesis became very popular, despite the fact that it never received any direct experimental support. In this respect, the following points should be emphasized:

(1) so far, the existence of an acyl intermediate in the transpeptidation reaction has remained completely hypothetical;
(2) the structural analogy between penicillins and the D-alanyl-D-alanine portion of the peptide is far from being perfect [3];
(3) enzymes exist that utilize as substrates peptides ending in a R-D-Ala-D-Ala sequence, but are not inhibited by penicillins [4];
(4) enzymes also exist that are strongly inhibited by penicillins, although they do not "recognize" true structural analogues of the donor peptide (for example R-D-Ala-D-Glu) [5];
(5) depending upon the enzyme, penicillins may behave as competitive or non-competitive inhibitors and the inactive enzyme-inhibitor complexes may be stable or unstable [6].

Obviously, a very careful study of the transpeptidation reaction and of its inhibition by penicillins was needed in order to unravel the mechanism of action of penicillins at the molecular level. In the early 1970's this study was made extremely difficult

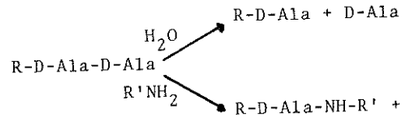
because, at that time, a transpeptidase could only be assayed by using complex systems consisting of membrane suspensions and of exogenously added precoursors UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-peptide precursors. With such systems, a series of complex reactions had to take place before the peptide cross-linking could occur so that it was not possible to study the transpeptidation reaction independently from the preceding steps in the biosynthesis.

In 1972, in our laboratory, *Actinomadura* strain R39 and several strains of *Streptomyces* were shown to be good sources of exocellular and membrane-bound enzymes that were able to catalyze transpeptidation reactions in simple, uncoupled systems, by using synthetic peptides as substrates [7,8]. On the basis that these "artificial" transpeptidation reactions thus catalyzed were identical or, at least, very similar to those occuring *in vivo*, it was postulated that the relevant transpeptidases were closely related or identical to the physiological ones.

2. THE SOLUBLE ENZYMES FROM *STREPTOMYCES* STRAIN R61 AND *ACTINOMADURA* STRAIN R39.

2.1. GENERAL PROPERTIES

The R61 and R39 enzymes that are excreted in the culture media during growth of the relevant strains, were purified to protein homogeneity [9,10]. In addition to the transpeptidation reaction, they catalyze a simple hydrolysis of the donor substrate:

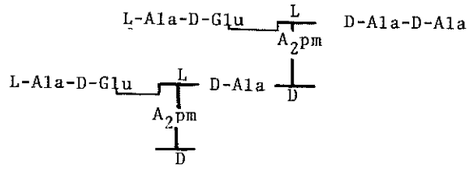


In fact, the two pathways are in competition with each other and an increase of the ratio of transpeptidation to hydrolysis can be obtained [11,12] by:

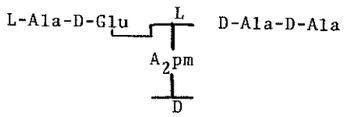
(1) increasing the acceptor concentration;
(2) increasing the pH;
(3) decreasing the water content of the reaction mixture (e.g. by replacing up to 90% of the water by a mixture of glycerol and ethylene glycol).

2.2. FORMATION OF PEPTIDE DIMERS AND POLYMERS

Substrates acting both as donor and acceptor in transpeptidation reactions are utilized by the R61 and R39 enzymes. Thus for example, the R39 enzyme [13] catalyzes the formation of the dimer



from the natural pentapeptide



This pentapeptide can be obtained from various bacilli. These bacteria contain a peptidoglycan that is very similar to that of *Actinomadura* R39. From the accumulated UDP-N-acetylmuramyl pentapeptide, the free pentapeptide is prepared by chemical removal of the UDP moiety followed by the enzymic splitting of the N-acetylmuramyl-L-alanyl linkage. The above dimerization is identical to the one which occurs when the nascent peptidoglycan of *Actinomadura* R39 becomes cross-linked *in vivo*.

A more complete study [14,15] of polymerization reactions was carried out with the R61 enzyme. With this latter enzyme, the synthetic tetrapeptide

was used as substrate and the following observations were made:
(1) the formation of dimers, trimers (Table 1) and of minute amounts of tetramers was demonstrated;
(2) the formation of trimers was not a random process. It occurred preferentially by reaction between a donor monomer and an acceptor dimer. The dimer was a very poor donor;
(3) tetramer formation could not be detected upon incubation of the intact dimer alone. Consequently, it was proposed that tetramer synthesis might proceed through the addition of a monomer to a trimer.

Table 1: Peptide polymerization by the Streptomyces R61 exocellular enzyme: substrate and products.

Substrate	
[14C]Ac-L-Lys-D-Ala-D-Ala-OH H-Gly (ε)	M.W. = 387 with [14C] = 389
Products	
- Hydrolyzed monomer [14C]Ac-L-Lys-D-Ala-OH H-Gly (ε)	M.W. = 316 with [14C] = 318
- Dimer [14C]Ac-L-Lys-D-Ala-D-Ala-OH (ε) [14C]Ac-L-Lys-D-Ala-Gly (ε) H-Gly (ε)	M.W. = 685 with [14C] = 689
- Hydrolyzed dimer [14C]Ac-L-Lys-D-Ala-OH (ε) [14C]Ac-L-Lys-D-Ala-Gly (ε) H-Gly (ε)	M.W. = 614 with [14C] = 618
- Trimer and hydrolyzed trimer [14C]Ac-L-Lys-D-Ala-(D-Ala)-OH (ε) [14C]Ac-L-Lys-D-Ala-Gly (ε) [14C]Ac-L-Lys-D-Ala-Gly (ε) H-Gly (ε)	M.W. = 983 or 912 with [14C] = 989 or 918

2.3. THE INTERACTION BETWEEN THE SOLUBLE ENZYMES AND PENICILLINS

2.3.1. REACTION PATHWAY

The kinetic data obtained with both R61 and R39 enzymes and various penicillins or cephalosporins are best explained on the basis of the reaction scheme :

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^{*} \xrightarrow{k_4} E + X$$

This model involves three steps

1) the reversible formation of complex EI (K = dissociation constant of EI). Complex EI is assumed to be in rapid equilibrium with free E (enzyme) and I (inhibitor, i.e. penicillin);

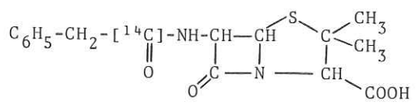
2) the irreversible transformation of EI into EI*, i.e. a rather stable complex in which both the enzyme and the antibiotic molecules are altered; k_3 is the first order rate constant for this transformation;

3) the irreversible breakdown of EI* into a native, fully active enzyme E and a penicillin metabolite deprived of any antibiotic activity; k_4 is the first order rate constant for the breakdown of EI*.

The most important and novel feature of this pathway is that complex EI* undergoes spontaneous breakdown with the concomitant release of a fully active enzyme. On this basis, a new mode of resistance against β-lactam antibiotics can be envisaged, which involves the destruction of the antibiotic by the enzyme target itself. The values of the various constants have been measured with several antibiotics. These values provide a physical basis for the understanding of the activity of a given antibiotic as an inhibitor of one specific enzyme [16,17].

2.3.2. THE DEGRADATION OF BENZYLPENICILLIN

The first studies were performed by using, as substrate, benzylpenicillin [^{14}C]-labelled in the side-chain



The radioactive degradation product was purified by taking advantage of the fact that it exhibited an abnormal behaviour by filtration on Sephadex G-25. An NMR spectrum in D_2O revealed no other bands than those characteristic of the protons of the phenyl ring and of two CH_2 groups, respectively. No methyl group appeared to be present. The metabolite was indistinguishable from phenylacetylglycine by co-chromatography, co-electrophoresis, co-crystallization and mass spectrometry (Fig. 4) [18].

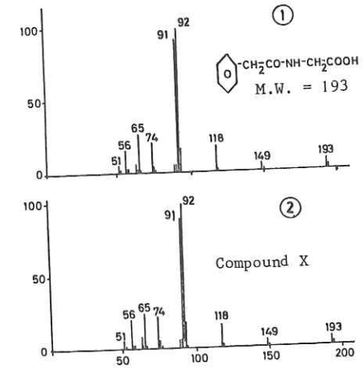


Figure 4: Mass spectra of authentic phenylacetylglycine (above) and of phenylacetylglycine arising from the EI complex (compound X) (from [18]). Reprinted by permission of Nature.*

The fate of the thiazolidine moiety of the molecule was investigated [19] by NMR analysis. After chromatography on Sephadex G-25, the fractions corresponding to the salt volume contained a compound which exhibited those bands characteristic of the methyl groups (Fig. 5). The distance between the bands, however, indicated that the thiazolidine ring had been opened. The spectrum was identical to that of N-formyl-D-penicillamine or

di-N-formyl-D-penicillamine disulphide. On the basis of various degradation experiments, the compound was identified as the disulphide derivative.

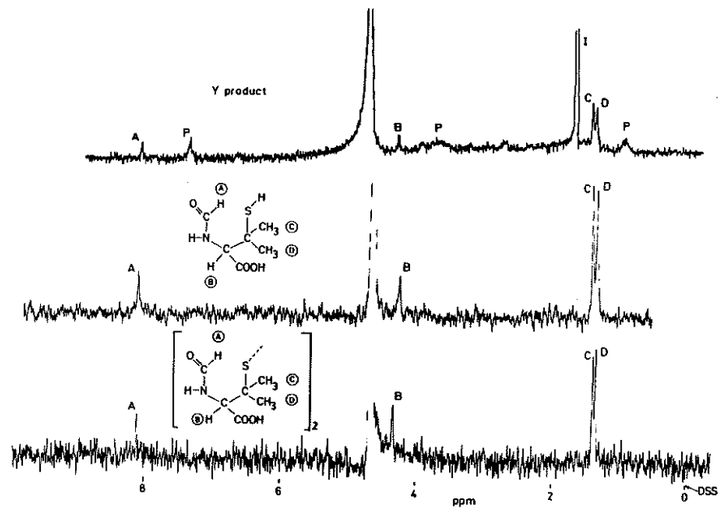
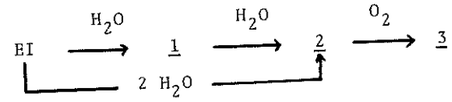
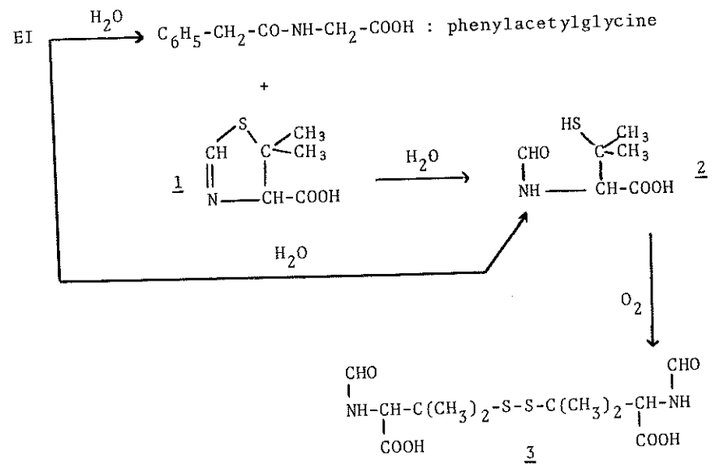


Figure 5: NMR spectrum of the compound obtained from the thiazolidine moiety of benzylpenicillin after interaction with the Streptomyces R61 exocellular enzyme. The areas under the bands labelled A,B,C and D were in the ratio 1/1/3/3. These bands were attributed to the released compound. Bands labelled P are due to a trace of penicilloic acid and band labelled I to an unknown impurity. The figure also shows the spectra of authentic N-formyl-D-penicillamine and of its disulphide (from [19]) Reprinted by permission of Nature.

Since this compound had been obtained after a prolonged incubation (several days) at 37°, the formation of the disulphide could be a secondary reaction. Consequently, [^{3}H]ß-methyl benzylpenicillin (obtained from P. Adriaens and H. Vanderhaeghe, Katholieke Universiteit, Leuven) was incubated with the enzyme for a short period of time and in a N_2 atmosphere. The tritiated

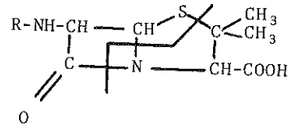
metabolite obtained under these conditions was N-formyl-D-penicillamine. This latter compound itself, however, might not be the primary product of the breakdown of complex EI . Indeed, in aqueous solutions, thiazolines are known to decay into a mixture of N-acyl and S-acyl derivatives[20] . It thus remains possible that the primary product to be released is a thiazoline derivative 1 which, in turn, is hydrolyzed into N-formyl penicillamine 2. Eventually, oxidation of this latter compounds yields the disulphide 3.





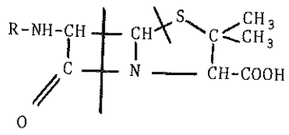
A similar fragmentation of penicillin V into phenoxyacetylglycine and N-formyl-D-penicillamine has been shown to occur. At present the nature of the degradation products arising from cephalosporins is not known.

In conclusion, penicillins which, on the basis of their biosynthetic pathway, can be regarded as the condensation products of N-acyl-L-cysteine and D-valine 4, are degraded through the action of the exocellular R61 and R39 enzymes into a mixture of N-acylglycine and N-formyl-D-penicillamine 5



N-acyl-L-cysteine D-valine

4



N-acyl-glycine N-formyl-D-penicillamine

5

3. THE MEMBRANE-BOUND ENZYMES

3.1. PROPERTIES OF THE MEMBRANE PREPARATIONS

Isolated cytoplasmic membranes from *Streptomyces* strains R61, K15 and *rimosus* and from *Streptococcus faecalis* ATCC 9790 contain a transpeptidase activity [8,21,22] which can be assayed by using the model system.
Ac_2-L-Lys-D-Ala-D-Ala + Gly-Gly → Ac_2-L-Lys-D-Ala-Gly-Gly + D-Ala. A striking property of the membrane suspensions from *Streptomyces* R61 is that they remain active in the frozen state down to a temperature of -35° [21]. Moreover, the temperature-activity relationship exhibits a discontinuity at the freezing point of the mixture (ca -5°), the frozen state being accompanied by a sharp increase in the total transpeptidase activity.

The various membrane preparations also exhibit a DD-carboxypeptidase activity and the ratio of transpeptidation to hydrolysis varies according to the strain. In addition, the membranes from *Streptococcus faecalis* contain a LD-transpeptidase catalyzing the transfer of the carbonyl group of the L-lysyl residue of Ac_2-L-Lys-D-Ala on a suitable acceptor: Ac_2-L-Lys-D-Ala + R-NH_2 → Ac_2-L-Lys-NH-R + D-Ala. Among all these activities only the LD-transpeptidase from *S. faecalis* is relatively insensitive to penicillins. The membrane-bound DD-transpeptidases and the DD-carboxypeptidases react with penicillins according to the same general pathway as the soluble enzymes. It is particularly important to note that the inhibited complexes also break down into an active enzyme and an inactivated antibiotic. A major difference, however, is that the degradation product which is released upon reactivation of the *Streptomyces* membranes is not an N-acylglycine derivative. The product released from ^{14}C benzylpenicillin (labelled in the side-chain), behaves as penicilloic acid on thin-layer plates and by paper electrophoresis. Degradation of the *Streptococcus faecalis* - [^{14}C]benzylpenicillin complex produces equimolecular amounts of [^{14}C]phenylacetylglycine and of a [^{14}C]-labelled compound behaving as penicilloic acid.

3.2. SOLUBILIZATION OF THE ENZYMIC ACTIVITIES WITH DETERGENTS

The membrane-bound activities can be solubilized with the help of proper detergents. The quaternary ammonium cetyltrimethylammonium bromide is used for the *Streptomyces* enzymes and the non-ionic Genapol X-100 is used for the *Streptococcus* enzymes. The solubilized transpeptidase and DD-carboxypeptidase activities from *Streptomyces* K15 can be partially separated from each other by molecular sieve chromatography (M. Leyh-Bouille, unpublished results). In other cases, the same technique failed to provide any separations between the two activities which therefore might be attributed to a single protein. All the solubilized enzymes are sensitive to penicillins and the enzyme-penicillin complexes break down into active enzyme and a degradation product deprived of antibiotic activity. Solubilization, however, might change the value of the first-order rate constant involved in the degradation of the complex. Similarly, the nature of the inactivated antibiotic also appears to depend upon the physico-chemical environment which prevails during the degradation of the complexes (M. Leyh-Bouille, J. Coyette and J. Dusart, unpublished results). Thus, for example, benzylpenicillin which is degraded into a penicilloic acid-like compound by the membrane-bound enzymes of *Streptomyces* strain R61 and *rimosus*, is degraded into phenylacetylglycine by the corresponding detergent-solubilized enzymes. The fate of the thiazolidine portion of the penicillin molecule during interaction with these solubilized enzymes is under investigation.

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DISCUSSION

WIELAND — Is penicilloic acid split hydrolytically by your bacterial enzymes to give the same products as penicillin gives?

FRERE — No, penicilloic acid seems to be quite stable in the presence of our enzymes.

MORLEY — Have you examined the new penicillins derived from glycine and tyrosine?

FRERE — We had not the opportunity to use these new penicillins.

RYDON — Do you get the same results with "amino-acyl penicillins" such as ampicillin?

FRERE — Interaction with ampicillin appears to follow the same general pathway as with benzylpenicillin. However we have not tried to isolate the corresponding N-acylglycine derivative.

GROSS — What do you obtain if you allow the glycine derivative and N-formylcysteine to react?

FRERE — The reaction EI $\longrightarrow$ enzyme + N-acyl-Gly + N-Formyl penicillamine seems to be irreversible. We have not yet studied the interaction between the enzyme and the fragmentation products. We intend to try it in the near future.

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