8. Interaction Between β-Lactam Antibiotics and the Enzymes of the Wall Peptidoglycan Crosslinking System

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I. THE PEPTIDOGLYCAN CROSSLINKING SYSTEM (PgCS)

The primary structures and possible three-dimensional arrangements of the bacterial wall peptidoglycan have been discussed in detail, as have the various reactions involved in the biosynthesis of the polymer (Ghuysen, 1977b). Linear glycan strands consisting of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid are held together by short peptides. In most bacteria, tetrapeptide units L-Ala-D-Glu$_\gamma$L-X-D-Ala (II) substitute the glycan strands through *N*-acetylmuramyl-L)alanine linkages, Depending upon the bacterial species, X is either a diamino acid residue (such as L-diaminobutyric acid, L-ornithine, L-lysine, LL- or *meso*-diaminopimelic acid), or a diamino acid the lateral chain of which is extended by one or several additional amino acid residues [such as N^ϵ-(L-Ala)-L-Lys, N^ϵ-(L-Ser-L-Ala)-L-Lys or N^ϵ-$(Gly)_5$-L-Lys]; variations are almost endless. Finally, adjacent glycan strands are cross-linked through peptide linkages which extend between the C-terminal D-Ala residue of one peptide to the ω-amino group of X in the other peptide. Peptide crosslinking causes the polymer to become water insoluble.

Peptidoglycan biosynthesis involves the formation of activated disaccharide pentapeptide units N^α-(β-1,4*N*-acetylmuramyl-*N*-acetylglucosamyl)-L-Ala-D-Glu$_\gamma$L-X-D-Ala-D-Ala, on the membrane carrier undecaprenyl phosphate (II). Chains consisting of multiple disaccharide–peptide

units grow on the exterior of the plasma membrane by addition of 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 (Ward and Perkins, 1973; Weston *et al.*, 1977). The membrane acceptor on which elongation of the nascent peptidoglycan chain occurs is unknown. Finally, the nascent peptidoglycan becomes water-insoluble, a process in which the peptidoglycan cross-linking system (PgCS)* is involved. The action of β-lactam antibiotics is to inactivate, at least temporarily, all or some of the PgCS constituents, causing morphological abnormalities and/or cell death and cell lysis. Synthesis of β-lactamase is one protective mechanism that bacteria have developed against the deleterious effects of such antibiotics. Considerable variations in sensitivity of bacteria to β-lactam antibiotics also result from variations in sensitivity of the corresponding PgCSs, a low sensitivity of the target leading to a high level of intrinsic resistance. The goal of this article is to review the mode of interaction between β-lactam antibiotics and the enzyme constituents of the PgCS.

A. Penicillin-sensitive Enzymes (PSEs) of the PgCS

Essentially, the reactions catalysed by the PgCS are nucleophilic attacks of pentapeptides L-Ala-D-Glu$_\gamma$L-X$_\omega$(H)-D-Ala-D-Ala and tetrapeptides L-Ala-D-Glu$_\gamma$L-X$_\omega$(H) on the carbon atom bearing the carbonyl group of the penultimate D-ala and L-X residues, respectively (Fig. 1). The nucleophile involved may be water; consequently, simple hydrolyses occur, with the formation of tetrapeptides (DD-carboxypeptidase activity; reaction no. 1) and tripeptides (LD-carboxypeptidase activity; reaction no. 2). Alternatively, the nucleophile may be an amino group NH_2—R; transpeptidations then occur with formation of L-Ala-D-Glu$_\gamma$L-X(H)-D-Ala-CO—NH—R (DD-transpeptidase activity; reaction no. 3) and L-Ala-D-Glu$_\gamma$L-X(H)—CO—NH—R (LD-transpeptidase activity; reaction no. 4). The reac-

* Abbreviations used here are:
PgCS: peptidoglycan-crosslinking system,
PBP: penicillin-binding protein,
PSE: penicillin-sensitive enzyme.

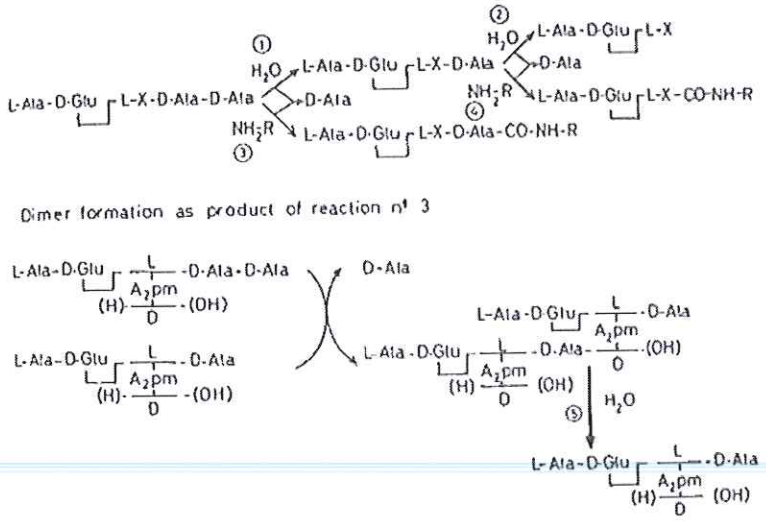


Fig. 1 Schematic representation of the enzyme activities catalysed by the peptidoglycan crosslinking system PgCS.

(1) DD-carboxypeptidase activity; (2) LD-carboxypeptidase activity; (3) DD-transpeptidase activity; (4) LD-transpeptidase activity; (5) DD-endopeptidase activity. In the natural substrates, the L-Ala residues substitute the glycan strands through *N*-acetylmuramyl-L-Ala linkages. X = a diamino acid, the lateral chain of which is either free or extended by one or several aminoacid residues (see Text). A_2pm = diaminopimelic acid.

tion product formed by DD-transpeptidase action on a pentapeptide acting as carbonyl donor and a tetrapeptide acting as acceptor through its N^{ω}-X amino group, is the peptide dimer (Fig. 1, bottom). The above reactions are "penicillin-sensitive" although to various extents (depending upon the enzymic activity, the bacterial species and the β-lactam antibiotic concerned) and free D-Ala is a reaction product which is common to all of them.

Peptide crosslinking between pentapeptide-substituted nascent peptidoglycan strands results in the polymer becoming insoluble in water. Such a process is a crucial event in the early stages of reversion of protoplasts to normal cells (Elliott *et al.*, 1975a, b); it may also occur, at least in part, during peptidoglycan extension in normal cells. The main function of the PgCS, however, is to attach the nascent peptidoglycan strands to the completed wall peptidoglycan, which is part of the pre-existing wall. The reaction catalysed is reaction no. 3 (Wise and Park, 1965; Tipper and Strominger, 1965). As shown by studies carried out

with membrane + cell wall preparations, in an assay system first devised by Mirelman and Sharon (1972), the direction of the transpeptidation between nascent and preformed peptidoglycans is not uniform among the bacteria. In Bacilli, the reaction proceeds from nascent peptidoglycan, the pentapeptide units of which act as carbonyl donors, to pre-existing wall peptidoglycan where the ω-amino groups of the L-X residues of tetrapeptide units function as acceptors (Ward and Perkins, 1974). Conversely, in *Gaffkya homari*, the nascent peptidoglycan acts as amino acceptor through tetrapeptides which have been produced by prior DD-carboxypeptidase action, whereas the pre-existing wall peptidoglycan acts as carbonyl donor via pentapeptide units which must have escaped hydrolysis and undergone wall incorporation by passive transpeptidation (Hammes, 1976; Hammes and Kandler, 1976; Hammes and Seidel, 1978). In *G. homari*, transpeptidation seems to require a definite number and correct alignment of penta-, tetra- and tri- peptides in the nascent peptidoglycan. Hence, if the idea is correct, a precise modulation of the activity of DD- and LD-carboxypeptidase is essential for the bacterium. In fact, specific inhibition of DD-carboxypeptidase by β-lactam antibiotics (under conditions where DD-transpeptidase activity is not affected) appears to be sufficient to cause cell death. The roles played by these hydrolases in bacteria other than *G. homari* are obscure. It is probably correct to assume that destruction of donor sites in the nascent peptidoglycan strands controls the extent of peptidoglycan crosslinking. Nevertheless, many observations suggest that in various Bacilli and in *Escherichia coli*, DD-carboxypeptidase is not vital for the cell. *B. subtilis*, and other Bacilli, are not killed by the specific inhibition of the DD-carboxypeptidase activity (Blumberg and Strominger, 1971; Reynolds *et al.*, 1978) and *E. coli* mutants containing only a fraction of the DD-carboxypeptidase activity found in wild-type strains show no detectable defects (Matsuhashi *et al.*, 1977, 1978). The possible role of the LD-transpeptidase activity is completely unknown; at present, *Streptococcus faecalis* is the only bacterium where such an activity has been detected (Coyette *et al.*, 1974).

The PgCSs also possess endopeptidase activity, which hydrolyses those peptide dimers previously formed by the action of DD-transpeptidase activity; peptide monomers are regenerated which, of course, lack the D-Ala-D-Ala donor group. Many endopeptidases escape penicillin action, except those which act on interpeptide bonds extending between D-Ala and another D-centre in α-position to a free carboxyl group (Ghuysen *et al.*, 1970). Such a structural feature fits well into the active site of some DD-carboxypeptidases which function primarily as DD-endopeptidases. Reaction no. 5 in Fig. 1 is the hydrolysis by DD-endopeptidase action of the C-terminal D-Ala-(D)-*meso*-diaminopimelic acid linkage which serves

to crosslink the peptide units in the wall peptidoglycans of Gram-negative bacteria and several Bacilli. Endopeptidase activity must control the extent of peptide crosslinking by acting at the level of the completed wall peptidoglycan. While it is thought to be involved in the remodelling of the wall during the bacterial cell cycle, its exact function is not known.

B. Penicillin-binding Proteins (PBPs) of the PgCS

With few exceptions, the enzyme activities catalysed by the PgCS are located in the plasma membrane of bacteria. Isolated membranes bind about 50–150 pmols of [^{14}C]benzylpenicillin per mg of protein. Consequently, another approach to study the constituents of the PgCS has involved the investigation of the membrane-bound proteins responsible for penicillin binding (Blumberg and Strominger, 1974). If the radioactive complexes formed between [^{14}C]benzylpenicillin and the proteins exhibit sufficient stability or can be stabilized by protein denaturation, the PBPs can be separated from each other on the basis of their individual molecular weights by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and detected by scintillation autoradiography (Spratt, 1975, 1977a). The technique has been applied to *B. megaterium* (Chase *et al.*, 1977; Chase and Reynolds, 1978), *B. subtilis* (Blumberg and Strominger, 1972a; Buchanan and Strominger, 1976), *B. stearothermophilus* (Yocum *et al.*, 1974; and P. E. Reynolds, personal communication) and *B. licheniformis* (Chase and Reynolds, 1978), *E. coli* (Spratt, 1975, 1977a), *Salmonella typhimurium* (Shepherd *et al.*, 1977), *Proteus* sp. (M. Matsuhashi, personal communication) and *Pseudomonas* sp. (Noguchi *et al.*, 1977), *Staph. aureus* (Kozarich, 1977) and *Strep. faecalis* (Coyette *et al.*, 1978) and *Streptomyces* sp. (J. Dusart and P. E. Reynolds, unpublished experiments). These taxonomically different bacteria represent a whole range of peptidoglycans of different primary structures. A selection of the results obtained is shown in Fig. 2. The PBP profiles much depend on the precise experimental conditions used. Resolution of PBPs 5 and 6 of *E. coli* K12 depend on the quality of the SDS (W. Zimmerman, personal communication). With a loosely crosslinked polyacrylamide gel, PBP 1 of the same organism is resolved in several components: one PBP 1A (6% of total PBPs) and three PBPs 1B (2%, altogether) (Spratt *et al.*, 1977; Tamaki *et al.*, 1977); similarly, PBP 5 of *Strep. faecalis* is resolved in two components: 5A (2.1%), and 5B (1.9%) (J. Coyette, unpublished data). Variations may occur from strain to strain: the major component in *Streptomyces* K15 is PBP 4, and that in *Streptomyces* R61 is PBP 5 (M. Leyh-Bouille and J. Dusart, unpublished data).

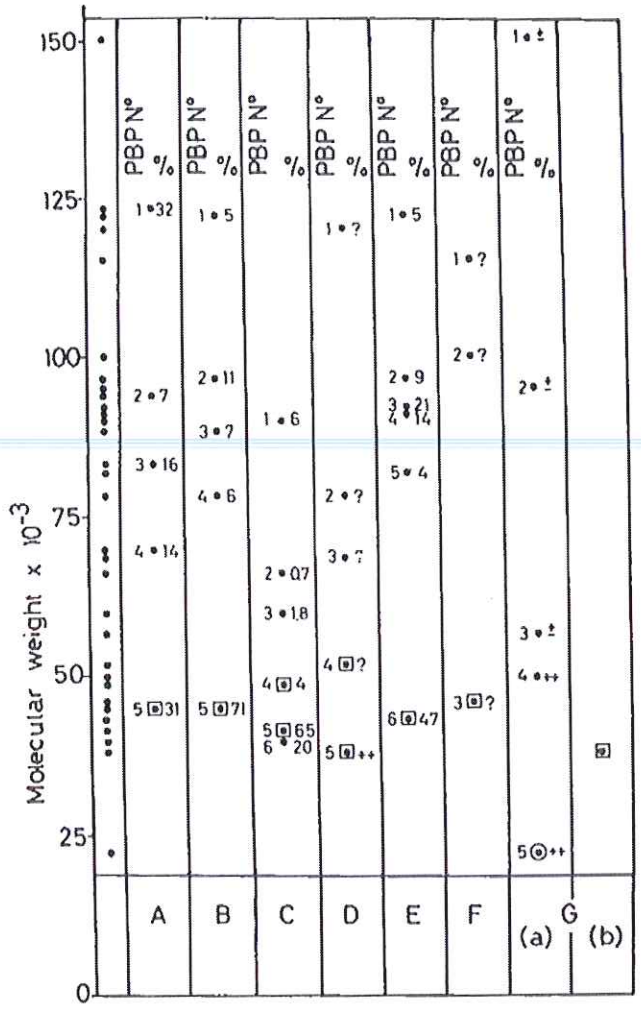


Fig. 2 Penicillin-binding proteins (PBPs) in bacteria.
(A) *B. megaterium* KM; (B) *B. subtilis* Porton; (C) *E. coli* K12; (D) *Salm. typhimurium*; (E) *Strep. faecalis* ATCC 9790; (F) *Staph. aureus* H; (G) (a and b) *Streptomyces* sp.
All PBPs are membrane-bound except that shown in column G (b) which is the lysozyme-releasable PBP from *Streptomyces*. The PBPs surrounded by white squares, or a white circle, have been identified as enzymes. Percentages refer to the sum of all the PBPs detected in each organism. For references, see Text.

PBPs may be lost during the preparation of the membranes: thus, for example, a PBP of molecular weight equal to 38 000 is found in, and can be isolated from, the lysozyme-releasable fractions obtained from *Streptomyces* R61 and K15 (column (b) in Fig. 2) (Leyh-Bouille *et al.*, 1977); a similar PBP is hardly detectable in the isolated membranes (column (a) in Fig. 2). Whatever the limitations of the technique, it is clear that bacteria possess multiple PBPs (from 3 to 8 or more depending upon the species). From an integration of all the data, the PBPs have molecular weights ranging almost without any discontinuity from 20 000 to 150 000 (left column in Fig. 2).

The current hypothesis is that PBPs and PSEs are synonymous (at least most of them). Few PBPs have been isolated, purified to the stage where one single PBP coincides with one single protein band, and characterized by the enzymatic activities they can perform *in vitro*. PBPs known to be PSEs are:

(1) PBP no. 5 (mol. wt 22 000) of *Streptomyces* R61 and K15 (white circle in Fig. 2) (J. Dusart, M. Leyh-Bouille and P. E. Reynolds, unpublished data); and

(2) those PBPs with between 40 000–50 000 mol. wt (white squares in Fig. 2) and originating from Bacilli: (Blumberg and Strominger, 1972b; Umbreit and Strominger, 1973); Yocum *et al.*, 1974; Chase *et al.*, 1977; Chase and Reynolds, 1978); Gram-negative organisms: (Nguyen-Distèche *et al.*, 1974a, b; Pollock *et al.*, 1974; Tamura *et al.*, 1976; Spratt and Strominger, 1976; Martin *et al.*, 1976; Matsuhashi *et al.*, 1977; Shepherd *et al.*, 1977; Schilf and Martin, 1977); *Strep. faecalis*: (Coyette *et al.*, 1978); *Staph. aureus*: (Kozarich, 1977); and *Streptomyces* sp. (Leyh-Bouille *et al.*, 1977), respectively. Among the PBPs of higher molecular weight, PBP 1 of *B. megaterium* and *B. licheniformis* is the only one which has been purified (Chase and Reynolds, 1978) but its possible enzymic function is unknown.

In addition to the above PBPs/PSEs, a few others are excreted during growth by bacteria (not shown in Fig. 2). Excretion of enzymes belonging to the PgCS seems to be a property unique to the actinomycetes. The exocellular PSEs excreted by *Actinomadura* R39: (i.e. the R39 enzyme, mol. wt = 53 000) (Frère *et al.*, 1974a); *Streptomyces* R61: (i.e. the R61 enzyme, mol. wt = 38 000) (Frère *et al.*, 1973a); and *Streptomyces albus* G: (i.e. the G enzyme, occurring in two forms, with mol. wt values of 10 000 and 20 000, respectively) (Duez *et al.*, 1978) have been purified to protein homogeneity. The R61 enzyme has been crystallized (Kelly *et al.*, 1977). Many of their chemical, physical and enzymatic properties are known (Ghuysen, 1977a).

C. DD-Carboxypeptidase, Transpeptidase and Endopeptidase Activities of Isolated PBPs/PSEs

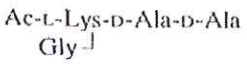
PSEs isolated from, or excreted by, the cells are necessarily dissociated from their natural peptidoglycan substrates. *In vitro*, they may act on simple D-Ala-D-Ala terminated peptides. Depending upon the enzymes, nucleotide precursors UDP-*N*-acetylmuramyl-pentapeptides, free pentapeptides or synthetic peptides such as Ac_2-L-Lys-D-Ala-D-Ala may be used as substrates. On the basis of the enzymic profiles that they exhibit both for the C-terminal D-amino acid of the peptide donor and the nucleophile, the isolated PSEs can be classified into different groups.

(1) PBP 5 of *Streptomyces* sp. (Marquet *et al.*, 1974; Dusart *et al.*, 1975; Leyh-Bouille *et al.*, 1977; Nguyen-Distèche *et al.*, 1977; Dusart *et al.*, 1977). This PBP (mol. wt 22 000, unpublished data) has a very low hydrolytic activity and performs with high efficiency transpeptidation reactions between Ac_2-L-Lys-D-Ala-D-Ala as peptide donor and Gly-Gly, various peptides possessing an N-terminal glycine residue and other amino compounds as nucleophile acceptors. The standard reaction catalysed is Ac_2-L-Lys-D-Ala-D-Ala + Gly-Gly → D-Ala + Ac_2-L-Lys-D-Ala-Gly-Gly. Because of this considerable specificity for amino nucleophiles instead of water, PBP 5 of *Streptomyces* sp. is regarded as a DD-transpeptidase; in fact, it is the only DD-transpeptidase which has ever been isolated. The membrane-bound DD-transpeptidase is able to perform transpeptidation reactions in a solid, frozen state. A temperature as low as −30°C is necessary to prevent the membrane from catalysing the reaction, suggesting that the enzyme functions in a lipid environment which remains remarkably fluid at low temperature. The DD-transpeptidase can be extracted from the membranes, or directly from the mycelia, with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide, or better with *N*-dodecyl-*N*,*N*,*N*-trimethylammonium chloride (which extracts PBP 5 with high selectivity; unpublished data) and then purified by molecular sieve exclusion chromatography. The water-soluble enzyme continues to function as a DD-transpeptidase with very low hydrolytic activity but is inactive in the frozen state.

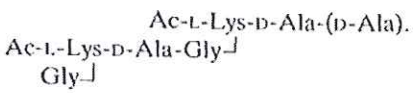
(2) The exocellular G enzyme (Leyh-Bouille *et al.*, 1970; Pollock *et al.*, 1972; Frère *et al.*, 1978b). This PSE is a DD-carboxypeptidase and H_2O is the only nucleophile that it is able to utilize. Another peculiarity of the G enzyme is its ability to hydrolyse with high efficiency a variety of C-terminal N^{α}-(D-Ala-D) peptide bonds irrespective of the structure of the lateral chain of the terminal D-amino acid residue. As a consequence, it is lytic for those walls where C-terminal N^{α}-(D-Ala-D) linkages serve to

crosslink the peptidoglycan subunits (reaction no. 5 in Fig. 1). It is thus as a DD-endopeptidase.

(3) PBP 5 of *B. megaterium*, PBP 5 of *B. subtilis*, PBP 6 of *Strep. faecalis*, PBP 3 of *Staph. aureus*, PBPs 4 and 5 of *E. coli*, *Salm. typhimurium* and *Proteus* sp., the lysozyme-releasable PBP of *Streptomyces* sp. and the exocellular R61 and R39 PSEs are also DD-carboxypeptidases. At least the R61 enzyme (Leyh-Bouille *et al.*, 1971; Nieto *et al.*, 1973) and the R39 enzyme (Leyh-Bouille *et al.*, 1972; Nieto *et al.*, 1973) are known to have a rather strict requirement for the occurrence of a D-Ala-D-Ala residue at the C-terminal position of the peptide donor. The isolated PSEs of group 3 exhibit great variations with respect to their abilities to utilize (in addition to H_2O) amino nucleophiles of increasing complexities. The R39 enzyme (Perkins *et al.*, 1973; Ghuysen *et al.*, 1973, 1974), the DD-carboxypeptidases from *E. coli* (Nguyen-Distèche, 1974a, b; Pollock *et al.*, 1974) and those from *Salm. typhimurium* (Shepherd *et al.*, 1977) are able to catalyse "natural model transpeptidations" leading to the formation of peptide dimers (reaction no. 3 in Fig. 1). Similarly, the R61 enzyme (Zeiger *et al.*, 1975; Frère *et al.*, 1976a), and the *Streptomyces* lysozyme-releasable DD-carboxypeptidase (Leyh-Bouille *et al.*, 1977) catalyse the dimerization of the tetrapeptide monomer



into hexa- and heptapeptides



A very high degree of enzymic selectivity is involved in these transpeptidations, and the reactions catalysed are identical to those which occur *in vivo* during peptidoglycan crosslinking in the corresponding bacteria. In marked contrast, the DD-carboxypeptidases from Bacilli (Wickus and Strominger, 1972; Nishino *et al.*, 1977; Chase *et al.*, 1977); *Strep. faecalis* (Coyette *et al.*, 1977a) and *Staph. aureus* (Kozarich, 1977) are unable to catalyse natural model transpeptidations and have transfer capabilities limited to simple amino compounds such as hydroxylamine, glycine and D-amino acids. These observations are difficult to interpret; the specificity of these enzymes might be such that they require concomitant peptidoglycan synthesis to enable peptide crosslinking to be catalysed.

In water and in the presence of a proper amino nucleophile (whatever its complexity), the above PSEs catalyse concomitantly the hydrolysis of

the peptide donor ~L-X-D-Ala-D-Ala to ~L-X-D-Ala (hydrolysis: reaction no. 1 in Fig. 1) and the formation of ~L-X-D-Ala-CO—NH—R peptides (transpeptidation: reaction no. 3 in Fig. 1). As shown with the R61 and R39 enzymes, partitioning of the total enzyme activity into either pathways is conditioned by the environmental conditions such as the pH value, the polarity of the medium and the concentrations of both the donor peptide and the amino acceptor (Frère *et al.*, 1973b; Ghuysen *et al.*, 1973, 1974). The exact roles that bifunctional PSEs may play *in vivo* are unknown; their functioning might be modulated by the properties of the microenvironment of the cell envelope where they are located.

Bimolecular transpeptidation reactions occurring concomitantly with the hydrolysis of the peptide donor may proceed through at least 10 different mechanisms (Frère, 1973). From the effects caused by the donor concentrations on the v_t/v_{Hy} ratios (v_t = rate of transpeptidation; v_{Hy} = rate of hydrolysis) measured with the R61 enzyme, the most likely mechanism for the transpeptidation reaction is an ordered pathway in which the acceptor binds first to the enzyme (Frère *et al.*, 1973b). However, non-symmetrical pathways, in which the amino nucleophile would bind first to the enzyme in the transpeptidation reaction whereas the donor would bind first to the enzyme in the hydrolysis reaction cannot be excluded (Frère *et al.*, 1973b). Another mechanism has been proposed by Nishino *et al.* (1977) for the DD-carboxypeptidases of *B. subtilis* and *B. stearothermophilus*; formation of an acyl-enzyme (R-D-Ala-E complex) would be the first step of the reaction and transpeptidation would then be the consequence of the partitioning of the intermediate between H_2O and amino nucleophile in a second step. Unfortunately, the v_t/v_{Hy} ratio was not shown to be independent of the concentration of the peptide donor, a condition which must be fulfilled if, as suggested, the donor binds first to the enzyme.

D. The Lethal Target of β-Lactam Antibiotics

The possibility that the PBP involved in the lethal-target of β-lactam antibiotics could act as a direct trigger for autolytic activity but lacks enzymic activity itself (Tomasz and Waks, 1975) remains an interesting hypothesis. The induction of β-lactamase synthesis which results from the irreversible binding of penicillin to Bacilli (Pollock, 1950) might involve a PBP which is not a PSE. However, *B. licheniformis* 94, a mutant deficient in β-lactamase and autolytic enzyme, has the same PBP profile as the wild strain 6346, suggesting that loss of β-lactamase or autolytic enzymic activities in this species is not associated with the loss of a PBP (Chase

and Reynolds, 1978). Hence it is more likely that the penicillin killing target is to be found among the PBPs/PSEs, because misfunctioning of the PgCS would result in cell lysis due to the continuous action of the autolytic enzymes. In addition, disorganization of the bacterial cell envelope might also result in the activation of the autolysins.

The use of (i) β-lactam antibiotics which bind to a single PBP (as mecillinam for PBP 2 in *E. coli*; Spratt, 1977b), (ii) studies of the relative affinities of different β-lactam antibiotics for the various PBPs (Spratt, 1975, 1977a), and (iii) the isolation and genetic analyses of mutants exhibiting morphological abnormalities which produce altered or defective forms of PBPs (Spratt, 1977c; Spratt *et al.*, 1977; Matsuhashi *et al.*, 1977; Tamaki *et al.*, 1977; Iwaya and Strominger, 1977; Matsuhashi *et al.*, 1978; Suzuki *et al.*, 1978) have been invaluable tools to dissect the roles that the PBPs may play in the growth of *E. coli*. PBP 2, the product of a gene located at 14.4 min on the genetic map of *E. coli* K12, and PBP 3, the product of a gene located at 1.8 min, are targets to which β-lactam antibiotics bind to cause production of spherical cells and filamentous cells, respectively. PBPs 1B, probably the products of a single gene located at 3.3 min, appear to be the target at which β-lactam antibiotics bind to inhibit cell elongation (resulting in cell lysis) and are supposed to be the "main" transpeptidase responsible for peptidoglycan crosslinking. Unfortunately, none of these "essential" PBPs have been isolated and their possible enzymic activities are unknown. PBPs 4 and 5 (i.e. the bifunctional DD-carboxypeptidases-transpeptidases of group 3) which together represent 69% of all the PBPs present in *E. coli*, PBP 6 (possibly another DD-carboxypeptidase, although this point remains to be established) which represents 20% of the total PBPs, and PBP 1A (function unknown) do not appear to be susceptible targets for the lethal action of β-lactam antibiotics. These PBPs may even be non-essential for cell growth. This apparent superfluity of DD-carboxypeptidases-transpeptidases in *E. coli* and other Gram-negative bacteria is puzzling. As shown with the *E. coli* and *S. typhimurium* enzymes, PBP 4 is a better DD-transpeptidase than PBP 5, at least *in vitro*. Conversely, PBP 5 is a better hydrolase than PBP 4, and finally PBP 4 is more sensitive to penicillin then PBP 5. It is difficult to suppose that so many enzymes exhibiting compensating potentialities are synthesized by the cell without performing important functions! The situation is therefore complex. PBPs, which are not killing targets in themselves, may enhance the effects produced by binding to other PBPs or may compensate for lack of, or defect in, other PBPs. As suggested by Tamaki *et al.* (1977), to resolve the mechanism of peptidoglycan crosslinking in more detail, mutants with defects in each of the PSEs and PBPs should be isolated and an *in vitro*

reconstitution system that would compensate for the defect of each of them could possibly be devised.

Both Bacilli and Gram-negative bacteria possess cell wall peptidoglycans which have essentially the same primary structure. To all appearances, however, Bacilli have only one DD-carboxypeptidase (PBP 5) and apparently, this enzyme fails to catalyse natural model transpeptidations. PBP 1 of *B. megaterium* reacts with benzylpenicillin at concentrations just sufficient to kill the cells and the characteristics of its interaction with the antibiotic are consistent with its being the protein which catalyses transpeptidation (Reynolds *et al.*, 1978). PBP 2, however, has been proposed to be the killing target in *B. subtilis* (Buchanan and Strominger, 1976). In *Strep. faecalis*, the characteristics of the interaction of the DD-carboxypeptidase PBP 6 with β-lactam antibiotics suggest that DD-carboxypeptidase inhibition is involved, at least in part, in the bactericidal effect (Coyette *et al.*, 1978). Finally, in *Streptomyces* sp., the DD-transpeptidase PBP 5 is the most likely candidate for being the target responsible for the bactericidal effect (Dusart *et al.*, 1973, 1977). In all cases, final proof must await the isolation and the study of mutants with characterized defects.

E. Kinetics of the Interaction Between β-Lactams and PSEs

The model which best explains the kinetics of the interaction between PSE and β-lactam antibiotics is $E+I \rightleftharpoons^{K} EI \rightleftharpoons^{k_3} EI^* \rightleftharpoons^{k_4} E+$ degraded antibiotic. Essentially, it is similar to that suggested previously by Smith *et al.* (1968). The free enzyme reversibly binds the β-lactam antibiotic I to form a first stoichiometric complex EI. In turn, complex EI undergoes an irreversible transformation into a second stoichiometric complex EI*. Finally, complex EI* breaks down, the active enzyme is regenerated (if breakdown occurs under non-denaturing conditions) and a degraded antibiotic molecule is released (Frère *et al.*, 1975a). The dissociation constant K (in M) and the first-order rate constants k_3 and k_4 (in s^{-1}) have been measured for the interaction between several β-lactam antibiotics and the R61 (Frère *et al.*, 1978a), R39 (Faud *et al.*, 1976) and G (Frère *et al.*, 1978b) enzymes, as well as the purified DD-carboxypeptidase PBP 6 of *Strep. faecalis* (Coyette *et al.*, 1978) (Table 1). When $[I] \ll K$, i.e. at low antibiotic concentrations, formation of EI* is dependent on the ratio k_3/K (a second-order rate constant, in $M^{-1} s^{-1}$). For technical reasons, it was not always possible to use antibiotic concentrations high enough to fulfil the conditions $[I] > K$ and to demonstrate that formation of complex EI

Table 1. Values of the constants involved in the interaction between isolated enzyme and β-lactam antibiotics $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + \text{degradation product(s)}$

		Formation of complex EI*				Breakdown of complex EI* (at 37°C)	
Enzyme	Antibiotic	k_3/K ($M^{-1} s^{-1}$)	K (mM)	k_3 (s^{-1})	T (°C)	k_4 (s^{-1})	half-life (min)
G+	Phenoxymethyl-penicillin	0.005	150	0.0008	37	9×10^{-5}	130
	Cephalothin	0.06	9.5	0.0005	37	3.3×10^{-5}	350
	Cephalosporin C	0.06	1.6	0.0001	37	8×10^{-5}	145
R61[b]	Cephaloglycine	22	0.4	0.009	37	3×10^{-6}	3800
	Ampicillin	107	7.2	0.77	37	1.4×10^{-4}	82
	Carbenicillin	820	0.11	0.09	37	1.4×10^{-4}	82
	Cephalosporin C	1150	>1	>1	37	1×10^{-6}	10000
	Phenoxymethyl-penicillin	1500	>1	>1	37	2.8×10^{-4}	40
	Benzylpenicillin	13700	13	180	25	1.4×10^{-4}	82
R39[c]	Cephalosporin C	67000	0.19	12.5	20	0.3×10^{-6}	38000
S. faecalis[d]	Benzylpenicillin	1000	0.025	0.025	37	2.8×10^{-5}	410

[a] Frère *et al.*, 1978b; [b] Frère *et al.*, 1975b; [c] Fuad *et al.*, 1976; [d] Coyette *et al.*, 1978.

was a two-step process; however, the k_3/K and k_4 values could be determined (Table 2). Hence, β-lactam antibiotics which are substrates of various β-lactamases, acylases and esterases, are also, in the strict sense, substrates of the enzymes of the PgCS.

The above model has the following implications:

(1) At low antibiotic concentrations ($[I] < K$), the concentration of active enzyme at the steady-state is a function of [I] and of both the k_3/K and k_4 values.

(2) A β-lactam antibiotic exhibiting a bactericidal effect at low concentrations of drug must have a high k_3/K value and a low k_4 value.

(3) With high k_3/K and k_4 values, the antibiotic is a good substrate.

(4) For the enzyme, intrinsic penicillin resistance may result from low k_3/K values under which conditions, formation of EI* can only occur at high antibiotic concentrations; at identical k_4 values, the lower the k_3/K value, the more resistant is the enzyme to penicillin inactivation. Intrinsic penicillin resistance may also result from high k_4 values under which conditions the enzyme is regenerated rapidly; at identical k_3/K values, the higher the k_4 value, the more resistant is the enzyme to penicillin.

Table 2. Values of the constants involved in the interaction between enzyme and β-lactam antibiotics $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E +$ degradation product(s)

Enzyme	Antibiotic	k_3/K ($M^{-1} s^{-1}$) (at the indicated temperature)	k_4 (s^{-1}) (at 37°C)
G	see Table 1	see Table 1	see Table 1
R61	see Table 1	see Table 1	see Table 1
R39[a]	Methicillin	1100 (20°C)	21×10^{-6}
	Carbenicillin	2920 (20°C)	5.4×10^{-6}
	Cephalexin	3000 (20°C)	2.4×10^{-6}
	Cephalosporin C	67000 (20°C)	0.28×10^{-6}
	Ampicillin	74000 (20°C)	4.4×10^{-6}
	Cephaloglycine	74000 (20°C)	0.8×10^{-6}
	Benzylpenicillin	>90000 (20°C)	2.8×10^{-6}
	Cephalosporin 87–312	3000000 (10°C)	1.5×10^{-6}
Purified[b] *Strep. faecalis* enzyme	Cloxacillin	0.8 (37°C)	8×10^{-5}
	Methicillin	3.6 (37°C)	7×10^{-5}
	Oxacillin	4.6 (37°C)	15×10^{-5}
	Carbenicillin	27 (37°C)	3.5×10^{-5}
	Ampicillin	600 (37°C)	1.3×10^{-5}
	Benzylpenicillin	1000 (37°C)	2.8×10^{-5}
	Phenoxymethylpenicillin	1200 (37°C)	8.6×10^{-5}
Membrane-bound DD-transpeptidase of *Streptomyces* R61[c]	Cephalosporin C	3 (37°C)	33×10^{-4}
	Cloxacillin	13 (37°C)	1.2×10^{-4}
	Carbenicillin	15 (37°C)	0.7×10^{-4}
	Benzylpenicillin	53 (37°C)	1.1×10^{-4}
	Phenoxymethylpenicillin	140 (37°C)	2.8×10^{-4}
	Ampicillin	400 (37°C)	33×10^{-4}
Purified enzyme from unstable L-form of *P. mirabilis*[d]	Benzylpenicillin	20000–80000 (37°C)	3.3×10^{-3}

[a] From Fuad *et al.*, 1976; [b] From Coyette *et al.*, 1978; [c] From Dusart *et al.*, 1977; [d] From Schilf *et al.*, 1978.

(5) Enzyme inhibition may appear to be irreversible in some cases (low k_4 values) and reversible in others (high k_4 values). Depending upon the enzyme and the antibiotic concerned, the k_4 values exhibit important variations. Thus, for example, the k_4 value for the interaction between cephalosporin C and the R39 enzyme is about $3 \times 10^{-7} s^{-1}$ (half-life of EI*: 40 000 min, at 20°C), whereas that for the interaction between benzylpenicillin and the purified DD-carboxypeptidase PBP 5 of the

unstable L-form of *Pr. mirabilis* is about $3 \times 10^{-3}\ s^{-1}$ (half-life of EI*: 3.5 min at 37°C) (Martin *et al.*, 1976).

(6) Enzyme regeneration through breakdown of complex EI* explains the well-known observations that bacteria previously saturated with penicillin are able to recover and grow when reincubated in a fresh, penicillin-free medium. Hence, the interaction between penicillin and the PBP involved in the lethal target, whatever its exact enzymic function, is essentially reversible, a property which is compatible with the proposed model.

1. *The penicilloyl–enzyme complex EI**

In complex EI* both PSE and the β-lactam antibiotic are modified. Formation of complex EI* between the R61 enzyme and benzylpenicillin is paralleled by an extensive alteration of the CD spectrum of the enzyme in the near UV and by a decrease of its fluorescence (Nieto *et al.*, 1973). Fluorescence quenching is also observed during interaction between the R39 enzyme and cephalosporins (Fuad *et al.*, 1976). These modifications reflect conformational changes caused to the enzymes. A serine residue is involved within the penicillin binding site of the R61 enzyme (Frère *et al.*, 1976c) and probably within that of the DD-carboxypeptidase PBP 5 of *B. subtilis* (Kozarich *et al.*, 1977).

Between 320 and 600 nm, the absorption spectrum of complex EI* formed between the R39 enzyme and the chromogenic cephalosporin 87-312 [3-(3,4dinitrostyryl)-(6R-7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer] is identical with that of cephalosporin 87-312 hydrolysed by β-lactamase (i.e. the maximum of the absorption spectrum shifts from 386 to 482 nm). Similarly, the molar extinction coefficients at 260 nm, of cephalexin, cephalosporin C and cephaloglycine decrease, as a result of their binding to the R39 enzyme, to an extent similar to that obtained by β-lactamase action (Frère *et al.*, 1974b). Hence, formation of complex EI* between the R39 enzyme and cephalosporins probably involves the hydrolysis of the β-lactam bond of the antibiotic molecule. However, whereas the $\varepsilon_{482}/\varepsilon_{386}$ ratio of both cephalosporin 87-312, either combined to the R39 enzyme or when hydrolysed by β-lactamase, is 2.4, the ratio of the R61 enzyme–cephalosporin 87-312 complex is only 1.2 (Frère *et al.*, 1974c). Breakdown of the latter complex causes the release of a product which has the same absorption spectrum as that of cephalosporin 87-312 hydrolysed by β-lactamase. The "blue shift" of the absorption spectrum of the R61 enzyme–cephalosporin 87-312 complex (maximum at 450 nm), when compared to that of the R39 enzyme–cephalosporin 87-312 complex (maximum at 482 nm), indicates differences between the two complexes

with regard to the electrostatic and/or the conformational changes involved.

Both EI* complexes formed between benzylpenicillin and the R61 and R39 enzymes, respectively, when denatured by heat, break down without enzyme regeneration but with release of benzylpenicilloate (C. Duez, unpublished data). The R39 enzyme is stable only at high ionic strength. Formation of complex EI* with benzylpenicillin and breakdown in 10 mM phosphate pH 7.0 (37°C) causes formation of benzylpenicilloate, but enzyme regeneration does not occur (Frère *et al.*, 1974b). Treatment with sodium borohydride, or treatment at pH 12.0, of complex EI* formed between benzylpenicillin and the DD-carboxypeptidase PBP 5 of *B. subtilis*, when denatured by heat or trichloracetic acid treatment results in the total release of the penicilloyl moiety (Kozarich *et al.*, 1977).

The simplest interpretation of all these observations is that formation of complex EI* probably consists of the penicilloylation of the PSE.

2. *Breakdown of the penicilloyl–enzyme complex EI**

The reaction products of the breakdown of complex EI*, under conditions of enzyme reactivation, depend upon the PSE that is used (Fig. 3). With the G enzyme (C. Duez, unpublished data), the membrane-bound DD-transpeptidase PBP 5 of *Streptomyces* sp. (Marquet *et al.*, 1974), the isolated DD-carboxypeptidases of *E. coli* (Tamura *et al.*, 1976) and of the unstable L-form of *Pr. mirabilis* (Schilf *et al.*, 1978), benzylpenicilloate is released. Hence, these enzymes behave as β-lactamases of low efficiency.

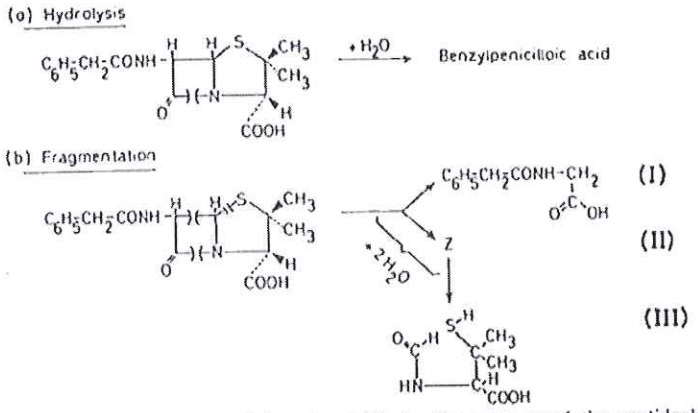
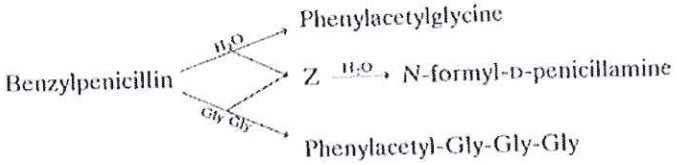


Fig. 3 Degradation pathways of benzylpenicillin by the enzymes of the peptidoglycan crosslinking system PgCS.

Water is assumed to be the only nucleophile involved. I, phenylacetylglycine; II unknown intermediate; III *N*-formyl-D-penicillamine.

With the R61 enzyme (Frère *et al.*, 1975c), the R39 enzyme (Frère *et al.*, 1975c), the isolated DD-transpeptidase PBP 5 of *Streptomyces* sp. (Dusart *et al.*, 1977), the isolated DD-carboxypeptidase of *Strep. faecalis* (Coyette *et al.*, 1977b) and the isolated DD-carboxypepidase of *B. stearothermophilus* (Hammarström and Strominger, 1975), phenylacetylglycine is released. Formation of phenylacetylglycine implies the fragmentation of the penicillin molecule. The primary product Z which arises from the thiazolidine molecule is not free D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (Adriaens *et al.*, 1978) as previously proposed by Hammarström and Strominger (1976). In 3 mM phosphate pH 7.5, at 37°C, the intermediate Z has a half-life of 10 min and gives rise to *N*-formyl-D-penicillamine (Frère *et al.*, 1976b; Adriaens *et al.*, 1978).

Cleavage of the C_5–C_6 bond of penicillin by the R61 enzyme with formation of a $—CH_2$-methylene group at C_6 has been followed by isotopic studies of the effects of D_2O on the fragmentation reaction (Frère *et al.*, 1978a). In complex EI*, the C_5–C_6 bond is intact; breakdown of the complex involves a rate-limiting reaction of unknown nature which is immediately followed by: (1) C_5–C_6 cleavage and formation of an activated *N*-acylglycyl fragment, and (2) the transfer of the *N*-acylglycyl fragment to a nucleophilic acceptor. The nucleophile may be either water, in which case *N*-acylglycine is released, or an amino group such as Gly-Gly (or D-Ala or Gly-L-Ala), in which case phenylacetyl-Gly-Gly-Gly or the other corresponding derivatives are formed (A. Marquet, unpublished data). Partitioning of the activated *N*-acylglycyl moiety between the two pathways depends on the nature and the concentration of the amino acceptor that is present. Whether regeneration of the free, active enzyme is comcomitant with the C_5–C_6 bond cleavage or with the transfer of the *N*-acylglycyl moiety is unknown. Whatever the case, the overall reactions are analogous to reactions 1 and 3 of Fig. 1.



F. Ternary Interaction Between PSE, Peptide Donor and Penicillin

Some PSEs hydrolyse penicillin to penicilloate, whereas others fragment the penicillin molecule. Most likely, penicilloylation occurs with both types of PSEs, but the penicilloate-producing PSEs appear to be unable

to carry out those subsequent steps through which the penicillin-fragmenting PSEs transform the penicilloyl-enzyme complex into an *N*-acylglycyl-enzyme complex. This idea is further supported by the fact that formation of the *N*-acylglycyl moiety by those PSEs which normally fragment penicillin can be suppressed by modifying the conformation of the enzyme or by denaturing the complex EI*. The DD-transpeptidase of *Streptomyces* sp. hydrolyses penicillin into penicilloate when it is membrane-bound (Marquet *et al.*, 1974). However, once it has been solubilized, the same enzyme catalyses penicillin fragmentation (Dusart *et al.*, 1977). The effect of heat on complexes EI* formed with the R61 and R39 enzymes and, with the R39 enzyme, the effect of a low ionic strength have already been mentioned. Similarly, breakdown at 22°C of the benzylpenicillin-R61 enzyme complex EI* in 6 M guanidinium chloride yields phenylacetylglycine (40%), benzylpenicilloate (40%) and another unknown product (20%) (J. M. Frère, unpublished data).

The question therefore arises whether the initial steps of the interactions between enzyme and penicillin, and between enzyme and peptide donor, occur on the same enzyme centre(s) or not. Investigations of the ternary interaction between enzyme, β-lactam antibiotic and peptide donor have been carried out by kinetic studies. A non-competitive model is the most general. In Fig. 4, D represents the peptide donor Ac_2-L-Lys-D-Ala-D-Ala; H_2O is assumed to be the only nucleophile involved and the two possible pathways of penicillin degradation are shown. If binding of

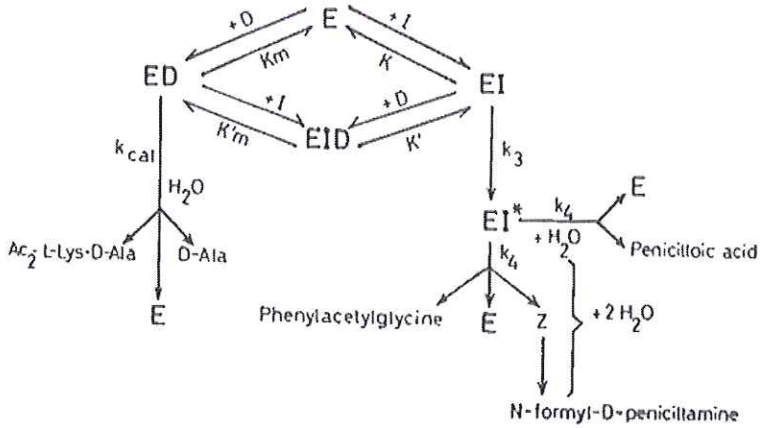


Fig. 4 Non-competitive interaction between penicillin-sensitive enzyme (E), benzylpenicillin (I) and tripeptide donor (D).
Water is assumed to be the only nucleophile involved.

penicillin and peptide donor is mutually exclusive, the ternary complex EID cannot be formed and the interaction is competitive.

With several PSEs, apparently competitive kinetics are obtained. However, a careful analysis shows that in systems involving the formation of a rather stable complex, such as EI*, the concentrations of EI and EID might be negligible, with the result that seemingly competitive Lineweaver–Burk plots are obtained even if the interaction is non-competitive (Frère *et al.*, 1975b). With the G enzyme, the k_3 value for the interaction with cephalosporin C is extremely low ($1 \times 10^{-4}\ s^{-1}$); this property made it possible to study the ternary interaction between enzyme, cephalosporin C and Ac_2-L-Lys-D-Ala-D-Ala by steady-state kinetics under conditions where EI* was virtually not formed (Frère *et al.*, 1978b). The result was that the interaction is non competitive with the constants K, K', K_m and K'_m exhibiting similar values of about 0.5 mM. Similarly, the DD-carboxypeptidase PBP 5 of the unstable L-form of *Pr. mirabilis*, with which both formation and breakdown of complexes EI* are fast processes, also reacts with benzylpenicillin and UDP-*N*-acetylmuramylpentapeptide in a non-competitive manner (Martin *et al.*, 1976; Schilf *et al.*, 1978). Hence, at least for some PSEs, distinct enzyme centres appear to be involved in the initial steps of the interactions with peptide donor and penicillin, respectively.

II. CONCLUSION

The enzymes of the PgCS are peculiar β-lactam degrading enzymes: the reaction is a slow, or a very slow, process and, depending upon the enzymes and/or the environmental conditions, the reaction results either in penicillin fragmentation or in formation of penicilloate. The latter pathway may be regarded as an abortive fragmentation. The reaction products arising from cephalosporins are unknown. There is no doubt that these antibiotics are also degraded during the reaction; the few observations made suggest that, at least, the amide β-lactam bond is hydrolysed.

From the data of Table 1, the following observations can be made:

(1) The G enzyme has a very low penicillin sensitivity, not because the complexes EI* are especially unstable but because the k_3/K values are extremely low. Thus formation of complex EI* requires high antibiotic concentrations. With phenoxymethylpenicillin, this relative lack of reactivity can be attributed in part to a high K value, but in all cases, it is

clear that the main cause of penicillin resistance is due to very low k_3 values;

(2) The decreased sensitivity of the R61 enzyme to carbenicillin, ampicillin and cephaloglycine when compared with benzylpenicillin is also due to decreased k_3 values;

(3) Whether the enzyme is highly sensitive to β-lactam antibiotics (the R39 enzyme); moderately sensitive (the R61 enzyme or the *S. faecalis* DD-carboxypeptidase); or highly resistant (the G enzyme), the K value is rarely low and is never very low. Hence, the recognition of the antibiotic by the PSE is never especially good. This property thus excludes the alternative mechanism proposed by Tipper and Strominger (1965) and Lee (1971), according to which penicillin might be viewed as being a "transition state" analogue of the normal enzymic process (in which case, penicillin should bind to the enzyme with an exceedingly low K value). These observations, together with the fact that with some PSEs the ternary interaction between enzyme, β-lactam antibiotic and peptide donor is non-competitive, are best explained on the basis of the view of Rando (1975) that penicillin would be a "k_{cat}" inhibitor or a "suicide" substrate. 6-Aminopenicillanic acid and Δ^3-7-aminocephalosporanic acid are poor antibacterial agents, indicating that the occurrence of an appropriate acyl side chain on the molecules is required for high activity. Similarly, most modifications of carboxyl group in penicillin and Δ^3-cephalosporin chemistry have led to disappointing antibiotic activity. Presumably, these groupings are the "handle" through which the PSEs apply the binding forces required to strain further the antibiotic and distort the β-lactam nitrogen to a planar shape, resulting in formation of complex EI*. The initial binding of penicillin may or may not interfere with the binding of the peptide donor; whatever the case, the enzyme is "frozen" and made unable to perform its normal functions. A decreased velocity of the "suicide" step, i.e. a decreased k_3 value, results in a decreased inhibitory activity of the antibiotic and therefore in an increased resistance of the enzyme to the β-lactam antibiotic. Alternatively, a rapid recovery following the "suicide" step, i.e. a rapid breakdown of complex EI* resulting in a rapid turnover of the antibiotic molecule, also causes resistance due to β-lactamase like action.

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