PENICILLIN-SENSITIVE ENZYMES IN PEPTIDOGLYCAN BIOSYNTHESIS

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I. INTRODUCTION

During the past few years, several meetings were organized to celebrate the 50th anniversary of Fleming's historical discovery of penicillin. Reviews were written[1-4] and books edited[5-12] covering the many fields in which penicillin-related research is now performed. From total synthesis and isolation of new β-lactams from culture filtrates to high resolution protein crystallography of penicillin-sensitive enzymes and β-lactamases, from electron microscopy observation of β-lactam-treated cells to nucleotide sequencing of genes coding for penicillin-binding proteins, it seems that an ever-expanding number of research projects attempt to deal with this rather simple molecule and its unique and, in many respects, still mysterious interactions with the bacterial cells. When he first noticed that area devoid of bacteria around what turned out to be a colony of *Penicillium notatum*, Fleming could certainly not imagine the tremendous research efforts that his observation would generate. In fact, he himself did not appear to immediately realize its therapeutic implications, since it took about 15 more years before the new antibiotic started its quasimiraculous medical career.

It might thus appear ambitious and maybe useless to write still another review on this subject. In fact, we feel that it is both difficult to be really original and nearly impossible to coherently discuss results obtained in so many different specialities. In consequence, we intend to restrict our analysis mainly to the studies of the interactions between the β-lactam family of molecules and sensitive enzymes or penicillin-binding proteins.

In 1957, it was widely recognized that penicillin interfered with the formation of the bacterial cell wall.[13] During the next few years, it became clear that the reaction which did not take place in the presence of the antibiotic was a transpeptidation. This reaction was one of the final steps in the biosynthesis of the major polymer of the cell wall: the peptidoglycan. We will thus briefly describe the structure and synthesis of this large polymer; putting most of the emphasis on the final, exocellular events, i.e., the transglycosylation and transpeptidation reactions. It has recently become clear that in some cases, exposure of cells to β-lactams induces effects apparently not directly connected with a decrease of transpeptidase activity: triggering of the autolytic system[14] and decrease in the degree of *O*-acetylation of the peptidoglycan polysaccharide moiety.[15-18] Phenomena of tolerance[19,20] have also been described, which still remain unexplained. Although perfectly conscious of the importance of these experimental data, we will not attempt to deal with them since the present knowledge on penicillin-sensitive enzymes does not allow a critical discussion of these results.

As soon as β-lactams were utilized as chemotherapeutic agents, some strains exhib-

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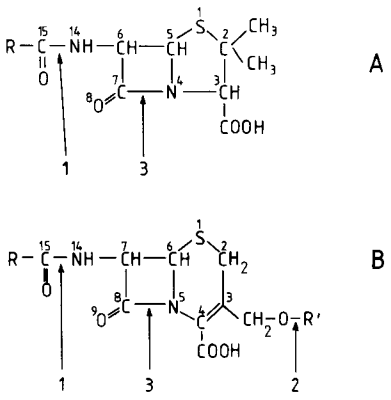


FIGURE 1. Activities of amidases (1, also called penicillin-acylases), esterases (2), and β-lactamases (3) on penicillins (A) and cephalosporins (B). Note that the side chains on C3 of cephalosporins might have a very different structure ($-CH_3$ in cephalexin, for instance). From Frère, J. M., Duez, C., Dusart, J., Coyette, J., Leyh-Bouille, M., Ghuysen, J. M., Dideberg, O., and Knox, J. R., *Enzyme Inhibitors as Drugs,* Sandler, M., Ed., Macmillan, New York, 1980, chap. 12. With permission.)

ited a high degree of resistance.[21] It is possible to grossly divide the resistance phenomena into three large categories: (1) permeability barriers such as the outer layers of the Gram-negative wall might keep the antibiotic from reaching its target(s); (2) the target enzyme(s) themselves might exhibit decreased sensitivity to the antibiotic; and (3) the bacteria might produce variable amounts of enzymes capable of destroying the antibiotic. If the first and second phenomena are clearly outside and inside the scope of this article, respectively, the situation with the third one is not as clear-cut. The activities of amidases and esterases (Figure 1) which can alter the efficiency of the β-lactam molecules are only of marginal interest to our discussion, but β-lactamases certainly share mechanistic and structural characteristics with the penicillin-sensitive enzymes and it is not impossible that β-lactamases might behave as penicillin-binding proteins under certain circumstances.

This article is thus divided into four main chapters: peptidoglycan structure and biosynthesis, sensitive enzymes (PSE), penicillin-binding proteins (PBP), and β-lactamases. It will be seen that the distinction between PBP and PSE might sometimes appear arbitrary, but it is the authors' conviction that these two approaches imply very different strategies and interests. Physiologists and geneticists relish the PBP approach and try to establish correlations between the absence or inactivation of a membrane-bound protein and cellular events such as septation, elongation, or lysis. Chemists and enzymologists prefer to search for penicillin-sensitive enzymatic activities, and try to isolate and study the enzymes. Both approaches can claim numerous complementary successes and clearly converge since numerous PBPs have been demonstrated to be PSE and vice versa.

II. STRUCTURE AND BIOSYNTHESIS OF PEPTIDOGLYCAN

Many excellent reviews have been published about cell wall structure and biosynthesis.[22-26] We will only discuss the structure and metabolism of the polymer which protects the bacterium from its own osmotic pressure: the peptidoglycan (also called mucopeptide, glycopeptide, or murein). A thick multilayered, three-dimensional net-

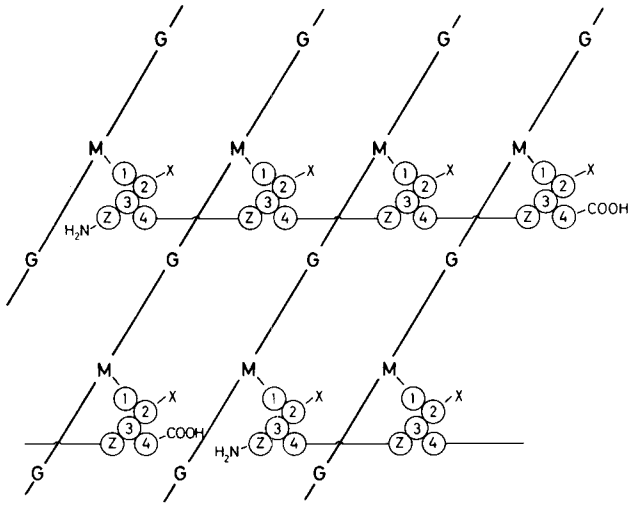


FIGURE 2. General structure of peptidoglycans of chemotypes I, II, and III. Z represents the cross-linking amino acid(s), which is (are) absent in chemotype I. G and M, *N*-acetylglucosaminyl and *N*-acetylmuramyl residues, respectively. 1 = L-Ala; 2 = D-Glu; 4 = D-Ala; X = OH or $-NH_2$. The circles represent amino acid residues or short peptides. For more details, see Figure 3. (Reproduced from Frère, J. M., *Biochem. Pharmacol.*, 26, 2203, 1977. With permission.)

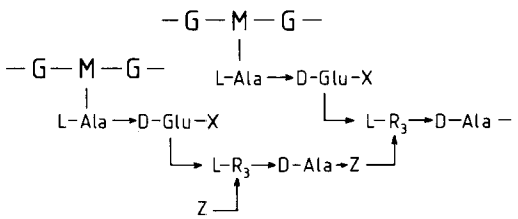


FIGURE 3. Detailed structure of the peptide cross-link in peptidoglycans of chemotypes I, II, and III. X = OH or NH_2. Residue L-R_3 can derive from a diaminomonocarboxylic (L-ornithine, L-lysine, L-α,δ-diaminobutyric acid) or from a diaminodicarboxylic acid (LL- or *meso*-diaminopimelic acid). The ω-amino group of this residue forms an amide (peptide) bond with the carboxylic group of a D-Ala residue of another peptide (in chemotype I, Z is absent in this case, R_3 is *meso*-diaminopimelic acid: Gram-negative bacteria, *Bacillus* and *Actinomadura* genera) or of the cross-linking peptide Z. In chemotype II, Z is one amino acid (Gly in *Streptomyces* genus) or a short peptide (Gly_5 in *Staphylococcus aureus*). In chemotype III, Z is formed by one or several tetrapeptide units, exhibiting the same sequence as the main tetrapeptide: L-Ala-D-Glu→L-R_3-D-Ala (*Micrococcus lysodeikticus, Sarcina lutea*). In chemotype IV peptidoglycans, a diamino acid cross-links the carboxylic group of the D-Ala with the α-carboxyl of the D-Glu residue (*Corynebacterium poinsettiae*).

work of peptidoglycan is the major component of the Gram-positive cell wall. Although quantitatively less important (only one or two molecular layers), it remains the major element in maintaining the shape and rigidity of the Gram-negative wall.

Figures 2 and 3 display schematic representations of this large polymer, for which the name "giant macromolecule" is neither a pleonasm nor an exaggeration, since it completely surrounds the cell.

Linear, saccharidic strands composed of alternating *N*-acetyl-glucosaminyl (G) and *N*-acetylmuramyl (*M*) residues are cross-linked by short peptides attached by an amide bond to the lactyl side chain of the *N*-acetylmuramyl residue. The structures of these peptides depend upon the bacterial species under consideration. Ghuysen[27] has distinguished four different chemotypes. Figure 3 shows a more detailed view of the peptide moiety in chemotypes I, II, and III. The alternation of L and D amino acid residues is

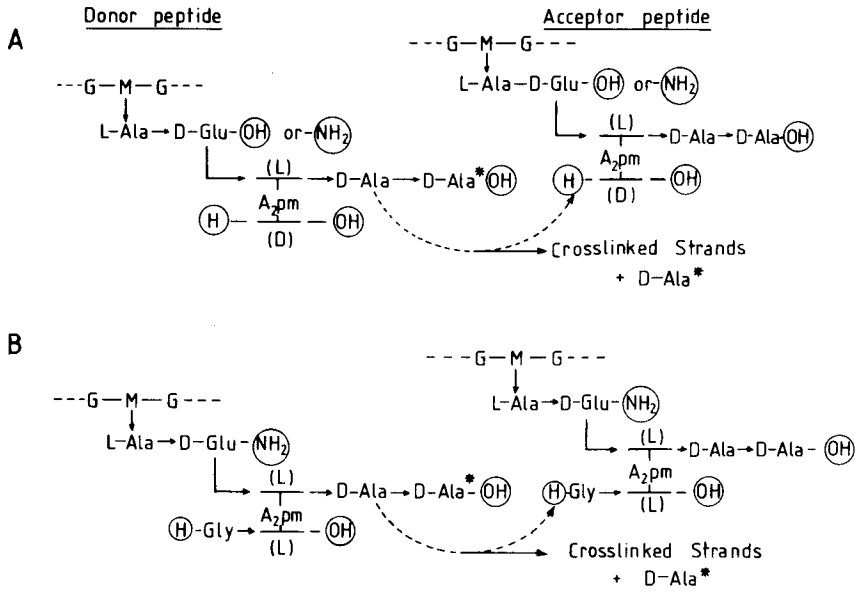


FIGURE 4. The transpeptidation reactions. (A) In Gram-negative bacteria, *Bacillus* and *Actinomadura* genera (chemotype I); (B) In *Streptomyces* genus (chemotype II). (From Ghuysen, J. M., *The Bacterial DD-Carboxypeptidase-Transpeptidase System*, University of Tokyo Press, Tokyo, 1977. With permission.)

a major characteristic of the structure, as well as the involvement of the γ-carboxyl group of the D-glutamyl (or isoglutaminyl) residue in a peptide bond along the main peptide backbone. In all three chemotypes, residue L-R_3 is derived from a diamino acid or a diamino diacid: it supplies the branching point necessary for the building of a cross-linked peptide. The degree of cross-linking also varies according to the species and can be influenced by the growth conditions. It is low in *Escherichia coli*, where 75% of the D-Ala residues exhibit a free carboxyl group, and high in *Staphylococcus aureus*, where this figure drops to 10%.[28]

The numerous steps in the biosynthesis of the polymer can be divided into three groups of events according to their cellular location:

1. Soluble enzymes in the cytoplasm synthesize activated precursors: UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-peptide.
2. These precursors successively react with a C_{55}-isoprenoidyl-phosphate membrane carrier to form an undecaprenyl-pyrophosphate-*N*-acetylmuramyl(peptide)-*N*-acetylglucosamine carrier-linked disaccharide. At this stage, modifications of the peptide can also occur.
3. The polymer is finally assembled in the extracellular space or on the external surface of the cytoplasmic membrane by (at least) two reactions catalyzed by membrane-bound enzymes: a transglycosylase and one or more transpeptidases. Figures 4 and 5 illustrate these two reactions.

Ward and Perkins[29] have studied the direction of growth of the saccharide strands in *Bacillus* and concluded that the new units were added at the reducing end of the growing chains. The "nascent", linear peptidoglycan thus obtained is then cross-linked to preexisting strands, or to other "nascent" strands by the transpeptidation reaction. It should be noted that the peptide units which are transported through the cytoplasmic membrane contain one additional D-alanine residue, thus exhibiting a D-alanyl-D-alan-

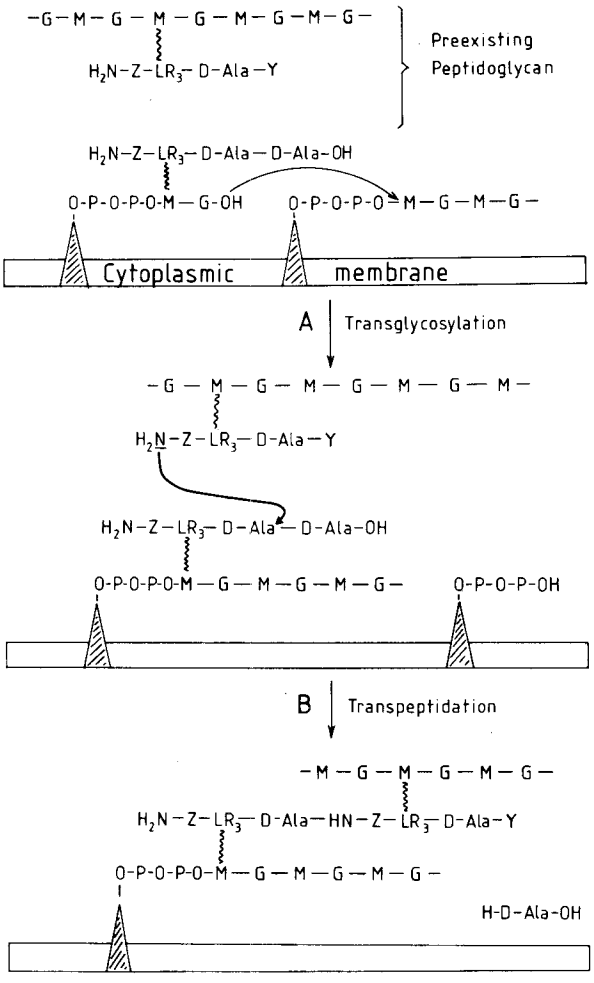


FIGURE 5. Possible scheme for the integration of new disaccharide-peptide units into nascent peptidoglycan by transglycosylation (A) and into preexisting peptidoglycan by transpeptidation (B). The peptide in the preexisting peptidoglycan which serves as an acceptor in the transpeptidation reaction might already be engaged in another cross-link (Y = —NH—Z—peptide), be intact (Y = D-Ala-OH), or have been shortened by DD-carboxypeptidase activity (Y = OH). In some cases, the L-R_3-D-Ala-OH peptide bond of the acceptor molecule has been hydrolyzed by LD-carboxypeptidase activity.

ine C-terminus (compare Figures 3 and 4). The transpeptidase utilizes the energy of this supplementary peptide bond to form a new amide bond between peptide units on neighboring glycan strands, leading to the incorporation of the nascent strand into the preexisting sacculus and releasing one D-alanine molecule for each cross-link formed. According to Mirelman,[25] further transpeptidation reactions might occur during the maturation of the polymer, and the transpeptidase involved in these latter events might be different from the first one. Under normal conditions, if one peptide unit has not been used as a D-alanyl donor, the C-terminal D-alanine is most often split by a D-alanyl-D-alanine carboxypeptidase which explains the absence of detectable D-alanyl-D-alanine termini in many peptidoglycans. The physiological role of this enzyme remains obscure. It could, e.g., control the degree of cross-linking by eliminating poten-

tial donor sites. When peptide units are assembled into trimers or tetramers, several lines of evidence suggest that the new, still monomeric unit often serves as a donor in the transpeptidation reaction. Ward and Perkins[30] obtained efficient integration of peptide units whose free amino groups had been acetylated, Frère et al.[31] showed that formation of trimer was much more efficient when the monomer was used as a donor and the dimer as an acceptor, and Oldmixon et al.[32] found the proportions of monomer, dimer, and trimer in the peptidoglycan of *Streptococcus faecium* in good agreement with a monomer addition model.

Figure 5 integrates all these data but the authors are fully conscious that this scheme, which is based on results obtained with very different organisms and sometimes with very simplified "in vitro" systems, might not be of general value (see below, for instance, the special situation in *Gaffkya homari* where carboxypeptidase must precede transpeptidase activity). That the D-alanyl-D-alanine caboxypeptidases and transpeptidases (in short, the DD-peptidases) are the penicillin-sensitive enzymes in peptidoglycan biosynthesis is now clearly established.

In 1964, after studying the composition of the peptidoglycan of penicillin-treated *Proteus mirabilis*, Martin[33] proposed that the transpeptidase was the target of the antibiotic. Although the actual experimental results later turned out to be doubtful,[34] the hypothesis itself was confirmed 2 years later. Wise and Park[35] and Tipper and Strominger[36] independently demonstrated that a large number of uncross-linked units accumulated in the peptidoglycan of *Staphylococcus aureus* cells grown in the presence of sublethal concentrations of penicillin. Moreover, this peptidoglycan contained an increased proportion of D-alanine. In 1966, two groups independently obtained cellular membrane preparations which could catalyze in vitro peptidoglycan biosynthesis from the UDP-activated precursors.[37,38] In the presence of penicillin, only uncross-linked peptidoglycan was formed. Moreover, if UDP-*N*-acetylglucosamine was omitted from the reaction mixture, a DD-carboxypeptidase activity could be detected on the sole UDP-*M*-peptide, an activity which also disappeared in the presence of penicillin. Transglycosylation remained generally unaffected by penicillin and the release of soluble, linear peptidoglycan in the culture medium was observed in several instances.[39-43]

A third enzymatic activity, which was later discovered to be penicillin sensitive,[44] was the so-called "endopeptidase" activity. This enzyme hydrolyzed the cross-links formed by the transpeptidase. To our knowledge, whenever this activity was found to be blocked by β-lactams, the name "endopeptidase" turned out to be a misnomer. In fact, the peptide bond which was hydrolyzed was always in a position α to a free carboxyl group on a D-center, and the activity was a DD-carboxypeptidase activity (Figure 6). That the side chain of the C-terminal amino acid happened to be very long and to have its ω amino and carboxyl groups engaged in another peptide chain (thus being the pivotal element in the cross-link) was irrelevant to the fact that no "endo" peptide bond was hydrolyzed by the enzyme. No true penicillin-sensitive endopeptidase has ever been described (with the possible exception of the R61 exocellular enzyme, see Section III.B.3). This does not mean that all D-alanyl-D-alanine carboxypeptidases are capable of catalyzing the hydrolysis of the peptide cross-link of Gram-negative bacteria. In fact, the carboxypeptidases exhibit varying degrees of specificity for the C-terminal amino acid that they can release.

What is the physiological role of the "endopeptidase"? The peptidoglycan should not be seen as a fixed structure, growing only at one end. It is probable that new growing sites are created in the polymer and the "endopeptidase" activity could create those sites by making new aminated acceptor groups available. But this is still a hypothetical notion.

This leaves only two different types of penicillin-sensitive enzymes: DD-carboxypep-

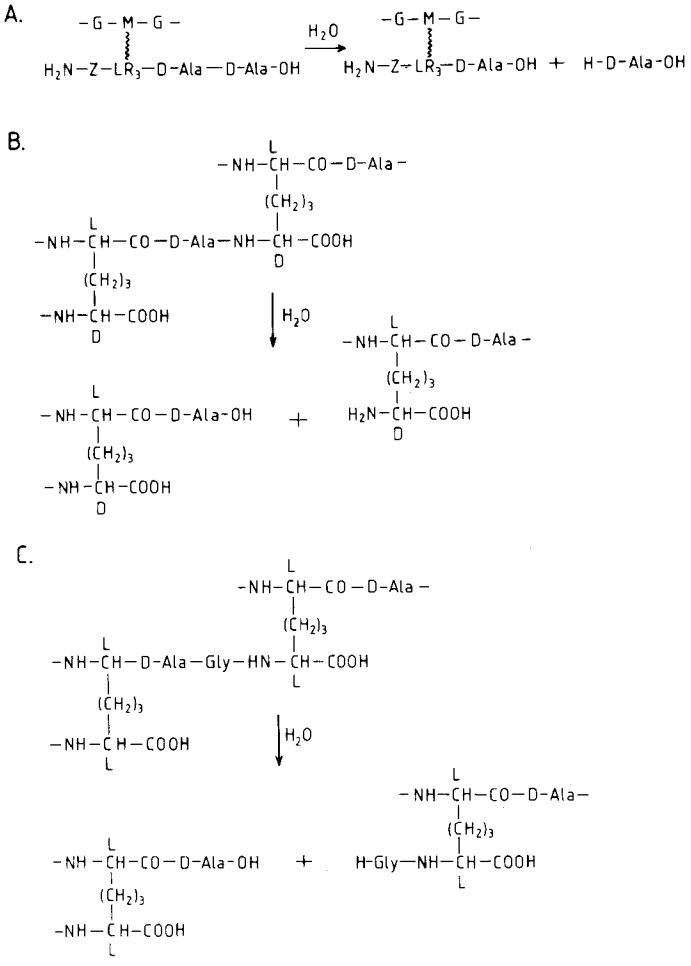
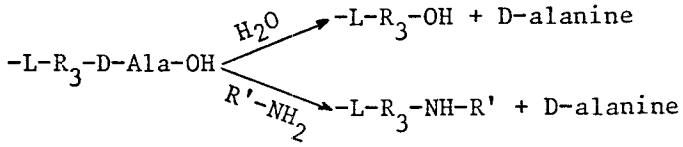


FIGURE 6. (A) D-alanyl-D-alanine carboxypeptidase. (B) D-alanyl-D-carboxypeptidase, often misnamed "endopeptidase". This enzymatic activity should be named "peptidoglycan hydrolyzing"DD-carboxypeptidase. (C) True endopeptidase.

tidases and DD-transpeptidases.* However, as noted above, and as will be discussed at length in the section dealing with PBP, different transpeptidases probably coexist in

* Exceptions to this general rule have been described. Other enzymes catalyze the removal of the penultimate D-alanine residue (L-D transpeptidase or carboxypeptidase II activities):



In most cases, the L-D peptidase activity is not sensitive to β-lactams. However, *Streptococcus faecium* membranes contain a L-D transpeptidase[45] whose physiological role is obscure, but which might synthesize atypical L-Lys-D-isoasparaginyl cross-links (as yet undetected) in the peptidoglycan of these bacteria. The sensitivity of this enzyme to penicillins (50% inhibition at 0.15 to 3 m*M*) is low, but some cephalosporins are better inhibitors (50% inhibition at 15 to 50 μ*M*). More recently, a penicillin-sensitive *N*-acetylglucosaminidase has been found in *Neisseria gonorrhoeae*.[46] This enzyme is 50% inhibited by concentrations of benzylpenicillin, ampicillin, methicillin, and mecillinam ranging from 10^{-7} to 10^{-6} *M*. In neither of these two cases has the mode of interaction between enzyme and β-lactams been established.

the bacterial cytoplasmic membranes: different enzymes appear to be involved in cell elongation, septation, and shape determination at least in Gram-negative bacteria. Moreover, the existence of "specialized" transpeptidases seems to be necessary to explain some paradoxical experimental observations: detailed examination of the peptidoglycan of *Neisseria gonorrhoeae* cells which had been killed or whose morphology had been grossly altered by penicillin revealed that peptidoglycan cross-linking was unaffected.[16] Similar results had been previously reported in the unstable L-forms of *Proteus mirabilis*.[15] As stated by Blundell and Perkins,[16] "This observation did not, of course, preclude some specialized subpopulation of peptidoglycan from having suffered decreased cross-linking . . . ". It is thus clear that different transpeptidases exist, but how these enzymes control the shape and the strength of the peptidoglycan network remains mysterious.

It seems that, in most cases, the transpeptidase, or one of several different transpeptidases, is the lethal target of penicillins. In Bacilli, a large proportion of the DD-carboxypeptidase can be inactivated without apparent damage to the cell.[47] In contrast, the *Gaffkya homari*[48,49] DD-carboxypeptidase appears to be both extremely physiologically important and the lethal target of the antibiotics (see Section IV.B.6).

III. SOLUBLE PENICILLIN-SENSITIVE AND RESISTANT CARBOXYPEPTIDASES AND TRANSPEPTIDASES

There is quite a difference between the demonstration of a penicillin-sensitive enzymatic activity and the isolation and study of a penicillin-sensitive enzyme. Penicillin-sensitive enzymatic reactions have often been studied using crude membrane preparations and, in retrospect, it has become evident that these preparations quite often contained several enzymes which could catalyze the same or very similar reactions. Little quantitative information can thus be extracted from these results. The isolation of the pure enzyme is a prerequisite for meaningful interpretation of the experimental results. This is, however, more easily said than done. As underlined above, the physiologically important enzymes are membrane bound and their solubilization most often requires the utilization of detergents. In some cases, a good yield can only be obtained with charged detergents, thus excluding the powerful techniques of ion exchange and electrophoresis in the subsequent purification procedures. Fortunately, covalent affinity chromatography, involving a β-lactam attached to an agarose matrix, has yielded excellent results. The presence of detergents in the final hopefully pure preparations constitutes, however, a major difficulty when crystallization of the protein is attempted, and the study of protein crystals by X-ray diffraction remains at present the best method to obtain a detailed view of the penicillin-binding site of an enzyme. Fortunately, some species of the Actinomycetes family came to the rescue of the biochemist: several strains of *Streptomyces* and one strain of *Actinomadura* were found to excrete during growth soluble enzymes which behaved as D-alanyl-D-alanine peptidases and exhibited various levels of sensitivity to β-lactam antibiotics.[50-52] Three of these enzymes were selected for detailed studies: the enzymes from *Actinomadura* R39 and *Streptomyces* R61 were highly penicillin-sensitive and catalyzed both carboxypeptidase and transpeptidase reactions.[53] The enzyme from *Streptomyces albus* G catalyzed carboxypeptidase and peptidoglycan hydrolyzing activities and was virtually insensitive to β-lactams. The reason for this insensitivity will become clear in Section III.D.3. More information is now available about these three enzymes than about any other D-alanyl-D-alanine peptidase. In particular, the R61 and the *albus* G enzymes have been crystallized;[54,55] the structure of the G enzyme is known at a resolution of 2.5 Å[56] and its complete amino acid sequence has been determined.[57] Although still less advanced, the studies on the R61 enzyme are expected to reach the same level in the near future

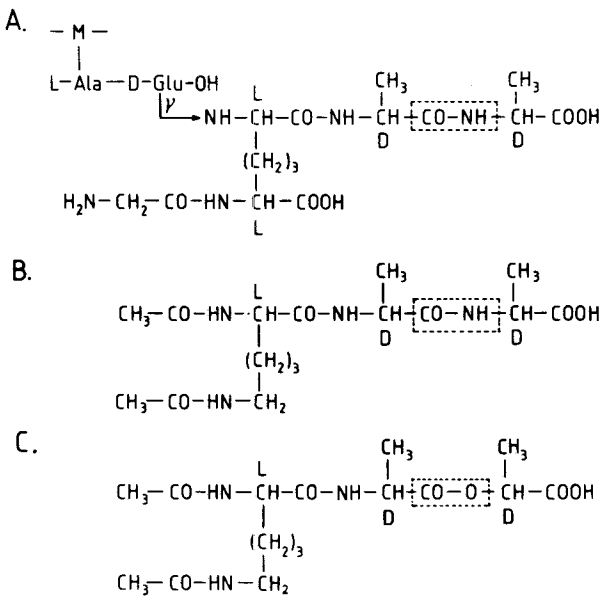


FIGURE 7. Comparison of the structures of the "natural" (A) peptide (*Streptomyces* sp.) and of the synthetic tripeptide (B) and depsipeptide (C).

and one can hope to obtain for the first time a detailed view of the penicillin-binding site of a penicillin-sensitive enzyme. Several membrane-bound peptidases have also been purified to protein homogeneity. In general, the results obtained with these enzymes were in good agreement with the models which had been deduced from the studies on the soluble enzymes. Moreover, the differences which were sometimes observed did not appear to depend upon the origin of the enzyme (soluble vs. membrane bound) or its ability to perform transpeptidation or not (strict carboxypeptidase vs. transpeptidase). These similarities and differences will be underlined in the present section, which mainly details results obtained with purified enzymes.

However, before starting this discussion, we will briefly describe the difficulties involved in the estimation of these enzymatic activities.

A. Estimation of the D-Alanyl-D-Alanine Peptidase Activities

1. The Substrates

The first experiments were performed using the [^{14}C]-labeled, natural precursors of peptidoglycan biosynthesis: UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-peptide.[37,38] These were obtained by growing bacteria in the presence of [^{14}C]D-alanine and sublethal concentrations of penicillin. In 1969 and 1970, Ghuysen et al.[50-52] compared these natural compounds with short, synthetic peptides as substrates for the soluble Actinomycetes enzymes. They demonstrated that some of these enzymes could hydrolyze tripeptides such as Ac_2-L-Lys-D-Ala-D-Ala (Figure 7) even better than the UDP-linked precursors. This tripeptide was an excellent substrate of the R61, R39, and *albus* G enzymes. It is, however, not a good substrate for many membrane-bound enzymes for which no alternative for the natural precursors presently exists.

More recently, Rasmussen and Strominger have utilized an ester derivative, the depsipeptide Ac_2-L-Lys-D-Ala-D-lactate[58] which appeared to be a better substrate for many enzymes than the usual D-alanine-terminated peptide.

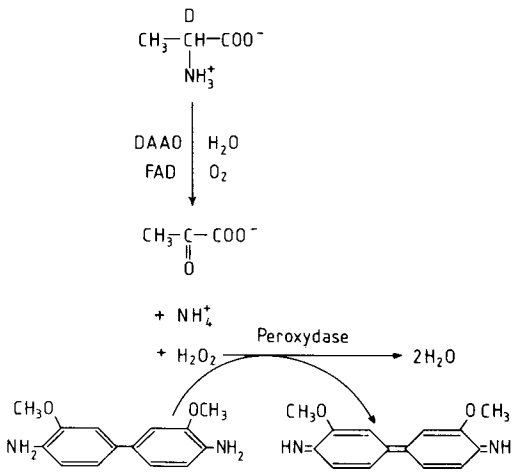


FIGURE 8. Principle of the enzymatic procedure for the estimation of D-alanine. DAAO = D-amino acid oxidase. (From Frère, J. M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M., and Perkins, H. R., *Methods Enzymol.*, 45, 610, 1976. With permission.)

2. The Assay: Carboxypeptidase Activity

With the [^{14}C]-labeled substrates, the reaction mixture was separated by paper chromatography and the intensities of the various spots were estimated either by autoradiography or by cutting the paper and determining the radioactivity of the strips by liquid scintillation.[37,38] The released D-alanine was also estimated by a chemical method, which involved transformation of the free alanine into the dinitrophenyl derivative, extraction, thin layer chromatography isolation, solubilization, and spectrophotometric determination of this derivative.[59] Both procedures were rather long, making it impossible to complete an assay in less than 4 to 6 hr.

The introduction of a rapid assay for the estimation of D-alanine was thus an important practical contribution. In the original procedure,[60] D-amino acid oxidase (DAAO) was used to transform D-alanine into pyruvate and hydrogen peroxide. In turn, this latter compound was used to oxidize *o*-dianisidine in the presence of horseradish peroxidase (Figure 8). Oxidized *o*-dianisidine exhibited a characteristic absorbance maximum at 460 nm. Since all the reagents could be added together to the D-alanine-containing sample, the assay time was reduced to about 10 min. It was important, however, to make sure that the D-amino acid oxidase preparation did not contain large amounts of catalase, which would compete with peroxidase for the hydrogen peroxide. A more sensitive procedure could be devised by replacing *o*-dianisidine by 2,2′-azino-di-[3-ethylbenzothiazoline sulfonate] (ABTS),[61] but in that case, the complete absence of catalase in the DAAO preparation became crucial, since the method involved two subsequent incubations, the first with DAAO and the second with peroxidase and ABTS.

The depsipeptide utilized by Rasmussen and Strominger was [^{14}C]-labeled in the acetyl residues. Quantification of the hydrolysis reaction was thus performed by determining the radioactivity after electrophoretic resolution of the reaction mixture. When cold substrate was utilized, D-lactate was used to reduce NAD^+ in the presence of D-lactate dehydrogenase and NADH was estimated either by measuring the absorbance at 340 nm or the fluorescence emission at 460 nm (excitation at 340 nm).[62]

3. The Assay: Transpeptidase Activity

Unfortunately, there is presently no easy assay for estimating the products of

Table 1
PHYSICOCHEMICAL, CHEMICAL, AND CATALYTIC CHARACTERISTICS OF THE SOLUBLE D-ALANYL-D-ALANINE PEPTIDASES[51,52,57,63,64,69,128]

	R39	R61	*albus* G
Molecular weight	53,000 ± 1,000	38,000 ± 1,000	22,076
Frictional ratio (f/f_0)	1.07	1.12	N.D.
Number of domains	N.D.	1	2
Number of polypeptide chains	1	1	1
Number of disulfide bridges	1 or 2	1 or 2	3
Isoelectric pH	<5	4.8	8.5
Basic residues (%)	6.3	8.1	10
Hydrophilic residues (%)	40	47	41
S-containing residues (%)	1	2.5	3.3
Maximum of UV spectrum	280 nm	280 nm	280 nm
$A^{1\%}_{1\ cm}$ at 280 nm	10	10	10
Maximum of fluorescence spectrum	339 nm	319 nm	350 nm
Digestion of chemotype I peptidoglycan	No	No	Yes
Catalysis of transpeptidation	Yes	Yes	No
Turnover number (min^{-1})			
On Ac_2-L-Lys-D-Ala-D-Ala	1,050	3300	180
On α-Ac-L-Lys-D-Ala-D-Ala	1,900	15	3
Km (Ac_2-L-Lys-D-Ala-D-Ala)	0.33 m*M*	14 m*M*	0.5 m*M*

transpeptidation reactions: a radioactive substrate is necessary and the reaction mixture must be resolved by paper electrophoresis or chromatography.[53] However, in the complete absence of parasitic or secondary carboxypeptidase activity, the transpeptidation can be monitored easily by estimating the release of free D-alanine, using the DAAO procedures described above.

B. The Exocellular D-Alanyl-D-Alanine Peptidases of *Streptomyces* R61 and *Actinomadura* R39: General Model of Interaction between β-Lactams and Penicillin-Sensitive Enzymes

1. Purification and Properties

These two enzymes were the first D-alanyl-D-alanine peptidases to be purified to protein homogeneity.[63,64] Table 1 summarizes their principal physicochemical and chemical characteristics. Relatively large amounts of these proteins have been obtained, but these preparations involved treatment of large (500 to 10,000 ℓ) culture volumes. The enzymes were first identified and isolated on the basis of their carboxypeptidase activities, but it was soon recognized that they could also catalyze transpeptidation reactions when supplied with a suitable aminated acceptor.[53,65-67] Under these conditions, they would concomitantly catalyze transfer and hydrolysis on a competitive basis. Careful studies of the activity profiles for donor and acceptor peptides[51,64-68] indicated a close correlation between the structures of the good synthetic substrates and those of the natural peptides involved in the cross-linking of peptidoglycan (see, e.g., Tables 7 and 8). Two typical examples can be rapidly discussed: a free ε amino group (i.e., at neutral pH, a positive charge) on the Lys residue of the donor tripeptide drastically reduced the activity of the R61 enzyme, but increased that of the R39 enzyme (Table 1), in excellent agreement with the absence of Z-bridge in the peptidoglycan of the *Actinomadura* strain (see Figure 4 for the reactions) and its presence (a Gly residue) in that of the *Streptomyces* strain. Similarly, peptides were utilized as acceptors by the R61 enzyme, while the R39 enzyme required the presence of a free carboxyl

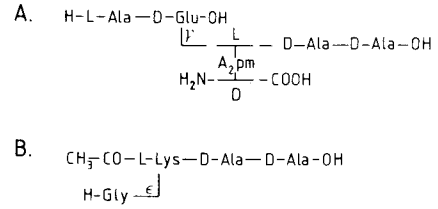


FIGURE 9. Donor-acceptor peptides which can be polymerized by the exocellular peptidases of R39 (A) and R61 (B).

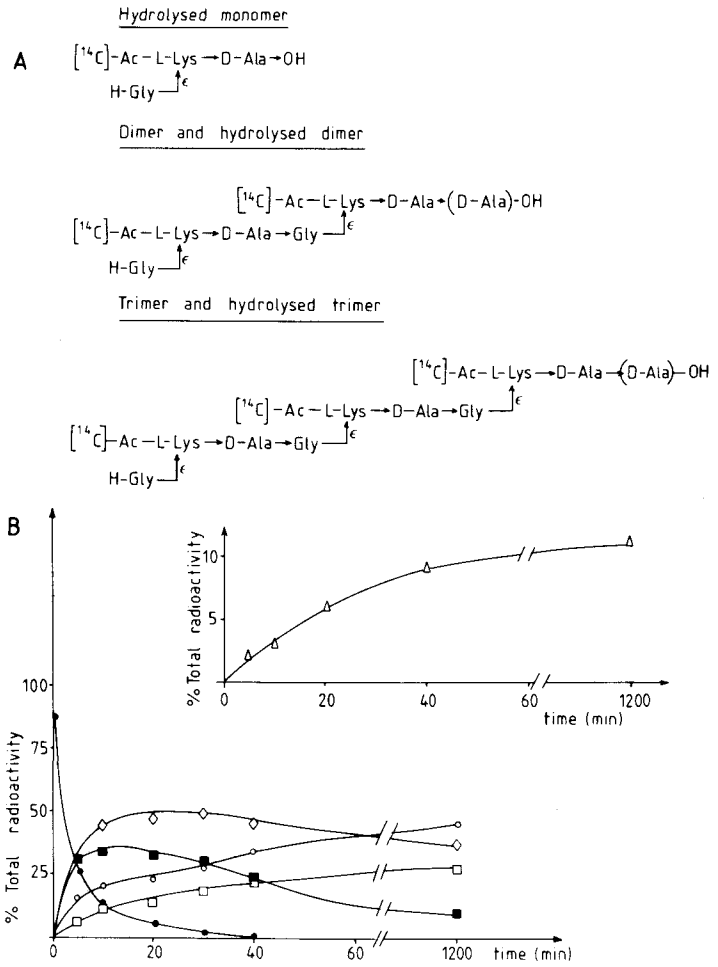


FIGURE 10. Structure of the products (A) and evolution of the reaction mixture (B) with time during the incubation of the R61 peptidase when the donor-acceptor peptide of Figure 9 is utilized. (•) Substrate. (△) Trimer. (■) Dimer. (□) Hydrolyzed dimer. (◇) Total dimers. (○) Hydrolyzed monomer.

in a position α to the accepting amino group (it only utilized D-amino acids), again in agreement with the structures of the type I peptidoglycan of *Actinomadura* and the type II peptidoglycan of *Streptomyces*. In addition, when supplied with bifunctional peptides (or donor-acceptor peptides, Figure 9), both enzymes could catalyze the formation of dimers and, sometimes, trimers (Figure 10).[31,69,70]

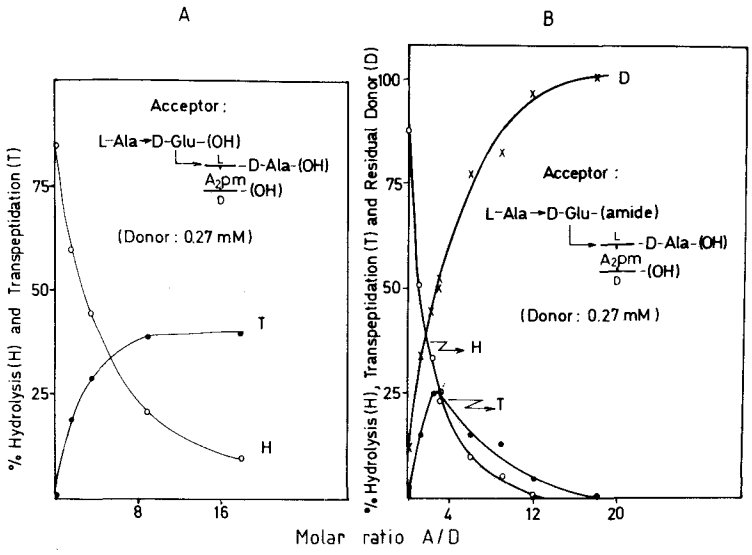


FIGURE 11. Influence of the amidation of the D-Glu residue of the tetrapeptide acceptor on the hydrolysis (H) and transpeptidation (T) reactions. (From Ghuysen, J. M., Leyh-Bouille, M., Campbell, J. N., Moreno, R., Frère, J. M., Duez, C., Nieto, M., and Perkins, H. R., *Biochemistry*, 12, 1243, 1973. With permission.)

2. Factors which Influence the Ratio of Transpeptidation to Hydrolysis[66,67]

As already mentioned, the ratio of transpeptidation to hydrolysis (T/H ratio) strongly depended upon the structure of the acceptor substrate. The concentration of the acceptor was also a determining factor. With simple D-amino acids and glycine, the T/H ratio was strictly proportional to the acceptor concentration, an observation in agreement with all simple bimolecular reaction models[71] (sequential or ping-pong). With substrates which more closely resembled the physiological acceptor (e.g., Gly-L-Ala for the R61 enzyme), substrate inhibition was observed at high acceptor concentrations, and the T/H ratio exhibited a hyperbolic dependence upon the acceptor concentration. These results indicated the possible presence of a second, inhibitory binding site for the acceptor substrate. This site might be involved in the control of the transpeptidation reaction. An extreme case of specific effect has been recorded with the R39 enzyme: amidation of the α-carboxyl of the D-Glu residue of the natural tetrapeptide transformed a "normal" acceptor (T/H proportional to acceptor concentration) into an inhibitory acceptor. At high concentrations of the Glu-amidated peptide, complete inhibition of both transpeptidation and hydrolysis was observed (Figure 11). Moreover, some acceptors had little or no effect on the total (T + H) reaction, while many acceptors similar to the physiological one influenced this total reaction. Both transpeptidation and hydrolysis by the R61 enzyme decreased in the presence of high ionic strength, but the T/H ratio showed little variation. On the contrary, pH had a dramatic effect on the transpeptidation reaction. With the R61 enzyme, in the absence of acceptor, hydrolysis remained virtually constant between pH 5 and 9. When an acceptor was added, however, transpeptidase activity sharply increased from pH 6 to 8, while the hydrolysis decreased proportionately, the total remaining constant. A higher transpeptidase activity at high pH would be expected, since the amino group of the acceptor must be in the base form to perform a nucleophilic attack on the carbonyl of the donor substrate (or of the acyl enzyme intermediate, see below). Experiments were performed with two acceptors whose amino groups exhibited rather different pK:

glycyl-L-alanine (pK ≅ 8.15) and *meso*-diaminopimelic acid (pK > 9.0). The curves of T vs. pH were similar and presented an inflexion point near pH 7.0. At that pH, both free acceptors were largely protonated and the observed value of 7 might therefore be assigned to a specific enzyme-substrate complex.

With the R39 enzyme, an increase in ionic strength not only favored the transpeptidation reaction, but also increased the optimum pH for hydrolysis from 6 to 8. Transpeptidation became important only at pH > 7.5. At pH 9, the yield of transpeptidation was still increasing, but experiments were not performed above this latter pH. With both enzymes, an important increase in the T/H ratio could also be obtained by partially replacing water by ethylene glycol or glycerol in the incubation mixture.

Finally, with the R61 enzyme, the T/H ratio decreased at high donor concentrations, an observation which was not explained by any of the simple kinetic models where the donor first binds to the enzyme.[71] However, Yocum et al. covalently labeled the protein after incubation with [^{14}C]Ac_2-L-Lys-D-Ala-D-lactate,[72] thus suggesting an acylation of the serine residue which also reacted with β-lactam antibiotics (see Section III.B.5.e). This observation implied a ping-pong mechanism. It is, as yet, not possible to decide whether such a mechanism is compatible with a preliminary binding of the acceptor in the transpeptidation reaction.

3. Irreversibility of the Transpeptidation Reaction[66]

When the in vivo transpeptidation reaction resulted in the attachment of a new peptide unit to preexisting peptidoglycan, the irreversibility could be explained by the insolubility of the polymer or by the diffusing away of free D-alanine. This was not the case when the experiment was performed in vitro with soluble peptides. Surprisingly, with the R61 enzyme, the transpeptidation reaction was found to be irreversible: no Ac_2-L-Lys-D-Ala-D-Ala could be detected after incubation of Ac_2-L-Lys-D-Ala-Gly-L-Ala with D-Ala in the presence of the enzyme. A tenfold increase in the concentration of enzyme did not alter that situation, but the formation of Ac_2-L-Lys-D-Ala was then observed, the rate of that reaction being independent of the presence of D-alanine. The usual specificity profile of the enzyme made it unlikely that the two C-terminal residues were hydrolyzed sequentially. That this low endopeptidase activity was not due to a contaminating enzyme was established by showing that the endopeptidase activity also disappeared in the presence of penicillin.[73] It was thus likely that the R61 DD-peptidase was also an inefficient but true endopeptidase. It did not, however, hydrolyze the *Streptomyces* peptidoglycan.

The meaning of the apparent irreversibility remained mysterious. One would expect a transpeptidation to be a reversible reaction, since the replacement of a peptide bond by another one should not involve a large variation in free energy. One could argue that the pK of the amino group of free D-alanine is higher than that of a peptide and that less of the free amine is available as a nucleophilic reagent. However, the yield of the transpeptidation reaction did not seem to be directly dependent upon the pK of the acceptor (see above). An alternative explanation would be that a D-alanyl-D-alanine C-terminus might have a particularly high free energy, but there is no theoretical ground on which such a hypothesis could rely.

4. Physiological Role of the Exocellular DD-Transpeptidases-Carboxypeptidases

For both the R61 and R39 strains, it is very unlikely that the exocellular enzymes are the killing targets of β-lactam antibiotics. The sensitivity profiles of the enzymes to about 12 antibiotics were not in good correlation with that of the strain itself.[74] In strain R61, the killing target of penicillin seemed to be a membrane-bound transpeptidase exhibiting a molecular weight of about 26,000 as determined by SDS gel electrophoresis (see Section IV.A.1). Unless this technique was, in this particular case, subject to gross artefacts, this result also ruled out the possibility that the excreted enzyme

(MW: 38,000) was a solubilized form of the membrane target. We do not have, so far, a rational explanation for the synthesis and excretion of these enzymes by Actinomycetes strains. However, they have proved to be excellent models for studying both the transpeptidation reaction and the interaction with β-lactam antibiotics. It will be seen that most of the mechanisms which were elucidated resulting from studies with the soluble enzymes were found to be valid for the membrane-bound lethal targets of penicillins and cephalosporins.

5. Interaction with Penicillins

a. Inactivation vs. Competitive Inhibition

Until 1974, the situation was rather confused: the original hypothesis was based on experimental data indicating an irreversible inactivation of the enzyme.[36] This was in agreement with the rather stable fixation of penicillin to cells observed in vivo.[75-77] However, other results were soon obtained, involving a competitive[51,52,78-80] or noncompetitive[52,81-83] interaction, when the data were analyzed on the basis of a reversible model. It was even thought, for some time, that depending upon the enzyme, a reversible or irreversible interaction could prevail.[84] This explanation was, however, ruled out when it was found that the R61 enzyme could exhibit both behaviors, depending upon the technique which was used to analyze the interaction;[85] after reacting the enzyme with penicillin, a rather stable complex could be isolated, but after incubation of ternary substrate-enzyme-inactivator mixtures, Lineweaver-Burk plots indicated a competitive inhibition.

It is, however, important to realize that, in these experiments, no demonstration was made that steady-state conditions prevailed in the presence of the β-lactam. The Lineweaver-Burk plots were thus constructed using product concentrations rather than initial velocities, a simplification which turned out to be both illicit and misleading. The same apparently paradoxical phenomenon was observed[79,80] with the *Bacillus subtilis* and *Bacillus stearothermophilus* DD-carboxypeptidases (see Section IV.B.3.d).

After incubation of the R61 enzyme in the presence of an excess of [^{14}C]benzylpenicillin, the labeled complex was isolated at 4°C by gel filtration and the molar ratio of bound penicillin to enzyme was 0.98:1. When the enzymatically inactive complex was maintained at 37°C, a first-order release of a radioactive degradation product of penicillin was observed, accompanied by a concomitant return of the enzymic activity. At 37°C, the half-life of the inactive complex was estimated at about 100 min on the basis of both criteria.

b. Kinetic Model of Interaction

Inactivation of the R61 enzyme by penicillin was accompanied by a decrease in the intensity of the fluorescence of the enzyme.[86] Stopped-flow studies[87] indicated that the formation of the R61 enzyme-benzylpenicillin complex was a two-step phenomenon, involving the rapid formation of a reversible complex (EI) followed by its irreversible transformation into the complex which could be isolated at low temperature. The overall mechanism of interaction could thus be represented by a three-step model (Scheme 1):

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P(s)^*$$

Scheme 1. Model for the interaction between enzyme (E) and β-lactam inactivator (I). The first reversible step is characterized by the dissociation constant of EI (K); k_2 and k_3 are first-order rate constants for the second and third irreversible steps.

* In the early publications, the first-order rate constants for the second and third steps were called k_3 and k_4, respectively. After 1980, we changed these appellations to k_{+2} and k_{+3}, respectively, to comply with the generally accepted nomenclature. Since no evidence was ever found for any of these steps to be reversible, we usually utilize the symbols k_2 and k_3.

The validity of the model was confirmed for the interaction between the same enzyme and other β-lactams and for the interaction between the R39 enzyme and several β-lactams.[88] The degradation product(s) [P(s)] were not always the expected penicilloic acid derivatives.[85,89] This point will be discussed in Section III.B.5.f.

On the basis of this model, the values of the three constants were measured for numerous β-lactams (Table 2). In many cases and for technical reasons, the individual values of k_2 and K could not be measured, but this was of little importance since it will be seen that the important parameter was the second-order rate constant k_2/K, which was determined in all studies.

For a β-lactam to be an efficient inactivator, the values of K and k_3 should be low, that of k_2 high. However, the measured values of K seldom fulfilled the first condition. This rather poor recognition of the antibiotic molecule was paralleled by the rather high Km values observed in the interaction of the enzymes with their peptide donor substrates. The efficiency of the inactivation was generally due to a high value of k_2: the value of 180 s^{-1} (at 25°C) recorded in the interaction between benzylpenicillin and the R61 enzyme was of the order of magnitude of a good catalytic turnover number; the enzyme thus appeared to catalyze its own inactivation. In many cases, rapid inactivation was observed for inactivator concentrations much lower than K and the second-order rate constant k_2/K became the important parameter governing the efficiency of the β-lactam. The values of k_3 were generally low, very seldom above 1×10^{-3} s^{-1} and it became rapidly apparent that, but for some exceptional cases, the third step would have little influence on the overall efficiency of the inactivator. It is, however, useful to remember that this third step represented a rather unexpected, possibly new type of bacterial resistance to a β-lactam antibiotic: the destruction of the antibiotic by the target enzyme itself.

The rate of inactivation of an enzyme could easily be deduced from the model (Equation 1):

$$\frac{[EI^*]}{E_o} = \frac{k_a\,[1 - e^{-(k_3 + k_a)t}]}{k_3 + k_a} \tag{1}$$

where

$$k_a = \frac{k_2[I]}{K + [I]} \tag{2}$$

and E_o was the total concentration of enzyme, i.e., [E] + [EI] + [EI*]. When t was much larger than $1/(k_3 + k_a)$, a steady state was reached. At the steady state:

$$\left(\frac{[EI^*]}{E_o}\right)_{ss} = \frac{k_a}{k_3 + k_a} \tag{3}$$

and the proportion of active enzyme was

$$\left(\frac{[E]}{E_o}\right)_{ss} = \frac{K}{[I] + K} \cdot \frac{k_3}{k_a + k_3} \tag{4}$$

However, due to the generally large values of K, the efficient antibiotics inactivated the enzymes at concentrations much lower than K. Under these conditions, Equations 2 and 4 simplified:

$$k_a = \frac{k_2}{K}[I] \tag{5}$$

$$\left(\frac{[E]}{E_o}\right)_{ss} = \frac{k_3}{k_3 + k_a} \tag{6}$$

Table 2 shows an extremely wide range of variations for the second-order rate constant k_2/K from 2.6×10^6 M^{-1} s^{-1} for the R39-nitrocefin to 0.06 M^{-1} s^{-1} for the R61-penicillanic acid interactions. The very large values which were often observed with the R39 enzyme can, however, be somewhat misleading; this is an extremely sensitive enzyme, the most sensitive one which has been described to date. In fact, an inactivator exhibiting a k_2/K value of 1000 M^{-1} s^{-1} can be considered a good one. As depicted in Table 3, a rather low concentration of such an inactivator (10 μM, i.e., about 4 $\mu gm\ell^{-1}$) induces a very rapid loss of enzyme activity and a low steady-state level of free enzyme. Under the same conditions, even a 10-fold lower value of k_2/K (100 M^{-1} s^{-1}) still induces a very rapid loss of activity ($t_{0.5}$ = 10 to 12 min). The table also shows that the third step becomes relevant only when k_3 is larger than 0.1 $k_2[I]/K$. In this latter case, a larger degree of inactivation can only be obtained by increasing the concentration of the inactivator and not by allowing the reaction to continue for a longer period. If one tries to extrapolate these data to the actual effect of the β-lactam on a growing population of bacteria, other factors must be taken into account: the proportion of active enzyme which is necessary to maintain the bacteria alive, and the rate of synthesis of new enzyme, which is correlated with the generation time of the population. No information is available about the first factor and, although a simple model can be derived to account for the second,[47] this discussion is probably beyond the scope of the present article.

c. Solution of the "Competitive Graphs Paradox"

After the various constants along the inactivation pathway were measured, it became possible to explain the apparent paradox of the competitive, linear Lineweaver-Burk plots obtained in those experiments where enzyme, inactivator, and substrate had been incubated together. On the basis of Scheme 2, Equation 7 could be derived,[94] which gave the amount of product formed after an incubation performed during t seconds.

$$\begin{array}{lcl} & E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P(s) \\ & + & \\ & S & \\ & \Downarrow & \\ k_{cat}, Km & ES & \\ & \downarrow k & \\ & E + P' & \end{array}$$

Scheme 2. Competitive model for the interaction between enzyme, inactivator, and substrate.

$$\frac{P}{P_M} = \frac{k_3}{b(k_3 + k_a')} + \frac{k_a'}{b(k_3 + k_a')^2 t}\left[1 - e^{-(k_a' + k_3)t}\right] \tag{7}$$

where P_M is the amount of product obtained in the absence of inactivator and under maximum velocity conditions, and

$$k_a' = \frac{k_2}{1 + \frac{K}{[I]}\left(1 + \frac{[S]}{Km}\right)} \tag{8}$$

$$b = 1 + \frac{Km}{[S]}\left(1 + \frac{[I]}{K}\right) \tag{9}$$

Table 2
KINETIC PARAMETERS FOR THE INTERACTIONS BETWEEN β-LACTAMS AND THE R61 AND R39 D-ALANYL-D-ALANINE PEPTIDASES (AT 37°C UNLESS OTHERWISE STATED)[74,87,88,90-93]

	R61				R39			
	K (m*M*)	k_2 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})	K (m*M*)	k_2 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})
Penams								
6-Aminopenicillanate	0.8	2×10^{-4}	0.25	$<6 \times 10^{-5}$	—	—	1,200	6×10^{-3}
Benzylpenicillin	13[a]	180[a]	13,000[a]	1.4×10^{-4}	—	—	300,000	3×10^{-6}
Phenoxymethylpenicillin	>1	>1	1,500	2.8×10^{-4}	—	—	[>70,000]	—
Carbenicillin	0.11	0.09	830	1.4×10^{-4}	—	—	6,000	5×10^{-6}
Ampicillin	7	0.8	110	1.4×10^{-4}	—	—	70,000[b]	4×10^{-6}
Methicillin	—	—	[15]	—	—	—	1,200[b]	3×10^{-5}
Oxacillin	—	—	[130]	—	—	—	[40,000]	—
Cloxacillin	—	—	[30]	—	—	—	[15,000]	—
Penicillanate	36	2×10^{-3}	6×10^{-2}	$<6 \times 10^{-5}$	—	—	14	6×10^{-3}
Quinacillin	No inactivation at 5 m*M*, 15 min				—	—	400	6×10^{-5}
Quinacillin sulfone	No inactivation at 5 m*M*, 15 min				—	—	10	6×10^{-5}
Mecillinam	—	—	0.22	$<2 \times 10^{-4}$	—	—	32	1×10^{-4}
β-Iodopenicillanate	4.0	3×10^{-3}	0.7	$<3 \times 10^{-5}$	—	—	7,600	$<10^{-5}$
Δ^3-Cephems								
7-Aminocephalosporanate	—	—	33	4×10^{-3}	—	—	200	1×10^{-6}
Cephalosporin C	>1	>1	1,500	1×10^{-6}	0.2[b]	12.5[b]	65,000[b]	0.3×10^{-6}
Cephalothin	—	—	[3,000]	—	—	—	[>70,000]	—
Cephaloglycine	0.4	9×10^{-3}	22	3×10^{-6}	—	—	70,000[b]	0.8×10^{-6}
Cephalexine	—	—	[4]	—	—	—	3,000[b]	2×10^{-6}
Nitrocefin	—	—	460[c]	3×10^{-4}	—	—	2.6×10^{6}[c]	1.5×10^{-6}
Benzyl-3-cephem[d]	—	—	80	5×10^{-6}	—	—	4,900	$<1 \times 10^{-7}$
RU 23345[e]	—	—	24	5×10^{-6}	—	—	2,300	$<2 \times 10^{-6}$
Cefotaxime	—	—	16	$<4 \times 10^{-6}$	—	—	2,600	$<2 \times 10^{-6}$
RU 25159[e]	—	—	23	$<2 \times 10^{-6}$	—	—	2,000	$<2 \times 10^{-6}$
HR 109[e]	—	—	1.5	4×10^{-6}	—	—	200	$<2 \times 10^{-6}$
HR 979[e]	—	—	1.5	1×10^{-6}	—	—	1,200	$<2 \times 10^{-6}$

Cefuroxime	—	—	350	4×10^{-6}	—	—	3,900	$<2 \times 10^{-6}$
RU 25238[e]	—	—	2	$<2 \times 10^{-6}$	—	—	2,300	$<2 \times 10^{-6}$
S 810592[e]	—	—	1.5	$<5 \times 10^{-6}$	—	—	450	$<5 \times 10^{-6}$
RU 25519[e]	—	—	1.5	$<2 \times 10^{-6}$	—	—	1,300	$<2 \times 10^{-6}$
Δ^2-Cephem								
Benzyl-2-cephem[d]	—	—	70	3×10^{-3}	—	—	52	$<2 \times 10^{-5}$
Cephamycine								
Cefoxitine	—	—	1,500	5×10^{-5}	—	—	7,000	$<3 \times 10^{-5}$
Penems								
Unsubstituted	—	—	670	0.05	—	—	1,750	8×10^{-3}
2-Methyl	—	—	800	0.01	—	—	5,400	8×10^{-3}
2-Phenyl	—	—	1,400	0.02	—	—	10,000	0.01
Monobactams								
Sulfazecin	0.032	1.3×10^{-3}	40	2×10^{-5}	—	—	6,500	$<1 \times 10^{-5}$
SQ 26180[f]	—	—	100	$<1 \times 10^{-5}$	—	—	2,500	$<1 \times 10^{-5}$
SQ 26324	—	—	70	2.5×10^{-5}	0.35×10^{-3}	2.9×10^{-3}	8,300	$<1 \times 10^{-5}$
Azthreonam	12	$<2 \times 10^{-5}$	<0.002	—	—	—	15	$<0.5 \times 10^{-5}$
Others								
Clavulanate	—	—	21	5×10^{-6}	—	—	32	$<10^{-6}$
N-Formimidoylthienamycin	—	—	1,000	7×10^{-6}	—	—	10,000	2×10^{-6}

Note: The structures of the various groups of compounds are given in Table 23. Values between square brackets were computed from ID_{50} values obtained in the absence of titration phenomena. —, not determined. Also from unpublished results (β-iodopenicillanate).

[a] At 25°C.
[b] At 20°C.
[c] At 10°C.
[d] Respectively, Δ^3- and Δ^2-desacetoxy-7-phenylacetamido cephalosporanate.
[e] For the complete structure.[93]
[f] For the complete structure.[92]

Table 3
STEADY-STATE LEVEL OF FREE ENZYME AND TIME NEEDED TO REACH 50% OF INACTIVATION ($t_{0.5}$) AT A 10 μM (I.E., ABOUT 4 $\mu g\ m\ell^{-1}$) CONCENTRATION OF INACTIVATOR, FOR VARIOUS VALUES OF k_2/K AND k_3 (K IS MUCH LARGER THAN 10 μM)

k_2/K ($M^{-1}\ s^{-1}$)	k_3 (s^{-1})	$([E]/E^\circ)_\infty$	$t_{0.5}$ (s)
1000	$\leqslant 10^{-5}$	$\leqslant 10^{-3}$	69
1000	10^{-4}	0.01	70
1000	10^{-3}	0.10	73
100	$\leqslant 10^{-6}$	$\leqslant 10^{-3}$	690
100	10^{-5}	0.01	696
100	10^{-4}	0.10	716
100	10^{-3}	0.50	—

Since the values of k_{cat} and Km had been measured in independent experiments, it became possible to calculate the amount of product formed under given conditions of substrate and β-lactam concentrations. These computed values were in good to excellent agreement with the observed ones. Surprisingly, at least to us, when the calculated values were used to build Lineweaver-Burk graphs (in the present case, P_M/P vs. $1/[S]$), the relationship was perfectly linear, without the slightest hint of curvature.

Since the equation of P_M/P vs. $1/[S]$ which could be deduced from Equation 7 was certainly not that of a straight line, one could wonder whether obtaining of apparently linear graphs was a coincidence, due to particular values of the various constants, or if it was a more general phenomenon. A computer-simulation study was performed[95] in which the values of K, k_2, and k_3 were widely varied. The results indicated that, provided that the P/P_M value was maintained above an experimentally realistic level ($P/P_M > 0.01$), all the curves obtained could very easily be confused with lines within the limits of experimental errors. Moreover, it was also found that a noncompetitive model, i.e., one in which the ternary ESI complex could be formed, would give rise to apparently competitive graphs if [I] was much smaller than K and if the dissociation constant of ESI ($\leftrightarrow$ ES + I) was of the same order of magnitude as K. Thus, the competitive Lineweaver-Burk graphs which had been obtained gave no indication that binding of penicillin and substrate occurred at the same enzyme site.

The existence of two distinct sites was proposed by Ghuysen and co-workers[96] on the basis of the noncompetitive kinetics observed with the R39 enzyme and of the high resistance of the *albus* G enzyme to β-lactams.[52,94,97] The first result was later shown to be due to a titration effect[89] and the second to be the consequence of the totally different, noncovalent catalytic mechanism utilized by the *albus* G enzyme.[98] As shown above, the study of the influence of β-lactams on the formation of product did not allow an unambiguous distinction between competitive and noncompetitive interaction. The hypothesis of an allosteric inactivation was finally falsified by results strongly suggesting that the same amino acid residue was acylated by both β-lactams and substrates[72] (see Section III.B.5.e).

*d. Effect of Substrates on the Formation of EI**

With the R39 enzyme, the Km for the usual donor substrate Ac_2-L-Lys-D-Ala-D-Ala was low enough to allow a study of the influence of this peptide on the formation of EI*.[88] A competitive interaction was found, but again the noncompetitive model could not be excluded with certainty. However, since the presence of a large concentra-

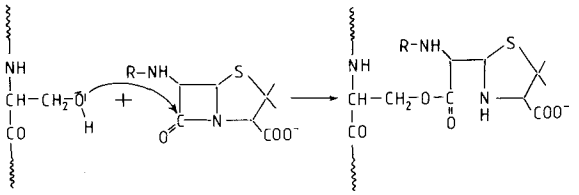


FIGURE 12. Penicilloylation of the serine residue.

tion (10 m*M*) of D-alanine, a good acceptor had no influence on the formation of the complex, it became clear that the β-lactam was only interfering with binding of the donor substrate.

e. Binding Site of Penicillins and Donor Substrates

The [^{14}C]benzylpenicillin-R61 enzyme complex was heat-denatured and digested by pronase. Paper high-voltage electrophoresis separated two labeled peptides. Prolonged digestion increased the proportion of the faster-moving peptide. Both peptides were isolated and their amino acid composition was Val, Gly, Ser for the slower and Gly, Ser for the faster one.[99] Leucine amino peptidase also transformed the first peptide into the second, with the concomitant release of one valine residue. The sequence of the slow peptide was H-Val-Gly-Ser-OH. The same tripeptide was obtained by digestion of the denatured complex in the presence of thermolysin. The electrophoretic mobility of the peptide indicated the presence of one negative charge and, in consequence, the only possible attachment site for the penicillin moiety was the hydroxyl group of the serine residue. The exact structure of the native complex was not directly established, but evidence has accumulated, indicating a penicilloylation of the hydroxyl group (Figure 12);

1. As shown by deuterium fixation during the formation or the degradation of the R61 enzyme — penicillin V complex, the C_5-C_6 bond of the penicillin was intact in the EI* complex.[100]
2. The nuclear magnetic resonance spectrum of the denatured benzylpenicillin — R61 enzyme complex presented a proton resonance characteristic of α-methylpenicilloate.[101]
3. The same denatured complex and the tripeptide obtained by pronase digestion slowly released the penicillin moiety as penicilloic acid.[102]

When the same digestion technique was applied to the R39 enzyme-[^{14}C]benzylpenicillin complex, a heptapeptide was isolated and its sequence determined (H-Leu-Pro-Ala-Ser-Asn-Gly-Val-OH). Again, the hydroxyl group of the serine was the only possible attachment site for the penicilloyl moiety.[103]

It was more difficult to directly demonstrate the implication of the same serine in the catalytic activity of the R61 enzyme. Attempts to trap a covalent intermediate using the [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala donor substrate remained unsuccessful. An ester analog of this peptide [^{14}C]Ac_2-L-Lys-D-Ala-D-lactate was then synthesized by Rasmussen and Strominger[58] and, upon denaturation of a mixture of enzyme and depsipeptide, some label remained attached to the protein. A technique of partial proteolytic digestion of the trapped [^{14}C]-labeled intermediate, followed by SDS gel electrophoresis and fluorography, yielded a "proteolytic map" characteristic of the substrate binding site. Such maps were obtained with various proteolytic enzymes. They were very similar to those obtained by partial digestion of the [^{14}C]benzylpenicillin-labeled enzyme,[72] indicating that substrate and penicillin were binding to the same residue, or to residues

extremely close to each other. However, the first possibility appeared to be the more reasonable one. Although these experiments supplied a much clearer indication of the involvement of the same serine residue in substrate and β-lactam binding than did all the kinetic data, the final demonstration of this point will be supplied by X-ray crystallographic results, which are expected in the very near future. The same limited proteolysis-gel electrophoresis technique was utilized to demonstrate the binding of monobactams to the same residue.[104]

Finally, the implication of a positively charged residue has also been suggested by chemical modification studies. However, inactivation was obtained by Georgopapadakou et al.[105] using arginine-specific reagents and by Charlier and Frere using dinitrofluorobenzene, which would be more likely to react with the ε-amino group of a Lys residue.[106] Although the presence of a positive charge in the binding site is extremely likely, the amino acid residue remains to be clearly identified.

f. Spontaneous Degradation of the EI Complex*

Surprisingly, the spontaneous degradation of the [^{14}C]benzylpenicillin — R61 or R39 enzyme complexes, when performed at pH 7.0 to 7.5, did not yield the expected [^{14}C]benzylpenicilloate.[85,89] Purification of the product was made relatively easy by its abnormal retention on a Sephadex® G-25 column: at low ionic strength, its elution volume was slightly larger than the total volume of the column, a peculiar property it shared with benzylpenicillin itself, but not with benzylpenicilloate. Acid hydrolysis yielded [^{14}C]phenylacetic acid and glycine. The product cocrystallized with authentic phenylacetylglycine and its mass spectrum was identical to that of the same reference compound.[88,107] Similarly, degradation of the R61 — penicillin V complex yielded phenoxyacetylglycine.[107] When the complexes had been previously denatured, penicilloates were obtained. The enzymes could thus catalyze a very slow breaking of the C_5-C_6 bond of the penicilloyl moiety of the complexes. The fate of the right-hand part of the molecule was explored using penicillin V [^{14}C]-labeled on C_5, kindly given by Professor H. Vanderhaeghe (Katholieke Universiteit Leuven, Belgium). *N*-formylpenicillamine was clearly identified,[108] but on the basis of rate of appearance of the free sulphydryl group, it became clear that the latter compound was not the primary product of the reaction.[109] By performing pulse-chase experiments with D_2O during the degradation process, it was also demonstrated that fixation of a proton on C_6 and enzyme reactivation were concomitant events.[100] These observations led us to propose the reaction scheme depicted in Figure 13. To date, the identification of Z remains doubtful. The most likely candidate is the thiazoline, but the half-life of Z, as deduced from the rate of appearance of the free -SH of *N*-formylpenicillamine, was shorter than that of the authentic thiazoline under the same conditions but in the absence of enzyme. It is not impossible that the presence of the enzyme might influence the rate of hydrolysis of the five-membered ring.

The presence of other nucleophiles in the solution during the degradation of the [^{14}C]benzylpenicillin-R61 complex may influence the fate of the penicilloyl moiety.[110] Two distinct families of nucleophiles were found; the first family which comprised amino acids and peptides ($R\text{-}NH_2$) competed with water for the hydrolysis of the phenylacetylglycyl enzyme. Adducts of general structure phenylacetyl-Gly-NHR were formed and the velocity of enzyme activity recovery was influenced little or not at all. Interestingly, larger amounts of adducts were formed with compounds which were good acceptors in the transpeptidation reaction when the standard donor peptide was used. However, some phenylacetylglycyl-L-alanine was obtained in the presence of L-alanine, which was not an acceptor with the standard donor. Hydroxylamine, ethyleneglycol, and glycerol behaved as members of a second family which could perform nucleophilic attack on both the phenylacetylglycyl enzyme and the intact penicilloyl enzyme itself. Three distinct products were obtained, in each experiment, which were

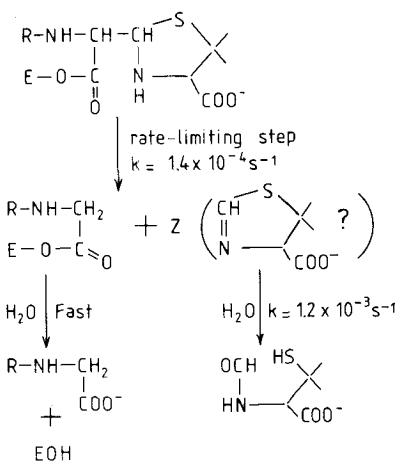


FIGURE 13. Proposed scheme for the degradation of the R61 enzyme-[^{14}C]benzylpenicillin complex.

tentatively identified as phenylacetylglycine and the phenylacetylglycyl and benzylpenicilloyl esters (or hydroxamates). When a 2:3 v/v glycerol-water mixture was used, the rate of enzyme activity recovery was increased by more than 60%. When a similar proportion of methanol was used, complex EI* was rapidly denatured, and only small amounts of phenylacetylglycine and α-methylpenicilloate were obtained.

All R61 and R39 enzyme-β-lactam complexes certainly do not decay through the fragmentation pathway. With cephalosporins, detailed studies have been made very difficult by the very long half-lives of most of the complexes. Indications have, however, been obtained that in some cases the product was the same as that obtained by the action of a β-lactamase on the cephalosporin.[73] With 7-aminocephalosporanic acid (7-ACA), the half-life of the complex formed with the R61 enzyme was quite short. Analysis of the reaction mixture after degradation of about 60 nmol of 7-ACA by the enzyme failed to reveal any trace of glycine (the product that should have arisen by fragmentation of the unsubstituted cephem) and at least 5 ninhydrin-positive spots were revealed after thin-layer chromatography or paper electrophoresis.[90] Similar results (no glycine, several ninhydrin positive spots) were obtained during the study of the R39-6-aminopenicillanic acid interaction.[90] Finally, we have recently performed a study of the influence of pH upon the value of k_3 using the R61-benzylpenicillin complex.[62] The value of k_3 remained relatively constant between pH 5 and 8, increased slightly at pH 9, and increased sharply at pH 10 (about 4 times the value at pH 7). At the highest pH, both phenylacetylglycine and penicilloate were released, the latter being the most abundant product. The results showed that the penicilloyl-enzyme complex was susceptible to nucleophilic attack by OH^- ions, but that, at low pH, the fragmentation pathway was faster, the direct attack of the penicilloyl-enzyme by OH^- becoming only detectable when the concentration of these ions increased above 10^{-5} *M*. [If $(k_3)_{pH\ 10} = 6 \times 10^{-4}\ s^{-1}$, then the second-order constant $k_{OH^-} = 5\ M^{-1}\ s^{-1}$.]

6. Sequence and Crystallography

The R61 enzyme is the only serine D-alanyl-D-alanine peptidase which has been succesfully crystallized. Several β-lactamases have also been crystallized,[111-113] but of all the penicillin-recognizing enzymes, the R61 enzyme is the one for which the X-ray diffraction studies are presently the most advanced. From a solution containing 14% polyethyleneglycol, it formed orthorhombic crystals (space group $P2_12_12_1$) which retained a low level of enzyme activity.[54] The unit cell dimensions were a = 5.11 nm, b =

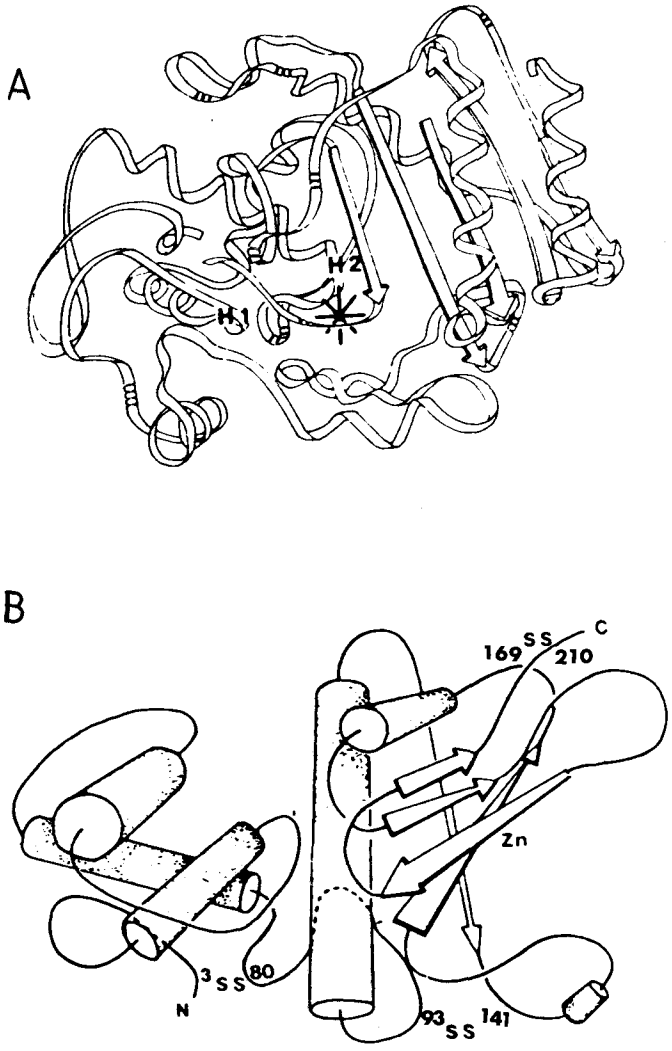


FIGURE 14. (A) Schematic drawing of the R61 serine enzyme. The star represents the binding site for cephalosporin C. (Reproduced with permission from Kelly, J. A., et al., *Science*, 218, 479, 1982. Copyright 1982 by the AAAS.) (B) Schematic drawing of the Zn^{++} enzyme. When compared to the sequence (Figure 15), the differences in numbering are due to small disagreements between the chemically established sequence and the interpretation of the electron density maps. (Reprinted by permission from *Nature (London)*, 299, 469, 1982. Copyright (C) 1982 Macmillan Journals Limited.)

6.74 nm, and c = 10.29 nm. The asymmetric unit contained one single molecule of protein. At a resolution of 2.8 Å, 8 helixes and one β-sheet, comprising 5 strands, were identified (Figure 14A). One common fixation site was visualized for *o*-iodophenylpenicillin, cephalosporin C, and 6,6-dichloro-4-desaza-2,2-didesmethyl penicillanic acid.[114,115] A peptide inhibitor of the enzyme (α-t Boc ε-TFA-L-Lys-D-Glu-D-Ala) could be tentatively located in the same area, but the Fourier difference map was not very satisfying.

The sequence of the *N*-terminus of the protein (29 residues) was determined,[102] as well as that of three large and two small cyanogen bromide fragments (out of a total

of 7[116]), adding up to a total of 140 residues. Unfortunately, none of these fragments contained the active serine residue, so our knowledge of the penicillin binding site is still limited to the tripeptide described above.* No homology was found between these peptides and (1) the published sequences of the N-terminal portion of the *Bacillus subtilis* D-alanyl-D-alanine carboxypeptidase,[117,118] (2) PBPs 3 and 5 of *Escherichia coli*,[119-121] and (3) serine β-lactamases of classes A and C.[122,123]

One can reasonably hope that both the complete sequence and a high resolution three-dimensional structure will become available in the near future. These results will shed light on a rather interesting, but confusing problem. It has been known for a rather long time that those side chains which promote good inactivation by a penicillin are not necessarily similar to those which promote a high catalytic efficiency toward the substrates; e.g., the phenylacetyl side chain conferred a high inactivating potency to a penicillin (benzylpenicillin), while phenylacetyl-D-alanyl-D alanine was a very poor substrate of the R61 enzyme and, conversely, the Ac_2-L-Lys side chain of the best substrate was 100-fold less favorable than the phenylacetyl group when attached to 6-aminopenicillanic acid.[73,87]

C. Exocellular and Lysozyme-Releasable DD-Carboxypeptidases-Transpeptidases of *Streptomyces* K15[124,125]

Fractionation of *Streptomyces* K15 culture filtrates on Sephadex® G-100 yielded two distinct penicillin-sensitive DD-carboxypeptidases-transpeptidases. The first one exhibited a molecular weight of 54,000, was very sensitive to benzylpenicillin, and was a rather poor transpeptidase. The second one (MW = 40,000) was less sensitive to benzylpenicillin and was a better transpeptidase (Table 4). This latter enzyme was also inhibited by antibodies prepared against the R61 exocellular enzyme. (This antiserum had no effect on the R39 enzyme.) A very similar protein (MW = 40,000) was released from the cells by lysozyme treatment and purified by immunoaffinity chromatography on agarose-coupled anti-R61 enzyme IgG. This latter preparation also contained a somewhat smaller protein (MW = 38,000) which could only be distinguished from the larger one by gel electrophoresis. On the basis of the amount of penicillin bound, the smaller protein represented about 25% of the mixture. These results indicated that the exocellular and lysozyme-released 40,000-MW proteins were probably identical and very similar to the R61 exocellular enzyme. Their relationship to the lysozyme-releasable 38,000-MW protein remained uncertain.

D. The Zn^{++} D-Alanyl-D-Alanine Peptidase of *Streptomyces albus* G

1. Substrate Specificity and Resistance to β-Lactams

This enzyme was, to our knowledge, unique in that it hydrolyzed the C-terminal D-alanine of D-alanyl-D- alanine-terminated peptides, but was very poorly inactivated by penicillins and cephalosporins.[68,97] The rate of formation of a rather stable adduct with [^{14}C]benzylpenicillin was only 10-fold higher than that reported for lysozyme or ribonuclease, and 10^6- to 10^7-fold lower than those observed with the R61 and R39 serine D-alanyl-D-alanine peptidases.[126,127] The *albus* G enzyme did not exhibit a high degree of specificity for the C-terminal amino acid: glycine or any residue in the D configuration was released with a reasonable efficiency.[68] In fact, it was first referred to as an "endopeptidase" because it was able to catalyze the lysis of some cell walls by hydrolyzing the peptide cross-link. Certainly, this was inappropriate since the hydrolyzed peptide bond was always in α position to a free carboxyl group. Interestingly, the enzyme was unable to lyse the peptidoglycan of the *Streptomyces* sp., which contains a D-Ala-Gly-L-(LL-A_2pm)-OH cross-link and, in consequence, could not serve as a true endopeptidase to provide new acceptor sites for the peptidoglycan during remodeling (growth or

* Note added in proof: a hexapeptide Val-Gly-Ser-Val-Thr-Lys has now been sequenced (see Figure 19).

Table 4
PROPERTIES OF EXOCELLULAR AND LYSOZYME-RELEASABLE DD-CARBOXYPEPTIDASES-TRANSEPTIDASES OF *STREPTOMYCES* K15[124,125]

		Carboxypeptidase activity[a]					Interaction with benzylpenicillin		
	MW	V μmol min^{-1} (mg protein)$^{-1}$	Km (m*M*)	T/H ratio[b]	Inhibition by anti-R61-antiserum	Degree of purification[c] (%)	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})	Released compound[d]
Exocellular 1	54,000	0.5	0.1	0.08	No	1.7	170,000	1.5×10^{-5}	Phenylacetylglycine
Exocellular 2	40,000	2	14	0.84	Yes	3.8	10,000	4.8×10^{-4}	Phenylacetylglycine
Lysozyme-releasable (mixture)	40,000 + 38,000	1.7	12	0.3	Yes	—	16,000	2.3×10^{-4}	—

[a] On Ac_2-L-Lys-D-Ala-D-Ala.
[b] Using α-Ac-ε-Gly-L-Lys-D-Ala-D-Ala as donor-acceptor substrate.
[c] Based on titration with [^{14}C]benzylpenicillin.
[d] The release of phenylacetylglycine was accompanied by enzymatic activity recovery.

Table 5
INTERACTION BETWEEN THE *ALBUS* G Zn^{++} D-ALANYL-D-ALANINE PEPTIDASE AND β-LACTAMS

	K (m*M*)	k_2 (s^{-1})	(k_2/K) ($M^{-1}s^{-1}$)	k_3	Ref.
6-Aminopenicillanate	—	—	0.01	—	90
Phenoxymethylpenicillin	150	8×10^{-4}	0.005	9×10^{-5} (T)	126
Benzylpenicillin	—	—	0.009	6×10^{-5} (H)	127
				$>1 \times 10^{-4}$ (T)	127
7-Aminocephalosporanate	0.2 (C)	$<3 \times 10^{-5}$	<0.15	—	90
Cephalosporin C	1.6 (NC)	1×10^{-4}	0.06	5×10^{-5} (T)	126, 129
				2.4×10^{-6} (H)	126, 129
Cephalothin	9.5 (NC)	5×10^{-4}	0.06	3×10^{-5} (T)	129
				5×10^{-6} (H)	129
Cephaloglycine	4.5 (C)	3×10^{-4}	0.07	3×10^{-5} (T)	129
				9×10^{-6} (H)	129
p-Iodo-7-β-phenylacetamido-cephalosporanate	3.5 (C)	6×10^{-4}	0.17	3×10^{-5} (T)	129
				1×10^{-6} (H)	129

Note: (C), competitive inhibition; (NC), noncompetitive inhibition; (H), in Hepes buffer, pH 8.0; (T), in 10 m*M* Tris-HCl buffer, pH 8, under these conditions, the product formed upon reactivation of the [^{14}C]benzylpenicillin-enzyme complex was the same as the adduct formed by incubation of penicillin alone in the presence of the buffer (probably penicilloyl-Tris), indicating a direct attack of the enzyme-linked penicillin by the nucleophilic buffer.

septation) or as an autolytic enzyme. Apart from the hypothetical limitation of the degree of cross-linking (due to the elimination of donor sites), this exocellular enzyme might be viewed as a weapon of *Streptomyces albus* G in the struggle for life since it can theoretically lyse the wall of many competitors such as Bacilli or *Actinomadura.* However, this hypothesis has not been confirmed. The inclusion of this enzyme in a review devoted to penicillin-sensitive enzymes might appear somewhat surprising. If the DD-carboxypeptidase activity is, however, more necessary to the survival of bacteria than is usually thought, the *albus* G enzyme represents a typical, if rare, example of the development of an intrinsically resistant enzyme.

2. Interaction with β-Lactams

a. The Low Values of k_2[126-29]

The purified enzyme[128] was partially inactivated by benzylpenicillin and penicillin V after a rather long incubation (2 hr or more) in the presence of very large (10 m*M* or more) concentrations of the antibiotic. Inactivation by some cephalosporins was a little more rapid, but still very inefficient (Table 5). However, the three-step model depicted by Scheme 1 also seemed to apply to this slow interaction. The high resistance of the *albus* G enzyme was due to very low values of k_2: 1, 5 and $8 \times 10^{-4} s^{-1}$ for cephalosporin C, cephalothin, and penicillin V, respectively.

b. β-Lactamase Activity[127]

Recovery of the activity, after removal of the excess of penicillin by gel filtration, was accompanied by the release of penicilloate, indicating that the enzyme behaved as an exceedingly poor β-lactamase. Respective turnover numbers of 7 and $8 \times 10^{-5} s^{-1}$ were calculated when benzyl- and phenoxymethylpenicillins were used as "substrates".

Isolation of a [^{14}C]benzylpenicillin-labeled complex was possible at 4°C. Inactivation of the enzyme was incomplete, but a molar ratio of 1.02 mol of bound benzylpenicillin per mole of inactivated enzyme was measured. Denaturation of the inactive,

labeled complex followed by proteolytic digestion with pronase did not yield any labeled peptide, the radioactivity being quantitatively recovered as benzylpenicilloate.

c. Steady-State Kinetics[126-129]

The low values of k_2 permitted the direct determination of K and of the type of inhibition by steady-state kinetic methods. Surprisingly, the inhibition was noncompetitive with cephalothin and cephalosporin C, and competitive with 7-aminocephalosporanic acid, cephaloglycine, and p-iodo-7-β-phenylacetamidocephalosporanic acid.

3. A Zn^{++} Enzyme[98]

Proton-induced X-ray emission experiments indicated the presence of one Zn^{++} ion per molecule of enzyme. Early experiments had shown that the activity was substantially inhibited by buffers which were capable of complexing divalent cations, and suppressed by 2 m*M* EDTA.[50] Surprisingly, the presence of 2 m*M* $MgCl_2$ appeared to be required for optimal activity and stability, while a similar concentration of $ZnCl_2$ was strongly inhibitory. After complete removal of the Zn^{++} in the presence of EDTA and isolation of the inactive apoenzyme by gel filtration, addition of a stoichiometric amount of Zn^{++} restored full activity. Again, an excess of Zn^{++}, amounting to a final concentration of free Zn^{++} larger than 0.1 m*M*, was inhibitory. Thus, the presence of $MgCl_2$ during all the purification steps of the enzyme only provided very low concentrations of Zn^{++}, an impurity that the enzyme was able to scavenge thanks to its very high affinity for that divalent cation.

These findings provided an explanation for the very low values of k_2 discussed above: if the enzyme was a Zn^{++} peptidase, comparable to carboxypeptidase A or thermolysin, a molecule of β-lactam, after binding to the active site it would not find a serine residue ready to be acylated. In fact, the nature of the amino acid residue responsible for the formation of the adduct which could be isolated remained unknown. The Zn^{++} ion, however, seemed to play a certain role in this reaction: formation of a similar adduct with the apoenzyme was at least 10 times slower than with the native enzyme and took place at a velocity comparable to that observed with lysozyme or insulin.

4. Sequence and Crystallography

a. General Features of the Molecule

Well-formed, large prismatic crystals were obtained from 12% polyethylene glycol[55] at pH 8.0, i.e., very close to the isoelectric pH of the protein. An experiment performed on a single, rather large crystal indicated that the protein retained 2 to 4% of the enzymatic activity of an equivalent amount of protein solution. Redissolution of the crystal yielded a preparation exhibiting 96 ± 5% of the specific activity of the initial one. X-ray analysis of the crystals indicated a P_{21} space group with unit cell dimensions: a = 5.11 nm, b = 4.97 nm, c = 3.87 nm, and $\beta = 1.6°$. There was one molecule per asymmetric unit.

While a 0.25-nm resolution map was constructed and interpreted,[130,131] the primary structure was established by classical cyanogen bromide and proteolytic digestion methods, followed by automatic or manual sequencing.[57] The complete sequence is given in Figure 15. Except for a few residues, situated outside the catalytic cavity, the two techniques (chemical sequencing and crystallography) were in excellent agreement. Several important features of the molecule could thus be determined (Figure 14B). The enzyme molecule had an overall dimension of 4.8 × 3.4 × 2.8 nm and was composed of two globular domains. The N-terminal, smaller domain comprised residues 1 to 84, the first disulfide bridge Cys 3-80, and 3 α-helices, containing 43% of the residues. The larger, C-terminal domain contained residues 85 to 212, 2 disulfide bridges

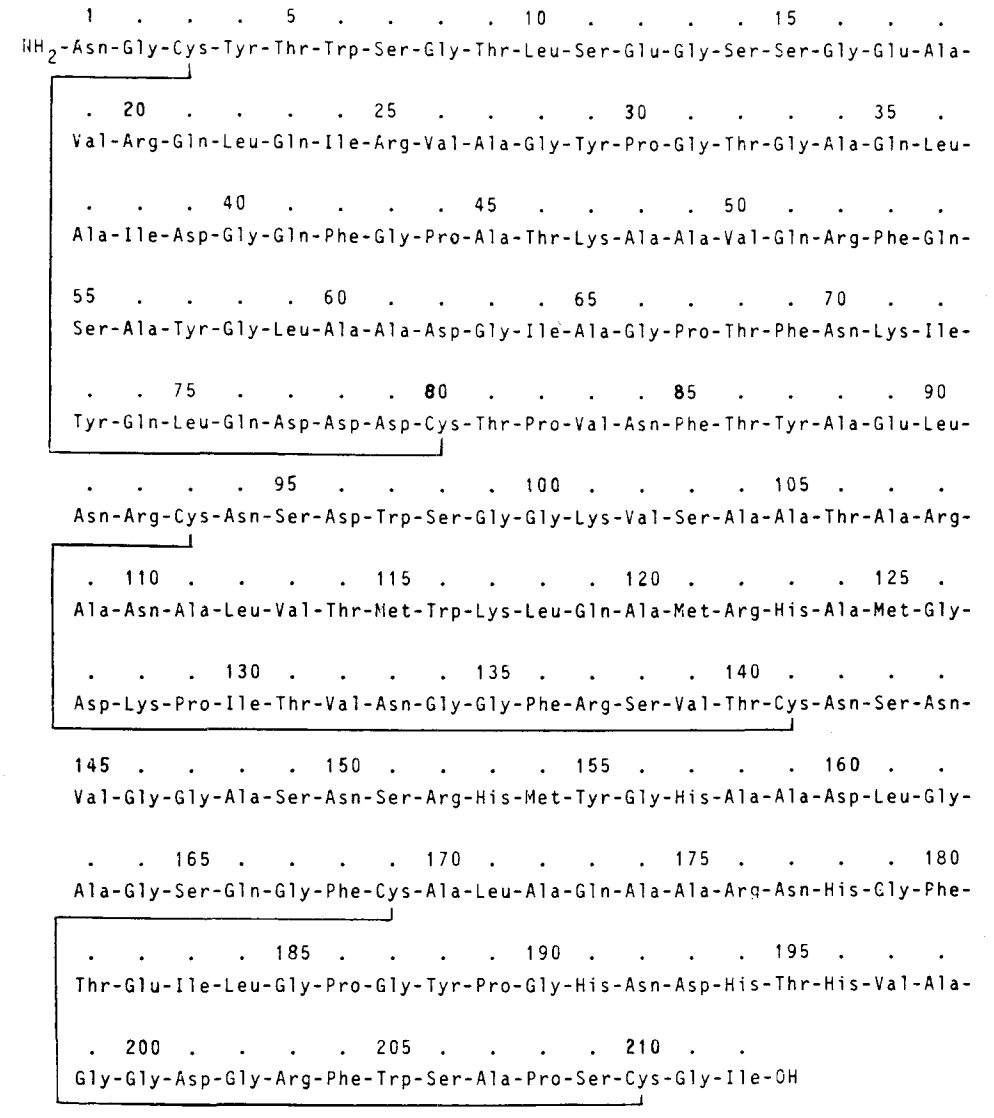


FIGURE 15. Complete sequence of the Zn^{++} D-alanyl-D-alanine peptidase as determined by chemical methods. On the electron density maps derived from the crystallographic data, an additional Trp residue was found between Arg 52 and Phe 53, and residues Asn 84 and Ser 209 were not visualized.

(Cys 93-141 and Cys 169-210), 3 α-helices (34%), and 5 β-strands (17%). (The numbering of the residues is that obtained by chemical sequencing.) The Zn^{++} cofactor was bound inside a wide-open cavity in the large domain. In that, this D-alanyl-D-alanine peptidase seemed rather exceptional since in multidomain enzymes, the catalytic centers are usually located at the junction of different domains. The Zn^{++} ligands of the protein were identified as His 152, His 193, and His 196.

b. Binding of Peptide and β-Lactam Inhibitors[130,131]

The dipeptide acetyl-D-Ala-D-Glu, a competitive inhibitor (K_i = 0.2 m*M*[132]), was found to bind in the cavity near the Zn^{++} ion. With the cephalosporins which behaved as competitive inhibitors, the Fourier difference maps at a resolution of 0.45 nm presented a positive density close to the Zn^{++} ion in the catalytic cavity, but the maps were

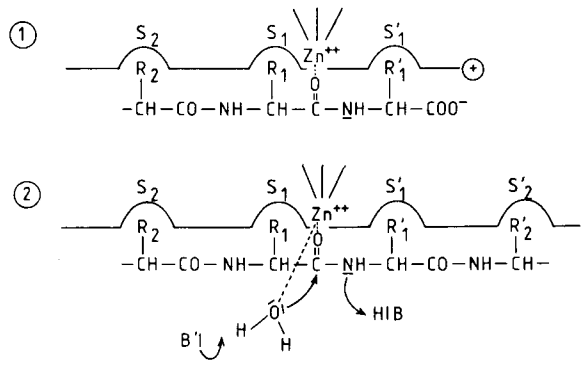


FIGURE 16. Hypothetical mechanism of Zn^{++} peptidases. The Zn^{++} ion binds the carbonyl oxygen and polarizes the C=O of the peptide bond to be hydrolyzed. Subsites S_2, S_1, S'_1, and S'_2 are specific for the side chains preceding (S_2,S_1) or following (S'_1,S'_2) that peptide bond. In the case of carboxypeptidase A (1) an arginine side chain (+ = Arg) interacts with the free carboxylate of the C-terminal amino acid. No such positive charge is found in thermolysin (2) which is an endopeptidase. The peptide bond is broken by the combined actions of a proton donor (Tyr in the case of carboxypeptidase A; His in the case of thermolysin) and a water molecule (Zn^{++}-bound ?) which performs a nucleophilic attack on the carbonyl carbon, while being activated by a base (B'|, Glu 270 in the case of carboxypeptidase A; Glu 143 in the case of thermolysin).

rather noisy and this finding requires confirmation under better experimental conditions. Unfortunately, those cephalosporins which behaved as noncompetitive inhibitors could not be located by Fourier difference maps since they completely shattered the crystals. In parallel to this, small-angle X-ray scattering in solution, which for the free enzyme yielded molecular parameters in good agreement with those calculated from the crystal data, did show that the presence of these noncompetitive inhibitors drastically altered the scattering behavior of the protein.[133] These results were interpreted as indicating either large conformational changes in the protein or, more likely, aggregation of the enzyme molecules, this latter hypothesis being confirmed by fluorescence polarization measurements.

c. Comparison with the Zn^{++} β-Lactamase

A Zn^{++}-containing β-lactamase has been described and the study of its mechanism of action and primary structure has been undertaken.[134] Since the Zn^{++} D-alanyl-D-alanine carboxypeptidase exhibited a weak β-lactamase activity, we compared the primary structure of the carboxypeptidase with a partial sequence of the β-lactamase.[135] No homology was found.

5. Hypothesis for a Mechanism of Action

The present knowledge of the mechanism of action of Zn^{++}-peptidases is mainly based on data obtained for carboxypeptidases A and B and thermolysin.[136-138] The main features of this mechanism are summarized in Figure 16. In addition to the subsites, which should be complementary of the various side chains of the residues, one could in all three cases identify a proton donor, and a basic group, probably responsible for the activation of a water molecule. These hypotheses could be deduced from pH activity curves and from detailed studies of crystals of enzyme-competitive inhibitor complexes. Electron density maps of such complexes are not yet available for the

G enzyme. However, a detailed atomic model, displaying all the side chains of the residues, has now been built for the native enzyme.[56] Peptide models could be fitted inside the catalytic cavity and, assuming that no major conformational change took place upon binding of the substrate, the following observations were made:

1. If the C=O of the peptide bond to be broken was positioned so that it became a fourth ligand of the Zn^{++}, a good salt bridge could also be formed between the positive charge of Arg 136 and the C-terminal carboxylate.
2. Subsite S_1' was a very large cleft, explaining the ability of the enzyme to accommodate C-terminal D-amino acids with very long side chains (the misnamed "endopeptidase" activity). Only side chains in the D-configuration could fit into the cleft.
3. Subsite S_1 was relatively small, allowing no larger side chain than the methyl group of D-alanine. The side chain of a L-alanine residue would collide with the Zn^{++} ligands. However, this did not explain why a glycine residue in that position caused such a large drop in activity.
4. There was certainly enough room to accommodate the large side chain of lysine further away from the Zn^{++} ion. It was difficult with the present data to identify subsite S_2. In particular, why a free ε-amino group induced a large decrease of activity, and how the catalytic cavity could accommodate a rather extended peptide chain beyond the Lys residue, remained to be explained.
5. His 190 was well located to play the role of a proton donor.
6. Ser 151 was well situated to form a hydrogen bond with a water molecule (or a hydroxyl ion) which could, at the same time, provide a fifth ligand to the Zn^{++} ion.

When k_{cat} and k_{cat}/Km were measured between pH 4.5 and 9.2,[73] curves were obtained exhibiting a plateau above pH 8.0 and dropping to low values at low pH, with inflection points at pH 5.9 to 6.0 (k_{cat}) and pH 6.7 (k_{cat}/Km). Although these pHs could be those of an imidazole group, these curves could only be reconciled with a proton-donating histidine residue by assuming that a second group, somehow involved in the nucleophilic attack of the C=O, had a pH of 9.5 or higher. This could be the pH of a Zn^{++}-bound water molecule or of an as yet undetected base which would be brought into the right position by a conformational change of the enzyme after binding of the substrate. Unfortunately, the instability of the enzyme at high pH did not allow the determination of the kinetic parameters above pH 9.5. As stated above, these considerations are only working hypotheses. The obtaining and detailed analyses of electron maps derived for peptide inhibitor-enzyme complexes should shed more light on the mechanism.

If one tried to fit penicillin and cephalosporin molecular models into the catalytic site, one found it impossible to align both the C=O of the β-lactam bond with the Zn^{++} ion and the free carboxylate with the positive charge of Arg 136. These two functional groups of cephalosporins could, however, supply two ligands to the Zn^{++}, explaining the lower values of K observed with these molecules. No indications on the nature of a group susceptible to form a covalent adduct with these molecules was found. Thus, although the study of this D-alanyl-D-alanine peptidase is now well advanced, many more experiments remain to be performed before clear models for its enzymatic activity and its (poor) interaction with β-lactams can be proposed.

6. Other Zn^{++}-Activated DD-Carboxypeptidases

A D-alanyl-D-alanine carboxypeptidase extracted from *Bacillus subtilis* membranes required 15 m*M* Zn^{++} for maximum activity.[139] Reaction of this enzyme with

[^{14}C]benzylpenicillin, however, resulted in the penicilloylation of a serine side chain (see Section IV.B.3.f). The requirement for Zn^{++} ions was thus not clearly understood, but it has been suggested that the divalent cation (the Zn^{++} could be replaced by Mn^{++}, Co^{++}, and Ni^{++}) might interact with UDP-*N*-acetylmuramyl pentapeptide, which was used as a substrate. In addition, no Zn^{++} requirement was mentioned in later publications where smaller substrates such as Ac_2-L-Lys-D-Ala-D-Ala or Ac_2-L-Lys-D-Ala-D-lactate were used.[58]

The behavior of the membrane-bound DD-carboxypeptidase of *Bacillus coagulans* was even more surprising;[140] the enzyme was solubilized by Genapol X-100 and purified by affinity chromatography on agarose-linked ampicillin. Since 0.8 *M* neutral hydroxylamine was needed to elute it, it could safely be assumed that the enzyme was covalently bound to the column. However, while enzyme activity was considerably increased by Pb^{++}, Zn^{++}, and inhibited by phosphate and citrate buffers, its stability was enhanced in the presence of 1 m*M* EDTA. Since the nucleotide precursor was also used as a substrate in these experiments, a more active metal-substrate complex could also be possible. Unfortunately, the authors did not mention whether similar results were obtained with the free pentapeptide, which was also a rather good substrate.

Finally, the *Salmonella typhimurium* membrane-bound DD-carboxypeptidase activity was stimulated by 25 m*M* $CaCl_2$, $ZnCl_2$, or $MgCl_2$.[141] The major carboxypeptidase, however, easily formed a covalent adduct with benzylpenicillin (see Section IV.A.2).

IV. MEMBRANE-BOUND PENICILLIN-BINDING PROTEINS AS PSEs

In 1972, Suginaka et al. demonstrated[142,143] that the membranes of *Bacillus subtilis* contained at least five distinct polypeptides which could covalently bind penicillin [penicillin-binding proteins (PBP)]. They were separated by gel electrophoresis in the presence of sodium dodecylsulfate (SDS) and numbered in order of increasing electrophoretic mobility (i.e., of decreasing molecular weight of the polypeptides). These reports were followed by similar findings in all eubacterial strains which were examined (but not in the wall-less mycoplasmatales). The studies were, however, impeded by the long and tedious experimental procedures that they required: the labeling was made with [^{14}C]benzylpenicillin, the gels were sliced, and the radioactivity of the slices determined by liquid scintillation, or the dried gel was submitted to autoradiography.

In 1975, Spratt and Pardee[144] proposed the utilization of flat-bed (slab) gel electrophoresis for the separation and of the fluorographic method of Bonner and Laskey[145] for the detection of the labeled protein. These improvements made the technique highly popular. The number of journal articles and of slides at meetings displaying PBP patterns started to increase exponentially. Some PBP had previously been described as membrane-bound enzymes and, conversely, enzymatic activities could later be attributed to a certain number of PBP. In some cases, labeling with [^{14}C]penicillin followed by gel electrophoresis revealed the presence of two PBPs in enzyme preparations which had been believed to be homogeneous. In the following pages, we will mainly describe the PBPs which have been clearly associated with an enzymatic activity and put the emphasis on their catalytic and structural properties. PBPs whose enzymatic properties are not more or less well understood will be discussed in Section V.

When one attempts to summarize the abundant data which have been collected about membrane-bound penicillin-sensitive enzymes, one can only regret that the sensitivity of the enzymes was too often only chracterized by an ID_{50} or a B_{50} value. The B_{50} value is that concentration of radioactive β-lactam which saturates the protein by 50% as estimated by SDS gel electrophoresis and fluorography. This method only measures complex EI* since EI is most probably dissociated upon denaturation. It is clear from Equation 1 that the B_{50} values strongly depend upon the time of contact.

The ID_{50} value is the concentration of β-lactam which, when added to enzyme and substrate, reduces the formation of product by 50%. Scheme 2 and Equation 7 show that this value depends upon Km, K, k_2, k_3, substrate concentration, and the duration of the incubation. It is not surprising that ID_{50} and B_{50} values can be very different. It is easy to realize that for the same incubation time, ID_{50} should always be larger than B_{50} values when [EI] is much smaller than [EI*]. Conversely, if K is very small, considerable enzyme inhibition can be obtained before any important labeling is observed.

Although ID_{50} values can be of practical interest in a first approach, or when one wishes to compare the inactivating efficiency of a series of β-lactams on the same enzyme, it is very difficult, on that basis alone, to compare the efficiency of the same inactivator towards various enzymes. A decreased ID_{50} can reflect a higher value of k_2/K, a lower value of k_3, or a higher value of Km. In addition, when the ID_{50} or B_{50} values are very low, they can result from a trivial titration of the enzyme, i.e., represent 50% of the enzyme concentration. These points will be discussed at length in Section V.A. In the present paragraph, we will try, whenever possible, to deduce k_2/K values from the ID_{50} or B_{50} values, but it should be realized that these computations can only be made when k_3 and Km have been independently determined and that these deduced values can, at best, be regarded as rough approximations, only valid within one order of magnitude.

A. Transpeptidases

1. The Membrane-Bound Transpeptidases of Streptomyces sp.

a. Streptomyces R61[74,124,146-150]

Isolated membranes of *Streptomyces* R61 catalyzed the model transpeptidation Ac_2-L-Lys-D-Ala-D-Ala + R-NH_2 → Ac_2-L-Lys-D-Ala-NH-R + D-Ala. In contrast to what was observed with the exocellular enzyme of the same strain, little or no hydrolysis of the donor was recorded whether an aminated acceptor was included in the incubation mixture or not. The soluble and membrane-bound activities appeared to recognize the same amino acids and peptides as acceptors in the transpeptidation reaction (Table 8). In both cases, good substrates were closely related to the natural peptide found in the peptidoglycan. However, the sensitivity profiles of the 2 activities to 12 different β-lactam antibiotics were rather different[74] and, since the membrane preparations contained very little β-lactamase activity, the differences could not be attributed to a preferential degradation of some β-lactams. The sensitivity of the bacteria themselves to the same antibiotics closely paralleled that of the membrane-bound transpeptidase, which thus became a very likely candidate for a killing target. The membrane suspensions exhibited a surprising property: they were enzymatically active in the frozen state.[147] Addition of a small amount of glycerol to the reaction mixtures permitted a comparison of some properties of the transpeptidase in the liquid and frozen state at the same temperature: the Km value for the acceptor remained unchanged, but freezing induced a tenfold decrease in the Km value for the donor, a threefold increase in the maximum velocity, and a tenfold increase in the ID_{50} value of benzylpenicillin. The catalytic efficiency (V/Km) was 25-fold higher in the frozen than in the liquid state at the same temperature! The transpeptidase activity was solubilized by Dusart et al. using the positively charged detergent cetyl-trimethylammonium bromide (Cetavlon) and partially purified by filtration on Sephadex® G-100.[147] The Km values for donor and acceptor substrates and the k_2/K for the interaction with benzylpenicillin measured with this preparation were, respectively, seven-, ten-, and sixfold lower than the corresponding values observed with the membrane suspensions in the liquid state. The properties of the detergent-solubilized enzymatic activity were thus surprisingly close to those of the membrane suspensions in the frozen state. In contrast to the membrane preparations which released penicilloic acid, spontaneous reactivation of the solubi-

lized enzyme-[^{14}C]benzylpenicillin complex resulted in the formation of phenylacetylglycine (the nature of the released compound was not investigated with frozen suspensions). Electrophoresis of penicillin-treated membranes of *Streptomyces* R61 in the presence of SDS yielded an unusual PBP pattern: about 85% of the bound radioactivity migrated as a rather diffuse band whose position corresponded to a molecular weight of 25,000.[150] The only other relatively abundant PBP (about 10%) exhibited a molecular weight of about 50,000. Interestingly, the membranes of *Streptomyces rimosus* yielded a similar PBP pattern and also contained a transpeptidase activity which could be solubilized with Cetavlon. The thermolability of the 25,000-MW protein corresponded to that of the transpeptidase activity, indicating that, unless the molecular weight determinations were gravely perturbed by unsuspected artefacts, *Streptomyces* R61 was very unusual in that the penicillin killing target was a very low molecular weight transpeptidase. From these results, it was also concluded that the exocellular DD-carboxypeptidase-transpeptidase could not be a solubilized form of the membrane-bound transpeptidase produced by proteolytic removal of a hydrophobic peptide from this latter enzyme. In addition, no inhibition of the membrane-bound transpeptidase activity was observed in the presence of the antiserum prepared against the exocellular enzyme.[148] With the R61 strain, quantitative data were obtained for the interactions of both membrane-bound and solubilized enzymes with several β-lactam antibiotics. These results could be interpreted on the basis of Scheme 1 and the results are displayed in Table 6. It should, however, be realized that the experimental data did not permit the computation of the individual values of k_2 and K, and that the existence of the first, reversible step of Scheme 1 remains to be demonstrated for this enzyme.

b. Streptomyces K15[124,148,149,151-153]

In addition to the four exocellular and lysozyme-releasable PBPs already described, five membrane PBPs (MW: 60,000, 54,000, 50,000, 39,000 and 26,000) were found in *Streptomyces* K15. The transpeptidase activity of the isolated membranes was 50% inhibited by the concentration of benzylpenicillin which, under the same conditions, induced the labeling of 50% of the 26,000-MW protein. This protein could be solubilized from the membranes or extracted from the mycelium by Cetavlon. It was purified 8,300-fold to 90% homogeneity by filtration on Sephadex® G-100 and affinity chromatography on ampicillin-linked CH Sepharose® 4B. Although about 50% of the extracted activity was finally recovered, the procedure only yielded 1 mg of protein from 100 ℓ of culture.[151] The purified enzyme exhibited a turnover number of 0.61 s^{-1}, using Ac_2-L-Lys-D-Ala-D-Ala-OH as donor and glycylglycine as acceptor. The carboxypeptidase activity, measured with the same donor, was 30-fold lower. To perform an accurate estimation of the hydrolysis, one had to include D-amino acid oxidase in the mixture, otherwise, as soon as some D-alanine was released, the enzyme started using it as an acceptor and hydrolysis was inhibited. The Km values for the donor were 2 m*M* in the hydrolysis and 6 m*M* in the transpeptidation.[154] Even in the absence of acceptor, very little acyl-enzyme could be trapped when the pure enzyme was incubated with labeled tripeptide.

The transpeptidation reaction was essentially complete and, moreover, no regeneration of the tripeptide was observed when the product tetrapeptide was incubated in the presence of large concentrations of D-alanine.[152] As with the R61 exocellular enzyme, the transpeptidation reaction thus appeared to be irreversible. The specificity profiles were determined for the donor and acceptor molecules. The results were very similar to those recorded with the R61 exocellular and membrane-bound enzymes (Tables 7 and 8). Again, the best substrates were those which most closely mimicked the natural cell wall precursor peptides. One surprising finding, however, was that the membrane-bound enzymes (K15 or R61) did not catalyze dimer formation with the donor-acceptor

Table 6
INTERACTION BETWEEN THE MEMBRANE-BOUND TRANSPEPTIDASES OF *STRETOMYCES* R61 AND K15 AND β-LACTAMS (AT 37°C)[74,146,149]

	Membrane-bound transpeptidase (R61)		Solubilized transpeptidase (R61) (partially purified)		Solubilized transpeptidase (K15) (partially purified)	
	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})
6-Aminopenicillanate	[10]	1.4×10^{-4}	—	—	—	—
Benzylpenicillin	53	1.1×10^{-4}	10	0.13×10^{-4}	30[a]	1.1×10^{-4}
Phenoxymethylpenicillin	140	2.8×10^{-4}	15	0.9×10^{-4}	30	1.6×10^{-4}
Ampicillin	400	3.3×10^{-3}	15	1.8×10^{-4}	15	6.8×10^{-4}
Carbenicillin	15	0.7×10^{-4}	8	0.9×10^{-4}	5	0.8×10^{-4}
Cloxacillin	13	1.2×10^{-4}	—	$<10^{-5}$	—	—
Cephalosporin C	5	3.3×10^{-3}	—	—	—	—
Methicillin	[30]	2.1×10^{-4}	—	$<10^{-5}$	—	—
Cephalothin	[30]	8.3×10^{-4}	—	—	—	—

Note: The values between square brackets were computed from the ID_{50} values. —, not determined.

[a] A larger value (150 $M^{-1}s^{-1}$) was obtained with the 90% pure enzyme.

Table 7
SPECIFICITY OF THE MEMBRANE-BOUND TRANSPEPTIDASE OF *STREPTOMYCES* K15 AND OF THE *STREPTOMYCES* R61 EXOCELLULAR DD-PEPTIDASE FOR THE DONOR PEPTIDE[51,152]

Donor	Enzyme	
	K15 membrane-bound after solubilization (transpeptidation with 2 m*M* Gly-Gly)	R61 exocellular (hydrolytic activity)
Ac_2-L-Lys-D-Ala-D-Ala	100	100
Ac_2-L-Lys-D-Ala-D-Leu	8	7
Ac_2-L-Lys-D-Ala-Gly	8	7
Ac_2-L-Lys-D-Ala-D-Lys	4	9
Ac_2-L-Lys-D-Ala-L-Ala	0	0
Ac_2-L-Lys-D-Leu-D-Ala	0	1
Ac_2-L-Lys-Gly-D-Ala	0	0.2
Ac_2-L-A_2bu-D-Ala-D-Ala	40	8
Ac_2-L-Orn-D-Ala-D-Ala	13	45

peptide N^{α}-Ac,N^{ϵ}-Gly, L-Lys-D-Ala-D-Ala, a reaction which was performed by several soluble enzymes. The kinetic parameters were determined for the interaction with various β-lactams (Table 6). The values of k_2/K were rather low, as observed with the R61 membrane-bound transpeptidase. The velocity of the inactivation was not influenced by the presence of the acceptor and varied with the donor concentration in a competitive manner, a result in good agreement with those obtained with the exocellular enzymes.

ID_{50} values were determined for some other β-lactams, among which cefoxitin and sulfazecin turned out to be four- and eightfold more active than benzylpenicillin, respectively. Reactivation of the enzyme at pH 7.5 and 37°C after complete inactivation by [^{14}C]benzylpenicillin was a first-order process ($k_3 = 1.1 \times 10^{-4}$ s^{-1}) and was concomitant with the degradation of the radioactive complex ($k_3 = 0.96 \times 10^{-4}$ s^{-1}). This process yielded a mixture of benzylpenicilloate and phenylacetylglycine[153] in a ratio of 5 to 7:1. (The first-order rate constants for the release of these compounds were 0.76 and 0.11 $\times 10^{-4}$ s^{-1}.)

In conclusion, the membrane-bound mainly differed from the exocellular enzymes by their lower and different sensitivities to β-lactams, their exceedingly low hydrolytic activities and lower k_{cat}, and their smaller sizes. It will be seen below that low k_{cat} values are general characteristics of the membrane-bound enzymes, both carboxypeptidases and transpeptidases. Although one can assume that optimum activity requires the interaction between a membrane-bound enzyme and a membrane-bound substrate, no experimental evidence has been obtained which supports this assumption.

2. PBPs 1, 4, and 5 from Salmonella typhimurium[141]

The cytoplasmic membranes of *Salmonella typhimurium* contain five [^{14}C]benzylpenicillin binding proteins. They exhibited a DD-carboxypeptidase activity when assayed both on UDP-*M*-pentapeptide and on the pentapeptide itself. The pentapeptide was that depicted on Figure 9A. Transpeptidation activity was not assayed at this stage. Extraction of the membrane by LiCl/Genapol (a nonionic detergent) yielded a supernatant significantly enriched in PBPs 1, 4, and 5. When incubated in the presence of pentapeptide, the supernatant catalyzed the formation not only of D-alanine and tetra-

Table 8
TRANSPEPTIDATION WITH VARIOUS ACCEPTORS CATALYZED BY SOME *STREPTOMYCES* ENZYMES[65,74,152]

	Enzyme					
	K15 Membrane-bound after solubilization		R61 Membrane-bound		R61 Exocellular	
Acceptor	[A] (m*M*)	% T	[A] (m*M*)	% T	[A] (m*M*)	% T
Amino acids						
Glycine	2	22	1.7	76	12.3	105
D-Alanine	2	60	1.7	40	—	—
	10	68	—	—	12.3	150
L-Alanine	2	1[a]	1.7	0	12.3	0
meso-Diaminopimelate	10	50	1.7	87	—	—
Dipeptides						
Glycylglycine	2	100	1.7	100	2.5	100
					12.3	150
Glycyl-D-alanine	10	3	1.7	20	12.3	12
Glycyl-L-alanine	2	101	1.7	140	12.3	64
D-Alanyl-D-alanine	10	0	1.7	2	12.3	0
D-Alanyl-L-alanine	10	0	1.7	14	—	—
L-Alanyl-D-alanine	10	0	1.7	1	—	—
D-Alanylglycine	10	0	1.7	20	—	—

[A] = acceptor concentration: % T = relative efficiency as acceptor compared to glycylglycine (= 100 at approximately 2 m*M*).

[a] Could be due to contamination by a low amount of D-alanine.

peptide, but also of dimers (Figure 17). When the incubation was prolonged, the dimers disappeared, yielding more tetrapeptide. The authors characterized this latter activity as an endopeptidase, but Figure 17 clearly shows that it was another manifestation of the carboxypeptidase activity. The solubilized mixture was fractionated by chromatography on ampicillin-affinose and DEAE ion exchangers. Fractions were thus obtained which contained PBP 4 or 5 as the sole PBP, respectively. A fraction enriched in PBP 1, but still contaminated by PBPs 4 and 5, was also prepared. The isolated PBP 4 represented the majority of the D-Ala-D-Ala peptidase activity of the extract. The initial ratio of hydrolysis to transpeptidation (H/T) was 3:2 and, upon prolonged incubation, the dimer disappeared. The isolated PBP 5 was a much less efficient transpeptidase, the initial H/T ratio being 12:1 and no dimer hydrolysis was observed. Finally, the enriched PBP 1 fraction also catalyzed D-alanine release and dimer formation. Unfortunately, the author's data did not permit the calculation of the specific activities of these preparations. Kinetic studies of penicillin binding and release, and, when it was possible, of enzyme inactivation and regeneration, permitted the computation of the characteristic constants for the interaction (Table 9). No indication of saturation was observed ($[I] \ll K$) and the second-order rate constants for the formation of EI* are expressed as k_2/K.

3. Proteus vulgaris[155] *and Proteus mirabilis* [81,82,156,157]

PBPs 4 and 5 of *Proteus mirabilis* and PBP 4 of *Proteus vulgaris* catalyzed concomitant hydrolysis and transpeptidation[155,157] using the pentapeptide of Figure 9A as donor and the tetrapeptide L-Ala → D-Glu(amide) L-meso-A_2 pm → D-Ala as an acceptor.

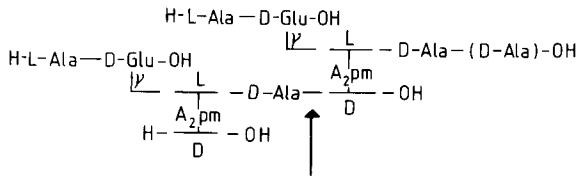


FIGURE 17. Products of transpeptidase activity of *Salmonella typhimurium* PBPs. Structure of the dimers formed by the LiCl/Genapol extract and site of hydrolysis by the misnamed "endopeptidase" activity (arrow). Two dimers were separated, one containing a D-alanyl-D-alanine C-terminus and the second one derived from the first by DD-carboxypeptidase activity removal of the terminal D-alanine. Once more, it is clear that the "endopeptidase" is in fact a carboxypeptidase activity.

Table 9
INTERACTION BETWEEN THE D-ALANYL-D-ALANINE PEPTIDASES OF *SALMONELLA TYPHIMURIUM* AND [^{14}C]BENZYLPENICILLIN AT 37°C[141]

	k_2/K ($M^{-1}s^{-1}$)		k_3 (s^{-1})	
	From [^{14}C] binding	From enzyme inactivation	From [^{14}C] release	From enzyme reactivation
PBP 1	550	—	4.8×10^{-4}	—
PBP 4	6,000	40,000	1.3×10^{-4}	1.3×10^{-4}
PBP 5	2,000	7,800	1.1×10^{-3}	1.2×10^{-3}

In addition, both *P. mirabilis* PBPs also catalyzed dimer formation when the pentapeptide was used as donor acceptor. In contrast, no transpeptidation was observed with PBP 5 of *P. vulgaris*, which behaved as a strict carboxypeptidase. Table 10 summarizes the properties of the four enzymes. Striking similarities exist between the corresponding PBPs of both species. Both PBPs 5 were characterized by a lower turnover number and a lower sensitivity to benzylpenicillin, which could partly be explained on the basis of a much higher value of k_3, i.e., a largely increased instability of the EI* complex. The much lower specific activities of the *P. vulgaris* enzymes could be due to incomplete purification, since the authors have demonstrated that their preparations contained one single PBP, but not one single protein. Interestingly, in both cases, PBP 4 could not be detected in the membranes of L-forms, spheroplast-like cells which are grown in the presence of high concentrations (0.1 to 0.3 m*M*) of penicillin. Nonetheless, the *P. mirabilis* unstable L-forms synthesize peptidoglycan whose overall cross-linking degree is similar to that of the bacterial form.[15] The cytoplasmic membranes of *P. vulgaris* stable L-forms did not contain any PBP 4, which was released in the culture filtrate. When membranes were prepared from unstable L-form spheroplasts of *P. mirabilis*, only PBPs 2, 5, and 6 were left in a form capable of binding radioactive penicillin. The other PBPs were permanently saturated by the benzylpenicillin present in the culture medium. On the basis of these results, Martin and colleagues have suggested that PBP 5 might be responsible for catalyzing peptide cross-linking during L-form growth. This hypothesis, however, appears unrealistic, since the high concentration of penicillin in the culture medium was sufficient to completely inactivate PBP 5. The appearance of free PBP 5 in the membrane preparations could be accounted for by the

Table 10
THE D-ALANYL-D-ALANINE PEPTIDASES OF *PROTEUS* [81,82,155-157]

	P. mirabilis		*P. vulgaris*	
	PBP 4 (H)	PBP 5 (L)	PBP 4	PBP 5
MW	49,000	43,000	46,000	43,000
Isoelectric pH	8.1	5.9	—	—
Dimer hydrolysis	Yes	No	—	—
T/H[a]	0.33	0.01	0.10	0
Km[b]	0.7 m*M*	0.5—0.9 m*M*	0.6 m*M*	—
Turnover number (min^{-1})	1,100	77	180	2.5 4.0 (L-forms)
Interaction with benzylpenicillin				
Molar ratio penicillin/enzyme	0.99	0.41	—	—
k_3 (s^{-1})	4×10^{-5}	1.6×10^{-3}	5×10^{-5}	1.1×10^{-3}
ID_{50} (μM)	0.02—0.04	0.6—1.0	0.01[d] 0.02[e]	1 —
k_2/K ($M^{-1}s^{-1}$)	$2—3 \times 10^{5}$[c]	20—80,000	—	—
Degradation product	Phenylacetylglycine	Penicilloate + 8% phenylacetylglycine	—	—

[a] For *P. mirabilis*, T represented dimer formation from the pentapeptide, for *P. vulgaris*, the donor was the pentapeptide and the acceptor a Glu-amidated tetrapeptide.
[b] Using UDP-*M*-pentapeptide as donor.
[c] The k_2/K value was computed by us on the basis of an ID_{50} of 0.03 μM and a reaction time of 15 min. Such a low ID_{50} might, however, be influenced by a titration effect.
[d] Measured as the concentration which saturates 50% of PBP 4.
[e] Measured as ID_{50} on the purified enzyme.

rapid degradation of the benzylpenicillin-PBP 5 complex during membrane isolation.

P. mirabilis stable L-forms, which are completely devoid of cell wall and which grow in the absence of penicillin, were also studied; their membranes had lost PBP 4 and this might be related to their permanent inability to revert to the bacterial form and to synthesize peptidoglycan.

Finally, one should note that the inactivation of the carboxypeptidase activity of PBP 5 by penicillins appeared to obey a noncompetitive model.[81,82,156] The significance of many of the results presented here remains unclear and it is certain that several problems must still be solved before a clear and convincing explanation of the properties of L-forms can be proposed.

4. Escherichia coli

Membrane fractions synthesized cross-linked peptidoglycan from UDP-*M*-pentapeptide and UDP-G.[158] In the absence of UDP-G, only hydrolysis of the C-terminal D-alanine was observed. Penicillin inhibited the release of D-alanine and the cross-linking of peptidoglycan.

Membrane extracts of *E. coli* strain 44 obtained using Brij 36 catalyzed hydrolysis of the pentapeptide (Figure 9A) as well as dimer formation and dimer hydrolysis.[159-162] Chromatography on DEAE-cellulose or ampicillin-affinose yielded two fractions, one retained on both supports, the other one eluted with the void volume. Both fractions catalyzed the three activities, although in variable proportions, and exhibited different sensitivities to β-lactams. Moreover, in one given preparation, the three activities were not equally sensitive to ampicillin, indicating the possible presence of different enzymes.[162] In the sections dealing with the *E. coli* PBP system (Sections IV.B.5 and V.B.1), we will try to propose correlations between the PBPs and the enzymatic activ-

ities. This is not an easy task, since many of the early experiments were performed on preparations containing more than one enzyme.

5. Others

Synthesis of peptidoglycan using the nucleotide precursors has also been demonstrated in many other strains, e.g., in *Bacillus megaterium*,[163,164] *Micrococcus luteus*,[165] *Staphylococcus aureus*,[166] *Sporosarcina ureae*,[167] and *Bacillus stearothermophilus*.[168] Unfortunately, little quantitative information can be deduced from these studies since transpeptidation represented the last reaction in a complex biosynthetic sequence. However, these studies clearly established the sensitivity of this latter reaction to β-lactam antibiotics.

B. Membrane-Bound D-Alanyl-D-Alanine Carboxypeptidases

Many of the carboxypeptidases which will be discussed in this section also catalyzed simple transfer reactions, using simple aminated acceptors such as glycine, hydroxylamine, or D-alanine. Generally, high acceptor concentrations were used and the yields of the transfer reactions were rather poor. Thus, we thought that it was necessary to make a clear distinction between these enzymes and what we consider as true transpeptidases, i.e., enzymes which catalyze the formation of natural dimers, which recognize aminated molecules whose structure closely mimicks that of the physiological acceptor, or which catalyze little or no hydrolysis.

1. Streptococcus faecium

The membranes of *Streptococcus faecium* ATCC 9790 (in the past, this strain was referred to as *S. faecalis*) contain seven to eight PBPs[169] and exhibit DD-carboxypeptidase and LD-transpeptidase activities.[45] The first of these activities could be entirely attributed to PBP 6[170] (MW: 43,000). When assayed at pH 6, no transpeptidation occurred, even in the presence of simple acceptors such as glycine. At pH 10, however, transpeptidation was observed with 10 m*M* D-alanine, glycine, and glycylglycine.[171] The initial rate of exchange with 5m*M* [^{14}C]D-alanine, was about twice that of the hydrolysis (these measurements were complicated by the fact that the product of the exchange reaction, Ac_2-L-Lys-D-Ala-[^{14}C]D-alanine, was itself a substrate for the hydrolysis). The T/H ratio was not altered by solubilization of the enzyme with Genapol X-100® and the subsequent, 130-fold purification to apparent protein homogeneity. Glycine and glycylglycine were, respectively, 1.5- and 4-fold less efficient than D-alanine. A disaccharide-peptide whose peptide moiety was identical to the natural acceptor was inactive. The specificity of the enzyme for the donor was also determined. In addition to the standard peptide Ac_2-L-Lys-D-Ala-D-Ala, peptides and disaccharide peptides which closely resembled the natural ones were very good donors (for instance, G-M-L-Ala-D-Gln$_{\Gamma}$→ L-Lys[ε-D-Asn]-D-Ala-D-Ala). In the hydrolysis reaction, at pH 10, the purified enzyme had a turnover number of 3 s^{-1} on Ac_2-L-Lys-D-Ala-D-Ala. The interaction of the enzyme with β-lactams was studied both on the membrane-bound form and the solubilized, purified enzyme.[170] Very similar values were obtained (Table 11). In addition, with the purified enzyme, the individual values of k_2 (0.005 s^{-1}) and K (24 μ*M*) were determined, indicating the validity of model 1 for this enzyme. Spontaneous degradation of the [^{14}C]benzylpenicillin-membrane complex at pH 7.5 yielded a mixture containing phenylacetylglycine, benzylpenicilloate, and an unidentified compound. Only the release of the first compound appeared to be concomitant with the reactivation of the enzyme.[172] Accordingly, reactivation of the purified enzyme under the same conditions yielded only phenylacetylglycine. With this latter preparation, *N*-formylpenicillamine was also identified as the fragment arising from the degradation of the thiazolidine moiety of the penicillin molecule. The rate of the reaction was too

slow, however, to decide whether this compound was directly released from the complex or whether it was formed by spontaneous degradation of a primary product.[172]

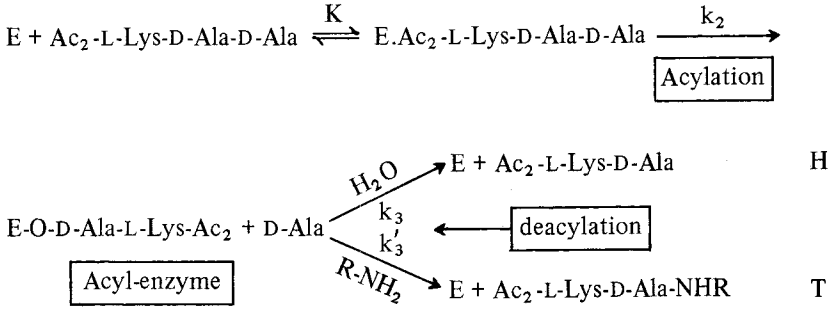
Trypsin treatment of the isolated membranes or of the Genapol-solubilized DD-carboxypeptidase (PBP 6) gave rise to various degraded protein fragments, the major one exhibiting a molecular weight of about 30,000. This fragment was water soluble in the absence of detergent, and no apparent alterations of the catalytic and penicillin-binding properties of the DD-carboxypeptidase were observed.[169]

2. Staphylococcus aureus PBP4

a. Catalytic Properties[173]

Among the four PBPs present in the membranes of *Staphylococcus aureus* H,[173] only PBP 4 exhibited a DD-carboxypeptidase activity. Ironically, this protein was not detected as a PBP in the early studies[174] because of the short half-life of the benzylpenicillin-enzyme complex. The enzyme was solubilized with Triton X-100® and purified by an affinity chromatography procedure which took advantage of that short half-life to separate it from the other PBPs.[173] The purified enzyme had a molecular weight of 46,000. Its carboxypeptidase activity was assayed using Ac_2-L-Lys-D-Ala-D-Ala and a turnover number of 20 min^{-1} was observed. With the same substrate as donor and glycine as acceptor, the enzyme behaved as an efficient transpeptidase, a 40-μM concentration of glycine yielding a H/T ratio of 1. Hydroxylamine could also be used as an acceptor, but the same H/T ratio was only reached at a 7-mM concentration. Unfortunately, no transpeptidation or dimer formation was attempted with acceptors structurally more similar to the natural one. D-alanine was shown to inhibit transpeptidation with glycine at low concentrations ($K_i \cong 50\ \mu M$), while L-alanine was inefficient even at large concentrations.

Both glycine and hydroxylamine increased the total utilization of the donor. This effect was interpreted[173] by assuming that the interaction with the substrate obeyed the model represented in Scheme 3.



Scheme 3. Model for the interaction between enzyme, donor, and acceptor substrates and water.

When water is the only potential acceptor present, the scheme is kinetically indistinguishable from Scheme 1. The kinetic parameters for the hydrolysis are

$$Km = \frac{k_3 K}{k_2 + k_3} \tag{10}$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{11}$$

Table 11
INTERACTION BETWEEN β-LACTAMS AND THE MEMBRANE-BOUND AND PURIFIED DD-CARBOXYPEPTIDASES OF *STREPTOCOCCUS FAECIUM* (AT pH 7.5 AND 37°C)[170]

	Membrane-bound form		Solubilized and purified enzyme	
	k_2/K (M^{-1} s^{-1})	k_3 (s^{-1})	k_2/K (M^{-1} s^{-1})	k_3 (s^{-1})
Benzylpenicillin	445	4.4×10^{-5}	1045	2.8×10^{-5}
Phenoxymethylpenicillin	560	10×10^{-5}	1220	9×10^{-5}
Ampicillin	230	1.5×10^{-5}	600	1.3×10^{-5}
Carbenicillin	19	4×10^{-3}	27	4×10^{-5}
Oxacillin	4.5	24×10^{-5}	4.5	15×10^{-5}
Cloxacillin	0.8	6×10^{-5}	0.8	8×10^{-5}
Methicillin	1.9	6×10^{-5}	3.6	7×10^{-5}

These equations simplify to

$$Km = K \tag{12}$$

and

$$k_{cat} = k_2 \tag{13}$$

if acylation is rate limiting ($k_2 \ll k_3$) and to

$$Km = \frac{k_3}{k_2} K \tag{14}$$

$$k_{cat} = k_3 \tag{15}$$

if deacylation is rate limiting ($k_2 \gg k_3$).

If the presence of the R-NH_2 acceptor increases the total turnover of the tripeptide (T + H), the acyl-enzyme represents a sizable proportion of the total enzyme at the steady state, i.e., acylation is not rate limiting.

Kinetic parameters were determined for the hydrolysis of the tripeptide depicted on Scheme 3 and of the depsipeptide Ac_2-L-Lys-D-Ala-D-lactate. The k_{cat} values for both substrates were similar (≅0.15 s^{-1}), but the Km of the depsipeptide was 100-fold lower than that of the peptide, data which were in agreement with a rate-limiting deacylation. Indeed, whatever the substrate, the acyl-enzyme would be the same and k_{cat} would be equal to k_3 (Equation 15); $(k_2)_{peptide}$ would be much larger than 0.15 s^{-1}, and k_2 would be 100-fold larger with the depsipeptide, thus explaining the lower Km for this latter substrate (Equation 14). Accordingly, utilization of a depsipeptide [^{14}C]-labeled in the acetyl groups allowed the detection of an acyl-enzyme exhibiting a bound substrate to enzyme ratio of 0.43. Both substrates also interfered with the binding of [^{14}C]benzylpenicillin, indicating a competition between substrates and inactivator for the same binding site.

Moreover, acetone precipitation of the membranes after a short contact with [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala followed by SDS gel electrophoresis permitted the detection not only of a [^{14}C]-labeled 46,000-MW PBP, but also of [^{14}C]-labeled PBPs of higher molecular weight. This result was rather surprising since none of these PBPs behaved as a D-alanyl-D-alanine peptidase in vitro.[175]

b. Interaction with β-Lactams

After the early experiments, a β-lactamase activity comparable to its peptidase activity was attributed to the enzyme and the product of the interaction with benzylpenicillin was identified as benzylpenicilloate.[173] A turnover number of 25 min^{-1} and a Km of 20 μM were found for the β-lactamase reaction. Later experiments, in which the half-life of the PBP 4-benzylpenicillin complex was directly measured (1.5 min) yielded a maximum turnover number of 0.46 min^{-1}.[175] These results indicated that more than 95% of the β-lactamase activity observed in the earlier experiments was probably due to a contaminating β-lactamase of high specific activity but present in the preparation as a trace which remained undetected as a protein. The Km of 20 μM and the nature of the product (benzylpenicilloate) were thus determined by the contaminating enzyme. (Note that the Km of the exocellular lactamase of *Staphylococcus aureus* is 5 μM for benzylpenicillin.) It would be particularly interesting to reexamine the structure of the degradation product after isolation of the complex, since PBPs 1 and 2 of the same strain were demonstrated to release phenylacetylglycine.[175] The utilization of a β-halogenopenicillanate to specifically inactivate the true β-lactamase might be useful for this purpose, since these compounds reacted rather slowly with the DD-carboxypeptidases-transpeptidases (see Table 2).

Another unexplained and curious result was that, upon inhibition of the transpeptidation by benzylpenicillin, the very low level of hydrolysis did not appear to decrease in parallel, so that the T/H ratio increased from 260 in the absence of penicillin to about 8 in the presence of 2.8 mM penicillin.[173]

Unfortunately, none of the published data obtained with strain H permitted the computation of the second-order rate constant for the formation of EI*. In experiments where the enzymatic activity was determined, the results were difficult to analyze because of the presence of the β-lactamase, and the measure of the affinity of PBP 4 for some antibiotics was made using a competition method, which is completely unreliable when the half-lives of the complexes are short.[176] From these latter experiments, however, it is clear that PBP 4 seemed to be highly sensitive to cefoxitin, the ID_{50} being 300- to 400-fold lower than that of benzylpenicillin. Whether this sensitivity could be at least partially attributed to an increased stability of the complex has not been determined. A reliable B_{50} value for penicillin (3 μM) has been obtained by Brown and Reynolds,[177] but the experiments were done using another strain and these authors did not measure the value of k_3. Assuming that k_3 was not very different from that observed with PBP 4 of strain H, a bimolecular rate constant of about 2000 M^{-1} s^{-1} at 37°C can be calculated. Electrophoretic patterns were compared after limited proteolysis of the benzyl-penicillin and substrate-labeled enzyme: they were similar, but not identical; several additional important bands were present in the case of the substrate-labeled enzyme.[72] The authors interpreted these differences by indicating that penicillin and substrate were bound at the same site, but that the bound penicillin and substrate differentially influenced cleavage at one or more sites adjacent to the active site residue.

c. Physiological Role

The enzyme first seemed a nonessential protein since mutants lacking PBP 4 were viable[178] and it was saturated by cefoxitin concentrations two orders of magnitude below the minimum inhibitory concentration (MIC). More detailed experiments,[179] however, revealed a marked reduction in the degree of cross-linking of the peptidoglycan, when PBP 4 was either absent (by mutation) or inactivated (by cefoxitin). Thus, PBP 4 may act as a transpeptidase involved in secondary cross-linking of the peptidoglycan, a reaction whose existence had been proposed before by Mirelman on the basis of experiments performed with wall-membrane preparations.[180]

3. The Low Molecular Weight PBPs of Bacillus subtilis and Bacillus stearothermophilus

Early experiments[181,182] indicated that the isolated membranes of *Bacillus subtilis* catalyzed a D-alanyl-D-alanine carboxypeptidase reaction which was penicillin sensitive. Penicillin binding to the membrane obeyed saturation kinetics, and a maximum of 0.05 nmol of benzylpenicillin was bound per milligram of membrane protein. Inhibition of the DD-carboxypeptidase paralleled the binding of penicillin. Both penicillin binding and enzymatic activities were inhibited by treatment with various thiol reagents. In the presence of neutral hydroxylamine, the bound penicillin was released and catalytic activity was recovered. The authors concluded that a sulphydryl group was involved in both catalysis and interaction with β-lactams.

Reynolds[183] described a similar DD-carboxypeptidase activity in the membranes of *Bacillus stearothermophilus*. These membranes were also active in the utilization of UDP-G and UDP-*M*-pentapeptide for the formation of cross-linked peptidoglycan. On the basis of their different penicillin sensitivities, it was established that the transpeptidase and carboxypeptidase activities were catalyzed by different enzymes.[184] The carboxypeptidase was solubilized by extraction with water-saturated butanol and the solubilized enzyme catalyzed transpeptidation using glycine and D-alanine as acceptors. Some properties of the membrane-bound DD-carboxypeptidase studied by Barnett[83] were rather peculiar: storage at 0 and −20°C differentially influenced the catalytic and penicillin-binding properties of the enzyme and the study of the interaction with penicillin indicated the formation of a ternary (enzyme-substrate-inhibitor), catalytically active complex.

a. Purification and Catalytic Properties of the Enzymes

Several penicillin-binding proteins were found in the membranes of both bacteria,[79,174] the smaller polypeptide (MW: 45,000 to 50,000) representing a very large proportion (80 to 90%) of the total. In studies performed by Strominger and co-workers, the proteins were solubilized using the neutral detergents Triton X-100® or Nonidet P40®.[79,139] Affinity chromatography on agarose-bound 6-aminopenicillanate eliminated the bulk of other proteins. The carboxypeptidases, which were identified as the low molecular weight PBPs (PBP 5), were separated from the other PBPs by ion-exchange chromatography. The major properties of the enzymes are summarized in Table 12. The molecular weights were those measured by sodium dodecylsulfate gel electrophoresis. In the presence of nonionic detergents, aggregates were formed containing a variable number of enzyme molecules. Valine was found as the N-terminal amino acid of the *B. subtilis* enzyme.[139] The specific activities measured with the various substrates were rather low when compared to those of the soluble Actinomycetes enzymes but were of the same order of magnitude as those of other membrane-bound D-alanyl-D-alanine peptidases. Comparisons are, however, made difficult by the facts that some enzyme activities were given in "cpm of D-Ala released per 30 min" and that in different articles different values were obtained for the kinetic parameters. For example, Nishino et al.[186] reported Km and V values of 0.55 m*M* and 1.6 μmol min^{-1} (mg protein)$^{-1}$, respectively, for the interaction between the *B. subtilis* enzyme and the synthetic peptide Ac_2-L-Lys-D-Ala-D-Ala-OH. For the same interaction, the corresponding values found by Rasmussen and Strominger[58] were 6.7 m*M* and 0.2 μmol min^{-1} (mg protein)$^{-1}$. When the catalytic efficiencies (V_{max}/Km) are computed from these data, a variation by two orders of magnitude is observed. It should also be noted that the surprising results of Barnett[83] were not reproduced with the purified preparations of *B. stearothermophilus* DD-carboxypeptidase. These results remained unexplained. The "hyperbolic competitive" kinetics observed in the presence of benzylpenicillin and substrate could be due to the presence in the membranes of a second, penicillin-insensitive DD-carboxypeptidase representing 20 to 30% of the total activity.

Table 12
PROPERTIES OF THE D-ALANYL-D-ALANINE PEPTIDASES OF *BACILLUS STEAROTHERMOPHILUS* AND *BACILLUS SUBTILIS* [58,79,80,185,186,189]

	B. subtilis	*B. stearothermophilus*
Molecular weight	50,000	46,500
Optimum pH	5—6	5.5—6 ?[a]
Optimum temperature	30—35	50—60
Solubilizing detergent	Nonidet P40	Triton X-100®
Interaction with substrates		
UDP-*M*-pentapeptide		
Km (m*M*)	0.3 (37°C)	1.4 (25°C)
V (μmol min^{-1} mg^{-1})	0.018 (Zn^{++})	0.12 (Zn^{++})
k_{cat}/Km (M^{-1} s^{-1})	58 (Zn^{++})	66 (Zn^{++})
Ac_2-L-Lys-D-Ala-D-Ala (25°C)		
Km (m*M*)	6.7 (0.55)[b] (25°C)	0.25
V (μmol min^{-1} mg^{-1})	0.2 (1.6)[b] (25°C)	0.046
k_{cat}/Km (M^{-1} s^{-1})	25 (2,400)[b] (25°C)	143
Ac_2-L-Lys-D-Ala-D-Lac		
Km (m*M*)	0.7 (25°C)	—
V (μmol min^{-1} mg^{-1})	3 (25°C)	—
k_{cat}/Km (M^{-1}s^{-1})	3,600 (25°C)	—
Dimer hydrolysis	0	0.2% of Cbase
Acceptors	NH_2OH	NH_2OH
	Glycine (very poor)	Glycine
		D-alanine
Interaction with benzylpenicillin		
K (m*M*)	0.1 (4°C)	—
k_2 (s^{-1})	0.12 (4°C)	—
k_2/K (M^{-1} s^{-1})	1,200 (4°C)	—
k_3 (s^{-1})	6×10^{-5} (37°C)	3×10^{-4} (37°)
Released product	Phenylacetylglycine	Phenylacetylglycine

[a] A sharp drop was recorded between pH 5.5 and 5.0, but the buffer was changed from sodium cacodylate to sodium acetate.

[b] The values are those of Reference 58, the values between round brackets those given in Reference 186. The authors do not supply any explanation for these large differences. The different Vs could be due to the utilization of preparations exhibiting different specific activities. We do not see any straightforward explanations for the different Km. In later publications, Waxman and Strominger only cite the first set of values.

Although such an activity was found in the membranes of *Streptomyces* K15,[151,154] it was never detected, to our knowledge, in the membranes of *B. stearothermophilus.* Strominger and co-workers agreed with Barnett on only one point: the Km for UDP-*M*-pentapeptide was of the order of 1.5 to 3 m*M*. Another property which remained unexplained was the activation of the enzymes by divalent cations. This activation disappeared when the synthetic peptides and esters were used and it was first supposed that the cations interacted with the UDP moiety of the nucleotide precursor. However, the activation did persist when the UDP was cleaved and a *M*-pentapeptide was used as a substrate. An interaction of the cations with the sugar moiety or with the -L-Ala-D-Glu→portion of the peptide (the free α-carboxylate of Glu?) might be assumed, but no clear answer can be supplied yet. The enzymes also released D-alanine from linear, uncross-linked peptidoglycan obtained by suspending cells in a penicillin-containing medium, inadequate for growth but suitable for cell-wall synthesis.[43] Unfortunately, the influence of divalent cations was not studied in this case.

When the enzymes were used as peptidases, the catalytic efficiency (V/Km) remained relatively constant for various substrates: UDP-*M*-pentapeptide (with L-R_3 = *meso*-A_2pm or L-Lys, see Figure 3), *M*-pentapeptide, and the synthetic peptide Ac_2-L-Lys-D-

Ala-D-Ala. When the terminal D-alanine was replaced by D-lactate (Figure 7) and the *B. subtilis* enzyme was used as an esterase, the V value increased by a factor of 15 and that of Km decreased by a factor of 9,[58] yielding a 135-fold increase in the catalytic efficiency. (See Table 12 the comparison is made using the first set of values for the peptide substrate; the conclusions are obviously wrong if the set of values between round brackets is used. We can offer no explanation for this discrepancy). On the basis of Scheme 3 and of what was known about the catalytic pathway of serine proteases, it was assumed that with the peptide substrate, k_3 was larger than k_2 (Equations 12 and 13), while with the ester, k_2 was larger than k_3 (Equations 14 and 15). Whether the substrate was a peptide, or an ester, the acyl-enzyme intermediate was the same and k_3 remained unchanged. For the esters, the value of k_2 was thus much larger than with the peptides, a property arising from the much higher reactivity of esters.

These considerations easily explained the observed variations. Moreover, assuming that K was little influenced by the replacement of a peptide by an ester bond, the increase in catalytic efficiency [$(k_{cat}/Km) = (k_2/K)$] reflected the increase of k_2 which would thus be more than 100-fold larger for the ester than for the peptide. On this basis, the following values were computed for the interaction between the *B. subtilis* enzyme and the two substrates: $K = 7$ m*M*; $k_3 = 2.5\ s^{-1}$; $(k_2)_{peptide} = 0.17\ s^{-1}$; $(k_2)_{ester} = 24\ s^{-1}$.

Both enzymes were partially inactivated by relatively high (0.1 to 200 m*M*) concentrations of various sulphydryl reagents (iodoacetamide, *N*-ethylmaleimide, dithio *bis*-nitrobenzoate, *p*-hydroxymercuribenzoate, $HgCl_2$). Strominger and co-workers suggested that a thiol group was present at the active sites of the enzymes.[79,80,139]

b. Transpeptidase Activity[184,186]

In agreement with the results of Reynolds and Barnett,[184] the *Bacillus stearothermophilus* purified enzyme catalyzed simple transpeptidation reactions using glycine and D-alanine as acceptors, although the T/H ratio at equivalent concentrations of glycine were much lower than those observed by the first authors. With the *Bacillus subtilis* enzyme, glycine was a much poorer acceptor and D-alanine was not utilized. Both enzymes catalyzed the transfer of the R-D-Ala moiety of the donor to hydroxylamine, yielding the corresponding hydroxamate. *Meso*-A_2pm, which was structurally very close to the natural acceptor, was not an acceptor, and negligible, if any, peptide polymer formation could be detected using the linear, uncross-linked peptidoglycan as donor-acceptor substrate.

Nishino et al.[186] studied the influence of the acceptor concentration on the release of D-alanine (T + H = total reaction) and on the transfer (T) and hydrolysis reactions (H). Table 13 depicts the results and their interpretation on the basis of Scheme 3. This simple scheme, however, did not explain two of the observations:

1. In some cases, the presence of the acceptor decreased the total reaction. This could not be attributed to a simple inhibition of the enzyme, since the same concentration of acceptor had no such effect in the presence of another donor.
2. The T/H ratio was not always proportional to the acceptor concentration. Such a proportionality should be expected in all simple bimolecular mechanisms.

c. Physiological Role

Experiments performed with agarose-bound ampicillin[187] indicated that a large proportion (2/3) of the enzyme was manifesting its activity on the outer face of the cytoplasmic membrane, a situation in agreement with a peptidoglycan-directed activity.

Inactivation of these enzymes, however, did not appear to be lethal for the cells.[47,79] Cephalothin killed the bacteria at concentrations which were too low to inactivate a sizable proportion of the enzymes. Conversely, 6-aminopenicillanate inactivated at

Table 13
B. SUBTILIS AND *B. STEAROTHERMOPHILUS* DD-CARBOXYPEPTIDASES: INFLUENCE OF THE ACCEPTOR ON HYDROLYSIS (H) AND TOTAL REACTION (T + H) WITH VARIOUS DONORS[186]

Enzyme	Donor	Acceptor	H	T + H	Interpretation on the basis of Scheme 3
B. stearothermophilus	*M*-pentapeptide[a]	NH_2OH	Constant	Increases	$k_2 \gg k_3$
B. subtilis	Ac_2-L-Lys-D-Ala-D-Ala	NH_2OH	Decreases	Constant	$k_2 \ll k_3$
B. stearothermophilus		Glycine	Decreases		
B. subtilis	Nucleotide precursor 1[b]	NH_2OH	Decreases	Increases	$k_2 \cong k_3$[d]
		NH_2OH	Decreases		
	M-pentapeptide[a]				
B. stearothermophilus	Ac_2-L-Lys-D-Ala-D-Ala	NH_2OH	Decreases	Decreases	?
	Nucleotide precursor 2[c]	NH_2OH	Decreases		

[a] *M*-pentapeptide = *M*-L-Ala-D-Glu→(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala.
[b] Nucleotide precursor-1 = UDP-*M*-L-Ala-D-Glu→(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala.
[c] Nucleotide precursor 2 = UDP-*M*-L-Ala-D-Glu→L-Lys-D-Ala-D-Ala.
[d] T/H not proportional to acceptor concentration.

Table 14
KINETIC CONSTANTS FOR THE INACTIVATION OF THE *B. SUBTILIS* DD-CARBOXYPEPTIDASE BY β-LACTAM ANTIBIOTICS[80]

	Membranes			Purified enzyme	
β-lactam	k_2 (s^{-1})[a]	K (m*M*)	k_2/K (M^{-1} s^{-1})	K (m*M*)	k_2/K (M^{-1} s^{-1})
Benzylpenicillin (4°C)	0.12	0.07	1,700	0.1	1,200
Phenoxymethylpenicillin (4°C)	0.06	0.075	800	0.13	600
Ancillin	0.1	3.2	31	5.5	20
Propicillin	0.4	1.1	360	2.0	200
Methicillin	0.11	10	11	14	7
Ampicillin	0.08	0.56	140	0.73	110
Cloxacillin	0.02	2.7	7	4.6	5
Oxacillin	0.1	7.5	13	12.7	8
Cephalosporin C	0.023	2.9	8	0.10	230
Cephalothin	0.1	13	8	28	4

Note: The model was $E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \overset{k_2}{\rightleftharpoons} EI^*$ where K is the dissociation constant of EI and k_2 a first-order rate constant. Experiments were performed at 37°C unless otherwise stated.

[a] The k_2 values were the same for the membrane-bound and the purified enzyme.

least 95% of the *B. subtilis* DD-carboxypeptidase at nonlethal concentrations. Moreover, inactivation of 95% of the carboxypeptidase did not influence the overall degree of cross-linking of the peptidoglycan.[188]

d. Interaction with β-Lactams

The formation of a covalent complex in a two-step reaction between the *B. subtilis* enzyme and penicillin was proposed by Blumberg and Strominger[47] and Umbreit and Strominger.[80] They compared the values of K and k_2 for a series of β-lactams using the membrane-bound and the purified enzymes (Table 14). If one excepts cephalosporin C, both preparations yielded very similar results indicating that solubilization had not drastically altered the properties of the protein. In contrast with the R61 exocellular enzyme, for which the efficiency of a given inactivator appeared to depend mainly upon the k_2 value,[87] the variations of the k_2/K ratio for the *B. subtilis* enzyme seemed

to depend mainly upon the K value. However, this conclusion should be considered with a good deal of care since (1) the degradation of EI* which at that time had not been discovered was not taken into account in the calculations and (2) from the published data, it seems that the extrapolation of the inverse graphs ($1/k_a$ vs. $1/[I]$) were very near the origin, making the individual values of k_2 and K unreliable.

When 1/P values were plotted vs. 1/S for various inactivator concentrations, linear Lineweaver-Burk graphs were obtained.[79,80] It seems very likely that the explanation which was proposed above (see Section III.B.5.c) for the R61 exocellular enzyme was also valid in the present case.

Complexes prepared with [^{14}C]benzylpenicillin and both *B. subtilis* and *B. stearothermophilus* enzymes were isolated. In the early experiments,[185] very low molar ratios of penicillin/enzyme were obtained (0.2 and 0.04 for the *B. subtilis* and *B. stearothermophilus* enzymes, respectively). Waxman and Strominger[117], however, very clearly demonstrated that a quasistoichiometric complex could be isolated and that the poor ratios were probably due to the choice of inappropriate conditions during the isolation of the labeled complexes.

As with the R61 exocellular enzyme, spontaneous degradation of the complexes was observed and the enzyme activity was concomitantly recovered.[185] The optimum pH for the reactivation of the *B. stearothermophilus* enzyme was 5.5 and, from the influence of temperature, an activation energy of 88 kJ/mol was calculated for the reaction. Denaturation of the complexes by 1% SDS at 25 to 37°C did not cause an important loss of radioactivity and suppressed any further release of the label. In contrast, denaturation at 100°C in the presence of SDS resulted in a very important loss of radioactivity.[185]

e. Nature of the Degradation Products

When [^{14}C]benzylpenicllin was used, the label was on the carbonyl group of the phenylacetyl moiety. Degradation of the native complex was accompanied by the release of a [^{14}C]-labeled compound which was not penicilloic acid and was later identified as phenylacetylglycine,[189] the same product as that obtained with the exocellular R61 and R39 enzymes upon reactivation. The fate of the thiazolidine moiety of the antibiotic was studied by Hammarström and Strominger.[190] They treated isolated membranes of *B. stearothermophilus* with cephalothin to saturate the other PBPs, then with [^{35}S]benzylpenicillin. The complex was completely degraded by a 50-min incubation at 55°C and pH 6.5. The product was tentatively identified as 5,5-dimethyl-Δ^2-thiazoline-1-carboxylate (see Figure 13). However, experiments performed with authentic 5,5-dimethyl-Δ^2-thiazoline-1-carboxylate demonstrated that the half-life of this compound under the same conditions was about 3 min: it underwent spontaneous degradation into *N*-formylpenicillamine.[109] It was thus very unlikely that Hammarström and Strominger had obtained any of the intact thiazoline. Moreover, the authentic compound did not crystallize in the solvent utilized by these authors for the cocrystallization of the labeled and the cold thiazoline, but *N*-formylpenicillamine precipitated quite well under the same conditions. The only possible explanation was that the labeled compound had cocrystallized with *N*-formylpenicillamine, present as an important impurity in the thiazoline used by Hammarström and Strominger. There was, however, no evidence indicating that the thiazoline had not been formed as an unstable intermediate during the degradation process. As with the R61 enzyme, this compound is probably the best candidate as a primary product of the reaction, but this hypothesis remains to be confirmed.

The presence of hydroxylamine[28] influenced the degradation pathway, and penicilloyl-hydroxamate was formed, in addition to phenylacetylglycine and a product tentatively identified as phenylacetylglycyl hydroxamate (Figure 18).

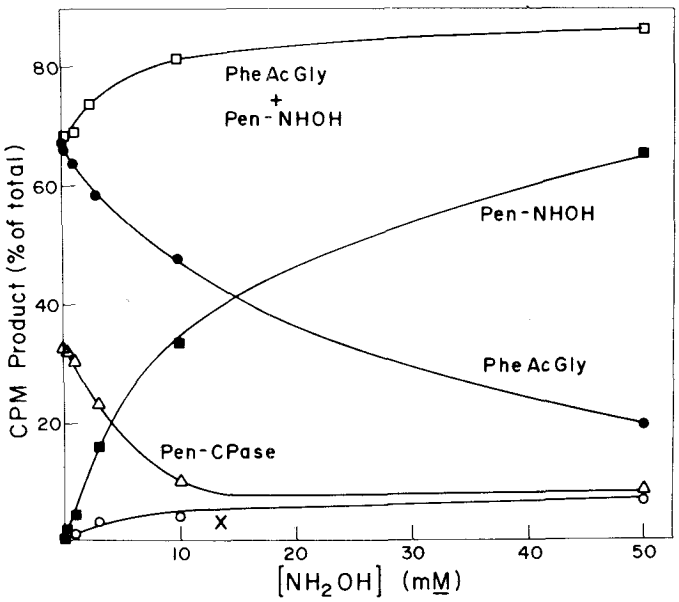


FIGURE 18. Influence of hydroxylamine on the degradation of the *Bacillus stearothermophilus* penicilloyl-carboxypeptidase complex. The isolated complex was incubated for 30 min at pH 7 and 55°C in the presence of various concentrations of hydroxylamine. Fragmentation products were separated by paper electrophoresis. Compound X is probably phenylacetylglycyl hydroxamate. (From Waxman, D. J. and Strominger, J. L., *The Chemistry and Biology of β-Lactam Antibiotics*, Vol. 3, Morin, R. B., Ed., Academic Press, New York, 1982, 209. With permission.)

f. Binding Site of β-Lactams and Substrates

On the basis of the reversal of penicillin binding by neutral hydroxylamine,[182] which cleaves thioesters but not normal esters, it was first proposed that penicilloylation of an essential sulphydryl group was responsible for the inactivation of the enzymes by β-lactams. This hypothesis was corroborated by the absence of sensitivity of the enzymes to typical serine protease inactivators such as diisopropylfluorophosphate and by their loss of activity and penicillin binding capacity in the presence of thiol reagents. Umbreit and Strominger[80] reported that, of the four sulphydryl groups of the *B. subtilis* enzyme which could be titrated by dithiobisnitrobenzoate, one disappeared after reaction with benzylpenicillin. This latter experiment was, however, performed with a very low concentration of enzyme (2.5 μM) and the results should be considered with caution. With both enzymes, indications were later obtained that the hydroxylaminolysis of the penicilloyl enzymes was enzymatically catalyzed: no such reaction occurred when the complexes were first denatured,[185,191] and these results were more in agreement with the penicilloylation of a hydroxyl group, i.e., of a Tyr, Thr, or Ser residue.

After digestion of the *B. subtilis* [^{35}S] or [^{14}C]benzylpenicillin-carboxypeptidase complex with pronase, Georgopapadakou et al.[192] isolated a labeled peptide whose composition was Asn, Ser, Gly_2, Ala, Ile with Gly as the N-terminal residue. Serine was thus the only residue to which the penicilloyl moiety could be attached. Digestion by trypsin yielded a larger peptide (11 residues) whose N-terminal residue was also Gly and which also contained a serine. These findings were in agreement with the previously reported demonstration of the penicilloylation of a serine residue in the R61 exocellular D-alanyl-D-alanine peptidase (although different enzymes might use different mechanisms).

More detailed information about the penicillin binding site was obtained by Yocum

Table 15
PROPERTIES OF THE DD-CARBOXYPEPTIDASES OF *BACILLUS MEGATERIUM* AND *BACILLUS LICHENIFORMIS*

Strain	PBP n°	MW	Acceptors for transpeptidase activity	Km for Ac_2-L-Lys-D-Ala-D-Ala	Interaction with benzylpenicillin: Temperature (°C)	Interaction with benzylpenicillin: k_2/K ($M^{-1}s^{-1}$)	Interaction with benzylpenicillin: k_3 (s^{-1})	Optimum pH	Ref.
B. megaterium KM	5	45,000	—	—	37	200	3×10^{-4}	—	
			—	0.8 m*M*	20	100	1.2×10^{-4}	5.2 6—7[a]	197—199
			Glycine	1.7 m*M*	25	—	0.6×10^{-4}	7	
			D-alanine		0	50	—	7	200
B. licheniformis 94	4	46,000	Glycine D-alanine	—	35	80	$<0.6 \times 10^{-4}$	—	184, 201

[a] The lower optimum pH was observed in the presence of divalent cations (3 m*M* $MgCl_2$), the higher in their absence. The activity of cation-deprived membranes was 30 to 60% of that observed in the presence of 3 m*M* $MgCl_2$, depending on the ionic strength.

membranes of their respective organisms. For the *B. megaterium* enzyme, there were some differences between the results of Diaz-Maurino et al.[199] and those of Marquet et al.,[200] but these could probably be attributed to the addition of divalent cations to the incubation mixtures by the first authors. A transpeptidase activity could only be demonstrated with glycine and D-alanine. No dimer formation was recorded with a "natural" donor-acceptor substrate and *meso*-diaminopimelic acid was not an acceptor. These DD-carboxypeptidases have been excluded as possible lethal targets of β-lactam antibiotics.[201]

True transpeptidase activity has been observed using membranes and wall-membrane preparations of these organisms.[184] This activity could be differentiated from the DD-carboxypeptidase by a much higher sensitivity to penicillins. No enzyme activity could be directly attributed to the high molecular weight PBPs of these strains. The indirect evidence relating some of the proteins with a possible killing target will be discussed in the section dealing with PBPs.

McArthur and Reynolds purified the membrane DD-carboxypeptidase of *B. coagulans* to homogeneity after solubilization with urea and Genapol.[140] The molecular weight of the protein (29,000) was markedly lower than that of the corresponding enzyme in other Bacilli. It catalyzed transpeptidation only with glycine and D-alanine as acceptors. It was activated by several divalent cations, Pb^{++} being the most effective at about 10 m*M*. The enzyme contained one sole sulphydryl residue, whose blocking by thiol reagents induced a loss of activity. Unfortunately, no more details are presently available.

Finally, a DD-carboxypeptidase activity has been described in the membrane of *B. sphaericus*, which exhibited an optimum pH of 8 and was slightly activated by Mg^{++}, Mn^{++}, and Co^{++} ions.[202]

5. PBPs 4, 5, and 6 of Escherichia coli

a. From Mixtures of Enzymes to Homogeneous Proteins

It has only recently become apparent that these three enzymes were distinct proteins and the products of different genes. Before 1980, many experiments were performed using mixtures of two or three of the enzymes, even when the authors assumed their preparations to be homogeneous.

It was rapidly recognized, on the basis of the reversibility of the penicillin inhibition, that the membrane DD-carboxypeptidase activity could not be assigned to the enzymes responsible for the cross-linking of the peptidoglycan.[159] The membranes also contained a LD-carboxypeptidase or carboxypeptidase II activity, which was not penicillin sensitive. In 1974, Nguyen-Distèche et al.[160-162] separated two distinct enzymatic fractions, both exhibiting DD-carboxypeptidase, transpeptidase, and dimer-hydrolyzing activities, and, in 1976, Tamura et al.[203] purified three DD-carboxypeptidases which they described as carboxypeptidases IA, IB, and IC. Only the method of extraction distinguished carboxypeptidase IB from carboxypeptidase IC; the former was solubilized from the membranes using LiCl, while the latter was obtained from the supernatant after disruption of the cells in a Ribi fractionator. Otherwise, the two proteins appeared to be identical. It was proposed that carboxypeptidase IB might be the membrane-bound precursor of IC and carboxypeptidase IB was identified as PBP 4 on the basis of its electrophoretic behavior and of mutation experiments (see below).

Enzyme IA solubilized by Triton X-100® and purified to "homogeneity" nonetheless behaved as a doublet upon gel electrophoresis in the presence of SDS. This doublet was identified as PBPs 5-6.[204] Amanuma and Strominger finally demonstrated in 1980 that carboxypeptidase IA was in fact a mixture of two distinct enzymes, generally exhibiting similar properties, but clearly distinguishable on the basis of the half-lives of the complexes they formed with benzylpenicillin and of the peptide maps obtained

after digestion with various proteinases.[205] Genetic data confirmed that the two proteins were the products of distinct genes.

An analysis of the properties of these enzymes is made rather difficult by the fact that some experiments, performed with the mixtures, were not repeated using the pure proteins.

b. Properties

Table 16 summarizes the properties of the proteins. For PBPs 5 and 6, some data are for the mixture, others for the pure proteins. There does not seem to be any major discrepancy if one excepts the specific activities on the synthetic peptides and depsipeptide that we computed for the separated proteins from the data of Amanuma and Strominger[205] and on the basis of the Km measured with the mixture.[203] Some V values are tenfold higher than those determined earlier with the mixture. We can offer no simple explanation for this difference. One could assume that the carboxypeptidase IA of Tamura et al.[203] contained inactive impurities, but with UDP-*M*-pentapeptide as substrate, the difference is much less important. Fractions A and B of Pollock et al.[160] and Nguyen-Distèche et al.[161] probably corresponded to carboxypeptidases IA and IB, respectively, as indicated by their behavior on DEAE-ion exchangers and their sensitivities to β-lactams. However, fraction A could (rather inefficiently) catalyze the formation and hydrolysis of peptide dimers, a property which was not observed with the purified carboxypeptidase IA or with PBPs 5 and 6. It is thus possible that fraction A contained other enzymes besides these PBPs. Comparing fractions A and B, it was also surprising to note that fraction B, which catalyzed true transpeptidation (dimer formation) more efficiently, was also less efficient in the utilization of glycine and D-alanine as acceptors. It is unfortunate that no attempt was made to catalyze the formation of dimers with purified carboxypeptidase IB.

With carboxypeptidase IA, the total reaction (T + H) increased or decreased in the presence of high concentrations of glycine or low concentrations of hydroxylamine, respectively.[203] This latter effect was considered as a simple inhibition, while the effect of glycine was explained on the basis of Scheme 3, assuming a rate-limiting deacylation.

This assumption was, however, in disagreement with the results obtained when the kinetic parameters for the depsipeptide were compared to those observed with the corresponding peptide (Table 16). The depsipeptide exhibited increased V and decreased Km values, which suggested a rate-limiting acylation with the peptide. The easier trapping of acyl-enzyme after reaction of the enzyme with pCMB also suggested a rate-limiting acylation (see Section IV.B.5.d). Thus, the acceleration of the total reaction at high glycine concentrations remained unexplained, as well as, conversely, the strong inhibition when hydroxylamine was used as an acceptor.

c. Reaction with Penicillin

PBP 4 was much more sensitive to benzylpenicillin than PBPs 5 and 6, for which the rates of inactivation were similar.[203,205] However, the velocity of complex degradation (k_3) was higher with PBP 5 (half-life at 25°C = 12 min) than with PBP6 (half-life = 40 min). Surprisingly, very poor binding of [^{14}C]benzylpenicillin was observed with carboxypeptidase IB (less than 1% of the stoichiometric amount), although the enzyme was clearly inactivated and the protein was well labeled and detected as a PBP.[203] In the first experiments, the labeling of carboxypeptidase IA was also rather low (10% of the stoichiometric amount), but nearly stoichiometric binding was later observed with the separated PBPs 5 and 6.[205] Tamura et al. also reported that penicilloic acid was formed upon degradation of the EI* complex with both carboxypeptidases IA and IB.[203] However, this compound was detected and identified after prolonged incubations of the enzymes with a large excess of benzylpenicillin and the presence of a small

amount of β-lactamase could not be excluded. The identification of the released product should be repeated after isolation of the EI* complex.

Both UDP-*M*-pentapeptide and Ac_2-L-Lys-D-Ala-D-Ala protected carboxypeptidase IA from inactivation by benzylpenicillin.[207] At concentrations close to the Km, this protection was even more than expected, the rate of inactivation being decreased by a factor 5 instead of 2. Using the [^{14}C]-labeled depsipeptide substrate and the limited proteolysis technique, Yocum et al.[72] obtained clear indications that benzylpenicillin and substrate were probably bound at the same site of PBP 6.

d. Presence of a Sulphydryl Group near the Active Site

Low concentrations (5 to 10 μ*M*) of pCMB efficiently inactivated carboxypeptidase IA.[203] With dithiobisnitrobenzoate and *N*-ethylmaleimide, inactivation was only observed at concentrations above 1 m*M*,[207] where the specificity of these reagents for sulphydryl groups should be considered as doubtful. When a ternary mixture (enzyme + pCMB + substrate) was incubated, the substrate UDP-*M*-pentapeptide (where L-R_3 was *meso*-A_2pm) exhibited a clear protective effect which again was rather disproportionate, a 6-μ*M* concentration (0.0015 times the Km value) decreasing the rate of inactivation by a factor of 3.[203] Surprisingly, pCMB had no effect on the rate of binding of benzylpenicillin, but it completely inhibited its release.[207] This latter inhibition was in good parallelism with that of the enzymatic activity. The interpretation of the authors was that a pCMB-sensitive sulphydryl group was involved in catalysis of the acyl-enzyme hydrolysis and not of its formation. In agreement with this hypothesis, Curtis and Strominger succeeded in labeling the enzymes (both PBPs 5 and 6) with [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala in the presence of pCMB, a reaction which had been very inefficient in the absence of the sulphydryl reagent.[207]

e. Sequence Data

Waxman et al. determined the sequence of 28 and 25 residues, starting from the N-terminus of PBPs 5 and 6, respectively, and recorded a homology of 36% between the two proteins.[213] This degree of homology was confirmed by Broome-Smith et al.,[121] who deduced the complete sequence of PBP 5 and of its signal peptide from the nucleotide sequence of the dacA gene. They utilized the known N-terminal sequence to align the start of the mature protein with the amino acid sequence derived from the DNA sequence. The protein contained 374 amino acids. Residues 9 to 52 exhibited a rather high degree of homology (45%) with residues 1 to 44 of the DD-carboxypeptidase (PBP 5) of *B. subtilis*. The homology was even more convincing in the area surrounding the active site Ser 36 of this latter enzyme (see Table 17 and Figure 19). The homology reached 60% for residues 20 to 44 of the *B. subtilis* enzyme (28 to 52 of *E. coli* PBP 5) and 80% for residues 35 to 44. Going further to the C-terminus, the homology became much less extensive (only about 20% in the next 27 residues). The homology with the first 40 residues of the *B. stearothermophilus* DD-carboxypeptidase was also very convincing. When compared to the two *Bacillus* enzymes, the first 25 residues of PBP 6 exhibited a lower degree of homology, but the area near the active serine was not known for PBP 6 and could thus not be included in the comparison. PBP 5 contained only one cysteine residue — Cys 115 — and, in consequence, no disulfide bridge.

f. Physiological Role and Genetic Data

Mutants were obtained in which the activity of carboxypeptidase IB-IC and PBP 4 had simultaneously disappeared.[209,214] In one of the mutants,[214] only 10% of the DD-peptidase activity of the parent was detected, which might indicate that carboxypeptidase IA had also been strongly decreased. Other mutants[209] behaved more as expected for a mutation involving only carboxypeptidase IB. After cell disruption, the DD-carboxyeptidase activity in the supernatant nearly disappeared and that in the particulate

Table 16
PROPERTIES OF THE LOW MOLECULAR WEIGHT PBPs OF *ESCHERICHIA COLI*

	PBP 4	PBP 5	PBP 6	Remarks and references
Molecular weight	41,000	34,000	32,000	203
	49,000	42,000	40,000	204, 206
		41,340		121 (from the sequence)
Identification	Cbases IB and IC fraction B[a]	Cbase IA Fraction A[a]		203 160—162
Interaction with DEAE ion exchangers	Retained	Not retained		160—162
Effect of pCMB	Activation	Inhibition		203
Effect of Me^{++}	Activation	Inhibition		203
Enzymatic properties				
Carboxypeptidase activity				
On UDP-*M*-pentapeptide[c]				
km (m*M*)	1—2	4.2		203
V [μmol min^{-1} mg P^{-1}]	2—4	1.0		203
		2.4	0.6	205[b]
On Ac_2-L-Lys-D-Ala-D-Ala-OH				
Km	—	14; 19		58, 203
V	Not detected	0.3; 0.42		58, 203
V		4.4	1.7	205[b]
On Ac_2-L-Lys-D-Ala-D-Lac				
Km	—	4.2		58
V	—	1.6		58
V	—	18	27	205[b]
Ratio of dimer hydrolysis to carboxypeptidase activity[f]	1.6	0.28		161 *Substrates:* UDP-*M*-pentapeptide (Cbase) and bidisaccharide peptide dimer (dimer hydrolysis)

Transpeptidation (acceptor = glycine)				
Glycine concentration for which T/H = 1				
Donor = UDP-*M*-pentapeptide	600—800 m*M*	100 m*M*		203—205
Donor = Ac_2-L-Lys-D-Ala-D-Ala	—	30 m*M*		203—205
		15 m*M*	20 m*M*	203—205
Transpeptidation (donor-acceptor = pentapeptide[e])				
Ratio of dimer formation to hydrolysis[f]	0.33	0.05		161
Interaction with benzylpenicillin				
k_2/K (M^{-1} s^{-1})	7,000 (37°C)	175 (37°C)		—[c]
		120 (25°C)	115 (25°C)	—[c]
k_3 (s^{-1})	0.4×10^{-3} (30°C)	2.3×10^{-3} (37°C)		203
		0.7×10^{-3} (25°C)		207
		1×10^{-3} (25°C)	0.3×10^{-3} (25°C)	205, 208
Gene	dacB (68 min)	dacA (13.7 min)	dacC	209—212
Polarity index	—	0.45	0.45	205
Optimum pH	>9	9—10	9—10	203, 205

[a] Although cross-contamination of the fractions was very unlikely, both might contain other enzymes. Indeed, some differences were observed in the sensitivities to ampicillin of the various activities of each fraction.

[b] Computed by us on the basis of the activities at low substrate concentrations given in Reference 205 and of the Km for the *mixture* given in Reference 203. For this reason, these values should be considered with a great deal of caution.

[c] Computed by us from the data of Tamura et al.[203] using Equation 7.

[d] From the data of Amanuma and Strominger.[205]

[e] The pentapeptide was that of Figure 9A at a concentration of 1.5 m*M*.

[f] Results obtained with fractions A and B. These results might reflect the activities of more than one enzyme.

Table 17
SEQUENCE HOMOLOGY BETWEEN *ESCHERICHIA COLI* PBPs 3, 5, AND 6 AND THE *BACILLUS SUBTILIS* AND *BACILLUS STEAROTHERMOPHILUS* DD-CARBOXYPEPTIDASES (PBPs 5)

Compared pairs and residues	Ratio of identical residues to total residues	Homology (%)
Escherichia coli PBP3 261—277 *Escherichia coli* PBP5 19—35	9/17	53
Escherichia coli PBP3 295—315 *Escherichia coli* PBP5 32—52	4/21	19
Escherichia coli PBP3 261—272 *Escherichia coli* PBP6 14—25	4/12	33
Escherichia coli PBP5 6—30 *Escherichia coli* PBP6 1—25	9/25	36
Escherichia coli PBP3 261—277 *Bacillus subtilis* PBP5 11—27	4/17	24
Escherichia coli PBP3 295—315 *Bacillus subtilis* PBP5 24—45	3/21	14
Escherichia coli PBP5 9—52 *Bacillus subtilis* PBP5 1—44	20/44	45
Escherichia coli PBP6 4—25 *Bacillus subtilis* PBP5 1—22	5/22	23
Escherichia coli PBP3 261—277 *Bacillus stearothermophilus* PBP5 11—27	8/17	47
Escherichia coli PBP3 295—311 *Bacillus stearothermophilus* PBP5 24—40	4/17	24
Escherichia coli PBP5 9—48 *Bacillus stearothermophilus* PBP5 1—40	16/40	40
Escherichia coli PBP6 4—25 *Bacillus stearothermophilus* PBP5 1—22	6/22	27
Bacillus subtilis PBP5 1—40 *Bacillus stearothermophilus* PBP5 1—40	24/40	60

Note: The numbering of the residues of *Escherichia coli* PBP 3 is that deduced from the gene nucleotide sequence, including the signal pepide. For the four other enzymes, numbering starts at the N-terminus of the mature protein.

fraction was significantly reduced. In these latter cases, not only PBP 4, but also the carboxypeptidase activity which was retained on DEAE ion exchangers, were absent. These dacB mutants grew normally under a wide range of conditions and synthesized normally cross-linked peptidoglycan. It was thus possible that alternative pathways might exist which could compensate for the lack of this enzyme. Interestingly, Tomioka and Matsuhashi observed in their mutants a residual dimer hydrolyzing activity which was not inhibited by 3 m*M* benzylpenicillin and might be due to a penicillin-

resistant cell-bound "endopeptidase" activity which was also found in *Escherichia coli.*[215]

In another mutant, deficient in carboxypeptidase IA (dacA) and isolated by Matsuhashi et al.,[210] the soluble carboxypeptidase and dimer hydrolyzing activities were unchanged, while the particulate carboxypeptidase was strongly reduced. The dacA mutants still possessed PBP 5, but the mutant protein no longer released the labeled antibiotic,[216] thus behaving as the pCMB-treated parent PBP 5. It was thus possible that the mutated protein was unable to catalyze hydrolysis of the acyl-enzyme, which would explain both its absence of carboxypeptidase activity and its retention of penicillin-binding capacity. The nucleotide sequence of the gene coding for the deacylation-impaired dacA mutant has recently been established.[217] Surprisingly, the mutation involved the replacement of Gly 105 by an Asp residue, but Cys 115 was unaltered. The influences of this residue and of the Gly-Asp substitution on the catalysis of the deacylation reaction are thus, more than ever, totally mysterious. A complete deletion of the dacA gene, involving the absence of PBP 5, was also obtained.[211]

DacA, dacB, and dacC, as well as double dacAdacB mutants, grew normally and their morphology did not significantly differ from that of the parent strain.[28,209-212] However, the dacA and dacC mutations resulted in elevated sensitivity to some β-lactams.[28,218] Moreover, cells overproducing PBP 5 grew as osmotically stable spheres and also exhibited an increase in the degree of cross-linking of the newly synthesized peptidoglycan.[219] If, as observed in *Gaffkya homari,* the transpeptidase involved in septation was assumed to require tetrapeptides as acceptors, increased conversion of pentapeptides into tetrapeptides would stimulate cross-linking by the cell division system. A similar implication of the carboxypeptidase activity in the switching between cell elongation and cell division has been proposed by Mirelman[180] on the basis of experiments showing a higher level of carboxypeptidase activity immediately before cell division. Nonetheless, the physiological role of the three DD-carboxypeptidases of *Escherichia coli* is far from being clearly understood. It would obviously be quite interesting to construct a mutant completely and simultaneously deleted in the dacA, dacB, and dacC genes. This would help in establishing whether growth in the total absence of carboxypeptidase activity is possible.

6. Gaffkya homari

Studies performed with wall-membrane preparations indicated that the peptidoglycan cross-linking system in *Gaffkya homari* involved three enzymes: a transpeptidase, a DD-carboxypeptidase, and a LD-carboxypeptidase.[48,49,220,221] Of these, the DD-carboxypeptidase was the most penicillin sensitive and the ID_{50} values were similar to the concentrations required to inhibit the growth of the bacteria by 50%. Incorporation of nascent into preexisting peptidoglycan by transpeptidation occurred not only when .UDP-G and UDP-*M*-pentapeptide were used as substrates, but also when UDP-*M*-tetrapeptide was used. The structure of the pentapeptide was L-Ala-D-Glu⌋→ L-Lys-D-Ala-D-Ala and when the free ε-amino group of Lys was acetylated, very little incorporation was observed. These results indicated that the incorporation was mainly due to a transpeptidation reaction in which pentapeptides in the preexisting peptidoglycan were used as donors and the tetrapeptides in the nascent chains as acceptors, in total contrast to the mechanism observed in Bacilli. The data also indicated that only a tetrapeptide could serve as acceptor, making the hydrolysis of the C-terminal D-alanine of some of the pentapeptides in the nascent peptidoglycan a prerequisite for the transpeptidation. Penicillin inhibited transpeptidation indirectly by blocking the D-alanyl-D-alanine carboxypeptidase, thus making the acceptor unavailable.[48,49] In fact, when the UDP-*M*-tetrapeptide was directly included in the assay mixture, inhibition of the transpeptidation occurred only at much higher concentrations of benzylpenicillin, more than 2500-fold that required to inhibit both the growth and the carboxypeptidase.

At the present time, this mechanism of incorporation has only been demonstrated in *Gaffkya* clearly putting this organism into a class by itself, for which the inactivation of a DD-carboxypeptidase is the lethal event in penicillin action.

Moreover, a LD-carboxypeptidase was also detected, splitting the L-Lys-D-Ala bond in tetrapeptides and probably acting at the level of the nascent peptidoglycan.[220,221] The presence of tripeptide units in that linear polymer appeared to be necessary for its efficient utilization as a transpeptidation acceptor. The only β-lactam which exhibited a notable inhibitory effect on the LD-carboxypeptidase was thienamycin ($ID_{50} \cong 10\ \mu M$; $k_2/K = 40\ M^{-1}s^{-1}$ if Scheme 1 is applicable and if k_3 is smaller than $10^{-4}s^{-1}$). The mechanism of this inhibition remains completely undetermined. It might be useful to remember that this carbapenem exhibited unique properties, being for example a substrate for the renal dehydropeptidase[222] and the only β-lactam whose side chain on C6 is in an α stereo position, and which is nonetheless active as a D-alanyl-D-alanine peptidase inactivator (see Table 2) and as an efficient antibacterial agent.

V. PBPs AS STUDIED BY OTHER METHODS

As already mentioned at the beginning of Section IV, a very large number of articles have been published presenting PBP patterns in a wide variety of bacterial species. A complete discussion of all these data is well beyond the scope of the present review. We will only try to present the results which have established a role for some PBPs in the maintenance of the shape, properties, and viability of the bacteria. Many of the meaningful results have been obtained by genetic and selective inactivation methods. The contribution of enzymology to these studies has been marginal at best. In fact, in the few cases where enzymatic activities could be attributed to the PBPs, they were exceedingly low and it was, on that sole basis, impossible to explain the clearly different physiological roles of the various proteins. For example, it will be seen that PBPs 1, 2, and 3 of *Escherichia coli* are, respectively, involved in elongation, shape determination, and septation of the bacterial cell. In vitro, they all exhibited nearly indistinguishable enzymatic activities. Although it is reasonable to assume that, in the future, the enzymatic properties of these proteins will be understood and related to their respective physiological roles, this particular field is still in its infancy.

The numerous experimental data which have been collected to date confirm the validity of Scheme 1 for the interaction between PBPs and β-lactams. However, the consequences of such a mechanism on the quantitative interpretation of the results have been too often overlooked. For this reason, before analyzing the data and their implications, we would like to present a critical discussion of the many pitfalls which should be avoided when conducting this type of study.

A. A Theoretical Approach to the Interpretation of PBP Patterns

In clear-cut situations when, for instance, the complete disappearance of one PBP after either a mutagenic treatment or a specific inactivation can be related to a clear morphological defect, the following points need not necessarily be considered with too much attention. If, however, quantitative estimations are to be used to explain differences in the sensitivity of a strain to various β-lactams or the higher resistance of certain mutants, great care should be exercised in the analysis.

1. General Remarks

It is evident that a radioactive molecule only reveals PBPs for which it has sufficient affinity. Recently, [^{14}C]cefotaxime was found to bind to at least five *Escherichia coli* membrane proteins which were little or not visualized by [^{14}C]benzylpenicillin.[223] Conversely, characterization of a protein as a PBP might be somewhat arbitrary: under conditions of high concentration and long contact times, insulin, lysozyme, and bovine

serum albumin did bind [^{14}C]benzylpenicillin.[224] In reference to Scheme 1, one could wonder which minimum value of k_2/K still qualifies a protein as a PBP. Finally, since the detection after electrophoresis involves the denaturation of the complex, a protein with a high affinity (low K) but low reactivity (low k_2) will be, as discussed above (Section IV), grossly underestimated.

2. Titration

The affinity of a PBP for a β-lactam is usually characterized by a B_{50} or an ED_{50} value. Direct measurements (B_{50}) are only possible when the β-lactam is radioactive. In the other cases, an ED_{50} (exclusion dose) value is determined by a competition experiment with a labeled, standard compound, most often benzylpenicillin. The ED_{50} is defined as the concentration of the cold β-lactam which reduces the binding of the labeled one by 50%.

When the sensitivity of a PBP is very high, the ID_{50}, the B_{50} and the ED_{50} may all be equivalent to half the concentration of the PBP present. This titration effect has already been discussed by Reynolds and by us.[89,201] Moreover, when very low concentrations of β-lactam and large quantities of membranes are used, the total number of PBP molecules might become larger than the number of β-lactam molecules available, for which the PBPs thus compete. This latter situation is well beyond a simple analytical treatment.

3. The Presence of β-Lactamases

Reynolds and Chase have clearly demonstrated that a decreased labeling or an increased B_{50} could result from an increase in the membrane protein concentration.[225] This can be explained by a titration effect as discussed above, but the presence of a membrane-bound β-lactamase which has been described in several strains can exacerbate this effect. Even if the initial concentration of β-lactam is much larger than the total concentrations of the PBPs, a β-lactamase can rapidly hydrolyze the excess of β-lactam and, eventually, the reaction with the PBPs will slow down and stop because of the depletion of reagent. Chase and Reynolds have advised to keep the β-lactam concentration constant while progressively diluting the membrane preparation until the B_{50} no longer decreases with further dilution. In the case of a very active β-lactamase, this might not be possible, and the utilization of a specific β-lactamase inactivator, such as clavulanate or β-iodopenicillanate, might become necessary. To determine whether a low amount of β-lactamase is present, the specific β-lactamase activity of the preparation should be measured and compared with the activity which can be deduced from the k_3 values and the concentrations of the various PBPs (see the example of *Staphylococcus aureus* PBP 4 in Section IV.B.2.b).

4. Kinetic Considerations

a. Direct Binding of a Radioactive β-Lactam

The reaction between a β-lactam and a PBP may be interrupted before or after the steady state has been reached, and the time required to reach this steady state varies for each protein. The relative labeling of different proteins in a mixture may change depending on the time of contact. Assume that, for protein E_1, $k_2 = 1\ s^{-1}$ and $k_3 = 10^{-3}\ s^{-1}$, while for protein E_2, $k_2' = 0.01\ s^{-1}$ and $k_3' = 10^{-6}\ s^{-1}$. If the value of K is similar for both proteins and $[I] = K \times 10^{-3}$, E_1 and E_2 are, respectively, 50 and 90% labeled at the steady state, but this steady state is reached after about 20 min for E_1 and only after 60 hr for E_2. Assuming a similar concentration for both proteins, only E_1 will be detected after 30 min, but after 40 to 50 hr, E_2 will be more heavily labeled. If, for a given protein, k_3 is of the same order of magnitude as k_2, or worse, if k_2 is smaller than k_3, the protein is never completely immobilized in the form of EI* complex, even if $[I] \gg K$. Indeed, under these circumstances:

$$\left(\frac{EI^*}{E_o}\right)_{ss} = \frac{k_2}{k_2 + k_3} \tag{16}$$

and all the other enzyme molecules occur as the reversible complex EI. Although the enzymatic activity is completely inhibited, the absolute abundance of the protein will be grossly underestimated, unless the individual values of k_a and k_3 are measured. Unfortunately, the value of k_3 is too rarely determined in PBP studies.

According to Model 1 and Equation 1, saturation curves should not expand over more than two orders of magnitude: starting from the B_{50} value, a tenfold increase or decrease in concentration should result in nearly complete saturation (>90%) or absence of labeling (<10%), respectively. "Flat" saturation curves must be considered as an indication of an abnormal situation. For instance, two or more different proteins might not be resolved, the quantification technique might be inappropriate, or Model 1 might not be applicable.

b. Direct Competition between Radioactive and "Cold" β-Lactams of Different Structures

When a protein is exposed to two β-lactams I_1 and I_2, the velocities of formation of complexes EI_1^* and EI_2^* and their individual concentrations at the steady state are reduced. Assuming, to simplify the discussion, that $[EI_1]$ and $[EI_2]$ remain negligible throughout the experiment, the influence of the time of contact can be illustrated under the following extreme conditions where $(k_a)_{I_1} \gg (k_a)_{I_2}$; $(k_3)_{I_1} \gg (k_3)_{I_2}$ and

$$\left(\frac{k_3}{k_a + k_3}\right)_{I_1} \cong \left(\frac{k_3}{k_a + k_3}\right)_{I_2} \cong 0.1$$

A pseudosteady state is reached rapidly with I_1, immobilizing about 90% of the protein as EI_1^*, and reducing the rate of the reaction with I_2 to 10% of its expected value in the absence of I_1. However, as the reaction approaches the true steady state, $[EI_1^*]$ and $[EI_2^*]$ become similar and smaller than the values that would be observed for each β-lactam acting individually. Thus, if the reaction is terminated before the true steady state is reached, $[EI_2^*]$ may be substantially lower and $[EI_1^*]$ larger than the concentrations observed under steady-state conditions.

c. Indirect Competition Between "Cold" and Radioactive β-Lactams of Different Structures

When this method involves washing of the membranes after the incubation with the first, nonradioactive β-lactam I_1, it has two main pitfalls:

1. If k_3 for I_1 is large, EI_1^* decays during the washing step and the following incubation with the radioactive β-lactam I_2. Hence, a substantial amount of free protein is regenerated, which becomes available for reaction with I_2. Consequently, EI_1^* is largely underestimated.
2. Removal of the unbound I_1 during the washing step causes dissociation of the reversible complex EI_1 present. Thus, only EI_1^* is measured (assuming it is stable) and the reaction with I_2 is no longer influenced by EI_1. When compared to the results obtained by direct competition, however, those obtained by this indirect competition procedure may allow a rough estimation of the dissociation constant of reversible complex EI_1, provided that the k_3 values for both EI_1^* and EI_2^* complexes are known.

This indirect competition technique is most often modified in that the washing step is omitted and the unbound, nonradioactive β-lactam I_1 is not eliminated. The results

thus obtained are difficult to interpret. After addition of the radioactive β-lactam I_2, the reaction with I_1 can continue. Any free protein which is regenerated by decay of EI_1^* can react with either I_1 or I_2. If a saturating concentration of I_2 is used, most of the free protein will react with I_2, yet the term "saturating concentration" has not the same implication for all penicillin-binding proteins.

In conclusion, in all the competition experiments, the knowledge of the kinetic constants governing the interaction between the labeled antibiotic and the proteins is very useful, and the knowledge of the k_3 values for the unlabeled antibiotic is essential.

5. Inhibition of the Deacylation Step

PBP 5 of *Escherichia coli* supplies an excellent example that a mutant PBP might lose its enzymatic activity without losing its penicillin binding capacity.[207,210] Again this situation can only be detected if the k_3 values are measured for the parent and mutant proteins.

6. In Vivo Labeling

This type of experiment poses additional problems due to three major factors: high values of k_3, permeability barriers, and the presence of β-lactamases. When k_3 is high, a PBP-β-lactam complex decays during the washing and membrane preparation steps and may eventually become undetectable. Reynolds and Chase have clearly shown that PBP 4 of *Staphylococcus aureus* could not be visualized after in vivo labeling, although it is the most abundant PBP present in the membranes of that organism.[225]

The outer membrane of Gram-negative bacteria behaves as an efficient permeability barrier against nonpolar, small molecules. Even the more simple wall of Gram-positive bacteria might also restrict access of the β-lactam to the targets in the membranes. As shown by Reynolds and Chase, the B_{50} values were significantly larger when replacing protoplasts of *Bacillus megaterium* by intact cells. An alternative explanation to such observations is the presence of a β-lactamase on the membranes of intact cells.[225]

Finally, and as noted in Section III.B.5.b, if the labeling of growing culture is attempted, the continuous synthesis and turnover of the proteins must also be considered.

7. Problems Connected with the Denaturation by SDS

Surprisingly, the stability of the protein-β-lactam complexes during the rather harsh denaturation treatment (1 min at 100°C in the presence of 1% SDS) has received very little attention. Release of the bound [^{14}C]penicillin from the *Bacillus* carboxypeptidases under such conditions was, however, demonstrated by Blumberg et al.[185] and, with the *Streptomyces* R61 and *Actinomadura* R39 exocellular enzymes, the stability of the denatured [^{14}C]benzylpenicilloyl-enzyme complexes depended upon the denaturation conditions.[89,102] On the other hand, too mild a denaturation might result in incomplete reduction of the disulfide bridges producing shorter protein-SDS rods which move faster and thus exhibit a lower apparent molecular weight upon electrophoresis.[128,227] In contrast, Stoker et al.[228] have observed that boiling in the presence of SDS induced aggregation of the rod A gene product. It might thus not be useless to closely examine the influence of the method of denaturation on the PBP patterns.

8. Conclusions

With this short discussion, the authors hope to convince the readers that interpreting PBP patterns may not be as straightforward as it is generally thought. We would like to reiterate the three aspects that we believe to be the most important:

1. The titration effect should be detected and, if possible, avoided or, at least, diagnosed as such.

2. The k_3 values should be determined.
3. When B_{50} values are given, they have little meaning if the time of contact is not mentioned.

Finally, one cannot put too much emphasis on the difficulties which arise if competition (ED_{50}) experiments are performed and one would want to obtain a clear and quantitative interpretation.

B. Specific Examples

1. The High Molecular Weight PBPs of Escherichia coli

Six PBPs were first found in the membranes of *Escherichia coli.*[144] A more efficient electrophoresis technique[229,230] succeeded in separating PBP 1A, a single polypeptide, from PBP 1B, a triplet of polypeptides which were, however, the products of one single gene.[229] Two additional bands, moving a little faster than PBP 3 (3A and 3B) were also detected, as well as smaller polypeptides (MW ≅ 30,000), PBPs 7 and 8.[231] These four supplementary PBPs received little attention. One should also note that, as mentioned above, Labia et al. identified at least five cefotaxime-binding proteins which were apparently different from the usual benzylpenicillin-binding proteins.[223] However, most of the attention has been devoted to proteins 1A, 1B, 2, and 3, whose properties are summarized in Table 18.

a. Physiological Roles

Two methods were used in these studies. Mutants characterized by unusual morphological features (altered shape, filamentation) were isolated. Some of these were thermosensitive (ts), exhibiting the defect only at 42°C and growing normally at 30°C. Alternatively, the same morphological defects could be induced by treatment of the bacteria with β-lactams exhibiting a preferential affinity for one of the PBPs. It had been known for a rather long time that morphological effects were concentration dependent, low concentrations of several β-lactams inhibiting cell division, and higher concentrations inducing cell lysis. In 1975, Spratt and Pardee demonstrated that mecillinam induced the formation of ovoid cells, at a concentration which inhibited the binding of [^{14}C]benzylpenicillin to the sole PBP 2.[144] Similar and mutation experiments by Spratt indicated that PBP 3 was responsible for septum formation and PBP 1 for cell elongation.[208,232] After PBPs 1A and 1B were individualized, it was possible to show that the cells could survive without either of them, but not without both. For example, a ponA^{ts}ponB^{-} mutant could grow normally at 30°C, but would lyse at 42°C.[233]

Simultaneous loss or inhibition of PBPs 2 and 3 resulted in "bulge" formation.[232] Sometimes, the loss of one PBP was accompanied by overproduction of another one and it seemed clear that certain PBPs could compensate for the absence of others.[208,229,234]

When more detailed studies of the structure of the peptidoglycan synthesized by mutants were performed, other interesting observations were made. For example, a strain devoid of PBP 1A, although morphologically normal, synthesized hypocross-linked peptidoglycan, while hypercross-linked new murein was obtained when PBP 2 was inactivated.[219,235]

Although the fine details of the interplay of the activities of the various proteins are not yet known, the major function of each of the high molecular weight proteins can thus be considered as well understood.

b. Purification and Enzymatic Properties

PBPs 1A, 1B, and 3 have been purified to apparent protein homogeneity using covalent affinity on penicillin-agarose and ion-exchange chromatography.[235-240] When

measured, the molar ratio of bound benzylpenicillin to pure enzyme was far from unity, but gel electrophoresis revealed the presence of only one protein band.

PBP 1B was independently isolated and assayed by three different groups.[234,236,238] In all cases, the preparations synthesized cross-linked peptidoglycan using undecaprenyl-pyrophosphoryl-disaccharide-pentapeptide (UPDP) as substrate. The protein thus appeared to be a bifunctional enzyme, capable of catalyzing both transglycosylation and transpeptidation. No transpeptidation occurred in the absence of transglycosylation, while this latter activity was sometimes activated when the cross-linking reaction was inhibited by penicillin. Conversely, none of the reactions could be observed after addition of macarbomycin or moenomycin, transglycosylation inhibitors. The demonstration of the enzymatic activities of PBPs 1A and 3 required the utilization of more exotic conditions: the reactions were performed on filter paper or in solvent mixtures containing methanol and glycerol.[237,239] The products could not be distinguished from those formed by PBP 1B. The degree of cross-linking varied with the protein, but it also varied with the same protein depending upon the experimental conditions. Hypercross-linked peptidoglycan was observed with PBP 1A,[235] a result which could be correlated to the in vivo formation of hypocross-linked peptidoglycan by a mutant lacking this protein.

PBP 2 was not purified. A mutant lacking PBP 1B was lysogenized with a thermoinducible phage carrying the PBP 2 and 5 genes.[241] Thermoinduction strongly elevated the levels of these PBPs in the membrane. The transpeptidase activities of all the other PBPs were then inhibited by cefmetazole, a cephamycin to which PBP 2 was highly resistant. Upon addition of nucleotide precursors, cross-linked peptidoglycan was synthesized by the membranes and cross-linking was severely inhibited by mecillinam: a 50% reduction of cross-linking was observed at a concentration of this β-lactam close to that which half-saturated PBP 2. Cefmetazole had no effect. Although the cross-linking could thus be attributed to PBP 2, the transglycosylation might have been carried out by the other PBPs and it is not certain yet that PBP 2 is a bifunctional enzyme.

None of the high molecular weight PBPs exhibited carboxypeptidase or dimer hydrolyzing activities. Their function thus appeared to be strictly synthetic. Although the turnover values observed in vitro were much too low to explain the rate of peptidoglycan biosynthesis in vivo, this discrepancy could tentatively be attributed to the absence of the natural membrane environment during the in vitro studies. The important result was that the expected enzymatic activities could at all be demonstrated with the surprising fact that the PBPs could also catalyze the transglycosylation reaction.

c. Sequence Data

Hirota and co-workers[120] have determined the entire nucleotide sequence of the ftsI gene (PBP 3), consisting of 1764 base pairs and coding for a polypeptide of 588 residues (MW = 63,850). At the N-terminus of this sequence, a signal peptide of 43 residues was tentatively identified. Cleavage of this peptide would yield a mature protein of about 60,000 dalton, which corresponds to the molecular weight of PBP 3. Attempts were made to find sequence homologies between PBP 3 and the other PBP whose sequence was known. Maruyama et al.[119] aligned Ser 307 and Lys 310 with Ser 36 and Lys 39 of the Bacilli PBP 5. The homology thus obtained with these bacillary DD-carboxypeptidases and PBP 5 of *Escherichia coli* was rather low (Figure 19 and Table 17). However, considering the five residues which are conserved in the two bacillary enzymes and the four known class A β-lactamases and allowing for two important insertions in *Escherichia coli* PBP 3, the authors found four identical residues in identical positions. They concluded that Ser 307 was probably the penicillin-binding residue. Although this conclusion might be true, we do not believe that the arguments concerning the homology were very convincing.

Table 18
PROPERTIES OF THE HIGH MOLECULAR WEIGHT PBPs OF *ESCHERICHIA COLI*[144,208,230-241]

	PBP 1A	PBP 1B	PBP 2	PBP 3
Molecular weight	90,000		66,000	60,000
Molecules per cell[a]	230 (about equivalent amounts of 1A and 1B)		20	50
Gene and location on chromosome	ponA, mrcA 73.5 min	ponB, mrcB 3.3 min	pbpA, mrdA[d] 14.4 min	ftsI, pbpB 1.8 min
Physiological role	Elongation		Shape	Division
Specific β-lactam	Cephaloridine		Mecillinam	Cephalexin
	Cephalexin			
In vitro enzymatic activities				
Nature	Transglycosylase and transpeptidase	Transglycosylase and transpeptidase	Transpeptidase (transglycosylase not demonstrated)	Transglycosylase and transpeptidase
Specific activities (μmol min^{-1} mg P^{-1}[b])	5×10^{-3}	$6\text{-}25 \times 10^{-3}$	—	1.4×10^{-3}
Turnover (min^{-1})[c]	0.45	0.54—2.2	—	0.084
Cross-linking (%)	Up to 39	16—24	18—26	10
Purification method	Affinity on 6-APA-agarose + ion exchange	Affinity on ampicillin-agarose and ion exchange	Not purified	Affinity on ampicillinagarose
Interaction with benzylpenicillin				
k_2/K (M^{-1} s^{-1})[e]	200		130	300
k_3 (s^{-1})	3×10^{-5}		1.4×10^{-4}	1.8×10^{-4}

Molar ratio bound penicillin/enzyme	0.35	0.06 0.17	—	—
Remarks	Doublet?	Triplet but one gene only		

[a] For PBPs 4, 5, and 6, the values are 110, 1800, and 570 molecules per cell, respectively. These three PBPs represent 90% of the total binding sites.[208]

[b] Micromoles of disaccharide-peptide units added.

[c] For the disaccharide addition reaction.

[d] The rodA gene codes for another membrane protein (MW: 31,000) which is also involved in shape determination. The rodA gene maps[242] next to the pbpA and dacA genes (5.6 k bases for the 3 genes).

[e] Computed by us on the basis of the data of Spratt.[208]

Broome-Smith et al.[121] found that a 17-residue peptide (residues 261 to 277) exhibited a rather high degree of homology with residues 19 to 35 of *Escherichia coli* PBP 5 (Figure 19 and Table 17). When so aligned, however, Ser 307 of PBP 3 no longer coincided with Ser 36 of PBP 5 and of the bacillary DD-carboxypeptidases. Moreover, comparisons of short protein segments can be somewhat misleading. A rapid search through the *Handbook of Protein Sequence*[243] allowed us to find 2 10-residue sequences (residues 181 to 190 of horse liver alcohol dehydrogenase and 23 to 32 of subtilisin BPN′) exhibiting a 50% homology with residues 306 to 315 of PBP 3. Any sequence homology between *Escherichia coli* PBP 3 and the D-alanyl-D-alanine peptidases thus remains rather questionable.

Broome-Smith and Spratt have now completed the determination of the nucleotide sequences of PBPs 1A and 1B.[217] In both, they found a sequence -Gly-Ser-X-X-Lys-Pro, similar to residues 306 to 311 of PBP 3. The involvement of these serine residues in the transpeptidase activity of these three high molecular weight PBPs still remains to be convincingly demonstrated by direct labeling with a β-lactam. Before that is done, they can only be considered as possible candidates.

2. The High Molecular Weight PBPs of Bacillus

Although *Bacillus subtilis* was the first species in which multiple penicillin-binding proteins were identified,[142,143] their role is less well understood than in *Escherichia coli.* This is due to the much more detailed knowledge of the genetics of this latter strain, and also to the absence of clear morphological changes observed after selective binding of β-lactams to particular PBPs in Bacilli. General properties of the PBPs of *Bacillus subtilis, Bacillus megaterium* KM, and *Bacillus licheniformis* are summarized in Table 19. After labeling of the *Bacillus megaterium* KM membranes with [^{125}I]ampicillin, Rodriguez-Tebar et al.[247] observed two additional PBPs (1B and 3′). It is not known whether the *Bacillus licheniformis* membranes really contain a smaller number of PBPs or if some proteins have yet escaped detection in this strain.

a. Is It Possible to Identify a Killing Target?

In *Bacillus subtilis,* PBPs 3 and 5 were rapidly eliminated as possible killing targets,[47,174] since their sensitivities to various β-lactams were quite different from that of the strain. Buchanan and Strominger[248] isolated a series of mutants whose resistance to cloxacillin increased stepwise. Only PBP 2 exhibited a resistance increasing in parallel to that of the mutants. Although these results clearly indicated the importance of PBP 2, they were probably not sufficient to definitively identify it as the sole killing target, as shown by the more complete data obtained with *Bacillus megaterium* KM. With this strain, PBP 1 was first recognized as a good candidate; it was the only PBP which was substantially labeled by [^{14}C]benzylpenicillin at a concentration which inhibited the cellular growth by 50%.[249] Moreover, a 0.3 μM concentration of penicillin inhibited the natural transpeptidation catalyzed by wall-membrane preparations by 50% and also resulted in 50% saturation of PBP 1. The rate of degradation of the PBP1-benzylpenicillin complex was also similar to the rate of recovery of this natural transpeptidation system after elimination of the excess of benzylpenicillin. However, Reynolds observed that saturation by some cephalosporins occurred at concentrations below or well above the MIC values and, after treatment with cefaclor, cephalexin and cephradine, and destruction of the excess β-lactam by a β-lactamase, the ability of toluenized cells to produce peptide dimers was recovered faster than the ability to produce higher oligomers.[225] These data indicated that two different enzymes might catalyze transpeptidation reactions, a conclusion which was in agreement with that of Rodriguez-Tebar et al.[247] who suggested that both PBPs 1 and 3 might be required for cellular growth and division. Clearly, PBP 1 is an important protein, but one can certainly not claim that it is the only one.

Table 19
THE PBPs OF *B. SUBTILIS, B. MEGATERIUM* AND *B. LICHENIFORMIS* (GENERAL PROPERTIES)[175,197,201,244-246]

		Interaction with benzylpenicillin			
	Molecular weight	Total binding (%)	k_2/K (M^{-1} s^{-1})[a]	k_3 (s^{-1})	Released product
B. subtilis			37°C	37°C	
1a-1b	120,000	7	38,000—48,000	1.1×10^{-3}	—[b]
2a	96,000	6	25,000	3×10^{-5}	
2b	94,000	4	2,500	3×10^{-5}	Phenylacetylglycine
2c	92,000	1	70,000	3×10^{-5}	
3	88,000	7	50	—	—
4	78,000	5	25,000	2×10^{-4}	Phenylacetylglycine
5	50,000	70	400	1×10^{-4}	Phenylacetylglycine
B. megaterium KM			37°C	37°C	
1	123,000	32	110,000	1.2×10^{-3}	
2	94,000	7	30,000	3×10^{-5}	
3	83,000	16	30,000	8×10^{-5}	—
4	70,000	14	30,000	1×10^{-5}	
5	45,000	31	500	3×10^{-4}	
B. licheniformis			35°C	35°C	
1	123,000	18	>10,000[c]	7×10^{-4}	
2	89,000	12	800	1×10^{-5}	
3	83,000	3	1,100	1×10^{-5}	—
4	46,000	68	80	1×10^{-5}	

[a] Computed by us on the basis of the B_{50} values given by the authors. It should be noted that only in the case of *B. megaterium* has the dilution method of Reynolds been used.[245] For the two other strains, titration effects and β-lactamase activity cannot be excluded.

[b] Phenylacetylglycine has been reported, but the authors could not exclude the possibility of penicilloate.[175]

[c] Clear titration effect observed by the authors.[245]

b. Purification and Interaction with β-Lactams

Several proteins have been purified to protein homogeneity by covalent affinity and ion-exchange chromatography methods: PBPs 1 and 2B of *B. subtilis*,[244,246] PBPs 1, 3, 4, and 5 of *B. megaterium*,[198] and PBP 1 of *B. licheniformis*.[201] High molar ratios of bound penicillin to enzyme were observed with PBPs 1 of *B. subtilis*[246] and *B. licheniformis*[201] (about 1 and 0.7, respectively), indicating a good conservation of at least some of the properties of the proteins during the purification procedure.

The interaction with β-lactams appeared to obey Model 1 and, whenever the nature of the degradation product of benzylpenicillin was studied, it was identified as phenylacetylglycine.[175] Indirect evidence indicated that binding of penicillin probably occurred by reaction with hydroxyl groups.[246]

c. Enzymatic Activities

No enzymatic activity had been attributed to the high molecular weight PBPs until Taku et al. purified the transglycosylase of *B. megaterium* 899. This protein utilized the UPDP to synthesize linear peptidoglycan.[250] Although this activity was unaffected

by benzylpenicillin, the protein could bind [^{14}C]benzylpenicillin covalently, and its electrophoretic mobility was similar to that of PBP 4 (MW = 61,000, the PBP pattern being somewhat different from that of strain KM).

Jackson and Strominger[251] more recently isolated mixtures of PBPs 1, 2, and 4 of *B. subtilis* and PBPs 1, 2, 3, and 4 of *B. stearothermophilus* which also catalyzed the formation of peptidoglycan from the UPDP, the reaction being performed on filter paper or in a complex solvent containing glycerol, ethyleneglycol, and methanol. With the *B. stearothermophilus* PBPs, some penicillin-sensitive peptide dimer formation was observed. *B. subtilis* PBPs 1, 2, and 4 were somewhat labeled by [^{14}C]Ac_2-L-Lys-D-Ala-D-lactate, but other proteins not identified as PBPs were also labeled. The depsipeptide did not inhibit the binding of benzylpenicillin to the PBPs.[246]

In all cases, synthesis of peptidoglycan was strongly decreased in the presence of transglycosylase inhibitors: β-lactams had no effect on the incorporation of the disaccharide units; and no carboxypeptidase activity was recorded. It is somewhat ironic that these proteins, identified thanks to their capacity to specifically bind penicillins, catalyze little or no penicillin-sensitive reaction in vitro.[246,250,251]

3. Staphylococcus aureus

Two major PBPs (MW = 115,000 and 100,000) were first identified in the membranes of *S. aureus* H.[174] The 100,000-MW band was later resolved in a doublet and modification of the labeling conditions[173] showed that a smaller molecule (PBP 4) had escaped detection because of the short half-life of the EI* complex (see Section IV.B.2). Some properties of the four PBPs are summarized in Table 20. As discussed above, PBP 4 appeared to be a transpeptidase involved in secondary cross-linking and mutants which had lost the protein were viable, sometimes growing as enlarged spheres.[178,179] No function could be attributed to PBP 1, which was eliminated without apparent damage to the cells, the only reported variation being a slight (two- to four-fold) increase in the sensitivity of the cells to some β-lactams.[252] Moreover, a mutant lacking PBP 1 and in which PBP 4 was selectively inactivated by cefoxitin grew normally, exhibiting only the expected decrease in the degree of cross-linking.[179,253] From a rather large body of mutational evidence, it was concluded that simultaneous inactivation of PBPs 2 and 3 was necessary to kill the bacterium. Apparently, each of these proteins could take over the function of the other one in its absence.[253,254] In one case of high resistance, the appearance of an additional, very resistant PBP 3 was observed.[177] The sensitivity of the bacterium would thus be determined by the sensitivity of the more resistant of these two proteins. Other data indicate that the resistance would depend upon the sensitivity of the septum-forming machinery,[255] and one could be tempted to conclude that PBPs 2 and 3 are involved in that particular process.

4. Streptococcus faecium ATCC 9790

In contrast to the rather clear understanding of resistance phenomena in *Staphylococcus aureus*, the situation in *Streptococcus faecium* appears to be very complex. Seven "primary" PBPs have been detected in the membranes.[169] Some of these were quite sensitive to endogenous proteases and spontaneously released soluble fragments which could still bind [^{14}C]benzylpenicillin. Protein 4 (MW = 80,000, see Table 20) was thus transformed into protein 4* (MW = 73,000) and protein 3B (82,000) into protein X (72,000). At 45°C and in a chemically defined medium, the strain had a generation time of 31 min and a MIC value for benzylpenicillin of 0.04 μg $m\ell^{-1}$. At that concentration, only PBP 3 was labeled, a result making the inactivation of PBP 3 a possible lethal event.[256] At 32°C, however, the generation time increased to 47 min and the MIC value to 4 μg $m\ell^{-1}$. At that concentration, PBPs 1, 2, 3, 4, and 6 were nearly saturated and it was impossible to correlate the inhibition of growth with the inactivation of any of them. A more detailed study of the growth conditions indicated that

Table 20
PROPERTIES OF THE PBPs of *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS FAECIUM* ATCC 9790

	MW	Total PBPs (%)	Interaction with benzylpenicillin		
			k_2/K (M^{-1} s^{-1})[b]	k_3 (s^{-1})[b]	Released product
Staphylococcus aureus			37°C	25°C	
1	115,000	—	3,800	1.3×10^{-4}	Phenylacetylglycine
2	100,000	—[a]	3,800	2.9×10^{-3}	Phenylacetylglycine[c]
3	95,000	—	1,200	$<2 \times 10^{-5}$	—
3′	80,000	—	—	—	—[d]
4	46,000	—	2,000	8×10^{-3}	?[e]

	MW	Total PBPs (%)		k_2/K (M^{-1} s^{-1})	k_3 (s^{-1})	Released product
		Memb.	Cells			
Streptococcus faecium				37°C	37°C	
1	140,000	12	10	1,500	3×10^{-5}	Penicilloate
2	90,000	10	6	2,000	—[f]	—
3a	85,000	5	0	27,000	—[f]	—
3b	82,000	19	15	27,000	—[f]	—
4	80,000	14	16	400	—[f]	—
5	75,000	2	0	<200	—[f]	—
4*	73,000	3	0	500	—[f]	—
6	43,000	39	52	500	2×10^{-5}	Phenylacetylglycine

[a] The density of blackening of the fluorographies indicated 3 > 2 > 1 > 4,[173] 2 > 3 > 4 > 1,[178] or 2 ≅ 4 > 1 > 3.[176] In most cases, PBP 4 was probably underestimated due to the short half-life of the complex.

[b] k_2/K from Brown and Reynolds;[177] k_3 from Waxman and Strominger.[175] It is not certain that the two groups utilized exactly the same strain.

[c] After denaturation of the complexes, penicilloic acid was released six- to eightfold more slowly.[175]

[d] This protein is only reported in one paper.[173]

[e] Penicilloic acid identified by Kozarich et al. is probably due to contamination by a β-lactamase.[173]

[f] Proteolytic degradation preceded complex decay.

From References 173, 175 to 178 (*Staphylococcus aureus*), and 169 (*Streptococcus faecium*).

sensitivity to β-lactams, PBP patterns, generation time, morphological characters, and peptidoglycan synthesis varied according to temperature and composition of growth medium, but no clear relationship could be established between these factors, demonstrating that the relative importance of each of the PBPs might depend upon the physiological status of the cells.[257] From selective inactivation experiments, Coyette et al. concluded that PBP 3 might be involved in septation (although maybe not as the only PBP) and PBP 2 and/or 6 in pole formation, but a mutant devoid of PBP 2 grew normally.[258] An increased concentration of PBP 5 was also found in the membranes of resistant mutants. This PBP reacted very slowly with benzylpenicillin (k_a at 20 μM = $2 \times 10^{-4} s^{-1}$). In the mutants, PBP 5 appeared to be the killing target, athough in the parent, only PBPs 1, 2, and 3 were saturated at the MIC value. However, the minimum bacteriolytic concentration was that which saturated PBP 5 in all cases.[259]

5. Conclusions

In recent years, the understanding of the respective functions of the various PBPs has rapidly progressed. One can tentatively differentiate these various proteins on the basis of their site of action and of the substrates on which they act. It has been

proposed that cocci might have only one site of insertion of new peptidoglycan, and rod-shaped bacteria at least two.[260] In cocci, insertion would take place in the area where the new septum is synthesized. Conversely, as proposed by Mirelman,[25,180] two types of transpeptidases might be involved in the cross-linking of peptidoglycan: the first one would form bridges between nascent saccharide chains concomitantly with their elongation and the second one would catalyze attachment of these new polymers to preexisting wall and, possibly, form new cross-links in "old" peptidoglycan. In several cases, one enzyme might take over the function of the other one if the latter disappears after either a mutation or reaction with a β-lactam. This compensation mechanism is not, however, a general phenomenon since, for instance, the secondary cross-linking disappeared when *S. aureus* PBP 4 was absent.

Enzymatic activities have been attributed to several PBPs. The most unexpected discovery was the dual transglycosylase and transpeptidase activities of some high molecular weight PBPs. Apparently, the two activities were coupled and transpeptidation could only take place concomitantly with or immediately after transglycosylation. These enzymes (PBPs 1A, 1B, and 3 of *E. coli* and the high molecular weight PBPs of Bacilli) might correspond to the first type of transpeptidases of Mirelman. Other enzymes, capable of catalyzing transpeptidation independently of transglycosylation, would correspond to the second type of transpeptidases. PBPs 4 of *E. coli* and *S. aureus* would belong to this latter class.

A more detailed study of these activities at the enzyme level will certainly present many difficulties. However, considering the recent progress in the study of the DD-carboxypeptidases, mainly due to the utilization of simple, synthetic substrates and of easy, rapid assay techniques, one can hope that the next few years will yield results that considerably improve our understanding of these more complex phenomena at the molecular level.

VI. β-LACTAMASES

The inclusion of a section dealing with β-lactamases in the present article might appear somewhat surprising. Indeed, these enzymes have never, to our knowledge, been conclusively found to be involved in peptidoglycan biosynthesis. In practice, however, the history of β-lactamases was closely associated with that of the therapeutic utilization of β-lactams, bacteria, and antibiotic research groups competing in a seemingly endless race which has been aptly described as "the β-lactamase cycle" by Sykes and Bush: "The emergence of resistant strains applied pressure to antibiotic research groups, which responded by discovering new antibiotics to which these strains were susceptible. In turn, utilization of these new antibiotics applied pressure to the microbial population, resulting in the emergence of new resistant strains, quite often producing β-latamases which could hydrolyze the new molecules."[261] Sometimes, the genetic information coding for the enzymes was carried by plasmids (R factors), which facilitated the rapid spread of the capacity for synthesizing the enzymes in the microbial world.

Penicillin-sensitive enzymes (PSE) and β-lactamases obviously share the ability to recognize β-lactams, but the result of the encounter is quite different depending upon the type of enzyme. However, we will show that β-lactamases can also fall victims to some treacherous β-lactams, and that the similarities between both families of enzymes extend far beyond the simple recognition of the penicillin molecule. Since several recent reviews have aptly covered the general field of β-lactamases,[134,261-264] we will mainly center our discussion on a comparison between β-lactamases and PSEs.

A. Function and Diversity

The existence of enzymes whose sole role would be to destroy penicillin has seemed

puzzling to some authors. The possible presence of β-lactams in natural ecological niches has been discussed and no definitive answer could be offered.[265,266] Although complete and permanent loss of the gene coding for the enzyme did not seem to affect bacterial growth in most cases,[265] the involvement of the enzyme in sporulation has been proposed together with the hypothesis that its penicillin-destroying ability may be coincidental and secondary to an as yet undiscovered function.[266,267]

However, some β-lactamases exhibit turnover numbers which range among the largest ones, and it seems unlikely that this enormous activity would only be a secondary and accidental one. If so, how could the main activity have yet escaped detection? Thus, it seems fairly safe to assume that β-lactamases mainly function as antibiotic detoxifiers.

The β-lactamases are also remarkable for the large number of different enzymes which have been discovered. On the basis of substrate specificity and sensitivity to inhibitors and inactivators, Richmond and Sykes have distinguished 5 classes and 14 subclasses among the enzymes produced by Gram-negative bacteria.[268]

B. Structural Data

The complete amino acid sequence of the *Bacillus licheniformis* β-lactamase was published in 1969.[269] By 1980, three additional primary structures had been determined: the plasmid-mediated RTEM β-lactamase from *E. coli,* the β-lactamase from *S. aureus,* and β-lactamase I from *Bacillus cereus.*[122,269-273] The four sequences exhibited a high degree of homology. In contrast to this wealth of sequence data, studies of the mode of action lagged far behind for a long time. One enzyme, β-lactamase II from *B. cereus,* contained an essential Zn^{++} ion, but remained a kind of unique curiosity[263] until the discovery in 1982 of another Zn^{++}-requiring β-lactamase produced by a strain of *Pseudomonas maltophilia.*[274] Before 1978, little was known about the catalytic mechanism of all the other enzymes. Although a low resolution structure of the RTEM enzyme had been published,[275] no high resolution X-ray diffraction data have yet been obtained.

The real progress started with the discovery, in 1976, of clavulanic acid, a powerful and specific β-lactamase inactivator, discovered in culture filtrates of *Streptomyces clavuligerus.*[276]

C. β-Lactamases as Serine Enzymes

Clavulanic acid was only the first of a rather long list of specific β-lactamase inactivators which, maybe not surprisingly, all turned out to be β-lactams and to behave as suicide substrates: carbapenems (thienamycin, olivanic acids, PS5),[277-279] β-halogenopenicillanates,[280] and sulfones.[264,281] These compounds became β-lactamases what diisopropylfluorophosphate and sulfonyl halides had been to serine proteases. Their utilization permitted the labeling of a homologous serine residue (Ser 70 in Ambler's numbering) in the RTEM,[282] *B. cereus* I,[283] and *S. aureus*[284] β-lactamases.

As summarized by Fisher et al.[264] the chemical features of a potential β-lactamase inactivator included

1. An appropriate β-lactam structure which could be recognized by the enzyme and allow acyl-enzyme formation
2. A 6-α proton of sufficient acidity
3. The presence of a leaving group for anti-elimination

Irreversible inactivation of β-lactamases by these suicide substrates thus appeared to involve a rearrangement of the acyl moiety of the acyl-enzyme, yielding in most cases a stable α-β unsaturated ester (see the example of β-iodopenicillanate, Figure 20).

One interesting result remained unexplained: some active carbapenems, such as

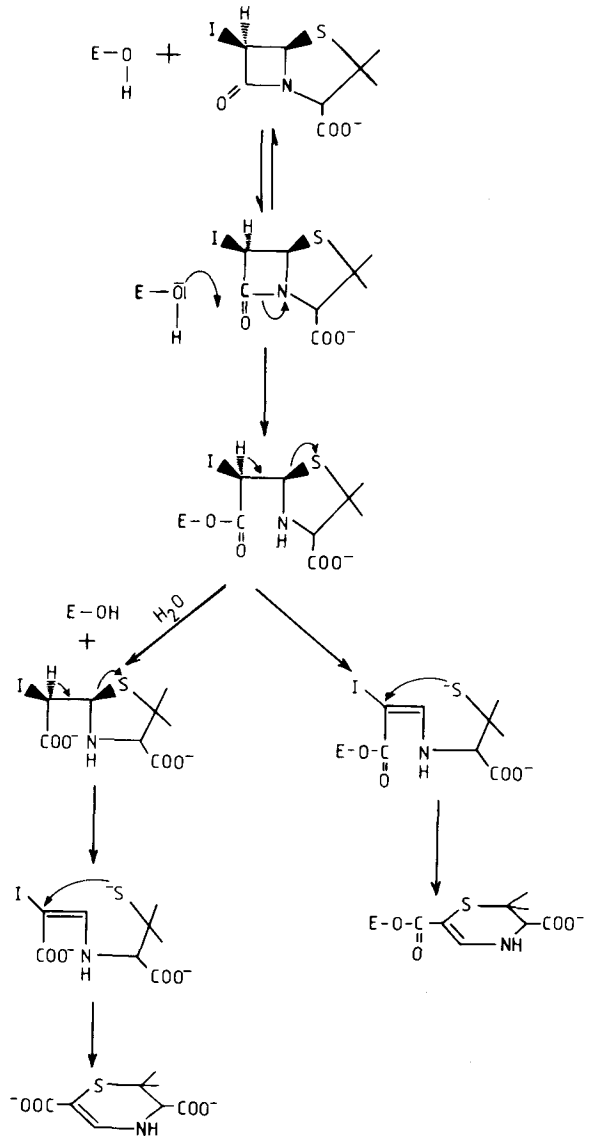


FIGURE 20. Proposed reaction pathway for the interaction between β-iodopenicillanate and the β-lactamases of *Streptomyces albus* G and *Actinomadura* R39.

thienamycin, had the wrong C6 stereochemistry. Indeed, antibiotic activity and capacity to serve as β-lactamase substrates and PSE inactivators appeared to strictly require the absence of any α-substituent. Although exceptions were already known in the cephalosporin family (cephamycins have an α-methoxy group on C7), the rule seemed to be more stringent for penicillins. Thus, the stereochemistry of thienamycins, which exhibited antibiotic properties and inactivating capacities against PSEs and β-lactamases, was a complete surprise.

That the labeled serine residue was also involved in the formation of a catalytically competent acyl-enzyme was suggested by trapping a covalent intermediate during the hydrolysis of a good substrate, dansyl-6-aminopenicillanic acid (k_{cat} = 2000 s^{-1}) by the *B. cereus* β-lactamase I.[285] Digestion of the denatured complex by pepsin yielded a

peptide whose composition and properties were consistent with the attachment of the fluorescent label to Ser 70. Anderson and Pratt[286] also obtained indications of the acylation of the *S. aureus* enzyme by a poor substrate (k_{cat} = 1 to 2 s^{-1}).

In 1981, Jaurin and Grundström determined the nucleotide sequence of the AmpC gene coding for the chromosomal β-lactamase of *E. coli* K12.[123] The amino acid sequence which was deduced exhibited no homology to that of the four previously known enzymes. Waley and co-workers[287] isolated the covalent complexes formed during the hydrolysis of a poor substrate, cloxacillin, by the same enzyme and by the β-lactamase of *Pseudomonas aeruginosa*. Proteolytic digestion and sequence determination indicated that a serine residue (Ser 80) had been labeled. Similar results were obtained by Joris et al.[288] with the *Enterobacter cloacae* P99 β-lactamase after inactivation by β-iodopenicillanate. The three enzymes exhibited extensive homology around the labeled serine residue (Figure 19).

On the basis of the amino acid sequence and catalytic properties, three classes of β-lactamases are now recognized: class A (RTEM and family) and class C (AmpC and family) are serine enzymes while the *B. cereus* β-lactamase II (class B) is a Zn^{++} enzyme. It is not known if the *Pseudomonas maltophilia* enzyme is homologous to *B. cereus* β-lactamase II and also belongs to class B. Zn^{++} enzymes are not sensitive to inactivation by the suicide substrates. The existence of additional classes is a distinct possibility. By specifically altering the nucleotide sequence of the gene coding for the β-lactamase of the pBR 322 plasmid,* Ser 70 was replaced by a cysteine residue.[289] The modified enzyme was active and sensitive to pCMB, which demonstrated that hydrolysis of the β-lactam nucleus could be catalyzed by a sulphydryl group. The natural occurrence of thiol β-lactamases is thus certainly not impossible.

D. Mechanisms of Inactivation

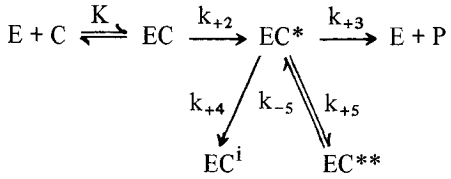
Schemes 4 and 5 present the various mechanisms which have now been identified.

Scheme 4, with $k_{+4} = 0$, characterizes a normal substrate (type S). Formally, this scheme is identical to Scheme 1 and if k_{+3} is very low, a substrate might behave as a transient inactivator. This seemed to be the case with cefoxitin and the RTEM β-lactamase;[290] k_{+3} was only 4.8×10^{-3} s^{-1} and the half-life of ES* was thus 144 s, a value very close to that observed in the interaction between benzylpenicillin and *S. aureus* PBP4 (90 s). With type A substrates, k_{+4} was different from zero, and a time-dependent slowdown of the hydrolysis was observed.[291] Cloxacillin, oxacillin, and methicillin appeared to react with β-lactamase I of *B. cereus* according to that pathway. E'S* was a complex in which an isomerization of the protein had taken place. As proposed by Waley and co-workers in this case, "the acyl-enzyme (ES*) is prone to unfolding".[263,292] Upon addition of a saturating concentration of a class S substrate, E'S* slowly regenerated ES* (k_{-4}), and a time-dependent increase in velocity was observed.

$$E + S \underset{}{\overset{K}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES^* \xrightarrow{k_{+3}} E + P$$
$$\qquad\qquad\qquad\qquad\quad k_{+4} \downarrow\uparrow k_{-4}$$
$$\qquad\qquad\qquad\qquad\quad E'S^*$$

Scheme 4. Model for the interaction between β-lactamases (E) and substrate (S).

* This β-lactamase is identical to the RTEM enzyme, but for one substitution[122] at position 39.



Scheme 5. Model for the interaction between β-lactamases (E) and inactivators or inactivating substrates (C).

Note that the difference between type A and type S substrates can also be given a purely kinetic explanation, k_{+2} being larger than or similar to k_{+3} for type A and much smaller than k_{+3} for type S and that more complicated schemes, involving the isomerization of free enzyme (E ↔ E′), have also been proposed.[291,293] However, this discussion bears little relationship to our present purpose. Finally, the second step might not be completely irreversible, as suggested by recent results of Anderson and Pratt.[294] In that case, ES* would more likely be a tetrahedral intermediate than an acyl-enzyme.

Scheme 5 represents the interaction between β-lactamases and the suicide substrates which have been described above: in that case, a rearrangement of the acyl moiety is responsible for the final inactivation. The complete model was not necessary to account for all studied interactions. When k_{+3} and k_{+5} were zero, or negligible when compared to k_{+4}, inactivation of the enzyme was the only observable phenomenon: this was found to happen when β-bromo- or β-iodopenicillanate was used to inactivate the *B. cereus* I[295] and *Streptomyces cacaoi* β-lactamases.[296] Inactivation and hydrolysis concomitantly took place in some other cases, without formation of EC** ($k_{+5} = 0$) upon interaction of various sulfones with the RTEM enzyme[264] or of β-iodopenicillanate with the *Actinomadura* R39 and *Streptomyces albus* G β-lactamases[297] (Figure 20). Complete inactivation was only obtained for an initial molar ratio of inactivator to enzyme equal to or larger than $(k_{+3} + k_{+4})/k_{+4}$. The complete model was needed to explain the interaction between clavulanate and the RTEM[298] and *Actinomadura* R39[299] β-lactamases. When the initial molar ratio of inactivator to enzyme was not sufficient, i.e., equal to or larger than $(k_{+3} + k_{+4} + k_{+5})/k_{+4}$ incomplete inactivation was followed by a partial recovery of the activity. The transiently inhibited complex EC** was produced through a rearrangement of the acyl-enzyme EC*. The exact structure of EC^i remained undetermined, as well as the fate of EC**, which might regenerate EC*, as shown in Scheme 5 or irreversibly decay into E + P′ (see the discussions in References 298 and 299). Results were aso obtained indicating that several different EC^i complexes were formed.[300] An interesting observation was that β-lactamases of classes A and C could be distinguished on the basis of their interaction with β-bromopenicillanate. Inactivation was much more rapid with class A (second-order rate constants $\cong 1.5 \times 10^4$ $M^{-1}s^{-1}$)[295,301] than with class C enzymes (second-order rate constants $\cong 100$ M^{-1} s^{-1})[73,302] but the pathway apparently was not branched ($k_{+3} = k_{+5} = 0$). The enzymes which reacted following the branched pathway were not metallo-proteases and one could wonder whether they did not belong to a fourth class.

Interestingly, the R61- and R39-soluble D-alanyl-D-alanine peptidases also reacted with β-iodopenicillanate following an unbranched pathway, and the acyl moiety underwent the same rearrangement as that observed with the β-lactamases.[73]

E. Comparison with PSEs

On the basis of their common ability to interact with β-lactams, an evolutionary

Table 21
HOMOLOGY BETWEEN RESIDUES 1 TO 44 OF THE *BACILLUS SUBTILIS* DD-CARBOXYPEPTIDASE, 9 to 52 OF *ESCHERICHIA COLI* PBP 5, AND 34 TO 78 OF CLASS A β-LACTAMASES

	Carboxypeeptidases	
β-lactamases	*B. subtilis*	*E. coli*
RTEM	12/44 = 0.27	12/44 = 0.27
S. aureus	11/44 = 0.25	12/44 = 0.27
B. licheniformis	6/44 = 0.14	9/44 = 0.20
B. cereus	7/44 = 0.16	9/44 = 0.20

Note: As in Figure 19, one deletion was allowed in the DD-carboxypeptidases and in three of the β-lactamases.

relationship was suggested between PSEs and β-lactamases.[36] A comparison of the sequences of class A β-lactamases with the 64 N-terminal residues of the *B. subtilis* DD-carboxypeptidase (PBP 5) appeared convincing to Waxman and Strominger who observed a high degree of homology in the immediate surroundings of the essential serine residue.[117] The same authors also discussed the extension of this homology to *E. coli* PBP 5, and a computer search detected a significant homology near the essential serine residues.[3] Using the method of Chou and Fassman, the sequence homology appeared to extend to the level of secondary structure. Table 21 and Figure 19 show that for the first 40 to 50 residues of the PBPs, which include the active serine, the homology is, however, rather weak, the best scores (27%) being obtained for the pairs *E. coli* PBP5/RTEM β-lactamase, *E. coli* PBP5/*S. aureus* β-lactamase, and *B. subtilis* PBP5/RTEM β-lactamase. The same evidence has been interpreted by Ambler as indicating a slight structural similarity near the active site, but no common evolutionary origin.[122] We will leave the reader free to choose sides. Nonetheless, an interesting feature emerges from this comparison: if one excepts the D-alanine-D-alanine peptidase of *Actinomadura* 39, a Lys residue is invariably found three residues after the essential serine, even when class C β-lactamases are included in the comparison. This suggests that the positive charge on the Lys side chain might be involved in an ion pair with the free terminal carboxylate of peptide substrates in PSEs or with the free carboxylate of β-lactams in both PSEs and β-lactamases.

It is also informative to compare the catalytic mechanisms. Serine β-lactamases and penicillin-sensitive enzymes appear to share the same pathway: recognition — acylation — hydrolysis of acyl enzyme. If one considers the interaction with β-lactams, the third step is obviously extremely different. We can now hope that the near future will show us why hydrolysis of acyl-enzyme is so efficient in one case (velocities larger than 1000 s^{-1} are not rare) and so slow in the other one (from 1×10^{-4} to 1×10^{-6} s^{-1} in most cases). Moreover, with DD-peptidases, deacylation sometimes utilizes a very different pathway, first involving the fragmentation of the β-lactams. At the present time, we do not even have the beginning of an explanation.

If one wishes to compare the acylation steps, the value of k_2/K can be directly deduced from inactivation experiments of penicillin-sensitive enzymes and is equal to k_{cat}/Km for β-lactamases. With the β-lactamase and the exocellular D-alanyl-D-alanine peptidase of *Actinomadura* R39, we had the unique opportunity to study the two types of enzymes produced by the same strain. It should be underlined that the *Actinomadura* R39 β-lactamase has not yet been demonstrated to be a serine enzyme, but its

Table 22
SECOND-ORDER RATE CONSTANTS FOR THE FORMATION OF THE ACYL-ENZYME WITH THE β-LACTAMASE AND THE D-ALANYL-D-ALANINE PEPTIDASE OF ACTINOMADURA R39[73,88,90-93,304,305]

	β-lactamase k_{cat}/Km (M^{-1} s^{-1}) at 30°C	D-alanyl-D-alanine peptidase k_2/K (M^{-1} s^{-1}) at 37°C unless otherwise stated
Benzylpenicillin	980,000	300,000
Phenoxymethylpenicillin	500,000	>70,000
Ampicillin	590,000	280,000
Carbenicillin	97,000	6,000
Methicillin	61,000	4,000
Oxacillin	380,000	40,000
Cloxacillin	46,000	15,000
Quinacillin	53,000	32
6-Aminopenicillanate	700,000	1,200
Penicillanate	127,000	14
Mecillinam	60,000	32
Unsubstituted penem	20,000	1,750
2-Methylpenem	64,000	5,400
2-Phenylpenem	150,000	10,000
Cephalosporin C	3,500	200,000
Cephaloglycine	50,000	74,000 (20°C)
Cephalexin	12,000	3,000 (10°C)
Cephalothin	300,000	>70,000
Nitrocefin	2,800,000	3,600,000 (10°C)
Δ^3-Deacetoxy-7-phenylacetamido cephalosporanate	7,500	5,000
7-Aminocephalosporanate	800	200
Δ^2-Deacetoxy-7-phenylacetamido cephalosporanate	25,000	52
Quinacillin sulfone	0.8[a]	10
Cefoxitin	0.8[a]	7,000
N-formimidoyl thienamycin	2,000[a]	10,000
Clavulanate	100,000[b]	30
β-Iodopenicillanate	1,000,000[b]	7,600

[a] Inactivation; the exact pathway has not been determined.
[b] Inactivation following branched pathway.
Compounds underlined with a continuous line belong to group 2; with a dashed line, to group 3; all the other ones, to group 1.

behavior in the presence of the specific inactivators of this family of enzymes was consistent with this hypothesis.[297,299,304] Table 22 shows a comparison of the $(k_2/K)_{peptidase}$ and $(k_{cat}/Km)_{\beta\text{-}lactamase}$ values for 27 β-lactams. The compounds could be divided into three groups. The largest group contained the β-lactams (18 out of 27) for which the values were similar within one order of magnitude. As a whole, cephalosporins in that group exhibited a preference for the peptidase, and the penicillins a preference for the β-lactamase. The second group (seven compounds) reacted much faster with the β-lactamase. Quinacillin was the only "normal" member of this group. Four of the other compounds lacked the usual amide side chain on C6 and the Δ^2-cephalosporin and mecillinam did not belong to the classical β-lactams depicted by Figure 1. It was, in fact, surprising to find that the enzymes reacted at all with the Δ^2-cephalosporin. These data indicated that a suitable side chain on C6 was more important for the DD-peptidase and, conversely, one was tempted to conclude that the β-lactamase exhibits a less defined specificity. This conclusion could not, however, be generalized, since thienamycin and 7-aminocephalosporanate, which have short side chains, be-

longed to the first group. The last group only comprised two compounds — cefoxitin and cephalosporin C — which interacted much faster with the DD-peptidase. It is obvious that the existence of such a group is encouraging if one considers its therapeutic implications, but this certainly does not supply an explanation to the apparently aberrant behavior of these β-lactams.

Although these comparisons are quite interesting, we do not feel that they can be easily generalized. Indeed, the good agreement which is observed for the majority of the tested compounds is probably a consequence of the extraordinarily high sensitivity of the R39 peptidase to β-lactams. It has been seen above that, for many DD-peptidases, the values of k_2/K ranged between 100 to 1000 M^{-1} s^{-1}, while for β-lactamases , k_{cat}/Km was often larger than 10^6 M^{-1} s^{-1}.

No interaction was recorded between β-lactamases and the peptide substrates of the peptidases.[306] However, using depsipeptide substrates, Pratt and Govardhan[307] have demonstrated an esterase activity by the three classes of β-lactamases, and a transacylase activity by classes A and C. The highest k_{cat}/Km (3800 M^{-1} s^{-1}) value was recorded for the hydrolysis of phenacetyl-Gly-D-mandelate [ϕ-CH_2-CO-NH-CH_2-CO-NH-$CH(C_6H_5)COO^-$] by the *Enterobacter cloacae* P99 β-lactamase, but this rather low value was mainly due to a very high Km (70 m*M*), the k_{cat} value (270 s^{-1}) being close to that observed for good β-lactam substrates of the enzyme. Interestingly, for the same enzyme, D-alanine was one of the best acceptors in the transacylation reaction.

In conclusion, β-lactamases and D-alanyl-D-alanine peptidases appear to share several important properties. The temptation is great to hypothesize that these common properties are a consequence of a common ancestry. Indeed, if the bacteria discovered the need to manufacture enzymes specialized in the hydrolysis of β-lactams, it can be argued that it was easier for them to modify enzymes which, by chance, were already performing the first two steps of the reaction than to start from scratch. The evidence presently available to support this hypothesis, however, remains ambiguous and the case of convergent evolution can also be sensibly defended. The elucidation of the three-dimensional structure of β-lactamases and DD-peptidases will certainly, in the near future, help to solve some of these ambiguities.

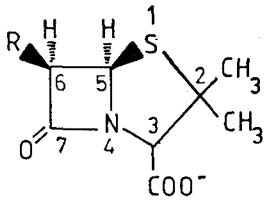
VII. CONCLUSIONS AND PERSPECTIVES

A. The New β-Lactams

Understanding how β-lactams kill bacteria has turned out to be a complex objective. Even the ideas about the structural features necessary to obtain an "active" molecule have drastically changed. Not a long time ago, it was generally believed that only penicillins and Δ^3-cephalosporins could be active (structures 1b and 6 in Table 23). The ingenuity of microorganisms and chemists gave birth to many other active molecules, the ultimate simplification being reached with monobactams[308] where penicillin was stripped down to its essential components: the β-lactam nucleus, bearing one negatively charged group on the nitrogen. We do not imply that all the structures depicted in Table 23 exhibited antibacterial properties, but they all interacted with some PSEs or β-lactamases. Compounds such as clavulanate and β-halogenopenicillanate had little if any activity on PSEs, but they inactivated β-lactamases so well that clavulanate is presently used for therapeutic purposes, in synergy with classical, β-lactamase-sensitive penicillins. Some molecules also exhibited a surprisingly high degree of specificity: of all the *Escherichia coli* PBPs, mecillinam reacted only with PBP2[144] and the monobactam azthreonam with PBP3.[309] Although some molecules might behave as good inactivators of most PSEs, these cases of high specificity are good examples demonstrating the difficulties of extrapolating data from one enzyme to another. Possible correlations between the intrinsic sensitivity of β-lactams to nucleophiles and their inactivating potency have been discussed,[91] but no clear pattern has emerged from these studies.

Table 23
RING SYSTEMS OF VARIOUS β-LACTAMS WHICH INTERACT WITH PSEs OR β-LACTAMASES OR WHICH EXHIBIT ANTIBACTERIAL ACTIVITY

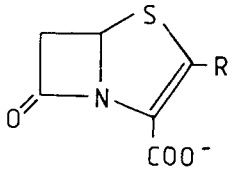
1—Penams



(a) Small substituent on C6 (R = H, NH_2, halogen); (b) penicillins R = R′–CO–NH–; (c) amidinopenicillins R = R′–CH=N–

2—Penams sulfoxides and sulfones
1 or 2 oxygens on S1

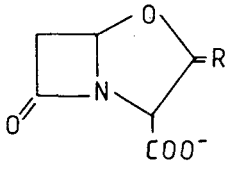
3—Penems



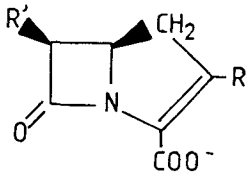
R = H, CH_3, C_6H_5

R = H, CH_3, C_6H_5

4—Oxopenams (clavulanate)



5—Carba-2-penems
(a) C6 substituent in β (olivanic acids)



(b) C6 substituent in α (thienamycin)

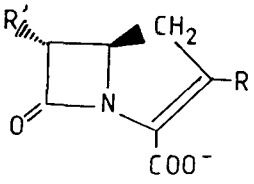
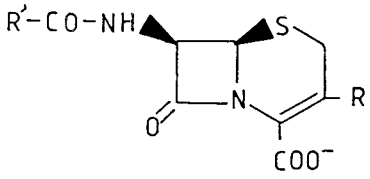


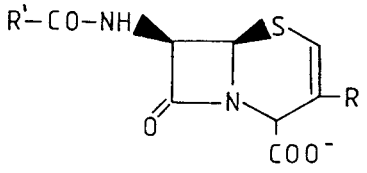
Table 23 (continued)
RING SYSTEMS OF VARIOUS β-LACTAMS WHICH INTERACT WITH PSEs OR β-LACTAMASES OR WHICH EXHIBIT ANTIBACTERIAL ACTIVITY

6—Δ^3-Cephems

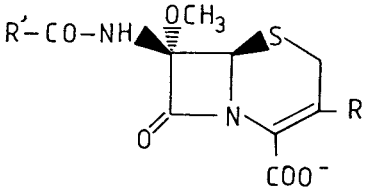


Sulfones and sulfoxides also exist

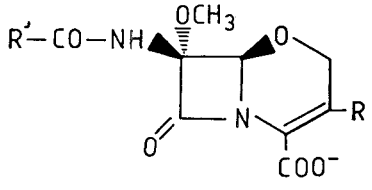
7—Δ^2-Cephems



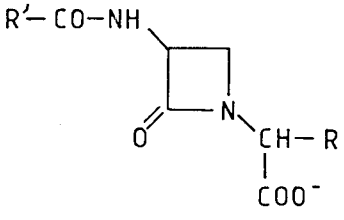
8—Cephamycins



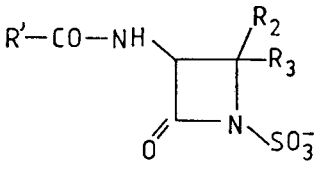
9—Oxocephamycins (moxalactam)



10—Nocardicins



11—Monobactams



B. Physiological Mechanisms

A detailed comprehension of the physiological events which follow the addition of a β-lactam to a growing culture would require a complete knowledge of the mechanisms governing cell growth and division. Many problems remain unsolved. What is the physiological importance of *O*-acetylation of the peptidoglycan? If autolysins are responsible for cell lysis, how does inactivation of PSEs by β-lactams trigger their activity? Can this role be generalized to all bacteria? What is the origin of tolerance phenomena?

In the case of *E. coli,* the physiological functions of the various high molecular weight PBPs are rather clear, but the enzymatic activities, as determined in vitro are nearly identical and it is still difficult to imagine how these similar activities could produce such different effects as septation or shape determination. One can assume that the enzymes perform their tasks at distinct locations on the cell membrane and that a local defect in peptidoglycan cross-linking, not evidenced by a global analysis, may be lethal to the cell, but this does not supply much of an explanation. It would also be important to know how the compensation mechanisms work and how much of a given PBP is really necessary for the well being of the cell. For example, and although the question might appear somewhat teleological, one cannot help wondering why the cells should manufacture such large amounts of apparently useless D-alanyl-D-alanine carboxypeptidases. Could these enzymes be more useful in natural environments than in the artificial, rich media where bacteria are grown as pure cultures in the laboratory?

Another tantalizing question is that of the specificity of some mechanisms for particular species (remember *Gaffkya homari*!) and, in consequence, of the extrapolation of conclusions from one genus to others.

C. Enzymatic Mechanisms

Considerable progress has been made in the study of penicillin-sensitive enzymes. For four of them, the acylation of a serine residue by β-lactams has been demonstrated, and it was clear, or very likely, that the reaction with substrates followed a covalent catalysis pathway involving transient acylation of the same serine. These results allowed a final choice between the competitive and noncompetitive models. One should realize that many important contributions were made in the framework of this controversy. From a kineticist's point of view, a very valuable conclusion was that, in the case of a model represented by Scheme 2, it is much more useful to try to determine the influence of the substrate concentration on the formation of EI* than to measure the influence of the inactivator on the appearance of product, unless this latter event can be continuously monitored, a type of experiment which is presently rather easy with β-lactamases,[292,299] but impossible with D-alanyl-D-alanine peptidases.

Table 24 summarizes the data which have been obtained for the interactions between pure, or nearly pure, D-alanyl-D-alanine peptidases and substrates or benzylpenicillin. The catalytic activities of these enzymes range from medium to very low, the highest turnover numbers being observed with the exocellular R39 and R61 enzymes. The identity of donor substrates and penicillin-binding sites might not be complete. As discussed by Ghuysen et al.,[1,2] those side chains (R′ in groups 1b and 6 of Table 23) which make a β-lactam a good inactivator are often very different from L-R_3 residues which confer good substrate properties to a peptide. These substituents play a pivotal role in catalysis and inactivation phenomena: 6-aminopenicillanic acid and the dipeptide D-alanyl-D-alanine react poorly or not at all with the peptidases. Moreover, the same side chain may influence reactivity in a different manner when different enzymes are studied (compare 6-aminopenicillanic acid and methicillin or 7-aminocephalosporanic acid and cephaloglycine in Table 2).

The fitting of substrate L-R_3 residues and β-lactam R′ side chains into specific subsites probably results in an adequate alignment of the serine hydroxyl group with the carbonyl of the substrate/inactivator. Whether this efficient geometry involves a con-

formation change of the enzyme or a distorsion of the inactivator (or both) remains to be determined. Penicillin has been successively hypothesized to be a substrate analog,[36] transition-state analog,[310] and a suicide substrate.[311] The data which are presently available appear to favor the third hypothesis. When penicillins and cephalosporins are compared to D-Ala-D-Ala-terminated peptide, neither the preferred conformations[61] nor the pattern of relative spatial disposition and strength of positive and negative potentials[312] indicate a clear structural analogy. The initial complementarity between the enzymatic active sites and both substrates and β-lactams is not especially good since the measured values of K and Km are generally rather high. One would expect a transition-state analog to exhibit a high affinity for the enzyme. β-lactams are good inactivators because their interaction with sensitive enzymes aborts at the stage of a "normal" intermediate complex, the word "normal" meaning that no rearrangement of the penicilloyl group occurs, in contrast to what happens in the case of several β-lactamase inactivators. The high stability of the penicilloyl-enzyme could be due to an interaction with a stabilizing site or to a direct purely geometric protection of the ester bond by the thiazolidine moiety. When this part of the inactivator is removed, by breaking of the C_5-C_6 bond, rapid hydrolysis of the acyl-enzyme is observed. Is the thiazolidine-binding site distinct from the acceptor site? The experimental evidence is rather ambiguous: one acceptor (D-alanine) did not interfere with the binding of benzylpenicillin to the R39 enzyme,[88] but acceptors were only transferred on the phenylacetylglycyl moiety of the benzylpenicillin-R61 complex (and not on the penicilloyl group itself), possibly indicating that the acceptor had only access to its own site after the thiazolidine portion had been removed.[110]

Are the mechanisms of transpeptidation and hydrolysis similar? In other words, does Scheme 3 apply to both types of reactions assuming that, for strict transpeptidases, k_3 is negligible?

For the R61 and R39 exocellular peptidases, this is a difficult question to answer since, in the absence of acceptor, any intermediate is rapidly processed through the hydrolysis pathway. The lack of acyl-enzyme trapping upon incubation with labeled peptide donor substrate could be explained either by a rate-limiting acylation, or by the fact that the rapid deacylation (at least 55 s^{-1} with the R61 enzyme) might be faster than binding of SDS and denaturation, or protein aggregation after addition of acetone. Very little trapping of acyl-enzyme was also observed with the K15 transpeptidase, even in the absence of an acceptor, under which conditions the hydrolytic activity was very low ($k_{cat} = 0.02\ s^{-1}$). It was thus unlikely that deacylation would significantly compete with denaturation or aggregation, and it seemed safe to assume that the low level of acyl-enzyme was due to rate-limiting acylation. However, the addition of acceptor increased the turnover number 30-fold and this could not be explained by an influence on the sole deacylation step. In this case, a simple model similar to that depicted by Scheme 3 is probably not valid.

The relatively higher activities of soluble enzymes have allowed the easy study of their interactions with a vast number of β-lactams (see Table 2). Its availability in large quantities, and its solubility in the absence of detergents, permitted the crystallization of the R61 enzyme which is expected to soon become the first penicillin-sensitive enzyme of known three-dimensional structure.

Unfortunately, it is presently difficult to display the same optimism about the determination of the tertiary structures of membrane-bound transpeptidases, particularly of the bifunctional PBPs. Even if the pure preparations which have been obtained yield suitable crystals in the near future, the very low in vitro catalytic activities exhibited by these enzymes will remain an important obstacle to a good understanding of their functioning in the lipid environment of the cytoplasmic membrane.

Table 24
KINETIC PARAMETERS GOVERNING THE INTERACTIONS BETWEEN D-ALANYL-D-ALANINE PEPTIDASES, SUBSTRATES, OR BENZYLPENICILLIN

	Interaction with substrate				Interaction with benzylpenicillin	
Enzyme	Type of reaction	Substrate(s)	k_{cat}/Km (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_2/K (M^{-1} s^{-1})	k_3 (s^{-1}) and product
Exocellular, *Streptomyces* R61	Cbase	a_2LAA	4,600	55	14,000 (25°C)	1.4×10^{-4} Φ
Exocellular, *Actinomadura* R39	Cbase	a_2LAA	22,500	18	300,000	3×10^{-6}
		α-aLAA	160,000	32		Φ
Exocellular, *Streptomyces albus* G	Cbase	a_2LAA	9,000	3	9×10^{-3}	6×10^{-5} P
Membrane-bound *Streptomyces* K15	Tpase	a_2LAA + glycylglycine	100[a]	0.6	150	1×10^{-4} Φ < P
PBP 4 *Proteus mirabilis*	Cbase	UDP-*M*-pentapeptide	26,000	18	200,000—300,000	4×10^{-5} Φ
PBP 5 *Proteus mirabilis*	Cbase	UDP-*M*-pentapeptide	1,900	1.3	20,000—80,000	1.6×10^{-3} Φ ≪ P
PBP 6 *Streptococcus faecium*	Cbase	a_2LAA	500	3	1,000	3×10^{-5} Φ
PBP 5 *Bacillus subtilis*	Cbase	a_2LAA	25	0.17	1,700 (4°C)	6×10^{-5}
		a_2LALa	3,600	2.5		Φ
PBP 5 *Bacillus stearothermophilus*	Cbase	a_2LAA	140	0.036	—	3×10^{-4}Φ
Membrane-bound *Bacillus coagulans*	Cbase	UDP-*M*-pentapeptide	1,500	3.7	—	—
PBP 4 *Staphylococcus aureus*	Cbase	a_2LAA	2	0.2	2,000	8×10^{-3}
		a_2LALa	130	0.12		P?
PBP 4 *Escherichia coli*	Cbase	UDP-*M*-pentapeptide	1,600	2.5	7,000	0.4×10^{-3} (30°C) P?
PBP 5 *Escherichia coli*	Cbase	a_2LAA	190	3.1	120 (25°C)	1×10^{-3} (25°C)
		a_2LALa	3,000	13		P?

PBP 6	Cbase	a_2LAA	70	1.1	115 (25°C)	0.3×10^{-3} (25°C)
Escherichia coli		a_2LALa	4,300	18		P?

Note: Cbase, carboxypeptidase; Tpase, transpeptidase; a_2LAA, $N^{\alpha}N^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine; α-aLAA, N^{α}-acetyl—; a_2LALa, $N^{\alpha}N^{\epsilon}$diacetyl-L-lysyl-D-alanyl-D-lactate; Φ, phenylacetylglycine; P, penicilloate; P?, penicilloate was identified but the presence of a β-lactamase could not be excluded. Data are at 37°C unless otherwise stated.

[a] The Km used was that for the donor substrate.

D. Resistance Phenomena

Two types of resistance mechanisms can now be studied at the enzyme level: intrinsic resistance and β-lactamase production. The *albus* G Zn^{++} carboxypeptidase represents an extreme example of an enzyme which has lost the sensitivity to β-lactams, but still interacts with D-alanyl-D-alanine-terminated peptides. From the available data on metalloenzymes, it seems, however, unlikely that such proteins could catalyze transpeptidation reactions. Table 24 shows that very large variations can occur in the reactivity of D-alanyl-D-alanine peptidases towards β-lactams. The study of the molecular basis of these variations will supply clues for the design of new molecules hopefully, exhibiting increased inactivating potency towards the target enzymes.

Several β-lactamases have now been crystallized, but little information is available yet about their three-dimensional structures and the catalytically important residues besides the serine. One cannot put too much emphasis on the importance of a clear understanding of the mechanism of action of β-lactamases. Indeed, it seems unlikely that we have seen the end of the β-lactamase cycle. The ingenuity of bacteria, who owe their survival to the acquisition of new resistance mechanisms, has been amply demonstrated in the past. There is no reason for scientists to worry about their future: their own ingenuity will, in turn, remain necessary if we want to successfully continue the fight against the pathogenic strains which still constitute one of the major therapeutic problems.

ACKNOWLEDGMENTS

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