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BACTERIAL TRANSPEPTIDASES AND PENICILLIN

by

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Abbreviations used in the text:

CTAB = cetyltrimethylammonium bromide.
EI = enzyme + inhibitor (an intermediate and inactive complex).
EI* = enzyme + inhibitor, both being modified.
ESI = enzyme + substrate + inhibitor (antibiotic).
UDP = uridine-5′-pyrophosphoryl.

Abbreviations used in the figures:

Ac = acetyl.
A_2pm = diaminopimelic acid.
G = N-acetylglucosamine.
M = N-acetylmuramic acid.

(N-acetylmuramic acid is N-acetylglucosamine substituted on C_3 by an ether-linked D-lactyl group).

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Key-words: Penicillin, Transpeptidase; *Streptomyces*, Bacteria, Membrane; Review.

I. — INTRODUCTION

Penicillins and cephalosporins, collectively called penicillin, constitute a group of antibiotics that are characterized by the presence in the molecule of a highly reactive β-lactam ring. When exposed to penicillin, bacteria that are actively growing undergo lysis. This phenomenon is caused by the specific inhibition of the synthesis of a particular heteropolymer, the peptidoglycan, which imparts to the wall its rigidity, shape and insolubility. In the absence of a continued synthesis of this peptidoglycan, the bacteria lose their osmotic protection and are lysed by their own autolysins. The aim of this paper is to discuss « how » penicillin stops the synthesis of the wall peptidoglycan.

A) Structure and biosynthesis of peptidoglycan

The structure of the peptidoglycan and its biosynthesis have been reviewed many times [24]. Peptidoglycans are networks in which linear strands of alternating β-1,4 linked pyranoside-N-acetylglucosamine and N-acetylmuramic acid are interconnected through peptide chains. The D-lactyl groups of the muramic acid residues in the glycan strands, or at least some of them, are substituted by tetrapeptide units L-Ala-γ-D-Glu-L-R_3-D-Ala. The L-R_3 residue varies according to the bacterial species. Often it is a diamino acid. The tetrapeptides belonging to adjacent glycan strands are in turn crosslinked through « specialized » bridges. Bridging between two peptides always involves the C-terminal D-alanine residue of one tetrapeptide and often, but not always, the ω-amino group of the L-R_3 residue of the second tetrapeptide. The composition and the location of the bridges have been used to divide bacterial peptidoglycans into four main chemotypes [19] and have been considered to be a criterion of taxonomic importance [45]. Figures 1,

2 and 3 show how two disaccharide peptide units are interlinked in those bacteria which were used for the present study. In *Escherichia coli* [49] and in *Streptomyces* R39 [21], the L-R_3 residue is *meso*-diaminopimelic acid. The interpeptide bridge is a direct linkage D-Ala-(D)-*meso*-diaminopimelic acid. In *Streptomyces* R61 [29] and K15, the L-R_3 residue is LL-diaminopimelic acid and the interpeptide bridge is mediated *via* one additional glycine residue. In *Streptococcus faecalis* ATCC 9790 [7], the L-R_3 residue is L-lysine

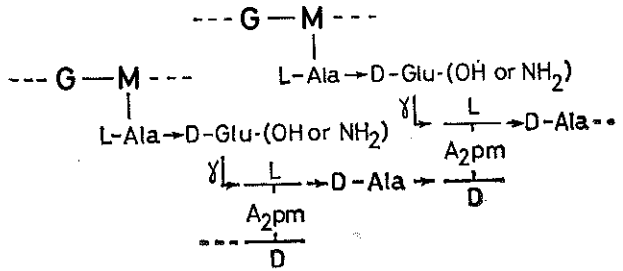


FIG. 1. — *Peptidoglycan of* E. coli *and* Streptomyces *R39.*

The α-carboxyl group of D-glutamic acid is free in *E. coli* and amidated in *Streptomyces* R39.

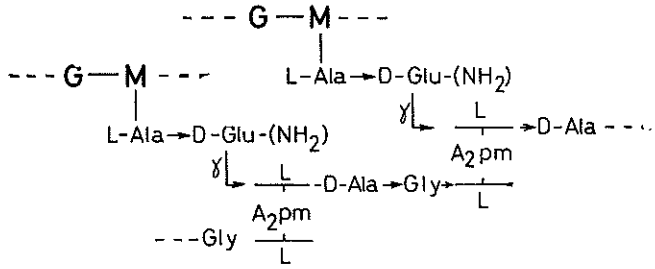


FIG. 2. — *Peptidoglycan of* Streptomyces *R61 and K15.*

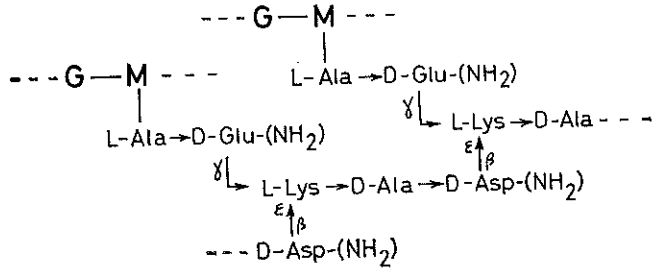


FIG. 3. — *Peptidoglycan of* S. faecalis *ATCC 9790.*

and the interpeptide bridge is mediated *via* an additional iso-D-asparagine residue.

Two groups of enzymes that are cytoplasmic and membrane-bound, respectively, are involved in the biosynthesis of the peptidoglycan [46]. The cytoplasmic enzymes catalyse the synthesis of two uridylic acid precursors: uridine-5′-pyrophosphoryl-(UDP)-N-acetylglucosamine and UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-L-R_3-D-Ala-D-Ala. Note that the peptide of this latter precursor ends in a D-Ala-D-Ala sequence. From these two precursors, a membrane-bound multi-enzyme complex, working in conjunction with a C-55 undecaprenyl phosphate coenzyme, catalyses a coordinated sequence of transfer reactions that lead to the formation of a « nascent » peptidoglycan. This nascent peptidoglycan, which is thought to emerge on the outer surface of the cytoplasmic membrane, consists of glycan strands substituted by uncrosslinked L-Ala-γ-D-Glu-L-R_3-D-Ala-D-Ala pentapeptides (in which one or several bridging amino acid residues have been incorporated, if necessary). The insolubilisation of the nascent peptidoglycan and its incorporation in the preexisting wall peptidoglycan are eventually carried out by transpeptidation [52, 47]. This reaction is catalyzed by a membrane-bound enzyme. The penultimate C-terminal D-alanine residue of a peptide *donor* is transferred to the amino group of another peptide *acceptor;* interpeptide bonds are formed and equivalent amounts of D-alanine are liberated from the donor peptides. The transpeptidation reaction as it must occur between two nascent peptides in *E. coli* and *Streptomyces* R39 is shown in figure 4 and the transpeptidation reaction as it must occur between two nascent peptides in *Streptomyces* R61 and K15 is shown in figure 5. The transpeptidation reactions as they are described above, are certainly oversimplified. It is likely that, at least in part, elongation of the nascent peptidoglycan by transglycosidation and its insolubilization by transpeptidation occur

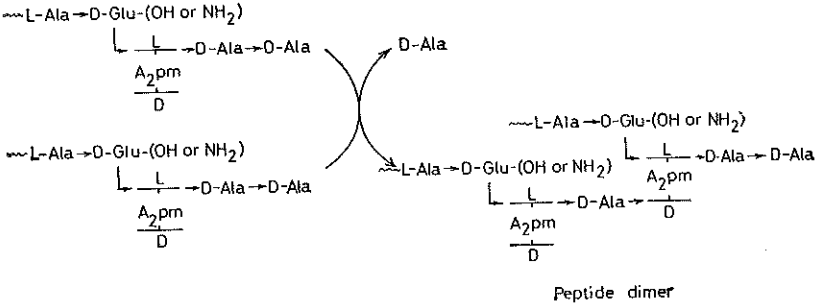


FIG. 4. — *Transpeptidation in* E. coli *and* Streptomyces *R39.*

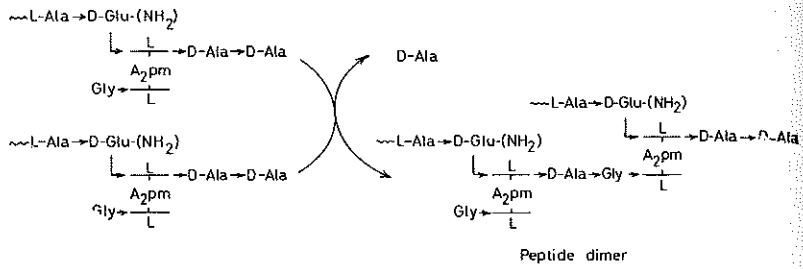


FIG. 5. — *Transpeptidation in* Streptomyces *R61 and K15.*

simultaneously [35, 50]. Moreover, transpeptidation probably also occurs between the nascent peptidoglycan and the preexisting wall peptidoglycan, hence causing the attachment of the former to the wall matrix.

B) SITES OF ACTION OF PENICILLIN

In 1965, Wise and Park [52] and Tipper and Strominger [47] showed independently that the closure of the interpeptide bridges was specifically inhibited by penicillin. In 1966, particulate preparations were obtained from *E. coli* and *Salmonella* which carried out peptidoglycan synthesis including the bridge-closure reaction, from the nucleotide precursors UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide [26, 1]. The only activity of these preparations to be inhibited by penicillin was the transpeptidase and, seemingly, this activity could not be restored by washing or treatment with penicillinase.

In 1968, Izaki and Strominger [27] demonstrated the occurrence in *E. coli* of a DD-carboxypeptidase activity which was also inhibited by penicillin and a LD-carboxypeptidase which was not sensitive to penicillin. The DD-carboxypeptidase hydrolyzes UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala into D-Ala and UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala and the LD-carboxypeptidase hydrolyzes this latter compound into D-Ala and UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-(L)-A_2pm. Subsequently, such activities were shown to occur in many bacteria. They might play an important role in the regulation of the biosynthesis of the peptidoglycan by limiting the number of peptide donors available for transpeptidation.

The most popular hypothesis concerning the mechanism of action of penicillin is that proposed by Tipper and Strominger in 1965 [47]. Penicillin would be a structural analog of the D-Ala-D-Ala donor site

of the nascent peptidoglycan and would be preferentially recognized by the transpeptidase (and the DD-carboxypeptidase). The initial interaction between the enzyme and penicillin would cause the cleavage of the β-lactam amide bond and concomitantly, the irreversible acylation of the enzyme (in the form of a stable penicilloyl-enzyme complex).

On the basis of this hypothesis, it was reasonable to assume that the isolation of cellular sites able to irreversibly fix penicillin might lead to the isolation of the transpeptidase. This approach was followed by Strominger and his colleagues and an exhaustive review of these studies has been recently published [4]. The main result was to show the diversity and multiplicity of the penicillin binding sites in bacteria. In particular, five such compounds were isolated from *Bacillus subtilis* and one of them, apparently the most abundant one, was isolated, purified and characterized as a DD-carboxypeptidase [3]. In no case, however, was a transpeptidase isolated.

C) The strategy

If one excepts those described in the present paper, no bacterial transpeptidase has ever been isolated and characterized. The reason for this situation is that the extraction of a transpeptidase from its membrane-bound multi-enzyme complex and its final isolation cannot be attempted unless the transpeptidase activity can be directly and specifically estimated with the help of a proper system of donor and acceptor peptides. Indeed, the nucleotide precursors UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide which had been used previously to show peptidoglycan synthesis and peptide bridging from bacterial particulate preparations, are not substrates of the transpeptidase. It was thus essential to develop substrate systems which allowed this enzyme to be estimated independently of the preceding biosynthetic reactions. We have used this approach and the results so far obtained are reviewed in the ensuing paragraphs. Two types of transpeptidases were studied. Some of them were membrane-bound; others were exocellular.

II. — THE EXOCELLULAR TRANSPEPTIDASES OF *STREPTOMYCES* R61 AND R39

In 1969, it was observed that certain strains of *Streptomyces* had the ability—apparently unique in the bacterial world—to

excrete a soluble, exocellular DD-carboxypeptidase during growth [20, 30, 31, 32]. These enzymes hydrolyzed the tripeptide Ac_2-L-Lys-D-Ala-D-Ala into D-Ala and Ac_2-L-Lys-D-Ala. In 1972, it was shown that some, but not all, of these DD-carboxypeptidases were also able to perform *in vitro* transpeptidation reactions with the same tripeptide Ac_2-L-Lys-D-Ala-D-Ala acting as donor [42]. The present review describes the transpeptidase-DD-carboxypeptidase enzymes excreted by *Streptomyces* R61 (of which the corresponding membrane-bound enzyme will be described below) and by *Streptomyces* R39. The particular interest of *Streptomyces* R39 is that the bridging in its wall peptidoglycan is very different from that of *Streptomyces* R61 (and probably most *Streptomyces* strains) but is identical to that in *E. coli* (and probably in all Gram-negative bacteria and in many *Bacillaceae*; see figures 1 and 4).

A) General properties

The exocellular enzymes of *Streptomyces* R61 and R39 (in short, the R61 and R39 enzymes) were purified to protein homogeneity on the basis of their DD-carboxypeptidase activity. They are globular proteins, each consisting of a single polypeptide chain [13, 17] with molecular weights of 38,000 and 53,000 for the R61 and R39 enzymes, respectively. The acidic residues (18.9-20.3 %) outnumber the basic ones (7.4-9.7 %). Hydrophobic residues represent 46 % of the total residues of the R61 enzyme and 56 % of the total residues of the R39 enzyme. The R61 enzyme undergoes aggregation by freezing and thawing (the phenomenon is, however, reversible) and the R39 enzyme undergoes aggregation and inactivation at low ionic strength. The fluorescence emission spectrum of the R61 enzyme exhibits a maximum at 318-320 nm (excitation: 273 nm) [36] and that of the R39 enzyme at 339-340 nm [17]. The Km and Vmax values for the tripeptide Ac_2-L-Lys-D-Ala-D-Ala (in the absence of acceptor) are 10 mM and 86 μmoles/min/mg protein for the R61 enzyme and 0.8 mM and 20 μmoles/min/mg protein for the R39 enzyme.

B) Specificity profile for peptide donors

The specificity of the R61 and R39 enzymes for peptide donors was determined on the basis of the release of the C-terminal R_1 residue from peptides presenting the general structure $\sim$ L-R_3-(L or D)R_2-(L or D)R_1. In each case, the C-terminal sequence giving the highest activity is $\sim$ D-Ala-D-Ala but the preceding L-R_3 residue

also has a considerable effect on the enzyme kinetics. The longer the side-chain of the L-R_3 residue (at least up to four —CH_2— as it occurs in L-lysine and diaminopimelic acid), the higher is the enzyme activity [22, 30, 32].

Peptides were synthesized which were analogs of the standard substrate Ac_2-L-Lys-D-Ala-D-Ala [22, 37]. Some of them (for example: Ac-D-Ala-D-Asp and acetyl-racemic-cyclodiaminoadipic acid) were good inhibitors of the R61 enzyme. On the basis of these studies, it was suggested that the C-terminal sequence-D-Ala-D-Ala (of both substrates and inhibitors) was mainly responsible for the initial interaction and binding with the enzyme, whereas the side-chain of the L-R_3 residue of the substrates was critical in inducing catalytic activity. The strong inhibitory activity of acetyl-racemic-cyclodiaminoadipic acid also suggested that the amide bond linking the two C-terminal D-Ala residues had a *cis* configuration when bound to the enzyme. Following the initial binding, the side-chain of the L-R_3 residue would induce a conformational change in the enzyme which, in turn, would result in the previously *cis*-amide linkage adopting a configuration intermediate between *cis* and *trans*, thus losing all double-bond character. This proposed mechanism strictly applies to the R61 enzyme. None of the peptide inhibitors of the R61 enzyme had any effect on the R39 enzyme. In fact, some of them were good substrates for this enzyme.

C) Specificity profile for peptide acceptors [21, 23, 41]

The specificity of the R61 and R39 enzymes for peptide acceptors was determined on the basis of the amount of transpeptidation product formed in reactions of the type Ac_2-L-Lys-D-Ala-D-Ala + acceptor → D-Ala + Ac_2-L-Lys-D-Ala-acceptor. At this stage, the fact that the tripeptide donor was simultaneously hydrolyzed through the DD-carboxypeptidase activity of these enzymes, was not taken into account. In order to function as an acceptor, a peptide must have a structure which resembles that of the natural peptide acceptor involved in the *in vivo* transpeptidation. Thus in strain R61, the cross-link is formed between the penultimate D-alanine of one primary side-chain and a glycine residue that is attached to the ε-amino group of the LL-diaminopimelic acid belonging to another side-chain (fig. 2 and 5). Correspondingly, glycine (also D-alanine, but not L-alanine) and various peptides possessing an N-terminal glycine residue (and other amino compounds) act as acceptors for the R61 enzyme [41]. In strain R39, on the other hand, the inter-

peptide linkage extends directly from a D-alanine residue to the amino group at the D-centre of *meso*-diaminopimelic acid (fig. 1 and 4). Correspondingly, suitable transpeptidation acceptors for the R39 enzyme must have an amino group in α-position to the carboxyl group of a D-amino acid (or glycine) [21]. Hence, the Ac_2-L-Lys-D-Ala-D-Ala + Gly-Gly system is especially suitable for the study of the R61 enzyme. D-Ala is released, and the tetrapeptide Ac_2-L-Lys-D-Ala-Gly-Gly is formed. This system, however, is not utilized by the R39 enzyme. This latter enzyme, on the other hand, catalyzes transfer reactions such as that shown in figure 6.

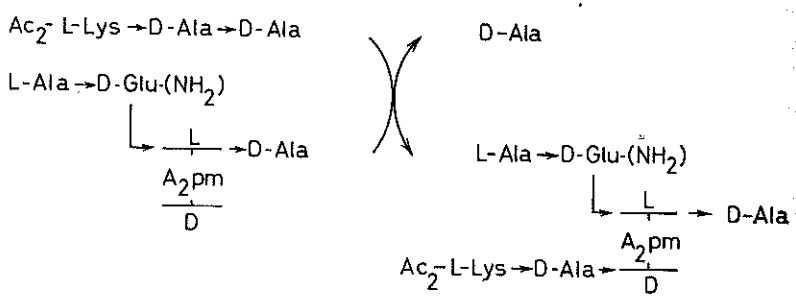


FIG. 6. — In vitro *transpeptidation reaction catalyzed by the exocellular transpeptidase of* Streptomyces *R39*.

More recently, systems were devised from which both R61 and R39 enzymes synthesized peptide dimers (and sometimes trimers) that were analogous or even virtually identical to those found in the walls of the parent strains. Thus the R61 enzyme utilizes the tetrapeptide

Ac-L-Lys-D-Ala-D-Ala
Gly ⌋

both as donor (through the D-Ala-D-Ala sequence) and acceptor (through the N-terminal glycine) and transforms it into the dimer

Ac-L-Lys-D-Ala-D-Ala
Ac-L-Lys-D-Ala-Gly ⌋
Gly ⌋

which, in turn, incorporates another peptide monomer to form a trimer [54]. Similarly, the R39 enzyme utilizes the system L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala and L-Ala-γ-D-Glu(NH_2)-

(L)-*meso*-A_2pm-(L)-D-Ala, giving rise to a peptide dimer [23] which, if one excepts that one of the two glutamic acid residues is not amidated, is identical to the peptide dimers of the walls of *Streptomyces* R39 (fig. 1 and 4).

D) Kinetics [11, 14, 21, 23]

When incubated with a mixture of Ac_2-L-Lys-D-Ala-D-Ala and a proper acceptor, the R61 and R39 enzymes catalyse concomitantly the hydrolysis of the tripeptide donor and the synthesis of the peptide Ac_2-L-Lys-D-Ala-acceptor (fig. 7). D-Ala is a reaction product that is common to both pathways. The activity of these enzymes can be channeled either to the hydrolysis pathway or to the transfer pathway by modifying the experimental conditions.

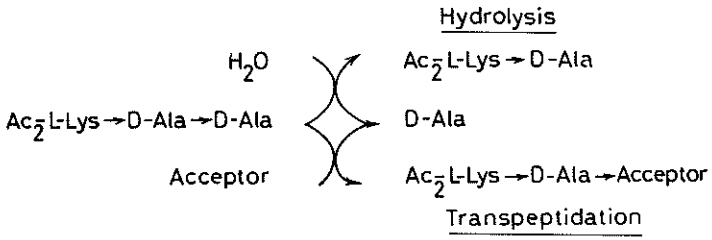


Fig. 7. — *Concomitant hydrolysis and transfer reactions catalyzed by the exocellular transpeptidases of* Streptomyces *R61 and R39.*

Thus transpeptidation is favoured by increasing the pH, by decreasing the water content of the reaction mixture (part of the water being replaced by a mixture of glycerol and ethylene glycol), by increasing the ratio of acceptor to donor (the acceptors behave as non-competitive inhibitors of the hydrolysis pathway) and, in the case of the R39 enzyme, by increasing the ionic strength of the medium.

Since concomitant hydrolysis of the donor also occurs during transpeptidation by the R61 and R39 enzymes, the kinetics are complex. Kinetic studies made with the R61 enzyme suggested that the reactions proceed through an ordered mechanism in which the acceptor molecule binds first to the enzyme [11, 14]. Whatever the mechanism, at infinite concentration of donor, it is possible to determine from Lineweaver-Burk plots a Km_{app} value for the

acceptor (*). The values obtained with the R61 enzyme were about 5 mM for *meso*-diaminopimelic acid as acceptor and 3 mM for the dipeptide Gly-L-Ala. Thus the « affinity » of this R61 enzyme for donor (Km: 10 mM) and acceptor is about the same [14].

When complex acceptors related to peptidoglycan fragments were used, it became apparent that certain features of these substances exerted a profound effect on the relative amount of transpeptidation and donor hydrolysis that occurred. Thus, for instance, the transpeptidase activity (as well as the DD-carboxypeptidase activity) of the R61 enzyme was found to be inhibited by high concentrations of Gly-L-Ala (but not by high concentrations of *meso*-diaminopimelic acid) [14]. Similarly, when the R39 enzyme was incubated in the presence of a fixed amount of Ac_2-L-Lys-D-Ala-D-Ala (0.27 mM) and of increasing concentrations of the tetrapeptide acceptor L-Ala-γ-D-Glu(NH_2)-(L)-*meso*-A_2pm-(L)-D-Ala, the transpeptidation rose to a maximum at an acceptor concentration of about 0.8 mM [21]. At higher concentrations, both transpeptidation and hydrolysis reactions were progressively inhibited until, eventually, the tripeptide donor present in the reaction mixtures remained unused. Thus a high concentration of amidated tetrapeptide acceptor can « freeze » the enzyme so that both substrates remain completely unattacked. This phenomenon was dependent on the α-amide group on the glutamic residue, since high concentration of the same, non-amidated tetrapeptide inhibited overall attack on the donor by inhibiting its hydrolysis, but did not decrease the amount of transpeptidation product formed (which remained at a constant maximal value). More extensive studies also carried out with the R39 enzyme [23] showed that when the donor site of the enzyme was saturated, the rate of the total reaction (hydrolysis + transpeptidation) was the same as the rate of hydrolysis alone when no acceptor was present. The enzyme had the same turnover number for D-alanine release whether or not acceptor was present. Moreover, the less the enzyme was saturated by the donor, the lower was the concentration of amidated tetrapeptide required to inhibit transpeptidation (as well as hydrolysis). These—and other— studies led to the conclusion that mechanisms able to change the (absolute and relative) acceptor

(*) The general equation for an enzyme-catalysed bimolecular reaction is

$$\frac{E_0}{V} = \Phi_0 + \frac{\Phi_A}{[A]} + \frac{\Phi_B}{[B]} + \frac{\Phi_{AB}}{[A][B]}.$$

Rigorously, the Km_{app} value for substrate A is equal to $\frac{\Phi_A}{\Phi_0}$.

and donor concentrations or to alter some particular structural feature of these peptides (such as the amidation of the glutamic acid residue) were important for the control of the activity of the R39 enzyme. Similar mechanisms could well be involved in the control of the peptide cross-linking system *in vivo*.

E) Interaction between enzyme and penicillin in the absence of substrate

1) *General scheme* [15, 16]

Both R61 and R39 enzymes react with penicillins and cephalosporins to form equimolar and inactive enzyme-antibiotic complexes. When incubated at 37° C in the absence of free antibiotic, these complexes undergo breakdown. If the incubation is carried out under conditions when the enzyme is stable, breakdown of the complexes causes reactivation of the enzyme and restoration of its penicillin sensitivity. In marked contrast, the released antibiotic is chemically modified and biologically inactive.

The half-lives of the enzyme-antibiotic complexes vary depending upon the enzyme, the antibiotic and the conditions of incubation. Thus, at 37° and in 10 mM phosphate buffer pH 7.0, the half-life of the R61 enzyme-benzylpenicillin complex is 80 min. Under the same conditions, the R61 enzyme-cephalosporin C complex is 100 times more stable [16]. At 37°, in 0.1 M Tris-HCl buffer pH 7.7 supplemented with 0.2 M NaCl and 0.05 M $MgCl_2$ (a high ionic strength is necessary for the stability of the R39 enzyme), the half-life of the R39 enzyme-benzylpenicillin complex is about 4,200 min and that of the R39 enzyme-cephalosporin C complex is higher than 40,000 min [15].

Within the limits of the techniques used, only one radioactive product was shown to be released from the complexes formed between the R61 and R39 enzymes and [^{14}C]benzylpenicillin [15, 16]. This product was neither benzylpenicillin nor benzylpenicilloic acid. Moreover, the presence of large amounts of penicillinase during breakdown of the complex neither affected the first order reaction rate for release of the compound and enzyme recovery nor caused formation of benzylpenicilloic acid.

2) *Choice among models*

E = active enzyme; I = inhibitor (*i. e.* the antibiotic); EI = an intermediate and inactive enzyme-inhibitor complex, EI* = inactive

enzyme-inhibitor complex in which both constituents are modified; X = released and chemically modified antibiotic; K = dissociation constant; k = rate constant.

The simple model $E + I \overset{K}{\rightleftarrows} EI^* \overset{k}{\rightarrow} E + X$ could be immediately discarded. Indeed, the rapid reversible breakdown of complex EI^* with benzylpenicillin in the presence of penicillinase would have given rise to benzylpenicilloic acid, a conclusion which was in conflict with the observed facts. The five following models were envisaged [12]:

$$\text{Model IA} : \quad E + I \overset{k_1}{\underset{k_2}{\rightleftarrows}} EI^* \overset{k_4}{\rightarrow} E + X$$

$$\text{Model IB} : \quad E + I \overset{k_1}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X$$

$$\text{Model IIA} : \quad E + I \overset{k_1}{\underset{k_2}{\rightleftarrows}} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X$$

$$\text{Model IIB} : \quad E + I \overset{k_1}{\rightarrow} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X$$

$$\text{Model IIC} : \quad E + I \overset{K}{\rightleftarrows} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X$$

In models of type II, enzyme and antibiotic react together to form an intermediate inactive complex EI which, in turn, undergoes isomerisation into complex EI^*. Models IA and IIA are general. Models IB and IIB are limiting cases where k_2 is much smaller than k_4 (model IB) or k_3 (model IIB). Since microscopic reversibility occurs in the systems, models IB and IIB are necessarily approximations. Finally, in model IIC, the first step of the interaction is a rapid equilibrium process characterized by a dissociation constant K.

Mathematical treatment of these models showed that under conditions where the enzyme concentration was much lower than that of the antibiotic, a choice among them could be made on the basis of the influence exerted by the antibiotic concentration on the apparent rate constant k_a for the formation of complex EI^* [12]. The condition $[E] \ll [I]$ was necessary in order to allow linearization of the differential equations and integration of the rate equations. For each antibiotic concentration used, the k_a value was estimated from plots of $\ln \left(1 - \frac{[EI^*]}{E_0}\right)$ *vs* time (where E_0 = total concentration of active and inhibited enzyme) and the concentrations of complex EI^* formed as a function of time were estimated in the case of the R39 enzyme by measuring the residual enzyme activity [unpublished results] and in the case of the R61 enzyme by measuring the emission fluorescence of the reaction mixture at 320 nm. Indeed, the fluo-

rescence of the R61 enzyme (but not that of the R39 enzyme) is decreased by 25-30 % as a result of its interaction with various penicillins [36] (benzylpenicillin, carbenicillin, ampicillin, penicillin V) and by 50-60 % as a result of its interaction with various cephalosporins (cephalosporin C, cephaloglycine) [12]. This particular property of the R61 enzyme allowed us to determine the k_a values with high accuracy by using, when necessary, stopped-flow techniques. At high antibiotic concentrations, the plots k_a *vs* [I] showed deviation from linearity and hence, models IA and B for which $k_a = k_1$ [I], were eliminated. At antibiotic concentrations causing non linearity in these plots k_a *vs* [I], the velocity of the formation of complex EI* was immediately maximal at time zero, a property which did not fit models IIA and B in which cases the velocity of the reaction should have been maximal only after a lag time. Hence model IIC was the simplest one which, within the limits of experimental errors, best explained all the experimental facts. On the basis of this model, the K and k_3 values were measured from the straight lines obtained by plotting $1/k_a$ *vs* 1/[I]. In such double reciprocal plots, the line intercepts the ordinate axis at the $1/k_3$ value and its slope is equal to the ratio K/k_3. Tables I and II give the K, k_3 and k_4 values (this

TABLE I. — *Values of the constants for the interaction between the exocellular transpeptidase of* Streptomyces *R61 and β-lactam antibiotics according to model IIC:*

$E + I \overset{K}{\rightleftarrows} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X$ *(at 37° C unless otherwise stated).*

Antibiotic	K (mM)	$k_3(s^{-1})$	$\frac{k_3}{K}(M^{-1}s^{-1})$	$k_4(s^{-1})$	Half-life of complex EI* (min)
Benzylpenicillin	13 (25° C)	179 (25° C)	13,700 (25° C)	0.21×10^{-4} (25° C)	530 (25° C)
				1.4×10^{-4}	80
Carbenicillin	0.109	0.091	830	1.4×10^{-4}	80
Ampicillin	7.2	0.77	107	1.4×10^{-4}	80
Penicillin V	> 1	> 1	1,500	2.8×10^{-4}	40
Cephalosporin C	> 1	> 1	1,150	1×10^{-6}	11,200
Cephaloglycine	0.4	0.0085	21	3×10^{-6}	3,700

Experiments were carried out in 10 mM phosphate pH 7.0.

TABLE II. — *Values of the constants for the interaction between the exocellular transpeptidase of* Streptomyces *R39 and β-lactam antibiotics according to model IIC:*

$$E + I \overset{K}{\rightleftarrows} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X.$$

Antibiotic	$\frac{k_3}{K}$ ($M^{-1}s^{-1}$) (at 20° C)	k_4 (s^{-1}) (at 37° C)	Half-life of complex EI* (at 37° C) (min)
Benzylpenicillin	100,000	2.7×10^{-6}	4,250
Carbenicillin	3,000	5.4×10^{-6}	2,125
Ampicillin	67,000	4.4×10^{-6}	2,600
Methicillin	1,100	2.1×10^{-5}	545
Cephalosporin C	35,400	$< 3 \times 10^{-7}$	> 40,000
Cephalexin	3,300	2.4×10^{-6}	4,800
Cephaloglycine	74,000	$< 3 \times 10^{-7}$	> 40,000

[Unpublished data]. These experiments were carried out by Miss Noha Fuad (University of Liège). The buffer used was 0.1 M Tris-HCl buffer pH 7.7 supplemented with 0.2 M NaCl and 0.05 M $MgCl_2$.

latter value being determined independently on the basis of the half-lives of the complexes) for the interactions between the R61 and R39 enzymes and various β-lactam antibiotics.

3) *Sensitivity and resistance.*

At relatively low concentrations of antibiotic, the important parameter for activity is the ratio k_3/K (tables I and II). The higher this ratio, the more active is the antibiotic. A high ratio value may be caused by a high k_3 value (R61 enzyme + benzylpenicillin) or by a low K value (R61 enzyme + carbenicillin). At very low concentrations of antibiotic, the activity of the inhibitor is also much dependent upon the k_4 value. Indeed, the higher the k_4 value, the smaller is that part of the total enzyme which is immobilized in the form of complex EI*. Therefore, at equal K and k_3 values, the enzyme is more resistant to an antibiotic for which the k_4 value is high than to another antibiotic for which the k_4 value is low. It thus follows that alterations in the structure and/or conformation of the transpeptidases that would not affect their catalytic activity but would alter the relevant K, k_3 or k_4 values, may lead to « resistance »

to antibiotics [12]. Comparison between the data of tables I and II and those of table III well illustrates this phenomenon. ID_{50} values —*i. e.* the antibiotic concentrations required to inhibit by 50 % the activity of the enzymes in the presence of a low concentration of tripeptide donor— had been used previously [9] to estimate the sensitivity of these enzymes. These ID_{50} values (table III) show that, depending upon the antibiotic, the R61 enzyme is 3 (for benzylpenicillin) to 2,750 (for cephalexin) times more « resistant » than the R39 enzyme. If, under the conditions where they were determined, these ID_{50} values were totally independent of the k_4 values for the interaction between the enzyme and the antibiotic, then they should be directly related to the reciprocals of the k_3/K values. Such a

TABLE III. — *Effect of β-lactam antibiotics on activity of the exocellular DD-carboxypeptidases-transpeptidases of* Streptomyces *R39 and R61.*

Antibiotic	ID_{50} value *(a)* (10^{-8} M)	
	R39 enzyme *(b)*	R61 enzyme
Benzylpenicillin	5	14
Carbenicillin	200	800
Ampicillin	3	2,600
Methicillin	50	14,000
Penicillin V	4	190
Cephalosporin C	5	45
Cephalexin	20	55,000
Cephaloglycine	6	6,900

a) The ID_{50} values are the antibiotic concentrations which decrease by 50 % both the amount of D-Ala released from Ac_2-L-Lys-D-Ala-D-Ala in the absence of acceptor and the amount of transpeptidation product formed from the system Ac_2-L-Lys-D-Ala-D-Ala + [^{14}C]Gly, under standard conditions as described in [9]. In both tests, the Ac_2-L-Lys-D-Ala-D-Ala concentration was 1.23 mM, *i. e.* about 10 times lower than the Km value. For the transfer reaction, the molar ratios of [^{14}C]Gly to the tripeptide were 10:1 for the R61 enzyme and 20:1 for the R39 enzyme.

b) With the R39 enzyme and benzylpenicillin, ampicillin, penicillin V, cephalosporin C and cephaloglycine, the enzyme concentration used in the experiments was very close to the ID_{50} values. Consequently, these values most probably reflect a titration phenomenon. The ID_{50} value is independent of the enzyme concentration only if $ID_{50} \gg$ enzyme concentration. Under conditions when $ID_{50} \simeq$ enzyme concentration, the ID_{50} value experimentally obtained is higher than the real one.

correlation should appear by comparing either the action of various antibiotics on one specific enzyme or the response of various enzymes to one specific antibiotic. From the data so far accumulated, table IV-A shows that, with both R61 and R39 enzymes, the ratios $\frac{k_3/K \text{ (for carbenicillin)}}{k_3/K \text{ (for other antibiotics)}}$ and the ratios $\frac{ID_{50} \text{ (for other antibiotics)}}{ID_{50} \text{ (for carbenicillin)}}$ are similar or at least are of the same order of magnitude. Similarly, table IV-B shows that, for the various antibiotics tested, the ratios $\frac{k_3/K \text{ (for the R39 enzyme)}}{k_3/K \text{ (for the R61 enzyme)}}$ and the ratios $\frac{ID_{50} \text{ (for the R61 enzyme)}}{ID_{50} \text{ (for the R39 enzyme)}}$ are also similar. This fair correlation between k_3/K and ID_{50} values is true with antibiotics for which the k_3/K values range from 21 to 13,700 $M^{-1}s^{-1}$ in the case of the R61 enzyme, and from 1,100 to 100,000 $M^{-1}s^{-1}$ in the case of the R39 enzyme.

4) *Penicillin as substrate of the exocellular transpeptidases.*

Both R61 and R39 enzymes convert the antibiotic into a biologically inactive compound, *i. e.* catalyze the reaction $I \rightarrow X$, with a low efficiency. The rate constant k_3 value for the isomerisation $EI \rightarrow EI^*$, however, may be very high (179 s^{-1} for the interaction between the R61 enzyme and benzylpenicillin; see table I) and similar to the $k_{cat.}$ value of a true enzymatic reaction. On the other hand, the breakdown of the complexes EI^* into $E + X$ is always a slow process. In fact, tables I and II show the specificity profiles of the R61 and R39 enzymes for various antibiotics considered as substrates [12].

The exact nature of the alteration undergone by the antibiotic molecule during its interaction with the R61 and R39 enzymes is still unknown. The radioactive compound X arising through the action of both enzymes on [^{14}C]benzylpenicillin has an electrophoretic migration at pH 6.0 which is intermediate between those of benzylpenicillin and benzylpenicilloic acid. Moreover, it does not react with the iodine reagent as benzylpenicilloic acid does. Several mg of this compound have been prepared. Its identification is under current study.

The structure of the modified antibiotic molecule as it occurs in the complexes EI^* (and before the release) is also unknown. Most likely, differences at this stage occur between the R61 and the R39 enzymes. Thus, for instance, by using the chromogenic cephalosporin 87-312 (3-[2,4-dinitrostyryl]-[6R-7R]-7-[2-thienylacetamide]-ceph-3-em-4-carboxylic acid, E isomer), the absorption spectrum of the complex EI^* formed with the R39 enzyme is identical to that of cephalosporin 87-312 hydrolyzed by penicillinase (*i. e.* the absorp-

TABLE IV. — *Correlation between the k_3/K values (tables I and II) and the ID_{50} values (table III) for the interaction between the R61 and R39 enzymes and β-lactam antibiotics.*

Antibiotics	A) Comparative action of the antibiotic on the enzymes						B) Comparative response of the enzymes to the antibiotics		
	R61 enzyme			R39 enzyme			a	b	$\frac{a}{b}$
	$(k_3/K)_{rel.}$ (*)	$(ID_{50})_{rel.}$ (**)	$\frac{(k_3/K)_{rel.}}{(ID_{50})_{rel.}}$	$(k_3/K)_{rel.}$ (*)	$(ID_{50})_{rel.}$ (**)	$\frac{(k_3/K)_{rel.}}{(ID_{50})_{rel.}}$	$\frac{k_3/K_{(R39)}}{k_3/K_{(R61)}}$	$\frac{ID_{50\ (R61)}}{ID_{50\ (R39)}}$	
Benzylpenicillin	0.061	0.0175	3.5	0.03	0.025	1.2	7.3	2.8	2.6
Carbenicillin	1	1	1	1	1	1	3.61	4	0.9
Ampicillin	7.75	3.25	2.4	0.045	0.015	3	626	867	0.72
Methicillin	—	—	—	2.7	0.025	11	—	—	—
Penicillin V	0.55	0.24	2.3	—	—	—	—	—	—
Cephalosporin C	0.72	0.056	12.8	0.085	0.025	3.4	31	9	3.4
Cephalexin	—	—	—	0.9	0.1	9	—	—	—
Cephaloglycine	39.5	8.62	4.5	0.04	0.03	1.33	3,523	1,150	3.1

(*) Ratio of the k_3/K value for carbenicillin to the k_3/K value for the other antibiotic.
(**) Ratio of the ID_{50} value for the antibiotic to the ID_{50} value for carbenicillin.

tion spectrum shifts from 386 to 482 nm) [15]. On the contrary, the absorption spectrum of the complex EI* formed with the R61 enzyme, has a maximum at 440 nm. In this case, a spectrum identical to that of cephalosporin 87-312 hydrolyzed by penicillinase, only appears after the breakdown of the complex accompanied by restoration of enzymic activity [16].

The possible analogy between transpeptidase and penicillinase is also a problem of interest. In addition to the transpeptidase, *Streptomyces* strain R39 excretes a soluble, exocellular penicillinase during growth. This penicillinase has been separated from the transpeptidase. Comparative biochemical studies of these two enzymes are in progress [28].

5) *Titration of the exocellular transpeptidases.*

Owing to the rapidity of formation of the complexes EI* and their relatively high stability, both R61 and R39 enzymes can be « titrated » by addition of a β-lactam antibiotic solution (and *vice versa*). Details of these techniques can be found elsewhere [15, 16, 36]. The titration of the enzymes may be based on the loss of enzyme activity, on the fluorescence quenching (with the R61 enzyme) or on the changes in the absorbance of the solution at a proper wavelength (by using cephalosporin 87-312 or cephaloridin).

F) Interaction between the R61 enzyme, penicillin and the peptide donor Ac_2-L-Lys-D-Ala-D-Ala

1) *Lineweaver-Burk kinetics.*

Early experiments had shown that by incubating together enzyme, tripeptide donor and antibiotic for some time (30-60 min) at 37°, and on the basis of the residual activity measured under these conditions, the R61 enzyme was « competitively » inhibited by the antibiotics [30]. With the R39 enzyme the inhibition seemed to be of an « allosteric » type [32]. These experiments had been carried out at a time when the enzymes available were only partially purified (and their molecular weight not known). It was found subsequently that the antibiotic concentrations that had been used were roughly equivalent to that of the enzyme. In fact the experiments carried out with the R39 enzyme should have been interpreted as a case of enzyme « titration ». With the R61 enzyme, however, the interpretation was more difficult and therefore additional experiments were made. The enzyme used had been purified to protein homogeneity and the antibiotic concentrations were much higher

than that of the enzyme. Once more, « competitive » inhibition was observed [16]. Thus for instance, Dixon plots gave with penicillin V, K_i values with regard to the peptide donor of 0.33 μM for the inhibition of hydrolysis in the absence of acceptor, 0.62 μM for the inhibition of hydrolysis in the presence of acceptor and 0.65 μM for the inhibition of transpeptidation. On the other hand, the inhibition of this latter reaction was non-competitive with regard to the acceptor [16].

2) *Competitive and non-competitive inhibition according to model IIC.*

The theory of competitive inhibition is based on the assumption that the step(s) in which the inhibitor is involved are rapid equilibrium processes. Hence, model IIC in which two steps are irreversible processes and in which an intermediate complex relatively stable is formed, could not give rise, at least at first sight, to competitive inhibition kinetics.

According to model IIC, a competitive inhibition (characterized by the fact that a ternary complex ESI cannot be formed) would proceed as follows:

$$\begin{array}{l} E + I \overset{K}{\rightleftarrows} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X \\ + \\ S \\ K_s \left\{ \begin{array}{ll} k_1' \downarrow\uparrow k_2' & \text{where P = hydrolysis product} \\ ES & \\ \downarrow k_3' & \\ E + P & \text{and } K_s = \dfrac{k_2' + k_3'}{k_1'} \end{array} \right. \end{array}$$

Assuming that P_M is the amount of hydrolysis product formed after time t if all the enzyme were in the form ES and P is the amount of hydrolysis product formed after the same time t in the presence of inhibitor (and in the presence of non-saturating concentrations of substrate), it can be shown that

$$\frac{P}{P_M} = \frac{k_4}{b(k_4 + k_a')} + \frac{k_a'}{b(k_4 + k_a')^2 t}\left[1 - e^{-(k_4 + k_a')t}\right]$$

If $[I] \ll K$, then $b = 1 + \dfrac{K_s}{[S]}$ and k_a' (*i. e.* the apparent rate constant for the formation of complex EI^*) $= \dfrac{k_3[I]}{K\left(1 + \dfrac{[S]}{K_s}\right)}$ [unpublished results]. The above equation is only valid if the enzyme concentration is much smaller than the antibiotic concentration and if the concen-

tration of the hydrolysis product formed is negligible when compared to the initial concentration of substrate.

On the basis of the preceding equation, a Lineweaver-Burk plot of $\frac{1}{[P]}$ *vs* $\frac{1}{[S]}$ for various concentrations of antibiotic should give rise to a series of curves. Unexpectedly, however, under the following conditions: 1) by giving to the K_s constant the Michaelis constant value of the R61 enzyme found experimentally for the tripeptide Ac_2-L-Lys-D-Ala-D-Ala (10 mM); 2) by assigning the K, k_3 and k_4 values of table I; and 3) by using the substrate concentrations, antibiotic concentrations and incubation times that had been actually used for the kinetics published previously [16] (in which cases both conditions $[E] \ll [I]$ and $[I] \ll K$ were fulfilled), it was found that the theoretical plots $\frac{1}{[P]}$ *vs* $\frac{1}{[S]}$ thus obtained for various antibiotic concentrations gave rise to series of « curves » that were indistinguishable from straight lines and that these lines converged to the same value of the ordinate axis. In other words, the plots were typical of classical competitive inhibitions. Moreover, the theoretical plots thus obtained coincided remarkably well with the experimental plots obtained previously (fig. 8) and the theoretical K_i values estimated from Dixon plots were identical or at least similar to the experimental ones:

— penicillin V: 0.33 μM (theoretical and experimental);
— carbenicillin: 0.73 μM (theoretical) and 1.05 μM (experimental);
— benzylpenicillin: 61.5 nM (theoretical) and 80 nM (experimental).

Competitive inhibition means that fixation of the substrate (*i. e.* the tripeptide donor) and fixation of the antibiotic on the same form of enzyme are mutually exclusive. Therefore, the question arose whether or not the above results excluded the possibility of the formation of the ternary complex enzyme-substrate-antibiotic (ESI). According to model IIC, a non-competitive inhibition would proceed as follows:

$$\begin{array}{ccccccc} E + I & \overset{K}{\rightleftarrows} & EI & \overset{k_3}{\rightarrow} & EI^* & \overset{k_4}{\rightarrow} & E + X \\ + & & + & & & & \\ S & & S & & & & \\ {\scriptstyle K_m}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K'_m} & & & & \\ ES + I & \overset{K'}{\rightleftarrows} & ESI & & & & \\ \downarrow{\scriptstyle k'_3} & & & & & & \\ E + P & & & & & & \end{array}$$

The four steps in the loop are rapid equilibrium processes and therefore $K_m K' = K K'_m$.

Surprisingly, mathematical treatment showed that if as above $[E] \ll [I] \ll K$ and if, in addition, $[I] \ll K'$, then such a non-competitive model gives rise to linear plots that are those of a classical competitive inhibition ! [unpublished results].

The above studies showed that the inhibition of the R61 enzyme by penicillin may appear to be competitive with regard to the peptide donor in Lineweaver-Burk plots although it involves the formation of an intermediate complex EI* exhibiting a rather long half-life. These studies also showed that these plots do not exclude the possibility that the inhibition proceeds through a non-competitive mechanism.

The above conclusion strictly applies to the inhibition of the R61 enzyme by β-lactam antibiotics. Most likely, however, it also applies to other similar enzymes, such as the DD-carboxypeptidases from *B. subtilis* and *B. stearothermophilus* for which both « competitive » inhibition by β-lactam antibiotics and formation of rather stable enzyme-antibiotic complexes were described [4, 5, 48, 53]. Contrary to a suggestion made by Blumberg and Strominger [4], the present studies support the idea that all DD-carboxypeptidases and transpeptidases are probably inhibited by penicillin according to the same general mechanism, although, depending upon the enzyme and/or the technique used, the inhibition may appear as « competitive » (in Lineweaver-Burk plots) or as « irreversible » (*i. e.* involving the formation of rather stable complexes EI*).

The present studies demonstrate that a structural analogy between penicillin and donor substrate cannot be justified on the basis of a « competitive » inhibition. The type of inhibition of the exocellular R61 enzyme by penicillin, however, remains undetermined since it is not possible to distinguish between competitive and non-competitive models. Results obtained in other laboratories with the membrane-bound DD-carboxypeptidases from *B. stearothermophilus* [2], *S. faecalis* [40] and *Proteus mirabilis* [34] would be best explained on the basis of a non-competitive model. These data, however, are still incomplete. Indeed, the stability of the complexes EI* and the molar ratios enzyme/antibiotic used in these experiments are not known. Under these conditions, a definite choice requires further work.

III. — THE MEMBRANE-BOUND TRANSPEPTIDASE OF *STREPTOMYCES* R61

A) Synthetic substrates

By analogy with what had been observed with the exocellular R61 enzyme, one could assume that the tripeptide Ac_2-L-Lys-D-Ala-

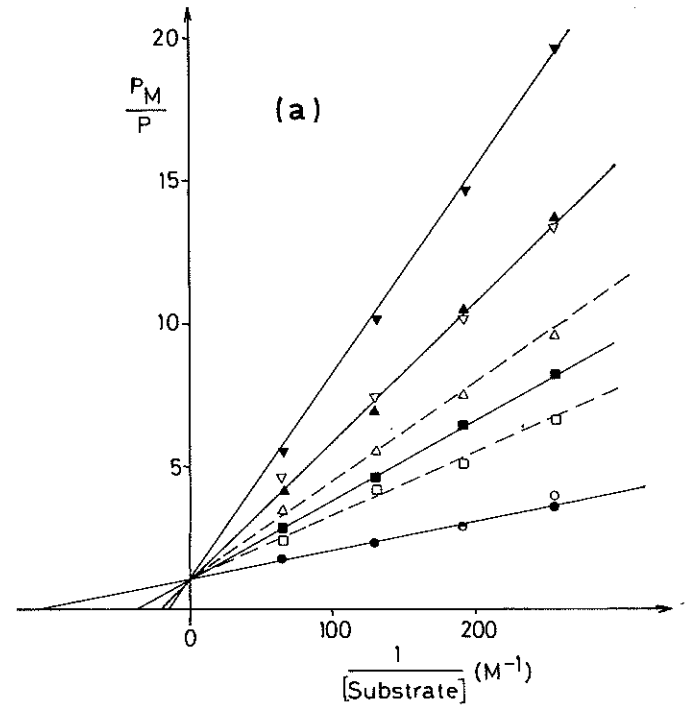

20
$\frac{P_M}{P}$
(a)
15
10
5
0
100
200
$\frac{1}{[\text{Substrate}]}$ (M^{-1})

FIG. 8 *a*.

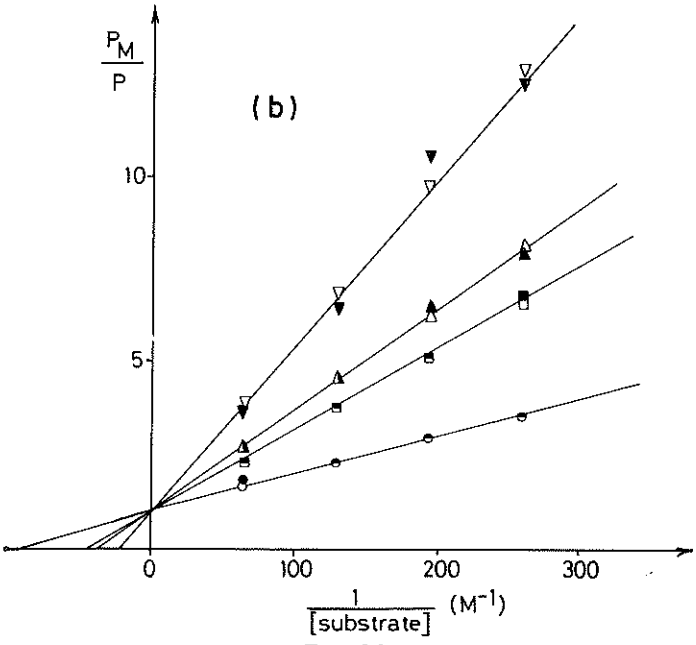

$\frac{P_M}{P}$
(b)
10
5
0
100
200
300
$\frac{1}{[\text{substrate}]}$ (M^{-1})

FIG. 8 *b*.

D-Ala (an analog of the natural peptide donor) and the dipeptide Gly-Gly (an analog of the natural peptide acceptor) would be also recognized and utilized by the membrane-bound transpeptidase. The assumption was right. By incubating together these two peptides with isolated plasma membranes, the tetrapeptide Ac_2-L-Lys-D-Ala-Gly-Gly was synthesized and an equivalent amount of D-alanine was liberated [9]. In addition to Gly-Gly, free glycine, D-alanine (but not L-alanine), various peptides possessing a N-terminal glycine residue and other amino compounds also functioned as acceptors for the reaction [9], *i. e.* the specificity profile of the membrane-bound transpeptidase for acceptors closely resembled that of the exocellular R61 enzyme. With the membrane-bound enzyme, however, the liberation of D-Ala from the tripeptide donor was dependent on the simultaneous presence of an acceptor. Within the limits of the technique used, a DD-carboxypeptidase activity could not be detected in the isolated membranes. Gly-Gly was one of the best acceptors. It has been used routinely as standard acceptor in the experiments described below.

B) Kinetics [10]

From Lineweaver-Burk plots, the Km_{app} value of the isolated membranes for the dipeptide acceptor (at infinite concentration

---

Fig. 8. — *Interaction between R61 enzyme, tripeptide Ac_2-L-Lys-D-Ala-D-Ala and β-lactam antibiotics. Theoretical and experimental plots of* $\frac{P_M}{P}$ *versus* $\frac{1}{[S]}$ *in the absence and in the presence of various concentrations of carbenicillin (a) and penicillin V (b).*

Filled symbols: theoretical plots (see text); open symbols: experimental plots [16]. The enzyme concentration was 17 nM. $\frac{P_M}{P}$: see text (P_M is the amount of hydrolysis product which would be formed at time t if all the enzyme were in the form ES; hence P_M is a constant).

a) The carbenicillin concentrations were: zero (○ and ●); 1.13 μM (□ and ■); 2.25 μM (△ and ▲) and 3.4 μM (▽ and ▼). The experimental plot at [I] = 3.4 μM (▽) and the theoretical plot at [I] = 2.25 μM (△) were practically superimposable.

b) The penicillin V concentrations were: zero (○ and ●); 0.53 μM (□ and ■); 0.76 μM (△ and ▲) and 1.52 μM (▽ and▼). In all cases, the theoretical and experimental plots were superimposable.

It should be noted that less than 13 % of substrate was utilized in each case. Hence, it was assumed that the substrate concentration remained constant throughout the experiment.

of donor) was 4 mM, and the relevant value for the tripeptide donor (at infinite concentration of acceptor) was 40 mM. At 37°, the Vmax value (at infinite concentrations of both donor and acceptor) was about 1.7 nmoles of transpeptidation product formed/min/mg protein.

Mixtures of substrates and membranes, once prepared, were immediately immersed in liquid nitrogen and then incubated in the frozen state at temperatures ranging from — 5° to — 70°. Surprisingly, the enzyme functioned normally at least down to a temperature of — 30° ! At lower temperatures, no transpeptidation product was formed. In the frozen state, the Km_{app} value for the acceptor was the same as that measured with liquid membrane suspensions but that for the donor was 4 mM. Experiments were carried out at — 5° simultaneously in the frozen state and in the liquid state (if not immersed in liquid nitrogen prior incubation, reaction mixtures remain liquid at — 5°). The Vmax value was 4 times higher in the frozen state than in the liquid state. Obviously, such a behaviour suggested that the membrane-bound transpeptidase functions in a lipid environment which remains remarkably fluid at very low temperatures. Moreover, the frozen state somehow imparts to the enzyme a conformation which favours both its interaction with the tripeptide donor and its catalytic activity.

C) Membrane-bound transpeptidase and penicillin binding site [33]

Isolated membranes fix 25 picomoles of [^{14}C]benzylpenicillin per mg of protein under the following conditions. Membranes and [^{14}C]benzylpenicillin were incubated for 20 min at 22° in phosphate buffer pH 7.0, washed twice with the same buffer by centrifugation at 4° and the radioactivity of the pellets was measured.

The fixation of other non-radioactive penicillins and cephalosporins by the isolated membranes was estimated by determining the amount of non-radioactive antibiotic required to exclude [^{14}C]benzylpenicillin from 50 % of its binding sites (Exclusion D_{50} values). These Excl. D_{50} values were found to be identical or at least very similar to those required to inhibit by 50 % the growth of *Streptomyces* R61 (LD_{50} values) and to inhibit by 50 % the *in vitro* activity of the membrane-bound transpeptidase (at a low concentration of tripeptide donor; ID_{50} values). This correlation was true with antibiotics of which the inhibiting concentration *in vivo* and *in vitro* ranged from $2\text{-}5 \times 10^{-6}$ M to $100\text{-}510 \times 10^{-6}$ M (table V). Thus,

most likely, the membrane-bound transpeptidase as revealed by the synthetic substrates Ac_2-L-Lys-D-Ala-D-Ala and Gly-Gly was the physiological one and the penicillin binding sites were, at least for a majority of them, the transpeptidase molecules.

TABLE V. — *Competition between β-lactam antibiotics for the binding sites of the* Streptomyces *R61 membrane* (Excl. D_{50} values) *and its correlation with the* in vitro *inhibition of the membrane-bound transpeptidase* (ID_{50} values) *and the* in vivo *inhibition of the growth of* Streptomyces *R61* (LD_{50} values) *by the same antibiotics.* Excl. D_{50}, ID_{50} and LD_{50} values are expressed in μM.

Antibiotics	Excl. D_{50} values	ID_{50} values	LD_{50} values	Excl. D_{50} / ID_{50}	Excl. D_{50} / LD_{50}
	μM				
Benzylpenicillin	3	2	5	1.50	0.60
Penicillin V	4	18	5	0.22	0.80
Ampicillin	9	8	5	1.10	1.80
6-Aminopenicillanic acid	13	80	18	0.16	0.72
Carbenicillin	30	34	33	0.90	0.91
Cephaloglycine	34	62	29	0.55	1.16
Methicillin	37	35	44	1.06	0.84
Cloxacillin	45	39	50	1.16	0.90
Oxacillin	65	88	47	0.74	1.40
Cephalothin	65	70	14	0.93	4.64
Cephalexin	78	60	21	1.30	3.70
Cephalosporin C	220	510	112	0.43	2

For more details, see text and ref. [33]. For the determination of the ID_{50} values, the Ac_2-L-Lys-D-Ala-D-Ala concentration was 1.35 mM (*i. e.* about 30 times lower than the Km_{app} value at infinite concentration of acceptor) and the acceptor [^{14}C]Gly-Gly concentration was 13.5 mM (*i. e.* about 3 times higher than the Km_{app} value at infinite concentration of donor).

D) PENICILLIN, A SUBSTRATE OF THE MEMBRANE-BOUND TRANSPEPTIDASE [33]

Membranes previously saturated (and inactivated) by a β-lactam antibiotic, were resuspended, in the absence of free antibiotic, in

phosphate buffer pH 7.0 and incubated at 37° in the presence of the substrates. As observed previously with the exocellular transpeptidase, the membranes underwent spontaneous reactivation and during the process, the antibiotic was released in a modified and biologically inactive form. At pH 7.0 and 37° the first order reaction rate constant s^{-1} varied depending upon the antibiotic initially fixed to the membranes (table VI). The value with benzylpenicillin was $1.1 \times 10^{-4} s^{-1}$. Remarkably, a same rate constant value for [^{14}C]benzylpenicillin was obtained on the basis of the release of the radioactivity from the membrane-[^{14}C]benzylpenicillin complex. Release of the antibiotic and reactivation of the enzyme was thus the same phenomenon and this observation gave further support to the idea that transpeptidase molecules and penicillin binding sites were the very same compounds. The characterization of the released compound thus also became a problem of major importance. Because of the paucity of the transpeptidase molecules in the membranes (as they were prepared), it was obvious that this problem could not be solved as long as isolated membranes were the only source of enzymes available.

E) Solubilization of the membrane-bound transpeptidase [10]

Osmotic shocks, treatments with urea, various non-ionic detergents, sodium dodecylsulphate and 1-butanol were either totally unsuccessful or at least not satisfactory. Eventually, cetyltrimethylammonium bromide (CTAB) was found to be a solubilizing agent of high efficiency. Moreover, treatment with CTAB could be applied directly to the washed mycelium. The yield in solubilized enzyme was even much higher than that obtained from the isolated membranes.

The CTAB-extracted transpeptidase does not sediment by centrifugation at $200,000 \times g$ for 2 hours; it passes through membranes devised to retain substances of molecular weight higher than 50,000 and it is included in gels of Sephadex G-100 (K_D value: 0.45). By using this latter technique, the enzyme could be separated from a bulk of contaminating proteins and a preparation exhibiting a Vmax value (at saturating concentration of both substrates) of 0.2 μmoles of transpeptidation product formed/min/mg protein was obtained (a value to be compared with that of 1.7 nmoles/min/mg protein with the isolated membranes).

The extracted transpeptidase has no activity in the frozen state. Its Km_{app} value for the donor (at infinite concentration of acceptor) is about 4 mM and that for the acceptor (at infinite concentration

TABLE VI. — *Breakdown of the complexes between β-lactam antibiotics and isolated membranes of* Streptomyces *R61 in 0.017 M K_2HPO_4 at 37° C.*

Antibiotics	Rate constants s^{-1}	Half-life (min)
Ampicillin	3.3×10^{-3}	3.5
Cephalosporin C	3.3×10^{-3}	3.5
Cephalothin	8.3×10^{-4}	14
Penicillin V	2.8×10^{-4}	41
Methicillin	2.1×10^{-4}	55
6-Methylpenicillin V	1.7×10^{-4}	68
6-Aminopenicillanic acid	1.4×10^{-4}	82.5
Cloxacillin	1.23×10^{-4}	94
Benzylpenicillin	1.10×10^{-4}	104
Carbenicillin	0.73×10^{-4}	160

(For more details, see [33]).

of donor) is smaller than 0.2 mM. These values are much smaller than those obtained with a liquid suspension of isolated membranes. If one takes into account that drastic changes have been caused to the environment of the enzyme as a result of its extraction, such differences are not surprising. Nevertheless, the sensitivity to benzylpenicillin of the extracted enzyme is identical to that of the membrane-bound enzyme in liquid suspension (ID_{50}: 5×10^{-6} M). In phosphate buffer pH 7.5 containing CTAB and at 37°, the complex formed between the extracted enzyme and ampicillin has a half-life of 60 minutes [unpublished results].

The extracted and partially purified enzyme (specif.: 0.2 μmoles/min/mg protein) possesses an activity that could not be detected with the membranes [unpublished results]. It reacts with the tripeptide donor and water in the absence of acceptor, according to the equation Ac_2-L-Lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2-L-Lys-D-Ala. Thus, the membrane-bound transpeptidase is also a DD-carboxypeptidase. The DD-carboxypeptidase activity, however, is low. The amount of D-alanine liberated in the absence of acceptor and in the presence of a saturating concentration of donor is about 8 % of the amount of D-alanine liberated when the enzyme functions in the presence of saturating concentrations of both donor and acceptor.

F) Membrane-bound transpeptidases of *Streptomyces* sp. other than *Streptomyces* R61 [unpublished results]

The membrane-bound transpeptidase of *S. rimosus* is very similar (but not identical) to that of *Streptomyces* R61. It is solubilized either from the membranes or from the washed mycelium with CTAB and it can be partially purified by chromatography on Sephadex G-100 (specific activity at saturating concentrations of substrates: 0.6 μmoles/min/mg protein). The extracted enzyme reacts with benzylpenicillin to form a complex of which the half-life at 37° and in phosphate buffer pH 7.5 containing CTAB, is about 200 min. Finally, it also performs a low DD-carboxypeptidase activity. The interest of *S. rimosus* rests upon the fact that its genome has been mapped, at least partially [18].

The isolated membranes of *Streptomyces* K15 possess two enzymes. One of them is essentially a transpeptidase which performs a low DD-carboxypeptidase activity. The other is essentially a DD-carboxypeptidase which performs a low transpeptidase activity. Both enzymes are extracted directly from the washed mycelium with CTAB. They can be separated from each other and partially purified by chromatography on Sephadex G-100. The K_D values are 0.35 for the DD-carboxypeptidase and 0.45 for the transpeptidase. At 37° and in phosphate buffer pH 7.5 containing CTAB, the half-life of the complex formed between the transpeptidase and benzylpenicillin is 100 min. The peptide crosslinking enzyme system in *Streptomyces* K15 is a bi-enzyme complex and hence is more complex than those of *Streptomyces* R61 and *S. rimosus*. When obtained in a state of protein homogeneity, the two K15 enzymes will provide useful models for the study of the nature of the « determinant » which forces this type of membranous enzyme molecules to react preferentially with an amino acceptor (transpeptidase activity) or with water (DD-carboxypeptidase activity).

IV. — MEMBRANE-BOUND TRANSPEPTIDASES FROM BACTERIA OTHER THAN *STREPTOMYCES*

Streptomyces are gram-positive eubacteria which form a characteristic mycelium and multiply by means of special spores. It was desirable to extend the present study to more « conventional » eubacteria. *Streptococcus faecalis* ATCC 9790, a Gram-positive

coccus, and *Escherichia coli* K12 strain 44, a Gram-negative rod-shaped bacterium, were selected because they are being used by others for very extensive studies concerning the cell division process and the topography of wall growth [25]. Moreover, *E. coli* K12 strain 44 (a mutant almost completely devoid of penicillinase) would be one of the best models if genetic studies were to be undertaken. The same approach as that used for the *Streptomyces* enzymes was used for the study of both *S. faecalis* and *E. coli* systems, which was to estimate directly the membrane-bound transpeptidase(s) with the help of appropriate, well-defined peptide substrates.

A) « Streptococcus faecalis » ATCC 9790

Like *Streptomyces* K15, *S. faecalis* possesses a membrane-bound DD-carboxypeptidase which catalyzes the reaction Ac_2-L-Lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2-L-Lys-D-Ala, and like the *Streptomyces* enzymes, the *S. faecalis* enzyme has a considerable specificity for peptides with a C-terminal L-R_3-D-Ala-D-Ala sequence and a long side-chain on the R_3 residue [8]. More recently, a transpeptidase activity which utilizes (in carbonate buffer pH 10.0; I = 0.05) the above tripeptide as donor and at least D-alanine and Gly-Gly as acceptors was shown to also occur in the isolated membranes of *S. faecalis* [unpublished results]. Its specificity profile for more complex acceptors is under study.

The peptide cross-linking enzyme complex in the membranes of *S. faecalis*, however, contrasts with that of the membranes of *Streptomyces* in that, in addition to the « classical » DD-carboxypeptidase-transpeptidase system, it also contains an LD-transpeptidase which catalyzes reactions of the type Ac_2-L-Lys-D-Ala + acceptor → D-Ala + Ac_2-L-Lys-acceptor [8]. Hydrolysis of the dipeptide Ac_2-L-Lys-D-Ala in the absence of acceptor (*i. e.* the corresponding LD-carboxypeptidase activity) was not detected. This LD-transpeptidase has a considerable specificity for peptide donors that have a C-terminal L-R_3-D-Ala sequence with a free ω-amino group at the end of a long side-chain on the R_3 residue, and a considerable specificity for amino group acceptors located on a D-carbon in α-position to a free carboxyl group. Since at least the majority of the peptide cross-linking bonds in *S. faecalis* extend between two D centers and are not in α-position to a free carboxyl group (fig. 3), it was hypothesized that this LD-transpeptidase might be involved in some « specialized » remodeling function as, for instance, wall thickening. In spite of many efforts, however, the possible physiological role of this enzyme is still unknown. Both interestingly and unexpectedly, the LD-trans-

peptidase, like the DD-carboxypeptidase and the « classical » transpeptidase, is inhibited by penicillin [8]. The relative sensitivities of these enzymes, however, differ according to the particular antibiotic used.

The *S. faecalis* enzymes are irreversibly inactivated by CTAB. The DD-transpeptidase is completely extracted from the membranes with the help of « Genapol X-100 » (in carbonate buffer pH 10.0). The purification of the extracted enzyme is under study. As observed with the *Streptomyces* transpeptidases, the benzylpenicillin-inactivated DD-transpeptidase of *S. faecalis*, when incubated at 37° in carbonate buffer pH 10.0 supplemented with Genapol X-100 and in the absence of free antibiotic, spontaneously undergoes reactivation. The half-life of the complex is 11 hours.

B) « E. coli » K12, strain 44

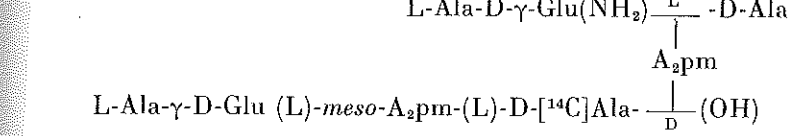
E. coli possesses two membranes in its cell envelope: the inner cytoplasmic membrane and the O-antigen containing outer membrane. A lipoprotein-peptidoglycan complex is sandwiched between these two membranes. The peptide crosslinking system in *E. coli* is exclusively located in the inner membrane [43]. It is exceedingly complex. Moreover, its specificity profile is such that it neither utilizes nor recognizes the tripeptide Ac_2-L-Lys-D-Ala-D-Ala. Hence, substrate systems specific to *E. coli* had to be devised.

1) *The* E. coli *multi-enzyme complex* [38, 39, 43].

UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala is a substrate of the *E. coli* membrane-bound DD-carboxypeptidase (and the hydrolysis product thus obtained is a substrate of the membrane-bound LD-carboxypeptidase; see the Introduction). The free pentapeptide L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala is also a substrate of the DD-carboxypeptidase (and the resulting free tetrapeptide a substrate of the LD-carboxypeptidase). Whereas the nucleotide precursor is not a substrate for transpeptidation, the free pentapeptide is also utilized both as a donor (through its D-Ala-D-Ala sequence) and as an acceptor (through its amino group of the D-center of *meso*-diaminopimelic acid) by the membrane-bound transpeptidase according to the reaction shown in figure 4. According to this reaction, the transpeptidation product formed is a nonapeptide. This nonapeptide, however, can lose one of its C-terminal D-alanine residue or even both of them under the sequential action

of the DD and LD-carboxypeptidases, thus giving rise to a mixture of various dimers: nonapeptide, octapeptide and heptapeptide. In fact, these dimers are identical to those which occur in the completed wall peptidoglycan [49, 51].

Since both the substrate for transpeptidation (*i. e.* the pentapeptide) and the transpeptidation product (*i. e.* the nonapeptide) are hydrolyzed by the DD-carboxypeptidase, a more specific test for the transpeptidase was devised. It consists of a mixture containing a low concentration of radioactive pentapeptide L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-[^{14}C]Ala-D-[^{14}C]Ala and a high concentration of non-radioactive, amidated tetrapeptide L-Ala-γ-D-Glu(NH_2)(L)-*meso*-A_2pm-(L)-D-Ala. D-[^{14}C]Ala is liberated and the mono-amidated octapeptide dimer is formed:



The advantages of this improved system are the following: 1) the amidated tetrapeptide functions only as an acceptor; 2) neither the amidated tetrapeptide acceptor nor the mono-amidated octapeptide dimer are substrates of the DD-carboxypeptidase; 3) at high concentration, as it is used, the amidated tetrapeptide acceptor inhibits, at least partially, the DD-carboxypeptidase activity on the pentapeptide; 4) the occurrence of one amide group in the radioactive transpeptidation product makes it readily separable by electrophoresis from the other radioactive compounds present in the reaction mixture, *i. e.* the liberated D-Ala, that part of the pentapeptide which is not utilized in the reaction and the radioactive tetrapeptide L-Ala-γ-D-Glu-(L)-A_2pm-(L)-D-[^{14}C]Ala produced by the residual DD-carboxypeptidase activity.

Although already complex, the situation is made still more complicated by the fact that the interpeptide bond D-Ala-(D)-*meso*-diaminopimelic acid made by transpeptidation extends between two D-centers and is in α-position to a free carboxyl group. In principle, such a bond is, in turn, susceptible to hydrolysis by a DD-carboxypeptidase functioning as an endopeptidase by hydrolyzing the peptide dimer in two monomers. In fact, such an endopeptidase activity also occurs in the cytoplasmic membrane of *E. coli*. It may play an important role in the « continued remodeling » of the wall peptidoglycan during the cell division cycle [44].

The membrane-bound activities described above were extracted

with the help of non-ionic detergents (Brij 36T and Genapol X-100) and the extracted enzymes were submitted to various fractionations. The results so far accumulated suggested the occurrence of: 1) a DD-carboxypeptidase which is mainly active on the pentapeptide either free or as it occurs in the form of the nucleotide precursor; 2) a transpeptidase of which the main function is to catalyze dimerization; 3) an endopeptidase of which the main function is to hydrolyze into monomers the dimers formed by transpeptidation. Although devised in order to perform one of these specific jobs with high efficiency, each of these enzymes, however, seems to be able to perform both other jobs with low efficiency. Obviously, this overlapping specificity profile makes the fractionation especially difficult.

Finally, in addition to the multi-enzyme complex just described and which acts specifically on D-D peptide bonds, the *E. coli* cytoplasmic membrane also possesses an LD-carboxypeptidase (see the Introduction) and probably a corresponding LD-transpeptidase. This latter enzyme activity has not yet been detected but its existence has been postulated. It would be responsible for the synthesis of the Nε-[*meso*-A_2pm-(L)]-L-lysine bonds through which a particulate lipoprotein of the outer membrane of the cell envelope is covalently linked to the underlying wall peptidoglycan [6].

2) *Penicillin sensitivity.*

The analysis of the membrane-extract obtained with the help of Brij 36T suggested that this multi-enzyme (DD-carboxypeptidase-transpeptidase-endopeptidase) complex may well occur in two distinct forms, one of them being sensitive to very low dose levels of ampicillin (and perhaps specifically involved in the septation of the cell) and the other being sensitive only to much higher dose levels of ampicillin (and perhaps specifically involved in the elongation of the cell). Actually, two fractions, each of them performing the three above activities but exhibiting largely different ampicillin sensitivities, were prepared from Brij 36T-membrane-extracts [38, 39, 43]. If such two groups of enzymes of low and high sensitivity to ampicillin, respectively, function *in vivo* (and are not artefacts caused by the isolation of the membranes and the various treatments to which they were submitted), the well-known observation that low dose levels of ampicillin induces the transformation of the rod-shaped *E. coli* into long filaments [44] would then rest upon a biochemical basis.

The DD-carboxypeptidase and the transpeptidase present in Genapol X-100 membrane extracts exhibit a high ampicillin sensi-

tivity (ID_{50} values: about 5×10^{-9} M). When incubated at 37° in 0.03 M Tris-HCl buffer pH 7.9, supplemented with Genapol X-100, and in the absence of free antibiotic, both ampicillin-enzyme complexes undergo spontaneous reactivation with a same half-life of about 90 min, *i. e.* behave as the *Streptomyces* enzyme-antibiotic complexes do [unpublished results].

V. — CONCLUSION

The use of peptides that are direct and, as much as possible, specific substrates of the membrane-bound activities involved in the wall peptidoglycan crosslinking enzyme system, revealed that the complexity of this system largely varies depending upon the bacteria. It consists, at least to all appearances, of one enzyme in *Streptomyces* R61 and *S. rimosus* (a transpeptidase with a low DD-carboxypeptidase activity), of two enzymes in *Streptomyces* K15 (a transpeptidase with a low DD-carboxypeptidase activity and a DD-carboxypeptidase with a low transpeptidase activity), whereas it is a multi-enzyme complex in *S. faecalis* and in *E. coli*. Very subtle mechanisms must exist in this latter organism in order to coordinate and control a system of very high complexity, some components of which have antagonistic actions. On the basis of the simplicity of their membrane-bound enzyme system and the ability of at least some of them, to excrete DD-carboxypeptidases-transpeptidases in soluble forms, *Streptomyces* sp are models of choice for the study of these enzymes. At present, the *Streptomyces* membrane-bound enzymes have not been studied as thoroughly as the exocellular ones. The results so far accumulated, however, show that the mechanisms through which these two types of enzymes interact both with the substrates and with the β-lactam antibiotics, respectively, are at least similar and are probably identical. In particular, β-lactam antibiotics are « substrates » of these enzymes. The interaction between these compounds and the exocellular enzymes involves three sequential steps: 1) formation through a rapid equilibrium process, of an equimolar inactive complex; 2) irreversible isomerization into a modified complex with a rate constant value which may be similar to that of a true enzymatic reaction; 3) spontaneous and irreversible breakdown of this latter complex with reactivation of the enzyme and the release of the antibiotic molecule into a chemically modified form. In all cases studied so far, this last step is a slow process. The values of the various constants involved in the interaction have been determined and the parameters

that are important for the activity of the antibiotics have been defined. Although it involves two irreversible steps and the formation of a rather stable intermediate, the above mechanism is compatible with kinetics of inhibition which, in plots of Lineweaver-Burk, may be of the competitive type with regard to the peptide donor. In spite of this behaviour, however, formation of a ternary complex (enzyme + peptide donor + antibiotic) cannot be excluded. Clearly, the identification of the chemical modifications undergone by the antibiotic during its interaction with these (exocellular and membrane-bound) enzymes as well as the identification of the enzyme centers involved in hydrolysis and transfer reactions and in the breakdown of the antibiotic molecule, respectively, are the next problems which must be solved in order to fully understand how penicillin kills bacteria.

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