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STRUCTURE–ACTIVITY RELATIONSHIPS IN THE β-LACTAM FAMILY: AN IMPOSSIBLE DREAM

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Abstract—The difficulty of establishing structure–activity relationships in the β-lactam family of antibiotics stems from the fact that: (1) The targets in various bacteria exhibit widely different sensitivities. (2) Some bacteria produce β-lactamases, enzymes capable of destroying the antibiotics. The rates of the reactions with the β-lactamases and the target enzymes are not necessarily related. (3) In Gram-negative bacteria, the diffusion rate through the outer membrane varies independently from the two other factors.

The first members of the β-lactam family of antibiotics which were discovered and widely utilized in chemotherapy were the penicillins (Fig. 1,**1**). The acyl side chain R—CO— offered the possibility of obtaining a wide variety of semi-synthetic compounds exhibiting different properties: stability under acidic conditions allowing per os administration, resistance to the first clinically obnoxious β-lactamase (the staphylococcal enzyme) and activity against Gram-negative strains. In the early fifties, cephalosporins (Fig. 1,**2**) supplied the chemists with a second possibility of structural variations, the R′ side chain on C_3. For about twenty years, it was commonly assumed that the two original squeletons shown on Fig. 1,**1** and **2**, were an absolute requirement for antibacterial activity. This dogma was shattered in the seventies, with the discovery of active cephamycins (Fig. 1,**3**), oxapenams (Fig. 1,**4**), carbapenems (Fig. 1,**7**), oxacephamycins (Fig. 1,**5**) and finally, nocardicin (Fig. 1,**9**) and the monobactams (Fig. 1,**8**) in which the only recognizable features remained the β-lactam nucleus and a negative charge within 3.5 Å of the amide nitrogen. Even the fused second ring appeared to be dispensable. The final straw came a few months ago, with the discovery of lactivicin (Fig. 1,**10**), a non-β-lactam compound which was shown to compete with β-lactams for their traditional—and up to that point—absolutely specific targets.

Those targets are membrane-bound enzymes, usually referred to as penicillin-binding proteins or PBPs, which play a vital role in the biosynthesis and remodelling of the cell wall peptidoglycan (for a review, see Ref. 1). They exhibit DD-peptidase activity and catalyse the closing of the interpeptide bridges which are responsible for the mechanical strength of the polymer. The particularity of that reaction is that the peptide substrate has a D-alanyl-D-alanine C-terminus which is specifically recognized by the enzymes (Fig. 2). The enzymes contain an essential serine residue which can be blocked by reaction with the β-lactam [2]. The general interaction pathway (Fig. 3) was first identified with model enzymes [3], but has been found to be valid for all essential PBPs studied so far [1, 4, 5]. With so many β-lactam compounds available, one might think that very reliable structure–activity relationships have been established in the β-lactam family. That is far from true, and the complication of the system explains that failure. Indeed, one would like to find correlations between the properties of a bicyclic structure and/or of the side chain and the concentration of the compound necessary to kill a given type of bacterial cell. Most often, in fact, this latter experimental fact is the only one available, as Minimum Inhibitory Concentration (MIC) or Minimum Bactericidal Concentration (MBC) values. The goal of this paper is to show that those values most often result from a complex interplay between various apparently unrelated factors.

SENSITIVITY OF THE TARGET ENZYME

The first factor to consider is the sensitivity of the target. On the basis of the model depicted in Fig. 3, one can deduce that the efficiency of the inactivator is increased by low values of K and k_3 and by high values of k_2. Often, but not always, K is rather large so that the proportion of complex EC remains negligible and the important parameter is the second-order rate constant k_2/K. The value of k_3 is generally low and, hence, irrelevant. It is easy to understand that if the half-life of the EC* complex is long compared to the generation time of the bacterium, its degradation will not strongly influence the overall phenomenon. However, some values of k_3 as high as $10^{-3}\,sec^{-1}$ have been reported [7] and, under those conditions, the concentration of β-lactam needed to inactivate a given proportion of target enzyme can increase significantly. The value of k_3 depends both upon the enzyme and the antibiotic and, so far, it is clear that the behaviour of the acyl–enzyme complexes formed with various antibiotics and one enzyme cannot be extrapolated to another PBP. Cephalosporins tend to form generally more stable complexes than penicillins, but that higher stability remains unexplained. Moreover, three distinct degradation pathways have been identified: (i) a

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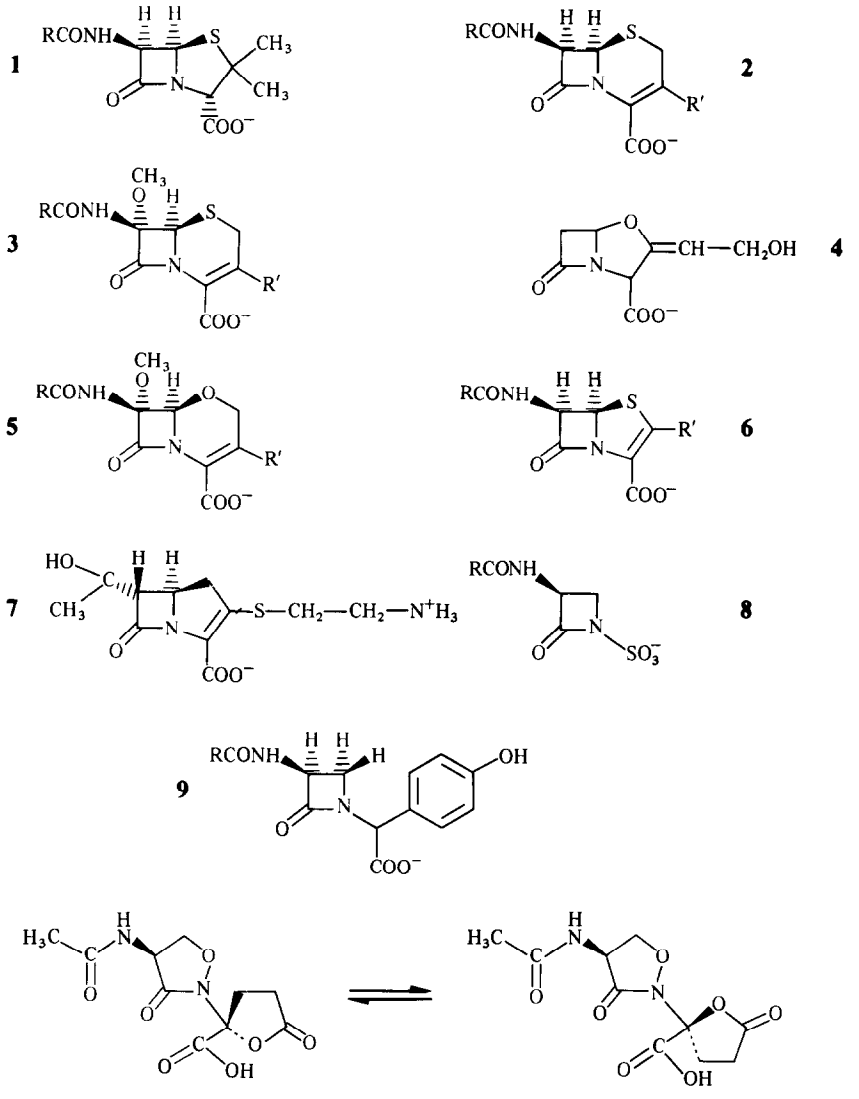


Fig. 1. Structures of β-lactam antibiotics (1–9) and of lactivicin (10). **1**: penicillins (penams), **2**: cephalosporins (Δ^3-cephems), **3**: cephamycins, **4**: oxapenams (clavulanate), **5**: oxacephamycins, **6**: penems, **7**: carbapenems (thienamycin), **8**: monobactams, **9**: nocardicin, **10**: lactivicin.

simple hydrolysis (Fig. 4A) of the ester bond [8]; (ii) expulsion of an electron-withdrawing substituent on the carbon atom attached to C_3 of the cephem nucleus (Fig. 4C): this yields a modified, more stable acyl–enzyme [9]; (iii) hydrolysis of the C_5—C_6 bond of penicillins [10, 11] immediately followed by hydrolysis of the acylglycyl enzyme thus formed (Fig. 4B). But all penicilloyl–enzyme complexes do not exhibit that behaviour.

The rate of formation of the acyl–enzyme (characterized by k_2/K) also strongly depends on the enzyme–β-lactam pair under investigation. Thus, mecillinam specifically inactivates PBP2 of *Escherichia coli* while cephalothin is more active on PBPs 2 and 3, cephaloridine on PBP1B and benzylpenicillin on PBP3 [12, 13]. It is clear that the knowledge of the 3-D structure of the various PBPs will help the understanding of those specificities, but the crystallography of those proteins is not even in its infancy! Moreover, very few reliable kinetic data are available for the interaction between the membrane-bound proteins and the β-lactams.

R′——D-Ala——D-Ala + R——NH_2⟶

R′——D-Ala——NH——R + D-Ala

Fig. 2. Transpeptidation reaction catalysed by penicillin-sensitive DD-peptidases.

Thus, a bacterium can be resistant because its target enzyme(s) is (are) intrinsically resistant. In the recent years, various resistant mutants have been described in which the resistance could be attributed to the appearance of a new PBP of extremely low sensitivity, which seems capable of taking over the activity of the original ones [14, 15]. There are thus at least as many target enzymes to hit as there are bacterial species that one might want to kill. Conversely, if one is dealing with hypertension and de-

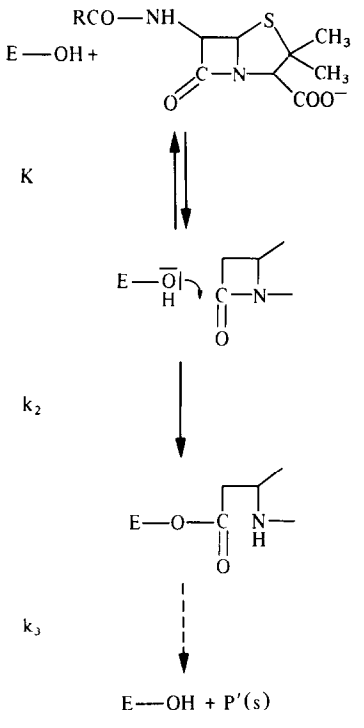


Fig. 3. General interaction pathway between penicillin-sensitive DD-peptidases (E-OH) and β-lactams. K is a dissociation constant, k_2 and k_3 are first-order rate constants.

cides to inhibit the angiotensin converting enzyme, one has to worry only about one enzyme in one species—homo sapiens.

β-LACTAMASES

It is true, however, that target enzymes can be rather similar in related genera: the PBP patterns and sensitivities in Enterobacteriaceae are not very different. But the intrinsic sensitivity of the target is certainly not the sole factor to be considered. As already mentioned above, many bacteria produce β-lactamases, enzymes which hydrolyse the β-lactam amide bond, yielding biologically inactive products (Fig. 5). In Gram-positive bacteria, those enzymes are excreted in the growth medium, and constitute a collective weapon, responsible for the well-known inoculum effect. In Gram-negative strains, they remain in the periplasmic space where, in some superproductive mutants, their concentration can be as high as 1 mM [16]. Moreover, they are extremely efficient enzymes, exhibiting turnover number values up to 2000 sec^{-1} [17]! In the recent years, a lot of interesting information has become available about those enzymes.

1. Two broad groups have been found so far: metallo (Zn^{2+}) and active-serine enzymes. A very large majority of enzymes belong to the second group: only three Zn^{2+} β-lactamases have been described, two of them very close relatives produced by two strains of *Bacillus cereus* [18, 19].

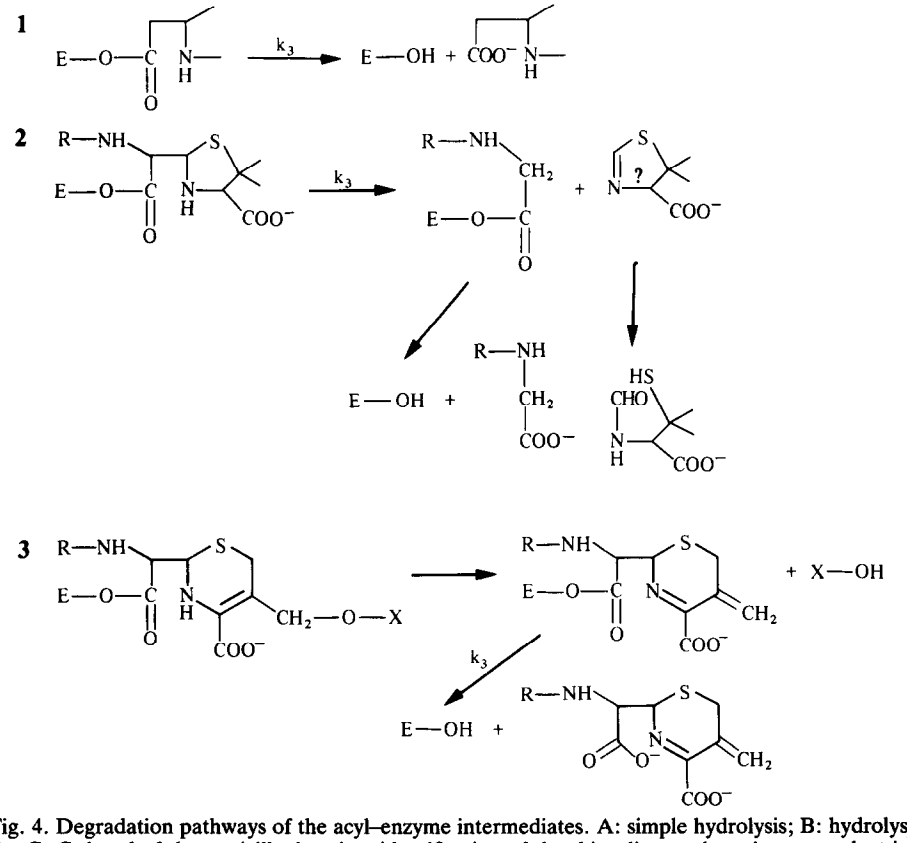


Fig. 4. Degradation pathways of the acyl–enzyme intermediates. A: simple hydrolysis; B: hydrolysis of the C_5-C_6 bond of the penicilloyl moiety identification of the thiazoline as the primary product is still tentative (?); C: rearrangement of the cephalosporoyl.

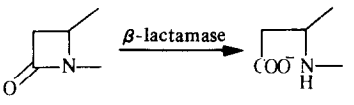


Fig. 5. Hydrolysis of a penicillin by a β-lactamase.

2. Active-serine β-lactamases have been divided into two classes, A and C, on the basis of their primary structures [20]. Very little sequence homology was found between the two classes, but both appear to share the same kinetic pathway, involving an acyl–enzyme intermediate and similar to that described above for DD-peptidases. There is, however, a major quantitative difference between DD-peptidases and β-lactamases, the value of k_3 being generally much higher, up to 10^8–10^9-fold, with the β-lactamases.

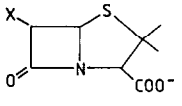
3. However, even with the β-lactamases, some acyl–enzyme adducts are particularly stable ($k_3 = 10^{-3}$–10^{-4}sec^{-1}) which makes the compound a β-lactamase inactivator and increases the similarity with the DD-peptidases (see, for example, Ref. 21). However, that situation is the rule with DD-peptidases and the exception with β-lactamases.

4. Significant sequence homology was found between a model DD-peptidase and the class C β-lactamases [22]. Unfortunately, no tertiary structure information is presently available about class C β-lactamases. Surprisingly, and despite the lack of sequence homology, a high degree of tertiary structure similarity was found between the DD-peptidase and several class A β-lactamases [23–25].

Attempts have been made to correlate the chemical reactivity of various molecules with the rates of acylation of DD-peptidases and β-lactamases. No clear correlation was found. For instance, modification of the substituent on C_6 of penicillin, going

Table 1. Second-order rate constants for the acylation of four enzymes by various penicillins and cephalosporins

		$(k_2/K)_{\text{DD-peptidase}}$ $(M^{-1}sec^{-1})$		$(k_{cat}/K_m)_{\beta\text{-lactamase}}$ $(M^{-1}sec^{-1})$	
	k_{OH^-} $(M^{-1}sec^{-1})$	Streptomyces R61	Actinomadura R39	Actinomadura R39	Streptomyces albus G
Benzylpenicillin	0.37	14,000	300,000	980,000	620,000
Ampicillin	0.40	110	280,000	590,000	513,000
Carbenicillin	0.37	830	6,000	97,000	47,000
6-Aminopenicillanic acid	0.15	0.25	1,200	700,000	600,000
Penicillanic acid	0.01	0.06	14	127,000	25,000
Cephalothin	0.42	3,000	>70,000	300,000	8,800
Cephaloglycine	0.70	22	74,000	50,000	7,200
7-Aminocephalosporanic acid	0.24	33	200	800	1,500



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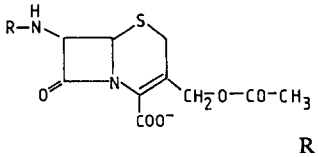

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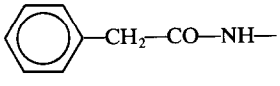
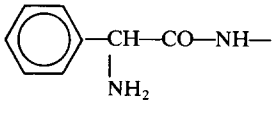
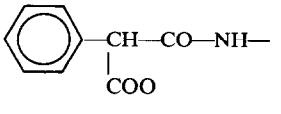
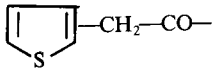
Benzylpenicillin 

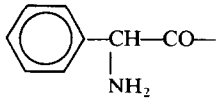
Ampicillin 

Carbenicillin 

6-Aminopenicillanic acid H_2N—

Penicillanic acid H—

Cephalothin 

Cephaloglycine 

7-Aminocephalosporanic acid H—

k_{OH^-} characterizes the rate of hydrolysis by OH^- ions. For the β-lactamases, the value of k_{cat}/K_m is equal to k_2/K. Data are from Refs 3, 26 and 27 and were obtained at 37° for the DD-peptidases and 30° for the β-lactamases with the exceptions of the pairs benzylpenicillin-R61 DD-peptidase (25°) and cephaloglycine-R39 DD-peptidase (20°).

from an *N*-acyl side chain to a free amino group to no substituent at all (Table 1), progressively decreases the chemical reactivity and the sensitivity to β-lactamases but has a disproportionate effect on the interaction with DD-peptidases (Table 1). In consequence, 6-aminopenicillanic and penicillanic acids have no antibiotic properties. The presence of an amino group on the side chain of ampicillin strongly decreases the rate of acylation of the DD-peptidase of *Streptomyces* R61 but has little effect on the interaction with the two β-lactamases and with the DD-peptidase of *Actinomadura* R39. Conversely, the presence of a carboxylate on the side chain of carbenicillin decreases the rate of acylation of the four enzymes. Acylation rate constants were compared for a DD-peptidase and a β-lactamase produced by the same strain *Actinomadura* R39. Some compounds acylated both enzymes with comparable efficiencies, but many others had more selective behaviors (see Ref. 1).

THE PERMEABILITY FACTOR

In Gram-negative bacteria, the situation is further complicated by the permeability barrier constituted by the outer membrane which slows down the diffusion of the antibiotics to the target enzymes. This factor has been elegantly studied by Zimmermann and Rosselet [28] and Nikaido and his colleagues [29] and it is now well established that the diffusion barrier is only significant if a β-lactamase is also present in the periplasm. Indeed, in the worst cases for which quantitative data are available and in the absence of β-lactamases, the periplasmic concentration of the antibiotic equilibrates with the external one within a period (a few minutes) which is short when compared to the generation time of the bacteria. With *E. coli*, Nikaido has measured half-equilibration times ranging from 0.1 to 5 sec [29].

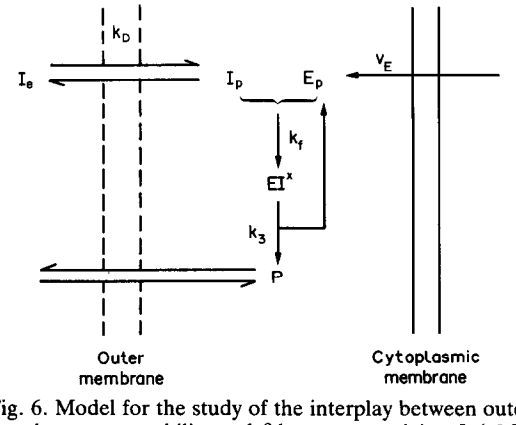


Fig. 6. Model for the study of the interplay between outer membrane permeability and β-lactamase activity. I_e (μM): concentration of β-lactam in the external medium; I_p (μM): concentration of β-lactam in the periplasm; E_p (μM): concentration of free β-lactamase; v_E (μM sec^{-1}): rate of synthesis and excretion of β-lactamase; *EI**: penicilloyl enzyme; P: penicilloic acid; k_f ($\mu M^{-1} sec^{-1}$): second-order rate constant for the formation of penicilloyl enzyme (corresponds to k_{+2}/K in Fig. 3); k_{+3} (sec^{-1}): first-order rate constant for penicilloyl-enzyme hydrolysis; k_D (sec^{-1}); first-order rate constant for β-lactam diffusion through the outer membrane.

However, other genera (*Enterobacter, Pseudomonas*) exhibit a generally lower permeability, accompanied by a decreased selectivity [29] and it remains possible that truly "impermeable" strains will be found. At present, however, this factor mainly increases the impact of the β-lactamase.

Three differential equations can be deduced from the simple model represented by Fig. 6:

$$\frac{d(I_p)}{dt} = k_D(I_e - I_p) - k_f(I_p)(E_p)$$

$$\frac{d(E_p)}{dt} = v_E + k_3\,(EI^*) - k_f(I_p)(E_p)$$

$$\frac{d(EI^*)}{dt} = k_f(I_p)(E_p) - k_3(EI^*)$$

where I_e and I_p are the external and periplasmic concentrations of β-lactam, v_E the rate of synthesis of the enzyme, E_p the periplasmic concentration of the enzyme, (EI^*) the concentration of acyl–enzyme. The values of k_D, a first-order rate constant for β-lactam diffusion through the outer membrane, can be computed from the half-equilibration times measured by Nikaido and his colleagues. Those of k_f, the second-order rate constant for acyl–enzyme formation, are obtained from the usual kinetic parameters of the enzyme ($k_f = k_2/K = k_{cat}/K_m$, and the values of k_3 are equal to or larger than k_{cat}. The rate of enzyme synthesis, v_E, can be deduced from the initial enzyme concentration in the periplasm (E_o) and the generation time (tg) of the bacterium. Indeed

$$v_E = \frac{E_o}{2\,tg}.$$

On the basis of those equations and assuming that the value of I_e remained constant, a situation which prevails when a MIC is determined, simulations were performed with the help of a numerical integration program. It was found that if k_3 was larger than $10^{-2} sec^{-1}$, the value of I_p rapidly reached a nearly stable value, close to that obtained under steady-state conditions when v_E was neglected. The simple model of Zimmerman and Rosselet, which involves the usual parameters K_m and k_{cat} can then be used. It is interesting to compute the I_e/I_p ratio necessary

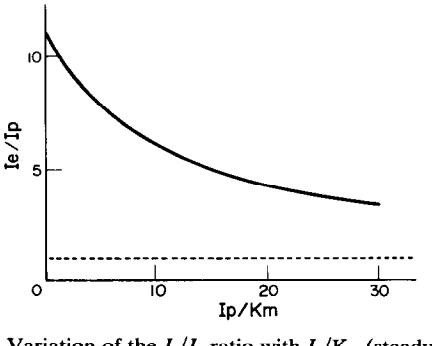


Fig. 7. Variation of the I_e/I_p ratio with I_p/K_m (steady-state model). The parameters are $k_{cat}E_o/k_D = 100$ μM and $K_m = 10$ μM. The dashed line represents the horizontal asymptote to the curve ($I_e/I_p = 1$).

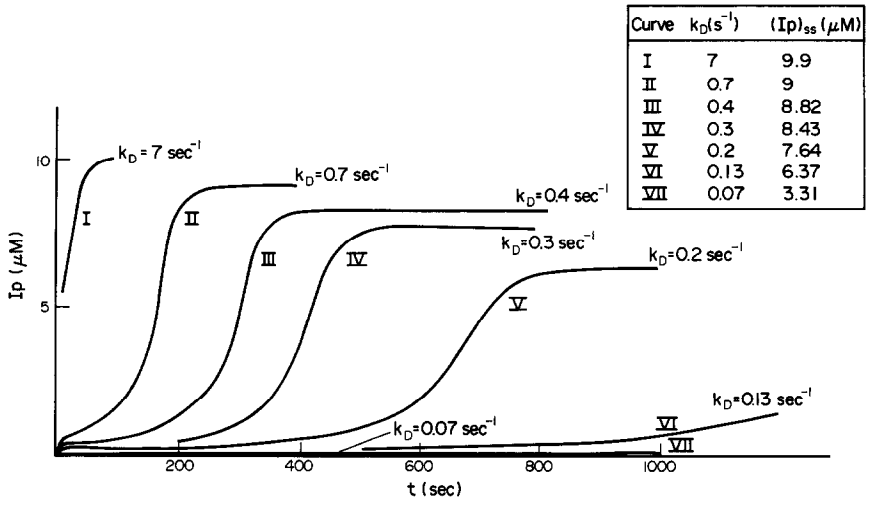


Fig. 8. Influence of the value of k_D on the accumulation of β-lactam in the periplasm. The parameters are $E_o = 950\,\mu M$; $v_E = 0.2\,\mu M$ sec^{-1} (tg = 42 min); $k_3 = 5 \times 10^{-4}$sec^{-1}; $k_f = 0.01\,\mu M^{-1}$sec^{-1} and $I_e = 10\,\mu M$. The values of $(I_p)_{ss}$ (insert) were computed with $v_E = 0$ and correspond to the steady-state situation.

to yield a pre-determined periplasmic β-lactam concentration

$$\frac{I_e}{I_p} = 1 + \frac{k_{cat} E_o}{K_m k_D \left(1 + \frac{I_p}{K_m}\right)}.$$

Since the value of I_p which is needed to kill the cell depends in turn on the sensitivity of the target enzyme(s), the equation clearly shows that the three factors cannot be considered independently: as shown by Fig. 7, the I_e/I_p ratio necessary to kill the bacterium is larger when the sensitivity of the target is high (low "killing" I_p value) if k_D and the amount and properties of the β-lactamase remain unchanged. The model, however, does not allow for the fact that E_o might be similar to or larger than I_p, in which case the Henri–Michaelis equation is not valid. This requires the utilization of more complex equations and is outside the scope of the present review.

For low values of k_3, the numerical integration program was used to study the influence of each of the kinetic parameters identified in Fig. 6. For example, Fig. 8 shows how the decrease of the permeability factor slows down the accumulation of the antibiotic in the periplasm.

Finally, the model can still be improved by taking account of the increase of the periplasmic volume in growing cells. Indeed, the differential equations as written above imply a continuous increase in the total enzyme concentration, $(E_p) + (EI^*)$. This effect is depicted by Fig. 9A where it has been exacerbated by choosing an exaggeratedly short generation time. With the corrected model (Fig. 9B), a steady state is eventually reached where the periplasmic concentrations of the various components remain stable. That complete model will allow the determination of the conditions needed to observe trapping phenomena, i.e. conditions where the antibiotic entering the periplasm is immediately locked into a stable acyl–enzyme complex by newly synthetized enzyme, a phenomenon which has been the source of intense controversy [29–31].

The difficulty of deriving structure–activity relationships in the β-lactam family is thus explained by (1) the variation of the "reactivity" of the targets, (2) the different properties and quantities of the β-lactamases, (3) the variations of the diffusion rate through the outer membrane of Gram-negative bacteria, and (4) the interplay between those three

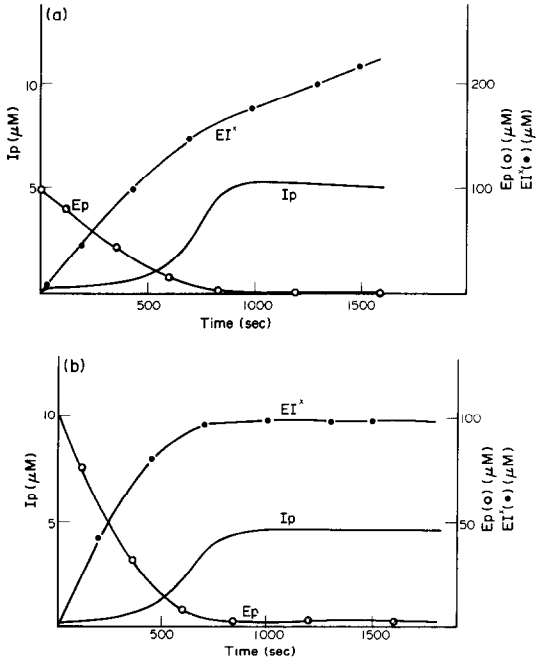


Fig. 9. Modification of the model allowing for the increase of periplasmic volume in growing cells. (A) Simulation obtained before correction: $E_o = 100\,\mu M$, $v_E = 0.159\,\mu M$ sec^{-1} (tg = 10.5 min), $k_D = 0.025$ sec^{-1}, $k_f = 0.01\,\mu M^{-1}$sec^{-1}, $I_e = 10\,\mu M$. (B) After correction. The values of the parameters are the same as above.

factors, the values of which appear to depend upon unrelated characteristics of the molecules.

Finally, it is interesting to note that, even the "safest" structural feature of the "active" β-lactams can be challenged. Experiments performed (Varetto and Frère, unpublished) with deacetylcephalosporin C lactone indicate that, despite its lack of negative charge within 3.0 Å of the β-lactam nitrogen, the compound acts as a DD-peptidase inactivator and a β-lactamase substrate. As a DD-peptidase inactivator, it is about 10-fold less efficient than deacetyl cephalosporin C but, paradoxically, it is a much better β-lactamase substrate. On the contrary, and according to the accepted dogma, the amide and methyl ester of benzylpenicillin are barely recognized by any of the enzymes.

Does there remain a single principle to be trusted?

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