Chapter 48

β-Lactamase-Induced Resistance

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Increased bacterial resistance to β-lactam antibiotics has usually been attributed to three causes: (i) a decreased sensitivity of the target enzyme(s), the membrane-bound DD-peptidases, or the synthesis of new, more resistant DD-peptidases; (ii) the synthesis and excretion through the cytoplasmic membrane of enzymes capable of hydrolyzing the amide bond of the β-lactam nucleus, i.e, β-lactamases; and (iii) the hindering of free diffusion of the antibiotic by the outer membrane of gram-negative bacteria (permeability barrier). The outer membrane also prevents the leaking of the β-lactamase into the culture supernatant, as happens with gram-positive strains.

The elegant work of Nikaido and his colleagues (7, 8, 14, 15) allowed estimations of the diffusion rates of various antibiotics through the outer membrane of *Escherichia coli* and other gram-negative bacteria. A first important conclusion of that work was that the permeability barrier was significant only if a β-lactamase was also present in the periplasm. Indeed, with *E. coli*, first-order rate constants for the penetration of the antibiotics in the periplasm ranged from 0.14 to 7 s^{-1}, values indicating a very rapid equilibration. Assuming a penetration rate of only 0.03% of the highest value (2×10^{-3} s^{-1}), a periplasmic concentration representing 50% of the external one would still be reached after only 6 min.

In this chapter we analyze the interplay between outer membrane permeability and β-lactamase activity in determining the periplasmic concentration of the antibiotic. The model (Fig. 1) is based on a realistic pathway (4, 5) for the β-lactamase-catalyzed hydrolysis of penicillins. It also assumes that the

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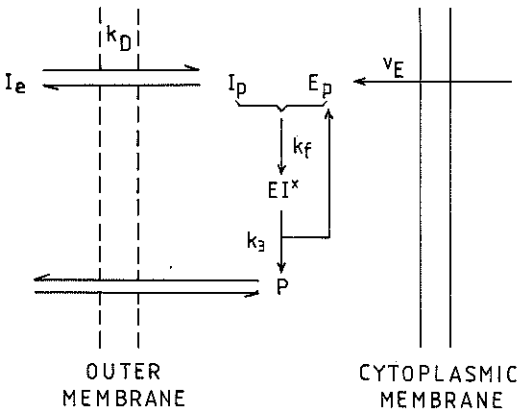


Figure 1. Complete model for the study of the interplay between outer membrane permeability and β-lactamase activity. I_e and I_p represent, respectively, the antibiotic concentrations in the culture medium and in the periplasm, E_p is the free enzyme in the periplasm, EI^* is the acyl enzyme intermediate, P is the hydrolyzed β-lactam, k_D is the first-order rate constant for the diffusion through the outer membrane, v_E is the rate of synthesis and excretion of the enzyme through the cytoplasmic membrane, k_f is the apparent second-order rate constant for acyl enzyme formation, and k_3 is the first-order rate constant for its hydrolysis. A more complete pathway for the β-lactamase-catalyzed reaction is:

$$E + I \overset{K}{\rightleftharpoons} EI \overset{k_2}{\rightarrow} EI^* \overset{k_3}{\rightarrow} E + P$$

In this analysis, we have assumed that I_p is $<<K$, in which case the concentration of EI remains negligible and the rate of formation of EI^* is given by $k_f(I_p)(E_p)$, where $k_f = k_2/K$. The units used in the simulations are as follows: concentrations, micromolar; k_f, micromolar^{-1} second^{-1}; k_3, second^{-1}; and v_E, micromolar second^{-1}.

experimental conditions are those which prevail during the determination of an MIC, i.e., that the cell density is very low and that throughout the experiment the external concentration of β-lactam, I_e, remains virtually constant.

Preliminary simulations (see below) indicated that two very different situations could arise. For high values of k_3, the value of I_p rapidly reached a steady state, and the synthesis of new enzyme was not relevant ($v_e \simeq 0$). The simple equations derived by Zimmermann and Rosselet (16) and Vu and Nikaido (14) supplied an adequate description of the phenomena. For low values of k_3 ($<10^{-2}$ s^{-1}), the appearance of new enzyme could no longer be neglected, a true steady state was never really attained, and the value of I_p could present considerable variations over periods corresponding to the generation time of the bacterium.

Steady-State Situation

The model was simplified by neglecting v_E. For the interaction with the enzyme, the usual Henri-Michaelis parameters were used. Some interesting features arise from a close study of equations giving $I_p = f(I_e)$ and $I_e = f(I_p)$.

Equation A: $I_e = f(I_p)$

As emphasized by Vu and Nikaido (14), the equation allows the computation of the external concentration of antibiotic, I_e, which is necessary to yield a predetermined concentration in the periplasm.

$$I_e = I_p + \frac{k_{cat}E_0 \cdot I_p}{k_D(K_m + I_p)} \quad (1)$$

where E_0 is the total concentration of β-lactamase. For a fixed value of I_p, I_e increases linearly with $k_{cat}E_0$ (i.e., V_{max} of the enzyme). Note that if I_p/K_m is low and k_D is high, the slope of the line can be rather shallow. I_e also increases linearly with $(k_D)^{-1}$. The variation with K_m is hyperbolic with upward concavity, and the equation of the horizontal asymptote is $I_p = I_e$. The most interesting influence is that of I_p itself. The variation is hyperbolic with downward concavity, and the equation of the oblique asymptote is:

$$I_e = I_p + \frac{k_{cat}E_0}{k_D} \quad (2)$$

The curve approaches the asymptote when I_p becomes $>>K_m$; I_e then varies linearly with I_p. In contrast, when I_p is not much larger than K_m, it must be emphasized that the relationship between I_e and I_p is not linear. This can be better illustrated by plotting I_e/I_p versus I_p/K_m (Fig. 2). In fact, the lower the desired I_p/K_m, the larger the I_e/I_p ratio necessary to reach that desired value of I_p. Thus, under such conditions, an increase in the sensitivity of penicillin-binding proteins (PBPs) does not proportionally decrease the MIC, and this effect is more pronounced if $k_{cat}E_0/k_D$ is large.

Equation B: $I_p = f(I_e)$

Equation 3 has been used by Zimmerman and Rosselet (16) to study the outer membrane permeability to β-lactams:

$$I_p = \frac{-N \pm \sqrt{N^2 + 4k_D^2 I_e K_m}}{2k_D} \quad (3)$$

where N equals $k_{cat}E_0 + k_DK_m - k_DI_e$.

Some extreme cases result in very simple equations. If $k_{cat}E_0$ is much larger than k_DK_m and k_DI_e, I_p is directly proportional to I_e (equation 4).

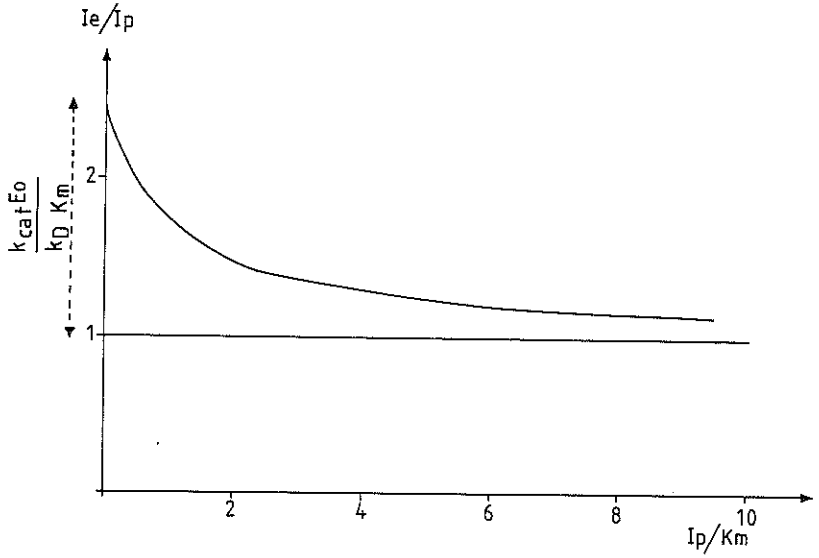


Figure 2. Influence of I_p/K_m on the ratio I_e/I_p. K_m, 1 μM; $k_{cat}E_0$, 10 μM s^{-1}; k_D, 7 s^{-1}.

$$I_p = \frac{k_DK_m}{k_{cat}E_0} I_e \quad (4)$$

This situation prevails in the presence of large concentrations of a very active enzyme ($k_{cat}E_0$ might be as high as 10^6 μM s^{-1}).

If I_e is much larger than K_m, I_p decreases linearly with E_0 (equation 5).

$$I_p = I_e - \frac{k_{cat}}{k_D} E_o \quad (5)$$

If, in addition, k_DI_e is much larger than $k_{cat}E_0$, I_p equals I_e.

With four variable parameters ($k_{cat}E_0$, k_D, K_m, and I_e), a detailed discussion would be too long. We will only point out the most striking feature.

Figure 3 shows the influence of $k_{cat}E_0$ on I_p for various values of k_D. Depending upon the value of $k_{cat}E_0$, it can be seen that the effect of k_D can be nearly negligible (compare curves I and II for $k_{cat}E_0$ of <5 μM s^{-1}) or quite spectacular (compare curves II and III for values of $k_{cat}E_0$ between 5 and 10 μM s^{-1}).

Since large concentrations of periplasmic β-lactamase (100 to 900 μM) have been observed in some superproducing strains (7; H. Martin, personal communication), rather large values of $k_{cat}E_0$ can be obtained even if k_{cat} is relatively low. Moreover, when the activity of the β-lactamase on a "resistant"

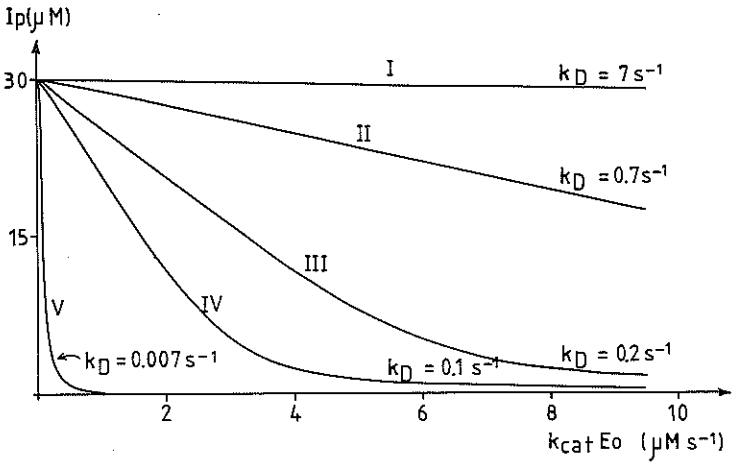


Figure 3. Variation of I_p with $k_{cat}E_0$ for various values of k_D. Other conditions: K_m, 1 μM; I_e, 30 μM.

substrate is less than 1% of that observed on a good substrate, it has often been considered as negligible. However, since k_{cat} for good substrates can be in the range of 1,000 to 3,000 s^{-1}, a rather high value of $k_{cat}E_0$ can be obtained even if k_{cat} is only 0.1% of that of the good substrate. It is thus important to measure accurately the kinetic parameters of the β-lactamase-substrate interaction even when the enzyme seems to transform the substrate very slowly.

Non-Steady-State Situation

Equations and Simulations

From the model presented in Fig. 1, the following differential equations can be deduced:

$$\frac{d(I_p)}{dt} = k_D(I_e - I_p) - k_f(I_p)(E_p) \quad (6)$$

$$\frac{d(E_p)}{dt} = v_E + k_3(EI^*) - k_f(I_p)(E_p) \quad (7)$$

$$\frac{d(EI^*)}{dt} = k_f(I_p)(E_p) - k_3(EI^*) \quad (8)$$

On that basis, simulations were performed with the help of a numerical integration program.

The value of v_E was estimated from the initial enzyme concentration in the periplasm (E_0) and the generation time of the bacterium (t_g). Indeed:

$$v_E = \frac{E_0}{2t_g} \quad (9)$$

However, the equations as written do not take account of the increase of the periplasmic volume in growing cells. This problem will be discussed below (see *The Problem of* v_E). In most cases, high values of E_0 (100 to 950 μM) were chosen to exacerbate the effects of the presence of the β-lactamase.

Reaching the Steady State: Importance of k_3

As stated above, k_3 appears to be the most important factor in determining the time before a situation approaching the steady state (computed with the assumption that v_E equals 0) is reached. Very low values of k_D and k_f do not significantly delay the reaching of a value of I_p close to $(I_p)_{ss}$ if k_3 is relatively high (0.2 s^{-1}) (Table 1). The values of $t_{0.5}$ and $t_{0.75}$ increase when k_f decreases but remain very low compared with the generation time even with a value of k_f as low as 10^{-4} μM^{-1} s^{-1}. Observed values of k_f for serine β-lactamases are usually in the range of 10^{-2} to 10 μM^{-1} s^{-1} (see, for example, references 1, 3, and 6).

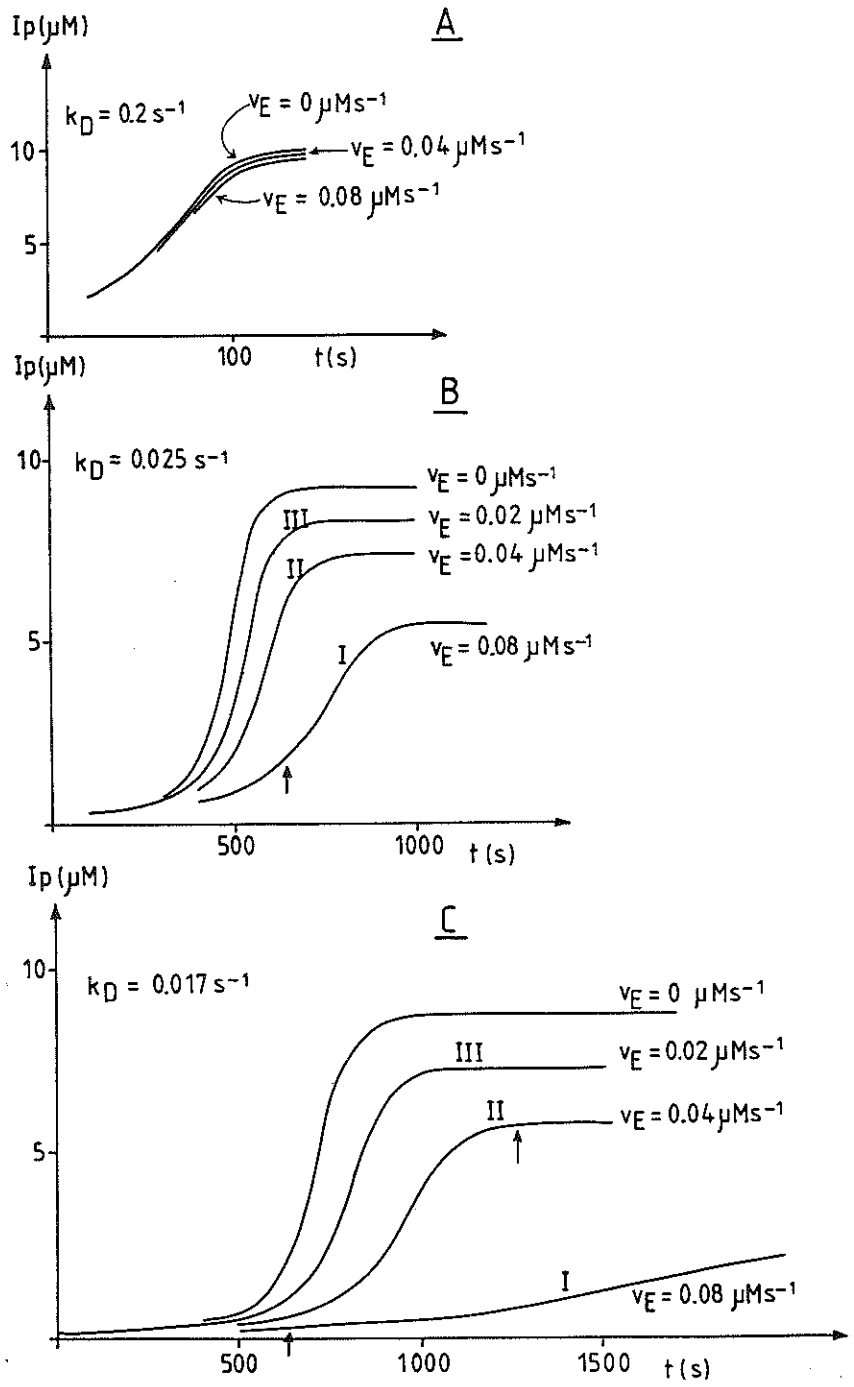
The Problem of v_E

If v_E is not zero in equation 7, a true steady state is never observed. Figure 4 depicts the effects of increasing values of v_E (i.e., decreasing generation times) for three different values of k_D. A significant effect is observed only for the

Table 1. Influence of low values of k_D and k_f on the time required to reach I_p values representing 50% ($t_{0.5}$) and 75% ($t_{0.75}$) of the steady-state value[a]

k_D (s^{-1})	k_f (μM^{-1} s^{-1})	I_e (μM)	$(I_p)_{ss}$[b] (μM)	$t_{0.5}$ (s)	$t_{0.75}$ (s)
1×10^{-3}	10^{-2}	10	1.07×10^{-3}		<1
5×10^{-3}	10^{-2}	100	0.053		<1
5×10^{-3}	10^{-3}	100	0.525	<1	<2
5×10^{-3}[c]	10^{-4}	100	5	7	14

[a]In all cases, E_0 is 950 μM, v_E is 0.2 μM s^{-1} (t_g = 42 min), and k_3 is 0.2 s^{-1}.
[b]$(I_p)_{ss}$, Value of I_p at the steady state, assuming v_E is 0.
[c]Represents 3% of the lowest value observed in *E. coli* by Nikaido and co-workers (7, 8).



smallest k_D, and this effect becomes drastic only when a rather unrealistic generation time of about 10 min is assumed (Fig. 4C). However, when k_D is 0.017 s^{-1} and t_g is 21 min, the periplasmic concentration of β-lactam is important only for a short time before cell division, and that might not be sufficient to inactivate the essential PBPs. Finally, with a generation time of 42 min, an I_p value of 7.4 μM (i.e., 0.74 I_e) prevails during 1,500 s before cell division.

As stated above, one important factor is neglected in the present approach: as new enzyme is synthesized and excreted, the cell is also growing and the periplasmic volume is increasing. The total enzyme concentration ($E_{tot} = E_p + EI^*$) in the periplasm thus remains constant and equal to E_0, whereas our simple model assumes an increase of E_{tot} ($= E_0 + v_E \cdot t$).

The building of a complete model which will include those more complicated factors and extend into several generations is one of our future goals, but the present simple model can supply very useful information. Indeed, as can be seen by comparing the three panels of Fig. 4, the influence of v_E is important only when k_D is small, and it is sufficient to remember that the real situation is intermediate between those based on the following assumptions: $v_E = 0$; $v_E = E_0/2t_g$.

Influence of k_D and k_3

For large values of k_D (>0.5 s^{-1}), I_p values representing 75% of $(I_p)_{ss}$ (computed with $v_E = 0$) are reached within a few seconds or a few minutes. Figure 5 depicts situations where k_D is low and the enzyme concentration is extremely high. A rather stable value of I_p is reached within at most 1,000 s (t_g = 42 min) if k_3 is $\leq 10^{-3}$ s^{-1}. When k_3 is smaller than 0.5×10^{-4} s^{-1}, the curves can nearly be superimposed on curve I, indicating that within 600 s all the enzyme is immobilized as EI^* and the periplasmic concentration of β-lactam is virtually in equilibrium with the external one. The value of I_p after 1,000 s, computed with $v_E = 0.2$ μM s^{-1}, is significantly different from $(I_p)_{ss}$ (computed with $v_E = 0$) only when k_3 is 1×10^{-3} to 2×10^{-3} s^{-1}. But when k_3 is larger (5×10^{-3} s^{-1}), $(I_p)_{ss}$ is smaller, and after only 140 s, I_p becomes $0.75(I_p)_{ss}$.

For Fig. 6, a low value of k_3 has been chosen, and k_D is varied from 0.07 to 7 s^{-1}. As seen above under steady-state conditions, k_D can have a disproportionate influence on I_p: after 500 s, for instance, I_p is 0.9 when k_D = 0.2

Figure 4. Influence of v_E on the value of I_p. In all cases, E_0 is 100 μM, k_f is 0.01 μM^{-1} s^{-1}; k_3 is 2×10^{-4} s^{-1}; and I_e is 10 μM. (A) k_D = 0.2 s^{-1}; (B) k_D = 0.025 s^{-1}; (C) k_D = 0.017 s^{-1}. The arrows indicate the time of cell division, assuming the cycle started at $t = 0$. The values of v_E correspond to the following generation times: (A) v_E = 0.02, t_g = 42 min; (B) v_E = 0.04, t_g = 21 min; (C) v_E = 0.08, t_g = 10.5 min.

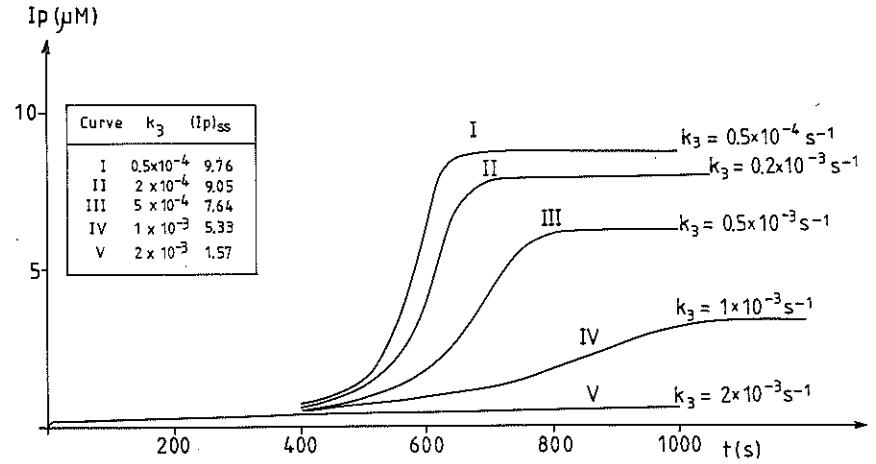


Figure 5. Influence of k_3 on I_p. In all cases, E_0 is 950 μM, k_f is 0.01 $\mu M^{-1}\ s^{-1}$, k_D is 0.2 s^{-1}, I_e is 10 μM, and v_E = 0.2 μM s^{-1} (t_g = 42 min). The insert shows the $(I_p)_{ss}$ values computed with v_E equal to 0.

s^{-1} and 7.5 when k_D = 0.3 s^{-1}. Thus, a 1.5-fold increase of k_D results in an eight-fold increase of I_p! Again, it can be noticed that v_E exhibits a strong influence only when k_D is small [compare curves VI and VII with the corresponding values of $(I_p)_{ss}$ in the insert].

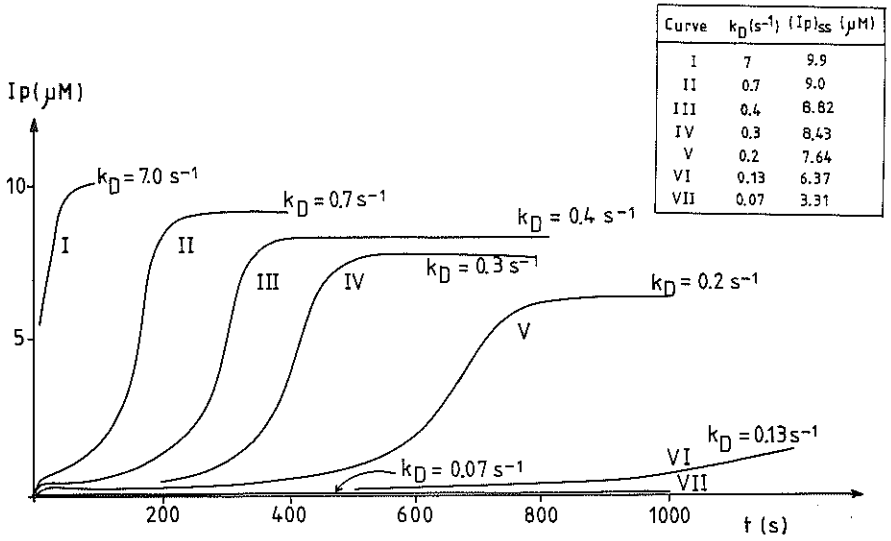


Figure 6. Influence of k_D. Conditions are as in Fig. 5, with k_3 equal to 5 × 10^{-4} s^{-1}.

Influence of k_f and E_0

Figure 6 represents simulations which were all performed using a k_f value of 0.01 $\mu M^{-1}\ s^{-1}$. In the cases of curves I, II, and III, a burst of antibiotic concentration in the periplasm is observed during the first few seconds. The size of the burst can be increased by increasing k_D (Fig. 6), decreasing k_f (Fig. 7), or decreasing E_0. Under the conditions described in the legend of Fig. 7, a k_f value of 10^{-4} $\mu M^{-1}\ s^{-1}$ results in an I_p/I_e ratio of 0.7 after a few seconds, even in the presence of an enormous (0.95 mM) concentration of β-lactamase. This example shows that an intact β-lactam can coexist with a very high periplasmic concentration of β-lactamase if k_f is low.

In examining the influence of E_0 (Fig. 8), the value of v_E was proportionally varied so that the generation time remained constant (ca. 42 min). Under some conditions, E_0 also has a disproportionate influence on the value of I_p: after 300 s, I_p is 0.4 μM when E_0 = 950 μM and 8.9 μM when E_0 is 400 μM. On the contrary, after 800 s, the influence of E_0 is negligible.

Trapping or Not Trapping?

Various authors (9, 13) have suggested that the periplasmic β-lactamase might prevent poor substrates from reaching their targets by trapping them as acyl enzyme complexes. This hypothesis has generated a lot of controversy (11). Nikaido (7) has also noted that the outer membrane barrier could reduce the rate of penetration so that it would become similar to the rate of new β-

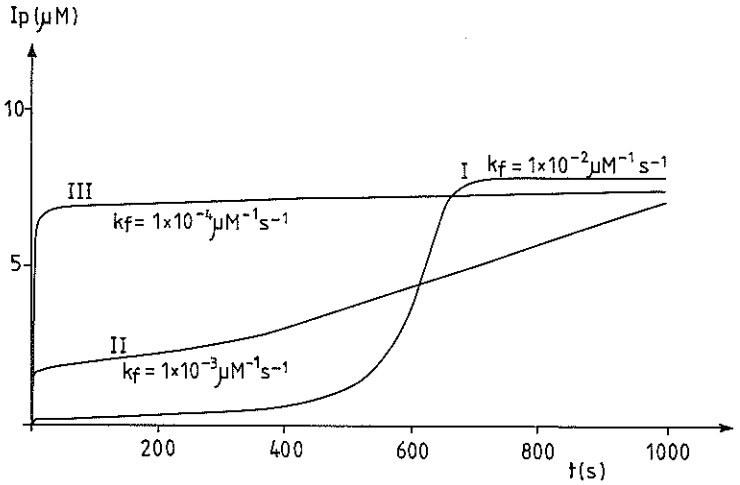


Figure 7. Influence of k_f. E_0, 950 μM; k_3, 2 × 10^{-4} s^{-1}; k_D, 0.2 s^{-1}; I_e, 10 μM; v_E, 0.2 μM s^{-1}.

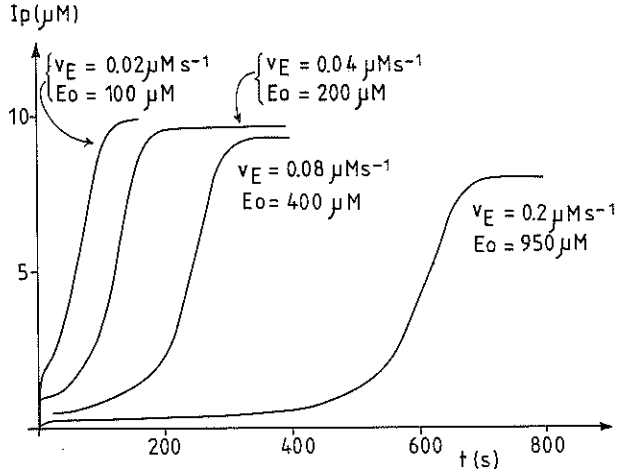


Figure 8. Influence of E_0 and v_E. k_f, 10^{-2} μM^{-1} s^{-1}; k_3, 2×10^{-4} s^{-1}; I_e, 10 μM; k_D, 0.2 s^{-1}.

lactamase synthesis. In the light of the results presented above, the following points might be stressed.

(i) High concentrations of β-lactamase result in rather high values of $k_{cat}E_0$ even if k_{cat} is as low as 10^{-2} s^{-1}, and hydrolysis, not trapping, might then be the relevant phenomenon.

(ii) When k_D is high, it is the β-lactamase which is rapidly trapped, and within a few seconds or a few minutes, I_p becomes similar to I_e (Fig. 6, curves I and II). The MIC then represents the intrinsic sensitivity of the essential target DD-peptidase.

(iii) When k_D is low, trapping can become a significant phenomenon (Fig. 9). The effect of the synthesis of new β-lactamase is also illustrated in Fig. 9 (compare curve I with curve II and curve III with curve IV). Again, small variations of k_D can induce rather large variations of I_p (compare curves II and IV). However, the restrictions outlined above about the increase of total periplasmic volume should be kept in mind. A complete answer must await the utilization of a more realistic model, and the simulation should be extended over several generations.

Another interesting question concerns the apparently nonessential, low-molecular-weight PBPs: could they perform trapping of the antibiotics and be responsible for resistance to low levels of antibiotics by protecting the essential, high-molecular-weight PBPs? In *E. coli* (2), only PBP 4 exhibits a value of k_f (0.7×10^{-2} μM^{-1} s^{-1}) considerably higher than that of PBPs 1, 2, and 3 (1×10^{-4} to 3×10^{-4} μM^{-1} s^{-1}). But the low number of PBP 4 molecules per cell (140 copies [12]) would not make the mechanism efficient. PBPs 5 and 6 are much more abundant (1,800 and 570 copies [12]), but in their cases, k_f is

Curve	k_D (s^{-1})	v_E (μMs⁻¹)
I	0.13	0
II	0.13	0.2
III	0.09	0
IV	0.09	0.2

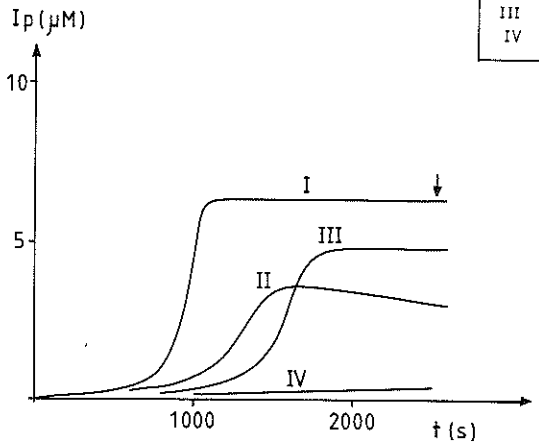


Figure 9. Influence of k_D and v_E. E_0, 950 μM; k_f, 10^{-2} μM^{-1} s^{-1}; k_3, 5×10^{-4} s^{-1}; I_e, 10 μM. The arrow indicates the time of cell division.

quite small (1.75×10^{-4} μM^{-1} s^{-1}), and it has been shown above that, with such a low value, even a very large number of "trapping" enzyme molecules per cell does not prevent a rapid accumulation of antibiotic in the periplasm (Fig. 7, curve III).

Unfortunately, quantitative data concerning the kinetic constants which characterize the interactions between PBPs and β-lactams are rather scarce. A possible candidate for trapping might be PBP 4 of *Proteus mirabilis* (k_f, 0.2 to 0.3 μM^{-1} s^{-1}; k_3, 4×10^{-5} s^{-1}), and a candidate for hydrolysis might be PBP 5 of the L form of the same species (k_f, 0.02 to 0.08 μM^{-1} s^{-1}; k_3, 1.6×10^{-3} s^{-1}) (2, 10). However, it is quite probable that the small number of those proteins present per cell might only marginally account for resistance (the values given above for the number of copies of PBPs per cell should be compared with values of the order of 10^5 and more copies per cell for β-lactamases) (7).

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Chapter 49

Induction of β-Lactamase in *Bacillus* spp., from Penicillin Binding to Penicillinase

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The induction of β-lactamase synthesis in *Bacillus* species was one of the first microbial regulatory systems to be investigated (14) and has continued to attract interest because of its unusual properties. Induction is delayed and protracted even though the half-life of the specific mRNA for the enzyme is short (4, 16). The continued presence of the inducer (a β-lactam) is not essential, and the level of inducer added determines the amplitude of the response, but not the timing (3). Furthermore, it has not been possible to induce production of the enzyme in protoplasts. Because of these features it was of considerable interest to identify and characterize the various components of the regulatory system.

Bacillus cereus and *Bacillus licheniformis* have received the greatest attention. The time course of induction is similar in the two species, although *B. licheniformis* forms only a single enzyme whereas *B. cereus* 569 makes three different β-lactamases that are coordinately regulated. We have focused

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