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QUANTITATIVE RELATIONSHIP BETWEEN SENSITIVITY TO β-LACTAM ANTIBIOTICS AND β-LACTAMASE PRODUCTION IN GRAM-NEGATIVE BACTERIA—II

NON-STEADY-STATE TREATMENT AND PROGRESS CURVES

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Abstract—A non-steady-state model is discussed for the study of the interplay between β-lactamase activity and outer membrane permeability with slowly hydrolysed β-lactams. The analysis shows: (1) that the simple, steady-state model presented in the accompanying paper remains valid as long as k_{cat} (i.e. k_3 with chromosome-encoded class C β-lactamases) is larger than 10^{-3}/sec (generation time = 20 min or more); (2) that among the β-lactam antibiotics studied here, the complete, non-steady-state model needs only be used in the case of aztreonam; (3) that the term "trapping" should be replaced by "formation of a covalent acyl-enzyme" and that such a phenomenon only contributes significantly to the resistance when penetration and hydrolysis are very slow and the periplasmic β-lactamase concentration is very high. Aztreonam seems to be the only compound which fulfils the first two conditions.

In the preceding paper [1], we have described a method for analysing the correlation between the MIC values for β-lactam antibiotics and the amount and properties of the periplasmic β-lactamases in Gram-negative bacteria. That analysis was rigorously valid when the half-life of the acylenzyme intermediate formed upon interaction between the β-lactam and the β-lactamase was significantly shorter than the generation time of the bacterium. If that condition is not fulfilled, the periplasmic concentration of β-lactam $[I_p]$ does not reach a stable value within one generation. A rigorous treatment then requires that the synthesis of new enzyme be taken into account, a factor which is particularly important in the case of superproducing strains. A first simple model has been described [2, 3] where v_E, the rate of enzyme synthesis, was computed on the basis of the generation time and of the observed concentration of β-lactamase in the periplasm. That model, however, failed to consider the increase of periplasmic volume (Vol) due to cellular growth. A more realistic model, based on Scheme 1 in the preceding paper is now proposed. However, for practical reasons, the branch representing the interaction between the β-lactamase and the β-lactam

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

must be slightly modified. When I_p is much smaller than K, the non-covalent complex EI does not accumulate and the reaction can be written

$$E + I_p \overset{k_2/K}{\longrightarrow} EI^* \overset{k_3}{\longrightarrow} E + P$$

In practice, one very seldom knows the individual values of K and k_2, but the ratio k_2/K can be obtained easily either directly or by measuring k_{cat}/K_m. Since the model applies to cases where k_3 is very low, one can also safely assume that $k_{cat} = k_3$. Thus, the following treatment strictly applies only when $I_p \ll K$. Since the values of K are generally rather high, the approximation is valid in most cases. When it is not valid, the accumulation of I in the periplasm becomes faster than computed.

Model and equations

The analysis rests on the following equations:

Periplasmic volume at time t

$$(Vol)_t = (Vol)_o e^{kt} \tag{1}$$

where k (= 0.69/generation time) is the first-order rate constant characteristic of the cellular growth.

Total quantity of β-lactamase at time t

$$(Q_E)_t = E_o(Vol)_t = E_o(Vol)_o e^{kt} \tag{2}$$

where E_o is the total periplasmic concentration of enzyme, which is assumed to remain constant.

Total rate of synthesis of enzyme

$$v_{QE} = dQ_E/dt = E_o(Vol)_o k\, e^{kt}. \tag{3}$$

X, Y and U being the total quantities of E, I_p and EI*, respectively

$$\frac{dX}{dt} = E_o(Vol)_o k e^{kt} + k_3 U - \frac{k_2}{K}\frac{XY}{e^{kt}}\frac{1}{(Vol)_o} \tag{4}$$

$$\frac{dY}{dt} = k_D(Vol)_o e^{kt}\left(I_e - \frac{Y}{(Vol)_o e^{kt}}\right) - \frac{k_2}{K}\frac{XY}{e^{kt}}\frac{1}{(Vol)_o} \tag{5}$$

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Table 1. Comparison between the values obtained for E_p and I_p at the steady-state with the complete and simple models

	Simple model		Complete model			
k_3 (/sec)	I_p (μM)	E_p	I_p (μM)	E_p	$(t_{0.9})_I$ (sec)	$(t_{0.9})_E$
0.0001	2.0	2.5	0.084	285	3700	3200
0.0003	1.0	14.5	0.057	450	3200	2400
0.0010	0.048	509	0.036	687	950	600
0.0030	0.029	835	0.026	943	<100	300
0.0100	0.026	951	0.026	953	<100	220

The values of the other parameters are as follows: $k_2/K = 0.02/\mu M/sec$, $k_D = 0.2/sec$, $I_e = 2.5\,\mu M$, $k = 0.575 \times 10^{-3}/sec$ (generation time = 20 min), $E_o = 1000\,\mu M$. $(t_{0.9})_I$ = time required for I_p to reach 90% of its steady-state value. $(t_{0.9})_E$ = time required for EI* to reach 90% of its steady-state value.

$$\frac{dU}{dt} = \frac{k_2}{K}\frac{XY}{e^{kt}}\frac{1}{(Vol)_o} - k_3 U \qquad (6)$$

where k_D is a first-order rate constant which characterizes the rate of antibiotic equilibration (see Ref. 1). The periplasmic concentrations of E, I_p and EI* at time t are obtained by dividing X_t, Y_t and U_t by $(Vol)_t$ [i.e. $(Vol)_o e^{kt}$]. Equations 4–6 can be simplified by taking $(Vol)_o$ equal to unity.

The phenomenon can then be simulated by choosing values for k_D, E_o, k, k_2, K and k_3 and using a numerical integration program (based on a Runge–Kutta method of the 4th order) which computes the values of E, I_p and EI* as a function of time. The accuracy of the numerical integration was checked by varying the integration step. If the step was small enough, its value did not significantly influence the results. Conversely, if the chosen value was too large, completely aberrant results were obtained. Moreover, when the value of k was set close to zero (absence of cellular growth), the values of the variables at the steady-state were identical to those which were directly calculated on the basis of the simple steady-state equation of Zimmerman and Rosselet's [4].

After a period of time which is mainly dependent upon the value of k_3 (Table 1), stable concentrations of I, E and EI* are predicted in the periplasm, although the total amount of enzyme (E + EI*) continues to increase proportionally to the total cellular mass.

As also shown by Table 1, those stable periplasmic concentrations can be very different from those which might be calculated on the basis of the simple steady-state model and equation of Zimmerman and Rosselet [4]. In the following discussion the latter will be referred to as the *simple* model and that resting on the numerical integration of the unsolved differential equations as the *non-steady-state* model.

Since the non-steady-state model is a continuously expanding model, it is also clear that it cannot be used over too many generations.

Visualization of non-hydrolytic trapping

Impressed by the very high concentrations of β-lactamase which can be present in the periplasm of derepressed producers, some authors [5–7] have suggested that "trapping" by the β-lactamase might contribute to a significant decrease of the periplasmic concentration of "non-hydrolysable" β-lactams which would be immobilized in the EI (Michaelis complex) and EI* (acyl-enzyme) intermediates. Since that hypothesis has generated a lot of controversy [8, 9], we believe that it is interesting to examine it in the light of our model.

(1) The term "non-hydrolysable" β-lactam is misleading. As pointed out by various authors [2, 9, 10], the important parameter is $k_{cat}E_o$, where a slow turnover (k_{cat}) might be compensated for by a very high enzyme concentration (E_o). For instance, if E_o = 1 mM and $k_{cat} = 10^{-3}/sec$, $k_{cat}E_o$ is $1\,\mu M/sec$ although k_{cat} is only 10^{-6}-fold the value observed for good substrates.

(2) As long as the half-life of the acyl-enzyme remains significantly shorter than the generation time, the steady-state model can be used (see Table 1) and trapping is irrelevant, since the entering β-lactam first saturates the periplasmic β-lactamase and then rapidly reaches its steady-state level [2]. This is due to the fact that, when a MIC is determined, the exterior volume is many orders of magnitude larger than the periplasmic volume and the quantity of available β-lactam is much larger than the quantity of enzyme, even if E_o is larger than I_e.

(3) As a result, the β-lactam can only be "trapped" by newly synthesized β-lactamase. This will only happen if the rate of entry ($k_D \times I_e$) is so slow that it becomes similar to the rate of new enzyme synthesis [9]. The simulation of such a situation is described by Fig. 1. After 12,000 sec, when I_p has stabilized, the entering antibiotic is partitioned as follows: 0.3% remains free and is responsible for "filling" the new periplasmic space formed by bacterial growth, 12.4% is hydrolysed and 87.3% immobilized as acyl-enzyme by newly synthesized enzyme. Thus, trapping only occurs if $k_3 < 10^{-3}/sec$ and $k_D I_e \leq kE_o$ (which means that the ratio of the half-equilibration time to the generation time must be similar to or smaller than the ratio I_e/E_o.

(4) In fact, among the compounds studied in this paper and the preceding one, aztreonam seems to be the only one for which a combination of low penetration rate and low k_3 value indicate that "trap-

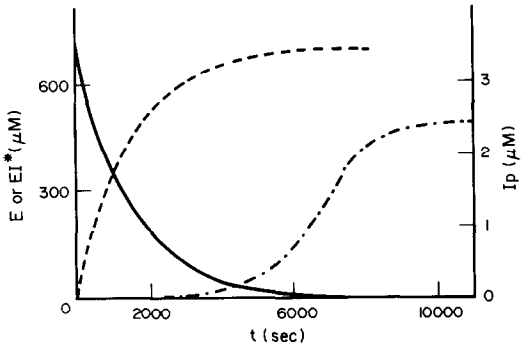

Fig. 1. Variation of [E], [EI*] and I_p with time. The values of the parameters are $k_2/K = 0.068/\mu M/sec$, $k_3 = 8.2 \times 10^{-5}/sec$, $k_D = 1.6 \times 10^{-3}/sec$, $I_e = 288\ \mu M$, $k = 0.000575/sec$ (generation time: 20 min), $E_o = 696\ \mu M$. After 12,000 sec, the following steady-state values are found: [E] = 2.74 μM, [EI*] = 693.3 μM, $I_p = 2.45\ \mu M$. By contrast, the steady-state values found with the simple model would be: $[E] = 3.32\ \mu M$ and $I_p = 252\ \mu M$. ———, [E]; – – – –, [EI*]; –·–·–, I_p.

ping" might be relevant.

(5) Finally, our model does not take account of the possible presence of the Michaelis complex EI. Indeed, all the available data indicate that with class C β-lactamases, low k_{cat} and low K_m values always reflect low k_3 values [11, 12] and that *the only relevant intermediate is the covalent acyl-enzyme*. A poor substrate for which the Michaelis complex would be prevalent remains to be found and there is presently no reason to assume, as does Sanders [7] that it might significantly contribute to resistance. The vague term "trapping" should thus be replaced by "formation of a covalent acyl-enzyme" [10].

Induction

At this stage, our model does not include the possible effect of induction of β-lactamase production by the presence of antibiotic in the periplasmic space. Since the simulations extend over several generations, and a non-negligible antibiotic concentration can prevail in the periplasm, the induction phenomenon might become relevant. However, the mechanism and kinetics of induction still remain mysterious and it is not yet possible to build a realistic model including an increase of the rate of β-lactamase synthesis. As will be seen below, this failure certainly determines some of the limitations of our study.

Utilization of the model to estimate k_D. *Two different approaches*

Bush *et al.* [13] have measured the MIC values of aztreonam for seven different *Enterobacter cloacae* strains. After treatment of whole cells with that monobactam during the same period of time (120 min), they have also measured the activity of the β-lactamase immediately after isolation and disruption of the cells and after full reactivation of the enzyme by spontaneous decay of the acyl-enzyme. They could thus calculate the proportion of acyl-enzyme at the end of the treatment of whole cells, which was clearly dependent upon the antibiotic penetration rate. The analysis of those data with the help of our model thus allows the computation of the k_D values by two independent approaches.

(1) Simulations can be performed under the conditions used in the whole cell treatment experiments: the k_D value is modified until the $[EI^*]/E_o$ ratio obtained after 7200 sec is equal to the observed value. The k_D value obtained for each strain is then used in other simulations to compute the value at which I_p stabilizes when I_e is equal to the MIC. This should supply the value of I_{pl}, i.e. the periplasmic aztreonam concentration necessary to inactivate the essential PBP(s). As seen on Table 3, that I_{pl} value for the WT strain (0.76 μM) was very close to the MIC, a result which was expected, since that strain

Table 2. Kinetic parameters for the E_2 and P99 β-lactamases as deduced from the data of Bush *et al.*

	Enzyme E_2			Enzyme P99		
	k_3 (/sec)*	K_m† (μM)	k_2/K (μM/sec)	k_3 (/sec)	K_m† (μM)	k_2/K (μM/sec)
Aztreonam	8.2×10^{-5}	0.0012	0.068	2.8×10^{-5}	0.0024	0.0116
Ceftazidime	0.077	1	0.077	0.17	3	0.057
Imipenem	0.0135	2.5	0.0054	0.0015	0.0051	0.011
Cephaloridine	650	810	0.802	845	580	1.46

* Computed from the relative V_{max} (cephaloridine = 100) or from the half-life of the acyl-enzyme. When both values were measured, the deduced k_3 values were in excellent agreement, except for the interaction between ceftazidime and the P99 β-lactamase: a value of 0.017/sec is reported from the relative V_{max} and a value > 0.11/sec from the half-life of the acyl-enzyme. Since it does not seem possible that acylation would be rate-limiting, the only explanation is that the relative V_{max} should be 0.02% of that observed with cephaloridine (and not 0.002%).

† With aztreonam, ceftazidime and imipenem, we have assumed $K_m = K_i$. The K_m and K_i values determined by Bush *et al.* sometimes differ by large factors, which can only be explained by the fact that their "initial rates" were strongly influenced by a burst corresponding to acyl-enzyme accumulation. The K_i values, measured after a 5 min preincubation were probably more significant and, in the case of the interaction between imipenem and the P99 β-lactamase, in much better agreement with our own measurements [11].

Table 3. Properties of the strains studied by Bush *et al.* [13] and k_D values determined by the direct method or deduced from the MIC

Strain	Enzyme type	E_o (μM)*	MIC for aztreonam (μM)	Method 1		k_D as deduced from the MIC (/sec)† (Method 2)
				k_D(/scc)	I_p at MIC (μM) = I_{pl} ?	
WT	E_2	6	1.12	0.011	0.76	0.011
DR	E_2	696 (1400)	288	ND	ND	0.0016–0.0030
Az^r	E_2	582	576	1.8×10^{-5}	<0.0005	0.0004–0.0009
Cf^r	E_2	600	288	ND	ND	0.0016
Mx^r	E_2	6.6 (20)	9.2	0.008	8 (7)	0.0005–0.0010
Az^rMx^r	E_2	781	1150	0.0009	350	0.0004‡
SC10435	P99	1000	58	0.17	54	0.010

* The values between round brackets are those expected if induction occurs at the MIC.

† Computed with $I_{pl} = 0.76$ μM for all strains.

‡ This case is a dramatic example of a disproportionate influence of a small variation of k_D on the stabilization value of I_p. For $I_e = 1150$ μM (= MIC) and $k_D = 4 \times 10^{-4}$/sec, the rate of penetration of aztreonam is close to the rate of synthesis of the β-lactamase, nearly all the entering aztreonam is immobilized as acyl-enzyme and I_p never reaches 0.1 μM. When k_D is larger than 4.5×10^{-4}/sec, a rapid increase of I_p is observed after the β-lactamase has been saturated. For instance, the values of I_p after 3600 sec rapidly increase with increasing values of k_D: 0.11 μM ($k_D = 4.5 \times 10^{-4}$/sec), 12 μM ($k_D = 5 \times 10^{-4}$/sec), 125 μM ($k_D = 6 \times 10^{-4}$/sec) and 212 μM ($k_D = 7 \times 10^{-4}$/sec). When $k_D = 4.5 \times 10^{-4}$/sec, a value of $I_p = 2.2$ μM (twice the WT MIC) is observed after 7200 sec.

ND = not determined.

is a very poor β-lactamase producer.

(2) Conversely, assuming that the PBP sensitivity is the same for all strains, simulations can be performed with an external aztreonam concentration equal to the MIC until k_D values are found which yield the same stabilized I_p value (= I_{pl}) for each strain. If k_D and the amount and/or properties of the β-lactamase(s) are the only factors which are altered in the different strains, both methods must essentially produce the same relative scale of permeabilities.

We have analysed the data of Bush *et al.*, with the following assumptions:

(1) the generation time of the bacteria was 20 min;

(2) the periplasmic concentration of enzyme could be calculated from the number of β-lactamase molecules per cell (2.3×10^5 for strain SC10435), a cell volume of 2 μm^3 and a periplasmic volume representing 20% of the total cellular volume. The calculation yielded a value of about 1 mM for E_o in strain SC10435;

(3) no induction occurred with strains WT and Mx^r at a concentration of 0.1 μg/ml;

(4) the values of k_3 and k_2/K could be obtained from the reported kinetic parameters as follows:

$$k_{cat} = k_3 = 0.69/t_{50}$$

where t_{50} is the half-life of the isolated acyl-enzyme;

$$k_2/K = k_{cat}/K_m = k_3/K_i$$

which yielded the values given in Table 2. Errors might arise from the fact that experiments with the bacteria were performed at 37° while the kinetic parameters of the enzymes were measured at 25°, but the differences should not exceed a factor of about 2;

(5) the aztreonam concentrations at which k_D was measured by the first method, were below the MIC value.

Figure 2 shows the results of the simulation for strain SC10435 at a concentration of aztreonam (1 μg/ml) resulting in about 40% of active enzyme after 2 hr of contact with growing cells (method 1). A k_D value of 0.17/sec yielded the expected E_p value after 7200 sec, a time at which, in this case, the value of E_p had nearly become stationary.

Table 3 presents a summary of the properties of the various strains studied by Bush *et al.*, and the values of k_D that we computed on the basis of their experimental data. The two methods yielded extremely different results: with method 1, k_D values spanned four orders of magnitude (roughly in agreement with the relative permeability ratios deduced by Bush *et al.*) and the results implied even larger variations of the PBP sensitivities (I_{pl} values ranging from 350 to $<5 \times 10^{-4}$ μM). With method 2, which assumed a constant I_{pl} value of 0.76 μM, the smallest k_D value was only 25-fold smaller than the largest. Two interesting points should be underlined:

(1) with the WT strain, and I_e = MIC and k_D = 0.011/sec, I_p stabilized at 0.76 μM after only 1100 sec. The MIC value was thus very close to I_{pl} and reflected the sensitivity of the PBP(s). As expected, the low amount of β-lactamase was nearly irrelevant. Moreover, a k_D value of about 0.01/sec (i.e. 1000-fold lower than that observed with a rapidly penetrating β-lactam in *E. coli*, see [1]) did not result in a large difference between I_{pl} and the MIC;

(2) the sensitivity of the PBP(s) of strain Az^r as computed by method 1 was so extreme that it did not seem to be realistic.

Since the two procedures utilized for the computation of k_D yielded such widely different results, we tried to obtain further information about the scale of relative permeabilities by analysing the data obtained by Bush *et al.* with more rapidly hydrolysed β-lactams for which the simple model could be utilized.

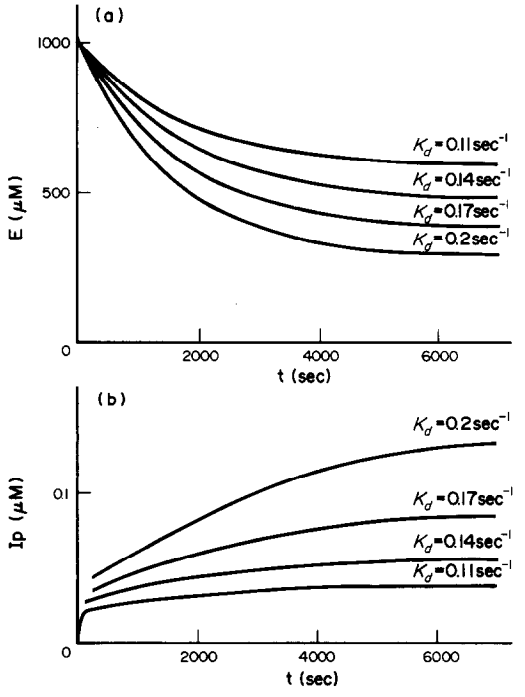

Fig. 2. Determination of the k_D value for aztreonam and strain SC10435 of Bush *et al.* (a) Variation of β-lactamase concentration [E] with time. (b) Variation of I_p with time. The following parameters were used: $k_2/K = 0.0116/\mu M/sec$, $k_3 = 2.8 \times 10^{-5}/sec$, $I_e = 2.3\ \mu M$, $k = 5.75 \times 10^{-4}/sec$ (generation time: 20 min), $E_o = 1000\ \mu M$. After 7200 sec, the k_D value of 0.17/sec yields the $[E]/E_o$ ratio of 0.4 observed by Bush *et al.*

Ceftazidime and imipenem

The k_D values were deduced from the MICs as described in the preceding paper. The kinetic parameters which were utilized are given in Table 2. When non-negligible induction was expected at the MIC, we also performed the calculations with concentrations of enzyme corresponding to the induced level at the MIC. Since different convergence points were observed for different pairs of curves, the calculations were performed with the extreme values. Table 4 summarizes those calculated values and shows that the relative permeabilities for ceftazidime are in good agreement with those derived from the MICs for aztreonam (method 2). The values derived from imipenem are completely different (see below).

With ceftazidime, it is interesting to note that the absolute k_D values which could be deduced from the $\bar{Z}$ values were in the range 0.004–0.4/sec, which was not very different from the values (0.05–0.32/sec) deduced in the preceding paper for *E. coli* from the data of Nikaido and Normark [14].

Similarly, from the data of Marchou *et al.* [15], a value of 0.2/sec/μM could be deduced for $k_D/(E_o)_S$. Since, in that case, the sensitive strain contained about 1000-fold less enzyme than the best producer, an $(E_o)_S$ value of about 1 μM could be assumed. This would yield a k_D value of 0.2/sec for strains 218S and 218R1 and of 0.04/sec for strain 218R2, which would also be in the range of those found here.

Moxalactam

Bush *et al.* report half-lives (60 and 210 min) for the acyl-enzymes formed with moxalactam and the E2 and P99 β-lactamases. Those data would imply the utilization of the non-steady-state model. However, by directly measuring the slow hydrolysis of moxalactam by the P99 β-lactamase, we have observed the characteristic features of a branched pathway and the k_{cat} value, after establishment of the steady-state was $9 \pm 1 \times 10^{-3}/sec$ [12]. In this case, further studies are needed to reconcile our data with those of Bush *et al.* and before the kinetic parameters can be utilized to explain the MIC values.

DISCUSSION

Why does method 1 fail?

The relative k_D values computed for aztreonam by method 2 were in excellent agreement with those computed for ceftazidime, assuming a constant PBP sensitivity in both cases. Why did method 1 fail? Cell densities used in that method are certainly much larger than those used in MIC determinations, but that would only invalidate the model if substantial hydrolysis of aztreonam occurred. This is not realistic since the values of k_{cat} were exceedingly low. Some of the data that we analysed according to method 1 were also obtained at aztreonam concentrations which were close to or above the MIC for the strain under study or which might result in non-negligible

Table 4. Relative values of k_D (WT = 1)

	Aztreonam		Ceftazidime*		Imipenem†	
Strain	Method 1	From the MIC (method 2)	Without induction	With induction	Without induction	With induction
WT	1	1	1	1	1	1
DR	ND	0.15–0.27	0.23–0.86	0.67–0.85	275	3.9
Az^r	0.0016	0.04–0.08	0.13–0.36	0.14–0.18	100	1.4
Cf^r	ND	0.15	0.06–0.18	0.07–0.09	250	3.5
Mx^r	0.7	0.04–0.09	0.05–0.13	0.86–1.08	0.075	0.021
Az^{r}Mx^r	0.08	0.04–0.08	0.04–0.11	0.04–0.05	4	0.057
SC10435	15	1	1–18	0.86–0.9	1250	18

* Extreme values obtained with the following values of I_{pl}: without induction 1.7 μM–0.2 μM or lower; with induction: 0.7 μM–0.2 μM or lower.

† With $I_{pl} = 1\ \mu M$.

ND = not determined.

induction. If induction took place, the rate of enzyme synthesis increased *faster* than the periplasmic volume and enzyme produced at the end of the experiment, which was in contact with aztreonam during a shorter time disproportionately decreased the percentage of acyl-enzyme. This might explain the unexpectedly high proportion of active β-lactamase observed by Bush *et al.* with strains WT and Mx^r at aztreonam concentrations above 0.1 μg/ml but does not seem to be important for strains SC10435 and Az^r. Finally, the proportion of active β-lactamase might also be overestimated if thermal inactivation occurred concomitantly with acyl-enzyme degradation. The shortcomings of method 1 show the dangers of calculating a permeability factor without examining the effects of its value on the periplasmic concentration of β-lactam which can be expected when I_e is equal to the MIC.

The behaviour of imipenem

It seems that imipenem had a very distinct behaviour. Strains DR, Az^r and Cf^r which were less permeable to the two other compounds were more permeable to imipenem. This conclusion was in agreement with that drawn in the accompanying paper [1] where the analysis of the data of Marchou *et al.* [15] also indicated the very particular behaviour of that compound. Our results also agree with those of Buscher *et al.* [16] who suggested a different permeability mechanism for imipenem, although the comparison should be made with great care since those authors studied *Pseudomonas*, a different genus.

The fact that the MIC for imipenem often appeared to be independent of the β-lactamase concentration suggested a very rapid equilibration (very high k_D). The MIC would essentially correspond to the sensitivity of the most sensitive essential PBP. In some cases however (strain Mx^r for instance), this latter factor, or the permeability might be modified.

Influence of the inaccuracies of MIC determination

As discussed in the preceding paper [1], the MIC values, as usually determined, can be inaccurate by a factor slightly lower than 2. Examination of Eqn (5) shows that, when I_e remains much larger than I_{pl}, the product $k_D I_e$ will be the important factor. In consequence, the k_D values computed from the MIC for aztreonam might be too low by a factor of at most 2 for all the strains but the WT strain. With that strain, for a I_e value of 0.6 μM, i.e. 55% of the MIC, the I_p value stabilizes at 0.24 μM, i.e. 40% of the I_e value (instead of 70% at the MIC). Those errors do not significantly modify the relative k_D values.

Induction and PBP reactivity

The inclusion in the comparison of strains where the production of β-lactamase was inducible further complicated the analysis when induction became detectable at antibiotic concentrations at or below the MIC. At the present time, the kinetics of induction remain difficult to include in the model and the behaviour of strains where the β-lactamase might be induced at the MIC is impossible to analyse rigorously.

Reaction of the PBPs with the periplasmic antibiotic is also not immediate. In fact, the scheme described for the interaction between the β-lactamase and the antibiotic is also valid for that between the latter molecule and the PBPs. In consequence, as soon as some free antibiotic is present in the periplasm, acylation of the PBPs starts to occur. Note that if k_2/K is not very large, non-negligible concentrations of β-lactam can coexist in the periplasm with large concentrations of β-lactamase (see for instance Fig. 2 and [2]). Cell death requires the inactivation of a still undetermined proportion of the essential PBPs. But it is quite likely that inactivation of a smaller proportion of the essential PBPs might impair cell function, influence the generation time and modify the β-lactamase production.

In relation with those two complicating factors, β-lactamase induction and sublethal PBP inactivation, one must realize that two very different situations might arise. The first is depicted by Fig. 1. After a sufficient period of time, all the β-lactamase is inactivated, and a rapid and dramatic increase in periplasmic β-lactam concentration occurs. In that case, β-lactamase induction and partial PBP inactivation are probably of reduced importance. The second situation is depicted by Fig. 2. Large concentrations of active β-lactamase remain present at the steady-state but the value of I_p can represent a non-negligible proportion of I_e. Indeed, Fig. 2B shows that, for a k_D value of 0.2/sec, I_p stabilizes at about 5.6% of I_e after about 6000 sec. If that value must be reached to induce a lethal inactivation of the essential PBPs, one must however realize that 50% of that periplasmic concentration was already present after less than 1500 sec.

The building of a more complete model must rely on further experimental work, which should involve the study of the kinetics of induction, of the proportions of essential PBPs which are necessary for cell survival and of the effects of the inactivation of sublethal proportions of the same PBPs on cell behaviour. Meanwhile, it remains dangerous to draw conclusions about the permeability factors based on a simple and intuitive reasoning. The approach which is presented in our contributions can be nicely complemented by the utilization of the procedure proposed by Waley [17] who suggests to compare the rate of β-lactam hydrolysis by intact and disrupted cells.

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