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

STEADY-STATE TREATMENT

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Abstract—A simple graphical method is proposed to decide whether a decreased sensitivity to β-lactam antibiotics can be explained by the sole increase of β-lactamase production or if other factors such as outer membrane permeability or target sensitivity must also be involved. The parameters which are necessary for the analysis can easily be measured: the relative amounts of β-lactamase produced by the various strains; the K_m of the enzyme for the various β-lactams under study and the susceptibility of each strain to the same antibiotics, measured as MIC values.

The resistance of a Gram-negative strain to a β-lactam antibiotic results from the interplay between three independent factors [1–3]:

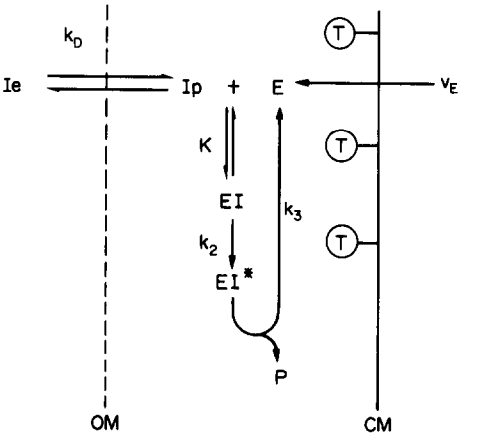
(1) the sensitivity of the target enzymes, the DD-peptidases, responsible for the closing of the interpeptide bridges in peptidoglycan;

(2) the concentration and properties of the periplasmic β-lactamases;

(3) the permeability of the outer membrane to the β-lactam under study.

Various types of mutants have been described, where both the periplasmic concentration of β-lactamase and the permeability barrier appeared to be modified. The analysis of those results, however, often suffers from the fact that the kinetic aspects of the phenomena are not rigorously considered. In that respect, the article by Nikaido and Normark [4] stands as a unique exception.

Two different situations can arise [3, 5], depending upon the stability of the acyl-enzyme EI* (Scheme 1). If its half-life ($0.69/k_3$) is short compared to the generation time of the bacterium, one can assume that the steady-state is rapidly established in the periplasm, and the synthesis of new β-lactamase can be neglected ($v_E = 0$). In the present article, we wish to propose a practical approach to situations where the steady-state model applies. In the accompanying paper, we examine the non-steady-state situation.



Scheme 1. General scheme for the study of the factors whose interplay determines the concentration of antibiotic in the periplasm (I_p). I_e represents the external concentration of antibiotic, E the periplasmic concentration of β-lactamase, EI a non-covalent complex and EI* the acyl-enzyme; v_E is the rate of synthesis and excretion of β-lactamase by the cell, k_D a first-order rate constant characteristic of the rate of diffusion of the antibiotic through the outer membrane (OM), K the dissociation constant of EI and k_2 and k_3 first-order rate constants. CM is the cytoplasmic membrane on the external face of which the target DD-peptidases can be found (Ⓣ—).

THE EQUATION OF ZIMMERMAN AND ROSSELET [6]

The original equation can be rearranged to express the external concentration I_e which is necessary to yield a pre-determined periplasmic concentration I_p:

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

where

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

and

$$K_m = \frac{k_3 K}{k_2 + k_3}.$$

Only two conditions need to be fulfilled for Eqn (1) to be valid:

(1) as stated above, the half-life of EI* must be significantly shorter than the generation time of the strain;

(2) I_e must remain constant, i.e. the amount of

antibiotic which is destroyed during the experiment must be negligible, a condition which is fulfilled when a MIC is determined (low cell density).

Several authors have based their analysis on Eqn (1), using different symbols (Table 1). Note that in computing $t_{\frac{1}{2}}$, Nikaido [7] considers the *total volume* of the cells which are used to measure V_{max}. In fact, if the β-lactam only penetrates in the periplasm, *Vol* should be the periplasmic volume and the $t_{\frac{1}{2}}$ values should be 5–10-fold lower than those deduced by that author.

Equation 1 is valid even when the total enzyme concentration E_o is similar to or larger than the external concentration of antibiotic I_e. Indeed, the derivation of Eqn 1 does not rest on the condition $I_p \gg E_o$. This is due to the fact that the value of I_p is maintained constant thanks to the diffusion of the compound through the outer membrane. An apparently paradoxical situation can thus arise, where the total concentration of the enzyme–substrate complexes (EI + EI*) is much larger than I_p, but all those concentrations nonetheless remain constant in time. That had not been realized before by several authors, including ourselves [3, 8].

GOAL OF PAPER. MATHEMATICAL BACKGROUND

Assume that, starting with a wild-type strain (WT), various mutants exhibiting increased resistance have been isolated. We wish to describe a simple graphical method which allows to decide whether an increased resistance can be solely attributed to an increase of the β-lactamase concentration or if a decrease of the permeability or an increase of target resistance are also involved. In practice, the easily accessible parameters are the MICs, the relative amounts of β-lactamase produced by the various mutants (as determined using a standard substrate, cephaloridine for example, at a saturating concentration) and the K_m values of the β-lactamase for the different β-lactams under investigation. The exact value of E_o, the periplasmic concentration of the enzyme is more difficult to estimate and a correct evaluation of k_{cat} requires the purification of the enzyme to protein homogeneity but one can easily measure the ratio R_i characterizing the increase of β-lactamase production for mutant i:

$$R_i = (\text{sp. act.})_i/(\text{sp. act.})_{WT} = (k_{cat}E_o)_i/(k_{cat}E_o)_{WT}.$$

For each mutant, an external concentration (I_e) equal to the MIC results in a periplasmic level of antibiotic (I_{pl}, where l stands for lethal) sufficient to induce the inactivation of the target enzyme(s).

$$MIC_i = I_{pl} + \frac{(k_{cat}E_o)_i I_{pl}}{k_D(K_m + I_{pl})}. \quad (2)$$

The value of I_{pl} is so difficult to measure that it has, in fact, never been rigorously determined. The antibiotic concentration which is needed to inactivate 50% of a given PBP over a given period of time has often been measured. The meaning of those I_{50} values has been discussed [9, 10]. To transform those data into I_{pl} values, one should know (i) which PBP is the lethal target and (ii) the proportion of the lethal target which is necessary to the survival of the cell. The first factor has seldom been determined and the second is generally unknown.

Equation (2) is then rearranged:

$$k_D = \frac{(k_{cat}E_o)_i I_{pl}}{(MIC_i - I_{pl})(K_m + I_{pl})} \quad (3)$$

and each member is divided by $(k_{cat}E_o)_{WT}$, yielding Eqn (4):

$$\frac{k_D}{(k_{cat}E_o)_{WT}} = \frac{(R_i)I_{pl}}{(MIC_i - I_{pl})(K_m + I_{pl})} \quad (4)$$

in the second member of which I_{pl} is the only unknown. However, the value of I_{pl} can be deduced as follows from the experimental data: for each mutant, a curve

$$Z = \frac{R_i I_p}{(MIC_i - I_p)(K_m + I_p)} \quad (5)$$

is calculated with I_p as independent variable. If the amount of β-lactamase is the only factor which distinguishes the mutants, the various curves should converge to a point of coordinates (I_{pl}, $\bar{Z}$). Indeed, if that condition is fulfilled, an I_p value equal to I_{pl} yields a Z value of $k_D/(k_{cat}E_o)_{WT}$ (see Eqn 4) and neither the abcissa nor the ordinate of the point depend upon the properties of the mutant (see Fig. 1 for a numerical example). Quite often, as observed by Nikaido and Normark [4], if the reference strain is a poor producer of β-lactamase, the value of I_{pl} will not be much smaller than the MIC. An even better approximation of I_{pl} can be obtained by comparing the MIC values in the presence and in the absence of a β-lactamase inactivator for which the MIC is rather high. Such a compound added at a concentration well below its own MIC should result in a MIC essentially equal to I_{pl} for any other β-lactam.

If the curves do not converge, three possible causes must be examined, besides the experimental errors whose effect will be discussed below:

(1) the permeability factor (k_D) has been altered,

(2) the sensitivity of the target enzyme (I_{pl}) has been modified,

(3) the value of R_i, established with the standard substrate, is not valid for the other β-lactams. That may happen if the compared strains do not produce the same β-lactamase (e.g. plasmid vs chromosome-encoded), or, alternatively, if a mutation, similar to that observed by Hall and Knowles [11] has changed the specificity profile of the β-lactamase.

Those last possibilities can easily be dealt with by measuring the specific activity of the various enzyme extracts on the β-lactams under study. A distinction between an increase of I_{pl} and a decrease of k_D is more difficult to perform, short of measuring the affinity of the PBP targets for the β-lactam or the value of k_D by a direct method. However, one might consider the following rule of thumb. A modification of the affinity of the PBP essential target is very unlikely to be the same for a series of very different β-lactam compounds. On the contrary, and particularly if one considers compounds exhibiting the same net charge, a porin-related modification of the permeability is likely to decrease the value of k_D by

Table 1. Comparison of our parameters and symbols with those utilized by other authors

	Zimmermann and Rosselet [6]	Nikaido and Normark [4]	Waley [1, 8]	This paper	Comments
Extracellular concentration of β-lactam	S_o	C_o	C_o	I_e	
Periplasmic concentration of β-lactam	S_e	C_p	C_p	I_p	
Lethal periplasmic concentration of β-lactam at MIC	—	C_{inh}	$\frac{2.3 \times 10^{-3}/\text{sec}}{k_T}$	I_{pl}	Waley characterizes the PBP sensitivity by a second-order rate constant k_T and assumes that the cell is killed if 90% of essential PBP is inactivated in 1000 sec
Permeability factor	$C = P \times A$	P(cm/sec) $t_{1/2}$(sec)	P (cm/sec)	k_D(sec)	$k_D = 0.69/t_{1/2} = \frac{P \times A}{Vol} = \frac{C}{Vol}$ where A and Vol are respectively, the surface and volume* of a given number of cells, N
Characteristics of the β-lactamase	V_{max} K_m	V_{max} K_m	$V_{max} = k_{cat}Q_E$ K_m $U = \frac{V_{max}}{K_m} = \frac{k_2}{K}Q_E$	k_{cat}(sec) K_m E_o (μM) $\frac{k_{cat}}{K_m} = \frac{k_2}{K}$	V_{max} expressed in μmoles/sec/N. N usually represents 1 mg of cell dry weight. Q_E is the corresponding quantity of enzyme
Other parameters	—	$\text{TAI} = \frac{C_{inh}}{\text{MIC} - C_{inh}}$	$Pn = \frac{P \times A}{U}$ Dimensionless	$\bar{Z} = \frac{P_n}{K_m}$ (/μM) $R_i = \frac{(E_o)_i}{(E_o)_{WT}}$	

* In fact, only the periplasmic volume should be considered; see text. Assuming that 1 mg dry wt = 4 mg wet wt, the value of A/Vol is 33,000 cm^{-1} if Vol is the total volume and 165,000 cm^{-1} if Vol is the periplasmic volume.

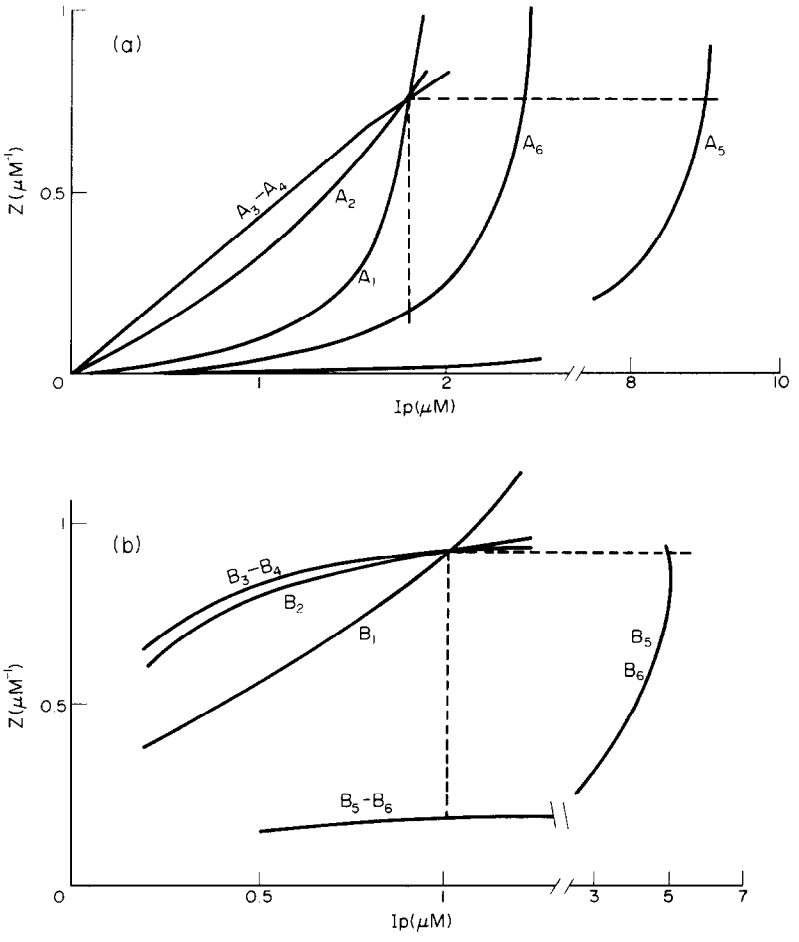


Fig. 1. Plots of Z vs I_p for two groups of strains using "exact" MIC values. The MIC values utilized for drawing the curves were calculated exactly on the basis of the following parameters. (a) Strains of group A: $K_m = 10\,\mu M$, $k_{cat} = 1/sec$, $(E_o)_{WT} = 1\,\mu M$. The value of k_D is 0.76/sec for strains A_1–A_5, 0.18/sec for strain A_6. I_{pl} is 1.8 μM for strains A_1–A_4 and A_6 and 9 μM for strain A_5. A_1: $R = 1$, MIC = 2.0 μM; A_2: $R = 10$, MIC = 3.8 μM; A_3: $R = 100$, MIC = 21.9 μM; A_4: $R = 1000$, MIC = 202.5 μM; A_5: $R = 1$, MIC = 9.63 μM; A_6: $R = 1$, MIC = 2.65 μM. (b) Strains of group B: $K_m = 0.1\,\mu M$, $k_{cat} = 1/sec$, $(E_o)_{WT} = 1\,\mu M$. The value of k_D is 0.9/sec for strains B_1–B_5 and 0.18/sec for strain B_6. I_{pl} is 1 μM for strains B_1–B_4 and B_6 and 5 μM for strain B_5. B_1: $R = 1$, MIC = 2 μM; B_2: $R = 10$, MIC = 11 μM; B_3: $R = 100$, MIC = 101 μM: B_4: $R = 1000$, MIC = 1001 μM; B_5: $R = 1$, MIC = 6.09 μM; B_6: $R = 1$, MIC = 6.05 μM.

a similar factor for all the β-lactams.

Finally, the value of k_D can be obtained if the absolute value of $k_{cat}E_o$ for the WT strain is directly measured (see below).

EFFECTS OF THE EXPERIMENTAL ERRORS

The major sources of errors lie in the determination of the MIC values. Those are usually measured using serial two-fold dilutions of the antibiotic. At worst, that means that two strains whose theoretical MICs differ by a factor slightly smaller than 2 might exhibit identical "observed" MICs. Figure 1 displays a series of theoretical curves showing the influence of variations of R, I_{pl} and k_D in cases where I_{pl} is smaller (A) or larger (B) than K_m. In both cases, curves 1–4 converge to a point of ordinate $\bar{Z}$ equal to $k_D/(k_{cat}E_o)_{WT}$ and of abcissa I_{pl}. Curves 5 and 6, corresponding respectively to an increase of I_{pl} and a decrease of k_D do not pass through that point. In practice, however, such clear-cut results will seldom be obtained (see below). Starting with the values of $\bar{Z} = k_D/(k_{cat}E_o)_{WT}$ and of I_{pl} determined from the coordinates of the convergence point [$\bar{Z}_m$ and $(I_{pl})_m$], one should recalculate the MIC values using Eqn (6) and compare them to the measured values (Table 2)

$$(MIC)_{calc} = (I_{pl})_m + \frac{R_i(I_{pl})_m}{\bar{Z}_m[K_m + (I_{pl})_m]}. \quad (6)$$

It is clear that the calculated MIC values for strains A_5, B_5 and B_6 are significantly lower than the expected values, indicating that the β-lactamase alone does not explain the resistance of the strains. In contrast, the calculated MIC for strain A_6 is close

Table 2. Parameters and comparison of "observed" and calculated MIC values for the theoretical examples of Figs 1 and 2

		Parameters			Comparison of MIC values (μM)					
					With exact MICs		With MICs from 2-fold dilution		With MICs from 1.5-fold dilution	
		R	k_D(/sec)	I_{pl} (μM)	$(MIC)_T$	$(MIC)_c$*	$(MIC)_o$	$(MIC)_c$†	$(MIC)_o$	$(MIC)_c$‡
K_m = 10 μM	1	1	0.76	1.8	2.0	2.0	3.85	**1.65**	2.28	2.24
A	2	10	0.76	1.8	3.8	3.8	3.85	3.86	5.1	4.4
	3	100	0.76	1.8	21.9	21.8	31	26	26	25.8
	4	1000	0.76	1.8	202.5	201.8	250	247	198	242
	5	1	0.76	9.0	9.63	**2.0**	15.5	**1.65**	11.6	**2.4**
	6	1	0.18	1.8	2.6	2.0	3.85	**1.65**	3.42	2.4
K_m = 0.1 μM	1	1	0.9	1.0	2	2	3.85	3.8	2.28	2.59
B	2	10	0.9	1.0	11	11	15.5	13.6	11.6	11.5
	3	100	0.9	1.0	101	101	125	112	131	106
	4	1000	0.9	1.0	1001	1001	1000	1092	1000	992
	5	1	0.9	5.0	6.09	**2**	7.7	**3.8**	7.7	**2.6**
	6	1	0.18	1.0	6.05	**2**	7.7	**3.8**	7.7	**2.6**

$(MIC)_T$ = theoretical MIC calculated from the various parameters and Eqn (4).
$(MIC)_o$ = observed MIC; $(MIC)_c$ = calculated MIC.
* Computed with the coordinates of the convergence points (Fig. 1) and Eqn (7).
† Computed from the data of Fig. 2 (panel 1) with A: I_{pl} = 1.4; $\bar{Z}$ = 0.5; B: I_{pl} = 2.7; $\bar{Z}$ = 0.88.
‡ Computed from the data of Fig. 2 (panel 2) with A: I_{pl} = 2; $\bar{Z}$ = 0.7; B: I_{pl} = 1.6; $\bar{Z}$ = 0.95.
The bold values disagree with the observed MIC. They indicate that the β-lactamase is not the only variable responsible for the modified MIC.

to the real value, and a four-fold decrease of k_D is not apparent under those conditions. It has been stressed before [3, 5] that relatively large variations of k_D have only minor effects if $k_{cat}E_o$ is $<k_D(K_m + I_p)$ and the MIC is not much larger than I_{pl}. Indeed, Eqn (2) shows that, for strain A_1, MIC = 1.8 + 0.2 and for strain A_6, MIC = 1.8 + 0.85. In both cases, the second term is smaller than the first one (I_{pl}) and, in consequence, the MICs are not very different.

In the example discussed above, the MIC values were supposed to be very accurately determined. Figure 2 (Panel 1) illustrates the results of an experiment where, starting with a 1 mM concentration of antibiotic, the MICs have been determined by serial two-fold dilutions. The observed MICs (Table 2) have been taken as the concentrations in the serial dilution just above the theoretical MICs. Equation (5) shows that choosing too large a MIC value will shift a curve downwards. When the theoretical MIC is close to I_{pl}, as with strain A_1, this error also significantly shifts the curve rightwards. Since the error is different for each MIC, curves A_1–A_4 and B_1–B_4 no longer exhibit a common convergence point. One can however estimate the value of I_{pl} on the basis of the following principles:

(1) The curves which are most likely to be close to the theoretical ones are those obtained with strains exhibiting a high R value (i.e. good β-lactamase producers). In fact, those curves can be shifted downwards by a factor of at most 2 when the corresponding MIC is $\gg I_{pl}$;

(2) The curves corresponding to strains with low R values and low MICs can be shifted rightwards by a factor of at most 2;

(3) If two curves converge for an I_p value larger than the MIC of the wild type strain, that is probably due to the error on the MICs for the corresponding strain or to variations of k_D or I_{pl}.

For group A, a reasonable assumption would be $\bar{Z}$ = 0.5 and I_{pl} = 1.4 and, for group B, $\bar{Z}$ = 0.88 and I_{pl} = 2.7. As shown on Table 1, the calculated MIC values are in fair agreement with the observed and theoretical MICs. Only strain A_5 exhibits a calculated MIC which is clearly too low. For strains A_1, A_6, B_5 and B_6, the values are slightly too low, but it is not possible to draw a definitive conclusion.

The situation can be improved by a more accurate determination of the MIC values. Starting with the same 1 mM concentration, the MICs have been determined a second time using serial 1.5-fold dilutions (Table 2). From the curves shown on Fig. 2 (Panel 2), the following assumptions could be made: group A: I_{pl} = 2, $\bar{Z}$ = 0.7; group B: I_{pl} = 1.6, $\bar{Z}$ = 0.95.

The calculated values, shown on Table 2, are now in much better agreement with the "observed" MICs. Clearly, strains A_5, B_5 and B_6 exhibit low calculated values, indicating a modification of either k_D or I_{pl}. Again, the fourfold decrease of k_D for strain A_6 escapes detection but, as explained above, such a result was expected. In fact, with the strains of group A, k_D should decrease to 0.07 (i.e. 11-fold) to double the theoretical MIC to 4.0 μM. If k_D decreases by a smaller factor, as in our example, even a very accurate measurement of the MIC will not lead to an unambiguous conclusion. Table 3 compares the theoretical and calculated parameters in each case.

DESCRIPTION OF THE METHOD

(1) With each mutant or strain, measure the MIC values for the various antibiotics and determine the relative amounts of β-lactamase.

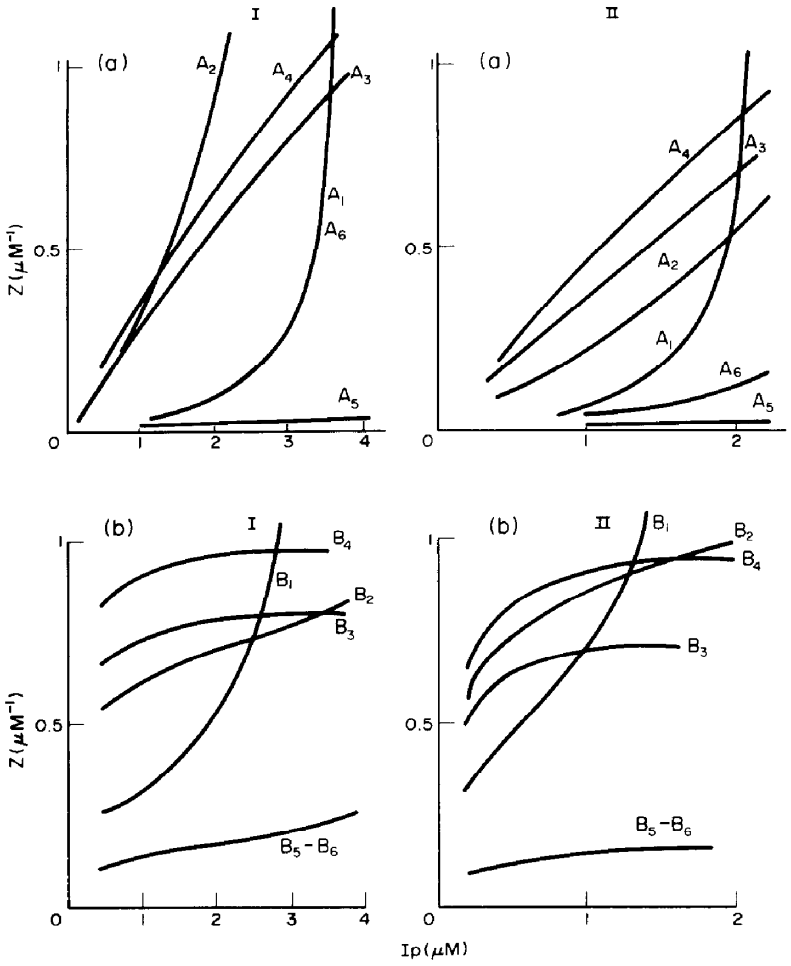


Fig. 2. Effects of the inaccuracies of MIC determinations. The other parameters are the same as in Fig. 1. Panel I: The MIC values were "determined" by serial twofold dilutions starting at 1000 μM. Panel II: The MIC values were determined by serial 1.5-fold dilutions starting at 1000 μM. Those "observed" values, taken as the antibiotic concentration just above the theoretical MIC are given in Table 1 for each strain.

Table 3. Comparison of theoretical and calculated parameters for the example of Fig. 2

	Theoretical values		Calculated values			
			2-fold dilution		1.5-fold dilution	
	I_{pl}	$\bar{Z}$	I_{pl}	$\bar{Z}$	I_{pl}	$\bar{Z}$
A	1.8	0.76	1.4 (1.9)	0.5 (0.6)*	2.0 (1)	0.7 (0.35)
B	1.0	0.90	2.7 (1.5)	0.88 (0.79)	1.6 (1)	0.95 (0.8)

* The values between round brackets also yield a good agreement between the calculated and "observed" MIC values.

I_{pl}: μM; $\bar{Z}$:/μM.

The I_{pl} and $\bar{Z}$ values correspond to strains A_1–A_4 and B_1–B_4. Those values *never* explain the behavior of strain A_5. With strains B_5 and B_6, the modified I_{pl} or k_D might escape detection in the two-fold dilution series. The four-fold decrease of k_D for strain A_6 is never clearly apparent.

(2) Determine the various K_m values.

(3) For each antibiotic, draw the curves $Z = f(I_p)$ corresponding to each strain.

(4) From the intersections, derive the values $(I_{pl})_m$ and $\bar{Z}_m$ corresponding to each β-lactam.

(5) Calculate the MICs using Eqn (6) and compare them to the observed values.

If one strain consistently exhibit significant differences between the observed and calculated MIC values, variations of k_D or I_{pl} for that strain must be suspected.

(6) As will be seen below, determination of k_D or P for one β-lactam requires the measurement of the corresponding value of k_{cat} and of E_o.

(7) Errors on the MICs can lead to an overestimation of I_{pl} by a factor of about 2. When k_D is high, and the β-lactam a poor substrate of the β-lactamase, variations of k_D can escape detection.

In the following paragraphs, the method is used to analyse some published data.

ANALYSIS OF THE DATA OF NIKAIDO AND NORMARK

Nikaido and Normark [4] have compared observed MIC values with values calculated from their own measurements of P, k_{cat} and K_m for each antibiotic and from values of I_{pl} (C_{inh}) taken from the literature, as the lowest of the I_{50} values obtained for any of the essential PBPs. Of the five strains under investigation, four produce various amounts of the AmpC chromosome-encoded β-lactamase (class C) and one the RTEM plasmid-encoded enzyme (class A).

Thirteen antibiotics were studied and 65 MIC values determined. The calculated values exactly coincided in 27 cases, differed by a factor of 2 in 16 cases and by a factor of 4 or more in 21 cases. In one case (carbenicillin and the TEM-producing strain), the MIC was too high to be determined. It should be noted that in five cases, the lowest observed MIC values (MIC_{min}) were *lower* than C_{inh}, which can only be explained if survival of the bacterium requires significantly more than 50% of the most sensitive PBP. Figure 3 shows the results obtained by applying our method to five of the 13 antibiotics studied by Nikaido and Normark, using only the relative quantities of β-lactamase (R_i), the observed MICs and the K_m for each substrate. The curves for the RTEM β-lactamase producer were drawn using the K_m values for the AmpC β-lactamase. This deliberate mistake was made in order to illustrate the consequences of comparing strains producing different enzymes. Two features are immediately noticeable:

(1) The behaviour of strain JF701 separates it from the other ones: in two cases, cephaloridin and cefazolin, the curves for that strain are close to those for the other good β-lactamase producers (LA51 and TE18). In a third case (ampicillin), the Z values are significantly lower which is surprising for an excellent β-lactamase producer ($R = 5000$). With cefoxitin and cephalothin, the Z values for JF701 are, respectively, 50 and 30–150-fold larger than for TE18. This indicates that strain JF701 probably produced a β-lactamase different from that of the other strains.

(2) The curves corresponding to the two other good producers (LA51, $R = 295$ and TE18, $R = 1177$) never differ by a factor larger than 2. They are nearly superimposable in the cases of cephalothin and ampicillin.

Table 4 shows the values of I_{pl} and $\bar{Z}$ estimated from graphs similar to those displayed on Fig. 3 for the 13 antibiotics.

With those values, and using Eqn (6), the MIC values were calculated and compared to the observed values (Table 5). The agreement is generally quite good. The few values which differ from the observed MIC by a factor of 2 or more are marked by a dagger (†). Table 4 also displays the C_{inh} values utilized by Nikaido and Normark. Our I_{pl} values are generally in good agreement with those C_{inh} values. In the cases where C_{inh} was lower than the smallest MIC, our I_{pl} values are, as expected, also lower than C_{inh}.

The values of permeability coefficients can be obtained from the $\bar{Z}$ values. Indeed, one can easily show that $P = (\bar{Z} \times V_{max})/1.32$ where P is expressed in dm per sec, $\bar{Z}$ in per μM, V_{max} in μmoles of substrate hydrolyzed per sec per mg dry weight and 1.32 is A expressed in dm^2 per mg dry weight.

Table 4 compares the values of P so calculated to those given by Nikaido and Normark. Out of the 13 compounds tested, the values of P agree very well in five cases (factor 2 or less), fairly in six cases (factors 4–10) and not at all in two cases (cefsulodin and carbenicillin). It is, however, worth noting that those are precisely the compounds for which the disagreements between Nikaido and Normark's measured and predicted MIC values were the largest. For the latter moreover, the authors acknowledge that "the true permeability of this compound might be much lower".

As underlined above, the behaviour of mutant JF701 indicates that it might produce another enzyme. In consequence, if one tries to calculate the MIC values using the $\bar{Z}$ and K_m values for the AmpC enzyme, nearly all the results are in very poor agreement with the experimental results (Table 5), particularly for cefaclor, cefotaxime and ceftazidime.

Since the enzymes are different, a calculation of the MIC requires the knowledge of the kinetic parameters of both enzymes for all the tested compounds.

Equation (7) can be easily derived from Eqn (6). For one antibiotic:

$$MIC_2 = I_{pl} + (MIC_1 - I_{pl}) \frac{k_{cat2}E_{o2}}{k_{cat1}E_{o1}} \frac{(K_{m_1} + I_{pl})}{(K_{m_2} + I_{pl})}. \quad (7)$$

In our case, enzymes 1 and 2 are, respectively, the AmpC and the TEM β-lactamases. We have chosen to compare strain JF701 to the best AmpC producer, TE18. Since cephaloridin is the reference substrate, the ratio for that substrate $(k_{cat2}E_{o2})/(k_{cat1}E_{o1}) = 5000/1277 = 3.87$. For the other substrates, one must first compute E_{o2}/E_{o1} by multiplying 3.87 by the cephaloridin k_{cat1}/k_{cat2} ratio, i.e. 152/740, which yields 0.795. For any substrate, $(k_{cat}E_o)_2/(k_{cat}E_o)_1$ is thus obtained by multiplying 0.795 by the corresponding k_{cat1}/k_{cat2} ratio. The rest of the calculation is straightforward.

By using Eqn (7) and the kinetic parameters measured by Nikaido and Normark, we have obtained the calculated MIC values shown in the last column of Table 4. They agree generally well with

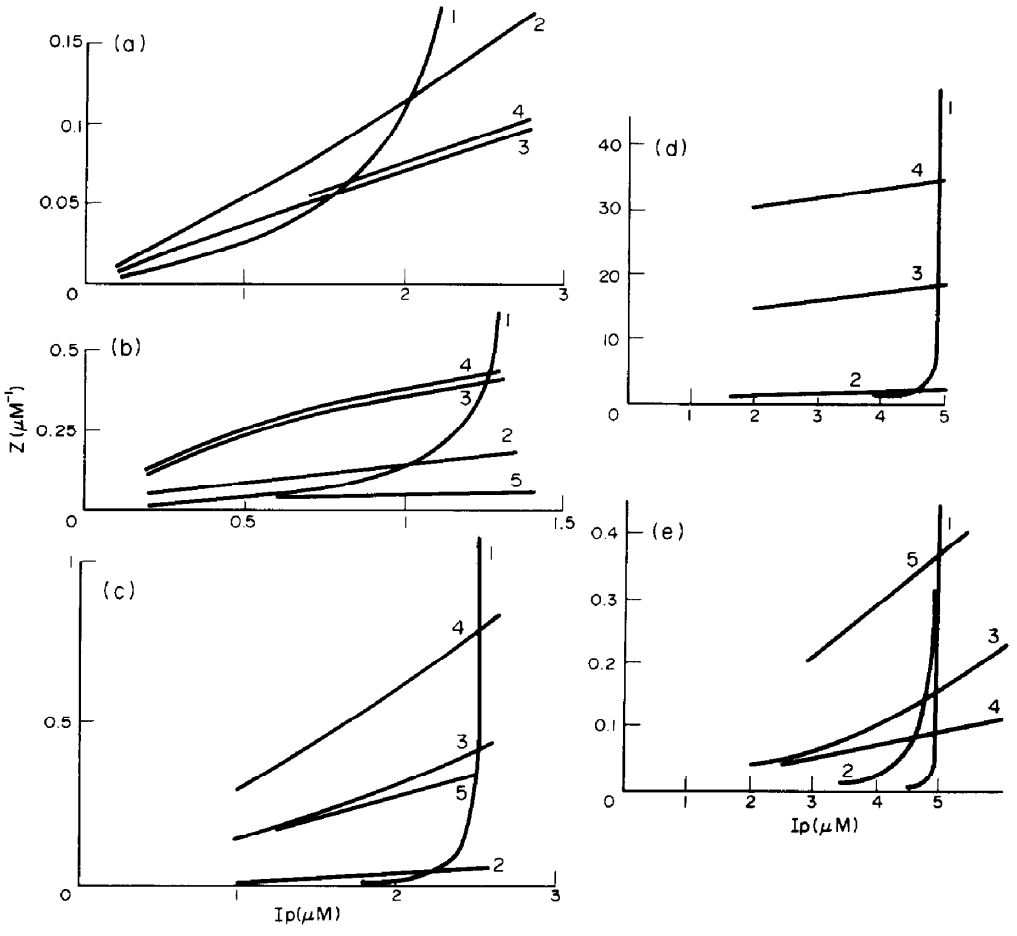


Fig. 3. Analysis of the data of Nikaido and Normark [4]. The strain numbering is as follows—(1): SNO3 (R = 1); (2): LA5 (R = 12.3); (3): LA51 (R = 295); (4): TE18 (R = 1277); (5): JF701 (R = 5000). Strains 1–4 produce the AmpC β-lactamase, strain 5 the TEM enzyme. The MIC and K_m values were from the same article. In the cases of cephalothin (a) and cefoxitine (d), the curves corresponding to strain 5 are completely out of scale (Z values 30–150-fold larger than with strain 4). a = cephalothin, b = ampicillin, c = cephaloridine, d = cefoxitin, e = cefazolin.

Table 4. Values of I_{pl} and $\bar{Z}$ deduced from the data of Nikaido and Normark

	C_{inh} (μM)	I_{pl} (μM)	$\bar{Z}$(/μM)	P_{calc} (cm/sec × 10^5)	P_{NN} (cm/sec × 10^5)
Cephaloridin	6	1.2–**2.4**	0.4–**0.8**	8	35.7
Cefazolin	11	2.5–**4.7**	0.05–**0.1**	6	6.2
Cephalothin	2.75	1–2	0.05–0.11	0.9–1.8	1.1
Cefamandole	0.63	0.3–0.6	0.9–**1.8**	0.7	1.1
Cefaclor	5	1.2–**2.4**	0.7–**1.4**	10	26.3
Cefsulodin	9	25–**35**	3–**5**	0.1	3.0
Cefoxitin	16	2.5–**5**	31–**35**	0.34	3.7
Cefotaxime	0.125	0.08–0.12	170–250	0.65–1.0	1.8
Ceftazidime	0.15	0.08–**0.15**	2.6–**5.7**	0.13	0.96
Penicillin G	2.28	1.6–3.2	0.19–0.23	0.8–1.0	0.2
Ampicillin	1.9	0.7–**1.25**	3–**4**	1.7	9.8
Carbenicillin	5.5	10–**20**	4–**4.5**	6×10^{-4}	0.075
Aztreonam	0.15	0.08–**0.14**	950–**1100**	0.043	0.33

The values of I_{pl} are compared to those of C_{inh}. From the values of $\bar{Z}$, a value of the permeability factor was calculated (P_{calc}) which is compared to that measured by Nikaido and Normark (P_{NN}). The bold values are those which give the best agreement between the observed and calculated MICs.

Table 5. Comparison between observed (O) and calculated (C) MIC values

	Strain															
	SNO3 $R = 1$			LA5 $R = 12.3$			LA51 $R = 295$			TE18 $R = 1277$			JF701 $R = 5000$			
Antibiotic	O	C_N	C_F	O	C_N	C_F	O	C_N	C_F	O	C_N	C_F	O	C_N	C_{F1}	C_{F2}
Cephaloridin*	2.5	5	1.2–**2.4**	5	5	1.4–2.6	10	10	**4–6.2**	20	20	18–19	160	40†	66†	47
Cefazolin*	5	10	2.53–**4.72**	5	10	2.82–**5**	10	40†	**13–13.3**	40	80	36.1–36.3	40	40	128†	28
Cephalothin	2.75	2.75	2–2.9	11	22	12–13	352	352	300–270	1406	1406	1280–1160	80	80	3800†	115
Cefamandole	0.65	0.65	0.32†	1.3	1.3	0.5–**0.8**	5.2	5.2	5.1–5.4	41.6	41.6	20.4–21.2	80	40	80	109 (58)
Cefaclor*	2.5	5	1.3–**2.5**	5	10	2.8–3.9	40	40	40–38	160	160	168–156	10	10	615†	14
Cefsulodin	40	10†	25–**35**	80	10†	26–35†	80	10†	38–45	80	10†	80	320	20†	205	977†
Cefoxitin*	5	10	2.5–**5**	10	10	2.9–**5.3**	20	10	11–13	40	10†	40	5	5	142†	5
Cefotaxime	0.15	0.15	0.08–0.12	0.15	0.15	0.08–0.14	0.63	0.63	0.66–0.62	2.5	2.5	2.6–2.3	0.15	0.15	10†	0.14
Ceftazidime	0.15	0.15	0.08–0.15	0.30	0.15	0.1–**0.17**	0.63	0.15†	0.63	2.52	0.63†	2.5–2.2	0.15	0.15	8.3†	0.15
Penicillin G	5.7	11.4	5.4–5.8	91	91	**48**–36	727	2900†	793–1120	2900	11,200†	4900–3400	10,000	10,000	13,600	8000
Ampicillin	1.4	1.4	0.8–1.4	5.6	2.8	2.5–3	45	11†	44	180	45†	187	5000	160†	726†	1200†
Carbenicillin	21	5†	10–**20**	42	5†	13–**27**	85	10†	84	340	42†	310	>5000	40,000?	1131†	10×10^6?
Aztreonam(1)	0.15	0.15	0.08–**0.14**	0.30	0.15	0.09–**0.15**	0.63	0.15†	0.36–0.39	1.26	0.3†	1.24	0.15	0.15	4.4†	0.25

The table compares the observed MIC values (O), those calculated by Nikaido and Normark (C_N) and by us (C_F). In the case of strain JF701, which is the only producer of TEM β-lactamase in the study, the calculations were performed with the kinetic parameters of the AmpC enzyme (C_{F1}) and with those of the TEM enzyme (C_{F2}). It can be seen that the agreement is much better in the second case.

* Compounds for which the MIC values are below the C_{inh} for strain SW03.

† Calculated values which differ from the observed MIC values by a factor larger than 2.

The bold values are those which give the best agreement between the observed and calculated MIC values.

the observed MICs, although less well than in the case of the four other strains. This is not surprising, since the number of measured factors introduced in the calculation is significantly larger. The extremely high value obtained with carbenicillin is due to the high k_{cat2}/k_{cat1} ratio (75,000) but since the MIC has not been measured a rigorous comparison remains impossible.

ANALYSIS OF THE DATA OF MARCHOU *et al.* [12]

Starting with clinical isolates of *Enterobacter cloacae* strains, susceptible to ceftriaxone (S clones), these authors have obtained resistant mutants, either by selection on agar containing a gradient of ceftriaxone (R1 clones) or by exposition to ceftriaxone therapy in a mouse model (R2 clones). All R1 and R2 mutants produced elevated amounts of β-lactamase. We have applied our method to the three mutants obtained for strains 218, 908 and 895. The β-lactams used in our calculations were the following: ceftriaxone and Sch34343 (K_m values: 0.14 and 0.3 μM, given by Marchou *et al.*), ceftazidime (K_m value: 2 μM, taken as the average of the two values given by Bush *et al.* [13] for two *E. cloacae* β-lactamases) and carbenicillin, moxalactam, azthreonam (K_m values: 0.01 μM, 0.07 μM and 0.001 μM, obtained by Galleni *et al.* [14, 15] with the *E. cloacae* P99 enzyme). Figure 4 represents the results obtained with strain 218S and the two corresponding resistant mutants. The results are consistent with the hypothesis that the permeability of mutant 218R2 is decreased by a factor of about 5. Indeed, if the intersection between curves S and R1 is assumed to yield the correct I_{pl} value, one can see that at the same I_{pl} value, the value of $\bar{Z}$ for mutant 218R2 is decreased by a factor 5.5 with ceftriaxone, 5.6 with ceftazidime, 4 with carbenicillin, 5.4 with moxalactam and 3.4 with azthroeonam. This excellent agreement clearly reinforces the conclusions of Marchou *et al.* The situation with strains 908 and 895 is far from being so clear. If one wishes to obtain I_{pl} values similar to those calculated for strain 218, one must assume that mutant R1 is the one whose permeability is modified (Table 6). However, the modification is not similar for all antibiotics. Table 6 also shows that the $\bar{Z}$ values of mutant R1 are not significantly decreased in all cases but only with moxalactam and carbenicillin (for both 908 and 895) and with azthreonam (for 908). Accordingly, the calculated MICs are only too low in the same cases. It would be tempting to hypothesize that, in the cases of mutants 908R1 and 895R1, the modified parameter is the sensitivity of the PBP to moxalactam, carbenicillin and azthreonam for the former and to moxalactam and carbenicillin for the latter. The sensitivity to the other antibiotics might also be changed, but by a factor which would not significantly exceed 2. However, it remains possible that some modifications of the porin channel might result in selective modifications of the permeability factor. To our knowledge, that type of effect has never been described. A better understanding of the behaviour of strains 908 and 895 would require the isolation of other mutants.

With Sch34343, the MIC values were not different for the "sensitive" strains and for the resistant mutants. This can only be explained by assuming a very high value of $\bar{Z}$ in all cases. For example, calculated MIC values ranging between 2 and 3 μM were obtained assuming $I_{pl} = 2.0$ μM and $\bar{Z} = 1250$–$2500/\mu M$. Assuming a periplasmic enzyme concentration of 0.5–1.0 μM for S strains, this would correspond to k_D values about 50-fold higher than those calculated for ceftazidime and ceftriaxone and 20-fold higher than that for moxalactam. The behavior of imipenem is even more surprising: in fact, with strains 218 and 895, the MICs are lower with mutants R2 than with the "sensitive" S strains and mutants R1, a result which remains impossible to explain.

It is extremely interesting to note that the I_{pl} values calculated here for ceftriaxone, ceftazidime, moxalactam and aztreonam are very similar to the antibiotic concentrations (I_{50}) required to reduce [^{14}C]benzylpenicillin binding by 50% to PBP3 for the same β-lactams [16]. Moreover, the I_{pl} value for carbenicillin is very similar to the I_{50} value of ticarcillin, a closely related compound.

Our calculations were made by assuming that induction of the β-lactamase did not occur during the measurement of the MIC. This assumption is probably justified when the steady-state model is used, since the periplasmic concentrations rapidly reach constant values which should be sufficient to kill the cells within one generation, while the induction phenomenon probably takes at least one generation before reaching a maximum. When the calculations were repeated by assuming that maximal induction took place immediately, the results became incoherent for strain 218 (curves R1 and R2 were consistently below curve S, implying three different permeabilities) and no improvement was recorded for strains 908 and 895. It thus seemed reasonable to assume that little induction of β-lactamase production occurred during the determination of the MICs.

CONCLUSIONS

The method which is described here is simple and practical to use since the experiments that are required are easy to perform. It also supplies an estimate of the periplasmic concentration of antibiotic which is necessary to inhibit the growth of a low density population of bacteria, an important factor which seems very difficult to measure directly. An evaluation of the relative penetration rates of a series of β-lactams can also be easily obtained.

As shown by our analysis of the data of Nikaido and Normark, the method works best when more than three strains or mutants are compared. If only two strains are compared, a variation of k_D or I_{pl} can be inferred if the curve corresponding to the better β-lactam producer consistently lies below that of the poorer producer, but one cannot decide which factor is affected. Conversely, if the two curves converge, it seems dangerous to draw firm conclusions.

The analyses presented here rely on the utilisation of a steady-state model. This implies that the value of I_{pl} depends only upon the interplay between hydrolysis and penetration. In other words, "trap-

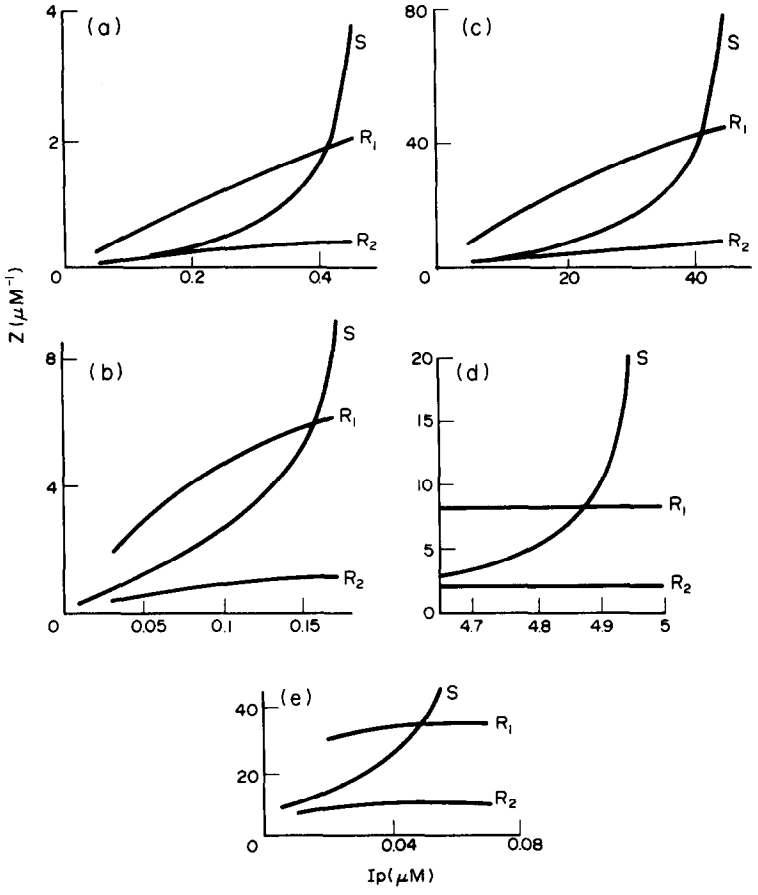


Fig. 4. Analysis of the data of Marchou *et al.* [12] for strain 218. (a) ceftazidime, (b) ceftriazone, (c) moxalactam, (d) carbenicillin, (e) aztreonam. Mutant S: $R = 1$, mutant R1: $R = 566$, mutant R2: $R = 522$.

Table 6. Analysis of the results of Marchou *et al.*

	Ceftriaxone		Ceftazidime		Moxalactam		Carbenicillin		Aztreonam	
	I_{pl}*	$\bar{Z}$*	I_{pl}	$\bar{Z}$	I_{pl}	$\bar{Z}$	I_{pl}	$\bar{Z}$	I_{pl}	$\bar{Z}$
218 S/R1	0.1–**0.16**	**4–6**	0.2–**0.4**	**1–2**	0.025–0.04	**30–43**	2.5–**5**	8	0.025–**0.05**	**35**
218 R2		0.8–1.1§		0.2–0.4§		5–7§		2§		9§
908 S/R2	0.05	1.2	0.15–**0.25**	0.25–**0.45**	0.04–**0.075**	28–**40**	3–**6**	4	0.015–**0.03**	13–**18**
908 R1		0.6		0.15–0.25		1.8–2.2§		0.6§		3–4§
895 S/R2	0.1	1.6	0.1–**0.2**	0.15–**0.3**	0.03–**0.06**	10–**15**	4–**8**	3	0.015–**0.03**	9–12
895 R1		1.0		0.1–0.2		1–2§		0.7§		7–10
I_{50} for PBP3 of 908S‡	0.025		0.15		0.125		4.8 (ticarcillin)		0.075	
$(k_D)/(E_o)_S = Z \times k_{cat}$†	0.17 ($\bar{Z} = 6$)		0.2 ($\bar{Z} = 2$)		0.39 ($\bar{Z} = 43$)		0.02 ($\bar{Z} = 8$)		0.01 ($\bar{Z} = 35$)	
Relative permeability	0.85		1		2		0.1		0.05	

*I_{pl} (μM)/(/μM).

† (/μM/sec). The values of k_{cat} were: ceftriaxone 0.029/sec; ceftazidime 0.1/sec [13]; carbenicillin 2.5×10^{-3}/sec [14]; moxalactam 0.009/sec and aztreonam 3×10^{-4}/sec [15].

‡ Then and Angehrn [16].

§ Values of $\bar{Z}$ which are significantly different from those obtained with the reference pairs of clones (S/R1 for strain 218, S/R2 for the two other strains).

The bold values are those which give the best agreement between the observed and calculated MICs.

ping" phenomena cannot affect the behaviour of systems obeying the steady-state model. In the accompanying paper, we analyse a non-steady-state model where such a phenomenon can be involved. Finally, it should be noted that the behaviour of aztreonam, for which the β-lactamases exhibit k_{cat} values lower than 10^{-3}/sec should be more rigorously analysed on the basis on that non-steady-state model.

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