

CEFOXITIN, *N*-FORMIMIDOYL THIENAMYCIN,
CLAVULANIC ACID, AND PENICILLANIC ACID SULFONE
AS SUICIDE INHIBITORS FOR DIFFERENT TYPES OF β -LACTAMASES
PRODUCED BY GRAM-NEGATIVE BACTERIA

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Cefoxitin, *N*-formimidoyl thienamycin (MK0787), clavulanic acid, and penicillanic acid sulfone (CP45,899) were studied to determine their potency as suicide inhibitors of three very different kinds of β -lactamases produced by Gram-negative bacteria: type Ib penicillinase (TEM-2 type), a typical cephalosporinase (the enzyme of *Proteus morgani*), and a cephalosporinase with broad substrate specificity (the enzyme of *Proteus vulgaris*). All these β -lactams were confirmed to be quite stable to the three β -lactamases. The absolute values of the turnover numbers (k_{cat}) for these enzyme-catalyzed hydrolyses were determined, the values ranged from 0.25 minute⁻¹ to 660 minute⁻¹. All the β -lactams studied, except cefoxitin, acted as suicide inhibitors of the typical cephalosporinase. Although cefoxitin did not exhibit such an effect, it was a powerful competitive inhibitor of this enzyme. Although the four β -lactams acted as suicide inhibitors of the *P. vulgaris* cephalosporinase, the inactivated enzyme partially regained its activity after removing the effect of the free inhibitor. Type Ib penicillinase was also inactivated by the four β -lactams, and the activity of the inactivated enzyme, except in the case of cefoxitin, was partially restored. The rate constants for enzyme inactivation or reactivation were calculated and are presented. The information obtained from this study suggests that the catalytic center of the *P. vulgaris* cephalosporinase is different not only from that of the penicillinase-type but also from that of the cephalosporinase-type β -lactamases.

It is well known that β -lactamase production in many pathogenic bacteria is closely associated with their resistance to β -lactam antibiotics. This association has created much interest in the development of β -lactamase-stable β -lactams or inhibitors of these enzymes as antibiotic-protecting agents. In the initial studies in this field, methicillin, oxacillin and its derivatives were discovered. These semisynthetic penicillins are very stable to many kinds of β -lactamases, but they show poor efficacy against Gram-negative bacteria because they are unable to penetrate the outer membrane of the bacterial cells^{1,2}).

Recently, new types of β -lactams with novel ring structures such as clavulanic acid³), cephamycins⁴), penicillanic acid sulfone⁵), carbapenems⁶), and 6- β -bromopenicillanic acid⁷) have been discovered. These β -lactams are stable to many types of β -lactamases, and it has been reported that some of them act as suicide inhibitors for the enzymes⁸⁻¹⁴). The kinetic investigations on these suicide inhibitors have mainly been performed using one group of β -lactamases, namely, the TEM-type penicillinases. We were much interested in studying the kinetic behavior of many different types of β -lactamases toward these β -lactams since we expected that such an investigation would offer useful information about the characteristics of the catalytic site of the individual types of β -lactamases. We report herein some aspects of the mode of interaction of cefoxitin, *N*-formimidoyl thienamycin (MK0787), clavulanic acid, and penicillanic acid sulfone (CP45,899) with three representative β -lactamases of Gram-negative bacteria: type Ib penicillinase (TEM-2 penicillinase), a typical cephalosporinase (the enzyme of *Proteus morgani*), and a cephalosporinase with broad substrate specificity (the enzyme of *Proteus vulgaris*).

Materials and Methods

Bacterial Strains

The bacterial strains employed in this study were selected from those reported previously¹⁵⁾, which had been prepared in order to evaluate the β -lactamase-stability of β -lactam antibiotics. *Escherichia coli* ML1410 RGN14 and *E. coli* ML1410 RGN823 harbor R plasmids, RGN14 and RGN823, respectively. These plasmids mediate the constitutive production of type Ia and Ib penicillinases¹⁶⁾, respectively. Type Ia and type Ib penicillinases correspond to TEM-1 and TEM-2 penicillinases of MATTHEW's classification¹⁷⁾, respectively. *E. coli* ML1410 is an *E. coli* K-12 substrain and is sensitive to β -lactam antibiotics.

P. vulgaris GN76/C-1 is a constitutive mutant with respect to its cephalosporinase production and was derived from the wild type strain GN76. The other two *P. vulgaris* strains, GN76/C-1/2 and GN76/C-1/3, were derived from strain GN76/C-1, and produce lower cephalosporinase activity than their parental strain.

P. morganii 1510 is a constitutive producer of a cephalosporinase; it was originally isolated from a clinical source by FUJII-KURIYAMA *et al.*¹⁸⁾. Strains 1510/3 and 1510/9, mutant strains with lower enzyme activity, were isolated by us from strain 1510.

β -Lactamases

The three kinds of β -lactamases, namely, type Ib penicillinase and two cephalosporinases produced by *P. vulgaris* GN76/C-1 and *P. morganii* 1510, were used in this study. These two cephalosporinases are assumed to be mediated by chromosomal gene(s), and thought to be species-specific β -lactamases of the respective species.

Type Ib penicillinase was purified by a procedure reported previously¹⁶⁾. The *P. morganii* cephalosporinase was purified according to the procedure of FUJII-KURIYAMA *et al.*¹⁸⁾. The purification of the *P. vulgaris* cephalosporinase was performed by adsorption and elution on CM-Sephadex C-50(Pharmacia Fine Chemicals) column and gel filtration on a Sephadex G-75(Pharmacia Fine Chemicals) column. The purity of these β -lactamase preparations was confirmed by polyacrylamide disc gel electrophoresis^{19, 20)}. Detailed information as to the properties of these three kinds of β -lactamases has been presented in a previous paper²¹⁾.

β -Lactams

The following β -lactams of established purities were kindly provided by the following pharmaceutical companies; benzylpenicillin and ampicillin, Meiji Seika Co., Tokyo, Japan; cephalothin and cephaloridine, Torii Pharmaceutical Co., Tokyo, Japan; cefoxitin and *N*-formimidoyl thienamycin (MK0787), Merck, Sharp & Dohme Research Laboratories, Rahway, N. J., U.S.A.; clavulanic acid, Beecham Pharmaceuticals, Surrey, U.K. Penicillanic acid sulfone (CP45,899) was synthesized in our laboratory from 6-aminopenicillanic acid.

Assay for β -Lactam Hydrolysis

For routine assay, β -lactamase activity was determined iodometrically at 30°C in 0.1 M phosphate buffer (pH 7.0) by a colorimetric assay²²⁾. The kinetic measurement of the β -lactamase reaction was performed by a spectrophotometric method²³⁾ by measuring the change in ultraviolet absorption of the β -lactam ring at 30°C in 0.05 M phosphate buffer (pH 7.0) with the aid of a Beckman model 24 spectrophotometer. The wavelengths used for the assay were 232 nm (benzylpenicillin), 265 nm (cephalothin), 270 nm (cefoxitin), 300 nm (MK0787), 230 nm (CP45,899), and 220 nm (clavulanic acid). These appropriate wavelengths were determined by complete hydrolysis of each β -lactam with a large excess of type Ib penicillinase or the *P. morganii* cephalosporinase.

Estimation of K_i Value

The K_i values of β -lactams for the β -lactamases were determined with benzylpenicillin (type Ib penicillinase) or cephalothin (the cephalosporinases) as substrate, and the values were estimated graphically from LINEWEAVER-BURK plots.

Assay of Enzyme Inactivation by Pre-incubation with Cefoxitin, MK0787, Clavulanic Acid, and CP45,899

The enzyme preparation in 500 μ l of 0.05 M phosphate buffer (pH 7.0) was pre-warmed at 30°C, and then mixed with 25 μ l of the β -lactam dissolved in the same buffer. The final enzyme concentration in the mixture was 1 μ M. The final β -lactam concentration was 1 μ M to 10 mM, and varied according to the sensitivity of the enzyme with respect to the β -lactam tested. The mixture was incubated at 30°C. At appropriate time intervals, a portion (50 μ l) was taken from the reaction mixture and diluted 600- to 1,000-fold with the phosphate buffer. It was confirmed that this dilution precluded any further effect of the β -lactam on the enzyme activity. The enzyme activity remaining in the diluted reaction mixture was measured by the spectrophotometric method with 100 μ M benzylpenicillin (in the case of penicillinase) or 100 μ M cephalothin (in the case of cephalosporinases) as substrate.

Antibiotic Sensitivity Testing

Antibacterial activity of β -lactams was determined by a serial dilution method and expressed as the minimal inhibitory concentration (MIC, μ g/ml). A detailed procedure has been reported.¹⁵⁾

Results and Discussion

Sensitivity of the β -Lactamase-producing Bacteria to β -Lactams

We measured the sensitivity of three groups of bacterial strains to the four β -lactams and to ampicillin and cephaloridine chosen as representatives of common β -lactam antibiotics. The three groups of bacteria produce different kinds of β -lactamases: type I penicillinase (the *E. coli* strains), a cephalosporinase with broad substrate specificity (the *P. vulgaris* strains) and a typical cephalosporinase (the *P.morganii* strains). All the bacterial strains in a given group produce the same or a similar kind of β -lactamase constitutively but the intracellular enzyme activity differs from strain to strain. The results shown in Table 1 confirm that the antibacterial activity of the new β -lactams is little affected by the bacterial production of β -lactamases. The antibacterial activity of cefoxitin, however, was slightly reduced by the typical cephalosporinase in *P.morganii*. In addition, *P. vulgaris* mutant strain, GN76/C-1/3, showed lower sensitivity to MK0787; the reason for this has not yet been determined.

Table 1. Antibacterial activity of β -lactam antibiotics and β -lactam analogues against β -lactamase-producing Gram-negative bacteria.

Strain ^b	β -Lactamase activity (units/mg dry weight of bacteria)	β -Lactam (MIC μ g/ml)					
		APC ^a	CER	CFX	MK	CVA	CP
<i>E. coli</i> ML1410 RGN823	16.7	>3200	25	3.1	0.2	25	200
<i>E. coli</i> ML1410 RGN14	0.6	800	6.3	3.1	0.2	25	100
<i>E. coli</i> ML1410	<0.003	3.1	3.1	3.1	0.2	25	100
<i>P. vulgaris</i> GN76/C-1	2.4	400	800	3.1	0.8	25	100
<i>P. vulgaris</i> GN76/C-1/3	0.03	25	200	3.1	3.1	25	100
<i>P. vulgaris</i> GN76/C-1/2	<0.01	1.6	3.1	3.1	0.4	25	100
<i>P.morganii</i> 1510	2.68	400	400	12.5	1.6	50	200
<i>P.morganii</i> 1510/3	0.07	50	100	6.3	0.8	50	200
<i>P.morganii</i> 1510/9	0.006	1.6	6.3	3.1	1.6	50	100

^a Abbreviations: APC, ampicillin; CER, cephaloridine; CFX, cefoxitin; MK, *N*-formimidoyl thienamycin (MK0787); CVA, clavulanic acid; CP, penicillanic acid sulfone (CP45,899).

^b See Materials and Methods for a description of the nature of the enzymes produced by the strains.

Hydrolysis of the β -Lactams by the β -Lactamases and their Affinity for the Enzymes

The four β -lactams, cefoxitin, MK0787, clavulanic acid, and CP45,899 are well known to be quite stable to many kinds of β -lactamases^{3,4,6,24}). Consequently, it was very difficult to determine the absolute values of the turnover number (k_{cat}) for the β -lactamase-catalyzed hydrolysis of these β -lactams. We could determine the k_{cat} values by incubating the β -lactams with a high concentration (1 μM) of the pure enzyme preparation. From the initial velocity of the enzyme reaction, the k_{cat} values were calculated and are listed in Table 2. Type Ib penicillinase and the *P. vulgaris* cephalosporinase showed relatively high hydrolytic activity against CP45,899 and clavulanic acid. However, these values were very low when compared with the k_{cat} values of good substrates for these enzymes. For example, the k_{cat} value for the hydrolysis of benzylpenicillin by type Ib penicillinase was reported to be 4.1×10^4 minute⁻¹²¹). Similarly, the k_{cat} values of cephaloridine hydrolysis by the *P. vulgaris* cephalosporinase and the *P. morganii* cephalosporinase were reported to be 2.0×10^4 minute⁻¹ and 3.3×10^8 minute⁻¹, respectively²¹).

In order to estimate the affinity between the β -lactams and the enzymes, the dissociation constant, K_i , of the enzyme- β -lactam complex was determined. The enzyme was used as a much more dilute solution for assaying β -lactamase activity in the presence of a good substrate. Therefore, the change in ultraviolet absorption due to hydrolysis of the inhibitors was negligible. The K_i values were also calculated from the initial velocities determined after a short reaction time (2 minutes) in order to avoid the suicide effect of the β -lactam on the enzyme activity. The K_i values obtained are listed in Table 2. An interesting tendency was found not only with respect to the hydrolytic activity of the three β -lactamases toward the β -lactams, but also with respect to their affinity for the β -lactams. Cefoxitin showed a very high K_i value for type Ib penicillinase, indicating its very low affinity for the enzyme. However, the affinity of cefoxitin increased greatly with the increasing cephalosporinase-type character of the enzyme. In contrast, clavulanic acid showed high affinity for the penicillinase but exhibited low affinity for the typical cephalosporinase, *i.e.*, the *P. morganii* cephalosporinase. A similar tendency was found in the case of CP45,899. The carbapenem β -lactam, MK0787, showed high affinity for all three kinds of β -lactamases tested.

It is also an interesting fact that the *P. vulgaris* cephalosporinase had a behaviour intermediate between the cephalosporinase-type and the penicillinase-type β -lactamases with respect to its affinity for the inhibitors as well as its substrate profile determined with common β -lactam antibiotics²¹).

Table 2. Kinetic constants of three β -lactamases using cefoxitin, MK0787, clavulanic acid, and CP45,899.

β -Lactam ^a	Type Ib penicillinase		Cephalosporinase of <i>P. vulgaris</i>		Cephalosporinase of <i>P. morganii</i>	
	k_{cat} ^b	K_i (μM)	k_{cat}	K_i (μM)	k_{cat}	K_i (μM)
Cefoxitin	0.25	556	0.50	4.2	0.94	0.28
MK0787	6.2	3.1	2.4	0.31	0.29	1.7
Clavulanic acid	660	0.5	42	0.35	3.4	1,100
CP45,899	350	0.5	180	2.1	0.63	8.9

^a The four β -lactams were used as substrates for determination of k_{cat} values and as inhibitors for determination of K_i values.

^b k_{cat} : molecules of β -lactam hydrolyzed per minute per molecule of β -lactamase at pH 7.0 and 30°C.

The Inhibition of the Three β -Lactamases by Cefoxitin, MK0787,
Clavulanic Acid and CP45,899

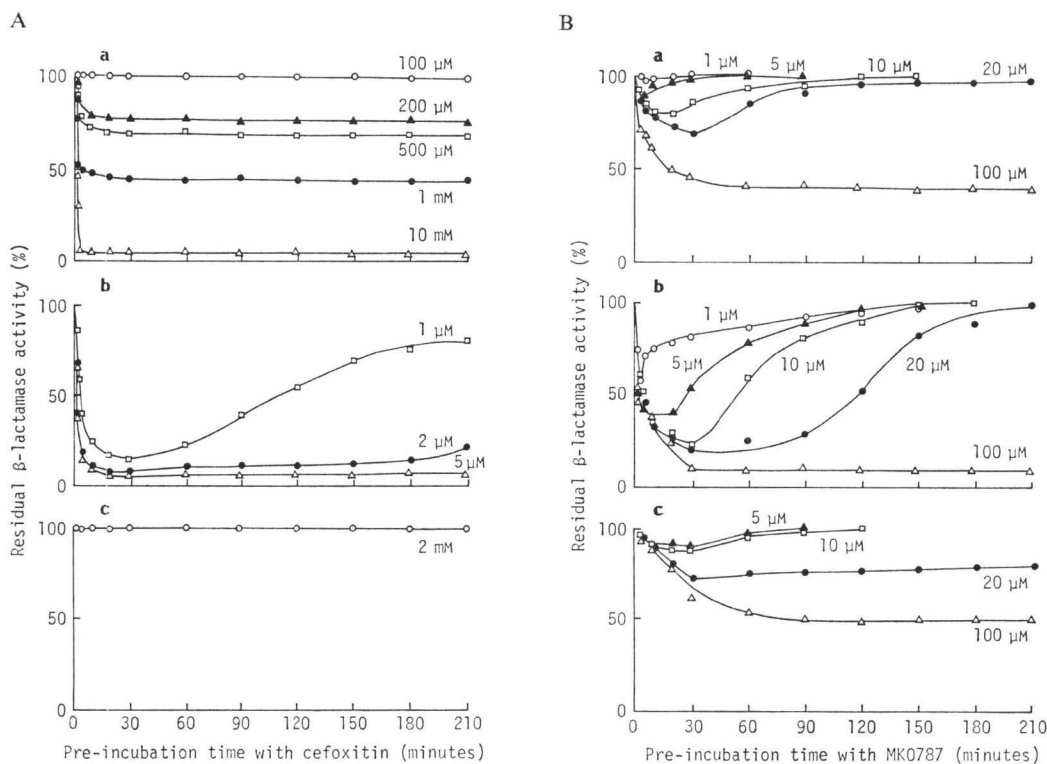
Clavulanic acid and CP45,899 have been reported to be irreversible inhibitors for some kinds of β -lactamases^{8,9}. These β -lactams are thought to act as 'suicide inhibitors or progressive inhibitors' of β -lactamases. The mechanisms of this reaction has been extensively studied for a limited number of β -lactamases, namely penicillinases⁸⁻¹⁴.

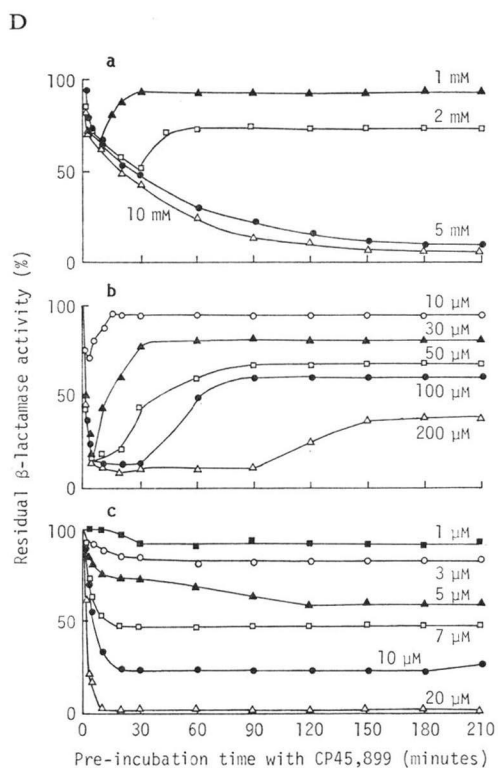
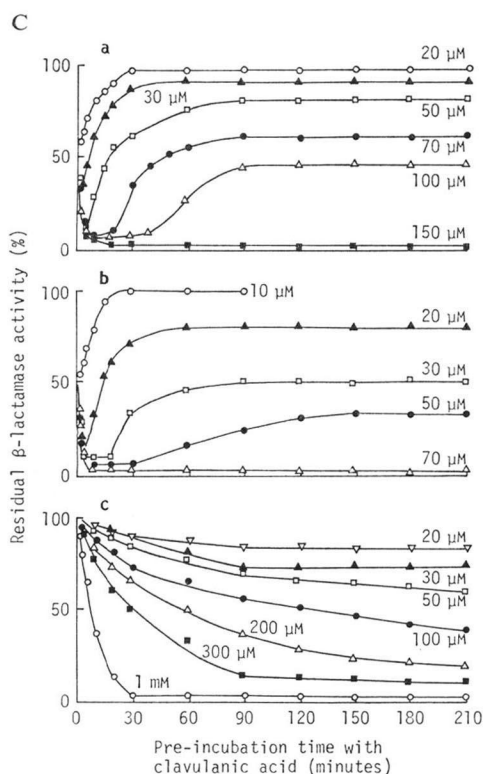
The irreversible inhibition of the three types of β -lactamases studied by the four β -lactams was measured by pre-incubating the enzyme with the β -lactam for different time periods according to the procedure described in Materials and Methods. The results are shown in Fig. 1.

Fig. 1A shows the effect of cefoxitin on the activity of the three kinds of β -lactamases. The activity of the penicillinase was completely reduced in the presence of 10 mM cefoxitin within a few minutes of preincubation. A stronger inhibitory effect was observed on the *P. vulgaris* cephalosporinase though recovery of enzyme activity was observed. Cefoxitin is a powerful competitive inhibitor of the *P. morganii* cephalosporinase². We did not observe an irreversible inhibitory effect on the cephalosporinase even with 2 mM cefoxitin. This finding is in agreement with the results shown in Table 1 and with our previous observations that the *E. coli* strains producing the R plasmid-mediated penicillinases and the

Fig. 1. Inactivation of the three kinds of β -lactamases with cefoxitin, MK0787, clavulanic acid, and CP-45,899.

The enzyme solution (1 μ M) was incubated at 30°C with the indicated concentrations of the β -lactam, and the remaining enzyme activity was measured after various incubation times by the procedure described in Materials and Methods. β -Lactams tested: Fig. 1A, cefoxitin; Fig. 1B, MK0787; Fig. 1C, clavulanic acid; Fig. 1D, CP45,899. β -Lactamases tested: (a), type Ib penicillinase; (b), the *P. vulgaris* cephalosporinase; (c), the *P. morganii* cephalosporinase.





P. vulgaris strains producing the cephalosporinase are highly sensitive to cefoxitin though the typical cephalosporinase-producers in *P. morgani*, *Citrobacter freundii*, and *Enterobacter cloacae* are highly resistant to cefoxitin¹⁵⁾. Such a phenomenon may be attributed to a difference in the mode of cefoxitin reaction with the enzymes, *i.e.*, suicide inhibition or competitive inhibition of the β -lactamases.

All the β -lactamases tested showed reduced activity when pre-incubated with MK0787, and it was found that the *P. vulgaris* cephalosporinase was most susceptible to the inhibitor (Fig. 1B). When treated with relatively low concentrations of MK0787, complete recovery of the activity was observed for all the β -lactamases tested. The pre-incubation experiment, however, indicated that MK0787 acted as a suicide inhibitor for all the β -lactamases tested. This finding is consistent with the recent observation by RICHMOND²⁵⁾.

More than 80% of the activities of type Ib penicillinase and the *P. vulgaris* cephalosporinase were inactivated with clavulanic acid concentrations higher than 50 μ M and 20 μ M, respectively, within a few minutes of pre-incubation (Fig. 1C). Clavulanic acid has been reported to be active against penicillinases, but not cephalosporinases²⁶⁾. Our observations confirmed that clavulanic acid is a potent inhibitor against the penicillinase-type enzyme, but we also showed that clavulanic acid is a powerful suicide inhibitor of the *P. vulgaris* cephalosporinase. The activity of the inactivated form of these two β -lactamases was partially restored at a relatively rapid rate. This fact is in striking contrast to that observed for the *P. morgani* cephalosporinase. This cephalosporinase was inactivated irreversibly by incubation with the inhibitor though a higher concentration and a prolonged incubation period were required for successful inhibition.

In contrast to clavulanic acid, CP45,899 was shown to be a powerful suicide inhibitor of the typical

cephalosporinase, the *P. morganii* cephalosporinase. Inactivation with CP45,899 was observed after pre-incubation for 30 minutes (Fig. 1D). The degree of apparent irreversible inactivation was proportional to the increase of CP45,899 concentrations. It is interesting to note that the potency of CP45,899 as a suicide inhibitor decreased as the penicillinase-type character of the enzyme increased.

Except in the case of the *P. morganii* cephalosporinase and cefoxitin, the activity of the β -lactamases tested decreased as the time of pre-incubation with the β -lactam increased. Since the plot of the % of residual activity (log scale) versus pre-incubation time is linear, the inactivation process was assumed to follow first order kinetics (Fig. 2). This time-dependent inactivation is good evidence of covalent-bond

Table 3. Kinetic parameters of inactivation and reactivation.

β -Lactam	Type Ib penicillinase		Cephalosporinase of <i>P. vulgaris</i>		Cephalosporinase of <i>P. morganii</i>	
	k_{inact} (minute ⁻¹) $t_{1/2}$ of inact. (minutes)	k_{react} (minute ⁻¹) $t_{1/2}$ of react. (minutes)	k_{inact} (minute ⁻¹) $t_{1/2}$ of inact. (minutes)	k_{react} (minute ⁻¹) $t_{1/2}$ of react. (minutes)	k_{inact} (minute ⁻¹) $t_{1/2}$ of inact. (minutes)	k_{react} (minute ⁻¹) $t_{1/2}$ of react. (minutes)
Cefoxitin	7.4×10^{-1} 0.94		3.5×10^{-1} 2.0	1.4×10^{-2} 50		
MK0787	1.9×10^{-2} 36	2.3×10^{-2} 30	6.5×10^{-2} 11	2.2×10^{-2} 32	1.7×10^{-2} 41	6.5×10^{-2} 11
Clavulanic acid	3.5×10^{-1} 2.0	8.4×10^{-2} 8.3	3.3×10^{-1} 2.1	1.4×10^{-1} 5.0	1.0×10^{-1} 6.9	
CP45,899	3.2×10^{-2} 22	1.1×10^{-1} 6.3	2.9×10^{-1} 2.4	5.5×10^{-2} 13	1.2×10^{-1} 5.8	

Fig. 2. Semilogarithmic plots of the rate of inactivation of the three β -lactamases in the presence of the four β -lactams.

Type Ib penicillinase (○), the *P. vulgaris* cephalosporinase (●), and the *P. morganii* cephalosporinase (△) were examined by the procedure described in Fig. 1 and the Materials and Methods section. Cefoxitin concentrations were: ○, 10 mM; ●, 5 μ M; △, 2 mM. MK0787 concentrations were: ○, 100 μ M; ●, 100 μ M; △, 100 μ M. CP45,899 concentrations were: ○, 10 mM; ●, 200 μ M; △, 10 μ M. Clavulanic acid concentrations were: ○, 150 μ M; ●, 70 μ M; △, 1 mM.

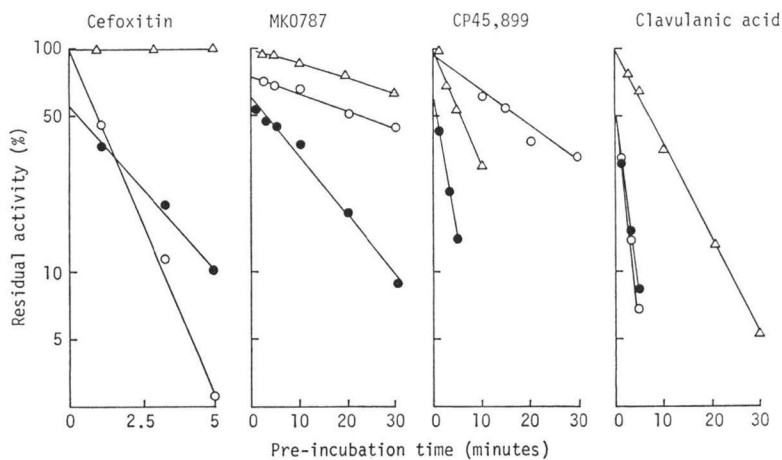
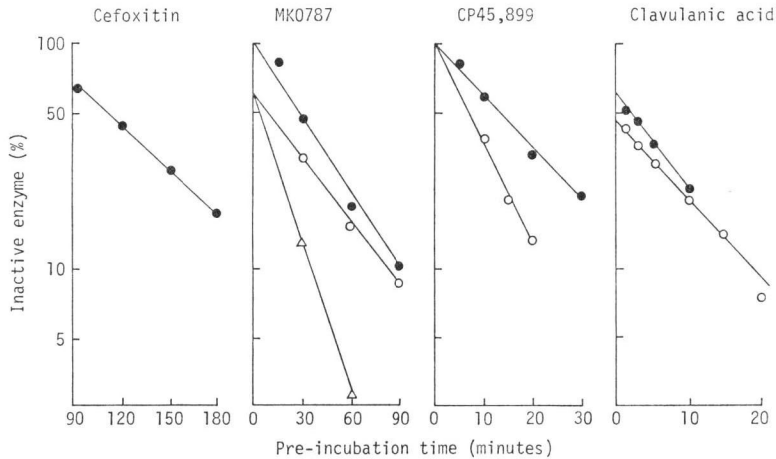


Fig. 3. Semilogarithmic plots of the reactivation rate of the inhibited β -lactamases.

The inactivated enzyme fraction of type Ib penicillinase (\circ), the *P. vulgaris* cephalosporinase (\bullet), or the *P. morgani* cephalosporinase (Δ) was plotted against the pre-incubation time. The inhibitor concentrations were as follows. Cefoxitin: \bullet , 1 μ M. MK0787: \circ , 20 μ M; \bullet , 5 μ M; Δ , 10 μ M; CP45,899: \circ , 1 mM; \bullet , 30 μ M. Clavulanic acid: \circ , 20 μ M; \bullet , 10 μ M.



formation between inhibitor and enzyme²⁷). The rate constants (k_{inact}) calculated from the experimental curves in Fig. 2, are shown in Table 3 together with the half-life ($t_{1/2}$) of inactivation. The k_{inact} value depends on the inhibitor concentration, therefore, a direct comparison of the values obtained at different inhibitor concentrations is unsuitable for an indication of inhibitory activity. However, the k_{inact} values in Table 3 may perhaps be used for an understanding of relative inhibitory activities of the β -lactams against the three β -lactamases.

As observed in Figs. 1A, 1B, 1C, and 1D, an apparent irreversible inhibition of the enzyme activity was demonstrated in the following three cases: cefoxitin inhibition of type Ib penicillinase, clavulanic acid and CP45,899 inhibition of the *P. morgani* cephalosporinase. For the other combinations, recovery of enzyme activity was observed after hydrolysis of the inhibitors in the medium. The regain of activity was found to depend on the β -lactam concentrations. Since the % of reactivated enzyme (log scale) versus pre-incubation time could be plotted on a straight line, this suggests that the reactivation process also followed first-order kinetics (Fig. 3). The calculated rate constants of reactivation, k_{react} , are shown in Table 3 together with the values of the half-life ($t_{1/2}$) of reactivation.

On the basis of the results of this comparative kinetic study involving three types of β -lactamases and four β -lactam inhibitors, we suggest that the catalytic center of the *P. vulgaris* cephalosporinase more closely resembles that of the penicillinase-type than that of the typical cephalosporinase-type β -lactamase.

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