INACTIVATION OF CEPHAMYCINS BY VARIOUS β-LACTAMASES FROM GRAM-NEGATIVE BACTERIA

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The enzymic inactivation of cephamycins, *i.e.* cefoxitin, cefmetazole, cefotetan and cefbuperazone, was investigated by means of bioassay, high pressure liquid chromatography (HPLC) and spectrophotometric analysis using three types of cephalosporinase (CSase, RICHMOND type Ia, Ib and Ic) and one penicillinase (PCase, TEM type). These four cephamycins were not inactivated by Ic CSase and TEM type PCase or producers of these enzymes. However, the inactivation of cefmetazole and cefoxitin was noted when they were incubated in the cultures of CSase (Ia and Ib)-producers or incubated with a large amount of these purified enzymes although the inactivation of cefbuperazone was not noted. HPLC of culture fluid or enzyme solution which contained cefmetazole or cefoxitin and were incubated at 37°C showed that metabolites of cefmetazole or cefoxitin appeared as the drug disappeared. In addition, the appearance of metabolites corresponded to the loss of the drug's bioactivities and the absorption of iodine. UV and IR spectra of cefmetazole which were taken after incubation with the purified CSase showed the cleavage of the β-lactam ring.

β-Lactamases (penicillin or cephalosporin β-lactam hydrolyzing enzymes) have been found in a number of bacteria and many of these enzymes have been purified from Gram-positive bacteria and Gram-negative species including R-factor specified β-lactamases.[1,2] Surveys on the substrate specificities and inhibition profiles of these enzymes have revealed that cephamycins are highly resistant to hydrolysis by various type of β-lactamases and have a potent inhibitory activity against some types of enzymes[3~7]. However, it has been reported that a cephalosporinase (CSase)-producing *Escherichia coli* was moderately resistant to cefoxitin despite high resistance to hydrolysis by *E. coli* CSase[8~10]. Furthermore, it has also been reported that bacteria in which there is impaired β-lactamase inducibility became resistant to β-lactamase-stable β-lactams following treatment with cefoxitin[11~13]. There has not yet been strong evidence supporting the view that hydrolysis could account for the resistance of β-lactamase-producing bacteria to β-lactamase stable β-lactams including cephamycins. Therefore, the alternative mechanism, that is, the trapping by β-lactamase has been proposed as the resistance mechanism in such cases[9,14].

This report deals with the enzymic inactivation of cephamycins including newly introduced agents using four types of β-lactamase and four different β-lactamase-producers.

Materials and Methods

Bacterial Strains and Media

E. coli GN5482[8], *Proteus vulgaris* T-178, *Enterobacter cloacae* H-27 and *E. coli* W3630Rms212$^+$

were used in this study. *E. cloacae* H-27 and *P. vulgaris* T-178 were clinical isolates and maintained in our laboratory. *E. coli* W3630Rms212$^+$ mediated the production of type I penicillinase (TEM type PCase)[2,15]. Brain heart infusion (BHI) broth (Eiken, Tokyo, Japan) was used as a liquid culture.

Antibiotics

Four cephamycins, *i.e.*, cefoxitin[3], cefmetazole[5], cefotetan[6] and cefbuperazone (T-1982)[4] were used. Cefazolin was used as a control drug which was easily hydrolyzed by β-lactamases. Cephaloridine and ampicillin were used as a substrate in determining β-lactamase activity. Cephalothin was used as a substrate in determining *Ki* values.

Preparation of Purified β-Lactamase

The procedure has been described previously[8,10,17]. The β-lactamase was purified by means of CM-Sephadex column (C-50, Pharmacia, Sweden). Cefmetazole (10 μg/ml) was used as an inducer in preparing β-lactamase from *E. cloacae* H-27 and *P. vulgaris* T-178.

β-Lactamase Assay

β-Lactamase activity was assayed by a spectrophotometric method[18]. Enzyme solution (0.05 ml) was added to 3 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing a substrate which was prewarmed at 30°C. The reaction mixture was incubated in a cuvette in a Hitachi spectrophotometer 100-50 (Hitachi, Tokyo, Japan). The decrease of UV absorption was recorded with Hitachi recorder model 200. Absorption coefficient specific to each β-lactam ring was obtained from differential spectra before and after complete hydrolysis by the purified enzyme. The absorption coefficients (ε) and wave length (nm) of each antibiotic used were as follows; cefmetazole (8,400, 275), cefoxitin (8,200, 266), cefotetan (5,700, 274), cefbuperazone (6,300, 277), cefazolin (7,700, 263), cephaloridine (10,200, 260), cephalothin (7,700, 262) and ampicillin (900, 235).

One unit of the enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of the substrate in 1 minute at 30°C.

A microiodometric method[19] was also employed when the hydrolysis of cephamycins by β-lactamase was not detectable by the spectrophotometric method.

Susceptibility Tests

Minimal inhibitory concentrations (MICs) of antibiotics were determined by the agar dilution method as described in the previous papers[1,8,16]. An overnight culture of an organism was diluted to give about 10^3 and 10^6 cells/ml and inoculated onto the agar plates (about 0.005 ml) by a microplanter (Sakuma, Tokyo, Japan). The MICs were determined after 18 hours incubation at 37°C.

Inactivation of Cephamycins in the Growing Culture

An overnight culture of a β-lactamase-producing strain was diluted 20-fold with fresh BHI broth and incubated with shaking at 37°C for 2 hours. The culture which reached about 10^9 cells/ml was divided into L-tubes (10 ml), and cephamycins were added to give a final concentration of 100 μg/ml. One ml of the culture was sampled at various times of incubation, diluted at once with the same volume of methanol, and centrifuged at 3,000 rpm for 20 minutes. The drug concentrations in the supernatant were determined by bioassay and by high pressure liquid chromatography (HPLC). The number of viable cells was counted at the same time.

Inactivation of Cephamycins by the Purified Enzyme

Each cephamycin (a final concentration of 100 μg/ml) was added to the purified β-lactamase solution (in 0.05 M phosphate buffer, pH 7.0) prepared from *E. cloacae* H-27. The reaction mixture was incubated at 37°C and then sampled at various times of incubation. The samples were diluted immediately with methanol and centrifuged at 3,000 rpm for 20 minutes. The residual drug concentrations in the supernatant was determined by either bioassay or HPLC.

Antibiotic Assay

The following two methods were used for the measurement of drug concentrations. The disk-plate diffusion method[20] was employed as bioassay. The test organisms and the test medium used are summarized in Table 1. Standard solutions for the measurement of antibiotic concentrations in the cultures and in the enzyme solution were prepared in BHI broth and 0.05 M phosphate buffer (pH 7.0),

Table 1. Summary of the antibiotics assay system by bioassay and HPLC.

Antibiotics	Bioassay		HPLC		
	Test organism	Test medium	Mobile phase		Retention time (minutes)
Cefmetazole	*Micrococcus luteus* ATCC 9341	Polypeptone 6.0 g Yeast extract 3.0 g Beef extract 1.5 g Agar 15.0 g H_2O 1,000 ml	CH_3CN 1 M CH_3COOH 1 M $CH_3COOH \cdot (C_2H_5)_3N$ H_2O	145 ml 14 ml 27 ml 1,000 ml	10
Cefbuperazone	*Klebsiella pneumoniae* ATCC 10031	Polypeptone 5.0 g Beef extract 3.0 g NaCl 2.5 g Glucose 1.0 g Agar 15.0 g H_2O 1,000 ml	CH_3CN 1 M CH_3COOH 1 M $CH_3COOH \cdot (C_2H_5)_3N$ H_2O	125 ml 14 ml 27 ml 1,000 ml	12
Cefazolin	*Bacillus subtilis* ATCC 6633	Polypeptone 5.0 g Beef extract 3.0 g Agar 15.0 g H_2O 1,000 ml	Same as cefmetazole		12
Cefotetan	*E. coli* NIHJ	Same as cefmetazole	NT		NT
Cefoxitin	Same as cefmetazole	Same as cefmetazole	Same as cefmetazole		12

NT: Not tested.

respectively, and diluted twofold with methanol. The disks were immersed and then dried at 37°C for 30 minutes to eliminate methanol before being placed on the plates seeded with the test organism. Plates were incubated at 37°C for 18 hours, and inhibition zones were measured.

HPLC assay was performed with a high pressure liquid chromatograph (Shimadzu, LC-3 model). Samples were run on a column (250 mm × 4 mmϕ) of LiChrosorb RP-18 at room temperature and a flow rate of 1.0 ml/minute. The mobile phase and retention times are shown in Table 1. The eluate was monitored at 254 nm. For the detection of the hydrolysis of cefmetazole and cefoxitin in the culture, and in the enzyme solution, a column treated with tetrabutylammonium bromide was used, and a mobile phase consisting of 240 ml of CH_3CN, 5 ml of 1 M CH_3COOH, 5 ml of 1 M CH_3COONa and 3 g of tetrabutylammonium bromide in 1,000 ml of H_2O. An adequate condition for the measurement of cefotetan concentrations could not be determined.

Iodine Absorption by the Hydrolyzed Cephamycins

Iodine absorption by the hydrolyzed cephamycins was determined essentially according to the microiodometry[19]. The reaction mixture containing 100 μg/ml (about 200 μM) of cephamycin and the purified enzyme of *E. cloacae* H-27 was incubated at 37°C. Samples were taken and the enzymic reaction was stopped by addition of 0.15 M sodium tungstate (in 0.2 M acetate buffer, pH 4.0). After stopping the enzymic reaction, a dilution was made for the measurement of the absorption of iodine. The absorption of iodine was determined by measuring optical density at 620 nm.

Inducer Activity of Cephamycins

The inducer activity of cephamycins was determined as described in the previous papers[17,21]. The drug concentration used in this study was 100 μg/ml.

Results

Resistance Levels and β-Lactamase Activity

The resistance levels of four β-lactamase-producing strains are shown in Table 2. *E. coli* GN-5482 which constitutively produced a CSase (RICHMOND type Ib) was rather susceptible to cefotetan and

Table 2. Resistance levels and β-lactamase activities.

Strain	Drug resistance (MIC, μg/ml)					State of enzyme production	Enzyme activity[a] (unit/mg of protein) with	
	Cefoxitin	Cefmetazole	Cefotetan	Cefbuperazone	Cefazolin		Cephaloridine	Ampicillin
E. coli GN5482	50 (100)[b]	25 (50)	6.25 (12.5)	1.56 (3.13)	50 (200)	Constitutive	0.25	0.02
E. cloacae H-27	200 (200)	100 (100)	100 (200)	12.5 (25)	>400 (>400)	Inducible	3.38	0.02
P. vulgaris T-178	6.25 (6.25)	3.13 (3.13)	0.39 (0.78)	1.56 (1.56)	>400 (>400)	Inducible	0.60	0.14
E. coli W3630Rms212$^+$	6.25 (12.5)	0.78 (1.56)	0.2 (0.39)	0.2 (0.39)	3.13 (12.5)	Constitutive	0.10	0.10
E. coli W3630	6.25 (6.25)	1.56 (1.56)	0.1 (0.2)	0.1 (0.1)	3.13 (3.13)	—	<0.005	<0.01

[a] β-Lactamase activity of the sonic extract of shake-cultured organisms was determined.

[b] The values in the parentheses indicate the MICs which were determined using one loopful of 10^8 cells/ml.

cefbuperazone but moderately resistant to cefoxitin, cefmetazole and cefazolin. *E. cloacae* H-27 inducibly produced a CSase (RICHMOND type Ia) and was resistant to all drugs other than cefbuperazone. This strain exhibited the highest resistance level and enzyme activity among the four strains tested. *P. vulgaris* T-178 which inducibly produced cefuroximase (CXase, RICHMOND type Ic) was susceptible to all drugs other than cefazolin. The enzyme from *P. vulgaris* T-178 hydrolyzed ampicillin at higher rate than did the above two typical CSases. *E. coli* W3630Rms212$^+$ which produced TEM type penicillinase (PCase) mediated by Rms212 was susceptible to all drugs tested. *E. coli* W3630 which lost PCase producing ability showed almost the same susceptibility to cephamycins as *E. coli* W3630Rms212$^+$. The enzyme from *E. coli* W3630Rms212$^+$ could hydrolyze cephaloridine and ampicillin equally readily but its CSase activity was the lowest among the enzymes tested. Two newly introduced cephamycins, *i.e.* cefotetan and cefbuperazone, were rather more active against these four β-lactamase-producing strains than cefoxitin and cefmetazole. The variation of inoculum size did not appreciably affect the activity of cephamycins against these strains.

β-Lactamase Induction

β-Lactamase induction by cephamycins for these four strains was determined at the drug concentration of 100 μg/ml (Table 3). All the cephamycins showed a high level of induction of β-lactamase activity in *E. cloacae* H-27. In each case the β-lactamase activity of this strain was about 50 to 100 times higher than that without inducer. With *P. vulgaris* T-178, the cephamycins also induced high enzyme production and the β-lactamase activity was about 10 to 30 times higher than without inducer. However, maximum value of enzyme activity for this strain was lower than that for *E. cloacae* H-27. The inducer activity of the cephamycins was similar to each other in these two strains. The production of β-lactamase in *E. coli* GN5482 and *E. coli* W3630Rms212$^+$ was not appreciably affected by these antibiotics.

Inactivation of Cephamycins in the Growing Cultures of β-Lactamase-producing Strains

The stability of cephamycins in the growing cultures of the β-lactamase-producing strains was studied by measuring the residual amount of antibiotic with bioassay and HPLC. Each antibiotic was added to logarithmically growing cultures to give a final concentration of 100 μg/ml (Fig. 1). There was no significant difference in the measured values between bioassay and HPLC. The four cephamycins were stable for at least 4 hours in the cultures of both *P. vulgaris* T-178 (B) and *E. coli* W3630-Rms212$^+$ (D). On the other hand, in the cultures of *E. coli* GN5482 (A) and *E. cloacae* H-27 (C), cefmetazole and cefoxitin were readily inactivated while cefotetan and cefbuperazone were relatively stable. The cephamycins were inactivated more rapidly by *E. cloacae* H-27 than by *E. coli* GN5482.

The growth curves of these four strains were determined simultaneously when the inactivation of the

Table 3. β-Lactamase induction by cephamycins in the β-lactamase-producing strains.

Strain	β-Lactamase activity (unit/mg of protein) of cells induced by					
	Without drug	Cefoxitin	Cefmetazole	Cefotetan	Cefbuperazone	Cefazolin
E. coli GN5482	0.25	0.27	0.29	0.18	0.18	0.25
E. cloacae H-27	0.02	3.65	3.51	2.56	2.13	0.58
P. vulgaris T-178	<0.02	0.53	0.55	0.18	0.69	0.56
E. coli W3630Rms212$^+$	0.10	0.08	0.10	0.06	0.14	0.16

Cephamycins were added to the culture at mid-log phase to give a final concentration of 100 μg/ml, and the incubation continued with shaking for 2 hours at 37°C. β-Lactamase activity of the sonic extract of the organisms was determined by spectrophotometric method using cephaloridine (100 μM) as a substrate.

Fig. 1. Inactivation of cephamycins in the growing cultures of β-lactamase-producing strains.

The residual activities of cefoxitin (□), cefmetazole (▲), cefotetan (○), cefbuperazone (●) and cefazolin (△) in the culture of *E. coli* GN5482 (A), *P. vulgaris* T-178 (B), *E. cloacae* H-27 (C) and *E. coli* W3630-Rms212$^+$ (D) were determined by bioassay. The drug concentration was 100 μg/ml.

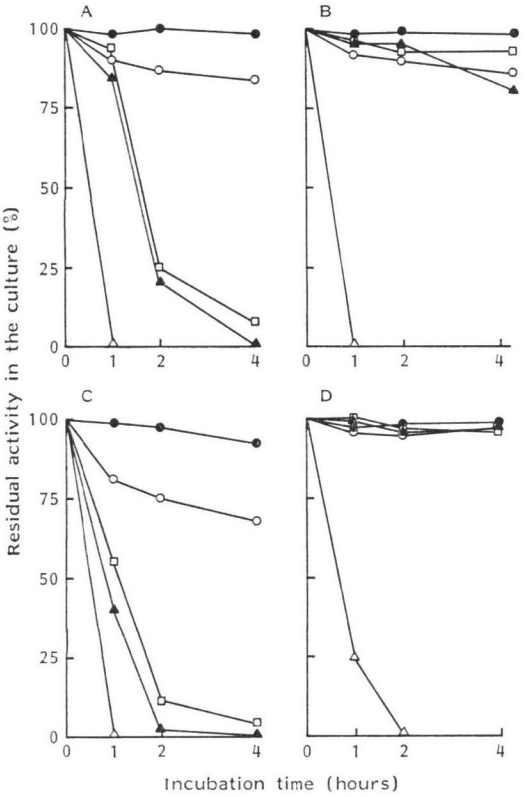
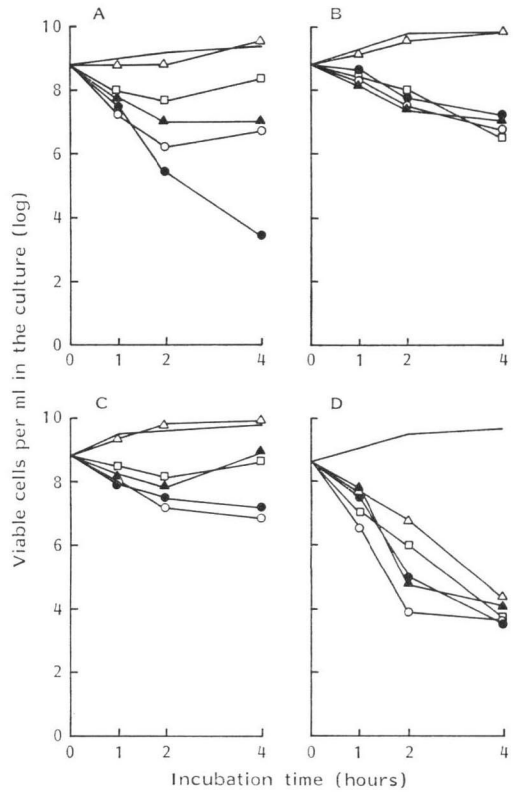


Fig. 2. Growth curves of β-lactamase-producing strains in the presence of 100 μg/ml of cephamycins.

The numbers of viable cells in the culture of *E. coli* GN5482 (A), *P. vulgaris* T-178 (B), *E. cloacae* H-27 (C) and *E. coli* W3630Rms212$^+$ (D) were counted at various times of incubation after addition of cephamycins.

Symbols are as follows; cefoxitin (□), cefmetazole (▲), cefotetan (○), cefbuperazone (●) and cefazolin (△).



cephamycins was studied (Fig. 2). The number of viable cells of each strain decreased at the drug concentration of 100 μg/ml. The order of the bactericidal activity of the cephamycins against *E. coli* GN5482 and *E. cloacae* H-27 (C) was approximately in the same order as their stability in these cultures.

Stability and Affinity of Cephamycins for β-Lactamase

Relative rates of hydrolysis (Vmax), and *Ki* values of the cephamycins with four purified β-lactamases are shown in Table 4. All the cephamycins were very stable to the enzymes tested. Hydrolysis by the enzymes of *P. vulgaris* T-178 and *E. coli* W3630Rms212$^+$ was not detectable even by microiodometry while cefazolin and cephaloridine were hydrolyzed very readily. The β-lactamase from *E. coli* GN5482 hydrolyzed all the cephamycins at a detectable rate. There was a difference in the rates of hydrolysis of each cephamycin by the CSases of *E. coli* GN5482 and *E. cloacae* H-27 although the rates of hydrolysis were very low and barely detectable. Cefmetazole and cefoxitin were less stable to the CSases of these strains than cefotetan and cefbuperazone. The stability of the cephamycins to the

Table 4. Kinetics of hydrolysis of cephamycins by β-lactamases.

	Kinetic parameters of hydrolysis by β-lactamase from											
	E. coli GN5482			*E. cloacae* H-27			*P. vulgaris* T-178			*E. coli* W3630-Rms212$^+$		
	Ki[a]	*Km*[b]	Vmax[c]	*Ki*	*Km*	Vmax	*Ki*	*Km*	Vmax	*Ki*	*Km*	Vmax
Cefmetazole	1.36	—	1.00	0.07	—	0.04	34	—	<0.01	37	—	<0.03
Cefoxitin	0.26	—	0.90	0.07	—	0.02	27	—	<0.01	26	—	<0.03
Cefotetan	0.20	—	0.25	0.06	—	<0.01	65	—	<0.01	23	—	<0.03
Cefbuperazone	0.89	—	0.12	0.16	—	<0.01	50	—	<0.01	24	—	<0.03
Cefazolin	—	705	265	—	450	133	—	130	33	—	256	12
Cephaloridine	—	136	100 (3.7)[d]	—	155	100 (11.5)	—	110	100 (2.6)	—	430	100 (0.9)

[a] *Ki* values were determined with cephalothin as a substrate by LINEWEAVER-BURK plots.
[b] *Km* values were determined by LINEWEAVER-BURK plots, and the hydrolysis was measured by a spectrophotometric method.
[c] Relative values when the hydrolysis of cephaloridine was taken as 100. Vmax values of cephamycins were obtained by microiodometry when the concentration of cephamycins was 100 μM.
[d] The values in parentheses are the specific activity (unit/mg of protein) with cephaloridine (100 μM).

enzymes corresponded well with their stability in the growing cultures of these β-lactamase-producers.

The *Ki* values of cephamycins were also determined. All the cephamycins showed high *Ki* values (*i.e.* low affinity) for enzymes of *P. vulgaris* T-178 and *E. coli* W3630Rms212$^+$, and low *Ki* values (*i.e.* high affinity) for the enzymes of *E. coli* GN5482 and *E. cloacae* H-27. The affinity of the cephamycins for the latter two enzymes was about 100 times higher than for the former two enzymes. There was no significant difference in affinity among each cephamycin.

The Hydrolysis of Cephamycins by the Purified Enzyme from *E. cloacae* H-27

The hydrolysis of the cephamycins was studied using β-lactamase of *E. cloacae* H-27 which produced relatively large amounts of enzyme. The cephamycins (a final concentration of 100 μg/ml) were added to the enzyme solution whose activity was about 3 units/ml (with cephaloridine) and was almost the same level as that induced by 100 μg/ml of cefmetazole. The residual amounts of the cephamycins were determined by HPLC and bioassay. Since there was no significant difference in the measured values between HPLC and bioassay, the results of HPLC assay are shown except in the case of cefotetan for which the bioassay results are used (Fig. 3). Cefoxitin and cefmetazole were as readily inactivated in the enzyme solution as they were in the culture of *E. cloacae* H-27. On the other hand, cefbuperazone was very stable in the enzyme solution.

In the course of HPLC assay, it was confirmed that the peaks of cefmetazole and cefoxitin appeared respectively at the retention times of 10 and 12 minutes, and that as the enzyme reaction progressed, they gradually disappeared and at the same time the new single peak appeared at the retention time of 3 minutes in the case of cefmetazole and the broad peak appeared at the retention time 3 to 5 minutes in the case of cefoxitin.

Further investigations were performed to determine whether cefmetazole (and cefoxitin) was inactivated due to hydrolytic cleavage of β-lactam ring. The change of two peaks at retention times of 3 minutes and 10 minutes corresponding to the hydrolyzed cefmetazole and cefmetazole is shown in Fig. 4. The disappearance of cefmetazole was closely related to the appearance of the hydrolyzed cefmetazole. The iodine absorption was also determined by microiodometry (Fig. 4). The absorption of

Fig. 3. Hydrolysis of cephamycins by the purified β-lactamase from *E. cloacae* H-27.

Cefoxitin (□), cefmetazole (▲), cefotetan (○), cefbuperazone (●) and cefazolin (△) were added to the enzyme solution to give a final concentration of 100 μg/ml, and the reaction mixture incubated at 37°C. The enzymic reaction was stopped by adding an equal volume of methanol. The residual antibiotic was determined by HPLC except for cefotetan. The data for cefotetan were obtained by bioassay.

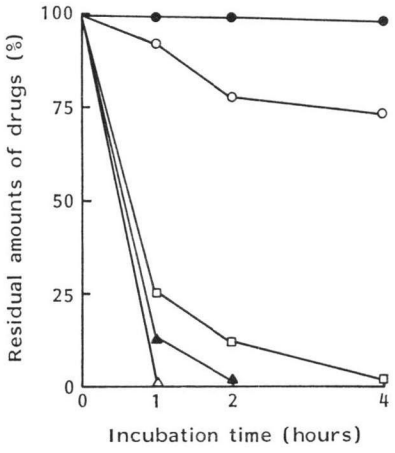


Fig. 4. Hydrolysis of cefmetazole by the purified β-lactamase from *E. cloacae* H-27.

The change of the peak high of cefmetazole (○) at 10-minute and the metabolite (●) at 3-minute in HPLC, and the absorption of iodine (□, change of optical density at 620 nm) were determined when cefmetazole (100 μg/ml) was incubated with the purified enzyme.

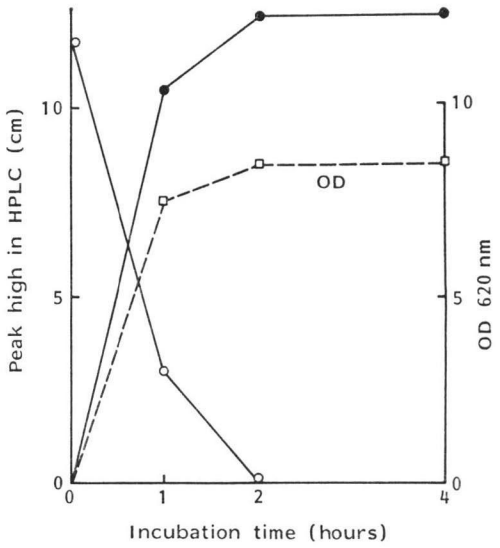


Fig. 5. IR and UV spectra of the hydrolyzed cefmetazole.

(A) IR spectra of the hydrolyzed cefmetazole was measured (------, KBr) and compared with that of cefmetazole (——).

(B) UV spectra of cefmetazole was measured at start (——) and after 2 hours of incubation (------) with the purified enzyme of *E. cloacae* H-27.

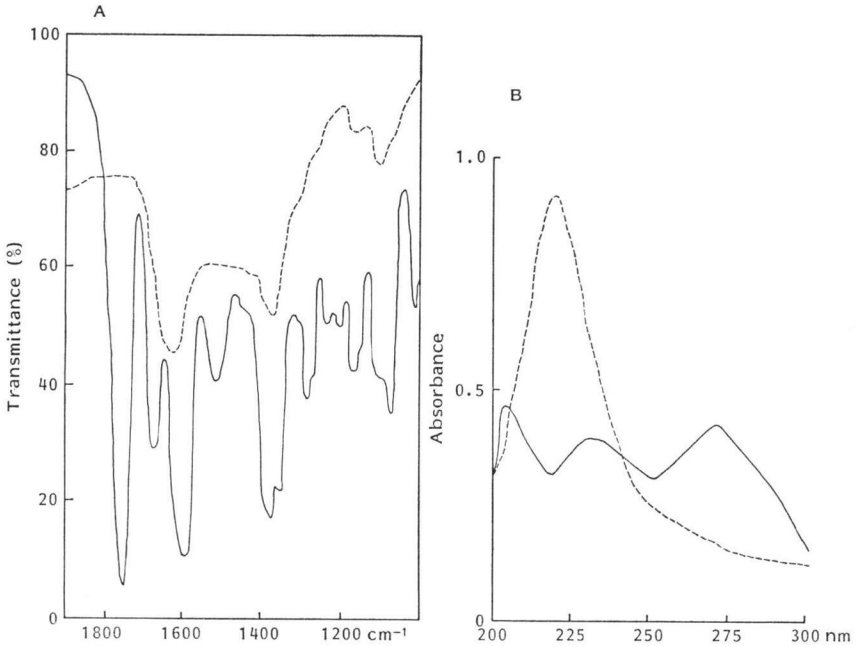
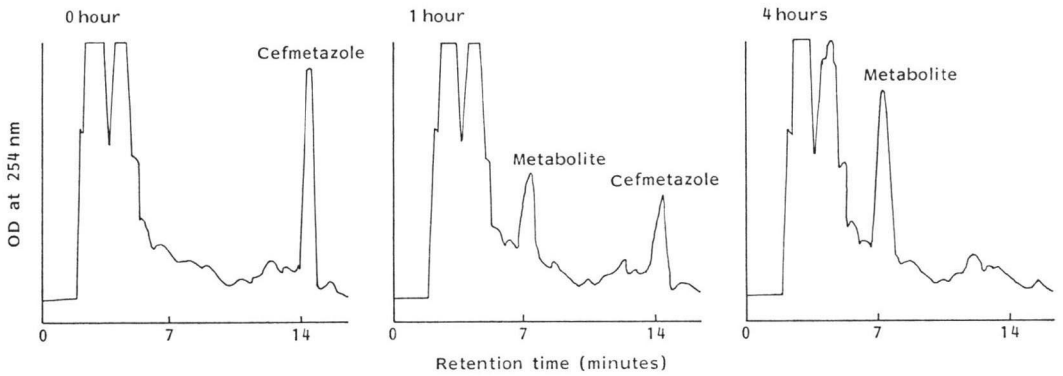


Fig. 6. Hydrolysis of cefmetazole in the growing culture of *E. cloacae* H-27.

Cefmetazole was added to the mid-log culture of *E. cloacae* H-27 to give a final concentration of 100 μg/ml. The culture was taken at 0-, 1- and 4-hour of incubation, and each sample was diluted twofold with methanol. The determination of cefmetazole and hydrolyzed cefmetazole was performed by HPLC.



iodine corresponded well with the amount of the hydrolyzed cefmetazole.

For the measurement of IR spectrum of the hydrolyzed cefmetazole, the enzymic reaction was carried out in distilled water, at pH between 7.0 and 7.5, the enzyme preparation having been deionized. After complete inactivation of cefmetazole was confirmed by HPLC, methanol was added to the reaction mixture and the resulting precipitate filtered off. Methanol was evaporated *in vacuo*, and the resulting aqueous solution lyophilized. The IR spectrum of the resulting colorless powder was measured and compared with that of cefmetazole (Fig. 5A). The band at about 1750 cm^{-1} specific to the carbonyl group of β-lactam ring was absent.

The UV spectrum of the reaction mixture was measured at the beginning of reaction and after 2 hours. The enzyme solution incubated without cefmetazole was used as the control. As shown in Fig. 5B, the absorption at 270 nm was recognized as being decreased after 2 hours of incubation.

The presence of the hydrolyzed cefmetazole in the culture of *E. cloacae* H-27 was also proved by HPLC analysis (Fig. 6). The hydrolyzed cefmetazole could be retained longer in a column (about 7 minutes) by forming the ion pair with tetrabutylammonium ion. The peak of the hydrolyzed cefmetazole (7 minutes) became higher as the peak of cefmetazole at about 14 minutes became lower.

Discussion

Cephamycins which has a 7α-methoxyl group in the cephalosporin nucleus has been shown to be highly resistant to hydrolysis by various types of β-lactamase[3~7]. However, recently, GOOTZ and SANDERS[22] have revealed that cefoxitin, the first semisynthetic cephamycin, was inactivated in the growing culture of CSase-producing *E. cloacae*. Our results also confirm this inactivation. Cefoxitin and cefmetazole were readily inactivated in the growing cultures of *E. coli* GN5482 and *E. cloacae* H-27 which produced the typical CSases (RICHMOND type Ib and Ia, respectively), but they were very stable in the growing cultures of a cefuroximase[16,28]-producer (*P. vulgaris* T-178, RICHMOND type Ic) and a TEM type PCase-producer (*E. coli* W3630Rms212$^+$). They had high affinity (very low *Ki* values) for Ia and Ib CSases but low affinity (relatively high *Ki* values) for Ic CSase and TEM type PCase. The inactivation of cefoxitin or cefmetazole was seemingly related to their affinity for the enzyme, that is to say cefoxitin and cefmetazole bound tightly to the enzyme to form a stable acyl-enzyme[24], and because of this, their bioactivity disappeared in the cultures of CSase-producers. However, this is not always the case

because cefotetan and cefbuperazone showed an affinity for Ia and Ib CSases as high as that of cefoxitin and cefmetazole yet they were not significantly inactivated in the growing cultures of these strains. It is therefore more reasonable to consider that the inactivation of cefoxitin and cefmetazole in the growing cultures of CSase-producers is due to hydrolysis by the enzymes, and that their inferior stability in the culture stems from their poorer stability to the enzymes. The rates of hydrolysis of cefoxitin and cefmetazole by CSases were higher than those of cefotetan and cefbuperazone though the values of hydrolysis rates were very small and barely detectable in the usual enzyme assay.

More convincing evidence for hydrolysis by CSase was obtained in the case of cefmetazole. A new peak appeared at a shorter retention time in HPLC than that of cefmetazole when it was incubated with *E. cloacae* H-27 or a large amount of the purified enzyme of this strain. The shorter retention time in this HPLC analysis using a column of LiChrosorb RP-18 indicated that this new compound was more hydrophilic than cefmetazole, and hence was probably the hydrolyzed cefmetazole. The increase of the new peak in HPLC of the enzyme solution containing cefmetazole corresponded well with the decrease of cefmetazole's peak in HPLC, the loss of bioactive cefmetazole, and the increase of iodine absorption. Similar results were obtained in the case of cefoxitin although metabolite of cefoxitin gave broad rugged peaks in HPLC. The hydrolyzed cefoxitin was likely to be more unstable than that of cefmetazole.

The measurements of UV and IR spectra of both cefmetazole and hydrolyzed compound demonstrated the cleavage of β-lactam ring of cefmetazole. The comparison of the UV spectrum of cefmetazole before and after incubation with the enzyme showed that the absorption at about 270 nm decreased with the disappearance of cefmetazole. The decrease of absorption at 270 nm indicates the cleavage of β-lactam ring. The IR spectrum of the metabolite also shows the cleavage of the β-lactam ring. The absorption band at about 1750 cm^{-1}, which is specific to the carbonyl group in β-lactam ring, completely disappeared in the IR spectrum of the metabolite.

All the evidence described above strongly supports the view that the inactivation of cefmetazole (probably cefoxitin) is not due to tight binding to the enzyme but due to hydrolytic cleavage of the β-lactam ring by the enzyme.

LABIA and GUIONIE[10] have already reported about the resistant mechanism of CSase-producing *E. coli* to cefoxitin. They determined a ratio, *Km*/Vmax which is related to the half life of β-lactam antibiotics at low concentration. From the fact that *Km*/Vmax value for cefoxitin was about the same of cefazolin, they concluded that cefoxitin was as susceptible to CSase action as cefazolin was. Furthermore, from the fact that a powerful cefoxitin-cloxacillin synergy and a good linear relation between MICs of cefoxitin and cephalothin were obtained with CSase-producing *E. coli*, they concluded that the cefoxitin resistance mechanism was the same as that involved for the other cephalosporins. Although our results clearly show the hydrolysis of cefmetazole (and cefoxitin) by the CSases or by the CSase-producers, the relationship between the hydrolysis of the cephamycins by β-lactamase and the resistance levels of β-lactamase-producers is still unclear. The MICs of each cephamycin against *E. coli* GN5482 and *E. cloacae* H-27 were likely related to their stability to the CSases of these strains or to their inactivation rates in the growing cultures. This does not, however, prove that the hydrolysis of the cephamycins by the CSases or the CSase-producers is responsible for the resistance of CSase-producers to these antibiotics. The order of antibacterial activity of each cephamycin against *E. coli* GN5482 was similar to that against *E. coli* W3630 which was a β-lactamase-less strain. The increase of resistance of *E. coli* GN5482 to each cephamycin was not related to the degree of stability to the CSase or the inactivation rate in the culture. For example, the MICs of cefbuperazone, cefmetazole and cefoxitin against *E. coli* GN5482 were sixteen fold those for the β-lactamase-less strain (W3630), despite the fact that cefbuperazone was more stable both in the growing culture of *E. coli* GN5482 and to the CSase than cefmetazole and cefoxitin.

Judging from the fact that the enzyme inactivation of cephamycins was observed only when a relatively large amount of enzyme was present and the fact that the CSase-producing *E. coli* was more resistant to cefotetan and cefbuperazone than the β-lactamase-less strain, even though they were very stable, it is still not clear whether the hydrolysis of cephamycins by CSases plays an effective role in the resistance of CSase-producers. These problems will be elucidated by further investigations.

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