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Activity of Cefoperazone Against Ampicillin-Resistant Bacteria in Agar and Broth Dilution Tests

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Examination of the activity of cefoperazone against ampicillin-resistant, gram-negative bacteria in agar dilution and simultaneously in broth dilution revealed that strains could be divided into three classes: class I strains were susceptible in agar (mean minimal inhibitory concentration [MIC], 0.5 mg/liter) as well as in broth dilution (mean MIC, 1.5 mg/liter), class II strains were susceptible in agar (MIC, 0.9 mg/liter), but resistant in broth dilution (MIC, 182 mg/liter); and class III strains were highly resistant in both test systems. Among 100 randomly selected ampicillin-resistant *Escherichia coli* cultures, 51 belonged to class I and 49 belonged to class II. Class III *E. coli* strains were much rarer. Similar results were obtained with cefamandole and cephalothin, but not with six other second- and third-generation cephalosporins. MICs of cefoperazone against cultures of all three classes were influenced by initial inoculum size. The inoculum effect was greatest with class II strains. Examination of bactericidal activity by cefoperazone showed killing of class I and class II *E. coli* strains and of class III strains of other genera during the first hours of incubation and regrowth after the drug was destroyed by the action of TEM β-lactamase (penicillinase). Representative class I bacteria produced 10 to 100 times less TEM β-lactamase than did class II strains. It appeared that the quantitative difference in TEM production was the reason for the different resistance phenotypes in class I and class II strains. *Salmonella* and *Klebsiella* strains of class III produced the same amounts of TEM β-lactamase as did class II *E. coli* strains. Probably, some factors other than β-lactamase contributed to the class III phenotype in these species.

Cefoperazone is a new semisynthetic, injectable cephalosporin similar in structure to both piperacillin and cefamandole. Worldwide extensive in vitro studies on the activity of this drug against more than 25,000 clinical isolates have been carried out (11). These studies indicated that cefoperazone has one of the widest antimicrobial spectra among antibiotics currently available. Strains resistant to cefoperazone were rarely observed. Many of the studies used agar dilution procedures to compare cefoperazone with other antimicrobial agents.

In a series of experiments 3 years ago on the antibacterial activity of this drug, using agar dilution, Kayser et al. (15) found only one cefoperazone-resistant *Escherichia coli* strain (minimal inhibitory concentration (MIC), 32 mg/liter) among 49 cultures examined; all other *E. coli* strains were highly susceptible.

Recently, we reexamined these isolates in a micro-broth dilution test system and realized that a considerable number of strains previously susceptible to cefoperazone in agar dilution now appeared highly resistant. Similar discrepancies between MICs obtained in different test systems have been described for cephalothin and cefamandole (1, 7, 9, 23). We therefore decided to study the phenomenon with cefoperazone in more detail. For comparative purposes, some data obtained with cephalothin, cefamandole, and additional cephalosporins are also given.

MATERIALS AND METHODS

Bacterial strains. Ampicillin-resistant *E. coli* strains were fresh, serial isolates from patient materials. Only hospital isolates were included in the study. An isolate or strain is defined here as the first culture obtained from a single patient. No further typing of the *E. coli* isolates was done. *Salmonella dublin* HK246 (13) and *Klebsiella pneumoniae* HK212 (26) were clinical isolates which harbored antibiotic resistance plasmids pFK17 (14) and pFK1 (26), respectively. Both plasmids determined resistance (MIC, 50 mg/liter) to ampicillin, carbenicillin, cephalothin, cefamandole, cefoperazone, chloramphenicol, gentamicin, kanamycin, sulfonamide, and tobramycin and a low level of resistance (MIC, 100 mg/ml) to streptomycin. In addition, plasmid pFK1 codes for resistance to tetracycline. *S. dublin* HK313 and *K. pneumoniae* HK198 were derivatives, cured of pFK17 and pFK1, respectively. *E. coli* K-12 (HK225) was the host of plasmids RP4 (6) and R6K (17).

Antibiotics. Laboratory standards of the following antimicrobial agents were kindly provided by various

pharmaceutical companies: cefoperazone (Pfizer Inc., New York, N.Y.); cephalothin, cefamandole, and moxalactam (Eli Lilly & Co., Indianapolis, Ind.); cefoxitin (Merck Sharp & Dohme, West Point, Pa.); cefuroxime, cephaloridine, and ceftazidime (Glaxo Laboratories, Ltd., Greenford, Middlesex, England); cefotaxime (Roussel UCLAF, Paris, France); and ceftriaxon (Hoffmann-La Roche Inc., Nutley, N.J.). Ampicillin and benzylpenicillin (penicillin G) were provided by the pharmacy of the university hospital.

Susceptibility tests. MICs were determined by serial twofold dilutions of the drugs in a standardized agar dilution test (28) or in micro-broth dilution (8). The inoculum for agar dilution was about 10^4 cells per spot, and for micro-broth dilution the inoculum was 5×10^5 to 10^6 cells per ml. The total volume was 0.1 ml per well in the broth dilution system. In some experiments, drug resistance was determined by the U.S. disk diffusion test (3). The media used were Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) and Mueller-Hinton broth (BBL). A strain was considered resistant to cefoperazone, cefamandole, or cephalothin if the MIC was $\geq$32 mg/liter.

The rate of killing of bacterial cultures by cefoperazone was examined by determination of viable cells. Flasks containing 50 ml of Mueller-Hinton broth were inoculated with approximately 10^6 bacteria per ml and incubated at 37°C. At appropriate time intervals, samples were withdrawn, diluted, and plated onto blood agar in several dilutions to determine colony-forming unit survival. In certain cases, to avoid a carry-over of bacteriostatic drug concentrations from the flask to the plate, a *Bacillus cereus* β-lactamase preparation in a dilution of 1:5 was added to the samples. The samples were then incubated for 5 min at 20°C to destroy cefoperazone (24).

Antibiotic assays. Quantitative determinations of cefoperazone and cefamandole in broth cultures or in broth alone were performed by agar diffusion assay with *Bacillus subtilis* ATCC 6633 as the test strain (5). All assays were performed in triplicate. In a preliminary experiment, samples from bacterial cultures were centrifuged and the supernatant was filtered through a Diaflo YM10 membrane (Amicon Corp., Lexington, Ky.) to remove bacteria and any β-lactamase in the test sample. This was done to avoid possible degradation of cefoperazone during incubation of the assay plates. Since no degradation by unfiltered samples was observed, this procedure was not used in later experiments.

Enzyme procedures. For quantitative determination of β-lactamase, bacteria were grown in ML-medium (ingredients per liter: tryptone, 10 g; yeast extract, 50 g; NaCl, 10 g; glucose, 4 g [pH 7.2]) until the end of logarithmic growth, harvested by centrifugation, and adjusted to a cell density of 10^{10} viable cells per ml in phosphate buffer (0.2 M, pH 5.9). Ten milliliters of cell suspension was sonicated at intervals for a total of 5 min at 70 W and 0°C (Branson Sonifier B 12; Branson Instruments Co., Danbury, Conn.). The cell debris was removed by centrifugation at 8,000 × *g* for 20 min. The supernatant served as the crude enzyme extract. Enzymatic activity was determined at 30°C and pH 5.9 with a 0.007 M solution of benzylpenicillin as substrate by using the iodometric method of Perret (22), modified according to Jack and Richmond (12). The amount of β-lactamase produced by 10 ml of suspension was expressed in katals, which are defined as moles of substrate hydrolyzed per second.

Substrate profiles were determined by the iodometric method with benzylpenicillin, ampicillin, cloxacillin, oxacillin, cephalexin, or cephaloridine as the substrate. The profiles were calculated against a value of 100 for the rate of hydrolysis of benzylpenicillin. Sensitivity of β-lactamase activity to *p*-hydroxychloromercuribenzoate was examined by mixing the crude enzyme preparation with *p*-hydroxychloromercuribenzoate in 0.1 M phosphate buffer, pH 7.0, to give a final molarity of 0.005 (27). Sensitivity to cloxacillin was measured by mixing cloxacillin, dissolved in the same buffer, with the enzyme to a final molarity of 0.007 (10). The mixtures were incubated at 30°C for 10 min. Residual enzyme activity was then estimated iodometrically with cephaloridine as substrate.

Isoelectric focusing of bacterial extracts was performed with an LKB 8100 electrofocusing column (LKB-Produkter AG, Bromma, Sweden) of 110-ml capacity, using the procedure described in the manufacturer's instruction manual. After electrofocusing was completed, the column contents were drained and collected in 2-ml fractions, which were adjusted to pH 5.9 and assayed iodometrically, with ampicillin as the substrate, for β-lactamase activity.

Conjugation experiments. R-plasmid transfers between the clinical isolates *K. pneumoniae* HK212 and *S. dublin* HK246 as donors and *E. coli* K-12 (HK225) as the recipient were performed in brain heart infusion broth. The recipient strain possessed high levels of resistance to streptomycin (MIC $\geq$ 3,200 mg/liter) and rifampin (MIC $\geq$ 200 mg/liter). Overnight cultures of donor and recipient were mixed 1:10, and the mixture was diluted in 10 times the volume of fresh brain heart infusion broth and incubated for 24 h. Transconjugants were selected by plating appropriate dilutions of the cultures onto agar plates containing either ampicillin (100 mg/liter) or chloramphenicol (50 mg/liter) for selection of the donor plasmid and either streptomycin (2,000 mg/liter) or rifampin (100 mg/liter) as counterselecting agents.

RESULTS

Drug resistance studies. In Table 1 the MICs of cephalosporins and related compounds against three ampicillin-resistant, gram-negative strains are shown. These strains represented typical examples of three classes of bacteria. *E. coli* HK407 belonged to class I, in which we included all strains susceptible to cefoperazone in both standard agar dilution and micro-broth dilution tests. *E. coli* HK288 was a typical class II strain, susceptible to cefoperazone in the agar dilution test, but resistant in the broth dilution test. Finally, *S. dublin* HK246 belonged to class III, which is composed of strains resistant to cefoperazone in agar as well as in broth dilution. The three strains showed these characteristics not only in tests with cefoperazone but also in tests with cefamandole and cephalothin (Table 1). Other second- and third-generation cephalospo-

TABLE 1. MICs of cephalosporins for three gram-negative strains in agar and micro-broth dilution tests

Antibiotic	MIC (mg/liter) for:					
	E. coli HK407		*E. coli* HK288		*S. dublin* HK246	
	Agar	Broth	Agar	Broth	Agar	Broth
Ampicillin	>256	>256	>256	>256	>256	>256
Cefoperazone	0.25	16	4	>256	256	256
Cefamandole	1	16	8	>256	128	256
Cephalothin	4	8	8	>256	128	256
Cefoxitin	2	4	1	4	1	2
Cefuroxime	4	4	1	4	4	8
Cefotaxime	<0.1	<0.1	<0.1	0.5	<0.1	0.5
Ceftazidime	<0.1	<0.1	<0.1	<0.1	0.5	0.5
Ceftriaxon	<0.1	<0.1	<0.1	0.1	0.25	<0.1
Moxalactam	<0.1	<0.1	<0.1	0.25	0.25	<0.1

rins examined, as well as moxalactam, were all highly active against the three strains.

We next examined the frequency of these classes among *E. coli* isolates resistant to ampicillin. For this purpose, we randomly selected 100 ampicillin-resistant *E. coli* strains and determined their MICs of β-lactam antibiotics. In Table 2 the geometric mean MICs of each drug are summarized. Fifty-one of the strains belonged to class I and, thus, were susceptible to cefoperazone in agar as well as in broth. Forty-nine of the strains belonged to class II and, thus, were susceptible in agar, but resistant in broth dilution. Class III strains were not found in this collection of isolates. Such strains are only occasionally observed among *E. coli* (15). They are more often encountered in other bacterial species (15).

TABLE 2. Geometric mean MICs of cephalosporins for 100 ampicillin-resistant *E. coli* strains of class I (51 strains) and class II (49 strains) determined in agar and micro-broth dilution tests

Antibiotic	Geometric mean MIC (mg/liter) for:			
	Class I		Class II	
	Agar	Broth	Agar	Broth
Ampicillin	229	308	501	572
Cefoperazone	0.5	1.5	0.9	182
Cefamandole	2.0	3.4	2.2	54
Cephalothin	16	29	9.6	40
Cefoxitin	4.4	2.8	2.7	1.4
Cefuroxime	5.7	1.5	3.5	0.8
Cefotaxime	0.1	0.08	0.06	0.06
Ceftazidime	0.2	0.1	0.08	0.06
Ceftriaxon	0.09	0.08	0.06	0.06
Moxalactam	0.1	0.06	0.07	0.06

Most class II strains were susceptible to cefoperazone not only in agar dilution but also in agar disk diffusion. Results of the examination of 50 strains are shown in Table 3. Data obtained with cefamandole and cephalothin are also given. Nearly all of the strains were classified as susceptible or intermediate in tests against cefoperazone and cefamandole. Only two strains (4%) were categorized as resistant. The results of tests with cephalothin were somewhat different. The majority of class I *E. coli* strains were susceptible to cefoperazone and cefamandole in disk diffusion and some were intermediate, but none was resistant. Class III enterobacteria were always resistant in the disk test (data not shown).

Resistance to ampicillin by most *E. coli* strains is known to be due to β-lactamase production. This property can result in a significant inoculum effect. We therefore examined the influence of the initial inoculum on results obtained with cefoperazone, cefamandole, and cephalothin. Table 4 summarizes our results with cefoperazone. A significant inoculum effect, especially in micro-broth dilution tests with class II strains, was observed. This effect was strongest in the inoculum range between 5×10^4 to 5×10^6 colony-forming units per ml, which is the range usually used in MIC determination (8, 20). A similar inoculum effect was also found with cefamandole and cephalothin (data not shown).

In studying the bactericidal activity of cefoperazone, we observed that the class I *E. coli* strain HK407 was initially killed even by subinhibitory concentrations of the drug, but began to regrow after several hours of incubation (Fig. 1A). Regrowth occurred even with the MIC of cefoperazone, but the number of viable cells

TABLE 3. Categorization of 50 class I and 50 class II *E. coli* strains with the agar disk diffusion test

Antibiotic (30 μg/disk)	% of strains classified[a] as:					
	Susceptible		Intermediate		Resistant	
	Class I	Class II	Class I	Class II	Class I	Class II
Cefoperazone	96	88	4	10	0	2
Cefmandole	96	86	4	12	0	2
Cephalothin	80	38	10	44	10	18

[a] Interpretation of inhibition zones: susceptible, ≥18 mm; resistant, ≤14 mm.

after 24 h of incubation was too low to result in visible turbidity. Regrowth resulted when most of the antibacterial activity in the culture medium was destroyed (see Table 5). We also examined the ability of cefoperazone to kill cells of a representative class II *E. coli* strain (HK288) during growth in broth and that strain's ability to destroy the drug (Fig. 1B; Table 5). At concentrations below the MIC, there was killing of cells by about 2 logs for 4 to 6 h. Then regrowth occurred, and after 12 h the number of viable cells equaled that of the control. Destruction of cefoperazone was complete after 6 h of incubation (Table 5).

Figure 1C demonstrates the growth that occurred on incubation of the class III *S. dublin* strain HK246. There was killing by 1 to 2 logs for 10 h of incubation, and then regrowth occurred. The kinetics of destruction of cefoperazone by *S. dublin* HK246 were similar to those of the class II *E. coli* strain HK288 (Table 5).

Enzymatic studies. Cefoperazone, cefamandole, and cephalothin are known to be hydrolyzed by TEM β-lactamases of gram-negative bacteria (19). Since we showed destruction of cefoperazone in broth cultures (Table 5), we selected appropriate strains of all three classes and examined them for presence and kind of β-lactamase produced. Enzymes were characterized in crude extracts by determining substrate profiles and inhibition of activity by *p*-hydroxychloromercuribenzoate and by cloxacillin and by measuring the isoelectric points. The β-lactamases of class I *E. coli* strains HK438, HK407, HK427, and HK442 hydrolyzed ampicillin, benzylpenicillin, and cephaloridine, but not cloxacillin or cephalexin. Hyrdolysis of cephaloridine was inhibited by cloxacillin, but not by *p*-hydroxychloromercuribenzoate. The isoelectric point of β-lactamase isolated from HK438 was 5.4, and the isoelectric points of the enzymes from HK407, HK427, and HK442 were each 5.6. Thus, HK438 produced TEM-1 β-lactamase, and the other three strains produced TEM-2 β-lactamase. No other β-lactamases were made by these cultures. As representative class II and class III strains, we selected those carrying plasmids known to code for production of TEM-1 β-lactamase (6, 13, 17, 26). Table 6 summarizes the MICs of cefoperazone and cefamandole for strains of all three classes as well as for plasmid-free derivatives of some of the strains. These derivatives did not produce detectable β-lactamase activity. It can be seen that resistance to cefoperazone and cefamandole in class II and III strains was indeed due to plasmid-mediated

TABLE 4. Effect of initial inoculum on MICs of cefoperazone

Test	Inoculum (colony-forming units/ml)	Class[a]					
		I		II		III	
		No.	MIC (mean)	No.	MIC (mean)	No.	MIC (mean)
Micro-broth dilution	5×10^3	10	0.25	50	0.7	2	16
	5×10^4	10	0.25	50	2.2	2	45
	5×10^5	10	5.1	50	182	2	362
	5×10^6	10	223	50	338	2	≥512
Agar dilution	10^3	5	0.25	50	0.8	2	16
	10^4	5	0.5	50	0.9	2	362
	10^5	5	0.5	50	8	2	≥512
	10^6	5	4	50	232	2	≥512

[a] Class I and class II strains were exclusively ampicillin-resistant *E. coli* strains. The two class III strains were *S. dublin* HK246 and *K. pneumoniae* HK212. Italicized inocula represent those recommended by the National Committee for Clinical Laboratory Standards (20).

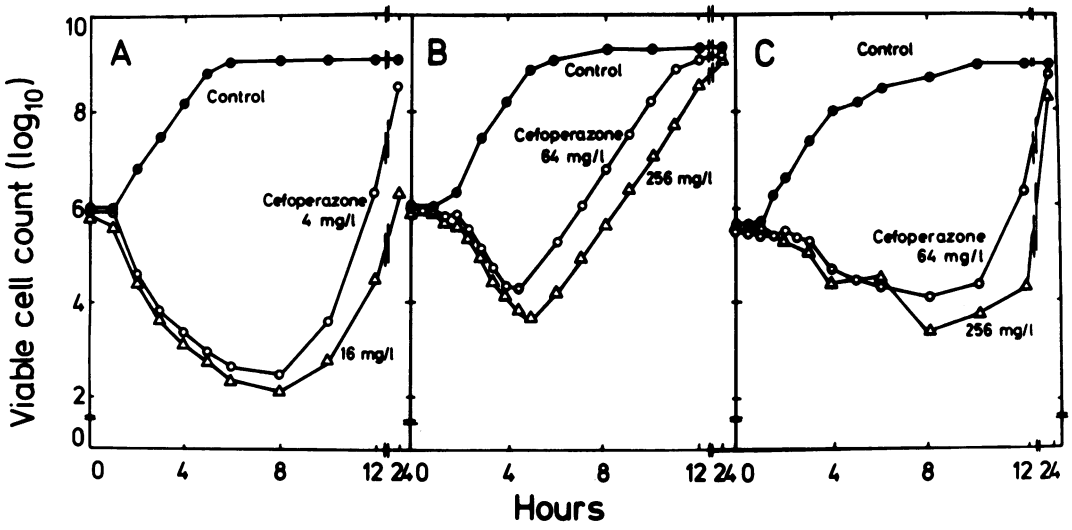

FIG. 1. Growth of bacteria of classes I, II, and III with and without cefoperazone. (A) *E. coli* HK407 (class I); (B) *E. coli* HK288 (class II); (C) *S. dublin* HK246 (class III).

TEM β-lactamase production. The four class I strains were not examined for the presence of R-plasmids coding for TEM β-lactamase.

We now had to explain why some TEM β-lactamase-producing, gram-negative strains were highly resistant to cefoperazone and to cefamandole in agar as well as in broth dilution, others were resistant only in broth dilution, and others were resistant in neither test. For this purpose we compared the amounts of TEM β-lactamase produced. The last column of Table 6 gives β-lactamase activity averaged from three determinations. These data show that the four class I *E. coli* strains produced less than 1/10 of the amount of enzyme produced by the four class II *E. coli* strains. *K. pneumoniae* HK212 and *S. dublin* HK246 produced approximately the same amounts of enzyme as the class II *E. coli* strains did, although these two strains belonged to class III. Transfer of the R-plasmids of these strains into *E. coli* K-12 yielded transconjugants (HK281 and HK319) which made amounts of TEM similar to those made by the donor strains, but which were typical class II strains.

DISCUSSION

The present investigation raises a question not new in susceptibility testing: What is the method

TABLE 5. Destruction of cefoperazone in broth cultures of *E. coli* HK407 (class I), *E. coli* HK288 (class II), and *S. dublin* HK246 (class III)

Culture	Cefoperazone concn (mg/liter)[a] at:					
	0 h	2 h	4 h	6 h	8 h	10 h
E. coli HK407	4	3.9	3.8	3.2	2.2	<2
	16	17.3	14.5	12.7	4.5	3.2
	64	64	56	48	41	36
E. coli HK288	16	13.6	4.8	<2	<2	<2
	64	58	31	<2	<2	<2
	256	152	108	<2	<2	<2
S. dublin HK246	16	13	9.5	<2	<2	<2
	64	47.5	42.5	15.5	<2	<2
	256	250	120	90	37.5	3.2
Control	64	60	58	55	58	52

[a] Residual concentration measured by a microbiological assay after incubation of 5×10^5 organisms per ml in Mueller-Hinton broth for the indicated times at 37°C. The control shows the spontaneous decay of the drug.

TABLE 6. MICs of cefoperazone and cefamandole for TEM β-lactamase-positive and TEM β-lactamase-negative strains and amounts of β-lactamase produced

Class	Bacterial strain	Plasmid	MIC (mg/liter)				β-Lactamase activity (nanokatals)
			Cefoperazone		Cefamandole		
			Agar	Broth	Agar	Broth	
I	*E. coli*						
	HK407	ND[a]	0.25	16	1	16	6
	HK427	ND	0.25	8	4	1	17
	HK438	ND	0.25	4	2	4	8
	HK442	ND	0.25	8	2	8	17
II	*E. coli* K-12						
	HK215	pR6K	0.25	256	1	256	154
	HK281	pFK1	4	>256	16	256	736
	HK288	RP4	4	>256	8	>256	691
	HK319	pFK17	2	128	8	128	340
III	*K. pneumoniae* HK212	pFK1	>256	>256	>256	256	461
	S. dublin HK246	pFK17	256	256	128	256	424
	K. pneumoniae HK198	Cured	<0.1	<0.1	0.5	<0.1	
	S. dublin HK313	Cured	0.25	<0.1	0.25	<0.1	
	E. coli K-12	None	<0.1	<0.1	0.25	<0.1	

[a] ND, Not determined.

of choice for obtaining clinically relevant susceptibility results? Recently, the National Committee for Clinical Laboratory Standards proposed methodologies for agar and broth dilution tests (20). By using these procedures in cefoperazone determinations of MICs, 49 of 100 ampicillin-resistant *E. coli* strains collected from hospitalized patients were categorized as resistant in agar dilution and susceptible in broth dilution tests. Results obtained with the disk diffusion test closely paralleled those of the agar dilution procedure and thus also differed significantly from the results obtained by broth dilution. This is not a minor problem, because *E. coli* is the most frequent pathogen isolated from hospitalized patients and strains are often resistant to ampicillin. In 1980 the incidence of *E. coli* hospital isolates among gram-negative enterobacteria, staphylococci, and enterococci in the Zurich, Switzerland, area was 28%, 33% of the more than 5,500 strains examined were resistant to ampicillin (16). In the United States the respective incidence in 1977 was 27%, and the percentage of resistance was 26 (2). Thus, the problem described could affect a considerable number of bacterial pathogens.

Discrepancies in the results obtained by using various methods of susceptibility testing of cephalosporins also have been observed by other authors. Greenwood and O'Grady (9) compared the activities of cephalothin and cephaloridine against ampicillin-resistant, gram-negative bacilli in a turbidimetric system and in conventional MIC testing on agar. They observed overnight regrowth in the turbidimetric system by many strains susceptible to the drugs in agar dilution. Findell and Sherris (7), Adams et al. (1), and Rylander et al. (23) have reported higher MICs of cefamandole for enterobacteria in conventional broth tests than in agar tests. Antibiotic destruction as well as high mutation rate to resistance were mentioned as possible causes of the discrepancies. For cefoperazone, Peddie et al. (21) also observed a difference between results obtained with the Stoke disk diffusion method and those obtained with a tube dilution procedure.

From the following information from previous enzymatic studies and data in this investigation, it can be concluded that the amount of TEM β-lactamase produced by ampicillin-resistant, gram-negative bacteria can play an important role in the expression of resistance to cefoperazone in vitro: (i) It has been shown that cefoperazone is rather unstable against TEM enzymes (19); (ii) we detected no β-lactamase other than TEM β-lactamase in the strains examined; (iii) representative class I isolates produced 10 to 100 times less of the β-lactamase than did class II cultures; and (iv) cefoperazone was more rapidly and more completely inactivated in cultures of the class II *E. coli* strain HK288 than in cultures of the class I strain HK407.

Gram-negative bacteria of class III, i.e., the *Klebsiella* and the *Salmonella* strains, produced the same amount of TEM β-lactamase as did class II *E. coli* strains. Probably, some additional factors, such as the ability of these drugs to

penetrate through the outer membrane or the location of the enzyme in the cell wall, contributed to the class III phenotype in these species.

Few data are available correlating different in vitro test results for cephalosporins with in vivo results. Recently, the efficacies of cefamandole and cefoxitin were compared in rats with acute or chronic pyelonephritis caused by a TEM β-lactamase-producing *E. coli* and its isogenic plasmid-free strain (18). It was found that the β-lactamase-producing *E. coli* was cleared more slowly from the kidney by treatment with cefamandole than was the nonproducer. The difference was not observed in animals treated with cefoxitin, a drug resistant to TEM β-lactamase. However, this says little about the value of cephalosporins with reduced resistances to TEM β-lactamase in infections caused by TEM β-lactamase producers in humans. As we have shown, there is initial killing of organisms of all three classes during the first 4 to 10 h of growth, even by subinhibitory concentrations of cefoperazone (see Fig. 1). This might be sufficient to result in a cure. In studies on the in vitro effects of cephalothin and cephaloridine, Benner et al. (4) demonstrated a marked inoculum effect and isolated resistant variants of *Klebsiella*. Despite this, these agents have proved useful therapeutically in *Klebsiella* infections. Nevertheless, it has been shown that certain infections in seriously ill patients, caused by organisms producing inactivating enzymes, were resistant to treatment with cefamandole (25). Either resistance in the causative organisms developed during therapy, or the agents showed susceptibility in the disk test but were resistant in tube dilution. For cefoperazone, combined clinical and laboratory studies are needed to reveal the clinical relevance of the phenomenon shown by class II gram-negative bacteria.

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