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Biological Properties of Three 3-Heterocyclic-Thiomethyl Cephalosporin Antibiotics

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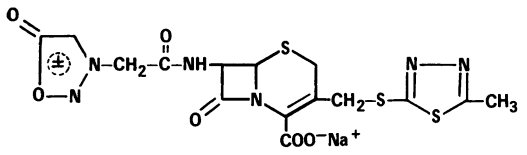
Three new cephalosporin antibiotics, prepared by substitution of heterocyclic groups on 7-aminocephalosporanic acid, possess certain desirable chemical or biological properties. All three compounds are active in vitro against a variety of gram-positive and gram-negative bacteria. Minimal inhibitory concentrations (MIC) of these bactericidal antibiotics were not significantly affected by changes in *p*H or NaCl content of nutrient broth, or by the use of different inoculum sizes. However, agar-dilution MIC values were generally two- to fourfold lower than the MIC values in comparable broth-dilution tests. Stability to cephalosporinase by two of the compounds extended their antibacterial spectra over cephalothin and cephaloridine to include strains of *Enterobacter* sp. and indole-positive *Proteus* sp. Binding to serum proteins of the new cephalosporins was intermediate between cephalothin and cephaloridine. Excellent concentrations of the antibiotics were attained in mouse blood, after subcutaneous administration of 20 mg per kg. In vitro biological characteristics of the antibiotics were verified by successful therapy of experimental mouse infections. Regression lines were calculated to show the correlation of agar-dilution MIC values with zones of inhibition by the disc testing procedure. Because each of the three new cephalosporins has certain advantageous properties over cephalothin and cephaloridine, additional toxicological and pharmacological data should be obtained for all three compounds.

The clinical efficacy of parenterally administered cephalosporin antibiotics, cephalothin and cephaloridine, has been well established. However, certain problems occur during therapy with these antibiotics. For example, because of pain on intramuscular injection, cephalothin is usually administered intravenously. Cephaloridine possesses excellent antibacterial activity and has low toxicity, but is associated, rarely, with an adverse effect on renal function. New cephalosporin antibiotics, for which laboratory data have provided evidence for possible advantages over cephalothin or cephaloridine, may prove to be clinically useful compounds. Three new cephalosporin antibiotics, prepared from 7-aminocephalosporanic acid by substitution of heterocyclic groups (heterocyclic cephalosporins), do possess certain chemical and biological properties that make them candidates for clinical evaluation. One of these antibiotics, cefazolin, has been investigated extensively in Japan (10, 12–15, 19). This communication summarizes the data from a laboratory study with cefazolin and two chemically related cephalosporin antibiotics. The chemical names for the three new compounds (i, ii, iii) shown in Fig. 1 are, respectively, 3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone) acetamido]-3-cephem-4-carboxylic acid, sodium salt; 7-D-mandelamido-3-(1-methyl-1H-tetrazol-5-ylthiomethyl)-3-cephem-4-carboxylic acid, sodium salt; and 3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(1H-tetrazol-1-yl)acetamido]-3-cephem-4-carboxylic acid, sodium salt. A generic name has not been assigned for the "mandelic-tetrazole" derivative (ii), thus, this antibiotic will be called "CMT."

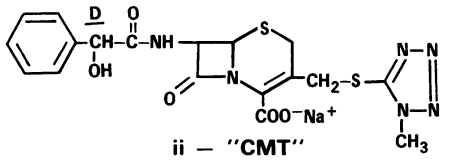
MATERIALS AND METHODS

Antibiotics. Sodium cephalothin and cephaloridine were employed for these experiments. The three new heterocyclic cephalosporin antibiotics used, cefazolin, cephanone, and CMT, were prepared in The Lilly Research Laboratories by W. B. Blanchard and C. W. Ryan.

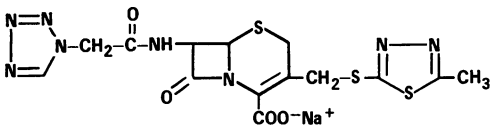
Bacterial cultures. Clinical isolates of diverse bacterial genera, both gram-positive and gram-negative, were employed. When several isolates of one micro-



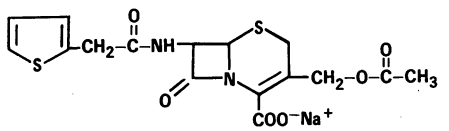
i — Cephanone (CSY)



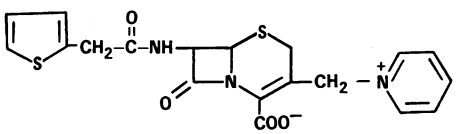
ii — "CMT"



iii — Cefazolin (CEZ)



iv — Cephalothin (CET)



v — Cephaloridine (CER)

FIG. 1. *Chemical structures of cephanone, "CMT," cefazolin, cephalothin, and cephaloridine.*

bial genus were used, each culture was shown to be of a different strain by phage typing, serology, biochemical reactions, or antibiotic susceptibility spectra.

Disc susceptibility testing. Strains of bacteria were tested for susceptibility to the antibiotics by the standardized disc technique described by the Food and Drug Administration (5). This procedure is a modification of the Bauer-Kirby (or Kirby-Bauer) disc susceptibility testing method (2).

MIC determinations. Broth-dilution or agar-dilution procedures were used to determine antibiotic minimal inhibitory concentrations (MIC) for all strains of bacteria. For aerobic gram-positive rods, staphylococci, pseudomonads, and *Enterobacteriaceae*, the agar-dilution method of the International Collaborative Study (ICS) as described by Ericsson and Sherris (6) was used. Mueller-Hinton agar (BBL) was the medium employed, and the plates were inoculated with a device similar to the Steers' replicator (20).

Modifications of broth- or agar-dilution methods were made to determine MIC values for fastidious aerobic or anaerobic bacteria. Trypticase Soy Broth (BBL) was used for *Corynebacterium* sp., *Streptococcus* sp., and *Diplococcus pneumoniae*. When strains of streptococci or pneumococci were tested, 5% defibrinated rabbit blood was added to the broth. Final inocula of 10^5 bacteria per ml of broth were used in these tests. For *Neisseria* sp. and *Haemophilus* sp., the ICS agar-dilution method was modified by using Trypticase Soy Agar with 5% rabbit blood and 1% IsoVitaleX (BBL). The agar was chocolatized for *Haemophilus* sp. Inoculated plates were incubated in a 10% CO_2 atmosphere. For *Bacteroides fragilis, Fusobacterium* (*Sphaerophorus*) *necrophorus*, and the clostridia, fluid thioglycolate medium was utilized, and the tubes were inoculated with one drop from overnight broth cultures.

MIC values for all procedures described above were determined after overnight incubation at 37 C.

Correlation of MIC values with disc test results. Zone diameters obtained with discs containing 30 μg of antibiotic were plotted against MIC values. The experimentally determined points fell along a regression line, which was calculated by the method of least squares.

Effects of variations of pH, inoculum size, or NaCl content of media on antibacterial activity. The broth-dilution susceptibility test was used to assess the effect on activities of the antibiotics caused by variations in media *p*H, size of inoculum used, or NaCl content of the broth. Nutrient broth (Difco) was the medium employed. Variations in medium *p*H were accomplished by preparing the media with phosphate buffer solutions (Harleco, Na^+, K^+ phosphate salts) instead of water. To determine the effects of NaCl on the MIC, various concentrations of NaCl in broth were utilized. The inoculum size used for the *p*H or NaCl studies was 10^4 bacteria per ml of broth. Inocula sizes of 10^3, 10^4, 10^5, and 10^6, in unmodified broth, were used to demonstrate any inoculum effects on MIC values.

Bactericidal activity. Two procedures were used to detect bactericidal activity of the antibiotics examined. In one of these methods, the surviving bacteria in all clear tubes of the broth-dilution MIC series were counted. A 1-ml sample from a 10-fold dilution of each tube was placed in 20 ml of melted Trypticase Soy Agar and poured into a 100-mm petri dish. After incubation for 48 hr at 37 C, the colonies were counted. Reduction of the original inoculum by 99.9% or more was considered to define bactericidal activity in the corresponding tube of the MIC test.

A second method used to estimate bactericidal activity originated from the cellophane transfer technique of Chabbert (3). However, instead of cellophane, 25-mm HA (0.45 μm) black membrane filters (Millipore Corp., Bedford, Mass.) were used. Antibiotic was incorporated in Mueller-Hinton agar to give final concentrations in a $\log_2$ dilution series. Filter discs were placed on the surface of the agar, and each filter was then seeded by pipetting one drop from an appropriate suspension of a bacterial culture. A glass rod was used to spread the bacterial suspensions over the surfaces of the discs. The plates were then incubated at 37 C. To determine the number of viable cells applied to each disc, an agar-plate count was made of the organisms contained in a drop delivered by the pipette.

After contacts for various periods of time at 37 C, the inoculated discs were transferred to plates containing antibiotic-free agar. To insure that no residual antibiotic remained, discs were transferred via two intermediate antibiotic-free agar plates. All plates to which discs were transferred were incubated at 37 C during the entire transfer time.

An MIC was determined from the filters that remained on the original antibiotic plates for 24 hr. The MIC was the lowest level of antibiotic that inhibited visual growth of the culture. Discs with no growth were then transferred, and incubation was continued. After sufficient incubation for visible colony formation, the colonies that grew on the surface of each filter were counted. Each colony represented a surviving organism, and the number of survivors was compared with the number of bacteria used to inoculate the membrane filter. From these data, bactericidal activity of the antibiotics was determined; i.e., the minimal bactericidal concentration (MBC) was obtained from the discs that were transferred to the antibiotic-free plates at 24 hr, and was the concentration of antibiotic that killed $\geq$99.9% of the inoculated bacteria. This MBC was compared with the MIC that was determined from the same series of discs at 24 hr. In addition to the MBC, the rate of bactericidal activity could be estimated by counting colonies on discs that were transferred to antibiotic-free agar at incubation times of less than 24 hr.

Microbiological assays. Disc-plate assays with *Sarcina lutea* strain PCI-1001-FDA or *Bacillus subtilis* strain ATCC 6633 were used. The greatest sensitivity was obtained by using the *B. subtilis* assay with Antibiotic Medium 5 (Difco) at *p*H 8.0 for cephalothin, the *B. subtilis* assay with the agar adjusted to *p*H 6.0 for the heterocyclic compounds, and the *S. lutea* assay with agar at *p*H 8.0 for cephaloridine.

Concentration of antibiotic in mouse blood or urine. The disc-plate assays described above were utilized to determine antibiotic concentration in mouse blood or urine. All antibiotics were administered to mice subcutaneously at equal doses of 20 mg per kg of body weight. The blood was collected from the orbital sinus in heparinized hematocrit tubes, which were allowed to fill by capillary action (23). Paper discs (6.35 mm, Schleicher & Schuell Co., Inc.) were saturated with blood and immediately placed on inoculated assay plates. Urine specimens were diluted in saline and assayed as soon as they were collected.

Chemical stability studies. The stability of the cephalosporin antibiotics was studied by incubating solutions of the antibiotics at 4, 25, or 37 C. Samples were withdrawn at intervals and immediately frozen with alcohol and dry ice. After all samples were collected, they were assayed by the assay procedures described above.

Metabolism of antibiotics. To assess the extent of metabolic degradation of the antibiotics, the paper chromatographic method of Hoehn and Pugh (9) was employed. Urine was collected from mice at 2 hr after subcutaneous administration of 20 mg of antibiotic per kg. The specimens were kept frozen until processed. Developed Whatman no. 1 sheets were treated for bioautography on *B. subtilis* plates.

Stability studies with bacterial enzymes. β-Lactamase enzymes (penicillinase and cephalosporinase) were used to evaluate stability of the antibiotics to enzymatic degradation. The enzymes were derived from several bacterial species, including *B. cereus, Staphylococcus aureus, Enterobacter* sp., *Proteus* sp., *Serratia* sp., and *Pseudomonas* sp. Penicillinase (Riker) was the only highly purified enzyme used; enzymes from other organisms were only partially purified preparations. Activity of the enzymes against each of the five cephalosporins was measured by a previously described assay method (21, 24).

Effect of human sera on antibacterial activity. The percentage of antibacterial activity bound by human serum was estimated by comparing serum and buffer standard curves from assays described above.

Therapy of experimental infections. The in vivo efficacy of the antibiotics was studied by therapy of experimental infections in mice. Groups of eight white mice (11 to 13 g) were treated subcutaneously at various times after intraperitoneal bacterial challenge. Antibiotics were usually administered at 1 and 5 hr post-infection; however, in some experiments, other dosage regimens were employed. Deaths and survivors were recorded for a period of seven days. The antibiotic dose effective in curing 50% of the infected mice (ED_{50}) was calculated by the method of Reed and Muench (16).

RESULTS AND DISCUSSION

The in vitro antibacterial activities of five cephalosporin antibiotics against representative gram-positive bacteria and *Neisseria* sp. are shown in Tables 1 and 2. All five of the compounds (Fig. 1) were active against strains of *Staphylococcus* sp., *Streptococcus* sp. (Viridans and A groups), *Diplococcus pneumoniae, Clostridium* sp., *Corynebacterium* sp., and *Neisseria* sp. (Table 1). In some instances, the three heterocyclic derivatives were slightly less active than cephalothin or cephaloridine.

Barber (1) and Chabbert (4) have shown that the addition of 5% NaCl to Trypticase Soy media provides conditions permitting the resistant cells within the heterogeneous population of methicillin-resistant *S. aureus* cultures to express their resistance. When this procedure was used, three of the five cephalosporin antibiotics shown were more active than methicillin itself against this type of *S. aureus* culture (Table 2). Nevertheless, a certain amount of "multiple resistance" between methicillin and all of these cephalosporins was evident.

Against gram-negative bacteria, CMT was the most active of the heterocyclic cephalosporins, followed in order by cephanone and cefazolin (Table 3). Of particular interest was the inhibition of certain cultures of *Enterobacter* sp. and *Proteus* sp. by CMT and cephanone. Inhibition of these organisms is very likely a result of increased stability to cephalosporinase. This possibility was

TABLE 1. *Susceptibility of representative gram-positive bacteria and Neisseria sp. to five cephalosporin antibiotics*

Organism	Special identity of culture	Minimal inhibitory concn (μg/ml)[a]				
		CEZ	CMT	CSY	CER	CET
Clostridium perfringens						
CP23	—	0.06	<0.03	0.12	0.5	0.25
CP24	—	0.12	0.06	0.12	0.5	0.5
CP25	—	0.12	0.5	0.12	0.5	0.5
C. tetani CT 1	Harvard H-12AW	0.06	<0.03	<0.03	<0.03	0.06
Corynebacterium diphtheriae						
CD 1	mitis	2.0	1.0	1.0	0.06	1.0
CD 2	gravis	2.0	1.0	1.0	0.06	1.0
Diplococcus pneumoniae						
DP 1	Type I	0.12	0.25	0.12	0.06	0.12
DP 2	Type II	0.12	0.12	0.12	0.03	0.12
DP14	Type XIV	0.25	0.25	0.25	0.06	0.25
Staphylococcus aureus (benzylpenicillin-susceptible)						
V92	Phage: 47, 53, 59, 73, 75	0.5	1.0	1.0	0.06	0.25
V104	29, 52, 52A, 6, 47, 54, 75, 80, 42B	0.25	0.25	0.25	<0.03	0.25
SS7	77, VA4, (29, 53)	0.25	0.25	0.25	<0.03	0.25
S. aureus[b] (penicillinase-producing, and methicillin-susceptible)						
H43	42B, 81	0.5	1.0	1.0	0.12	0.5
V57	42B, 52, 80, 81	0.5	0.5	1.0	0.12	0.5
H356	52A, 79	0.5	0.5	0.5	0.06	0.5
S. epidermidis						
3064	Benzylpenicillin-resistant	0.5	0.5	0.5	0.06	0.5
3078	Benzylpenicillin-susceptible	0.25	0.25	0.25	<0.03	0.12
3079	Benzylpenicillin-susceptible	0.25	0.25	0.25	<0.03	0.12
Streptococcus pyogenes (group A)						
C203	—	0.12	0.03	0.03	0.008	0.06
12385	ATCC culture	0.12	0.06	0.06	0.008	0.12
10389	ATCC culture	0.12	0.06	0.06	0.008	0.06
Streptococcus sp. (Viridans group)						
9943	—	0.5	0.5	0.5	0.06	0.5
9961	—	1.0	0.5	0.5	0.03	0.5
K7	—	0.5	1.0	0.25	0.25	0.5
Streptococcus sp. (group D)						
9901	—	32	32	16	16	32
9960	—	32	32	16	16	16
Neisseria gonorrhoeae 5GH	—	0.25	0.125	0.06	4.0	1.0
N. meningitidis OS	—	0.25	0.125	0.03	4.0	0.5

[a] See Materials and Methods for procedures used to determine minimal inhibitory concentrations. The abbreviations used for the antibiotics are spelled out in Fig. 1.

[b] Data for "methicillin-resistant" *S. aureus* are given in Table 2.

TABLE 2. *Activities of methicillin and five cephalosporin antibiotics against "methicillin-resistant" S. aureus isolates*

S. aureus isolate[a]	Minimal inhibitory concn (μg/ml)[b]											
	Methicillin		CEZ		CMT		CSY		CER		CET	
	0.5%[c]	5.0%[c]	0.5%	5.0%	0.5%	5.0%	0.5%	5.0%	0.5%	5.0%	0.5%	5.0%
3130	8	128	64	>128	8	32	32	128	4	8	8	64
3131	8	128	8	>128	4	32	16	128	1	16	1	32
3132	8	32	8	>128	4	32	4	128	2	2	2	16
3133	8	128	32	>128	8	32	32	128	4	16	2	64
3134	8	64	64	>128	16	32	64	128	8	16	8	64
3135	8	64	8	>128	4	32	8	128	1	8	2	32
3137	>128	>128	>128	>128	32	64	128	>128	8	32	64	128
3138	16	>128	128	>128	16	32	64	128	8	16	8	64
3139	8	>128	32	128	8	16	32	64	1	4	1	32
966	8	64	32	>128	8	64	16	128	0.25	0.25	1	32
561	8	128	64	>128	8	64	32	128	4	16	2	64
3055	1	2	0.25	0.5	0.5	0.25	0.5	0.25	0.03	0.06	0.25	0.5
H232	2	2	1	1	0.25	1	0.25	1	0.06	0.06	0.5	0.5

[a] All cultures except 3055 and H232 are "methicillin-resistant." Culture 3055 is sensitive to both methicillin and benzylpenicillin; H232 is sensitive to methicillin but produces penicillinase.

[b] Minimal inhibitory concentrations were determined by the agar-dilution method. The abbreviations used for the antibiotics are spelled out in Fig. 1.

[c] NaCl content in Trypticase Soy Agar.

studied, and the results of the experiments are presented later.

The MIC of the cephalosporin antibiotics for 16 fresh clinical isolates of *H. influenzae* was compared with the MIC of ampicillin. In vitro susceptibility of *Haemophilus* sp. differs considerably with variations in methodology, even when the same test is replicated. For this reason, the meaning of an MIC value for a *Haemophilus* culture is difficult to determine; i.e., prediction of clinical efficacy of an antibiotic in therapy of infections caused by these organisms should not be made on the basis of an MIC value alone. However, ampicillin is used in humans for therapy of *Haemophilus* infections, and MIC values for this antibiotic by the method used ranged from 0.12 to 0.5 μg per ml. All of the cephalosporin antibiotics were less active in vitro than ampicillin, but the MIC values of CMT (0.12 to 1.0 μg/ml) approached those of ampicillin.

Except for cephalothin, no significant changes in MIC values were obtained with variations of the *p*H of nutrient broth, or with different inoculum sizes employed. *Escherichia coli* MIC values were twofold lower when 2% NaCl was added to the broth; however, media that are commonly used for susceptibility testing contain less than 1% NaCl. Differences in MIC observed for cephalothin are probably a result of chemical changes during incubation (17, 22). Unlike the other four cephalosporins (Fig. 1), cephalothin possesses an acetyl group that can be removed by hydrolysis. Variations in the degree of deacetylation of cephalothin that occur with different test procedures can account for changes in MIC values. At temperatures less than 37 C, all five antibiotics were stable for at least 24 hr.

The MIC values of five cephalosporin antibiotics for an *E. coli* and an *S. aureus* culture, obtained with the use of several media, are presented in Table 4. Final inocula of 10^4 bacteria per ml were used for the broth tests. This inoculum size was particularly important for cephalothin because of the deacetylation problem (discussed above). Nevertheless, the highest MIC values were for cephalothin with Brain Heart or Heart Infusion broth. Values for all antibiotics tested in either broth or agar media were consistent against the *S. aureus* culture. However, except for cephaloridine, agar-dilution results with the *E. coli* strain were two- to fourfold lower than in each comparable broth. Variables other than type of media were minimized in this experiment because all tests were performed by the same technician, on the same day, with the same starting cultures. The variation in MIC values for the different media or methods is evidence of the need for a standardized method with which to compare results with the diverse procedures used for susceptibility testing. Greater end-point stability and re-

TABLE 3. *Susceptibility of representative gram-negative bacteria to five cephalosporin antibiotics*

Organism	Special identity of culture	Minimal inhibitory concn (μg/ml)[a]				
		CEZ	CMT	CSY	CER	CET
Bacteroides sp.						
B-1	—	4.0	8.0	2.0	4.0	8.0
B-2	—	2.0	2.0	1.0	2.0	4.0
Citrobacter freundii CF17		>128	>128	>128	>128	>128
Enterobacter sp.						
EB 5	cloacae	>128	8.0	128	>128	>128
EB 17	aerogenes	8.0	1.0	1.0	32	16
Escherichia coli						
EC 9	Variant *E. coli*	4.0	1.0	2.0	4.0	32
EC31	O11a, O111b, B4NM	2.0	1.0	1.0	2.0	8.0
EC35	O127:B8	2.0	1.0	1.0	2.0	16
EC38	O55:B5	2.0	0.5	0.25	2.0	4.0
Fusobacterium necrophorus FN 1	—	1.0	1.0	0.5	16	0.5
Haemophilus influenzae						
HI 1[b]		4.0	0.5	4.0	4.0	1.0
HI 2[b]		2.0	0.5	2.0	2.0	0.5
Herellea sp. HE28	—	>128	32	>128	>128	>128
Klebsiella pneumoniae						
KL 3[b]		2.0	0.5	0.5	2.0	1.0
KL25[b]		2.0	1.0	1.0	4.0	2.0
Mima polymorpha MP 1	—	1.0	0.25	0.25	1.0	0.5
Proteus mirabilis						
PR 6[b]		8.0	1.0	4.0	8.0	4.0
PR10[b]		8.0	2.0	8.0	8.0	8.0
P. morganii						
PR 1[b]		>128	1.0	64	>128	>128
PR15[b]		>128	1.0	64	>128	>128
P. rettgeri						
PR 7[b]		64	0.5	2.0	>128	>128
PR 9[b]		32	2.0	32	128	>128
P. vulgaris						
PR27[b]		128	32	64	>128	>128
PR28[b]		64	8.0	32	>128	>128
Pseudomonas aeruginosa						
PS 9	Gentamicin-susceptible	>128	>128	>128	>128	>128
PI25	Gentamicin-resistant	>128	>128	>128	>128	>128
P. pseudomallei PS121	Viet Nam isolate	>128	64	>128	>128	>128
Salmonella sp.						
SA 4	typhimurium	4.0	1.0	4.0	4.0	4.0
SA 12	typhosa	4.0	1.0	4.0	4.0	4.0
Serratia sp. (non-pigmented)						
SE 3	—	>128	>128	>128	>128	>128
SE 7	—	>128	>128	>128	>128	>128
Shigella sp.						
SH 3	flexneri 2a	8.0	2.0	4.0	8.0	8.0
SH10	sonnei I	8.0	2.0	4.0	8.0	8.0

[a] See Materials and Methods for procedures used to determine minimal inhibitory concentrations. The abbreviations used for the antibiotics are spelled out in Fig. 1.

[b] Isolates judged as different strains because of differences in drug susceptibility spectra.

TABLE 4. *Minimal inhibitory concentrations of five cephalosporin antibiotics with several different growth media*

Medium[b]	Minimal inhibitory concn (μg/ml)[a]									
	Escherichia coli EC38					*Staphylococcus aureus* H43				
	CEZ	CMT	CSY	CER	CET	CEZ	CMT	CSY	CER	CET
Mueller-Hinton (BBL)										
Broth	4.0	0.5	0.5	4.0	4.0	1.0	1.0	0.5	0.12	0.25
Agar	1.0	0.25	0.5	4.0	1.0	0.5	0.5	0.5	0.12	0.25
Trypticase Soy (BBL)										
Broth	4.0	1.0	1.0	4.0	8.0	1.0	1.0	0.5	0.12	0.25
Agar	1.0	0.5	0.5	4.0	4.0	0.5	1.0	0.5	0.25	0.25
Brain Heart Infusion (Oxoid)										
Broth	4.0	1.0	1.0	4.0	16.0	1.0	1.0	1.0	0.12	0.5
Agar	1.0	0.5	0.5	4.0	4.0	1.0	1.0	1.0	0.25	1.0
Heart Infusion (Difco)										
Broth	4.0	2.0	1.0	4.0	16.0	1.0	1.0	1.0	0.12	0.5
Agar	1.0	0.5	0.5	4.0	2.0	0.5	1.0	0.5	0.25	0.25
Sensitivity Test (Oxoid)										
Broth	4.0	0.5	1.0	4.0	8.0	0.5	0.5	0.5	0.12	0.25
Agar	1.0	0.5	0.5	8.0	4.0	0.5	0.5	0.5	0.25	0.25
Nutrient (Difco)										
Broth	4.0	0.5	1.0	4.0	8.0	0.5	1.0	0.5	0.12	0.25
Agar	1.0	0.5	0.5	8.0	2.0	0.5	0.5	0.25	0.25	0.12
Nutrient (Difco) with 0.5% NaCl added										
Broth	4.0	0.5	1.0	4.0	8.0	0.5	0.5	0.5	0.12	0.12
Agar	1.0	0.5	0.5	4.0	2.0	0.25	0.5	0.25	0.25	0.12

[a] Inocula for broth-dilution tests were 10^4 cells per ml. For the agar-dilution tests, each drop applied to the agar surface contained 5,000 organisms.

[b] For agar medium, 1.5% agar (Difco) was added to each corresponding broth.

producibility of MIC values with the agar-dilution method make it the preferred technique (18). This does not imply that other methods such as broth-dilution, streak-plate, gradient-plate, or microtiter procedures are in error. However, to evaluate susceptibility data from different laboratories, a comparison of MIC values should be made with results obtained by use of the ICS agar-dilution technique that is described by Ericsson and Sherris (6). In Table 4, the ICS values are those shown for Mueller-Hinton agar. Additional standardization is possible by using ICS agar-dilution results for preparing regression lines correlating MIC values with zone diameters by the disc method (discussed later).

Viable-cell counts from broth-dilution tests show that the cephalosporin MIC values for gram-positive and gram-negative bacteria were usually the bactericidal levels also. This bactericidal activity was confirmed by the more elaborate "filter-transfer" technique, performed with the two representative organisms (Table 5). With this latter method, the killing of *E. coli* became evident after 3 hr, but activity against *S. aureus* was somewhat delayed.

All five cephalosporin antibiotics were resistant to degradation by penicillinase derived from either *B. cereus* or *S. aureus* (Table 6). Cephaloridine, cefazolin, and cephanone were slightly affected only by the high concentrations of the enzymes used, but cephalothin and CMT were essentially unchanged.

Antibiotic CMT was very stable to cephalosporinases derived from several gram-negative bacilli, and cephanone was more stable than cephalothin (Table 7). Resistance of *Enterobacter* sp. and indole-positive *Proteus* sp. to cephalothin may be due to cephalosporinase production by these bacteria. All cultures from these two genera that were tested (Table 7) were susceptible to CMT, which was also stable to enzymes produced by the same organisms. Both CMT and cephanone were more stable than cephalothin to β-lactamase from *Serratia* sp. or *Pseudomonas* sp., but these particular isolates were still resistant to the antibiotics by other mechanisms. In 1963, Fleming and his associates (7) stated, "The varying activity of the enzyme against different cephalosporins suggests the possible development of cephalosporinase-resistant cephalosporins." Additional

TABLE 5. *Summary of data obtained by the membrane filter technique for determining bactericidal activities of five cephalosporin antibiotics*

Representative bacterium	Antibiotic	24-hr MIC (μg/ml)[a]	24-hr MBC (μg/ml)[b]	Reduction of inocula by concn equal to 24-hr MBC			
				3 hr	6 hr	12 hr	24 hr
				%	%	%	%
Escherichia coli (strain EC38)	Cefazolin	1.0	1.0	NE[c]	98.5	99.6	99.9
	CMT	1.0	1.0	97.7	99.7	99.9	>99.9
	Cephanone	0.5	0.5	NE	98.6	>99.9	>99.9
	Cephaloridine	4.0	4.0	98.6	99.6	99.8	>99.9
	Cephalothin	8.0	8.0	NE	99.6	99.9	>99.9
Staphylococcus aureus (strain H43)	Cefazolin	0.5	0.5	NE	NE	90	99.9
	CMT	0.5	0.5	NE	NE	90.2	>99.9
	Cephanone	0.5	0.5	NE	NE	96.9	99.9
	Cephaloridine	0.12	0.12	NE	NE	96.7	>99.9
	Cephalothin	0.25	0.25	NE	NE	90.5	99.9

[a] MIC = lowest concentration of antibiotic to which there was no visible growth on the filter after 24 hr of incubation.

[b] MBC = lowest concentration of antibiotic that killed ⩾99.9% of the inoculated bacteria in 24 hr.

[c] NE = the bactericidal effect could not be determined because the number of colonies on the filter discs were too numerous to count.

TABLE 6. *Stability of cephalosporin antibiotics to penicillinase*

Antibiotic	Amt of antibiotic degraded in 1 hr[a]	
	Penicillinase from *B. cereus*[b]	Penicillinase from *S. aureus*[c]
	μg	μg
Benzylpenicillin	>192	>192
Cefazolin	2	10
Cephaloridine	10	5
Cephanone	1	5
CMT	0.5	1
Cephalothin	0	0

[a] All antibiotics were tested against an amount of enzyme sufficient to degrade 320 units (192 μg) of benzylpenicillin.

[b] Penicillinase (Riker).

[c] The staphylococcal penicillinase used was an acetone-precipitated, water-dialyzed preparation from a clinical isolate of *S. aureus*.

studies with CMT may verify that this derivative fits this category.

Peak concentrations of cefazolin and cephanone in mouse blood exceeded those obtained with similar doses of cephalothin or cephaloridine (Table 8). The half-lives for cefazolin (36.2 ± 3.5 min) and cephanone (29.5 ± 2.2 min) were similar to that for cephaloridine (33.8 ± 4.1 min). On the other hand, CMT concentrations in blood were about equal to those of cephaloridine, but the CMT half-life (20.5 ± 2.9 min) was as rapid as for cephalothin (15.8 ± 4.1 min).

Binding of antibacterial activity of the five antibiotics, as estimated by comparing standard assay curves prepared in either saline or human serum, followed several patterns (Table 9). The activity of cephaloridine was essentially the same in saline or serum, indicating very little binding. Binding of CMT was not as great as with cephalothin; however, the amount of binding for both of these antibiotics did not change with concentrations. Serum binding of cefazolin and cephanone was concentration-dependent, increasing as the concentration decreased. Kind et al. (11) reported binding of cephalothin and cephaloridine as 65 and 13%, respectively, in pooled human serum, by the ultrafiltration method. Nishida et al. (13), using essentially the same method, showed a higher percentage of binding for cephalothin (79%) and cephaloridine (31%), and reported cefazolin as 74%. The ratio of 79% to 74% for cephalothin and cefazolin by these latter investigators compares with the 53% to 48% ratio at the 5-μg level as shown in Table 9. However, binding of activity against different organisms can vary (8), and may not accurately predict protein binding. Therefore, additional studies with procedures other than those employed in this experiment may be necessary to predict the degrees of binding to serum proteins.

ED_{50} values for subcutaneous administration of five cephalosporins in therapy of experimental infections in mice are shown in Tables 10 and 11. To interpret properly the meaning of the two-dose ED_{50} values in Table 10, the in vitro activity, concentrations in serum, excretion rates, and meta-

TABLE 7. *Comparison of in vitro activity (MIC) of three cephalosporin antibiotics with their stability to β-lactamase (cephalosporinase) from gram-negative bacilli*

β-Lactamase derived from	Cephalothin		CMT		Cephanone	
	Enzyme potency[a]	MIC (μg/ml)	MIC (μg/ml)	Enzyme stability[b]	MIC (μg/ml)	Enzyme stability[b]
Enterobacter cloacae EB24	1:64	>128	2	64	8	16
E. aerogenes EB 6	1:128	>128	1	64	1	8
Proteus morganii PR15	1:32	>128	8	32	>128	8
P. rettgeri PR 9	1:8	128	2	16	32	4
P. vulgaris PR27	1:16	>128	32	4	64	1
Serratia marcescens Se 2	1:64	>128	32	32	>128	8
Pseudomonas aeruginosa PS 9	1:256	>128	>128	64	>128	8

[a] Enzyme potency is expressed as the greatest dilution of enzyme retaining the ability to degrade a known amount of cephalothin under the test conditions used (see Materials and Methods).

[b] Relative cephalosporinase stability compared with cephalothin, which was arbitrarily assigned the value of 1.

TABLE 8. *Concentrations of cephalosporin antibiotics in the blood or urine of mice after subcutaneous administration of 20 mg/kg*

Antibiotic	Mouse body fluid assayed	Antibiotic concn (μg/ml)						
		5 min	10 min	20 min	30 min	60 min	90 min	120 min
Cefazolin	Blood[a]	11.3	12.5	12.0	10.2	5.4	2.8	1.9
	Urine[b]	ND[c]	ND	ND	438	269	87	9
CMT	Blood	4.9	7.2	5.9	4.8	1.9	0.5	0.2
	Urine	ND	ND	ND	4,500	1,680	1,070	217
Cephanone	Blood	7.7	10.2	10.9	9.5	4.0	2.1	1.1
	Urine	ND	ND	ND	605	397	273	103
Cephaloridine	Blood	6.4	8.1	7.5	7.2	3.5	1.7	1.1
	Urine	ND	ND	ND	2,031	1,080	280	312
Cephalothin	Blood	5.1	5.8	3.9	2.6	0.5	0.2	0.1
	Urine	ND	ND	ND	1,308	573	117	50

[a] Blood antibiotic concentration values are the averages from four mice.

[b] Urine antibiotic concentration values are from pooled urine specimens.

[c] Not determined.

TABLE 9. *Estimation of antibacterial activity bound by human sera, as determined by comparing standard curves prepared in saline or serum*

Antibiotic[a]	Range of assay curves (μg/ml)	Diluent	Standard curve, $y = a + bx$		Per cent less active in serum than saline at					
			Slope (b)	Intercept (a)	0.6[b]	1.2	2.5	5.0	10	20
Cefazolin	2.5–20	Saline	5.06	9.72	ND[c]	ND	ND	48	40	31
		Serum	6.13	4.73						
CMT	0.31–10	Saline	5.20	20.12	27	28	30	31	33	ND
		Serum	4.96	18.62						
Cephanone	1.25–20	Saline	5.29	13.64	ND	ND	50	42	32	ND
		Serum	6.36	9.14						
Cephalothin	0.31–10	Saline	4.65	21.15	ND	54	53	53	52	ND
		Serum	4.72	17.55						
Cephaloridine	0.31–10	Saline	5.06	19.63	ND	15	4	0	0	ND
		Serum	5.66	18.94						

[a] See Materials and Methods for assay procedures.

[b] Concentration in micrograms per milliliter.

[c] Not determined.

TABLE 10. *Activity of five cephalosporin antibiotics on experimental bacterial infections in mice*

Bacterium	Challenge LD_{50}[a]	Subcutaneous ED_{50}[b]				
		CEZ	CMT	CSY	CER	CET
Staphylococcus aureus 3055[c]	7,750	3.1	6.3	2.9	1.0	3.1
S. aureus 3074[d]	5.1	3.8	13.3	3.3	2.0	26.2
Streptococcus pyogenes C203	1,580	0.6	0.6	0.3	<0.1	0.9
Diplococcus pneumoniae type I	630	1.6	15.7	1.4	0.9	20.7
Escherichia coli EC38	10,000	12.8	15.9	9.3	20.6	95
Klebsiella pneumoniae KL14	100	6.7	12.1	2.6	7.5	27.2
Proteus mirabilis PR6	475	9.7	6.7	3.7	8.7	11.4
P. morganii PR15	100	>166	41.5	83	>166	<166
Salmonella typhosa SA12	820	8.5	7.9	6.1	11.6	33.6
Shigella flexneri 2a SH3	1,280	19.3	18.6	14.4	13.5	144.5

[a] One LD_{50} = infecting dose of bacteria required to kill 50% of the mice.
[b] The ED_{50} value is expressed as milligrams per kilogram in two treatments (1 and 5 hr postinfection). The abbreviations used for the antibiotics are spelled out in Fig. 1.
[c] Benzylpenicillin-susceptible.
[d] Benzylpenicillin-resistant.

TABLE 11. *Multiple-dose therapy of experimental D. pneumoniae infections with five cephalosporin antibiotics*

Time of administration (hr post-infection)	ED_{50} (mg per kg per dose)[a]				
	CEZ	CMT	CSY	CER	CET
1	22.0	114	19.3	5.3	100
1, 2	1.6	15.7	1.4	0.9	20.7
1, 2, 3, 4, 5	0.3	1.7	0.2	0.2	4.2

[a] All antibiotics were administered subcutaneously. The abbreviations used for the antibiotics are spelled out in Fig. 1.

bolic patterns for all of the compounds must be compared.

Peak serum concentrations of cefazolin and cephanone were higher than those of cephaloridine, and the excretion rates were similar (Table 8). Therefore, the infecting bacteria were exposed in the animal to these antibiotics for similar periods of time; ED_{50} values (Table 10) for these three antibiotics can be compared. Cephaloridine, cefazolin, and cephanone are metabolically stable and are excreted unchanged in urine. Both cefazolin and cephanone are less active in vitro than cephaloridine against some gram-positive cocci (Table 1). Against gram-negative bacilli, the heterocyclic compounds have activities equal to or twice those for cephaloridine (Table 3). In addition, cephanone exhibits a degree of stability to β-lactamase derived from certain gram-negative bacilli (Table 7). When all of these facts about cefazolin and cephanone are considered, the expected ED_{50} values for these antibiotics would be about equal to cephaloridine against *S. aureus* and *D. pneumoniae* infections, at least twice that for cephaloridine against *Streptococcus pyogenes* infections, and one-half to about equal to those for cephaloridine in therapy of infections caused by *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Salmonella typhosa*, and *Shigella flexneri*. Cefazolin was not expected to be effective in treatment of infections caused by the β-lactamase-producing *P. morganii*, but therapy with cephanone would be successful. The data presented in Table 10 confirmed these predictions.

On the other hand, peak serum concentrations of CMT are equal to those of cephaloridine, but the excretion rate is rapid, like that of cephalothin (Table 8). Therefore, the infecting bacteria are in contact with cephalothin or CMT in the mouse for only short periods of time; ED_{50} values (Table 10) for these two antibiotics can be compared. Also, unlike cephalothin, CMT is metabolically stable. Antibiotic CMT is slightly less active than cephalothin in vitro against *S. aureus* or *D. pneumoniae*, but about equal in activity against *S. pyogenes* (Table 1). Against gram-negative bacilli, CMT is from 2 to >100 times more active than cephalothin (Table 3). In addition, CMT is stable to β-lactamases from gram-negative bacilli (Table 7). When these facts about CMT were considered, the ED_{50} values shown in Table 10 were expected; i.e., ED_{50} values for CMT were about one-half of those for cephalothin in therapy of all of the experimental infections shown. Antibiotic CMT was effective in treating *P. morganii* infections, most likely because of its stability to β-lactamase.

Based on the known efficacy of cephalothin and cephaloridine in therapy of bacterial infections in humans, the in vitro and in vivo data accumulated

TABLE 12. *Susceptibility of Klebsiella sp., Enterobacter sp., and Proteus sp. to 30-μg discs of five cephalosporin antibiotics*

Bacterial isolate	Expected susceptibility with 30-μg cephalothin disc	Susceptibility to cephalosporins with 30-μg discs[a] (zone diam in mm)				
		CEZ	CMT	CSY	CER	CET
Klebsiella pneumoniae						
KL1	Susceptible	25	34	30	27	28
KL3	Susceptible	26	33	29.5	24	26
Enterobacter aerogenes						
EB 1	Resistant	—[b]	25	17.5	—	—
EB 6	Resistant	10	23.5	18	—	—
EB11	Resistant	—	24.2	17	—	—
E. cloacae						
EB 5	Resistant	—	23.5	10	—	—
EB 8	Resistant	—	18	13	—	—
EB 9	Resistant	—	23	16.3	—	—
EB10	Resistant	—	23.6	16.4	—	—
EB19	Resistant	18.7	22.8	20	—	—
EB21	Resistant	—	22	12.8	—	—
EB24	Resistant	9.8	26.3	22	—	—
Proteus mirabilis						
PR3	Susceptible	22.3	27	24.2	24.7	22.7
PR4	Susceptible	23.8	30.6	25.7	25.0	28.0
P. morganii						
PR1	Resistant	—	25.2	—	—	—
PR15	Resistant	—	29.8	—	—	—
P. rettgeri						
PR2	Resistant	—	22.6	—	—	—
PR7	Resistant	12.5	32.5	25	—	—
PR9	Resistant	12.7	23.8	15.7	—	—
P. vulgaris						
PR27	Resistant	—	11	—	—	—
PR28	Resistant	10.3	22.6	10.4	—	9

[a] Disc susceptibility by the FDA standardized method. For cephalothin, isolates having zones of 14 mm or less are interpreted as resistant, those having zones of 15 to 17 mm are of intermediate susceptibility, and those having zones of 18 mm or greater are considered susceptible. The abbreviations used for the antibiotics are spelled out in Fig. 1.

[b] No zone surrounding the 6-mm paper disc.

for the heterocyclic cephalosporins may be interpreted to predict probable clinical efficacy. Successful therapy with cefazolin has already partially supported this prediction (19). However, the size and frequency of dosage needed in human therapy may vary with each antibiotic. This is evident from the study (Table 11) in which three dosage schedules were employed for therapy of experimental *D. pneumoniae* infections. When only one dose was administered, or if the antibiotic concentrations were allowed to decrease between two dosages, the less rapidly excreted antibiotics (cephaloridine, cefazolin, and cephanone) appeared superior to cephalothin or CMT. However, when the antibiotic concentrations were maintained by hourly administration, all five cephalosporin antibiotics were effective at respectable dose levels.

A recent proposed ruling of the Food and Drug Administration (5) has required that, whenever possible, the susceptibility to structurally related antibiotics with similar activity spectra be tested against only one member of the antibiotic family. Susceptibility to cephalothin, cephaloridine, cephaloglycin, and cephalexin may be determined by using one test disc that contains 30 μg of cephalothin (25). Accordingly, unless there is a difference in activity spectrum, susceptibility to any new cephalosporin antibiotic might also be determined with the cephalothin disc. Judging on the basis of MIC values (Tables 1–3) and the disc data presented in Table 12, there may be a slight extension of the activity spectrum for cefazolin, and the spectra of CMT and cephanone can include bacterial species that produce cephalosporinase. In addition, serum concentrations attainable in humans with the heterocyclic cephalosporins may be very high (13). For both of these reasons, plans

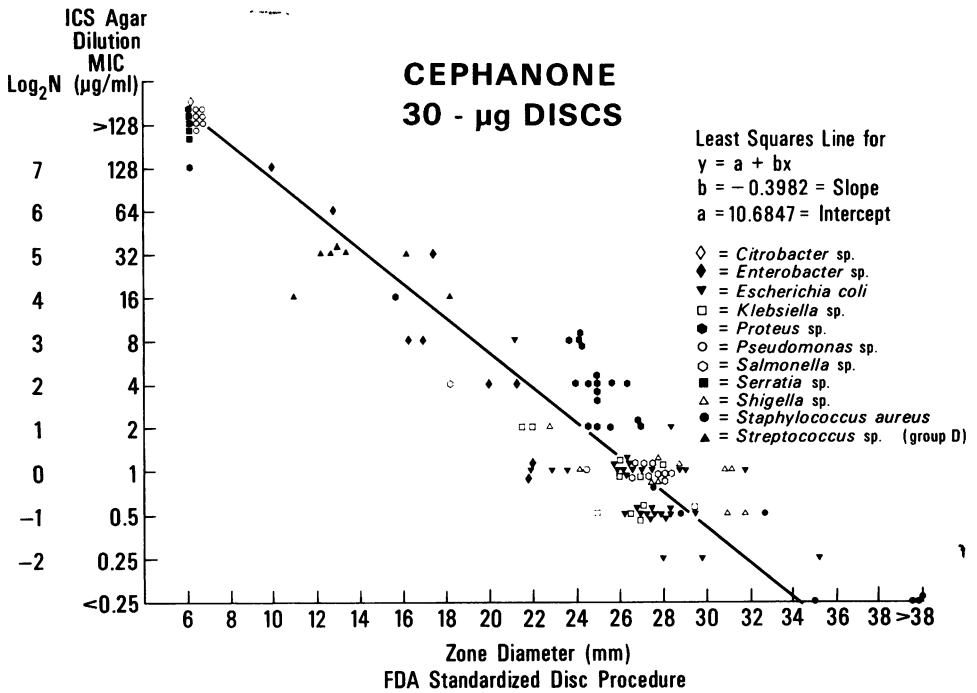


FIG. 2. *Relationship of zone diameters (30-μg discs of cephanone) by the Food and Drug Administration standardized disc procedure to ICS agar-dilution MIC values.*

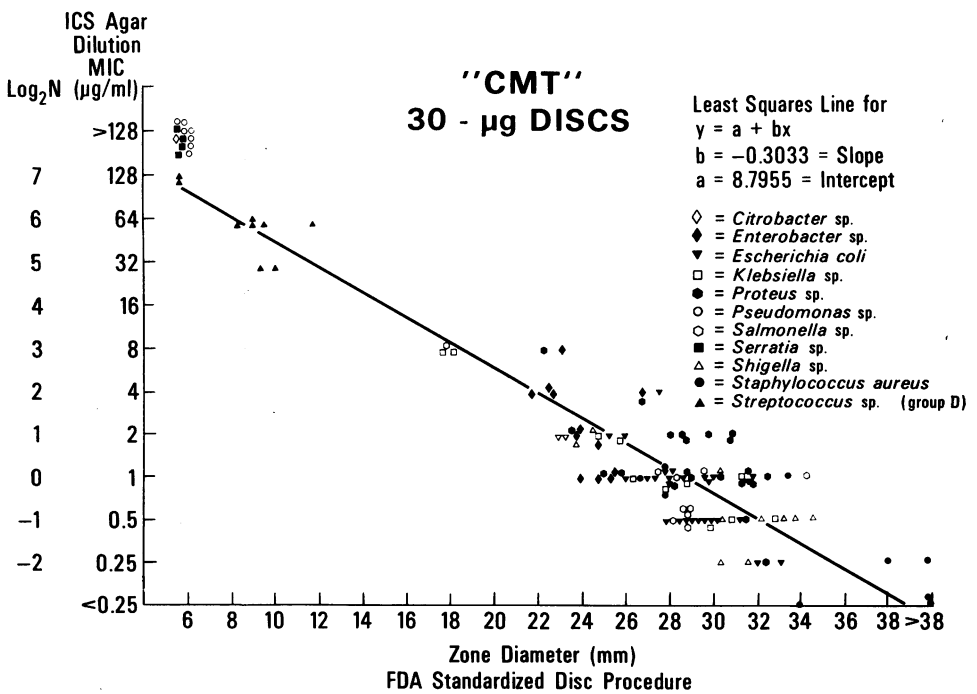


FIG. 3. *Relationship of zone diameters (30-μg discs of "CMT") by the Food and Drug Administration standardized disc procedure to ICS agar-dilution MIC values.*

to use the representative 30-μg cephalothin disc for these new cephalosporins should be deferred. Discs for each of the heterocyclic cephalosporins should be used, along with cephalothin discs, should these antibiotics be used in clinical trials.

Regression lines and equations correlating MIC values by the ICS agar-dilution method with zone diameters obtained by the Food and Drug Administration standardized disc procedure are shown in Fig. 2, 3, and 4. Data obtained by these two standardized procedures should be used to select a zone size that divides susceptible from resistant organisms. However, the zone diameter selected must be based on an MIC value below which successful therapeutic results are expected. Therefore, zone diameters and MIC values that determine susceptible bacteria must be judged on the basis of data collected during a clinical trial.

Procedures other than the two standardized ones discussed above are often used for susceptibility testing. When they are, the type of medium or the size of inoculum are two variations most commonly causing differences in end points. Differences in MIC values with various media used for broth- or agar-dilution tests have been discussed previously (Table 4). When other media were substituted for Mueller-Hinton agar in the Food and Drug Administration disc procedure, zone diameters were about equal with Trypticase Soy Agar (BBL), 1 mm smaller with Sensitivity Test Agar (Oxoid), and 4 mm larger with Nutrient Agar (Difco). A 1:100 dilution of an overnight Trypticase Soy Broth culture approximates the turbidity of the $BaSO_4$ standard used to adjust inocula sizes for the Food and Drug Administration disc test. For each 10-fold change in inoculum size, zone diameters varied by about 2 mm.

The original purpose of these comparative studies with five antibiotics was to select one of the heterocyclic cephalosporin antibiotics that might warrant clinical trial. However, analysis of the data presented above makes that selection difficult. In fact, each of the three newer cephalosporins has certain advantageous characteristics. For example, all three antibiotics have excellent in vitro activity against gram-negative bacilli, peak serum concentrations of cefazolin and cephanone are very high, and the better in vitro activity and stability to cephalosporinase of CMT easily compensates for its generally lower blood levels. Thus, all three of the newer cephalosporins are promising, and additional toxicological and pharmacological data should be obtained.

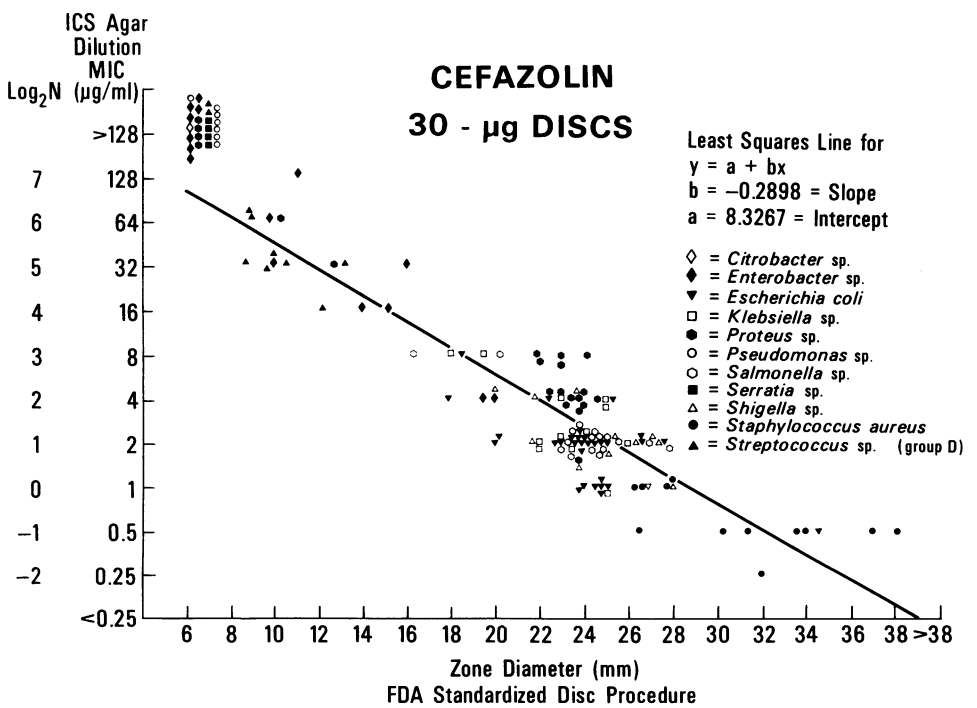


FIG. 4. *Relationship of zone diameters (30-μg discs of cefazolin) by the Food and Drug Administration standardized disc procedure to ICS agar-dilution MIC values.*

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