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BL-S 640, a Cephalosporin with a Broad Spectrum of Antibacterial Activity: Properties In Vitro

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BL-S 640 was evaluated in vitro by comparison with cephalothin, cephaloridine, cefazolin, and cephalexin. The new compound was more active than the control cephalosporins against most major gram-negative and some gram-positive species. Moreover, its antibacterial spectrum included strains of *Enterobacter*, *Proteus morganii*, *P. rettgeri*, and *Providencia stuartii*, species generally resistant to the other cephalosporins. BL-S 640 was an effective bactericidal agent for strains of various species of *Enterobacteriaceae*. In human plasma, the compound was 58% protein bound.

BL-S 640 (Fig. 1) is a new semisynthetic cephalosporin with a broad spectrum of antibacterial activity. The compound is well absorbed parenterally and orally by rodents and is effective by either route in the treatment of various experimental infections of mice. Biological and physicochemical properties of BL-S 640 were compared with those of four cephalosporins used clinically in the United States—namely, cephalothin, cephaloridine, cefazolin, and cephalexin. Antibacterial spectrum and other properties determined in vitro are the subject of the present paper; pharmacokinetics and therapeutic efficacy in rodents will be discussed in a companion paper (3).

MATERIALS AND METHODS

Cephalosporins. BL-S 640, 7-[R-α-amino-α-(4-hydroxyphenyl)acetamido]-3-(1,2,3-triazol-5-ylthiomethyl)ceph-3-em-4-carboxylic acid, was synthesized by members of the Antibiotic Chemistry Department, Bristol Laboratories. Cephalothin, cephaloridine, and cephalexin were products of Eli Lilly and Company; cefazolin was obtained from Fujisawa Pharmaceutical Company and Smith Kline and French Laboratories.

Bacteria. The organisms, 612 strains of gram-negative and 177 strains of gram-positive species, preponderantly of recent clinical origin, were obtained from numerous sources of broad geographical distribution. The organisms were stored as follows: suspensions of *Neisseria gonorrhoeae* in liquid nitrogen; *Haemophilus influenzae*, *Neisseria meningitidis*, *Vibrio cholerae*, *Pasteurella multocida*, *Streptococcus pneumoniae*, *S. viridans*, and *S. pyogenes* as lyophilized preparations; strains of the other organisms as a dry film on porcelain beads (insulators, Honeywell Inc.) (1).

Antibiotic spectrum. The growth inhibitory activity of BL-S 640 and the control compounds was determined on solid medium by the antibiotic dilution technique. Except for *Haemophilus* and *Neisseria*, Mueller-Hinton medium (Difco) was used in these assays. For fastidious organisms, i.e., *Streptococcus*, *Listeria*, *Pasteurella*, *Bordetella*, and *Vibrio*, the medium was supplemented with 4% defibrinated sheep blood. The antibiotic susceptibility of *Haemophilus* and *Neisseria* was determined in GC Medium Base (BBL) supplemented with 1% hemoglobin (BBL) and 1% Isovitalex (BBL).

Overnight broth cultures or an exponentially growing culture (*Neisseria*) served as the source of inoculum. A volume of approximately 0.003 ml of the undiluted or diluted culture was applied to the surface of the antibiotic-containing agar plates with the inoculator of Steers et al. (8). Cultures of *Neisseria*, *S. pneumoniae*, *S. viridans*, and *S. pyogenes* were used without dilution; those of all other organisms were diluted 100-fold. The inoculum contained 10^3 viable cells of *Neisseria*, 10^5 of *S. pneumoniae* and *S. pyogenes*, 10^6 of *S. viridans*, and 10^4 of all other species. The culture plates were incubated at 37 C either overnight or for 24 h (*Haemophilus*), and the minimum inhibitory concentration, i.e., the lowest concentration of antibiotic that prevents visible growth, was recorded.

Effect of inoculum size. The effect of the initial cell concentration on the growth inhibitory activity of the cephalosporins was assessed by using three inocula, containing 10^6, 10^4, and 10^2 viable cells.

Bactericidal activity. Effect on viability was determined in medicated Mueller-Hinton broth (Difco). An inoculum of 10^4 cells was incubated overnight at 37 C with various concentrations of antibiotic in 1 ml of broth. The number of cells remaining viable was determined by plating 0.1 ml of culture on Mueller-Hinton medium. The minimum bactericidal concentration was defined as the lowest concentration of antibiotic giving a count of less than 10 colonies per plate.

Susceptibility to β-lactamase. The rate of hydrolysis by β-lactamase-containing cell-free extracts was measured according to the iodometric assay of Perret

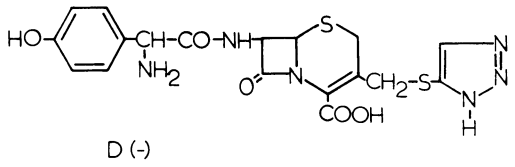

FIG. 1. *Structure of BL-S 640.*

(4) with the following modifications: gelatin was omitted and the buffer was adjusted to pH 7.0. The extracts of gram-negative organisms were obtained by sonic disruption of cell suspensions, followed by the removal of cell debris by centrifugation. The preparations were stored at −20 C for extended periods of time without significant loss of β-lactamase activity. Staphylococci, grown in the presence of methicillin, were extracted with acetone and ether, and the resulting preparations were stored at 4 C.

Binding to plasma proteins. The degree of binding to human plasma proteins was estimated by means of the antibiotic diffusion technique of Scholtan and Schmid (6). The assays were performed in 95% pooled human plasma. The range of cephalosporin concentrations was as follows: 2 to 50 μg/ml for BL-S 640 and cephalexin; 1 to 20 μg/ml for cephalothin; 0.5 to 5 μg/ml for cephaloridine; and 4 to 100 μg/ml for cefazolin.

Stability in solution. Stability at 37 C was determined at pH 2.0 in 0.002 M citric acid-hydrochloride buffer, and at pH 7.4 in 0.005 M barbital (BL-S 640, cephalexin), or 0.005 M phosphate buffer (cephalothin, cephaloridine, cefazolin, cephalexin). The initial antibiotic concentration was 0.2 mg for cephalothin, 0.6 mg for cefazolin, and 2 to 4 mg for the other cephalosporins. Residual antibiotic activity was determined periodically over a 24-h period by a turbidimetric assay procedure or by an antibiotic diffusion technique (cephalothin).

RESULTS

Antibiotic spectrum. The growth inhibitory activity of BL-S 640 and the four control cephalosporins is illustrated in Fig. 2 to 4 and Table 1.

Against 96 strains of *Escherichia coli*, BL-S 640 was two to four times more active than cephaloridine, cephalexin, and cephalothin but only half as active as cefazolin (Fig. 2a). Against *Klebsiella*, BL-S 640 was nearly twice as active as cefazolin and about four times as active as the other three cephalosporins (Fig. 2b). At an antibiotic concentration of 16 μg/ml, BL-S 640 inhibited 40%, whereas the control compounds inhibited a negligible percentage of the *Enterobacter* strains (Fig. 2c). *Proteus mirabilis* was four to eight times more susceptible to BL-S 640 than to the control compounds (Fig. 2d). Moreover, all strains were inhibited by 16 μg/ml of BL-S 640, whereas the other cephalosporins failed to inhibit all 42 strains even at a concentration of 125 μg/ml. BL-S 640 was more active than the other cephalosporins against 12 of the 17 strains of *Proteus vulgaris* (Fig. 2e). The remaining five strains were highly resistant to all cephalosporins. Four of the fifteen strains of *Proteus morganii* were inhibited by 8 μg of BL-S 640 per ml, but all strains were highly resistant to the control compounds (Fig. 2f). At an antibiotic concentration of 16 μg/ml, slightly more than half of the *Proteus rettgeri* strains were inhibited by cefazolin, and nearly half were inhibited by BL-S 640 (Fig. 2g). The other compounds were significantly less active. Against *Salmonella* and *Shigella*, BL-S 640 was more active than the control cephalosporins by about two- to eightfold (Fig. 3a and b). Over 50% of the *Providencia stuartii* strains were inhibited by 16 μg of either BL-S 640 or cefazolin per ml (Fig. 3c). At the same concentration, cephalexin inhibited 17%, and cephalothin and cephaloridine inhibited less than 10% of the strains. Although most strains of *H. influenzae*, including seven ampicillin-resistant strains, were more susceptible to cephalothin than to BL-S 640, the latter inhibited the growth of all 24 strains at a lower concentration (4 μg/ml) than did cephalothin (8 μg/ml) (Fig. 3d). *N. gonorrhoeae* was about equally susceptible to BL-S 640, cephalothin, and cefazolin (Fig. 3e), but *N. meningitidis* was most susceptible to BL-S 640 (Fig. 3f). *Edwardsiella tarda*, *Arizona hinshawii*, *Erwinia* sp., *V. cholerae*, *P. multocida*, and five of twelve strains of *Alcaligenes* sp. were susceptible to all cephalosporins (Table 1). Of the remaining strains of *Alcaligenes*, two were susceptible to BL-S 640 and cephalexin only, and five were susceptible to none of the cephalosporins. BL-S 640 alone was highly effective against four strains of *Citrobacter* sp. The fifth strain of *Citrobacter* and all strains of *Serratia marcescens* and *Pseudomonas aeruginosa* were resistant to the five compounds.

BL-S 640 was more active than cephalexin but less active than cefazolin, cephalothin, and particularly cephaloridine against *S. aureus* (Fig. 4a and b). Nevertheless, all strains were inhibited by 2 μg of BL-S 640 per ml. The relative activities of the five cephalosporins against *S. pneumoniae* and *S. viridans* paralleled those seen with staphylococci (Fig. 4c and d). All pneumococcal strains were inhibited by 0.25 μg of BL-S 640 per ml, and all strains of *S. viridans* were inhibited by 2 μg of BL-S 640 per ml. *S. pyogenes* was highly susceptible to BL-S 640, which inhibited all strains tested at a concentration of 0.063 μg/ml (Fig. 4e). By contrast, at 16 μg/ml, BL-S 640 inhibited only half of the strains of *Streptococcus faecalis*, a species relatively insensitive to cephalosporins (Fig. 4f). *Listeria monocytogenes* was resistant

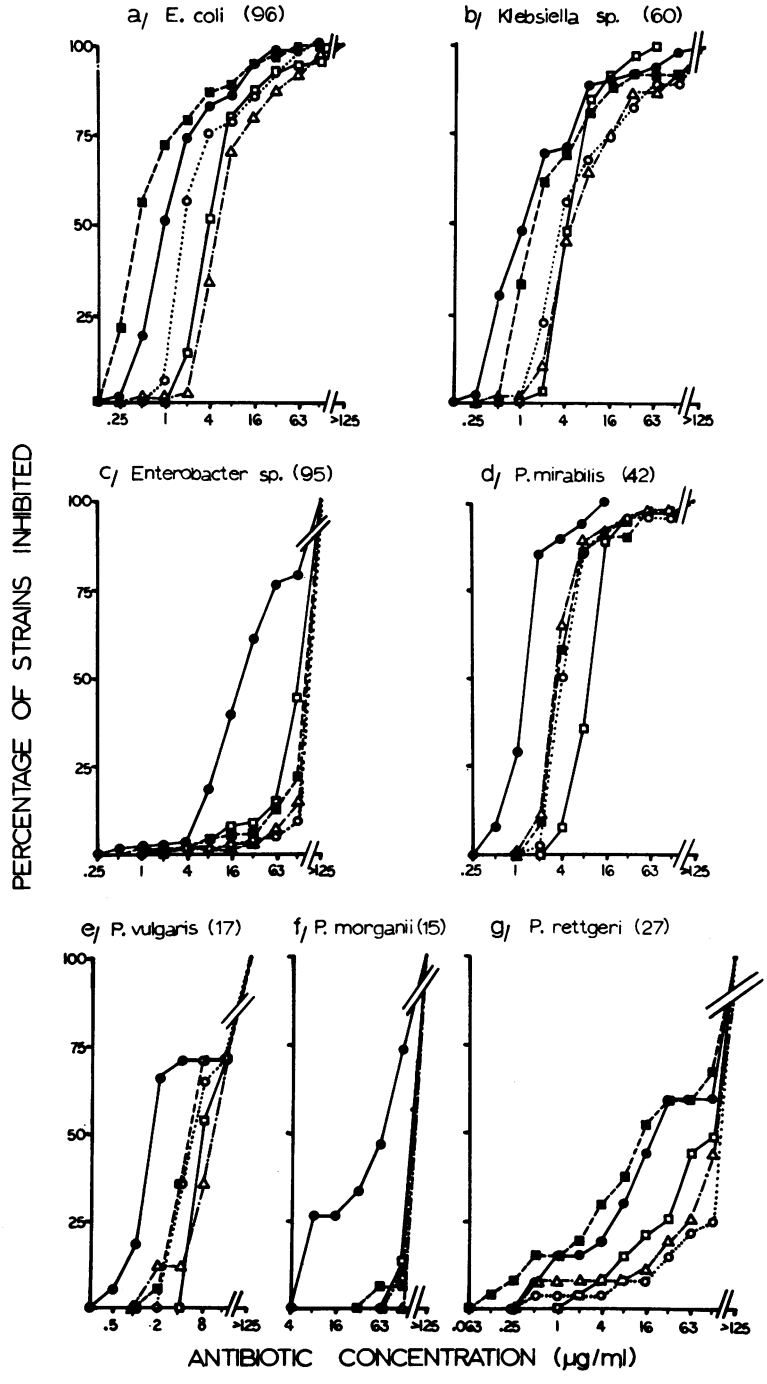

FIG. 2. *Growth inhibitory activity of BL-S 640 (●), cephalothin (△), cephaloridine (○), cefazolin (■), and cephalexin (□) against strains of various Enterobacteriaceae. The numeral in parenthesis indicates the number of strains.*

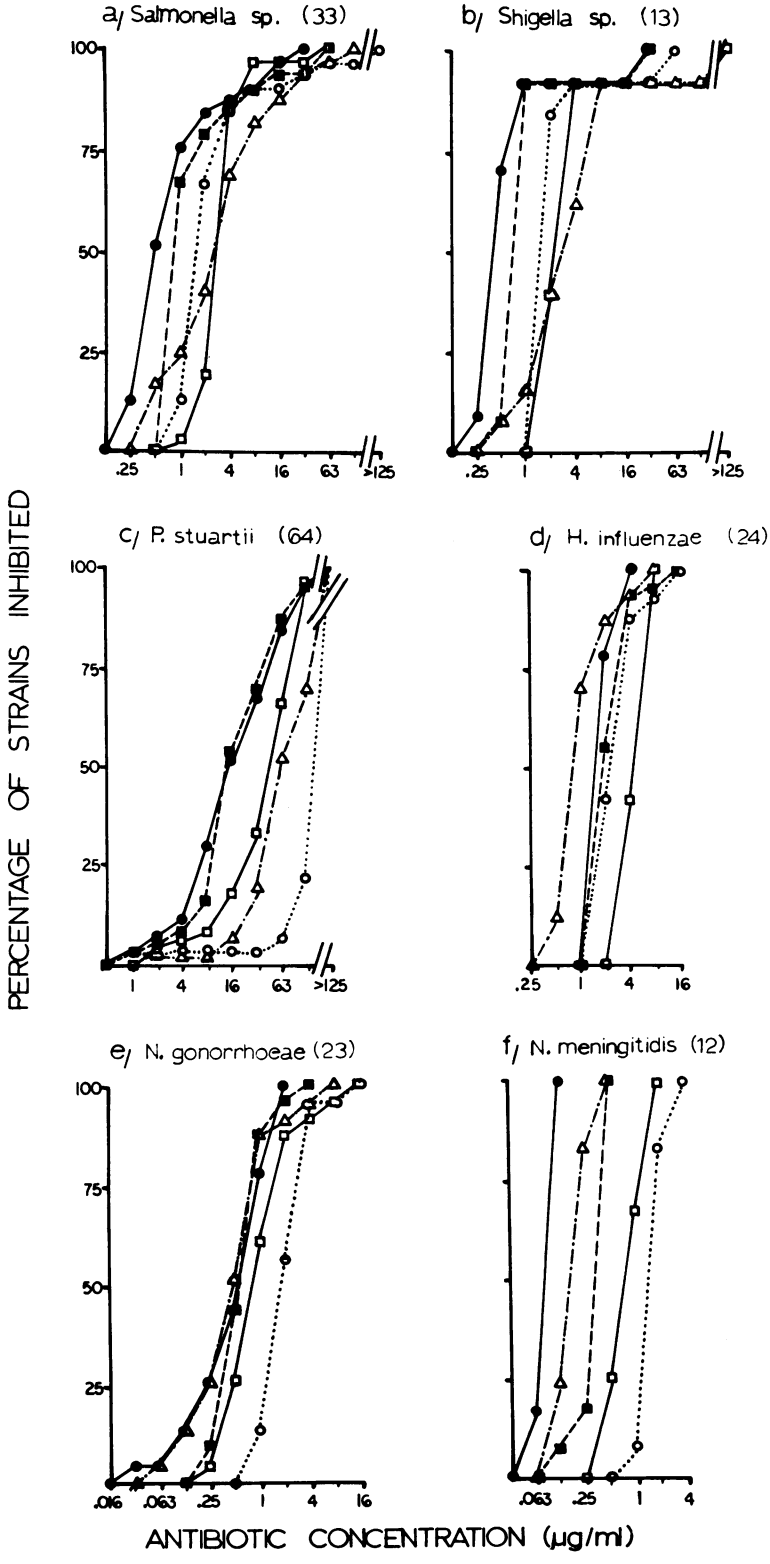

FIG. 3. *Growth inhibitory activity of BL-S 640 (●), cephalothin (△), cephaloridine (○), cefazolin (■), and cephalexin (□) against strains of various gram-negative organisms. The numeral in parenthesis indicates the number of strains.*

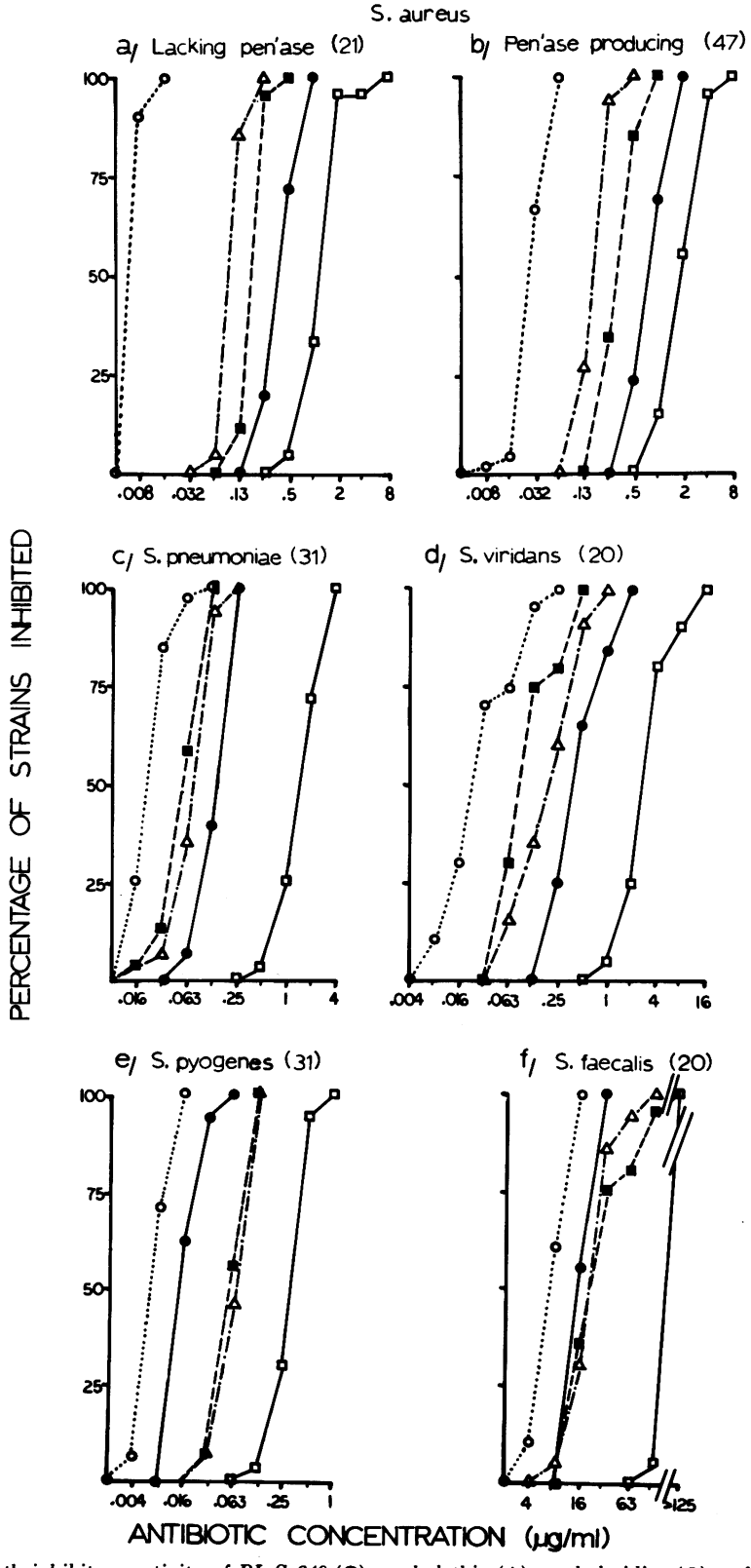

FIG. 4. *Growth inhibitory activity of BL-S 640 (●), cephalothin (△), cephaloridine (○), cefazolin (■), and cephalexin (□) against strains of various gram-positive organisms. The numeral in parenthesis indicates the number of strains.*

to cephalexin but susceptible to BL-S 640, cephalothin, cephaloridine, and cefazolin (Table 1).

Effect of inoculum size. Variations in the initial cell concentration of *Enterobactericaeae* affected in a similar fashion the growth inhibitory activity of BL-S 640 and of the control compounds (Table 2). Thus, when the inoculum was increased from 10^2 to 10^6 organisms, the activity of all cephalosporins generally declined

TABLE 1. *Growth inhibitory activity against strains of miscellaneous organisms*

Organism	No. of strains	Minimum inhibitory concentration[a] (μg/ml)				
		BL-S 640	Cephalothin	Cephaloridine	Cefazolin	Cephalexin
Edwardsiella tarda	2	0.5	0.71	0.5	0.5	2
Arizona hinshawii	2	0.5	2	2	1	4
Citrobacter sp.	4	2.8	32	105	45	63
Citrobacter sp.	1	63	250	125	250	125
Serratia marcescens	30	>125	>125	>125	>125	>125
Erwinia sp.	6	3.2	6.3	2.5	1.8	9
Vibrio cholerae	1	4	1	8	4	8
Pasteurella multocida	2	0.18	0.13	0.35	0.35	1
Pseudomonas aeruginosa	30	>125	>125	>125	>125	>125
Alcaligenes sp.	5	0.87	1.5	6.1	7	5.3
Alcaligenes sp.	2	4	>125	>125	>125	2.8
Alcaligenes sp.	5	>125	>83	>125	>125	>125
Bordetella bronchiseptica	1	32	8	32	125	125
Listeria monocytogenes	7	4	2	0.67	1	63

[a] Geometric mean when applicable.

TABLE 2. *Effect of inoculum size on the susceptibility of Enterobacteriaceae*

Organism	Strain	Minimum inhibitory concentration (μg/ml)[a]									
		BL-S 640		Cephalothin		Cephaloridine		Cefazolin		Cephalexin	
		A	B	A	B	A	B	A	B	A	B
Escherichia coli	1	1	8	2	32	1	8	0.5	16	4	16
	2	2	8	4	32	2	16	2	16	8	16
	3	1	2	2	16	2	4	0.5	2	8	8
	4	2	4	4	16	2	4	1	2	8	16
	5	2	4	2	32	2	8	1	2	8	16
	6	1	4	8	16	2	8	1	2	8	16
	7	1	4	0.5	4	1	4	0.5	2	2	8
Klebsiella pneumoniae	1	0.5	1	1	4	1	2	1	2	4	4
	2	0.5	4	1	32	2	4	0.5	2	4	8
	3	0.5	16	1	32	2	16	0.5	8	4	8
	4	0.5	4	1	4	2	8	0.5	2	4	8
Enterobacter cloacae	1	1	>256	16	>256	8	>256	2	>256	4	>256
	2	2	>256	16	>256	128	>256	16	>256	32	>256
Hafnia alvei	1	1	>256	4	>256	2	>256	2	>256	4	>256
	2	8	>256	256	>256	16	>256	32	>256	16	>256
Proteus mirabilis	1	2	16	4	64	4	32	4	32	4	64
	2	2	16	4	64	4	16	4	32	8	32
Proteus vulgaris	1	0.5	4	1	4	4	16	4	128	8	16
	2	2	64	8	256	8	256	8	>256	16	128
Proteus rettgeri	1	0.25	128	0.25	>256	4	256	0.25	>256	2	128
	2	0.25	>256	0.13	>256	0.25	>256	0.13	>256	1	256

[a] A, Inoculum of 10^2 organisms; B, inoculum of 10^6 organisms.

slightly to moderately against strains of *E. coli*, *K. pneumoniae*, and *P. mirabilis*, and considerably against strains of *Enterobacter cloacae*, *Hafnia alvei*, and *P. rettgeri*. BL-S 640 and three of the control cephalosporins were similarly affected in their activity by a variation in the inoculum of *P. vulgaris* strains.

The effect of inoculum on the susceptibility of staphylococci to cephalosporins is illustrated in Table 3. The susceptibility of strains that lack penicillinase varied little with initial cell concentration. Penicillinase-producing strains, however, were not homogeneous in this respect. With some strains, cell concentration affected only slightly the growth inhibitory activity of the cephalosporins, except cephaloridine; with others, the effect was marked except on cephalothin.

Bactericidal activity. BL-S 640 and the control compounds were effective bactericidal agents for strains of *E. coli*, *K. pneumoniae*, and *P. vulgaris* (Table 4). With the exception of cephalexin, they were also very effective against strains of *P. mirabilis*. Against most strains of *E. cloacae*, however, the growth inhibitory activity itself was low; hence, no significant bacte-

TABLE 3. *Effect of inoculum size on the susceptibility of Staphylococcus aureus*

Organism type	No. of strains	MIC_B/MIC_A[a]				
		BL-S 640	Cephalothin	Cephaloridine	Cefazolin	Cephalexin
Lacking penicillinase	3	2.0	1.0	1.6	1.6	1.3
Penicillinase producer						
Susceptibility slightly affected[b]	14	2.3	2.1	7.5	1.9	1.9
Susceptibility markedly affected[b]	13	15	2.4	26	7.1	6.4

[a] MIC_A, Minimum inhibitory concentration with an inoculum of 10^2 organisms; MIC_B, minimum inhibitory concentration with an inoculum of 10^6 organisms.

[b] Susceptibility to BL-S 640 was slightly or markedly affected by cell concentration.

TABLE 4. *Minimum growth inhibitory and bactericidal concentrations for Enterobacteriaceae*[a]

Organism	Strain	BL-S 640		Cephalothin		Cephaloridine		Cefazolin		Cephalexin	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	1	1	2	8	16	2	4	2	2	8	8
	2	4	8	16	16	8	8	8	8	16	16
	3	1	1	4	4	2	2	1	1	8	8
	4	2	4	8	32	4	4	1	4	16	16
	5	2	4	16	16	4	4	1	1	16	16
	6	2	4	8	8	4	4	2	2	8	8
Klebsiella pneumoniae	1	0.5	1	0.5	1	1	2	1	1	4	4
	2	1	2	2	8	2	4	1	4	4	4
	5	1	8	8	8	4	8	2	4	4	8
	6	0.5	1	2	4	2	2	2	4	4	4
Enterobacter cloacae	3	2	16	32	64	16	16	4	8	8	8
	4	16	256	256	>256	256	>256	256	>256	>256	>256
	5	64	256	256	256	128	128	128	128	64	64
	1	128	128	>256	>256	>256	>256	256	>256	256	256
Proteus mirabilis	1	2	4	8	8	4	8	8	8	8	16
	3	2	8	4	16	8	8	4	8	16	64
	4	2	8	8	16	8	16	8	8	16	256
	5	2	8	8	32	4	16	4	8	8	128
	2	2	16	8	32	8	8	4	8	16	128
Proteus vulgaris	1	1	1	1	1	4	8	2	4	8	8
	2	4	16	4	16	8	16	4	16	16	32

[a] MIC, Minimum growth inhibitory concentration; MBC, minimum bactericidal concentration; expressed as micrograms per milliliter. The inoculum was 10^4 cells.

TABLE 5. *Relative susceptibility to hydrolysis by β-lactamase in cell-free extracts*

Enzyme[a]		Organism	Strain	Relative rate of hydrolysis (benzylpenicillin = 100)				
Class	Type			BL-S 640	Cephalothin	Cephaloridine	Cefazolin	Cephalexin
I	a	*Enterobacter cloacae*	214	200	2,100	7,900	10,000	900
	b	*Escherichia coli*	719	28	480	370	760	55
II	a	*Proteus mirabilis*	1266	<1	<1	2	<1	<1
III	a	*E. coli*	TEM	7	9	93	12	<1
IV	a	*Klebsiella pneumoniae*	53	11	8	100	12	<1
	b	*K. pneumoniae*	1169	48	43	50	31	4
		Staphylococcus aureus	2	<0.5	<0.5	<0.5	<0.5	<0.5
		S. aureus	4	<0.5	<0.5	<0.5	<0.5	<0.5

[a] Enzyme classification according to Richmond and Sykes (5).

ricidal activity could be expected.

Susceptibility to β-lactamase. Susceptibility to enzymic hydrolysis was assessed by determining relative rates of hydrolysis by cell-free preparations of various β-lactamases (Table 5). BL-S 640 was slightly less susceptible than cephalexin and much less susceptible than the other cephalosporins to hydrolysis by β-lactamases of type Ia and Ib. All cephalosporins were poor substrates for type IIa and staphylococcal β-lactamases. Enzyme of type IIIa and IVa split the β-lactam bond of BL-S 640, cephalothin, and cefazolin at about 10% the rate they hydrolyzed benzylpenicillin and cephaloridine; cephalexin was highly resistant to these enzymes. BL-S 640, cephalothin, cephaloridine, and cefazolin were about equally susceptiable to the type IVb enzyme. Cephalexin was relatively resistant to this enzyme also.

Binding to plasma proteins. In 95% human plasma, BL-S 640 was 58%, cephalothin 67%, cephaloridine 30%, and cefazolin 76% protein bound. Results with cephalexin obtained by the antibiotic diffusion technique were variable, ranging from 16 to 32%. By a centrifugal ultrafiltration method, C. W. Dixon (Department of Drug Metabolism and Pharmacokinetics) found cephalexin 17% protein bound, which is in agreement with values reported in the literature (2, 7).

Stability in solution at 37 C. At pH 2.0, the half-life of BL-S 640 and cephalexin was greater than 24 h. At pH 7.4, the half-life of BL-S 640 activity was 6 h, and that of the other cephalosporins was greater than 24 h.

DISCUSSION

BL-S 640 compared favorably with four cephalosporins currently in clinical use when tested against a broad spectrum of bacteria. The compound was more active than the control cephalosporins against most major gram-negative and some gram-positive pathogenic species. Moreover, the antibacterial spectrum of BL-S 640 includes strains of *Enterobacter*, *P. morganii*, *P. rettgeri*, and *P. stuartii*, species generally resistant to the other cephalosporins. In most instances, BL-S 640 and the four control compounds were similarly affected in their growth inhibitory activity by variations in the inoculum. BL-S 640 was also an effective bactericidal agent for strains of several species of *Enterobacteriaceae*. How these antibacterial properties translate into therapeutic efficacy is the subject of a subsequent paper (3).

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Addendum in Proof

Cefatrizine is the non-proprietary name of BL-S 640, recently approved by the USAN Council.

LITERATURE CITED

1. Hunt, G. A., A. Gourevitch, and J. Lein. 1958. Preservation of cultures by drying on porcelain beads. J. Bacteriol. **76**:453-454.
2. Kind, A. C., D. G. Kestle, H. C. Standiford, and W. M. M. Kirby. 1969. Laboratory and clinical experience with cephalexin, p. 361-365. Antimicrob. Agents Chemother. 1968.
3. Leitner, F., D. R. Chisholm, Y. H. Tsai, G. E. Wright, R. G. DeRegis, and K. E. Price. 1975. BL-S 640, a cephalosporin with a broad spectrum of antibacterial activity: bioavailability and therapeutic properties in rodents. Antimicrob. Agents Chemother. **7**:306-310.
4. Perret, C. J. 1954. Iodometric assay of penicillinase. Nature (London) **174**:1012-1013.
5. Richmond, M. H., and R. B. Sykes. 1973. The β-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. **9**:31-88.
6. Scholtan, W., and J. Schmid. 1962. Die Bindung der Penicilline an die Eiweisskörper des Serums und des Gewebes. Arzneim. Forsch. **12**:741-750.
7. Shimizu, T. 1974. Studies on protein binding of cefazolin and other antibiotics. Jpn. J. Antibiot. **27**:296-301.
8. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307-311.