The relative susceptibility of four cephalosporins to a beta-lactamase from *Bacillus cereus*

A. D. RUSSELL AND J. R. FURR

From the Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cathays Park, Cardiff

SYNOPSIS The effects of purified beta-lactamase from *Bacillus cereus* on the activity of four cephalosporins are described. Drug inactivation was measured spectrophotometrically and confirmed by microbiological assay and by microbiological techniques involving determinations of minimum inhibitory concentrations and growth curves of *Staphylococcus aureus* NCTC 6571 (Oxford) in broth containing drug $\pm$ enzyme. Cephaloridine was the most sensitive of the four cephalosporins, followed by cephalothin and the sodium salt of 7-cyanacetamidocephalosporanic acid, with cephalixin considerably resistant to inactivation. The possible use of this beta-lactamase in the sterility testing of cephaloridine and cephalothin is examined.

Cephalosporins are more resistant to beta-lactamases from Gram-positive bacteria such as staphylococci than are benzylpenicillin, phenoxymethylpenicillin, or ampicillin (Pollock, 1967), although the relative instability of cephaloridine to staphylococcal penicillinase has been described (Benner, Bennett, Brodie, and Kirby, 1965; Ridley and Phillips, 1965). Kuwabara and Abraham (1967) have reported that the zinc-dependent beta-lactamase II enzyme, obtained from a strain of *Bacillus cereus* preferentially destroyed cephalosporins.

In this paper, we describe the comparative susceptibility of four cephalosporins to a highly purified commercial beta-lactamase obtained from *B. cereus* and show some possible laboratory uses.

Methods

ANTIBIOTICS

These were the sodium salt of 7-cyanacetamidocephalosporanic acid (Cephacetrile (CAA), a gift from Ciba Laboratories, Horsham, Sussex) and cephaloridine, cephalothin, and cephalixin, which were kindly provided by Glaxo Research Laboratories Ltd, Greenford, Middlesex.

BETA-LACTAMASE PREPARATION

This was an injectable preparation of a highly purified freeze-dried form of *B. cereus* penicillinase, as an 800 000 unit vial (Neutrapen, Riker

Laboratories, Loughborough, Leicestershire). One unit inactivates 1 unit of benzylpenicillin per min at 25°C and pH 7.

HYDROLYSIS OF CEPHALOSPORINS

Solutions (2×10^{-4} M) of cephalosporins in 6.7×10^{-3} M phosphate buffer, pH 7.0, were mixed with equal volumes of Neutrapen solutions in this buffer, all solutions having previously been allowed to equilibrate at 37°C. Changes in extinction at 255 nm for cephaloridine (O'Callaghan, Kirby, and Wishart, 1968; Jack and Richmond, 1970) and at 260, 263, and 263 nm, respectively, for cephalothin, cephalixin, and CAA during subsequent incubation at 37°C ($\pm 0.1^\circ$ C) were followed by means of an automatic SP 600 recording spectrophotometer fitted with a temperature control. The blank cell contained buffer only.

ASSAYS

A biological assay was used to determine the effect of Neutrapen (0, 160 or 320 units/ml, final concentration) on the four cephalosporins (25 μ g/ml final concentration) in buffer, pH 7, at 37°C. Residual antibiotic activity was determined, after quenching enzyme action in aliquots by iodine (O'Callaghan, Morris, Kirby, and Shingler, 1972), by the agar (Oxoid Ltd, London) cup-plate method, using *Bacillus subtilis* NCTC 8236 as test organism.

INFRARED ANALYSES

These were undertaken on the four cephalosporins

in the presence and absence of Neutrapen using Nujol as a vehicle on sodium chloride plates. In addition, a potassium bromide disc was used with the CAA-Neutrapen mixture. Analyses were made with the Perkin-Elmer 397 infrared spectrophotometer, using a normal slit width and medium scan speed.

BACTERIAL GROWTH

The test organism, *Staphylococcus aureus* NCTC 6571 (Oxford), was grown overnight at 37°C in 10 ml broth (Oxoid Ltd, London). Minimum inhibitory concentrations (MICs) of the drugs were determined as follows: 0.2 ml of overnight *S. aureus* cultures were added to 10 ml broth containing various concentrations of a cephalosporin $\pm$ Neutrapen. The presence or absence of growth was noted after incubation for 24 hours at 37°C.

The effect of cephalosporins on the rate of growth of *S. aureus* in broth was determined as follows: 1 ml of an overnight 37°C broth culture was added to 39 ml broth (prewarmed to 37°C) containing the desired drug concentration $\pm$ Neutrapen. Growth during subsequent incubation at 37°C was followed by means of a Unicam SP 600 spectrophotometer at a wavelength of 660 nm. This wavelength was chosen because of the red colour (absorption maximum 550 nm) which occurs when CAA is incubated with some beta-lactamases, including Neutrapen (Russell, 1972).

STERILITY TEST OF CEPHALORIDINE, CEPHALOTHIN, AND CAA

Various quantities of Neutrapen were added to broth (final volume 10 ml) containing 50 mg of an antibiotic. After incubation for one hour at 30°C (British Pharmacopoeia, 1968), 0.1 ml (50-100 viable cells) of a 10^{-8} dilution of an overnight 30°C culture of *S. aureus* 6571 was added, the containers were incubated at 30°C, and the presence or absence of growth was noted during a subsequent seven-day incubation at 30°C.

COLONY FORMATION OF *S. aureus*

Of a 1 in 10^7 dilution of an overnight 37°C broth culture 0.1 ml was spread onto dried agar plates containing a cephalosporin $\pm$ Neutrapen. Colonies were counted after incubation for 48 hours at 37°C.

Results

The rates of inactivation of the four cephalosporins by Neutrapen are shown in figure 1a, b, c, d. These results indicated that cephaloridine is the most rapidly hydrolysed, followed by cephalothin and CAA, with cephalixin the most resistant. The results

(table I) of the biological assays, in which a cephalosporin was treated for 15 and 30 min with 160 or 320 units/ml of Neutrapen, support this finding.

Infrared spectrophotometric analyses suggested that the decrease in extinction (fig 1) at the stated wavelengths was the result of an opening of the beta-lactam ring by enzyme attack.

Antibiotic	Time (min)	Percentage Antibiotic Remaining after Incubation with Neutrapen (units/ml) ¹		
		0	160	320
Cephaloridine	5	104	22.8	
	15	104		
	30	97.6	22.8	6.5
Cephalothin	15	108	70.4	70.4
	30	108	76	73.2
CAA	15	100	98.5	
	30	93.1	92.8	84.1
Cephalixin	30	Nd ²	Nd	Nd

Table I Rates of inactivation of four cephalosporins by Neutrapen in phosphate buffer, pH 7, at 37°C

¹Initial antibiotic concentration 25 μ g/ml; ²Nd no destruction

GROWTH OF *S. aureus*

The MICs of cephaloridine, cephalothin, cephalixin, and CAA against *S. aureus* were 0.05, 0.5, 5-10, and 0.5 μ g/ml respectively. The effects of various concentrations of Neutrapen on 25 and 50 μ g/ml of a cephalosporin in broth inoculated with *S. aureus* are shown in table II. Cephaloridine is obviously the most sensitive of the four drugs to Neutrapen; CAA is rather more resistant than is cephalothin, whereas cephalixin shows a very high resistance to breakdown by this beta-lactamase. These findings confirm those shown in figure 1.

Table III describes colony formation of *S. aureus* on agar containing a cephalosporin $\pm$ Neutrapen. No colonies developed on plates containing 25 or

Antibiotic	Concentration (μ g/ml)	Minimum Concentration of Neutrapen (units/ml) to Overcome Growth-inhibitory Activity
Cephaloridine	25	Between 0 and 40
	50	
Cephalothin	25	80
	50	80-160
CAA	25	160
	50	320
Cephalixin	25	2400
	50	3200

Table II Effect of Neutrapen on the growth-inhibitory activity of four cephalosporins against *S. aureus* in broth at 37°C

Fig 1 Neutrapen-induced inactivation of four cephalosporins (10^{-4} M, final concentration) as measured by changes in extinction at the stated wavelength: (a) cephaloridine, 255 nm (b) cephalothin, 260 nm (c) CAA, 263 nm (d) cephalixin, 263 nm. Figures are enzyme concentration (units/ml)

Fig 2 Rate of growth of *S. aureus* in broth containing 25 or 50 µg/ml of (a) cephaloridine, (b) cephalothin, (c) CAA, (d) cephalixin. Antibiotic 25 µg/ml and Neutrapen (units/ml) 80, △—△; 160, ▲—▲; 320, □—□; 1280, ▽—▽. Antibiotic 50 µg/ml and Neutrapen (units/ml) 160, ■—■; 320, ●—●; 640, ●—●; 2560, ☆—☆. Growth in broth alone, ○—○.

Antibiotic	Concentration ($\mu\text{g/ml}$)	Neutrapen Concentration (units/ml)	Colonies at 48 Hours
Cephaloridine	50	0	0
	50	25	195
	50	50	205
	50	75	420
	50	100	408
	50	250	398
Cephalothin (or CAA)	25	0	0
	25	50 or 100	0
	50	50, 100 or 250	0
Cephalexin	25	0, 50, 100 or 250	0
None	—	250	420

Table III Effect of Neutrapen concentration on colony formation of *S. aureus* 6571 on nutrient agar containing a cephalosporin

50 $\mu\text{g/ml}$ cephaloridine, but colony formation on plates containing cephaloridine + enzyme. No colonies were produced on agar containing Neutrapen and 25 or 50 $\mu\text{g/ml}$ of CAA, cephalothin, or cephalixin.

The rate of growth at 37°C of *S. aureus* in broth containing 25 or 50 $\mu\text{g/ml}$ of a cephalosporin and varying concentrations of Neutrapen is shown in figure 2. Growth in broth containing Cephaloridine plus the higher concentrations of Neutrapen begins within the first five hours; in contrast, no visible growth of the organism occurs within this period in broth containing Neutrapen plus CAA or cephalothin, and virtually no growth after 24 hr when cephalixin replaces the other cephalosporins.

Table IV describes the results of the sterility tests carried out at 30°C with the three injectable products, cephaloridine, cephalothin, and 7-cyanacetamidocephalosporanic acid. Similar results were obtained at 37°C. Low concentrations of Neutrapen were needed to inactivate a 50 mg sample of cephaloridine, but these could not inactivate cephalothin and CAA to such an extent as to allow *S. aureus* to grow within the seven-day incubation period, although higher Neutrapen concentrations also inactivated cephalothin. Samples from those containers showing growth were tested for their Gram-staining reaction, cultural purity, and sensitivity to beta-lactam antibiotics: no differences from the parent culture were observed.

Cephalosporin (50 mg/10 ml)	Minimum Concentration of Neutrapen (μml) Allowing Growth after (days) ¹			
	One	Three	Five	Seven
Cephaloridine	9000	1000	500	200
Cephalothin	>80 000	40 000	20 000	5000
CAA	>90 000	>90 000	>90 000	>90 000

Table IV Sterility testing of three cephalosporins

¹Of *S. aureus* NCTC 6571 at 30°C

Discussion

Our initial interest in studying the effect of this enzyme on the four cephalosporins was to determine whether Neutrapen could be used (a) as a simple inactivating agent (Russell, 1964) in performing viable counts on cephalosporin-treated bacteria; (b) as an inactivating agent in sterility testing of cephalosporins. The results shown in figs 1 and 2 and tables I-IV indicate that cephaloridine is quite sensitive to inactivation by Neutrapen which confirms and extends the findings of Newsom and Walsingham (1973), whereas CAA, cephalothin, and especially cephalixin are more resistant. Low concentrations of Neutrapen, are needed for colony formation of *S. aureus* in agar containing cephaloridine, and Neutrapen appears to be suitable for use as an inactivating agent in the sterility testing (Russell and Gilbert, 1964) of cephaloridine, and possibly of cephalothin, but not of 7-cyanacetamidocephalosporanic acid. Cephalexin is given orally, and no sterility test was thus made with this compound. The present pharmacopoeial method of testing 'penicillinase-resistant' beta-lactam antibiotics is to use a filtration technique. From the results presented here and from those of Newsom and Walsingham (1973) it seems that this could be supplemented by an inactivation method for cephaloridine and even for methicillin.

References

- Benner, E. J., Bennett, J. V., Brodie, J. L., and Kirby, W. M. M. (1965). Inactivation of cephalothin and cephaloridine by *Staphylococcus aureus*. *J. Bact.*, **90**, 1599-1604.
- British Pharmacopoeia (1968). Pharmaceutical Press, London.
- Jack, G. W., and Richmond, M. H. (1970). A comparative study of eight distinct β -lactamases synthesized by Gram-negative bacteria. *J. gen. Microbiol.*, **61**, 43-61.
- Kuwabara, S., and Abraham, E. P. (1967). Some properties of two extracellular β -lactamases from *Bacillus cereus* 569/H. *Biochem. J.*, **103**, 27C.
- Newsom, S. W. B., and Walsingham, B. M. (1973). The use of β -lactamases in the clinical laboratory. *J. med. Microbiol.*, **6**, 59-66.
- O'Callaghan, C. H., Kirby, S. M., and Wishart, D. R. (1968). Microbiological assay of mixed cephalosporins. *Antimicrob. Agents and Chemother.*, **1967**, 716-722.
- O'Callaghan, C. H., Morris, A., Kirby, S. M., and Shingler, A. H. (1972). Novel method for detection of β -lactamases by using a chromogenic cephalosporin substrate. *Antimicrob. Agents and Chemother.*, **1**, 283-288.
- Pollock, M. R. (1967). Origin and function of penicillinase: a problem in biochemical evolution. *Brit. med. J.*, **4**, 71-77.
- Ridley, M., and Phillips, I. (1965). Relative instability of cephaloridine to staphylococcal penicillinase. *Nature (Lond.)*, **208**, 1076-1078.
- Russell, A. D. (1964). Damaged bacteria: recovery and revival. *Lab. Pract.*, **13**, 114-122.
- Russell, A. D. (1972). Interaction of a new cephalosporin, 7-cyanacetamidocephalosporanic acid, with some Gram-negative and Gram-positive β -lactamase-producing bacteria. *Antimicrob. Agents and Chemother.*, **2**, 255-260.
- Russell, A. D., and Gilbert, R. J. (1964). The sterility testing of pharmaceutical products. *Mfg. Chem. and Aerosol News*, **35**, 34-42.