THE USE OF β -LACTAMASES IN THE CLINICAL LABORATORY

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THE use of β -lactamases (penicillinases) to destroy penicillin in culture media (Harper, 1943) is well established. These enzymes can also be used to destroy penicillin in body fluids to allow the assay of another antibiotic, such as streptomycin. A chance observation (Newsom, 1967) led to the realisation that some commercially available enzymes destroyed cephaloridine, methicillin, and cloxacillin. Many more β -lactam antibiotics and β -lactamases are now available and this investigation was undertaken to study them all. The commercially available "penicillinases" are filtrates of cultures of *Bacillus* species, usually *Bacillus cereus* or *B. licheniformis*. The activity of the different preparations is very variable and the situation is more confused because the units used by the makers to express it are nearly all calculated by different methods.

The zinc-dependent β -lactamase II from *B. cereus*, strain 569/H (Kuwabara and Abraham, 1967) and the β -lactamases made by some enterobacteria (Jack and Richmond, 1970), especially *Enterobacter cloacae* (Fleming, Goldner and Glass, 1963), preferentially destroy cephalosporins. Crude enzymes were therefore made from some enterobacteria and from a highly carbenicillin-resistant strain of *Pseudomonas aeruginosa* (Newsom, Sykes and Richmond, 1970) to compare their activity with that of the bacillus enzymes.

MATERIALS AND METHODS

The antibiotics tested fell into three groups. The "penicillinase-sensitive" group included: benzylpenicillin, ampicillin, carbenicillin, amoxycillin, and BRL 2288—a carbenicillin-like compound. The "penicillinase-resistant" group included: methicillin, cloxacillin, flucloxacillin, Magnapen (flucloxacillin and ampicillin), and Ampiclox (cloxacillin and ampicillin). The cephalosporin group included cephaloridine, cephalexin, and cephalothin. The antibiotic stock solutions were stored at -20°C and working dilutions of 5, 50, and 500 μg per ml were made in horse serum.

Enzymes. Bacillus-enzyme preparations were obtained from the following firms: Whatman Biochemicals Ltd, Springfield Mill, Maidstone (β -lactamase 1+11); Riker Laboratories, Loughborough ("Neutrapen"); Baltimore Biological Laboratories, York House, Empire Way, Wembley (penicillinase concentrate); Difco Laboratories, Central Avenue, East Molesey, Surrey ("Penase" concentrate); AVM Pharmaceuticals Ltd, PO Box 79, Wrexham, Denbigh (penicillinase); Wellcome Reagents Ltd, Beckenham, Kent (penicillinase), and Koch-Light Laboratories, Colnbrook, Bucks (penicillinase). The Whatman, Riker, and AVM products were freeze dried; the others were liquid. The Riker enzyme was made for administration to man, the AVM for addition to milk and dairy products, and the others for

Received 23 June 1972; accepted 12 July 1972.

laboratory use only. Crude enzyme extracts were made by us from strains of *Enterobacter aerogenes*, *Klebsiella aerogenes*, *Escherichia coli*, and the aforementioned strain of *P. aeruginosa*, which were all obtained from routine laboratory cultures. The organisms were grown

TABLE I
β-lactamases tested—specifications and results of iodometric assay

Make	Information from makers		Results of iodometric assay; μmoles of indicated antibiotic destroyed per hr	
	Unit specification	Concentration (units); organism of origin	<i>Benzylpenicillin</i>	<i>Cephaloridine</i>
Whatman Biochemicals	1 unit destroys 1 μmole per hr at pH 7.0 and 30°C	50,000 units per vial; <i>B. cereus</i>	28,000 per vial	480 per vial
Riker	1 unit destroys 1 unit of benzylpenicillin per min. at 25°C and pH 7.0	800,000 units per vial; <i>B. cereus</i>	100,000 per vial	730 per vial
Baltimore Biological Laboratories	1 Kersey unit destroys 0.39 unit benzylpenicillin at 30°C and pH 7.0	1,000,000 units per ml; <i>B. cereus</i>	5400 per ml	64 per ml
Difco	Kersey unit (as above)	2,000,000 units per ml; ?	2600 per ml	40 per ml
AVM	1 unit destroys 2–3 units benzylpenicillin at 37°C and pH 7.0	100,000 units per vial; <i>B. cereus</i>	1200 per vial	96 per vial
Wellcome	1 unit destroys 1 unit of benzylpenicillin	100,000 units per ml; <i>B. cereus</i>	52 per ml	0
Koch-Light	1 unit destroys 1 unit of benzylpenicillin	100,000 units per ml; <i>B. licheniformis</i>	280 per ml	0
Papworth 1	...	...; <i>Klebsiella</i>	2000 per ml	1680 per ml
2	...	...; <i>Enterobacter</i>	1600 per ml	1200 per ml
3	...	...; <i>Esch. coli</i>	10 per ml	0
4	...	...; <i>Pseudomonas</i>	660 per ml	112 per ml

for 18 hr on 10-in. assay dishes containing nutrient agar. The growth was resuspended in 40 ml of distilled water and disrupted for 5 min. in an ultrasonic cell disintegrator (Dawe Instruments Ltd). The extract was centrifuged at 15,000 r.p.m. for 5 min. and the supernatant fluid was sterilised by passing it through a 0.22-μm membrane-filter. The freeze-dried commercial enzymes were reconstituted in 2 ml of phosphate-buffered saline, pH 5.9, which was used as buffer throughout. Stock solutions of enzymes were stored at -70°C. Dilutions of 1 in 10, 1 in 100, and 1 in 1000 of the enzymes were made in buffered saline.

The biochemical activity of the enzymes was estimated by the iodometric method of Perret (1954). Benzylpenicillin and cephaloridine were used as substrates, and the calculations took account of the fact that cephalosporins react with half as much iodine as do the penicillins. The results are expressed as μ moles of substrate destroyed per hour.

The biological activity of the enzymes was measured by mixing 0.05-ml volumes of enzyme dilutions and of antibiotics in a "Microtiter" (Cooke) tray and assaying the residual antibiotic after 1 hr at room temperature (18°C). Some additional tests were done after shorter reaction times. The whole of each antibiotic-enzyme mixture was transferred to a well in a 10-in.-square dish containing nutrient agar overlaid with a standardised suspension of *Sarcina lutea* no. NCIB8553 (no. ATCC9341) in agar (Grove and Randall, 1955). Antibiotic controls were included. The results were scored as positive only when there was complete destruction of the antibiotic; inhibition zones of reduced size were ignored. Streptomycin was assayed in the presence of penicillins and β -lactamases by a similar method, but with *B. subtilis* var. *globigii* no. NCTC10073 as the test organism.

RESULTS

Table I shows the results of the biochemical assay of the enzymes and compares these with the data provided by the makers. The Whatman enzyme was defined in μ moles of substrate destroyed per hour measured iodometrically, the Difco and Baltimore Biologicals enzymes were in Kersey units (Kersey *et al.*, 1955) and the others in units defined by their makers. The Baltimore Biologicals enzyme had been received 1 yr before testing, was stored in accordance with the makers' instructions, and was said to have an indefinite shelf life; the results of the iodometric assays were 75 per cent. lower than those done when this enzyme was first received, but the enzyme was still very active. The results of the iodometric assays of all the enzymes showed that there was a wide range of activity, and that the "Gram-negative" enzymes—except the *Escherichia* enzyme—were highly active against cephaloridine.

The results of the biological tests for destruction of penicillinase-sensitive penicillins are seen in table II. The term "penicillinase sensitive" is used loosely here, for it is meant to refer to the sensitivity of the antibiotics to the amounts of penicillinase produced *in vivo* by staphylococci. The enzymes all destroyed 500 μ g per ml of benzylpenicillin, although the *Esch. coli*, Wellcome, and Koch-Light enzymes acted on this concentration of antibiotic only neat or at a dilution of 1 in 10. The action of the enzymes on other members of the group was very similar to that on benzylpenicillin. The enterobacterial enzymes were slightly less active against ampicillin, the Wellcome enzyme was less active against carbenicillin, and the *Pseudomonas* enzyme was predictably most active against carbenicillin.

Table III records the action of the enzymes on the "penicillinase-resistant" penicillins. The enzymes did not destroy these as well as the previous group. The weaker ones could not destroy 500 μ g per ml of cloxacillin or flucloxacillin. The strongest enzymes destroyed these antibiotics at a dilution of 1 in 100. Cloxacillin and flucloxacillin were equally resistant to destruction; Ampiclox and Magnapen—containing half the amount of cloxacillin and flucloxacillin—were less resistant and methicillin, although the most readily destroyed was considerably more resistant than benzylpenicillin.

TABLE II
Destruction of "penicillinase-sensitive" penicillins

Make of enzyme	Log ₁₀ dilution of enzyme causing complete destruction of antibiotic in an equal volume of the indicated solution													
	Benzylpenicillin per ml <div>5 μg 50 μg 500 μg</div>			Ampicillin per ml <div>5 μg 50 μg 500 μg</div>			Carbenicillin per ml <div>5 μg 50 μg 500 μg</div>			Amoxycillin per ml <div>5 μg 50 μg 500 μg</div>			BRL 2288 per ml <div>5 μg 50 μg 500 μg</div>	
Whatman	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Riker	3	3	3	3	3	3	3	3	3	3	3	3	3	3
BBL	3	3	3	3	3	3	3	3	3	3	3	3	3	3
AVM	3	3	2	3	3	3	3	3	3	3	3	3	3	3
Difco	3	3	2	3	3	3	3	3	3	3	3	3	3	2
Wellcome	2	2	1	2	1	1	2	1	0	2	1	3	1	0
Koch-Light	2	1	1	2	2	1	2	1	0	2	1	3	1	1
Papworth, from:														
<i>Klebsiella</i>	3	3	3	2	2	2	3	3	1	3	3	3	3	2
<i>Enterobacter</i>	3	3	2	2	3	3	3	3	3	3	3	3	3	2
<i>Pseudomonas</i>	3	2	2	3	3	3	3	3	3	3	3	3	3	2
<i>Esch. coli</i>	1	1	0	0	NA	NA	0	0	NA	1	0	2	1	0

3 = Dilution of 1 in 1000 or more; 2 = dilution of 1 in 100; 1 = dilution of 1 in 10; 0 = undiluted; NA = no activity.

TABLE III
Destruction of "penicillinase-resistant" penicillins

Make of enzyme	Log ₁₀ dilution of enzyme causing complete destruction of antibiotic in an equal volume of the indicated solution														
	Cloxacillin per ml			Flucloxacillin per ml			Methicillin per ml			Ampiclox per ml			Magnapen per ml		
	5 μg	50 μg	500 μg	5 μg	50 μg	500 μg	5 μg	50 μg	500 μg	5 μg	50 μg	500 μg	5 μg	50 μg	500 μg
Whatman	2	2	1	3	2	1	3	3	2	3	2	2	3	2	1
Riker	2	2	1	2	1	1	3	3	2	3	2	2	2	2	1
BBL	1	1	0	1	1	0	3	2	2	2	1	1	2	1	1
AVM	1	1	0	1	1	0	1	0	0	2	1	1	2	1	0
Difco	1	1	0	1	1	0	2	2	1	2	1	1	2	2	1
Wellcome	0	NA	NA	0	NA	NA	1	0	0	1	NA	NA	0	NA	NA
Koch-Light	0	NA	NA	0	NA	NA	2	1	0	0	NA	NA	1	NA	NA
Papworth, from:															
<i>Klebsiella</i>	2	1	1	2	1	1	3	2	1	3	2	1	2	2	1
<i>Enterobacter</i>	2	1	1	2	2	1	3	2	2	3	2	1	2	2	2
<i>Pseudomonas</i>	1	0	0	1	0	0	2	1	NA	2	0	0	2	1	0
<i>Esch. coli</i>	NA	NA	NA	NA	NA	NA	0	NA	NA	0	NA	NA	0	NA	NA

3 = Dilution of 1 in 1000 or more; 2 = dilution of 1 in 100; 1 = dilution of 1 in 10; 0 = undiluted; NA = no activity.

The action of the enzymes on the cephalosporins is seen in table IV. The ranges of enzyme activity and substrate resistance are greater than in the previous experiments. The weaker enzymes could not destroy 5 μg per ml of cephalixin, and only two of the bacillus enzymes destroyed 500 μg per ml of this antibiotic. The klebsiella and enterobacter enzymes had the greatest effect on cephalixin, which correlates well with the iodometric assay. Cephaloridine was the most easily destroyed and cephalothin was more resistant to destruction by the enzymes.

TABLE IV
Destruction of cephalosporins

Make of enzyme	Log ₁₀ dilution of enzyme causing complete destruction of antibiotic in an equal volume of the indicated solution								
	Cephaloridine per ml			Cephalexin per ml			Cephalothin per ml		
	5 μg	50 μg	500 μg	5 μg	50 μg	500 μg	5 μg	50 μg	500 μg
Whatman	3	2	1	1	0	0	3	2	2
Riker	3	2	2	0	0	0	2	1	0
BBL	2	2	1	0	0	NA	1	0	NA
AVM	2	1	1	NA	NA	NA	1	0	NA
Difco	1	0	0	0	NA	NA	1	0	NA
Wellcome	0	0	NA	0	NA	NA	1	0	NA
Koch-Light	0	0	0	NA	NA	NA	1	0	NA
Papworth, from:									
<i>Klebsiella</i>	2	2	1	2	1	1	2	2	1
<i>Enterobacter</i>	2	2	2	1	1	0	2	2	2
<i>Pseudomonas</i>	1	0	0	0	NA	NA	1	0	0
<i>Esch. coli</i>	0	NA	NA	0	0	NA	1	0	NA

3 = Dilution of 1 in 1000 or more; 2 = dilution of 1 in 100; 1 = dilution of 1 in 10; 0 = undiluted; NA = no activity.

Tests of shorter reaction times showed that 1 in 100 dilutions of all the stronger enzymes inactivated 50 μg per ml of benzylpenicillin when equal volumes of the reagents were mixed actually in the well of the assay dish; i.e., the reaction was almost instantaneous. The sizes of zones of inhibition of *B. subtilis* produced by 50, 40, 30, 20, 10, and 5 μg per ml of streptomycin were identical with those produced by streptomycin after incubation for 1 hr with equal volumes of cloxacillin solution 100 μg per ml and Riker "neutrapen" diluted 1 in 100.

DISCUSSION

A wide range of β -lactam antibiotics is now available and many of these are described as "penicillinase resistant". This term correctly describes the in-vivo situation with regard to staphylococci, but it is not surprising that it does not hold *in vitro* where concentrated enzyme preparations, often produced from non-pathogenic bacteria, can be used. The number of β -lactamases available was greater than might be expected and several of them destroyed

all the antibiotics against which we tested them. The total biological activity of the stronger enzymes and the relative resistance of the substrates were not estimated precisely because the ability to destroy 500 μg per ml is all that is relevant in the laboratory. The amount of benzylpenicillin destroyed by the commercial enzymes did tally with their makers' specifications. Nevertheless, the existence of so many ways of expressing β -lactamase activity is undesirable and an internationally acceptable unit is needed. These results confirm an earlier report (Newsom, 1967) and those of Sabath *et al.* (1968) and Waterworth (1972), which suggested that β -lactamases can be used to neutralise antibiotics other than benzylpenicillin for laboratory procedures. Stroy and Preston (1971) have also used an *Enterobacter cloacae* culture filtrate to neutralise cephalosporins, although they do not comment on its action against penicillins. This is, however, presumably a weaker enzyme than one made by cell disruption, because most of the β -lactamase produced by *Enterobacter* is inside the cell (Hennessey, 1967). We have used the Riker enzyme for several years to neutralise cloxacillin or cephaloridine in the serum of patients, when assaying an aminoglycoside. The enzyme activity against a stock solution of 100 μg per ml of cloxacillin or cephaloridine is used as a control, because there have been occasional failures due to inadequate storage of the enzyme. There is also a possibility that different batches of enzyme vary in potency, for their production is a biological process.

The alternative method for assaying an aminoglycoside in the presence of a β -lactam antibiotic is to use a marker organism that is sensitive only to the aminoglycoside. *Esch. coli* is usually recommended, but its resistance to β -lactam antibiotics is carried on an episome and may not be stable. The destruction of the β -lactam antibiotic is probably a more reproducible method.

The home-made klebsiella and enterobacter enzymes were very simple preparations, but nevertheless they possessed more activity against the cephalosporins than did the bacillus enzymes. The strain of *Klebsiella* was unusual in that it was resistant to 100 μg per ml of cephalosporin C and was an active producer of "cephalosporinase". A suitably purified and concentrated klebsiella extract might provide the most potent of all enzymes.

Finally the results of the timed tests with benzylpenicillin showed that a strong enzyme destroyed the antibiotic before any had time to kill the indicator organism. The technique of pre-incubation is simple to use for performing antibiotic assays, but the rapid destruction of the antibiotic would be very valuable for destroying penicillin in blood that is to be cultured.

SUMMARY

Eleven β -lactamases were tested for their ability to destroy the currently available β -lactam antibiotics. The biochemical activities of the enzymes against cephaloridine and benzylpenicillin were compared by iodometric assay. Their biological activity was tested by mixing various amounts of enzymes and of 13 different β -lactam antibiotic preparations and assaying the residual antibiotic by an agar-diffusion method, with *Sarcina lutea* as the test organism.

All the enzymes destroyed 500 μg per ml of benzylpenicillin and the more potent ones also destroyed "penicillinase-resistant" penicillins and cephalosporins. Crude enzyme extracts from a strain of *Enterobacter aerogenes* and of *Klebsiella aerogenes* compared favourably with the bacillus enzymes in their action on cephalosporins. Suitable β -lactamases can therefore be used to destroy all the available β -lactam antibiotics in specimens from patients, either in blood cultures or to allow the assay of other antibiotics in patients being treated concurrently with both.

We would like to thank Beecham Laboratories Ltd, Glaxo Ltd, and Whatman Biochemicals Ltd for supplies of reagents. This work was done with the help of a grant from the East Anglian Regional Hospital Board.

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