Properties of β -Lactamases Produced by Three Species of Mycobacteria

By J. E. KASIK* AND LIN PEACHAM
Sir William Dunn School of Pathology, University of Oxford

(Received 17 October 1967)

1. *Mycobacterium smegmatis* (N.C.T.C. 8158), *M. fortuitum* and *M. phlei* (MPI) produce a constitutive β -lactamase that has penicillinase and cephalosporinase activity. 2. The β -lactamases of these three species of acid-fast bacteria were mainly cell-bound, only small amounts of activity being liberated into the extracellular fluid. The total β -lactamase activity of these mycobacteria was much lower than that of certain Gram-positive organisms, but comparable with that reported for species of Gram-negative bacteria. 3. The β -lactamases of intact cells of the mycobacteria were not freely accessible to any of the substrates tested, but the apparent crypticity factor to benzylpenicillin was greater than that to cephaloridine and cephalosporin C. 4. Attempts to induce β -lactamase activity in *M. smegmatis* and *M. phlei* failed even with high concentrations of inducer. 5. The β -lactamases obtained from the three species of mycobacteria showed different substrate specificities, including different relative activities as cephalosporinases and penicillinases respectively. 6. Certain derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid were found to be resistant to hydrolysis by β -lactamases of *M. smegmatis* and *M. fortuitum*. 7. The β -lactamase of *M. smegmatis* was competitively inhibited by a number of β -lactamase-resistant derivatives of 6-aminopenicillanic acid, but not by similar derivatives of 7-aminocephalosporanic acid.

Several species of mycobacteria, including strains of *Mycobacterium tuberculosis*, have been reported to inactivate benzylpenicillin (Woodruff & Foster, 1945; Kirby & Dubos, 1947; Soltys, 1952). This inactivation is caused by an enzyme (Iland & Baines, 1949) that has been identified as a β -lactamase (Kasik, 1965). Though the role of this enzyme in mycobacterial resistance to benzylpenicillin has not been established, it has been shown that mycobacteria share several of the properties of certain other penicillinase-producing penicillin-resistant bacteria, such as a population effect when their sensitivity to benzylpenicillin is tested (Kirby & Dubos, 1947) and frequently greater sensitivity, in conventional tests, to penicillins more resistant than benzylpenicillin to the β -lactamase they produce (Kasik, 1964). Methicillin has been reported to be stable in cultures of *M. tuberculosis* that inactivate benzylpenicillin (Kasik, 1965), and it has been noted that methicillin and other β -lactamase-resistant penicillins inhibit the inactivation of benzylpenicillin in mycobacterial cultures (Kasik, Weber & Freehill, 1967) in a manner similar to that noted with certain non-acid-fast bacteria (Crompton, Jago, Crawford, Newton

& Abraham, 1962; Hamilton-Miller & Smith, 1964). Dicloxacillin, a β -lactamase-resistant penicillin that inhibits the enzyme in mycobacteria, has also been shown *in vivo* to be synergistic with benzylpenicillin against *M. tuberculosis* (Kasik, Weber, Winberg & Barclay, 1966).

Studies of β -lactamases obtained from a variety of bacteria have demonstrated that there may be great differences in the amount of enzyme produced, the accessibility of the enzyme to substrates, the substrate specificity of the enzyme and the effect on it of inhibitors (for review see Citri & Pollock, 1966). Though these factors have been studied extensively in non-acid-fast bacteria little has been published about the properties of mycobacterial β -lactamases. This paper describes some of the properties of the β -lactamases obtained from three species of the Runyon group IV mycobacteria (Corpe, Runyon & Lester, 1963).

MATERIALS AND METHODS

Cultures. Three species of the Runyon group IV mycobacteria, *Mycobacterium smegmatis* (N.C.T.C. 8158), *M. fortuitum* (a clinical isolate; Wells, Agius & Smith, 1955) and *M. phlei* (strain MPI), maintained on solid Lowenstein-Jensen medium (Jensen, 1932), were adapted to grow on liquid Sauton's medium (Willis & Cummings, 1952), which

* Present address: Departments of Medicine and Pharmacology, University of Chicago, Chicago, Ill. 60637, U.S.A.

was modified by substituting sodium glutamate (4.0g./l.) for asparagine. The bacteria were grown in Roux bottles containing 70ml. of medium incubated at 37°, after the addition of a heavy inoculum from a liquid starter culture. *M. smegmatis* and *M. phlei* were allowed to grow for 7–10 days and *M. fortuitum* for 14–16 days; the resulting heavy surface growth was harvested by filtration on hardened paper and washed with a small amount of water. The harvested bacteria (approx. 1g. wet wt. recovered/100ml. of culture), which had the consistency of a hard wax, were resuspended in 0.005% sorbitan mono-oleate (Tween 80; Honeywell Atlas Ltd., London, W. 1) by addition of 1g. wet wt. of bacteria to 6ml. of solution. This suspension of organisms was employed where intact bacteria were studied and was used for the preparation of crude mycobacterial β -lactamase. The dry weight of the bacteria in this suspension was obtained by drying a measured quantity of the suspension *in vacuo* over silica gel to a constant weight.

Enzyme preparation from disrupted cells. Preparations of mycobacterial β -lactamase (pH 6.2) were made from bacterial suspensions (in Tween 80, as described above) by ultrasonic disruption of the cells at 1° (MSE ultrasonic disintegrator, 60 w, 20 kcyc./sec.). Preliminary experiments, with benzylpenicillin as a substrate, showed that enzyme activity of ultrasonically treated suspensions of *M. smegmatis* progressively increased during the first 30 min. of ultrasonic treatment. Continued treatment up to 60 min. had no further effect on enzyme activity. Acid-fast staining and microscopic examination of the suspensions subjected to 30 min. ultrasonic treatment revealed efficient disruption of the organisms with only occasional intact acid-fast bacteria remaining. *M. fortuitum* was more difficult to disrupt than *M. smegmatis* and required 60 min. of ultrasonic treatment to release maximum enzyme activity. Microscopic examination of this ultrasonically treated suspension indicated that disruption of the bacterial cell walls was at least 98% complete. *M. phlei* was more resistant to ultrasonic treatment than both *M. fortuitum* and *M. smegmatis*. After 60 min. of ultrasonic treatment at least 5% of these bacterial cells were still acid-fast, but enzyme activity of the ultrasonically treated suspension did not change with further treatment.

Enzyme activity. β -Lactamase activity was assayed by the manometric method of Henry & Housewright (1947) at 30° and pH 7.0, with 2ml. of culture filtrate, 1ml. of intact bacteria suspended in Tween 80, as described above, or 0.5 ml. of the crude enzyme preparation.

Dialysis experiments. A 5 ml. sample of the crude enzyme preparation was dialysed overnight in a Visking cellulose acetate tube against running tap water, or in 50 ml. of distilled water at 4° with constant agitation. The dialysed preparation was assayed with benzylpenicillin or cephalosporin C, with or without added ZnCl_2 (3 mm). Control assays were carried out with an undialysed sample of the same preparation of enzyme. On some occasions, disodium EDTA (0.27 mm) was added to the crude undialysed enzyme preparation just before assay.

Enzyme location. In an attempt to locate β -lactamase in the mycobacterial cell, the crude enzyme preparation of *M. smegmatis* was successively centrifuged at 3000g for 30 min., 10000g for 1 hr. and 144000g for 3 hr. The resulting three sediments were resuspended in 1% (w/v) gelatin, and, together with the final supernatant fluid, assayed for

β -lactamase activity. A drop of each of these samples was also acid-fast stained in the conventional way and examined.

Substrates and inhibitors. Sodium benzylpenicillin was purchased from Boots Pure Drug Co. Ltd., Nottingham. Sodium methicillin (2,6-dimethoxyphenylpenicillin), sodium oxacillin [6-(5-methyl-3-phenylisoxazole-4-carbamido)-penicillanic acid], sodium cloxacillin [6-(3-*o*-chlorophenyl-5-methylisoxazole-4-carbamido)penicillanic acid] and sodium dicloxacillin [6-(3-2',6'-dichlorophenyl-5-methylisoxazole-4-carbamido)penicillanic acid] were provided by Bristol Laboratories, Syracuse, N.Y., U.S.A. Ampicillin (*D*- α -aminobenzylpenicillin), BRL 1577 [6-(5-methyl-3-*p*-chlorophenylisoxazole-4-carbamido)penicillanic acid] and quinacillin (3-carboxy-2-quinoxalinylnicillin) were obtained from Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey. Phenoxymethylpenicillin, provided by the Distillers Co. (Biochemicals) Ltd. Manchester, was converted into the potassium salt and crystallized from aqueous acetone. Potassium DL- α -phenoxypropylpenicillin was given by Pfizer Ltd., Sandwich, Kent.

Cephalosporin C, cephalosporin C_A, cephaloridine, cephaloglycine, cephalothin [7-(thien-2-ylacetamido)-cephalosporanic acid] and 7-(2,5-dimethoxybenzamido)-cephalosporanic acid were provided by Eli Lilly and Co., Indianapolis, Ind., U.S.A.

7-(2,6-Dimethoxybenzamido)cephalosporanic acid, 7-(2,6-dichlorobenzamido)cephalosporanic acid, 7-(3-2',6'-dichlorophenyl-5-methylisoxazole-4-carbamido)-cephalosporanic acid and 3-azido-7-benzylthioacetamido-cephalosporanic acid were obtained from Glaxo Laboratories Ltd., Greenford, Middx.

Enzyme induction. In an attempt to induce β -lactamase activity in *M. smegmatis* and *M. phlei*, 4g. wet wt. of bacteria was aseptically resuspended in 200 ml. of modified Sauton's medium containing 0.05% (w/v) Tween 80 and 0.25% (w/v) bovine serum albumin. Benzylpenicillin or cephaloridine, in concentrations given below, were added as inducers. Resuspended bacteria incubated without antibiotics were used as controls. The cultures were incubated at 37° and daily samples were appropriately diluted and assayed for extinction with a Spekker absorptiometer with neutral grey filter H 508. When growth was re-established, as indicated by an increase in the extinction (>10%), the bacteria were harvested by centrifugation, weighed, resuspended, disrupted by ultrasonic treatment and assayed for β -lactamase activity. Benzylpenicillin concentrations in the culture supernatant were determined daily by the chemical method of Brandriss, Denney, Huber & Steinman (1962). No antibiotic remained at harvesting.

RESULTS

β -Lactamase activity

As shown in Table 1, only trace amounts of β -lactamase activity were found in culture supernatants of the three species of mycobacteria studied, despite the relatively long incubation period used to grow the organisms. With benzylpenicillin as a substrate, intact cells of the mycobacteria showed more activity than their supernatant fluid, but considerably greater β -lactamase

Table 1. *β -Lactamase activity of culture supernatants and of intact and disrupted mycobacteria*

Methods of culture, suspension of bacteria and ultrasonic treatment are given in the Materials and Methods section. The intact cells used were provided by suspending the bacteria in 0.005% Tween, and contained the same concentration of bacteria as the ultrasonically treated material. The volumes of CO₂ recovered were adjusted to compensate for the greater volume of gas liberated/mole of cephalosporin C and cephaloridine (2 moles of CO₂/mole of substrate). Activities are expressed as μ moles of substrate utilized/hr./ml. of enzyme-containing fluid. Substrate concentrations: benzylpenicillin, 5.6 mM; cephalosporin C, 4.9 mM; cephaloridine, 4.8 mM. —, Not determined.

Mycobacteria	Compound assayed	Activity			Activity ratio (disrupted/intact)
		Supernatant	Intact bacteria	Disrupted bacteria	
<i>M. smegmatis</i>	Benzylpenicillin	<2.7	6.2	79.3*	12.8
	Cephalosporin C	<1.4	4.0	22.0	5.5
	Cephaloridine	<1.4	33.2	69.6	2.1
<i>M. fortuitum</i>	Benzylpenicillin	<2.7	<2.7	14.7	>5.5
	Cephalosporin C	<1.4	—	12.6	—
	Cephaloridine	<1.4	—	13.4	—
<i>M. phlei</i>	Benzylpenicillin	<2.7	<2.7	5.6†	>2.1
	Cephalosporin C	<1.4	<1.4	6.4	>4.5
	Cephaloridine	<1.4	6.7	16.0	2.4

* 2.9 units/mg. dry wt. of bacteria.

† 0.3 unit/mg. dry wt. of bacteria.

activities were obtained after disruption of the bacterial cell wall by ultrasonic treatment. However, even after ultrasonic treatment, the activity from *M. smegmatis* was only 2.9 units/mg. dry wt. of bacteria (expressed in the way proposed by Pollock, 1960). Ultrasonically treated *M. phlei* showed an even smaller activity of 0.3 unit/mg. dry wt.

Comparison of the β -lactamase activities found in culture supernatants, whole cells and disrupted cells of *M. smegmatis*, with benzylpenicillin as a substrate, demonstrated that most of the enzyme activity was inside the bacterial cell, or bound to it, and became available to its substrate after disruption of the cell wall by ultrasonic treatment.

Though an increase in β -lactamase activity occurred on ultrasonic treatment, this increase was not uniform for all substrates. The quotient (β -lactamase activity with disrupted cells/ β -lactamase activity with intact cells) was lower with cephaloridine than with benzylpenicillin (Table 1).

With a view to obtaining information about the possible influence the *N*-acyl side chain and the pyridinium group of the cephaloridine molecule on the accessibility of the cellular enzyme to this substance, cephalothin and cephalosporin C_A (pyridine) were also assayed with suspensions of intact and disrupted cells respectively of *M. smegmatis*. With both compounds the quotient (activity with disrupted cells/activity with intact cells) was considerably higher than with cephaloridine and similar to the quotient obtained with

benzylpenicillin. This indicated that neither the pyridinium group, present in cephaloridine and in cephalosporin C_A, nor the thien-2-ylacetyl group, found in cephalothin and cephaloridine, was responsible alone for the apparently increased accessibility of the enzyme to cephaloridine. Further, the enzyme appeared to be more accessible to cephalosporin C, which contains an α -amino-adipoyl group in place of the thien-2-ylacetyl group and an acetoxyl group in place of a pyridinium group, than to either cephalothin or cephalosporin C_A.

Effect of dialysis. On dialysis, the crude enzyme preparation from *M. smegmatis* retained about 90% of its activity with either benzylpenicillin or cephalosporin C as substrate. No significant change in activity was noted when EDTA was added before dialysis or when the enzyme was assayed in the presence of zinc chloride (3 mM) before or after dialysis.

Constitutive nature of the enzyme. The attempts to induce additional β -lactamase activity in *M. smegmatis* and *M. phlei* failed in spite of the exposure of the bacteria to high concentrations of benzylpenicillin (1000 and 100 μ g./ml. respectively) or cephaloridine (250 μ g./ml.). These concentrations of inducer were used after preliminary experiments with lower concentrations had produced no increase in enzyme activity. These concentrations of inducer were much above those reported to produce maximum induction in Gram-positive organisms (Richmond, 1963), but were similar to those

reported by Sabath, Jago & Abraham (1965) to be effective with *Pseudomonas pyocyanea*. With the highest concentrations of benzylpenicillin (100 and 1000 $\mu\text{g./ml.}$) the enzyme activity/ml. of bacterial suspension was about 85% of that from cultures of *M. smegmatis* and *M. phlei* to which no inducer had been added.

Hydrolysis of benzylpenicillin and cephaloridine occurred in the cultures of *M. smegmatis*, but daily assay of benzylpenicillin by the method of Brandriss *et al.* (1962) indicated that substantial concentrations of this inducer were present for at least 48 hr. Thus cultures that initially contained 1000 $\mu\text{g./ml.}$ contained 600 $\mu\text{g./ml.}$ after 23 hr. and 80 $\mu\text{g./ml.}$ after 48 hr. incubation. In view of the slow growth of *M. smegmatis* this hydrolysis may have obscured enzyme induction, and a firm conclusion as to the inducibility of mycobacterial β -lactamases should perhaps be withheld until an attempt is made to induce the enzyme with a β -lactamase-resistant penicillin.

Distribution of β -lactamase activity on centrifugation of disrupted cells. Suspensions of disrupted *M. smegmatis* were centrifuged under different conditions and the resulting sediments were

resuspended. The sediments and the final supernatant were assayed for β -lactamase activity with benzylpenicillin as a substrate. Acid-fast staining and microscopic examination of the sediments and supernatant showed that centrifugation at 3000g resulted in a sediment composed mainly of intact bacteria, some empty cell walls and scattered acid-fast particles. The sediment obtained at 10000g was composed almost entirely of non-acid-fast material having the appearance of empty cell walls, and that obtained at 144000g was composed of an amorphous particulate material. The final supernatant appeared to be free of particulate material. β -Lactamase activity was found mainly (80%) in the clear supernatant fraction and, except for the initial sediment containing whole bacteria (15%), only minor amounts of activity (3%) were present in the remaining fractions.

Substrate profiles of β -lactamases from different mycobacteria

Enzyme from M. smegmatis. The relative activity of this β -lactamase with different substrates is given in Table 2. Phenoxymethylpenicillin,

Table 2. *Rates of hydrolysis of some derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid by β -lactamase from M. smegmatis*

For details of the procedures used see the Materials and Methods section. Values of K_m and V_{max} were obtained from plots of $1/v$ against $1/[S]$ (Lineweaver & Burk, 1934). V_{max} is given in $\mu\text{moles/hr./ml.}$ of enzyme.

Substrate	Concn. of substrate (mM)	Rate of hydrolysis		K_m (mM)	V_{max}
		(relative to benzylpenicillin)	($\mu\text{l. of CO}_2\text{/hr./ml. of enzyme}$)		
Benzylpenicillin	3.7	100	1716	1.7	122
Phenoxypropylpenicillin	3.5	64	1098		
Phenoxymethylpenicillin	3.8	88	1518		
Ampicillin	3.7	68	1181		
Methicillin	19.9	< 1	< 10		
Oxacillin	19.6				
Cloxacillin	17.8				
Dicloxacillin	16.8				
Quinacillin	80.0				
BRL 1577	17.8				
Cephalosporin C*	4.0	12	204	2.2	11
Cephalothin*	4.0	22	375	2.1	27
Cephaloglycine*	4.8	56	630		
Cephaloridine*	3.2	77	1332	0.85	72
7-(2,5-Dimethoxybenzamido)cephalosporanic acid	4.6	18	312		
7-(2,6-Dimethoxybenzamido)cephalosporanic acid	4.6	14	240		
7-(3,2',6'-Dichlorophenyl-5-methylisoxazole-4-carbamido)cephalosporanic acid	3.6	< 1	< 10		
3-Azido-7-benzylthioacetamidocephalosporanic acid*	4.2	69	1194	0.89	72
7-(2,6-Dichlorobenzamido)cephalosporanic acid	5.7	< 1	< 10		

* CO_2 recovery values were adjusted to compensate for the fact that 2 moles of gas had been shown to be liberated/mole of the cephalosporin concerned.

phenoxypropylpenicillin and ampicillin were hydrolysed somewhat less rapidly than benzylpenicillin. Methicillin, oxacillin, cloxacillin, dicloxacillin and quinacillin were not detectably hydrolysed during 20 min. incubation, even when tested in relatively high concentrations. However, BRL 1577, which differs from cloxacillin only in containing a *p*-chlorophenyl substituent in place of *o*-chlorophenyl substituent in its isoxazole side chain, behaved as a substrate, though a much poorer one than benzylpenicillin. No significant hydrolysis of BRL 1577 occurred in a control solution from which enzyme had been omitted.

At the substrate concentrations used most of the cephalosporins tested were hydrolysed by *M. smegmatis* β -lactamase, but less rapidly than benzylpenicillin. Only with cephaloridine did the rate approach that with some of the derivatives of 6-aminopenicillanic acid. Among the derivatives of 7-aminocephalosporanic acid used were 7-(2,6-dimethoxybenzamido)cephalosporanic acid and 7-(2,5-dimethoxybenzamido)cephalosporanic acid, the first of which had the same *N*-acyl side chain as methicillin. These cephalosporins were hydrolysed, in contrast with methicillin, at a significant rate, which was approximately the same as that obtained with cephalosporin C. However, the 7-aminocephalosporanic acid analogue of dicloxacillin, 7-(3-2',6'-dichlorophenyl-5-methylisoxazole-4-carbamido)cephalosporanic acid, and also 7-(2,6-dichlorobenzamido)cephalo-

sporanic acid, were resistant to hydrolysis under the conditions used.

Table 2 also gives values for K_m and relative values for V_{max} with benzylpenicillin and several cephalosporins. The K_m values were of the same order but the value of V_{max} for benzylpenicillin was more than 10 times that for cephalosporin C.

Enzyme from M. fortuitum. β -Lactamase from *M. fortuitum* showed relatively more cephalosporinase activity than that from *M. smegmatis*. Thus crude enzyme preparations of *M. fortuitum* hydrolysed a number of penicillins less rapidly than did similar preparations from *M. smegmatis*, whereas the *M. fortuitum* preparations hydrolysed some of the cephalosporins at rates that were equal to, or greater than, the rates obtained with *M. smegmatis* preparations (Tables 2 and 3).

K_m values obtained with some of the substrates and the enzyme from *M. fortuitum* were similar to those obtained with the same substrates and the enzyme from *M. smegmatis*. Those penicillins that resisted hydrolysis by the β -lactamase from *M. smegmatis* were also stable in the presence of the enzyme from *M. fortuitum* (Table 3). In addition, BRL 1577, which was hydrolysed by the enzyme preparation from *M. smegmatis*, underwent no detectable hydrolysis in the presence of a corresponding preparation from *M. fortuitum*.

Enzyme from M. phlei. The enzyme preparations from *M. phlei* were less active than those from the other two mycobacteria (Table 4), and were studied

Table 3. Rates of hydrolysis of some derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid by crude β -lactamase from *M. fortuitum*

For details of the procedures used see the Materials and Methods section and Table 2.

Substrate	Concn. of substrate (mM)	Rate of hydrolysis		K_m (mM)	V_{max}
		(relative to benzylpenicillin)	(μ l. of CO ₂ /hr./ml. of enzyme)		
Benzylpenicillin	5.6	100	300	0.57	15
Phenoxypropylpenicillin	3.5	72	240		
Phenoxymethylpenicillin	3.8	172	570		
Methicillin	19.9	< 1	< 10		
Oxacillin	19.6				
Cloxacillin	17.8				
Dicloxacillin	16.8				
BRL 1577	19.5				
Cephalosporin C*	4.9	74	245	1.1	15
Cephalothin*	5.0	116	384	4.4	25
Cephaloglycine*	4.8	227	750		
Cephaloridine*	4.8	91	300		
7-(2,5-Dimethoxybenzamido)cephalosporanic acid	4.6	47	156		
3-Azido-7-benzylthioacetamidocephalosporanic acid*	4.2	129	426		

* CO₂ recovery values were adjusted to compensate for the fact that 2 moles of gas had been shown to be liberated/mole of the cephalosporin concerned.

Table 4. *Rates of hydrolysis of some derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid by crude β -lactamase from M. phlei*

For details of the procedure used see the Materials and Methods section and Table 2.

Substrate	Concn. of substrate (mm)	Rate of hydrolysis	
		(relative to benzylpenicillin)	(μ l. of CO ₂ /hr./ml. of enzyme)
Benzylpenicillin	5.6	100	126
Phenoxypropylpenicillin	3.5	71	90
Phenoxymethylpenicillin	3.8	71	90
Cephalosporin C*	4.9	109	138
Cephalothin*	5.0	47	60
Cephaloglycine*	4.8	113	168
Cephaloridine*	4.8	285	360
7-(2,5-Dimethoxybenzamido)cephalosporanic acid	4.6	—	< 25
3-Azido-7-benzylthioacetamidocephalosporanic acid*	4.2	76	96

* CO₂ recovery values were adjusted to compensate for the fact that 2 moles of the gas had been shown to be liberated/mole of the cephalosporin concerned.

less extensively. The β -lactamase of *M. phlei*, like that of *M. fortuitum*, appeared to be relatively more effective as a cephalosporinase than as a penicillinase. But the rates of hydrolysis of most of the penicillins and cephalosporins tested as substrates were low and only with cephaloridine was the rate above that obtained with preparations from *M. fortuitum*. Since *M. phlei* was difficult to disrupt and a significant fraction of the cells remained intact after ultrasonic treatment, the low enzyme activities may have resulted from the persistence of a barrier between enzyme and substrate. This possibility is supported by the observation that cephaloridine was hydrolysed more rapidly than any other compound tested by the enzyme preparation from *M. phlei*, since the enzyme of intact cells of this organism appeared to be more accessible to cephaloridine than to benzylpenicillin (Table 1).

Effects of inhibitors on the β -lactamase from M. smegmatis

Several of the β -lactamase-resistant penicillins, such as methicillin, inhibit the conversion of benzylpenicillin into benzylpenicilloic acid in cultures of *M. tuberculosis* (Kasik *et al.* 1967). The results of quantitative studies of the inhibition of the β -lactamase from *M. smegmatis* by a variety of enzyme-resistant penicillins and cephalosporins and with benzylpenicillin and several cephalosporins as substrates are given in Table 5. All the derivatives of 6-aminopenicillanic acid that were not hydrolysed by this β -lactamase were effective inhibitors of the enzyme. Chlorine substitution in the phenyl group of the side chain of oxacillin, to

give cloxacillin and dicloxacillin, appeared to result in an increase in inhibitory activity.

Analysis of the data by the methods of Hunter & Downs (1945) and Lineweaver & Burk (1934) showed that inhibition, when observed, was competitive in nature. The K_i values for methicillin as an inhibitor were of the same order with several cephalosporins as substrates as they were with benzylpenicillin.

None of the cephalosporin derivatives that was stable to, or was hydrolysed only slowly by, *M. smegmatis* β -lactamase inhibited the enzyme to a measurable extent in the concentrations employed. Estimates of the minimum K_i values for these compounds are given in Table 5.

DISCUSSION

In general, Gram-positive organisms that produce a β -lactamase have been reported to synthesize relatively large amounts of an accessible, loosely bound or extracellular, and inducible enzyme (Pollock, 1956; Richmond, 1963). In contrast, many Gram-negative bacteria produce smaller amounts of a less accessible, cell-bound and usually, though not always, constitutive enzyme (Jago, Migliacci & Abraham, 1963; Ayliffe, 1963, 1964; Hamilton-Miller, 1963; Smith, 1963). With respect to these properties the three species of mycobacteria studied in the present work produced β -lactamases that resembled those of Gram-negative bacteria, though they differed from a β -lactamase from *Pseudomonas pyocyanea* (Sabath & Abraham, 1966) in that their activity was not enhanced by Zn²⁺. The enzymic activity of the mycobacteria was low in comparison with that of Gram-positive organisms

Table 5. *Behaviour of some derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid as inhibitors of the β -lactamase from M. smegmatis*

Penicillinase and cephalosporinase activities were measured with benzylpenicillin (5.6 mM) and cephalosporin C (9.6 mM) respectively as substrates. K_i values were determined by the method of Hunter & Downs (1945). The minimum K_i values listed for the derivatives of 7-aminocephalosporanic acid were calculated from the finding that these derivatives had no detectable effect on the rate of hydrolysis in the concentrations used. These concentrations were limited by the amounts of the inhibitors available.

Substrate	K_m (mM)	Inhibitor	K_i (mM)
Benzylpenicillin	1.7	Methicillin	0.09
		Oxacillin	0.1
		Cloxacillin	0.05
		Dicloxacillin	0.03
		Quinacillin	Inhibited
		7-(2,5-Dimethoxybenzamido)cephalosporanic acid	> 8.0
		7-(2,6-Dimethoxybenzamido)cephalosporanic acid	> 8.0
		7-(3-2',6'-Dichlorophenyl-5-methylisoxazole-4-carbamido)cephalosporanic acid	> 30
Cephalosporin C*	0.85	7-(2,6-Dichlorobenzamido)cephalosporanic acid	> 30
		Methicillin*	Inhibited
Cephaloridine	0.85	Methicillin	0.15
Cephalothin	2.7	Methicillin	0.065
		7-(3-2',6'-Dichlorophenyl-5-methylisoxazole-4-carbamido)cephalosporanic acid	> 4.0
		7-(2,6-Dichlorobenzamido)cephalosporanic acid	> 4.0

* Concentration of methicillin in this experiment was 0.1 mM and that of cephalosporin C was 11 mM.

(Pollock & Richmond, 1962; Richmond, 1963). The β -lactamase activity found with *M. smegmatis* was 2.9 units/mg. dry wt. of bacteria, whereas a corresponding value with *Bacillus cereus* 569/H was 2000 units/mg. (Kogut, Pollock & Tridgell, 1956). On the other hand, the value with *M. smegmatis* was in the same range as that of 7.1 units/mg. for a penicillin-resistant strain of *Escherichia coli* (calculated from the data of Smith, 1963). In their non-inducibility, under the conditions used, the β -lactamases from *M. smegmatis* and *M. phlei* resembled those from *E. coli*, *Aerobacter aerogenes* and *Klebsiella* (Ayliffe, 1965; Percival, Brumfitt & DeLouvois, 1963).

Suspensions of disrupted cells of the mycobacteria showed much greater β -lactamase activity with benzylpenicillin as substrate than comparable suspensions of intact cells. The quotient (activity with disrupted cells/activity with intact cells) has been termed the permeability or crypticity factor (Hamilton-Miller, 1965, 1966; Citri & Pollock, 1966). The nature of this factor has not been elucidated but it presumably provides a measure of a barrier between the substrate and the β -lactamase of the cell, and it may be one of the factors that determine the resistance of certain strains of bacteria to penicillins and cephalosporins (Hamilton-Miller, 1965; Hamilton-Miller, Smith & Knox, 1965). With the mycobacteria studied here the crypticity factor for cephaloridine was greater

than 1 but lower than that for benzylpenicillin. On the assumption that the cellular β -lactamase had the same properties as the β -lactamase obtained in solution after ultrasonic treatment this indicated that there was a barrier between the cellular enzyme and cephaloridine, but that the latter could reach the enzyme more easily than benzylpenicillin. Others have observed a more striking difference between cephaloridine and other penicillins and cephalosporins with certain Gram-negative bacteria, whose β -lactamase appeared to be freely accessible to cephaloridine (Hamilton-Miller *et al.* 1965). With the mycobacteria the low value for the crypticity factor for cephaloridine appeared to be associated with the general structure and ionic character of the molecule, since the value increased when either the pyridinium group was replaced by an acetoxy group or the thienyl group was replaced by an α -aminoadipoyl group.

Substrate profiles showed that *M. smegmatis*, *M. fortuitum* and *M. phlei* produced different β -lactamases or, in the absence of evidence to the contrary, several β -lactamases, which resulted in different substrate profiles for each species. In general, the enzyme or enzymes from *M. smegmatis* showed less cephalosporinase activity, relative to penicillinase activity, than the enzymes from the other two organisms. Penicillins with bulky side chains that could give rise to steric hindrance, such as methicillin and the isoxazolylic penicillins, were

highly resistant to hydrolysis by mycobacterial β -lactamases. The penicillins tested that were resistant to the β -lactamase from *M. smegmatis* were also powerful competitive inhibitors of this enzyme, and in this respect the latter resembled β -lactamases from certain Gram-negative bacteria that are inhibited by methicillin and isoxazolympenicillins (Hamilton-Miller, Smith & Knox, 1964; Sabath *et al.* 1965). In contrast with the relative behaviour of ampicillin and its cephalosporin analogue, cephaloglycine, the cephalosporin analogue of methicillin was less resistant to the enzyme from *M. smegmatis* than methicillin itself. None of the cephalosporins with structurally related side chains showed affinities for this enzyme (as reflected by values of $1/K_i$) that were comparable with those of the enzyme-resistant penicillins. However, the difference in the affinity of these two groups of penicillins and cephalosporins did not appear to extend to all compounds with the 6-aminopenicillanic acid and 7-aminocephalosporanic acid ring systems respectively. On the assumption that values of K_m for benzylpenicillin, cephalosporin C, cephalothin and cephaloridine were close to those for the dissociation constants of the enzyme-substrate complexes, the affinities of all these compounds were similar.

We thank Professor E. P. Abraham for his advice and for his generous help during the preparation of the manuscript. We are also indebted to Dr R. L. Vollum and Mr N. Smith for supplying the organisms used and for their advice. We acknowledge the many helpful suggestions and the advice of Dr G. G. F. Newton. We also thank Eli Lilly and Co., Glaxo Research Laboratories Ltd., Beecham Research Laboratories, Distillers Co. (Biochemicals) Ltd. and Bristol Laboratories for supplying the penicillins and cephalosporins used in these studies. This work was conducted during the tenure of a Fulbright Scholarship (under the Fulbright-Hays Act, Public Law 87-256) generously provided for J.E.K. by the U.S.-U.K. Education Foundation, London.

REFERENCES

- Ayliffe, G. A. J. (1963). *J. gen. Microbiol.* **30**, 339.
 Ayliffe, G. A. J. (1964). *Nature, Lond.*, **201**, 1032.
 Ayliffe, G. A. J. (1965). *J. gen. Microbiol.* **40**, 119.
 Brandriss, M. W., Denney, E. L., Huber, M. A. & Steinman, H. G. (1962). In *Antimicrobial Agents and Chemotherapy*, p. 626. Ed. by Sylvester, J. C. Ann Arbor: American Society for Microbiology.
 Citri, N. & Pollock, M. R. (1966). *Advanc. Enzymol.* **28**, 237.
 Corpe, R. F., Runyon, E. H. & Lester, W. (1963). *Amer. Rev. resp. Dis.* **87**, 459.
 Crompton, B., Jago, M., Crawford, K., Newton, G. G. F. & Abraham, E. P. (1962). *Biochem. J.* **83**, 52.
 Hamilton-Miller, J. M. T. (1963). *Biochem. J.* **87**, 209.
 Hamilton-Miller, J. M. T. (1965). *J. gen. Microbiol.* **41**, 175.
 Hamilton-Miller, J. M. T. (1966). *Biochem. J.* **100**, 675.
 Hamilton-Miller, J. M. T. & Smith, J. T. (1964). *Nature, Lond.*, **201**, 999.
 Hamilton-Miller, J. M. T., Smith, J. T. & Knox, R. (1964). *Nature, Lond.*, **201**, 867.
 Hamilton-Miller, J. M. T., Smith, J. T. & Knox, R. (1965). *Nature, Lond.*, **208**, 235.
 Henry, R. J. & Housewright, R. D. (1947). *J. biol. Chem.* **167**, 553.
 Hunter, A. & Downs, C. E. (1945). *J. biol. Chem.* **157**, 427.
 Iland, C. N. & Baines, S. (1949). *J. Path. Bact.* **61**, 329.
 Jago, M., Migliacci, A. & Abraham, E. P. (1963). *Nature, Lond.*, **199**, 375.
 Jensen, K. A. (1932). *Zbl. Bakt.* **125**, 222.
 Kasik, J. E. (1964). In *Antimicrobial Agents and Chemotherapy*, p. 315. Ed. by Sylvester, J. C. Ann Arbor: American Society for Microbiology.
 Kasik, J. E. (1965). *Amer. Rev. resp. Dis.* **91**, 117.
 Kasik, J. E., Weber, M. & Freehill, P. J. (1967). *Amer. Rev. resp. Dis.* **95**, 12.
 Kasik, J. E., Weber, M., Winberg, E. & Barclay, W. R. (1966). *Amer. Rev. resp. Dis.* **94**, 260.
 Kirby, W. M. M. & Dubos, R. J. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 120.
 Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 568.
 Percival, A., Brumfitt, W. & DeLouvois, J. (1963). *J. gen. Microbiol.* **32**, 77.
 Pollock, M. R. (1956). *J. gen. Microbiol.* **15**, 154.
 Pollock, M. R. (1960). In *The Enzymes*, p. 269. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
 Pollock, M. R. & Richmond, M. H. (1962). *Nature, Lond.*, **194**, 446.
 Richmond, M. H. (1963). *Biochem. J.* **88**, 452.
 Sabath, L. D. & Abraham, E. P. (1966). *Biochem. J.* **98**, 110.
 Sabath, L. D., Jago, M. & Abraham, E. P. (1965). *Biochem. J.* **96**, 739.
 Smith, J. T. (1963). *J. gen. Microbiol.* **30**, 299.
 Soltys, M. A. (1952). *Tubercle*, **33**, 120.
 Wells, A. Q., Agius, E. & Smith, N. (1955). *Amer. Rev. resp. Dis.* **72**, 53.
 Willis, H. S. & Cummings, M. M. (1952). *Diagnostic and Experimental Methods in Tuberculosis*, p. 129. Springfield, Ill.: Charles C. Thomas.
 Woodruff, H. B. & Foster, J. W. (1945). *J. Bact.* **49**, 7.