Inducible β -lactamase in *Enterobacter*

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SUMMARY

Production of β -lactamase by nine strains of the genus *Enterobacter* (eight *Enterobacter cloacae*, one *E. aerogenes*) was studied to determine its inducibility. Induction was observed with benzylpenicillin (500 μ g./ml.) in all except one strain. Cultures were examined to locate the enzyme; it was found that in exponential growth the enzyme was cell-bound, and in stationary phase cultures much of it was in the culture medium. Maximum enzyme activity was only demonstrated after cell-breakage. Substrate profiles of crude enzyme preparations were examined and it was observed that the enzymes were 20-80 times more active against cephalosporins than against benzylpenicillin. Evidence is presented which suggests that one strain of *E. cloacae* produced two β -lactamases, an inducible cephalosporinase and a constitutive penicillinase.

INTRODUCTION

Abraham & Chain (1940) were the first to describe the production of penicillinase (β -lactamase) by *Escherichia coli* and since then there have been many reports of the synthesis of this type of enzyme by other Gram-negative species (see Citri & Pollock, 1966). Unlike the β -lactamases from Gram-positive species, however, these enzymes are not usually inducible (Smith & Hamilton-Miller, 1963; Citri & Pollock, 1966) and where induction has been shown to occur, it is of a low order (Hamilton-Miller, 1963; Ayliffe, 1964, 1965; Citri & Pollock, 1966). *Pseudomonas aeruginosa* has, in contrast, been shown to produce an inducible β -lactamase, but only in the presence of high concentrations of inducer (Sabath, Jago & Abraham, 1965). The term β -lactamase includes two types of enzyme—penicillinases and cephalosporinases—which frequently show overlapping specificities. Among Gram-negative bacteria, *Klebsiella aerogenes* and *Proteus mirabilis* produce penicillinases (Ayliffe, 1965), while members of the genera *Enterobacter* (*Aerobacter*), *Serratia*, *Hafnia*, and *Proteus morgani* and *Pseudomonas aeruginosa* produce cephalosporinases (Fleming, Goldner & Glass, 1963; Ayliffe, 1965; Sabath *et al.* 1965). Furthermore, many species of Enterobacteriaceae can become infected with R-factors and some, at least, of these carry genes responsible for synthesis of penicillinase (Datta & Kontomichalou, 1965). The present paper describes the production of one or more types of β -lactamase by nine *Enterobacter* strains—either *Enterobacter cloacae* or *E. aerogenes*—and reports an investigation of the kinetics of enzyme production to see whether it is inducible in any of these strains. Whereas Smith & Hamilton-Miller (1963) studied six *Enterobacter* (*Aerobacter*) strains and found them all to produce constitutive penicillinase, Fleming and his collaborators showed that a strain of *E. cloacae* produced a high concentration of

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cephalosporinase, presumably constitutively (Fleming *et al.* 1963). All the strains of *Enterobacter* examined in the present work produced cephalosporinase and in eight of these production was inducible. Enzyme synthesis was increased at least 50-fold in four strains. Examination of the substrate profile of one of the inducible strains suggests that, in addition to inducible cephalosporinase, it produced another β -lactamase constitutively.

METHODS

Bacterial strains and identification. *Enterobacter cloacae* strains 214, 249, 251, 252, 255, 256 and 257 were isolated in 1964 and 1965 from patients at Hammersmith Hospital. With the exception of strains 251 and 252, which were conceivably different isolates of the same strain, the organisms were unrelated to each other. *Enterobacter cloacae* P99 was kindly supplied by Dr Cynthia O'Callaghan (Glaxo Laboratories) and *E. aerogenes* 229 by Miss Pamela Waterworth (Department of Bacteriology, Royal Postgraduate Medical School).

Species were identified according to the scheme of Cowan & Steel (1965), whose basis for distinction between the genera *Enterobacter* and *Klebsiella* included two criteria: (a) motility—enterobacters are motile and klebsiellas are non-motile; (b) production of ornithine decarboxylase—enterobacters produce this enzyme and klebsiellas do not.

Antibiotic resistance. Minimum inhibitory concentrations (m.i.c.) of benzylpenicillin, ampicillin and cephaloridine were determined by using two-fold dilutions in nutrient agar with two inoculum sizes (about 10^6 and 10^3 organisms). All the *Enterobacter cloacae* strains were resistant to these drugs at $1000 \mu\text{g./ml.}$, regardless of the size of the inoculum. *Enterobacter aerogenes* strain 229 was 10–20 times less resistant than the *E. cloacae* strains.

Resistance patterns were examined by using Multodisks (Oxoid Ltd.) containing the following compounds: chloramphenicol (Cm) $50 \mu\text{g.}$; colomycin (Col) $200 \mu\text{g.}$; nitrofurantoin (Nitro) $200 \mu\text{g.}$; sulphafurazole (Sul) $500 \mu\text{g.}$; ampicillin (Amp) $25 \mu\text{g.}$; kanamycin (Kan) $30 \mu\text{g.}$; streptomycin (Sm) $25 \mu\text{g.}$; tetracycline (Tet) $50 \mu\text{g.}$; The resistance patterns were: strain 229, Amp, Sul; 249, Amp, Tet, Sul, Sm; P99, Amp, Tet, Sul, Sm, Cm; 214, 251, 252, 255, 256 and 257, Amp, Tet, Sul, Sm, Nitro.

There was no evidence that resistance to the two penicillins and cephaloridine could be transferred to *Escherichia coli* K12. This was tested by growing each strain overnight in mixed culture with *E. coli* K12 and heavily inoculating the mixture on a defined medium containing appropriate antibiotic and dulcitol as sole carbon source. Enterobacters could not, whereas *E. coli* K12 could, utilise dulcitol and grow on this medium. Conjugation and genetic transfer could be demonstrated by the method used, for resistance to chloramphenicol and tetracycline was transferred from strain P99. Moreover, an R-factor carrying a determinant for resistance to chloramphenicol was introduced by conjugation into strain 214, and the resulting culture could transfer chloramphenicol resistance to *E. coli* K12, but no mobilization of genes mediating β -lactamase production was demonstrated.

Media. Oxoid Nutrient Broth No. 2 was used for all fluid cultures. The medium was solidified when required by adding 1.5% (w/v) Oxoid agar.

Penicillins and cephalosporins. Commercial preparations of the following were used: benzylpenicillin and cephaloridine (Glaxo Laboratories Ltd., Greenford, Middlesex)

and ampicillin (Beecham Research Laboratories, Brockham Park, Betchworth, Surrey). Benzylpenicillin will be referred to as penicillin. Other compounds were received as gifts from the manufacturers: methicillin, cloxacillin, phenethicillin, 6-aminopenicillanic acid from Beecham Research Laboratories; quinacillin from Boots Pure Drug Company, Nottingham; phenoxymethylpenicillin from Eli Lilly and Company, Indianapolis, Ind., U.S.A.; cephalothin, cephalosporin C, 7-phenylacetamidocephalosporanic acid, cephaloridine from Glaxo Laboratories, Stoke Poges, Bucks.

Growth experiments. The bacteria were grown in conical flasks shaken in a water-bath at 37°. The volume of culture was one fifth of the flask capacity. Extinctions were measured with a Unicam SP 600 spectrophotometer at wavelength 675 m μ . Conversion of extinction to bacterial dry weights was made by reference to a standard curve prepared for *Enterobacter cloacae* strain 214. Exponential cultures referred to in the text were cultures grown as above and harvested after 3 hr.

Induction of β -lactamase. In experiments to determine the inducibility of β -lactamase, an overnight culture was diluted 20-fold into fresh medium and grown with shaking at 37°. After 1 hr, inducer was added and shaking continued for a further 2 hr before the culture was harvested.

Where a large yield of enzyme was required, as in the study of substrate profiles, overnight cultures were used. In some, inducer was added to obtain sufficient enzyme for the purpose. Two additions of penicillin were made to 600 ml. culture at 1 and 4 hr after subculture (300 mg. at each addition) because penicillin itself was hydrolysed and it was thought likely that greater induction would result if the inducer was replenished. After the second addition of penicillin, growth was continued for 16–18 hr.

Harvesting procedure. Normally, cultures were centrifuged at 5000 g at 4° for 20 min, the deposited organisms washed once with 0.01 M-phosphate buffer (pH 7.0) and re-suspended in buffer, giving a final bacterial concentration equivalent to 5–15 mg. dry wt/ml. When a preparation was required for substrate specificity experiments, a concentration equivalent to 20–40 mg. dry wt/ml. was used.

Breakage of organisms. Organisms were disrupted in 5 ml. volumes of suspension for 4 min. with a Mullard ultrasonic disintegrator. Excessive heating was prevented by immersing the preparation in a bath of flake ice.

Substrate specificity experiments. Suspensions of broken organisms were centrifuged at 40,000 g at 4° for 60 min. and the supernatant fluid used for determining the relative rates of hydrolysis of different substrates.

Induction experiments. Suspensions of broken organisms were used without further treatment (i.e. without high speed centrifugation).

Enzyme assays. The iodometric method of Perret (1954) was used but when cephalosporin hydrolysis was measured, the calculation was based on the observation of Alicino (1961) that 1 mole of cephalosporin C hydrolysed is equivalent to 4 equivalents of iodine and not approximately 8 equivalents as with penicillins. Enzyme activity was assayed in culture fluids, whole culture samples, concentrated suspensions of bacteria, preparations of broken bacteria, or supernatant fluid from these preparations. Corrections were made for non-specific uptake of iodine by substrates and by bacterial extracts by testing controls in which enzyme preparation was added to substrate after the addition of iodine reagent. Enzyme activities are expressed in units similar to those defined by Pollock & Torriani (1953) but at pH 6.0. Substrate concentration was 0.007 M in 0.2 M-phosphate buffer (pH 6.0).

RESULTS

 β -lactamase

The absolute activities of enzyme produced by log-phase organisms which had been disrupted are shown in Table 1. Two strains (P99, 214) were distinctly more active in hydrolysing cephaloridine than the remaining strains. Strain 249 alone catalysed the hydrolysis of ampicillin and the difference between penicillin and cephaloridine hydrolysis was less with this strain than with the others, which predominantly produced cephalosporinase.

Table 1. *Absolute activities of β -lactamase produced by 9 Enterobacter strains*

Strain	Substrate		
	Penicillin	Ampicillin	Cephaloridine
	<i>β-lactamase (units/mg. dry wt bacteria)</i>		
P99	24	—	1380
214	3	—	200
249	19	28	32
251	—*	—	3
252	—	—	2.3
255	—	—	1.6
256	—	—	1.8
257	—	—	49
229	—	—	6

* — = less than 0.3 units/mg. dry wt

Location of enzymes

Measured volumes of cultures in the exponential or in the stationary phase (3 or 16–18 hr, respectively) were used to locate β -lactamase activity. Total activities were calculated for whole cultures, supernatant fluids, intact and broken organisms. The distribution of enzyme activity is shown in Table 2. Also shown is the 'crypticity factor' which is the ratio of activities in broken:intact organisms. In log-phase cultures the proportion of enzyme in the supernatant fluid was less than 12% of the total activity (supernatant fluid activity + activity in broken organisms) whereas in stationary-phase cultures the supernatant fluid contained 40–60%. Addition of penicillin did not increase the amount of extracellular enzyme. In most cases maximum enzyme activity was only evident after breaking the organism.

Induction

To test whether penicillin would induce β -lactamase in *Enterobacter cloacae*, strain 256 was grown in the presence of a wide range of concentrations (10 μ g.–12 mg./ml.). The activity of broken organisms was measured against penicillin, ampicillin and cephaloridine; ampicillin was not hydrolysed and therefore is not recorded (Table 3).

Maximal induction was observed with penicillin, 500 μ g./ml., and hydrolysis of cephaloridine was increased 117 times with this concentration. Although concentrated suspensions of bacteria were tested, the basal rate of hydrolysis of penicillin, in the absence of inducer, was so low that it was not possible to assess the induction ratio for penicillinase activity. In the presence of inducer, however, the increased rate of hy-

hydrolysis of penicillin was comparable with that of cephaloridine and was consistent with the view that both penicillinase and cephalosporinase activities were manifestations of the same enzyme. Induction took place even when penicillin concentration (6 mg./ml.) caused lysis. In all subsequent induction experiments the inducer concentration was 500 μ g./ml.

Table 2. *Distribution of β -lactamase activities in cultures*

	Time of harvest (hr)	Strain no.	'Cephalosporinase'			'Penicillinase'		
			Total activity (units)*	% total activity in sf.†	'Crypticity factor'‡§	Total activity (units)*	% total activity in sf.†	'Crypticity factor'‡§
Untreated	3	P99	91,300	6	2.1	1,300	—¶	3.3
	3	214	11,200	5	2.3	190	—	1.4
	3	249	3,055	3	1.1	2,247	3	9.9
	3	257	1,610	11	1.0	< 18	—	—
	16-18	257	25,850	66	1.0	nt	nt	nt
+ Penicillin (500 μ g./ml.)	3	P99	209,500	3	2.8	2,500	2	3.2
	3	214	118,200	2	6.5	1,640	4	3.9
	3	256	21,800	2	1.1	360	—	2.0
	16-18	256	13,800	40	1.4	220	41	1.5
	16-18	252	68,200	44	1.6	940	48	1.0
	16-18	255	82,400	27	1.8	nt	nt	nt
	16-18	257	127,100	64	1.9	nt	nt	nt

The substrates tested were cephaloridine and penicillin.

* Total activity = activity in broken cells + activity in culture supernatant.

† sf = supernatant fluid.

‡ Ratio of disrupted: intact cell activities.

¶ = Activity too low for measurement.

nt = not tested.

Table 3. *Induction of β -lactamase in Enterobacter cloacae strain 256*

Inducer	Concentration (μ g./ml.)	'Penicillinase'* units/mg. dry wt	'Cephalosporinase'*	
			units/mg. dry wt	Induction ratio†
None	—	< 0.3	2	—
Penicillin	10	< 0.3	6	3
	100	0.5	25	12.5
	500	3	234	117
	1,000	2	121	60.5
	6,000	< 0.3	16	8
	12,000	< 0.3	< 2	—
Ampicillin	500	1	92	46
Cephaloridine	500	3	198	99
Methicillin	500	< 0.3	0.4	0.2

* Hydrolysis of penicillin and cephaloridine respectively.

† Ratio of activities induced:uninduced preparations.

Ampicillin and cephaloridine also induced enzyme production in strain 256 but methicillin inhibited cephaloridine hydrolysis (see Table 3).

The results of induction experiments with the remaining 8 strains are recorded in Table 4, included in which are the relevant figures of strain 256 for comparison. Hydrolysis of ampicillin is omitted since only one strain (*Enterobacter cloacae* 249)

hydrolysed this substrate. Strain P99 was the only one which showed no induction. When strain 249 was grown in the presence of penicillin, the rate of hydrolysis of cephaloridine increased compared with the uninduced control but there was no change in the rates of hydrolysis of either ampicillin or penicillin. In all other strains, increase in penicillinase and cephalosporinase activities paralleled each other.

The effect of methicillin on cultures of P99 and 214 was not tested for the following reason. There was no detectable hydrolysis of methicillin by crude enzyme preparations and, further, incubation of these preparations with methicillin (2 mM final concentration) for 15 min. completely abolished activity. The nature of this inhibition has not been determined. From Table 4, however, it will be seen that methicillin inhibited β -lactamase of strains 229 and 256, had no effect on 249 or 255, but induced 251, 252 and 257. Thus, 8 of 9 strains examined produced β -lactamase inducibly.

Table 4. *Induction of β -lactamase in 9 Enterobacter strains. Hydrolytic activities in units/mg. dry wt of bacteria*

Strain	Uninduced		Induced			
	Substrate		Penicillin (500 μ g./ml.)		Methicillin (500 μ g./ml.)	
	Cephaloridine*		Substrate		Substrate	
	Penicillin*	Cephaloridine*	Penicillin	Cephaloridine	Penicillin	Cephaloridine
P99	24	1300	26 (1.0)†	1325 (1.0)	nt	nt
214	3	200	16 (5.3)	945 (4.7)	nt	nt
251	< 0.2	3	6	328 (109)	1	78 (26)
252	< 0.2	2	3	238 (119)	2	132 (66)
255	< 0.2	2	1	104 (52)	< 0.2	2 (1.0)
256	< 0.3	2	3	234 (117)	< 0.3	0.4 (0.2)
257	0.4	28	4 (10)	249 (9.7)	1 (2.5)	71 (2.5)
249	20	28	21 (1.0)	100 (3.6)	21 (1.0)	30 (1.0)
229	0.25	6	2 (8)	49 (8.2)	< 0.1	< 0.06

* Hydrolysis of penicillin and cephaloridine respectively; measured in preparations from concentrated suspensions of organisms

† () Ratio of induced:uninduced activities.

nt = not tested.

Relative activities of crude enzyme preparations

Crude enzyme preparations from induced and uninduced cultures were examined for their relative activity against different penicillins and cephalosporins (Table 5). Methicillin, cloxacillin and quinacillin were also tested; they were resistant to hydrolysis. Strains P99, 214, 251, 252, 255, 256 and 257 (all *Enterobacter cloacae*) had broadly similar profiles but strain 257 may have been significantly different with respect to its action on cephalosporin C. Strain 229 (*E. aerogenes*) was not as active against either cephalosporin C or cephaloridine as were the above 7 strains. Hydrolysis of ampicillin, phenoxymethylpenicillin, phenethicillin and 6-aminopenicillanic acid by strain 249 did not change significantly when an induced preparation was tested, but destruction of the cephalosporins did increase. It seems likely therefore that this strain produced more than one β -lactamase.

DISCUSSION

One of the difficulties in comparing the work described here with earlier studies on β -lactamase in Gram-negative bacteria stems from taxonomic confusion. In the present work the classification of Cowan & Steel (1965) has been used. That members of the

Table 5. Relative activities of *Enterobacter* crude enzyme preparations from uninduced and induced (IND) cultures

Substrate	Strains										
	P 99	214	251	251 (IND)	252 (IND)	255 (IND)	256 (IND)	257 (IND)	229	229 (IND)	249 (IND)
Penicillin	100	100	100	100	100	100	100	100	100	100	100
Ampicillin	—*	—	—	—	—	—	—	—	—	—	147
Phenoxymethylpenicillin	79	69	—	68	90	87	79	80	—	50	56
Phenethicillin	—	—	—	—	—	—	—	—	—	—	24
6-Aminopenicillanic acid	—	—	—	—	—	—	—	—	—	—	176
Cephaloridine	7400	8030	6560	6550	8750	8000	7400	7000	4200	2420	133
Cephalothin	1180	2640	2360	1990	2700	2370	2400	2130	2340	2560	15
Cephalosporin C	6550	6180	6560	5950	7120	7610	6660	3970	3775	1900	14
7-Phenylacetamido-cephalosporanic acid	1730	2220	—	1800	2200	2120	2290	1965	2680	2130	11
Absolute rate of hydrolysis of penicillin (units/mg. dry wt)	20	8.6	0.014†	2.6	0.6	1.1	1.3	2.3	0.03†	2.2	10.7

* — Activity too low for measurement.

† These values were calculated from the induction ratio observed with cephaloridine as substrate.

tribe Klebsiellae have been called by a variety of names may well account for the fact that the strains reported here synthesized cephalosporinase while strains identified as members of the genus *Aerobacter* (*Enterobacter*) by Hamilton-Miller, Smith & Knox (1965) synthesized penicillinase. The enzymes described in the present paper are all cell-bound, at least in exponential growth. Breakage of the organisms liberated some β -lactamase; the fact that 40–60% of the enzyme was liberated into the culture medium of overnight cultures may reflect lysis of stationary-phase organisms. Because cell damage readily causes liberation of enzyme, penicillin in high concentrations might be expected to have this effect by making the organisms rather more leaky. This argument has been used by Sabath *et al.* (1965) to explain the presence of large quantities of enzyme in culture fluids of *Pseudomonas aeruginosa* grown exponentially in the presence of 10 mg. penicillin/ml. In contrast, no such enzyme liberation was detected with the *Enterobacter* cultures examined here, even after treatment with 500 μ g. penicillin/ml.

Comparison of the substrate profiles of crude enzyme preparations from the 9 *Enterobacter* strains showed two main types of pattern. Strains P99, 214, 251, 252, 255, 256, 257 (all *E. cloacae*), and perhaps 229 (*E. aerogenes*) showed broadly similar cephalosporinase activities which could well represent the same, or very closely related, enzymes and which might be characteristic of the genus. Strain 249 (*E. cloacae*), on the other hand, was decidedly different and had a broader spectrum of activity. Of the 9 strains examined, the enzymes of strains 251, 252, 255 and 256 were highly inducible by penicillin and had induction ratios greater than 50 (Table 4). With the exception of strain 249, cephalosporinase and penicillinase activities were likely to be manifestations of the same enzyme. Cephalosporinase activity of strain 249 was increased approximately four-fold in the presence of penicillin and there was some evidence that the strain produced two β -lactamases, inducible and constitutive. The induction ratio for this strain cannot be accurately calculated since it is not possible to assess the relative contribution of each enzyme in any given preparation.

The substrate profiles reported here are different from that reported by Datta & Richmond (1965) for *Escherichia coli* carrying an R-factor, indeed there is no evidence that these enterobacters carry R-factors controlling β -lactamase synthesis. The β -lactamase produced by the *Pseudomonas aeruginosa* strain used by Sabath, *et al.* (1965) was similar to those described here in that it was relatively more active against cephalosporins, but it differed in being active against ampicillin. Two *Proteus* strains examined by Hamilton-Miller *et al.* (1965) appeared to make enzymes which bore some resemblance to *Enterobacter* enzymes. *Enterobacter cloacae* P99 came indirectly from Dr P. C. Fleming in Toronto and was the same strain about which he and his colleagues reported (Fleming *et al.* 1963).

No studies in the genera *Enterobacter*, *Proteus* or *Pseudomonas* have located genes for β -lactamase production at specific chromosomal sites; it remains possible therefore that all these genes are extrachromosomal, but the close similarity of β -lactamases of *E. cloacae* strains described here, however, suggests that the enzyme was species-specific.

The absence of highly inducible β -lactamase from strains of Enterobacteriaceae may reflect on the methods used for investigating this property. Hitherto inducer concentrations have been low as compared with what appears to be optimal for *Pseudomonas aeruginosa* (Sabath *et al.* 1965) and the *Enterobacter* strains reported here.

Penicillin is itself hydrolysed (albeit slowly) by these enzymes and this might explain why such high concentrations are required for induction. The kinetics of induction with methicillin, which is not attacked, may shed light on this observation.

If inducible β -lactamase in Gram-negative bacteria is a rarity, then it raises interesting questions about the evolution of these enzymes. Clearly some control of protein synthesis is necessary for the cell's economy and if this is not effected by repression, then what is the mechanism?

If non-inducible β -lactamase is the rule in Gram-negative bacilli, then either regulatory genes have undergone mutation, or they were never present. Although mutation in a regulatory locus should be a rare event, selection pressures encountered in hospitals could encourage survival of such mutants; this might explain the origin of the constitutive strain P99 or the non-inducible strains of *Escherichia coli* producing large amounts of enzyme described by Hamilton-Miller *et al.* (1965). Where low levels of β -lactamase were produced even in the presence of potential inducers, as in klebsiellas (unpublished observations), and in *E. coli* (Smith & Hamilton-Miller, 1963; Ayliffe, 1965), some form of regulation, presumably repression, is likely and induction cannot be ruled out just because the conditions required have not been determined.

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REFERENCES

- ABRAHAM, E. P. & CHAIN, E. B. (1940). An enzyme from bacteria able to destroy penicillin. *Nature, Lond.* **146**, 837.
- ALICINO, J. F. (1961). Iodometric assay of natural and synthetic penicillins, 6-aminopenicillanic acid and cephalosporin C. *Analyt. Chem.* **33**, 648.
- AYLIFFE, G. A. J. (1964). Induction of cephalosporinase and penicillinase in *Proteus* species. *Nature, Lond.* **201**, 1032.
- AYLIFFE, G. A. J. (1965). Cephalosporinase and penicillinase activity of Gram-negative bacteria. *J. gen. Microbiol.* **40**, 119.
- CITRI, N. & POLLOCK, M. R. (1966). The biochemistry and function of β -lactamase (penicillinase). *Advanc. Enzymol.* **28**, 237.
- COWAN, S. T. & STEEL, K. J. (1965). *Manual for the Identification of Medical Bacteria*, 1st ed. p. 61. Cambridge University Press.
- DATTA, N. & KONTOMICHALOU, P. (1965). Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature, Lond.* **208**, 239.
- DATTA, N. & RICHMOND, M. H. (1965). The purification and properties of a penicillinase whose synthesis is mediated by an R-factor in *Escherichia coli*. *Biochem. J.* **98**, 204.
- FLEMING, P. C., GOLDNER, M. & GLASS, D. G. (1963). Observations on nature, distribution and significance of cephalosporinase. *Lancet* **i**, 1399.
- HAMILTON-MILLER, J. M. T. (1963). Inducible penicillinase in *Proteus morgani*. *Biochem. biophys. Res. Commun.* **13**, 43.
- HAMILTON-MILLER, J. M. T., SMITH, J. T. & KNOX, R. (1965). Interaction of cephaloridine with penicillinase-producing Gram-negative bacteria. *Nature, Lond.* **208**, 235.
- PERRET, C. J. (1954). Iodometric assay of penicillinase. *Nature, Lond.* **174**, 1012.
- POLLOCK, M. R. & TORRIANI, A. M. (1953). Purification and physicochemical characteristics of penicillinase from *Bacillus cereus*. *C. r. Séanc. Acad. Sci., Paris* **237**, 276.
- SABATH, L. D., JAGO, M. & ABRAHAM, E. P. (1965). Cephalosporinase and penicillinase activities of a β -lactamase from *Pseudomonas pyocyanea*. *Biochem. J.* **96**, 739.
- SMITH, J. T. & HAMILTON-MILLER, J. M. T. (1963). Differences between penicillinases from Gram-positive and Gram-negative bacteria. *Nature, Lond.* **197**, 976.