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The Purification and Properties of a Penicillinase whose Synthesis is Mediated by an R-Factor in *Escherichia coli*

By NAOMI DATTA
Bacteriology Department, Postgraduate Medical School, Ducane Road, London, W. 12
AND M. H. RICHMOND*
Bacterial Physiology Division, National Institute for Medical Research, Mill Hill, London, N.W. 7

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1. The penicillinase (β-lactamase) from *Escherichia coli* strain TEM has been purified and its activity against a range of penicillin and cephalosporin derivatives measured. 2. The enzyme shows little resemblance to penicillinases from *Bacillus cereus*, *Bacillus licheniformis* and *Staphylococcus aureus*. 3. The molecular weight of the enzyme is $16700 \pm 5\%$, which is about half the value obtained for other penicillinases. 4. The enzyme is most similar in properties to a crude preparation of a penicillinase from *Klebsiella* (*Aerobacter*) *aerogenes*, but clearly different from crude enzyme preparations from other strains of *E. coli*. 5. Since penicillinase synthesis in *E. coli* strain TEM is mediated by an R-factor known to infect many other species of Enterobacteriaceae, the appearance of similar enzymes in other Gram-negative species is not surprising.

A number of penicillinases (penicillin β-lactamases; EC 3.5.2.6) from Gram-positive species [i.e. *Bacillus cereus* (Kogut, Pollock & Tridgell, 1956; Pollock, Torriani & Tridgell, 1956), *Bacillus licheniformis* (Pollock, 1965*a*) and *Staphylococcus aureus* (Richmond, 1963, 1965)] have been purified and their properties examined, but hitherto no penicillinase from Gram-negative species has been purified to any extent. The isolation by Kontomichalou (Datta & Kontomichalou, 1965) of a strain of *Escherichia coli* (strain TEM) that synthesized relatively large amounts of penicillinase was a convenient opportunity to attempt the isolation of a penicillinase from a Gram-negative species. The strain has the added interest that the genome responsible for penicillinase synthesis is carried on an extrachromosomal R-factor (Datta & Kontomichalou, 1965). The enzyme should not therefore be regarded strictly as 'the penicillinase of *E. coli*', but rather as the penicillinase synthesized by *E. coli* when it is carrying this particular R-factor. It is known that R-factors, including that present in strain TEM, can be transferred to a wide range of Enterobacteriaceae, but whether the properties of the enzyme are modified by the transfer of the gene to a new host is unknown.

The purified enzyme shows a number of differences from penicillinases obtained from Gram-positive bacteria; perhaps the most striking is the markedly lower molecular weight (approx. 16700 as against about 30000 for the penicillinases from the Gram-positive species). In addition, enzyme synthesis in *E. coli* strain TEM is constitutive, which is unlike the situation found with all strains of Gram-positive bacteria that synthesize penicillinase except *B. cereus* strain 5 (Sneath, 1955).

METHODS AND MATERIALS

Organism and medium. The purification has been developed with *E. coli* strain TEM, a strain in which the penicillinase gene is carried on an R-factor (Datta & Kontomichalou, 1965). This strain is convenient since it synthesizes a relatively large amount of penicillinase (about 80 enzyme units/mg. dry wt. of organisms). The organism was grown in 1% CY medium (Novick, 1962*a*), which has the following composition: sodium β-glycerophosphate, 0·12M; $MgSO_4$, 1mM; trace-metal solution, 0·02ml./l.; yeast extract (Difco), 1·0% (w/v); acid-hydrolysed casein (Difco), 1·0% (w/v); glucose, 0·4%. The trace-metal solution contained: $CuSO_4,5H_2O$, 0·5%; $ZnSO_4,7H_2O$, 0·5%; $FeSO_4,7H_2O$, 0·5%; $MnCl_2,4H_2O$, 0·2%; conc. HCl, 10% (v/v). The glucose and β-glycerophosphate were autoclaved separately and added immediately before use. Agar (2·0%, w/v) was added to 1% CY medium when required.

Column materials. DEAE-cellulose powder (formerly known as grade DE50, now as DE11) was obtained from Whatman Products, Reeve Angell, London. Sephadex G-75 (bead form) and Sephadex G-50 (medium grade) were obtained from Pharmacia, Uppsala, Sweden.

Growth of organisms. The R-factor is lost from *E. coli* strain TEM at a frequency of about 1/1000 divisions, and this strain was therefore maintained on 1% CY agar plates

* Present address: Department of Molecular Biology, University of Edinburgh.

containing 25 μg. of ampicillin/ml. For routine growth purposes a colony was picked from the ampicillin–agar plate and grown overnight in 100 ml. of 1% CY medium on a shaker at 35°. In the morning this culture was diluted to a density of about 0·2 mg. dry wt. of organisms/ml. in fresh 1% CY medium and shaken at 35° until the culture density reached 2–3 mg. dry wt. of organisms/ml. At this point the organisms were collected by centrifugation and used for stage 1 of the purification procedure (see the Results section). For large-scale preparations the cultures were shaken at 35° in 5 l. conical flasks containing not more than 1100 ml. of medium.

Enzyme assays. Penicillinase activities are expressed in the units defined by Pollock & Torriani (1953) for *B. cereus* penicillinase: 1 unit ≡ 1 μmole of benzylpenicillin hydrolysed/hr. at 30° and pH 7·0. Penicillinase was assayed iodometrically at pH 7·0 by the method of Perret (1954) as modified by Novick (1962*a*). The hydrolysis of cephalosporin derivatives was measured similarly except that the amount of cephalosporin destroyed was calculated on a basis of 1 mole of hydrolysed cephalosporin ≡ 4 equiv. of iodine (Alicino, 1961). Michaelis constants were determined by the microiodometric assay (Novick, 1962*b*).

Preparation of chromatography and Sephadex columns. DEAE-cellulose was suspended in 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, and the fines were removed. The column was then poured in 0·1 M-phosphate buffer, pH 7·0, and washed with this buffer until the effluent had reached pH 7·0. The column was prepared for use by washing with about 1 l. of water. The DEAE-cellulose columns used in these experiments were about 1·5 cm. diam. × 15–20 cm. long, and were used at 2°.

The Sephadex columns (approx. 2 cm. × approx. 140 cm.) were prepared as described in the booklet issued by the makers. The columns were poured in water and then equilibrated against 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, before use. They were used at room temperature. In between periods of use the columns were either equilibrated against 0·1 N-acetic acid or 0·1 M-phosphate buffer saturated with $CHCl_3$.

Protein estimations. Protein was normally estimated by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), with an aqueous solution of bovine serum albumin as standard. Elution of proteins from columns was followed by the extinction at 280 mμ.

Starch-gel electrophoresis. Separation of proteins by starch-gel electrophoresis was carried out at pH 8·0 in the discontinuous buffer systems described by Poulik (1957).

RESULTS

Location and liberation of penicillinase in E. coli *strain TEM*

As with other species of Gram-negative bacilli that produce penicillinase, the enzyme of *E. coli* strain TEM is associated with the cells, and normally none is found in the culture supernatant. The enzyme in the intact organisms does not appear to be readily accessible to substrate, since a 130-fold increase in enzyme activity occurs on ultrasonic disruption under the conditions described below. Other treatments designed to disrupt the bacterial surface, such as toluene, EDTA at pH 8·0 (Repaske, 1958) or EDTA plus 20% (w/v) sucrose at pH 8·0 (Neu & Heppel, 1964), liberated up to 35% of the total enzyme in a soluble form. The fact that the enzyme is liberated from the bacteria under conditions in which the cytoplasmic membrane is protected (Neu & Heppel, 1964) suggests that penicillinase may be one of the 'periplasmic enzymes' of *E. coli*.

Since the most efficient liberation of enzyme was achieved by ultrasonic disintegration, this was the method chosen as a preliminary step in purification of the enzyme.

Purification of the penicillinase

The recoveries at each stage are shown in Table 1. All phosphate buffers were made from disodium hydrogen phosphate and potassium dihydrogen phosphate.

Stage 1. Organisms from the late exponential phase of growth were harvested by centrifugation, washed well in 0·1 M-phosphate buffer, pH 7·0, and resuspended in a similar buffer to a density of about 100 mg. dry wt. of organisms/ml. The

Table 1. *Summary of the purification of penicillinase from* E. coli

Experimental details are given in the text. The starting material was 3 l. of culture containing 11·1 g. dry wt. of organisms.

Stage	Procedure	Enzyme activity recovered (units)	Specific enzyme activity (units/μg. of enzyme)	Recovery (%) Per stage	Recovery (%) Overall
1	Ultrasonic disintegration	683000	1	100	100
2	Centrifugation and dialysis	674000	0·82	98·6	98·6
3	Adsorption and elution on DEAE-cellulose	491800	14·7	72·9	72·0
4	Chromatography on DEAE-cellulose	298200	41·2	60·6	43·6
5	Sephadex G-50	261000	68·3	87·6	38·2
6	Sephadex G-75	234000	81·2	89·6	34·2
7	Rechromatography on DEAE-cellulose	147400	80·7	62·9	21·6

suspension was stored at 2° and 8·0ml. portions were broken for 2min. at 2° in a Mullard 25kcyc./sec. ultrasonic disintegrator. The temperature was kept low by packing the transducer cup in a vacuum flask filled with crushed ice. The broken cells were stored at 2° until all the suspension had been broken and then centrifuged at 5000*g* for 20min. at 2° to remove whole cells and cell debris.

Stage 2. The supernatant from the low-speed centrifugation was centrifuged again (first at 30000*g* for 4hr. at 2°, then at 105000*g* for 2hr. at 2°) and the pellets were discarded. The supernatant from these centrifugation steps was then dialysed twice for 16hr. each at 2° against 0·01M-phosphate buffer, pH 7·0. During this step a slight precipitate formed, which was removed by centrifuging at 5000*g* for 15min. at 2°.

Stage 3. The enzyme solution, after dialysis, was loaded on to a DEAE-cellulose column (prepared as described in the Methods and Materials section) and washed on the column with about 500ml. of water. A great deal of protein and some pigmented material passed straight through the column at this stage and these were discarded. The enzyme was then eluted (rate of flow, 1·0ml./min.) with 200ml. of 0·03M-phosphate buffer, pH 7·0, and 6ml. fractions were collected. The fractions containing the enzyme were combined, freeze-dried, dissolved in about 5·0ml. of water and dialysed overnight against 0·01M-phosphate buffer, pH 7·0, at 2°.

Stage 4: chromatography on DEAE-cellulose. The enzyme preparation was reloaded on to a DEAE-cellulose column prepared as described in the Methods and Materials section and washed with 200ml. of water. The enzyme was then eluted (6ml. fractions) with a concentration gradient of phosphate buffer, pH 7·0. In an experiment in which about 600000 units of enzyme were loaded on to a column 1·2cm. diam. × 20cm. long, the gradient was constructed with 300ml. each of 0·003M- and 0·05M-phosphate buffer. Fig. 1 shows a typical elution pattern. The fractions containing the enzyme (20–30, Fig. 1; total vol. 66ml.) were pooled, freeze-dried, dissolved in about 2·0ml. of water and dialysed overnight against 0·1M-phosphate buffer, pH 7·0, at 2°.

*Stage 5: separation through Sephadex G-*50. The enzyme was loaded on to a Sephadex G-50 column (2·1cm. × 138cm.) that had already been equilibrated against 0·1M-phosphate buffer, pH 7·0, and eluted (5·8ml. fractions) with similar buffer. Fig. 2 shows the elution diagram obtained. Fractions containing penicillinase at a specific activity greater than 65units/μg. of protein were pooled, freeze-dried, dissolved in 2·0ml. of water and dialysed overnight against 0·1M-phosphate buffer, pH 7·0, at 2°.

*Stage 6: separation through Sephadex G-*75. The enzyme was loaded on to a Sephadex G-75 column (1·9cm. × 157cm.) that had already been equilibrated against 0·1M-phosphate buffer, pH 7·0, and the enzyme eluted with similar buffer. Fig. 3 shows the elution pattern obtained at this stage. About 85% of the total enzyme activity was obtained in three 6ml. fractions with specific enzyme activities of 79·6, 81·2 and 80·3units/μg. of protein, and this suggests that the enzyme is nearly pure. The purity of the enzyme was checked by electrophoresis in starch gel (see the Methods and Materials section), where only one protein band which corresponded with enzyme activity could be detected. Examination of the purified enzyme in the

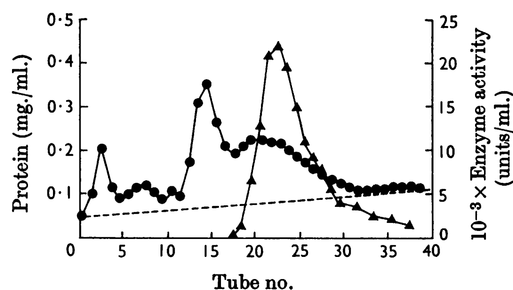

Fig. 1. Chromatography of *E. coli* penicillinase on DEAE-cellulose. Samples (6·0ml.) were collected and assayed for penicillinase (▲) and protein (●) content. The broken line indicates the non-specific reaction obtained in a dummy run with a column of similar dimensions. Protein estimations were carried out by the Lowry method (Lowry *et al.* 1951).

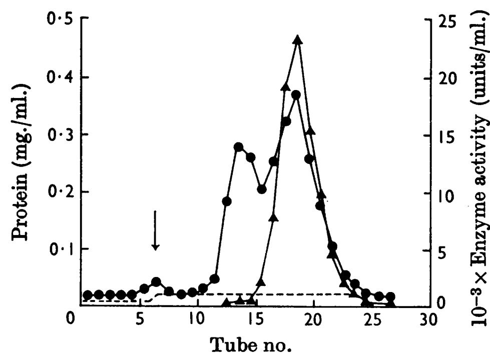

Fig. 2. Separation of *E. coli* penicillinase through Sephadex G-50 equilibrated against 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0. Each fraction (5·8ml.) was assayed for protein (●) and enzyme (▲) content. The broken line indicates non-specific reaction. The arrow indicates the exclusion volume of the column. About 15 fractions were discarded at the beginning of the run. Protein estimations were carried out by the Lowry method (Lowry *et al.* 1951).

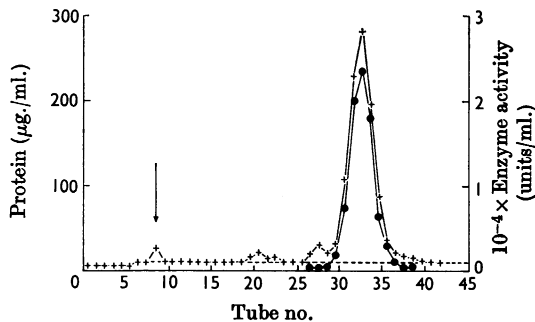

Fig. 3. Separation of *E. coli* penicillinase through Sephadex G-75 equilibrated against 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0. Each fraction (6·0 ml.) was assayed for protein (+) and enzyme (●) content. The broken line indicates non-specific reaction. The arrow marks the exclusion volume of the column. About 15 fractions were discarded at the beginning of the run. Protein estimations were carried out by the Lowry method (Lowry *et al.* 1951).

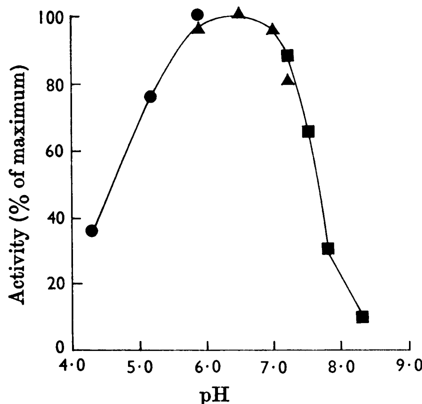

Fig. 4. pH–activity curve for the purified penicillinase from *E. coli* strain TEM. Sodium acetate buffers (0·1M) were used for the range pH 4·3–5·9 (●), Na_2HPO_4–KH_2PO_4 buffers (0·1M) for the range pH 5·9–7·2 (▲) and tris–HCl buffers (0·1M) for the range pH 7·2–8·3 (■).

analytical ultracentrifuge (enzyme concn., 8·8 mg./ml. in 0·03M-phosphate buffer, pH 7·0) showed a single symmetrical peak.

Stage 7: rechromatography on DEAE-cellulose. To check the purity of the enzyme the preparation was chromatographed once more on DEAE-cellulose, as described in stage 4. The protein peak coincided with the peak in enzyme activity exactly, and the specific activity of the enzyme across the peak was nearly constant (min. 76·4, max. 82·1 units/µg. of protein).

Properties of the penicillinase

Molecular weight. The molecular weight of the enzyme was found by the equilibrium-ultracentrifugation method of Yphantis (1964) to be 16700 ± 5%. This value agrees well with the fact that the enzyme was almost completely excluded by Sephadex G-50. The enzyme had $S_{20,w}$ 1·85 s when compared with lysozyme by centrifugation in a sucrose density gradient (Charlwood, 1964).

pH–*activity curve.* The pH–activity curve (Fig. 4) was determined over the range pH 4·3–8·3 in various buffer mixtures. The enzyme has a broad optimum over the range pH 5·8–7·0. As with the penicillinase of *S. aureus* (Richmond, 1963), the enzyme is remarkable for a sharp fall in activity at alkaline pH values.

Adsorption to surfaces. The purified enzyme was only poorly adsorbed on to sand, sintered, powdered or scratched glass. In this respect the enzyme is like the penicillinase from *B. licheniformis* (Collins, 1964; Pollock, 1965*a*) but unlike those from *S. aureus* (Richmond, 1963) and *B. cereus* (Kogut *et al.* 1956).

Substrate-specificity and Michaelis constants. The purified enzyme was tested for hydrolysis of various penicillin and cephalosporin derivatives, and $V_{max.}$ and K_m values are shown in Table 2. The enzyme is somewhat similar to penicillinase from *B. licheniformis* strain 749 (Collins, 1964) but sharply different from the penicillinase of *S. aureus* (Richmond, 1963), since it has a higher affinity for methicillin than benzylpenicillin, although the $V_{max.}$ value is lower. In general the enzyme shows a broad specificity of action against both penicillin and cephalosporin derivatives. Thus cephaloridine is hydrolysed almost as rapidly as benzylpenicillin.

Inhibitors. A number of compounds that are known to inhibit enzymes were tested for their effect on this purified penicillinase. Unless otherwise stated the enzyme was incubated in the presence of 1mM-inhibitor for 15 min. at room temperature, and then assayed for enzyme activity. An equal quantity of enzyme, incubated without the inhibitor, acted as control. Disodium EDTA had no effect at pH 7·0 on the enzyme; *p*-chloromercuribenzoate produced about a 15% inhibition at 1mM. Smith (1963*a*) has reported that the penicillinase of *Aerobacter cloacae* is exceptional among penicillinases in being sensitive to *p*-chloromercuribenzoate. The *Aerobacter* enzyme is about 50% inhibited by 2µM-*p*-chloromercuribenzoate at pH 7·4, and this is about 500-fold less than the concentration needed to inhibit the enzyme from *E. coli* strain TEM by 15%. Probably, therefore, the enzyme in *E. coli* strain TEM contains no reactive thiol groups.

The enzyme from *E. coli* strain TEM is extremely

Table 2. *Kinetics of hydrolysis of various penicillin and cephalosporin derivatives by purified penicillinase from* E. coli

Experimental details are given in the Methods and Materials section. $V_{max.}$ values are expressed as moles of substrate hydrolysed/mole of enzyme/min. at 30° and pH 7·0. The $V_{max.}$ value for benzylpenicillin (2×10^4 moles of penicillin/mole of enzyme/min.) corresponds to a specific enzyme activity of 81 enzyme units/μg. of protein.

Substrate: approved name and chemical name	$V_{max.}$ (turnover no.)	K_m (μM)
Benzylpenicillin [6-(phenylacetamido)penicillanic acid]	2×10^4	22
Phenoxymethylpenicillin [6-(phenoxyacetamido)penicillanic acid]	1×10^4	—
Phenethicillin [DL-6-(α-phenoxypropionamido)penicillanic acid]	$0·8 \times 10^4$	—
6-Aminopenicillanic acid	$1·7 \times 10^4$	22
Methicillin [6-(2,6-dimethoxybenzamido)penicillanic acid]	2×10^2	3·2
Cloxacillin [6-(3-*o*-chlorophenyl-5-methylisoxazole-4-carboxamido)penicillanic acid]	$5·4 \times 10^3$	
Ampicillin {6-[D(−)-α-aminophenylacetamido]penicillanic acid}	$2·2 \times 10^4$	2·3
Quinacillin [6-(3-carboxyquinoxalin-2-ylamido)penicillanic acid]	6×10^2	260
Cephalosporin C [7-(5-amino-5-carboxyvaleramido)cephalosporanic acid]	$1·6 \times 10^2$	—
Cephaloram (7-phenylacetamidocephalosporanic acid)	$3·2 \times 10^3$	—
Cephaloridine {7-[(thien-2-yl)acetamido]-3-(pyrid-1-ylmethyl)-3-cephem-4-carboxylic acid betaine}	$1·3 \times 10^4$	600

Table 3. *Comparison of the properties of purified penicillinase preparations from different species*

Data are taken from Pollock *et al.* (1956), Kogut *et al.* (1956) and M. R. Pollock (personal communication) for *B. cereus*, from Collins (1964) and Pollock (1965*a*) for *B. licheniformis* and from Richmond (1963, 1965) for *S. aureus*. 'Physiological efficiency' is defined by Pollock (1965*b*).

		Mol. wt.	Turnover no. (moles of substrate hydrolysed/ mole of enzyme/min.)	'Physiological efficiency' ($V_{max.}/K_m$)	Wt. of penicillinase/ dry wt. of organisms (%)
Gram-negative:	*E. coli* (strain TEM)	16700	$1·9 \times 10^4$	$3·6 \times 10^6$	0·1
Gram-positive:	*B. cereus* (569/H)	31600	$1·6 \times 10^5$	$5·9 \times 10^6$	1·1
	B. cereus (5)	35700	$1·5 \times 10^5$	$4·9 \times 10^6$	0·9
	B. licheniformis (749/C)	28000	$1·1 \times 10^5$	$6·6 \times 10^6$	1·2
	B. licheniformis (6346/C)	28000	$2·1 \times 10^4$	$5·7 \times 10^6$	1·1
	S. aureus (types A and C): constitutive mutant	29600	$2·0 \times 10^4$	$1·6 \times 10^7$	0·75–0·9

sensitive to iodine–potassium iodide solution. The enzyme was about 50% inhibited by a solution made 30μN with respect to iodine, and was completely inhibited within 2 min. by a solution made 1 mN with respect to iodine.

DISCUSSION

The main difference, so far, between the penicillinase made by *E. coli* strain TEM and the other penicillinases that have been purified is the size of the molecule. At a value of approx. 16700, the molecular weight of this penicillinase is about half that of the other penicillinases (Table 3). In terms of turnover numbers, however, the enzyme from *E. coli* is similar in specific activity against benzylpenicillin to the enzyme from *S. aureus* rather than those from the *Bacillus* spp. (Table 3), all but one of which are about 6–10 times as active. Pollock (1965*b*) has introduced the term 'physiological efficiency' (defined as $V_{max.}/K_m$) as a measure of the efficiency of an enzyme at hydrolysing various substrates under physiological conditions. By this parameter all the penicillinases are almost equally 'efficient' at hydrolysing benzylpenicillin under conditions where the substrate concentration is limiting (Table 3). A point where *E. coli* is clearly distinct from the Gram-positive species is in the amount of penicillinase protein synthesized by the culture when unrepressed. An enzyme content of

80 enzyme units/mg. dry wt. of organisms corresponds to a penicillinase protein content of $1\mu g$. of protein/mg. dry wt. of bacteria, and this proportion of the total dry weight of the organisms (0·1%) is about one-tenth of the value found in the Gram-positive species (Table 3).

A number of crude preparations of penicillinases from Gram-negative species have been tested for their relative rates of hydrolysis of penicillin and cephalosporin derivatives (e.g. Smith, 1963*a*,*b*; Smith & Hamilton-Miller, 1963*a*,*b*; Hamilton-Miller, 1963; Fleming, Goldner & Glass, 1963), but it is not possible to identify for certain the enzyme studied here with any of those enzymes. This enzyme is clearly not the one studied in *Aerobacter cloacae* (now called *Enterobacter cloacae*) (Smith, 1963*a*) because of its insensitivity to *p*-chloromercuribenzoate, and it is also different from the one studied in *E. coli* strain 214T (Smith, 1963*b*) because of the clear differences between the relative rates of hydrolysis of benzylpenicillin and 6-aminopenicillanic acid by the two enzymes. Nor is it the enzyme found in *Enterobacter cloacae* (Fleming *et al.* 1963), which is predominantly a cephalosporinase and only slightly active against benzylpenicillin. The enzyme described in the present paper is most similar to those studied in *Klebsiella* (*Aerobacter*) *aerogenes* by Smith & Hamilton-Miller (1963*b*; Hamilton-Miller, 1963). The fact that a penicillinase in *E. coli* should be similar to an enzyme in *K. aerogenes* is not surprising, since the enzyme synthesis in *E. coli* strain TEM is mediated by an R-factor that is known readily to infect other genera of Enterobacteriaceae (Datta & Kontomichalou, 1965). Unfortunately, it is not yet known whether the penicillinase genes in the *K. aerogenes* strains studied by Smith & Hamilton-Miller (1963*a*,*b*) were carried on R-factors.

It is likely that penicillinase genes in Gram-negative species can either be chromosomal or carried on R-factors (Datta, 1965), and in view of the infectivity of R-factors among the Enterobacteriaceae it is important that no penicillinase should be regarded as 'species-specific' until its synthesis is proved to be controlled by chromosomal genes.

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