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Enterobacter cloacae P99

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tuents as methicillin and cloxacillin (5/1 and 291/1). The value of K_i for P99 enzyme, 5/1 inhibitor and cephaloridine substrate is 0.128 μM. The structure of the acyl substituent of the inhibitor is the main determining factor; the nucleus has little effect and analogous penicillins and cephalosporins have similar inhibitory activity. For cephalosporins, the structure of the group in the 3 position can affect β-lactamase inhibition, and it has been suggested that a stable group which does not readily accept electrons gives the cephalosporin a low affinity for the enzyme and thus reduces its effect as a competitive inhibitor.[5] Some substrate inhibition of P99 enzyme by cephaloridine has been detected for substrate concentrations greater than 0.5 mM.

The hydrolysis of cephaloridine by the P99 enzyme is 5% and 30% inhibited by 10 μM and 0.1 mM p-chloromercuribenzoate, respectively, when inhibitor is added to enzyme immediately before substrate (37°, spectrophotometric assay). It is completely inhibited by 0.1 mM mercuric chloride and by 0.01 mM iodine. Iodoacetate or divalent cations other than mercuric (Mg^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}) have no inhibitor effect at concentrations between 1 μM and 1 mM, even when incubated with the enzyme at 37° for 1 hr before assay. Mechanical shaking (2 min) inactivates the enzyme, but this can be prevented by addition of 0.5% gelatin.

[53f] β-Lactamases (Actinomycetes Species)

By KENNETH JOHNSON, COLETTE DUEZ, JEAN-MARIE FRÈRE, and JEAN-MARIE GHUYSEN

Strains and Culture

Strains. The aerobic euactinomycetes are true gram-positive bacteria which form a characteristic mycelium and multiply by means of special spores like the fungi. Based on the structure of their wall peptidoglycan, the various genera are divided into several groups.[1] The two strains used are soil isolates[2,3] and are assigned arbitrary designations. *Streptomyces albus* G (in fact probably a *Streptomyces griseus*)[3] has a peptidoglycan of the general chemotype II with LL-diaminopimelic acid and glycine.[4,5] Although strain R39 (origin: Ruwenzori; soil sample No.

[1] K. H. Schleifer and O. Kandler, *Bacteriol. Rev.* **36**, 407 (1972).
[2] M. Welsch, *Rev. Belge Pathol. Med. Exp.* **18**, Suppl. 2, 1 (1947).
[3] M. Welsch and A. Rutten-Pinckaers, *Bull. Soc. Roy. Sci. Liege* **3–4**, 374 (1963).
[4] J. M. Ghuysen, *Bacteriol. Rev.* **32**, 425 (1968).
[5] M. Leyh-Bouille, R. Bonaly, J. M. Ghuysen, R. Tinelli, and D. J. Tipper, *Biochemistry* **9**, 2944 (1970).

XVI)[3] also has the appearance of a *Streptomyces*, its peptidoglycan is of the general chemotype I, with *meso*-diaminopimelic acid but no glycine.[4,6] The selected strains produce both an exocellular β-lactamase[7,8] and an exocellular DD-carboxypeptidase.[9] The DD-carboxypeptidase from strain R39 liberates the C-terminal D-Ala residue from R-D-Ala-D-Ala peptides (R-D-Ala-D-Ala + $H_2O \rightarrow$ D-Ala + R-D-Ala) and catalyzes bimolecular transfer reactions in which R-D-Ala-D-Ala peptides act as acyl donors (R-D-Ala-D-Ala + NH_2-R′ $\rightarrow$ D-Ala + R-D-Ala-R′).[9] β-Lactam antibiotics react with the R39 DD-carboxypeptidase-transpeptidase to form equimolar and inactive antibiotic–enzyme complexes.[10] In Tris·HCl buffer pH 7.7 containing 0.2 *M* NaCl and 50 m*M* $MgCl_2$ and at 37°, the half-life of the benzyl penicillin–enzyme complex is 70 hr. During dissociation, the enzyme undergoes reactivation whereas the released antibiotic molecule is neither benzylpenicillin nor benzylpenicilloic acid.[10] While DD-carboxypeptidase from strain *albus* G also hydrolyzes the R-D-Ala-D-Ala peptides, it does not catalyze transpeptidation reactions and is virtually unaffected by β-lactam antibiotics.[9]

Culture Media. Peptone oxoid medium contains 1% peptone oxoid, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$, and 0.05% KCl.

Agar-APG medium contains per liter of final volume: agar 20 g; asparagine, 0.5 g; peptone oxoid 0.5 g; glucose 10 g; and K_2HPO_4, 0.5 g.

Agar KC medium contains per liter of final volume: agar, 20 g; partially hydrolyzed keratin from white hens' feathers, 2.5 g; partially hydrolyzed casein, 2.5 g; NaCl, 0.5 g; $CaCO_3$, 0.1 g; $MgSO_4 \cdot 7H_2O$ 0.1 g; K_2HPO_4, 1 g; and $FeSO_4 \cdot 7H_2O$, 0.1 g. Final pH is 7.5. Hydrolyzed keratin and casein are prepared as follows: 100 g of dried, white hens' feathers are treated for 1 hr at 100° with 1 liter of *N*/8 KOH. After centrifugation, casein (100 g) is added to the supernatant fraction and dissolved by heating at 70°. The pH of the mixture (final volume: 1 liter) is adjusted to 7.5–8.0; 25 ml contain 2.5 g of both partially hydrolyzed keratin and casein.

Maintenance of Strains. Strain R39 is grown at 28° on slants of agar KC and strain *albus* G on slants of agar APG. Abundant sporulation occurs after 4–5 days. The strains are then maintained at 4°.

[6] J. M. Ghuysen, M. Leyh-Bouille, J. N. Campbell, R. Moreno, J. M. Frère, C. Duez, M. Nieto, and H. R. Perkins, *Biochemistry* **12**, 1243 (1973).
[7] M. Welsch, *Proc. 4th Int. Congr. Microbiol., Copenhagen, 1947,* Section 1, p. 144.
[8] K. Johnson, J. Dusart, J. N. Campbell, and J. M. Ghuysen, *Antimicrob. Ag. Chemother.* **3**, 289 (1973).
[9] J. M. Ghuysen, M. Leyh-Bouille, J. M. Frère, J. Dusart, A. Marquet, H. R. Perkins, and M. Nieto, *Ann. N.Y. Acad. Sci.* **235**, 236 (1974).
[10] J. M. Frère, J. M. Ghuysen, P. E. Reynolds, R. Moreno, and H. R. Perkins, *Biochem. J.* **143**, 233 (1974).

Assay Method for β-Lactamases[11]

Unit. One unit of β-lactamase catalyzes the hydrolysis of 1 μmole of benzylpenicillin per minute at 30°.

Reagents

Sodium acetate buffer, 1 *M*, pH 3.6

Color reagent: equal volumes of a water-soluble starch solution (0.8%, w/v) and of a 240 μ*M* I_2 + 4.8 m*M* KI solution.

Procedure. The following is a microscale adaptation of the technique of Novick and Dubnau.[12] Benzylpenicillin (0.3 μmoles) is incubated with the enzyme preparation in 30 μl (final volume) of 25 m*M* sodium phosphate buffer, pH 7.0. The benzylpenicillin concentration in the mixture is 10 m*M* and, hence, any β-lactamase with a K_m value for this antibiotic equal to or lower than 1 m*M* is saturated. After 10–30 min, to the reaction mixture are added in sequence 200 μl of 1 *M* acetate buffer, and then 200 μl of color reagent. After 10 min at 25°, the optical density of the solution is measured at 620 nm. Control consisting of the same mixture lacking enzyme is incubated as above. Acetate buffer, color reagent, and finally the same amount of enzyme as used in the test are added, and the optical density at 620 nm is determined. A decrease of the optical density of 0.1 corresponds to about 0.37 nmole of hydrolyzed benzylpenicillin.

Determination of K_m and V_{max} Values for Various β-Lactam Antibiotics

Reagents

Sodium acetate buffer, 5 *M* pH 3.6

Color reagent: the same as above

Procedure for Benzylpenicillin. Enzyme and benzylpenicillin are incubated together at 30° in 190 μl, final volume, of 25 m*M* sodium phosphate buffer pH 7.0. After 10–30 min, to the reaction mixtures are added 40 μl of 5 *M* acetate buffer and then 200 μl of color reagent. The remainder of the procedure is as described above. This technique allows the determination of a K_m value as low as 30 μ*M*. With initial concentrations of benzylpenicillin ranging from 9 to 90 μ*M* (i.e., from 0.3 to 3 × a K_m value of 30 μ*M*), a 10% utilization of the substrate corresponds to decreases of the absorbance of the final solutions ranging from 0.046 to 0.460.

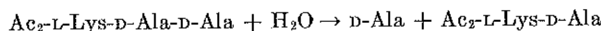
[11] This volume [5].

[12] R. P. Novick, *J. Gen. Microbiol.* 33, 121 (1963).

Procedure for Other β-Lactam Antibiotics. The same procedure as for benzylpenicillin is used. However, the products of β-lactamase action upon 6-aminopenicillanic acid and its derivatives and those obtained from 7-aminocephalosporanic acid and its derivatives have not the same iodine uptake. A decrease of the absorbance at 620 nm of 0.1 corresponds to more hydrolyzed cephalothin (about 0.58 nmole) than to hydrolyzed benzylpenicillin. Standard curves [hydrolyzed β-lactam antibiotic vs iodine uptake] must be used as references.

Assay Methods for DD-Carboxypeptidases

Reaction



$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + H_2O \rightarrow \text{D-Ala} + Ac_2\text{-L-Lys-D-Ala}$$

Unit. One unit of DD-carboxypeptidase catalyses the hydrolysis of 1 μmole of N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine into D-alanine and N^{α},N^{ε}-diacetyl-L-lysyl-D-alanine, per minute at 37°.

Substrates. Nonradioactive tripeptide Ac_2-L-Lys-D-Ala-D-Ala is prepared as described by Nieto and Perkins.[13] The same peptide radioactively labeled with ^{14}C in both acetyl groups (specific activity 10,000 dpm/nmole) is prepared as described by Perkins *et al.*[14]

Standard Incubation Conditions. Ac_2-L-Lys-D-Ala-D-Ala (0.25 μmole) is incubated with the relevant enzyme (K_m values: 0.8 and 0.33 m*M* for R39 and *albus* G enzymes, respectively) at 37° in 30 μl (final volumes) of 30 m*M* Tris·HCl buffer, pH 7.5, supplemented with 3 m*M* $MgCl_2$. Hydrolysis products (D-Ala or Ac_2-L-Lys-D-Ala dipeptide) are then estimated by using one of the following procedures.

Chemical Estimation of Free Alanine

The following is a modification of the technique of Ghuysen *et al.*[15]

FDNB Reagent. Fluorodinitrobenzene, 130 μl in 10 ml 100% ethanol.

Procedure. Samples containing 10–50 nmoles of alanine are mixed with 10% $K_2B_4O_7$ and water to give a total volume of 100 μl of 1% $K_2B_4O_7$. FNDB reagent (10 μl) is added. The solutions are mixed and incubated at 60° for 30 min. After acidification with 50 μl of 12 *N* HCl, the DNP-alanine is extracted three times with 200 μl of ether. The ether extracts are evaporated in a stream of hot air and dried *in vacuo*. The residues are dissolved in methanol and chromatographed at room temperature on thin-layer plates of silica gel G in chloroform:methanol:acetic acid

[13] M. Nieto and H. R. Perkins, *Biochem. J.* **123**, 789 (1971).

[14] H. R. Perkins, M. Nieto, J. M. Frère, M. Leyh-Bouille, and J. M. Ghuysen, *Biochem. J.* **131**, 707 (1973).

[15] J. M. Ghuysen, D. J. Tipper, and J. L. Strominger, this series, Vol. 8, p. 685.

(220:25:5, v/v/v). DNP-alanine moves faster than DNP-Tris. After drying, the DNP-alanine spots are transferred to 1-ml tubes and eluted by vigorous mixing with 500 μl of water:ethanol:25% (0.91) ammonia (100:100:054, v/v/v). After centrifugation, the optical density of the supernatant fractions is measured at 360 nm. The molar extinction coefficient for DNP-alanine is about 15,000.

Enzymic Estimation of D-*Alanine*

The following modifications of the technique of Ghuysen *et al.*[15] permit one to carry out many simultaneous tests in a very short time.

Reagents

o-Dianisidine (Merck, pro analysis): 10 mg/ml in methanol (freshly prepared)
K pyrophosphate buffer, 0.1 *M*, pH 8.3
FAD (monosodium; Boehringer): 0.3 mg/ml in pyrophosphate buffer
Peroxidase (Boehringer. Reinheitsgrad 1. Für analytische Zwecke; suspension 10 mg/ml) to be diluted to 10 μg/ml in H_2O
D-Amino acid oxidase (Boehringer. Für analytische Zwecke; Kristallsuspension: 5 mg/ml)
Enzymes and coenzyme mixture (freshly prepared): pyrophosphate buffer:FAD solution:diluted peroxidase solution:D-amino acid oxidase suspension (20:10:5:1, v/v/v/v).

Procedure. Samples (30 μl) containing 5–40 nmoles of D-alanine are mixed with 5 μl of *o*-dianisidine solution and 70 μl of the enzymes–coenzyme mixture. To such solutions, incubated 5 min at 37°, is added 400 μl of methanol:water (v/v). Following an additional 2-min incubation at 37°, the absorbance at 460 nm is immediately measured. (Coloration of the solution is slightly labilized after addition of the methanol–water solution.) Blanks consist of the same mixtures as above lacking Ac_2-L-Lys-D-Ala-D-Ala tripeptide. Controls are blanks containing known amounts of D-alanine.

Estimation of Radioactive [^{14}C]Ac_2-L-*Lys*-D-*Ala Dipeptide*

Reagents

Collidine–acetic acid–water (9.1:2.65:1000, v/v/v), buffer pH 6.4
Liquid scintillation: 2,2-*p*-phenylenebis (5-phenyloxazole) (POPOP), 100 mg; 2,5-diphenyloxazole, (PPO), 4 g; toluene, 1 liter

Procedure.[14] Samples (30 μl) containing 10,000–20,000 dpm are diluted with 40 μl of water. These are spotted as bands, 30 cm from the cathode on 4 cm × 1.5 m strips of Whatman 3MM paper, and subjected to electrophoresis at pH 6.4 for 4 hr at 60 V/cm, under a Sol T Shell. A Gilson high voltage, 10,000 V, Electrophoretor Model DW equipped with a cooling device is used as power source. Residual [^{14}C]Ac$_2$-L-Lys-D-Ala-D-Ala and the reaction product [^{14}C]Ac$_2$-L-Lys-D-Ala move 65 and 75 cm, respectively, toward the anode. The radioactive compounds are located on the dried strips with a Packard Radiochromatogram Scanner Model 7201. Cuts of the radioactive spots (10 mm section) are placed in vials, to each of which is added 0.75 ml of the scintillation liquid. Counting is performed in a Packard Tri-Carb liquid scintillation spectrometer.

Excretion of β-Lactamase and DD-Carboxypeptidase by *Streptomyces* Strains

Streptomyces can be grown aerobically in 1-liter flasks containing 500 ml of peptone oxoid medium on a New Brunswick shaker at 28°. Inoculation is made with a 20-ml suspension of actively growing *Streptomyces.* Maximal β-lactamase activity (about 3 to 7 × 10^{-2} units/ml) and maximal DD-carboxypeptidase activity (about 2.5 to 10 × 10^{-3} units/ml) occur after 48–72 hr of culture. Both activities then disappear progressively and are negligible after 6 days, at which time mycelium production is maximal (1.5–2 g dry weight per liter). With time, the two enzyme activities increase and decrease independently of each other.

Streptomyces can also be grown in 500-liter tanks. After two successive subcultures of increasing size, 100 liters of culture of actively growing *Streptomyces* are used to inoculate 400 liters of medium contained in a 500-liter tank. This culture is grown at 28° for 72–96 hr with mechanical stirring (120 rpm) and an air flow rate of 100 liter/min at an air pressure of 1.5 kg/cm^2. Silicone A emulsion (Dow Corning, 20 ml) is used as antifoam. Maximal β-lactamase activity and maximal DD-carboxypeptidase activity are comparable to those obtained with cultures carried out in 1-liter flasks.

Adsorption and Storage of Crude Enzymes. Both β-lactamase and DD-carboxypeptidase in 500 liters of culture fluid are adsorbed either on 5 kg of moist diethylaminoethyl (DEAE)-cellulose previously equilibrated against 50 m*M* sodium phosphate buffer pH 7.0 (*Streptomyces* R39), or on 10 kg of Amberlite CG50 or XE64 H$^+$ by adjusting the pH to 5 with acetic acid (*Streptomyces albus* G). The enzyme–resin complexes are collected by centrifugation with a Sharpless type MU865P33 (17,000 *g*). They can be stored for months at 0° without loss of activity.

Isolation of *Streptomyces* β-Lactamases

Concentration of Enzyme Preparations

Unless otherwise stated, concentration is carried out by ultrafiltration through UM10 membranes with an Amicon apparatus (Amicon Corporation, Lexington, Massachusetts).

Preparative Polyacrylamide Gel Electrophoresis

It is carried out with a Shandon preparative apparatus No. SAE-2782. The gel, polymerized in the presence of 0.14% ammonium persulfate, is prepared in Tris-glycine buffer pH 8.3, 42 m*M* with respect to Tris. Height of the gel column: 3 cm. Electrophoresis: 2 hr at 320 V (40 mA/tube) at 10°. Fractions reaching the bottom of the gel are collected by means of a flow of 0.5 *M* Tris-acetate buffer pH 8.3.

Phosphate Buffer

Unless otherwise stated, 50 m*M* sodium phosphate buffer, pH 7.0, is used.

General Remarks

During fractionation, it is essential to monitor the collected fractions for β-lactamase and DD-carboxypeptidase activity and to keep for further processing those fractions preferentially enriched in β-lactamase. The β-lactamase and DD-carboxypeptidase from *Streptomyces* R39 are anionic proteins at pH 8.3. At this pH, the β-lactamase of *Streptomyces albus* G is anionic whereas its DD-carboxypeptidase is cationic. The procedures described below give rise to preparations devoid of any detectable DD-carboxypeptidase activity after periods up to 24 hr of incubation with Ac_2-L-Lys-D-Ala-D-Ala.

Procedures

Unless otherwise stated, all the operations described below are carried out at 4°. Recoveries and specific activities with benzylpenicillin as substrate are given in Table I.

β-Lactamase from Strain R39

Step 1. The enzyme–DEAE–cellulose complex (640 g; corresponding to about 60 liters of culture fluid) is extracted twice with 1200 ml of

TABLE I
SUMMARY OF PURIFICATION PROCEDURES[a]

Strain	Step	Total units	Total protein (mg)	Specific activity (unit/mg protein)	Purification (fold)	Percent activity recovered	Total units DD-carboxy-peptidase
R39	Culture filtrate (60 l)	2180	346,104	0.0063	—	100	690
	Step 1	2180	12,867	0.169	27	100	690
	Step 2	1506	177	8.52	1400	69	0
	Step 3	686	3.5	198	31,500	31	0
albus G	Culture filtrate (10 l)	466	39,840	0.012	—	100	30
	Step 1	252	210	1.2	10.0	55	—
	Step 2	144	16	9	750	30	—
	Step 3	24	0.9	33	2,250	5	0

[a] Proteins are estimated by measuring the amount of total amino groups available to fluorodinitrobenzene after 6 *N* HCl hydrolysis (100°, 17 hr), with bovine serum albumin as standard.

phosphate buffer containing 1 M NaCl. The combined extracts (1 volume) at 0° are slowly added with 1.60 volume of acetone previously cooled at —20°, the precipitate collected by centrifugation and dissolved in 200 ml of phosphate buffer containing 0.2 M NaCl. The solution is centrifuged and concentrated to 80 ml.

Step 2. After step 1, the enzyme is adsorbed onto a 180 ml column of DEAE-cellulose equilibrated against phosphate buffer containing 0.2 M NaCl. After washing with the same buffer until only traces of material absorbing at 280 nm remain, most of the DD-carboxypeptidase is eluted with phosphate buffer containing 0.33 M NaCl. β-Lactamase is eluted in its turn with an increasing convex gradient of NaCl (mixing flask at constant volume: 750 ml phosphate buffer containing 0.33 M NaCl; adding solution: phosphate buffer containing 0.45 M NaCl). Active fractions (from 1550 to 2000 ml) are pooled and concentrated by ultrafiltration to 25 ml. The concentrated solution is filtered through a 700-ml column of Sephadex G-100 in phosphate buffer containing 0.2 M NaCl. β-Lactamase is eluted at a K_D value of 0.21 and is at least partially separated from two yellow and brown pigments of lower and higher K_D values, respectively. Active fractions (from 340 to 390 ml) are pooled.

Step 3. After step 2, the enzyme solution is submitted to additional chromatography on DEAE-cellulose and filtration on Sephadex G-100 as described in step 2. Active fractions are pooled, concentrated by ultrafiltration to 5 ml, and the concentrated solution is filtered through a 100 ml column of Sephadex G-75 in phosphate buffer containing 0.2 M NaCl. All the active fractions exhibit the same high specific activity. They are pooled and concentrated by ultrafiltration. The final enzyme solution (3 mg of protein/ml) is stored at 0° in the presence of thymol.

β-Lactamase from Strain Albus G

Step 1. The enzyme–Amberlite complex (200 g; corresponding to about 10 liter of culture fluid) is suspended in 0.15 M K_2HPO_4 and the pH is adjusted to 8.0 with concentrated ammonia. The extract (1 liter) is adjusted to 40% saturation by the addition of 226 g of solid $(NH_4)_2SO_4$, and after removal of the precipitate, is adjusted to 75% saturation by the addition of 222 g of solid $(NH_4)_2SO_4$. The precipitate is dissolved in phosphate buffer (final volume: 50 ml), dialyzed, and filtered on a 2 liter column of Sephadex G-75 in phosphate buffer. The fractions containing β-lactamase activity (elution volume: 800–1300 ml) are pooled and concentrated to a final volume of 30 ml.

Step 2. After step 1, the enzyme (30 ml) is adsorbed onto a 200-ml column of DEAE-cellulose equilibrated against phosphate buffer and,

(100°, 17 hr), with bovine serum albumin as standard.

after washing with the same buffer, is eluted with an increasing NaCl gradient from 0 to 0.25 *M* in phosphate buffer (total elution volume: 500 ml). Most of the β-lactamase (usually 75% of the total activity) is eluted between 0 and 0.1 *M* NaCl and fractions containing maximal activity are about 50 m*M* with respect to NaCl. They are pooled, concentrated, dialyzed, and subjected to an additional chromatography on the 200 ml column of DEAE-cellulose as above except that the elution is carried out with an increasing NaCl gradient from 0 to 0.1 *M* in phosphate buffer (total elution volume: 1 liter). The active fractions (elution volume: 300–500 ml) are pooled and concentrated.

Step 3. After step 2, the enzyme is transferred into 20 m*M* Tris·HCl buffer pH 7.0 by filtration through Sephadex G-75, and is dialyzed against this buffer. Residual pigment is then removed by preparative polyacrylamide gel electrophoresis at pH 8.3. The β-lactamase fractions are pooled, concentrated, dialyzed against phosphate buffer containing 10% glycerol (v/v) and stored in 100-μl aliquots at −20°.

Properties of *Streptomyces* β-Lactamases

Stability. They are completely inactivated by heating at 60° for 5 min. The *albus* G β-lactamase is sensitive to dilution; activity is retained and assays are performed in the presence of 10% glycerol (w/v; final concentration).

pH and Salt Optimum. pH optimum is between 6 and 8 in 30 m*M* phosphate buffer. At pH 7, rate of hydrolysis is maximal between 10 and 30 m*M* phosphate buffer. At pH 8, activity is higher in phosphate buffer than in Tris·HCl buffer.

Metal Ion Requirement. Sodium EDTA, Mg^{2+} Mn^{2+}, Ca^{2+}, and Zn^{2+}, each cation being used as its chloride at 2 m*M*, have no effect. Cu^{2+} (2 m*M*) inhibited both R39 and *albus* G enzymes by 50 and 90%, respectively.

Sensitivity to -SH Group Reagents. *p*-Chloromercuribenzoate, iodoacetate, and *p*-aminobenzoate (1 m*M*) have no effect on the enzymes.

Sensitivity to Iodine. Unlike most β-lactamases, neither the R39 nor the *albus* G enzymes are inactivated by 2.5 m*M* iodine/KI solution, for 30 min at 30°.

Physical Properties. Both R39 and *albus* G β-lactamases are anionic at pH 8.3. Polyacrylamide gel electrophoresis at this pH, in 25 m*M* Tris-glycine buffer[16] with respect to Tris, discloses only one band of protein

[16] M. Leyh-Bouille, M. Nakel, J. M. Frère, K. Johnson, J. M. Ghuysen, M. Nieto, and H. R. Perkins, *Biochemistry* 11, 1290 (1972).

(detected with Coomassie blue) which migrates toward the anode with the buffer discontinuity. β-Lactamase activity is associated with this band. Isoelectric points and molecular weights are not known.

Specificity. Typical Michaelis-Menten kinetics are observed over a wide range of antibiotic concentrations. K_m, V_{max}, and efficiency values (Table II) are determined in 30 m*M* sodium phosphate pH 7.0 and 30°. The K_m values are well within the norm of other β-lactamases. The V_{max} values of the enzyme preparations, as they are obtained, and therefore, their physiological efficiencies, are low compared with other β-lactamases. Based on their substrate profiles, the two *Streptomyces* β-lactamases differ from one another and from other β-lactamases of bacterial origin.

Effect of DD-*Carboxypeptidase Substrates and Inhibitors.* N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine, N^{α}-acetyl-L-lysyl-D-alanyl-D-alanine and the disaccharide pentapeptide-pentaglycine [N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(pentaglycyl)-L-lysyl-D-alanyl-D-alanine] are substrates of both R39 and *albus* G DD-carboxypeptidases. Acetyl-D-alanyl-D-glutamic acid, N^{α},N^{ϵ}-disuccinyl-L-lysyl-D-alanyl-D-glutamic acid, and L-lysyl-D-glutamyl-D-alanine inhibit the *albus* G DD-carboxypeptidase but have no effect on the R39

TABLE II
HYDROLYSIS OF β-LACTAM ANTIBIOTICS BY β-LACTAMASES R39 AND *albus* G

	R39			*albus* G		
Substrate	K_m (m*M*)	V_{max} (μmoles/mg protein/min)	Efficiency[a]	K_m (m*M*)	V_{max} (μmoles/mg protein/min)	Efficiency[a]
Benzylpenicillin	0.07	198	2835	0.74	33	45
9-Aminopenicillanic acid	0.07	152	2175	0.60	25	42
Penicillin V	0.28	452	1615	0.62	27	44
Ampicillin	0.57	1038	1820	0.90	70	78
Carbenicillin	0.26	78	300	1.00	3.3	3.3
Oxacillin	0.54	646	1200	0.33	6.2	19
Cloxacillin	0.42	60	140	0.25	0.6	2.5
Methicillin	0.29	55	190	0.77	0.5	0.7
Cephalosporin C	1.05	12	10	3.33	2	0.7
Cephaloglycin	2.86	429	150	3.84	2.5	0.7
Cephalexin	0.91	35	40	4.53	0.4	0.1
Cephalothin	0.12	111	920	1.33	0.8	0.6

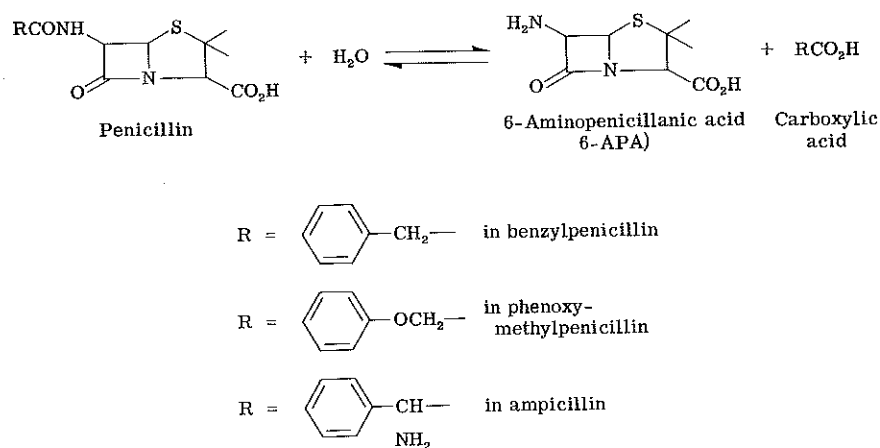
[a] Efficiency: V_{max} divided by K_m.

DD-carboxypeptidase-transpeptidase.[17] The above peptides (1.1–1.7 mM, final concentrations) neither inhibit nor activate the *Streptomyces* β-lactamases (using benzylpenicillin at concentrations near the relevant K_m values).

[17] M. Nieto, H. R. Perkins, M. Leyh-Bouille, J. M. Frère, and J. M. Ghuysen, *Biochem. J.* **131**, 163 (1973).

[54a] Penicillin Acylase (Assay)

By M. Cole, T. Savidge, and H. Vanderhaeghe



The enzyme catalyzing the above reversible reaction is usually called penicillin acylase and is produced by many types of microorganisms in intracellular and extracellular forms. It was called penicillin amidase in early literature and has been given the Enzyme Commission number EC 3.5.1.11. For a discussion of enzyme nomenclature and early work, the reviews by Hamilton-Miller[1] and Cole[2] can be consulted. The enzyme is not specific for penicillins but can also hydrolyze certain cephalosporins[3,4] and a variety of acylamino acids, amides, and esters.[5] The

[1] J. M. Hamilton-Miller, *Bacteriol. Rev.* **30**, 761 (1966).
[2] M. Cole, *Process. Biochem.* **2**, 35 (1967).
[3] M. Cole, *Biochem. J.* **115**, 733 (1969).
[4] B. Sjoberg, L. Nathorst-Westfelt, and B. Ortengren, *Acta Chem. Scand.* **21**, 547 (1967).
[5] M. Cole, *Biochem. J.* **115**, 741 (1969).