Binding of β-Lactam Antibiotics to the Exocellular DD-Carboxypeptidase–Transpeptidase of Streptomyces R39

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Benzylpenicillin and cephaloridine reacted with the exocellular DD-carboxypeptidase– transpeptidase from Streptomyces R39 to form equimolar and inactive antibiotic–enzyme complexes. At saturation, the molar ratio of chromogenic cephalosporin 87-312 to enzyme was 1.3:1, but this discrepancy might be due to a lack of accuracy in the measurement of the antibiotic. Spectrophotometric studies showed that binding of cephaloridine and cephalosporin 87-312 to the enzyme caused opening of their β-lactam rings. Benzylpenicillin and cephalosporin 87-312 competed for the same site on the free enzyme, suggesting that binding of benzylpenicillin also resulted in the opening of its β-lactam ring. In Tris–NaCl–MgCl2 buffer at pH 7.7 and 37°C, the rate constants for the dissociation of the antibiotic–enzyme complexes were 2.8×10⁻⁶, 1.5×10⁻⁶ and 0.63×10⁻⁶s⁻¹ (half-lives 70, 130 and 300 h) for benzylpenicillin, cephalosporin 87-312 and cephaloridine respectively. During the process, the protein underwent reactivation. The enzyme that was regenerated from its complex with benzylpenicillin was as sensitive to fresh benzylpenicillin as the native enzyme. With [¹⁴C]benzylpenicillin, the released radioactive compound was neither benzylpenicillin nor benzylpenicilloic acid. The Streptomyces R39 enzyme thus behaved as a β-lactam-antibiotic-destroying enzyme but did not function as a β-lactamase. Incubation at 37°C in 0.01 M-phosphate buffer, pH 7.0, and in the same buffer supplemented with sodium dodecyl sulphate caused a more rapid reversion of the [¹⁴C]benzylpenicillin–enzyme complex. The rate constants were 1.6×10⁻²s⁻¹ and 0.8×10⁻⁴s⁻¹ respectively. Under these conditions, however, there was no concomitant reactivation of the enzyme and the released radioactive compound(s) appeared not to be the same as before. The Streptomyces R39 enzyme and the exocellular DD-carboxypeptidase–transpeptidase from Streptomyces R61 appeared to differ from each other with regard to the topography of their penicillin-binding site.

On the basis of its specificity profile for donor and acceptor substrates, the exocellular DD-carboxypeptidase–transpeptidase of Streptomyces R39 might be a soluble form of the membrane-bound transpeptidase which catalyses peptide cross-linking during the last stages of the wall peptidoglycan biosynthesis (Leyh-Bouille et al., 1972; Ghuysen et al., 1973, 1974; Perkins et al., 1973). The Streptomyces R39 enzyme was inhibited by very low concentrations of benzylpenicillin and the interaction between the protein and the antibiotic appeared to be reversible at least with regard to the enzyme activity (Leyh-Bouille et al., 1972). The binding of benzylpenicillin and of various cephalosporins to the Streptomyces R39 enzyme and the dissociation of the complexes thus formed were further studied. The results of these investigations are presented in the present paper.

Materials and Methods

DD-Carboxypeptidase–transpeptidase from Streptomyces R39

The enzyme used in most experiments was 90% pure and had a specific activity of 17.1 units/mg of protein as determined in the DD-carboxypeptidase assay (AC₃₈-L-Lys-D-Ala-D-Ala+H₂O → D-alanine +AC₂₈-L-Lys-D-Ala) (preparation A after step 4; Frère et al., 1974). In some experiments, a 100%- pure enzyme (specific activity 19.8 units/mg of protein; Frère et al., 1974) was used.

§ Abbreviation: Ac, acetyl.
\(\beta\)-Lactamase

This enzyme was purchased from Riker Laboratories, Loughborough, Leics., U.K. One penicillinase unit hydrolysed 1 \(\mu\)mol of benzylpenicillin to benzylpenicilloic acid/min at pH 7.0 and 25°C. The concentration of benzylpenicillin was 1 mm.

Antibiotics

Benzylpenicillin was purchased from Rhone-Poulenc, Paris, France, cephaloridine from Glaxo Research Ltd., Greenford, Middx., U.K., and \([^{14}C]\)benzylpenicillin (45 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. Chromogenic cephalosporin 87-312 [i.e. 3-(2,4-dinitrostyryl)-(6R-7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer] was a gift from Dr. O'Callaghan, Glaxo Research Ltd. (O'Callaghan et al., 1972). Solutions of cephalosporin 87-312 (usually about 0.1 mm) were made by dissolving the antibiotic in 1 ml of dimethylformamide and the volume of the solution was adjusted to 250 ml with the Tris–NaCl–MgCl₂ buffer, pH 7.7 (see below). The final concentration was estimated by measuring the extinction at 386 nm and by using a molar coefficient \(\varepsilon_{386}^{\text{em}}\) of 17 500 (O'Callaghan et al., 1972).

\([^{14}C]\)Benzylpenicilloic acid

\([^{14}C]\)Benzylpenicilloic acid was prepared by incubation of \([^{14}C]\)benzylpenicillin with \(\beta\)-lactamase.

Separation of \([^{14}C]\)benzylpenicillin and \([^{14}C]\)benzylpenicilloic acid

This was done as described by Marquet et al. (1974) by t.l.c. on silica-gel plates with butan-1-ol–acetic acid-water (4:3:3, by vol.) as solvent and by paper electrophoresis at pH 6.5 (collidine-acetic acid–water, 9:1:2.65:1000, by vol.) for 60 min at 60 V/cm. The \(R_f\) values were 0.82±0.03 for \([^{14}C]\)benzylpenicillin and 0.67±0.03 for \([^{14}C]\)benzylpenicilloic acid. The electrophoretic migrations toward the anode were 16 cm for \([^{14}C]\)benzylpenicillin and 22 cm for \([^{14}C]\)benzylpenicilloic acid.

Spectra

Spectra were determined with a Cary 17 double-beam recording spectrophotometer with automatic slit-width adjustment.

Titration and kinetic data

From the plotted data, all the lines were drawn according to the best fit by least-squares regression analyses (P < 0.001).

**Tris–NaCl–MgCl₂ buffer**

This buffer was 0.1 M in Tris, 0.2 M in NaCl and 0.05 M in MgCl₂; adjusted to pH 7.7 with HCl. In this buffer, the *Streptomyces* R39 enzyme was very stable. No loss of activity was observed after 100 h of incubation at 37°C (enzyme concentration 0.2 mg of protein/ml).

**Measurement of the Streptomyces R39 enzyme concentration**

This was done on the basis of a \(E_{\text{1cm}}^{\text{1%}}\) value at 280 nm of 9.7 (Frère et al., 1974).

**Measurement of radioactivity on chromatograms and electrophoretograms**

The radioactive compounds on t.l.c. plates and electrophoresis papers were located by using a Packard Radiochromatogram Scanner. The radioactivity was determined by cutting or scraping the radioactive areas which were counted in a Packard Tri-Carb liquid-scintillation spectrometer.

Results

**Binding of benzylpenicillin to the R39 enzyme**

Binding of benzylpenicillin to the exocellular DD-carboxypeptidase–transpeptidase from *Streptomyces* R61 caused quenching of the fluorescence of the enzyme and affected its near-u.v. circular dichroism (Nieto et al., 1973). In marked contrast with these observations, the fluorescence emission and circular-dichroism spectra of the *Streptomyces* R39 enzyme were not modified by benzylpenicillin. Binding of benzylpenicillin to the *Streptomyces* R39 enzyme was studied by measuring the inhibition of the DD-carboxypeptidase activity caused by the antibiotic. Samples (10–5 μl) of a 5 μM-benzylpenicillin solution in Tris–NaCl–MgCl₂ buffer were added stepwise to 110 μl of a solution containing 15 μg of the *Streptomyces* R39 enzyme (100% purity) in the same buffer. After each addition, the solution was mixed, maintained at room temperature for 5 min and a sample (2–10 μl) was removed and used for the measurement of the DD-carboxypeptidase activity (on Ac₂-L-Lys-D-Ala-D-Ala). After correction for the decrease in the amount of enzyme owing to the removal of the samples, the end-point for titration of 15 μg of *Streptomyces* R39 protein, on the basis of the disappearance of the enzyme activity, occurred after the addition of 58.5 μl of the antibiotic solution (i.e. 0.294 nmol of benzylpenicillin). Assuming that one benzylpenicillin-binding site occurred per molecule of protein, the above result gave a molecular weight of 50 500 for the *Streptomyces* R39 enzyme. From this value and from those obtained by physical methods (Frère et al., 1974), an average molecular weight of 53 300±1700 was assigned to the enzyme. From this average value, the end-point for titration of the *Streptomyces* R39 enzyme in the above experiment occurred at a molar ration of benzylpenicilline to enzyme of 1.04±0.03:1 (Fig. 1).
In a second experiment, the *Streptomyces* R39 enzyme preparation used was at 90% purity and the time of incubation with benzylpenicillin was prolonged. Samples containing 0.0119 nmol of protein were incubated at room temperature with increasing amounts of benzylpenicillin in 30 μl (final volumes) of Tris–NaCl–MgCl₂ buffer. After 60 min, Ac₂-L-Lys-D-Ala-D-Ala was added to each sample and the dd-carboxypeptidase activity was measured. On the basis of an average molecular weight of 53 300, complete inhibition of the enzyme occurred at a molar ratio of benzylpenicillin to enzyme of 0.91±0.02:1 (or after correction for the presence of about 10% impurities in the enzyme preparation, at a molar ratio virtually equal to 1:1; Fig. 1).

Reversion of the [¹⁴C]benzylpenicillin–Streptomyces R39 enzyme complex

Reversion of the complex was studied under conditions in which, in the absence of the antibiotic, the enzyme was perfectly stable (i.e. in the Tris–NaCl–MgCl₂ buffer) and under conditions where it underwent spontaneous inactivation. [¹⁴C]Benzylpenicillin–Streptomyces R39 enzyme complex (5.5 nmol; 0.28 μCi; 41 mCi/mmoll; in 1 ml of Tris–NaCl–MgCl₂ buffer) was prepared by mixing the *Streptomyces* R39 enzyme with a twofold excess, on a molar basis, of [¹⁴C]benzylpenicillin and was separated from the unbound [¹⁴C]benzylpenicillin by filtration on a 25 ml column (0.8 cm×13 cm) of Sephadex G-25 equilibrated against the same Tris–NaCl–MgCl₂ buffer. The solution containing the isolated [¹⁴C]benzylpenicillin–enzyme complex was then maintained at 37°C for 4 days. At increasing time-intervals during the incubation, samples were removed which showed a progressive recovery of the enzyme activity (dd-carboxypeptidase assays) and a parallel release of the radioactivity (as revealed by both t.l.c. and paper electrophoresis; see the Materials and Methods section). In both these latter tests, the enzyme-bound radioactivity remained at the origin. On the basis of the enzyme activity recovered and the radioactivity released, the half-lives of the complex were almost identical, 65 and 73 h respectively (Fig. 2). The reactivated enzyme (after 90 h of incubation at 37°C; 63% recovery) was identical with the native one with regard both to the $K_m$ value for Ac₂-L-Lys-D-Ala-D-Ala (dd-carboxypeptidase assay) and to the rates of concomitant hydrolysis and transfer reactions that
it catalysed (transpeptidase assay, with Ac-O-Lys-D-Ala-D-Ala as donor and meso-diaminopimelic acid as acceptor; Frère et al., 1973). Moreover, the regenerated enzyme reacted with fresh benzylpenicillin as did the native one and formed an equimolar and inactive antibiotic–enzyme complex which did not migrate on t.l.c. and paper electrophoresis.

Reversion of the $^{14}$C benzylpenicillin–enzyme complex was also studied in 0.01 m-sodium phosphate, pH 7.0, under the following conditions: (1) at 37°C and in the absence of sodium dodecyl sulphate (Fig. 2); (2) at 37°C and in the presence of sodium dodecyl sulphate (Fig. 2) and (3) at 100°C. Under these conditions, the free enzyme either exhibited a low stability (at 37°C) or was very rapidly destroyed (at 100°C). On the basis of release of the radioactivity, the half-lives of the complex were 11–14 h, 147 min and less than 5 min respectively. In all cases, recovery of enzyme activity did not occur.

*Nature of the radioactive compound(s) released from the $^{14}$C benzylpenicillin–Streptomyces R39 complex*

Dissociation of the complex was carried out at 37°C either in Tris–NaCl–MgCl$_2$ buffer or in 0.01 m-sodium phosphate buffer, pH 7.0. Control experiments were carried out with free $^{14}$C benzylpenicillin and free $^{14}$C benzylpenicilloic acid. After 100 h of incubation in both buffers, $^{14}$C benzylpenicilloic acid had unchanged $R_f$ and electrophoretic-mobility values (see the Materials and Methods section). After 40 h of incubation in phosphate buffer, $^{14}$C benzylpenicillin was degraded into a compound which behaved as $^{14}$C benzylpenicilloic acid on both chromatography and electrophoresis. After 15 h of incubation in Tris–NaCl–MgCl$_2$ buffer, $^{14}$C benzylpenicillin gave rise to two compounds: a main one (80% of the original radioactivity) which was less anionic than $^{14}$C benzylpenicillin and a minor one (20%) which had the same electrophoretic mobility as $^{14}$C benzylpenicilloic acid (Fig. 3). Chromatographically, however, the main degradation compound was indistinguishable from intact $^{14}$C benzylpenicillin whereas the minor one still behaved as $^{14}$C benzylpenicilloic acid. Both compounds appeared to be degradation end-products, since their relative proportion after 96 h of incubation was the same as after 15 h. Although it was not identified, the main breakdown compound was probably caused by aminolysis of the benzylpenicillin molecule by the Tris buffer, i.e. a benzylpenicilloyl–Tris derivative (Schwartz, 1969).

Dissociation of the $^{14}$C benzylpenicillin–enzyme complex in Tris–NaCl–MgCl$_2$ buffer, i.e. under conditions where the free enzyme was very stable and where recovery of the enzyme activity from the inhibited complex occurred, gave rise to a compound which behaved chromatographically as $^{14}$C benzylpenicillin (Fig. 4) and electrophoretically as $^{14}$C benzylpenicilloic acid and thus, clearly, was neither benzylpenicillin nor benzylpenicilloic acid nor a product arising from a spontaneous degradation of free benzylpenicillin in Tris–NaCl–MgCl$_2$ buffer.

Dissociation of the complex in phosphate buffer, i.e. under conditions where recovery of the enzyme

![Fig. 3. Co-electrophoresis at pH 6.5 of intact $^{14}$C benzylpenicillin and of its degradation products that appeared after incubation of free $^{14}$C benzylpenicillin in Tris–NaCl–MgCl$_2$ buffer for 15 h at 37°C.](image.png)

From left to right, the compounds were: at $-3 \text{ cm}$, impurity present in the $^{14}$C benzylpenicillin; at $+12 \text{ cm}$, main degradation product (this compound behaved as $^{14}$C benzylpenicillin on t.l.c.); at $+16 \text{ cm}$, intact $^{14}$C benzylpenicillin (control); at $+22 \text{ cm}$, minor degradation product with the same mobility as $^{14}$C benzylpenicilloic acid (this compound also behaved as $^{14}$C benzylpenicilloic acid on t.l.c.). Scanning conditions: 3000 c.p.m. at full scale; 0.5 cm/min; time constant, 10 s; slit width, 0.5 cm.
activity did not occur, gave rise to a compound which behaved as \textsuperscript{14}Cbenzylpenicilloic acid on both chromatography and electrophoresis. Dissociation of the complex in the same phosphate buffer but in the presence of 1% (w/v) sodium dodecyl sulphate was also studied. Chromatographically, the observed increase in the released \textsuperscript{14}Cbenzylpenicilloic acid-like compound was equivalent to the decrease in the radioactivity remaining at the origin of the plates. Electrophoretically, however, the \textsuperscript{14}Cbenzylpenicilloic acid-like compound represented only a fraction of the total released radioactivity whereas various compounds of slower mobility on electrophoresis progressively accumulated (Fig. 5).

**Effects of thiol-group reagents on enzyme activity and \textsuperscript{14}Cbenzylpenicillin binding**

*Iodoacetate.* Incubation of the *Streptomyces* R39 enzyme (0.10 nmol) in 100 µl of Tris–NaCl–MgCl₂ buffer containing 1 mM-iodoacetate for 16 h at 37°C had no effect on the enzyme activity or on the ability of the protein to bind benzylpenicillin.

*5,5′-Dithiobis-(2-nitrobenzoic acid).* Samples (40 µl) of a 1 mM-5,5′-dithiobis-(2-nitrobenzoic acid) solution in Tris–NaCl–MgCl₂ buffer were added stepwise and at room temperature to 0.4 ml of a solution containing 4.5 nmol of *Streptomyces* R39 enzyme protein (90% purity) in the same buffer. The same experiment was carried out in the presence of 5 mM-guanidinium chloride (previously neutralized with 5 mM-NaOH to pH 7.5). Finally, controls consisted of the same mixtures as above but without the *Streptomyces* R39 enzyme. Assuming a molar extinction coefficient at 420 nm of 13.6×10³ for the thiol-5,5′-dithiobis-(2-nitrobenzoic acid) derivative (Umbreit & Strominger, 1973) and that two free thiol groups occur per protein molecule (amino acid analyses had revealed the presence of two half-cystine residues; Frère *et al.*, 1974), the above conditions were such that a reaction would have caused an increase in the E₄₂₀ of 0.25. In fact, no differences between the samples and the controls were observed even when the experiment was carried out in the presence of guanidinium chloride. Moreover, 5,5′-dithiobis-(2-nitrobenzoic acid)-treated *Streptomyces* R39 protein (in the absence of guanidinium) exhibited the same enzyme activity and the same benzylpenicillin-binding capability as the native enzyme.

**Binding of cephalosporin 87-312 to the Streptomyces R39 enzyme**

When exposed to the *Streptomyces* R39 enzyme, a solution of cephalosporin 87-312 turned red immediately. This change of colour was observed even when both reagents were cooled to 0°C before being mixed. Since the hydrolysis of the β-lactam ring of cephalosporin 87-312 was known to cause a shift of the absorption maximum from 386 to 482 nm

![Graph](image-url)
(O'Callaghan et al., 1972), the above observation suggested that the exposure of cephalosporin 87-312 to the *Streptomyces* R39 enzyme resulted in the opening of its \(\beta\)-lactam ring. In agreement with this conclusion, the absorption spectrum of the *Streptomyces* R39 enzyme-treated cephalosporin 87-312 was virtually identical with that of the \(\beta\)-lactamase-treated cephalosporin 87-312 (Fig. 6). Hence cephalosporin 87-312 allowed the two reagents involved in the reaction to be titrated simultaneously, the protein by estimation of its enzyme activity and the \(\beta\)-lactam antibiotic by measurement of the change in the absorption spectrum. Portions of a 58.8 \(\mu\)M-cephalosporin 87-312 solution in Tris–NaCl–MgCl\(_2\) buffer were added stepwise and at room temperature to 0.4 ml of a solution containing 13.3 nmol of *Streptomyces* R39 protein (90% purity) in the same buffer. After each addition, the extinction of the solution was measured both at 386 nm (to measure intact cephalosporin) and at 482 nm (to measure modified cephalosporin) and the enzyme activity was measured in a 2 \(\mu\)l sample. The end-points of the titration as revealed by the three procedures used occurred at a molar ratio of cephalosporin 87-312 to *Streptomyces* R39 protein of 1.27:1 (Fig. 7). Unexpectedly, this value was more than 30% higher than that obtained when benzylpenicillin reacted with the same (90%-purity) enzyme preparation (see

![Fig. 6. Absorption spectrum of cephalosporin 87-312 after treatment with the *Streptomyces* R39 enzyme and with \(\beta\)-lactamase.](image)

--- Solution containing 15.5 nmol of *Streptomyces* R39 protein (100% purity) in 0.4 ml of Tris–NaCl–MgCl\(_2\) buffer was mixed with 0.2 ml of a 67.7 \(\mu\)M solution of cephalosporin 87-312. The mixture was incubated for 5 min at room temperature before the absorption spectrum was determined. --- Solution containing 6 units of \(\beta\)-lactamase in 0.4 ml of Tris–NaCl–MgCl\(_2\) buffer was mixed with 0.2 ml of the same solution of cephalosporin 87-312 as above. The mixture was incubated at 37°C until the absorbance at 482 nm was a maximum (less than 5 min). The broken line represents the difference spectrum between cephalosporin treated with *Streptomyces* R39 enzyme and cephalosporin treated with \(\beta\)-lactamase. Note the change of scale.

![Fig. 7. Titration of the *Streptomyces* R39 protein with cephalosporin 87-312, based on the inhibition of enzyme activity and on the alteration of the antibiotic molecule.](image)

\(v_v\), Residual enzyme activity; \(\bigcirc\) and ☻, determination of modified cephalosporin 87-312 (two experiments); ● and ■ (two experiments), determination of intact cephalosporin 87-312. For conditions see the text. The extinction values at 386 and 482 nm were transformed into concentrations (and from these into nmol) of intact and modified cephalosporin 87-312 from the equations:

\[\varepsilon_{386} = 17500 \text{ [intact cephalosporin]} + 7700 \text{ [modified cephalosporin]};\]
\[\varepsilon_{482} = 2600 \text{ [intact cephalosporin]} + 16700 \text{ [modified cephalosporin]}\]

where the values 17500 and 2600 were the molar extinction coefficients of intact cephalosporin 87-312 and the values 7700 and 16700 were the molar extinction coefficients of modified cephalosporin 87-312 at the relevant wavelengths. The \(\varepsilon_{386}\) value for intact cephalosporin 87-312 was that given by O'Callaghan et al. (1972). The other coefficients were determined experimentally. The \(\varepsilon_{386}\) and \(\varepsilon_{482}\) values for the modified cephalosporin 87-312 were determined after complete hydrolysis of cephalosporin 87-312 with \(\beta\)-lactamase and were assumed to be identical with those of the cephalosporin 87-312 treated with the *Streptomyces* R39 enzyme. The validity of this assumption was supported by the data of Fig. 6.

1974
above). At present, the reason for this discrepancy is not understood since, as shown in the next paragraph, benzylpenicillin and cephalosporin 87-312 were found to compete for the same site on the enzyme.

**Competition between \[^{14}C\text{-benzylpenicillin and cephalosporin 87-312 for the Streptomyces R39 enzyme}**

Cephalosporin 87-312 (26nmol) and *Streptomyces* R39 protein (15.5 nmol; 90% purity) were mixed together (final volume 300μl of Tris–NaCl–MgCl₂ buffer). After 5 min at room temperature, \[^{14}C\text{-benzylpenicillin (55nmol; 2.5 μCi) was added to the mixture, the solution incubated at 37°C for 20min and then filtered at room temperature on a 25ml column (0.8 cm × 13 cm) of Sephadex G-25 equilibrated against the same Tris–NaCl–MgCl₂ buffer. As shown in Fig. 8, all the modified cephalosporin 87-312 was eluted with the protein at the void volume of the column, whereas the small amount of radioactivity found associated with these fractions corresponded to less than 0.01 mol of benzylpenicillin/mmol of enzyme. In another experiment, benzylpenicillin (10nmol) and *Streptomyces* R39 enzyme (5nmol) were mixed together (final volume 300μl of Tris–NaCl–MgCl₂ buffer). After 10 min at 37°C, 90μl of a 80μM solution of cephalosporin 87-312 in the same buffer was added. Since no variations in the \(E_{430}/E_{482}\) ratio occurred, the β-lactam ring of the cephalosporin 87-312 must have remained intact.

**Binding of cephaloridine to the Streptomyces R39 enzyme**

Hydrolysis of the β-lactam ring of cephaloridine caused a 70% decrease of the absorbance at 250 nm (O'Callaghan et al., 1969). Hence cephaloridine offered another possibility to study the interaction between the *Streptomyces* R39 enzyme and a cephalosporin-type antibiotic by measuring the alterations undergone by both reagents. Samples of 0.5mm cephaloridine in Tris–NaCl–MgCl₂ buffer were added stepwise and at room temperature to 0.4ml of a solution containing 6.5nmol of *Streptomyces* R39 protein (90% purity) in the same buffer. After each addition, the extinction of the solution was measured at 250nm and the enzyme activity was measured in a 2μl sample. A plot of the increased extinction of the solution at 250nm as a function of the amount of cephaloridine added yielded two straight lines intersecting at a point. On the basis of these spectrophotometric data and the determinations of enzyme activity, the end-points of the reaction occurred at molar ratios of cephaloridine to enzyme of 0.85 and 0.98 respectively (Fig. 9). The average value of 0.915 was identical with that obtained when benzylpenicillin reacted with the same enzyme preparation and was about 30% lower than that obtained with cephalosporin 87-312.

**Reversion of the cephalosporin 87-312–Streptomyces R39 enzyme and cephaloridine–Streptomyces R39 enzyme complexes**

The experiments were carried out in Tris–NaCl–MgCl₂ buffer and at 37°C. The antibiotics left in excess after reaction with the enzyme were destroyed by β-lactamase and the dissociation of the antibiotic–enzyme complexes was followed by measuring the recovery of the enzyme activity. A control experiment with benzylpenicillin showed that the rate constant for the dissociation was identical with that obtained when the benzylpenicillin–enzyme complex was separated from the excess of benzylpenicillin by chromatography on Sephadex G-25. Hence the presence of β-lactamase during dissociation of the complex had no effect on the kinetics of the process. Enzyme (90% purity; 2.5 nmol) and 10nmol of either cephalosporin 87-312 or cephaloridine in 100μl (final volumes) of Tris–
NaCl–MgCl₂ buffer were incubated together for 10 min at room temperature. β-Lactamase (6 units) was added to the mixtures which were maintained at 37°C for several days. On the basis of enzyme recovery, the rate constants for the dissociation of the antibiotic–enzyme complexes were 1.5 × 10⁻⁶ s⁻¹ (half-life 130 h) with cephalosporin 87-312 and 0.63 × 10⁻⁶ s⁻¹ (half-life 300 h) with cephaloridine.

**Discussion**

Saturation of the *Streptomyces* R39 enzyme with benzylpenicillin and cephaloridine occurred when 1 mol of antibiotic was added to 1 mol of protein (mol wt. 53300). The same conclusion can probably be extended to chromogenic cephalosporin 87-312, although in this case saturation occurred at a molar ratio of antibiotic to enzyme of about 1:3:1. One possible explanation for this discrepancy might be that the amounts of cephalosporin 87-312 used were estimated from extinction values of the solutions by using a ε₂⁵⁰₆ value of 17500 (O’Callaghan et al., 1972). An underestimation of this coefficient would of course result in an overestimation of the amount of antibiotic required to saturate the enzyme.

The titration of the *Streptomyces* R39 enzyme with the three antibiotics yielded very clear-cut end-points. These experiments are in contrast with those carried out with the purified di-carboxypeptidase from *Bacillus subtilis* (Umbreit & Strominger, 1973), in which about 20% of the enzyme activity remained detectable after the addition of 2–3 times the theoretical amount of benzylpenicillin. The present results also offer an explanation for the unusual kinetics previously observed for the inhibition of the *Streptomyces* R39 enzyme by benzylpenicillin (Leyh-Bouille et al., 1972). It is now clear that the benzylpenicillin concentrations used in these preliminary studies were very close to that of the enzyme itself, so that the upward concavity exhibited by the Dixon plots of 1/v versus [benzylpenicillin] simply reflected the decrease in the concentration of the residual free enzyme.

The isolated β-lactam antibiotic–*Streptomyces* R39 enzyme complexes, when incubated under conditions where the free enzyme itself was stable, underwent dissociation with the concomitant reactivation of the enzyme. Hence, and as previously observed (Leyh-Bouille et al., 1972), inhibition of the *Streptomyces* R39 enzyme by β-lactam antibiotics was reversible at least with regard to the enzyme itself. Spectrophotometric studies, however, gave direct evidence that binding of cephaloridine and cephalosporin 87-312 to the *Streptomyces* R39 enzyme resulted in the opening of the β-lactam ring. Since binding of cephalosporin 87-312 and benzylpenicillin were mutually exclusive, there was very little doubt that both antibiotic molecules competed for the same site on the free enzyme, and therefore it seemed reasonable to assume that binding of benzylpenicillin to the *Streptomyces* R39 enzyme also resulted in the opening of its β-lactam ring.

The nature of the chemical alteration undergone by the antibiotic molecule during its interaction with the *Streptomyces* R39 protein remains a matter for speculation. The following observations made with [¹⁴C]benzylpenicillin are important for the final elucidation of the problem. (1) Reversion of the [¹⁴C]benzylpenicillin–enzyme complex in Tris–NaCl–MgCl₂ buffer, under conditions where concomitant reactivation of the enzyme occurred, yielded a released radioactive compound which was not benzylpenicillin, nor benzylpenicillioic acid, nor a product which could have arisen from a spontaneous degradation of free [¹⁴C]benzylpenicillin. Hence the *Streptomyces* R39 enzyme, although behaving as a β-lactam-antibiotic-destroying enzyme, did not function toward benzylpenicillin as a (primitive) β-lactamase. Moreover the regenerated enzyme was as sensitive to fresh benzylpenicillin as the native one, hence excluding the theoretical possibility that the released penicillin derivative was carrying with it some labile part of the protein.
(2) Reversion of the complex in 0.01M-phosphate buffer without concomitant reactivation of the enzyme yielded a radioactive compound which behaved as benzylpenicilloic acid. However, when sodium dodecyl sulphate was present during the dissociation of the complex, the benzylpenicilloic acid-like compound behaved as an unstable intermediate, decaying into several other compounds. Hence the conditions which prevailed during the dissociation of the \[^{14}C\]benzylpenicillin–enzyme complex were important for the fate of the antibiotic molecule (as they were for the enzyme). The chemical alterations undergone by the antibiotic molecule appeared to depend on whether or not the enzyme was released in an active form.

The nature of the bond formed between the \(\beta\)-lactam antibiotics and the Streptomyces R39 enzyme also remains a matter of speculation except that no thiol group appeared to be involved in the binding. This situation is comparable with that previously observed for the exocellular \(\delta\delta\)-carboxypeptidase–transpeptidase from Streptomyces R61 (Nieto et al., 1973) and stands in marked contrast with the \(\delta\delta\)-carboxypeptidase of \(B.\ subtilis\) (Umbrecht & Strominger, 1973), in which case a thioester bond was apparently formed on reaction with benzylpenicillin. In Tris–NaCl–MgCl\(_2\) buffer, i.e. under conditions where reversion of the benzylpenicillin–Streptomyces R39 enzyme complex was accompanied by the reactivation of the enzyme, the half-life of the complex was about 70h at 37°C. This value corresponded to a first-order rate constant for the dissociation of \(2.8 \times 10^{-6}_s^{-1}\). In 0.01M-phosphate buffer reactivation of the enzyme did not occur, and parallel to this, the rate constant for the dissociation considerably increased to \(1.6 \times 10^{-5}_s^{-1}\). Addition of sodium dodecyl sulphate to the mixture caused, in turn, an additional increase of the constant value to \(0.8 \times 10^{-4}_s^{-1}\). These results suggest that the bond formed between the \(\beta\)-lactam antibiotics and the Streptomyces R39 enzyme, whatever its exact nature, is probably buried within the protein molecule and that denaturation of the protein greatly facilitates the exposure of the bond to the solvent and the subsequent release of the modified antibiotic molecule.

The exocellular \(\delta\delta\)-carboxypeptidase–transpeptidase from Streptomyces R61 differs from the Streptomyces R39 enzyme in that the complex formed with benzylpenicillin is very unstable even under conditions where reactivation of the enzyme occurred. Strikingly, the rate constant for the dissociation (\(1 \times 10^{-4}_s^{-1}\); Nieto et al., 1973) is almost identical with the rate constant for the dissociation of the benzylpenicillin–Streptomyces R39 enzyme complex in the presence of sodium dodecyl sulphate (\(0.8 \times 10^{-4}_s^{-1}\)). The Streptomyces R61 enzyme also altered the \(\[^{14}C\]\)benzylpenicillin molecule and the released radioactive compound was neither benzylpenicillin nor benzylpenicilloic acid (J. M. Frère, M. Leyh-Bouille, J. M. Ghuyesen & H. R. Perkins, unpublished work). Hence a major difference between the Streptomyces R61 and R39 enzymes might reside in the topography of the site on which binding of the \(\beta\)-lactam antibiotics occurs and which would make the bond thus formed more or less accessible to the action of the solvent. It might also be possible that the different molecular weights of the two enzymes (38000 for the R61 enzyme; 53300 for the R39 enzyme) are relevant to this problem.

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