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Interaction between β-Lactam Antibiotics and Exocellular DD-Carboxypeptidase-Transpeptidase of *Streptomyces* R61

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On the basis of steady-state kinetics, inhibition of the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61 by β-lactam antibiotics was competitive with regard to the donor substrate. However, the complexes formed between the *Streptomyces* R61 enzyme and various β-lactam antibiotics were relatively stable, exhibiting half-lives of 40 to 80 min at 37 °C and neutral pH. During breakdown of the complexes the protein underwent reactivation, whereas the released antibiotic molecule was chemically altered. With [^{14}C]benzylpenicillin, the released compound was neither benzylpenicillin nor benzylpenicilloic acid. The properties of the *Streptomyces* R61 enzyme · β-lactam antibiotic complexes were compared with those of the complexes formed between the same antibiotics and either the membrane-bound transpeptidase from *Streptomyces* R61 or the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R39.

Streptomyces strain R61 excretes during growth an exocellular DD-carboxypeptidase-transpeptidase which is thought to be a soluble form of the membrane-bound transpeptidase involved in the biosynthesis of the wall peptidoglycan [1]. Kinetically, benzylpenicillin was found to inhibit the DD-carboxypeptidase activity of this exocellular enzyme in a competitive manner with a K_i value of 80 nM [2]. Fluorimetric studies also suggested that the interaction between the *Streptomyces* R61 enzyme and benzylpenicillin was of the type

$$E + P \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EP$$

where k_f (the second-order constant for the forward reaction) had a value of $10^4\ l \times mol^{-1} \times s^{-1}$ and k_r (the first-order constant for the reverse reaction) a value of $10^{-4} \times s^{-1}$ [3]. The K_i value (k_r/k_f) thus obtained (at 25 °C) was equal to 10 nM and was in fair agreement with the above K_i value obtained by steady-state kinetics (at 37 °C). However, the value of the constant for the reverse reaction was much too small to account for a rapid equilibrium of the reaction system and suggested that the complex formed between the *Streptomyces* R61 enzyme and the antibiotic had a lifetime long enough to allow its actual isolation. The effects of various β-lactam antibiotics on both the DD-carboxypeptidase and the transpeptidase activities of the *Streptomyces* R61 enzyme and the properties of the complexes formed between the enzyme and the antibiotics were investigated. The results are presented in this paper. Similar studies dealing with the properties of the complexes formed between β-lactam antibiotics and the membrane-bound transpeptidase from the same *Streptomyces* strain R61 [4] as well as between β-lactam antibiotics and the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R39 [5] were recently reported.

Abbreviation. A_2pm, diaminopimelic acid.

Enzyme. β-Lactamase or penicillinase (EC 3.5.2.6).

MATERIALS AND METHODS

Enzymes

The *Streptomyces* R61 DD-carboxypeptidase-transpeptidase had a specific activity of 86 units/mg protein as determined in the carboxypeptidase assay (Ac_2-L-Lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2-L-Lys-D-Ala-D-Ala) [6]. One unit of enzyme catalyses the hydrolysis of 1 μequiv of D-alanyl-D-alanine linkage per min at 37 °C and pH 7.5 [6]. β-Lactamase was purchased from Riker Laboratories (Loughborough,

U.K.); one unit of enzyme catalyses the hydrolysis of 1 μmol of benzylpenicillin to benzylpenicilloic acid per min at 25 °C and pH 7.0.

Antibiotics

[^{14}C]Benzylpenicillin (45 or 41 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). Chromogenic cephalosporin 87-312 [*i.e.* 3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E isomer] was a gift from Dr O'Callaghan (Glaxo Research Ltd, Greenford, Middlesex, U.K.) [7]. 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 brought to the desired volume with 5 mM sodium phosphate buffer, pH 7.0. The final concentration was estimated by measuring the absorbance at 386 nm using a molar absorption coefficient ε_{386} of 17500 $M^{-1}cm^{-1}$ [7]. Benzylpenicillin was purchased from Rhône-Poulenc (Paris, France), ampicillin was obtained from Bristol Benelux, S.A. (Brussels, Belgium), carbenicillin from Beecham Research Laboratories (Brentford, U.K.) and penicillin V was a gift of Imperial Chemical Industries Ltd (Macclesfield, U. K.).

[^{14}C]Benzylpenicilloic Acid

[^{14}C]Benzylpenicilloic acid was prepared by hydrolysing [^{14}C]benzylpenicillin with penicillinase.

Substrates of Streptomyces *R61 Enzyme and Estimation of Reaction Products*

The DD-carboxypeptidase activity was estimated by measuring the hydrolysis of non-radioactive Ac_2-L-Lys-D-Ala-D-Ala to D-Ala and Ac_2-L-Lys-D-Ala [6]. The tripeptide [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala, radioactively labelled in the acetyl groups (26.6 mCi/mmol), was prepared as described by Perkins *et al.* [8]. The transpeptidase activity was estimated by measuring the amount of [^{14}C]Ac_2-L-Lys-D-Ala transferred from the above tripeptide (the donor) to *meso*-diaminopimelic acid (the acceptor) to form [^{14}C]Ac_2-L-Lys-D-Ala-D-A_2pm [9]. Before use, the solutions of *meso*-diaminopimelic acid were neutralised with concentrated NaOH. In the presence of *meso*-diaminopimelic acid as the acceptor, both the transfer and the hydrolysis reactions occurred concomitantly and the acceptor behaved as a non-competitive inhibitor of the hydrolysis pathway [9]. After incubation with the enzyme, residual tripeptide donor, hydrolysis product ([^{14}C]Ac_2-L-Lys-D-Ala) and transpeptidation product ([^{14}C]Ac_2-L-Lys-D-Ala-D-A_2pm) were separated from each other on paper electrophoresis at pH 6.5, the radioactive compounds were located on the paper strips and the radioactivity was measured as described previously [6,9].

Separation of [^{14}C]Benzylpenicillin and Other Radioactive Derivatives

This was carried out by thin-layer chromatography with 1-butanol – water – ethanol – acetic acid (10:4:3:3 by vol.) and by paper electrophoresis at pH 6.5 as described previously [4,5].

Spectra

Spectra were recorded with a Cary 17 double-beam recording spectrophotometer with automatic slit-width adjustment (volume of the cells: 0.5 ml; optical pathway: 1 cm).

Kinetic Measurements

The experiments were carried out under conditions where the enzyme concentration (about 10 – 20 nM) was considerably lower than the concentrations of the antibiotics (0.3 μM was the lowest concentration utilized). Substrate, antibiotic (when present), enzyme and buffer were precooled and mixed together at 0 °C. The final solutions were then incubated at 37 °C for 60 min after which time the reaction products were estimated.

Hydrolysis Reaction in the Absence of Transfer Reaction

Ac_2-L-Lys-D-Ala-D-Ala (3.9 – 15.5 mM) and enzyme (15 ng or 1.3 munit) were incubated for 60 min at 37 °C in 40 μl (final volume) of 6 mM sodium phosphate buffer pH 7.5 in the absence and in the presence of either carbenicillin (up to 3.4 μM) or penicillin V (up to 1.5 μM). At the most, 13% of the tripeptide was hydrolysed.

Concomitant Hydrolysis and Transfer Reactions with Variable Donor Concentration. [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (from 2 to 7.6 mM), *meso*-diaminopimelic acid (at a fixed, 14.3 mM concentration) and enzyme (30 ng or 2.6 munit) were incubated together for 60 min at 37 °C in 40 μl (final volume) of 6 mM sodium phosphate buffer in the absence and the presence of penicillin V (up to 0.8 μM).

Concomitant Hydrolysis and Transfer Reactions with Variable Acceptor Concentration. [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (at a fixed, 3.2 mM, concentration), *meso*-diaminopimelic acid (from 3.6 to 14.5 mM) and enzyme (30 ng or 2.6 munit) were incubated for

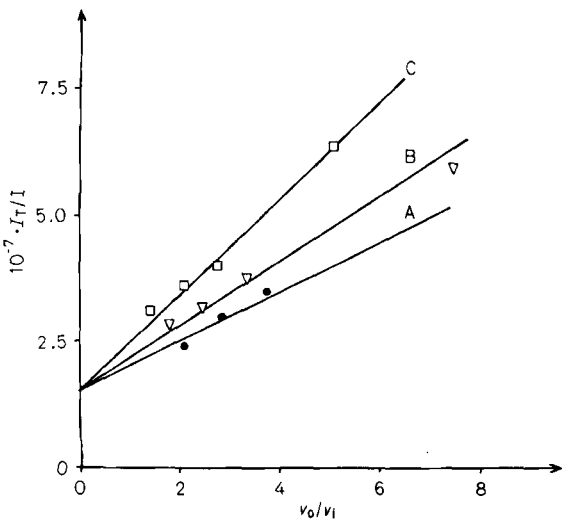

Fig. 1. *Inhibition of the* DD-*carboxypeptidase activity of the* Streptomyces *R61 enzyme by benzylpenicillin, in the absence of acceptor.* Representation of the data of Leyh-Bouille *et al.* [2] according to the graphical method of Morrisson and Henderson [10, 11], by using the equation

$$\frac{I_t}{i} = E_t + K_i \left(\frac{[S] + K_s}{K_s}\right) \frac{v_0}{v_i}$$

with v_0 = velocity of the reaction in the absence of benzylpenicillin; v_i = velocity of the reaction in the presence of benzylpenicillin; E_t = total (active + inhibited) enzyme concentration; $i = \frac{v_0 - v_i}{v_0}$; I_t = concentration of total (free + bound) inhibitor; K_s = 11 mM (see Leyh-Bouille *et al.* [2]); [S] = concentration of substrate Ac_2-L-Lys-D-Ala-D-Ala. For experimental conditions see [2]. The K_i value obtained from the slope of the various lines was 40 nM. Concentrations of substrate (Ac_2-L-Lys-D-Ala-D-Ala): (A) 2.1 mM; (B) 4.3 mM; (C) 6.6 mM

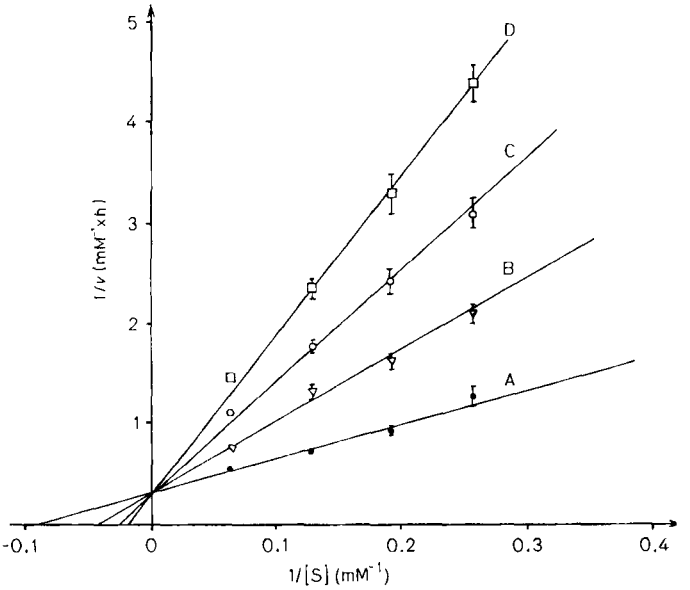

Fig. 2. *Inhibition of the* DD-*carboxypeptidase activity of the* Streptomyces *R61 enzyme by carbenicillin in the absence of acceptor.* Lineweaver-Burk plots of reciprocal of initial velocity of hydrolysis *vs* reciprocal of substrate concentration (Ac_2-L-Lys-D-Ala-D-Ala) for various concentrations of carbenicillin. Concentrations of carbenicillin: (A) 0; (B) 1.13 μM; (C) 2.25 μM and (D) 3.40 μM. For conditions see Materials and Methods. Error bars are standard deviations (4 measurements)

60 min at 37 °C in 40 μl (final volume) of 6 mM sodium phosphate buffer in the absence and in the presence of penicillin V (up to 0.95 μM).

In all reactions involving *meso*-diaminopimelic acid, the concentration of NaCl was adjusted to 29 mM. At the most, 5% of the acceptor and 13% of the tripeptide donor were utilized except in the experiments carried out in the absence of penicillin V, in which cases up to 18% of the tripeptide donor was utilized.

Symbols

v_T = Velocity of the transfer reaction; v_{Hy} = velocity of the hydrolysis reaction; $v_p = v_T + v_{Hy}$: velocity of total reaction. [D] = Concentration of donor. [A] = Concentration of acceptor.

RESULTS

Steady-State Kinetics. Inhibition by Benzylpenicillin of the Hydrolysis Reaction Catalysed by Streptomyces *R61 Enzyme in the Absence of Acceptor*

The effect of benzylpenicillin on the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala by the *Streptomyces* R61 enzyme had been studied previously [2]. However, when the molecular weight of the *Streptomyces* R61 enzyme was known (38000) [6], it appeared that these steady-state kinetics had been carried out under conditions where the concentration of benzylpenicillin had not been much greater than the concentration of enzyme. The data of Leyh-Bouille *et al.* [2] were plotted according to the graphical method devised by Morrisson [10] and Henderson [11] for the study of tight-binding inhibitors. The inhibition was again competitive (Fig. 1) with a K_i value of 40 nM. This latter value was of the same order of magnitude as that obtained previously and was even closer to the K_i value obtained when the interactions between the *Streptomyces* R61 enzyme and benzylpenicillin were studied by optical methods [3] (see introductory paragraph).

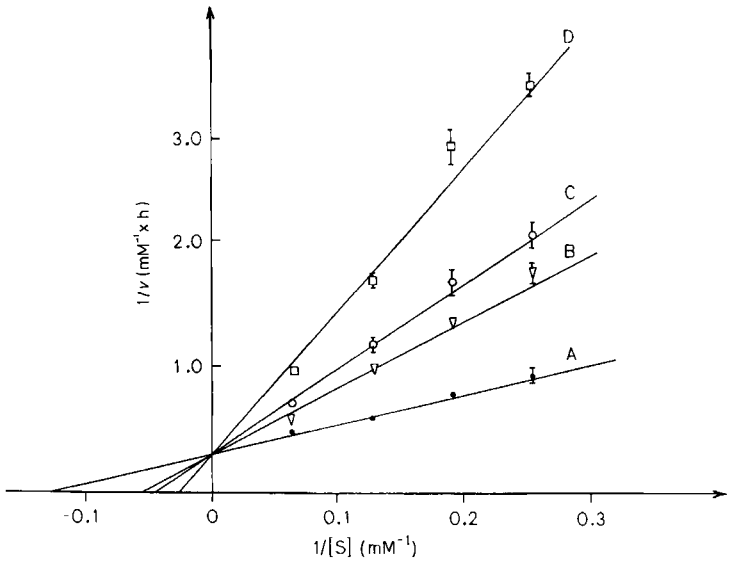

Fig. 3. *Inhibition of the* DD-*carboxypeptidase activity of the* Streptomyces *R61 enzyme by penicillin V in the absence of acceptor.* Lineweaver-Burk plots of reciprocal of initial velocity of hydrolysis *vs* reciprocal of substrate concentration (Ac_2-L-Lys-D-Ala-D-Ala) for various concentrations of penicillin V. Concentrations of penicillin V: (A) 0; (B) 0.54 μM; (C) 0.75 μM and (D) 1.50 μM. For conditions see Materials and Methods

Steady-State Kinetics. Inhibition by Carbenicillin and Penicillin V of the Hydrolysis Reaction Catalysed by Streptomyces *R61 Enzyme in the Absence of Acceptor*

Because it is based on differences between measured values, the Morrisson-Henderson plot is not very accurate. Inhibition of *Streptomyces* R61 enzyme was carried out by using β-lactam antibiotics which were much less active than benzylpenicillin and, consequently, could be used at concentrations considerably higher than that of the enzyme. The inhibition of the carboxypeptidase activity of the *Streptomyces* R61 enzyme by carbenicillin and penicillin V was competitive (Fig. 2 and 3). Dixon plots of the same data yielded K_i values of 1.05 μM for carbenicillin and 0.33 μM for penicillin V.

Steady-State Kinetics. Inhibition by Penicillin V of Concomitant Hydrolysis and Transfer Reactions Catalysed by Streptomyces *R61 Enzyme*

Variable Donor Concentration. The plots $1/v_{Hy}$, $1/v_T$ and $1/v_p$ *vs* 1/[D] at a fixed concentration of acceptor (14.3 mM) and various concentrations of penicillin V showed that inhibition was competitive with regard to the donor (Fig. 4). Dixon plots yielded K_i values of 0.65 μM for v_T, 0.62 μM for v_{Hy} and

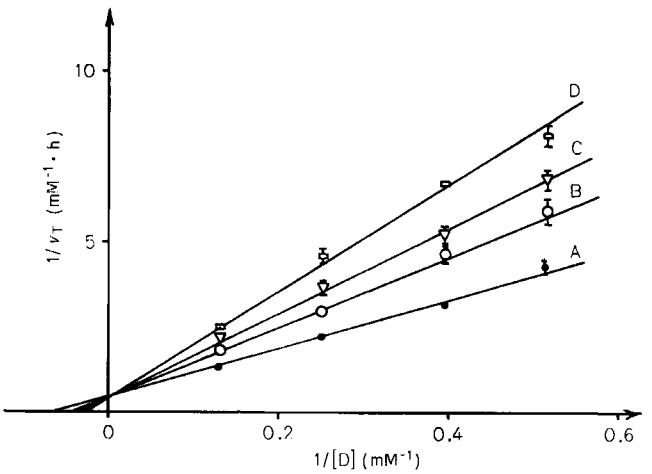

Fig. 4. *Inhibition of the transpeptidase activity of the* Streptomyces *R61 enzyme by penicillin V under conditions of concomitant hydrolysis and transfer reactions. Variable donor concentration.* Lineweaker-Burk plots of reciprocal of initial velocity of transfer *vs* reciprocal of donor concentration (Ac_2-L-Lys-D-Ala-D-Ala) in the presence of a fixed concentration of acceptor (14.3 mM *meso*-diaminopimelic acid), for various concentrations of penicillin V. Concentrations of penicillin V: (A) 0; (B) 0.303 μM; (C) 0.505 μM; (D) 0.755 μM. For conditions see Materials and Methods. Each point corresponds to two experiments. Error bars indicate the variations between the two measurements. Similar double-reciprocal plots $1/v_{Hy}$ and $1/v_p$ *vs* 1/[D] also showed that inhibition by penicillin V was competitive

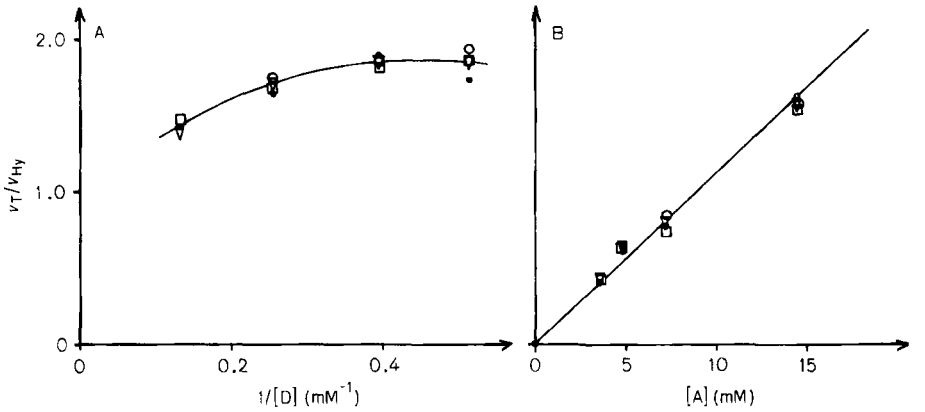

Fig. 5. *Effect of penicillin V on both* DD-*carboxypeptidase and transpeptidase activities of the* Streptomyces *R61 enzyme.* (A) Plots of v_T/v_{Hy} ratios *vs* reciprocal donor concentration (Ac_2-L-Lys-D-Ala-D-Ala) in the presence of a fixed concentration of acceptor (14.3 mM) for various concentrations of penicillin V. (B) Plots of v_T/v_{Hy} ratios *vs* acceptor concentration (*meso*-diaminopimelic acid) in the presence of a fixed concentration of donor (3.2 mM) for various concentrations of penicillin V. Concentrations of penicillin V: (A): 0 (●); 0.303 μM (○); 0.505 μM (▽) and 0.755 μM (□). (B): 0 (●); 0.38 μM (○); 0.63 μM (▽) and 0.95 μM (□)

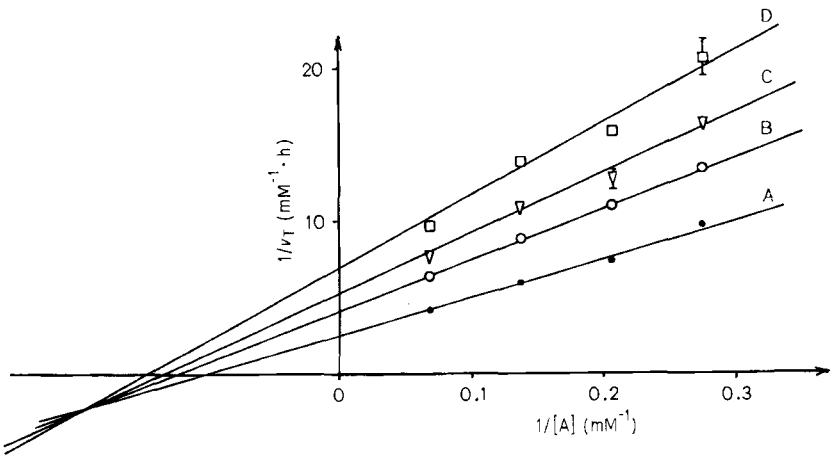

Fig. 6. *Inhibition of the transpeptidase activity of the* Streptomyces *R61 enzyme by penicillin V under conditions of concomitant hydrolysis and transfer reactions. Variable acceptor concentration.* Lineweaver-Burk plots of reciprocal of initial velocity of transfer *vs* reciprocal of acceptor concentration (*meso*-diaminopimelic acid) in the presence of a fixed concentration of donor (13.2 mM Ac_2-L-Lys-D-Ala-D-Ala) for various concentration of penicillin V. Concentrations of penicillin V: (A) 0; (B) 0.38 μM; (C) 0.63 μM; (D) 0.95 μM. For conditions see Materials and Methods

0.77 μM for v_p. It was also observed that penicillin V had no effects on the plots v_T/v_{Hy} *vs* 1/[D] (Fig. 5A).

Variable Acceptor Concentration. (a) Effect on v_T: the plots of $1/v_T$ *vs* 1/[A] at a fixed concentration of donor (3.2 mM) and for various concentrations of penicillin V, showed that inhibition was non-competitive (Fig. 6). (b) Effect on v_{Hy}: the acceptor by itself is a non-competitive inhibitor of the hydrolysis pathway [9]. The Dixon plots of $1/v_{Hy}$ *vs* [A] at a fixed concentration of donor (3.2 mM) and for various concentrations of penicillin V (Fig. 7A) and of $1/v_{Hy}$ *vs* [penicillin V] at a fixed concentration of donor (3.2 mM) and for various concentrations of acceptor (Fig. 7B) showed that the inhibitory effects of penicillin V and *meso*-diaminopimelic acid on the hydrolysis pathway were cumulative. (c) Effect on v_p: with *meso*-diaminopimelic acid as acceptor, the velocity of the total reaction in the absence of antibiotic ($v_p = v_T + v_{Hy}$) was found to be independent of the acceptor concentration [9]. As expected, the plots of $1/v_p$ *vs* [penicillin V] at a fixed concentration of donor (3.2 mM) gave rise to one single line irrespective of the concentration of acceptor used, and the plots of $1/v_p$ *vs* [acceptor] at a fixed concentration of donor (3.2 mM) gave rise to a series of lines which paralleled the abscissa and intersected the ordinate axis at increased

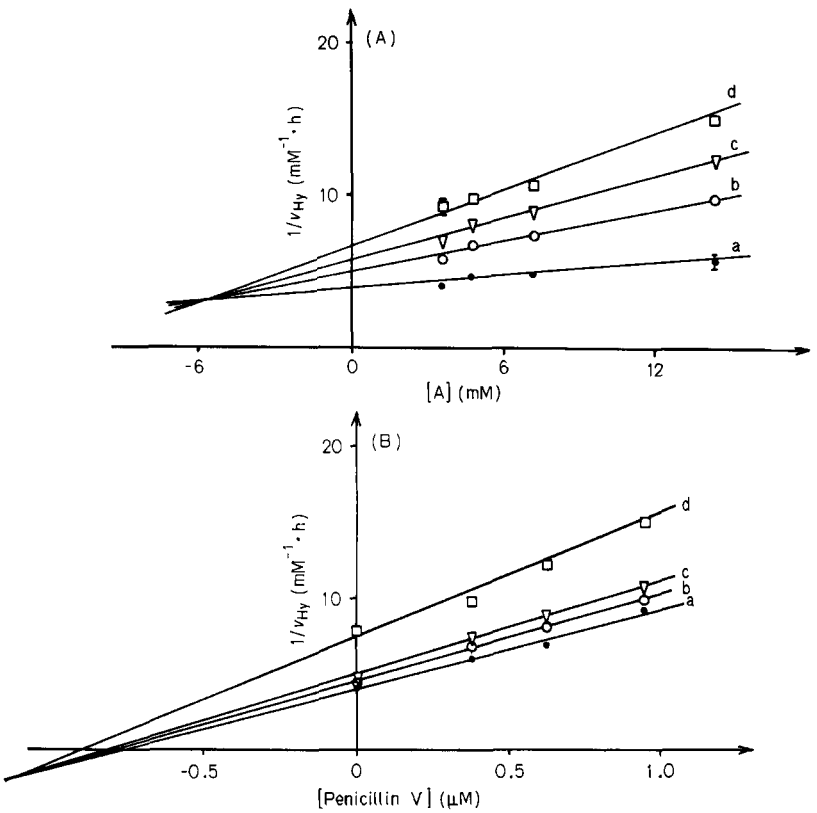

Fig. 7. *Cumulative inhibitory effects of penicillin V and* meso*-diaminopimelic acid on the* DD*-carboxypeptidase activity of the* Streptomyces *R61 enzyme.* (A) Dixon plots of reciprocal of initial velocity of hydrolysis *vs* acceptor concentration (*meso*-diaminopimelic acid) in the presence of a fixed concentration of donor (3.2 mM Ac_2-L-Lys-D-Ala-D-Ala) for various concentrations of penicillin V. Concentrations of penicillin V: (a) 0; (b) 0.38 μM; (c) 0.63 μM; (d) 0.95 μM. For conditions see Materials and Methods. (B) Dixon plots of reciprocal of initial velocity of hydrolysis *vs* penicillin V concentration in the presence of a fixed concentration of donor (3.2 mM Ac_2-L-Lys-D-Ala-D-Ala) for various concentrations of acceptor (*meso*-diaminopimelic acid). Concentrations of acceptor: (a) 3.63 mM; (b) 4.83 mM; (c) 7.25 mM; (d) 14.5 mM. For conditions see Materials and Methods

$1/v_p$ values as the concentration of penicillin V increased. (d) Effect on v_T/v_{Hy}: the plots of v_T/v_{Hy} *vs* [acceptor] gave rise to one single line passing through the origin irrespective of the concentrations of penicillin V (Fig. 5B).

Isolation and Stability of the Streptomyces *R61 Enzyme · [^{14}C]Benzylpenicillin Complex*

The enzyme (8 nmol) was incubated for 10 min at 37 °C with 25 nmol of [^{14}C]benzylpenicillin in a final volume of 400 μl of either 5 mM sodium phosphate buffer pH 7.0 or 20 mM Tris-HCl buffer pH 7.7. The enzyme · [^{14}C]benzylpenicillin complex was isolated by filtration at 0 °C on an 18-ml column of Sephadex G-25 (1.5 × 10 cm) in the same phosphate or Tris-HCl buffers as above. The filtration was performed in less than 5 min; under these conditions the enzyme · benzylpenicillin complex did not undergo any dissociation. The isolated complex exhibited a ratio of benzylpenicillin to enzyme of 0.98 to 1 and was maintained at 37 °C. Samples were removed after increasing times of incubation and were used for the estimation of enzyme activity and for the measurement of released radioactivity. Both procedures showed that the *Streptomyces* R61 enzyme · benzylpenicillin complex underwent spontaneous breakdown and that the process continued to completion of the reaction. On the basis of the released radioactivity, the plots of log c_t/c_o *vs* time (where c_o is the concentration of the original complex and c_t the concentration of the residual complex after time t), indicated a half-life for the complex of 90 or 105 min in phosphate buffer (as determined by thin-layer chromatography and paper electrophoresis, respectively) and of 83 or 105 min in Tris-HCl buffer (also as determined by thin-layer chromatography and paper electrophoresis, respectively). For the measurement of enzyme activity, the samples (10 μl) removed at increasing times t were

incubated for 10 min at 37 °C with 80 nmol of Ac_2-L-Lys-D-Ala-D-Ala (final volumes: 30 µl of 5 mM sodium phosphate buffer pH 7.0) and the amount A_t of hydrolysis product (DD-carboxypeptidase activity) formed in each case was estimated. An approximate first-order kinetic constant value k^* for the breakdown of the enzyme · benzylpenicillin complex was calculated by plotting $\log A_t/A_0$ *vs* time and measuring the slope of the line thus obtained (A_0 represented the amount of hydrolysis product obtained with the same concentration of active enzyme). The exact percentage of enzyme recovery after each time t was then calculated by using the following formula (for explanation, see legend of Fig. 8), which took into account the fact that breakdown of the original complex and enzyme recovery continued to proceed during the 10 min incubation (*i.e.* 600 s) with the tripeptide substrate:

Percentage enzyme recovery at time t

$$\left(\frac{A_t}{A_0} + \frac{1 - e^{-k^* \times 600}}{k^* \times 600} - 1\right) \Big/ \left(\frac{1 - e^{-k^* \times 600}}{k^* \times 600}\right).$$

From these corrected data, the plots of $\log c_t/c_0$ *vs* time yielded a half-life for the complex of 105 min both in phosphate and in Tris-HCl buffers (Fig. 8). This value was virtually identical to that found on the basis of the released radioactivity.

Samples removed after 90 min of breakdown of the enzyme · benzylpenicillin complex were also incubated with [^{14}C]Ac-L-Lys-D-Ala-D-Ala (3 mM; 0.5 mCi/mmol) in the presence of *meso*-diaminopimelic acid (5 mM). Estimation of the reaction products showed that the enzyme had recovered its transpeptidase activity. The v_T/v_{Hy} ratio thus obtained was 0.6, a value which was identical to that obtained with the untreated enzyme.

Nature of the Radioactive Compound Released from Streptomyces *R61 Enzyme · [^{14}C]Benzylpenicillin Complex*

The *Streptomyces* R61 enzyme · [^{14}C]benzylpenicillin complex was immobile both by paper electrophoresis at pH 6.5 and by thin-layer chromatography. By paper electrophoresis (60 V/cm), the radioactive compound released from the complex had a mobility towards the anode of 22 ± 1 cm/h. Under the same conditions, [^{14}C]benzylpenicillin and [^{14}C]benzylpenicilloic acid migrated 16 and 25 cm/h, respectively. By thin-layer chromatography, the released radioactive compound had an R_F value of 0.78 ± 0.02. It was easily distinguishable from [^{14}C]benzylpenicilloic acid (R_F 0.71) but not from [^{14}C]benzylpenicillin (R_F 0.82). Incubation with a large excess of penicillinase did not alter the R_F value of the released radioactive compound. Moreover the rate of the spontaneous breakdown of the *Streptomyces* R61 enzyme · [^{14}C]benzylpenicillin complex was not modified by adding penicillinase to the reaction mixture. In this latter experiment, 13 units of penicillinase were used in 200 µl, final volume, of 5 mM sodium phosphate buffer pH 7.0 containing 0.05 nmol of complex.

Stability of the Complexes Formed between the Streptomyces *R61 Enzyme and β-Lactam Antibiotics other than Benzylpenicillin*

Enzyme (0.1 nmol) and antibiotic (1 nmol of cephalosporin 87-312; 50 nmol of either benzylpenicillin, carbenicillin, ampicillin, or penicillin V) were incubated together for 10 min at 37 °C in 140 µl of 6 mM sodium phosphate pH 7.5. Penicillinase (20 units) was added, the reaction mixtures were maintained at 37 °C and samples were removed at increasing time intervals. Recovery of enzyme activity was measured as described above and the results are shown in Fig. 8. Because of the very large amount of penicillinase used, the time needed to destroy the excess of antibiotic which had not reacted with the enzyme was considered as being negligible. Table 1 gives the half-lives of various *Streptomyces* R61 enzyme · antibiotic complexes and the rate constants for their spontaneous breakdown. It also compares these values with those obtained previously with the complexes formed between β-lactam antibiotics and either the corresponding membrane-bound transpeptidase of *Streptomyces* R61 [5] or the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R39 [4].

Competition between [^{14}C]Benzylpenicillin and Cephalosporin 87-312 for the Streptomyces *R61 Enzyme*

The following experiment was performed in order to decide whether benzylpenicillin and cephalosporin 87-312 would bind to the enzyme in a mutually exclusive way or to distinct binding sites.

In a control experiment, 0.26 nmol of *Streptomyces* R61 enzyme and 1.2 nmol of [^{14}C]benzylpenicillin were incubated for 10 min at 37 °C in 30 µl of 5 mM sodium phosphate pH 7.0. After filtration on a 7-ml column of Sephadex G-25 (1.5 × 4 cm) in the same buffer, 0.235 nmol of [^{14}C]benzylpenicillin was found in the excluded peak, associated with the protein. In a second experiment, 0.26 nmol of *Streptomyces* R61 enzyme was first incubated with 0.75 nmol of cephalosporin 87-312 for 10 min at room temperature in 30 µl of the same phosphate buffer and then with

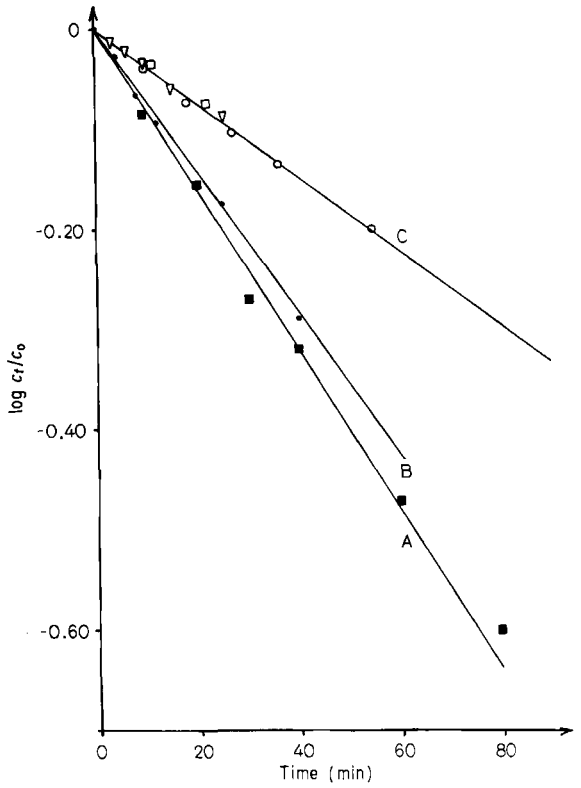

Fig. 8. *First-order plot of the breakdown of various* Streptomyces *R61 enzyme · β-lactam antibiotic complexes at 37 °C and in 5 mM phosphate buffer pH 7.0.* The kinetics were based on the recovery of enzyme activity. For conditions see text. (A) Cephalosporin 87-312; (B) penicillin V; (C) (○) benzylpenicillin, (▽) carbenicillin, (□) ampicillin. c_0: concentration of the original complex; c_t: concentration of the residual complex after time t of incubation. Enzyme activity was estimated by incubating the samples at 37 °C for 600 s in the presence of the tripeptide donor. Since enzyme recovery continued to proceed during the 600 s of incubation with the substrate, the exact percentage of enzyme recovery at time t was calculated by using the equation given in the text. This equation was based on the following mathematical development where: t = time of recovery in seconds; E_t = the concentration of active enzyme present after t seconds of recovery; EP_t = the residual concentration of inactive complex after t seconds of recovery; E_0 = the total (active and inhibited) concentration of enzyme. E_0 is equal to $E_t + EP_t$; t_1 = the time of incubation of the sample with the substrate (*i.e.* 600 s); $A_0 = NE_0t_1$: amount of hydrolysis product that a concentration E_0 of active enzyme would produce after 600 s of incubation with the substrate. N = proportionality factor including the concentration of substrate and the K_s value. The mixture containing $E_t + EP_t$ is incubated with the substrate at time t. At any time t' between t and $t + t_1$, the concentration of active enzyme is $E_{t'} = E_t + E_\tau$ where $\tau = t' - t$ and the amount of hydrolysis product (A) formed during the incubation between time t and time t_1 is

$$A = NE_t t_1 + N\int_0^{t_1} E_\tau d\tau$$

where $E_\tau = E_t[1 - e^{-k^*\tau}]$ (for the meaning of k^*, see text). Therefore

$$\frac{A_t}{A_0} = \frac{E_t}{E_0} + \frac{1}{E_0 t_1}\int_0^{t_1} E_\tau d\tau$$

and after substitution and integration

$$\frac{A_t}{A_0} = 1 - \frac{1 - e^{-k^*t_1}}{k^*t_1} + \frac{E_t}{E_0}\left(\frac{1 - e^{-k^*t_1}}{k^*t_1}\right)$$

1.2 nmol of [^{14}C]benzylpenicillin for 10 min at 37 °C. After filtration of the solution on Sephadex G-25, 0.026 nmol of [^{14}C]benzylpenicillin was found to be associated with the excluded protein. On the basis of the rate constant for the breakdown of the *Streptomyces* R61 enzyme · cephalosporin 87-312 complex (Table 1), 17% of the original complex must have decayed during the 10 min of incubation at 37 °C with [^{14}C]benzylpenicillin. Since at the beginning of this second incubation the ratio of free [^{14}C]benzylpenicillin to free cephalosporin 87-312 was 12/5, and assuming similar rate constants for the formation of both enzyme · benzylpenicillin and enzyme · cephalosporin 87-312 complexes, it was calculated that under the above conditions about 12% of the *Streptomyces* R61 enzyme should have undergone binding with [^{14}C]benzylpenicillin, a value which was almost identical to that found experimentally (10%).

Table 1. *First-order rate constants* k *and half-lives of various* DD-*carboxypeptidase-transpeptidase enzyme · β-lactam antibiotic complexes*
Temperature was 37 °C

Enzyme	Antibiotic	First-order rate constant	Half-life
		s^{-1}	min
Streptomyces R61 exocellular enzyme (for conditions see text)	benzylpenicillin	1.4×10^{-4}	81
	carbenicillin	1.4×10^{-4}	81
	ampicillin	1.4×10^{-4}	81
	penicillin V	2.72×10^{-4}	42
	cephalosporin 87-312	3.07×10^{-4}	38
Streptomyces R61 membrane-bound enzyme [4]	carbenicillin	0.73×10^{-4}	160
	benzylpenicillin	1.1×10^{-4}	104
	penicillin V	2.8×10^{-4}	41
	ampicillin	3.3×10^{-3}	3.5
Streptomyces R39 exocellular enzyme [5]	cephaloridin	0.63×10^{-6}	18000
	cephalosporin 87-312	1.5×10^{-6}	7800
	benzylpenicillin	2.8×10^{-6}	4200

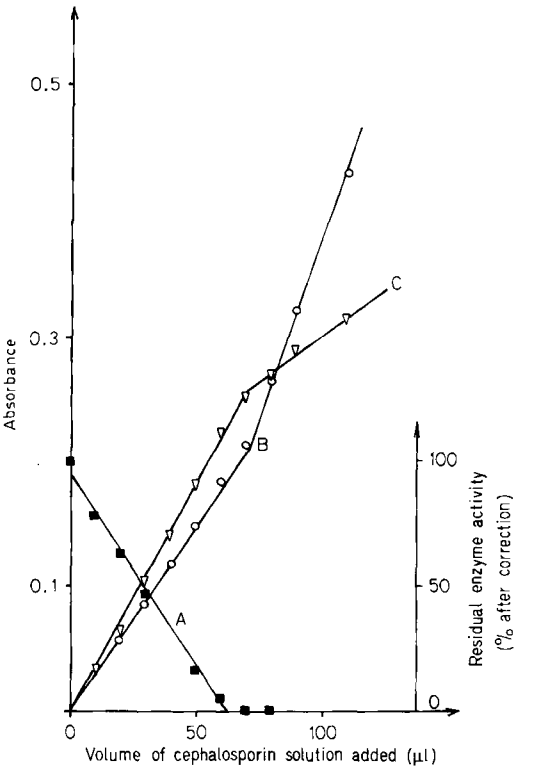

Fig. 9. *Titration of* Streptomyces *R61 enzyme by cephalosporin 87-312.* Portions (10 µl) of a 0.15 mM solution of cephalosporin 87-312 in 5 mM sodium phosphate buffer pH 7.0 were added to 7.9 nmol of enzyme dissolved in 400 µl of the same phosphate buffer. For other conditions see text. (A) Inactivation of the enzyme. The experimental data were corrected for recovery of enzyme activity during incubation with the substrate according to the formula:

Residual activity (%) after addition of x nmol of cephalosporin 87-312

$$= \left(\frac{A_t}{A_0} + \frac{1 - e^{-k_4 t}}{k_4 t} - 1 \right) \Big/ \left(\frac{1 - e^{-k_4 t}}{k_4 t} \right)$$

where A_t is the amount of hydrolysis product formed, A_0 the amount of hydrolysis product obtained with the same concentration of uninhibited enzyme, t the time of incubation (in s) with the substrate at 37 °C and k_4: $3.07 \times 10^{-4} \times s^{-1}$ (see Table 1). (B) Increase of the absorbance of the solution at 386 nm. (C) Increase of the absorbance of the solution at 482 nm. After each addition of cephalosporin 87-312, the absorbance of the solution is normalised for a final volume of 500 µl. In all cases the end-point of the titration occurred after the addition of 65–70 µl of the 0.15 mM cephalosporin 87-312 solution. Note that the molar absorption coefficient of cephalosporin 87-312 is 17500 $M^{-1} \times cm^{-1}$ at 386 nm and 2600 $M^{-1} \times cm^{-1}$ at 482 nm, and the molar absorption coefficient of cephalosporin 87-312 hydrolysed by penicillinase is 7700 $M^{-1} \times cm^{-1}$ at 386 nm and 16700 $M^{-1} \times cm^{-1}$ at 482 nm. Hence the molar absorption coefficient of hydrolysed cephalosporin 87-312 at 482 nm is equal to that of intact cephalosporin 87-312 at 386 nm

Titration of Streptomyces *R61 Enzyme by Cephalosporin 87-312*

Hydrolysis of the β-lactam ring of cephalosporin 87-312 by penicillinase caused a shift of the absorption maximum from 386 to 482 nm [7]. Portions of a 0.15 mM solution of cephalosporin 87-312 in 5 mM sodium phosphate pH 7.0 were added stepwise and at room temperature to 0.4 ml of a solution containing 7.9 nmol of *Streptomyces* R61 enzyme. After each addition the mixture was maintained at room temperature for 5 min, after which time the absorbance of the solution was measured both at 386 nm and at 482 nm and the residual enzyme activity was estimated on a 5-µl sample. As revealed by the three procedures, the end-points of the titration occurred at a molar ratio of cephalosporin 87-312 to *Streptomyces* R61 enzyme of 1.25 to 1 (Fig. 9). The same high ratio had also been obtained in similar experiments carried out with the *Streptomyces* R39 DD-carboxypeptidase-transpeptidase [5]. It might be due to the fact that the amount of cephalosporin 87-312 used was estimated on the basis of an ε_{386} value of 17500 $M^{-1} \times cm^{-1}$. An underestimation of this coefficient would result in an overestimation of the amount of antibiotic required to estimate the enzyme.

Interaction between Streptomyces *R61 Enzyme and Cephalosporin 87-312*

The $\varepsilon_{482}/\varepsilon_{386}$ ratio of cephalosporin 87-312 hydrolysed by penicillinase was found to be equal to 2.40. In marked contrast, the $\varepsilon_{482}/\varepsilon_{386}$ ratio of the *Streptomyces* R61 enzyme · cephalosporin 87-312 complex was 1.20 (Fig. 9). This value was considerably lower than that normally expected if the β-lactam

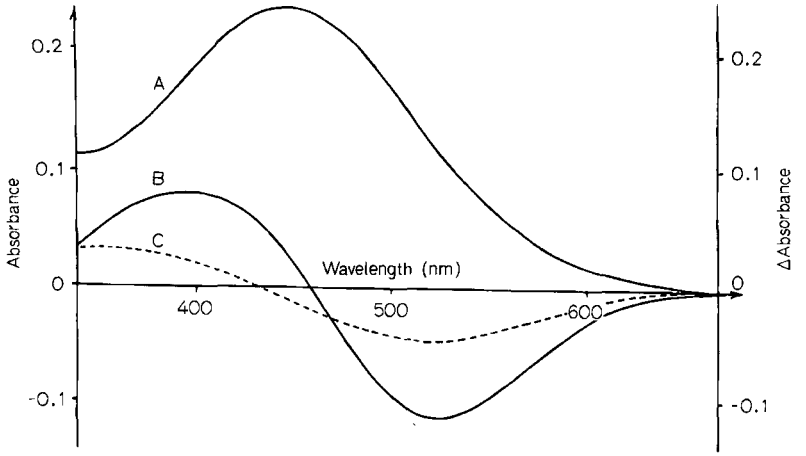

Fig. 10. *Absorption spectrum of cephalosporin 87-312 after treatment with the* Streptomyces *R61 enzyme and with β-lactamase.* The spectra were recorded with 0.5-ml cells and an optical pathway of 1.0 cm. Buffer: 5 mM sodium phosphate pH 7.0. For other conditions see text. (A) Absorption spectrum of a freshly prepared R61 enzyme · cephalosporin 87-312 complex. Concentration of the complex: 15 μM. (B) Difference spectrum between the freshly prepared R61 enzyme · cephalosporin 87-312 complex and an equivalent amount of cephalosporin 87-312 hydrolysed by penicillinase. The solutions contained 7.5 nequiv of cephalosporin 87-312 (either combined to the enzyme or hydrolysed by penicillinase) in a final volume of 500 μl. (C) Difference spectrum between the same complex as in (B) maintained for 70 min at 37 °C and an equivalent amount of cephalosporin 87-312 hydrolysed by penicillinase

ring of the antibiotic molecule had been hydrolysed as occurred with penicillinase. *Streptomyces* R61 enzyme (8 nmol) dissolved in 450 μl of 5 mM sodium phosphate buffer pH 7.0 was added to 7.5 nmol of cephalosporin 87-312 dissolved in 50 μl of the same buffer. After 5 min at room temperature, the solution exhibited an absorption maximum at 440 nm (ε_{440} = 16500 $M^{-1} \times cm^{-1}$) (Fig. 10A). Moreover, a difference spectrum against the same concentration of cephalosporin 87-312 hydrolysed by penicillinase exhibited extrema at 390 nm and 525 nm (Fig. 10B). The *Streptomyces* R61 enzyme · cephalosporin 87-312 complex solution was then maintained at 37 °C for 60 min. After this time 65% of the initially inhibited activity had recovered and, parallel to this, the intensities of the two extrema in the difference spectrum had decreased to about 60% of the original values (Fig. 10C). Hence, at 37 °C and pH 7.0 the spontaneous breakdown of the *Streptomyces* R61 enzyme · cephalosporin 87-312 complex, under conditions where reactivation of the enzyme occurred, proceeded through the release of the antibiotic molecule in a degraded form which exhibited the same absorption spectrum as that of cephalosporin 87-312 hydrolysed by penicillinase. In another experiment the *Streptomyces* R61 enzyme · cephalosporin 87-312 was isolated by filtration on Sephadex G-25 in 5 mM sodium phosphate buffer pH 7.0, and then maintained at 37 °C in the same buffer. The absorption maximum of the solution was at 440 nm as soon as the complex had been isolated. However, the maximum shifted to 457 nm after 30 min, to 472 nm after 60 min and to 480 nm after 90 min of incubation at 37 °C. Finally, it was determined that the *Streptomyces* R61 protein that had been released from its complex with cephalosporin 87-312 had not only recovered its enzyme activity, but had also recovered its initial ability to bind fresh cephalosporin 87-312.

The above experiments revealed a marked difference between the *Streptomyces* R61 DD-carboxypeptidase-transpeptidase and the *Streptomyces* R39 DD-carboxypeptidase-transpeptidase. Indeed, the interaction of the *Streptomyces* R39 enzyme with cephalosporin 87-312 had been shown to give rise to a' complex which immediately exhibited the same absorption spectrum as that of cephalosporin 87-312 hydrolysed by penicillinase [4]. The "blue shift" of the absorption spectrum of the *Streptomyces* R61 enzyme · cephalosporin 87-312 complex, when compared with that of the *Streptomyces* R39 enzyme · cephalosporin 87-312 complex, might possibly be linked to differences in the degree of protonation of the secondary amine that might be produced through hydrolysis of the β-lactam ring during complex formation. In order to check this hypothesis, cephalosporin 87-312 dissolved in 5 mM sodium phosphate buffer pH 7.0 was hydrolysed with penicillinase. The solution was diluted with 0.1 M glycine to a 40 μM concentration in hydrolysed cephalosporin 87-312 and then titrated to pH 1.4 with 1 N HCl. The absorption maximum of the solution progressively shifted from 482 nm at pH 7.0 to 440 nm at pH 1.4. However, the ε_{440} value at

pH 1.4 of the cephalosporin 87-312 hydrolysed by penicillinase ($3500\ M^{-1} \times cm^{-1}$) was five times lower than the ε_{440} value at pH 7.0 of the *Streptomyces* R61 enzyme · cephalosporin 87-312 complex ($16500\ M^{-1} \times cm^{-1}$). Hence, the nature of the alteration undergone by cephalosporin 87-312 during its interaction with the *Streptomyces* R61 enzyme (as well as with the *Streptomyces* R39 enzyme) remains to be established.

DISCUSSION

As revealed by steady-state kinetics, the inhibition of the *Streptomyces* R61 DD-carboxypeptidase-transpeptidase by penicillin V was competitive with regard to the peptide donor whether the enzyme worked as a carboxypeptidase or as a transpeptidase, and was non-competitive with regard to the acceptor in the transpeptidation pathway. The K_i values with regard to peptide donor were very similar both in hydrolysis and in transpeptidation, suggesting that the antibiotic had the same affinity for the free enzyme E (or the $E \cdot H_2O$ complex) and for the enzyme · acceptor complex. The antibiotic had no effect on the ratios v_T/v_{Hy} *vs* [D] and v_T/v_{Hy} *vs* [A], also suggesting that it did not alter the basic mechanism through which the reactions occurred. Previous experiments had shown that the reaction probably proceeded through an ordered mechanism in which the acceptor molecule binds first to the enzyme [9]. In the present experiments penicillin V was used because of its relatively low efficiency as an inhibitor, which made it possible to use it at concentrations much higher than that of the enzyme. The results obtained with carbenicillin and benzylpenicillin, which is a very potent inhibitor, supported the idea that the conclusions obtained with penicillin V could probably be extended to β-lactam antibiotics in general.

The competitive inhibition of the *Streptomyces* R61 enzyme caused by β-lactam antibiotics with regard to the peptide donor might suggest that inhibitor and peptide donor competed for the same site on the free enzyme. However, the complexes formed between the enzyme and the various β-lactam antibiotics had long half-lives of 40 to 80 min (at 37 °C and neutral pH). Such a property was in complete conflict with the hypothesis of a rapid equilibrium upon which the competitive inhibition theory is based. At neutral pH, both in Tris-HCl and in phosphate buffers, the isolated *Streptomyces* R61 enzyme · antibiotic complexes underwent spontaneous and complete breakdown, which was paralleled by the concomitant recovery of the enzyme activity. The radioactive compound released from the *Streptomyces* R61 enzyme · [^{14}C]-benzylpenicillin complex was neither [^{14}C]benzylpenicillin nor [^{14}C]benzylpenicilloic acid and had the same chromatographic and electrophoretic properties as those of the radioactive compound released from the *Streptomyces* R39 enzyme · [^{14}C]benzylpenicillin complex (under conditions where recovery of enzyme activity occurred) [4]. The values of the first-order kinetic constants for the breakdown of the various *Streptomyces* R61 enzyme · antibiotic complexes so far tested were all of the same order of magnitude ($1-3 \times 10^{-4} \times s^{-1}$). Except for ampicillin, these values were roughly comparable with those obtained for the breakdown of the complexes formed between the same antibiotics and the corresponding membrane-bound transpeptidase of *Streptomyces* R61 ($0.3-3 \times 10^{-4} \times s^{-1}$) [5], but they were about a factor of 100 higher than those obtained for the breakdown of the exocellular *Streptomyces* R39 enzyme · antibiotic complexes ($0.6-3 \times 10^{-6} \times s^{-1}$) [4]. Hence both *Streptomyces* R61 and *Streptomyces* R39 enzymes behaved as penicillin-destroying enzymes but did not function as penicillinases. Their effectiveness differed greatly but was always very low and could not be compared with that of penicillinases. In a brief report by Strominger *et al.* [12], it was claimed that the DD-carboxypeptidases from both *Bacillus stearothermophilus* and *Bacillus subtilis* hydrolysed benzylpenicillin to benzylpenicilloic acid and, hence, catalysed a reaction which was that of a penicillinase. Further experiments, however, did not confirm these preliminary findings and showed that benzylpenicilloic acid was not the reaction product (P. M. Blumberg, personal communication). It thus appears that in this respect the *Bacillus* and the *Streptomyces* enzymes behave similarly.

Whereas the absorption spectrum of the *Streptomyces* R39 · cephalosporin 87-312 complex, once formed, had a maximum at 482 nm and was indistinguishable from that of cephalosporin 87-312 hydrolysed by penicillinase [4], the absorption spectrum of the *Streptomyces* R61 · cephalosporin 87-312 exhibited a maximum at 430–440 nm. Breakdown of this latter complex, however, gave rise to a released compound exhibiting an absorption spectrum very similar to that of cephalosporin 87-312 hydrolysed by penicillinase. Hence, the complexes formed between cephalosporin 87-312 and both *Streptomyces* R61 and *Streptomyces* R39 enzymes probably differed from each other with regard to the electrostatic forces and/or the conformational changes involved. The above experiments also suggested that the various strains to which cephalosporin 87-312 was submitted during its interaction with the *Streptomyces* R61 enzyme were relieved during reactivation of the enzyme. It is impossible to state, however, whether or not the β-lactam ring of the antibiotic molecule was

hydrolysed during its interaction with the *Streptomyces* R61 enzyme.

The inhibition of both *Streptomyces* R61 and *Streptomyces* R39 enzymes by β-lactam antibiotics appeared to proceed through one of the two following reactions:

$$E + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EP \xrightarrow{k_3} E + X \qquad (1)$$

$$E + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EP \xrightarrow{k_3} EP^* \xrightarrow{k_4} E + X, \qquad (2)$$

where E is the active enzyme, P the antibiotic molecule, EP and EP* inactive complexes and X the altered antibiotic molecule. If reaction (1) represented the real mechanism, the rate constant k_2 should be much smaller than the rate constant k_3, since intact penicillin was never detected during the breakdown of the enzyme · antibiotic complexes so far tested. The attainment of steady-state kinetics which, when expressed in Lineweaver-Burk plots, seemingly show typical competitive inhibitions, can only be explained by determining the exact pathway of the reaction and measuring the values of the various constants. The elucidation of the reaction pathway will also reveal the exact physical meaning of the K_i values obtained here and by optical techniques [3] (see introductory paragraph). This problem is under current study and should be solved in the near future.

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