Biochem. J. (1979) **177**, 909–916
Printed in Great Britain

Effects of Nucleophiles on the Breakdown of the Benzylpenicilloyl–Enzyme Complex EI* Formed between Benzylpenicillin and the Exocellular DD-Carboxypeptidase–Transpeptidase of *Streptomyces* Strain R61

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(*Received* 16 *June* 1978)

Serine is one of the enzyme residues with which benzylpenicillin collides as a result of its binding to the *Streptomyces* strain-R61 DD-carboxypeptidase–transpeptidase enzyme. Nucleophilic attack occurs on $C_{(7)}$ of the bound antibiotic molecule with formation of a benzylpenicilloyl–serine ester linkage, i.e. formation of the benzylpenicilloyl–enzyme EI* complex. To reject the bound penicilloyl moiety and consequently to recover its initial activities, the strain-R61 enzyme has developed two possible mechanisms. Pathway A is a direct attack of the serine ester linkage by an exogenous nucleophile, resulting in the transfer of the benzylpenicilloyl moiety to this nucleophile. In pathway B, the benzylpenicilloyl moiety is first fragmented by $C_{(5)}$–$C_{(6)}$ cleavage and the enzyme-bound phenylacetylglycyl residue thus produced is in turn transferred to the nucleophile. Pathway B occurs with water, glycylglycine and other amino compounds. Both pathways A and B occur with glycerol, other ROH nucleophiles and neutral hydroxylamine. The nucleophilic attacks are enzyme-catalysed.

The exocellular DD-carboxypeptidase–transpeptidase (EC 3.4.2.12) of *Streptomyces* R61 (hereafter called the 'R61 enzyme') (E) forms with benzylpenicillin (I) a stoicheiometric complex EI* where the penicilloyl moiety of the antibiotic molecule is ester-linked to a serine residue of the enzyme (Frère *et al.*, 1976*a*). In 3mM-sodium phosphate, pH7.5, and at 37°C, complex EI* has a half-life of 80min (Frère *et al.*, 1975*b*). Under these conditions, breakdown of complex EI* results in the regeneration of a free active enzyme and the release of both PhAc-Gly (phenylacetylglycine) and an intermediate compound that in turn gives rise to *N*-formyl-D-penicillamine (Frère *et al.*, 1975*a*; Frère *et al.*, 1976*b*; Adriaens *et al.*, 1978; Frère *et al.*, 1978). The reaction involves a rate-limiting step of an unknown nature that is immediately followed by (1) cleavage of the $C_{(5)}$–$C_{(6)}$ bond with formation of a $-CH_2-$ (methylene) group at $C_{(6)}$ and (2) transfer of the PhAc-Gly moiety thus formed to water. In the present study, the effects of various nucleophiles on the breakdown of complex EI* have been investigated.

Materials and Methods

The R61 enzyme was 95% pure (Frère *et al.*, 1973*a*). [^{14}C]Benzylpenicillin (with the radioactive label on the carbonyl group of the PhAc side chain; 51Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Standard phenylacetylglycine (PhAc-Gly) was prepared as described by Frère *et al.* (1975*a*). [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala was that used previously (Perkins *et al.*, 1973). Standard phenylacetylglycylglycylglycine (PhAc-Gly-Gly-Gly) was synthesized by coupling between PhAc-Gly and the dipeptide Gly-Gly. A solution of PhAc-Gly (1g in 50ml of dichloromethane) was supplemented with 850mg of hydroxybenzotriazole and 1.5g of dicyclobenzylcarbodi-imide. The dicyclobenzylurea thus formed was eliminated by filtration, the supernatant fraction evaporated to dryness and the residue dissolved in 50ml of dry dimethylformamide. The solution was supplemented with 900mg of Gly-Gly and 0.97ml of triethylamine, stirred slowly for 15h at 22°C, evaporated to dryness and the residue dissolved in ethyl acetate. The organic phase, washed first with 0.1M-HCl and then with a saturated NaCl solution, was dried over solid $MgSO_4$, and evaporated to dryness. The product thus obtained was finally purified by chromatography on a column of Merckogel OR500 in methanol. The PhAc-Gly-Gly-Gly was homogeneous (i) by t.l.c. on Silica-gel G60 plates in both solvent A ($R_F = 0.71$) and solvent B ($R_F = 0$), (ii) by ascending chromatography on Whatman no. 1 paper in solvent A ($R_F = 0.60$), and (iii) by paper electrophoresis in collidine/acetic acid/water buffer, pH7.5, at 60V/cm, under which conditions it migrated at 18cm/h toward the anode.

Abbreviation used: PhAc, phenylacetyl.

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Solvent A was butan-1-ol/acetic acid/ethanol/water (10:3:3:4, by vol.) and solvent B was chloroform/methanol/acetic acid (88:10:2, by vol.). Detection of the peptide was performed by using the Cl_2/I_2 method.

Complex EI* can be made extemporaneously by incubating equimolar amounts of enzyme and [^{14}C]benzylpenicillin for 1min at 37°C. Indeed, formation of complex EI* (a two-step process:

$$E+I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_{+3}} EI^*$$

where K is a dissociation constant and k_{+3} a first-order rate constant) (Frère *et al.*, 1975*b*) is characterized by a high k_{+3}/K ratio of $13700\,M^{-1}\cdot s^{-1}$ (at 25°C), with the result that complex-formation is virtually complete under the above conditions. In most experiments, however, complex EI* was prepared and isolated as described by Frère *et al.* (1975*a*). Samples of the purified complex were freeze-dried and stored at −20°C until use (within 1–2 days). The operation must be carried out with great care, and in the frozen state, to avoid partial breakdown of complex EI*. Residual radioactive complex EI* and the various radioactive reaction products arising from nucleophilic attack of complex EI* (total radioactivity about 25000c.p.m. per expt.) were separated from each other by electrophoresis on strips of Whatman 3MM paper for 90min, in collidine/acetic acid/water (5:2.6:1000, by vol.) at pH6.5 and 60V/cm with a high-voltage Electrophorator (Gilson model DW) and refrigerated tanks. The radioactive spots were located on the strips with a Packard Radiochromatogram scanner, model 7201, and estimated with a Packard Tri-Carb liquid-scintillation spectrometer. Table 1 shows the relative mobility of the compounds studied.

Results

Release of a free active enzyme from the [^{14}C]-benzylpenicilloyl–enzyme complex EI* may proceed by nucleophilic attack on $C_{(7)}$ either before $C_{(5)}$–$C_{(6)}$ cleavage, i.e. on the non-fragmented complex EI* (pathway A), or after $C_{(5)}$–$C_{(6)}$ cleavage, i.e. on the PhAc-Gly–enzyme complex (pathway B) (Scheme 1). As demonstrated below, the pathway that is used depends on the nature of the nucleophilic reagent (HY) involved. With water, pathway B occurs exclusively and [^{14}C]PhAc-Gly is formed. The effects caused by nucleophiles other than water were necessarily examined in aqueous media and therefore the reactions studied were always of a competitive nature. It should also be noted that, since the benzylpenicillin used was ^{14}C-labelled in the PhAc side chain, the fate of the thiazolidine moiety of the antibiotic molecule (i.e. the reaction $\rightarrow Z \rightarrow$ *N*-formyl-D-penicillamine) was not investigated during the present studies.

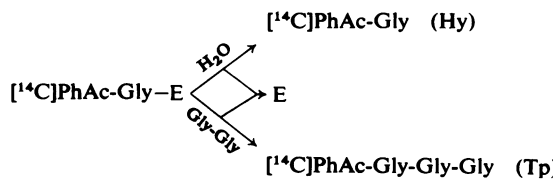
Table 1. *Electrophoretic mobility at pH6.5 of* [^{14}C]*PhAc-Gly and other* [^{14}C]*penicillin metabolites*

Electrophoresis was performed on Whatman 3MM paper with a high-voltage apparatus (60V/cm; 90min) in refrigerated tanks. The migration of PhAc-Gly was of 35cm/h toward the anode. All compounds are anionic except the PhAc-Gly esters of glycerol and ethylene glycol and PhAc-Gly hydroxamate, which are neutral (the small migration observed is due to electro-osmosis). The various benzylpenicilloyl esters of methanol, ethylene glycol and glycerol formed by nucleophilic attack of complex EI* had almost the same electrophoretic mobility, suggesting that both glycol and glycerol had one single benzylpenicilloyl substituent.

Compound		$R_{PhAc-Gly}$
PhAc-Gly-	L-Ala	0.78
	D-Ala	0.78
	Gly-Gly	0.69
	Gly-L-Ala	0.66
	Gly-D-Ala	0.64
	L-Ala-L-Ala	0.59
	racemic (DD+LL) diaminoadipic acid	0.58
PhAc-Gly esters of	ethylene glycol	−0.11
	glycerol	−0.11
Benzylpenicilloyl esters of	methanol	0.55–0.60
	ethylene glycol	0.55–0.60
	glycerol	0.55–0.60
PhAc-Gly-hydroxamate		−0.14
Benzylpenicilloylhydroxamate		0.58
Benzylpenicillin		0.60
Benzylpenicilloic acid		1.26

Use of nucleophilic reagents whose action occurs at the level of the PhAc-Gly–enzyme complex

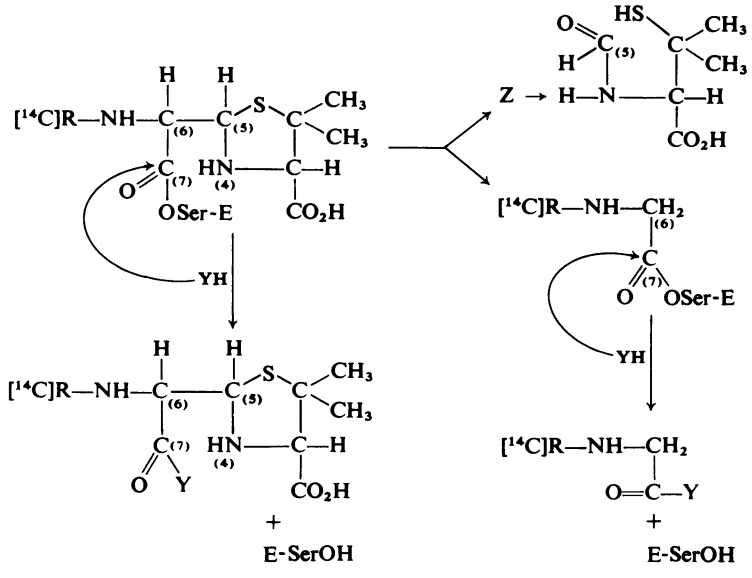
Like water, Gly-Gly and other amino compounds belong to this class of reagents. Within the limits of detection, the benzylpenicilloyl–enzyme complex EI* is exclusively channelled through pathway B and the PhAc-Gly–enzyme complex is partitioned as follows:



(where E = enzyme, Hy = hydrolysis and Tp = transpeptidation).

The transpeptidated product was isolated by paper electrophoresis and eluted from the paper strip. It was found to be identical with standard PhAc-Gly-Gly-Gly in all respects.

The buffers used were sodium cacodylate/HCl (pH5–6), sodium phosphate (pH6–8), Tris/HCl (pH8–9) and L-alanine/NaOH (pH9–10) at a final



Scheme 1. *Pathways A and B for the breakdown of the benzylpenicilloyl–R61 enzyme complex EI**
In the original complex, the benzylpenicilloyl complex is ester-linked to a serine residue of the enzyme (Ser-E). HY, a nucleophilic reagent.

I 0.005. In the absence of Gly-Gly, the rate of formation of PhAc-Gly remained constant from pH 5 up to 10, suggesting that H^+ or OH^- ions were not involved in the rate-limiting step of breakdown of complex EI*. That protons were not involved in this reaction had also been suggested by previous isotopic studies made in ^2H_2O (Frère *et al.*, 1978). In the presence of 2.5 mM-Gly-Gly, the rate of formation of PhAc-Gly-Gly-Gly from complex EI* (25 μM) increased as the pH of the reaction mixture increased from 5 to 7.5 and then remained constant at higher pH values, suggesting that the unprotonated species of Gly-Gly was the active nucleophilic reagent. For this reason, all the ensuing experiments were carried out at 37°C in 3 mM-sodium phosphate buffer, pH 7.5.

Neither the presence of a large amount of non-radioactive benzylpenicillin (250 μM), either alone or supplemented with 50 mM of the natural substrate analogue Ac_2-L-Lys-D-Ala-D-Ala, nor the presence of a large amount of benzylpenicilloic acid had any influence on the rates of release of PhAc-Gly and PhAc-Gly-Gly-Gly from complex EI* (25 μM) in 3 mM-sodium phosphate buffer, pH 7.5, containing 25 mM-Gly-Gly.

Breakdown of complex EI* (25 μM) in the absence and the presence of Gly-Gly showed that: (1) Increasing Gly-Gly concentrations up to 25 mM favoured the transpeptidation pathway and inhibited the hydrolysis pathway. Higher Gly-Gly concentrations (up to 100 mM) had no further effect (Fig. 1). Hence, hydro-

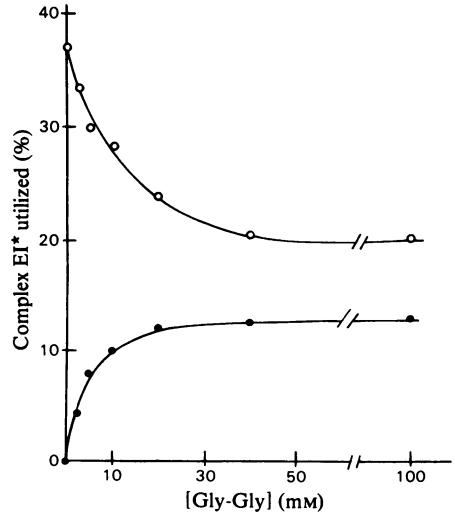


Fig. 1. *Breakdown of complex* [^{14}C]*EI** (25 μM) *in* 3 mM-*sodium phosphate, pH* 7.5, *for* 1 *h at* 37°C *in the absence and in the presence of increasing concentrations of Gly-Gly*
○, Hydrolysis (formation of PhAc-Gly); ●, transpeptidation (formation of PhAc-Gly-Gly-Gly).

lysis could not be completely inhibited under conditions where the enzyme acceptor site was saturated by Gly-Gly, an observation that suggested that Gly-Gly and water did not compete for the same enzymic site. (2) Progress curves for the release of PhAc-Gly and PhAc-Gly-Gly-Gly are shown in Fig. 2(*a*). Within the limits of experimental error, release of PhAc-Gly (Fig. 2*a*) and release of the sum of PhAc-Gly and PhAc-Gly-Gly-Gly appeared to be linear with time. This observed zero-order for up to 40% reaction is probably due to a lack of accuracy in the estimation of the products released. Finally, the velocity with which complex EI* was utilized was proportional to the initial EI* concentrations (assays made at 25 and 100 μM; not shown in Fig. 2). (3) At first sight the rates of breakdown of complex EI* were rather similar whether the reaction was exclusively channelled into hydrolysis (i.e. in the absence of Gly-Gly; half-life 80min) or was partitioned between hydrolysis and transpeptidation (i.e. in the presence of Gly-Gly). However, as shown in Fig. 2(*b*), Gly-Gly slightly increased the stability of complex EI*. This observation was supported by another independent experiment where two series of ten identical assays were submitted to a statistical analysis that showed that in 60min and in the absence of Gly-Gly 39.9% (S.D. 2.85) of complex EI* was converted into PhAc-Gly, whereas in the presence of 5mM-Gly-Gly 34.38% (S.D. 1.87) of complex EI* was utilized both for hydrolysis (29.34%) and for transpeptidation (5.04%).

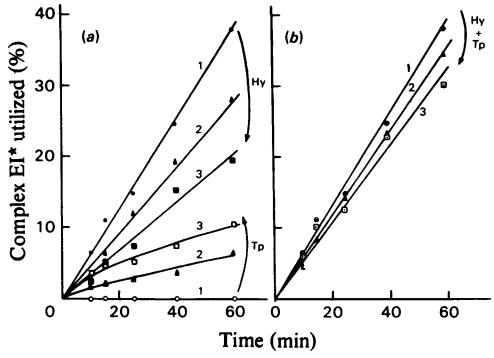


Fig. 2. *Time course of breakdown of complex* [^{14}*C*]*EI** (25 μM) *in* 3mM-*sodium phosphate, pH* 7.5, *and at* 37°*C, in the absence* (1) *and in the presence of* 5- *and* 25mM-*Gly-Gly* (2 *and* 3 *respectively*)

(*a*) Hy, hydrolysis; Tp, transpeptidation. (*b*) Hy+Tp, total breakdown of complex EI* due to both hydrolysis and transpeptidation. From the data of Fig. 2(*b*), it appears that Gly-Gly slightly decreased the rate of breakdown of complex EI*. Identical kinetics were observed by using an initial EI* concentration equal to 100 μM.

Transfer of PhAc-Gly to amino compounds other than Gly-Gly was also studied. The reaction products were identified on the basis of their electrophoretic mobilities. The specificity profile thus revealed is shown in Table 2.

Use of nucleophilic reagents whose action occurs both at the level of the benzylpenicilloyl–enzyme complex EI and at the level of the PhAc-Gly–enzyme complex*

Unlike water or the amino compounds listed in Table 2, glycerol in part attacks directly the benzylpenicilloyl–enzyme complex EI* and, for the other part, attacks the PhAc-Gly–enzyme complex produced by further fragmentation of complex EI*. The PhAc-Gly–enzyme complex is thus partitioned between water and glycerol. Consequently, the reaction products arising from both pathways A and B are the [^{14}C]benzylpenicilloyl ester of glycerol, [^{14}C]PhAc-Gly and the [^{14}C]PhAc-Gly ester of

Table 2. *Function of various amino nucleophiles in the transfer of* (*A*) *the* [^{14}*C*]*PhAc-Gly moiety from complex* [^{14}*C*]*EI** *and* (*B*) *the* [^{14}*C*]*Ac*$_2$-L-*Lys*-D-*Ala moiety from* [^{14}*C*]*Ac*$_2$-L-*Lys*-D-*Ala*-D-*Ala*

Complex EI* (25 μM) and the amino nucleophile HY (25mM) were incubated in 3mM-sodium phosphate, pH 7.5, for 1h at 37°C. Ac_2-L-Lys-D-Ala-D-Ala (1.7mM) and the amino nucleophile HY (1.7mM, except with racemic diaminoadipic acid, which was used at a 4.2mM concentration) were incubated in 17mM-sodium phosphate buffer, pH 8, for 1h at 37°C (Perkins *et al.*, 1973). (1), Formation of PhAc-Gly; (2), formation of PhAc-Gly-Y; (3), formation of Ac_2-L-Lys-D-Ala-Y (Perkins *et al.*, 1973).

	A			B
Amino nucleophile (HY)	Hydrolysis (1)	Transpeptidation (2)	Ratio (2)/(1)	Transpeptidation (3)
D-Alanine	3.4	32	9.4	7.5
Gly-L-Ala	10.2	34.6	3.4	25.5
Racemic (DD+LL) diaminoadipic acid	14.2	19.5	1.35	54.5
Gly-Gly	28.5	10.7	0.38	18.4
L-Alanine	31	3	0.10	0
L-Ala-L-Ala	38	3	<0.1	0.2
Gly-D-Ala	37	3	<0.1	3.5
None	40	0	0	0

the contrary, the release of benzylpenicilloyl ester of glycerol. The two esters were identified on the basis of their electrophoretic mobilities. Moreover, on subsequent treatment with 0.01 M-NaOH for 10 min at 37°C, the [^{14}C]benzylpenicilloyl ester of glycerol and the [^{14}C]PhAc-Gly ester of glycerol were quantitatively hydrolysed into compounds that had the same electrophoretic mobilities as [^{14}C]benzylpenicilloate and [^{14}C]PhAc-Gly respectively. Under the same conditions, the intact radioactive complex EI* gave rise to [^{14}C]benzylpenicilloate (without regeneration of the enzymic activity).

Breakdown of complex EI* (25 μM) for 1 h at 37°C in 3 mM-sodium phosphate, pH 7.5, in the absence and in the presence of glycerol (2–50%, v/v) showed that increasing concentrations of glycerol caused a progressive decrease of the amount of PhAc-Gly released and a progressive increase of the two free esters under consideration. The observed effects were almost maximal at 20% glycerol concentration (Fig. 3). Within the limits of experimental error, the decreased amounts of PhAc-Gly and the increased amounts of PhAc-Gly ester of glycerol released as a consequence of increasing concentrations of glycerol were commensurate with the result that the sum of both of them was virtually constant and equal to the amount of PhAc-Gly released in the absence of glycerol. Time-course experiments (Fig. 4) often showed an initial burst of breakdown of complex EI*, after which time the release of PhAc-Gly and PhAc-Gly ester of glycerol proceeded linearly. On glycerol rapidly ceased to be operational, and on prolonged incubations the amount of free ester very slightly decreased as a result of a slow, spontaneous hydrolysis. The amounts of benzylpenicilloate thus formed were hardly detectable, demonstrating that the observed stop in the formation of benzylpenicilloyl ester of glycerol was a real phenomenon and was not due to a rate of hydrolysis that would be roughly equal to the rate of formation. These observations suggest that the rate-limiting step of the fragmentation process may be preceded by a relatively fast reaction that is complete within 10–15 min and the product of which is an altered EI* complex that escapes attack by glycerol. Finally, depending on the experiments, the amounts of benzylpenicilloyl ester of glycerol released during the first 10–15 min of breakdown of complex EI* varied from 20 to 30% (in terms of complex EI* utilized). Both pathways A and B cause breakdown of complex EI*. Consequently, the enzyme was regenerated much more rapidly in the presence of glycerol (half-life of complex EI* about 40 min) than in its absence (half-life about 80 min).

Qualitatively, ethylene glycol (ethane-1,2-diol) and neutral 0.4 M-hydroxylamine behaved as glycerol, but, on the basis of the yields of PhAc-Gly and the corresponding esters or hydroxamates released, the effectiveness of these compounds as nucleophilic reagents was lower than that of glycerol (Table 3). Methanol had a pronounced denaturing effect on complex EI*, strongly increasing the stability of the linkage between the protein and the penicilloyl moiety.

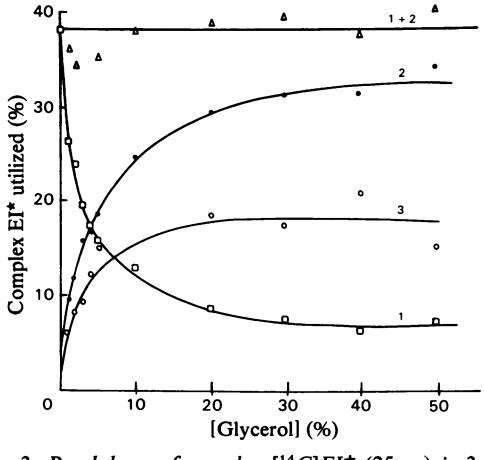


Fig. 3. *Breakdown of complex* [^{14}C]*EI** (25 μM) *in* 3 mM-*sodium phosphate, pH* 7.5, *for* 1 *h at* 37°C *in the absence and in the presence of increasing concentrations of glycerol*
The Figure shows the release of PhAc-Gly (curve 1), PhAc-Gly ester of glycerol (curve 2), benzylpenicilloyl ester of glycerol (curve 3) and the sum of PhAc-Gly and PhAc-Gly ester of glycerol (curve 1+2).

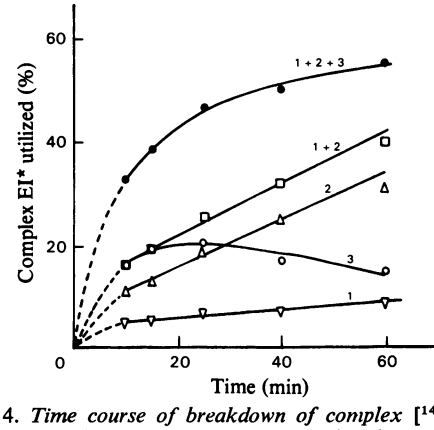


Fig. 4. *Time course of breakdown of complex* [^{14}C]*EI** (25 μM) *at* 37°C *and in* 3 mM-*sodium phosphate, pH* 7.5, *supplemented with* 20% (*v*/*v*) *glycerol*
The Figure shows the release of PhAc-Gly (curve 1), PhAc-Gly ester of glycerol (curve 2), benzylpenicilloyl ester of glycerol (curve 3), the sum of PhAc-Gly and PhAc-Gly ester of glycerol (curve 1+2) and the sum of the three penicillin metabolites (curve 1+2+3).

Table 3. *Function of various ROH nucleophiles and neutral* 0.4M*-hydroxylamine on breakdown of complex* [^{14}C]*EI**
All the reactions were carried out with 25 μM-complex EI* in 3 mM-sodium phosphate, pH 7.5, and at 37°C. 1, PhAc-Gly; 2, PhAc-Gly esters of ethylene glycol or glycerol; 3, benzylpenicilloyl esters of methanol, ethylene glycol or glycerol; 4, PhAc-Gly-hydroxamate; 5, benzylpenicilloylhydroxamate; Σ, sum of all penicillin metabolites released during the corresponding experiments. The penicillin metabolites obtained in the presence of methanol, ethylene glycol and neutral hydroxylamine were tentatively identified as the described esters or hydroxamates on the basis of their electrophoretic mobilities.

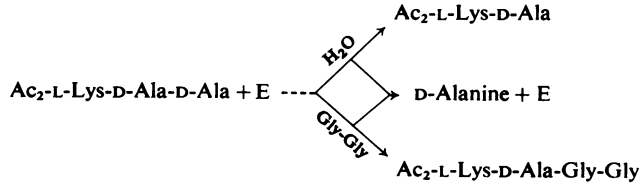
Nucleophile	Time of incubation (min)	Compound ...	Complex EI* utilized (%) for the formation of: 1	2	3	4	5	Σ
Water	30		24	0	0	0	0	24
Water	60		40	0	0	0	0	40
Methanol/water (2:3, v/v)	30		5	0	5	0	0	10
Ethylene glycol/water (2:3, v/v)	30		5	17	4	0	0	26
Glycerol/water (2:3, v/v)	30		6	20	14	0	0	40
Hydroxylamine (neutral; 0.4M)	60		8	0	0	14	18	40

Breakdown of denatured [^{14}C]*EI* complex*

Breakdown of complex EI* previously boiled for 3 min resulted in the release of only benzylpenicilloate whether the incubation of the denatured complex was carried out in 3 mM-sodium phosphate buffer alone or supplemented with Gly-Gly or with 20% glycerol. Moreover in water the denatured complex EI* had a half-life of several days, considerably higher than that of the native complex (80 min). Hence both fragmentation of the benzylpenicilloyl moiety and the nucleophilic attacks on $C_{(7)}$ either before or after fragmentation are enzyme-catalysed reactions, and, most probably, the slow release of benzylpenicilloate from the heated EI* complex is of a chemical nature.

Comparison between benzylpenicillin and the natural substrate analogue Ac_2-L-*Lys*-D-*Ala*-D-*Ala*

The R61 enzyme also catalyses nucleophilic attacks on the carbonyl carbon of the penultimate D-alanine residue of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala. In the presence of Gly-Gly, partitioning occurs as follows (Frère *et al.*, 1973*b*):



i.e. in a way that is comparable with that observed with the PhAc-Gly–enzyme complex. However, the two systems exhibited great quantitative differences, not only with respect to the specificity profiles for amino groups (Table 2), but also with respect to the effects observed with glycerol. Glycerol, which was an excellent nucleophilic reagent for both benzylpenicilloyl–enzyme and PhAc-Gly–enzyme complexes, was found to be a very poor acceptor for the enzyme-catalysed transfer of [^{14}C]Ac_2-L-Lys-D-Ala from [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (Table 4; Expts. 1 and 2). On the basis of competitive experiments where aqueous solutions of Gly-L-Ala supplemented or not with glycerol were used as nucleophilic reagents (Table 4; Expts. 3 and 4), glycerol had virtually no effect on the formation of Ac_2-L-Lys-D-Ala and Ac_2-L-Lys-D-Ala-Gly-L-Ala from Ac_2-L-Lys-D-Ala-D-Ala; moreover, Gly-L-Ala prevented the formation of the small amount of neutral compound that was detected in the absence of the dipeptide and tentatively identified as Ac_2-L-lysyl-D-alanyl ester of glycerol. Conversely, when similar experiments were carried out with complex EI* (and with Gly-Gly instead of Gly-L-Ala), the following observations were made (Table 5): (i) glycerol had a pronounced inhibitory effect on the formation of both PhAc-Gly and PhAc-Gly-Gly-Gly; (ii) these observed decreases were paralleled by the formation of an equivalent amount of PhAc-Gly ester of glycerol, with the result that the total extent of transfer of the PhAc-Gly moiety from the PhAc-Gly–enzyme complex (40% in terms of complex EI* utilized) was independent of the nucleophiles used (water, Gly-Gly, glycerol; either alone or combined); and (iii) the same proportion (25%) of the original benzylpenicilloyl–enzyme complex EI* underwent direct attack by glycerol whether Gly-Gly was present or not.

In previous studies, glycerol and ethylene glycol had already been used for the study of the enzymic properties of the R61 enzyme. Thus, for example, in a medium of low polarity such as water, ethylene glycol and glycerol (6:9:1.5, by vol.), the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala to Ac_2-L-Lys-D-Ala was found to be inhibited to a much higher extent than the transpeptidation reaction (formation of Ac_2-L-Lys-D-Ala-Gly-Gly). In the course of these studies, neutral compounds that might be the Ac_2-L-lysyl-D-alanyl esters of glycerol and ethylene glycol were not formed or were formed in such small amounts that they escaped attention. However, when

Ac-L-Lys-D-Ala-D-Ala
Gly ⌟

was utilized both as carbonyl donor and amino acceptor by the R61 enzyme in the same low-polarity medium (Zeiger *et al.*, 1975), small amounts of a compound (designated as compound VI) were formed transitorily. Its electrophoretic mobility was compatible with that of the N^{α}-acetyl-N^{ε}-glycyl-L-lysyl-D-alanyl esters of glycerol or ethylene glycol.

Discussion

Benzylpenicillin, once ester-linked to a serine residue of the R61 enzyme in the form of a benzylpenicilloyl derivative (the so-called EI* complex), may undergo a direct enzyme-catalysed nucleophilic attack on $C_{(7)}$, resulting in the transfer of the benzylpenicilloyl moiety and enzyme regeneration (pathway A). Alternatively, the benzylpenicilloyl moiety may be further transformed through $C_{(5)}$–$C_{(6)}$ cleavage and protonation of $C_{(6)}$ into an enzyme-linked PhAc-Gly residue that in turn may undergo an enzyme-catalysed nucleophilic attack on the original $C_{(7)}$, resulting in the transfer of PhAc-Gly and enzyme regeneration (pathway B). With water, Gly-Gly and other amino nucleophiles, pathway A does not occur, or occurs at such a small rate when compared with pathway B that the benzylpenicillin molecule is entirely fragmented. With glycerol, other ROH nucleophiles and neutral hydroxylamine, the benzylpenicilloyl moiety of complex EI* is partitioned between both pathways A and B. These various reactions are enzyme-catalysed. Heat-denatured complex EI*

Table 4. *Effects of glycerol and Gly-L-Ala on the transfer of* [^{14}C]Ac_2-L-*Lys*-D-*Ala from* [^{14}C]Ac_2-L-*Lys*-D-*Ala*-D-*Ala*
The R61 enzyme (0.5 μg) and [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (1.5 mM) were incubated in the absence or in the presence of Gly-L-Ala (25 mM), for 15 min at 37°C in 40 μl (final vol.) of 3 mM-sodium phosphate, pH 7.5, either as such or containing glycerol at the indicated concentrations.

Expt. no.	Nucleophilic mixture	[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala utilized (%) for the formation of: Ac_2-L-Lys-D-Ala	Ac_2-L-Lys-D-Ala-Gly-L-Ala	Neutral compound
1	Water	63	0	0
2	Glycerol/water (2:3, v/v)	65	0	5*
3	25 mM-Gly-L-Ala	10†	21†	0
4	Glycerol/water (1:5, v/v) containing 25 mM-Gly-L-Ala	8	18.5	0

* This small amount of ^{14}C-labelled neutral compound detected by paper electrophoresis might be the [^{14}C]Ac_2-L-lysyl-D-alanyl ester of glycerol.

† It is known that the presence of an appropriate amino compound such as Gly-L-Ala inhibits considerably the overall attack of the tripeptide donor Ac_2-L-Lys-D-Ala-D-Ala when the latter is used at non-saturating concentrations (Frère *et al.*, 1973*b*).

Table 5. *Competitive effects of glycerol and Gly-Gly on the breakdown of complex* [^{14}C]*EI**
All the reactions were carried out with 25 μM-complex EI* in 3 mM-sodium phosphate, pH 7.5, for 1 h at 37°C. Compounds: 1, PhAc-Gly; 2, PhAc-Gly-Gly-Gly; 3, PhAc-Gly ester of glycerol; 4, benzylpenicilloyl ester of glycerol.

	Nucleophilic mixture				Complex EI* utilized (%) for the formation of:					
Expt. no.	Gly-Gly (20 mM)	Glycerol (20%, v/v)	Water	Compound ...	1	2	3	4	Σ (1, 2, 3)	Σ (1, 2, 3, 4)
1	0	0	+		42	0	0	0	42	42
2	+	0	+		27	11	0	0	38	38
3	0	+	+		11	0	33	25	44	69
4	+	+	+		8	3	28	26	39	65

also breaks down, but an active enzyme is not regenerated, and benzylpenicilloate is very slowly released. Fixation of benzylpenicillin to the DD-carboxypeptidase of *Bacillus subtilis* also involves the formation of a penicilloyl ester bond, and the hydroxylaminolysis of the complex is thought to be enzymically catalysed (Kozarich *et al.*, 1977). Similarly, degradation of benzylpenicillin to PhAc-Gly by the *Streptococcus faecalis* DD-carboxypeptidase requires a native EI* complex (Coyette *et al.*, 1978).

The experiments reported here show that with the R61 enzyme and benzylpenicillin, the processing of the enzyme-bound and hydrolysed β-lactam depends on the nature of the nucleophilic reagent used. One may therefore postulate that the processing (either pathway A or B) of a β-lactam antibiotic is also governed by both the structure of the antibiotic itself and the enzyme that is used. DD-Carboxypeptidase–transpeptidases are known where the reaction product, arising from benzylpenicillin through breakdown of complex EI* in water, and under conditions of enzyme reactivation, is benzylpenicilloate (Marquet *et al.*, 1974; Tamura *et al.*, 1976; Schilf *et al.*, 1978). Hence, these enzymes behave as β-lactamases of low efficiency. Interestingly, the membrane-bound DD-transpeptidase of *Streptomyces* R61 is a β-lactamase of this type (Marquet *et al.*, 1974), but the same enzyme, once solubilized with the help of cetyltrimethylammonium bromide, catalyses the fragmentation of the benzylpenicillin molecule (Dusart *et al.*, 1977).

Finally, the question arises as to whether the nucleophilic attacks of (1) the carbonyl $C_{(7)}$ of the enzyme-bound benzylpenicilloyl moiety, (2) the same carbon of the enzyme-bound phenylacetylglycyl moiety, and (3) the carbonyl carbon of the penultimate D-alanine residue of the natural substrate analogue Ac_2-L-Lys-D-Ala-D-Ala, respectively, are catalysed by one or more than one enzyme site. The three reactions exhibit great differences with respect to their specificity profiles for amino and ROH nucleophiles, rather suggesting the specific involvement of distinct sites. Alternatively, however, one may also postulate that the same grouping of amino acid residues is involved in the three processes but that each of the carbonyl carbon donor substrates induces a different alignment of the catalytic groups, thus creating active sites with different conformations.

This work was supported in part by the National Institutes of Health, Washington (contract no. 1 RO1 AI 13364-02 MBC). A. Marquet is an EMBO Long-Term Fellow.

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