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Benzylpenicillin cleavage with polyelectrolytes

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ABSTRACT: Kinetic studies of the aminolysis of benzylpenicillin (BP) in poly(ethylenimine) (PEI) showed a complicated rate behaviour owing to the strong substrate–polyelectrolyte interaction. The results were interpreted by the formation of an unreactive and reactive complex which is converted into the poly(ethylenimine)penicilloylamide. In the presence of KCl the PEI behaves as a simple amine and the second-order rate constants (k_N) of the nucleophilic attack on β-lactam were calculated at various pH values. The Brønsted β value is consistent with a stepwise mechanism in which the rate-determining step is the $T^{\pm}$ intermediate decomposition in absence of general acid–base catalysis. The PEI reveals its catalytic ability not only by binding the substrate to the polymer, but also by increasing the reactivity of the reactive complex. A negligible effect on the alkaline hydrolysis of BP was found in the presence of poly(diallyldimethylammonium) chloride (PDDA). A parallel between PEI and human serum albumin (HSA) in the BP aminolysis is proposed. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: benzylpenicillin; aminolysis; acyltransfer; poly(ethylenimine); polyelectrolytes

INTRODUCTION

Much work on the mechanism of the hydrolysis[1] of β-lactams (catalysed by metal ions, cyclodextrins and micelles) and both experimental and theoretical[2,3] aminolysis[1] studies has been reported. However, to date the study of the polyelectrolyte effect on the rate of the β-lactam ring opening is lacking. Owing to the importance of β-lactams and the relevance of their aminolysis in understanding the allergies[4] sometimes induced by these drugs, we carried out a kinetic study with the aim of elucidating the mechanism involved in the acyl-transfer reaction in the presence of poly(ethylenimine) (PEI), a highly branched polyelectrolyte[5] containing about 25% primary, 50% secondary and 25% tertiary amino groups.

It seems that the major determinant in penicillin allergy is the penicilloyl group bound to the amino group of the L-lysine residue present in the carrier protein.[4] Recent studies identified peptides containing benzylpenicilloyl moieties in different binding regions of human serum albumin (HSA) involving several L-lysine residues.[6–8] PEI can be considered as a mimic of the carrier protein containing the L-lysine residue. In fact, the branched polymeric structure contains multiple primary amino groups which can simulate the L-lysine site binding residue of HSA (at least six different lysine residues out of a total of 59 can be penicilloylated[7]).

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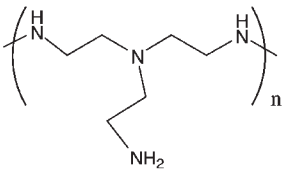
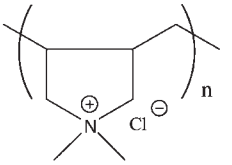
Moreover, in order to clarify the polyelectrolyte influence in the aminolysis reaction of BP, we also investigated the effect of poly(diallyldimethylammonium) chloride (PDDA), a polycation not containing protonated or free amino groups, on the hydrolysis of BP.



PEI



PDDA

RESULTS AND DISCUSSION

Aminolysis of benzylpenicillin in PEI

The aminolysis of BP, performed at 30 °C, was followed at various pH values in the range 5.20–9.40 and the pseudo-first-order rate constants (k_{obs}) were plotted vs PEI concentration (Fig. 1). The plots showed that at pH < 7.80 k_{obs} initially increases, reaches a maximum and then decreases, indicating an apparent polyelectrolyte inhibition. A simple mechanism explaining this behaviour involves the formation of a 'reactive complex', [BP–PEI]*, between the substrate and polyelectrolyte, which evolves to the poly(ethylenimmine)penicilloylamide (**P**), in addition to an 'unreactive complex', [BP–PEI], as shown in Scheme 1. Only the reactive complex [BP–PEI]* is able to decompose to the products or it can

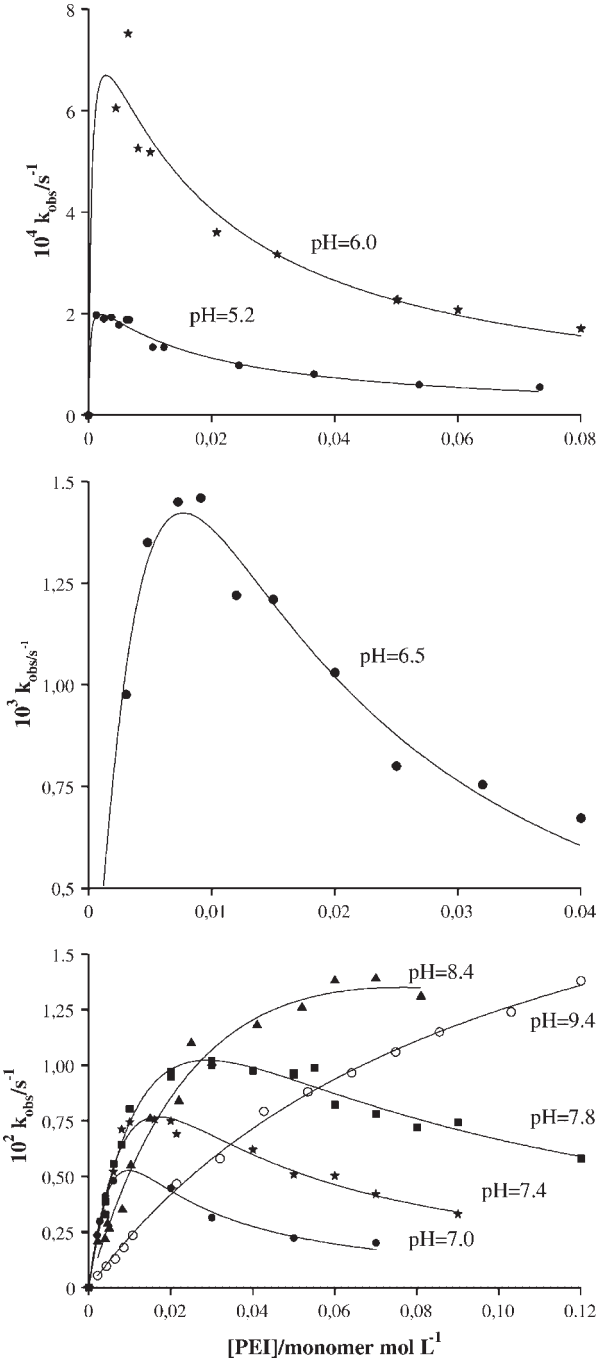
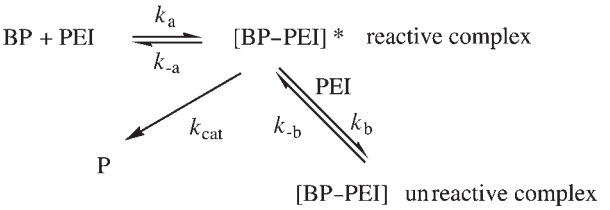


Figure 1. Dependence of pseudo-first-order rate constants (k_{obs}) on PEI concentration for the aminolysis of BP at various pH values at 30 °C. The points are experimental and the solid curves are calculated with parameters reported in Table 1 obtained by means of Eqn (1)



Scheme 1

associate with another PEI macroion to give the unreactive complex [BP–PEI].

The results can be qualitatively interpreted in terms of multiplicity of catalytic sites. When the polyelectrolyte is largely protonated ($5.20 < pH < 7.80$), on increasing the PEI concentration the rate constant increases because the positive charges on the macroion, which attract the BP in anionic form ($pK_{COOH} = 2.73$ at 25 °C),[9] increase and the maximum rate is then attained. Subsequently, the rate decreases because the number of protonated sites and vicinal doublet and triplet charges considerably increase,[5a,b] increasing the probability that the substrate binds itself at sites far away from the nucleophilic amino groups responsible for the aminolysis reaction. At $pH > 7.80$, the plots show a hyperbolic trend because on increasing the PEI concentration the anionic substrate is progressively less attracted to the polymer surface (owing to the decreased electrostatic potential) and a large number of free amino groups with increasing nucleophilicity are present.

Assuming the treatment previously reported for the aminolysis of some phenylacetates,[10] the kinetic behaviour can be explained by the following equation:

$$k_{obs} = k_{cat}K_1[PEI]/(1 + K_1[PEI] + K_1K_2[PEI]^2) \quad (1)$$

where $K_1 = k_a/k_{-a}$ is the binding constant for the formation of [BP–PEI]* reactive complex, k_{cat} is the first-order rate constant of its decomposition to the reaction product (**P**) and $K_2 = k_b/k_{-b}$ is the inhibition constant (Scheme 1).

The good fitting of experimental values to the Eqn (1) is consistent with the proposed mechanism in Scheme 1. The values of K_1, K_2 and k_{cat} (Table 1) were calculated by a non-linear curve fitting of k_{obs} vs [PEI] by using the FigP2.7 program (Biosoft). Since K_1 and K_2 increase when the pH decreases, the maximum of the curves is shifted to a lower PEI concentration, according to the equation $[PEI]_{max} = 1/\sqrt{(K_1K_2)}$.[10]

A diagnostic tool to investigate the reaction mechanism is the Brønsted-type relationship, which correlates the rate constant with the basicity of the nucleophile and is a measure of the reaction sensitivity to the base strength.[11] Unlike simple amines, the apparent pK_N of PEI changes with the degree of ionization of the macroion, owing to the strong interactions between charged and uncharged vicinal amino groups in the chain,[5a,b,12] and it also depends on both the PEI concentration and the ionic strength of the solution.[12] A Brønsted-type relationship can be obtained by using the rate constants k_{cat} (see Table 1), which reflect the reactivity of the [BP–PEI]* complex. The dependence of the complex reactivity on the PEI basicity can be expressed by the $k'_{cat} = k_{cat}/(1 - \alpha)$ where $1 - \alpha$ is the fraction of free amino groups

Table 1. Kinetic data for the aminolysis of benzylpenicillin in the presence of PEI at 30 °C

[PEI][a] monomer (mol l^{-1})	pH	α	pK_N[b]	K_1 (M^{-1})[c]	k_{cat} (s^{-1})[c]	K_2 (M^{-1})[c]	Runs
(1.22–73.3) $\times 10^{-3}$	5.20	0.78	5.74	2100 ± 410	(2.63 ± 0.11) $\times 10^{-4}$	75 ± 9	26
(4.4–80) $\times 10^{-3}$	6.00	0.66	6.30	950 ± 240	(8.90 ± 0.2) $\times 10^{-4}$	60 ± 3	26
(0.30–40) $\times 10^{-3}$	6.50	0.62	6.71	67 ± 5	(6.84 ± 0.24) $\times 10^{-3}$	240 ± 16	24
(2.04–70) $\times 10^{-3}$	7.00	0.48	7.04	96 ± 3	(1.6 ± 0.1) $\times 10^{-2}$	111 ± 5	20
(4–90) $\times 10^{-3}$	7.40	0.44	7.29	46 ± 23	(2.75 ± 0.1) $\times 10^{-2}$	76 ± 37	28
(4–120) $\times 10^{-3}$	7.80	0.33	7.49	44 ± 9	(2.63 ± 0.35) $\times 10^{-2}$	27 ± 5	34
(2.2–81) $\times 10^{-3}$	8.40	0.20	7.80	21 ± 7	(2.88 ± 0.75) $\times 10^{-2}$	7 ± 5	30
(2.12–120) $\times 10^{-3}$	9.40	0.04	8.02	10.2 ± 0.7	(2.47 ± 0.01) $\times 10^{-2}$	—	30

[a] Total polyamine concentration.
[b] ± 0.05, mean value in the concentration range, calculated from pK_N = pH + logα/(1 − α), where α is the degree of ionization (see text).
[c] Values calculated from Eqn (1) and standard errors are reported.

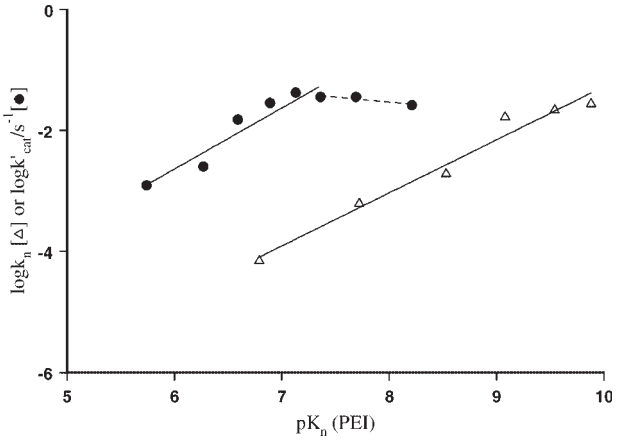


Figure 2. Brønsted-type plots for the aminolysis of BP with PEI in aqueous solution in absence of KCl (●) and in the presence of 1 M KCl (Δ)

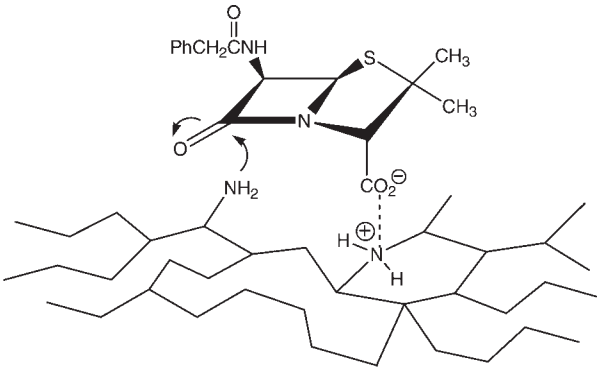


Figure 3. Nucleophilic attack of a primary amino group of PEI on the *re* face of β-lactam carbonyl

responsible for the aminolysis reaction.[10,13] The Brønsted-type relationship, not statistically corrected,[10] is linear with a slope $\beta_N = 0.96 \pm 0.12$ (Fig. 2):

$$\log k'_{cat} = (0.96 \pm 0.12)pK_N - (8.40 \pm 0.8) \qquad (2)$$

The k'_{cat} deviation observed at pK_N = 7.80 and 8.02 (not computed for the Brønsted plot) is probably due to the different nature of the electrostatic interaction between the anionic substrate and the poorly charged PEI (in fact α = 0.2 and 0.04, respectively). The break of the linearity is not ascribable to a change in the mechanism or rate-determining step[11a,b,14] because in the presence of 1 M KCl (see later) the Brønsted plot is linear up to pK_N = 9.88.

The large dependence of the rate constant on the PEI basicity ($\beta = 0.96 \pm 0.12$) indicates that the reactive complex could proceed stepwise with the formation of a zwitterionic tetrahedral intermediate by nucleophilic attack of PEI primary or secondary amino groups on the β-lactam carbonyl (Fig. 3). However, from the experimental data it is not easy to understand the mechanism involved because many catalytic sites are present on the PEI and other pathway cannot be excluded. In fact, a Brønsted β value of ca 1 has been observed in the aminolysis of BP with simple primary amines, a stepwise process which occurs by nucleophilic and general base catalysis.[15a,b] Evidence for intramolecular general acid catalysis with 1,2-diaminoethane monocation has also been reported.[15a,b]

Aminolysis of benzylpenicillin by PEI in the presence of KCl

In order to understand the kinetically complex aminolysis of BP in PEI, we also performed measurements in the presence of KCl. This is because in the presence of strong electrolytes the substrate–polyelectrolyte electrostatic interactions, responsible for complex kinetic behaviour, are strongly reduced.[16,17] The added salt partially shields the charges on the chain and chloride ions will compete with anionic substrate for the position close to the polyelectrolyte. Under these conditions the PEI behaves as a simple amine. In fact, kinetic studies carried out at 30 °C in the pH range 6.13–11.20 in the presence of PEI and 1 M KCl showed a linear dependence of the rate constant vs [PEI] (Fig. 4, Table 2) which can be expressed by the following equation:[17]

$$k_{obs} = k_0 + (k_N + k'_{OH}[OH])[PEI](1 - \alpha) \qquad (3)$$

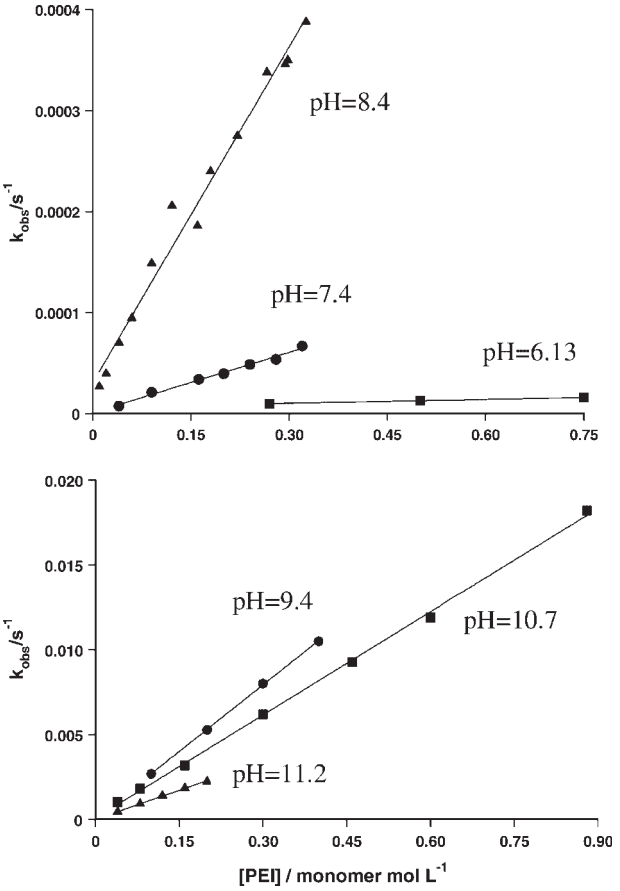


Figure 4. Dependence of pseudo-first-order rate constants (k_{obs}) on PEI concentration for the aminolysis of BP at various pH values at 30 °C in the presence of 1 M KCl

where k_0 is the spontaneous hydrolysis rate constant, k_N is the nucleophilic uncatalyzed rate constant, k'_{OH} is the hydroxy ion-catalysed aminolysis rate constant, [PEI] is the polyelectrolyte concentration expressed in monomer mol l^{-1} and α is the PEI ionization degree at a fixed pH. The term k'_{OH}[OH], which in this case cannot be measured because the basicity of PEI changes on changing the pH, can be neglected on the basis of the following estimation. Actually, from the k'_{OH} value of taurine[15a] ($1.41\ s^{-1}\ mol^{-2}\ l^{-2}$), which at pH 9.40 shows the same basicity of PEI, one can calculate a contribution of hydroxy ion-catalysed nucleophilic attack lower than 0.5% in comparison with k_N.

In order to ascertain the presence of a second-order term in amine, we increased the PEI concentration up to 0.86 monomer mol l^{-1} at pH 11.2, where 95% of the free PEI amino groups are present. However, in spite of this, intermolecular general base and/or general acid catalysis was not observed, the converse of that found in the aminolysis of BP with simple primary and secondary amines, their contribution to the catalysis being relevant.[15a]

By plotting $\log k_N$ vs pK_N of PEI, the following linear Brønsted-type relationship is obtained:

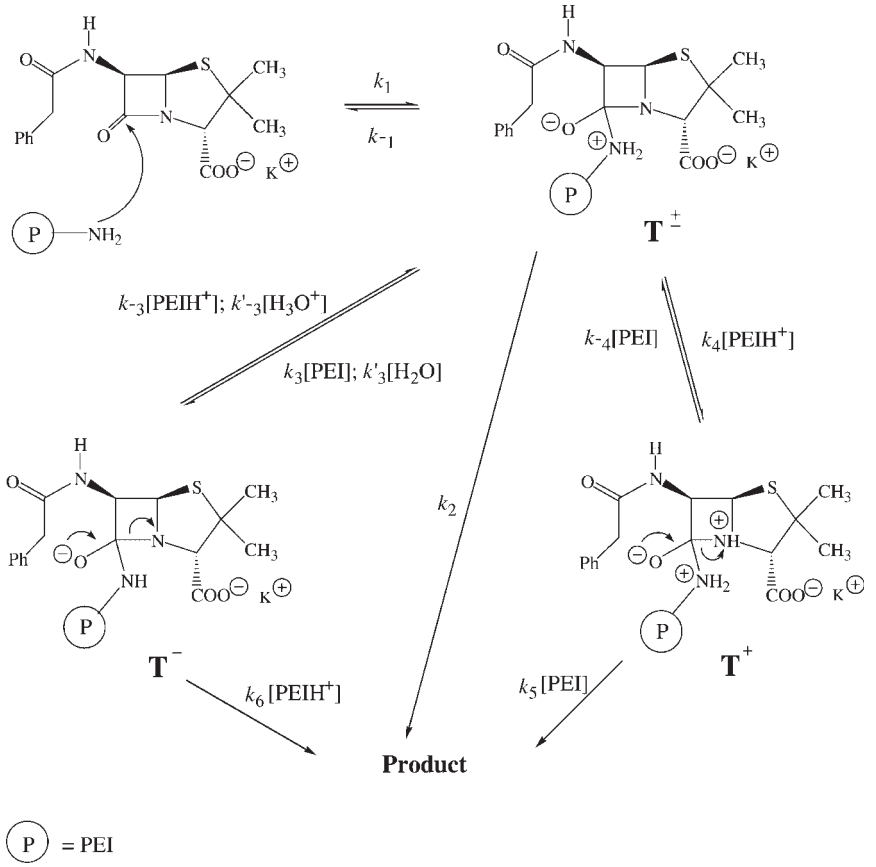
$$\log k_N = (0.88 \pm 0.06)pK_N - (10.0 \pm 0.05) \quad (4)$$

At this point, the accelerating effect due to the electrostatic nature of the phenomenon can be estimated by means of the ratio $K_1k'_{cat}/k_N$, which is 8.7×10^3 at pH 7.4 and about 2.2×10^5 at pH 5.2, k_N being calculated from Eqn (4). The catalytic efficiency of PEI in the [BP–PEI]* complex, which is reflected in k_{cat}, can be estimated from the ratio k'_{cat}/k_N, which formally represents an effective molarity (EM).[18] From the k_{cat} values (Table 1) and those of k_N, calculated from Eqn (4), we obtain EM = 107 M at pH 5.20, 189 M at pH 7.40 and 22 M at pH 9.40. These values are too large to be ascribed only to an effective concentration because the reactants occupy definite exclusion volumes.[19] This result, then, suggests that the nature of polymer domain modifies the reactivity in the [BP–PEI] complex.

Table 2. Kinetic parameters for the aminolysis of benzylpenicillin in the presence of PEI and 1 M KCl or PDDA at 30 °C

[PEI][a] monomer (mol l^{-1})	[PDDA][a] monomer (mol l^{-1})	pH	α	pK_N[b]	$10^4 k_{obs}(s^{-1})$	$10^4 k_N(s^{-1}\ M^{-1})$[c]	Runs
0.27–0.75		6.13	0.82	6.79	0.0984–0.159	0.7 ± 0.005	10
0.04–0.32		7.40	0.68	7.72	0.075–0.564	6.21 ± 0.04	12
0.01–0.326		8.40	0.54	8.47	0.268–3.88	19.1 ± 0.9	20
0.04–0.20		9.40	0.32	9.15	4.27–22.4	167 ± 4	10
0.04–0.88		10.70	0.06	9.54	10.1–182	276 ± 6	14
0.1–0.4		11.20	0.05	9.88	10.5–26.8	218 ± 7	8
	0.06[d]	1.01			89.2		4
	0.06[d]	1.36			36.4		4
	0.06[d]	1.50			30.5		4
	0.06[d]	1.90			14.3		4
	0.005–0.08[e]	11.04			2.56–4.55		10

[a] Total polyelectrolyte concentration.
[b] ± 0.05, mean value in the concentration range, calculated from $pK_N = pH + \log\alpha/(1-\alpha)$, where α is the degree of ionization (see text).
[c] Values calculated from Eqn (3) and standard errors are reported.
[d] In 0.1–0.02 M HCl buffer carrier.
[e] In 0.01 M Na_2CO_3–$NaHCO_3$ buffer carrier.



Scheme 2. Mechanism of β-lactam ring cleavage by a primary amine group of PEI

The β value (Fig. 2) compares well with 0.96 ± 0.12 found in the absence of KCl and with 1 ± 0.08 found for uncatalysed aminolysis of BP with simple amines.[15a] This means that the sensitivity of the bimolecular process towards the PEI basicity is similar to that of the [BP–PEI]* complex and it is independent of the nature of PEI catalysing primary or secondary amino groups.

According to both the rate law [Eqn (3)] and the Brønsted β value, the most probable mechanism is the k_2 pathway reported in Scheme 2. The good linearity of the plot, indicating a constant structure of the transition state,[14a,b,20] in spite of the presence of different catalytic groups, would exclude the possibility that general acid and/or base catalysis can operate over all the pH range investigated. The slope of the Brønsted plot is consistent with the development of a unitary charge in the attacking amino group of PEI and a significant amount of the C═O bond cleavage in the transition state, even if a concerted mechanism cannot be excluded. This is consistent with a stepwise mechanism where a zwitterionic tetrahedral intermediate $T^{\pm}$ is formed in the reaction pathway and its breakdown to the product is the rate-determining step (Scheme 2). A rate-determining step for the formation of the tetrahedral intermediate should give lower β values, namely in the range 0–0.3, as found for the aminolysis of esters with simple amines.[14,21] Assuming the steady-state treatment for the hypothetical tetrahedral intermediate $T^{\pm}$ formation, $k_N = k_2 K_e$, with $k_{-1} \gg k_2$, where $K_e = k_1/k_{-1}$. In acyl-transfer reactions the expulsion of a nucleofuge from a zwitterionic tetrahedral intermediate via k_2 is not significantly dependent on the basicity or the nature of the amine mojety.[14a,b,21] In addition, the pK_N of the leaving group is not affected by the presence of polyelectrolyte: in fact, the value of the ionization constant of butylpenicilloylamide ($pK_N = 4.3$), taken as a model of PEI-penicilloylamide, is almost coincident both in oxyanions and in the presence of PEI (see Experimental). Hence the higher reactivity of PEI in comparison with the isobasic primary amines[15a] is due to larger values of K_e. Unfortunately, to date, it is not possible to ascertain if the larger K_e value is attributable to a better nucleophilic attack of PEI on the β-lactam (k_1) or to the worse nucleofugality of PEI (k_{-1}) from the $T^{\pm}$ intermediate. However, the latter hypothesis is supported by the results already obtained for ester aminolysis in the presence of PEI.[17]

These findings do not exclude other possible processes: in fact, the deprotonation of $T^{\pm}$ could also occur by general base catalysis of PEI itself (k_3), or by water (k'_3), to yield the anionic intermediate T^{-}, which quickly decomposes to the product (path k_6). Also the k_4 path, where $T^{\pm}$ is protonated to give T^{+} according to general acid catalysis exerted by [PEI·H^{+}], cannot be ignored. The importance of various pathways can be evaluated

from the estimation of the theoretical acidity constant of the $T^{\pm}$ intermediate and comparison of the microscopic rate constants k_3 and k_{-3}, k_4 and k_{-4} with the uncatalyzed pathway k_2. On these bases, the acidity constant of the tetrahedral intermediate $T^{\pm}$ is $pK_N(T^{\pm}) = pK_N(PEI) - 0.8$ (see Appendix). Then, at each pH, the proton transfer from $T^{\pm}$ to PEI, to give T^-, is thermodynamically favoured and the value of the microscopic rate constant $k_3 = 2 \times 10^9\ s^{-1}\ M^{-1}$ can be used.[22]

Starting with k_3, at the highest pH value investigated (11.20), one calculates $k_3[PEI](1-\alpha) = 2.10^9 \times 0.4 \times 0.95 = 7.6 \times 10^8\ s^{-1}$, [PEI] being 0.4 monomer $mol\,l^{-1}$ and $\alpha = 0.05$. Analogously, at the lowest pH value (6.13), [PEI] being 0.75 monomer $mol\,l^{-1}$ and $\alpha = 0.82$, $k_3[PEI](1-\alpha) = 2.10^9 \times 0.75 \times 0.18 = 2.7 \times 10^8\ s^{-1}$.

The proton transfer from $T^{\pm}$ to water is never favoured because $pK_N(T^{\pm}) = 6.79 - 0.8 = 6$ and $pK(H_3O^+) = -1.75$. Therefore, the backward path k_{-3} is favoured and we can assume that under the best conditions at pH 6.13, $\log k'_3 = \log k'_{-3} - pK_N(T^{\pm}) + pK(H_3O^+) = 9.3 - 6 - 1.75 = 1.55$, $k'_3 = 33.5\ s^{-1}\ M^{-1}$ and then $k'_3[H_2O] = 33.5 \times 55.5 = 1.9 \times 10^3\ s^{-1}$. For the spontaneous decomposition of $T^{\pm}$ it is reasonable to assume a value of $k_2 = 1.5 \times 10^6\ s^{-1}$ found for primary amines.[15a] Thus, while the estimated values for general base catalysis are 5×10^2- and 1.8×10^2-fold higher than k_2, the water reaction described by k'_3 is not significant.

The negative charge on the oxygen and the uncharged amine moiety on T^- would make the nucleofuge expulsion from T^- (path k_6) faster than that from $T^{\pm}$ (path k_2).

The general acid catalysis (k_4) also deserves consideration. If the proton transfer occurs stepwise, the back-reaction k_{-4} is favoured with respect to k_4 because at pH 11.20 $pK_N(PEI) = 9.88$ and the β-lactam nitrogen $pK^*_N(T^{\pm}) = 1.5$ (see Appendix). Taking $k_{-4} = 2.0 \times 10^9\ s^{-1}\ M^{-1}$, one can calculate $\log k_4 = \log k_{-4} - \Delta pK = 9.3 - (9.88 - 1.5) = 0.92$, i.e. $k_4 = 8.3\ s^{-1}\ M^{-1}$. Since at pH 11.20 [PEI] = 0.4 monomer $mol\,l^{-1}$ and $\alpha = 0.05$, one calculates $k_4[PEI]\alpha = 8.3 \times 0.4 \times 0.05 = 0.166\ s^{-1}$. Analogously, at pH 6.13, since $pK_N(PEI) = 6.79$ and $pK^*_N(T^{\pm}) = -0.4$, one can calculate $\log k_4 = 9.3 - (6.79 + 0.4) = 2.11$, i.e. $k_4 = 1.3 \times 10^2\ s^{-1}\ M^{-1}$. Therefore, [PEI] being 0.75 and $\alpha = 0.82$, $k_4[PEI]\alpha = 1.3 \times 10^2 \times 0.75 \times 0.82 = 80\ s^{-1}$.

Therefore, the intramolecular proton transfer from $T^{\pm}$ to the β-lactam nitrogen is unimportant in comparison with k_2. The k_5 value can be expected to be larger than k_{-4} because the C—N bond breaking is favoured by the positive charge on the β-lactam nitrogen in T^+ and by the energy gain due to the opening of the strained four-membered ring structure giving the protonated benzylpenicilloylamide followed by a fast diffusion-controlled deprotonation.

Hence the microscopic rate constants calculated above suggest the presence of intermolecular general base catalysis not supported by kinetic experimental data. We believe that most probably the absence of these terms in the polymeric system can be ascribed to a slower rate of proton transfer in comparison with simple amines due to the repulsion effects of the large number of positive charges on the polymer surface and hydrogen bonding.[22,23] In addition, the proton transfer from $T^{\pm}$ to PEI could be sterically inhibited because $T^{\pm}$ may be buried inside the network polymeric matrix. The intramolecular proton transfer on the charged polymer surface requires the motion of the proton along the hydrogen bonds (found by x-ray studies[24,25] in the crystal lattices of linear PEI) and the reaction, which occurs in a medium less polar than water, is not favoured because it involves considerable solvent reorganization.[23]

Hydrolysis of benzylpenicillin in the presence of PDDA

BP reacts with aqueous solution of PDDA to give benzylpenicilloic acid in high yield.[1] As shown in Table 2, in acidic solutions the pseudo-first-order rate constants are not modified by the presence of 0.06 monomer $mol\,l^{-1}$ of PDDA. At pH 11.04, the apparent first-order rate constant for the alkaline hydrolysis does not depend linearly on [PDDA] and saturation behaviour was observed (Fig. 5).

According to Eqn (2) in Ref. 26, the values $K_1 = (37 \pm 10)\ M^{-1}$ and $k_{cat} = (3.43 \pm 0.3) \times 10^{-4}\ s^{-1}$ were obtained. The substrate–polyelectrolyte binding constant (K_1) causes an increase of only 60% in the alkaline hydrolysis (k_{cat}) with respect to the spontaneous hydrolysis (k_0) of BP. This means that although the reagents are attracted to the polymer surface by electrostatic interactions, the bimolecular alkaline hydrolysis rate is not appreciably modified because, unlike PEI, PDDA does not allow the stabilization of a transition state.

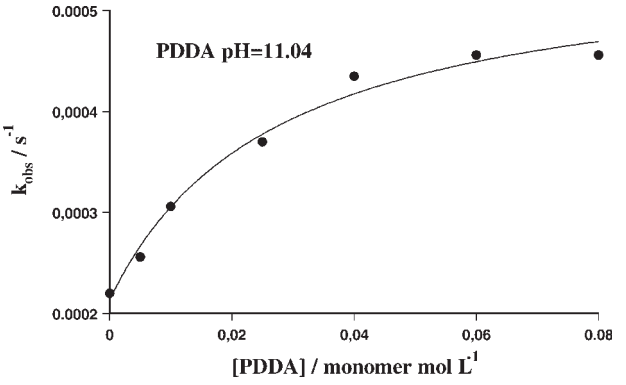


Figure 5. Dependence of pseudo-first-order rate constant (k_{obs}) on PDDA concentration for the alkaline hydrolysis of the BP at 30 °C. The points are experimental and the solid curve is calculated with the parameters reported in Table 2 (see text)

CONCLUSION

The aminolysis of BP in the presence of PEI occurs with a large rate enhancement and proceeds with a stepwise mechanism in which the rate-determining step is the $T^{\pm}$ intermediate decomposition. The polyelectrolyte reveals its catalytic ability not only by binding the substrate to the polymer (K_1), but also by increasing the reactivity of the [BP–PEI] complex (k'_{cat}). Most probably, the substrate, attracted to the polyelectrolyte, goes in a lesser polar environment where the $T^{\pm}$ intermediate is stabilized through electrostatic interactions and/or hydrogen bonds which prevent the rapid reversion to the starting material. In the presence of KCl, the chloride anion, with high charge density, not only shields the positive charges on the macroion but also causes strong electrostatic ordering of water molecules near the polyelectrolyte.[27] As a consequence, the hydrogen bonds are broken and the stabilizing interactions are reduced. The conclusion is in agreement with the electrostatic stabilization of ionic transition state hypothesized by Warshel *et al.*[28] for enzymatic catalysis.

Finally, it is interesting to observe that the analogy between the aminolysis of BP with PEI and with human serum albumin (HSA) suggests that a similar mechanism may operate. In fact, the HSA penicilloylation, at least *in vitro*, does not proceed through an intermediate BP–protein complex, analogous to that which occurs in PEI in the presence of KCl. In addition, the rate constants were found to be first order with respect to both BP and PEI and they are comparable to those found with HSA although at different ionic strengths. In fact, the second-order rate constants for the penicilloylation of HSA at 37 °C and at pH 7.35 and 9.65 ($k = 3 \times 10^{-3}$ and $6.8 \times 10^{-2}\,s^{-1}\,M^{-1}$, respectively)[29] are just about four times higher than the values found in PEI at pH 7.40 and 9.40 at 30 °C.

There are indications by x-ray structure[30] and NMR studies[31] that the principal binding pockets are located in the II and III domains of HSA where penicillins interact mainly by means of electrostatic and hydrophobic interactions. Moreover it has been ascertained that L-Lys-199 is principally responsible for the nucleophilic attack of BP, even though penicilloyl group-containing peptides involving other L-lysine residues in different regions of HSA were found.[7,8] MD simulations have also shown that the most likely configuration for the pairs L-Lys-195/L-Lys-199, situated at the beginning of the binding site of the protein, corresponds to a neutral ε-amino group of L-Lys-199 and protonated form of L-Lys-195.[3b] Most probably, the anionic BP is firstly electrostatically attracted by the positively protonated residue L-Lys-195[3b] and then it undergoes nucleophilic attack by the near free NH_2 group of L-Lys-199,[3b] giving a tetrahedral zwitterionic intermediate $T^{\pm}$ which evolves to the HSA–penicilloyl product.

EXPERIMENTAL

Materials

The potassium salt of BP was purchased from Fluka, PEI (47.6% by weight 'Polymin P', monomer weight 59) from BDH and PDDA (15% aqueous solution, monomer weight 197) from Polyscience. Other products (from Aldrich or Merck) were of analytical grade. Buffer solutions of PEI and PDDA were prepared as reported previously.[10,17,26] Benzylpenicilloic acid was obtained as reported previously.[32]

pK_N determination

The pK_N of PEI in the presence and absence of KCl was determined potentiometrically by using a microburette syringe apparatus and a Knick pH-meter equipped with an Ingold U402 combined glass electrode standardized according to Ref. 33. The values reported in Tables 1 and 2 were determined according to the equation $pK_N = pH - \log[(1-\alpha)/\alpha]$, where α is the degree of ionization calculated by the equation $\alpha = ([H^+]_{add} - [H^+]_{free} + [OH^-]_{free})/[PEI]_{tot}$.[12] The p$K_N$ of butylbenzylpenicilloylamide (synthesized according to Ref. 32) was determined by potentiometric titration of a 5×10^{-3} M solution [in 13% (w/w) aqueous methanol] with 0.1 M NaOH under nitrogen because the spectrophotometric determination was unsuccessful (the molar extinction coefficients of mono-, di- and non-protonated species being very similar). The apparent pK_N value, measured both in the presence of PDDA (0.1 monomer mol l^{-1}) and in the absence of polyelectrolyte, was 4.3 ± 0.05.

Kinetics

Kinetic measurements, performed at 30 °C by adding 20–40 µl of a stock solution of BP (3×10^{-2}–5×10^{-3} M) to 3 ml of PEI buffer solution in a thermostated cell, were carried out spectrophotometrically on a Perkin-Elmer Lambda 6 spectrophotometer at 232 or 240 nm. The pH of the solutions at the end of the reaction showed a maximum decrease of 0.05–0.1. The rate constants are mean values of two or three runs distributed over a 3% range. The pseudo-first-order rate constants (k_{obs}) were calculated on the basis of the equation $OD_t = OD_0 + (OD_\infty - OD_0)[1 - \exp(-kt)]$ by using a non-linear least-squares routine (FigP2.7 program supplied by Biosoft). The OD_∞ value was determined after at least 10 half-lives.

Reaction product analysis

The product of the aminolysis of BP with PEI, i.e. benzylpenicilloyl poly(ethylenimine), was ascertained

through two penamaldate assays[32] performed at pH 6.35 and 11.35. To a solution of PEI (0.557 g, 4.5 mmol) in 5 ml of water, acidified with 6.5 M HCl until pH 6.35, the potassium salt of BP (0.2 g, 0.54 mmol) dissolved in 2 ml of water was added. The buffered solution was stirred at 35 °C until the reaction was complete. The solution was dialysed against water and then freeze-dried. To the crystalline powder (0.016 mmol) dissolved in 2 ml of water, 3 ml of phosphate buffer (0.1 M, pH 7), 5 ml of 2×10^{-4} M aqueous $HgCl_2$ and 20 ml of water were added. After about 20 min, maximum absorbance was recorded at 287 nm. The same λ_{max} value was found for the penamaldate of a sample obtained by carrying out the aminolysis at pH 11.35.

The hydrolysis products of BP in the presence of PDDA (benzylpenicilloate salt or/and penicilloic acid) were determined by comparison of the UV spectra and HPLC analysis with those of an authentic sample.

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APPENDIX

$pK_N(T^{\pm})$ (Scheme 2) can be approximately estimated by using inductive parameters of the substituents bonded to the tetrahedral carbon atom,[34] as in the case of simple monomeric amines, and assuming that the inductive effects are determining factors on the acid properties of intermediates. In addition, the strong electrostatic potential in a neighbouring chain which could affect the p*K* of the chromophore bonded to the electrolytes is practically cancelled out by the presence of KCl.[35,36] The structure **3** (Fig. A1) was taken to simulate the intermediate ($T^{\pm}$).

The introduction of the $CH_3CH(O^-)$—at the nitrogen of the aminium ion of the PEI in structure **1** increases the pK_N by 2.2 units[14c] and then $pK_N(\mathbf{1}) = pK_N(PEI) + 2.2$. The substitution in **1** of CH_3 ($\sigma_I = -0.01$)[37] with $CH_2NHCOCH_3$ ($\sigma_I = 0.09$), assumed equivalent to $CH_2NHCOCH_2Ph$,[37] considering that the sensitivity of the pK_N of aminium ion $>CX—N^+H<$ to the σ_I is

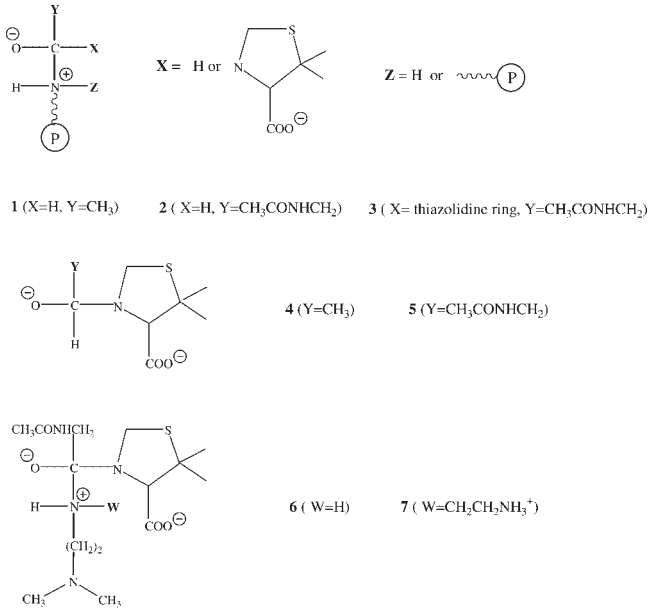

Figure A1. Structures of species **1**–**7**

$\rho_I = -9.2 \pm 0.4$,[37] gives $pK_N(\mathbf{2}) - pK_N(\mathbf{1}) = -9.2\,[0.09 - (-0.01)] \approx -0.9$, then $pK_N(\mathbf{2}) = pK_N(PEI) + 2.2 - 0.9 = pK_N(PEI) + 1.3$. $pK_N(\mathbf{3})$ is obtained by replacing H ($\sigma_I = 0$) in **2** with a 3-carboxy-2,2-dimethylthiazolidine ring ($\sigma_I = 0.23$) calculated from $\sigma_I = \sigma^*/6.23$.[38] The value of $\sigma^* = 1.43$ is calculated from the secondary amines correlation[39] $pK_N = 10.59 - 3.23\Sigma\sigma^*$, the pK_N of the 3-carboxy-2,2-dimethylthiazolidine ring in water being 5.98.[40] Because $\rho_I = -9.2$, one obtains $pK_N(\mathbf{3}) - pK_N(\mathbf{2}) = -9.2 \times 0.23 = -2.1$, then $pK_N(\mathbf{3}) = pK_N(PEI) + PEI) + 1.3 - 2.1 = pK_N(PEI) - 0.8$.

The $pK_N^{\ddagger}$ of the β-lactam nitrogen can be estimated following the same procedure. Starting from $pK_N = 5.98$ of the 3-carboxy-2,2-dimethylthiazolidine ring in water,[40] the introduction of the $CH_3CH(O^-)$— anion to the thiazolidine nitrogen increases the pK_N by 2.2 units, then $pK_N(\mathbf{4}) = 5.98 + 2.2 \approx 8.2$. Now, by substituting CH_3 in **4** with the $CH_3CONHCH_2$ group ($\sigma_I = 0.09$)[37] and with $\rho_I = -9.2$, one calculates $pK_N(\mathbf{5}) - pK_N(\mathbf{4}) = -9.2\,[0.09 - (-0.01)] \approx 0.9$, i.e. $pK_N(\mathbf{5}) = 8.2 - 0.9 = 7.3$.

In alkaline solution, we start from structure **5** because **6** can be considered mimic of the $T^{\pm}$ intermediate. The σ_I of—$NH^+{}_2CH_2CH_2$–$N(CH_3)_2$ is estimated to be 0.63, starting from the $\sigma_I = 0.6$[37] of—NH_3^+ and substituting the H with the—$CH_2CH_2N(CH_3)_2$ group whose $\sigma_I = 0.4 \times 0.4 \times 0.17 - 0 \approx 0.03$, where 0.17 is the σ_I of—$N(CH_3)_2$.[38] Then, $pK_N(\mathbf{6}) = pK_N(\mathbf{5}) - 9.2 \times 0.63 = 7.3 - 5.8 = 1.5$, ρ_I being -9.2.[37] In acidic solution, when the nucleophilic attack occurs preferentially with the secondary group of PEI, structure **7** was taken as a model. The $pK_N^{\ddagger}$ of the β-lactam nitrogen is obtained by substituting hydrogen on **6** with—$CH_2CH_2NH_3^+$, whose σ_I is 0.21.[37] Then, $pK_N(\mathbf{7}) - pK_N(\mathbf{6}) = -9.2\ (0.21 - 0)\ -1.9$ and $pK_N(\mathbf{7}) = pK_N(\mathbf{6}) - 1.9 = 1.5 - 1.9 = -0.4$.