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Nucleophilic phosphate-catalyzed degradation of penicillins: demonstration of a penicilloyl phosphate intermediate and transformation of ampicillin to a piperazinedione

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Summary

The catalytic effect of phosphate buffers on the rate of degradation of benzylpenicillin and ampicillin in neutral aqueous solutions has been shown to be due to a nucleophilic reaction mechanism involving formation of a penicilloyl phosphate intermediate through reaction of monohydrogen phosphate anion with the β-lactam group. Subsequent to its formation the benzylpenicilloyl phosphate undergoes hydrolysis to yield benzylpenicilloic acid. With ampicillin the corresponding α-aminobenzylpenicilloyl phosphate undergoes a rapid intramolecular aminolysis to yield a stable piperazine-2,5-dione derivative. This compound was found to be the major product of degradation of ampicillin in phosphate buffers and specific spectrophotometric methods are described for its quantitation.

Introduction

Degradation of penicillins in aqueous solution has been studied for more than 30 years and several kinetic studies examining the influence of various factors such as pH, buffers, ionic strength and temperature on the rate of degradation of penicillins have been reported (for ref., see Yamana et al., 1974, 1977).

Although many decomposition products have been identified (Schwartz, 1969) the mechanisms of penicillin degradation in aqueous solution and of reactions with inactivating agents have not been fully explored. Special attention has recently been focused upon mechanism and schemes of penicillin degradation in acidic and neutral

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TABLE 1

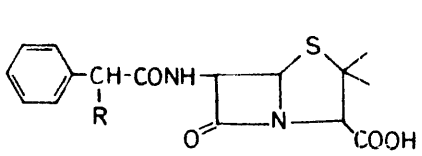
CATALYTIC RATE CONSTANTS FOR PHOSPHATE-CATALYZED DEGRADATION OF VARIOUS PENICILLINS AT 35°C AND $\mu = 0.5$

Penicillin	$k_{HPO_4^{2-}}$ ($M^{-1} h^{-1}$)	$k_{H_2PO_4^-}$ ($M^{-1} h^{-1}$)	Ref.
Benzylpenicillin	0.82 [a]	0.06 [a]	Finholt et al. (1965)
	0.078	0	Yamana et al. (1974)
Phenethicillin	0.094	0	Schwartz et al. (1962)
Cloxacillin	0.092	0.008	Bundgaard and Ilver (1970)
Ampicillin	0.166	0.077	Hou and Poole (1969)
Cyclacillin	0.355	0	Yamana et al. (1974)
Amoxycillin	0.287	0.072	Zia et al. (1977)

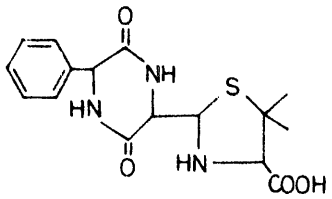
[a] At 60°C.

aqueous solutions (Blaha et al., 1976; Yamana et al., 1977; Mitsumori et al., 1977; Degelaen et al., 1979; Bundgaard, 1980a) and on reaction of penicillins with carbohydrates (Bundgaard and Larsen, 1978a and b, 1979; Larsen and Bundgaard, 1978; Bundgaard, 1980b; Wellmann et al., 1980). The latter studies showed that the accelerating effect of various carbohydrates and polyhydric alcohols on the rate of degradation of penicillins in neutral and alkaline aqueous solutions is due to a nucleophilic reaction mechanism involving an intermediate formation of penicilloyl esters.

We have observed that a similar nucleophilic reaction mechanism is involved in the phosphate-catalyzed degradation of penicillins (I, benzylpenicillin; II, ampicillin) in neutral aqueous solutions. Phosphate buffers have previously been shown to exhibit a strong catalytic effect on the rate of degradation of various penicillins (Table 1) but the mechanism has not been elucidated. In the present paper, conclusive evidence for the involvement of a nucleophilic catalysis mechanism is given along with the demonstration of the formation of a piperazine-2,5-dione derivative (III) from degradation of ampicillin in phosphate buffer solutions.



I R = H
II R = NH_2



III

Materials and methods

Chemicals

Ampicillin sodium, benzylpenicillin sodium, amoxycillin trihydrate, epicillin and

cyclacillin all with a purity better than 97% were commercial products or from lots used in a previous study (Bundgaard, 1977). The piperazine-2,5-dione derivative (III) of ampicillin was available from a previous study (Bundgaard and Larsen, 1979). 2,4,6-Trinitrobenzenesulphonic acid was obtained from Sigma Chemicals, St. Louis. Buffer substances and all other chemicals used were of reagent grade.

General procedures and apparatus

Ultraviolet and visible spectral measurements were performed with a Perkin-Elmer 124 spectrophotometer, using 1 cm cuvettes. Infrared spectra were recorded using the potassium chloride disc technique on a Unicam SP 200 spectrophotometer. The pH measurements were made at the temperature of the study using a Radiometer Type PHM 26 instrument. Melting points were determined by the capillary method.

High-performance liquid chromatography (HPLC) was done with a Spectra-Physics model 3500B instrument equipped with a 10 μl loop injection valve. The column used, 10 cm long and 4.7 mm i.d., was packed with LiChrosorb RP-8 (5 μm particles).

Analytical procedures

Penicilloic acid and penicilloyl ester. The concentrations of penicilloic acid and penicilloyl ester in the reaction solutions of benzylpenicillin were determined on the basis of the spectrophotometric penamaldate assay (Schneider and de Weck, 1966; Schwartz and Delduce, 1969) as previously described (Bundgaard and Larsen, 1978a and b). The assay involves treatment of aliquot portions of the reaction solution at pH 7.0 (0.1 M phosphate buffer solution) with mercury(II) chloride and subsequent measurement of absorbance-stability at 282 nm.

Piperazine-2,5-dione derivative (III). This product was determined by the modified penamaldate assay as previously described (Bundgaard and Larsen, 1979). This assay permits distinction between penicilloic acid, penicilloyl derivatives (esters and amides) and the piperazine-2,5-dione derivative and allows the simultaneous determination of all 3 products.

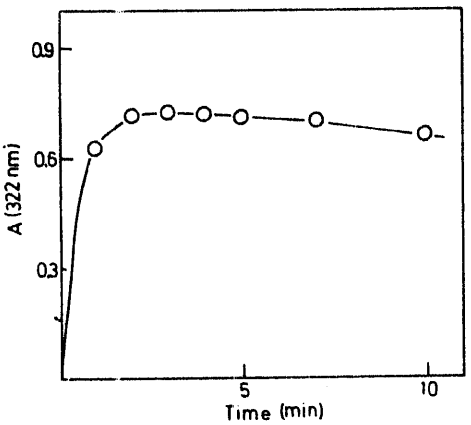


Fig. 1. Time course of production of absorbance at 322 nm in solution consisting of 100 μl of the piperazine-2,5-dione (8.0×10^{-4} M; ethanol) and 2000 μl of 1 M sodium hydroxide (22°C).

The piperazine-2,5-dione derivative was further quantitated by the following method. It was observed that on treatment with 0.5–2 M sodium hydroxide, the derivative is rapidly transformed into a product showing a strong absorption with maximum at 322 nm. Fig. 1 shows the time course of this reaction in 1 M sodium hydroxide at 22°C. The maximum absorbance occurring after 3–4 min was shown to be directly proportional to the concentrations of III. For analysis of the amounts of III formed during the reactions of ampicillin, a 100 μl sample of reaction solution (eventually diluted with water prior to analysis) was mixed with 2000 μl of 1 M sodium hydroxide. The solution was transferred to a cuvette and the absorbance increase was monitored on a recorder connected to the spectrophotometer. The concentration of III was determined from the maximal absorbance produced after 3–4 min and by reference to a standard curve. The molar absorptivity of the piperazine-2,5-dione product in this assay was found to be 18.8×10^3 at 322 nm. It was confirmed that no interference was made by ampicillin or its penicilloic acid in concentrations at least 20 times higher than those of III giving an absorbance of 0.05.

Primary amino groups. Primary amino group quantitation was done by using the colorimetric 2,4,6-trinitrobenzenesulphonic acid assay previously described (Bundgaard, 1976a). An aliquot of 100 μl of the reaction solution containing ampicillin or another amino-penicillin of an initial concentration of about 10^{-3} M was added to 1000 μl of a 0.2 M phosphate buffer solution, pH 7.6. Thereafter, 1000 μl of a 0.2% w/v aqueous solution of 2,4,6-trinitrobenzenesulphonic acid were added, and after standing for 30 min at 20–25°C the absorbance of the resulting orange-coloured solution was measured at 420 nm against a reagent blank. A molar absorptivity of 11.9×10^3 (420 nm) was found for ampicillin.

HPLC. A previously described (Bundgaard and Larsen, 1979) HPLC procedure was further used to identify and quantitate the piperazinedione derivative and penicilloic acid derived from ampicillin. The method also allowed the simultaneous determination of undegraded ampicillin.

Kinetic measurements

All rate studies were performed in aqueous solutions at 37.0 ± 0.2°C. The initial concentration of the penicillins was $1-5 \times 10^{-3}$ M and the reaction progress was followed by analyzing aliquots, withdrawn at suitable intervals, according to the procedures described above. Pseudo-first-order rate constants (k_{obs}) were calculated in the usual ways from the time courses of penicillin disappearance or product appearance.

Results and discussion

Kinetics of phosphate-catalyzed degradation

Previous work on the effect of phosphate buffers on the rate of hydrolysis of penicillins showed that the degradation rate increased linearly with increasing phosphate concentration at constant pH and that monohydrogen phosphate ion is

the primary catalytic species (Table 1). In the present study the effect of phosphate buffers on the rate of ampicillin degradation was studied in aqueous solutions at 37°C and an ionic strength (μ) of 0.5 (when possible). Within the pH range of 6.3–7.4 and at total phosphate buffer concentrations up to 0.5 M the degradation showed a first-order dependency on the buffer concentration. The observed pseudo-first-order rate constant, k_{obs}, for the overall degradation of the penicillin may be expressed by the following equation:

$$k_{obs} = k_0 + k_{HPO_4^{2-}}[HPO_4^{2-}] + k_{H_2PO_4^-}[H_2PO_4^-] \quad (1)$$

where k_0 represents the rate constant at zero buffer concentration as obtained from linear plots of k_{obs} vs total phosphate concentration at constant pH. The concentration of mono- and dihydrogen phosphate ions was calculated from the following equations:

$$[HPO_4^{2-}][H^+]/[H_2PO_4^-] = K_a \quad (2)$$

and

$$[HPO_4^{2-}] + [H_2PO_4^-] = [\text{total phosphate}] \quad (3)$$

where K_a was determined to be $10^{-6.60}$ at 37°C and $\mu = 0.5$.

The following values of the catalytic rate constants were obtained: $k_{HPO_4^{2-}} = 0.25\ M^{-1}\ h^{-1}$ and $k_{H_2PO_4^-} \sim 0$. Thus, the only significant catalytic species is monohydrogen phosphate ion. As it appears from Table 1, Hou and Poole (1969) have previously reported that the dihydrogen phosphate ion also exhibits a marked catalytic effect on ampicillin degradation. However, these authors have used a pK_a value of 7.21 for dihydrogen phosphate ion in their calculation. At 35–37°C and an ionic strength of 0.5 the correct pK_a value is 6.6 and by recalculating the rate data obtained by Hou and Poole using this value, a $k_{HPO_4^{2-}}$ value of 0.23 $M^{-1}\ h^{-1}$ is obtained while $k_{H_2PO_4^-}$ is negligible.

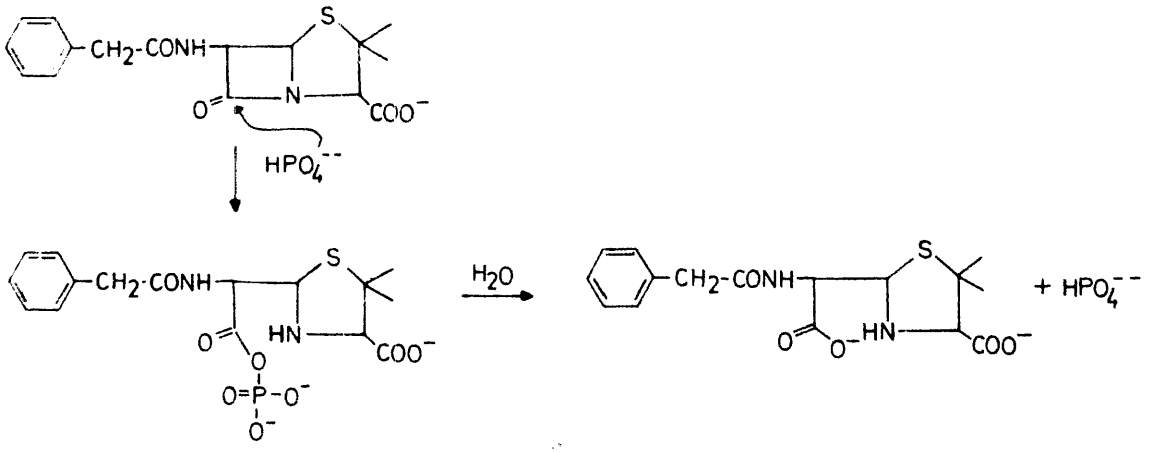
Mechanism of the phosphate catalysis

The catalytic effect of phosphate buffers (i.e. monohydrogen phosphate ion) on penicillin degradation may represent a general base-catalyzed hydrolysis or be due to a nucleophilic catalysis mechanism, or both. These two different types of reactions are kinetically equivalent, but they may be distinguished by product analysis. The nucleophilic pathway should involve a formation of penicilloylated phospate with at least a transitory existence while a general base catalytic mechanism would result in the formation of only penicilloic acid.

Evidence of a nucleophilic reaction mechanism was provided in the following ways.

Benzylpenicillin was allowed to degrade in 0.5 or 0.9 M phosphate buffer solutions of pH 7.43. Submitting aliquots of the reaction solutions to the penamaldate assay procedure at various intervals showed a rapid formation of a product behaving

like a penicilloate ester and a slower formation of penicilloic acid. The penamaldate stability (i.e. the penamaldate absorbance value measured at 10 min from the time of addition of mercury(II) chloride in relation to the absorbance measured at 0 time (Schneider and de Weck, 1966)) of the product was 94–96%, which is characteristic of penicilloyl derivatives (amides or esters). The corresponding value for penicilloic acid is 20–25%. Fig. 2 shows the time course for the formation and subsequent disappearance of the reaction product behaving like a penicilloyl ester. Penicilloic acid was shown to be formed simultaneously and at the end of penicillin degradation, its concentration corresponded to 100 ± 5% of the initial penicillin concentration. As can be seen from Fig. 2 the maximum concentration of penicilloyl ester reached during the reactions is proportional to the phosphate concentration. These observations indicate the presence of a penicilloate ester of phosphate in the reaction pathway and the occurrence of the reactions depicted in Scheme 1. Further evidence



Scheme 1

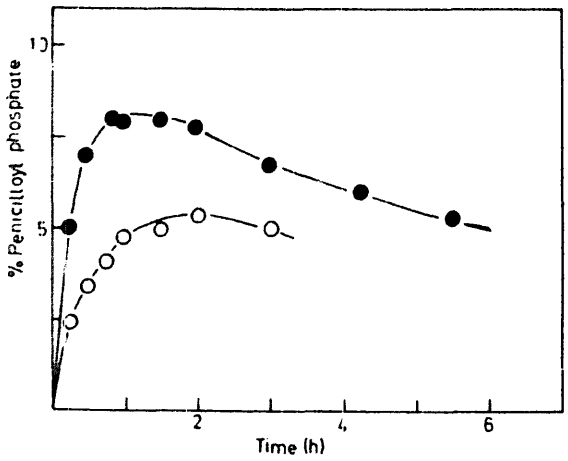


Fig. 2. Time courses for the formation and disappearance of benzylpenicilloyl phosphate in the decomposition of benzylpenicillin sodium (3.7×10^{-3} M) in 0.5 M (○) and 0.9 M (●) phosphate buffer solution of pH 7.43 at 37°C. The concentrations at various times, expressed as mol% in relation to the initial penicillin concentration, were determined by the penamaldate method.

for the formation of a penicilloyl phosphate intermediate was obtained from experiments with ampicillin.

Degradation of ampicillin in phosphate buffers of pH 6.3–7.4 was found to result in the formation of a stable piperazine-2,5-dione derivative (III) besides α-aminobenzylpenicilloic acid (IV). As will be discussed below, the formation of III must proceed via penicilloyl phosphate intermediate and the appearance of III is thus indicative of a nucleophilic mechanism. Identification of III as a product produced from reaction of ampicillin with phosphate was accomplished as follows. (a) After complete degradation at 37°C of ampicillin sodium (2.5 g) in 50 ml of 0.5 M phosphate solution of pH 7.4 the solution was acidified to pH 2.0 and cooled. The precipitate formed was filtered and recrystallized from ethanol–water to give 0.4 g of a compound with melting point, infrared spectrum and chromatographic behaviour identical to that of an authentic sample of 2-(6′-phenylpiperazin-2′,5′-dion -3′-yl)5,5-dimethylthiazolidine-4-carboxylic acid (III) (Bundgaard and Larsen, 1979). (b) Penamaldate analysis of reaction solutions containing ampicillin ($1-5 \times 10^{-3}$ M) and phosphate (0.1–0.5 M, pH 6.3–7.4) revealed the formation of the piperazinedione III. This assay in the modification described previously (Bundgaard and Larsen, 1979) is capable of distinguishing between III, penicilloyl ester and penicilloic acid. (c) Finally, the formation of III was demonstrated by the sodium hydroxide assay and by HPLC as described in the Experimental section.

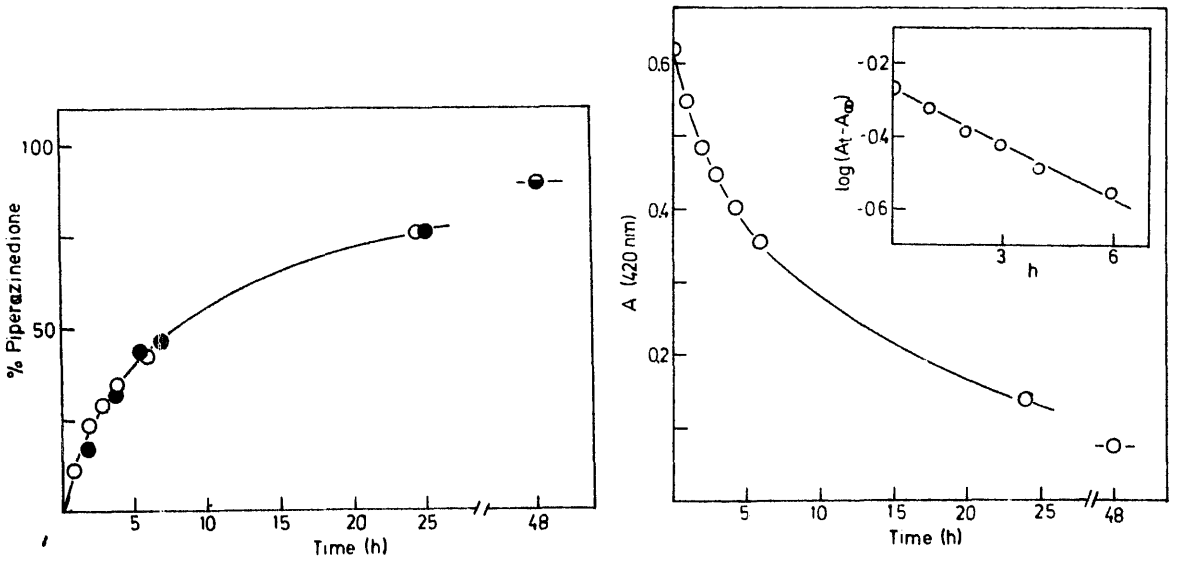


Fig. 3. Time course for the formation of the piperazine-2,5-dione derivative in the decomposition of ampicillin sodium ($1-4\times10^{-3}$ M) in 0.5 M phosphate buffer solution of pH 7.43 at 37°C. The concentrations at various times, expressed as mol% in relation to the initial ampicillin concentration, were determined both by the penamaldate method (●) and the sodium hydroxide assay (○).

Fig. 4. Time course for primary amino group consumption during degradation of ampicillin ($\sim 10^{-3}$ M) in 0.5 M phosphate buffer of pH 7.43 at 37°C. A (420 nm) refers to the absorbances produced by subjecting equal aliquots (100 μl) of the reaction solution to the trinitrobenzenesulphonic acid assay. The inset is a first-order plot of the data of the figure.

The rate and extent of formation of the piperazinedione III from ampicillin were determined in various phosphate buffers using several of the analytical methods mentioned. Fig. 3 shows the time course of formation of III in a 0.5 M phosphate solution of pH 7.43 as determined by the penamaldate method and the sodium hydroxide assay. Likewise, an indirect way of following the formation of III is illustrated in Fig. 4 showing a first-order consumption of the side-chain primary amino group in ampicillin during the decomposition.

As seen from Table 2 the values of k_{obs} determined by using different assay procedures are in good agreement. At complete ampicillin degradation these procedures showed a yield of 88–93% of the piperazinedione derivative, the remaining product being α-aminobenzylpenicilloic acid (Table 2). Under the reaction conditions referred to in Table 2 the spontaneous degradation of ampicillin (the k_0-term in Eqn. (1)) accounts for 1–2% of the total reaction. Thus, the data obtained show that the reaction of monohydrogen phosphate ion with ampicillin results in an almost quantitative formation of III. It should be pointed out that no significant (i.e. < 2–4%) formation of piperazinedione was observed when ampicillin was allowed to degrade in aqueous solutions of pH 6–10 with no content of phosphate or other buffers, thus demonstrating the role of phosphate for the production of III.

A phosphate-catalyzed piperazine-2,5-dione formation was likewise demonstrated for other amino-penicillins (amoxycillin, epicillin and cyclacillin) using the penamaldate and sodium hydroxide assays. With cyclacillin a yield of 98–100% of the corresponding piperazinedione was observed in 0.5 M phosphate buffer of pH 7.43, penicilloic acid being formed in only trace amounts.

The most plausible mechanism for the phosphate-catalyzed degradation of ampicillin to yield the piperazinedione III is a nucleophilic reaction mechanism involving formation of penicilloyl phosphate by attack of monohydrogen phosphate ion on the

TABLE 2

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE DEGRADATION OF AMPICILLIN IN 0.5 M PHOSPHATE BUFFER SOLUTIONS AT 37°C AND YIELDS OF PRODUCTS FORMED [a]

Buffer	k_{obs} (h^{-1})	Piperazine-2,5-dione (%)	Penicilloic acid (%)
0.5 M phosphate, pH 6.29	0.040	93	10
0.5 M phosphate, pH 7.02	0.091	90	8
0.5 M phosphate, pH 7.43	0.11	90	10
	0.11 [b]	88 [b]	
	0.12 [c]	89 [c]	
	0.12 [d]	90 [d]	11 [d]

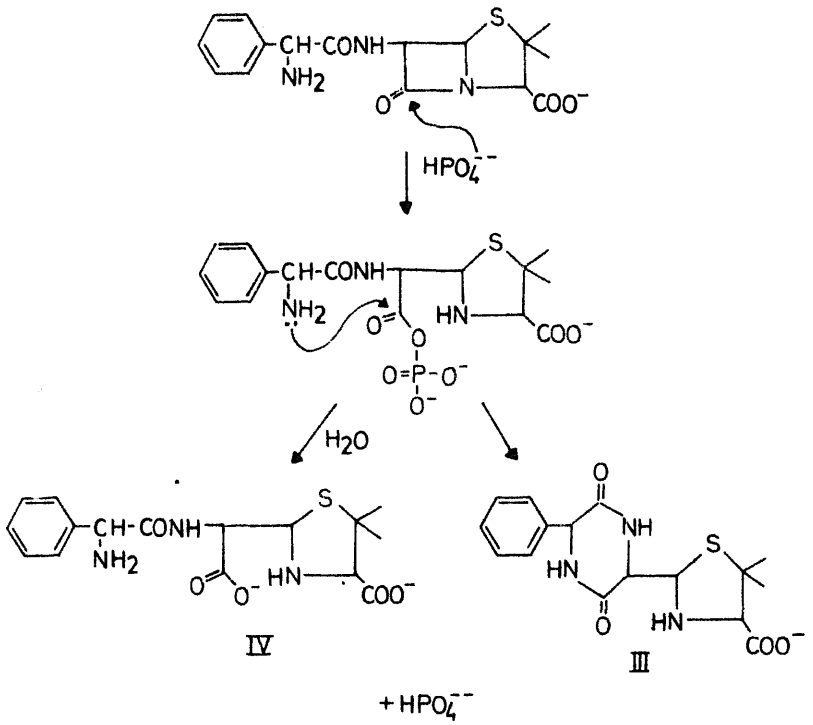
[a] The rate constants and products were determined by using the penamaldate method if not otherwise indicated.

[b] These figures were obtained from the primary amino group analysis.

[c] These figures were obtained from analysis of the piperazine-2,5-dione by the sodium hydroxide assay.

[d] These figures were obtained by HPLC analysis.

penicillin β-lactam carbonyl moiety. In a subsequent fast step the penicilloyl phosphate (a mixed acid anhydride) undergoes an intramolecular aminolysis by the side-chain amino group and, to a small extent, hydrolysis into penicilloic acid (Scheme 2).

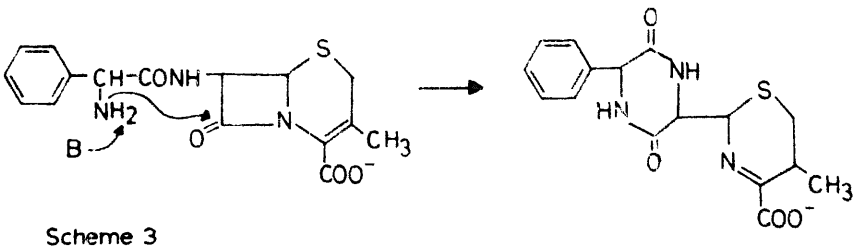


Scheme 2

Evidence for this proposed pathway of formation of the piperazinedione is provided by our previous study on the reaction of ampicillin with carbohydrates (Bundgaard and Larsen, 1979). It was demonstrated that the piperazinedione III, formed as a major reaction product, arose from an intramolecular aminolysis of a penicilloyl–carbohydrate ester intermediate. Furthermore, previous studies (Koshland, 1951, 1952; Kurz and Gutsche, 1960; Di Sabato and Jencks, 1961) have shown that acetyl phosphate, an analogue to the penicilloyl phosphate, is capable of undergoing aminolysis by various amines in neutral and alkaline aqueous solutions at the carbonyl moiety to yield the corresponding acetamides. Intramolecular aminolysis of an acyl phosphate appears not to have been reported before.

Direct intramolecular aminolysis by the side-chain amino group on the β-lactam moiety in ampicillin is not possible to any significant extent as evidenced from the degradation experiments in buffer-free solutions. This has been attributed to the steric hindrance exhibited by the gem-dimethyl group and the 3-hydrogen since such aminolysis is possible in 6-epi-ampicillin (Roets et al., 1973) as well as in cephalosporins with a primary amino group in the side-chain (Cohen et al., 1973; Indelicato et al., 1974, 1977; Bundgaard, 1976a). When the β-lactam ring is opened as in penicilloyl esters and penicilloyl phosphate such steric hindrance does not exist. The intramolecular aminolysis of cephalexin and cephaloglycin to piperazine-2,5-dione derivatives (for cephalexin, see Scheme 3) has been shown to be catalyzed by

phosphate and other buffers as well as to proceed spontaneously in neutral aqueous solution (Bundgaard, 1976a and b) but in these cases the catalytic effect is of the general base catalysis type, the effect possibly being exhibited by removing a proton from the amino group by the bases. The occurrence of such a mechanism in the ampicillin reactions can be excluded because of the inability of ampicillin per se to rearrange into the piperazinedione derivative.



Scheme 3

In conclusion, the demonstration of the piperazinedione III as being the major product of reaction of ampicillin with phosphate buffers and the observation of a penicilloyl phosphate intermediate in the degradation of benzylpenicillin provide conclusive evidence for the involvement of a nucleophilic catalysis mechanism in the monohydrogen phosphate anion-catalyzed degradation of penicillins. Other oxygen anions or compounds known to catalyze the degradation of penicillins in aqueous solutions may also react via nucleophilic mechanisms and it seems that ampicillin (or cyclacillin) may be a highly useful compound for the detection of such mechanism because of the side-chain amino group being capable of trapping a reactive penicilloyl intermediate as a piperazinedione derivative which can be readily determined. In fact, preliminary experiments along which line have shown that the reaction of ampicillin with phenols and imidazole also proceeds by a nucleophilic mechanism, resulting in piperazinedione formation through reactive intermediate penicilloyl derivatives.

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