*J. Pharm. Pharmac.*, 1975, **27**, 771

# COMMUNICATIONS

## The reaction of benzylpenicillin with amines containing hydroxyl groups

Although the principal antigenic determinant in pencillin allergy is known to be the penicilloyl group which results from aminolysis of the penicillin with ϵ-amino group of protein (see reviews by Schwartz, 1969; Schneider, 1970), the simple reaction between penicillin and primary amines under physiological conditions proceeds slowly. It thus appears that catalysis is likely to play a considerable part in the biochemical antigenicity reactions of penicillins. It has recently been suggested that compounds containing both nucleophilic and general acid-base groups are capable of exhibiting high reactivity in the formation of a penicilloyl conjugate at neutral pH (Schneider & de Weck, 1968; Schwartz, 1969; Schneider, 1970). In 1968, Schneider & de Weck and Schwartz reported such an instance in the reaction with aliphatic diamine derivatives. Most recently, we have reported (Yamana, Tsuji & others, 1975b) another possibility for intramolecularly catalysed penicilloylation in the reaction between aminoimidazole compounds and several penicillins.

We now report an example of penicilloylation in which a hydroxyl group assisted intramolecularly by general-base catalysis of the amino group in aminoalcohols reacts with benzylpenicillin (BPC). Schneider & de Weck (1968) have demonstrated that the reactions of BPC with *N*-carbobenzoxy- and *N*-acetyl-serines proceed at appreciable rates even under the physiological conditions through the formation of *O*-penicilloyl serine derivatives. However, it is doubtful whether the observed reactions would be the result of intramolecular catalysis because an oxygen attack assisted by the *N*-acylated moiety is unlikely.

In aqueous buffers of aminoalcohol derivatives (see Table 1), the first-order rate constants, $k_{obs}$, for the degradation of BPC were determined at 35° and 60° ($\mu = 0{\cdot}5$)

Table 1. *The catalytic rate constants for the reactions of benzylpenicillin with various aminoalcohols and other amines not containing a hydroxyl group.*[1]

| Amine | pKa[2] | Temp. °C | $k_1$ ($M^{-1}h^{-1}$) | $k_3$ ($M^{-2}h^{-1}$) |
|---|---|---|---|---|
| Aminoalcohol | | | | |
| Ethanolamine .. .. .. .. | 9·49 | 35 | 5·0 | 305 |
| | 8·86 | 60 | 25·0 | 510 |
| Diethanolamine .. .. .. | 8·48 | 60 | 35·0 | — |
| DL-Serine .. .. .. .. | 8·33 | 60 | 10·0 | — |
| Tris-(hydroxymethyl)-aminomethane .. | 7·50 | 60 | 8·5 | — |
| Triethanolamine .. .. .. | 7·45 | 60 | 5·6 | — |
| Other amines | | | | |
| Glycine .. .. .. .. .. | 9·41 | 35 | 0·40 | 90 |
| | 8·87 | 60 | 2·5 | 148 |
| 2-Methoxyethylamine .. .. .. | 8·72 | 60 | 2·6 | 186 |
| Glycylglycine .. .. .. .. | 7·45 | 60 | 0·25 | 11·2 |
| $H_2O$ .. .. .. .. .. .. | | 35 | $0{\cdot}27 \times 10^{-4}$ [3] | |
| | | 60 | $1{\cdot}30 \times 10^{-4}$ [3] | |

[1] All the catalytic rate constants are at $\mu = 0{\cdot}5$, and were evaluated from equation 1 using $k_{obs}$ determined by the iodometric method. The initial concentration of penicillin was $5 \times 10^{-3}$ M.
[2] Determined by the half neutralization method under experimental conditions.
[3] Second-order rate constant for the water-catalysed hydrolysis. The value was obtained by division of the first-order rate constant by 55·5.

by iodometric analysis and found to obey the general law (Yamana, Tsuji & others, 1975a) of equation 1

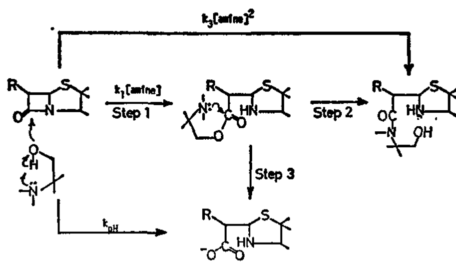
$$k_{obs} = k_{pH} + k_1[\text{amine}] + k_3[\text{amine}]^2 \quad .. \quad .. \quad .. \quad (1)$$

where $k_{pH}$ is the first-order rate constant in the absence of amine. Ethanolamine represented both $k_1$ (second-order rate constant for nucleophilic reaction) and $k_3$ (third-order rate constant for general base-catalysed nucleophilic reaction) terms. Other aminoalcohols exhibited only the $k_1$ term probably due to steric hindrance. These catalytic rate constants are in Table 1, together with those for other amines.

The products resulting from the reactions between BPC and aminoalcohols were analysed by the specific penamaldate assay (Schwartz & Delduce, 1969). The reactions with ethanolamine, tris(hydroxymethyl)aminomethane and DL-serine produced predominantly the corresponding penicilloylamides, which were stable and not hydrolysed to benzylpenicilloic acid (BPO) under these conditions. The rate constants of amide formation (determined by penamaldate assay) were in good agreement with those for β-lactam bond cleavage (determined by the iodometric method). The rate contants, $k_1$, for these aminoalcohols are 5–20 times greater than those for amines of the same basicity which do not contain a hydroxyl group (see Table 1). The observed $k_1$ reaction may proceed predominantly through nucleophilic attack by an oxygen rather than a nitrogen atom toward penicillin β-lactam, as shown in Scheme 1. Although there is no direct confirmation for the existence of penicilloyl ester as the intermediate in these reactions, the formation of such an ester was observed in the reaction with triethanolamine, the hydroxyl group of which can serve only as a nucleophile because the direct attack by the tertiary amine moiety is prevented sterically as in the reaction with triethylamine (Yamana & others, 1975a). The time-courses for the reaction of BPC catalysed by triethanolamine are shown in Fig. 1, indicating the formation and the decomposition of *O*-(penicilloyl)triethanolamine ($\epsilon = 2{\cdot}38 \times 10^4$ at $\lambda_{max}$ 285 nm). The curves in Fig. 1 were generated by analogue computer, programmed according to Scheme 1, and appear to fit the experimental data reasonably well.

The hydroxyl group of these aminoalcohols is $10^5$ times as reactive as the hydroxyl group of water (see $k_1$ in Table 1). In our opinion, the initially formed penicilloyl esters (Step 1) can be rapidly converted by intramolecular attack by the neighbouring amine to the stable penicilloylamides. The amide formation (Step 2) can occur easily with an aminoalcohol having a primary amine group. However, for *O*-(penicilloyl)-triethanolamine Step 2 of the reaction may be prevented sterically, and then the ester hydrolysis (Step 3) proceeds exclusively (see Fig. 1).

When BPC was reacted with 1 M aminoalcohols (0·5 M for DL-serine because of the low solubility) at pH 7·4 and 35°, the rates of β-lactam cleavage were accelerated about 10–$10^2$ times (see Table 2) compared with the rates of the solvent-catalysed hydrolysis and the rates for the reaction with ε-aminocaproic acid under the same conditions.




Scheme 1

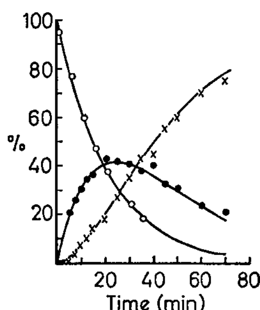
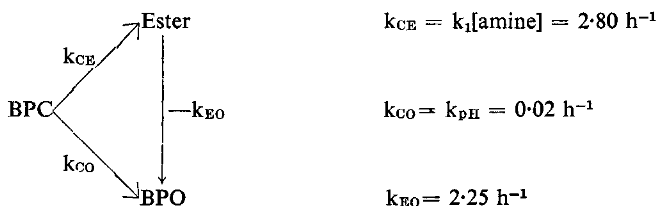



FIG. 1. Time courses* for benzylpenicillin (BPC, ○), *O*-(penicilloyl) triethanolamine (ester, ●) and benzylpenicilloic acid (BPO, X) in the reaction between BPC ($5 \times 10^{-3}$ M) and triethanolamine (0·5 M) at pH 7·45 and 60°. The concentrations at various times were determined by the iodometric method for BPC and by the penamaldate assay for ester and BPO.

* The curves were generated by the analogue computer-simulation according to the following Scheme and kinetic parameters:




$k_{CE} = k_1[\text{amine}] = 2{\cdot}80\ h^{-1}$

$k_{CO} = k_{pH} = 0{\cdot}02\ h^{-1}$

$k_{EO} = 2{\cdot}25\ h^{-1}$

Rate enhancement by a similar mechanism is also expected for the reaction between penicillins and mercaptoamine derivatives. The reaction of BPC in 0·1 M solution of 2-aminoethanethiol or cysteine at pH 7·4 and 35° is fairly rapid with a half-life of only about 1 min (see Table 2), as has already been demonstrated (at 23°) by Nakken, Eldjarn & Pihl (1960). The data suggest that a thiol attack by intramolecular general

Table 2. *The reaction of benzylpenicillin with* M *amino-alcohols and* 0·1 M *amino-thiols at pH* 7·4 *and* 35°.

| Compounds present in the reaction mixture | pKa[1] | $10^2k_{obs}$[2] ($h^{-1}$) | $T_{1/2}$[3] (h) |
|---|---|---|---|
| Ethanolamine | 9·37 | 19·2 | 3·6 |
| DL-Serine[4] | 9·21[5] | 2·5 | 28 |
| Diethanolamine | 9·04 | 20·8 | 3·3 |
| DL-Threonine | 8·89[5] | 0·83 | 83 |
| Tris-(hydroxymethyl) aminomethane | 8·02 | 52 | 1·3 |
| Triethanolamine | 7·75 | 60 | 1·2 |
| 2-Aminoethanethiol[6] | 10·5[7] | 2290 | 0·03 |
| Cysteine[6] | 10·8[7] | 3220 | 0·02 |
| ε-Aminocaproic acid | 10·6[5] | 0·45[8] | 154 |
| $H_2O$ | — | 0·15 | 462 |

[1] Dissociation constant of amino group. Determined by the half-neutralization method under the experimental conditions (35° and $\mu = 1$).
[2] The pseudo-first-order rate constants ($k_{obs}$) were determined by the iodometric analysis. The initial concentration of benzylpenicillin was $5 \times 10^{-3}$ M. The reactions were carried out at $\mu = 1$ in the presence of EDTA ($5 \times 10^{-3}$ M).
[3] Half-life.
[4] Total amine concentration was 0·5 M.
[5] Determined at $\mu = 0{\cdot}5$.
[6] Determined by the hydroxamic acid assay. The kinetic runs were carried out under oxygen-free nitrogen.
[7] Determined at 25°.
[8] Calculated value from Yamana & others (1975a).

base catalysis of the primary amine produces the thioester which may be converted to the stable penicilloylamide.

Although several pathways for the formation of penicilloylamide antigenic determinant have been proposed (see reviews by Schwartz, 1969; Schneider, 1970), the present results indicate the possibility that *in vivo* penicilloylation of a hydroxyl and/or thiol group suitably placed on protein should occur rapidly with the assistance of general acid-base catalysis toward penicillin $\beta$-lactam to yield the corresponding penicilloyl esters, which are then rapidly converted to penicilloylamide by nucleophilic attack by a proximate $\epsilon$-amino-group of lysine on protein. Schneider & de Weck (1968) have excluded such a possibility for the thiol reaction *in vivo* on the grounds that no protein with an *N*-terminal cysteine has been identified. However, protein with an *N*-terminal serine or cysteine is not necessarily required, what is needed is the combination of three-dimensionally and closely located functional groups on protein.

We wish to thank Meiji Seika Kaisha, Ltd. for the generous supply of potassium benzylpenicillin.

*Faculty of Pharmaceutical Sciences,*
*Kanazawa University,*
*Takara-machi, Kanazawa 920,*
*Japan*

TSUKINAKA YAMANA
AKIRA TSUJI
ETSUKO MIYAMOTO
EMI KIYA

October 5, 1974

REFERENCES

NAKKEN, K. F., ELDJARN, L. & PIHL, A. (1960). *Biochem. Pharmac.*, **3**, 89–100.

SCHNEIDER, C. H. (1970). In: *Penicillin Allergy, Clinical and Immunological Aspects*, pp. 23–58. Editors: Stewart, G. T. & McGovern, J. P. Springfield: C. C. Thomas.

SCHNEIDER, C. H. & DE WECK, A. L. (1968). *Biochim. biophys. Acta*, **168**, 27–35.

SCHWARTZ, M. A. (1968). *J. pharm. Sci.*, **57**, 1209–1211.

SCHWARTZ, M. A. (1969). *Ibid.*, **58**, 643–661.

SCHWARTZ, M. A. & DELDUCE, A. J. (1969). *Ibid.*, **58**, 1137–1139.

YAMANA, T., TSUJI, A., MIYAMOTO, E. & KIYA, E. (1975a). *J. Pharm. Pharmac.*, **27**, 56–58.

YAMANA, T., TSUJI, A., MIYAMOTO, E. & KIYA, E. (1975b). *Ibid.*, **27**, 283–287.

# A simple, new method for testing impurities in cephalexin

The British Pharmacopoeia (1973) specifies a paper electrophoresis method to measure the phenylglycin and 7-AMCA (7-amino-3-methyl-3-cephem-4-carboxylic acid) impurities present in cephalexin (7-(D-α-amino-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid). However, not all drug quality control laboratories possess electrophoresis equipment. Therefore we have developed a comparable but simpler method.

The method is based on that of Dévényi (1970). 20 $\times$ 20 cm chromatography plates (coated with 50% Dowex 50 $\times$ 8, Fixion, Chinoin, Budapest) were used in $Na^+$ cycle (Dévényi & Zoltán 1970). The plates were washed for 48 h according to the method of Decker (1951) with sodium citrate, pH 3·28, 0·02 N $Na^+$ (see Fig. 1), and then dried at room temperature (when dry the plates can be stored at room temperature for several months).

The buffer solutions used were:

| | pH 5·28 (0·35 N $Na^+$) | pH 4·59 (0·35 N $Na^+$) | pH 3·42 (0·2 N $Na^+$) |
|---|---|---|---|
| Citric acid monohydrate | 24·6 g | 19·35 g | 14·1 g |
| Sodium hydroxide | 14·0 g | 11·0 g | 8·0 g |
| Hydrochloric acid 37% (sp. gr. 1·19) | 6·5 ml | 9·4 ml | 12·3 ml |
| Each solution was made up to 1000 ml with ion-free distilled water | | | |