Metal Ion Catalysis in the Aminolysis of Penicillin

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The copper(II) ion catalysed hydrolysis and aminolysis of benzylpenicillin in water at 30° shows saturation kinetics. A 1 : 1 complex is formed between the metal ion and benzylpenicillin which is attacked by hydroxide ion and amine *ca.* 10^7-fold faster than unco-ordinated benzylpenicillin. The Brønsted β value for nucleophilic attack by amines is 0.87. The copper(II) ion catalysed reactions of the methyl ester of benzylpenicillin show much smaller rate enhancements indicating that the site of co-ordination for the antibiotic involves the ionised carboxy-group. The mechanism of the catalysed reactions does *not* involve the intermediate formation of benzylpenicillenic acid or a keten. The reactions are inhibited by buffers which co-ordinate to copper(II) ions. Trifluoroethylamine forms a 1 : 1 complex with copper(II) ion with an equilibrium constant of 15.6 l mol^{-1}.

THE aminolysis of penicillin (I) is of interest because the major antigenic determinant of penicillin allergy is the penicilloyl group bound by an amide linkage to ε-amino-groups of lysine residues in proteins.[1] There have been several discussions as to whether these penicilloyl haptenic groups are formed by the direct aminolysis of penicillin [2] or by amino-groups reacting with penicillenic acid (II), formed from a rearrangement of penicillin [3] (Scheme). An alternative route which also deserves consideration involves the intermediate formation of a keten which then reacts rapidly with an amine (Scheme). The base-catalysed isomerisation of penicillins proceeds by proton abstraction at C-6 to give an intermediate carbanion.[4] This carbanion could then form a keten so that the overall aminolysis reaction would be of the elimination–addition type.[5]

An unequivocal proof of the mechanism of formation of the hapten–protein conjugate requires a study of the kinetics of all these possible steps (Scheme) under conditions which simulate physiological conditions. As a contribution towards understanding this process we

[1] B. B. Levine and Z. Ovary, *J. Exp. Medicine*, 1961, **114**, 875; A. L. DeWeck and G. Bulm, *Internat. Arch. Allergy Appl. Immunol.*, 1965, **27**, 221; C. W. Parker, J. Shapiro, M. Kern, and H. N. Eisen, *J. Exp. Medicine*, 1962, **115**, 821.

[2] C. H. Schneider and A. L. DeWeck, *Helv. Chim. Acta*, 1966, **49**, 1695, 1707; F. R. Batchelor, J. M. Dewdney, and D. Gazzard, *Nature*, 1965, **206**, 362.

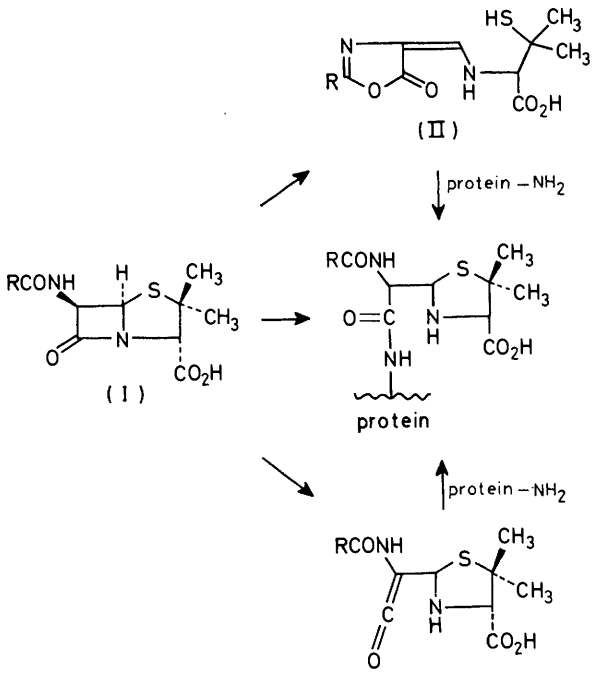
[3] B. B. Levine, *Arch. Biochem. Biophys*, 1961, **93**, 50; H. Bundgaard, *Tetrahedron Letters*, 1971, 4613; A. L. DeWeck, *Internat. Arch. Allergy Appl. Immunol*, 1962, **21**, 20; for a review see M. A. Schwartz, *J. Pharm. Sci.*, 1969, **58**, 643.

[4] D. A. Johnson, D. Mania, C. A. Panetta, and H. H. Silvestri, *Tetrahedron Letters*, 1968, 1903; J. P. Clayton, J. H. C. Mayler, R. Southgate, and E. R. Stove, *Chem. Comm.*, 1969, 129; S. Wolve and W. S. Lee, *ibid.*, 1970, 1067; B. G. Ramsey and R. J. Stoodley, *ibid.*, 1971, 450.

[5] A. Williams and K. T. Douglas, *Chem. Rev.*, 1975, **75**, 627.

have studied the aminolysis and hydrolysis of benzylpenicillin in the presence of copper(II) ions since there is *ca.* 10^{-6} g of copper per cm^3 of human serum.[6]

Penicillin-resistance in bacteria is due, in part, to the hydrolysis of the antibiotic β-lactam ring catalysed by β-lactamases. Some of these enzymes are metal-ion dependent[7] and this report may be of relevance to the mechanism of action of these β-lactamases. The function of the metal-ion in these enzymes may be similar to its catalytic effect proposed in this investigation.



SCHEME

The susceptibility of penicillins to attack by nucleophiles has been attributed to strain in the β-lactam ring[8] and to the nonplanarity of the system which inhibits the usual amide resonance.[9] This non-planarity may lead to the greater availability, compared with that in normal amides, of the lone pair of electrons on the β-lactam nitrogen for metal ion co-ordination. Penicillin may therefore act as a bidentate chelating ligand, through the β-lactam nitrogen and the adjacent carboxy-group, to a metal ion which could affect its reactivity. We report here evidence that co-ordination of copper(II) ions to benzylpenicillin increases the rates of aminolysis and hydrolysis of the antibiotic by factors $>10^6$.

[6] B. Sarkar and T. P. A. Kruck in ' The Biochemistry of Copper ', eds. J. Peisach, P. Aisen, and W. F. Blumberg, Academic Press, New York, 1966, p. 183.

[7] L. D. Sabath and E. P. Abraham, *Biochem. J.*, 1966, **98**, 11c; S. Kuwabara and E. P. Abraham, *ibid.*, 1967, **103**, 27c; L. D. Sabath and M. Finland, *J. Bacteriol.*, 1968, **95**, 1513.

[8] J. L. Strominger, *Antibiotics*, 1967, **1**, 706.

[9] R. B. Woodward in ' The Chemistry of Penicillin ', eds. H. T. Clarke, J. R. Johnson, and R. Robinson, Princeton University Press, Princeton, 1949, p. 443.

[10] H. C. Rapson and A. E. Bird, *J. Pharm. Pharmacol. Suppl.*, 1963, **15**, 222T.

EXPERIMENTAL

Materials.—Benzylpenicillin and benzylpenicillenic acid were of general reagent grade and other materials were of AnalaR grade. Benzylpenicilloic acid was prepared by the method of Rapson and Bird.[10]

Methyl ester of benzylpenicillin. The sodium salt of benzylpenicillin was acidified, extracted into ether, and treated with diazomethane.

Kinetic Measurements.—The pH of all solutions was checked before and after a kinetic experiment and if it had changed by >0.03 the experiment was rejected. Unless otherwise stated the ionic strength was made up to *I* 0.5M by adding sodium perchlorate.

(i) *Spectrophotometric method.* The rates of reaction of penicillin were determined by following the change in absorbance on a Gilford 240 recording spectrophotometer having the cell compartment controlled at 30 ± 0.05°. For reactions following a first-order course the initial penicillin concentration was normally 1—2 × 10^{-4}M and the corresponding rate constants were calculated using a generalised least squares program which treated the first-order rate constant and the absorbances at both time zero and infinity as disposable parameters.[11] The aminolysis reactions were generally studied at 245, the hydrolysis reactions at 265—275, and penicillenic acid formation at 318 nm. Some of the slower reactions were studied by the initial rate method and these initial slopes and other straight-line relationships were analysed using a linear least-squares method. The rates of formation of penicillenic acid were determined in the absence and presence of a concentration of mercury(II) chloride equal to that of the initial penicillin concentration. Mercury(II) chloride retards further reactions of penicillenic acid.[2,12]

(ii) *pH-stat method.* The kinetics of the penicillin reactions were also studied by maintaining a constant pH using a Radiometer TTT60 automatic titrator. Because of precipitation of metal ion complexes the rates were measured at low concentrations of copper(II) ions (*ca.* 10^{-4}M) in the presence of excess of penicillin. The rate of addition of sodium hydroxide to maintain constant pH followed first-order kinetics. The total reaction required the addition of one mole of sodium hydroxide per mole of copper.

Equilibrium Constant Measurements.—Trifluoroethylamine–Cu^{II} *complex and acetate–*Cu^{II} *complex.* The formation constants were determined at 30° and *I* 0.5M ($NaClO_4$) using a spectrophotometric method, at 235 nm, suitable for cases where the extinction coefficient of the complex is not known.[13] Both metal ion and ligand concentrations were varied independently.

RESULTS AND DISCUSSION

Hydrolysis.—The pH–rate profile for the decomposition of benzylpenicillin in water is shown in Figure 1 and the relevant data in Table 1. It has been shown previously that the nature of the products varies with pH as follows. Between pH 0 and 2 penilloic acid is formed

[11] W. E. Derning, ' Statistical Adjustment of Data ', Dover, New York, 1964; W. E. Wentworth, *J. Chem. Educ.*, 1965, **42**, 96, 162.

[12] M. A. Schwartz and G. M. Wu, *J. Pharm. Sci.*, 1966, **55**, 550; M. W. Brandriss, E. L. Denny, M. A. Huber, and H. G. Steinman in 'Antimicrobial Agents and Chemotherapy ', ed. J. C. Sylvester, American Soc. Microbiol., Ann Arbor, 1962, p. 626.

[13] R. W. Ramette, *J. Chem. Educ.*, 1967, **44**, 647.

from the decarboxylation of penicilloic acid (III), the product of hydrolysis of the penicillin β-lactam residue; [14] between pH 2 and 4 penicillenic acid (II) is formed and

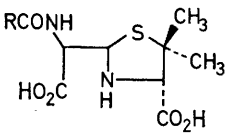
TABLE 1

The kinetics of the decomposition of benzylpenicillin in water at 30.0° (I 0.5M)

pH	$10^5 k_{obs}/s^{-1}$	$10^5 k_{calc}{}^a/s^{-1}$
1.03	836	709
1.39	330	330
1.51	238	259
1.75	153	163
1.96	136	113
2.32	58.0	65.2
2.62	34.1	39.9
3.00	35.0	21.5
3.52	10.6	8.05
3.73	5.96	5.12
4.42	1.11	1.11
5.40	0.079 0	0.118
5.95	0.027 3	0.033 3
9.45	0.690	0.663
9.80	1.51	1.48
10.07	3.30	2.76
11.01	22.4	24.0
11.83	161	159
12.87	1 470	1 740

[a] Calculated from the expression, $k_{calc} = k_1 (10^{-pH})^2/(10^{-pH} + K_a) + k_2 10^{-pH}/(10^{-pH} + K_a) + k_3 K_w/10^{-pH}$ with k_1 7.18 × 10^{-2} l mol^{-1} s^{-1}, k_2 5.18 × 10^{-4} s^{-1}, k_3 8.54 × 10^{-2} l mol^{-1} s^{-1} K_a 1.74 × 10^{-3} mol l^{-1} and K_w 1.48 × 10^{-14}.

this then reacts to give penillic acid and penamaldic acid; [15,16] between pH 4 and 8 the initial product,



(III)

penicillenic acid, undergoes a rapid base-catalysed hydrolysis to penicilloic acid (III) [16] and between pH 8 and 14 penicilloic acid (III) is formed from the hydroxide ion catalysed hydrolysis of penicillin.[17]

Between pH 3 and 8 the small first-order rate constants k for penicillenic acid formation were calculated from the initial increase in absorbance at 318 nm by means of equation (1) where ε is the molar absorption

$$k = -\frac{dA}{dt} \cdot \frac{1}{\varepsilon[\text{penicillin}]_0} \qquad (1)$$

coefficient of penicillenic acid and has the value 24 500 l mol^{-1} cm^{-1} and dA/dt is the slope of the linear plot of absorbance against time. When comparison was possible, the rate constants determined by this method agreed (±10%) with those determined by the conventional manner.

In the presence of copper(II) ions there is an enormous enhancement in the rate of decomposition of penicillin in water. In acetate buffers the apparent first-order rate constant k_{obs} for the loss of penicillin in the presence of an excess of copper(II) ions shows a complex dependence upon the metal ion concentration (Figure 2).

At low metal-ion concentration the apparent first-order rate constant is approximately first-order in metal

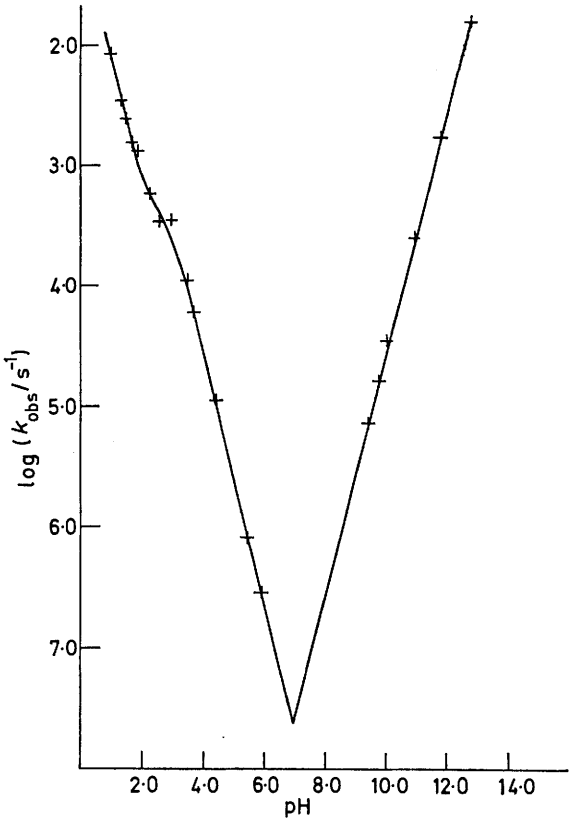


FIGURE 1 Plot of the rate of decomposition of benzylpenicillin as a function of pH. The solid line is calculated using the constants given in Table 1

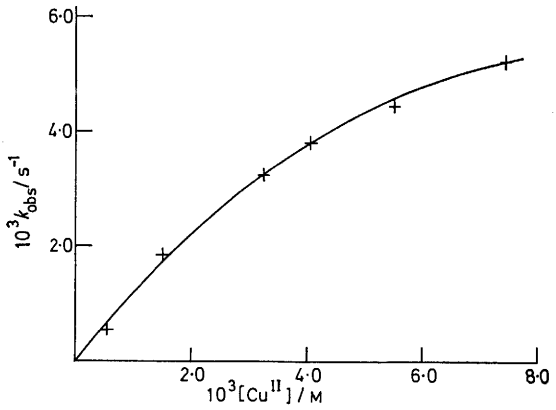


FIGURE 2 Plot of the apparent first-order rate constant for the hydrolysis of benzylpenicillin at pH 5.53 and 30.0° (I 0.50M) as a function of total metal ion concentration. The solid line is calculated from the constants given in the text

ion but at high concentrations of copper(II) ions it is nearly independent of metal ion concentration. This

[14] D. W. Dennen and W. W. Davis, 'Antimicrobial Agents and Chemotherapy,' Amer. Soc. for Microbiol, Ann Arbor, 1961, p. 531; J. M. Blaha, A. M. Knevel, D. P. Kessler, J. W. Mincy, and S. L. Hern, *J. Pharm. Sci.*, 1976, **65**, 1165.

[15] M. A. Schwartz, *J. Pharm. Sci.*, 1965, **54**, 472.

[16] J. L. Longridge and D. Timms, *J. Chem. Soc.* (*B*), 1971, 852.

[17] R. Mozingo and K. Folkers in ref. 7, p. 535.

saturation phenomenon is interpreted in terms of formation of a penicillin–metal ion complex which then breaks down to the products of the reaction. The sole product of this reaction is the hydrolysis product, penicilloic acid (III). In the presence of copper(II) ions but with an excess of penicillin the apparent first-order rate constant again shows a saturation phenomenon with varying penicillin concentration which supports the hypothesis of penicillin–metal ion complex formation. The kinetics of this reaction are further complicated by the decrease in the apparent first-order rate constant with increasing concentration of acetate ion but with pH, ionic strength, and penicillin and copper(II) ion concentrations all constant (Table 2). This is interpreted in terms of an effective decrease in the metal ion

TABLE 2

The kinetics of the hydrolysis of benzylpenicillin in acetate buffers in the presence of copper(II) ions at 30.0° (I 0.5M). Initial penicillin concentration 2×10^{-4}M; initial copper(II) concentration 5×10^{-3}M

pH	10^2 [AcO^-]/ M	10^4 [M_o–MB] [a]/ M	10^5 [MP] [a]/ M	10^4 k_{obs}/ s^{-1}	10^4 k_{calc} [b]/ s^{-1}
4.49	2.5	23.03	5.903	15.6	16.4
4.49	5.0	14.53	4.171	10.9	11.6
4.49	7.5	10.56	3.210	8.8	9.02
4.49	10.0	8.29	2.606	7.11	7.34
4.49	12.5	6.82	2.192	6.96	6.19
4.49	15.0	5.79	1.890	5.34	5.35
4.49	17.5	5.02	1.662	4.55	4.80
4.63	1.2	32.50	7.442	28.10	28.6
4.63	1.8	27.37	6.653	29.80	25.6
4.63	2.4	23.57	6.000	26.20	23.1
4.63	3.0	20.65	5.456	22.20	21.1
4.63	6.0	12.64	3.726	14.5	14.5
4.63	9.0	9.74	2.818	10.9	11.6
4.63	12.0	7.07	2.264	8.43	8.82
4.63	18.0	4.89	1.623	6.57	6.34
4.63	24.0	3.74	1.264	5.59	4.94
4.63	27.0	3.35	1.138	4.98	4.44
4.90	3.75	17.85	4.895	31.6	35.20
4.90	7.50	10.56	3.210	25.5	23.20
4.90	15.00	5.79	1.890	14.3	13.70
4.90	22.5	3.98	1.338	10.4	9.82
4.90	30.0	3.03	1.035	8.05	7.85
5.04	4.0	17.08	4.731	41.6	46.3
5.04	8.0	10.01	3.068	28.0	30.2
5.04	16.0	5.45	1.792	15.8	17.7
5.04	24.0	3.74	1.264	11.2	12.3
5.41	9.0	9.074	2.818	76.9	66,0
5.41	18.0	4.895	1.623	48.1	38.2
5.41	27.0	3.348	1.138	25.4	26.8
5.41	36.0	2.544	0.876 6	17.3	20.7

[a] Calculated using equations (6) and (7) with K_1 186.6 l mol^{-1} and K_2 53.2 l mol^{-1}. See text for definition of symbols. [b] k_{calc} determined using equation (8) and the constants given in the text.

concentration with increasing acetate ion concentration because of the known complex formation between acetate and copper(II) ions.[18] This interpretation is supported by a decrease in the rate constant when chloride ion, which is known to form complexes with copper(II) ions,[19] is used to maintain constant ionic strength instead of perchlorate ion. A kinetic scheme compatible with these observations is shown in equation (2) where M represents

$$\begin{array}{ccccc} M + P & \overset{K_1}{\rightleftharpoons} & MP & \xrightarrow{k'_2} & \text{products} \\ {\scriptstyle -B}\downarrow\uparrow{\scriptstyle K_2} & & & & \\ MB & & & & \end{array} \qquad (2)$$

copper(II) ion, P is penicillin, B is acetate ion, MP is the penicillin–copper(II) ion complex, and MB is the acetatocopper(II) complex. The concentrations of these two complexes are given by equations (3) and (4) where $[M_o]$, $[B_o]$, and $[P_o]$ are the initial concentrations of metal ion, acetate ion, and penicillin respectively.

$$[MP] = K_1([M_o] - [MP] - [MB])([P_o] - [MP]) \quad (3)$$

$$[MB] = K_2([M_o] - [MP] - [MB])([B_o] - [MB]) \quad (4)$$

Under the condition of $[B_o] \gg [M_o]$, $[M_o] \gg [P_o]$, and therefore $[M_o] \gg [MP]$, the observed apparent first-order rate constant is given by equation

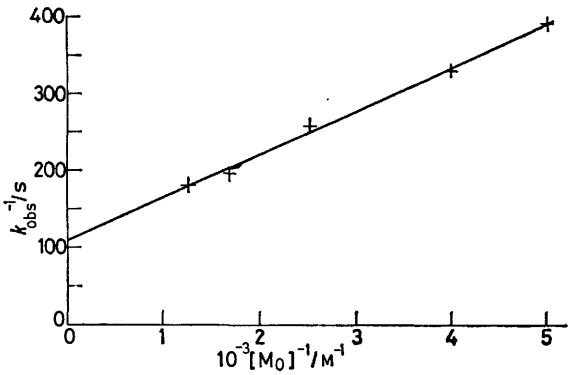


FIGURE 3 Plot of the reciprocal of the apparent first-order rate constant for the hydrolysis of benzylpenicillin at pH 4.92 and 30.0° as a function of the reciprocal of the metal ion concentration. Total acetate concentration 0.05M; initial penicillin concentration 2×10^{-4}M

(5), assuming that the rate of dissociation of MP is greater than k_2'. A plot of $1/k_{obs}$ against $1/[M_o]$ should

$$k_{obs} = k'_2K_1[M_o]/(1 + K_2[B_o] + K_1[M_o]) \quad (5)$$

give $1/k'_2$ as the intercept and this is shown in Figure 3. However, the majority of the data was obtained under the conditions of $[B_o] \gg [M_o]$ with the concentrations of the two complexes being given by equations (6) and (7) where $X = K_1K_2[B_o]/(1 + K_2[B_o])$.

$$[MB] = K_2[B_o][M_o - MP]/(1 + K_2[B_o]) \quad (6)$$

$$[MP]^2(X - K_1) + [MP](1 + K_1[P_o] + K_1[M_o] - [P_o]X - [M_o]X) - [M_o][P_o](K_1 - X) = 0 \quad (7)$$

The equilibrium constant for complex formation K_2 was determined spectrophotometrically [13] under the conditions used for the kinetic studies. At 235 nm the extinction coefficients of acetate ion, copper(II) ion, and acetatocopper(II) complex were found to be 2.05 ± 0.05,

[18] 'Stability Constants of Metal Ion Complexes', Chem. Soc. Special Publications, No. 17, 1964; No. 25, 1971.

[19] H. McConnell and N. Davidson, *J. Amer. Chem. Soc.*, 1950, **72**, 3164, 3168.

178.8 ± 5.0, and 2 350 ± 100 l mol^{-1} cm^{-1} respectively. The value of K_2 was determined to be 53.2 ± 2.0^{-1} l mol^{-1} which may be compared with literature values of 113 [18] and 54 [20] and with 41.0 l mol^{-1} determined kinetically using equation (5). The thermodynamic formation constant K_1 for the copper(II)–penicillin complex has been previously determined as 407.4 l mol^{-1}.[21] Values of [MP] and thence [MB] and [M_o — MB], examples of which are shown in Table 2, were calculated using equations (6) and (7). The apparent first-order rate constant is given by equation (8) and a plot of $1/k_{obs}$ against $1/[M_o - MB]$ gives an intercept of

$$k_{obs} = k'_2K_1[M_o - MB]/(1 + K_1[M_o - MB]) \quad (8)$$

$1/k'_2$ and a slope of $1/k'_2K_1$. Such graphs are shown in Figure 4 at different pH values. k'_2 is pH dependent and is first order in hydroxide ion with $k'_2/[OH^-]$ being 1.29 ± 0.2 × 10^7 l mol^{-1} s^{-1}. The hydroxide ion concentrations were calculated from the observed pH and

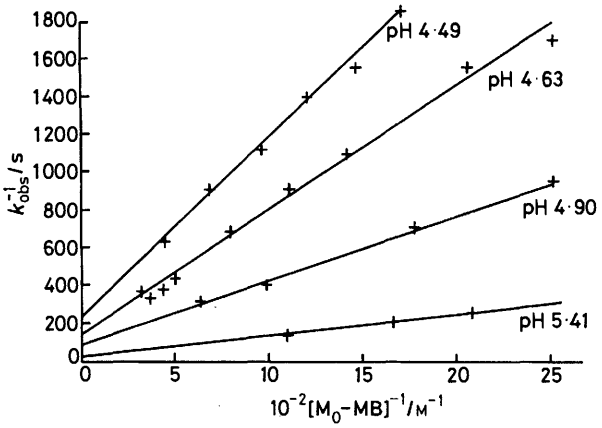


FIGURE 4 Plot of the reciprocal of the apparent first-order rate constant for the hydrolysis of benzylpenicillin in acetate buffers at 30.0° and the pH values indicated as a function of the reciprocal of the free metal-ion concentration

defined * as antilog (pH — pK_w) with pK_w 13.83 at 30°.[23] K_1, the equilibrium constant for penicillin–copper(II) ion complex formation, is 266 ± 40 l mol^{-1} which agrees well with the previously determined value [21] and may be compared with 187 l mol^{-1} determined from equation (5).

Reusing this value of K_1 in equation (7) has negligible effect upon the value of $k'_2/[OH^-]$. The second-order rate constant $k'_2/[OH^-]$ determined from equation (5) is 1.22 ± 0.2 × 10^7 l mol^{-1} s^{-1}. The second-order rate constant for hydroxide ion attacking unco-ordinated benzylpenicillin is 0.15 l mol^{-1} s^{-1} and so metal ion complexation increases the rate of reaction by a factor of *ca.* 9 × 10^7.

The solvent isotope effect for the hydrolysis reaction was determined from kinetic data obtained in acetate buffers in D_2O. The deuterioxide concentrations were calculated from the observed pH using the relationship [24] pD = pH (meter reading) + 0.40 and a value of p$K_w^{D_2O}$ of 14.70 at 30°.[25] The equilibrium constants for complex formation between copper(II) ion and acetate ion and penicillin, K_2 and K_1, in D_2O were found to be 68.3 and 419.8 l mol^{-1} respectively. Using equation (5) the rate constant for deuterioxide ion attack on the metal ion–penicillin complex was found to be 2.12 × 10^7 l mol^{-1} s^{-1} which gives a solvent isotope effect $k_{OH^-}^{H_2O}/k_{OD^-}^{D_2O}$ of 0.54. This may be compared with a value of 0.645 for the base-catalysed hydrolysis of benzylpenicillin itself.[26]

Before discussing these results in detail the metal ion catalysed aminolysis of penicillin will be described.

Aminolysis.—The kinetics of the aminolysis of penicillin have been previously described [27] and there is no detectable reaction of the weakly basic trifluoroethylamine with penicillin. However, in aqueous solution in the presence of copper(II) ion penicillin reacts with trifluoroethylamine to form the corresponding penicilloyl amide. The kinetics of this reaction show a saturation phenomenon with the concentration of metal ion and are also complicated by the complexation of copper(II) ions with trifluoroethylamine. Unlike the situation with acetate ion, when trifluoroethylamine is used as the buffer the apparent first-order rate constant increases with the concentration of trifluoroethylamine when the other parameters are kept constant [see Supplementary Publication No. SUP 22187 (6 pp.)].†

In water at 30° at ionic strength I 0.5M maintained with sodium perchlorate, trifluoroethylamine forms a 1 : 1 complex with copper(II) ion. This is shown by the method of continuous variation [28] and is illustrated in Figure 5. This method gave unsatisfactory results for the value of the equilibrium constant and has been previously criticised.[29] However the spectrophotometric method described by Ramette [13] was found to be suitable and the equilibrium constant for formation of the 1 : 1 complex between copper(II) and trifluoroethylamine was found to be 15.6 ± 2.0 l mol^{-1}. It is interesting to note that with copper(II) ion the more basic amines such as ethylamine form predominantly 1 : 4 complexes.[18] The extinction coefficient, at 235 nm, of the metal–amine complex was found to be 4 500 ± 500 l mol^{-1} cm^{-1}.

* No correction for ionic strength (0.5) was applied to K_w since it cannot be independently measured.[22] Activities rather than concentrations of L_3O^+ and LO^- have been used to evaluate the rate constants.

† For details of Supplementary Publications see Notice to Authors No. 7 in *J.C.S. Perkin II*, 1977, Index issue. Items less than 10 pp. are supplied as full size copies.

[20] M. H. Hutchinson and W. C. E. Higginson, *J.C.S. Dalton*, 1973, 1247.
[21] W. A. Cressman, E. T. Sugita, J. T. Doluisio, and P. J. Niebergall, *J. Pharm. Pharmac.*, 1966, **18**, 801.
[22] Myung-un Choi and E. R. Thornton, *J. Amer. Chem. Soc.*, 1974, **96**, 1428.
[23] R. A. Robinson and R. H. Stokes in ' Electrolyte Solutions,' Butterworths, London, 1959.
[24] P. Glasoe and F. A. Long, *J. Phys. Chem.*, 1960, **64**, 188.
[25] ' Handbook of Chemistry and Physics ', 49th edn., The Chemical Rubber Co., Cleveland, 1969, p. D-92.
[26] N. Gensmantel, unpublished observations.
[27] A. F. Martin, J. J. Morris, and M. I. Page, *J.C.S. Chem. Comm.*, 1976, 495; J. J. Morris and M. I. Page, unpublished observations.
[28] P. Job, *Ann. Chim (France)*, 1928, **9**, 113.
[29] F. Woldbye, *Acta Chem. Scand.*, 1955, **9**, 299.

A kinetic scheme compatible with the observed data is given in equation (9) where B represents trifluoroethyl-

$$\begin{array}{ccccc} M + P & \overset{K_1}{\rightleftharpoons} & MP & \xrightarrow{k_3[B]} & \text{aminolysis products} \\ -B \Big\downarrow K_2 & & \Big\downarrow k_2[OH^-] & & \\ MB & & \text{hydrolysis products} & & \end{array} \qquad (9)$$

amine and the other symbols maintain their previous significance. The apparent first-order rate constant for the disappearance of penicillin is given by equations (10) and (11). Using the experimentally determined value of

$$k_{obs} = \frac{k_3K_1[M_o][B]}{1 + K_2[B] + K_1[M_o]} + \frac{k_2K_1[M_o][OH^-]}{1 + K_2[B] + K_1[M_o]} \qquad (10)$$

$$k_{obs} = \frac{k_3K_1[M_o - MB][B]}{1 + K_1[M_o - MB]} + \frac{k_2K_1[M_o - MB][OH^-]}{1 + K_1[M_o - MB]} \qquad (11)$$

15.6 l mol^{-1} for K_2, values of $[M_o - MB]$ were calculated as previously described. The relevant data are given in SUP 22187 and examples of the plots $1/k_{obs}$ against $1/[M_o - MB]$, as a function of pH, are shown in Figure 6. The intercepts of these plots give values of $(k_3[B] + k_2[OH^-])^{-1}$ and intercept/slope gives K_1, which is 179.5 $\pm$ 20 l mol^{-1} in good agreement with the previously determined value in acetate buffers. Thus a plot of these intercept values against the reciprocal of the trifluoroethylamine concentration gives k_3 from the slope

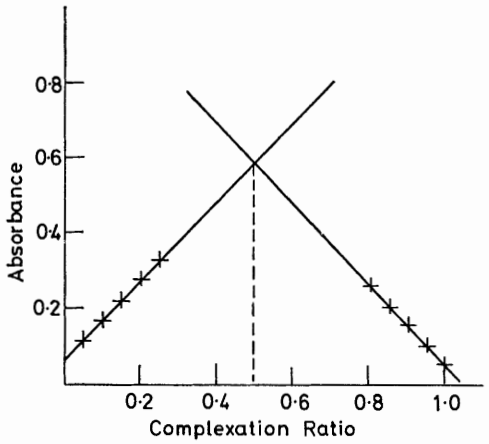


FIGURE 5 Plot using the method of continuous variation to determine the complexation ratio between copper(II) ion and trifluoroethylamine at 30.0°. The absorbance changes were measured at 235 nm

and $k_2[OH^-]$ as the intercept. The mean value of k_3 is found to be 2.86 $\pm$ 0.5 l mol^{-1} s^{-1} and that of k_2 to be 1.24 $\times$ 10^7 l mol^{-1} s^{-1} which compares favourably with the value 1.29 $\times$ 10^7 l mol^{-1} s^{-1} determined in acetate buffer.

The solvent isotope effect for the aminolysis reaction was determined from kinetic data obtained in trifluoroethylamine buffers in D_2O. Using equation (10) the rate constant for trifluoroethylamine attack on the metal ion–penicillin complex was found to be 4.24 $\pm$ 0.2 l mol^{-1} s^{-1} which gives a solvent isotope effect k^{H_2O}/k^{D_2O} of 0.67. This value is consistent with nucleophilic attack

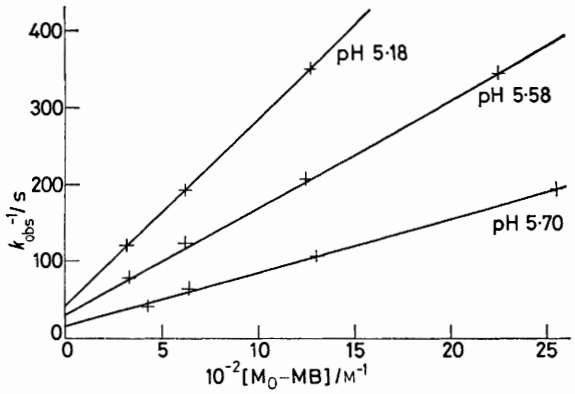


FIGURE 6 Plot of the reciprocal of the apparent first-order rate constant for the decomposition of benzylpenicillin in trifluoroethylamine buffers at 30.0° and the pH values indicated as a function of the reciprocal of the free metal ion concentration

of the amine rather than the kinetically equivalent mechanism of the amine acting as a general base catalyst for hydrolysis.

To further elucidate the mechanism of the metal ion catalysed reactions the reaction of penicillin with more basic amines was studied. Because of the rapid rate of these reactions and complications caused by precipitation of copper(II)–hydroxy complexes at high pH the kinetic studies were performed at low pH using a pH-stat. The enormous rate enhancement brought about by copper(II) ion is appreciated when aminolysis of penicillin occurs, for example, with propylamine at pH 4 when the concentration of free propylamine is only *ca.* 10^{-7}—10^{-8}M. A kinetic scheme compatible with the observed data is given in equation (12).

$$\begin{array}{ccccc} BH^+ & & & & \\ \Big\downarrow K_a & & & & \\ B + M & \overset{K_1}{\rightleftharpoons} & MP & \xrightarrow{k_3[B]} & \text{aminolysis products} \\ \Big\downarrow K_2 & & \Big\downarrow k_2[OH^-] & & \\ MB_4 & & \text{hydrolysis products} & & \end{array} \qquad (12)$$

Under the conditions of $[B_o] > [P_o] > [M_o]$, the apparent first-order rate constant is given by equation (13). Because $[H^+] \gg K_a$, where K_a is the dissociation

$$k_{obs} = \frac{k_3K_1[P_o][B] + k_2K_1[P_o][OH^-]}{1 + K_2[B]^4 + K_1[P_o]} \qquad (13)$$

constant of the amine, term $K_2[B]^4$ is given by equation (14), where B_o is the total concentration of amine, and is

$$K_2[B]^4 = K_2(K_a[B_o]/[H^+])^4 \qquad (14)$$

negligible compared with the other terms in the denominator of equation (13). Hence equation (13) is reduced

to equation (15). Using equation (15) the mean value

$$k_{obs} = \frac{k_3K_aK_1[P_o][B_o] + k_2K_wK_1[P_o]}{[H^+] + K_1[P_o][H^+]} \quad (15)$$

of k_3 for propylamine is found to be 4.14×10^4 l mol^{-1} s^{-1} and that for methoxyethylamine to be 1.40×10^4 l mol^{-1} s^{-1}.

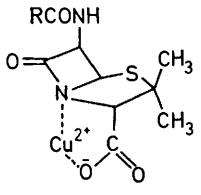
The observed pseudo-first-order rate constant k_{obs} for the aminolysis of benzylpenicillin is given by equation (16),[27] where k_o is the first-order constant for the hydrolysis reaction. The values of k_1 for propylamine and

$$k_{obs} = k_o + k_1[RNH_2] + k_2[RNH_2]^2 + k_3[RNH_2][OH^-] \quad (16)$$

methoxyethylamine are 1.0×10^{-2} and 1.4×10^{-3} l mol^{-1} s^{-1} respectively.[27] The rate enhancements of these amines reacting with the penicillin–copper(II) complex compared with their reaction with penicillin alone are therefore *ca.* 4×10^6 and 10^7 respectively. The reaction of trifluoroethylamine with penicillin is very slow but the value of k_1 estimated from initial slopes is 1.31×10^{-7} l mol^{-1} s^{-1} which may be compared with the value 1×10^{-7} l mol^{-1} s^{-1} extrapolated from the Brønsted plot of more basic amines.[27] The rate enhancement of trifluoroethylamine reacting with the penicillin–copper(II) complex compared with its reaction with penicillin alone is therefore *ca.* 3×10^7. These rate enhancements are similar to that for hydroxide ion, *ca.* 9×10^7, described earlier.

These large rate enhancements are attributed to the copper(II) ion complexing with penicillin as shown, (IV). The normal amide resonance is reduced in the β-lactam residue of penicillin because of the non-planarity of the molecule and, consequently, the β-lactam nitrogen is probably basic enough to act as a ligand. It has recently been suggested that this is the mode of complexation based on n.m.r. studies [30] but other modes have been proposed.[31] We believe that (IV) is the kinetically important complex for the following reasons.

(a) Esterification of the free carboxylate group in benzylpenicillin (I) to the methyl ester reduces the rate of reaction in copper(II) ion–trifluoroethylamine and copper(II) ion–acetate systems by *ca.* 10^3 and *ca.* 5×10^3, respectively. There is no kinetic dependence upon the



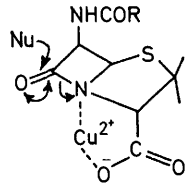
(IV)

concentration of trifluoroethylamine which indicates that aminolysis does not occur in this reaction. These observations may be rationalised by metal ion co-ordination to the carboxy-group in penicillin which is reduced or does not occur in the methyl ester. However, the rate of reaction of the methyl ester is increased by *ca.* 10^3 in the presence of 2×10^{-3}M-copper(II) ions and the rate is apparently first-order in metal ion. This may be due to either weak co-ordination, compared with penicillin itself, of the metal ion to the β-lactam nitrogen and the methoxycarbonyl group or to binding at another site of the penicillin molecule. The product of the reaction of the methyl ester in the presence of copper(II) ions is not known.

(b) The rate-limiting step for the uncatalysed reaction of amines with penicillin is thought to be breakdown of the tetrahedral intermediate, *i.e.* rate-limiting carbon–nitrogen bond fission.[27] This is compatible with the similar rate enhancement of *ca.* $3—9 \times 10^7$ for both trifluoroethylamine and hydroxide ion despite their large difference in basicity. However, this similar rate enhancement is probably incompatible with metal ion co-ordination to the β-lactam carbonyl group since such a situation may be expected to give rise to different rate enhancements for the two nucleophiles.

Incidentally, the second-order rate constant, at 30° and *I* 0.5M, for the hydrolysis of the β-lactam ring catalysed by hydroxide ion is 0.15 ± 0.02 l mol^{-1} s^{-1} for benzylpenicillin and 2.49 ± 0.15 l mol^{-1} s^{-1} for the methyl ester of benzylpenicillin. This rate difference of 17 also supports rate-limiting carbon–nitrogen bond fission in the hydroxide ion catalysed hydrolysis of penicillin, but could, of course, also reflect a simple electrostatic effect because the reaction between hydroxide ion and benzylpenicillin is one between two negatively charged ions.

It is proposed that nucleophilic attack by hydroxide ion or amine occurs on the β-lactam carbonyl group as



(V)

shown in (V) with the metal ion presumably facilitating both this step, by increasing the electrophilicity of the carbonyl carbon, and also the bond-breaking step. Protonation of the β-lactam nitrogen may not occur in the uncatalysed aminolysis of penicillin [27] because of the large amount of strain energy of the four-membered ring which is released upon carbon–nitrogen bond fission.[9] In this case the function of the copper(II) ion in the metal ion catalysed reaction may be regarded as stabilisation of the amine anion leaving group thus increasing the rate of carbon–nitrogen bond fission. If

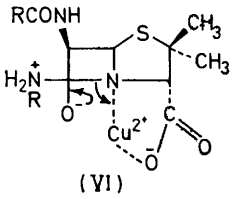
[30] G. V. Fazakerley and G. E. Jackson, *J.C.S. Perkin II*, 1975, 567.

[31] W. A. Cressman, E. T. Sugita, J. T. Doluisio, and P. J. Niebergall, *J. Pharm. Sci.*, 1969, **58**, 1471.

protonation of the β-lactam nitrogen does occur in the uncatalysed reaction, which seems unlikely,[27] then the function of the metal ion could simply be that of a 'super-acid' by substituting the role of the proton. Alternatively, because the metal ion facilitates carbon–β-lactam nitrogen bond fission the rate-limiting step of the reaction could change from breakdown of the tetrahedral intermediate for the uncatalysed reaction [27] to formation of the intermediate in the metal ion catalysed reaction.

A distinction between these mechanisms is made possible by examining the dependence of the rate constants for the attack of amine on the metal ion–penicillin complex upon the basicity of the attacking amine. A plot of the logarithm of these rate constants against the pK_a of the amine gives a Brønsted β value of 0.87 ± 0.06. This is taken to indicate that there is approximately a unit positive charge on the amine nitrogen in the transition state. A mechanism consistent with this involves the rate-limiting breakdown of the tetrahedral intermediate (VI).

It is worth noting that in equation (16) the k_1 term, which represents the uncatalysed reaction, is a minor pathway for the overall aminolysis of penicillin.[27] In the rate law for the metal ion catalysed reaction there is no evidence of a term second order in amine which, if present, would indicate general base catalysis by a



(VI)

second molecule of amine. The co-ordination of the metal ion to penicillin apparently makes this normally dominant mode of catalysis unnecessary.

If the mechanisms of the reactions of penicillin involves the intermediate formation of a keten (Scheme) then the products of the reaction should show deuterium incorporation at C-6 if the reactions are carried out in D_2O. The n.m.r. spectra of benzylpenicilloic acid obtained from the hydrolysis of benzylpenicillin in sodium deuterioxide–D_2O or in acetate buffers in D_2O in the presence of copper(II) ion show no incorporation of deuterium at C-6. This indicates that the elimination–addition mechanism is not a major pathway for either of these reactions.

The possibility arises that the metal ion catalysed aminolysis and hydrolysis of penicillin occurs through the intermediate formation of penicillenic acid (II) (Scheme). The formation of this rearrangement product is thought to occur by intramolecular nucleophilic attack of the side-chain amido-group upon the β-lactam carbonyl group and carbon–β-lactam nitrogen bond fission.[9,32] It is therefore conceivable that the rate of this rearrangement could also be facilitated by metal ions. However, the presence of copper(II) ion has little effect upon the rate of formation of penicillenic acid from benzylpenicillin. Furthermore, the rates of hydrolysis and aminolysis with trifluoroethylamine of benzylpenicillenic acid are retarded in the presence of copper(II) ion. Since the observed rates of hydrolysis and aminolysis of penicillin in the presence of copper(II) ion is at least 10^3 times faster than the rate of formation of penicillenic acid from penicillin these reactions cannot occur through the intermediate formation of penicillenic acid. The rate of penicillenic acid formation is presumably not increased by copper(II) ion since this reaction is thought to occur through the conjugate acid of the β-lactam carbonyl group [32] and metal ion co-ordination is not competitive with this pathway. This is further support for the suggested kinetically important site of co-ordination (IV).

Finally the proposed mechanism may be of relevance to the mechanism of the β-lactamase catalysed hydrolysis of penicillins in those cases where the enzyme is metal ion dependent. The role of the metal ion in these enzymes may be similar to its presently proposed function in the hydrolysis and aminolysis of penicillin (V).

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[7/1162 *Received*, 4*th July*, 1977]

[32] M. A. Schwartz, *J. Pharm. Sci.*, 1965, **54**, 472; H. Bundgaard, *ibid.*, 1971, **60**. 1273.