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Metal-ion Catalysed Hydrolysis of Some β-Lactam Antibiotics

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The metal(II)-ion catalysed hydrolysis of some pencillin and cephalosporin derivatives in water at 30° shows saturation kinetics. A 1 : 1 complex is formed between the metal ion and penicillin which is attacked by hydroxide ion up to 10^8 fold faster than the unco-ordinated compound. The site of co-ordination of the penicillins and copper(II) ions is the β-lactam nitrogen and the carboxylate group. The association constants for the cephalosporins and metal ions are greater than those for the penicillins but the transition states for the cephalosporin reaction with hydroxide ion bind less tightly to metal ions. The order of rate enhancement brought about by the metal ion is $Cu^{II} > Zn^{II} > Ni^{II} \sim Co^{II}$. The metal ions are thought to stabilise the tetrahedral intermediate formed by hydroxide ion attack on the β-lactam. Some comments are made about metal ions as electrophilic catalysts in enzymes.

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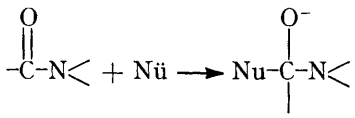
BACTERIAL resistance to β-lactam antibiotics is due mainly to the enzymic hydrolysis of the β-lactam ring of the antibiotic.[1] Many bacteria produce β-lactamases that hydrolyse both penicillins and cephalosporins to the harmless penicilloic and cephalosporoic acids although the relative maximum rates of hydrolysis of these substrates vary with the source of the enzyme.[2] Zinc(II) ion has been shown to be a cofactor for the demonstration of 'penicillinase' and 'cephalosporinase' activity of an enzyme from *Bacillus cereus* 569/H called β-lactamase II.[3]

The susceptibility of penicillins to attack by nucleophiles has been attributed to strain in the β-lactam ring[4] and to the non-planarity of the system which inhibits the usual amide resonance.[5] This non-planarity should lead to a greater availability, compared with that in normal amides, of the non-bonded pair of electrons on the β-lactam nitrogen for metal-ion co-ordination. Benzylpenicillin has in fact been shown to act as a bidentate chelating ligand, co-ordination to copper(II) ions was suggested to occur through the β-lactam nitrogen and the adjacent carboxy-group[6] although other co-ordination sites have been suggested.[7] Nucleophilic addition to the β-lactam carbonyl group to give a tetrahedral intermediate causes a large change in the basicity



(1)

of the β-lactam nitrogen (*ca.* 15 pK units) [equation (1)] so a metal-ion co-ordinated to this site should greatly increase the stability of the intermediate. We report here evidence that copper(II) ions co-ordinate to the β-lactam nitrogen and the carboxy-group of penicillins, the relative catalytic efficiency of various metal ions in increasing the rate of hydrolysis of penicillins and cephalosporins, and a comparison of the data with that for β-lactamase II.

EXPERIMENTAL

Materials.—Benzylpenicillin and 6β-aminopenicillanic acid were of general reagent grade, cephalosporins were kindly provided by Glaxo Limited, and other materials were of AnalaR grade.

Benzylpenicilloic acid was prepared by the method of Rapson and Bird.[8]

Benzylpenicillin methyl ester. Benzylpenicillin sodium salt (3 g) were dissolved in ice-cooled water (50 cm^3). The aqueous solution was acidified to pH 2.0 using molar perchloric acid, the free acid was then extracted into ether (3 × 75 cm^3) and dried over $MgSO_4$, and the volume reduced to 50—75 cm^3. To the dry ether solution of benzylpenicillin, cooled below 5 °C in a salt–ice–water-bath, was added an ethereal solution of diazomethane until gas evolution stopped and the solution remained pale yellow. The solution was left overnight to allow excess diazomethane to decompose slowly and ether to evaporate. The compound was redissolved in ether (30 cm^3), washed with hydrogencarbonate to remove an unchanged acid, and the ether layer separated and dried over $MgSO_4$. Evaporation of the ether left a thick oil which was triturated with hexane and a crystalline solid (50%) was obtained which was recrystallised from carbon tetrachloride, m.p. 94—95°; $\nu_{max.}$ (Nujol) 1 797, 1 775, 1 757, 1 744, 1 697, 1 678, and 1 520 cm^{-1}; δ($CDCl_3$) 1.45 (3 H, s, α-CH_3), 1.47 (3 H, s, β-CH_3), 3.65 (2 H, s, CH_2), 3.80 (3 H, s, OCH_3), 4.40 (1 H, s, 3-H), 5.45 (1 H, d, J 4.5 Hz, 5-H), 5.76 (1 H, d, J 4.5 Hz, 6-H), and 7.38 (5 H, s, aromatic) (Found: C, 58.25; H, 5.6; N, 7.9; S, 9.2. Calc. for $C_{16}H_{18}N_2O_4S$: C, 58.6; H, 5.7; N, 8.0; S, 9.3%).

6α-Chloropenicillanic acid. 6β-Aminopenicillanic acid (4.32 g) was dissolved in water (40 cm^3) to which was added methanol (140 cm^3) and concentrated HCl (20 ml), keeping the solution at 0 °C. A solution of sodium nitrite (1.6 g) in water (15 cm^3) was added in one portion and the solution stirred while warming to room temperature. The solution was then diluted with $CHCl_3$ and shaken with water. The organic layer was separated and dried over $MgSO_4$. The evaporated organic phase gave a yellow oil of 6α-chloropenicillanic acid. The oil was further treated by dissolving in ether cooled below 5 °C and adding 1 mol. equiv. benzylamine in ether solution to produce the benzylammonium salt. The salt (60%) was recrystallised from butanol–water, m.p. 145—146 °C; $\nu_{max.}$ (Nujol) 1 780 and 1 622 cm^{-1}; δ(D_2O) 1.50 (3 H, s, CH_3), 1.58 (3 H, s, CH_3), 4.26 (2 H, s, CH_2), 4.38 (1 H, s, 3-H), 5.06 (1 H, d, J 1.5 Hz, 6-H), 5.42 (1 H, d, J 1.5 Hz, 5-H), and 7.6 (5 H, s, aromatic) (Found: C, 52.75; H, 5.5; N, 8.3; S, 9.2; Cl, 10.5. Calc. for $C_{15}H_{19}ClN_2O_3S$; C, 52.6; H, 5.5; N, 8.2; S, 9.3; Cl, 10.4%).

6α-Bromopenicillanic acid. Sodium bromide (2.06 g) was dissolved in water (70 cm^3) and cooled to 0 °C. Concentrated HBr solution (9.0 cm^3) were added followed by

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6β-aminopenicillanic acid (4.32 g). To this cooled solution was added a solution of sodium nitrite (1.67 g) in water (6 cm^3) dropwise, the solution being rapidly stirred. Care was required as the solution foamed. After the addition of the sodium nitrite, 10 min was allowed for the completion of the reaction. Ether (50 cm^3) was added, the mixture shaken, and the layers separated. The aqueous layer was further extracted with ether (2 × 50 cm^3) and the ether layers combined, washed with water, and separated. The organic layer was shaken with sodium chloride solution (saturated; 15 cm^3), separated, and dried over Na_2SO_4. The ether was removed by vacuum distillation leaving a brown gum (3.82 g). The compound was redissolved in ether (50 cm^3), cooled to 20 °C, and a solution of benzylamine in ether was added dropwise to form the benzylammonium salt. The crude product (60%) was filtered, washed with ether, and dried in a desiccator, $\nu_{max.}$ (Nujol) 1 778 and 1 625 cm^{-1}; δ(D_2O) 1.50 (3 H, s, CH_3), 1.58 (3H, s, CH_3), 4.18 (2 H, s, CH_2), 4.34 (1 H, s, 3-H), 5.02 (1 H, d, 6-H), 5.43 (1 H, d, 5-H), and 7.46 (5 H, s, aromatic).

Penicillanic acid. In the hydrogenolysis of 6α-halogeno-compounds it is necessary to use large amounts of catalyst due to the poisoning of the catalysts by sulphur byproducts. The free acid (4 g) was dissolved in methanol (10 cm^3) and water (5 cm^3) was added. The solution was cooled in ice and the pH adjusted to 7 with M-sodium hydroxide. The solution was then hydrogenated using of pre-reduced 5% Pd–$CaCO_3$(4 g) for 2 h.

The catalyst was filtered off and washed with methanol–water. The methanol was then removed by vacuum distillation and the aqueous phase covered with chloroform. The pH of the solution was then adjusted to 2, the layers separated, and the aqueous layer extracted with chloroform (2 × 50 cm^3). The organic layer was then dried over $MgSO_4$, filtered, and evaporated to yield a yellow oil (2.26 g, 75%), $\nu_{max.}$ (oil) 1 785 and 1 620 cm^{-1}; δ($CDCl_3$) 1.54 (3 H, s, CH_3), 1.72 (3 H, s, CH_3), 3.02 (1 H, 2d, $J_{5,6\beta}$ 1.5 Hz, 6β-H), 3.50 (1 H, 2d, $J_{5,6}$ 4, $J_{6\alpha,6\beta}$ 16 Hz, 6α-H), 4.22 (1 H, s, 3-H), and 5.25 (1 H, 2d, 5-H) (Found: C, 47.15; H, 5.4; N, 6.5. Calc. for $C_8H_{11}NO_3S$: C, 47.7; H, 5.45; N, 6.95%).

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

The rates of reaction of the β-lactam antibiotics 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 β-lactam 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.[9] The hydrolysis reactions were generally studied at 265—275 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.

Equilibrium Constant Measurements.—Penicilloate- and cephalosporate-metal ion and buffer-metal ion complexes. 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.[10] Both metal-ion and the ligand concentrations were varied independently.

Some equilibrium constants were also determined kinetically as described in the text.

TABLE 1

Summary of the rate and association constants for the copper(II) catalysed hydrolysis of β-lactam derivatives in water at 30° (*I* 0.5M)

β-Lactam	k_{OH} [a]/l mol^{-1} s^{-1}	*K* [b]/l mol^{-1}	k_2^{OH} [c]/l mol^{-1} s^{-1}	k_2^{OH}/k_{OH}
Benzylpenicillin	0.154	187	1.22×10^7	8×10^7
Benzylpenicillin methyl ester	2.51	— [d]	$\leqslant 3 \times 10^4$ [d]	$< 1.5 \times 10^4$
6β-Aminopenicillanic acid	6.35×10^{-2}	232	5.15×10^6	8×10^7
Penicillanic acid	7.40×10^{-3}	120	1.58×10^6	2×10^7
Cephaloridine	0.526	2 080	1.64×10^4	3×10^4
3-Methyl-7β-phenylacetamidoceph-3-em-4-carboxylic acid	2.41×10^{-2}	2 400	1.56×10^3	7×10^4

[a] Second-order rate constant for the hydroxide-ion catalysed hydrolysis. [b] Association constant for metal ion and β-lactam. [c] Second-order rate constant for the hydroxide-ion catalysed hydrolysis of metal-ion bound β-lactam. [d] $k_2^{OH}K$, saturation was not observed.

RESULTS

The pH–rate profile for the decomposition of benzylpenicillin (I) in water has been reported previously.[6] Above pH 4 the product is benzylpenicilloic acid (II) which is formed directly above pH 7 but partially *via* penicillenic acid below this pH.[11] The reaction is very slow around neutral pH, for example, the half-life at pH 7 is *ca.* 1 year.[6] The second-order rate constants for the hydroxide-ion catalysed hydrolysis of the cephalosporins (III) are given in Table 1. The immediate product of hydrolysis is the cephalosporoic acid (IV) or the corresponding imine (V) if there is a good leaving group, such as pyridine or acetate, at C-3.[12]

In the presence of zinc(II) ions there is a large enhancement in the rate of decomposition of penicillin in water. In acetate and cacodylate buffers the apparent first-order rate constant, k_{obs}, for the loss of penicillin in the presence of an excess of zinc(II) ions shows a complex dependence upon the metal-ion concentration [Figure 1 and Supplementary Publication No. SUP 22869 (11 pp.)*].

At low metal-ion concentration the apparent first-order rate constant is approximately first order in metal ion but with increasing concentration of zinc(II) ions it has a decreasing dependence upon metal-ion concentration. This saturation phenomenon is interpreted in terms of formation

* For details of Supplementary Publications see Notice to Authors No. 7 in *J.C.S. Perkin II*, 1979, Index Issue.

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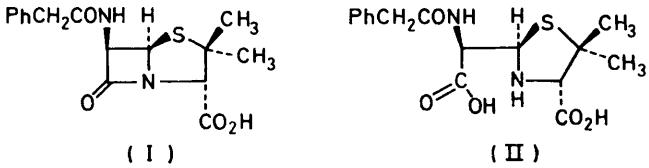
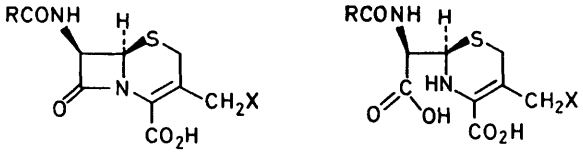
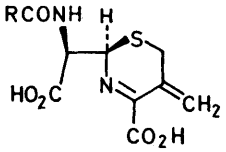
of a penicillin–metal-ion complex which then breaks down to the products of the reaction. The sole product of this reaction is the hydrolysis product, penicilloic acid (II). The kinetics of this reaction are further complicated by the decrease in the apparent first-order rate constant with



(I) (II)



(III) (IV)



(V)

increasing concentration of buffer but with pH, ionic strength, penicillin, and zinc(II) ion concentrations all constant. This is interpreted in terms of an effective decrease in the metal-ion concentration with increasing buffer concentration because of the known complex formation between acetate ion and zinc(II) ion. A kinetic

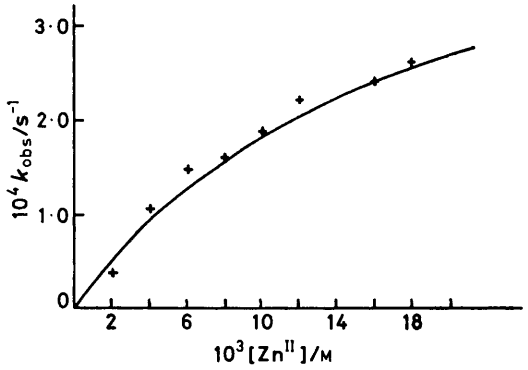


FIGURE 1 Plot of the apparent first-order rate constant for the hydrolysis of benzylpenicillin at pH 5.81 and 30.0° (I 0.50M) as a function of total zinc(II) ion concentration. The solid line is calculated from the constants given in the text

scheme compatible with these observations is as shown in equation (2) where M represents the metal(II) ion, P is

$$\begin{array}{l} M + P \underset{}{\overset{K_1}{\rightleftharpoons}} MP \xrightarrow{k_2'} \text{products} \\ \quad -B \Big\| K_2 \\ \quad MB \end{array} \qquad (2)$$

penicillin, B is acetate or cacodylate ion, MP is the penicillin–metal(II)-ion complex and MB is the buffer–metal(II)-ion complex. The concentrations of these two complexes are given by equations (3) and (4) where M_0, B_0, and P_0 are the initial concentrations of metal ion, buffer anion, and penicillin respectively. Under the condition of $B_0 \gg$

$$MP = K_1(M_0 - MP - MB)(P_0 - MP) \qquad (3)$$

$$MB = K_2(M_0 - MP - MB)(B_0 - MB) \qquad (4)$$

M_0, $M_0 \gg P_0$ and so therefore $M_0 \gg$ MP, the observed apparent first-order rate constant is given by equation (5).

$$k_{obs} = \frac{k'_2 K_1 M_0}{1 + K_2 B_0 + K_1 M_0} \qquad (5)$$

A plot of $1/k_{obs}$ against $1/M_0$ should give a straight line with $1/k_2'$ as the intercept and this is shown in Figure 2. The slope of this line when plotted against B_0 (Figure 3) yields $1/k_2'K_1$ as the intercept and $K_2/k_2'K_1$ as the slope. K_1' the equilibrium constant for penicillin–zinc(II)-ion complex formation was found to be 109 ± 10 l mol^{-1}. The value of K_2, the equilibrium constant for zinc(II)-ion–cacodylate-ion complex formation was found to be 21.0 l mol^{-1} and this may be compared with a value of 20.0 l mol^{-1} obtained from a Brønsted plot of the complex formation constants of zinc(II) ions with oxygen bases.[13] k_2' is pH-dependent and is first-order in hydroxide ion with $k_2'/[OH^-]$ being 6.25×10^4 l mol^{-1} s^{-1}. The hydroxide ion concentrations were calculated from the observed pH and defined as antilog(pH − pK_w) with pK_w 13.83 at 30°.[14]

The data were also analysed under the conditions of $B_0 \gg M_0$ using equation (5) with MB calculated as described previously.[6] The value of K_1 using this method

$$k_{obs} = \frac{k_2' K_1(M_0 - MB)}{1 + K_1(M_0 - MB)} \qquad (5)$$

was found to be 119 l mol^{-1} and that of $k_2'/[OH^-]$ to be 5.70×10^4 l mol^{-1} s^{-1}; both values are in reasonable agreement with those obtained using equation (4).

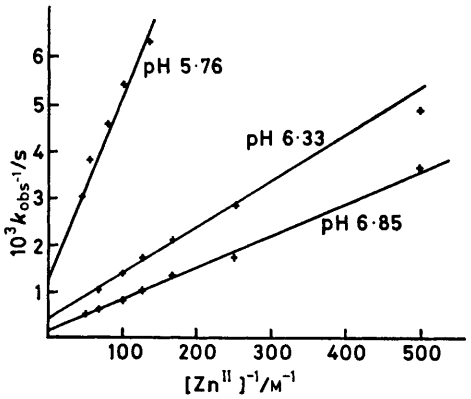


FIGURE 2 Plot of the reciprocal of the apparent first-order rate constant for the hydrolysis of benzylpenicillin in cacodylate or acetate buffers at 30.0° and at the pH values indicated as a function of the reciprocal of the zinc(II) concentration

A summary of the association constants for benzylpenicillin and cephaloridine with several metal ions is given in Table 2 together with the second-order rate constants for the hydroxide-ion catalysed hydrolysis of the metal-bound antibiotic.

In order to elucidate the site of co-ordination of the metal ion to the β-lactam antibiotic the kinetics of the copper(II)

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ion catalysed hydrolysis of some penicillin and cephalosporin derivatives were determined. The binding and rate constants are summarised in Table 1.

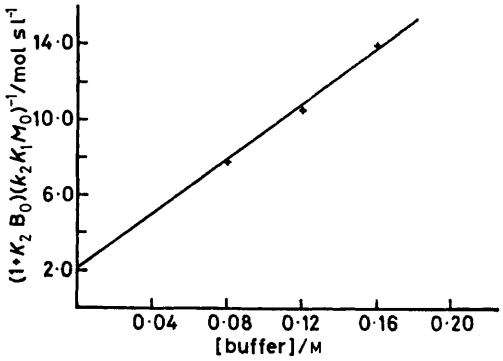


FIGURE 3 Plot of the slopes of the lines of Figure 2 against the total buffer concentration

DISCUSSION

Cobalt(II), nickel(II), zinc(II), and copper(II) ions cause an enormous increase of up to 10^8, in the rate of the hydroxide-ion catalysed hydrolysis of the β-lactam antibiotics (Tables 1 and 2). A 1 : 1 complex between the metal ion and the antibiotic is formed but before discussing the nature of the rate enhancement the site of co-ordination will be described.

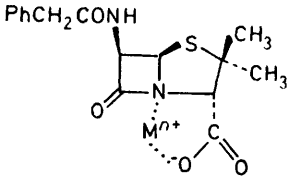
TABLE 2

Summary of the rate and association constants for the metal ion catalysed hydrolysis of benzylpenicillin and cephaloridine in water at 30° (I 0.5M)

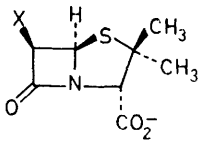
	benzylpenicillin		cephaloridine	
Metal ion	k_2^{OH} [a]/l mol^{-1} s^{-1}	K [b]/l mol^{-1}	k_2^{OH} [a]/l mol^{-1} s^{-1}	K [b]/l mol^{-1}
Cu^{II}	1.22×10^7	187	1.64×10^4	2 080
Zn^{II}	6.0×10^4	109	8.75×10^2	2 181
Ni^{II}	5.9×10^3	119		
Co^{II}	4.1×10^3	178		

[a] Second-order rate constant for the hydroxide-ion catalysed hydrolysis of the metal bound β-lactam. [b] Association constant for metal ion and β-lactam.

(a) *Site of Metal-ion Co-ordination.*—We have previously suggested [6] that copper(II) ion co-ordinates to the carboxylate group and the β-lactam nitrogen of benzylpenicillin [complex (VI)] but other sites have been proposed.[7] Co-ordination occurs to the carboxylate group because esterification of this group decreases the rate enhancement by *ca.* 5×10^3. Nonetheless, the rate of the hydroxide-ion catalysed hydrolysis of the copper(II) bound methyl ester of benzylpenicillin is still 1.5×10^4-fold faster than the rate of hydrolysis of the unco-ordinated ester, although the product of the reaction is not known. The i.r. spectrum of copper(II)–β-lactam solid mixtures shows a decrease in the carboxylate carbonyl stretching frequency of *ca.* 40 cm^{-1} compared with that of the sodium salt.[15]



(VI)



(VII)

Molecular models indicate that one of the conformations of cephalosporins would be very suitable for metal-ion co-ordination between the carboxylate group and the β-lactam carbonyl oxygen. However, in neither the cephalosporins nor the penicillins is there any change in the β-lactam carbonyl stretching frequency in solid mixtures of zinc(II) or copper(II) ions and the β-lactam. The ^{1}H n.m.r. spectrum of D_2O solutions of benzylpenicillin and metal ions shows a change in the chemical shift of the CH attached to the carboxylate group.[15] These observations are consistent with metal-ion co-ordination to the carboxylate group. Benzylpenicillin and cephaloridine (III; X = pyridyl) probably act as bidentate chelating ligands as the binding constants to copper(II) ions of 187 and 2 080 l mol^{-1}, respectively, are much greater than that predicted, *ca.* 10 l mol^{-1}, for a carboxylic acid of pK_a *ca.* 2.7 from a Brønsted plot of binding constants against pK_a.[13] (log K = 0.42 pK_a − 0.17). It is intuitively logical that the second co-ordination site is the β-lactam nitrogen in the penicillins. However, in the cephalosprins we cannot exclude the possibility that co-ordination occurs between the carboxylate group and the β-lactam carbonyl oxygen.

It has been suggested that copper(II) ions co-ordinate to the 6-acylamino side chain and the β-lactam carbonyl group.[7] Replacement of the acylamino side-chain by the more basic amino-group, (VII; X = NH_2), has little effect upon the binding constant and the rate enhancement for the hydroxide-ion catalysed hydrolysis is very similar to that for benzylpenicillin (Table 1). Furthermore, complete removal of the amido side chain as in penicillanic acid (VII; X = H), also gives similar binding constants and rate enhancements (Table 1). It is apparent that copper(II) ions do not bind to the amido-side-chain in penicillins.

Model studies of the binding of metal ions to α-amido-carboxylic acids indicate that co-ordination between the carboxylate group and the amide nitrogen does occur although the evidence is not unambiguous.[16] Furthermore, of course, the thermodynamically favoured binding site is not necessarily the kinetically important one.

(b) *Rate Enhancement.*—The hydroxide-ion catalysed hydrolysis of benzylpenicillin probably proceeds by the formation of the tetrahedral intermediate (VIII). The pK_a of the bridgehead nitrogen in (VIII) is estimated to be 8.0 [17] so there is a change of many pK units (>12) compared with that of the nitrogen in the β-lactam. The role of the metal ion in the hydroxide-ion catalysed hydrolysis could then be simply to stabilise the tetrahedral intermediate. An estimation of the binding constant of copper(II) ions to (VIII) can be made from a comparison with model compounds. Based on a Brønsted plot (not shown) for the binding of model ligands such as thioproline, proline, and glycine the estimated

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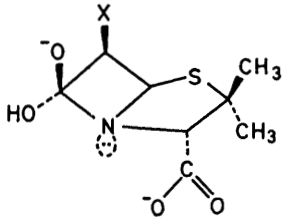
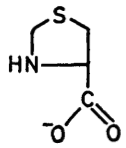
association constant for copper(II) and (VIII) is $10^{7.2}$ l mol^{-1}. This may be compared with the ground state equilibrium constant of $10^{2.27}$ l mol^{-1} for (I) which has a pK_a for nitrogen-protonation of < -1.



(VIII)



(IX)

The rate of the hydroxide-ion catalysed hydrolysis of copper(II)-bound benzylpenicillin is 8×10^7 faster than that of unco-ordinated benzylpenicillin.[6] A better estimation of the stabilisation of the transition state by the metal ion is from the comparison of the third-order rate constant, k_2K_1, for the metal and hydroxide ion catalysed hydrolysis with the second-order rate constant for the hydroxide-ion catalysed hydrolysis. For copper(II) ions and benzylpenicillin this ratio is 1.2×10^{10} mol l^{-1} (equivalent to 58.1 kJ mol^{-1} at 30°) and, of course, is dependent upon the choice of standard state in which to express the rate constant. Copper(II) ion thus stabilises the transition state for hydroxide ion catalysis by 58.1 kJ mol^{-1} at 30° compared with an estimated value of 41.5 kJ mol^{-1} (RTln $10^{7.2}$) for the stabilisation of the tetrahedral intermediate (VIII). The discrepancy of 16 kJ mol^{-1}, a factor of $10^{2.9}$, could arise from an underestimation of the basicity of the tertiary nitrogen in the tetrahedral intermediate (VIII) or because the latter is not a good model for the transition state. If the rate limiting step involved C–N bond fission the nitrogen would be more basic than that estimated for (VIII) and copper(II) ions would bind more tightly.

Figure 4 illustrates the free-energy changes diagramatically. Initial binding of penicillin to copper(II) stabilises the system by 12.6 kJ mol^{-1} and as the difference in free energy of activation for hydroxide ion catalysed hydrolysis of unco-ordinated and co-ordinated penicillin is 45.4 kJ mol^{-1}, copper(II) ion must stabilise the transition state by 58.0 kJ mol^{-1}.

(c) *Effect of Transition Metal Ion.* The rate enhancements brought about by various metal ions for the hydrolysis of penicillins and cephalosporins are summarised in Tables 1 and 2. There is no correlation between the binding constant of the β-lactam antiobiotic with the metal ion and the rate enhancement. Spectrophotometric methods to measure the metal ion binding constants of the hydrolysis products, penicilloic and cephalosporoic acids, were unsuccessful. Thioproline (IX) is probably a reasonable model for the transition state, where the ring nitrogen will be more amine-like than amide-like and Figure 5 shows a plot of the third-order rate constants for the metal-ion and hydroxide-ion catalysed hydrolysis against the association constants for metal ion and thioproline.[18] The order of reactivity is that of the Irving–Williams series,[19] $Co^{II} < Ni^{II} < Cu^{II} > Zn^{II}$.

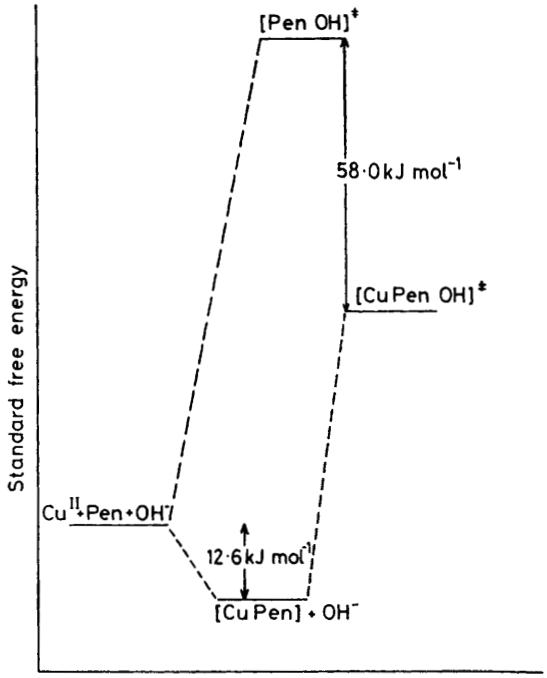


FIGURE 4 Plot of the standard free-energy change for the copper(II) catalysed hydrolysis of benzylpenicillin compared with the hydroxide-ion catalysed reaction at a standard state of 1M

(d) *Effect of* β-*Lactam.*—Copper(II) ions bind *ca.* 10-fold more tightly to cephalosporins than to penicillins (Table 1). To us, this is surprising in view of the greater non-planarity of the penicillin molecule (greater

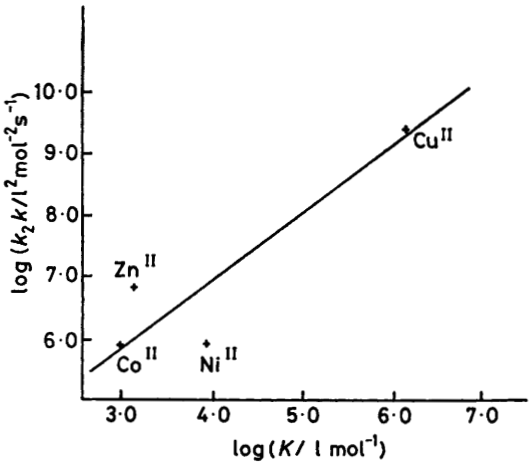


FIGURE 5 Plot of the logarithm of the third-order rate constants at 30° for the metal-ion and hydroxide-ion catalysed hydrolysis of benzylpenicillin against the logarithm of the association constants of thioprolinate and metal ion at 25°

reduction in amide resonance?), the possibility of enamine type conjugation in cephalosporins and the less favourable geometry (sp^2 C at C-3 and -4) in cephalosporins for

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metal-ion co-ordination to the β-lactam nitrogen and the carboxylate group. Based on, perhaps, the rather naive view that the strength of metal-ion co-ordination would be dependent upon the basicity of the β-lactam nitrogen we had erroneously anticipated penicillins to be a better ligand. Nonetheless, the rate of hydroxide ion catalysed hydrolysis of copper(II)-bound cephaloridine (III; X = pyridyl, R = $C_4H_3SCH_2$) is *ca.* 3×10^4 faster than that for the unco-ordinated compound. This may be compared with a rate enhancement of 8×10^7 for benzylpenicillin. The third-order rate constant for the copper(II) ion, hydroxide-ion catalysed hydrolysis of cephaloridine, k_2K, is 1.6×10^8 mol l^{-1} fold greater than the second-order rate constant for hydroxide-ion catalysed hydrolysis of the same substrate. The corresponding ratio for benzylpenicillin is 1.2×10^{10} mol l^{-1}. The transition state for cephaloridine hydrolysis is therefore stabilised by copper(II) ions *ca.* 100-fold less than that for penicillin hydrolysis, but both transition states are greatly stabilised by the metal ion. Again, *ad hoc* explanations for this difference may be found in the lower basicity of the ring nitrogen in the tetrahedral intermediate formed from cephaloridine and/or a less favourable geometry.

Whether or not the X group of the cephalosporin (III) is expelled to give (IV) or (V) makes little difference to the rate enhancement brought about by the metal ion. The 3-methyl derivative (III; X = H) has a similar association constant for copper(II) ion binding to that for cephaloridine (III; X = pyridyl) (Table 1). The rate enhancement brought about by copper(II) ion is the same within a factor of 2.

The zinc(II) ion dependent β-lactamase from *B. cereus* 569/H catalyses the hydrolysis of benzylpenicillin to benzylpenicilloic acid. At pH 7.0 and 30° the half-life for benzylpenicillin bound to β-lactamase II is *ca.* 5×10^{-4} s [20] compared with one of 100 s when it is bound to zinc(II) ions. This comparison involves the effectiveness of 10^{-7} mol l^{-1} hydroxide ion with an intramolecular nucleophile (unknown) in the enzyme. It is not known whether benzylpenicillin is co-ordinated to the zinc(II) of β-lactamase II but the apparent binding constant to the enzyme is similar to that for zinc(II) ion. The protein must provide significant binding energy in the transition state to bring about its large catalytic advantage compared with simple zinc(II) ions.[21]

β-Lactamase (II) increases the rate of hydrolysis of benzylpenicillin by a factor of *ca.* 10^{13} l mol^{-1} from a comparison of the rate of hydrolysis of 2.5×10^{-8} s^{-1} at pH 7 [6] with k_{cat}/K_m *ca.* 2×10^5 l mol^{-1} s^{-1} for the enzyme catalysed reaction.[20] Zinc(II) ions increase the rate of hydrolysis of benzylpenicillin by a factor of 2.5×10^7 l mol^{-1} s^{-1} at pH 7 ($k_2^{OH}K[OH^-]/2.5 \times 10^{-8}$). *If* β-lactamase (II) utilizes Zn^{II} in a similar mechanism to that illustrated in this work [(VI)] then the protein increases the efficiency of Zn^{II} catalysis by 4×10^5.

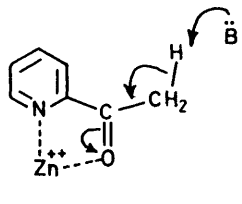
Metal Ions as Electrophilic Catalysts in Enzymes.—The co-ordination of electron donors to metal ions is an exergonic process. It is often suggested that one role for metals in metalloenzymes is to act as electrophilic catalysts by stabilising the increased electron density or negative charge that is often developed during reactions.[22] Although this is undoubtedly true it has been frequently stated that because the metal ion may be multiply charged it is a better catalyst than a proton [23] and metal ions have been referred to as ' superacids '.[24] Unfortunately there is little evidence to support this claim either for the binding or the activation of substrates. A proton binds more tightly to monodentate and even some bidentate ligands than do most mono-, di-, or even tri-positively charged metal-ions. The equilibrium of equation (6) is invariably more favourable than that of equation (7) and this is also often true even

$$L + H_3O^+ \rightleftharpoons HL^+ + H_2O \quad (6)$$

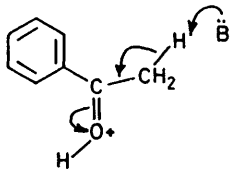
$$L + M(H_2O)_x^{n+} \rightleftharpoons ML(H_2O)^{n+}_{x-1} + H_2O \quad (7)$$

if L is a bidentate ligand.[25] A proton ' binds ' to an electron more tightly than any metal ion as indicated by its ionisation potential.[26] This is not surprising in view of the electron density surrounding the nucleus of a metal ion compared with that of the ' bare ' proton; the latter in fact could be called a ' hyperacid '.

A related consequence of the tight binding of a proton to basic sites is that such co-ordination increases the reactivity of adjacent bonds more so than does co-ordination of a metal ion. For example, co-ordination of a proton to a water molecule changes its pK_a from 15.7 to −1.7 whereas co-ordination of a divalent metal-ion changes it to *ca.* 8 to 9 and even a tripositively charged ion only to *ca.* 2 to 4.[27] There is very little evidence that the binding of metal ions to substrates causes a larger rate enhancement than do protons. For example, although metal ions greatly increase the rate of enolisation of suitably structured ketones they are no more efficient than the hydronium ion. Zinc(II) ions greatly increase the rate of proton abstraction from 2-



(X)



(XI)

acetylpyridine (X) [28] but it is no more efficient than the co-ordination of a proton to the carbonyl group (XI).

Metal ions are effective electrophilic catalysts for a wide variety of reactions [23] but they generally owe this efficiency to one of two factors. (1) The model substrates used invariably have a second co-ordination site available that is much more basic than the reactive site. If these two sites are suitably situated the substrate can act as a bidentate ligand. When reaction occurs this is usually accompanied by an increase in basicity of the reactive site co-ordinated to the metal ion which

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leads to more favourable binding and consequently a lowering of the activation energy. The metal ion binds more tightly to the transition state than it does to the ground-state structure of the substrate. (2) In neutral aqueous solution the concentration of the hydronium ion is limited. For example at pH 7 the hydronium-ion concentration is 10^{-7}M whereas the metal-ion concentration may be orders of magnitude higher than this.

Extrapolation of observations obtained from model systems to enzymes must be treated with caution. Metalloenzymes do not operate with high concentrations of metal ions and for those cases that have been studied the substrate usually acts as a monodentate ligand when it is directly co-ordinated to a metal ion, *e.g.*, the carbonyl oxygen of the amide link to be cleaved co-ordinates to the zinc atom of carboxypeptidase.[29] The protein itself is probably responsible for a large fraction of the binding energy resulting from the interaction of the substrate with the metalloenzyme.[30]

When the metal ion of the metalloenzyme acts as an electrophilic catalyst it serves an important function of stabilising the negative charges developed in the substrate. However, there is little evidence to suggest that it is markedly more efficient at this task than proton donors in the protein. For example, an indication of the stabilisation of a negative charge on oxygen brought about by a metal ion can be estimated from the binding energies of this species resulting from co-ordination to the metal ion. The equilibrium constant for zinc(II) ion binding hydroxide ion [equation (8)] is *ca.* 10^5 estimated from K_a/K_w where K_a is the dissociation constant for the ionisation of zinc-bound water [27] and K_w is the dissociation constant of water. The ' stabilisation ' of hydroxide-ion by a proton donor HA may be

$$Zn(H_2O)_n^{2+} + OH^- \rightleftharpoons Zn(H_2O)_{n-1}OH^+ + H_2O \quad (8)$$

$$H\text{–}A + OH^- \rightleftharpoons H_2O + A^- \quad (9)$$

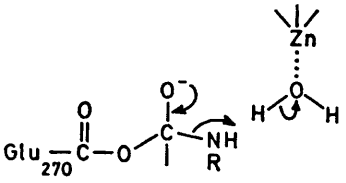
estimated from equation (9) where the equilibrium constant is given by K_a'/K_w where K_a' is the dissociation constant of the acid HA. For a pK_a' of 7 for HA the equilibrium constant for equation (4) is *ca.* 10^7. Although the stabilisation of the negatively charged oxygen by the zinc(II) ion is considerable it is no better than a weak proton donor.

In carboxypeptidase the carbonyl oxygen of the amide substrate is co-ordinated to the zinc(II) of the enzyme. The metal ion will stabilise the tetrahedral intermediate by binding to the alkoxide anion [equation (10)].

$$R\text{–}\underset{\underset{NHR}{|}}{C}{=}O \cdots Zn^{2+} \xrightarrow{Nü} Nu\text{–}\overset{\overset{R}{|}}{\overset{+}{C}}\text{–}O^- \cdots Zn^{2+} \quad (10)$$

Incidentally such stabilisation would only definitely lead to a rate enhancement if formation of the intermediate were rate limiting. If breakdown of the intermediate is the rate-limiting step the advantage of co-ordination is not so obvious. Although co-ordination would increase the concentration of the tetrahedral intermediate it would decrease its rate of breakdown. The net effect would depend on the relative charge density on oxygen in the transition state.

The binding of the carbonyl oxygen of amides to zinc(II) of carboxypeptidase is based on *X*-ray studies of inhibitors,[29,31] but, of course, the amides studied may be inhibitors because they bind incorrectly. An alternative mechanism could involve metal-ion co-ordination to the amide nitrogen as described herein for the hydrolysis of penicillin. Another possibility is general acid catalysed breakdown of the tetrahedral intermediate [32] by zinc(II)-bound water (XII). This mechanism has the



(XII)

advantage of explaining the *relative* insensitivity of the peptidase activity to the nature of the metal ion in carboxypeptidase.[29,31] However, the stereochemistry of carboxypeptidase catalysed enolisation of ketones indicates that zinc(II) co-ordinates to the carbonyl oxygen.[33]

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