The Copper(II) Promoted Hydrolysis of Benzylpenicillin. Evidence for the Participation of a Cu–OH Species in the Hydrolysis of the β-Lactam Ring

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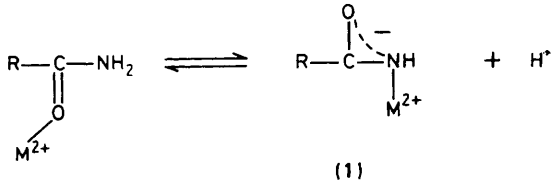
The pK_a of benzylpenicillin (HL $\rightleftharpoons$ L^- or H^+) relating to the carboxyl group ionisation has been determined to be 2.67 ± 0.005 at 10 °C and 2.60 ± 0.008 at 5 °C and I = 0.1 mol dm^{-3}. At a 1 : 1 ligand-to-metal ratio the interaction of copper(II) with benzylpenicillin can be described by the equilibria (i) and (ii) at 10 °C. The pK for the ionization $[CuLH_{-1}] \rightleftharpoons [CuLH_{-2}]^- + H^+$ is 4.92 at

$$L^- + Cu^{2+} \rightleftharpoons [CuLH_{-1}] + H^+; \log \beta_{11-1} = -1.41 \pm 0.1 \quad (i)$$

$$L^- + Cu^{2+} \rightleftharpoons [CuLH_{-2}]^- + 2H^+; \log\beta_{11-2} = -6.33 \pm 0.08 \quad (ii)$$

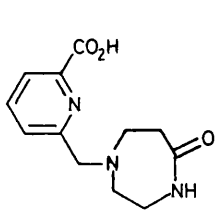
10 °C and I = 0.1 mol dm^{-3}. No potentiometric evidence was obtained for a species $[CuL]^+$. Possible structures for $[CuLH_{-1}]$ and $[CuLH_{-2}]^-$ are considered involving deprotonation of the side chain amide group and a water molecule co-ordinated to copper(II). The copper(II)-promoted hydrolysis of benzylpenicillin to penicilloic acid has been studied over the pH range 4.3—5.3 at 30 °C and I = 0.5 mol dm^{-3} using non-complexing 2,6-dimethylpyridine-3-sulphonic acid buffers. The dependence of the rate on the copper concentration and the pH strongly suggests that the complex $[CuLH_{-2}]^-$ is the active species in the reaction. Hydrolysis of the lactam ring of benzylpenicillin occurs by intramolecular attack of a Cu–OH species on the carbonyl group and not by intermolecular attack of 'free' hydroxide ion on the complex. The hydrolysis of $[CuLH_{-2}]^-$ at pH 6 is some 6 × 10^6 fold faster than the hydrolysis of the anion L^- at 30 °C.

The catalytic role of various divalent metal ions in hydrolytic metalloenzymes has been a topic of considerable interest. Although large rate enhancements have been observed in model systems for ester hydrolysis[1] and nitrile hydration,[2] until recently there have been few examples of significant catalysis of amide bond cleavage, except for reactions involving cobalt(III) complexes.[3] A prime reason for this lack of reactivity is that many metal complexes of primary and secondary amides deprotonate in weakly basic solution to give complexes of the type (**1**)[4] which are not susceptible to nucleophilic attack by hydroxide ion.[3,5]



(1)

Recently metal ions have been shown to promote the hydrolysis of the lactam (**2**)[6] which gives 1:1 complexes with Cu^{II}, Zn^{II}, and Co^{II} of the type (**3**). In the case of the copper(II) complex, the pK_a of the co-ordinated water molecule is *ca.* 7.6. Hydrolysis of the copper(II) complex is 1.6 × 10^6 fold faster than that of the free lactam at pH 7.6, while a rate enhancement of 1.9 × 10^5 fold occurs with Zn^{II}. Nucleophilic attack by metal bound hydroxide ion was considered to be the most plausible pathway for hydrolysis. Further studies[7] with the Zn^{II} complex (**4**) provide strong evidence for intramolecular hydrolysis *via* nucleophilic attack by zinc bound hydroxide ion, with a rate enhancement of 1.4 × 10^7 at pH 7 and 50 °C. Recent studies by



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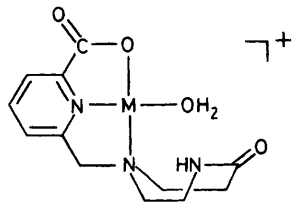

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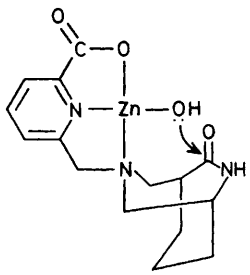

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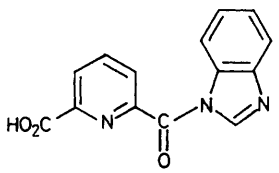
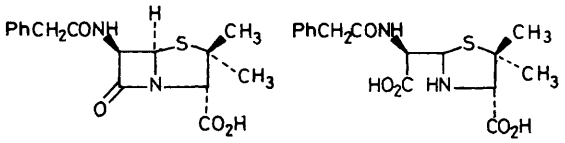


(5)

Fife and Przystas[8] have also provided evidence for intramolecular attack by co-ordinated Cu–OH in the metal promoted hydrolysis of *N*-(6-carboxypicolinoyl)benzimidazole (**5**), at 30 °C. In addition, hydrolysis of 1-acetylimidazole in the presence of exchange-inert metal complexes $[M(NH_3)_5(OH)]^{2+}$ (M = Cr^{III} or Co^{III}) has been shown to occur by a nucleophilic rather than a general base-catalysed pathway.[9]



(6) (7)

Page and co-workers[10,11] have previously shown that the hydrolysis of benzylpenicillin (**6**), to penicilloic acid (**7**), is strongly promoted by copper(II) with a rate enhancement of the order of 10^7 fold. The reaction was considered to involve attack by external hydroxide on the 1:1 complex but the mechanism was not well defined. In addition, the reaction was studied using acetate buffers and complications arose due to the formation of acetato complexes. Acetate forms quite stable complexes with copper(II) ($\log K_1 = 2.40$ at 30 °C).[12] The formation of mixed-ligand complexes with acetate could obscure any intramolecular pathway involving Cu–OH. For this reason we have re-examined this reaction using 2,6-dimethylpyridine-3-sulphonic acid buffers. The latter buffer is essentially non-complexing with copper(II) ($K \sim 2$ dm^3 mol^{-1}).[13] In addition, we have studied the interaction of copper(II) with benzylpenicillin by potentiometric techniques in an attempt to identify, in conjunction with the kinetic studies, the catalytically active complexes in solution.

It is known that β-lactamase II produced by *Bacillus cereus* which catalyses the hydrolysis of the β-lactam ring of penicillins and cephalosporins displays maximal activity in the presence of zinc(II), but significant activity is also observed in the presence of Co^{II}, Cd^{II}, and Mn^{II}.[14,15] Studies of the metal-promoted reaction may help to define some aspects of the mechanism of the enzymatic reaction.

Experimental

The potassium salt of benzylpenicillin (Pen G, $C_{16}H_{17}KN_2O_4S$) was obtained from Beecham Research Laboratories and was used without further purification.

2,6-Dimethylpyridine-3-sulphonic acid (dmps) was prepared by sulphonation of 2,6-dimethylpyridine essentially as described by McElvain and Goese[16] for the sulphonation of pyridine. The compound was twice recrystallised from hot water after prior treatment with charcoal. The sulphonic acid derivative does not have a sharp melting point, melting in the range 305—310 °C (Found: C, 45.1; H, 4.8; N, 7.5. Calc. for $C_7H_9NO_3S$: C, 44.9; H, 4.85; N, 7.5%). Molecular weight by potentiometric titration, 187 (calc. 187.2). The pK_a of dmps was estimated by potentiometric titration of a 9.79×10^{-3} mol dm^{-3} solution (50 cm^3) with sodium hydroxide (0.2 mol dm^{-3}) at $I = 0.5$ mol dm^{-3} (KNO_3) and 25 °C. The practical pK_a is 4.86 ± 0.01, in good agreement with the value of 4.80 ± 0.05 quoted in the literature.[13]

Kinetics.—The copper(II)-promoted hydrolysis was studied using dmps–NaOH buffers. Elias and co-workers[13] have shown that this is an excellent non-co-ordinating buffer which minimises metal–buffer interactions {$K = 1.6$ dm^3 mol^{-1} for Cu^{2+} + dmps ⇌ [Cu(dmps)]}. Copper(II) solutions were prepared from AnalaR $Cu(NO_3)_2 \cdot 6H_2O$ and were standardised iodometrically prior to use. Hydrolysis in the presence of copper(II) was monitored spectrophotometrically using the increase in absorbance at 270 nm. The ionic strength was adjusted to $I = 0.5$ mol dm^{-3} with $NaClO_4$. The buffer concentration was maintained at 0.02 mol dm^{-3}. All reactions were carried out at 30 °C. The pH of solutions prior to, and on completion of hydrolysis, were checked using a Radiometer PHM-64 Research pH meter. The maximum pH variation was ±0.02 unit.

Absorbance changes were logged directly by an Apple IIe computer interfaced with a Gilford 2400S spectrophotometer. Plots of $\log (A_\infty - A_t)$ were linear for 2—3 half-lives, and values of $k_{obs.}$ were evaluated directly using the computing system. Reactions were initiated by injecting a concentrated ethanolic solution of Pen G into the appropriate solution. The substrate concentration was 1.52×10^{-4} mol dm^{-3} in all the runs.

Potentiometric Measurements.—Potentiometric titrations of benzylpenicillin in the absence and presence of copper(II) (as the perchlorate salt) were carried out in a fully automatic system controlled by an Apple IIe computer. The equipment consists of: (*i*) a Radiometer PHM84 research pH meter equipped with a Beckman Futura glass electrode and an Ingold saturated sodium chloride–calomel reference electrode fitted in an Ingold bridge; (*ii*) a Radiometer ABU80 Autoburette, equipped with a 2.5/0.25 cm^3 B280 burette assembly; (*iii*) a Metrohm thermostatted cell; and (*iv*) a Huber MINISTAT digital thermostat. Typical concentrations used were in the range (0.5—1.0) $\times 10^{-3}$ mol dm^{-3}. The details of the experimental procedure have been published elsewhere.[17] The data were processed on a VAX 11/780 computer using the MINIQUAD program.[18] In the titration curves $-\log [H^+]$ was plotted *versus* B/L the ratio of moles of standard base (B) per mole of the ligand (L). Negative values indicate excess of acid.

Results and Discussion

The ionisation of benzylpenicillin (HL) in aqueous solution ($I =$ 0.1 mol dm^{-3} $NaClO_4$) was investigated by potentiometric titration at 10.0 ± 0.1 and 5.0 ± 0.1 °C. Benzylpenicillin behaves as a monoprotic ligand according to equilibrium (1),

$$L^- + H^+ \rightleftharpoons HL; \log \beta_{101} \qquad (1)$$

with $\log \beta_{101} = pK_a = 2.67 \pm 0.005$ at 10 °C and 2.60 ± 0.008 at 5 °C.

The interaction of copper(II) with benzylpenicillin (molar ratio 1:1) at 5 and 10 °C was also investigated by potentiometric titration. The titration curves of benzylpenicillin in the absence and in the presence of Cu^{2+} are exactly superimposable upon each other in the B/L region −1 to 0 indicating that no complexation occurs within this pH region, Figure 1, a result which excludes complexes of the type $[CuL]^+$. The best fit to the experimental data is given by the series of

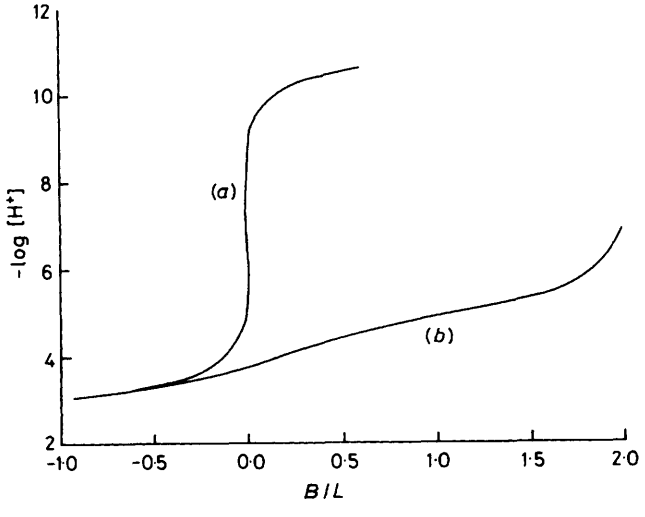


Figure 1. Titration curves for (*a*) benzylpenicillin and (*b*) benzylpenicillin and copper(II) perchlorate (1:1)

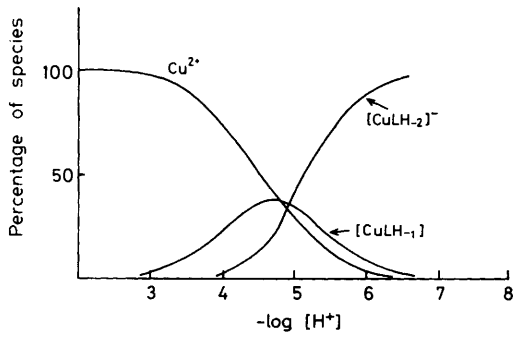


Figure 2. Species distribution curve for copper(II) complexes of benzylpenicillin at a 1:1 ligand-to-metal ratio, $[Cu^{2+}] = 1 \times 10^{-3}$ mol dm^{-3}

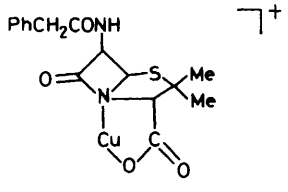
equilibria (2) and (3), with $\log \beta_{11-1} = -1.41 \pm 0.1$ at 10 °C

$$L^- + Cu^{2+} \rightleftharpoons [CuLH_{-1}] + H^+; \log \beta_{11-1} \quad (2)$$

$$L^- + Cu^{2+} \rightleftharpoons [CuLH_{-2}]^- + 2H^+; \log \beta_{11-2} \quad (3)$$

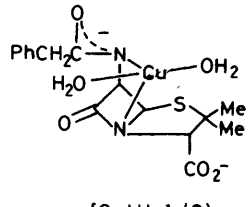
(-1.59 ± 0.2 at 5 °C) and $\log \beta_{11-2} = -6.33 \pm 0.08$ at 10 °C (-6.37 ± 0.08 at 5 °C). The β_{lmh} values are the corresponding formation constants, where l is the stoicheiometric coefficient of the ligand, m that of the metal, and h that of the hydrogen ion in the complex. The distribution curves are shown in Figure 2.

A surprising feature of the potentiometric investigation is that no evidence was found for a complex of the type $[CuL]^+$. Such a complex with the lactam nitrogen and the carboxylate group acting as donors, (**8**), was considered by Page *et al.*[10] to be



(**8**)

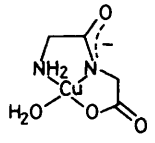
the active species in the metal-ion-promoted reaction. The potentiometric data indicate that the deprotonated complexes $[CuLH_{-1}]$ and $[CuLH_{-2}]^-$ are the main species in solution, a result suggesting that deprotonation of the amide side chain on the β-lactam ring is occurring with the complex $[CuLH_{-1}]$ having the structure (**9**), with the deprotonated amide and



$[CuLH_{-1}]$ (**9**)

possibly the lactam nitrogen acting as donors.* The complex $[CuLH_{-2}]^-$ is likely to be a hydroxo complex in which one of the water molecules on copper(II) has ionised. The pK_a for the process $[CuLH_{-1}] \rightleftharpoons [CuLH_{-2}]^- + H^+$ is given by ($\log \beta_{11-1} - \log \beta_{11-2}$) = 4.92 at 10 °C.

The deprotonation of amides and peptides in the presence of copper(II) is well documented and ionisation of the amide nitrogen commonly occurs in the pH range 4—5 as is observed in the present case. A typical example is the deprotonation of glycylglycine in the presence of copper(II) to give the complex (**10**) in the pH range 4—5,[4] with $\lambda_{max.}$ for the *d*–*d* band at 625



(**10**)

nm. Complex (**9**), representing $[CuLH_{-1}]$, involves a stable five-membered chelate ring if the lactam nitrogen acts as a donor. A five-membered chelate ring would also occur if the thioether sulphur of the thiazolidine ring, or the lactam O, acted as a donor. However, previous studies have indicated that the Cu–S(thioether) bond is readily cleaved in aqueous solution,[19] and on this basis the lactam O may well be the second donor site.

Kinetic Studies.—The hydrolysis of benzylpenicillin was studied over the pH range 4.31—5.37 at various copper(II) concentrations at 30 °C and $I = 0.5$ mol dm^{-3}. The copper(II) concentration was always in at least a four-fold excess over the substrate concentration. Values of the observed first-order rate constant ($k_{obs.}$) as a function of the copper(II) concentration at various pH values are listed in Table 1. Solutions were buffered using dmps–NaOH which effectively does not interact with copper(II). Plots of $k_{obs.}$ *versus* $[Cu^{2+}]$ begin to display saturation kinetics even at pH 4.31, with the reaction becoming independent of $[Cu^{2+}]$, Figure 3.

The rate constant $k_{obs.}$ in the absence of copper(II) is *ca.* 1×10^{-5} s^{-1} [10] so that the plot effectively passes through the origin. At constant pH, the kinetics are consistent with a scheme of the type shown in equations (4) and (5), where bzpen =

$$Cu^{2+} + \text{bzpen} \overset{K}{\rightleftharpoons} [Cu(\text{bzpen})]^{2+} \quad (4)$$

$$[Cu(\text{bzpen})]^{2+} \xrightarrow{k} \text{Products} \quad (5)$$

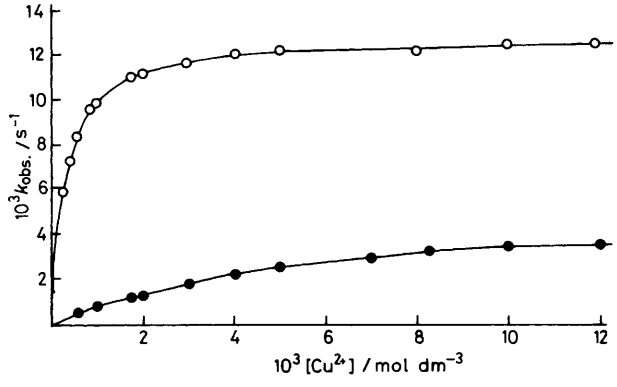


Figure 3. Plot of $k_{obs.}$ *versus* $[Cu^{2+}]$ for the copper(II)-promoted hydrolysis of benzylpenicillin at 30 °C in dmps buffer pH 5.19 (○), 4.31 (●)

* It should be recognised that we have no specific potentiometric evidence for benzylpenicillin acting as a bidentate ligand. The lone pair on N points away from the Cu^{II} so that a Cu–N interaction of the type (**9**) does not appear very probable.

Table 1. Rate constants $k_{obs.}$ for the copper(II)-promoted hydrolysis of benzylpenicillin in dmps buffers at $I = 0.5$ mol dm^{-3} ($NaClO_4$) and 30 °C. Total ligand concentration 1.52 × 10^{-4} mol dm^{-3}

	$10^3[Cu^{2+}]$/ mol dm^{-3}	$10^3k_{obs.}$/s^{-1}	$10^3[Cu^{2+}]$/ mol dm^{-3}	$10^3k_{obs.}$/s^{-1}
pH 4.31	0.55	0.44	5.0	2.50
	1.0	0.75	7.0	2.80
	1.75	1.18	8.25	3.20
	2.0	1.30	10.0	3.38
	3.0	1.78	12.0	3.51
	4.0	2.13		
pH 4.64	0.55	0.74	4.0	3.78
	1.0	1.24	5.0	4.35
	1.75	2.06	7.0	5.21
	2.0	2.30	8.25	5.70
	3.0	3.11	12.0	6.70
pH 5.00	0.55	1.06	4.0	4.87
	0.84	1.46	5.0	5.69
	1.0	1.89	8.0	7.0
	1.75	2.80	10.0	7.41
	2.0	3.19	12.0	7.72
	3.0	4.03		
pH 5.19	0.25	5.83	3.0	11.64
	0.40	7.19	4.0	11.91
	0.55	8.29	5.0	12.12
	0.84	9.46	8.0	11.97
	1.0	9.84	10.0	12.42
	1.75	10.94	12.0	12.40
	2.0	11.13		
pH 5.37	0.025	1.79	2.0	12.20
	0.40	9.83	3.0	12.34
	0.55	10.31	4.0	12.59
	0.84	11.09	5.0	12.67
	1.0	11.50	8.0	13.17
	1.75	12.04	10.0	12.99
			12.0	12.92

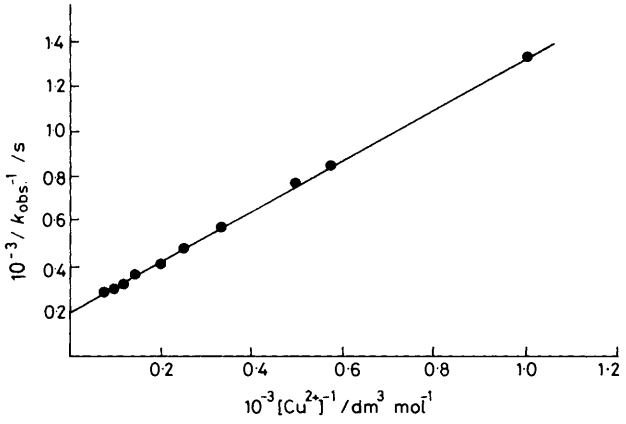


Figure 4. Double reciprocal plot of $1/k_{obs.}$ *versus* $1/[Cu^{2+}]$ for the copper(II)-promoted hydrolysis at pH 4.31 and 30 °C

$$k_{obs.} = \frac{kK[Cu^{2+}]}{(1 + K[Cu^{2+}])} \quad (6)$$

$$\frac{1}{k_{obs.}} = \frac{1}{kK} \cdot \frac{1}{[Cu^{2+}]} + \frac{1}{k} \quad (7)$$

benzylpenicillin. The variation of $k_{obs.}$ with $[Cu^{2+}]$ can be expressed by equation (6), which can be rearranged to give equation (7). A plot of $1/k_{obs.}$ *versus* $1/[Cu^{2+}]$ should be linear of slope $1/kK$ and intercept $1/k$. Such plots are indeed linear,

Table 2. The constants k and K determined from double reciprocal plots

pH	10^2k/s^{-1}	K/dm^3 mol^{-1}
4.31	0.55	159
4.64	1.10	130
5.00	1.18	180
5.19	1.28	3 300
5.37	1.32	6 300

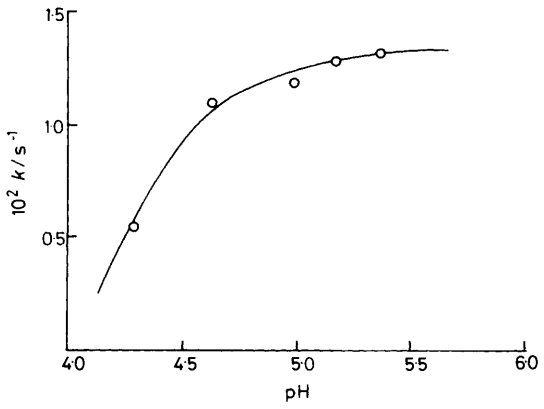


Figure 5. Plot of the limiting values of $k_{obs.}$ *versus* pH for the copper(II)-promoted reaction

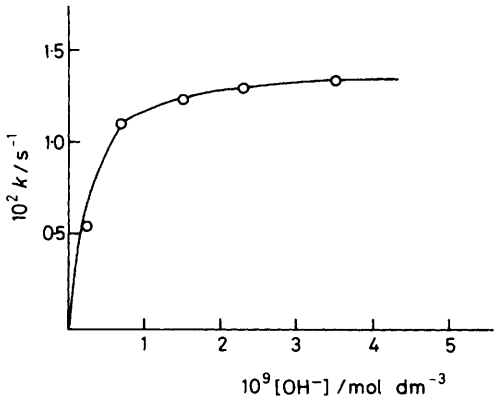
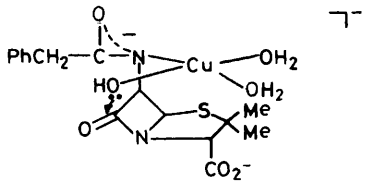


Figure 6. Plot of the limiting values of $k_{obs.}$ *versus* the hydroxide ion activity at 30 °C

Figure 4, and at pH 4.31 least-squares analysis gives $k = 5.46 \times 10^{-3}$ s^{-1} (the limiting rate constant) and $K = 159$ dm^3 mol^{-1} at 30 °C. The equilibrium constant K is a conditional constant determined at a single pH and is not directly comparable with the potentiometrically determined values. Values of k and K obtained by this procedure at various pH values are summarised in Table 2.

Values of k reach a plateau value as the pH is increased, Figure 5, and the rate of hydrolysis becomes essentially independent of pH above pH 6 where $[CuLH_{-2}]^-$ is completely formed. This effect is clearly seen in a plot of k *versus* $[OH^-]$, where $[OH^-]$ = antilog (pH − pK_w) and pK_w = 13.83 at 30 °C (Figure 6). The kinetic behaviour is consistent with the view that $[CuLH_{-2}]^-$ is the active species in the copper(II)-promoted reaction. As the reaction becomes independent of the hydroxide ion concentration above pH 6,

hydrolysis must occur by intramolecular attack of a co-ordinated hydroxide ion at the lactam carbonyl group as illustrated by complex (**11**). A mechanism involving bimolecular



(**11**)

attack of OH^- on the metal complex is excluded by the kinetic behaviour.

For the intramolecular hydrolysis of $[CuLH_{-2}]^-$ at 30 °C, $k \approx 1.3 \times 10^{-2}$ s^{-1}. Page and co-workers[10] have determined that k_{OH} for the base hydrolysis of the lactam ring of the anion L^- is 0.15 dm^3 mol^{-1} s^{-1} at 30 °C. At pH 6, the observed first-order rate constant ($k_{obs.}$) for the hydrolysis of the anion can be calculated using the expression $k_{obs.} = k_{OH}[OH^-]$, where $[OH^-] = 1.5 \times 10^{-8}$ mol dm^{-3}. The calculated value of $k_{obs.} = 2.2 \times 10^{-9}$ s^{-1} at 30 °C. The hydrolysis of $[CuLH_{-2}]^-$ is thus some 6×10^6 fold faster than the hydrolysis of L^- at pH 6.

Acknowledgements

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