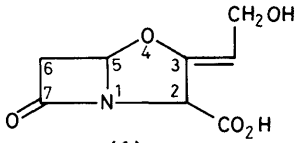
Studies on Clavulanic Acid. Part 2.† The Catalytic Effect of Metal Ions on the Hydrolysis of Clavulanic Acid

Javier Martin,* Rosa Méndez, and Francisco Salto
Departamento de Bioquímica y Biología Molecular, Facultades de Biología y Veterinaria, Universidad de León, 24071 León, Spain

The metal(II)-ion-catalysed hydrolysis of clavulanic acid in water shows saturation kinetics. A 1:1 complex is formed between the metal ion and clavulanic acid, which is attacked by hydroxide ion *ca.* 2×10^6 times as fast as the unco-ordinated clavulanic acid. The catalytic effect produced by the copper(II) ion on the decomposition of the methyl ester of clavulanic acid shows a much smaller rate enhancement, indicating that the site of co-ordination for the clavulanic acid involves the ionized carboxylate group. The co-ordination sites of the clavulanic acid are the β-lactam nitrogen and the carboxylate group. The association constants between clavulanate ion and several transition metal ions have been determined. The order of rate enhancement brought about by the metal ion is $Cu^{II} > Zn^{II} > Cd^{II} > Co^{II} > Ni^{II} > Mn^{II}$. The reaction is inhibited by the presence of anions co-ordinating with the copper(II) ion.

Clavulanic acid, (*Z*)-(2*R*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, (**1**) is a



(**1**)

potent inhibitor of β-lactamases from a variety of Gram-positive and Gram-negative bacteria.[1] This compound was isolated in 1975 from a culture broth of *Streptomyces clavuligerus*,[2] and is often used mixed with penicillins to protect them against the action of β-lactamase.

Some of these enzymes are metal-ion dependent[3] and this report may be of relevance to the mechanism of action of these β-lactamases. We also seek to ascertain the influence of the presence of metals on the *in vitro* stability, as well as on the *in vivo* distribution, storage, biotransformation, and elimination of clavulanic acid.

We report here evidence that copper(II) ions co-ordinate with the β-lactam nitrogen and the carboxylic group of clavulanic acid. The relative catalytic efficiency of various metal ions towards increasing the rate of hydrolysis of clavulanic acid is also discussed.

Experimental

Materials.—Lithium clavulanate (98.8%) was supplied by Antibióticos S.A. (León, Spain).

The methyl ester of clavulanic acid was prepared according to a literature method.[4]

The metal ions were in the form of chloride salts. Solutions of metal ions were prepared from reagent-grade metal salts. These solutions were standardized by titration with a standard EDTA solution, dilutions to the required concentration of metal ion were all made from these primary solutions. Other materials were of reagent grade. All water used in this work was distilled and deionized, and of 18 MΩ resistance. All solutions were prepared immediately prior to use.

Kinetic Procedure.—A constant pH was maintained all through the kinetic study by using a pH-stat (a titrimeter assembly consisting of an E-614 Impulsomat, a 655 Dosimat, and an E-632 pH-meter from Metrohm, Herisau, Switzerland). All reactions were conducted at 35.0 ± 0.1 °C and the ionic strength was adjusted to 0.5 mol l^{-1} with sodium perchlorate.

The rates of reaction of the clavulanate ion were determined by monitoring the absorbance at 312 nm of the product resulting from the reaction of clavulanate ion and imidazole,[5] on a Spectronic 2000 spectrophotometer (Bausch–Lomb). The methyl ester of clavulanic acid was analysed by h.p.l.c. The liquid chromatograph (Laboratory Data Control. Constametric II) was equipped with a u.v. detector (Waters Mod. 441) set at 229 nm. The stationary phase used was μ-Bondapack C18, 10 μm, prepacked into a stainless steel column 25 cm long and 4.6 mm inner diameter. The mobile phase was a mixture of aqueous 0.05M-KH_2PO_4–methanol (60:40 v/v; final pH 4.50), the flow rate of which was maintained at 2.0 ml min^{-1}. All chromatographic operations were carried out under ambient conditions.

Results and Discussion

In all our experiments, the ionic strength was adjusted to 0.5 mol l^{-1} using sodium perchlorate. This salt was used instead of the chloride because chloride ion has a higher tendency to form complexes with the metal ions.[6]

In order to ascertain the influence of the chloride and perchlorate ions, we measured the time, t_{10}, in which 10% 2.0×10^{-4}M-lithium clavulanate had been hydrolysed in the presence of 2.0×10^{-5}M-Cu^{II} at pH 6.00 and at a temperature of 35.0 ± 0.1 °C. When the ionic strength of the medium is adjusted to 0.5 mol l^{-1} with KCl, t_{10} is *ca.* 14 min. This value is reduced to 8 min with $NaClO_4$.

The hydrolysis of lithium clavulanate in the presence of a metal ion follows pseudo-first-order kinetics under the conditions of this study.[7] The variation in the pseudo-first-order rate constant with the concentration of metal ion is shown in Figure 1. At low concentrations of Zn^{II} ion, this constant has a linear relationship with the metal-ion concentration, while an

† Part 1, preceding paper.

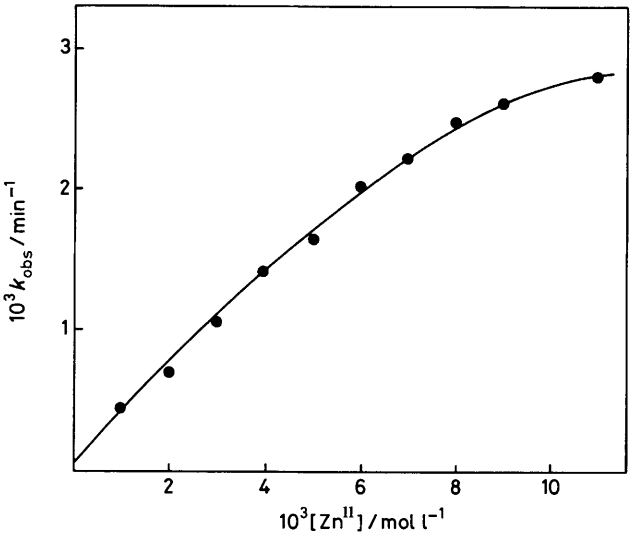


Figure 1. Pseudo-first-order rate constants, k_{obs}, *versus* total zinc(II)-ion concentration for the hydrolysis of lithium clavulanate at pH 6.00, 35.0 °C, and 0.5M ionic strength

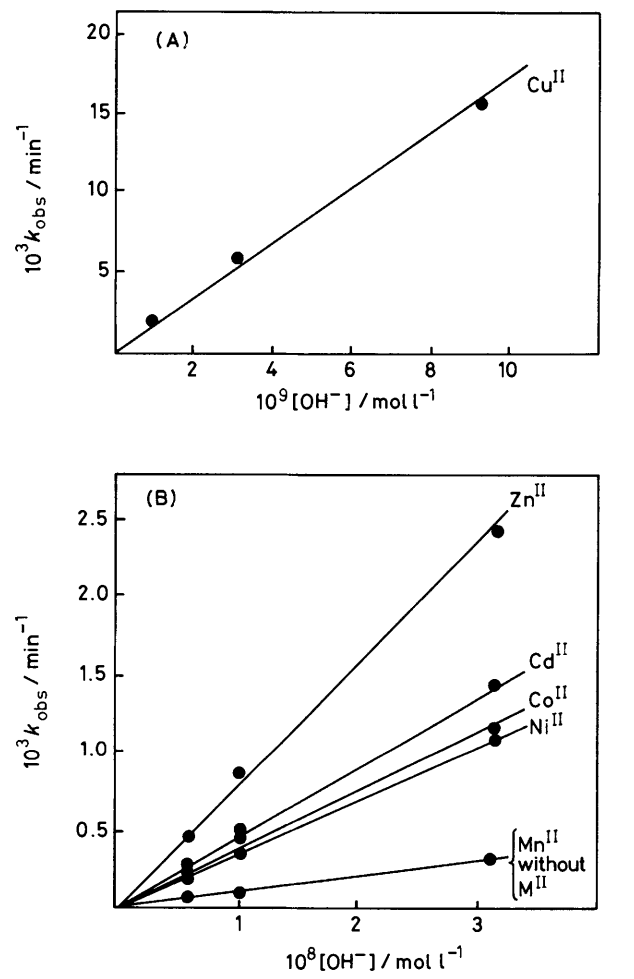
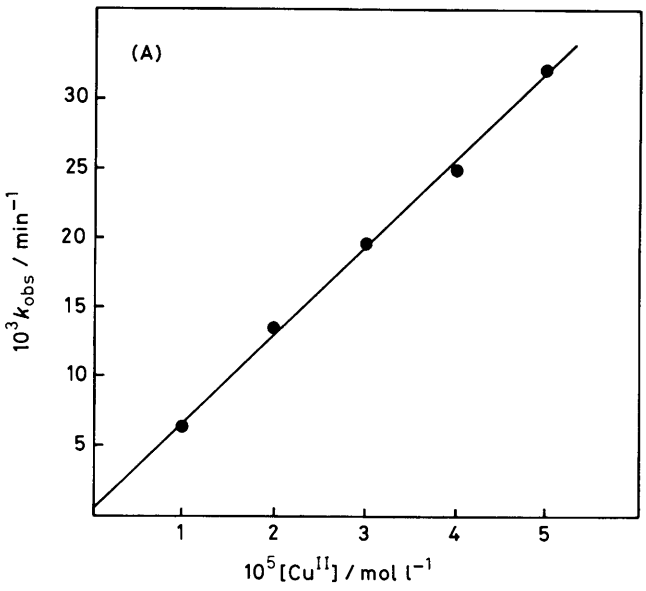


Figure 2. Pseudo-first-order rate constants, k_{obs}, *versus* hydroxide-ion concentration for the hydrolysis of lithium clavulanate at 35.0 °C and 0.5M ionic strength. Copper(II)-ion concentration 2×10^{-5} mol l^{-1} (A). Metal-ion concentration 2×10^{-3} mol l^{-1} (B)



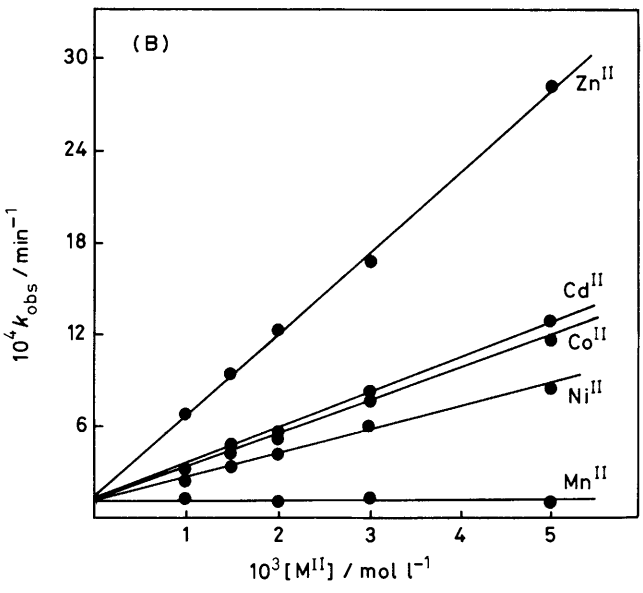


Figure 3. Pseudo-first-order rate constants, k_{obs}, *versus* total metal-ion concentration for the hydrolysis of lithium clavulanate at 35.0 °C, 0.5M-ionic strength, and pH 6.00. In presence of copper(II) ion (A). In presence of other metal ions (B)

increase in the ZN^{II}-ion concentration is accompanied by a slight loss of linearity. This saturation phenomenon is interpreted in terms of the formation of a co-ordination compound between the Zn^{II} and clavulanate ions which subsequently gives the reaction products.

Figures 2 and 3 show the variation in the pseudo-first-order rate constant with concentration of hydroxide and metal ions. These variations are linear for the interval studied.

Bearing in mind all these results, the following kinetic scheme is proposed:[8,9]

$$M + C \underset{k'_1}{\overset{k_1}{\rightleftharpoons}} MC \xrightarrow{k_2[OH^-]} M + \text{products} \qquad (1)$$

where M and C represent, respectively, the metal and clavulanate ions and MC is the co-ordination compound, which is attacked by the hydroxide ion and decomposes into the metal ion and products.

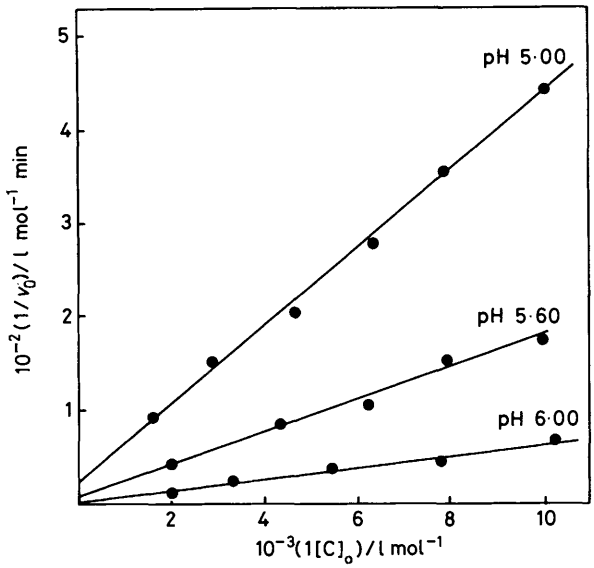


Figure 4. Plot of the reciprocal of the initial rate for the hydrolysis of lithium clavulanate at 35.0 °C, 0.5M-ionic strength, and the pH values indicated, as a function of the reciprocal of the initial clavulanate concentration

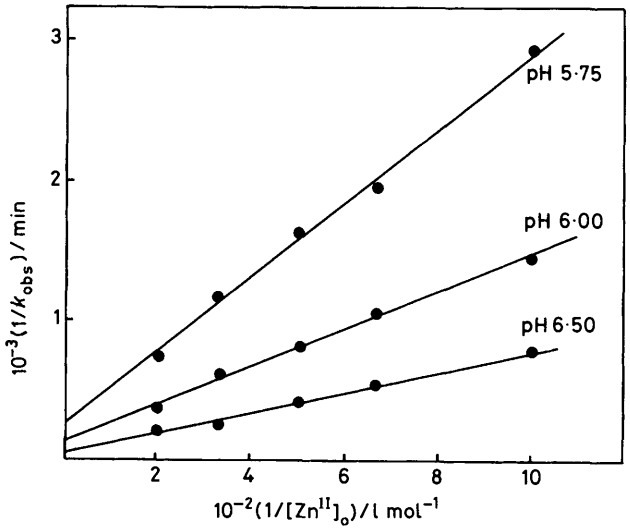


Figure 5. Plot of the reciprocal of the pseudo-first-order rate constants for the hydrolysis of lithium clavulanate at 35.0 °C, 0.5M-ionic strength, and the pH values indicated, as a function of the reciprocal of the metal-ion concentration

Table. Summary of the rate and formation constants for the metal-ion-catalysed hydrolysis of lithium clavulanate in water at 35 °C and ionic strength 0.5 mol l^{-1}. Data obtained according to equations (5) and (7)

Metal ion	$K \pm s^a$/l mol^{-1}	$k_2 \pm s^a$/l mol^{-1} min^{-1}
Cu^{II}	555 ± 30	(1.79 ± 0.36) × 10^8
Zn^{II}	89 ± 9	(6.88 ± 1.13) × 10^5
Cd^{II}	85 ± 9	(3.83 ± 0.35) × 10^5
Co^{II}	101 ± 8	(2.87 ± 0.45) × 10^5
Ni^{II}	114 ± 10	(2.06 ± 0.18) × 10^5
Mn^{II}	—	—

[a] Standard deviation.

Let us assume that the metal ion exists either free, M, or as the complex, MC. We obtain:

$$[C]_0 = [C] + [MC] \quad (2)$$

$$[M]_0 = [M] + [MC] \quad (3)$$

where $[C]_0$ and $[M]_0$ are the initial concentrations of clavulanate and metal ions, respectively.

On the condition $[C]_0 \gg [M]_0$, $[M] = [M]_0 - [MC]$, and $[C] \approx [C]_0$, and applying the steady-state hypothesis, the rate constant observed will be:

$$k_{obs} = \frac{k_1 k_2 [M]_0 [OH^-]}{k'_1 + k_1 [C]_0} \quad (4)$$

assuming that $k'_1 \gg k_2[OH^-]$.

A plot of k_{obs} as a function of the $[OH^-]$ at constant $[C]_0$ and $[M]_0$ gives a straight line (Figure 2), as does the representation of k_{obs} as a function of the concentration of metal ions, $[M]_0$, at constant pH and $[C]_0$ (Figure 3) when the system is not saturated.

Equation (4) can be re-written as:

$$\frac{1}{v_0} = \frac{1}{k_2[OH^-]K[M]_0[C]_0} + \frac{1}{k_2[OH^-][M]_0} \quad (5)$$

where $K = k_1/k'_1$ is the formation constant of the MC compound and v_0 is the initial rate.

At constant pH and $[M]_0$, the plot of $1/v_0$ *versus* $1/[C]_0$ is a straight line of slope $1/k_2[OH^-]K[M]_0$ and intercept $1/k_2[OH^-][M]_0$. This was the method used to determine the catalytic effect of the Cu^{II} ion.

The influence of other ions was studied under the conditions $[M]_0 \gg [C]_0$, $[C] = [C]_0 - [MC]$, and $[M] \approx [M]_0$. The rate constant observed is:

$$k_{obs} = \frac{k_1 k_2 [M]_0 [OH^-]}{k'_1 + k_1 [M]_0} \quad (6)$$

assuming that $k'_1 \gg k_2[OH^-]$.

By analogy with the previous treatment, the plot of the constants k_{obs} as functions of $[OH^-]$ at a constant $[M]_0$, or as a function of $[M]$ at a constant $[OH^-]$, is a straight line, as shown in Figure 2. Equation (6) can be re-written as:

$$\frac{1}{k_{obs}} = \frac{1}{k_2[OH^-]K[M]_0} + \frac{1}{k_2[OH^-]} \quad (7)$$

At constant pH and $[C]_0$, the plot of $1/k_{obs}$ *versus* $1/[M]_0$ is a straight line of intercept $1/k_2[OH^-]$ and slope $1/k_2[OH^-]K$. Plots of the data obtained for the metal ions studied, according to equations (5) and (7), gave linear variations in all cases at all pH values. Figures 4 and 5 show these plots for Cu^{II} and Zn^{II} ions. The values obtained for k_2 and K are shown in the Table.

The second-order rate constant k_2 for the hydroxide ion attacking the clavulanate ion co-ordinated with Cu^{II} is 1.79×10^8 l mol^{-1} min^{-1}, and the constant for the hydroxide ion attacking the clavulanate ion [10] is 1.02×10^2 l mol^{-1} min^{-1}, so the complexing of the Cu^{II} ion increases the rate of reaction by a factor of *ca.* 2×10^6. The value of the formation constant of the clavulanate ion–Cu^{II} complex is 555 l mol^{-1}, which may be compared with the values [11] of 407 and [8] 266 l mol^{-1} obtained for penicillin–Cu^{II}. For the remaining metal ions, this effect is much smaller.

These large increases in the rate are attributed, as has already been mentioned, to the complexation of metal ions with the clavulanate ion. We shall now go on to describe the co-ordination sites and to discuss the nature of the increase in the rate.

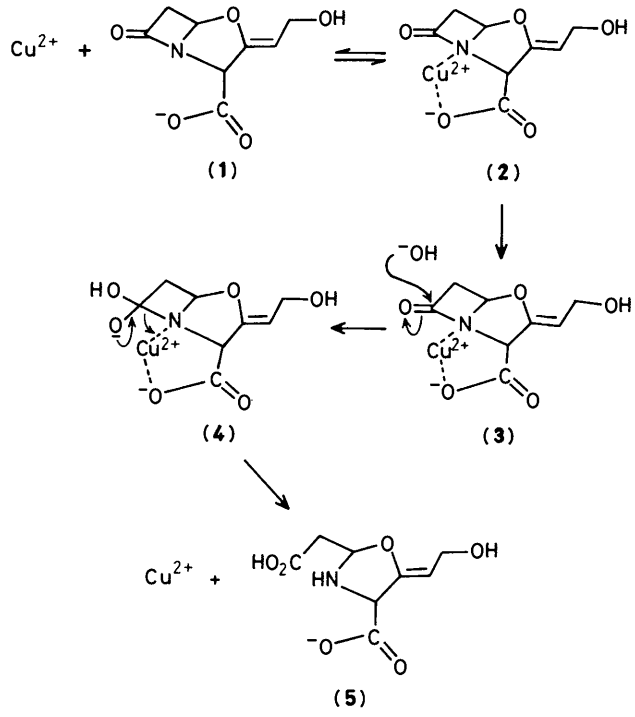
Co-ordination Sites.—The co-ordination of the metal ions with the clavulanate must occur *via* the carboxylate group, as is demonstrated by the fact that Cu^{II} has practically no catalytic effect in the decompositon of the methyl ester of clavulanic acid. The rate constant for the decomposition of the ester in the presence of Cu^{II}, 1.0×10^{-3} mol l^{-1} at pH 5.50, is $k_{obs} = 2.7 \times 10^{-3}$ min^{-1}, while, in the absence of Cu^{II}, all other things being equal, the clavulanate-ion decomposition has a rate constant[10] of *ca.* 2×10^{-4} min^{-1}. This difference could be the result of weak co-ordination, compared with that observed for clavulanate ion, of the metal ion with nitrogen and the methoxycarbonyl group. It could also be caused by the intermediate state of the ester being more stable, or it may reflect a simple electrostatic effect, as the reaction between the hydroxide ion and the clavulanate ion is a process involving two negatively charged ions.

The clavulanate ion must act as a bidentate ligand on the Cu^{II}, as the formation constant, K, for this co-ordination compound is 555 l mol^{-1}. Indeed, according to Gensmantel *et al.*[12] K is related to the pK_a of the carboxy group through log $K = 0.42pK_a - 0.17$, which for clavulanic acid (pK_a 2.4) would give a value for K of about 7 l mol^{-1}, which is much lower than that found by us.

In the light of this, and taking into account the studies carried out by Gensmantel *et al.*[12] and by Fazakerley and Jackson[13] on other β-lactam antibiotics in which they found the formation of 1:1 co-ordination compounds between the metal ion and the β-lactam, it is reasonable to conclude that, in our case, the co-ordination takes place in a similar manner, *i.e.*, a clavulanate–metal ion co-ordination compound occurs and the co-ordination takes place with the intervention of the carboxylate group and the nitrogen of the β-lactam.

Bearing in mind all these observations, we postulate the kinetic model shown in equation (1).

Rate Increase.—Presumably, and in line with Gensmantel's findings,[12] the rate-limiting step for the uncatalysed reaction of hydroxide ion with clavulanate ion is the severing of the carbon–nitrogen bond (Scheme 1). The nucleophilic attack of the hydroxide ion would be effected on the carbonyl carbon of the β-lactam ring, according to what is indicated for compound (**3**)



Scheme 1.

(Scheme 1). The metal ion would presumably facilitate this step by increasing the electrophilic character of the carbonyl carbon, and would also facilitate the step of severing the carbon–nitrogen bond.

The uncatalysed hydrolysis of clavulanate ion may not involve the protonation of the nitrogen, owing to the great amount of energy released in the breakdown of the carbon–nitrogen bond, in which case the function of the metal ion in the catalysed reaction would be to stabilize the intermediate anion (**4**) (Scheme 1), thus increasing the rate of rupture of the carbon–nitrogen bond. In the unlikely event of nitrogen protonation occurring in the uncatalysed reaction,[14] then the role of the metal ion would simply be that of a 'super-acid' replacing the proton.[8]

In the light of these observations, and as the metal ion facilitates the rupture of the carbon–nitrogen bond, the rate-

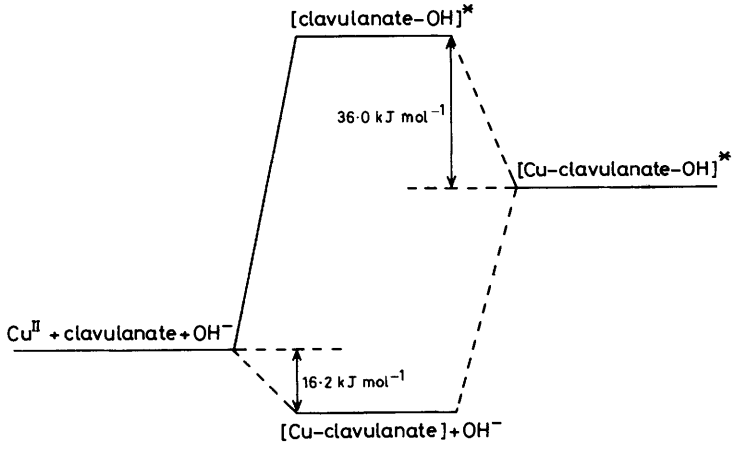


Figure 6. Plot of the change in free energy for the copper(II)-catalysed hydrolysis of lithium clavulanate compared with the hydroxide-ion-catalysed reaction at a standard state of 1 mol l^{-1}

limiting step of the reaction could change from being the breakdown of the tetrahedral intermediate (**4**) (Scheme 1) for the uncatalysed reaction, to being the formation of the tetrahedral intermediate in the metal-ion-catalysed reaction.

The stabilization of the transition state by the metal ion may be estimated from a comparison of the third-order rate constant, Kk_2 for the hydrolysis catalysed by the metal ion and the hydroxide ion, with the second-order rate constant for the hydrolysis catalysed by the hydroxide ion. For example, for the decomposition of clavulanate in the presence of Cu^{II}, this ratio is 7.1×10^8 mol l^{-1}, a very high value equivalent to 52.2 kJ mol^{-1} at 35 °C.

The bond formed initially between the clavulanate ion and Cu^{II} stabilized the system by 16.2 kJ mol^{-1} (RT ln 555) and, as the difference in the free energy activation for the hydroxide-ion-catalysed hydrolysis of co-ordinated and unco-ordinated clavulanate is 36.0 kJ mol^{-1}, the Cu^{II} must stabilize the transition state by 52.2 kJ mol^{-1}. Figure 6 represents the various changes in free energy that take place in the decomposition process.

A comparison of the relativities of clavulanic acid (C) and benzylpenicillin (P) leads to the following conclusions. (*a*) The ratios of the rate constants, k_2, for the decomposition of these compounds in the presence of Cu^{II}, Zn^{II}, Ni^{II}, and Co^{II} ions, namely $(k_2)_P/(k_2)_C$, are in the range 1—5. (*b*) The ratios of the formation constants for the 1 : 1 complexes between C and P and these metal ions, K_C/K_P, vary between 1 and 2.

All these results are nicely consistent with the model of ion-promoted decomposition of benzylpenicillin set forth by Gensmantel *et al.*[8,12]

Finally, we suggest that the metal-ion-catalysed hydrolysis of clavulanate is a likely model for a number of decomposition processes occurring in living systems.

Acknowledgements

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