Studies on Clavulanic Acid Part 3. Catalysis of Hydrolysis and Aminolysis of Clavulanic Acid by Metal Chelates

Javier Martín,* Rosa Méndez, and Francisco Salto
Departamento de Bioquímica y Biología Molecular, Universidad de León, 24071 León, Spain
Manuel Castillo
Departamento de Química Inorgánica, Universidad de Sevilla, Sevilla, Spain

The Zn^{II}–tris(hydroxymethyl)aminomethane (Tris) system has a large catalytic effect on the hydrolysis and aminolysis of the clavulanate ion, (*Z*)-(2*R*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate. In order to ascertain the mechanism of this catalysis we have analysed the effects of other metal ions (Cd^{II}, Co^{II}, Cu^{II}, Ni^{II}, and Mn^{II}), of amines structurally related to Tris, and of blocking the carboxylate group of the clavulanate ion. From these studies, we conclude that only the Cd^{II}–Tris and Co^{II}–Tris systems have any substantial catalytic effect, although this is not as important as that of Zn^{II}–Tris. Studies with methyl clavulanate indicate that co-ordination of the metal ion by the carboxylate group is necessary. We suggest that catalysis takes place *via* a ternary complex in which the metal ion plays a double role by (*a*) placing the clavulanate ion and the amino alcohol in the right position for the reaction and (*b*) lowering the pK_a of the hydroxide group of Tris, which is co-ordinated with the metal ion generating a strong nucleophile.

It is generally well known that metal ions promote degradation of some β-lactams and that a number of hydrolytic enzymes contain metal ions in their active sites. The β-lactamase II from *Bacillus cereus* 569/H is known to be zinc-ion dependent.[1] It has previously been shown that the zinc ion in the presence of tris(hydroxymethyl)aminomethane (Tris) buffer is a very efficient catalyst for the hydrolysis and aminolysis of clavulanate ion.[2]

This investigation was aimed at determining the mechanism of the decomposition reaction, its scope, and the roles played by other metal ions and by the esterification of the carboxylic group.

Experimental

Materials.—Lithium clavulanate (98.8%) was supplied by Antibióticos S. A. (León, Spain). The methyl ester of clavulanate was prepared as previously described.[3] 2-Amino-2-methylpropane-1,3-diol was recrystallized from ethanol, 2-amino-2-methoxyethanol and 2-amino-2-methylpropan-1-ol were redistilled. The metal ions were in the form of chloride salts, stock solutions for each 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 study was distilled and deionized, and of 18 Mohm resistance, all solutions being prepared immediately prior to use.

Kinetic Procedure.—The pH of the kinetic solution during the reaction was kept constant by means of a pH-stat (Titrimeter assembly consisting of a E-614 Impulsomat, a E-655 Dosimat and a 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 dm^{-3} with sodium perchlorate.

The rate constants of degradation of the clavulanate ion and its methyl ester were determined following the methods used in the previous papers.[4,5]

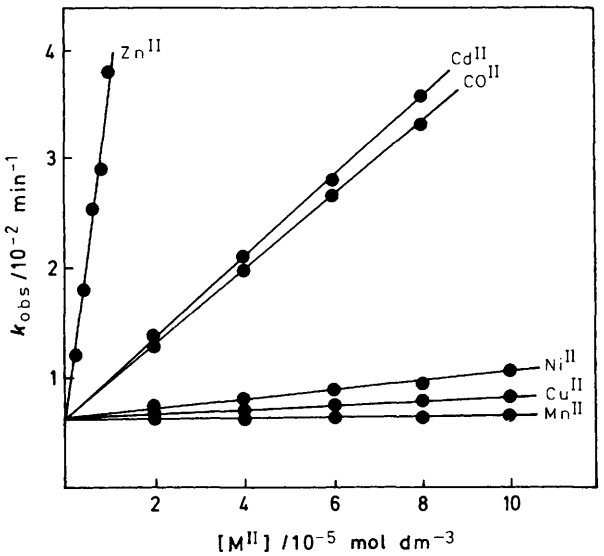


Figure 1. Effect of concentration of the metal ion on k_{obs} for the decomposition of lithium clavulanate (5.0×10^{-4} mol dm^{-3}) in the presence of Tris (0.100 mol dm^{-3}) at pH 8.00, 0.5 mol dm^{-3} ionic strength ($NaClO_4$) and $T = 35 \pm 0.1$ °C.

When the stability of the clavulanate ion in the presence of metal ions and amines was studied, the reaction was blocked with EDTA (final concentration 2×10^{-4} mol dm^{-3}), before the analysis of the remaining clavulanate.

Results and Discussion

The decomposition of lithium clavulanate and its methyl ester in aqueous solution of an excess of amines at constant pH, follows pseudo-first-order kinetics. The pseudo-first-order rate constant, k_{obs}, was obtained from the slope of the semilogarithmic plots of the residual concentration *versus* time by a least-squares treatment.

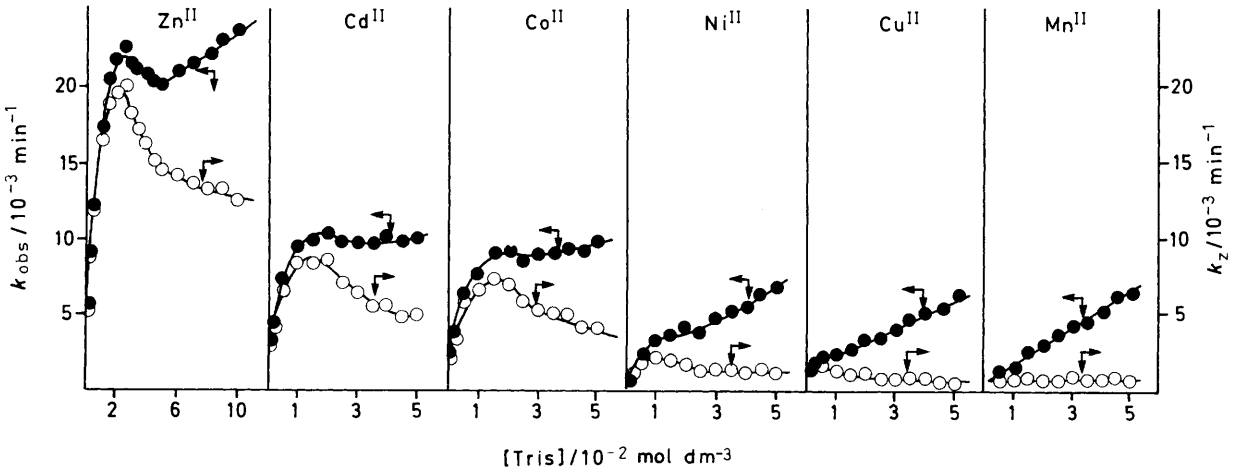


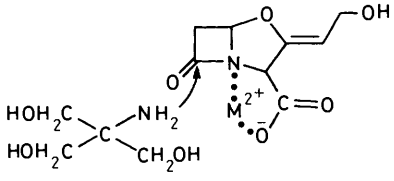
Figure 2. Dependence of the constants k_{obs} and k_z on the concentration of Tris for the decomposition of lithium clavulanate (5.0×10^{-4} mol dm^{-3}) in the presence of metal ions, at pH 8.00, 0.5 mol dm^{-3} ionic strength ($NaClO_4$) and $T = 35 \pm 0.1$ °C ([Zn^{2+}] = 5.0×10^{-6} mol dm^{-3}, [metal ions] = 1.0×10^{-5} mol dm^{-3}).

Table. Effect of the metal ion on the rate of decomposition of clavulanate in aqueous Tris at pH 8.00. $[M]_o$ is the metal ion concentration.

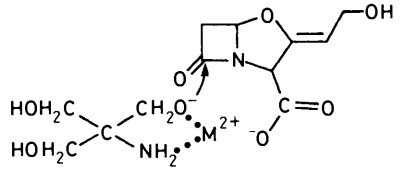
Metal ion	$\{k_z(max)/[M]_o\}$ / 10^2 dm^3 mol^{-1} min^{-1}	Relative activity
Zn^{II}	40	100
Cd^{II}	8.4	21
Co^{II}	7.4	19
Ni^{II}	2.0	5
Cu^{II}	1.7	4
Mn^{II}	—	—

Figure 1 shows the effects of the concentration of the metal ions Zn^{II}, Cd^{II}, Co^{II}, Ni^{II}, Cu^{II}, and Mn^{II} on the decomposition rate constant, k_{obs}, of lithium clavulanate in an aqueous solution of Tris. The relationship is observed to be linear in all cases. Note also the large catalytic effect brought about by the Zn^{II}–Tris system on the breakdown of clavulanate.

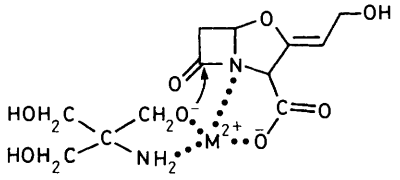
In order to explain the catalytic effect of the metal ions, especially of the zinc(II) ion, in the presence of Tris, we invoke a mechanism similar to that suggested by Gensmantel *et al.*[6] for the cupric-ion-catalysed aminolysis of benzylpenicillin by trifluoroethylamine, propylamine, and methoxyethylamine, whereby the Zn^{II} ion exerts Lewis-acid catalysis by activating the bond between the β-lactam carbonyl and the nitrogen of the clavulanate ion, and by facilitating the nucleophilic attack on the β-lactam carbonyl by neutralization of the charge in the reagents and/or transition state.



Another possible explanation of the same effect is based on the increase in the nucleophilic capacity of Tris on co-ordination with the Zn^{II} ion *via* the amine and one of the hydroxide groups. The pK_a of the co-ordinated hydroxide group may decrease substantially, by about 3 or 4 units, while the alkoxide ion formed maintains the nucleophilicity of a group with a higher pK_a.[7] In this way, these groups acting as chelates may become quite strongly nucleophilic in the hydrolysis of β-lactam antibiotics at around pH 7.



Finally, we could also postulate a third model based on the ideas developed by Schwartz[8] to explain the hydrolysis and aminolysis of benzylpenicillin in the presence of the Zn^{II}–Tris system. The Zn^{II} ion would facilitate, *via* co-ordination with Tris and the clavulanate ion, a spatial arrangement of the reacting species which is very favourable to the nucleophilic attack on the β-lactam carbonyl of the clavulanate ion by the bound, ionized hydroxy group of Tris to form a Tris ester. The effect produced by the metal ion in these cases is known as the 'template effect'.[7]



In order to decide which of these catalytic models offers a better explanation of the catalytic action of Tris–Zn^{II} on clavulanate, we need, among other things, information regarding the effect of substitution of other ions for the Zn^{II}, and of other similarly structured amines for the Tris. We also need to ascertain the effect of esterification of the carboxylate group on the clavulanate ion.

Figure 2 shows the dependence of the rate constants of the decomposition of clavulanate, k_{obs} and k_z, on the concentration of Tris in the presence of each of the metal ions. The constant k_z is obtained from k_{obs} using equation (1). Therefore, k_z in this

$$k_z = k_{obs} - k_{amine}[\text{Amine}] \quad (1)$$

case is the observed rate constant corrected for the rate in the presence of Tris alone. The representation of k_z *versus* Tris

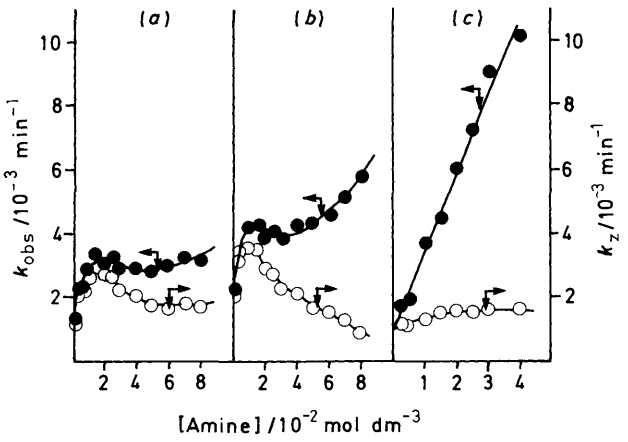


Figure 3. Dependence of the constants k_{obs} and k_z on the concentration of each amine for the decomposition of lithium clavulanate (5.0×10^{-4} mol dm^{-3}) in the presence of Zn^{II} (5.0×10^{-6} mol dm^{-3}) at pH = pK_a, 0.5 mol dm^{-3} ionic strength ($NaClO_4$) and $T = 35 \pm 0.1$ °C: (*a*) 0.100 mol dm^{-3} 2-amino-2-methylpropane-1,3-diol, pH 8.62; (*b*) 0.100 mol dm^{-3} 2-amino-2-methylpropan-1-ol, pH 9.52; (*c*) 0.05 mol dm^{-3} 2-methoxyethylamine, pH 9.35.

concentration shows how the catalytic action of each metal on the clavulanate degradation changes as a function of the Tris concentration.

The curves obtained in Figure 2, for Cd^{II} and Co^{II} are similar in shape to that of Zn^{II}, while those of Cu^{II}, Ni^{II}, and Mn^{II} are different. Cu^{II} has a greater catalytic effect at low Tris concentrations, probably because at high concentrations, it forms co-ordination compounds with Tris,[9] thus preventing its co-ordinating with clavulanate. Mn^{II} does not appear to exert any appreciable catalytic effect at any concentration of Tris.

For the interpretation of these results, it is useful to consider the following equilibria given in equations (2)–(4) where M

$$M^{II} + T \overset{K_1}{\rightleftharpoons} MT^{II} \quad (2)$$

$$MT^{II} + T \overset{K_2}{\rightleftharpoons} MT_2^{II} \quad (3)$$

$$MT^{II} + C^- \overset{K_3}{\rightleftharpoons} MTC^{I} \quad (4)$$

represents the metal(II) ion, T is Tris, MT^{II} and MT_2^{II} are Tris–metal(II) ion complexes, C^- is the clavulanate ion, and MTC^{I} is the ternary complex. At low concentrations of Tris the MT^{II} complex may react with the clavulanate ion, while at high concentrations of Tris the rate decreases, probably because the clavulanate ion cannot compete with a second Tris molecule for co-ordination with the metal ion and the relative amount of ternary complex that could be formed is reduced. As can be seen in Figure 2, the rate constant of degradation of clavulanate ion, k_z, is maximal at about 0.015–0.025 mol dm^{-3} for the different metal ions.

The Table shows the maximum values of the constant k_z for each metal ion divided by its concentration $[M]_o$. From these data, the relative activity was determined for each ion. Under the conditions of these assays, the catalytic activity of Zn^{II}–Tris is much greater than that of the other metal ions. It is not possible to explain these differences using the model proposed by Gensmantel, as the constant for the complex formed by the clavulanate ion and Zn^{II} does not differ sufficiently from that formed by the remaining metal ions.[5] Nor does the second model explain the results, given that the effect of the metal ions on the pK_a of the hydroxy group of Tris and the steric hindrance are sufficiently different to give these results.

Effect of the Structure of the Amine.—In order to determine

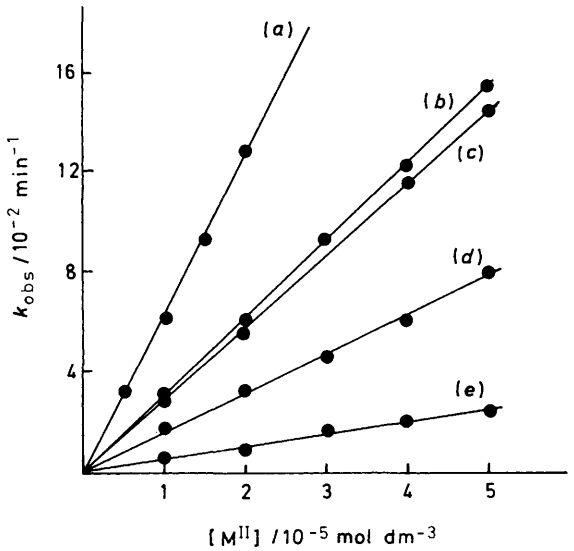


Figure 4. Variation of the rate constant k_{obs} corrected to intercept zero as a function of the concentration of the Zn^{II} ion for the amines used at pH = pK_a. Assays were performed at 0.5 mol dm^{-3} ionic strength and 35 ± 0.1 °C with a clavulanate concentration of 5.0×10^{-4} mol dm^{-3}. (*a*), (*b*) pH 9.00 and 8.00, respectively, 0.100 mol dm^{-3} Tris; (*c*) pH 8.62, 0.100 mol dm^{-3} 2-amino-2-methylpropane-1,3-diol; (*d*) pH 9.52, 0.100 mol dm^{-3} 2-amino-2-methylpropan-1-ol; (*e*) pH 9.35, 0.05 mol dm^{-3} 2-methoxyethylamine.

the effect of the structure of the ligand on the catalytic activity,[10] we determined the rate of decomposition of clavulanate in the presence of the Zn^{II} ion and certain amino alcohols, notably 2-methoxyethylamine, in which the hydroxide group is blocked. The values of the rate constants, k_{obs} and k_z, *versus* the amine concentration were determined at pH = pK_a, and are shown in Figure 3. Here again, the rate constants were corrected for the rate in the presence of each amine alone. As can be seen, the shapes of these curves for the compounds 2-amino-2-methylpropan-1,3-diol and 2-amino-2-methylpropan-1-ol are analogous to that of Tris under the same conditions. The presence of Zn^{II} has practically no influence on the hydrolysis and aminolysis of clavulanate in an aqueous solution of 2-methoxyethylamine.

One explanation of these facts is that the reaction may take place *via* the ternary co-ordination compound amino alcohol–Zn^{II}–clavulanate, and not because of the nucleophilic attack of the amine on the co-ordination compound Zn^{II} ion–clavulanate, since if the latter were the major pathway, no great difference in the rate of reaction between 2-amino-2-methylpropan-1-ol and 2-methoxyethylamine, which have very similar pK_a values would be observed, unless the latter amine has a great affinity for the Zn^{II} ion. Nevertheless, it would be reasonable to expect 2-amino-2-methylpropan-1-ol to have a greater affinity as it can form a bidentate co-ordination compound with the hydroxide group.

These data are therefore consistent with a mechanism taking place *via* the ternary co-ordination compound in which the hydroxide group of the amino alcohol, linked with the metal ion, is the attacking species. 2-Methoxyethylamine cannot form this ternary compound and act as a nucleophile in a zinc-ion-catalysed aminolysis. The order of reactivity would appear to be determined mainly by the number of hydroxide groups available for bonding with the metal ion, a statistical effect which would be reasonable to expect if the propensity for the metal ion to form the ternary compound were the most important factor involved.

The general effect may be observed in Figure 4, which shows the variations in the rate constants with the Zn^{II} ion

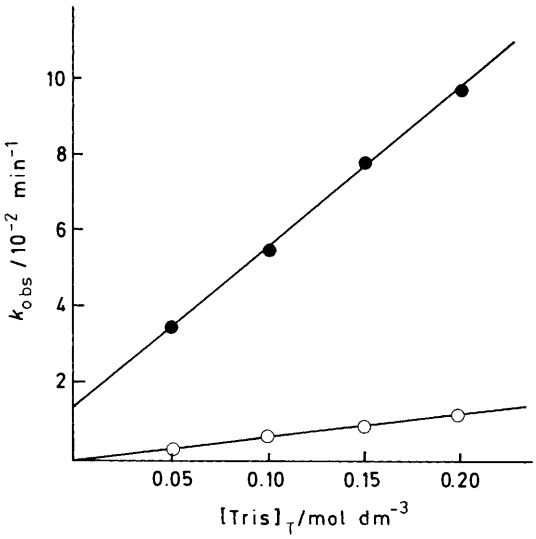


Figure 5. Dependence of the pseudo-first-order rate constant, k_{obs}, on the total concentration of Tris for the hydrolysis and aminolysis of lithium clavulanate (5.0×10^{-4} mol dm^{-3}) (○) and of the methyl ester of clavulanic acid (5.0×10^{-4} mol dm^{-3}) (●) at pH 8.00, 0.5 mol dm^{-3} ionic strength ($NaClO_4$) and $T = 35 \pm 0.1$ °C.

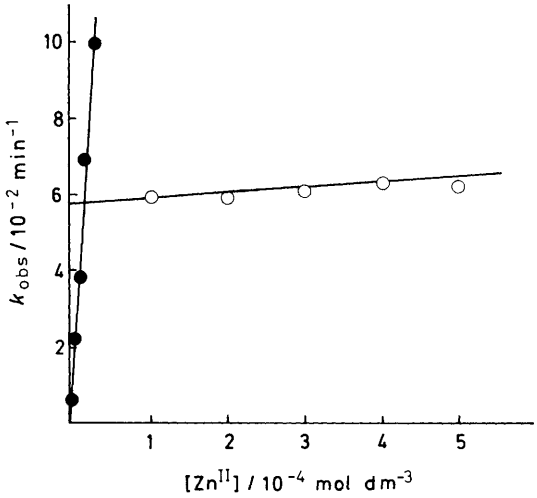


Figure 6. Effect of the concentration of the Zn^{II} ion on the constant k_{obs} for the decomposition, in the presence of Tris, of lithium clavulanate (5.0×10^{-4} mol dm^{-3}) (●) and of the methyl ester of clavulanic acid (5.0×10^{-4} mol dm^{-3}) (○) at pH 8.00, 0.5 mol dm^{-3} ionic strength ($NaClO_4$) and $T = 35 \pm 0.1$ °C.

concentration for these amines at pH = pK_a. Here we see that the slope is steeper for those amino alcohols with a greater number of hydroxide groups, although these experiments were performed at a lower pH, as is indicated in Figure 4. The influence of the pH may be observed by comparing the results obtained for Tris at pH 8.00 and 9.00.

In the case of 2-methoxyethylamine, the variation in the constant with the Zn^{II} ion concentration is very small. The mechanics explaining these results could be the nucleophilic attack by this amine on the co-ordination compound Zn^{II}–clavulanate.

Effect of Esterification of the Carboxylate Group.—In the absence of Zn^{II}, the ester reacts faster with Tris than does clavulanate (Figure 5). The ester is more liable to be attacked in the β-lactamic carbonyl than is the clavulanate, probably because the negative charge tends to repel the nucleophile (the hydroxide ion or Tris).

The presence of Zn^{II} has a slight effect on the decomposition of the ester in Tris (Figure 6). The rate constant is a linear function of the Zn^{II} ion concentration at the concentrations indicated, but the slope of this straight line is very small compared with that of the clavulanate ion under the same circumstances.

All these results are to be expected if the clavulanate is co-ordinated with Zn^{II} through the carboxylate ion. The blocking of the carboxylate group in the ester prevents the formation of a ternary complex, and if the ester bonds with Zn^{II}, it will be with substantially less affinity than the carboxylate, the predominant species in dissolution being the chelates formed by Tris with the Zn^{II} ($[ZnT]^{2+}$ and $[ZnT_2]^{2+}$) ions. A possible mechanism with the ester is an intermolecular nucleophilic attack by these chelates on the carbonyl carbon.[11] It is interesting to note that this mechanism could also be valid for the clavulanate, but at such a low rate that decomposition by this means becomes insignificant in relation to that *via* the ternary complex.

In summary, the above results are consistent with a mechanism of reaction *via* the ternary complex,[8] in which the Zn^{II} bonds with clavulanate and Tris acts as a template placing the substrate and nucleophile in the right position for the attack of the alkoxide group of Tris on the β-lactam carbonyl. The pK_a of the co-ordinated hydroxide group must decrease, in the presence of a strong nucleophile at slightly alkaline pH.

Acknowledgements

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