Catalysis of Hydrolysis and Aminolysis of Non-classical β-Lactam Antibiotics by Metal Ions and Metal Chelates

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The Zn^{2+}–tris (hydroxymethyl)aminomethane (Tris) system has a great catalytic effect on the hydrolysis and aminolysis of some β-lactam antibiotics. In order to ascertain the mechanism of this catalysis we have analysed the effects of the β-lactam antibiotic structure. First we studied the kinetics of the decomposition of imipenem, SCH 29482, aztreonam and nocardicin A in aqueous solution of Tris at 35.0 °C, 0.5 mol·dm^{-3} ionic strength and in the presence of metal ions (Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Mn^{2+}). From these studies, we conclude that Tris and metal ions (in separate solutions) exert a great catalytic effect on the hydrolysis of imipenem and SCH 29482. We suggest that in metal ion solutions a 1 : 1 complex is formed between the metal ion and β-lactam antibiotic, which is attacked by hydroxide ions.

Studies of the degradation of the antibiotics studied in solutions of Tris and metal ions together indicate that the systems Cd^{2+}–Tris and Zn^{2+}–Tris have a great catalytic effect on the hydrolysis and aminolysis of imipenem and SCH 29482. We suggest that this catalysis takes place *via* a ternary complex in which the metal ion plays a double role by (a) placing the antibiotic and the Tris in the right position for the reaction and (b) lowering the pK_a of the hydroxide group of Tris, which is coordinated with the metal ion, generating a strong nucleophile.

Keywords β-lactam antibiotic; imipenem; penem; SCH 29482; aztreonam; nocardicin A; aminolysis; hydrolysis; metal-ion catalysis

Introduction

It is generally well known that metal ions promote the degradation of some β-lactams and that a number of hydrolytic enzymes contain metal ions at their active sites. The β-lactamase II from *Bacillus cereus* 569/H is known to be zinc-ion dependent.[1] A number of systems have been proposed as models for these enzymes.[2] An important example is the mixture of Zn^{2+} ions with tris(hydroxymethyl)aminomethane (Tris).[3-5] This research is aimed at determining 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 β-lactam antibiotics.

In a previous paper,[5] we demonstrated the rapid decomposition undergone by clavulanic acid in the presence of aminoalcohols, metal ions and metal chelates. In the present work, we determine the catalytic effect exerted by these species on the decomposition of imipenem, SCH 29482, aztreonam and nocardicin A (Fig. 1).

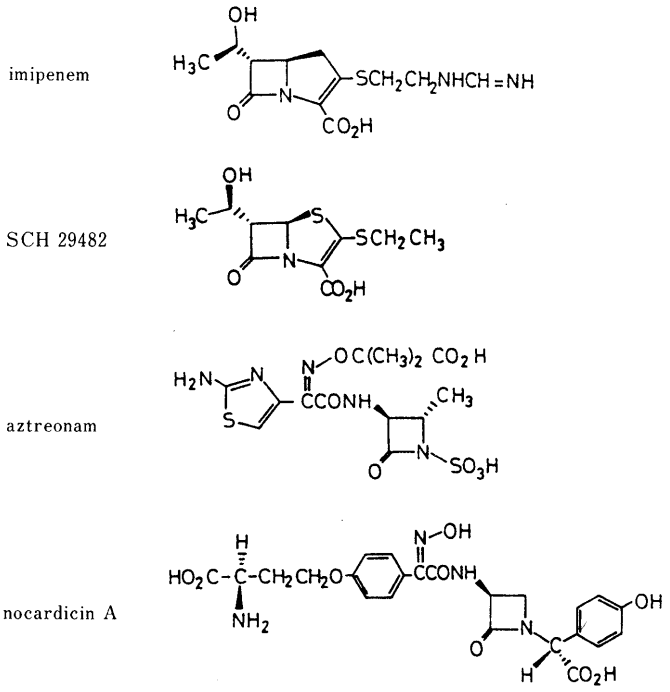

Fig. 1. Chemical Structures of the Antibiotics Studied

Experimental

Materials Imipenem (*N*-formimidoylthienamycin) was supplied by Merck (Sharp & Dohme of Spain, S. A.); SCH 29482 was supplied by the Schering Corporation (Bloomfield, U.S.A.); aztreonam by E. R. Squibb & Sons, Inc. (New Jersey, U.S.A.); and nocardicin A by the Fujisawa Pharmaceutical Co. (Osaka, Japan).

All the water used was purified with a Milli-Q-reagent water system (Millipore, Bedford, MA, U.S.A.).

Buffer Solutions The buffer solutions used in the kinetic studies were prepared freshly from 0.050 mol·dm^{-3} KH_2PO_4 (pH 7.50) and 0.050 mol·dm^{-3} Tris (pH 7.50—9.00) adjusted to an ionic strength of 0.5 mol·dm^{-3} with sodium perchlorate monohydrate, with which they were dissolved in purified water, the pH of the phosphate being adjusted with concentrated sodium hydroxide, and that of the Tris with hydrochloric acid. The pH's were measured at 35 °C by means of a pH meter with a combination electrode.

Solutions of Metal Ions Stock solutions of reagent-grade metal salts ($CdCl_2 \cdot 2.5\ H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnCl_2$, $CuCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$), obtained from Farmitalia Carlo Erba (Milan, Italy), were diluted to the required concentration of metal ion.

Analytical Procedures 1) Liquid Chromatography: A reverse-phase high-performance liquid chromatographic (RP-HPLC) method was used to follow the kinetics of the degradation of imipenem, aztreonam and nocardicin A. The HPLC system consisted of a Konik KNK-500A liquid chromatograph, a Rheodyne 7125 loop injector (volume 20 μl), a Water 441 UV detector and a Varian 4290 computing integrator. The detector was set at 313 nm for the analysis of imipenem and at 280 nm for the determination of aztreonam and nocardicin A. The separation was carried out using a Spherisorb ODS-2 RP-18 column (10 μm; 25 × 0.4 cm i.d.) with the following mobile phases: phosphate (0.1 mol·dm^{-3}, pH 7.0)–methanol (93 : 7) for imipenem, (96 : 4) for nocardicin A and (90 : 10) for aztreonam. A pre-column (3 × 0.4 cm i.d.) packed with μ-Bondapak C18 (30 μm) was used to guard the main column. The flow-rate was 1.0 ml·min^{-1}. All chromatographic operations were carried out under ambient conditions.

Analyses of the experimental reproducibility and of the various time plots indicate the relative uncertainty of the observed rate constant, k_{obs}, to be 10—15% at the 95% confidence level.

2) Spectrophotometric Method: A spectrophotometric method was used to follow the kinetics of the degradation of SCH 29482. This is based on

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the spectrophotometric measurement at 386 nm of the reaction product with imidazole formed at 35 °C in a 1.5 mol·dm^{-3} imidazole/2×10^{-3} mol·dm^{-3} mercuric chloride solution at pH 7.50 for 15 min.[6]

Kinetic Procedure Weighed amounts of the antibiotic were dissolved in the buffer solutions preheated to a desired temperature to produce a final antibiotic concentration of 2×10^{-4} mol·dm^{-3}. Aliquots (10 ml) of the solutions were each sealed in a glass vial. All reactions were conducted in a constant-temperature water bath at 35.0±0.1 °C with the total buffer concentration greatly exceeding the reacting substrate concentration to maintain pseudo-first-order kinetics. Aliquots of the solution were withdrawn at appropriate time intervals and assayed immediately. The pH values of the reaction solutions were measured at the experimental temperature initially and at the end of each experiment on a pH meter. No significant changes in pH were observed.

We determined the degradation rate constants of the antibiotics studied in metal ion solutions following the methods described above. For fast kinetics the reaction was blocked with EDTA (final concentration 2×10^{-4} mol·dm^{-3}), before the analysis of the remaining antibiotic.

Results and Discussion

Stability in Tris Solutions Over the pH range of 7.50—9.00, the decomposition of antibiotics studied in aqueous solution in the presence of an excess of Tris and a constant pH follows pseudo-first-order kinetics. The pseudo-first-order rate constant, k_{obs}, obtained from the slope of the semilogarithmic plots of the residual concentration *versus* time by a least-squares treatment, is the sum of a first-order hydrolysis rate, k_{pH} (Eq. 1), and the first-order rate constant depending on the concentration of Tris, k_{Tris}.

$$k_{pH}=k_{H_2O}+k_H[H^+]+k_{OH}[OH^-] \quad (1)$$

The equation

$$k_{obs}=k_{pH}+k_{Tris} \quad (2)$$

allows for the determination of k_{Tris} once the value of k_{pH} is known for each pH. Table I includes data concerning k_{H_2O}, k_{OH} and k_H, from which K_{pH} has been determined.

In order to study the influence of the chemical species present in the aqueous solution of Tris on the stability of the antibiotics studied, and to determine their catalytic constants, experiments were carried out in which k_{obs} was experimentally determined as a function of the concentration of the Tris, while the pH, ionic strength, temperature, and the concentration of EDTA were kept constant. The representation of k_{obs} and k_{Tris} *versus* the total Tris concentration, $[Tris]_T$, for each pH, is linear (Fig. 2), with k_{Tris} increasing greatly with pH. For amines, amino alcohols and amino acids, these pseudo-first-order rate constants apparently obey the general relationship previously reported for penicillins, cephalosporins and clavulanic acid[10-13] and which for amino alcohol Tris is expressed as:

$$k_{Tris}=k_1[Tris]+k_2[Tris]^2+k_3[Tris][OH^-] \quad (3)$$

Equation 3 can be re-written as:

$$\frac{k_{Tris}}{[Tris]_L}=k_1+k_3[OH^-]+k_2[Tris]_L \quad (4)$$

where k_1 represents the unassisted or water-catalysed reaction of free amine on the β-lactam moiety, k_2 represents the general base-catalysed and nucleophilic reaction assisted by a second molecule of amine, k_3 is the hydroxide-ion-catalysed third-order rate constant of nucleophilic displacement by the amine, and $[Tris]_L$ represents the unprotonated Tris concentration. The pK_a of Tris at 35 °C with an ionic strength of 0.5 mol·dm^{-3}, is 8.012.[3] Equation 4 predicts that plots of $K_{Tris}/[Tris]_L$ *versus* $[Tris]_L$ will provide k_2 as the slope and $k_1+k_3[OH^-]$ as the intercept. The plots of these intercepts *versus* hydroxide-ion concentration provide k_3 as the slope and k_1 as the intercept.

Representations of Eq. 4 for imipenem are shown in Fig. 3. As can be seen, the constant k_{Tris} is practically independent of the $k_2[Tris]_L^2$ and $k_3[Tris]_L[OH^-]$ terms.

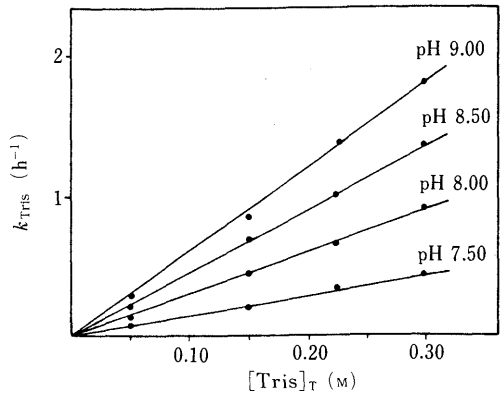

Fig. 2. Plot of the Rate Constants, K_{Tris}, in the Degradation of Imipenem *versus* the Total Tris Concentration

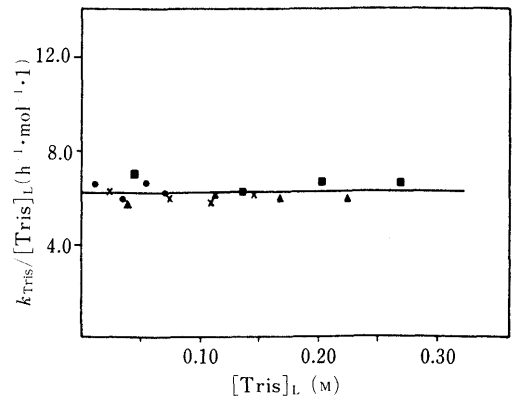

Fig. 3. Plot of Eq. 4 for the Aminolysis of Imipenem in an Aqueous Solution of Tris

●, pH 7.50; ×, pH 8.00; ▲, pH 8.50; ■, pH 9.00.

TABLE I. Catalytic Rate Constants of the Water Species in β-Lactam Antibiotic Hydrolysis at 35 °C and μ=0.5 mol·dm^{-3}

β-Lactam antibiotic	k_H (mol^{-1}·dm^3·h^{-1})	k_{H_2O} (h^{-1}×10^3)	k_{OH} (mol^{-1}·dm^3·h^{-1}×10^{-2})	Ref. No.
Imipenem	1207±220	6.61±1.63	89.16±17.31	7
SCH 29482	16.2±2.06	2.17±0.13	14.97±1.06	8
Aztreonam	2.55±0.299	0.221±0.0189	21.17±1.19	9
Nocardicin A	0.293±0.082	0.119±0.0083	0.0781±0.0113	9

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The intercepts are almost equal for the different pHs studied, their representation against hydroxide ion concentrations therefore producing a straight slope equal to k_3 with a value of practically zero. Similar results are obtained for SCH 29482, aztreonam and nocardicin A. The constants k_2 and k_3 are practically zero, probably owing to steric hindrance in the simultaneous nucleophilic attack of two molecules of Tris or Tris and hydroxide-ion.[14] The values of the constant k_1 are summarized in Table II. The values of both k_{pH} and k_1 (Tables I and II) are higher for imipenem than for the other antibiotics studied.

These results show how high the reactivity of Tris is in the decomposition of imipenem, which must be motivated mainly by the inhibition of amide resonance and the tension of the rings in its structure. It is interesting to observe that the reactivity of aztreonam to aminolysis is similar to that of SCH 29482 and significantly different to that of nocardicin A. Given that aztreonam shows no inhibition of amide resonance, it would seem that this reactivity is produced owing to the need of the sulphonic group to increase the electrophilic character of the carbonyl carbon of the β-lactam ring and favour the cleavage of the tetrahedral compound formed. This would be in agreement with Page's finding,[15] whereby the inhibition of amide resonance is one factor influencing the reactivity of β-lactam antibiotics, but not the decisive one. On the other hand, bearing in mind that the tension of the β-lactam ring in aztreonam and in nocardicin A must be similar, it would not seem likely that the reactivity of aztreonam is due to this factor.

These results strongly suggest that the fundamental process leading to the opening of the β-lactam moiety is the nucleophilic attack of the carbonyl group by the amino nitrogen (Chart 1).

Stability in Metal Ions We studied the stability of these β-lactam antibiotics in aqueous solution in the presence of the metal ions Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+} at concentrations ranging from 0.5×10^{-5} and 4×10^{-5} $mol\cdot dm^{-3}$ at pH 7.50, ionic strength $0.5\,mol\cdot dm^{-3}$ (adjusted with $NaClO_4\cdot H_2O$), at 35 °C with a buffer solution of phosphates at $0.050\,mol\cdot dm^{-3}$ to keep the pH constant.

Figures 4, 5 and 6 show the rate constants observed *versus* the metal ion concentration for imipenem, SCH 29482 and aztreonam, respectively. As may be seen, it is in the degradation of imipenem where the effect of the metal ions, especially Cu^{2+}, is at its greatest. Thus, a concentration of Cu^{2+} ($2\times10^{-5}\,mol\cdot dm^{-3}$) reduces the semi-reaction period of imipenem from 36 to 5 h. This is not a great catalytic effect when compared with that of the same ion on benzylpenicillin,[16] which has a semi-reaction period

Table II. Aminolysis Constants of the Antibiotics Studied in Aqueous Solution of Tris

Antibiotic	k_1 ($h^{-1}\cdot mol^{-1}\cdot l$)	Antibiotic	k_1 ($h^{-1}\cdot mol^{-1}\cdot l$)
Imipenem	6.37	Aztreonam	0.581
SCH 29482	1.39	Nocardicin A	2.65×10^{-3}

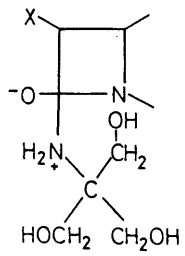

Chart 1

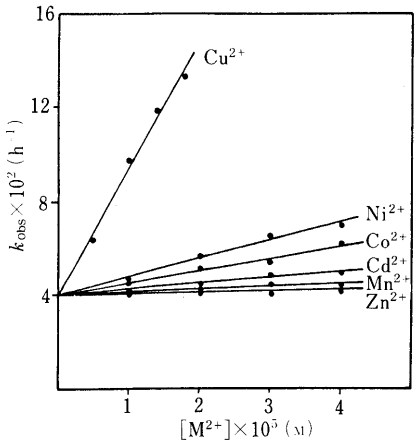

Fig. 4. Plot of the Rate Constants Observed, k_{obs}, for the Degradation of Imipenem *versus* the Concentrations of the Ions Shown

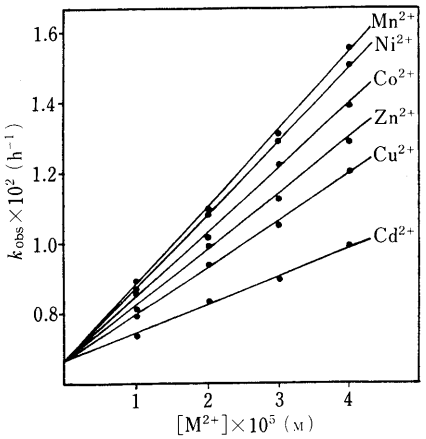

Fig. 5. Plot of the Rate Constants Observed, k_{obs}, for the Degradation of SCH 29482 *versus* the Concentrations of the Ions Shown

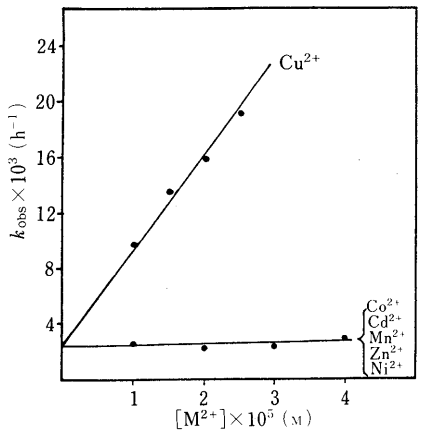

Fig. 6. Plot of the Rate Constants Observed, k_{obs}, for the Degradation of Aztreonam *versus* the Concentrations of the Ions Shown

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ranging from 11 weeks down to 0.1 s in the presence of Cu^{2+} at pH 7.00, or on clavulanic acid,[17] for which a concentration of 2×10^{-5} mol·dm^{-3} of Cu^{2+} in water reduces the semi-reaction period from 50 h to 14 min.

The Cu^{2+} ion also has a great catalytic effect on aztreonam, though not so great as on imipenem. The catalytic effect of the other metal ions on these antibiotics is small.

The catalytic effect of the above metal ions on SCH 29482 is similar bur lesser than on imipenem. In the case of nocardicin A, the effect was practically null in all cases.

In order to explain the catalytic effect of the metal ions, we invoke a mechanism similar to that suggested by Gensmantel *et al.*[18] for metal-ion catalysed hydrolysis of β-lactam antibiotics, whereby the metal ion exerts Lewis-acid catalysis by activating the bond between the carbonyl carbon and the nitrogen of the β-lactamic ring, and by facilitating the nucleophilic attack on the β-lactam carbonyl by neutralization of the charge in the reagents and/or transition state. In this model the antibiotic must act as a chelate binder on the metal ions. The coordination of the metal ions with the antibiotic must occur with the interaction of the carboxylate group, as is demonstrated by the fact that these metal ions show almost no catalytic effect on the decomposition of the esters of some β-lactam antibiotics,[16-18] the second point of coordination being the β-lactamic nitrogen.[19] This model described for penicillins and clavulanic acid may be applicable to imipenem and SCH 29482, as both compounds have a carbonate skeleton similar to that of the others. In the case of aztreonam, the probable coordination sites favoring the nucleophilic attack on the β-lactamic ring must be the sulphonate group and the β-lactamic nitrogen or the oxygen of the carbonyl group. For nocardicin A these coordination sites should be the nitrogen of the β-lactamic ring and the carboxylate group situated near it. They must, however, become differently arranged in space from these points in the penicillins, there being no coordination with the metal ion since no catalytic effect of the metal ions studied is noticed.

It is interesting to note that for imipenem, aztreonam and nocardicin A, other coordination points exist on the side chain which could lower the catalytic action of these metal ions in the degradation of these antibiotics. In the degradation of imipenem, the Cu^{2+} ion may coordinate with amino groups in the side chain, which would explain why this ion has a significantly lesser catalytic effect than it has on the degradation of clavulanic acid and benzylpenicillin, as has already been pointed out.

The nucleophilic attack of the hydroxyl ions would occur on the carbonyl carbon of the β-lactamic ring (compound **1** in chart 2). According to Gensmantel *et al.*'s[16,18] findings that the rate-limiting step for the non-catalyzed reaction of the hydroxyl ion with the antibiotic is the cleavage of the C–N link of the intermediate tetrahedron (compound **2** in Chart 2). The coordination of the metal ion with the antibiotic would probably facilitate the nucleophilic attack by increasing the electrophilic capacity of the carbonyl carbon and also the cleavage step of the β-lactamic ring. In this case the function of the metal ion in the catalyzed reaction would be the stabilization of the intermediate compound (compound **2**), with a consequent increase in the rate of cleavage of the C–N link. If nitrogen protonation occurred in the non-catalyzed reaction,[20] then the function of the metal ion would simply be that of a superacid taking over the role of the proton.[16] In line with these observations and owing to the fact that the metal ion facilitates the cleavage of the C–N link, the rate-limiting step of the reaction could change from being the cleavage of the tetrahedral intermediate for the non-catalyzed reaction to being the formation of the intermediate in the reaction catalyzed by metal ions.

Stability in Metal Chelates The degradation of the antibiotics studied in an aqueous solution of Tris (0.050 mol·dm^{-3}, ionic strength 0.5 mol·dm^{-3}, at 35 °C and pH 8.00) in the presence of concentrations ranging from 2×10^{-6} and 4×10^{-5} mol·dm^{-3} of the ions Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Mn^{2+} takes place following pseudo-first-order kinetics. The rate constants have been represented against the concentrations of these ions in Figs. 7, 8, 9 and 10, there being observed in all cases a linear dependence of these constants on the concentration of metal ions in the interval studied.

As may be observed, the catalytic effect exerted by these ions in Tris buffer solution is greater than in a phosphate solution. These differences are especially noticeable for imipenem and SCH 29482, where the Tris rate constants can be of the order of 200 or 400 times greater than in

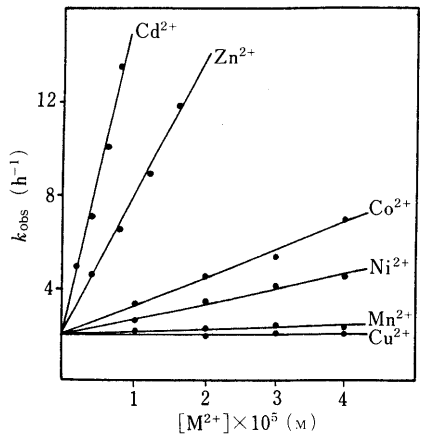

Fig. 7. Plot of the Rate Constants Observed for the Degradation of Imipenem *versus* the Concentrations of the Ions Shown, in Tris Buffer Solution

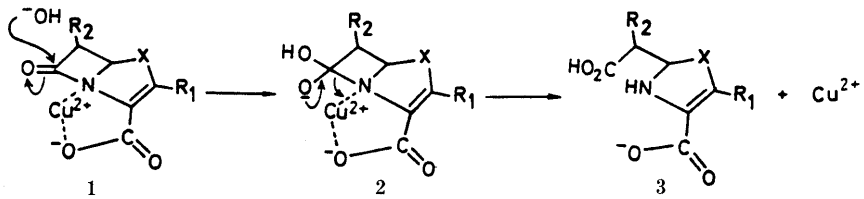

Chart 2

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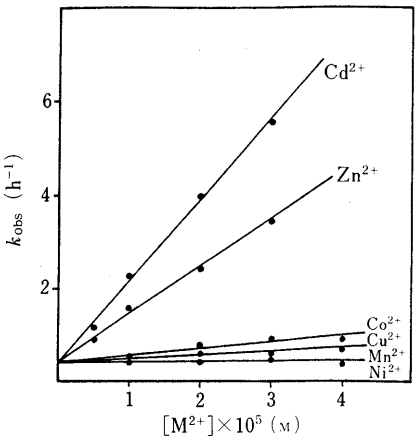

Fig. 8. Plot of the Rate Constants Observed for the Degradation of SCH 29482 *versus* the Concentrations of the Ions Shown, in Tris Buffer Solution

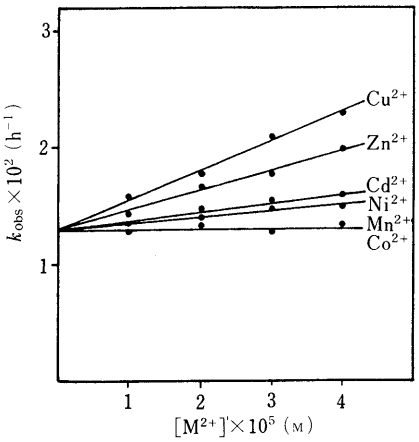

Fig. 9. Plot of the Rate Constants Observed for the Degradation of Aztreonam *versus* the Concentrations of the Ions Shown, in Tris Buffer Solution

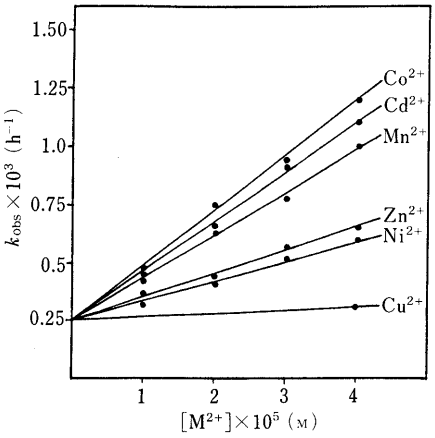

Fig. 10. Plot of the Rate Constants Observed for the Degradation of Nocardicin A *versus* the Concentrations of the Ions Shown, in Tris Buffer Solution

phosphates, if we compare the values of these constants of the most catalytic metal ions. Thus, the period of semi-decomposition of, for example, imipenem in phosphates is about 17 h at a concentration of 1×10^{-5} mol·dm^{-3} of Zn^{2+} or Cd^{2+} ions and is 7 and 15 min respectively in Tris with Zn^{2+} and Cd^{2+} at the same concentration. A similar

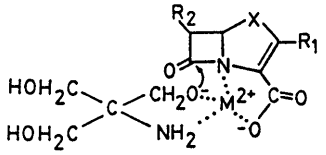

Chart 3

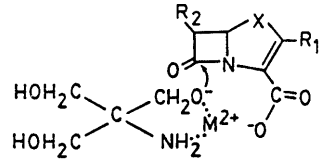

Chart 4

variation is observed in the reactivity of SCH 29482 under the same conditions. k_{obs} for the degradation of imipenem in Tris buffer solution containing EDTA (0.183 h^{-1}) is considerably lower than it is in a similar solution without EDTA (1.94 h^{-1}) and with a zero metal ion concentration, as the absence of EDTA causes the trace concentrations of metal ions (Cd^{2+}, Zn^{2+}) present to have a catalytic effect, thus raising k_{obs}. It is also interesting to note that Cu^{2+}, the ion with the greatest catalytic effect on the degradation of imipenem in phosphates, has no such effect in Tris buffer solution (Fig. 7), probably owing to its forming complexes with the Tris.[21]

Several papers exist concerning the catalytic effect of certain transition metal ions on β-lactamic antibiotics. Of special interest are those by Gensmantel *et al.*,[16,18] Schwartz[3] and Tomida and Schwartz[4] on the catalysis of hydrolysis and the aminolysis of benzylpenicillin in the presence of some of these ions, together with work carried out more recently in our laboratory with clavulanic acid,[5,17] where different mechanisms are suggested to explain the catalytic effect of these ions. In line with these studies, we suggest that the decomposition of imipenem and SCH 29482 in the presence of the Zn^{2+}–Tris and Cd^{2+}–Tris systems must be carried out according to the mechanism implying the formation of a ternary complex (Chart 3), in which the metal ion would have a template effect; that is, it would put the reactive groups of the reacting molecules in the right position and direction, in a way analogous to what happens with benzylpenicillin[3,4] and clavulanic acid.[5]

Other models have also been suggested, one of them analogous to the one set out in Chart 4, where the Zn^{2+} exerts a Lewis's acid catalysis. Another possible model is based on the increase in the nucleophilic capacity of Tris on coordination with the metal 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.[22] In this way, these groups acting as chelates may become quite strongly nucleophilic in the hydrolysis of β-lactam antibiotics at around pH 7.0. This model, however, would be the one to explain the results obtained in the degradation of aztreonam and nocardicin A, as in the two previous models, there has to be coordination between the β-lactamic compound and the metal ions, although this coordination obviously does not occur with aztreonam and nocardicin A, with the

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exception of Cu^{2+} and aztreonam, if we bear in mind the results obtained in the previous section.

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