Pharmaceutical Research, Vol. 4, No. 3, 1987

Research Article

Kinetics and Mechanism of Zinc Ion-Mediated Degradation of Cephalosporins in Tromethamine Solution

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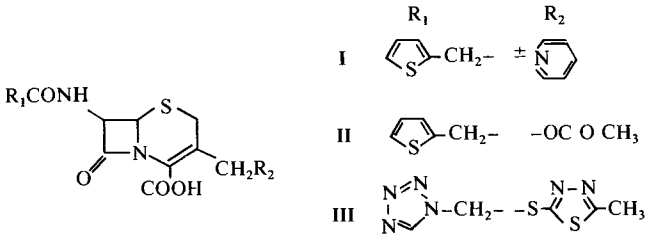
Received June 3, 1986; accepted March 10, 1987

Earlier studies of the hydrolysis and aminolysis of penicillin, in the presence of zinc ion and tromethamine (Tris), revealed a very rapid catalysis mediated by a ternary complex in which the metal ion brought the reactants into close proximity in a suitable configuration for reaction. In the present work similar studies with a group of cephalosporins show not only much slower rates of reaction but a different mechanism in which the zinc ion–tromethamine complex functions as a nucleophile in a bimolecular reaction. Evidence for the differences in mechanism includes not only the different dependence of rate upon tromethamine concentration, but comparable rates of reaction of methyl esters of a penicillin and a cephalosporin and the reaction products observed by high-performance liquid chromatography.

KEY WORDS: catalysis; zinc ion; hydrolysis; aminolysis; cephalosporins.

INTRODUCTION

Studies of the rate of degradation of penicillin in the presence of zinc ion and tromethamine at pH 7–10 had shown that the high rate accelerations observed were mediated by a ternary complex in which both tromethamine and penicillin were bound to zinc ion (1). Within the ternary complex a rapid nucleophilic attack by alkoxide ion derived from the ionization of the tromethamine hydroxyl group bound to zinc ion could occur, yielding *O*-(penicilloyl)tromethamine as a degradation intermediate. This mechanism has been supported by further studies in which the effects of structural changes in the penicillin side chain, other amines, and other metal ions were investigated (2). In the present study, similar experiments were carried out with some representative cephalosporins [cephaloridine (I), cephalothin (II), and cefazolin (III)]. These antibiotics contain a six-membered dihydrothiazine ring, in contrast with the five-membered thiazolidine ring of the penicillins. Since reaction via the formation of a ternary complex appears to be largely affected by steric factors within the ternary complex such as the distance between the metal ion-coordination sites on the β-lactam antibiotics (3–6) and the orientation of the catalytic group for intracomplex nucleophilic attack on the β-lactam carbonyl, one might expect different reaction rates and/or reaction mechanisms than observed with the penicillins.



MATERIALS AND METHODS

Materials

The cephalosporins studied and some derivatives were used as received from the suppliers: sodium cephalothin (Eli Lilly), sodium cefazolin (Fujisawa Pharmaceutical), cephaloridine, sodium deacetylcephalothin, and cephalothin methyl ester (Shionogi). Tromethamine (Trizma) was from Sigma Chemicals). Zinc chloride solution was prepared from reagent-grade zinc metal (Aldrich Chemical) as previously described (1). All other chemicals and solvents used were of reagent grade.

Kinetics

All rate measurements were carried out at 35°C with the ionic strength brought to 0.5 by the addition of potassium chloride. Tromethamine, pK_a 8.01 at 35°C and ionic strength 0.5, acted as its own buffer. The buffers were prepared just before use with calculated amounts of tromethamine and standard hydrochloric acid solution. In some cases where the buffer capacity was too low, the pH was maintained constant using a pH-stat (Model PCA270, Radiometer, Copenhagen, Denmark). The rates of loss of the cephalosporins

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from solution were followed by one of the following two methods. There was no significant difference in rate constants as obtained by these methods.

Direct Spectrophotometric Method. The reaction solutions (3 ml) were kept in 1-cm cuvettes placed in the thermostated compartment of a Cary 219 spectrophotometer (Varian Instruments, Palo Alto, Calif.) equipped with a stirrer accessory. The reaction was initiated by the addition of 20 μl of cephalosporin solution and the decrease in absorbance at 260 nm (7) was recorded continuously. The initial cephalosporin concentration in reaction solutions was 6–8 $\times 10^{-5}$ *M*. Pseudo-first-order rate constants were calculated from the absorbance–time curves by the method of Guggenheim (8).

Sampling Method. The reaction solutions (43 ml) were kept at a constant temperature in a screw-capped test tube or in a jacketed glass cell. After the addition of cephalosporin solution (1 ml) to give an initial concentration of 1–2 $\times 10^{-4}$ *M*, samples of 1 ml were taken at appropriate intervals, diluted with 4 ml of 2×10^{-4} *M* disodium edetate to quench the reaction in an ice-cooled water bath, and analyzed by the high-performance liquid chromatographic (HPLC) method as described below.

Liquid Chromatography

The liquid chromatography was performed with a Shimadzu Model LC4A instrument equipped with a Rheodyne (Model 7125) injection valve fitted with a 20-μl loop. The column used, 25 cm long and 4.6 mmin i.d., was packed with Zorbax ODS (5-μm particles). Three different mobile-phase systems were used to obtain excellent resolution of intact cephalosporin and its degradation products: (A) a mixture consisting of 70% (v/v) 0.00857 *M* phosphate buffer, pH 7.50, containing 0.0114 *M* tetrabutylammonium bromide (TBAB) and 30% (v/v) acetonitrile for cephalothin; (B) a mixture consisting of 60% (v/v) 0.01 *M* phosphate buffer, pH 7.50, containing 0.0113 *M* TBAB and 40% (v/v) acetonitrile for cephalothin methyl ester; and (C) a mixture consisting of 82% (v/v) 0.00732 *M* phosphate buffer, pH 7.50, containing 0.00976 *M* TBAB and 0.1 *M* potassium chloride and 18% (v/v) acetonitrile for cephaloridine and cefazolin. The flow rate of the mobile phase was 1.0 ml/min and the column was maintained at 30–32°C. The column effluent was monitored by UV absorption at 240 nm and peak height measurements were used for quantitation. Peak height was proportional to concentration.

RESULTS AND DISCUSSION

Degradation of Cephalosporins in Tromethamine Solution

The reaction of the cephalosporins with excess tromethamine in the absence of metal ions followed first-order kinetics and the observed rate constant (k_{obs}) was a linear function of the stoichiometric concentration of tromethamine as shown in Fig. 1. In measuring these rates, disodium edetate (1×10^{-4} *M*) was included in the reaction solutions to prevent small amounts of possible metal ion contaminants from exerting any influence on the reaction. The extrapolated values at zero tromethamine concentration coincided with those reported under the same conditions by Yamana and Tsuji (7).

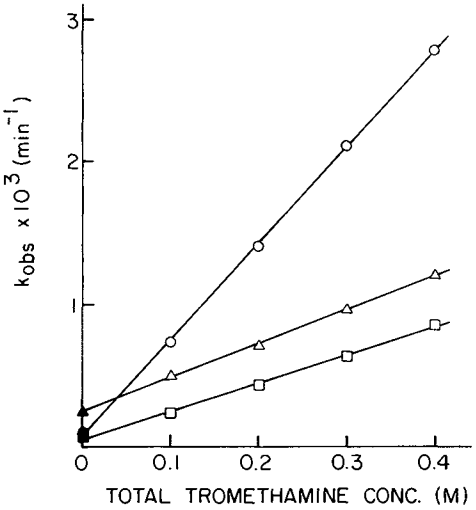


Fig. 1. Effect of the tromethamine buffer concentration on the pseudo-first-order rate constant (k_{obs}) for the degradation of cephaloridine (○), cephalothin (△), and cefazolin (□) at pH 8.00. The rate constants for cephaloridine were divided by two and the filled symbols on the ordinate indicate the data from Yamana and Tsuji (7).

From the slopes of the straight lines in Fig. 1, obtained by linear regression, the second-order rate constants for the aminolysis of the cephalosporins by tromethamine base were calculated and are listed in Table I, together with hydroxide ion-catalyzed second-order rate constants. Inspections of these rate constants shows that cephaloridine is three to six times more reactive than the other cephalosporins and has almost the same reactivity as or a slightly higher reactivity (three times in k_{OH}) than benzylpenicillin.

Effect of Zinc Ion

Although the penicillin reaction was greatly enhanced in tromethamine solutions with 10^{-6}–10^{-5} *M* zinc ion (1), no significant effect on the rate constant was observed at those zinc ion concentrations in the case of cephalosporins.

At much higher concentrations of zinc ion, however, there was observed a significant effect on the rate of loss of cephalosporins from solution, as shown in Fig. 2. The ob-

Table I. Second-Order Rate Constants[a] for the Reactions of Tromethamine and Hydroxide Ion with Some Cephalosporins and Benzylpenicillin in Aqueous Solution at 35°C and Ionic Strength 0.5

Compound	$k_{tromethamine}$	k_{OH}[b]
Cephaloridine	2.77×10^{-2}	64.7
Cephalothin	4.83×10^{-3}	17.7
Cefazolin	4.45×10^{-3}	19.0
Benzylpenicillin	3.2×10^{-2}[c]	19.8

[a] Rate constants in M^{-1} min^{-1}.
[b] From Yamana and Tsuji (7).
[c] From the previous study (1).

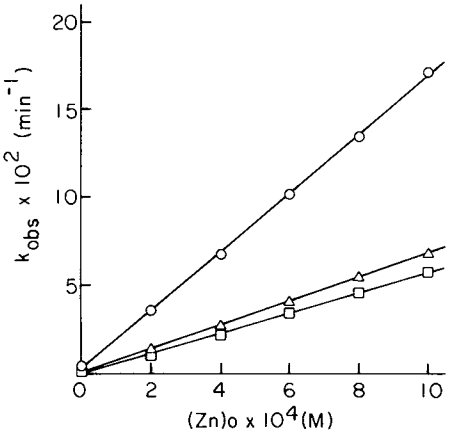


Fig. 2. Effect of the total zinc ion concentration on the pseudo-first-order rate constant (k_{obs}) for the degradation of cephaloridine (○), cephalothin (△), and cefazolin (□) in pH 8.00, 0.30 *M* tromethamine buffer solution.

served first-order rate constant is found to be a linear function of the total zinc ion concentration up to 10^{-3} *M* in every case.

In Fig. 3 is shown the dependence of the first-order rate constant (k_c) on the tromethamine base concentration at pH 8.00 and 6.00 × 10^{-4} *M* zinc ion for the three cephalosporins. Here, k_c is the rate constant corrected for the rate with tromethamine alone. For the cephalosporins, k_c increases rapidly with increases in the concentration of the base and then reaches a plateau at more than 0.1 *M* in every case. It is of interest to note that this dependence is quite different from that observed previously with penicillins (1,2). In the latter, the rate constants showed a clear maximum at about 0.02 *M* tromethamine base, followed by a gradual decrease at the higher concentration. This is shown in Fig. 3 for benzylpenicillin as a typical example.

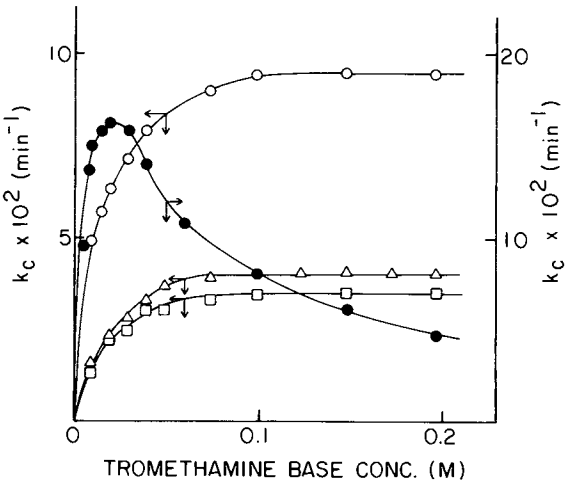


Fig. 3. Dependence of the rate constant (k_c) for the degradation of cephaloridine (○), cephalothin (△), and cefazolin (□) on the tromethamine base concentration at pH 8.00 and 6.00 × 10^{-4} *M* $(Zn)_o$. The rate constant (k_c) was corrected for the reaction with tromethamine alone. The filled circles refer to the rate constant for benzylpenicillin at pH 8.00 and 5.00 × 10^{-6} *M* $(Zn)_o$ as reported previously (2).

The dependence of the rate constant of cephalosporin degradation on the tromethamine base at various pH's in the presence of a constant zinc ion concentration (6.0 × 10^{-4} *M*) was further examined with cephalothin. As can be seen in Fig. 4, the rate constants increase with increasing pH. When the logarithm of the rate constant at plateau (k_p) divided by the total zinc ion concentration, $[k_p/(Zn)_o]$, is plotted against the pH, a straight line with a slope of almost unity is obtained at pH values up to about 8, but at higher pH the rate constants increase less rapidly as shown in Fig. 5. As described previously in the case of penicillin (1), this behavior usually indicates that an ionization of a catalytic species is involved in the degradation reaction prior to the rate-determining step.

Effect of Esterification of the Carboxyl Group

The effect of esterification of the carboxyl group of cephalosporin on the degradation rate was investigated by employing the methyl ester of cephalothin. It was found that the degradation of the ester is also enhanced in the zinc ion–tromethamine system and k_c shows a dependence on the tromethamine base concentration similar to those of the other cephalosporins as shown in Fig. 6. The plateau rate constant for the cephalothin ester is about 17 times lower than for free cephalothin at each pH studied. This can be contrasted with the great difference in the case of benzylpenicillin, where the esterification of the carboxyl group resulted in a 1400-fold decrease in the rate constant at the maximum under the same experimental conditions (2). Here it should be noted that the nucleophillic attack in both bases is at the β-lactam carbonyl, not the ester moiety.

Product Analysis

The degradation products of hydrolysis and aminolysis of cephalosporins have been extensively studied, and possible chemical structures of the products proposed, by Hamilton-Miller *et al.* (9,10). According to these workers, those cephalosporins carrying a leaving group at C-3, such as cephaloridine, cephalothin, and cefazolin, expel that group

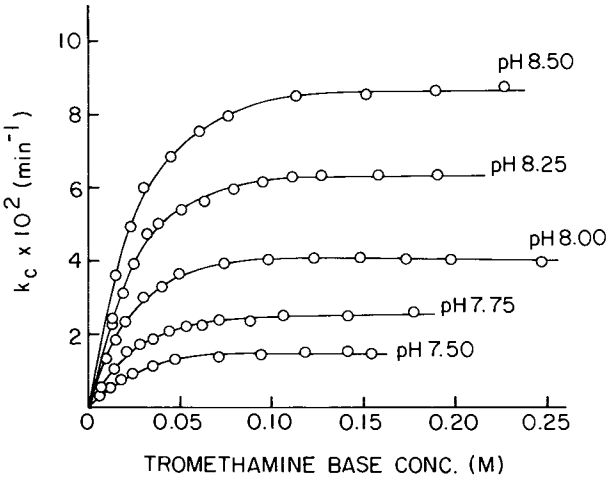


Fig. 4. Dependence of the rate constant for the degradation of cephalothin on the tromethamine base concentration at various pH's. The total concentration of zinc ion in the reaction solutions was 6.00 × 10^{-4} *M* throughout.

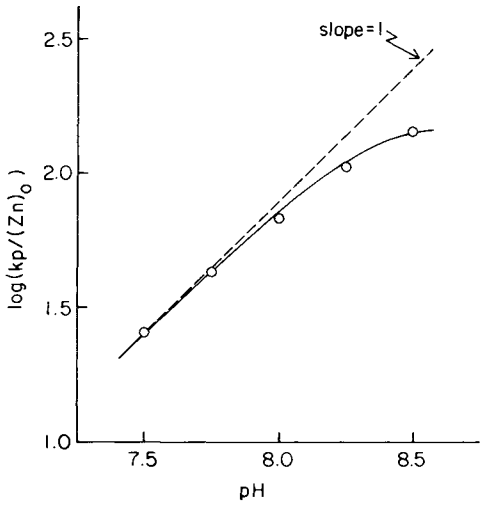


Fig. 5. The logarithm of the second-order rate constants $[k_p/(Zn)_O]$ for the zinc ion–tromethamine-catalyzed degradation of cephalothin plotted as a function of pH.

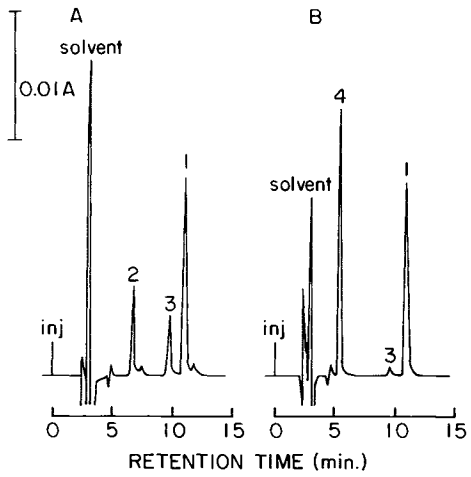
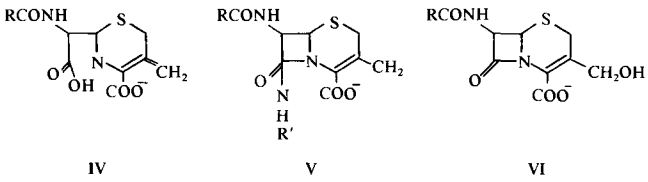


Fig. 7. Reverse-phase high-performance liquid chromatograms of the reaction solutions of cephalothin at 50% degradation. (A) In pH 10.0, 0.1 *M* $NaHCO_3$–Na_2CO_3 buffer; (B) in pH 8.00, 0.30 *M* tromethamine buffer containing 6.00×10^{-4} *M* zinc ion.

almost simultaneously with the cleavage of the β-lactam ring, yielding compound IV by hydrolysis or compound V by aminolysis as a degradation intermediate. These products may further degrade to small molecular fragments.



In the present study, the degradation products were separated by a high-performance liquid chromatographic (HPLC) technique. Figure 7 demonstrates typical chromatograms in the two different reaction solutions at the point where 50% of the initial cephalothin decomposed. By hydroxide ion-catalyzed hydrolysis (Fig. 7A), two peaks (peaks 2 and 3) were observed as well as an intact cephalothin peak (peak 1). Peak 2 could be assigned to deacethylcephalothin (VI) resulting from 3-acetoxy moiety hydrolysis, and peak 3 to the imine-type hydrolysis product (IV). These were confirmed from HPLC of authentic samples of IV and VI and/or from their UV absorption spectra. On the other hand, in the zinc ion–tromethamine system, cephalothin yielded mainly the corresponding amide compound (V) (peak 4 in Fig. 7B), with a trace amount of the hydrolysis product (IV).

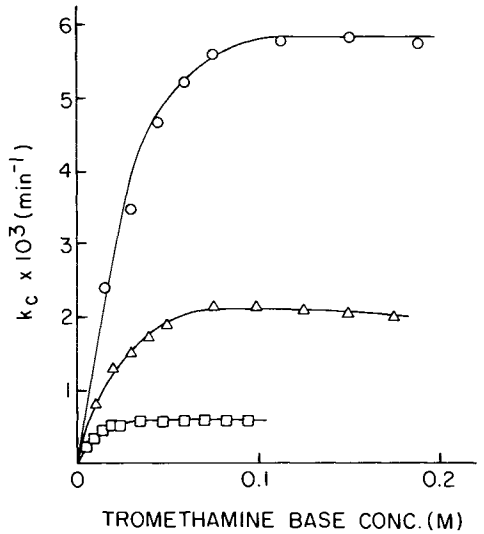


Fig. 6. Dependence of the rate constant for the degradation of the methyl ester of cephalothin at pH 8.50 (○), 8.00 (△), and 7.50 (□). The total concentration of zinc ion in the reaction solutions was 6.00×10^{-4} *M* throughout.

Mechanism and Stoichiometry

While the results previously observed with zinc ion–tromethamine-catalyzed degradation of penicillins suggested the involvement of a ternary complex (penicillin–Zn^{2+}–tromethamine) (1,2), the differences with cephalosporins appear to indicate a different reaction mechanism. As mentioned above, the three principal points of distinction are (a) the fact that the second-order rate constants, $k_c/(Zn)_o$, of penicillins are significantly larger (about 210–560 times) than those of cephalosporins; (b) the difference in the nature of the dependence of rates upon the tromethamine base concentration; and (c) the difference in effect of estrification of the carboxyl group of the β-lactam on the rate constant.

If a similar mechanism were involved, one would expect the rates of the two reactions to be much closer, since these two lactams are susceptible to attack of nucleophilies such as hydroxide ion and amines at almost the same or only slightly different rates (11–13). Also, if a ternary complex were mediating the cephalosporin reaction, one would expect to find the same kind of maximum in the dependence of the rate constant upon the tromethamine base concentration as was found with penicillins. This maximum was the result of a competition between penicillin and a second molecule

of tromethamine base for the second coordination site on the known zinc ion–tromethamine chelate (14,15).

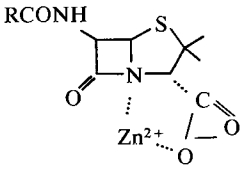
It seems, therefore, that one or both of the following mechanisms might account for the observed results with cephalosporins:

(a) bimolecular nucleophilic attack by one or more zinc–tromethamine chelates (ZnT^{1+} or ZnT_2^{1+}) and

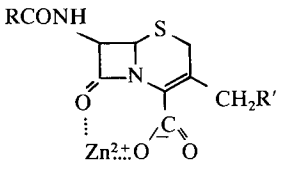
(b) nucleophilic attack by tromethamine base on a zinc–cephalosporin complex (ZnC^{1+}).

While the second mechanism may contribute to the overall reaction rate and was proposed by Gensmantel *et al.* (6) as the primary mechanism with non-hydroxyl-containing bases used as buffers, it is very likely that its contribution is insignificant in the present case. At high tromethamine concentrations, one would expect most, if not virtually all, of the zinc ion to be in a zinc–tromethamine complex, with very little zinc in a zinc–cephalosporin complex. For example, even at only 0.05 *M* tromethamine, one can calculate from estimated constants (1,6) that less than 2% of the total zinc ion exists as ZnC^{1+}. Since the observed rates did not decrease as the tromethamine concentration increased, as would be expected if mechanism b were important, one can conclude that this mechanism contributes very little to the overall degradation of the cephalosporin.

Hence, the most probable mechanism is nucleophillic attack by one or both of the zinc–tromethamine complexes. The contribution of the ZnT^{1+} species is somewhat questionable, however, since it can interact with the cephalosporins to form a ternary complex but one in which reaction cannot readily take place. As shown by Gensmantel *et al.* (5), in the complex of metal ions with cephalosporins the metal ion is bound to the carboxyl and the β-lactam carbonyl oxygen (VIII) rather than the β-lactam nitrogen as in the penicillins (VII). Thus, in the cephalosporin complex the tromethamine hydroxyl bound to the metal ion would be some distance from the point of nucleophilic attack. This difference between penicillins and cephalosporins is readily observed in molecular models.

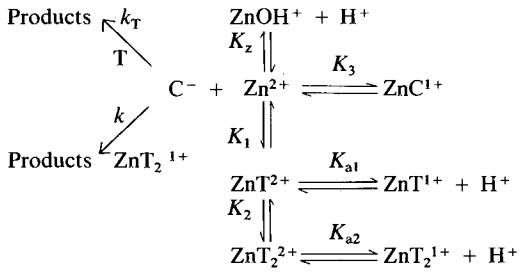


VII



VIII

Hence, it seems likely that the predominant reaction is nucleophilic attack of the species ZnT_2^{1+} with the cephalosporin. The equilibria and reactions involved are written as the following scheme, which also takes into account the hydrolysis of Zn^{2+} in the pH range of interest.



where K_1, K_2, and K_3 are the formation constants for the respective chelates. K_{a1} and K_{a2} are the dissociation constants of the tromethamine hydroxyl group coordinating to zinc ion and K_z is the ionization constant of the zinc ion–aquo complex. The constants k and k_T are the second-order rate constants for the depicted reactions.

The observed first-order rate constant corrected for the rate with tromethamine alone, k_c, is written as

$$k_c = k(ZnT_2^{1+}) \tag{1}$$

Using the appropriate equilibrium constants,

$$k_c = \frac{kK_{a2}K_1K_2}{(H^+)}(T)_0^2(Zn^{2+}) \tag{2}$$

where $(T)_0$ is the stoichiometric concentration of tromethamine base and

$$(T)_0 \gg (C)_0; \qquad (T)_0 \gg (Zn)_0$$

The stoichiometric concentration of zinc ion in the system, $(Zn)_0$, will be the sum of all species present:

$$(Zn)_0 = (Zn^{2+}) + (ZnOH^+) + (ZnT^{2+}) + (ZnT^{1+}) + (ZnT_2^{2+}) + (ZnT_2^{1+}) + (ZnC^{1+}) \tag{3}$$

$$= \frac{(Zn^{2+})}{(H^+)}[(H^+) + K_z + K_1(H^+)(T)_0 + K_{a1}K_1(T)_0 + K_1K_2(H^+)(T)_0^2 + K_{a2}K_1K_2(T)_0^2 + K_3(H^+)(C^-)] \tag{4}$$

Substituting into Eq. (2),

$$k_c = \frac{kK_{a2}K_1K_2(T)_0^2(Zn)_0}{(H^+) + K_z + [(H^+) + K_{a1}]K_1(T)_0 + [(H^+) + K_{a2}]K_1K_2(T)_0^2 + K_3(H^+)(C^-)} \tag{5}$$

Since the concentration of cephalosporins was low enough that first-order kinetics was observed in all of these studies, we may eliminate the last term in the denominator. Eliminating this term and rearranging give

$$\frac{(Zn)_0(T)_0^2}{k_c} = a + b(T)_0 + c(T)_0^2 \tag{6}$$

where

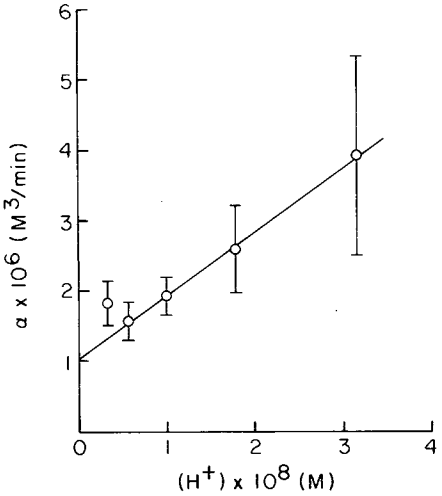


Fig. 8. Plot of a from Eq. (7) as a function of (H^+). The error bars represent standard deviations.

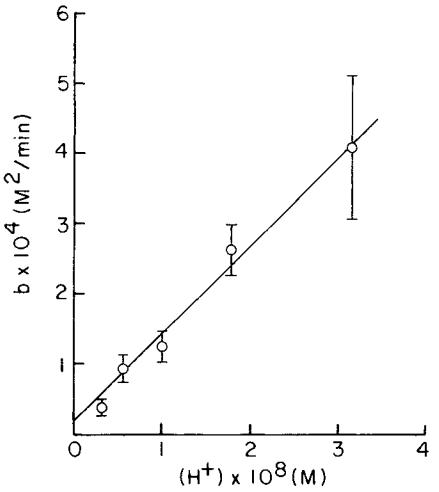


Fig. 9. Plot of b from Eq. (8) as a function of (H^+). The error bars represent standard deviations.

$$a = \frac{(H^+) + K_z}{kK_{a2}K_1K_2} \quad (7)$$

$$b = \frac{(H^+) + K_{a1}}{kK_{a2}K_2} \quad (8)$$

$$c = \frac{(H^+) + K_{a2}}{kK_{a2}} \quad (9)$$

The data for cephalothin were fitted to Eq. (6) by nonlinear least-squares, yielding values of a, b, and c for each pH studied. Figures 8, 9, and 10 show plots of the linear form of Eqs. (7)–(9) from which the parameters tabulated below were estimated.

$k = 364\ M^{-1}\ \text{min}^{-1}$
$K_{a1} = 1.5 \times 10^{-9}\ M;\ pK_{a1} = 8.8$
$K_{a2} = 2.6 \times 10^{-9}\ M;\ pK_{a2} = 8.6$
$K_1 = 138\ M^{-1}$
$K_2 = 86\ M^{-1}$
$K_z = 1.14 \times 10^{-8};\ pK_z = 7.94$

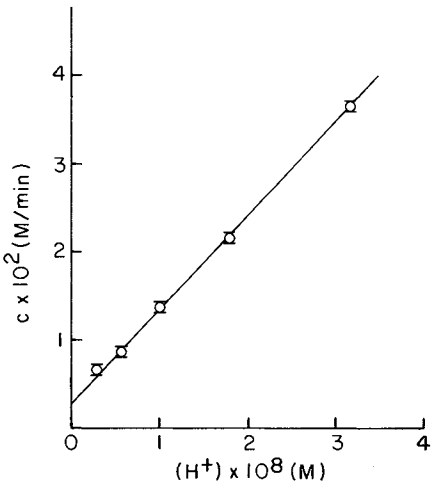


Fig. 10. Plot of c from Eq. (9) as a function of (H^+). The error bars represent standard deviations.

Obviously, these estimates are imprecise since they were derived from tertiary data. However, the pK_a's for the zinc-bound hydroxyl in ZnT and ZnT_2 are in the range one might expect based on the fact that binding to zinc often reduces the pK_a of a hydroxyl group by 3–5 units. The values also agree within reason with those obtained previously (1).

The dependence upon tromethamine concentration for degradation of the methyl ester of cephalothin shows a similar pattern to that of the free drug. Here, however, it is likely that both ZnT^{1+} and ZnT_2^{1+} are acting as nucleophiles since the blocking of the carboxyl group would reduce the affinity of the cephalosporin for zinc ion and/or zinc ion–tromethamine complexes. The fact that the rate of reaction with the ester of cephalothin is about 17-fold lower than that with the free drug also fits the proposed mechanism. It would be expected that the positively charged zinc ion–tromethamine complexes would react somewhat more rapidly with the negatively charged cephalothin than its neutral ester.

In summary, although zinc ion together with tromethamine greatly accelerates the degradation rate of β-lactam antibiotics, it is a much less effective catalyst for cephalosporins than for penicillins. It has been concluded that the predominant reaction of cephalosporin degradation in this system is a simple bimolecular reaction by a nucleophilic attack of a zinc ion–tromethamine complex. Also, in this study some of the hydrolysis and aminolysis products of the cephalosporins were successively separated by high-performance liquid chromatography.

ACKNOWLEDGMENTS

This work was supported in part by grants from Merck Sharp and Dohme Research Labs and Schering-Plough.

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