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Catalysis of Hydrolysis and Aminolysis of Benzylpenicillin Mediated by a Ternary Complex with Zinc Ion and Tris(hydroxymethyl)aminomethane

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It was discovered that the combination of zinc ion and Tris in the pH range 7.5–10 is a very effective true catalyst for hydrolysis and aminolysis (by Tris) of benzylpenicillin. Both Cu^{2+} and Ni^{2+} ions were nonreactive in this system. The rate of loss of penicillin from solution was found to be a linear function of zinc ion at concentrations up to 10^{-5} *M*, while the dependence upon Tris concentration is maximal at about 0.02–0.03 *M* at each pH studied. At high penicillin concentrations saturation kinetics was observed. Product assays showed the major product to be a penicilloic acid at low Tris concentrations and *N*(*penicilloyl*)-Tris at high Tris concentrations. A mechanism is proposed which suggests that the reaction is mediated by a ternary complex in which the metal ion acts to bring the reactants (penicillin and Tris) into close proximity and to lower the pK_a of a Tris hydroxyl, creating a strong nucleophile. This mechanism also explains the results of the product assays and the lack of reactivity of the other metal ions tested. This reaction may be related to the mechanism for a known zinc-dependent β-lactamase.

Renewed interest in the mechanism(s) of metal ion-catalyzed degradation of penicillins and cephalosporins was stimulated by the discovery some years ago of a zinc ion-mediated β-lactamase from *Bacillus cereus* 569 (*1*). In the course of studying rates of loss of penicillin in the presence of certain peptides it was found that a metal ion contaminant was responsible for the very rapid rates observed in tris(hydroxymethyl)aminomethane (Tris) buffers around pH 8, warranting further exploration of the mechanism of this catalysis. Initial screening of metal ions indicated that, under the conditions of the experiment (pH 8.0, 35°C, 0.2 *M* Tris buffer), Cu^{2+} and Ni^{2+} were inactive at a concentration of 10^{-3} *M*. This result was somewhat surprising considering the known relatively strong effect of cupric ion on penicillin hydrolysis and aminolysis (*2, 3*). On the other hand, zinc ion was found to be a very effective catalyst even at concentrations as low as 10^{-6}–10^{-5} *M*. It has been reported that zinc ion does catalyze hydrolysis of benzylpenicillin but at a lower rate than cupric ion (*3b*). The present report covers results of studies to elucidate the mechanism of the catalysis taking place in the zinc–Tris system.

[1] Part of this work was done while the author was at the School of Pharmacy, SUNY at Buffalo. The technical assistance of Ms. Joan Blindenhofer and Ms. Holly Chen is gratefully acknowledged.

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EXPERIMENTAL

Materials

All water used in this work was deionized and distilled and of 18 Mohm resistance. Solutions of $ZnCl_2$ were prepared from reagent-grade zinc metal dissolved by warming in a slight excess of dilute HCl and made up to 0.1 *M* concentration. This was standardized by titration with a standard EDTA solution using Eriochrome Black T as indicator. Dilutions to the required concentration of zinc ion were all made from this primary solution. Tris was TRIZMA (Sigma Chemical Co., St. Louis, Mo.). All other materials were reagent grade.

Rate Studies

Measurements of rate of loss of benzylpenicillin from solution were made either by following the change in absorbance of a thermostated solution at 232 nm on a Cary 118C spectrophotometer (*4*) or by the following general method:

To a solution, (18.0 ml) containing all compounds except penicillin, in a jacketed glass cell of a Radiometer pH-stat assembly, was added 2.0 ml of a penicillin solution to start the reaction. Samples were taken at appropriate intervals and assayed for residual benzylpenicillin by the method of Brandriss *et al.* (*5*).

All measurements were made at 35°C maintained by a Bronwill Constant Temperature Circulator. In all cases ionic strength was 0.5 maintained by including appropriate amounts of KCl. Initial penicillin concentration was 1×10^{-3} *M* in all cases except when the dependence of initial rate upon penicillin concentration was being measured. In the altter studies 8–10 samples were taken during the first 5–10% of the reaction and the initial rate estimated from the initial slope of a linear plot of concentration vs time.

In the other cases conditions were such that the reaction obeyed first-order kinetics. In measurements of rate in absence of metal ion, 1×10^{-5} *M* EDTA was included in the reaction solution.

Both methods gave essentially the same results when used to measure rate under the same conditions. The spectrophotometric method could not be used above pH 9 where the buffering effect of Tris was too weak.

The pK_a of Tris was determined to be 8.012 at 35°C and ionic strength 0.5, by potentiometric titration using a Radiometer M-26 pH meter.

Product Assay

When reaction was complete, the solution, which initially had contained 1×10^{-3} *M* benzylpenicillin was diluted fivefold with water and assayed by the previously described penamaldate method (*6*).

RESULTS

The reaction of benzylpenicillin with excess Tris in the absence of any metal ion followed first-order kinetics, and the observed rate constant was a linear function

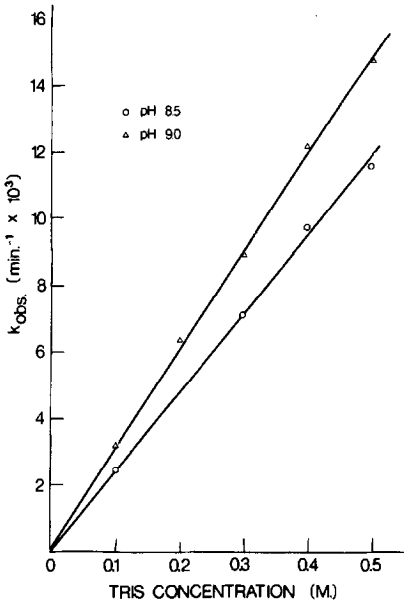


FIG. 1. Dependence of observed rate constant for penicillin loss on Tris concentration in absence of zinc ion.

of the stoichiometric Tris concentration as shown in Fig. 1. In measuring these rates a low concentration of EDTA was used to prevent small amounts of possible metal ion contaminants from exerting any influence. From the slopes of these lines as a function of pH it was evident that Tris free base was the reacting species with a second-order rate constant 0.032 M^{-1} min^{-1}. With zinc ion in the system at concentrations ranging up to 10^{-5} M in 0.2 M Tris buffer the rates were first order, and the rate constants were observed to be a linear function of zinc ion concentration as shown in Fig. 2.

It is of interest to examine the magnitude of the rate enhancement produced by the combination of zinc ion and Tris. In water alone at pH 8 at 30°C the half-life of penicillin is about 50 days (*3a*). From other data (*3b*) one may calculate an approximate half-life of penicillin in presence of 5×10^{-6} M zinc ion at 30°C at pH 8 to be about 4 hr. From our own data in Fig. 1 the half-life in presence of 0.02 M Tris base at pH 8 at 35°C is about 17 hr.

Table 1 shows that at pH 8 at 35°C in presence of both 5×10^{-6} M zinc ion and 0.02 M Tris the half-life is less than 5 min. Thus the Zn–Tris combination appeared to show unique catalytic properties.

The dependence of the observed first-order rate constants (k_{obs}) on Tris base concentration is given in Table 1 and depicted in Figs. 3 and 4. In the latter k_{obs} were corrected for the rate with Tris alone and normalized with respect to zinc ion. In order to better show the fit to the drawn curves the plots include only those points up to 0.1 M Tris, but all of the points in Table 1 were utilized to determine the best fit to Eq. [1] which was derived from a mechanism proposed below in the

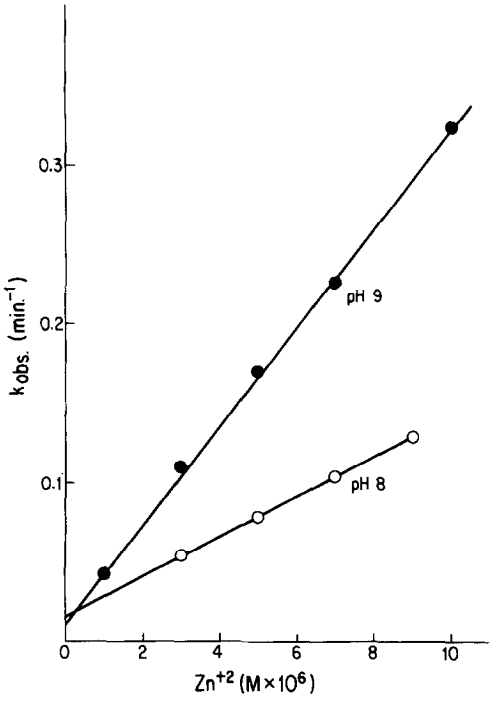


FIG. 2. Dependence of observed rate constant for penicillin loss on zinc concentration at 0.2 *M* Tris (total) at pH shown.

discussion. The fit was carried out by a nonlinear least-squares computer program[2] giving the data shown in Table 2.

$$\frac{k_T}{(Zn)_0} = \frac{aT}{1 + bT + cT^2}, \qquad [1]$$

where k_T refers to the observed first-order rate constant corrected for rate in presence of Tris alone, and $(Zn)_0$ is the stoichiometric concentration of zinc ion. The data points at pH 10 are not shown, because they fall very close to those at pH 9.5. It should be noted that the nonlinear least-squares fit is not very good. The relative imprecision of the results is probably attributable to variation of zinc ion concentration. Despite all efforts to control this factor it is quite possible that very small differences in zinc ion concentration from extraneous sources could, and did, occur and, at the level of these experiments (1×10^{-6}–1×10^{-5} *M*), were significant.

It is evident, however, that there is a maximum rate at one particular Tris concentration at each pH and that the values for all three parameters, *a*, *b*, and *c* in the table vary with pH. The latter two are clearly decreasing with increasing pH, while *a* increases sharply at lower pH and then becomes independent of the pH values.

[2] Program ZXSSQ, International Mathematic and Statistics Library, Houston, Texas.

TABLE 1

RATE CONSTANT AS A FUNCTION OF pH AND TRIS CONCENTRATION

pH:	7.5		8.0		8.5	
$(Zn)_0(M)$:	1×10^{-5}		5×10^{-6}		2×10^{-6}	
	Tris[a]	k_{obs}[b]	Tris	k_{obs}	Tris	k_{obs}
	0.00235	0.043	0.0015	0.0425	0.00151	0.038
	0.0047	0.077	0.00225	0.084	0.00227	0.0456
	0.0094	0.084	0.0039	0.095	0.00302	0.066
	0.0118	0.099	0.00493	0.083	0.00378	0.057
	0.0141	0.096	0.0059	0.122	0.00529	0.0792
	0.0141	0.100	0.0099	0.138	0.00675	0.103
	0.0165	0.099	0.0141	0.151	0.00755	0.113
	0.0188	0.095	0.0158	0.158	0.01133	0.124
	0.0188	0.105	0.0173	0.142	0.01133	0.126
	0.0235	0.105	0.0197	0.147	0.0151	0.133
	0.0235	0.096	0.0197	0.166	0.0302	0.124
	0.0329	0.092	0.022	0.145	0.0378	0.11
	0.0353	0.095	0.0247	0.161	0.0755	0.084
	0.0564	0.0815	0.0493	0.22	0.151[c]	0.047
	0.0658	0.0727	0.0592	0.118		
	0.0752	0.0636	0.0740	0.112		
			0.099	0.085		

pH:	9.0		9.5		10.0	
$(Zn)_0(M)$:	1×10^{-6}		1×10^{-6}		1×10^{-6}	
	Tris	k_{obs}	Tris	k_{obs}	Tris	k_{obs}
	0.00181	0.029	0.00455	0.0636	0.005	0.055
	0.00272	0.035	0.0097	0.115	0.01	0.103
	0.0054	0.0506	0.0194	0.130	0.02	0.133
	0.00907	0.0797	0.0291	0.130	0.025	0.139
	0.0145	0.098	0.0291	0.133	0.028	0.130
	0.0181	0.926	0.0339	0.135	0.028	0.148
	0.020	0.105	0.0388	0.131	0.03	0.139
	0.0272	0.102	0.0485	0.128	0.035	0.132
	0.0454	0.0930	0.0581	0.125	0.04	0.126
	0.0907	0.0745	0.0775	0.090	0.072	0.118
	0.181[c]	0.0447	0.0969	0.087	0.10	0.099
	0.272	0.037	0.193[c]	0.050	0.16	0.083
	0.363	0.037	0.291	0.050	0.20	0.079
	0.454	0.037	0.388	0.0500	0.30	0.066
			0.485	0.0504	0.40	0.067
					0.50	0.068

[a] Tris as free base, calculated from pK_a and pH.

[b] min^{-1}.

[c] Points below the line were not included in plots.

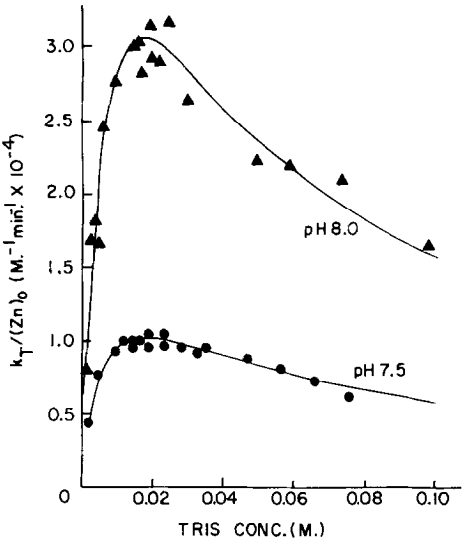


FIG. 3. Dependence of rate constant, corrected for rate with Tris alone, and normalized with respect to zinc ion concentration, on Tris concentration at pH shown.

All of the above measurements were made using an initial penicillin concentration of $1 \times 10^{-3} M$. One series of runs was made in which the initial concentration of penicillin was varied and initial rate of loss measured. In Fig. 5 is a plot of reciprocal of initial rate against reciprocal of initial concentration using zinc ion at $5 \times 10^{-7} M$ and $0.04 M$ Tris at pH 8.0. The linearity of this plot is an indication of saturation kinetics and involvement of penicillin in the formation of an intermediate complex prior to the rate-determining step.

Product assays were carried out following runs at pH 8.0 with zinc ion at $1.0 \times 10^{-6} M$ and varying Tris concentration. As shown in Table 3, the relative proportion of original penicillin eventually becoming amide is a function of the concentration of Tris. When Tris concentration was only four times that of penicillin most of the product was penicilloic acid, the normal hydrolysis product of penicillin, rather than amide. At very high Tris levels virtually all of the penicillin was ultimately converted to amide.

TABLE 2

BEST FIT PARAMETERS FOR DEPENDENCE OF RATE ON TRIS CONCENTRATION (EQ. [1])

pH	$a(\times 10^{-6})$	b	c
7.5	2.7	159	3000
8.0	7.6	132	3400
8.5	12.	75	3100
9.0	14.5	62	1550
9.5	16.6	51	1350
10.0	16.3	55	950

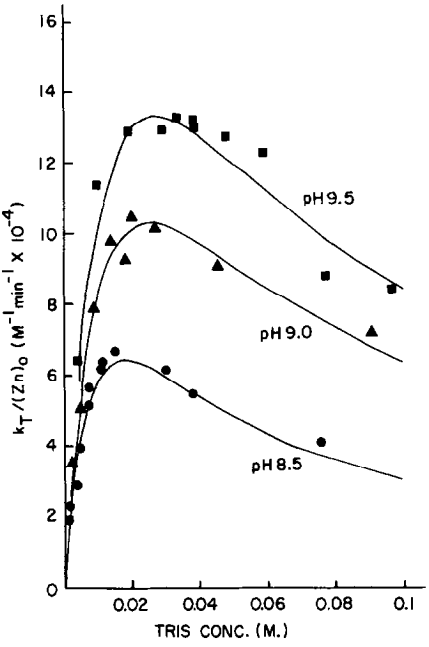


FIG. 4. Dependence of rate constant, corrected for rate with Tris alone, and normalized with respect to zinc ion concentration, on Tris concentration at pH shown.

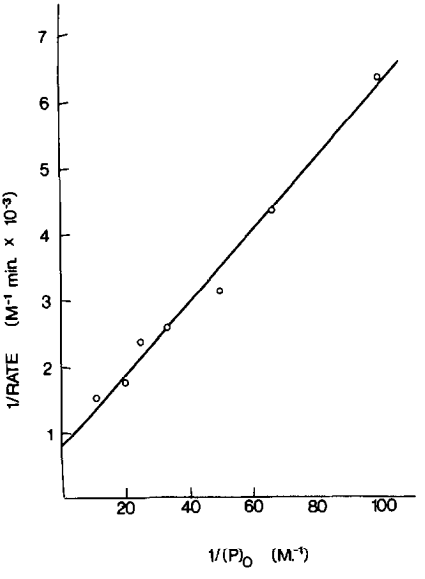


FIG. 5. Double-reciprocal plot of 1/rate vs 1/penicillin concentration at pH 8, $(Zn)_0 = 5 \times 10^{-7}$ *M*, Tris (total) = 0.04 *M*.

TABLE 3

PRODUCT ASSAYS[a]

Tris (*M*)	Percentage found in product	
	Penicilloic acid	Penicilloyl Tris
0.004	62.0	38.0
0.008	27.6	72.4
0.016	21.8	78.2
0.032	15.5	84.9
0.080	4.0	96.0

[a] At pH 8.0; $P_0 = 0.001$, $(Zn)_0 = 1 \times 10^{-6}$ *M*.

The only other metal ions tested for activitiy in this system were Cu^{2+} and Ni^{2+}. With neither was there any measurable loss of penicillin after 10 min at pH 8.0 in 0.2 *M* Tris buffer, using 10^{-3} *M* metal ion.

DISCUSSION

The marked enhancement of rate of penicillin loss in presence of zinc ion and Tris over that with either species alone is consistent with either of two mechanisms:

(a) Nucleophilic attack by Tris on a zinc–penicillin complex in which the penicillin is made much more susceptible to such attack. This is the mechanism proposed by Gensmantel *et al.* (*3a*) for the cupric ion-catalyzed aminolysis of benzylpenicillin by trifluoroethylamine, propylamine, and methoxyethylamine.

(b) Formation of a ternary complex in which the metal ion binds both penicillin and Tris in a conformation in which reaction is facilitated, i.e., nucleophilic attack by the bound ionized (*7*) hydroxyl group of Tris upon the β-lactam carbonyl of penicillin to form a Tris ester of penicilloic acid as an intermediate product. The ester subsequently hydrolyzes to penicilloic acid or reacts with another Tris to form the penicilloamide, the partitioning being dependent upon Tris concentration.

The evidence suggests that the latter mechanism, via the ternary complex, is predominant for a number of reasons. Before discussing these, however, it is useful to consider the mechanism involving the ternary complex in more detail as follows:

$$Zn^{2+} + T \overset{K_1}{\rightleftharpoons} ZnT^{2+}, \qquad [2]$$

$$ZnT^{2+} + T \overset{K_2}{\rightleftharpoons} ZnT_2^{2+}, \qquad [3]$$

$$ZnT^{2+} + P^- \overset{K_3}{\rightleftharpoons} ZnTP^{1+}, \qquad [4]$$

$$ZnTP^{1+} \overset{K_p}{\rightleftharpoons} ZnTP^0 + H^+, \quad [5]$$

$$ZnTP^0 \xrightarrow{k} \text{ester} \begin{matrix} \xrightarrow{(T)} \text{penicilloamide} \\ \xrightarrow{(OH^-)} \text{penicilloic acid} \end{matrix}, \quad [6]$$

where T is the free base of Tris, ZnT^{2+} and ZnT_2^{2+} are the known chelates of Tris (*8*), and $ZnTP^{1+}$ is a ternary complex with $ZnTP^0$ as its conjugate base. The constants K_1, K_2, and K_3 represent the association constants for the respective complexes, K_p the dissociation constant of the ternary complex, and k the rate constant.

Also to be taken into account is the possible dissociation of each zinc–Tris chelate, i.e.,

$$ZnT^{2+} \overset{K_{a1}}{\rightleftharpoons} ZnT^{1+} + H^+, \quad [7]$$

$$ZnT_2^{2+} \overset{K_{a2}}{\rightleftharpoons} ZnT_2^{1+} + H^+. \quad [8]$$

Additionally, it is known that zinc ion hydrolysis occurs in the pH range of interest (*9*):

$$Zn^{2+} + H_2O \overset{K_z}{\rightleftharpoons} ZnOH^+ + H^+. \quad [9]$$

The rate of reaction would be proportional to the concentration of $ZnTP^0$.

$$\text{Rate} = k(ZnTP^0) \quad [10]$$

$$= \frac{kK_p}{(H^+)}(ZnTP^{1+}) \quad [11]$$

$$= \frac{kK_p}{(H^+)} K_1K_3(Zn^{2+})(T)(P). \quad [12]$$

Under the condition $T \gg P \gg (Zn)_0$, the stoichiometric concentration of zinc ion in the system $(Zn)_0$ will be the sum of all species present:

$$(Zn)_0 = (Zn^{2+}) + (ZnT^{2+})\left[1 + \frac{K_{a1}}{(H^+)}\right] + (ZnT_2^{2+})\left[1 + \frac{K_{a2}}{(H^+)}\right] + (ZnTP^{1+})\left[1 + \frac{K_p}{(H^+)}\right] + (ZnOH^+) \quad [13]$$

Using the equilibrium relationships from Eqs. [1]–[5] and [7]–[9]:

$$(Zn)_0 = (Zn^{2+})\left\{1 + K_1(T)\left[1 + \frac{K_{a1}}{(H^+)}\right] + K_1K_2(T)^2\left[1 + \frac{K_{a2}}{(H^+)}\right] + K_1K_3(T)(P)\left[1 + \frac{K_p}{(H^+)}\right] + \frac{K_z}{(H^+)}\right\} \quad [14]$$

Substituting into Eq. [12]:

$$\text{Rate} = \frac{\frac{kK_p}{(H^+)} K_1 K_3 (Zn)_0 (T)(P)}{1 + \frac{K_z}{(H^+)} + K_1(T)\left[1 + \frac{K_{a1}}{(H^+)}\right] + K_1 K_2 (T)^2 \left[1 + \frac{K_{a2}}{(H^+)}\right] + K_1 K_3 (T)(P)\left[1 + \frac{K_p}{(H^+)}\right]} \quad [15]$$

In the cases where the initial concentration of penicillin $(P)_0$ was 10^{-3} M, only first-order kinetics were observed. It seems reasonable therefore that the last term in the denominator may be neglected under those circumstances. Eliminating this term, dividing numerator and denominator by $(1 + K_z/H^+)$ and rearranging gives

$$\frac{k_T}{(Zn)_0} = \frac{\frac{kK_p K_1 K_3 (T)}{K_z + (H^+)}}{1 + K_1(T)\left[\frac{(H^+) + K_{a1}}{(H^+) + K_z}\right] + K_1 K_2 (T)^2 \left[\frac{(H^+) + K_{a2}}{(H^+) + K_z}\right]}. \quad [16]$$

A comparison of Eqs. [1] and [16] gives the following relationships:

$$a = \frac{kK_p K_1 K_3}{(H^+) + K_z} \quad [17]$$

$$b = \frac{K_1[(H^+) + K_{a1}]}{(H^+) + K_z} \quad [18]$$

$$c = \frac{K_1 K_2[(H^+) + K_{a2}]}{(H^+) + K_z} \quad [19]$$

From Eq. [17] it can be seen that a plot of log a vs pH will give the typical curve for an ionizing species, and when $(H^+) = K_z$, a will be one-half of its maximum value. Such a plot is shown in Fig. 6. Also, Eq. [17] may be rearranged to

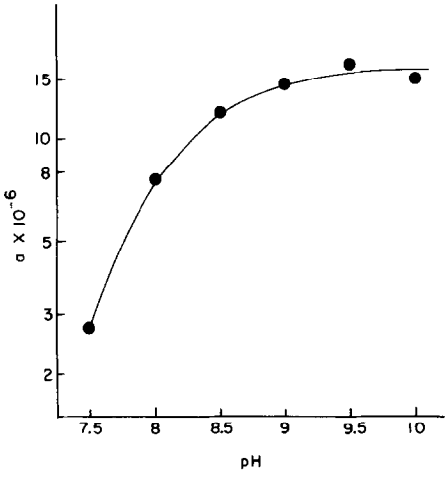


FIG. 6. Plot of log a from Eq. [1] as a function of pH.

$$aK_z + a(H^+) = \text{constant}, \quad [20]$$

or

$$a = \frac{-a(H^+)}{K_z} + \frac{\text{constant}}{K_z}. \quad [20a]$$

A plot of a vs $a(H^+)$ should be linear with slope $= -1/K_z$. From such a plot in Fig. 7, K_z was estimated to be approximately 6.4×10^{-9} ($pK_z = 8.19$).

Equation [18] may be rearranged to

$$b[(H^+) + K_z] = K_1(H^+) + K_1K_{a1}. \quad [21]$$

From a plot of $b[(H^+) + K_z]$ vs (H^+) one may estimate K_1 from the slope and K_{a1} from the intercept divided by slope. From the plot in Fig. 8, it was estimated that $K_1 = 180$ and $K_{a1} = 1.7 \times 10^{-9}$.

It should be possible by applying the same process to Eq. [19] to estimate the product of K_1K_2 and K_{a2}. Because of the great variability of the estimates of c this simply could not be done. The best that can be said of K_1K_2 is that it lies somewhere in the range of 3000–5000 M^{-2}, and hence $K_2 = 15$–$30\ M^{-1}$. It is also impossible to estimate K_{a2}, but one might speculate that it should be near the value for K_{a1}.

Using Eq. [17] an average value for the product kK_pK_3 was calculated to be 6.1×10^{-4} min^{-1}. A value for K_3 can be obtained from the slope (54 min) and intercept (750 M^{-1} min) of Fig. 5 and Eq. [15]. The value thus obtained is $K_3 = 28\ M^{-1}$. The value for kK_3K_p from this data is 7.6×10^{-4}, which is about 20% different from that noted above. This is deemed a satisfactory agreement in light of the relatively high experimental error. The value for kK_p is estimated to be in the range of 2.2×10^{-5}–$2.7 \times 10^{-5}\ M^{-1}$. It is impossible from the data to estimate K_p and hence k. If it is assumed that K_p is of the same order of magnitude as K_{a1}, i.e., 10^{-9}, then k would be about 2.5×10^4 min^{-1}.

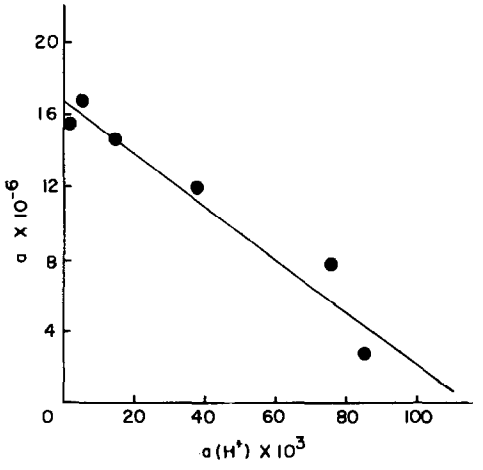


FIG. 7. Plot of Eq. [20a] from which K_z was determined.

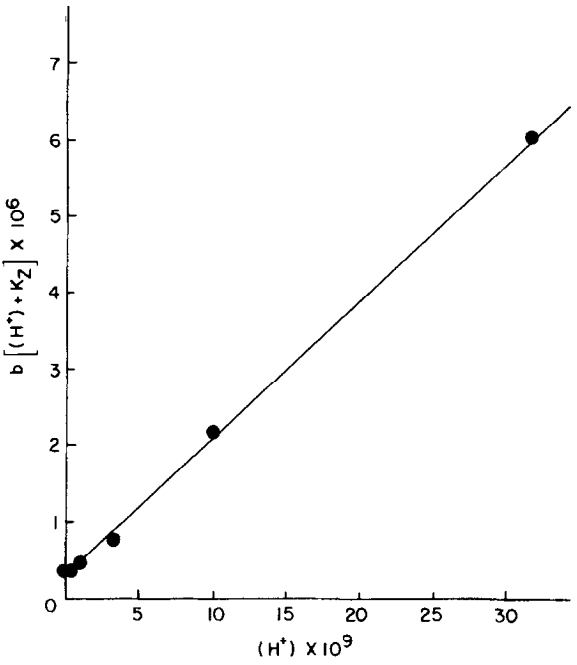


FIG. 8. Plot of Eq. [21] from which K_1 and K_{a1} were estimated.

The alternative mechanism involving attack by Tris on a zinc–penicillin complex is shown as follows:

$$\begin{array}{ccc} Zn^{2+} + P^- \overset{K_a}{\rightleftharpoons} ZnP^{1+} & \xrightarrow{k_a(T)} & \text{penicilloamide} \\ {\scriptstyle (T)}\downarrow\uparrow K_1 & & \\ ZnT & \underset{\downarrow}{} k_1(OH^-) & \\ {\scriptstyle (T)}\downarrow\uparrow K_2 & & \text{penicilloic acid} \\ ZnT_2 & & \end{array} \qquad [22]$$

One may approximate the expected rates of this reaction from the data of Gensmantel *et al.* (*3a, b*) as follows:

For the reaction of penicillin with amines at 30°C, catalyzed by cupric ion, k_2 for propylamine was $4.14 \times 10^4\ M^{-1}\ sec^{-1}$, and k_2 for trifluoroethylamine was $2.86\ M^{-1}\ sec^{-1}$. Assuming a linear relationship between rate constant and basicity the calculated k_2 for Tris would be about $200\ M^{-1}\ sec^{-1}$. The ratio of k_1 values for Zn^{2+}/Cu^{2+} was 4.9×10^{-3}. Assuming this same ratio holds for k_2, the value for Tris in presence of zinc ion would be about $1.0\ M^{-1}\ sec^{-1}$. At pH 8.0, the first-order rate constant for loss of penicillin in $0.02\ M$ Tris at 30°C with $5 \times 10^{-6}\ M$ zinc ion, assuming $K_1 = 180\ M^{-1}$ and ignoring K_2, would be about $2.4 \times 10^{-6}\ sec^{-1}$ for the aminolysis and about $1.2 \times 10^{-5}\ sec^{-1}$ for the hydrolysis. The total rate constant would be about $1.4 \times 10^{-5}\ sec^{-1}$ or $8.6 \times 10^{-4}\ min^{-1}$. As shown in Table 1 the aminolysis rate is $0.15\ min^{-1}$ or about 175 times that calculated above. This large calculated difference supports the ternary complex interpretation, in which one would expect a much higher rate than in a bimolecular reaction. Also note that

in the actual case (Figs. 3 and 4) the rate constant in absence of Tris (hydrolysis) hardly contributes to the overall rate.

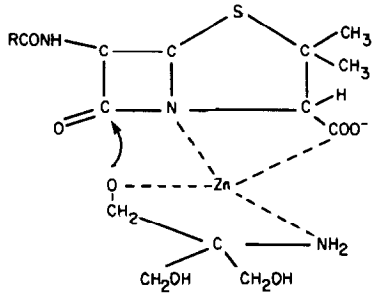
By means of the same approach as above, the equation for the mechanism [22] was developed and is given in Eq. [23], which ignores the ionization of the zinc–Tris chelates:

$$k_{obs} = \frac{\dfrac{kK_3(H^+)(T)}{K_z + (H^+)}}{1 + K_1(T) + K_1K_2(T)^2} \qquad [23]$$

Note that this result requires a different pH dependence for the numerator than that actually observed, while the latter is consistent with the suggested ternary complex pathway.

The product assays offer additional support for the ternary complex. As shown in Table 3, when Tris concentration was 0.016 *M* almost 80% of the product was amide. This result is inconsistent with the calculated hydrolysis rate relative to aminolysis for the bimolecular mechanism, wherein one would expect the product to be over 80% penicilloic acid. It is quite consistent with the ternary complex mechanism, however, for the following reasons:

Examination of the schematic representation of the ternary complex,



shows that the expected product would be a Tris ester of penicilloic acid. These esters are known to hydrolyze rather readily and are also subject to aminolysis (*10, 11*). Thus it would be expected that this intermediate would partition to the acid and amide with the relative proportion of the latter increasing with increasing Tris concentration but independent of the rate of penicillin loss. Thus the results of the product assays are totally consistent with the ternary complex pathway and contradict the pathway involving amine attack on a zinc–penicillin complex.

It should be noted that within the ternary complex, as well as in ZnT and ZnT_2, ionization of a bound hydroxyl group should be expected with a pK_a in the range 8–10 based on the known lowering of pK_a of such groups by 3–4 units upon binding to zinc (*7*). The second pK_a of Tris could not be found in the literature, but a value in the range 12–13 may be estimated from the known values for other polyhydric alcohols.

The binding of penicillin to zinc ion is shown to take place at the carboxylate ion and β-lactam nitrogen. This is analogous to the mode of binding proposed for the

TABLE 4

FORMATION CONSTANTS FOR TRIS COMPLEXES

Metal ion	Log K_1	Log K_2
Cu^{2+}	3.98	3.49
Ni^{2+}	2.80	2.10
Zn^{2+}	2.26	(1.4)

Cu^{2+}–penicillin complex based on NMR studies (*12*) and seems reasonable, since the binding constants for the two ions are similar (*3b*).

The lack of reactivity of Cu^{2+} and Ni^{2+} ions in this system at pH 8 and metal ion concentrations up to 10^{-3} *M* seems to be the result of chelation of the metal ion by Tris, essentially preventing interaction with penicillin. The formation constants for the metal ion–Tris chelates are shown in Table 4. The data for Cu^{2+} and Ni^{2+} were from the literature (*13*), while those for zinc ion were estimated kinetically in the present study. Under the conditions of our experiment (0.2 *M* Tris, 10^{-3} *M* penicillin, 10^{-3} *M* metal ion), it is obvious in the case of Cu^{2+} that virtually all of the metal ion would be bound by Tris. An approximate calculation for Ni^{2+} shows that well over 99% of the metal ion would be in the form of Tris complexes. Also it has been shown that the hydrolysis rate of penicillin complexed to Ni^{2+} is only about $\frac{1}{10}$th that of a zinc–penicillin complex (*3b*).

A very similar mechanism has been suggested fro the reaction of 2-cyanopyridine with Tris catalyzed by Ni^{2+} (*14*). In this case the metal ion is the focal point of a ternary complex in which there is a close approximation of the two reacting molecules. The metal ion also functions as a Lewis acid in coordination with the nitrile nitrogen and the reaction pathway involves attack by an ionized Tris hydroxyl group. This reaction, however, required an excess of metal ion, while the zinc–Tris–penicillin reaction showed turnover of substrate at very low metal ion concentrations. A key factor in catalysis is the apparent relatively weak binding of metal ion by the reaction product compared to substrate.

It was known quite early that zinc and other metal ions catalyze the degradation of penicillins in alcohols, producing the corresponding esters of penicilloic acid (*15*). It was suggested that the mechanism involved coordination of the metal to the sulfur of the thiazolidine ring, but this is clearly incorrect in view of the present and earlier studies (*2, 3*). It seems more likely that the metal-catalyzed alcoholysis involves nucleophilic attack by the alcohol on the penicillin, the latter having been activated by coordination to the metal ion. It may also be possible that some alcohols are also coordinated to the metal ion in a ternary complex which would accelerate the reaction via approximation. Further study of these reactions is needed.

Several years ago a zinc ion-dependent β-lactamase was discovered (*1*) and has since received a good deal of attention. It is interesting to note that in some of the experiments the activity of the enzyme was reconstituted, after removal of zinc ion, by treatment with a Zn^{2+}–Tris buffer containing 10^{-3} *M* zinc ion at pH 7.

Based on the present work, considerable apparent enzyme-like activity would be observed even if enzyme were not present. Some of the earlier data may therefore be questionable. It has also been reported that the binding of Tris to zinc ion is negligible with $K = 0.5–2.0\ M^{-1}$ (*16*). This result is in marked contrast to several published reports of the formation constant being in the range $\log K_1 = 2.3–2.4$ at 25°C (*17*).

In the sense that the Zn^{2+}–Tris system catalyzes the same reaction, it may be considered a model for the zinc-dependent β-lactamase. A perhaps better model would require a ligand other than Tris which would not be incorporated in the product and yield only penicilloic acid as the product.

REFERENCES

1. L. D. SABATH AND E. P. ABRAHAM, *Biochem. J.* **98,** 11c (1966).
2. W. A. CRESSMAN, E. T. SUGITA, J. T. DOLUISIO, AND P. J. NIEBERGALL, *J. Pharm. Pharmacol.* **18,** 101 (1966).

3a. N. P. GENSMANTEL, E. W. GOWLING, AND M. I. PAGE, *J. Chem. Soc. Perkin Trans. 2,* 335 (1978).

3b. N. P. GENSMANTEL, P. PROCTOR, AND M. I. PAGE, *J. Chem. Soc. Perkin Trans. 2,* 1725 (1980).

4. S. G. WALEY, *Biochem. J.* **139,** 789 (1974).
5. M. W. BRANDRISS, E. L. DENNY, M. A. HUBER, AND H. G. STEINMAN, "Antimicrobial Agents and Chemotherapy" (J. C. Sylvester, Ed.), p. 626, Amer. Soc. Microbiol, Ann Arbor, Mich., 1962.
6. M. A. SCHWARTZ AND A. J. DELDUCE, *J. Pharm. Sci.* **58,** 1137 (1969).
7. M. F. DUNN, *Struct. Bonding* **23,** 61 (1975).
8. R. L. DOTSON, *J. Inorg. Nucl. Chem.* **34,** 3131 (1972).
9. C. E. BAES, JR. AND R. E. MESMER, "The Hydrolysis of Cations," p. 288, Wiley, New York, 1976.
10. H. BUNDGAARD AND C. LARSEN, *Arch. Pharm. Chemi. Sci. Ed.* **6,** 184 (1978).

11a. D. E. TUTT AND M. A. SCHWARTZ, *J. Amer. Chem. Soc.* **93,** 4960 (1971).

11b. C. H. SCHNEIDER AND A. L. DEWECK, *Immunochemistry* **4,** 331 (1967).

12. G. V. FAZAKERLEY AND G. E. JACKSON, *J. Inorg. Nucl. Chem.* **37,** 2371 (1975).
13. L. G. SILLEN AND A. E. MARTELL, "Stability Constants of Metal Ion Complexes," Parts II, III, Chem. Soc., London, 1964, 1970.
14. R. BRESLOW AND M. SCHMIR, *J. Amer. Chem. Soc.* **93,** 4960 (1971).
15. E. CHAIN AND F. J. PHILPOT, *Arch. Biochem.* **18,** 171 (1948).
16. R. B. DAVIES AND E. P. ABRAHAM, *Biochem. J.* **143,** 129 (1974).
17. D. P. HANLON, D. J. WATT, AND E. W. WESTHEAD, *Anal. Biochem.* **16,** 225 (1966).