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Further Studies on the Catalysis of Hydrolysis and Aminolysis of Benzylpenicillin by Metal Chelates

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Abstract □ It was suggested previously that the very rapid catalysis of benzylpenicillin hydrolysis and aminolysis by zinc ion and tris(hydroxymethyl)aminomethane (tromethamine) was mediated by a ternary complex in which the metal ion not only held the substrate and tromethamine in close proximity but also lowered the pK_a of a bound tromethamine hydroxyl group making it a very powerful nucleophile. In this study the scope of this reaction was explored further by examining the effects of changes in substrate side chain, metal ion, and amino alcohols. All of the penicillins studied showed about the same rate of reaction. Of the other metal ions examined Cu^{2+} and Ni^{2+} showed no activity, Mn^{2+} very slight activity, and Cd^{2+} and Co^{2+} somewhat greater activity. The latter was the most effective of this group but was 40 times slower than zinc. The results with a number of amino alcohols provided additional evidence for the ternary complex mechanism. Studies with the methyl ester of benzylpenicillin indicated that the metal ion is bound to the antibiotic at the carboxylate site and that a different mechanism is involved in the slower catalysis observed with the ester. Some comparison is made with a zinc-dependent β-lactamase.

Keyphrases □ Benzylpenicillin—mechanism of hydrolysis and aminolysis, catalysis by divalent cations and amino alcohols, ternary complex formation □ Metal-ion catalysis—hydrolysis and aminolysis of benzyl penicillin, mechanism, ternary complex formation

It was previously shown that zinc ion in the presence of tromethamine buffer in the 7–10 pH range is a very facile catalyst for the hydrolysis and aminolysis of benzylpenicillin (1). Evidence was offered to show that the mechanism of this catalysis most probably involved a ternary complex in which zinc ion acts as a template to bring the reactants, tromethamine and penicillin, together. In this complex the bound hydroxyl group of tromethamine can become a very powerful nucleophile, by virtue of a lowering of its pK_a as a result of coordination of zinc ion, and attack the β-lactam carbonyl of penicillin. The resulting tromethamine ester then hydrolyzes to penicilloic acid or reacts with another molecule of tromethamine to form an amide.

The present study was undertaken to explore the scope of this reaction, *i.e.*, the activity of other metal ions and other ligands, as well as the effects of structural changes in the substrate side chain.

EXPERIMENTAL

Materials—Benzylpenicillin[1], phenethicillin[1], and methicillin[1] were used as received. Methylpenicillin was prepared by acetylating 6-aminopenicillanic acid as reported previously (2). The methyl ester of benzylpenicillin was prepared as follows.

Potassium benzylpenicillin and methyl iodide were stirred in dimethylsulfoxide for 4 hr at room temperature. The mixture was diluted with water, extracted with ether, and then the extract was dried and the solvent removed *in vacuo*. A small volume of ethyl acetate was added to the resulting oil and the solution chromatographed on silica gel 60[2] with ethyl acetate as the developing solvent. The fractions containing ester were combined and solvent removed *in vacuo*. The resulting oil crystallized on trituration with *n*-hexane and was recrystallized twice from carbon tetrachloride. Its structure was confirmed by IR and NMR spectroscopy.

Tromethamine was a very pure grade[3]; ethanolamine, diethanolamine, and triethanolamine were all reagent grade[4]; 2-amino-2-methyl-1,3-propanediol was recrystallized from ethanol; 2-methoxyethanolamine and 2-diethylaminoethanol were redistilled prior to use.

Zinc chloride solution was prepared from reagent grade zinc metal as previously described (1). The other metal ions were in the form of chloride salts and were reagent grade.

Imidazole was recrystallized twice from benzene and washed with ether.

Kinetics—All rate measurements were carried out at 35° with the ionic strength brought to 0.5 by the addition of potassium chloride. The amines normally acted as their own buffers. In some cases where buffer capacity was too low, the pH was maintained constant on a radiometer pH-stat.

Rate of loss of penicillin from solution was followed either by following the decrease in absorbance at 235 nm (3) or by sampling the reactant solution and assaying for residual penicillin by the method of Bundgaard and Ilver (4). Initial concentration of penicillin was 5×10^{-4} *M* in the

[1] Supplied by Bristol Laboratories.
[2] E. Merck, Germany.
[3] Trizma, Sigma Chemical Co.
[4] J. T. Baker Chemical Co.

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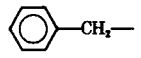
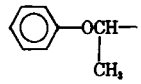
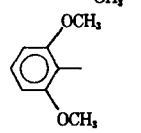
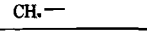
Table I—Catalytic Rate Constants [a] for Aminolysis by Tromethamine and Hydrolysis of Penicillins

Penicillin	Side Chain, R	k_T [b]	k_{OH} [c]
Benzylpenicillin		0.032 [d]	12.5
Phenethicillin		0.039	12.6
Methicillin		0.0263	6.5
Methyl penicillin		0.0294	10.6

[a] Rate constants in M^{-1} min^{-1}. [b] Reactions carried out at 35°, I = 0.5 with tromethamine alone. [c] Reactions carried out at 31.5°, I = 0.2 (2). [d] From Ref. (1)

direct spectrophotometric method and 2.75×10^{-4} *M* in the sampling method. When the methyl ester was used, the reaction mixture included 4.5% acetonitrile to keep the ester in solution.

Ionization Constants—The apparent pK_a of each of the amines was determined at 35° and ionic strength 0.5 by potentiometric titration.

RESULTS AND DISCUSSION

Effect of Penicillin Side-Chain Structure —The rates of the reaction of several penicillins with tromethamine were measured in both the absence and presence of zinc ion. Table I presents the second-order rate constants for the aminolysis of the penicillins by tromethamine in the absence of zinc ion and compares these with the alkaline hydrolysis rate constants. It can be seen that the order of reactivity is the same in both cases, reflecting the relatively small effect of the side chain on the susceptibility of the β-lactam ring to nucleophilic attack.

In Fig. 1 is shown the dependence on tromethamine concentration of the rate of loss of penicillins in the presence of zinc ion at pH 8.0. In these studies 3×10^{-6} *M* zinc ion was used, and the first-order rate constants were corrected for rate with tromethamine alone and normalized with respect to zinc ion.

It can be seen that differences in the side-chain structure exert only a very small effect on the rate of β-lactam cleavage. The rates with all the penicillins are very rapid relative to the rate in the absence of zinc ion, but there is only a less than twofold difference in rate. This result would be expected if binding of the drug to zinc ion were at the carboxyl group of the thiazolidine ring as has been proposed (5, 6). To further test this hypothesis, rates of reaction under similar conditions were carried out with the methyl ester of benzylpenicillin.

Effect of Esterification of Carboxyl Group—In the absence of zinc ion the methyl ester of benzylpenicillin reacts more rapidly with tromethamine than the free penicillin, as shown in Fig. 2. It should be noted that the reaction in both cases is at the β-lactam carbonyl. The ester is more susceptible to attack at this point than the free penicillin, because

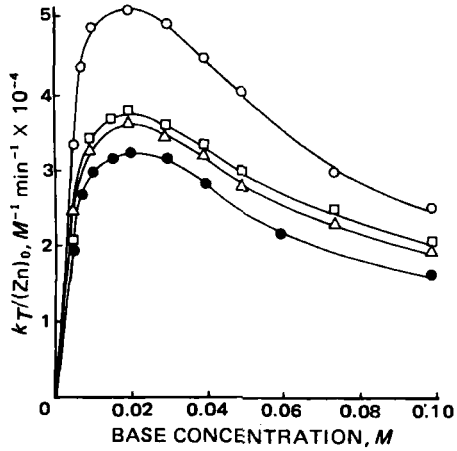


Figure 1—*Dependence on tromethamine concentration of rate constant for penicillin loss at pH 8.0. Key: (●) benzylpenicillin, (△) methicillin, (□) phenethicillin, (○) methylpenicillin.*

Table II—Effect of Zinc Ion on Rate of Loss of Benzylpenicillin and Its Methyl Ester in Tromethamine Buffer [a]

	Tromethamine-Base Concentration, *M*	$[Zn]_0$, *M*	$10^3 k_{obs}$, min^{-1}
Benzylpenicillin	0.02	0	0.526
	0.02	3×10^{-6}	110
	0.10	0	2.50
	0.10	3×10^{-6}	72.3
Methyl ester	0.02	0	2.02
	0.02	3×10^{-6}	2.33
	0.10	0	9.85
	0.10	3×10^{-6}	10.3

[a] Reactions run at pH 8.0, 35°, containing 4.5% acetonitrile with initial concentration of penicillin 2.75×10^{-4} *M*.

the negative charge on the latter tends to repel nucleophiles such as hydroxyl ion (7) and tromethamine. The presence of 3×10^{-6} *M* zinc ion causes, as shown in Table II, only a very small effect on the methyl ester of penicillin relative to the free penicillin. Again, if penicillin is coordinated to zinc ion at the carboxylate ion one would expect the result obtained.

At much higher concentrations of zinc ion there is observed a significant effect on the loss of methyl ester of penicillin in the presence of tromethamine, as shown in Fig. 3. Here the rate constant is a linear function of zinc ion concentration up to 10^{-3} *M*. The dependence of the rates of this reaction on tromethamine concentration is shown in Fig. 4, where it is compared with that of free benzylpenicillin.

Both curves show a maximum near 0.02 *M* tromethamine, but there are significant differences in slope of the curve as well as the magnitude of the rates. At the maximum, the rate constant for the ester is about 1400 times slower than that of the free penicillin. At tromethamine concentrations above 0.02 *M* the rate constant for the free penicillin decreases much more rapidly than that of the ester. These results, coupled with the great difference in rate, indicated that the reaction with the ester involves a different mechanism than the reaction with penicillin. In the latter it was proposed that catalysis is mediated by a ternary complex, in which both tromethamine and penicillin are bound to a zinc ion with nucleophilic attack by a tromethamine hydroxyl on the β-lactam carbonyl taking place within the complex. At high tromethamine concentrations a second tromethamine would compete with penicillin for the binding site of the zinc ion and reduce the reaction rate by reducing the relative amount of ternary complex that could be formed. Blocking of the penicillin carboxyl in the ester prevents the formation of this ternary complex, and, even if the ester binds to zinc it must be with a much lower affinity than the free acid, and the predominant species in solution would be chelates of tromethamine with zinc ion (ZnT and ZnT_2). With the ester one possible mechanism suggested by the tromethamine dependence is a direct intermolecular nucleophilic attack by these chelates on the β-lactam carbonyl. When tromethamine is coordinated to zinc ion through the amine and one of the hydroxyl groups, the pK_a of the coordinated hydroxyl group can be lowered substantially (*i.e.*, 3 or 4 units), but the alkoxide ion thus formed will retain the nucleophilicity of a group with the higher pK_a (8). Thus, these groups in the chelates can become relatively powerful nucleophiles at a pH close to neutrality. Also, it would be expected that the pK_a of the hydroxyl group in ZnT and ZnT_2 would

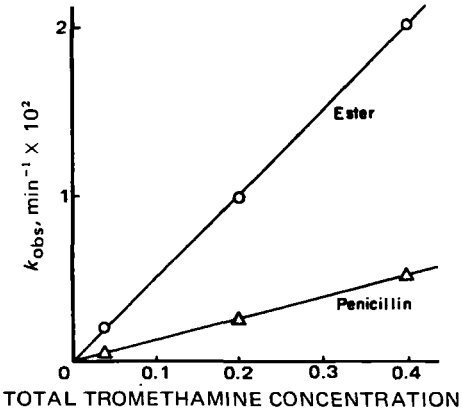


Figure 2—*Dependence of observed rate constant (k_{obs}) on tromethamine concentration, pH 8.0, EDTA 1×10^{-5} M, 4.5% acetonitrile. Key: (△) benzylpenicillin, (○) methyl ester.*

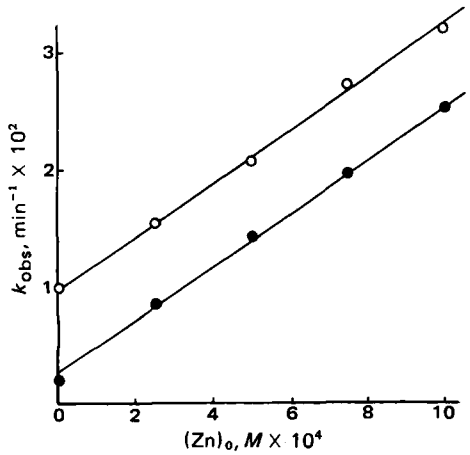


Figure 3—*Dependence of observed rate constant for loss of methyl ester of benzylpenicillin on zinc ion. Key: (○) tromethamine base 0.1* M, *(●) tromethamine base 0.02* M.

not be very different, and hence the rate constants for their reaction with the penicillin ester would be expected to be similar. That they are is evidenced by the tromethamine dependence shown in Fig. 4, where the decrease in rate at high tromethamine is relatively small. We may express the overall rate constant as follows:

$$k_T/[Zn]_0 = k_1[ZnT^{1+}] + k_2[ZnT_2^{1+}]$$

where k_T is the observed rate constant corrected for the rate with tromethamine alone, and $[Zn]_0$ is the stoichiometric total zinc ion concentration. Using values previously estimated for the affinity constants of the zinc–tromethamine chelates (1), and assuming the pK_a value for the two chelates are equal (pK_a = 8.7), values for the rate constants were calculated as $k_1 = 220\ M^{-1}\ min^{-1}$ and $k_2 = 120\ M^{-1}\ min^{-1}$. These values are in the range one would expect for nucleophiles with pK_a values ~12–13 (9), which is probably in the pK_a range of uncomplexed tromethamine.

It should be noted that this type of reaction probably also could occur with the free penicillin, but with a rate so low that it would be insignificant relative to that of the ternary complex pathway.

Effects of Other Metal Ions—It was of interest to examine the effects of other metal ions in this system. Rates of loss of benzylpenicillin in solution in the presence of Co^{2+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, and Ni^{2+} were measured at pH 8, at constant tromethamine concentration. The results are shown in Fig. 5 where these are compared with Zn^{2+}. Both Cu^{2+} and Ni^{2+} showed no activity at concentrations up to 10^{-4} M. The other metal ions all show a linear dependence on metal ion concentration and much lower rates than zinc ion.

In Fig. 6 is shown the dependence of the rate of penicillin loss on tromethamine concentration in the presence of three metal ions. Here again, the rate constants were corrected for the rate with tromethamine alone and normalized with respect to metal ion concentration. While the curve for Co^{2+} ion shows a maximum similar to that of zinc ion, Cd^{2+} ion shows only a plateau, and the curve for Mn^{2+} reveals only a steady increase in

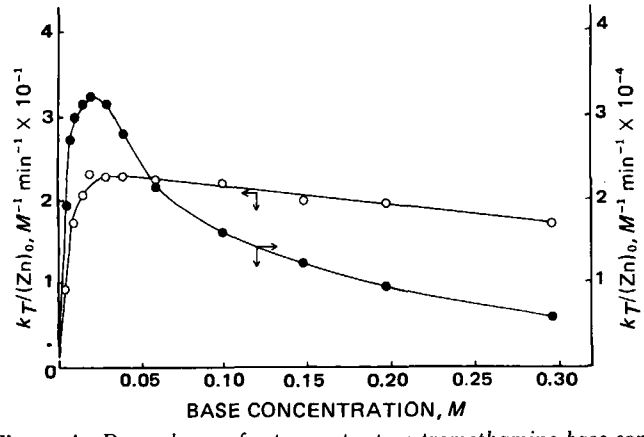


Figure 4—*Dependence of rate constant on tromethamine base concentration at pH 8.0. Key: (○) methyl ester, (●) penicillin.*

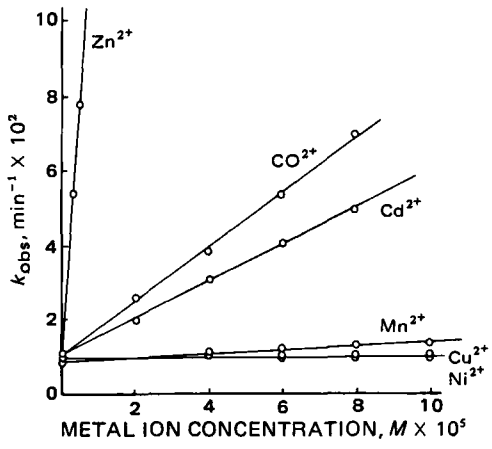


Figure 5—*Effect of concentration of metal ions on observed rate constant for penicillin loss at pH 8.0, 0.2* M *tromethamine buffer.*

rate with increasing tromethamine. Based on the mechanism proposed for zinc ion with benzylpenicillin, the differences in these curves might be thought to result from differences in the formation constants of the metal chelates with tromethamine. This appears to be the case particularly with Co^{2+}, but with Cd^{2+} the plateau suggests a different mechanism, perhaps similar to that proposed for the reaction of zinc–tromethamine chelates with penicillin methyl ester.

The rates of loss of the methyl ester of benzylpenicillin with these metal ions were also measured and are reported in Table III as the rate constant at the maximum in the tromethamine dependence curve divided by the metal ion concentration, except in the case of Mn^{2+} where no maximum was reached. Here the rates are compared at the same tromethamine concentration. In all cases the rate for the penicillin is much greater than that of the ester but these differences decrease markedly in the order shown in Table III.

Such a result may be expected if the reaction with the free penicillin, as proposed at least with zinc and cobalt, is mediated by a ternary complex in which an appropriate conformation of tromethamine and substrate would be necessary for optimum reaction rate. If an optimum fit is obtained with zinc ion, then large differences would be expected with other ions of different size. On the other hand, with the ester, where the reaction may involve bimolecular nucleophilic attack by the metal chelate, smaller differences would be expected based more on the effect of the metal ion on pK_a of the tromethamine hydroxyl, steric factors, *etc.* Thus, while these data are not inconsistent with the proposed mechanism, a good deal more information on the nature of the tromethamine complexes and the effect of pH on reaction rate would be necessary to make more definitive conclusions.

In terms of this system as a potential model for the zinc-dependent β-lactamases, it is interesting to note that other metal ions may replace zinc in the enzyme with some retention of activity (10). It has been reported that replacement with Co^{2+} ion gives an enzyme with 12.6% of the activity of the zinc-containing enzyme. With Mn^{2+} and Cd^{2+} the activity was reduced to 6.7 and 1%, respectively. No enzymic activity was noted

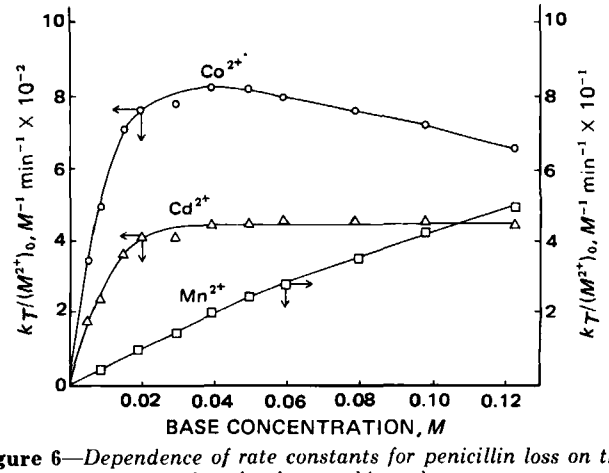


Figure 6—*Dependence of rate constants for penicillin loss on tromethamine concentrations for the metal ions shown.*

Table III—Effect of Metal Ions on Rate of Loss of Benzylpenicillin and Its Methyl Ester in Tromethamine Buffer [a] at pH 8.0

Metal Ion	Benzylpenicillin k_{max}/M_0	Benzylpenicillin Relative Activity	Methyl Ester [b] k_{max}/M_0	Methyl Ester [b] Relative Activity	Ratio of Penicillin–Ester
Zn^{2+}	3.22×10^4	100	23.6	100	1360
Co^{2+}	8.20×10^2	2.55	4.38	18.6	187
Cd^{2+}	451	1.40	8.78	37.2	51
Mn^{2+}	49.5	0.14	2.6	11.0	19
Cu^{2+}	0 [c]	0	0		
Ni^{2+}	0 [c]	0	0		

[a] Tromethamine base was 0.12 *M*. [b] Reaction solution contained 4.5% acetonitrile. [c] Up to 1×10^{-4} *M* metal ion.

Table IV—Effect of Amine Structure on Reaction Rate In Absence and Presence of Zinc Ion

Amine	pK_a	k_{amine} [a], M^{-1} min^{-1}	$k_{max}/[Zn]_0$ [b], M^{-1} min$^{-1} \times 10^{-4}$
Tromethamine	8.01	0.032	10.3
2-Amino-2-methyl-1, 3-propanediol	8.68	0.251	5.5
Ethanolamine	9.21	0.833 [c]	2.8
Diethanolamine	8.94	0.591	7.0
Triethanolamine	7.85	0.017	0.37
2-Diethylaminoethanol	9.86	0.600	10.7 [d]
2-Methoxyethylamine	9.30	—	0

[a] Rate constant in absence of Zn^{2+}. [b] Done at pH 9. [c] From Ref. (11). [d] At base conc. 0.069 *M* (no maximum observed).

with Cu^{2+} and Ni^{2+}. Thus, there is a considerable degree of similarity between the relative order of activity of metal ions in the enzyme and this model system.

Effect of Ligand Structure—As an initial approach to determining the effect of ligand structure on catalytic activity, the rates of loss of benzylpenicillin were measured in the presence of zinc ion with several amino alcohols and one in which the hydroxyl group was blocked. The results are shown in Figs. 7 and 8 and summarized in Table IV.

These data provide further evidence for the intermediacy of a ternary complex (amine–zinc ion–penicillin) in this type of reaction, as opposed to nucleophilic attack by amine on a metal ion–penicillin complex. If the latter were the predominant pathway, one would not expect to observe a great difference in rate between ethanolamine and methoxyethylamine, since their pK_a values are very close, unless the latter had a much greater affinity for the metal ion. One would expect however, that ethanolamine would show the greater affinity, because of the opportunity for a bidentate complex with the free hydroxyl group.

On the other hand, these data are entirely consistent with the ternary complex mechanism in which the hydroxyl of the ethanolamine, coupled to metal ion, is the attacking species. Methoxyethylamine cannot enter into a ternary complex, in which it can behave as a nucleophile, because of the blocked hydroxyl group.

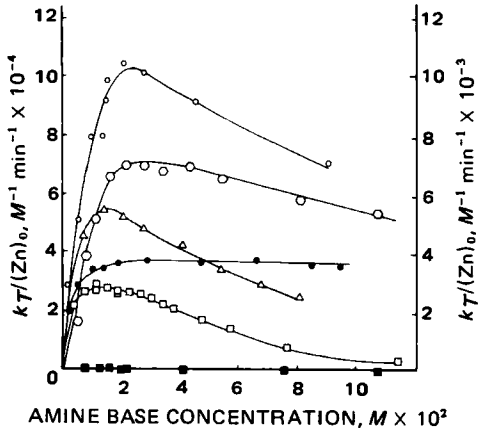


Figure 7—*Dependence of rate constant on amine concentration. Key: Left ordinate: (○) tromethamine, (△) 2-amino-2-methyl-1,3-propanediol (□) ethanolamine, (○) diethanolamine; right ordinate: (●) triethanolamine (■) 2-methoxyethylamine.*

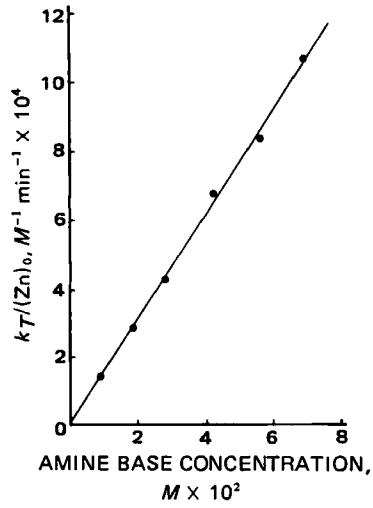


Figure 8—*Dependence of rate constant on concentration of 2-diethyl-aminoethanol.*

The relative values of the rate constants for tromethamine, 2-amino-2-methyl-1,3-propanediol and ethanolamine also lend support to the ternary complex hypothesis. If the other mechanism were predominant, one would expect the rates to increase with increasing pK_a, as they do in the absence of metal ion. The order of reactivity, however, seems to be determined more by the number of hydroxyl groups available for binding to the metal ion (*i.e.*, a statistical effect), which would be expected if the affinity for metal ion in a ternary complex was the most important factor involved.

The same general effect is seen in comparing the rate constants for diethanolamine with ethanolamine. Though the latter has a higher pK_a and reacts more rapidly with penicillin in the absence of zinc ion, its rate in the presence of zinc ion is significantly lower. Again, the rate constants with zinc ion are related more to the number of hydroxyl groups and this factor further supports the ternary complex mechanism.

With triethanolamine two factors are of interest: the relatively low rates with zinc ion and the shape of the concentration-dependence curve. The latter may indicate that triethanolamine forms only a 1:1 complex with zinc as it does with cupric ion (12). The plateau indicates either that the same species is present throughout the concentration range studied or that a higher complex has the same activity. The former seems more likely, in view of the fact that Cu^{2+} forms only a 1:1 complex with triethanolamine. The inability of triethanolamine to form higher complexes is probably due to steric inhibition. The lower rate with penicillin may reflect the same kind of steric inhibition of formation of a ternary complex with substrate.

The concentration dependence of rates with diethylaminoethanol is of interest, because it shows no maximum up to 0.1 *M*. This apparently results from formation of only a 1:1 complex perhaps due to steric inhibition or to a relatively low affinity of the compound for zinc ion. Yet the rates are relatively high, and it may be of interest to further study the concentration and pH dependence of this reaction. This type of compound may also be of more interest as a β-lactam model, since the product, with a tertiary amine, should be only penicilloic acid as with the enzyme rather than a mixture of the acid with an amine as with primary amines.

In summary, additional evidence presented here provides further support for the previously proposed mechanism for the catalysis of hydrolysis and aminolysis of penicillin by zinc ion and tromethamine, involving a ternary complex intermediate. The relative rates of reaction of other metal ions substituted for zinc are in the same general order as in a zinc-dependent β-lactamase. Thus, this system may be a model for the enzyme.

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Interaction of Nitroglycerin With Human Blood Components

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Abstract □ Nitroglycerin is rapidly lost from solution when incubated with red blood cells or whole blood. The assumption that the loss is enzymatic in nature may not be true, since no major metabolite is detected during this incubation. Explanation on the basis of a chemical reaction is also difficult, since the products of the chemical hydrolysis of nitroglycerin are the same as the metabolic products. After an initial rapid loss, nitroglycerin disappearance at 37° follows an apparent first-order process in the concentration range of 10–480 ng/ml when incubated with washed red blood cells suspended in normal saline solution. The half-life for the reaction of the apparent first-order phase varies with the initial concentration and increases as the concentration increases (4 min at 10 ng/ml, 52 min at 480 ng/ml), suggesting a mixed kinetic mechanism. Metabolites of nitroglycerin (1,2- and 1,3-dinitroglycerin) react similarly to nitroglycerin in terms of an apparent initial, fast step, a secondary first-order dependence, and concentration-dependent rate effects; however, the rate of the reaction is much slower ($t_{1/2}$ = 33 min at 10 ng/ml) for the metabolite. These data suggest the possibility of a physical mechanism for the loss of nitroglycerin. Since the loss to red blood cells can be rapid, it seems that the mechanism should be delineated, and that the rate of disappearnce be considered in an analysis of the pharmacodynamics of the drug.

Keyphrases □ Kinetics—nitroglycerin loss to red blood cells □ Nitroglycerin—rate of loss to red blood cells □ 1,2- and 1,3-Dinitroglycerin—rate of loss to red blood cells □ Disposition—reactivity of nitroglycerin and its metabolites with blood

It has been reported that when nitroglycerin is incubated with whole blood (fresh or outdated), resuspended red blood cells, serum, or plasma, the drug is lost from solution by an apparent first-order process. A relatively rapid loss occurred in blood or resuspended cells (1–3) ($t_{1/2}$ = 6 min) and rat serum ($t_{1/2}$ = 20 min) (4) with a slower loss occurring in plasma, $t_{1/2}$ = 53 (1) or 175 min (5). The rate of loss in the presence of erythrocytes approaches the biological half-life of organic nitrate (1.9 min) (1). The interaction of nitroglycerin with blood cells, serum, or plasma has been assumed by other workers to be an enzymatic reaction. However, our earlier work using an assay with resuspended red blood cells that could detect the major nitroglycerin metabolites (1,2-dinitroglycerin and 1,3-dinitroglycerin) showed that there was no metabolite in the medium over the entire time course of the loss of nitroglycerin (3). This observation led to the conclusion that the loss of nitroglycerin may not be enzymatic in nature. In this paper further studies are reported which probe the mechanism of loss of nitroglycerin when the drug is incubated with red blood cells. Since the rate of loss of nitroglycerin is rapid, it would appear that a knowledge of the mechanism of its loss would have significant impact on an analysis of the disposition kinetics of the drug and possibly its physiological activity as well.

EXPERIMENTAL

Nitroglycerin stock solutions were prepared from an alcoholic extract of a 10% aqueous solution of lactose adsorbate[1]. The major metabolites of nitroglycerin, 1,2-dinitroglycerin and 1,3-dinitroglycerin, were prepared from 2,3-dibromopropanol[2] and 1,3-dibromopropane-2-ol[3] using the method of Dunstan *et al.* (6). The purity of the compounds was determined by high-performance liquid chromatography (HPLC) and TLC and the solutions standardized as reported by Yuen *et al.* (7). All other chemicals used were obtained commercially and were reagent grade or better.

An electron capture gas chromatograph[4] was used with a data processor[5]. The GLC assay for nitroglycerin was reported earlier (9% QF-1[6] on 60–80 mesh Supelcoport[7]) (3). The column was silanized glass 2 mm × 0.915 m and the temperatures of the injection port, the column, and the detector were 150, 125, and 200°, respectively. The carrier gas was 5% methane in argon used at a flow rate of 21 ml/min. Under these conditions the retention times were: mixture of 1,2-dinitroglycerin and 1,3-dinitroglycerin, 3 min; nitroglycerin, 6.3 min; and internal standard (1-fluoro-2,4-dinitrobenzene[8]) 7.57 min. The system does not separate the two isomeric metabolites but does optimize their combined detection.

For the assay of 1,2- and 1,3-dinitroglycerin a method was used that was a slight modification of our earlier method (8). The column (silanized glass, 2 mm × 50 cm) consisted of 3% Carbowax 20M-TPA[9] on 60–80 mesh Supelcoport[7]. The injection port, column, and detector were at 150, 135, and 200°, respectively and the flow rate of the carrier gas was 20 ml/min. Using these conditions the two metabolites could be separated from each other and from nitroglycerin. The approximate retention times in minutes were: nitroglycerin, 2.7; *o*-iodobenzyl alcohol[10] (internal standard), 3.4; 1,2-dinitroglycerin, 6.5; and 1,3-dinitroglycerin, 8.2.

The concentration of nitroglycerin or its metabolite in the various

[1] Nitroglycerin 10% (w/w) in lactose, lot K17-O-H, ICI Americas, Atlas Chemical Div, Wilmington, DE 19899.
[2] Eastman Kodak, lot A5A, Rochester, NY 14650.
[3] Eastman Kodak, lot B6C, Rochester, NY 14650.
[4] 3700 Series dual column gas chromatograph, Varian Instrument Div., Palo Alto, CA 94303.
[5] Shimadzu Recording Data Processor. Chromatopac C-RIA Shimadzu Scientific Instruments, Columbia, MD 21045.
[6] Analabs, No. Haven, CT 06473.
[7] Supelco, Bellefonte, PA 16823.
[8] Eastman Organic Chemicals, Distribution Products Ind. Rochester, NY 14650.
[9] Applied Science Labs, State College, PA 16801.
[10] Aldrich Chemical Co., Milwaukee, WI 53233.

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