Model Catalysts Which Simulate Penicillinase I

Effect of Ionic Interaction on Catalysis of Penicillin Hydrolysis by Certain Catecholamines

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Further studies on the nature of the catalysis of hydrolysis of penicillin G by 3,6-bis-(dimethylaminomethyl)catechol (CDM) show that the active species of this compound (+1 charge) interacts by electrostatic attraction with the negatively charged penicillin. The 3,5-substituted catechol shows no pH of maximum rate, as does CDM, and much lower catalytic rate, indicating that both aminomethyl groups must be *ortho* to the hydroxyl groups for maximum catalytic effect. The possible relationships of CDM to the enzyme penicillinase are discussed.

It is often proposed that the high catalytic activity of certain hydrolytic enzymes may result in part from concerted polyfunctional catalysis by both nucleophilic and electrophilic groups at the active site of the enzyme. There have been suggested as models for these enzymes a number of systems in which these groups are situated in optimum position for exerting maximum catalytic effect. The monocatecholate anion, for example, is a more efficient catalyst than phenolate ion for the hydrolysis of phenyl chloroacetate and acyl halides (1, 2) and also reacts more rapidly than phenolate ion with isopropyl methylphosphonofluoridate (sarin) (3). Since the reactivity of resorcinol, hydroquinone, and guaiacol are all of the same order of magnitude as phenol, the *ortho* hydroxyl group of the catechol is clearly implicated as a participant. More recently, it has been shown that the presence of charged aminoalkyl groups on the ring of the catechol enhances its reactivity toward sarin (4). This high reactivity has been attributed to a charge effect which is related to the distance between cationic and anionic sites in the catecholamine and which is purported to modify the normally expected Bronsted relationship between basicity of the catechols and their nucleophilicity toward phosphorus. Charged aminoalkyl substituents on imidazole are also known to enhance its catalytic effectiveness for hydrolysis of *p*-nitrophenyl acetate (5). In this case polarization of the ester carbonyl, increasing the electrophilicity of the carbon, was assumed to be responsible for the increased rates.

One requisite of enzymatic catalysis notably lacking in most of the model systems studied thus far is a binding between substrate and catalyst such as is thought to occur in most enzymatic reactions. Electrostatic interaction between substrate and catalyst has recently been demonstrated, however, in the catalysis of hydrolysis of a negatively charged phenyl ester by polymers containing pyridine or imidazole groups (6, 7).

The author has recently reported (8) catalysis of penicillin hydrolysis by catechol and 3,6-bis-(dimethylaminomethyl)catechol (CDM) and noted particularly rapid rates and a bell-shaped pH–rate profile with the latter compound. It is now suggested that CDM represents a model for the enzyme penicillinase, which catalyzes the same reaction, and evidence is presented here to demonstrate an ionic interaction between CDM and penicillin and also a degree of specificity of the catalyst for the substrate.

EXPERIMENTAL

Materials.—Catechol was purified by sublimation. Pyrogallol was Fisher certified reagent. Potassium penicillin G[1] was recrystallized from water–*n*-butanol. The methyl ester of penicillin G was prepared by the method of Barnden *et al.* (9), m. p. 97–98°. The substituted catecholamines were prepared by methods described by Epstein *et al.* (4) with two exceptions.

The 3,5-bis(dimethylaminomethyl)catechol was prepared from the corresponding guaiacol derivative by treatment with 10% HBr in acetic acid, forming the hydrobromide salt, which was recrystallized from isopropyl alcohol. 1,4-Bis(dimethylaminomethyl)-2,3-dihydroxynaphthalene was prepared by treating 2,3-dihydroxynaphthalene in alcohol with dimethylamine and formaldehyde (3 equivalents of each) and removing the solvent under reduced pressure. The residue was slurried with a small amount of ether and then taken up in hot methanol and HCl gas admitted. On standing, crystals of the product separated out and were recrystallized from water–methanol, m.p. 218–220° dec.

Kinetic Studies.—The rates of hydrolysis of penicillin were followed by titrating the acid produced in the reaction with a Radiometer TTT-1 pH-stat

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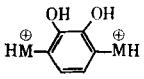
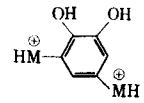
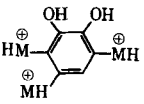
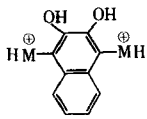
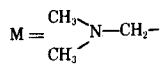
TABLE I.—COMPOUNDS STUDIED

Compd.	Code Name	pK_1'	pK_2'	pK_3'	Mol. Wt. Calcd.	Found
	CDM	6.35	9.65		297.3	297
	CMM	7.3	9.7		386.3	391
	CTM	4.95	7.10	10.35	528	512
	DHM	5.07	9.7		347.3	362



and SBR-2 recorder. All the runs were conducted in a nitrogen atmosphere in a jacketed glass cell maintained at constant temperature by water circulating from a thermostated bath. All of the present studies were conducted at 31.5 ± 0.1°. From the recorded curves of volume of titrant (standard NaOH solution) as a function of time, Guggenheim plots were constructed and the rate constants obtained from these. Generally the reactions were followed over at least three half-lives, and one equivalent of acid was produced in all the runs. The ionic strength was maintained at 0.2 by the addition of potassium chloride.

In the case of the penicillin G methyl ester, the solvent used was 13.3% by volume acetone in water. Since only one equivalent of acid was produced, it was obviously from the hydrolysis of the β-lactam, since ester hydrolysis would produce penicillin G which would subsequently hydrolyze to produce a second mole of acid.

The apparent acid dissociation constants Ka' were determined by potentiometric titration under the same conditions under which the rate studies were performed. Ultraviolet absorption spectra were recorded on a Beckman DB spectrophotometer.

RESULTS

From the preliminary results (8) with CDM and penicillin G, it appeared that an ionic interaction between substrate and catalyst might be responsible for the very high hydrolytic rates observed. Therefore, a number of compounds similar to CDM but with certain structural variations were prepared in order to study some of the factors influencing this interaction. These compounds are listed in Table I along with their dissociation constants (pKa'). The molecular weights were determined by potentiometric titration with standard alkali. The code names are utilized for brevity in the subsequent discussion.

Each kinetic run showed apparent first-order dependence upon penicillin concentration at constant pH. Figure 1 shows the first-order dependence of observed rate constant upon CDM concentration at pH 8.0. All the observed rate constants were corrected when necessary for alkaline hydrolysis of substrate. No correction for penicillin G was necessary below pH 9 since the rate of alkaline hydrolysis was slow. In the case of the methyl ester of penicillin, however, there was a substantial correction involved. The rate of hydrolysis of penicillin G methyl ester is plotted as a function of hydroxyl ion concentration in Fig. 2. The intercept represents a spontaneous hydrolysis and the slope the second-order rate constant for reaction of the ester with hydroxyl ion (k_{OH^-}).

The dependence of rate of penicillin G hydrolysis upon pH in the presence of each of the compounds listed in Table I is shown in Fig. 3. Here the ob-

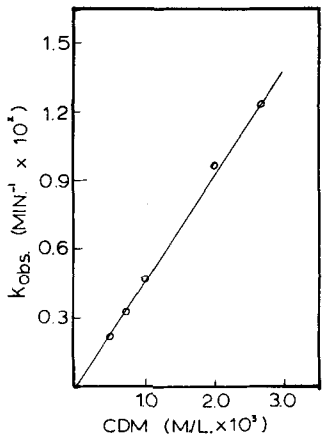


Fig. 1.—Plot showing first-order dependence of observed rate of penicillin hydrolysis on CDM concentration at pH 8.0.

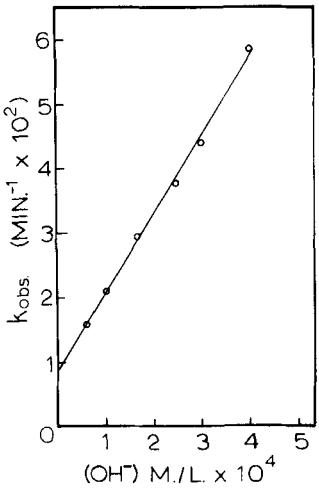


Fig. 2.—Dependence of rate of hydrolysis of penicillin G methyl ester on hydroxyl ion concentration.

served apparent first-order rate constants have been divided by the total molar concentration of catalyst to obtain k_C. Also shown in the uppermost curve is the dependence on pH of the rate of hydrolysis of penicillin G methyl ester catalyzed by CDM. From the bell-shaped curve for CDM catalysis of penicillin G hydrolysis, it is apparent that the singly charged ion is the most active catalytic species, the decrease at higher pH being due to formation of the neutral form. Calculations show that the latter has some catalytic activity, since the rate at pH 10 is somewhat higher than could be accounted for by the constant for the CDM^{+1}. The compound DHM follows the same general pattern as CDM. In the curve for CTM, however, the maximum covers a relatively broad pH range from 7 to 9.5, indicating that both the doubly charged and singly charged species are about equally active. In the case of CMM there is no maximum, but by examination of the dissociation constants for this compound, it can be seen that both the singly charged ion and neutral form are catalytic species, with the latter being slightly more active. From the curve for reaction of the methyl ester of penicillin with CDM, it is clear that both forms of the catalyst (+1 and neutral) are reactive.

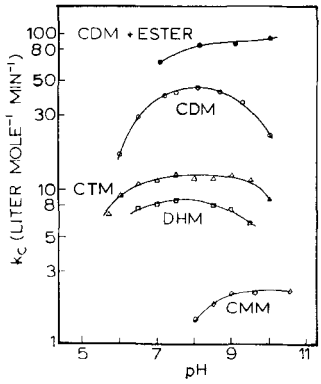
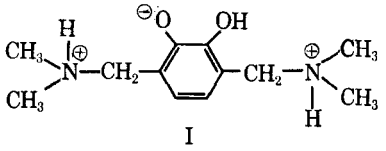


Fig. 3.—pH–rate profiles for hydrolysis of penicillin G by the catalysts used in this study. The uppermost curve is for hydrolysis of penicillin G methyl ester in presence of CDM.

To help ascertain the nature of the various species produced by dissociation of the catecholamines, ultraviolet spectra were recorded over most of the pH range covered by the rate studies. In Fig. 4 are shown the ultraviolet absorption spectra for CDM taken at pH 1 and pH 8, the latter representing the point of maximum concentration of singly charged species based on the titrimetric dissociation constants. The bathochromic shift in the wavelength of maximum absorption is typical of the ionization of phenols (10). The species (I) then



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probably accounts for a high proportion, if not all, of the singly charged form. Similar shifts were noted with the other catecholamines.

To evaluate catalytic rate constants for each species in solution, the following equations were

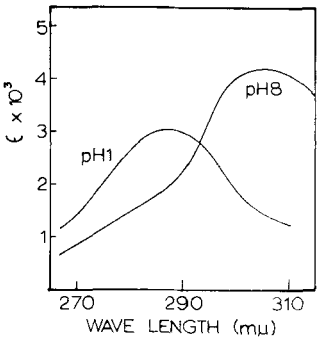


Fig. 4.—Ultraviolet absorption spectra for CDM at pH 1 and pH 8. ϵ = molar absorptivity.

utilized to calculate the fraction of the total concentration of each compound in the designated ionic state:

$$f_{\mathrm{I}} = \frac{[H^+]^2}{[H^+]^2 + K_1'[H^+] + K_1'K_2'}$$

$$f_{\mathrm{II}} = \frac{K_1'[H^+]}{[H^+]^2 + K_1'[H^+] + K_1'K_2'}$$

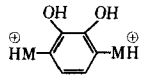
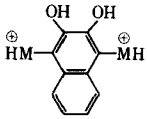
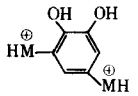
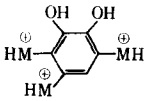
$$f_{\mathrm{III}} = \frac{K_1'K_2'}{[H^+]^2 + K_1'[H^+] + K_1'K_2'}$$

where f_{I} represents the fraction in the fully protonated form, f_{II} the fraction with +1 charge, and f_{III} the fraction as neutral species. These equations are applicable to all the compounds except CTM, where three dissociation constants must be considered. For this case similar equations may be derived with the denominator:

$$[H^+]^3 + K_1'[H^+]^2 + K_1'K_2'[H^+] + K_1'K_2'K_3'$$

By considering each observed rate to be the sum of the individual rates for each of the various species

TABLE II.—CATALYTIC RATE CONSTANTS

Catalyst or Reactant[a]	Code Name	pKa′	Net Charge	Catalytic Rate Constant (L. mole^{-1} min.$^{-1}$) with Substrate Penicillin G	Penicillin G Methyl Ester	Sarin[b]
OH^-		15.5	−1	12.5	112	1500
Catecholate ion		9.23	−1	3.8	66	589
Pyrogallol mono-anion		9.12	−1	15		1436
	CDM	6.35 9.65	+1 0	45 [c]	80 100	71.2
	DHM	5.07 9.7	+1 0	8.5 [c]		
	CMM	7.3 9.7	+1 0	2.0 2.2		63.4
	CTM	4.95 7.10 10.35	+2 +1 0	11 13 [c]		

[a] M, dimethylaminomethyl. [b] From the work of Epstein *et al.* (4). [c] Too low to measure accurately, but are all estimated to be in the range 1–4.

involved, catalytic rate constants for each species were calculated and are listed in Table II.

DISCUSSION

In considering the high rate of catalysis of penicillin G hydrolysis by CDM account must be taken of the possible contributions of each of the functional groups on the catalyst. It can be seen from the data in Table II that there is a parallel in the reactivity of both sarin and penicillin G with the negatively charged nucleophiles, hydroxyl ion, catecholate ion, and pyrogallol mono-anion. Probably, therefore, the undissociated *ortho* phenolic hydroxyl group of catechol participates in a similar manner with penicillin and penicillin methyl ester as it does with sarin (4), phenyl chloroacetate (1), and acyl halides (2).

A comparison of the catalytic rate constants for reaction of CDM^{+1} and catecholate ion with both sarin and penicillin G as depicted in Table II shows that CDM is relatively much more reactive with the penicillin. The same relationship holds for comparison between penicillin and its methyl ester, but to a lesser extent. Since the penicillin G is negatively charged while the other two substrates are neutral, these results lead one to the conclusion that the high catalytic rate for catalysis of penicillin G hydrolysis by CDM^{+1} is due to an ionic interaction between the two species. Additionally, both the singly charged and neutral forms of CDM are about equally effective catalysts for penicillin methyl ester hydrolysis, while the neutral CDM is much less effective than CDM^{+1} with the free penicillin. This result is in accord with the ionic interaction hypothesis, since such an interaction could not occur with the neutral ester or with the neutral CDM.

Further insight into the nature of the catalysis by CDM^{+1} is gained by comparison of its reactivity

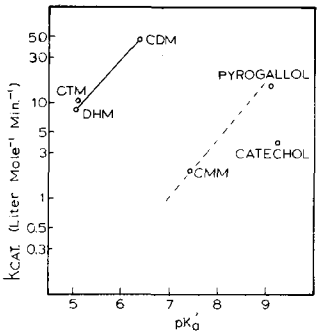


Fig. 5.—Bronsted plot of catalytic rate constant against apparent pKa′.

with penicillin G to that of CMM^{+1}. Both species carry the same net charge but differ only in the position of one of the aminomethyl groups. Despite the higher basicity of CMM^{+1}, its rate constant is only about $^1/_{20}$th that of the corresponding CDM species. It thus appears that ionic interaction favorable for catalysis occurs only with the compound having the aminomethyl group in position *ortho* to the hydroxyl group, while interaction with the *meta* aminomethyl group leads to relatively unfavorable conformation of substrate and catalyst.

The effect of basicity of the catalytic species may be seen in Fig. 5, which is a Bronsted plot of log catalytic rate constant for hydrolysis of penicillin G against pKa′. The solid line is drawn through those points which represent the two compounds having both charged substituents in positions *ortho* to the hydroxyl groups. Also inserted for reference are points for the other catalysts studied. It can be seen that CMM mono-anion has a higher rate constant than would be expected from its basicity rela-

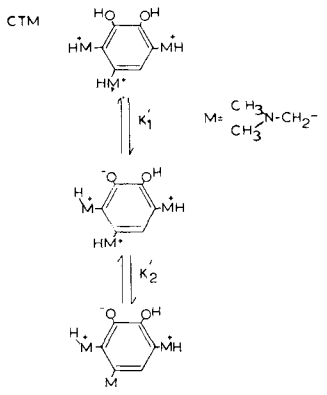


Fig. 6.—Proposed mode of dissociation of CTM.

tive to catecholate ion. Also a line drawn parallel to the line above, but through the point for CMM, would pass very close to the point for pyrogallol. Although definite conclusions cannot be drawn from such comparisons, it does seem probable that the charged group *ortho* to the ionized phenolic group participates in catalysis of penicillin hydrolysis by CMM^{+1}. Both the latter species and pyrogallol mono-anion have in common two electrophilic groups in addition to the nucleophilic phenolate.

The compound CTM fits into this general pattern. Its doubly charged form has about the same pKa′ and rate constant as DHM. The species with the +1 charge has about the same rate constant as the CTM^{+2}. It might be suspected therefore that CTM^{+1} has the same configuration as CDM^{+1}. That is, that loss of proton in the second ionization step is at the nitrogen of the 4-aminomethyl group as shown in Fig. 6. If this 4-aminomethyl group does not participate in the catalysis of penicillin hydrolysis by the CTM^{+2}, then removal of its proton would not change the nature of the existing active species. It seems quite probable that this is the case since the alternative removal of proton from the 6-aminomethyl group would be expected to reduce the rate considerably as it does in the formation of CDM^{0}.

From these results a consistent pattern emerges. In the catalysis of hydrolysis of penicillin G by CDM^{+1} and similar species, it appears that all four functional groups are necessary for optimum catalytic activity. One of the charged amino groups clearly participates by ionic interaction with the negatively charged substrate, while the other functions are involved directly in the catalytic process. Examination of molecular models shows that upon close approach of the negatively charged carboxylate of the penicillin to the positively charged aminomethyl group at the 3-position of the catalyst (assuming the ionized phenol is numbered 1), there is also close approach of the phenolate ion to the β-lactam carbonyl of the penicillin. Furthermore, both the 2-hydroxyl group and the 6-aminoalkyl group will also be in position to take part in the reaction.

The exact mechanism by which the hydrolysis proceeds has not yet been elucidated, although one might speculate that nucleophilic catalysis forms an intermediate penicilloate ester of the catecholamine which is subsequently hydrolyzed rapidly. Studies are presently under way to investigate this possibility.

It is tempting to speculate also on a possible relationship of a compound like CDM to the enzyme penicillinase. It is certainly not expected that the catecholamine grouping is to be found at the active site of the enzyme, but it is reasonable to suppose that a combination of nucleophilic, electrophilic, and charged groups situated on the enzyme surface in a manner similar to that in CDM^{+1} could be partly responsible for enzymatic activity.

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