steroid in the nonvolatile vehicle. The eventual decrease in penetration was the result of steroid precipitation from the supersaturated solution. In those cases where evaporation was prevented, the penetration from each solvent system remained at a low level.

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Model Catalysts Which Simulate Penicillinase IV: Steric and Electronic Effects in the Catalysis of Hydrolysis of Penicillins and Cephalothin by Aminoalkylcatechols

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Abstract □ Aminoalkylcatechols have been shown to catalyze the hydrolysis of penicillin at relatively rapid rates at neutral pH by a mechanism similar to that postulated for several hydrolytic enzymes. The present study was concerned with the effect on catalytic rates of varying the penicillin side chain and nucleus. Accordingly rates of hydrolysis of a number of penicillins and cephalothin were measured in the presence of several 3,6-bis(aminoalkyl)catechols where the amino group was varied in both size and basicity. Rates of alkaline hydrolysis were measured for comparison. There was little indication of steric hindrance of catalysis between penicillins with bulky side chains and catalysts with large amino groups. Electrostatic effects were much more prominent as exemplified by faster rates with carbenicillin (α-carboxybenzylpenicillin) than expected and changes in the pH-rate profile with ampicillin (α-aminobenzylpenicillin). With cephalothin the catalyst was virtually ineffective, probably because interaction between the carboxyl of a cephalosporin and a charged amine on the catalyst would leave the phenolate ion out of position for nucleophilic attack upon the β-lactam.

Keyphrases □ Aminoalkylcatechols—penicillinase simulation □ Penicillins, hydrolysis—aminoalkylcatechols, catalysts □ Cephalothin hydrolysis—aminoalkylcatechols, catalysts □ UV spectrophotometry—hydrolysis determination

Previous studies in this laboratory have shown that 3,6-bis(dimethylaminomethyl)catechol (CDM) (1) and other bis(aminoalkyl)catechols (2) are powerful catalysts of penicillin hydrolysis. Many of the characteristics of this catalysis resemble those observed with a number of hydrolytic enzymes. These include a maximum in the pH-rate profile (1), the presence of an acyl-catalyst intermediate (3), and some specificity in the structure of catalyst necessary for optimum activity (1). It has been shown that both the basicity of the amine and the susceptibility of the β-lactam to nucleophilic attack are factors influencing reaction rate.

The present study is concerned with the possible role of the penicillin side chain in the interaction with catalyst. A bulky side chain might sterically inhibit interaction of the penicillin with catalyst, whereas side chains containing certain groups might interact with the charged amino group and thus enhance catalytic rate. Therefore, the rates of hydrolysis of a number of penicillins were determined in presence of several 3,6-bis(aminomethyl)catechols in which the basicity and size of the amino group were varied. Also studied was cephalothin, one of the cephalosporins, a group of β-lactam antibiotics which differ from the penicillins in the distance between the carboxylate ion and β-lactam carbonyl group.

EXPERIMENTAL

Substrates—Cephalothin and penicillin V,[1] nafcillin,[2] carbenicillin,[3] ancillin,[4] and other penicillins,[5] were all used. Two of the penicillins were prepared as follows:

Methylpenicillin—(0.1 mole) 6-Aminopenicillanic acid (6-APA) was dissolved in water containing 0.5 mole $NaHCO_3$ and 0.15 mole acetic anhydride added with stirring. After 90 min., the mixture was cooled, acidified to pH 2 with phosphoric acid, and extracted three

[1] Eli Lilly and Co.
[2] Wyeth Laboratories.
[3] Beecham Research Labs.
[4] Smith Kline & French Labs.
[5] Bristol Laboratories.

times with ether. The combined ether extracts were washed once with cold water and dried over anhydrous sodium sulfate. To it was added excess 30% sodium 2-ethylhexanoate in butanol, precipitating the sodium salt of the penicillin. This was recrystallized from butanol–water.

Diphenylmethylpenicillin—To a suspension of 0.1 mole 6-APA in $CHCl_3$ was added 0.1 mole triethylamine bringing the 6-APA into solution. Excess diphenylacetyl chloride in $CHCl_3$ was added slowly with stirring while the mixture was cooled in an ice bath. After 1 hr., the reaction mixture was brought to pH 2 with aqueous H_3PO_4 and after agitation the $CHCl_3$ layer was separated, washed once with cold water, and dried with anhydrous Na_2SO_4. The potassium salt of the penicillin was precipitated by addition of excess 30% solution of potassium 2-ethylhexanoate in butanol and recrystallized from butanol–water.

The formation of a penicillin in each case was confirmed by the IR band at 5.65 μ characteristic of the β-lactam.

Catalysts—CDM and CDM_0 [(3,6-bis(morpholinomethyl)catechol·2HCl)] were the same materials used in previous studies (1, 2). The new compound, 3,6-bis(piperidinomethyl)catechol dihydrochloride (CDP) was prepared by the same method used to prepare CDM_0 (2). Its pKa's determined by potentiometric titration were $pK_1 = 6.39$, $pK_2 = 9.87$. Mol. wt. (by titration): Calcd: 377.4; Found: 380.

Rate Measurements—Rates of hydrolysis of penicillins were measured as previously described (1) by following the rates of acid formation on the radiometer (TTT-1) pH-stat with a recorder (SBR-2). Rates of alkaline hydrolysis were determined for each penicillin at several pH values between 10 and 11.5. In the presence of catalysts, rates were measured at pH 8.0 when CDM and CDP were used, and at pH 6.5 with CDM_0. These points had been found to be the pH of maximum rate. With ampicillin and CDM_0 the pH range 5–8 was covered.

Rate of hydrolysis of cephalothin was determined by following the loss of absorbance at 260 mμ which is characteristic of opening of the β-lactam (4). The reaction solution was kept at constant pH on the pH-stat, samples removed at appropriate intervals, diluted with 0.2 *M* pH 6 phosphate buffer, and absorbance read on a spectrophotometer (Hitachi Perkin Elmer model 139).

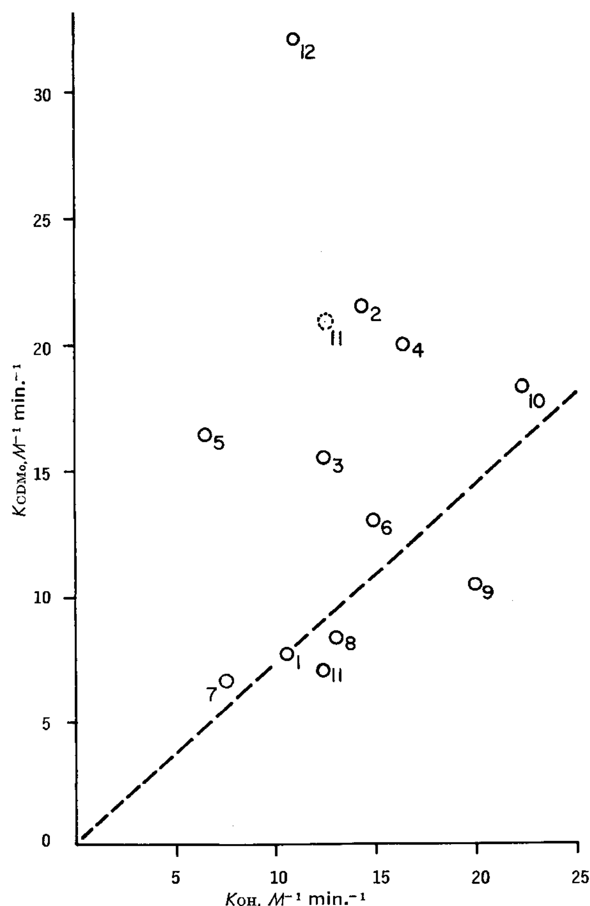


Figure 1—*Comparison of* k_{CDM_0} *with* k_{OH}. *The numbers correspond to those assigned to the penicillins in Table I.*

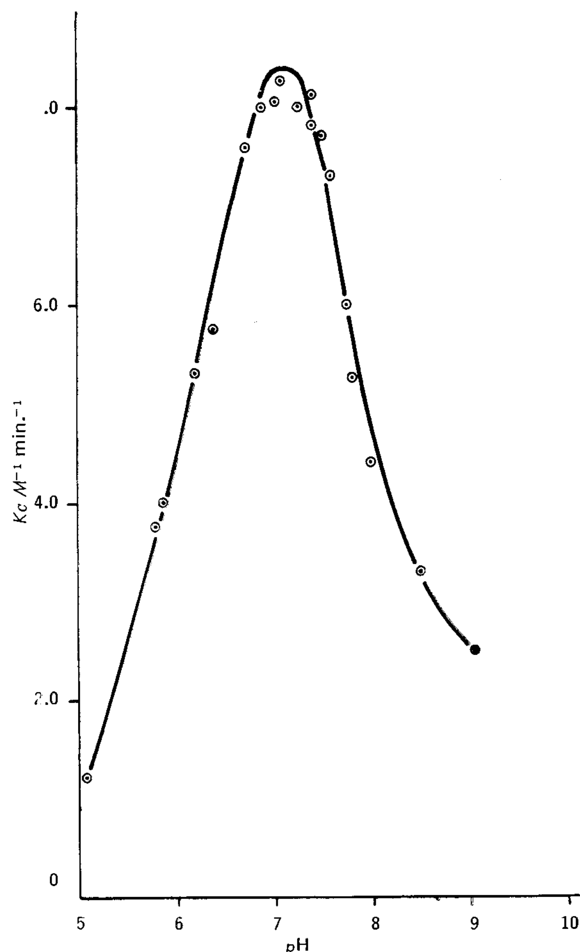


Figure 2—*Log* $k_{obs.}$*-pH profile for ampicillin hydrolysis catalyzed by CDMo.*

The concentration of catalyst was generally 0.001 *M* and that of substrate 0.008 *M*. Ionic strength was brought to 0.2 *M* with potassium chloride and all measurements were done at 31.5°. Titrant was 0.2 *M* NaOH solution.

RESULTS AND DISCUSSION

In all the runs apparent first-order kinetics were observed and the rate constants, $k_{obs.}$, were determined from the slopes of Guggenheim plots of the data. Specific rate constants for alkaline hydrolysis, k_{OH}, were calculated by dividing $k_{obs.}$ by K_w/a_H where a_H, is hydrogen ion activity determined with the glass electrode. For each substrate the mean of several determinations at varying a_H is reported.

Specific rate constants for the catalysts were calculated by dividing $k_{obs.}$, obtained at the pH of maximum rate, by the concentration of species with +1 charge present at that pH. The data are presented in Table I.

The values for k_{OH}, it is assumed, are a measure of the susceptibility of substrate to nucleophilic attack since approach of hydroxyl ion to the β-lactam carbonyl would not be noticeably affected by the side chain. It can be seen from these data that the side chain of a penicillin has very little influence upon alkaline hydrolysis rate, there being only about a threefold difference in rate between the slowest and fastest.

Methylpenicillin was included in this study as a standard for comparison since the small side chain would not be expected to interfere with the approach of the catalysts. Since CDM and CDP have about the same basicity it was expected that the ratio of the rate constants for these species would be close to unity unless some

Table I—Reaction Rate Data

Penicillin		Side Chain, R	Specific Rate Constants, M^{-1} min.$^{-1}$ k_{OH}	k_{CDM}[b]	k_{CDP}[b]	k_{CDM_0}[a]	k_{CDM}/k_{CDM_0}	k_{CDM}/k_{CDP}	k_{CDM}/k_{OH}
Methyl	1	CH_3-	10.6	25.7	30	7.7	3.3	0.86	2.4
Diphenylmethyl	2	$(C_6H_5)_2CH$-	14.4	77	90	21.5	3.6	0.86	5.4
Benzyl	3	$C_6H_5CH_2$-	12.5	45	64	15.5	3.1	0.70	3.6
Penicillin	4	$C_6H_5OCH_2$-	16.5	63.5	96.5	20	3.2	0.66	3.8
Methicillin	5	2,6-dimethoxyphenyl-	6.5	33.8	53.5	16.5	2.1	0.63	5.2
Nafcillin	6	2-ethoxy-α-naphthyl-	15	46	52	13	3.5	0.89	3.1
Ancillin	7	*O*-phenylphenyl	7.6	29	27	6.5	4.5	1.1	3.8
Oxacillin	8	3-phenyl-5-methyl-4-isoxazolyl	13	39	39.5	8.3	4.7	0.99	3.0
Cloxacillin	9	3-(2-chlorophenyl)-5-methyl-4-isoxazolyl	20	46	43	10.5	4.4	1.1	2.3
Dicloxacillin	10	3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolyl	22.3	63	81	18.3	3.5	0.78	2.8
Ampicillin	11	$C_6H_5CH(NH_2)$-	12.3	49	51	7	7.0	0.96	4.0
Carbenicillin	12	$C_6H_5CH(COO$-$)$-	11	96	117	32	3.0	0.82	8.7
Cephalothin	13	See Structure in Text	8.0	0.021					0.003

[a] Obtained at pH 6.5. [b] Obtained at pH 8.0.

specific steric hindrance was involved with the larger piperidine compound. The average value of k_{CDM}/k_{CDP} for all the penicillins was 0.86, which is close to unity, and the very small deviations indicate that steric effects only negligibly affect, if at all, the catalyst-substrate interaction.

A comparison of the specific rate constants for CDM_0 with k_{OH} is shown in Fig. 1. The numbers correspond to the penicillins in Table I and a dashed line was arbitrarily drawn through the point for methylpenicillin for reference. Points below the line should indicate some inhibition of interaction while points above the line would indicate an enhanced degree of interaction between CDM_0 and the substrate. Compound 12 is a special case and will be discussed below. Compounds 2–5 all fall well above the line indicating some interaction with CDM_0 which assists catalysis. Since the values for k_{CDM}/k_{CDM_0} for Compounds 2–5 are about the same, a similar interaction occurs with CDM. The nature of this interaction is difficult to ascertain from the data at hand. It may be that the charged amine adjacent to the ionized phenol forms weak hydrogen bonds with the π-electrons of a benzene ring or oxygen of the substrate. The points for Compounds 6–10 in Fig. 1 all lie very close to the line, indicating both a lack of interference of side chain with the kind of interaction seen with methylpenicillin and no apparent interaction between side chain and the charged amine as noted with Compounds 2–5. The latter effect may be due to the bulky aryl groups in the side chains of Compounds 6–10 which prevent these side chains from being in a conformation in which interaction with the charged amine of the catalyst could occur.

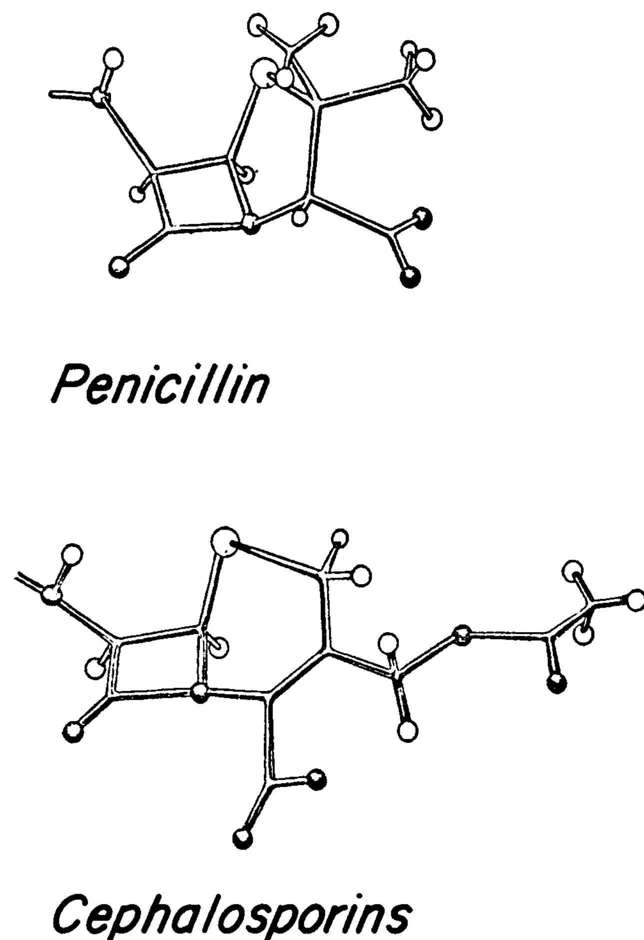
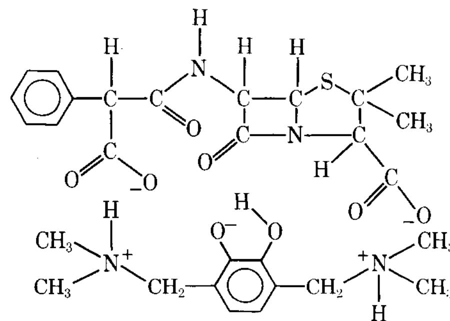


Figure 3—*Comparison of wire models of ring systems of penicillins and cephalosporins.*

With carbenicillin, Compound 12, the rate constants for all of the catecholamines are much larger than would be expected from its alkaline hydrolysis rate. These high rates are most likely the result of electrostatic interaction between the side chain carboxylate ion of the penicillin and the positively charged amine adjacent to the phenolate ion of the catalyst as shown below. The additional electro-



static attraction facilitates nucleophilic attack by the catecholate ion, increasing reaction rate.

The ratio k_{CDM}/k_{CDM_0} is higher for ampicillin than for the other penicillins. This is due to repulsion at pH 6.5 of CDM_0^{+1} by the positively charged amino group of pKa 7.2 (5) in the side chain of ampicillin. The repulsion of CDM^{+1} by the drug is less because the rate constants for CDM^{+1} were measured at pH 8.0, where only a small fraction of the amine on ampicillin is protonated. In contrast, at pH 6.5, where rate constants for CDM_0^{+1} were determined, most of the amine is protonated.

These electrostatic effects are reflected in the pH-rate profile for ampicillin hydrolysis catalyzed by CDM_0^{+1} shown in Fig. 2 where the maximum is shifted to higher pH than seen with the other penicillins (2). The overall rate in the case may be written as the

sum of rates for catalysis by each species of CDM_0 on both forms of ampicillin as follows:

$$k_C = k_1 (f_{A^\pm})(f_{C^{+1}}) + k_2 (f_{A^-})(f_{C^{+1}}) + k_3 (f_{A^-})(f_{C^0}) \quad \text{(Eq. 1)}$$

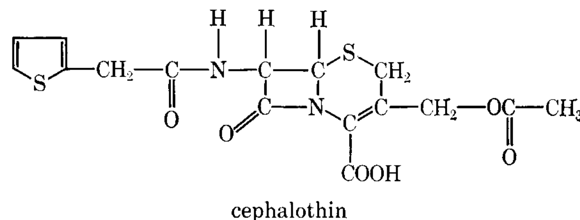
where $A^\pm$ represents the zwitter ion of ampicillin, A^- the negative ion, and C represents CDM_0. The term in k_2 is the kinetic equivalent of a term containing $(f_{A^\pm})(f_{C_0})$ and only one is necessary for this treatment. The f's represent the fraction of the total concentration as the designated species. Since all the pKa's are known, all the f's can be easily calculated at each pH and best fit values of the constants k_1, k_2, and k_3 obtained by solution of simultaneous equations. The curve in Fig. 2 was constructed using the values $k_1 = 5.7$, $k_2 = 21$, $k_3 = 2.2$, and the points are those determined experimentally. There probably is some hydrogen bond formation between the protonated amine on the catalyst and unprotonated amine of ampicillin and it should, therefore, fit better in Fig. 1 with Compounds 2–5 rather than near the line through Compound 1.

Using the value of $k_2 = 21$, which represents the interaction of positively charged catalyst with negatively charged substrate, is more appropriate for Fig. 1 than the $k_{obs.}$ value, since it represents the same interaction being considered for the other penicillins. The dotted circle in the figure shows that ampicillin does indeed fit in the same category with Compounds 2–5.

From Eq. 1 it should be possible to calculate the pH of maximum rate ampicillin hydrolysis catalyzed by CDM_0. Setting the first derivative of k_C with respect to a_H equal to zero leaves an equation very difficult to solve analytically. By inserting numerical values for the constants, an iterative approach may be utilized. When this was done, a value pH 7.2 was calculated. This is in excellent agreement with the experimental result.

The most profound effect of structural modification of substrate is seen with cephalothin. While the alkaline hydrolysis rate of this compound is about the same as that of the penicillins, catalysis of hydrolysis by CDM^{+1} is very low being at least 1000-fold less than for penicillins. A reasonable explanation for this large difference may be seen by examination of Fig. 3 showing the spatial relationships in the two molecules (6). It can be seen that there is a large difference in the relationship of the carboxylate ion to the β-lactam carbonyl group between the two molecules. Examination of molecular models shows that interaction of the charged amine of CDM^{+1} with the penicillin carboxylate ion places the phenolate ion very close to the β-lactam carbonyl in excellent position for nucleophilic attack. A similar electrostatic interaction with cephalothin, however, leaves the phenolate ion in poor position to attach to β-lactam carbonyl. This result further confirms the hypothesis that electrostatic attraction is a principal factor in the catalysis of penicillin hydrolysis (1).

On the basis of these observations, one may speculate as to the differences noted in the activity of a penicillinase and a cephalosporinase. Each enzyme has some, albeit very little, activity on the natural substrate of the other (7). The differences may simply be due to a different location of a positive charge relative to the catalytic functional groups in the active site of the enzyme. It is suspected that a positively charged group is located in the active site of the enzyme (8).



cephalothin

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