the first time, a synthetic method for labeling the carbon skeleton of tropine, for synthesizing tropine-labeled atropine, and for synthesizing labeled intermediates between arabinose and tropine. Further studies will be published in the third paper of this series.

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Model Catalysts Which Simulate Penicillinase II

Mechanism of Hydrolysis of Penicillins Catalyzed by Catechol

By MICHAEL A. SCHWARTZ and GERALD R. PFLUG

Studies have been made of the rates of loss of benzylpenicillin and methicillin from solutions containing catechol. The dependence upon pH of the specific rate constants for these hydrolyses catalyzed by monocatecholate ion is nonlinear, demonstrating the presence of metastable intermediates in the reaction pathway. The rates of penicillin loss at the higher pH's studied are faster than the rates of acid formation in the same systems indicating the presence of a covalent intermediate, probably catechol monopenicilloate. Analogy of this mechanism to that of hydrolyses of penicillins catalyzed by 3,6-bis(dimethylaminomethyl)catechol and by penicillinase is discussed.

IT WAS PREVIOUSLY reported that the compound 3,6-bis(dimethylaminomethyl)catechol (CDM) rapidly catalyzed the hydrolysis of penicillin to penicilloic acid (1). Pyrocatechol itself, as the monoanion, was also shown to be catalytic but at a much lower rate than CDM. Studies of the relationship between structure of the catalyst and its reactivity revealed that the catalytic efficiency of CDM was at least partly due to an ionic interaction between catalyst and substrate, similar to the type of interaction which might be expected in formation of an enzyme–substrate complex. It was speculated at that time that this reaction

proceeded by covalent catalysis (2) to form a penicilloate ester of the catecholamine which would then be rapidly hydrolyzed to product, releasing free catalyst.

penicillin + CDM → ester →
penicilloic acid + CDM
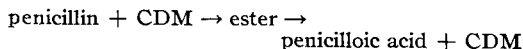
The above-mentioned studies were conducted by measuring the rate of acid production by means of a pH-stat. The presence of an ester intermediate might be demonstrated, however, by also following loss of penicillin. If the latter were more rapid than acid formation there would be no doubt as to the presence of an intermediate in the reaction pathway. The present report concerns the results of such studies in which pyrocatechol was utilized as the catalyst rather than CDM because it was felt that ester hydrolysis would be slower with pyrocatechol than with

CDM where the amino groups might accelerate the reaction by intramolecular general acid catalysis. As will be seen, the data provide evidence not only for an ester intermediate but also for metastable intermediates in the reaction pathway prior to ester formation

EXPERIMENTAL

Materials and Apparatus—Potassium benzylpenicillin and sodium methicillin were supplied by Bristol Laboratories. Pyrocatechol, mercuric chloride, and buffer ingredients were all reagent grade. pH measurements were made with the Radiometer TTT-1 meter in a glass cell thermostated at the temperature of the kinetic studies. The meter was standardized with both pH 6.83 and 9.18 buffers. The Hitachi Perkin-Elmer model 139 spectrophotometer was used in the penicillin assays and the Radiometer TTT-1 pH-stat with SBR-2C recorder used for measurement of acid formation rates.

Kinetic Studies—All the rate measurements were carried out at 31.5° at ionic strength 0.2 maintained by addition of sufficient potassium chloride to the reaction mixture. All solutions were prepared with water which had been freshly boiled and cooled under nitrogen and a nitrogen atmosphere was maintained throughout the rate measurements to minimize oxidation of catechol.

Rates of acid formation from penicillin in the presence of catechol were measured on the pH-stat as previously described (1).

Rates of penicillin loss were measured as follows. Catechol solution containing sufficient potassium chloride was placed in the reaction cell of the Radiometer pH-stat and allowed to come to bath temperature. The pH was adjusted to the desired value by addition of NaOH solution and the reaction was initiated by addition of a solution of the penicillin which had been kept at bath temperature. Samples were removed at appropriate intervals and transferred to a 0.2 M pH 2.7 glycine buffer containing 0.01 M mercuric chloride. After standing a specified time (80 min. for benzylpenicillin, 130 min. for methicillin), the absorbance was read at 322 mμ for benzylpenicillin or 328 mμ for methicillin. This assay for residual penicillin in solution has been described previously (3).

The rates of alkaline hydrolysis of the penicillins in absence of catechol were determined by following acid production rates on the pH-stat.

RESULTS

Figure 1 shows the typical first-order dependence of rate of penicillin loss upon concentration of benzylpenicillin at constant pH. Similar linear plots were obtained for methicillin. The rate constants ($k_{obs.}$) obtained from the slopes of these plots were corrected for alkaline hydrolysis of the penicillin and converted to specific rate constants for monocatecholate ion (k_c) by Eq. 1:

$$k_c = \frac{k_{obs.} - k_{OH}[OH^-]}{\dfrac{K_a[C]_t}{K_a + [H^+]}} \quad \text{(Eq. 1)}$$

where $[C]_t$ is the total of all catechol species in the system and K_a is the dissociation constant of cate-
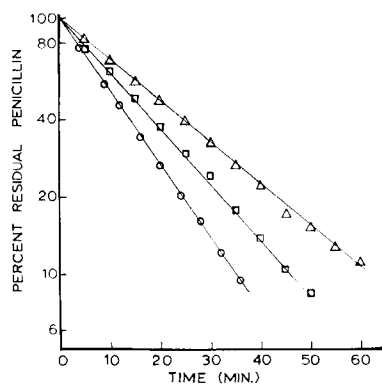

Fig. 1—First-order rate of loss of benzylpenicillin at various pH's. Catechol concentrations: ○, 0.0435 M, pH 9.15; □, 0.0174 M, pH 9.50; △, 0.0087 M, pH 10.0.

chol. The data are tabulated in Table I. The pKa of catechol under the conditions of these experiments was 9.40. The alkaline hydrolysis rate constants (k_{OH}) were: benzylpenicillin 12.5 and methicillin 6.0 L. mole^{-1} min.$^{-1}$.

At a single pH the observed first-order rate constants were found to be directly proportional to catechol concentration as shown in Fig. 2.

TABLE I—RATES OF PENICILLIN LOSS FROM SOLUTION IN PRESENCE OF CATECHOL

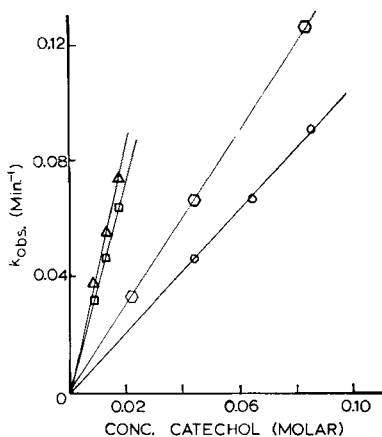| pH	Initial Penicillin Concn., $M \times 10^4$	Total Catechol Concn., M	$k_{obs.}$, min.$^{-1}$	k_c, L. mole^{-1} min.$^{-1}$
Benzyl Penicillin				
8.35	2.0	0.067	0.0210	3.82
8.50	2.0	0.131	0.0577	3.95
9.00	2.0	0.044	0.0507	4.02
	20.0	0.044	0.0507	4.02
9.15	2.0	0.0435	0.0666	4.22
	20.0	0.0435	0.0666	4.22
	20.0	0.022	0.0333	4.22
	2.0	0.083	0.126	4.19
9.35	2.0	0.022	0.0478	4.56
9.42	2.0	0.0174	0.0436	4.83
9.50	2.0	0.0174	0.0477	4.85
9.60	2.0	0.0176	0.0546	4.98
10.00	2.0	0.0174	0.0745	5.22
	2.0	0.0087	0.0380	5.24
	2.0	0.0129	0.0555	5.20
10.25	2.0	0.0172	0.0912	5.81
10.50	2.0	0.0083	0.0564	6.55
Methicillin				
8.50	2.0	0.129	0.0495	3.41
9.00	2.0	0.044	0.0468	3.71
	2.0	0.085	0.0912	3.74
	2.0	0.065	0.0673	3.65
9.15	2.0	0.043	0.0602	3.88
9.35	2.0	0.022	0.0420	4.02
9.50	2.0	0.022	0.0533	4.34
9.75	2.0	0.0174	0.0533	4.40
10.00	2.0	0.0086	0.0321	4.51
	2.0	0.0172	0.0641	4.51
	2.0	0.0129	0.0471	4.48
10.25	2.0	0.0085	0.0365	4.67
10.35	2.0	0.0081	0.0369	4.75
10.50	2.0	0.0086	0.0408	4.73

Fig. 2—*Plot showing first-order dependence of rate of penicillin loss on catechol concentration at constant pH. Key: △, benzylpenicillin, pH 10.0; ○, benzylpenicillin, pH 9.15; □, methicillin, pH 10.0; ○, methicillin, pH 9.0.*
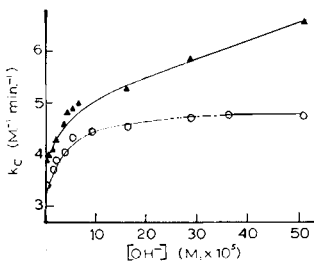

Fig. 3—*Dependence of k_C upon hydroxyl-ion concentration. Curves were fitted to Eqs. 2 and 3 for benzylpenicillin and methicillin, respectively. The points are the values determined experimentally. Key: ▲, benzylpenicillin; ○, methicillin.*

It was found that the values of k_c determined from rate of penicillin loss were not constant over the pH range studied, but varied as shown in Fig. 3.

In the case of benzylpenicillin the data were fitted to a curve of the form of Eq. 2:

$$k_c = \frac{a + b \ [OH^-] + c \ [OH^-]^2}{d + [OH^-]} \quad \text{(Eq. 2)}$$

where a, b, c, and d are constants. For methicillin the data were fitted to Eq. 3:

$$k_c = \frac{a + b(OH^-)}{d + (OH^-)} \quad \text{(Eq. 3)}$$

The curves in Fig. 3 were constructed from the values of the constants given in Table II, while the points are the actual values obtained experimentally. In both cases when (OH^-) is zero, $k_c = a/d$, whereas at high (OH^-):

benzylpenicillin: $k_c = b + c(OH^-)$
methicillin: $k_c = b$

The rates of acid formation were apparently first order and rate constants $k_{obs.}$ were determined from Guggenheim plots of the data. These values are given in Table III.

DISCUSSION

The specific rate constants (k_c) for loss of benzylpenicillin from solution, catalyzed by monocatecholate ion, are not independent of hydroxyl-ion concentration. This variation from a high degree of dependence at pH below 9.5 to only slight dependence at higher alkalinity is indicative of a change in mechanism of the reaction with increasing pH.

When $(OH^-) = 0$ the rate expression is:

$$\text{rate} = \frac{a \ (P)(C^-)}{d} \quad \text{(Eq. 4)}$$

which is the typical expression for nucleophilic catalysis by (C^-).

At high $[OH^-]$ the rate expression becomes:

$$\text{rate} = b \ [P][C^-] + c \ [P][C^-][OH^-] \quad \text{(Eq. 5)}$$

Equation 5 contains terms showing both nucleophilic catalysis by monocatecholate ion and by dicatecholate ion. The second term could alternatively be attributed to specific base-catalyzed

TABLE II—CONSTANTS USED TO FIT EQS. 2 AND 3

	a	b	c	d
Benzylpenicillin	18.2×10^{-5}	5.35	3.0×10^3	4.74×10^{-5}
Methicillin	9.30×10^{-5}	4.90	...	3.00×10^{-5}

TABLE III—RATES OF ACID FORMATION[a]

|	Benzylpenicillin			Methicillin	
pH	Catechol Concn., M	$k_{obs.}$, min.$^{-1}$	pH	Catechol Concn., M	$k_{obs.}$, min.$^{-1}$
8.50	0.129	0.051	8.50	0.089	0.0344
9.00	0.124	0.130	8.75	0.088	0.0550
9.50	0.053	0.105	9.00	0.088	0.0912
10.00	0.049	0.139	9.25	0.087	0.0937
10.25	0.048	0.151	9.50	0.044	0.0667
10.50	0.049	0.169	9.75	0.0178	0.0377
			10.0	0.0178	0.0423
			10.5	0.0089	0.0289

[a] Initial penicillin concentration was 0.02 *M*.

nucleophilic attack by monocatecholate ion, which is kinetically equivalent. At intermediate hydroxyl-ion concentrations a complex expression holds. This situation can only occur with the involvement of intermediates which are in acid-base equilibrium (4).

Such a mechanism is shown in Scheme I. C^{2-} represents dicatecholate ion, K_{2c} the second dissocia-
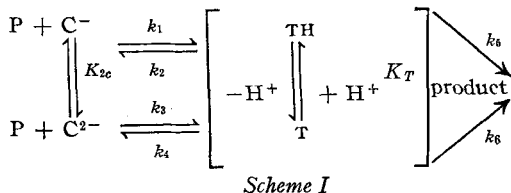
Scheme I

tion constant of catechol, and TH and T represent tetrahedral addition intermediates formed by nucleophilic attack of the catecholate ions on the penicillin.[1] These intermediates are depicted in Scheme II. The dissociation constant for TH is represented by K_T.

Tetrahedral Intermediates
Scheme II
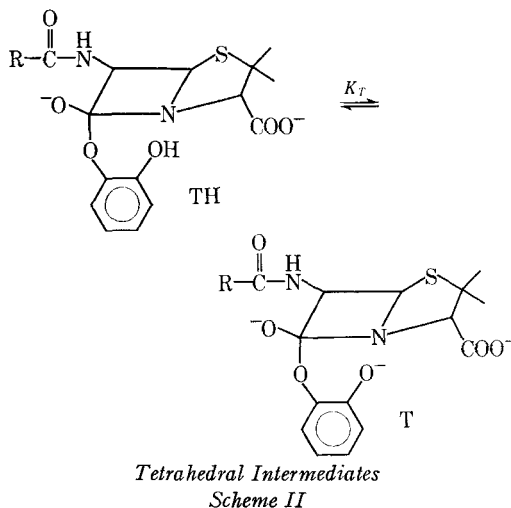
Taking a steady state in both TH and T, since these would be metastable intermediates, gives the following expression for k_c:[2]

$$\frac{\text{rate}}{[P][C^-]} = k_c =$$

$$\frac{\dfrac{k_1k_5K_w}{(k_4+k_6)K_T} + \dfrac{k_1k_6K_T+k_3k_5K_{2c}}{(k_4+k_6)K_T}[OH^-] + \dfrac{k_3k_6K_{2c}}{(k_4+k_6)K_w}[OH^-]^2}{\dfrac{(k_2+k_5)K_w}{(k_4+k_6)K_T} + [OH^-]}$$

(Eq. 6)

This equation is the same form as Eq. 2 showing that this mechanism fits the experimental data.

In the case of methicillin there is a plateau reached above pH 9.5 where k_c becomes independent of hydroxyl-ion concentration. This would occur if the pathway through dicatecholate ion were not

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[1] Attempts were made to find other mechanisms which would fit the experimental data, but none could be found.
[2] See *Appendix* for derivation of Eq. 6.

contributing significantly to the over-all reaction. If k_3 and k_4 are both set equal to zero, Eq. 6 becomes:

$$k_c = \frac{\dfrac{k_1k_5K_w}{k_6K_T} + k_1[OH^-]}{\dfrac{(k_2+k_5)K_w}{k_6K_T} + [OH^-]} \quad \text{(Eq. 7)}$$

which is of the form of Eq. 3 to which the data for methicillin were fitted. From Eq. 7 it can be seen that when $[OH^-] = 0$:

$$k_c = \frac{k_1k_5}{k_2+k_5}$$

and when $[OH^-]$ is high:

$$k_c = k_1$$

For methicillin k_1 equals 4.9 L. mole^{-1} min.$^{-1}$ and therefore:

$$\frac{k_5}{k_2+k_5} = 0.7 \text{ or } k_5 = 2.3\ k_2$$

It is difficult at this time to explain the differences in reactivity toward dicatecholate ion shown by benzylpenicillin and methicillin. The latter, judging from its lower rate of reaction with hydroxyl ion in the absence of catechol, is generally less susceptible to nucleophilic attack and this difference may be sufficient to obscure any contribution made by dicatecholate ion in the pH range studied.

For both penicillins the apparent first-order rate constants for acid formation at the higher pH values are lower than those for penicillin loss at the same catechol concentration. This is a clear indication of a buildup of a covalent intermediate in the reaction pathway.

This intermediate is apparently the monopenicilloate ester of catechol. Thus, the over-all reaction scheme at one pH may be depicted as:

$$P + C^- \xrightarrow{k_1'} \text{ester} \xrightarrow{k_2'} \text{product} + C^-$$
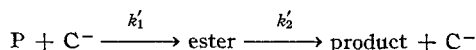
It is quite difficult to determine the values of k_2' from the present data because the concentration of intermediate ester must be determined by difference. Thus, small errors in the amount of acid formed and residual penicillin could lead to large errors in ester concentration. Approximate values of k_2' were determined nevertheless, from the maxima in plots of ester concentration *versus* time. At the time when the concentration of intermediate ester is maximal:

$$k_1'[P] = k_2'[E]$$

The values of k_2' thus calculated were found to be fairly constant for both penicillins in the pH range 9.5–10.5. For methicillin the values were about 0.2 min.$^{-1}$ and for benzylpenicillin about 0.4 min.$^{-1}$.

It has been shown that the rates of hydrolysis of catechol monobenzoate (5) and catechol monoacetate (6) depend on the concentration of anion, and the rapid rates of these hydrolyses are attributable to intramolecular general base catalysis by the *ortho*-hydroxyl ion.

In the two reactions cited the rates were almost constant at pH values where all of the *ortho*-hydroxyl group was ionized. The pKa of the acetate and

benzoate esters were 8.56 and 8.70, respectively, and the pKa of the penicilloate ester is therefore probably in the range 8.5–9.0. Thus, it would be expected that its rate of hydrolysis above pH 9.5 would become almost independent of pH as has been observed experimentally.

The ratio of k_2' for benzylpenicillin to that for methicillin is about 2 which is the same as the ratio of the k_{OH} values. The difference probably reflects the effect of the side chain on the susceptibility to nucleophilic attack of the β-lactam carbonyl. A similar mechanism has been proposed for the catechol-catalyzed hydrolysis of phenyl chloroacetate (7). Here it was shown that catechol monochloroacetate hydrolyzed much faster than phenyl chloroacetate, implicating the catechol monoester as a reaction intermediate.

In the catalysis of hydrolysis of benzylpenicillin by CDM it was shown (1) that one of the charged amino groups was responsible for binding the penicillin through ionic interaction with the carboxylate ion on the latter. The role of the other amino group, which apparently participated in the reaction, was not as clear. If the mechanism of hydrolysis catalyzed by catechol may be extrapolated to the situation with CDM, then there are revealed several possible functions for this second amino group. It may act to polarize the carbonyl group of the β-lactam increasing the susceptibility of the latter to nucleophilic attack by the adjacent hydroxyl ion. The amino function may also interact with the negatively charged oxygen of the tetrahedral intermediate thus stabilizing the latter relative to the reactants. Each of these actions would increase the rate of formation of ester relative to that observed with catechol. The amino group in all probability would also enhance the rate of hydrolysis of the ester by intramolecular hydrogen bonding to the ester carbonyl as has been shown in the hydrolysis of tertiary aminoalkyl acetates (8).

ANALOGY TO PENICILLINASE

The mechanism of action of the enzyme penicillinase and the nature of the functional groups involved in its active site are not known (9). Some speculation on these problems may, however, be based on consideration of the mechanism of hydrolysis of penicillins catalyzed by catechol and the aminocatechol derivatives previously reported (1).

While it would not be expected that catecholamines or even catechol groups will be found in the active site of the enzyme it would seem likely that a combination of nucleophilic and electrophilic (general acid) functions, located so as to act in concerted fashion could be responsible for its catalytic action. The binding of substrate to the enzyme is probably partly due to an ionic interaction between the carboxylate ion of the penicillin and a positively charged group in the active site of the enzyme. It has been shown that conversion of the carboxyl group to carboxamide decreases its susceptibility to enzymic hydrolysis (10, 11). Also, evidence for the presence of a positively charged group on the enzyme active site has been presented (12).

It has been shown that some penicillins resistant to the enzyme (such as methicillin) are competitive inhibitors of benzylpenicillin (13, 14) and activity is recovered only very slowly. In the light of the mechanism of catalysis of benzylpenicillin by

catechol it may be that the competitive inhibition is due to a covalent compound formed by acylation of the enzyme by methicillin, analogous to the catechol ester. If the rate of hydrolysis of the "methicilloylenzyme" were slow, then the slow recovery of enzymic activity would be explained. In the catechol ester the adjacent groups which assist hydrolysis are fixed in position. In the penicilloylenzyme this need not be so and the side chain may cause conformational changes as a result of which the groups catalyzing deacylation may be shifted to a position in which they can no longer influence hydrolysis rate.

One further point meriting attention is the difference in enzymic activity toward penicillins and cephalosporins (15). The cephalosporins are gener-
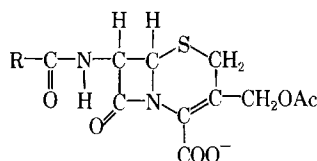
Cephalosporins

ally resistant to hydrolysis by penicillinase and are also relatively resistant to hydrolysis catalyzed by CDM as shown by preliminary studies in this laboratory. It can be readily seen with molecular models that in the cephalosporins the steric relationship between the carboxylate ion and the β-lactam carbonyl is quite different from that of the pencillins. Thus, even though CDM may associate with the cephalosporins by ionic interaction, the nucleophilic hydroxyl ion would not be in an optimum position to exert its catalytic action. This argument may account for the simultaneous lack of catalytic activity on cephalosporins by penicillinase and the observed competitive inhibition of the enzyme by these compounds (15).

APPENDIX

Derivation of Eq. 6

$$\text{rate} = \frac{d \, (\text{product})}{dt} = k_5 \, [\text{TH}] + k_6[\text{T}]$$

$$\text{TH} = \frac{[\text{H}^+]}{K_T + [\text{H}^+]} \, [\text{T}]_T; \quad \text{T} = \frac{K_T}{K_T + [\text{H}^+]} \, [\text{T}]_T$$

where $[\text{T}]_T$ represents $[\text{TH}] + [\text{T}]$

$$\text{rate} = \frac{k_5 \, [\text{H}^+] + k_6 K_T}{K_T + [\text{H}^+]} \, [\text{T}]_T$$

$$\frac{d \, [\text{T}]_T}{d_t} = k_1 \, [\text{P}][\text{C}^-] + k_3 \, [\text{P}][\text{C}^{2-}] -$$

$$[k_2 + k_5] \, [\text{TH}] - [k_4 + k_6][\text{T}] = 0 =$$

$$k_1 \, [\text{P}][\text{C}^-] + \frac{k_3 K_{2C} \, [\text{P}][\text{C}^-]}{\text{H}^+} -$$

$$\frac{(k_2 + k_5) \, [\text{H}^+] - [k_4 + k_6] \, K_T}{K_T + [\text{H}^+]} \, [\text{T}]_T$$

$$\text{T}_T = \frac{\left[k_1 + \frac{k_3 K_{2C}}{[\text{H}^+]} \right][K_T + \text{H}^+] \, [\text{P}][\text{C}^-]}{(k_2 + k_5) \, [\text{H}^+] + (k_4 + k_6) \, K_T}$$

rate =

$$\left\{ \frac{k_1 k_5 \, [\text{H}^+] + k_1 k_6 K_T + k_3 k_5 K_{2c} + \dfrac{k_3 k_6 K_T K_{2c}}{[\text{H}^+]}}{(k_2 + k_5) \, [\text{H}^+] + (k_4 + k_6) \, K_T} \right\} \times$$

$$[\text{P}][\text{C}^-]$$

Substituting $K_w/[OH^-]$ for $[H^+]$ and dividing numerator and denominator by $(k_4 + k_6)K_T$:

$$\frac{\text{rate}}{[P][C^-]} =$$

$$\frac{\dfrac{k_1 k_5 K_w}{(k_4 + k_6)K_T} + \dfrac{k_1 k_6 K_T + k_3 k_5 K_{2c}}{(k_4 + k_6)K_T}[OH^-] + \dfrac{k_3 k_6 K_{2c}}{(k_4 + k_6)K_w}[OH^-]^2}{\dfrac{(k_2 + k_5)K_w}{(k_4 + k_6)K_T} + [OH^-]}$$

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Bactericidal Properties of Straight-Chained Alkyltrimethylammonium Bromides in a Simple Emulsion System

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The individual constituents of the potent bactericidal agent cetrimide B.P. are among those alkyltrimethylammonium bromides (C_8 through C_{18}), which have been synthesized by classical methods. Each of these quaternary compounds was then emulsified with six (C_8 through C_{18}) fatty alcohols. The bactericidal properties of these emulsions are compared by the critical killing dilution method. When emulsified, only the C_{12} and C_{14} quaternary compounds exhibited any appreciable bactericidal action. All of the quaternary compounds in the series are inactivated when emulsified with myristyl alcohol.

THE OFFICIAL COMPOSITION of the quaternary ammonium bactericidal agent cetrimide B.P. has gradually changed over the last decade (1–3). Other investigators (4, 5) have recorded significant effects on their studies brought about by these changes.

Similar effects were noted in this laboratory during *in vitro* bactericidal testing of an experimental product. This product, a medicated cream, was an oil-in-water emulsion which utilized cetrimide B.P. as its bactericidal agent. The

disperse phase was comprised mainly of stearyl alcohol.

The erratic bactericidal results were discovered to have been caused by a shift in the predominant constituent in the cetrimide utilized. Cetrimide conforming to the 1963 B.P. monograph, *i.e.*, "comprised mainly of tetradecyltrimethylammonium bromide . . ." afforded excellent bactericidal activity. Cetrimide which conformed to the 1953 B.P. monograph, *i.e.*, "comprised mainly of hexadecyltrimethylammonium bromide . . ." was completely inactive in the product. Some anomalous bactericidal results were also obtained when alcohols other than stearyl were introduced into the formula.

The present study was initiated to determine: (*a*) the bactericidal properties of the component compounds which comprise cetrimide when they are incorporated into a simple emulsion system, and (*b*) if a pattern of relationship can be deter-