Cupric Ion-Catalyzed Hydrolysis of Penicillins: Mechanism and Site of Complexation

W. A. CRESSMAN*, E. T. SUGITA, J. T. DOLUISIO†, and P. J. NIEBERGALL

Abstract □ The presence of Cu(II) in penicillin solutions, through the pH range 4.0–6.0, has been shown previously to promote the degradation of the penicillins studied to their corresponding penicilloic acids. This study was undertaken to further substantiate the reaction mechanism and to postulate the catalytic site of complexation of Cu(II) with penicillin.

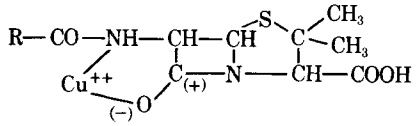
Keyphrases □ Penicillin hydrolysis—cupric ion-catalyzed □ Hydrolysis, penicillin—mechanism, site of complexation □ Ionic strength effect—penicillin degradation □ pH effect—penicillin degradation □ Temperature effect—penicillin degradation □ UV spectrophotometry—identification □ IR spectrophotometry—identification

Based on additional experimental observations, *i.e.*, the effect of ionic strength, pH, and temperature, the reaction mechanism proposed previously is modified to:

$$\text{Cu(II)} + \text{penicillin} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{Cu(II)-penicillin}$$

$$\text{Cu(II)-penicillin} + OH^- \xrightarrow{k_3} \text{Cu (II)-penicilloic acid}$$

On the basis of thermodynamic, kinetic and neighboring group effects, and observations obtained with model compounds the probable catalytic site of complexation of Cu(II) with intact penicillins is felt to be the following:



In previous publications it was stated (1, 2) that the effect of Cu(II) on the penicillins was to promote their degradation to coordination complexes of Cu(II) and the corresponding penicilloic acids. Complexation was assumed to occur between Cu(II) and the intact penicillins, followed by a rate limiting hydrolysis of the complex into the corresponding penicilloic acid-Cu(II) complex. This reaction mechanism enabled the authors to evaluate the stability constants for the interaction between Cu(II) and benzyl- and phenoxymethylpenicillins by analysis of the kinetic data obtained under pseudo-first-order conditions. This communication presents further work in support of the proposed mechanism and attempts to elucidate the site of complexation of Cu(II) with intact penicillins.

EXPERIMENTAL

Materials—All chemicals, other than the penicillins, were of reagent grade. Solutions were prepared in water that had been deionized after distillation and then degassed by boiling for 30 min. The purity of the water was checked on a Barnstead conductivity meter. All water used contained less than 0.01 p.p.m. total solids, expressed as sodium chloride.

The commercial penicillins used throughout the study were provided by various manufacturers. The purity of the samples was based on the ratio between the stated activity and the maximum possible activity. The penicillins used were: potassium benzylpenicillin,[1] stated activity 1595 units/mg., and potassium phenoxymethylpenicillin,[2] stated activity 1530 units/mg. The melting points and UV spectra of the compounds were run to test for the possibility of any trace contamination or degradation which might have occurred in handling or transit. In all cases the melting points before and after recrystallization from an acetone–water system were the same. The spectra demonstrated no apparent degradation to penicillenic acid as evidenced by a lack of any 322 mμ peak.

Sodium methicillin, 2,6-dimethoxyphenylpenicillin (supplied by Bristol Laboratories), 6-aminopenicillanic acid (supplied by Wyeth Laboratories), and a small sample of pure penicillanic acid (potassium salt)[3] were also used. Synthesis of the following compounds: potassium phenylpenicillin, potassium *p*-chlorophenylpenicillin, potassium *p*-nitrophenylpenicillin, and 6-bromopenicillanic acid (potassium salt), is described below.

Phenylpenicillin (Potassium Salt)—The general procedure for this synthesis was described by Doyle *et al.* (3). To 6-aminopenicillanic acid (16.2 g., 0.075 mole), which had been dissolved in 150 ml. of ice-cold sodium bicarbonate solution (24 g. sodium bicarbonate in 150 ml. of water), was added dropwise to 11.2 g., 0.08 mole of benzoyl chloride in 25 ml. of reagent grade acetone over a period of 30 min. on an ice bath. After the addition was completed, the mixture was stirred on the ice bath for 30 min. and then warmed to 20° and stirred at room temperature for 30 min. Then 50 g. of activated charcoal was added and removed 15 min. later by vacuum filtration. The filtrate containing the sodium phenylpenicillin was washed with 500-ml. portions of ether, cooled to 5–10°, overlayed with 500 ml. of ether and acidified to pH 2.0 with 10 *N* sulfuric acid. The phenylpenicillin was extracted into the ether. The ether phase was then washed with two 200-ml. portions of cold water and then dried over anhydrous sodium sulfate. Slow addition of 70 ml. of a 50% w/v solution of potassium 2-ethylhexanoate in butanol (prepared by reacting 61.2 g. of potassium metal with 200 ml. of butanol, then adding 250 ml. of 2-ethylhexanoic acid and sufficient butanol to make 500 ml.) caused the separation of an oil. The ether was decanted, the oil washed with 50-ml. portions of anhydrous ether and cooled on an ice bath with stirring. A solid precipitate of the potassium phenylpenicillin was obtained in about 30 min. This was collected, washed with acetone, and dried in a vacuum desiccator over magnesium perchlorate. The suggested solvent system for recrystallization was acetone–water. However, no satisfactory solvent system could be found. The material was purified by dissolving 12 g. in 15–20 ml. of cold acetone–water (9:1) and then adding 60 ml. of acetone. The substance which crystallized was potassium benzoate. The filtrate was then evaporated to dryness in a vacuum at room temperature. After three such purifications 4 g. of a solid was obtained which melted with decomposition at 111–113°. The IR spectrum of the compound confirmed the presence of an intact β-lactam ring by the presence of a peak at 5.55–5.65 μ. IR spectra of succeeding purifications indicated the loss of peaks at 6.4–6.5 and 14.2 μ. These peaks were similar to those noted on a spectrum of potassium benzoate. Elemental analysis of the compound gave: C, 45.67; H, 4.90; and N, 6.91. Calculated values for potassium phenylpenicillin containing two waters of crystallization were: C, 45.67; H, 4.85; and N, 7.10. The presence of two waters of crystallization was verified by Karl Fischer titration.

p-Chlorophenylpenicillin (Potassium Salt)—The synthesis of this compound is described by Doyle *et al.* (3) as Example 7. The procedure described by Doyle *et al.* was followed with the following

[1] Wyeth Laboratories, Inc., Philadelphia, Pa.
[2] Eli Lilly & Co., Indianapolis, Ind.
[3] Graciously synthesized and supplied by Dr. M. Claesen of the Rega Institute, Belgium, for which the authors are greatly indebted.

alterations. The oil obtained by the addition of 7 ml. of 50% potassium 2-ethylhexanoate was removed by decanting the ether. This oil was treated with 130 ml. of methyl isobutyl ketone and ether (3:7) and 3 ml. of butanol. A white precipitate of potassium *p*-chlorophenylpenicillin was obtained, washed with acetone, and dried in a vacuum over magnesium perchlorate. The compound melted at 173–176° with decomposition, literature value 174–176°, was soluble in water, and could not be recrystallized from acetone–water solvent systems. An IR spectrum of the compound confirmed the presence of an intact β-lactam ring. The compound was not further purified. Elemental analysis gave: C, 44.62; H, 4.01; and N, 6.66. Calculated values for potassium *p*-chlorophenylpenicillin $\cdot H_2O$ were: C, 43.84; H, 3.43; and N, 6.82.

p-Nitrophenylpenicillin (Potassium Salt)—This compound was prepared by the general method described by Perron *et al.* (4) using Method A. To a cooled stirred solution of 54 g. (0.25 mole) of 6-aminopenicillanic acid in 1200 ml. of water containing 105 g. of sodium bicarbonate was added a solution of 60 g. of *p*-nitrobenzoyl chloride in 100 ml. of acetone, in 1 min. The resulting mixture was stirred vigorously for 20 min. while the temperature was maintained at 10–15°. The stirring was stopped and the solution allowed to stand for several minutes. The supernatant solution was decanted and extracted two times with 300-ml. portions of methyl isobutyl ketone, cooled to 10–15°, overlayed with 500 ml. of methyl isobutyl ketone, and acidified to pH 2.0 with cold 10 *N* sulfuric acid. The cloudy methyl isobutyl ketone solution obtained was separated and centrifuged. The clear methyl isobutyl ketone layer was then washed with 200 ml. of cold water and dried over anhydrous sodium sulfate. To the dry methyl isobutyl ketone solution was slowly added 50 ml. of a 50% solution of potassium 2-ethylhexanoate in butanol. The further addition of potassium 2-ethylhexanoate is to be avoided since the precipitate obtained redissolves if more is added. The yellow precipitate of potassium *p*-nitrophenylpenicillin was collected, slurried with acetone, filtered, and dried in a vacuum desiccator over magnesium perchlorate. The compound melted with decomposition at 151–154°. It could not be recrystallized from the suggested acetone–water solvent. However, Perron *et al.* (4) stated that most of the derivatives obtained by this method were analytically pure. The IR spectrum of the compound indicated the presence of an intact β-lactam ring. Elemental analysis of the compound gave: C, 44.70; H, 3.80 and N, 10.27. Calculated values for potassium *p*-nitrophenylpenicillin were: C, 44.65; H, 3.49 and N, 10.42.

6-Bromopenicillanic acid (Potassium Salt)—The dibenzylethylenediamine salt of 6-bromopenicillanic acid was prepared by the method of Cignarella *et al.* (5) by diazotization of 6-aminopenicillanic acid in dilute hydrobromic acid. After several recrystallizations the melting point was constant at 160.5–161.5° with decomposition; the literature value was 164–165°. The structure was confirmed by the agreement of the IR spectrum for the compound with the spectrum published by Cignarella *et al.* The potassium salt of 6-bromopenicillanic acid was obtained from the above compound by the method Claesen (6) which was as follows. A suspension of 3.5 g. of the dibenzylethylenediamine salt of 6-bromopenicillanic acid in 25 ml. of water and 25 ml. of ether is chilled on ice and then acidified with agitation to pH 2 with 9.2 ml. of 1 *N* hydrochloric acid. The mixture is agitated for 30 min., then the dibenzylethylenediamine dihydrochloride is separated by centrifugation. The ether is separated, the aqueous solution is washed with ether, and the organic phases are combined, washed with a little water, and dried over anhydrous sodium sulfate. By slowly adding 3.9 ml. of a 50% solution of potassium 2-ethylhexanoate dissolved in butanol one obtains an oil which crystallizes on agitation. The precipitate is centrifuged and washed with ether. Pure potassium 6-bromopenicillanic acid is obtained by this method (6).

Procedure—Titrations were performed with a Radiometer TTT-1c titrator and Radiometer Titragraph model SBR-2c. The titration vessel was maintained at a temperature of 30.0 ± 0.05° by means of a water-jacketed holder. Sodium hydroxide 0.0200 *N* and a 0.5-ml. syringe were used for all titrations.

The general procedure for a titration was as follows. Using a 1.00×10^{-2} *M* stock solution of penicillin and sufficient water to give a final volume of 49.0 ml., reaction mixtures were prepared in 100-ml. beakers. The final concentrations were calculated on the basis of a total volume of 50.0 ml. The beaker was placed in the water-jacketed holder and the pH of the solution adjusted with the titrator. After the pH of the solution had been adjusted a time "zero" line was drawn on the chart paper. Then 1.00 ml. of a 5.00×10^{-3} *M* solution of Cu(II), cupric chloride, was added quickly by blow-out pipet and at the same time the titrator was activated.

The reaction was followed to completion in all instances, as indicated by the consumption of one equivalent of base and the production of one equivalent of penicilloic acid for each equivalent of Cu(II) added. The data were plotted according to Eqs. 5 and 7. All slopes and intercepts were evaluated using the method of least squares.

Effect of Ionic Strength—The rates of degradation of 30.00×10^{-4} *M* phenoxymethylpenicillin solutions in the presence of 1.00×10^{-4} *M* Cu(II) were followed under varying conditions of ionic strength. Ionic strengths were varied from 33.00×10^{-4} *M* to 433.00×10^{-4} *M* using potassium chloride. All other conditions were as described above.

Effect of pH—A series of experiments were performed at pH 4.00, 4.50, 5.00, 5.50, and 6.00. Phenoxymethylpenicillin was maintained constant at 20.00×10^{-4} *M*, Cu(II) at 1.00×10^{-4} *M*, and ionic strength at 0.01 *M* by the addition of potassium chloride.

The extension of the study to pH values above 6.00 or below 4.00 was hindered by the following limitations: below pH 4.00 the reaction proceeded too slowly to follow conveniently and was complicated by non-Cu(II)-catalyzed degradation of the penicillin; above pH 6.00 copper-hydroxo formation became a problem and the reaction proceeded too rapidly for the instrument to follow.

Effect of Temperature—The effect of temperature on the Cu(II)-catalyzed degradation of phenoxymethylpenicillin was studied at pH 5.50. The reaction was studied at 23.4, 29.4, 32.8, and 38.3° ± 0.05°. At higher temperatures the reaction rates became too rapid to follow.

THEORETICAL

The overall reaction scheme tentatively assumed in the previous work (2), while valid for determining the association constants for the interaction between Cu(II) and intact penicillins, is not sufficiently detailed for a complete analysis of the reaction mechanism. A step wise degradation scheme which conforms to the overall assumption of complexation followed by hydrolysis would be:

$$\text{Cu(II)} + \text{penicillin} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cu(II)-penicillin} \qquad \text{(Eq. 1)}$$

$$\text{Cu(II)-penicillin} + \text{OH}^- \xrightarrow{k_3} \text{Cu(II)-penicilloic acid} \qquad \text{(Eq. 2)}$$

$$\text{Cu(II)-penicillin} + \text{H}_2\text{O} \xrightarrow{k_4} \text{Cu(II)-penicilloic acid} + \text{H}_3\text{O}^+ \qquad \text{(Eq. 3)}$$

In the above scheme, Cu(II)-penicillin (hereafter referred to as Cu-Pen), is a complex of Cu(II) and intact penicillin, Cu(II)-penicilloic acid (hereafter referred to as Cu-Poic) is a complex of Cu(II) with the degradation product penicilloic acid, k_3 is the rate constant for hydroxyl ion attack upon Cu-Pen and k_4 is the rate constant for the spontaneous hydrolysis of Cu-Pen. Hydronium ion catalysis is omitted since the spectrophotometric data [Fig. 5 of the previous publication (1)] indicated that the rate of degradation increased with increasing pH.

From the above mechanism the following rate equation for the addition of base to the system can be obtained:

$$\begin{aligned} \frac{d(\text{OH}^-)_a}{dt} &= k_3(\text{OH}^-)\,(\text{Cu-Pen}) + k_4(\text{Cu-Pen}) \\ &= k_3'\,(\text{Cu-Pen}) + k_4(\text{Cu-Pen}) \\ &= k_5(\text{Cu-Pen}) \end{aligned} \qquad \text{(Eq. 4)}$$

in which $d(\text{OH}^-)_a/dt$ is the rate of addition of base to the system; (OH^-) is the concentration of hydroxyl ions in the system and is a function of the pH of the system; (Cu-Pen) is the concentration of the intact penicillin-Cu(II) complex present; and the other terms have the meanings described previously. If the equilibrium for complexation with intact penicillin is established much more rapidly than the degradation of the complex into products, and the penicillin concentration is much greater than that of cupric ion (so that it remains essentially constant), an equilibrium constant, K_s, may be given by:

$$K_s = \frac{k_1}{k_2} = \frac{(\text{Cu-Pen})}{(\text{Pen}_0)\,(\text{CuII})} \qquad \text{(Eq. 4}a\text{)}$$

in which (Pen_0) represents the initial concentration of penicillin and (CuII) represents the concentration of free Cu(II) in the system. If the pH is maintained at a constant value, the following equation can be obtained using the approach outlined previously (2):

$$\log[(Cu_0) - (OH^-)_a] = \log(Cu_0) - \frac{k_5K_s(Pen_0)t}{2.303 + 2.303K_s(Pen_0)} \quad \text{(Eq. 5)}$$

where (Cu_0) is the initial Cu(II) concentration, (Pen_0) is the initial penicillin concentration and $(OH^-)_a$ is the concentration of base added to the system. Therefore, the rate of addition of base to the system should be first-order with a slope, M, given by:

$$M = \frac{k_5K_s(Pen_0)}{2.303 + 2.303K_s(Pen_0)} \quad \text{(Eq. 6)}$$

This equation can be inverted to give:

$$\frac{1}{M} = \frac{2.303}{k_5K_s(Pen_0)} + \frac{2.303}{k_5} \quad \text{(Eq. 7)}$$

If one were to obtain the first-order rate constants for the consumption of base for a number of solutions in which (Cu_0) was kept constant but (Pen_0) was varied [always keeping (Pen_0) much greater than (Cu_0)] the following treatment could be applied to the data. A plot of $1/M$ *versus* $1/(Pen_0)$ should yield a straight line. This line will have a slope equal to $2.303/k_5K_s$ and an intercept equal to $2.303/k_5$. Dividing the intercept by the slope would give a value for K_s, the association constant for the unstable complex of Cu(II) and intact penicillin. Dividing the value of the intercept into 2.303 would give the rate constant, k_5, for the degradation step of the proposed mechanism.

It should be pointed out that this proposed reaction mechanism is also in agreement with the spectrophotometric data (1). If the mechanism depicted in Eqs. 1–3 is used to develop the differential equation for the rate of penicillin loss, in systems containing equimolar amounts of penicillin and Cu(II), the following is obtained:

$$-\frac{d(\text{penicillin})}{dt} = +\frac{d(OH^-)_a}{dt} = k_5K_s(\text{CuII})(\text{Pen}) \quad \text{(Eq. 8)}$$

$$= k''(\text{Pen})^2 \quad \text{(Eq. 9)}$$

where k'' is a constant which is a function of the rate constant for hydroxyl ion attack, k_3; the rate constant for spontaneous hydrolysis, k_4; and the hydroxyl ion concentration of the system.

Therefore, the observation of second-order kinetics which is a function of (OH^-) would be expected. This is what was found [as shown in Fig. 5 of the previous publication (1) in which penicillin loss was followed spectrally].

The values obtained for the association constant, K_s(2), were:

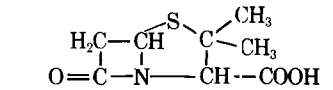
Compound	log K_s
phenoxymethylpenicillin	2.24 (in the absence of ionic strength control)
benzylpenicillin	2.63 (ionic strength of 0.01 *M*)
phenoxymethylpenicillin	2.09 (ionic strength of 0.01 *M*)

Certain inferences may be drawn concerning the site of complexation of Cu(II) with intact penicillins, based on a comparison of association constants with those obtained by Weiss *et al.* (7, 8) for several substituted amino acids. The necessary association constants are summarized in Table I.

If Compounds I, II, and III are compared with penicillin, it would appear that the sulfur of the thiazolidine ring is not involved to any significant extent in the complexation of Cu(II). The possibility of Cu(II) complexation with either of the two amide nitrogens in the penicillin molecule is suggested by the work of Manyak *et al.* (9) and Dobbie and Kermack (10), who found that Cu(II) can complex with the amide nitrogen of polypeptides. Molecular models of intact penicillin indicate that it is not possible for both the amide nitrogens to complex with Cu(II) at the same time. According to Johnson *et al.* (11), the normal resonance hybrids are not favored in the β-lactam ring of penicillin due to the bicyclic nature of the ring. Since no resonance structures are affected, complexation with the ring amide nitrogen should have little effect on promoting hydroxyl ion attack at the carbonyl function.

It does seem likely, however, that Cu(II) must have some direct interaction with the β-lactam ring of penicillin in order to bring about such a marked decrease in stability. Interaction between Cu(II) and the carbonyl oxygen of the β-lactam ring would place a partial positive charge on the carbonyl carbon atom. This would then accelerate the rate of hydroxyl ion attack on the β-lactam ring. It is also interesting to note that a five-membered chelate can be constructed using Cu(II), the side-chain amide nitrogen and the β-lactam carbonyl group.

One way to eliminate the possibility of the ring nitrogen being involved in catalytic complexation would be to study the effect of Cu(II) on the rate of degradation of penicillanic acid:

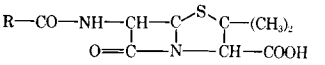


If the rate of hydrolysis of this compound is markedly increased in the presence of Cu(II), such as is true for the penicillin structure, the ring nitrogen could be involved in the complex. If, however, the stability of this compound is not markedly affected by the presence of Cu(II) the involvement of the side chain could be investigated by using penicillanic acids substituted in the six position with such functional groups as Br- or NH_2-.

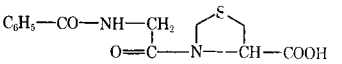
Another reaction series which might prove useful would be the effect of substituents on the benzene ring of phenylpenicillin. If the side chain nitrogen were involved in complexation with Cu(II) the presence of inductively withdrawing groups on the ring should reduce the value of K_s.

Table I—Association Constants of Various Compounds with Structures Similar to the β-Lactam Ring of Penicillin with Cu(II)

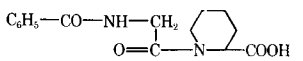
Penicillin — phenoxymethylpenicillin log K = 2.09; benzylpenicillin log K = 2.63



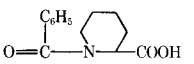
Compound I: *N*-hippuryl-thiazolidine-4-carboxylic acid — log K = 1.8



Compound II: *N*-hippurylpipecolinic acid — log K = 2.1



Compound III: *N*-benzoylpipecolinic acid — log K = 1.8



RESULTS AND DISCUSSION

Effect of Ionic Strength—Bronstead (12) assumed that in any bimolecular reaction a complex was formed by the reactants and that it was the rate limiting degradation of this complex, in equilibrium with the reactants, on which the rate of the reaction depended. In systems in which no stable complex has been postulated the complex is considered to be a transition state "activated complex."

In the Cu(II)-penicillin system the formation of a true complex was assumed, which then undergoes a rate limiting degradation. Based on the mechanism proposed in Eqs. 1–3 at constant pH, the following can be obtained:

$$\log(\text{initial rate}) = 1.02(Z_{C_1}Z_P)\sqrt{\mu} + \log k_6 \quad \text{(Eq. 10)}$$

in which k_6 is a constant resulting from the product of k_5, K_s, (CuII), and (Pen_0). The data obtained for phenoxymethylpenicillin are plotted in Fig. 1 in which the solid line represents the theoretical slope and the points are the experimental points. Positive deviation from the line at higher ionic strengths is expected since the Debye-Huckle equation used in the derivation is only for dilute solutions.

The slope of the least squares line for the data in Fig. 1 was −1.84 when all the experimental points were used and −2.21 when

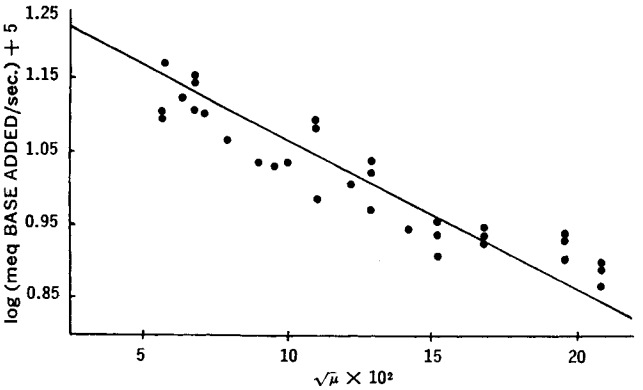


Figure 1—*Effect of ionic strength on the rate of degradation of phenoxymethylpenicillin in the presence of Cu(II). Data obtained with phenoxymethylpenicillin 30.00 × 10⁻⁴ M and Cu(II) 1.00 × 10⁻⁴ M at pH 5.50 and 30.0°.*

only those points obtained at ionic strength values less than 0.02 *M* were used. In any case these values are well within the ±20% suggested by Moelwyn-Hughes (13) as being "quantitatively significant" of the types of ions involved in the reaction. The agreement between theoretical and experimental values implies that the suggestion of an initial rapid equilibrium involving free Cu(II), $Z_{Cu} = +2$, and penicillin, $Z_P = -1$, followed by a rate limiting degradation is valid.

Dependence of Rate upon Hydroxyl Ion Concentration—If this proposed mechanism is correct the following treatment of the rate equations should be valid. From Eq. 6 the observed first-order rate constant, $k_{obs.}$, would be

$$k_{obs.} = k_3(OH^-)k_7 + k_4k_7 \qquad \text{(Eq. 11)}$$

where,

$$k_7 = \frac{K_s(Pen_0)}{1 + K_s(Pen_0)} \qquad \text{(Eq. 12)}$$

If $k_{obs.}$ is plotted *versus* $1/(H_3O^+)$ a straight line should be obtained with a slope equal to $k_3k_7K_w$ and an intercept of k_4k_7. The value of k_7 can be obtained by using Eq. 12 and the known values for K_s and initial penicillin concentration. With k_7 calculated it would then be possible to evaluate k_3 and k_4. A check of the data would be obtained if the value of $[k_3(OH^-) + k_4]$ was calculated and agreed with the experimental value obtained by the reciprocal plotting method. Additional substantiation of the proposed mechanism could be obtained by the following rearrangement of Eq. 11:

$$\log (k_{obs.} - k_4k_7) = \log k_3k_7K_w + pH \qquad \text{(Eq. 13)}$$

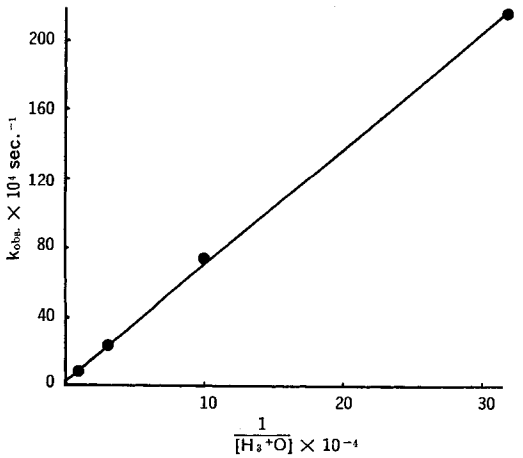


Figure 2—*Effect of hydronium ion concentration on the observed first-order rate constant for penicillin loss. Data obtained with phenoxymethylpenicillin 20.00 × 10⁻⁴ M, Cu(II) 1.00 × 10⁻⁴ M, and ionic strength 0.01 M at 30.0°.*

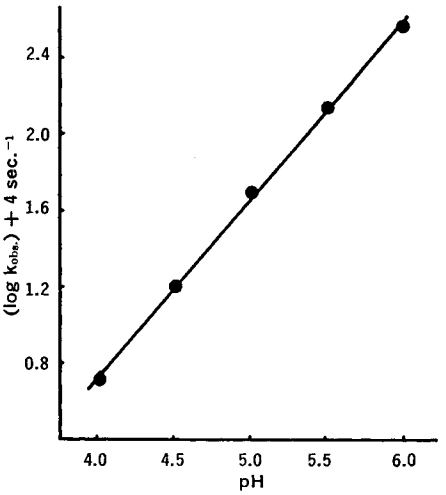


Figure 3—*Effect of pH on the observed first-order rate constant for penicillin loss. Data obtained with phenoxymethylpenicillin 20.00 × 10⁻⁴ M, Cu(II) 1.00 × 10⁻⁴ M, and ionic strength 0.01 M at 30.0°.*

If log $(k_{obs.} - k_4k_7)$ is plotted *versus* pH one should obtain a straight line with a slope of +1.00.

The data obtained are plotted in Fig. 2 as $k_{obs.}$ *versus* $1/(H_3O^+)$. The slope and intercept of this plot, calculated by the method of least squares were:

$$\text{slope} = 6.79 \times 10^{-8} \text{ 1/mole-sec.}$$
$$\text{intercept} = 3.85 \times 10^{-4} \text{ sec.}^{-1}$$

A *t* test of the intercept showed that it was not significantly different from zero at the 0.05 level of significance. Therefore, the value of k_4 can be considered, for all practical purposes, to be zero. This implies that there is no significant spontaneous water hydrolysis of the Cu(II)-intact penicillin complex. The value for k_7 calculated from the association constant for phenoxymethylpenicillin at constant ionic strength was 0.196 using $K_s = 1.22 \times 10^{+2}$ and $Pen_0 = 20.00 \times 10^{-4}$ *M*. This enabled k_3 to be calculated as follows:

$$k_3 = \frac{\text{slope}}{k_7K_w} = 3.46 \times 10^7 \text{ 1/mole-sec.}$$

Calculation of the theoretical value for k_5 at pH 5.50 and constant ionic strength gives:

$$k_4 + k_3(OH^-) = 0 + 10.91 \times 10^{-2} = 10.91 \times 10^{-2} \text{ sec.}^{-1}$$

which compares favorably with the experimentally determined value of 10.84×10^{-2} sec.$^{-1}$

A further check of the proposed reaction mechanism would be to plot the data according to the following modification of Eq. 13:

$$\log k_{obs.} = \log k_3k_7K_w + pH$$

This is shown in Fig. 3 and should be applicable since k_4 was shown not to be significantly different from zero. The slope of this line is +0.92, calculated by the method of least squares, and compares favorably with the theoretical value of +1.00.

Effect of Temperature—Arrhenius plots of the data obtained for the degradation of phenoxymethylpenicillin in the presence of Cu(II) at pH 5.50 are shown in Figs. 4 and 5 for K_s and k_3', respectively; each point represents the average of three determinations. The calculated enthalpy change for the complexation between cupric ion and intact penicillin was 27.4 ± 0.8 kcal./mole, the change in free energy was −1.9 ± 0.8 kcal. at 26.6°, and the reaction was accompanied by an entropy change of +97.8 e.s.u. The value of +97.8 e.s.u. for ΔS is in good agreement with the observations of Martin (14), who stated that "entropy changes of the order of +100 e.s.u. are to be expected for chelate formation. The increase in ΔS is due to the water molecules which are normally associated with the ligand and metal being 'squeezed out' when the complex is formed. This decreases the orderly arrangement of the solvent molecules around these ions and the entropy of the system increases. Decreases in ionic charge, which accompany chelation, will decrease hydration of the complex and contribute to an increase in the en-

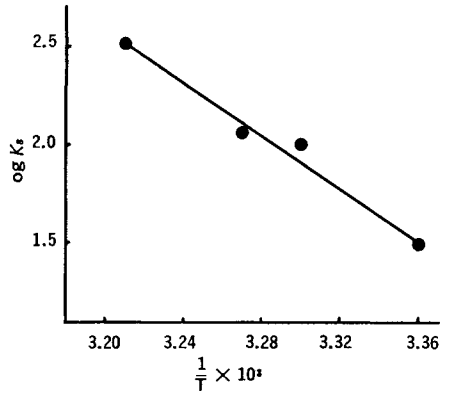


Figure 4—*Effect of temperature on the association constant,* K_s, *for phenoxymethylpenicillin and Cu(II) at pH 5.50. Data obtained with Cu(II) 1.00* $\times$ 10^{-4} M *and ionic strength 0.01* M.

tropy of the system." The change in entropy, therefore, supports the assumption of initial complexation between Cu(II) and intact penicillins, and suggests the possibility of chelation. This might be expected to place a severe strain on the β-lactam ring of penicillin, increasing its lability. This possibility will be considered in greater detail in later discussion.

Analysis of the data obtained for k_3', using the method of least squares, gave an activation energy of approximately zero (0.2 ± 0.6 kcal./mole) and a change in free energy of +19.2 ± 0.6 kcal. at 26.6°. Since the change in free energy for K_s is much smaller than for k_3', the rate limiting step would appear to be hydroxyl ion attack upon the Cu(II)-penicillin complex.

Therefore, the effects of ionic strength, hydroxyl ion concentration, and temperature support the theory of rapid complexation between Cu(II) and intact penicillin, followed by a rate limiting hydroxyl ion attack upon the complex, splitting the β-lactam ring.

Site of Complexation—Titration of penicillanic acid in the presence and absence of Cu(II) indicated that no degradation occurred, in either case, for at least 20 min. No base was added to the system over this time period and the Pan test (15) of the reaction mixtures produced no characteristic blue color. It was therefore concluded that penicillanic acid was relatively stable in the presence of Cu(II), since the half-life of the compound was greatly in excess of 20 min. at pH 5.50. This result indicates that the side chain is involved in some manner in the Cu(II) promoted degradation of penicillin.

Titrations for the determination of the effect of Cu(II) on 6-aminopenicillanic acid could only be used for semiquantitative estimations. The presence of the free amino group interfered with the titrations due to the release of a proton when Cu(II) complexes with this function. Comparison of the results of the Pan test (15) performed at the end of the titration with values obtained for simulated reaction mixtures indicated that one equivalent of penicilloic acid was produced. The half-life of 20.0 $\times$ 10^{-4} *M* reaction mixtures of 6-aminopenicillanic acid in the presence of 1.00 $\times$ 10^{-4} *M* Cu(II) was estimated from the time required to consume one-half of the total

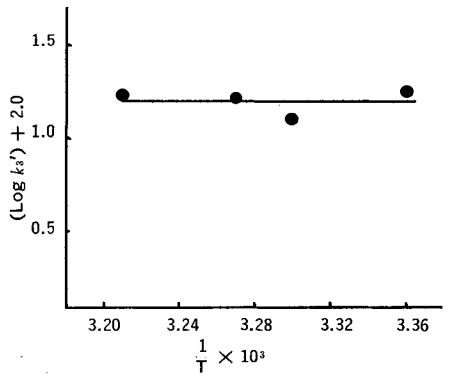


Figure 5—*Effect of temperature on* k_3' *at pH 5.50. Data obtained with Cu(II) 1.00* $\times$ 10^{-4} M *and ionic strength 0.01* M.

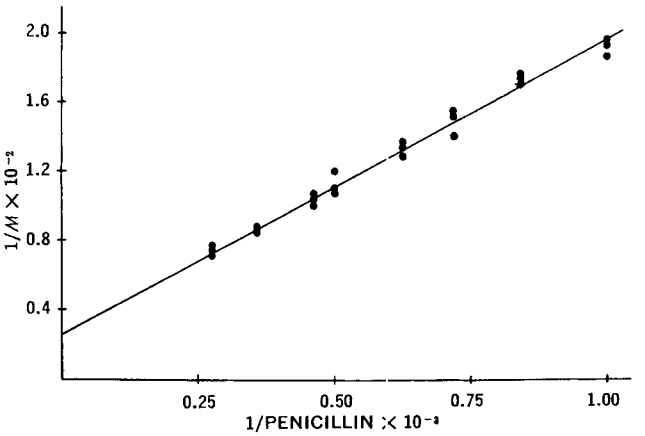


Figure 6—*Reciprocal plot to evaluate* K_n *for phenylpenicillin. Data obtained with Cu(II) 1.00* $\times$ 10^{-4} *M at pH 5.50 and ionic strength 0.01* M, *at 30.0°.*

amount of base added. The half-life was approximately 40–50 sec. This is comparable to the half-lives of 35 and 20 sec. noted for phenoxymethylpenicillin and benzylpenicillin, respectively, under the same conditions.

Cu(II) reacted with 6-bromopenicillanic acid to produce penicilloic acid. The half-life of the reaction was 23 min., calculated from the rate constant obtained for the first-order degradation at a penicillin concentration of 20.00 $\times$ 10^{-4} *M*. These data are summarized below:

Compound	$t_{1/2}$ at pH 5.50, 30.0°, 20.00 $\times$ 10^{-4} *M* Penicillin with 1.00 $\times$ 10^{-4} *M* Cu(II)
penicillanic acid	$\gg$ 20 min.
6-bromopenicillanic acid	23 min.
6-aminopenicillanic acid	40–50 sec.
penicillin	20–35 sec.

Two things appear evident from these data. First the presence of a side chain substituent is necessary for Cu(II) promoted degradation to occur at an appreciable rate. Second, the presence of nitrogen in the six position increases the rate of degradation. This suggests that, perhaps, the instability caused by bromination is enhanced when the ability to form a complex or chelate is introduced.

The association constants for Cu(II) with phenyl-, *p*-nitrophenyl-, *p*-chlorophenyl-, and 2,6-dimethoxyphenylpenicillin were obtained with Cu(II) 1.00 $\times$ 10^{-4} *M* at pH 5.50, 30.0°, and ionic strength of 0.01 *M*. The penicillin concentrations were varied from 10.00 $\times$ 10^{-4} to 40.00 $\times$ 10^{-4} *M*. The first-order slopes, *M*, were obtained using the Guggenheim method as described by Frost and Pearson (16). The values for K_s were obtained by plotting the slopes, *M*, according to Eq. 7. The data obtained for phenylpenicillin are shown in Fig. 6, and are typical of the results obtained for all of the penicillins studied. The constants obtained on triplicate runs for each penicillin are shown in Table II. Due to the complexity of the systems and the inter-atomic distances involved, the effects are not as marked as

Table II –Association Constants for the Degradation of Various Penicillin Derivatives and the pKa[a] of the Respective Side-Chain Acid Present in the Penicillin

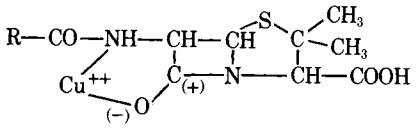
Penicillin	pKa of Side-Chain Acid	$K_s \times 10^{-1}$
Phenylpenicillin	4.20	14.19
p-Chlorophenylpenicillin	3.98	9.29
2,6-Dimethoxyphenylpenicillin	3.44[b]	12.51
p-Nitrophenylpenicillin	3.44	10.21
Benzylpenicillin	4.31	2.63
Phenoxymethylpenicillin	3.17	2.09

[a] See *Reference* 19. [b] See *Reference* 20.

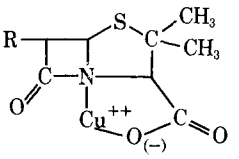
those that might be obtained for more ideal systems, such as benzoic acid esters. However, it does appear from the data in Table II that K_s has a tendency to increase with pKa. Increasing the electron density at the side-chain amide nitrogen by decreasing the strength of the side chain acid, should result in K_s increasing with pKa if indeed the side chain nitrogen is involved in complexation with Cu(II).

The inductive effect of a positive charge on the side chain nitrogen, however, does not explain the factor of 10^7 by which Cu(II) accelerates the hydrolysis of the penicillins. Protonation of glycine ethyl ester, for example, increases its rate of hydrolysis by a factor of 10^2 (17). In the presence of Cu(II), the rate of glycine ethyl ester hydrolysis increases by a factor of 10^6 (18). This has been suggested as being due to a "super acid" catalysis brought about by the inductive effects of the positive charge of the metal ion being introduced into the molecule at a reactive position where a proton is not normally found at a similar pH, *i.e.*, on the carbonyl oxygen in this example. In the case of penicillin the formation of a chelate between the side chain nitrogen and the beta lactam oxygen would place a severe strain on the lactam ring. Such a chelate would also be conducive to the production of "super acid" catalysis.

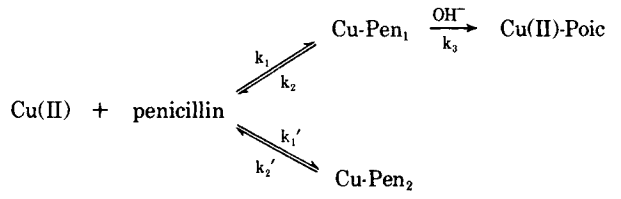
Therefore, the effect of side chain substitution, apparent "super acid" catalysis, and the +97.8 e.s.u. change in entropy which is indicative of chelate formation (14), leaves little doubt that catalytically Cu(II) interacts with penicillin through the formation of a five-membered chelate of the following type.



In addition to the catalytic site of complexation the work of Weiss *et al.* (7, 8) and Johnson *et al.* (11) leads to the possibility (as pointed out by the reviewer) that the following type of chelate also exists:



If this chelate does form it is *noncatalytic*, as shown by the kinetic experiments with penicillanic acid. Such a chelate would, however, decrease the amount of free Cu(II) available for chelation at the catalytic site. The overall reaction scheme would then be modified, as pointed out by the reviewer, as follows:



The following equation can readily be derived using the previous approach:

$$\log [(Cu_0) - (OH^-)_a] = \log (Cu_0) - \frac{k_5 K_s (Pen_0) t}{2.303 + 2.303(K_s + K_r)(Pen_0)}$$

in which $K_r = k_1'/k_2' = (\text{Cu-Pen}_2)/(\text{Pen}_0)\,(\text{CuII})$. Thus, a plot of $1/M$ *versus* $1/(\text{Pen}_0)$ should yield a straight line with a slope equal to $2.303/k_5K_s$, and an intercept equal to $2.303\,(K_s + K_r)/k_5K_s$.

This alternate mechanism is in agreement with the data for the dependence of the reaction rate upon ionic strength and hydroxyl ion concentration. In the alternate mechanism, the sum of the two association constants are being compared as a function of temperature, rather than K_s alone. It would be expected, however, that the stability constant K_r would be fairly constant over the range of penicillin compounds studied and would not be affected by the structure of the side-chain group. In addition, over the range of temperature used (15°), it would be reasonably safe to assume that K_r is some constant multiple of K_s. Thus, Fig. 4 would be a plot of XK_s *versus* $1/T$, and Fig. 5 would become a plot of log Yk_3' *versus* $1/T$ where X and Y are constants. If this were true, then X and Y would act as scalar functions and the thermodynamic constants presented would be the correct values for either mechanism. The effect of this modification of the reaction scheme on the association constants reported previously (2) would be to make the previous estimates of K_s larger than their true value. If, however, one assumes that K_r and K_s are multiples then log K_s of (2) is now equal to log K_s + log X. If log X is < log K_s then the effect on the reported (2) values for K_s would be relatively small. Since K_s is of the order of 10^2 (7, 8) this assumption appears feasible.

REFERENCES

(1) P. J. Niebergall, D. A. Hussar, W. A. Cressman, E. T. Sugita, and J. T. Doluisio, *J. Pharm. Pharmacol.*, **18,** 729(1966).

(2) W. A. Cressman, E. T. Sugita, J. T. Doluisio, and P. J. Niebergall, *ibid.*, **18,** 801(1966).

(3) F. P. Doyle, J. H. C. Nayler, and G. M. Rolinson, British pat. 880,400, Oct. 18, 1961.

(4) Y. G. Perron, W. F. Minor, C. T. Holdrege, W. J. Gottstein, J. C. Godfrey, L. B. Crast, R. B. Babel, and L. C. Cheney, *J. Am. Chem. Soc.*, **82,** 3934(1960).

(5) G. Cignarella, G. Pifferi, and E. Testa, *J. Org. Chem.*, **27,** 2668(1962).

(6) M. J. Claesen, personal communication.

(7) A. Weiss, S. Fallab, and H. Erlenmeyer, *Helv. Chim. Acta*, **40,** 611(1957).

(8) A. Weiss and S. Fallab, *ibid.*, **40,** 576(1957).

(9) A. R. Manyak, C. B. Murphy, and A. E. Martell, *Arch. Biochem. Biophys.*, **59,** 373(1955).

(10) H. Dobbie and W. O. Kermack, *Biochem. J.*, **59,** 246(1955).

(11) J. Johnson, H. Clarke, and R. Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 421.

(12) J. N. Bronsted, *Z. Physik. Chem.*, **102,** 169(1922).

(13) E. A. Moelwyn-Hughes, "The Kinetics of Reactions in Solution," 2nd ed., Oxford University Press, New York, N. Y., 1947, pp. 93–102.

(14) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, pp. 423–425.

(15) S. C. Pan, *Anal. Chem.*, **26,** 1438(1954).

(16) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed., Wiley, New York, N. Y., 1961, pp. 49–50.

(17) H. L. Conley and R. B. Martin, *J. Phys. Chem.*, **69,** 2914 (1965).

(18) R. W. Westheimer, *Trans. N. Y. Acad. Sci.*, **18,** 15(1955).

(19) G. Körtum, W. Vogel, and K. Andrussow, "Dissociation Constants of Organic Acids in Aqueous Solutions," International Union of Pure and Applied Chemistry, Butterworths, London, England, 1961.

(20) M. M. Davis and H. B. Hexter, *J. Phys. Chem.*, **61,** 125(1957).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 28, 1967 from the *Department of Pharmacy, The Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104*

Accepted for publication August 13, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Abstracted from a thesis submitted by William A. Cressman to the Graduate School, The Philadelphia College of Pharmacy and Science, in partial fulfillment of the Doctor of Philosophy degree requirements.

This investigation was supported in part by the United States Public Health Service Research grant AI-05321, from the National Institute of Allergy and Infectious Diseases, and in part by a Public Health Service fellowship 1-F1-GM-32,237-01 from the National Institute of General Medical Sciences. Present address: McNeil Laboratories Inc., Fort Washington, PA 19034

† Present address: University of Kentucky, College of Pharmacy, Lexington, Ky.