Effect of Copper (II)–Glycine Chelates on Degradation of Penicillin in Mildly Acid Solution

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Abstract □ This study was undertaken to elucidate by *in vitro* kinetic analysis the role that Cu (II) ions, through complexation with an amino acid, may have in the degradation of the penicillins and the formation of the penicilloyl determinant. The data obtained indicated that in the presence of the Cu (II)–glycine chelates, penicillin is quantitatively hydrolyzed to penicilloic acid. This finding is in direct contrast to the much slower reaction between penicillin and glycine in the absence of Cu (II), in which the major degradation product is the penicilloamide. The overall observed rate constant can be represented by:

$$k_0 = K_m[M][OH^-] + K_{mg}[MG][OH^-]$$

in which K_m and K_{mg} are the catalytic coefficients for Cu (II) and the 1:1 Cu (II)–glycine chelate, respectively, and $[M]$ and $[MG]$ represent the concentrations of free Cu (II) and the 1:1 chelate, respectively. The postulated mechanism involves the rapid formation of a ternary penicillin–Cu (II)–glycine complex, followed by a rate-limiting hydroxyl-ion attack.

Keyphrases □ Penicillin, hydrolysis to penicilloic acid—effect of copper (II)–glycine chelates, mechanism □ Copper (II)–glycine chelates—effect on penicillin hydrolysis

Copper (II) ions were shown to inactivate penicillins extremely rapidly (1, 2). There is approximately 1 mcg. of copper present in 1 ml. of human serum, existing in three forms: (*a*) most is bound to the copper protein ceruloplasmin; (*b*) about 5% is loosely bound to albumin; and (*c*) the last fraction is bound to amino acids. The latter form of serum copper is considered to be the transport form of copper in the blood (3)

In light of the rapid catalysis of penicillin hydrolysis by Cu (II), the fact that these ions are largely bound *in vivo*, and the fact that metal chelates were shown to have a high degree of catalytic activity (4–10), it was felt that the effect of Cu (II) should be studied under more nearly physiologic conditions, *i.e.*, bound to proteins or amino acids.

A system containing Cu (II) and an amino acid is relatively easy to characterize when compared to a system containing protein and/or protein fragments along with Cu (II). Glycine was chosen as the amino acid since its degradative activity upon the penicillins was reported (11, 12), it is the least complicated amino acid, and it can be obtained in a highly purified form.

EXPERIMENTAL

Materials—All chemicals except the penicillins and glycine were of reagent grade. All solutions were prepared with deionized distilled water which had been degassed before use.

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-nm. peak.

The glycine[3] obtained was dried at 110° for at least 2 hr. before use.

Reagents—*Sodium p-Chloromercuribenzoate Stock Solution*—Sodium *p*-chloromercuribenzoate (0.27 g.) was dissolved in a minimum amount of sodium hydroxide solution (5.0 *N*). Sufficient 0.05 *M* carbonate buffer at pH 9.2 was added to make 50.0 ml. of solution. The resulting solution was 1.4×10^{-2} *M* with respect to sodium *p*-chloromercuribenzoate.

Methods[4]—Benzylpenicilloic acid was determined by adding 1 ml. of the Pan (13) reagent to 5 ml. of the solution to be tested. This solution was allowed to stand for 200 sec. and then diluted with 10.0 ml. of 0.025 *N* sulfuric acid. Absorbance, when desired, was determined at 800 nm. immediately after the addition of the acid.

The pKa of glycine was determined at 30.0° and an ionic strength of 0.01 using the method of Niebergall (14). The value obtained was 9.700 ± 0.019.

The association constants for the Cu (II)–glycine chelates were determined by the methods described by Irving and Rossotti (15). The logarithm of the association constant for the 1:1 chelate was found to be 8.461 ± 0.073. The logarithm of the association constant for the 1:2 chelate was found to be 7.046 ± 0.046. Both constants were obtained at 30.0° and an ionic strength of 0.01.

Due to the strong interaction between Cu (II) and the buffers normally used at physiologic pH, the optical rotation studies were carried out at 30.0° in a 0.01 *M* acetate buffer at pH 5.05 using a polarimeter[5]. Initial rates were obtained graphically from plots of optical rotation *versus* time[6]. The reaction in most instances appeared to be complete within 5 min.

Degradation Products—Characterization of the degradation products was carried out through the pH range 5.0–8.0 using both benzyl- and phenoxymethylpenicillin. The results and conclusions were the same through the pH range studied. The results obtained at pH 7.50 for benzylpenicillin are presented as being typical.

The following five solutions were made:

Solution A	Benzylpenicillin	4.6×10^{-4} *M*
Solution B	Benzylpenicillin	4.6×10^{-4} *M*
	Cupric nitrate	5.8×10^{-4} *M*
Solution C	Benzylpenicillin	4.6×10^{-4} *M*
	Cupric nitrate	5.8×10^{-4} *M*
	Glycine	11.6×10^{-4} *M*
Solution D	Benzylpenicilloic acid prepared by the method of Birner (16)	4.6×10^{-4} *M*
Solution E	Cupric nitrate	5.8×10^{-4} *M*
	Glycine	11.6×10^{-4} *M*

[1] Eli Lilly and Co., Indianapolis, Ind.
[2] Wyeth Laboratories, Inc., Philadelphia, Pa.
[3] Matheson, Coleman and Bell (99.5% minimum assay as stated on the label).
[4] Spectral curves were obtained using a Beckman DB spectrophotometer in conjunction with a Photovolt linear/log model 43 recorder. A Beckman DU or Hitachi-Perkin-Elmer model 139 spectrophotometer was utilized for single wavelength determinations.
[5] Perkin-Elmer model 141.
[6] The initial rate in terms of degrees minute^{-1} was converted into terms of mole liter^{-1} minute^{-1} by dividing by the molar rotation of the penicillin used. The molar rotation had previously been obtained by plotting degrees *versus* molarity for a number of penicillin solutions of varying concentration. The slope of the resulting straight line gave the molar rotation in terms of degrees liter mole^{-1}.

One milliliter of the Pan (13) reagent was added to 5 ml. of each solution. Lack of a blue color after 200 sec. indicated that no penicilloic acid was present. Solutions B, C, and D gave an intense blue color. Solution E did not undergo any color change, nor did it absorb at 800 nm. Solution A gave a slight blue color (absorbance = 0.015), which was probably due to some degradation caused by the reagent.

A solution containing benzylpenicillin (2.5×10^{-3} *M*), cupric nitrate (1.25×10^{-3} *M*), and glycine (2.5×10^{-3} *M*) was prepared and allowed to stand at 25°. The pH was held constant at 7.50 by use of a controller[7] in conjunction with an automatic buret and a pH meter[8]. At various time intervals, 1-ml. samples were withdrawn and added to 5 ml. of a 0.05 *M* carbonate buffer at pH 9.20; then 0.1 ml. of the sodium *p*-chloromercuribenzoate stock solution was added. Ten minutes later, the UV spectrum of the solution was obtained. According to Levine (17) and Schneider and deWeck (18), any penicilloamide present in the reaction mixture under these conditions would be converted to the corresponding penamaldate, which would absorb strongly between 280 and 285 nm. Figure 1 indicates that no significant peak characteristic of penamaldate appears to develop within this region through a time period well beyond that used in our kinetic studies. The slight increase in absorbance within the 280–285-nm. region and the more marked increase in absorbance within the 258–264-nm. region is similar to the results obtained by Niebergall *et al.* (19) for the reaction between benzylpenicillin and Cu (II) ions, which resulted in the formation of a Cu (II)-benzylpenicilloic acid complex probably in equilibrium with the corresponding penamaldate. Preliminary optical rotation studies showed that no reaction between penicillin (both benzyl and phenoxymethyl) and glycine in neutral or slightly acid solution occurred through a time period well beyond that used in these kinetic studies. Thus, the slight increase in absorbance within the 280–285-nm. region is probably not due to penicilloamides.

Cressman *et al.* (2) showed that Cu (II) interacts with penicillin to form two complexes, one stable and the other susceptible to hydroxyl-ion catalyzed hydrolysis. It is reasonable to assume that the 1:1 Cu (II)-glycine chelate with a +1 charge and in which the Cu (II) ion is not completely coordinated can also form two mixed complexes with penicillinate anion. Thus, a possible degradation scheme might be:

$$M + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MP \qquad K_{mp} = \frac{[MP]}{[\bar{M}][\bar{P}]} \qquad \text{(Eq. 1)}$$

$$M + P \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} MP' \qquad K_{mp}' = \frac{[MP']}{[\bar{M}][\bar{P}]} \qquad \text{(Eq. 2)}$$

$$MP + OH^- \xrightarrow{k_3} MPA \qquad \text{(Eq. 3)}$$

$$MG + P \underset{k_5}{\overset{k_4}{\rightleftharpoons}} MGP \qquad K_{mgp} = \frac{[MGP]}{[\bar{MG}][\bar{P}]} \qquad \text{(Eq. 4)}$$

$$MG + P \underset{k_5'}{\overset{k_4'}{\rightleftharpoons}} MGP' \qquad K_{mgp}' = \frac{[MGP']}{[\bar{MG}][\bar{P}]} \qquad \text{(Eq. 5)}$$

$$MGP + OH^- \xrightarrow{k_6} \text{products} \qquad \text{(Eq. 6)}$$

in which *MP* represents the reactive complex between Cu (II) and penicillin, *MP'* is the stable complex between Cu (II) and penicillin, *MPA* is the complex between Cu (II) and penicilloic acid, *MGP* is the reactive complex between the 1:1 Cu (II)-glycine chelate and penicillin, *MGP'* is the stable complex between the 1:1 Cu (II)-glycine chelate and penicillin, and the products are assumed to be mixed chelates of Cu (II), glycine, and penicilloic acid.

If k_3 and k_6 are assumed to be rate controlling:

$$\text{initial rate} = k_3[MP][OH^-] + k_6[MGP][OH^-] \qquad \text{(Eq. 7)}$$

If the concentration of free penicillin is always kept much greater than that of free Cu (II) or the 1:1 Cu (II)-glycine chelate:

$$\text{initial rate} = k_3K_{mp}[OH^-][M]P_0 + k_6K_{mgp}[OH^-][MG]P_0 \qquad \text{(Eq. 8)}$$

[7] Model 77, Luft Instruments, Inc., Lincoln, Mass.
[8] Leeds and Northrup model 7401.

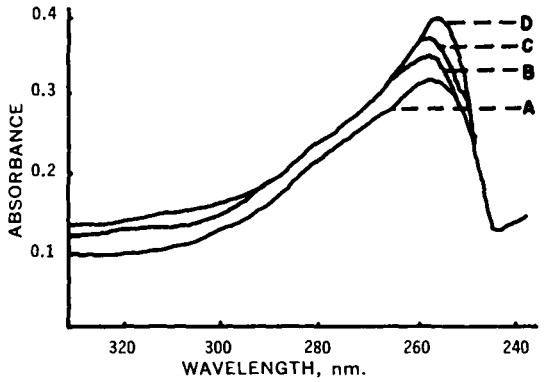

Figure 1—*Use of the sodium* p*-chloromercuribenzoate solution to determine if penicilloamides form upon mixing benzylpenicillin (2.5×10^{-3} M), cupric nitrate (1.25×10^{-3} M), and glycine (2.5×10^{-3} M). Curve A indicates 17 min., curve B 38.7 min., curve C 62.5 min., and curve D 88.5 min. after the reaction was started.*

Dividing both sides of the equation by $[OH^-][MG]P_0$ results in:

$$\frac{k_0}{[\bar{MG}][\bar{OH}^-]} = K_m\frac{[M]}{[\bar{MG}]} + K_{mg} \qquad \text{(Eq. 9)}$$

in which k_0 is the observed rate constant (initial rate divided by P_0), K_m is the Cu (II) catalytic coefficient and is equal to the product of k_3K_{mp}, and K_{mg} is the 1:1 Cu (II)-glycine chelate catalytic coefficient and is equal to the product k_6K_{mgp}. A plot of Eq. 9 should yield a straight line with a slope equal to K_m and an intercept equal to K_{mg}.

Calculation of the molar concentrations of free Cu (II) and of the 1:1 Cu (II)-glycine chelate was accomplished by doing a mass balance on penicillin, Cu (II), and glycine, taking into account all equilibria including the small interaction between Cu (II) and the acetate buffer, as follows:

$$T_m = [M] + [MG] + [MG_2] + [MP] + [MP'] + [MGP] + [MGP'] + [MA_c] \qquad \text{(Eq. 10)}$$

$$T_g = [HG] + [G] + [MG] + 2[MG_2] + [MGP] + [MGP'] \qquad \text{(Eq. 11)}$$

$$T_p = [P] + [MP] + [MP'] + [MGP] + [MGP'] \qquad \text{(Eq. 12)}$$

in which T_m, T_g, and T_p refer to total metal, glycine, and penicillin, respectively; *MG* represents the 1:1 Cu (II)-glycine chelate; MG_2 represents the 1:2 Cu (II)-glycine chelate; MA_c is the 1:1 Cu (II)-acetate complex (higher order complexes being omitted due to the very small association constants and the fact that the acetate concentration would be squared, *etc.*, thus making the concentrations very small in this system); *HG* is the glycine zwitterion; and the other terms have the meanings given previously. Equations 1 and 2 yield:

$$[MP] = K_{mp}[M][P] \qquad \text{(Eq. 13)}$$

$$[MP'] = K_{mp}'[M][P] \qquad \text{(Eq. 14)}$$

in which the sum of $[MP]$ and $[MP']$ can be given as:

$$[MP] + [MP'] = (K_{mp} + K_{mp}')[M][P] = K_{smp}[M][P] \qquad \text{(Eq. 15)}$$

In a similar manner, Eqs. 4 and 5 give:

$$[MGP] + [MGP'] = (K_{mgp} + K'_{mgp})[MG][P] = K_{smgp}[MG][P] \qquad \text{(Eq. 16)}$$

Since the assumption was made that the free penicillin concentration is always much greater than that of the free Cu (II) or the 1:1 Cu (II)-glycine chelate, the concentration of all species in solution could have been calculated without doing a mass balance on total penicillin. However, since the calculations were done on an IBM 360-75 digital computer system, the mass balance on total penicillin was included as a means of justifying the above assumption.

Utilizing Eqs. 1, 2, 4, and 5 and the usual definitions for describing the equilibria involved in the formation of *MG*, MG_2, MA_c, and the dissociation of *HG* gives:

$$T_m = [M] + \beta_1[M][G] + \beta_2[M][G]^2 + K_{smp}[M][P] + K_{smgp}\beta_1[M][G][P] + K_{mac}[A_c] \qquad \text{(Eq. 17)}$$

Table I—Data for Obtaining K_m and K_{mg} at 30° Assuming $K_{smgp} = 1.23$

pH	Total Penicillin[a] × 10^3 M	Total Cu (II) × 10^3 M	$[M]$ × 10^4 M	$[MG]$ × 10^3 M	k_0, $min.^{-1}$ × 10^2	Y^b, l. $mole^{-1}$ $min.^{-1}$ × 10^{-10}	X^c × 10^1
5.75	4.00	3.00	0.274	1.131	6.20	0.66	0.24
5.05	4.00	3.00	1.190	1.750	4.04	1.40	0.68
5.17	8.00	1.00	0.715	0.607	2.29	1.73	1.18
5.12	4.00	2.00	0.971	1.208	4.17	1.78	0.80
4.65	8.00	3.00	2.381	1.857	2.62	2.15	1.28
4.65	4.00	2.00	2.297	1.284	2.71	3.22	1.79

[a] In all instances, P_0, the initial concentration of free penicillin, was found to be equal to total penicillin to the fourth significant figure. Thus, although total penicillin and total Cu (II) do not differ to any great extent, P_0 is always much greater than $[M]$. [b] $Y = k_0/[MG][OH^-]$. [c] $X = [M]/[MG]$.

$$T_g = \left(\frac{[H_3O^+]}{K_g} + 1\right)[G] + \beta_1[M][G] + 2\beta_2[M][G]^2 + K_{smgp}\beta_1[M][G][P] \quad \text{(Eq. 18)}$$

$$T_p = [P] + K_{smp}[M][P] + K_{smgp}\beta_1[M][G][P] \quad \text{(Eq. 19)}$$

in which β_1 and β_2 are the association constants for the 1:1 and 1:2 Cu (II)-glycine chelates, respectively; K_{mac} is the association constant for the 1:1 Cu (II)-acetate complex; K_g is the second dissociation constant for glycine hydrochloride; and the other terms have the meanings given previously. Equations 17–19 were solved using the Newton-Raphson method of successive approximations as given in the *Appendix*.

RESULTS AND DISCUSSION

Penicilloic acid in the absence of Cu (II) ions has a significant molar rotation. To justify the use of polarimetry for obtaining the initial rates, a series of studies was run in an effort to determine whether, in this system, the change in optical rotation was indeed a measure of the rate of change of intact penicillin.

According to the results of Niebergall *et al.* (19), the reaction in a solution containing phenoxymethylpenicillin (8.0×10^{-3} M) and free Cu (II) plus Cu (II)-penicillin complex equal to 7.7×10^{-4} M (calculated as shown previously for a solution containing 8.0×10^{-3} M penicillin and 1.0×10^{-3} M cupric nitrate in a 0.01 M acetate buffer at pH 5.05) should cease when 9.7% of the penicillin had degraded. The initial rotation of the penicillin solution was

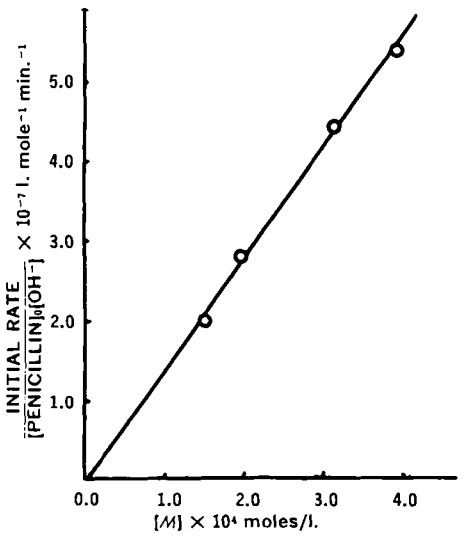

Figure 2—*Determination of the Cu (II) catalytic coefficient using the optical rotation technique. Initial penicillin concentration was 8.0×10^{-3} M in 0.01 M acetate buffer, pH 5.05, at 30°.*

Table II—Data for Obtaining K_m and K_{mg} at 30° Assuming $K_{smpg} = 12.3$

pH	Total Penicillin[a] × 10^3 M	Total Cu (II) × 10^3 M	$[M]$ × 10^4 M	$[MG]$ × 10^3 M	k_0, $min.^{-1}$ × 10^2	Y^b, l. $mole^{-1}$ $min.^{-1}$ × 10^{-10}	X^c × 10^1
5.75	4.00	3.00	0.264	1.104	6.20	0.68	0.24
5.05	4.00	3.00	1.153	1.704	4.04	1.44	0.68
5.17	8.00	1.00	0.678	0.576	2.29	1.83	1.18
5.12	4.00	2.00	0.941	1.176	4.17	1.83	0.80
4.65	8.00	3.00	2.257	1.761	2.62	2.27	1.28
4.65	4.00	2.00	2.236	1.249	2.71	3.31	1.79

[a] In all instances, P_0, the initial concentration of free penicillin, was found to be equal to total penicillin to the fourth significant figure. Thus, although total penicillin and total Cu (II) do not differ to any great extent, P_0 is always much greater than $[M]$. [b] $Y = k_0/[MG][OH^-]$. [c] $X = [M]/[MG]$.

2.526. This fell to an equilibrium value of 2.286 after the Cu (II) was added. Since the fall in optical rotation was equal to 9.5%, it was felt that the Cu (II)-penicilloic acid complex was not contributing to the observed optical rotation. This experiment was repeated with a solution for which the reaction should have proceeded to the extent of 4.7%. The optical rotation fell from 2.540 to 2.420, representing a 4.7% decrease.

To substantiate further the use of polarimetry in this system, the authors decided to obtain an estimate of K_m from a study in which glycine was omitted from the system but in which all other conditions were identical to the study in the presence of glycine. In the presence of Cu (II) alone:

$$\text{initial rate} = k_3[MP][OH^-] \quad \text{(Eq. 20)}$$

which can be rearranged to:

$$\frac{\text{initial rate}}{P_0[OH^-]} = k_3K_{mp}[M] = K_m[M] \quad \text{(Eq. 21)}$$

in a similar manner as was used to develop Eq. 9.

The initial rates of reaction for solutions containing only phenoxymethylpenicillin (8.0×10^{-3} M) and cupric nitrate (1.0×10^{-3} M) in a 0.01 M acetate buffer at pH 5.05 were obtained by measuring the change in optical rotation of the solution at 365 nm. The data are plotted according to Eq. 21 in Fig. 2. The slope of the line, equal to K_m, as determined by the method of least squares was found to be $1.42 \pm 0.15 \times 10^{11}$ l.^{2} mole^{-2} min.$^{-1}$. A t test showed that at the 95% confidence level this value of K_m is not significantly different from the value of 1.32×10^{11} l.^{2} mole^{-2} min.$^{-1}$ obtained from the data of Cressman (20), in which a pH-stat technique and an integrated rate expression were utilized to obtain rather well-de-

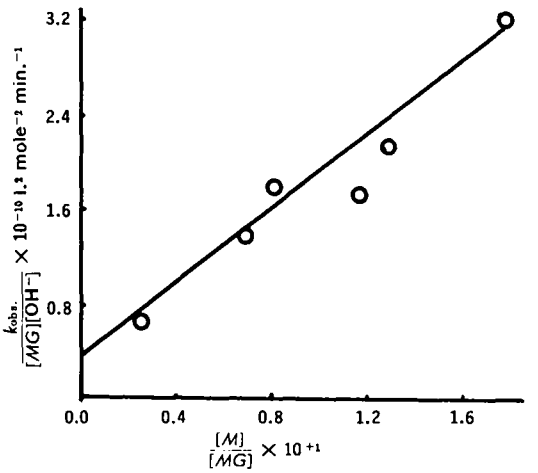

Figure 3—*Determination of the Cu (II) and Cu (II)-glycine catalytic coefficients at 30° and K_{smgp} = 1.23. Each point represents the average of three or more determinations.*

Table III—Effect of Altering K_{smgp} upon the Catalytic Coefficients, K_m and K_{mg}

Constant	95% Confidence Limits—Lower	Upper
$K_{smgp} = 1.23$	—	—
$K_m = 1.55 \times 10^{11}$[a]	1.32×10^{11}	1.77×10^{11}
$K_{mg} = 3.80 \times 10^{9}$	0.96×10^{9}	6.63×10^{9}
$K_{smgp} = 12.3$	—	—
$K_m = 1.59 \times 10^{11}$	1.36×10^{11}	1.81×10^{11}
$K_{mg} = 4.05 \times 10^{9}$	1.22×10^{9}	6.88×10^{9}

[a] Units of K_m and K_{mg} are $l.^2$ $mole^{-2}$ $min.^{-1}$.

fined values of K_m. The slope of the line given by Eq. 21 would be sensitive to systematic errors [such as neglecting the possible contribution of the Cu (II)–penicilloic acid complex to the observed rotation] in estimating the initial rates. The close agreement between the values of K_m found in this study and the data of Cressman (20) would tend to substantiate further the validity of using optical rotation for obtaining initial rates in this system.

Determination of K_{mg}—The only value needed for calculating the concentration of all species in the penicillin–Cu (II)–glycine system which was unknown was K_{smgp}. It has been shown that the interaction between intact penicillin and Cu (II) has an association constant, K_{smp}, of 1.23×10^2 (1, 2). In the interaction between glycine and Cu (II) to form the 1:1 chelate, electrons were donated to the Cu (II) by the ligand, thus tending to decrease the electrostatic interaction of Cu (II) with further ligands. Thus, it was felt that K_{smgp} should be less than K_{smp}. Arbitrary values of 12.3 and 1.23 (10 and 100 times less than K_{smp}) were chosen in an effort to determine whether an accurate knowledge of this constant is necessary for the evaluation of K_m and K_{mg} within the limitations of the experimental procedure.

Table I gives the data and Fig. 3 shows a plot of Eq. 9 used to determine K_m and K_{mg} assuming a value of 1.23 for K_{smgp}; Table II gives the data and Fig. 4 shows the plot assuming a value of 12.3 for K_{smgp}. The catalytic coefficients and their 95% confidence limits as determined by the method of least squares are shown in Table III. The close agreement between both sets of catalytic coefficients, and the fact that both the sets of K_m obtained in the presence of glycine were shown not to be significantly different from those obtained in the absence of glycine, would appear to indicate that an accurate knowledge of K_{smgp} is not required for this system.

APPENDIX

Newton–Raphson Method of Successive Approximations—If Eqs. 17–19 are rearranged to the form:

$$F(x,y,z) = 0 \quad \text{(Eq. A1}a\text{)}$$
$$G(x,y,z) = 0 \quad \text{(Eq. A1}b\text{)}$$
$$H(x,y,z) = 0 \quad \text{(Eq. A1}c\text{)}$$

and initial estimates x_0, y_0, and z_0 are available, a correction Δx, Δy, and Δz, can be applied such that improved values for x, y, and z would be:

$$x = x_0 + \Delta x \quad \text{(Eq. A2}a\text{)}$$
$$y = y_0 + \Delta y \quad \text{(Eq. A2}b\text{)}$$
$$z = z_0 + \Delta z \quad \text{(Eq. A2}c\text{)}$$

for which:

$$F(x_0 + \Delta x, y_0 + \Delta y, z_0 + \Delta z) = 0 \quad \text{(Eq. A3}a\text{)}$$
$$G(x_0 + \Delta x, y_0 + \Delta y, z_0 + \Delta z) = 0 \quad \text{(Eq. A3}b\text{)}$$
$$H(x_0 + \Delta x, y_0 + \Delta y, z_0 + \Delta z) = 0 \quad \text{(Eq. A3}c\text{)}$$

Expanding the set of Eqs. A3 by the Taylor theorem and neglecting all terms of order higher than one give:

$$F_x \Delta x + F_y \Delta y + F_z \Delta z = -F \quad \text{(Eq. A4}a\text{)}$$
$$G_x \Delta x + G_y \Delta y + G_z \Delta z = -G \quad \text{(Eq. A4}b\text{)}$$
$$H_x \Delta x + H_y \Delta y + H_z \Delta z = -H \quad \text{(Eq. A4}c\text{)}$$

in which:

$$F_x = \partial F/\partial x;\ F_y = \partial F/\partial y;\ F_z = \partial F/\partial z \quad \text{(Eq. A5}a\text{)}$$
$$G_x = \partial G/\partial x;\ G_y = \partial G/\partial y;\ G_z = \partial G/\partial z \quad \text{(Eq. A5}b\text{)}$$
$$H_x = \partial H/\partial x;\ H_y = \partial H/\partial y;\ H_z = \partial H/\partial z \quad \text{(Eq. A5}c\text{)}$$

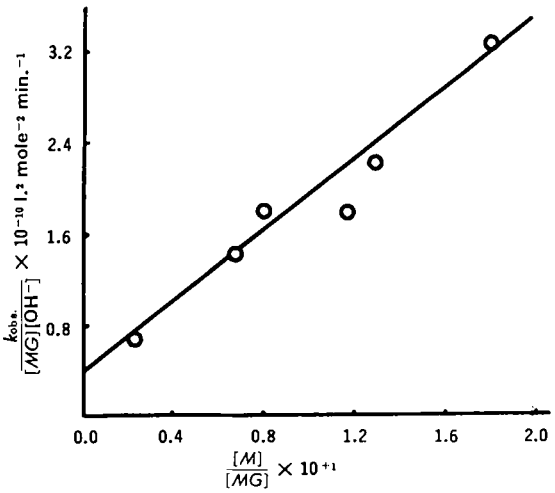

Figure 4—*Determination of the Cu (II) and Cu (II)–glycine catalytic coefficients at 30° and* $K_{smgp} = 12.3$. *Each point represents the average of three or more determinations.*

The set of Eqs. A4 can be solved utilizing Cramer's rule, or any other convenient method, for Δx, Δy, and Δz. These corrections are then applied to x_0, y_0, and z_0 to give new approximations to x, y, and z. The process is continued until the changes in x, y, and z become negligible. In actual practice, the initial estimates for x, y, and z can be taken to be zero, although the process converges much more rapidly if reasonable values are estimated for x_0, y_0, and z_0.

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Analgesic Activity as Determined by the Nilsen Method

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Abstract □ The Nilsen method for determining analgesic activity (pain stimulus and electrical pulsations to the mouse tail) was compared with the hot-plate procedure for several compounds including "pure" analgesics (agonists) (morphine, codeine, and meperidine), a known "pure" antagonist (naloxone), several so-called mixed agonist–antagonists (nalorphine, pentazocine, and cyclazocine), one antagonist that appears to be naloxonelike, and two compounds of relatively unknown pharmacology. The results obtained confirm the validity of the Nilsen test for the agonists and indicate its superior predictive value (to the hot-plate, Smith D'Amour, and perhaps writhing methods) for man with those substances possessing antagonist properties. The Nilsen technique may also be complementary to other procedures now used to predict "pure" antagonism. Refinements in methodology and instrumentation are described.

Keyphrases □ Analgesic activity—determination by Nilsen method, compared to hot-plate procedure □ Nilsen method for determination of analgesic activity—compared to hot-plate procedure

Since the discovery that the narcotic antagonist, nalorphine, also has analgesic (agonist) properties (1, 2) and particularly since the emergence of the much weaker antagonist, pentazocine (3), as a clinically useful, pain-relieving agent without substantial abuse liability (4, 5), a main thrust of research to develop improved analgesics has been on structures possessing a mixture of agonist and antagonist components. The hot-plate method (6, 7) of testing for analgesia, although of excellent predictive value for compounds (*e.g.*, morphine, codeine, meperidine, and methadone) displaying almost exclusively agonist activity mediated by the CNS, is not, in general, as sensitive for the antagonist–agonists [*e.g.*, nalorphine, cyclazocine, and pentazocine (3)].

An assay method first described by Nilsen (8) and modified by Helsley *et al.* (9) was suggested as being simple, economical, and predictive for the narcotic antagonists. This procedure involves applying electrical pulsations as the pain stimulus through the tissue of the tail of suitably selected mice. This test method was installed and what are believed to be improvements in instrumentation and methodology are reported. The method's reliability, predictive value, and reproducibility for narcotic antagonists as well as for the purer agonists have also been confirmed.

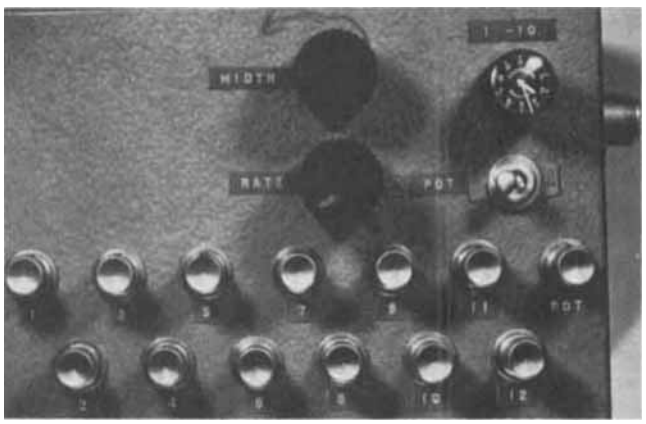

Figure 1—*Pulse control box. Pulse rate and duration may be controlled and monitored on an oscilloscope* via *coaxial connector seen on upper right-hand side of box. Voltage levels may be selected by push buttons or, alternatively, varied continuously by means of dial potentiometer in upper right corner of box.*

EXPERIMENTAL

Apparatus and Procedure—The voltage pulse (electrical stimulus) is derived from the box shown in Fig. 1. The electrical circuit is illustrated in Fig. 2. The stimulus is applied to the mouse by piercing the tail with gold-coated electrodes spaced 12 mm. apart. The electrodes are mounted in a Lucite spring clip modeled after a spring-type wooden clothespin. The clip is mounted in such juxtaposition to a mouse holder that when the mouse is in the holder the electrodes enter the tail approximately 25 mm. from the base of the tail. The electrodes are mounted somewhat off-center, so that they do not strike the bone of the tail, and at such an angle that they enter the tail with a slight compressive force toward the center. Details of the arrangement are illustrated in Figs. 3 and 4.

A "lazy-susan" type of mouse "dispenser" markedly reduces operator fatigue, speeds the processing of the mice, and reduces the chance of error in the order in which the mice are tested. This device consists of two concentric series of wells of radii of 19.5 and 24.5 cm., respectively. Each well is 8.25 cm. in diameter × 11.7 cm. deep; there are 12 wells to each circle, each fitted with expanded metal floors and all carried by a 62-cm. diameter, circular Lucite