# Kinetics of Reactions Involved in Penicillin Allergy I

## Mechanism of Reaction of Penicillins and 6-Aminopenicillanic Acid with Glycine in Alkaline Solution

By MICHAEL A. SCHWARTZ and GAY-MAY WU

**The principal antigenic determinant of penicillin allergy, penicilloyl-protein, may be formed either by direct reaction of penicillin with protein, or through penicillenic acid, a rearrangement product of penicillin. As part of a study of the mechanism of the direct reaction, the kinetics of reaction of glycine with a series of penicillins and 6-aminopenicillanic acid has been investigated. The aminolysis of penicillins by glycine is general base catalyzed, while the reaction with 6-APA is uncatalyzed. The latter is probably due to intramolecular general-base catalysis. The rates of reaction with glycine of all the penicillins studied are of the same order of magnitude. This is in contrast to the rates of penicillenic acid formation which are highly dependent upon the nature of the penicillin side chain. The pertinence of these results to the mechanism of formation of penicillin antigen is discussed.**

It is generally agreed that the principal antigenic determinant of penicillin allergy is the penicilloyl group covalently bound to body protein, probably through $\epsilon$-amino groups of lysine residues (1, 2). As shown in Scheme I, there are two pathways by which the antigen may form. One involves the known rearrangement of penicillin to penicillenic acid (II) which is known to react rapidly with amines. The other is a direct reaction of protein with penicillin. The latter will produce the $\alpha$-diastereoisomer of the penicilloamide while the penicillenic acid route requires isomerization of the asymmetric carbons (marked with asterisk in Scheme I) of the $\beta$-lactam ring to produce a mixture of diastereoisomers.

The evidence for the penicillenic acid route was recently summarized by Levine (3). (*a*) Penicillenic acid has been found in penicillin preparations (4); (*b*) while penicillin does react rapidly with $\epsilon$-aminocaproic acid at pH 11.5, this reaction would be slow at physiological pH; (*c*) penicillenic acid is a very reactive compound chemically (5); (*d*) studies on the nature of antibody to penicillin G indicated specificity toward the mixture of diastereoisomers of the benzylpenicilloyl haptenic group rather than for the $\alpha$-diastereoisomer alone (6).

On the other hand, evidence has been accumulated to show that the direct reaction of penicillin with protein may play a more important role than previously thought. Thiel, Mitchell, and Parker (7) found that the sera of most of 114 patients allergic to penicillin contained antibody specific for the $\alpha$-diastereoisomer of penicilloamide. This is in direct contrast to Levine's results cited above (6). More recently two groups (8, 9) have found that penicillins can indeed combine with protein at neutral pH at 37° *in vitro*. These included penicillins which were known to produce penicillenic acid at relatively low rates. Also included was 6-aminopenicillanic acid (6-APA), the nucleus common to all penicillins, which cannot form a penicillenic acid. It is known that 6-APA is antigenic in rabbits, and it has been suggested as an agent causing penicillin allergy (10).

The present work was undertaken to investigate the rates and mechanisms of reactions of penicillin which may be involved in penicillin allergy. Rather than a complex protein, glycine was selected as a model amine for study because of its ready availability in high purity, and its high solubility. The rate of penicillenic acid formation as a function of pH has also been measured for several penicillins.

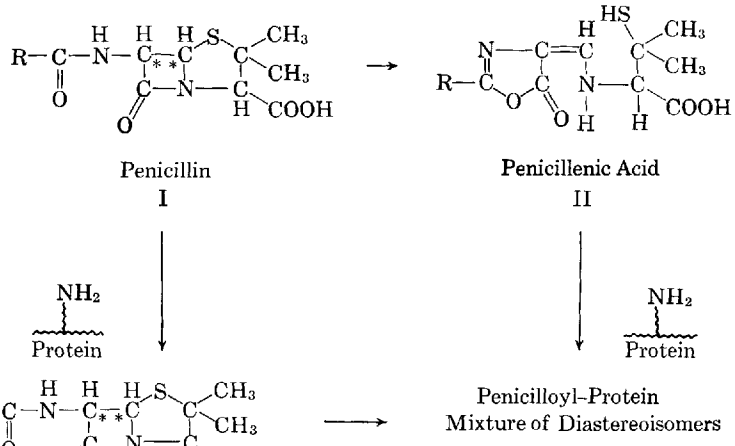
Received January 19, 1966, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo.

Accepted for publication February 28, 1966.

Presented to the Medicinal Chemistry Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

The authors acknowledge the technical assistance of Miss Antoinette Gornicki.

This study was supported by grant AI-06173 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.




Formation of Penicillin Antigen

Scheme I

## EXPERIMENTAL

The penicillins were all commercial products.[1] Glycine and mercuric chloride were Fisher certified reagent, as were the other reagents used.

The rates of formation of penicillenic acid from penicillin were measured by following the increase in absorbance at 322 mμ as a function of time. This wavelength is characteristic of the penicillenic acid chromophore. The rates were determined in a series of buffers extending over the pH range 1–4 at 34° on a Beckman DB recording spectrophotometer with thermostated cell compartment. Mercuric chloride was added to the buffer in a concentration equal to the initial penicillin concentration. It was known that mercuric chloride stabilizes the penicillenic acid (11). The authors' preliminary studies showed that mercuric chloride had no effect, however, upon the *rate* of penicillenic acid formation.

The rates of reaction of the penicillins with glycine were determined by following the changes in optical rotation accompanying opening of the β-lactam ring of the penicillin (12) with a Perkin-Elmer model 141 photoelectric polarimeter using a mercury vapor source with filter for 365 mμ. The glycine itself acted as buffer and maintained pH within less than 0.1 unit during a single determination. Ionic strength of the buffers was brought to 0.5 by addition of potassium chloride. Initial penicillin concentration was 0.001 or 0.002 *M*. The temperature in these studies was maintained at 50° by water circulating through the polarimeter cell from a thermostated bath.

The pH of the buffers was measured at 50° with a Beckman expanded scale pH meter. The pKa′ of glycine at 50°, ionic strength 0.5, was determined from the pH of the buffers to be 9.10 ± 0.02.

The rates of alkaline hydrolysis of the penicillins in the absence of glycine were determined at 50° by measurement of the rate of acid production on a Radiometer TTT-1 titrator-pH stat equipped with SBR-2, SBU-1 recorder. Ionic strength in these runs was brought to 0.5 by addition of KCl. The rates for each penicillin were measured at several pH's.

## RESULTS

**Penicillenic Acid Formation.**—The first-order rate constants for penicillenic acid formation were calculated by dividing the initial slope of a plot of absorbance at 322 mμ *versus* time by the initial concentration of penicillin.

$$k_{obs.} = \frac{\text{initial slope}/26{,}600}{\text{initial concn. of penicillin}}$$

The factor 26,600 is the molar absorptivity of penicillenic acid (5). Figure 1 shows these rate constants as a function of pH. These curves follow the same pattern previously observed for penicillin G (13) where the rate of penicillenic acid formation was found to be dependent upon the concentration of undissociated penicillin acid (HP):

$$\frac{d\,(\text{penicillenic acid})}{dt} = k_{obs.}\,(\text{HP}) = \frac{k_E(\text{H}^+)}{(\text{H}^+) + Ka}\,(\text{P})_{total}$$

where $k_E$ represents the specific pH-independent rate constant. At high pH where $(H^+) \ll Ka$, the observed first-order rate constant will be directly proportional to $(H^+)$ and, as shown in the dashed lines of Fig. 1, will decrease rapidly with increasing pH, and approach very low values at physiologic pH. There is a large difference in the magnitude of these rates for the various penicillins, the rate for methicillin being about 20 times that for oxacillin and about 50 times that of penicillin V. It is known that penicillin G fits in between oxacillin and methicillin, and much closer to the latter (13). Ampicillin produces penicillenic acid even more slowly than penicillin V (11).

**Reaction of Penicillins with Glycine.**—The optical rotation was followed until no further change

[1] The authors are grateful to Eli Lilly and Co., Indianapolis, Ind., for a supply of potassium penicillin V and to Bristol Laboratories for the other penicillins and 6-APA used in this work.

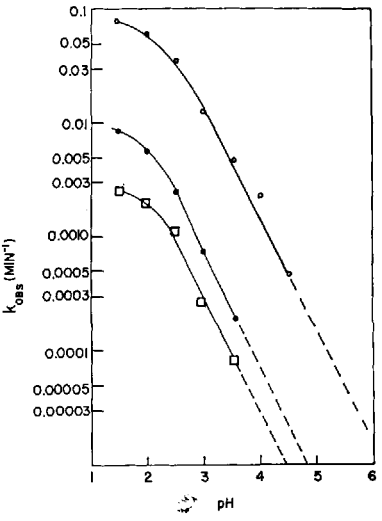



Fig. 1.—Rate of formation of penicillenic acid as a function of pH. Key: ○, methicillin; ●, oxacillin; □, penicillin V.

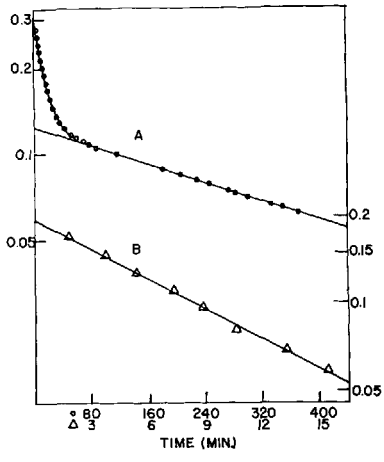



Fig. 2.—First-order plot for reaction of methicillin with 0.3 *M* glycine buffer, pH 9.47 at 50°. Key: ●, $(R - R_\infty)$ *vs.* *t*, left ordinate; △, residuals, right ordinate. Ordinates are log scale.

occurred and this value was designated $R_\infty$. Plots of log $(R_t - R_\infty)$ against time were expected to show apparent first-order kinetics since the concentration of glycine was in large excess over that of penicillin. As seen in Fig. 2, curve A, large deviations from linearity were observed. This particular curve depicts the reaction of methicillin with 0.3 *M*, pH 9.48 glycine buffer, but is typical of all the studies.

It is known that reaction of penicillin with amines yields the α-penicilloamide which can undergo mutarotation to a mixture of diastereoisomers. The mixture is known to have a lower specific rotation than the α-diastereoisomer and the specific rotation of the penicillin is higher than that of the α-diastereoisomer (14). Essentially the following is the situation:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \qquad \text{(Eq. 1)}$$

where *A* represents the original penicillin, *B* the α-diastereoisomer, and *C* the mixture of diastereoisomers. The apparent first-order rate constants are $k_1$ and $k_2$.

In this system:

$$A = A_0 e^{-k_1 t} \qquad \text{(Eq. 2)}$$

$$B = \frac{k_1 A_0}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \qquad \text{(Eq. 3)}$$

$$C = A_0\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \qquad \text{(Eq. 4)}$$

Let $r_A$, $r_B$, $r_C$ represent the molar rotations of the respective species. The rotation at any time during the course of the reaction, $R_t$, will be the sum of the rotations of the individual species:

$$R_t = r_A(A) + r_B(B) + r_C(C) \qquad \text{(Eq. 5)}$$

$$R_t = r_A A_0 e^{-k_1 t} + \frac{r_B k_1 A_0}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) + r_C A_0\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \qquad \text{(Eq. 6)}$$

Combining terms with the same exponential:

$$R_t = \left[r_A A_0 + \frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_2}{k_1 - k_2}\right] e^{-k_1 t} - \left[\frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_1}{k_1 - k_2}\right] e^{-k_2 t} + r_C A_0 \qquad \text{(Eq. 7)}$$

At infinite time $R = r_C A_0 = R_\infty$

$$R_t - R_\infty = \left[r_A A_0 + \frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_2}{k_1 - k_2}\right] e^{-k_1 t} - \left[\frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_1}{k_1 - k_2}\right] e^{-k_2 t} \qquad \text{(Eq. 8)}$$

Since $k_1$ is faster than $k_2$, at some time where $e^{-k_1 t}$ approaches zero there will remain a term in $e^{-k_2 t}$ and the plot of $\ln(R_t - R_\infty)$ *versus* time will be linear with slope $-k_2$. This linear portion of the curve may be extrapolated to zero time and the values of $(R_t - R_\infty)$ along this portion of the line represent the contribution of the term in $e^{-k_2 t}$ to the over-all value. Thus subtraction of this term from the observed $(R_t - R_\infty)$ will leave only the term in $e^{-k_1 t}$. Then a plot of logarithm of these residuals against time should be linear with slope $-k_1$, and this is shown as curve B in Fig. 2.

The rate constant $k_1$ was determined for each penicillin and 6-APA, and was corrected for alkaline hydrolysis in the absence of buffer:

$$k_C = k_1 - k_{OH}(OH^-) \qquad \text{(Eq. 9)}$$

It was found, for the penicillins, that $k_C$ was not a linear function of glycine concentration at constant pH but was a linear function of the square of the glycine concentration as shown in Fig. 3. These particular lines represent reaction of ampicillin but are typical of those observed with all of the penicillins studied. In the case of 6-APA, on the other hand, there *was* a linear relationship between rate constant $(k_C)$ and concentration of glycine as shown in Fig. 4.

The slopes of the lines in Fig. 3 represent the proportionality constant between $k_C$ and the square of

the total glycine concentration and are designated $k_G$. It can be seen that $k_G$ increases with increasing pH implicating the glycine anion, $NH_2CH_2COO^-$, as the reactive species. Figure 5 shows the direct dependence of $k_G$ on the square of the fraction of the total glycine which exists as anion:

$$k_G = k_G^-(f_G^-)^2 \qquad \text{(Eq. 10)}$$

Thus the entire rate may be attributed to a third-order reaction with no second-order term in evidence.

In the case of 6-APA the slopes of the lines in Fig. 4 were found to be proportional to the fraction of the total glycine existing as anion:

$$k_G = k_G^-(f_G^-) \qquad \text{(Eq. 11)}$$

Table I gives both $k_G$ and $k_G^-$ for all the penicillins studied and 6-APA. Also included are the rate constants for alkaline hydrolysis. Each of the latter represents the average of 3 or 4 determinations.

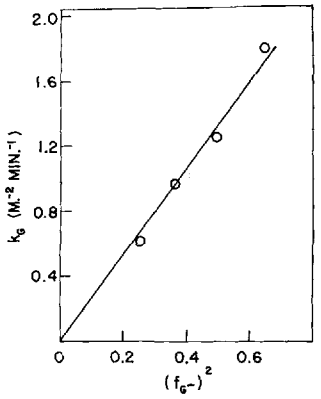



Fig. 5.—Plot showing glycine anion as reactive species.

## DISCUSSION

The requirement of 2 molecules of glycine anion for reaction with the penicillins is indicative of a general base-catalyzed nucleophilic attack of glycine on the β-lactam carbonyl. General base-catalyzed aminolysis of esters (15, 16) and δ-thiolvalerolactone (17) have been reported. In the case of aminolysis of phenyl acetate by glycine (16) both general base-catalyzed and an uncatalyzed reaction were detected. In the present study an uncatalyzed aminolysis could not be detected, although one would suspect that such a reaction should occur in an aqueous medium. Probably the rate of such a reaction is so small that its contribution to the over-all rate could not be observed.

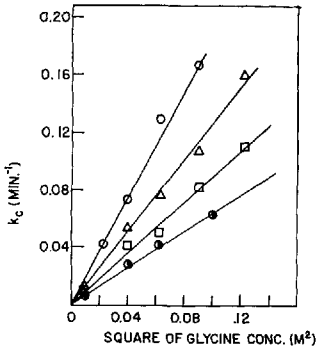



Fig. 3.—Dependence of rate of reaction of ampicillin with glycine upon the square of the glycine concentration. Key: O, pH 9.70; △, pH 9.48; □, pH 9.29; ◑, pH 9.10.

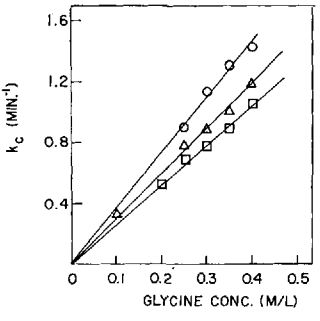



Fig. 4.—Dependence of rate of reaction of 6-APA upon glycine concentration. Key: O, pH 9.70; △, pH 9.48; □, pH 9.29.

Also absent from the rate law for reaction of penicillins with glycine was a term indicating general acid-catalyzed aminolysis as was noted with δ-thiolvalerolactone (17) in addition to the general base-catalyzed reaction. Again this might make too small a contribution to the over-all rate to be noticed or might be absent entirely.

A mechanism for general base-catalyzed aminolysis of penicillin may be described schematically as follows:

$$P + G^- \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} PG^-$$

$$PG^- + G^- \xrightarrow{k_4} G^{\pm} + \text{product} \qquad \text{(Eq. 12)}$$

where P represents penicillin, $G^-$ the glycine anion, and $G^{\pm}$ the zwitter ion. The intermediate produced in the first reaction could be a tetrahedral addition product or a complex. The driving force in the second step is the removal of a proton from the intermediate by the second glycine anion. Assuming a steady state in the intermediate:

$$\frac{d(PG^-)}{dt} = k_3(P)(G^-) - [k_{-3} + k_4(G^-)]\,PG^- = 0 \qquad \text{(Eq. 13)}$$

$$(PG^-) = \frac{k_3(P)(G^-)}{k_{-3} + k_4G^-} \qquad \text{(Eq. 14)}$$

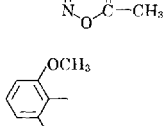
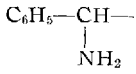
$$K_C = \frac{1}{P}\frac{dP}{dt} = \frac{k_4k_3(G^-)^2}{k_{-3} + k_4(G^-)} \qquad \text{(Eq. 15)}$$

when $k_{-3} \gg k_4(G^-)$

$$K_C = \frac{k_4k_3}{k_{-3}}(G^-)^2 \qquad \text{(Eq. 16)}$$

At sufficiently high $(G^-)$ it might be thought from Eq. 15 that the rate would reduce to first order in

TABLE I.—$k_G$ AND $k_G^-$ FOR PENICILLINS AND 6-APA AND RATE CONSTANTS FOR ALKALINE HYDROLYSIS

| Penicillin | Side Chain, R in I, Fig. 1 | $k_{OH}$ (L. mole$^{-1}$ min.$^{-1}$) | $k_G$ at Designated pH (L.$^2$ mole$^{-2}$ min.$^{-1}$) 9.70 | 9.47 | 9.29 | 9.10 | $k_G^-$ (L.$^2$ mole$^{-2}$ min.$^{-1}$) |
|---|---|---|---|---|---|---|---|
| G | $C_6H_5CH_2$— | 43 | 1.48 | 1.10 | 0.82 | 0.56 | 2.3 |
| V | $C_6H_5OCH_2$— | 63 | 2.44 | 1.77 | 1.30 | ... | 3.7 |
| Oxacillin |  | 52 | 1.34 | 1.00 | 0.77 | 0.56 | 2.1 |
| Methicillin |  | 32 | 1.18 | 0.76 | 0.58 | 0.37 | 1.6 |
| Ampicillin |  | 56 | 1.80 | 1.15 | 0.89 | 0.65 | 2.7 |
| 6-APA | ... | 12 | 0.036[a] | 0.030[a] | 0.026[a] | ... | 0.44[a] |

[a] Units are L. mole$^{-1}$ min.$^{-1}$.

glycine anion. This was not observed at concentrations up to 0.3 *M*, as would be expected for the rapid equilibration between reactants and intermediate which would take place in this case.

In the case of 6-APA only over-all second-order kinetics was observed. This might be due to intramolecular general base catalysis by the free amino group on the carbon adjacent to the β-lactam carbonyl, or a relatively large contribution by the uncatalyzed aminolysis, or both.

From these results one might speculate that compounds containing both general base and nucleophilic groups on the same molecule, diamines for example, would react much more rapidly with penicillin than glycine. This is currently under investigation, and preliminary results indicate that this is indeed the case. The results of these experiments will be the subject of a future communication (18). Intramolecular general base catalysis could provide a rationale for rapid reaction of penicillin with proteins at physiological pH, which is pertinent to the question of penicillin antigen formation. On protein molecules there could be many sites where intramolecular general base catalysis of aminolysis could occur at rates rapid enough to compete with the elimination of penicillins from the body where the half-life is about 30–60 min.

Also of pertinence to the mechanism of penicillin antigen formation is the fact that the rates of reaction of all these penicillins with glycine are of the same order of magnitude, a factor of about 2.3 separating the slowest (methicillin) from the most rapid (penicillin V). This shows a lack of influence of the side chain upon reactivity toward nucleophiles of the β-lactam carbonyl. This is also apparent in the values for $k_{OH}$. On the other hand, the side chain has a great effect on the rates of penicillenic acid formation from penicillins. Although clinical experience with some of these penicillins is rather limited, allergic reactions to all the penicillins have been reported (19). Even though many of these are probably due to cross-reaction with antibody to penicillin G, the new penicillin must still combine covalently with protein to provide an antigen. From the relative rates reported here it would seem more likely that the direct reaction between penicillin and protein is taking place rather than the penicillenic acid route.

While the side chain (R in I) appears to have little influence on reaction of penicillins with small molecules, the situation with proteins might be quite different where steric effects of the side chain might play a role. Such effects are observed in the relative affinities of the various penicillins for the enzyme penicillinase. Methicillin and oxacillin have very little affinity for the enzyme while penicillin G, penicillin V, and ampicillin are relatively tightly bound. Only a hint of steric effect was seen in the present work where the rate constant for reaction of oxacillin with glycine is slightly higher than that of penicillin G, while the reverse is true of the $k_{OH}$ values. Oxacillin has the bulky phenyl group on the carbon adjacent to the point of attachment which might interfere somewhat with the approach of the glycine more than with hydroxyl ion. The magnitude of the differences is too small, however, to draw definite conclusions and further studies of steric effects are contemplated.

In contrast to the potentially relatively rapid rates of reaction of penicillins with proteins are the very low rates of penicillenic acid formation calculated for neutral pH. For example, the half-life for penicillenic acid formation from methicillin at pH 7.4 at 34° would be about 1000 days. It would seem then that if the penicillenic acid route to antigen is of any consequence the penicillenic acid would have to be present in a preparation *prior* to parenteral administration. This is possible as a result of decomposition under normal storage conditions particularly in liquid dosage forms. Since acid is produced upon hydrolysis of penicillins the pH will be lowered, thus increasing the rate at which penicillenic acid is formed. It would seem important, therefore, that parenteral penicillin preparations be buffered to minimize this route of degradation. With oral preparations penicillenic acid is probably formed quite rapidly in the acid stomach. The limiting factor would then become the absorption of penicillenic acid. Since oral administration of penicillins has produced the least number of clinical allergy problems of any route, this might be considered relatively unimportant.

REFERENCES

(1) Levine, B. B., and Ovary, Z., *J. Exptl. Med.*, **144**, 875 (1961).
(2) Parker, C. W., *Federation Proc.*, **24**, 51(1965).
(3) Levine, B. B., *ibid.*, **24**, 45(1965).
(4) Levine, B. B., *Nature*, **187**, 939(1960).
(5) Levine, B. B., *Arch. Biochem. Biophys.*, **93**, 50(1961).
(6) Levine, B. B., and Price, V. H., *Immunology*, **7**, 527 (1964).
(7) Thiel, J. A., Mitchell, S., and Parker, C. W., *J. Allergy*, **35**, 399(1964).
(8) Batchelor, F. R., Dewdney, J. M., and Gazzard, D., *Nature*, **206**, 362(1965).
(9) Schneider, C. H., and De Weck, A. L., *ibid.*, **206**, 57 (1965).
(10) Wagelie, R. G., Dukes, C. D., and McGovern, J. P., *J. Allergy*, **34**, 489(1963).
(11) Brandriss, M. W., Denny, E. L., Huber, M. A., and Steinman, H. G., in "Antimicrobial Agents and Chemotherapy," Sylvester, J. C., ed., American Society for Microbiology, Ann Arbor, Mich., 1962, p. 626.
(12) Nakken, K. F., Lorentz, E., and Pihl, A., *Biochem. Pharmacol.*, **3**, 89(1960).
(13) Schwartz, M. A., *J. Pharm. Sci.*, **54**, 472(1965).
(14) Levine, B. B., *J. Med. Pharm. Chem.*, **5**, 1025(1962).
(15) Bunnett, J. R., and Davis, G. T., *J. Am. Chem. Soc.*, **82**, 665(1960).
(16) Jencks, W. P., and Carriuolo, J., *ibid.*, **82**, 675(1960).
(17) Bruice, T. C., Bruno, J. J., and Chou, W. S., *ibid.*, **85**, 1659(1963).
(18) Schwartz, M. A., unpublished data.
(19) Grieco, M. H., Dubin, M. R., Robinson, J. L., and Schwartz, M. J., *Ann. Internal Med.*, **60**, 304(1964); and references quoted therein.

# Polarographic Study of Pteridines

By MILTON LAPIDUS and MARVIN E. ROSENTHALE

**The electronegativity of the half-wave potentials of a series of pteridine congeners was found to be related to the substituent groups. The 2,4,7-triaminopteridines, 7-substituted 4-amino-2-aryl-6-pteridinecarboxamides, and 4,7-diamino-2-aryl-6-pteridinecarboxamides were characterized, in that order, by decreasingly lower electronegative half-wave potentials.**

A NUMBER of attempts have been made to correlate pharmacological activity with the oxidation-reduction potentials of a homologous series of compounds (1). Relationships have seldom been demonstrated; however, there is a report that acridines with reduction potentials ($E^{\circ}_{h}$) more negative than $-0.400$ v. have greater antiseptic activity (2) than those with less negative potentials. That study showed the active acridines to have reduction potentials so electronegative that no physiological system could reduce (inactivate) them. Evidently, with the acridines, the maintenance of the oxidized configuration is necessary for biological activity. Another study showed that the degree to which members of two homologous series of acridine antimalarials inhibit the diamine–oxidase enzyme system parallels the values of the reduction potential of the compounds (3). However, no evidence was found that inhibition of diamine–oxidase is necessary to antimalarial action (4).

The possible importance of the oxidation-reduction potential prompted the authors to determine it for a large number of pteridines which have been under pharmacological review. Some of these pteridines are useful diuretic agents (5–8).

Received January 14, 1966, from Wyeth Laboratories, Inc., Radnor, Pa.
Accepted for publication March 31, 1966.
The authors acknowledge the technical assistance of Mr. D. D. Austin.

## METHOD

**Material.**—The pteridines studied were synthesized by Osdene *et al.* (9).

**Polarographic Analysis.**—A Leeds and Northrup recording polarograph equipped with a dropping mercury electrode was used for determining reduction potentials. The H-type electrolysis cell consisted of a saturated calomel half-cell connected to the test solution through an agar bridge and a fritted-glass diaphragm. Under a potential of $-0.50$ v. the dropping mercury electrode delivered 2.43 mg. of Hg/sec. with a drop time of 4.31 sec. All measurements were made at 25°. Unless otherwise noted, the solutions for polarographic analysis contained $5 \times 10^{-4}$ *M* pteridine, 0.1 *M* phosphate buffer (pH 2.0), and 0.005% gelatin. The calibrating solution, which contained zinc chloride ($1 \times 10^{-3}$ *M*) in place of the pteridine, gave an $E^{1}_{1/2}$

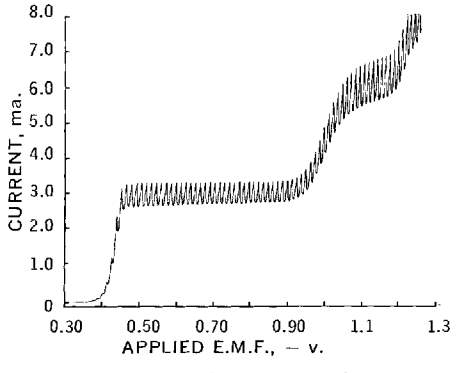



Fig. 1.—Polarographic reduction of Wy-4437 (pH 2.0).