STUDIES ON PENICILLINASE

II. MANOMETRIC METHOD OF ASSAYING PENICILLINASE AND PENICILLIN, KINETICS OF THE PENICILLIN-PENICILLINASE REACTION, AND THE EFFECTS OF INHIBITORS ON PENICILLINASE*

BY RICHARD J. HENRY AND RILEY D. HOUSEWRIGHT

(From the Technical Laboratories of Camp Detrick, Frederick, Maryland)

(Received for publication, September 29, 1946)

On alkaline hydrolysis, penicillins are converted into penicilloic acids (1). Penicillinase appears to bring about the same transformation, in which a carboxyl group of pK 4.7 is generated (2). Foster (3) allowed penicillinase to act on penicillin in bicarbonate buffer in the Warburg respirometer and observed the evolution of CO2.

Since such a reaction can be followed simply and with considerable accuracy, and since enzyme reaction velocities within certain limits are proportional to enzyme concentration, it was believed that a rapid and accurate assay for penicillinase, free of the numerous difficulties and inaccuracies of bioassay methods, could be developed by the manometric method. It was further considered that should the CO2 be released in stoichiometric proportion to the amount of penicillin present a direct chemical method of assaying penicillin itself might be developed. Furthermore, such a method lends itself very readily to a study of the kinetics of the reaction.

Materials and Methods

Constant volume Warburg respirometers were used; the main space of the vessel contained 1 ml. of the enzyme solution to be assayed and 3 ml. of sodium bicarbonate solution to give a total volume of 4 ml. The concentration of bicarbonate used depended on the pH desired and the per cent CO2 of the gas mixture with which the system was equilibrated (4) (actual composition determined by gas analysis).1 All experiments were carried out at pH 7.0 except where otherwise noted. In the side arm was placed 0.5 ml. of a penicillin-bicarbonate mixture, the bicarbonate concentration of which was the same as that existing in the main space. The manometers were shaken through a to and fro distance of 3 cm. at a rate of 100 cycles per minute. Experiments showed that a rate of at

* With the technical assistance of A. S. Herring, II. Clemons, and J. C. Hartley.
1 It was found that 5 per cent CO2 in either 95 per cent O2 or 95 per cent N2 gave the same results. Thus the reaction is uninfluenced by the presence or absence of O2. The CO2-N2 mixture was used throughout this investigation.
least 90 cycles per minute was required to give maximum CO₂ evolution from the reaction system. After equilibration with the gas mixture, the system was closed and readings taken until constant, at which time the penicillin from the side arm was tipped into the main space, allowing the reaction to begin. Blanks run without penicillinase evidenced no change in gas volume following admixture of the penicillin as long as the penicillin was dissolved in the same concentration of bicarbonate as was present in the main space.

Except where otherwise noted, the penicillin used was commercial penicillin sodium, Pfizer, Lot 585C.

Three different preparations of penicillinase, all produced by Bacillus cereus NRRL B-569² were employed: (1) corn steep penicillinase, crude; (2) corn steep penicillinase, alcohol- and acetone-precipitated, dialyzed; (3) “Casamino acid basal medium” penicillinase, crude (5). Unsterile preparations occasionally lost potency, apparently because of gross contamination. Many preparations were sterilized by filtering through sintered glass filters. Toluene, which could be used to keep down bacterial multiplication, was found to have no deleterious effect on the enzyme.

The bioassay method used for determining penicillinase activity is described in Paper I of this series (5). It is a tube dilution method in which Bacillus anthracis is the test organism. By statistical analysis this method has an experimental error on one single determination of ±75 per cent. By running replicates, the error of the estimate can be reduced considerably. With but few exceptions such bioassays were performed in duplicate or triplicate and in some cases with as many as fifteen replicates.

This method also was used for assaying penicillin. In this case, however, the penicillin is diluted 2-fold in series.

Varying Enzyme Concentrations; Manometric Assay of Penicillinase—Fig. 1 shows the log of reaction rate \(k\), calculated on the basis of a zero order reaction, plotted against the log concentration of penicillinase in arbitrary units from a typical experiment done at 36° with 5000 Oxford units of commercial penicillin sodium as substrate.³

The relationship is linear up to an enzyme concentration equivalent to and including penicillinase assaying 1:1024. At this concentration the substrate begins to be saturated with enzyme, and the reaction rate

² Obtained from Dr. R. D. Coghill, Northern Regional Research Laboratory, Peoria, Illinois.

³ This type of experiment was run numerous times with crude penicillinase obtained in the Casamino acid basal medium, crude penicillinase in corn steep medium, and alcohol-precipitated, dialyzed penicillinase. In all cases the same results were obtained. The commercial penicillin sodium used in these experiments was obtained from Pfizer, Lot 585C, 817 Oxford units per mg., and Lot 86C.
reaches a maximum. Several experiments with concentrations of enzyme greater than that assaying 1:512, in which the evolution of CO₂ was read at 30 second intervals, indicated that the rate was still increasing with increasing enzyme concentration, but the increase was not linear and approached a maximum in the neighborhood of penicillinase assaying 1:8192. The slope of the line in Fig. 1 is 1.00, and the slope is the same at 26°, 16°, and 6°.

When penicillinase is added to penicillin in the Warburg flask, there is usually a lag period of 1 to 4 minutes before the rate of CO₂ evolution becomes constant. With 5000 units of penicillin as substrate the reaction is of zero order over approximately 80 to 90 per cent of the reaction, except

at low concentrations of enzyme at which it approaches a first order reaction. The activity in concentrations assaying between 1:4 and 1:512 can be assayed directly. Below this range of concentrations the amount of CO₂ evolved in 1 or 2 hours is not considered sufficient for accurate assay. For penicillinase assaying 1:512 two or three readings at 5 minute intervals cover the complete reaction. For lower concentrations, the CO₂ evolution is measured at 5 or 10 minute intervals for 1 hour and, when an objective estimate of the reaction rate is desired, the slope of the straight portion of the line is determined by the method of least squares. The reaction rate thus determined is in terms of c.mm. of CO₂ per minute.

The precision of the method as determined from two experiments of ten replicates each is ±9.6 per cent (±2 standard deviations). All of

![Fig. 1. Penicillinase activity as a function of penicillinase concentration. Penicillinase concentration expressed in arbitrary units. From left to right the points represent penicillinase bioassaying 1:4, 1:16, 1:64, 1:256, 1:1024, and 1:4096.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ by guest on November 6, 2017
the CO₂ evolved is "acid CO₂;" i.e., is released from the bicarbonate buffer because of an increase in acid in the reaction system. This was determined in several experiments by adding excess sulfuric acid to the buffer system before and after reaction. The difference always equaled the CO₂ released by the reaction within a very few per cent. Further evidence that the release of CO₂ paralleled the destruction of penicillin lay in the fact that after CO₂ evolution stopped in a system containing 5000 units of commercial penicillin sodium and penicillinase assaying 1:1024 (approximately 40 minutes) more than 99.2 per cent of penicillin activity had been destroyed as determined by the bioassay method. It is true that in such a test the penicillinase activity cannot be stopped completely, but a dilution of 1:200 is made immediately for the bioassay, and this dilution of the enzyme would have a very slow rate of activity.

Since commercial penicillin sodium is a mixture of uncertain composition, the rates of enzyme activity at several enzyme concentrations on various pure penicillins were determined. The rate of reaction with penicillin G was arbitrarily designated as 1.0. The reaction rates with the other penicillins were adjusted in ratio to this arbitrary standard. These ratios are the k for any particular penicillin divided by the k for penicillin G, and are recorded in Table I as "k ratios (penicillin G = 1.0)." The results represent averages of at least six experiments. No significant difference was noted when penicillinase was assayed against the various penicillins by the tube dilution method with Bacillus anthracis as the test organism. This method, however, as previously shown, is not very precise.

The observation that penicillinase inactivates purified penicillin K at approximately the same rate as the other fractions rules out the possibility that the relatively low activity in vivo of penicillin K (6, 7) might be a result of its rapid destruction by penicillinase in vivo.

Interfering Substances—Two types of interference were considered: naturally occurring specific activators and inhibitors, and substances which could bring about erroneous results because of CO₂ retention. Fe⁴⁺⁺ in concentrations of 5 and 50 γ per ml. caused 25 and 95 per cent inhibition of the reaction, respectively. Ca²⁺ in concentrations of 12.5 and 25 γ per ml. caused 30 and 65 per cent inhibition. Co²⁺, Mg²⁺, and Mn²⁺ had no effect in concentrations up to 100 γ per ml. Cu²⁺, Cu⁺, and Zn²⁺ caused a large release of CO₂ when the substrate was tipped in from the side arm as a result of their combination with bicarbonate ion, and, therefore, their effects on the reaction could not be studied. No effect on penicillinase was observed with 5 × 10⁻² m methionine or 6.7 × 10⁻⁵ to 1.3 × 10⁻³ m dl-phenylalanine.

No retention of CO₂ was observed by crude corn steep filtrate, Casamino
acid basal medium filtrate, or ascitic fluid (50 per cent). Assay in the presence of horse serum (50 per cent), however, was low because of significant CO₂ retention. This, of course, can be corrected for by running the necessary blanks (4).

### Table I

**Penicillinase on Commercial Penicillin Sodium and Purified Penicillins**

<table>
<thead>
<tr>
<th></th>
<th>Commercial penicillin Na*</th>
<th>Penicillin Na F†</th>
<th>Penicillin Na G*</th>
<th>Penicillin Na K</th>
<th>Penicillin Na X‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>k ratios (penicillin G = 1.0)</td>
<td>0.78</td>
<td>0.78</td>
<td>1.0</td>
<td>0.69†</td>
<td>1.0</td>
</tr>
<tr>
<td>CO₂ evolved per Oxford unit</td>
<td>0.032</td>
<td>0.037</td>
<td>0.038</td>
<td>0.0216†</td>
<td>0.030†</td>
</tr>
<tr>
<td>Calculated No. molecules per unit</td>
<td>0.99</td>
<td>1.02</td>
<td>(0.81 × 10⁵)</td>
<td>1.72</td>
<td>(0.81 × 10⁵) ‡</td>
</tr>
<tr>
<td></td>
<td>× 10¹⁵</td>
<td>× 10¹⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apparent mol. wt.</td>
<td>391</td>
<td>354</td>
<td>366‡</td>
<td>409</td>
<td></td>
</tr>
<tr>
<td>Theoretical mol. wt.††</td>
<td>334.2</td>
<td>356.2</td>
<td>364.3</td>
<td>372.2</td>
<td></td>
</tr>
</tbody>
</table>

* Chas. Pfizer and Company, Inc., Lot 585C, 817 Oxford units per mg. Assuming only G and K penicillins present, differential assay indicated them to be present in approximately equal amounts (personal communication, Dr. F. J. Stock, Chas. Pfizer and Company).

† Obtained from Dr. G. F. Cartland of The Upjohn Company, Lot Research 67-ERH-2, crystalline, approximately 1500 units per mg.

‡ Obtained from Dr. R. D. Coghill of the Abbott Laboratories, Lot PP75-4 + 5C, analytically pure, standard *Staphylococcus aureus* assay 1667 units per mg.

§ Obtained from Dr. A. F. Langlykke of the Northern Regional Research Laboratory, NRRL Lot 1717-39-A, pure, *Staphylococcus aureus* assay 850 units per mg.

‖ Obtained from Dr. A. F. Langlykke of the Northern Regional Research Laboratory, Lot 1818-42-A, *Staphylococcus aureus* NRRL B-313 cup plate assay approximately 2635 units per mg.

¶ Obtained from Pfizer through Dr. C. J. Van Slyke of the National Institute of Health, United States Public Health Service, Lot 5/2/46, analytically pure, Oxford plate *Staphylococcus aureus* assay 2190 units per mg.

** Average of two samples above.

†† As calculated from empirical formulae in (1).

**Varying Substrate Concentration; Manometric Assay of Penicillin**—In Fig. 2 two experiments at 36° with penicillinase assaying 1:256 are shown with the reaction rate k plotted against varying amounts of substrate (commercial penicillin sodium). This type of curve is typical of enzyme reactions in which the substrate concentration is varied.

Since the total volume of CO₂ released is proportional to the amount of substrate, the procedure affords a chemical method of assaying peni-
cillin. The precision with which this assay can be carried out is ±4.6 per cent (±2 standard deviations, determined from 52 replicates, each with 5000 units of commercial penicillin sodium), a precision at least equal to that of existing bioassay methods (9). For assaying penicillin, a highly active concentration of penicillinase is used so that the reaction goes to completion very rapidly. The minimum amount of penicillin which can be thus assayed is approximately 100 units per ml., although at such low concentrations the precision is considerably less. 3 ml. of the peni-

![Diagram of penicillinase activity at various substrate concentrations.

Fig. 2. Penicillinase activity at various substrate concentrations. Abscissae, total units of penicillin in 4.5 ml. of reaction system. Results of two experiments.

- A chemical assay for penicillin based on alkalimetric titration of the acidity produced by penicillinase inactivation of penicillin has been reported previously (8).
high concentration, and is further limited to solutions of low buffering capacity.

The amount of CO$_2$ evolved per Oxford unit varied with the different purified penicillin fractions, as is shown in Table I. From this information and with the assumption that each newly formed carboxyl group brings about the release of 1 molecule of CO$_2$, the number of molecules of the penicillin per Oxford unit and its molecular weight were calculated. The molecular weights for penicillins G and K thus calculated agree remarkably well with those recently published (1). The values for penicillin sodium F and penicillin sodium X show some discrepancy, but only two experiments were run with these penicillins because of the limited amounts available.

It is to be noted also that differential analysis of the commercial penicillin sodium used indicated that it was composed of approximately equal amounts of penicillins G and K. If the values of c.mm. of CO$_2$ evolved per Oxford unit for penicillins G and K are averaged, each being given equal weight in the averaging, the value obtained is that found for the commercial penicillin. This immediately suggests the possibility that this type of analysis may be used in a differential assay of commercial mixtures made up largely of only two penicillins.

Such a chemical method for assaying an antibiotic can be of value only if absolutely specific for the chemotherapeutically active component. To determine whether penicillinase is specific for penicillin and has no activity on the degradation product of penicillin resulting from thermal decomposition, a commercial penicillin solution of 20,000 units per ml. was heated at different temperatures for varying lengths of time and then assayed by the manometric technique and the tube dilution bioassay method. After being autoclaved at 121° for 30 minutes, the penicillin was completely decomposed, as assayed by both methods. When solutions were heated at 100° for 10 and 30 minutes, the decomposition was 25 and 75 per cent respectively as estimated by both methods. A solution of 10,000 units per ml. of penicillin sodium G after being heated at 100° for 2 minutes showed 30 per cent decomposition by the manometric method and 50 per cent by bioassay. Thus, within the experimental error of these experiments, the penicillin inactivated by heat does not react with

---

5 Since the different penicillins result in different volumes of CO$_2$ given off and different lots of commercial penicillin vary with respect to the relative amounts of these penicillins (6), this method for assaying penicillin is obviously invalid when penicillin solutions of different lots are compared. On the other hand, the same criticism holds for bioassay methods, since the relative potency of the various penicillins is not constant from organism to organism (7, 10, 11). A completely satisfactory and valid assay for penicillin can be devised only when each penicillin can be assayed in the presence of all the others.
penicillinase in the Warburg system to give off CO₂. This does not rule out the possibility, however, of the existence of other penicillin degradation products which, although chemotherapeutically inactive, might react with penicillinase to give off CO₂, or vice versa.

It has been reported (12) that crude commercial penicillin preparations contain impurities which inhibit penicillinase, but these should only affect the rate of reaction and therefore would not interfere with the manometric assay of penicillin, since it depends on the total change in the reaction system and not on the rate of reaction.

Effect of pH on Reaction Rate—Fig. 3 shows the results of one of six experiments on the effect of pH on the penicillin-penicillinase reaction, all of which agreed very well. In some of the other experiments points intermediate between pH 6.9 and pH 7.6 were included, revealing a rather flat plateau within this pH range, and suggesting an optimum in bicarbonate buffer at approximately pH 7.2. This agrees very well with previous reports (12, 13).

Effect of Temperature on Reaction Rate—Reaction velocities were measured at 6°, 16°, 26°, 30°, 36°, and 46° with 5000 units of commercial penicillin sodium (Pfizer, Lot 585C) as substrate in the reaction system (1111 units per ml.). The penicillinase used was a crude preparation produced in Casamino acid basal medium and filtered through a sintered glass filter. As a check for enzyme inhibitors or activators in the preparation, 1 ml. of crude enzyme solution, boiled for 5 minutes, was included in the reaction system containing unheated active enzyme. No difference was
observed in enzyme activity with or without this boiled enzyme preparation.

Fig. 4 shows the velocity of reaction of five different concentrations of enzyme plotted against the reciprocal of absolute temperature. Since the substrate concentration used was 1111 units of penicillin per ml., reactions were of zero order during most of the reaction. Observations of CO₂ evolved were recorded every 10 minutes and the velocity constant for each experiment determined by the method of least squares. Each point in Fig. 4 represents one or the average of two to seven experiments (80 in all).

The optimum is seen to occur at approximately 36°C. This agrees well with previous reports on the optimal temperature as determined by bioassay methods (2, 12). To obtain the best estimate of the temperature coefficient (Q₁₀) of the reaction and the temperature velocity coefficient or "apparent activation energy" (μ), the slopes of the lines through observations at 6°C, 16°C, and 26°C were determined by the method of least squares. The weighted average of the five slopes is -0.162 ± 0.016. This gives a value and limits for μ of 7460 ± 750 calories per mole, and a Q₁₀ between 6-16°C and between 16-26°C of approximately 1.56. It must be pointed out that such kinetic data may vary with pH and pressure (15), and therefore these values probably are valid only for the conditions used in this investigation (pH 7.0, 1 atmosphere of pressure).

An attempt was made to determine whether the heat inactivation at 46°C is reversible to any extent. The rate of CO₂ evolution was observed for 30 minutes to obtain the reaction rate at 46°C and then the water bath temperature was changed to 36°C. No increase in the reaction rate was observed, but it should be noted that the enzyme was exposed to the higher temperature for approximately 1 hour before reversal was attempted.

Inactivation of penicillinase by exposure to temperatures of 50°C and above has been reported previously (12). Representative data on heat denaturation at 60°C and 100°C of crude penicillinase produced in the Cas-

---

6 The variance of the experimental points (forty in number) about each of the five lines was computed and the five estimates compared by Bartlett's χ² test for homogeneity of the variance (14). This test indicated heterogeneity and an examination of the data suggested that a few of the points at 26°C on Curve C, Fig. 4, which inexplicably were considerably more dispersed from their average than any similar set of points, might be responsible for the heterogeneity. With these points omitted, the χ² test indicated homogeneity of variance. An analysis of variance showed that the individual slopes agreed with each other within the sampling error. To obtain the best estimate of slope the five slopes were combined; similarly, the variances of the points about each line were combined to give a pooled variance. From these two statistics the standard error of the regression coefficient or slope was calculated. Limits given for the combined slope and μ represent ±2 standard errors.
amino acid basal medium are shown in Fig. 5 where log \( k \) is plotted against time of exposure. Since active penicillinase concentration is directly proportional to the reaction rate of the enzyme action on penicillin, \( k \) is an index of enzyme concentration. The reaction rates were determined manometrically. If heat denaturation of penicillinase is a first order reaction, as is heat denaturation of many proteins (16), the data in Fig. 5 should follow a straight line. The data obviously are not linear and it was thought at first that this may be due to the presence of impurities in the enzyme preparation. The experiment was repeated with the same preparation after dialysis against distilled water. No increased tendency toward linearity was noted but the rates of heat denaturation were increased in the dialyzed preparation.

![Graph showing inactivation of penicillinase by heat.](image)

**Fig. 5.** Inactivation of penicillinase by heat. Activity of penicillinase, expressed as reaction velocity \( (k) \), after exposure to 60° and 100° for various lengths of time.

### Specificity of Enzyme Activity

To be of value a method of assay should be specific for the substance assayed. The manometric assay method depends on the formation of a carboxyl group from the ring carbonyl group in the penicillin molecule which is adjacent to a ring nitrogen (1). This particular configuration occurs also in certain purines, pyrimidines, and other naturally occurring substances. In two experiments penicillinase assaying 1:1024 was added to the stated concentrations of the following substances: xanthine 0.28 mg. per ml., adenine sulfate 0.011 mg. per ml., guanine 0.11 mg. per ml., riboflavin 0.013 mg. per ml., sodium pyruvate 5.55 mg. per ml., uracil 1/9 saturated. In no case was there a significant change in the gas phase upon addition of the penicillinase over a period of 1 hour. It would appear, therefore, that penicillinase is rather
specific and certainly the presence of the above substances in solutions
to be assayed for penicillinase or penicillin by the manometric method
will not influence the results.

_Effects of Inhibitors on Penicillinase—_Pepsin (U. S. P.) and papain
activated by cysteine individually at pH 5.0 (37° for 6 hours) inactivated
penicillinase 50 to 95 per cent as measured by the manometric assay and
the bioassay.

The following inhibitors had no effect on penicillinase activity as meas-
ured by the manometric assay:7 0.15 M sodium azide, 0.01 M potassium
cyanide, 6 \times 10^{-3} M diethyl dithiocarbamate, 0.8 M ethyl urethane,
8 \times 10^{-4} M sodium sulfadiazine, 0.2 M NaF, 0.5 M formaldehyde, 4.4 \times 10^{-3}

![](https://example.com/fig6.png)

**Fig. 6.** Activity of penicillinase after exposure to oxidants and reductants. 1,
H₂ activated with platinized asbestos, 20 minutes exposure at room temperature; 2,
0.5 M cysteine hydrochloride, 24 hours at room temperature; 3, 6.4 \times 10^{-2} M cysteine
hydrochloride, 24 hours at room temperature; 4, 5 \times 10^{-2} M ascorbic acid + 10^{-2} M
CuSO₄, 30 minutes at room temperature, followed by exposure to catalase for 1 hour;
5, 6 \times 10^{-3} M diethyl dithiocarbamate, 1 hour at 36°; 6, 4.4 \times 10^{-3} M thioglycolate
sodium, 1 hour at 36°; 7, control, nothing added; 8, 4.5 \times 10^{-2} M iodoacetate, 1 hour
at 36°; 9, 6 \times 10^{-2} M K₂Fe(CN)₆, included in the bioassay tubes; 10, 0.44 M H₂O₂,
1 hour at room temperature, followed by exposure to catalase for 1 hour; 11, 8.3 \times
10^{-2} N I₂, 1 hour at 8°; 12, 8.3 \times 10^{-2} N I₂, 1 hour at 8°.

M sodium thioglycolate, 10^{-3} M sodium p-chloromercuribenzoate, 0.05 M
iodoacetamide, 0.045 M sodium iodoacetate, 0.001 per cent yeast nucleic
acid, 0.001 per cent thymonucleic acid, 8 0.05 M ascorbic acid, 0.01 M

7 Except where otherwise noted there was contact between the inhibitor and peni-
cillinase for approximately 1 hour at 36° at pH 7.0 before penicillinase activity was
measured by adding commercial penicillin sodium (final concentration of 1111 units
per ml.) to the system. Activity was measured for at least 1 hour.

8 Desoxyribonucleic acid but not ribonucleic acid is capable of forming relatively
stable complex salts with basic proteins (Weissman, N., and Grof, L. H., unpublished
work). Since no inhibition of penicillinase activity was observed with either com-
 pound, it is probable that penicillinase is not a basic protein, although there is a
possibility that such a complex may retain enzymatic activity.
ethanol, $8 \times 10^{-3} \text{ M}$ acetone, and $3.4 \times 10^{-3} \text{ M}$ amyl acetate. Potassium ferricyanide, $6 \times 10^{-3} \text{ M}$, had no effect as measured by the bioassay method.

The stability of penicillinase at various oxidation-reduction potentials (measured by a Beckman pH-Eh meter) was determined by exposing the enzyme to various reagents for 1 hour and then testing for activity by the bioassay method (Fig. 6). Hydrogen activated by platinized asbestos gave an $E_h$ of $-200$ millivolts and completely destroyed penicillinase. Cysteine hydrochloride, 0.5 and 0.064 $\text{ M}$, with potentials of 0 and +100 millivolts respectively, inactivated penicillinase 75 and 50 per cent. This exposure lasted 24 hours, during which time the cysteine was oxidized to cystine. Penicillinase was stable from an $E_h$ of $+150$ millivolts (ascorbic acid) to $+600$ millivolts (potassium ferricyanide). Hydrogen peroxide, 0.44 $\text{ M}$, gave an $E_h$ of $+660$ millivolts and inactivated the enzyme 80 per cent. Before assaying, the peroxide was removed by catalase. Iodine, $8.3 \times 10^{-3} \text{ N}$, gave a potential of $+720$ millivolts, and during exposure for 1 hour at $8^\circ$ completely inactivated the enzyme.

The failure of azide, cyanide, and diethyl dithiocarbamate to inhibit penicillinase rather definitely indicates that it contains no iron or copper essential for activity nor does it require either of these ions in the function of an activator. It is also rather definite that the enzyme does not have free sulfhydryl groups essential for activity since none of the inhibitors of sulfhydryl enzymes (17) had any effect on penicillinase. Furthermore, nitroprusside tests, with and without guanidine denaturation, on various penicillinase preparations were all negative. The failure of formaldehyde to inactivate penicillinase indicates that free amino groups are not essential for activity in this case (17).

**SUMMARY**

1. A chemical, manometric method of assaying penicillinase is described which is rapid, specific as far as is known, does not require sterile conditions, and has relatively high precision.

2. A similar chemical, manometric method is also described for assaying penicillin, but it is limited to concentrations greater than 100 Oxford units per ml. and has certain other limitations.

3. Penicillinase was shown to inactivate penicillins G and X with approximately equal rates of reaction, and penicillins F and K at somewhat slower rates.

4. The molecular weights of penicillins F, G, K, and X as determined from data obtained agreed remarkably well with those previously published.

5. The pH optimum of penicillinase at $36^\circ$ was found to be approximately 7.2.
6. The temperature optimum of penicillinase at pH 7.0 was approximately 36°.

7. The temperature coefficient of the reaction was calculated to be 1.56 and the apparent activation energy 7460 ± 750 calories per mole.

8. Penicillinase was rather rapidly inactivated at temperatures of 46° and above.

9. Penicillinase manifested high specificity for the configuration of the basic penicillin molecule. The activity of penicillinase was checked against several compounds having chemical groupings similar to that grouping in penicillin which is attacked by penicillinase, and no reaction was observed.

10. A study of the effects of various inhibitors on penicillinase activity indicated the following conclusions. (a) Penicillinase is not a copper or iron enzyme. (b) It is a protein or has a protein component essential for activity. (c) It is probably not a basic protein. (d) Neither free amino groups nor sulfhydryl groups are essential for enzymatic activity. (e) It does not have an activator among the more common metallic ions. (f) It is fairly resistant to oxidation but susceptible to reduction.

BIBLIOGRAPHY

STUDIES ON PENICILLINASE: II. MANOMETRIC METHOD OF 
ASSAYING PENICILLINASE AND PENICILLIN, KINETICS OF THE 
PENICILLIN-PENICILLINASE 
REACTION, AND THE EFFECTS OF 
INHIBITORS ON PENICILLINASE 
Richard J. Henry and Riley D. Housewright 