Spectropolarimetric and Circular Dichroism Methods for Determining the Activity of Penicillin

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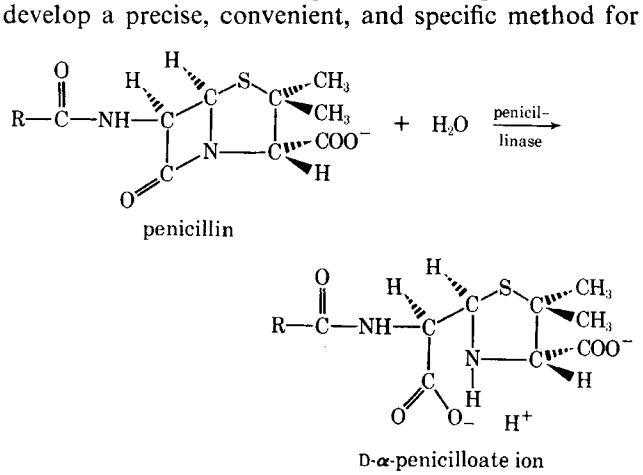
Abstract □ Experimental and mathematical analysis of an analytical spectropolarimetric method for determination of penicillins shows that this approach can yield precisions of the order of one part per thousand or better. The method, based on optical rotatory changes elicited by enzymatic catalysis, is sufficiently sensitive to detect losses in drug activity occurring in solution at room temperature in periods of less than 1 hr. Applications of the procedure to both penicillins sensitive and resistant to penicillinase catalysis and their mixtures are presented.

Keyphrases □ Penicillin, activity—determination using spectropolarimetry and circular dichroism □ Spectropolarimetry—determination, penicillin activity □ Circular dichroism—determination, penicillin activity

An interesting polarimetric assay procedure specific for penicillins susceptible to penicillinase catalysis was proposed by Levy (1) in 1951. This method, in essence, related the penicillin concentration to the changes in optical rotation of an aqueous solution of penicillin observed after hydrolysis of the β-lactam ring by penicillinase (Scheme I). The change in rotation was determined on a custom-built recording polarimeter (2), which used a mercury vapor lamp and two filters to isolate the mercury line (5460 Å). The polarimetric assay procedure never became too popular since most measurements were made in the visible region where the sensitivity was low. In addition, the change in rotation due to the mutarotation of the penicilloate ion was relatively large. The selection of a proper end-point was never determined, thus reducing the precision of this assay procedure.

The specific aim of the present investigation was to develop a precise, convenient, and specific method for the analyses of penicillins based on the use of a more sensitive, commercially available recording spectropolarimeter. Any changes in optical activity resulting from the addition of penicillinase, a specific enzyme for the hydrolysis of the β-lactam ring, to an aqueous solution of penicillin is a direct result of the action of this enzyme on the penicillin. The presence of other optically active materials has no effect on the assay, since the assay relates the change in optical activity arising from the cleavage of the β-lactam ring by this specific enzyme to the penicillin concentration.

These studies included application of the method to the analyses of both nonresistant and resistant classes of penicillin. The structural formulas of those studied and the names used in this paper are given in Table I.



Scheme I

PRESENT ASSAY METHODS

Several chemical methods are available for the analysis of penicillin (3). Among these, the iodometric and hydroxamic acid methods are the two most widely used. Results from these two chemical methods are in good agreement with microbiological data for a relatively pure substance, but poor agreement was obtained with samples containing relatively large amounts of degraded penicillin.

The principle of the iodometric method (3–5) is that the penicilloate ion takes up iodine, while there is no consumption of iodine by the intact penicillin molecule. This method also can be made relatively specific by utilizing enzymatic cleavage of the β-lactam ring. This method is not, however, applicable to penicillins, such as cloxacillin, that possess unsaturation in their side chain, since the side chain takes up iodine regardless of whether the β-lactam ring is intact (4). The iodine consumption appears to depend upon pH and iodine concentration even for systems where it is used (3). The stoichiometry of the reaction is not known exactly, but it appears that 8–9 atoms of iodine are taken up by each molecule of benzylpenicilloate (4). Because of this variation, the iodine equivalence must be determined by analyzing samples of pure penicillin under the same assay conditions to be used in assaying the unknown samples. A blank estimate also must be made.

In the hydroxamic acid method, hydroxylamine reacts with the β-lactam ring of penicillin at pH 7 to form a hydroxamic acid (3–5). Addition of ferric ions to the hydroxamic acid solution produces a color which is stable for about 5 min. (4). Different penicillins produce different color intensities (4). Hydroxylamine reacts only with the intact β-lactam ring and not with penicilloate. On the other hand, hydroxylamine reacts with other compounds, such as esters, that contain a carbonyl group.

EXPERIMENTAL

Apparatus—Measurements of optical rotation and circular dichroism (CD) were made on a spectropolarimeter and with a CD accessory[1]. It was found that a 5-cm. cell could be removed,

[1] Cary 60 recording spectropolarimeter and Cary model 6001 circular dichroism accessory.

shaken, and replaced without causing a significant variation in the spectropolarimetric reading. Because this was not true for the 1-cm. cell, the 1-cm. cell was securely fastened to the cell holder with tape; the cell holder, with the cell in it, was then shaken.

Chemicals and Reagents—*Bacillus cereus* penicillinase solutions[2] were prepared by dissolving one vial (800,000 units/vial) in 10 ml. of distilled water for the penicillinase-sensitive penicillin and one vial in 0.5 ml. of distilled water for the penicillinase-resistant penicillin. The penicillins used were potassium benzylpenicillin[3], phenoxymethylpenicillin acid[4], ampicillin B[5], and sodium cloxacillin[6].

Potassium benzylpenicillin[7] and sodium cloxacillin[8] were twice recrystallized by two different methods. In the first method, the penicillin salt was dissolved in a minimal amount of water and was precipitated by slowly adding approximately 20 volumes of 1-butanol (6, 7). The precipitate was then washed with ethyl acetate. In the second method, the penicillin salt was dissolved in a minimal amount of methanol and was precipitated by slowly adding approximately 5 volumes of ethyl acetate (6, 7). The precipitate was then washed with ethyl acetate.

Commercial grade ampicillin was once recrystallized by dissolving it in a minimal amount of 1 *N* hydrochloric acid at 70° and was precipitated by adding 2 *N* sodium hydroxide (8) at 70°. The precipitate was washed with boiling ethyl acetate.

Reagent grade potassium phosphate, monobasic and dibasic, sodium acetate, acetic acid, sodium carbonate, sodium hydroxide, and hydrochloric acid were used to prepare the buffer solutions.

Procedure for Penicillinase-Sensitive Penicillin Using Optical Rotation—An aqueous solution of the penicillin to be studied was prepared by rapidly dissolving 0.10–0.15 g., accurately weighed, of penicillin in 500 ml. of 0.1 *M* phosphate buffer solution, pH 7.0. A 5-cm. cell was filled with this aqueous penicillin solution and placed in the spectropolarimeter. The assay was carried out at a constant wavelength of 255 nm. (or 247 nm.), with a spectral bandwidth of 40 Å (slitwidth of 2.4 mm.), a chart speed of 25 sec./division, and usually a full range scale of 0.4 deg. The pen center was set at zero, and the pen was adjusted with the manual zero and zero suppression to the 1.0 line on the chart paper.

With the filled cell in place, the rotation was recorded for 1–2 min. Then 50 μl. of penicillinase solution (80,000 units/ml.) was rapidly injected into the cell, which was shaken and then replaced, and the recording was reinitiated.

Since spontaneous degradation of penicillin begins as soon as an aqueous solution is prepared, the time between the preparation of the solution and the assay was recorded. The timing was started as soon as the penicillin was dissolved and recorded when the cell was shaken. A kinetic curve of the rapid drop in rotation *versus* time was recorded (Fig. 1). When the pen reached zero on the chart paper, the pen center was switched from zero to 1.0 so that the change in rotation covered twice the width of the chart paper. The pen continued to drop rapidly until all the penicillin was degraded by the penicillinase. This was followed by a slow reduction in the rotation of the solution.

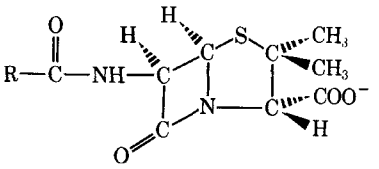
After each assay, the cells were filled with 3 *N* nitric acid and allowed to stand for a few minutes to inactivate the residual enzyme.

Procedure for Penicillinase-Sensitive Penicillin Using CD—For this situation, a wavelength of 231 nm. was used. This required that a smaller concentration of penicillin and a full range of 0.2 deg. be used. Approximately 3.5 mg., accurately weighed, of benzylpenicillin was dissolved in 50 ml. of buffer solution. Except for these modifications, the procedure described for the penicillinase-sensitive system using optical rotation was followed.

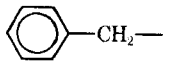
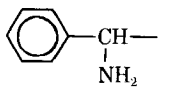
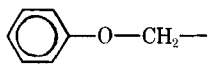
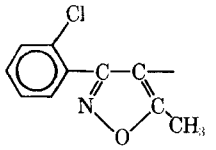
Procedure for Penicillinase-Resistant Penicillins Using Optical Rotation—For these samples, somewhat greater concentrations of both penicillin and penicillinase were used along with a reduction in the pathlength. Approximately 50–60 mg., accurately weighed, of the penicillin to be studied was dissolved in 100 ml. of 0.1 *M* phosphate buffer solution, pH 7.0, and a 1-cm. cell, which was affixed in a permanent position to the cell holder, was filled with this solution. Fifty microliters of penicillinase solution (1,600,000 units/ml.) was injected into the cell. Except for these modifications, the procedure described for the penicillinase-sensitive system using optical rotation was followed.

Procedure for Assaying a Mixture of a Penicillinase-Sensitive and a Penicillinase-Resistant Penicillin—Approximately 0.085–0.090 g. of the mixture, accurately weighed, was dissolved in 100 ml. of 0.1 *M* phosphate buffer solution, pH 7.0. A 1-cm. cell, securely fastened to the cell holder, was filled with this solution, and a baseline for the rotation of the active mixture was run on the spectropolarimeter. Ten microliters of penicillinase solution (80,000 units/ml.) was rapidly injected into the solution in the cell, the cell and cell holder were shaken and then replaced, and the recording was reinitiated. A kinetic curve of the rapid drop in rotaton *versus* time was re-

Table I—Structure of Penicillins Used in This Study



general formula for penicillin

Side Chain (R)	Penicillin
	Benzylpenicillin
	Ampicillin
	Phenoxymethylpenicillin
	Cloxacillin

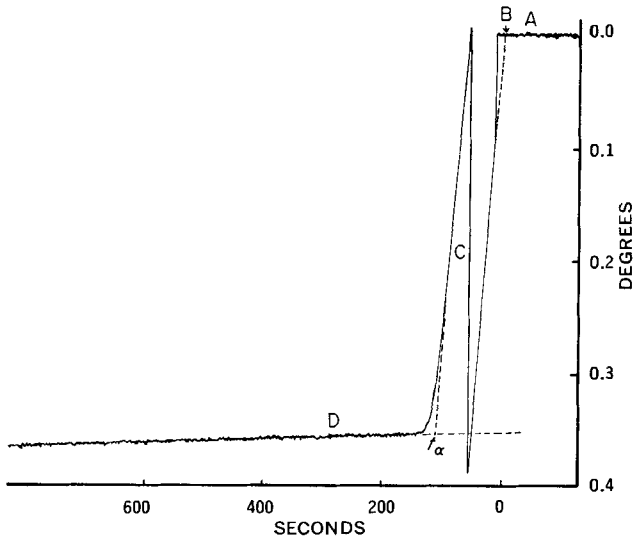


Figure 1—*Typical polarimetric assay recording of benzylpenicillin, with the extrapolated lines showing how the proper end-point was selected.*

[2] Donated by Riker Laboratories, Northridge, Calif., Neutrapen injectable.
[3] Wyeth's house standard, 1591 units/mg., control No. W620248-S1.
[4] Wyeth's house standard, 1664 units/mg., control No. E16-1.
[5] Wyeth's reference standard, 961 mcg./mg., lot No. C-10351. Wyeth samples were donated by Wyeth Laboratories.
[6] Ayerst reference standard, 905 mcg./mg. as the free acid, lot No. BRL-1621, and commercial grade, both furnished by Ayerst Laboratories.
[7] Purchased from Cal-Biochem.
[8] Ayerst commercial grade.

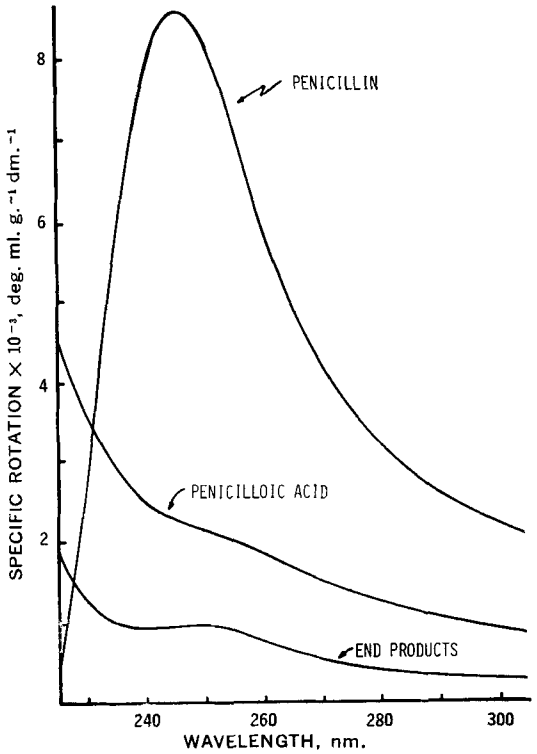


Figure 2—*ORD spectra of benzylpenicillin,* D-α-*benzylpenicilloate, and the final equilibrated mixture in a 0.1* M *phosphate buffer solution, pH 7.00.*

corded. This curve represented the degradation of the penicillinase-sensitive penicillin by penicillinase.

For the determination of the amount of penicillinase-resistant penicillin, the special 1-cm. cell was filled with a fresh sample of the above solution and a baseline of the active penicillins was run. Then 50 μl. of penicillinase solution (1,600,000 units/ml.) was

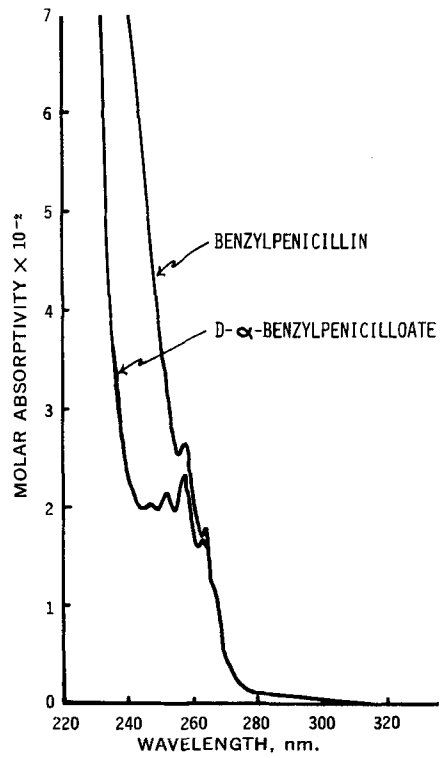


Figure 3—*UV spectra of benzylpenicillin and* D-α-*benzylpenicilloate ion in 0.1* M *phosphate buffer solution, pH 7.00.*

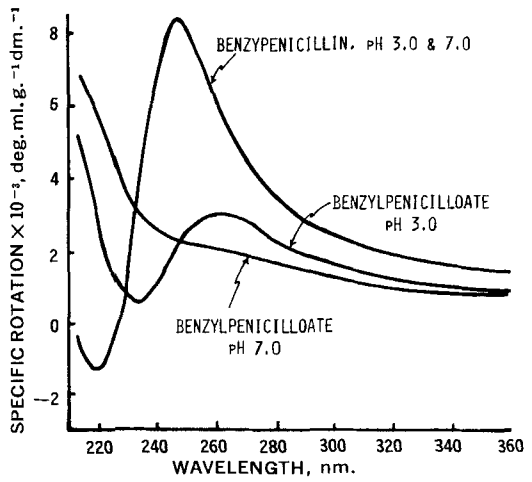


Figure 4—*ORD spectra of benzylpenicillin and its penicilloic acid derivative at pH 3.00 and 7.00.*

injected, and the recording was reinitiated. The observed curve represented the degradation of both the penicillinase-sensitive penicillin and the penicillinase-resistant penicillin.

RESULTS AND DISCUSSION

Typical Polarimetric Assay Curve—Figure 1 shows a typical polarimetric recording obtained at 255 nm. as a function of time for a sample of benzylpenicillin following the addition of penicillinase. Segment A represents the optical rotatory activity of the penicillin prior to the addition of the enzyme. At Point B, penicillinase solution was injected, producing the observed rapid linear decrease in rotation (Segment C) corresponding to the enzymatic cleavage of the β-lactam ring to form D-α-benzylpenicilloate. This was followed by a very slow decrease in rotation seen in the terminal phase (Segment D) due to the mutarotation of D-α-benzylpenicilloate (9). This observed behavior is typical of all penicillinase-sensitive penicillins.

Optical Rotary Dispersion (ORD) and UV Spectra for Benzylpenicillin and Its Degraded Products—ORD and UV spectra of benzylpenicillin and its degraded products were recorded in order to select an appropriate wavelength for the assay. The ORD spectra for benzylpenicillin, D-α-benzylpenicilloate, and the final equilibrated mixture of the degraded product are shown in Fig. 2.

The optical activities of penicillins in the UV and visible regions are largely due to the $n-\pi^*$ transition (10) of the carbonyl group in the β-lactam ring. It can be seen from the CD spectrum of benzylpenicillin (Fig. 11) that this transition has its maximum at 231 nm. When penicillins are degraded by penicillinase, the β-lactam ring is cleaved, thus causing a large reduction in the optical rotation in the UV and visible regions. The degraded product, D-α-benzylpenicilloate ion, then undergoes mutarotation giving the final ORD spectrum of the equilibrated mixture.

Figure 2 shows that the maximum change in optical rotation, arising from the cleavage of the β-lactam ring, occurs at the maximum in the ORD spectrum of benzylpenicillin, 247 nm. In this region, the change in optical rotation ascribable to the mutarotation is relatively small compared to the change in optical rotation due to the cleavage reaction. In the visible region, however, the magnitude of the mutarotation is almost the same as the magnitude of the cleavage reaction. However, the rate of change for the mutarotation step is much slower. This means that the relative magnitude of the terminal change in Fig. 1 would be much greater in the visible region.

Figure 3 shows the UV spectrum for benzylpenicillin and its degraded products. There is a rapid increase in absorption below 255 nm., somewhat limiting the concentration of penicillin that can be used in the assay. For this reason, the assays were carried out at both 247 and 255 nm.

Effect of pH on ORD Spectra of Benzylpenicillin and Its Degraded Products and on Change in Specific Rotation—Figure 4 shows the ORD spectra of benzylpenicillin and its penicilloic acid derivative at pH 7.00 and 3.00. The ORD spectra of the intact

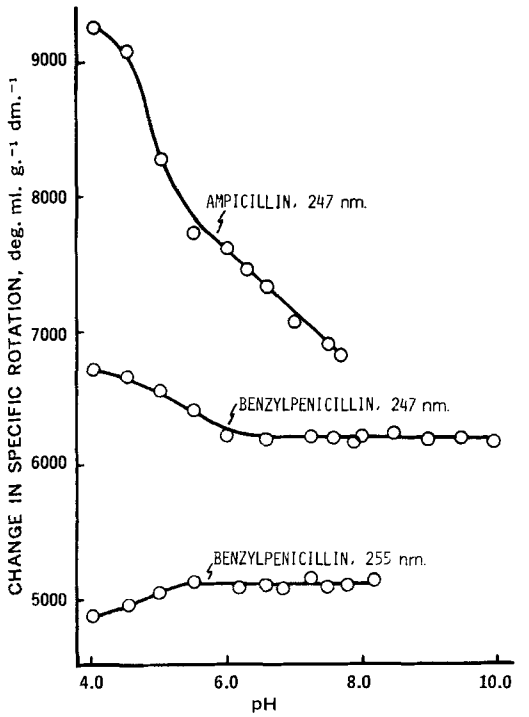


Figure 5—*Effect of pH on the change in specific rotation for benzylpenicillin at 247 and 255 nm. and for ampicillin at 247 nm. due to the catalytic hydrolysis of the β-lactam ring by penicillinase.*

penicillin at these two pH's are essentially the same, while there is a significant difference in the spectra of benzylpenicilloate. At pH 7.00, the carboxyl group formed by the cleavage of the β-lactam ring (pKa = 4.7) (11, 12) is essentially unprotonated; at pH 3.00, this carboxyl group exists mainly in its protonated form. When this carboxyl group is protonated, there is a positive cotton effect around 243 nm., while the unprotonated species has a positive plain ORD curve in this region.

Figure 5 shows the pH profiles for the change in specific rotation due to the hydrolysis of the β-lactam ring for benzylpenicillin at 247 and 255 nm. and for ampicillin at 247 nm.

Selection of End-Point—To convert the results from a polarimetric curve (Fig. 1) to an accurate quantitative measure of the amount of penicillin contained in the original sample, the penicillin concentration must be related directly to the decrease in the optical rotatory properties of the solution produced by the action of the enzyme. Levy (1) simply used the intersection of an extrapolated line from the initial rapid apparent zero-order cleavage reaction and an extrapolated line from the slower mutarotation. However, this point is dependent on the amount of enzyme used, particularly in the visible region where the relative change in the mutarotation step is large. In this section, the problem is analyzed theoretically to yield the best way of estimating the theoretical ΔR equivalent to an instantaneous cleavage reaction.

The overall rate of the catalyzed cleavage reaction obeys the classical Michaelis-Menten mechanism for enzymatic systems (13):

$$\underset{(S)}{\text{penicillin}} + \underset{(E)}{\text{penicillinase}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(ES)}{\text{enzyme–substrate complex}}$$

$$\underset{(ES)}{\text{enzyme–substrate complex}} \xrightarrow{k_2} \underset{(P)}{\text{penicilloate}} + \underset{(E)}{\text{penicillinase}}$$

$$\underset{(P)}{\text{penicilloate}} \xrightarrow[\text{(mutarotation)}]{k_3} \underset{(P')}{\text{diastereoisomers}}$$

It can readily be shown that the rate of degradation in these systems is given by (14):

$$\frac{-d[S]}{dt} = \frac{k_2 E_T [S]}{K_m + [S]} \qquad \text{(Eq. 1)}$$

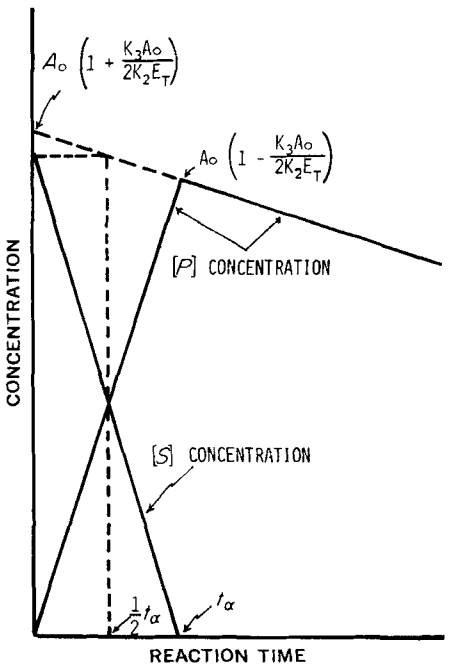


Figure 6—*Schematic drawing of the penicillin and its penicilloic acid derivative concentrations as a function of time for a penicillinase-sensitive penicillin.*

where [S] is the concentration of the penicillin, K_m is the Michaelis constant, k_2 is the rate constant for the breakdown of the penicillin–enzyme complex, and E_T is the total concentration of the enzyme. Since K_m for the penicillinase-sensitive penicillins is always very small, it can be neglected in the denominator except at very low substrate concentrations. Thus, at higher residual penicillin concentrations, a zero-order kinetic equation is obtained: $(-d[S]/dt) = k_2E_T$.

For Penicillinase-Sensitive Penicillin—Schematic plots of penicillin and penicilloic acid concentrations *versus* time for a penicillin–penicillinase system having a low K_m value are shown in Fig. 6. To obtain a precise measurement for the change in rotation due only to the cleavage of the β-lactam ring by penicillinase, a point must be selected on the extrapolated mutarotation curve of the penicilloic acid which represents the concentration of penicilloic acid equivalent to the initial concentration of penicillin and which has not undergone any mutarotation. In the following derivation, it is shown that this time can be approximated at $^1/_2t_\alpha$, where t_α is the hypothetical time required to complete the cleavage on the assumption that the reaction is strictly zero order.

In the strictly zero-order range, the kinetic rate equation for the disappearance of [S] can be approximated by: $S_0 - [S] = k_2E_Tt$, the integration of Eq. 1. When $t = t_\alpha$, $[S] = 0$; therefore:

$$t_\alpha = \frac{S_0}{k_2E_T} \qquad \text{(Eq. 2)}$$

Since the secondary change in rotation is presumably pseudo-first order, the differential rate equation describing [P] between $t = 0$ and $t = t_\alpha$ is:

$$\frac{d[P]}{dt} = k_2E_T - k_3[P] \qquad \text{(Eq. 3)}$$

Upon integrating and solving for [P]:

$$[P] = \frac{k_2E_T}{k_3}(1 - e^{-k_3t}) \qquad \text{(Eq. 4)}$$

By expanding the exponential part in a power series and evaluating the expression at t_α, the following equation is obtained:

$$[P]_{t=t_\alpha} = S_0 - \frac{k_3S_0^2}{2k_2E_T} + \ldots \qquad \text{(Eq. 5)}$$

For $t > t_\alpha$, after all the penicillin has been degraded to penicilloic

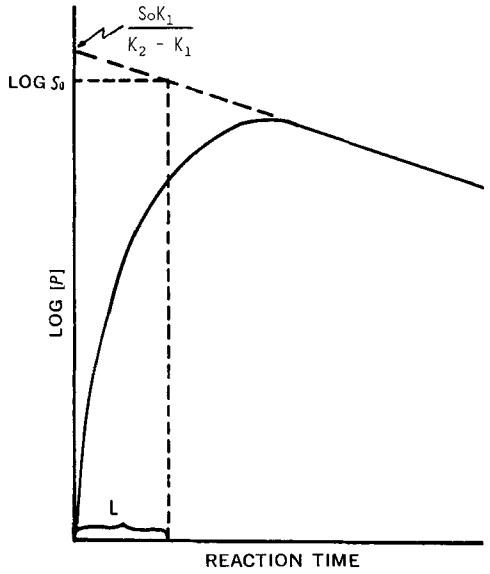


Figure 7—*Schematic drawing of the logarithm of the penicilloate-ion concentration* versus *reaction time for a penicillinase-resistant penicillin.*

acid, the secondary change in rotation is described by:

$$\frac{d[P]}{dt} = -k_3[P] \quad \text{or} \quad [P] = Ce^{-k_3t} \qquad \text{(Eq. 6)}$$

The integration constant, C, can be evaluated by equating Eqs. 4 and 6 at t_α:

$$Ce^{-k_3t_\alpha} = \frac{k_2E_T}{k_3}(1 - e^{-k_3t_\alpha}) \qquad \text{(Eq. 7)}$$

By solving for C, expanding the exponential in a power series, and substituting the expression for t_α (Eq. 2), Eq. 8 is obtained:

$$C = S_0 + \frac{k_3S_0^2}{2k_2E_T} + \ldots \qquad \text{(Eq. 8)}$$

The integration constant, C, represents the hypothetical concentration of $[P]$ required at $t = 0$ for a hypothetical system in which there is only P going to P' (*i.e.*, no S going to P) to give the same concentration changes for $t > t_\alpha$ in the above $S \rightarrow P \rightarrow P'$ system.

Now the problem is to select some time on the extrapolated P curve that represents the concentration of P equivalent to S_0. This point can be approximated by using Eq. 6, with a power series expansion of the exponential, and Eq. 8. Or it can be more easily seen graphically by extrapolating the right-hand portion of the mutarotation curve back to zero time. This point does not yield a concentration value corresponding to the initial penicillin concentration but is too high by the amount $(S_0^2k_3/2k_2E_T)$ (Eq. 8). On the other hand, the intersection of the initial zero-order cleavage

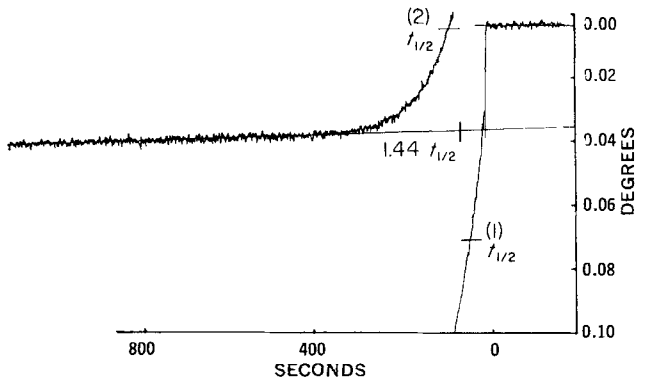


Figure 8—*Typical polarimetric assay recording with the extrapolated mutarotation line for cloxacillin in a 0.1* M *phosphate buffer solution, pH 7.00.*

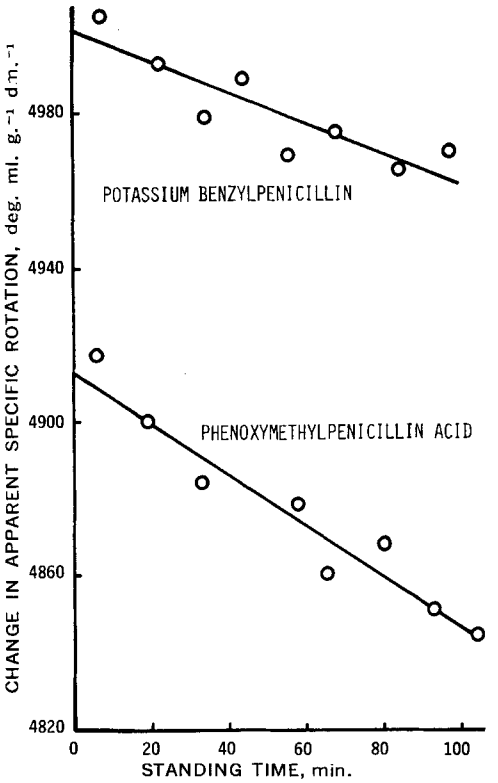


Figure 9—*Plot showing the decrease in the assay values for benzylpenicillin and phenoxymethylpenicillin as a function of the time the solutions were standing at room temperature prior to assaying.*

reaction and the slow mutarotation step occurring at t_α give a concentration value too low by the same amount (Eq. 5). On this basis, it is proposed that the optical rotational value of penicilloate that can be correlated with the initial penicillin concentration occurs at $^1/_2t_\alpha$ on the optical activity–time plot (Fig. 1) for penicillins that cleave largely through zero-order kinetics. The change in optical rotation related to the penicillin concentration was simply obtained by subtracting the value for the rotation at $^1/_2t_\alpha$ on the extrapolated mutarotation line from the initial rotation for the intact penicillin.

For Penicillinase-Resistant Penicillin—Since these penicillins did not follow the conditions already stated but instead exhibited pseudo-first-order kinetics with respect to the penicillin for the cleavage reaction, a different approach must be used. In Fig. 7, a behavior of this type is evident in a polarimetric plot obtained during the cleavage of cloxacillin in the presence of *Bacillus penicillinase.*

In these instances, there are two consecutive first-order reactions:

$$S \xrightarrow{k_1} P \xrightarrow{k_2} P'$$

where k_1 is the pseudo-first-order rate constant for the catalytic hydrolysis of the β-lactam ring, and k_2 is the first-order rate constant for the mutarotation.

The concentration of D-α-penicilloate, P, is given by (15):

$$[P] = \frac{S_0k_1}{k_2 - k_1}(e^{-k_1t} - e^{-k_2t}) \qquad \text{(Eq. 9)}$$

Since $k_1 >>> k_2$, e^{-k_1t}, at sufficiently large time, is very small compared to e^{-k_2t} and can be neglected. Under these conditions:

$$[P] = \frac{S_0k_1}{k_2 - k_1}e^{-k_2t} \qquad \text{(Eq. 10)}$$

and a plot of log $[P]$ against t is linear. If this linear portion of the curve is extrapolated back (Fig. 7), it passes through a point where log $[P]$ = log S_0. The time at which log $[P]$ = log S_0, L, is related to the rate constants by:

$$1 = \frac{k_1}{k_2 - k_1}e^{-k_2L} \qquad \text{(Eq. 11)}$$

Table II—Apparent Change in Specific Rotation for Some Penicillinase-Sensitive Penicillins Due to Catalytic Hydrolysis of β-Lactam Ring by Penicillinase

Penicillin	Concentration, mcg./ml.	Number of[a] Assays	$[\Delta\alpha]^{25°}_{255\ nm.}$[b]	Standard Deviation[c] from Line
Potassium benzylpenicillin				
Commercial house standard,	320.14	8	5003.3 ± 6.0	8.3
1591 units/ml.	279.71	8	5029.2 ± 6.1	7.7
	197.40	8	5020.6 ± 6.3	17.6
	260.35	8	5038.2 ± 5.8	7.2
	313.00	8	5000.8 ± 6.0	7.4
	56.90	8	5058.0 ± 5.8	14.6
	316.99	9	4999.8 ± 5.5	6.9
Recrystallized from methanol	305.20	3	5035.5 ± 8.5	11.4
with ethyl acetate	242.18	5	5061.4 ± 7.6	7.6
Recrystallized from water with	240.28	9	5061.7 ± 5.2	8.0
n-butanol	283.30	5	5101.5 ± 6.8	16.8
	276.60	10	5009.2 ± 5.2	9.7
Ampicillin B				
Commercial reference standard,	264.60	8	5688.0 ± 6.1	8.4
961 mcg./mg.	264.60	8	5665.7 ± 5.9	12.4
	255.13	3	5692.2 ± 9.9	13.9
	251.94	5	5670.0 ± 9.3	7.3
	222.00	14	5622.9 ± 4.3	20.8
Recrystallized from water	263.18	8	5533.1 ± 5.6	27.7
	263.18	8	5591.4 ± 5.3	27.3
Phenoxymethylpenicillin acid				
Commercial house standard,	297.71	8	4904.9 ± 5.8	5.8
1664 units/mg.	297.56	9	4901.3 ± 5.7	6.2
	290.47	8	4911.7 ± 5.6	7.2
	308.01	5	4906.5 ± 16.4	8.1
	303.31	8	4912.6 ± 5.7	6.8
	291.15	8	4919.5 ± 5.9	7.3
	301.78	8	4926.3 ± 7.1	12.1

[a] The number of times the solution was assayed. [b] The change in specific rotation at zero standing time was obtained from the intercept of a regression line through a plot of $[\Delta\alpha]^{25°}_{255\ nm.}$ against the time the solution was standing before being assayed (Fig. 9). These values are not corrected for purity or dilution. The standard error in the intercept (*Reference 13*) was also recorded. [c] This value represents the deviation of the experimental points for the regression line (Fig. 9).

If this identity is expanded in a power series:

$$k_1 - k_2 = k_1(1 - k_2L + \ldots) \qquad \text{(Eq. 12)}$$

and all terms greater than the linear term are neglected, the following expression is obtained:

$$L = \frac{1}{k_1} = \frac{(1/0.693)}{t_{1/2}} = 1.44t_{1/2} \qquad \text{(Eq. 13)}$$

where $t_{1/2}$ is the first half-life of the reaction ($S \rightarrow P$).

Thus, in Fig. 8, the value of rotational change that would be affected only by the initial penicillin concentration and independent of other variables in the system would occur only at the indicated point. Since the zero time is not known, the time between the first two half-lives was determined, multiplied by 0.44, and added to the first half-life time to obtain the proper end-point.

Correction for Spontaneous Degradation of Samples—Another important factor, which must be considered in the development of any really precise analytical method for these relatively unstable antibiotics, is the significant spontaneous degradation that takes place as the sample stands. The loss is usually negligible for dry samples but becomes significant in aqueous solutions. Figure 9 shows a typical plot for the enzyme-induced change in apparent specific rotation at room temperature and pH 7.0 with standing time for a 0.1 *M* phosphate buffer solution of benzylpenicillin and phenoxymethylpenicillin. In this study, assays were carried out on benzylpenicillin, ampicillin, phenoxymethylpenicillin, and cloxacillin. At room temperature and under the conditions generally employed, losses of approximately 1% were observed in 170 min. for benzylpenicillin and cloxacillin and in 90 min. for ampicillin and phenoxymethylpenicillin. The intercept represents the change in apparent specific rotation for the original dry sample before any spontaneous degradation of an aqueous solution occurs. Because of the much greater precision of the proposed method, this approach is necessary to take full advantage of its capability. With conventional methods, the losses in sample activities on standing are essentially ignored, even though the time involved may be considerable.

Precision and Accuracy of Assay Procedure—The precision is a measure of the deviation of the individual results from their mean value (16). Here, standard deviation is used to express the precision of the assay. To determine the precision of this procedure, assays were carried out several times on the same solution. Since the change in apparent optical rotation decreased with the time the sample was standing prior to assay, due to spontaneous degradation of the penicillin (Fig. 9), the standard deviation from a mean value cannot be used. Instead, the standard deviation from a regression line is used as a measure of the precision. The values given in the last column in Tables II and III represent the standard deviation of the experimental points from the regression line and, therefore, are measures of the precision for a single measurement.

Table III—Apparent Change in Specific Rotation at Zero Standing Time for a Pencillinase-Resistant Penicillin Due to Catalytic Hydrolysis of β-Lactum Ring by Penicillinase

Sodium Cloxacillin	Concentration, mcg./ml.	Number of Assays	$[\Delta\alpha]^{25°}_{255\ nm.}$[b]	Standard Deviation[c] from Line
Commercial grade	503.65	12	2681.8 ± 8.8	15.9
Commercial reference standard, 905 mcg./mg. as free acid	496.85	5	2799.0 ± 13.2	5.0
	503.70	5	2808.9 ± 13.2	14.5
Recrystallized from water with *n*-butanol	608.25	9	2877.1 ± 9.6	14.5
Recrystallized from methanol with ethyl acetate	678.45	3	2792.7 ± 16.0	2.7

[a,b,c] See corresponding footnotes in Table II.

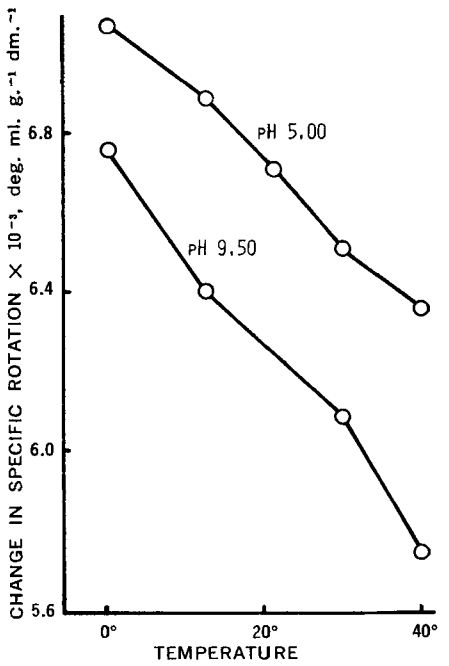


Figure 10—*Plot showing the effect of temperature on the change in specific rotation at 247 nm. due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase at pH 5.00 and 9.50.*

The joint estimate of standard deviation[9] of the change in apparent specific rotation for the experimental points from their regression lines for the penicillinase-sensitive penicillin was 13.3 deg. ml. g.$^{-1}$ dm.$^{-1}$, and it was 14.2 deg. ml. g.$^{-1}$ dm.$^{-1}$ for the penicillinase-resistant penicillin. This represents a precision of 0.27 and 0.50%, respectively. The instruction manual for the spectropolarimeter (17) claims a precision of 0.2% full-range scale [25.4 cm. (10 in.)] for a single reading when not limited by noise. Most of this error can be attributed to chart printing error. Since two readings and the switching of the pen center are required in this assay procedure, a precision much greater than 0.3% should not be expected if readings are taken off the chart.

Thus, the precision offered by this polarimetric method appears to be far superior to any of the presently available assay procedures. The hydroxylamine and iodometric methods do not give precisions greater than 1–2%, whereas the microbiological methods are at best semiquantitative procedures. In this polarimetric assay procedure, a precision of at least 0.3% was obtained. A far greater precision probably could be obtained by introducing some modifications into the method to improve the accuracy of the readout. Intrinsically, the method appears to be capable of readily yielding precisions of the order of a few parts per 10,000 since polarimetric readings can discern differences of a few tenths of a millidegree.

These polarimetric assay procedures are based on relating the intercept value, obtained from a linear plot of the change in apparent specific rotation against standing time for the aqueous solution, to the corresponding values for an absolute pure compound. Thus, the accuracy for these assay procedures depends on the determination of the absolute changes in specific rotation expected for the various penicillins. The intercept value of the regression lines along with the standard error in determining the intercept are given in Tables II and III for nonresistant and resistant penicillins, respectively. The values accepted in this investigation for the absolute change in specific rotation for the pure penicillins used in this study are given in Table IV. They were obtained by correcting the intercept values (Tables II and III) for the purity of the dry sample and averaging them. The value for benzylpenicillin appears to be very close to the true value. The value accepted here was the overall average for all the assays on the benzylpenicillin recrystallized both from water with 1-butanol and from methanol with ethyl acetate. This value was slightly larger than the average value obtained from the commercial reference standard[3]. The values for ampicillin[5], phenoxymethylpenicillin acid[4], and sodium cloxacillin[6] were obtained from a single reference standard, so the accuracy of these values are dependent on the labeled value of their respective reference samples and the purity at the time of assay. These values are probably quite accurate but not as dependable as the value given to potassium benzylpenicillin.

Table V shows a comparison of the spectropolarimetric penicillinase assay procedure to microbiological and iodometric assay procedures. Ampicillin samples, whose microbiological assays were in good agreement with the chemical assays, were received[10] along with the microbiological and chemical assay data. The values from the spectropolarimetric penicillinase method were obtained approximately 1 month after the microbiological and chemical assays.

For any real precise assay method, there is another important source of error: the sampling error. Several lots of the penicillin with varying purity are mixed together, so the batch may not be completely homogeneous in purity. Since only very small samples (usually less than 150 mg. to prepare a 500-ml. solution) are used, the sampling error can become significant. This sampling error could account for some of the variation shown in Table V.

Application of Optical Procedure for Assaying a Mixture of Penicillinase-Sensitive Penicillin and Penicillinase-Resistant Penicillin—The results from assaying mixtures of ampicillin (a nonresistant penicillin) and cloxacillin (a resistant penicillin) by optical rotation at 255 nm. are given in Table VI. In this table, a comparison was made of the concentration of the penicillins used to prepare the solutions and the activity of the penicillins determined by the assay. The activities of the penicillins used for preparing the mixtures were separately determined from the optical rotatory assay procedure for each respective penicillin.

The mixtures were prepared from known amounts of each penicillin. The ampicillin concentration was assayed by injecting 10 μl. of penicillinase solution (80,000 units/ml.) into the mixture. The selection of an end-point was obtained by adjusting the point at zero time (*i.e.*, the time at which the penicillinase solution was injected) on the extrapolated slower decreasing terminal phase. A small correction was made by extrapolating from the end-point at zero time to $^1/_2t_\alpha$ on a line with a slope obtained by averaging the slopes for the mutarotation phase from the assays of ampicillin alone. This correction was only an increase of 0.3–0.4% over the assayed value for ampicillin obtained at zero time.

The concentration of cloxacillin in the mixture was determined by subtracting the change in optical rotation determined for ampicillin from the total change in optical rotation for both penicillins. The total change in optical rotation was obtained by injecting 50 μl. of penicillinase solution (1,600,000 units/ml.) into the mixture. The selection of an end-point for the total change in rotation was essentially the same as the procedure described previously for penicillinase-resistant penicillins.

From Table IV, it can be seen that the change in specific rotation for ampicillin is about twice the change in specific rotation for

Table IV—Values for Change in Specific Rotation at 255 nm. Assigned to Pure Penicillin

Penicillin	$[\Delta\alpha]^{25°}_{255\ nm.}$[a], deg. ml. g.$^{-1}$ dm.$^{-1}$
Potassium benzylpenicillin	5053.9
Ampicillin (anhydrous acid)	5897.8
Penicillin V acid	5006.3
Sodium cloxacillin	2956.3

[a] The values given here are averages of the change in apparent specific rotation given in Tables I and II corrected for purity but not for the dilution caused by injecting 0.05 ml. of a penicillinase solution into 16 ml. of the penicillinase-sensitive penicillin solution or 0.05 ml. of a penicillinase solution injected into 3.2 ml. of the penicillinase-resistant penicillin solution.

[9] $$\sigma = \sqrt{\frac{\Sigma_i(n_i - 2)s_i^2}{\Sigma_i(n_i - 2)}}$$

where n_i is the number of experimental points used to determine the regression line, and s_i is the standard deviation of the points from their regression line. The summation is over all the regression lines to be combined.

[10] From Ayerst Laboratories, Rouses Point, N. Y.

Table V—A Comparison of Penicillinase–Spectropolarimetric Method to Microbiological and Iodometric Methods for Analyzing Activity for Several Samples of Ampicillin

Ampicillin Sample	Microbiological Assay[a] —Commercial Laboratory— Number 1	Number 2	Iodometric Method[a] ——Commercial Laboratory—— Number 3	Number 1	Number 4	Penicillinase–Spectropolarimetric Method		
Trihydrate	840	848	850	824	—	814	810	—
Trihydrate	843	746	855	839	—	819	812	824
Trihydrate	860	848	863	835	—	824	818	—
Trihydrate	841	848	860	840	—	818	813	—
Trihydrate	846	840	858	—	—	830	827	—
Trihydrate	821	826	847	836	—	823	823	—
Anhydrous	956	—	976	950	961	957	957	—

[a] Data furnished by Ayerst Laboratories, Rouses Point, N. Y.

cloxacillin. Therefore, any error in determining the ampicillin concentration would have twice the effect on the determination of the cloxacillin concentration. For example, if the ampicillin concentration was underestimated by 0.5%, then the cloxacillin concentration would be overestimated by 1%.

General Comments on These Assay Procedures—No effect on the change in apparent specific rotation due to the hydrolysis of the β-lactam ring by penicillinase was found when the ionic strength of the penicillin solution was changed from 0.005 to 1.0 *M*. Figure 10 shows the effect of temperature on the change in apparent specific rotation at 247 nm. due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase at pH 5.00 and 9.50.

The cell compartment in the recording spectropolarimeter has an operating temperature of 27°, while the room temperature was kept at 25°. Thus, there may have been a small change in rotation due to temperature change. The time required for the cleavage reaction to go to completion was approximately 2 min. Usually less than 5 min. elapsed from the time the solution was placed in the cell compartment until the completion of the cleavage reaction. Sometimes there was a slight decrease in the baseline for the intact penicillin due to temperature change. Later, when a temperature bath was used to bring the buffer solution to 27° prior to the preparation of the penicillin solution, this slight decrease in the baseline was almost completely eliminated.

At a constant pH and temperature, it was found that the spectropolarimetric penicillinase assay method for benzylpenicillin follows a Beer's-type law from a concentration of 1×10^{-5} to 1.3×10^{-3} *M*. At lower concentrations, there was greater deviation on both sides of the line since a small full range (0.02 deg.) was used, thus increasing the noise. A concentration of 2×10^{-6} *M* for benzylpenicillin and ampicillin was close to the smallest concentration that could be detected.

The presence of any other optically active material, such as sugars, does not affect the assay since the assay procedure relates the penicillin concentration to the changes in optical rotation caused by the cleavage of the β-lactam ring by penicillinase. The enzyme is very specific for the β-lactam ring, so the presence of degraded penicillin does not affect the assay. This was checked by assaying several samples of ampicillin which were subjected to degradation study at room temperature.

Sodium benzylpenicillin tablets, 250,000 or 400,000 units, were dissolved in 1 l. of the buffer solution. Without filtering, this solution with the suspended starch from the tablet was assayed at 255 nm. in a 5-cm. cell with a full range of 0.2 and 0.4 deg., respectively. The degradation curves were a little noisy due to the scattering of the light by the suspended starch, but an accurate assay could be made. When the solution was filtered, there was some loss of activity.

Since the change in specific rotation for ampicillin has a greater variation with pH than benzylpenicillin (Fig. 5), it is conceivable that a mixture of these two penicillins could be assayed by making measurements at two pH's, *e.g.*, at pH 7.50 where the difference between the change in specific rotation for these two penicillins is small and at pH 4.00 where this difference is significant. From these two readings, it should be possible to calculate the concentrations of both penicillins in the mixture.

Penicillin with UV absorption too great in the range of the present optical rotation measurement (247 or 255 nm.) can be assayed at a

Table VI—Assays of Mixtures of Ampicillin and Cloxacillin

Penicillin[a]	Weighed Concentration, mcg./ml.	Activity[b], mcg./ml.	Change in Rotation, deg.	Activity Measured, mcg./ml.	Percent Activity Assayed
Cloxacillin	342.00	332.7	0.0983	332.5	99.9
Ampicillin	405.00	381.9	0.2204	373.6	97.8
Cloxacillin	386.25	375.8	0.1116	377.5	100.4
Ampicillin	541.45	510.6	0.2940	498.5	97.6
Cloxacillin	380.20	359.3	0.1062	359.2	100.0
Ampicillin	453.70	427.8	0.2474	419.5	98.0
Cloxacillin	234.10	221.2	0.0639	216.1	97.7
Ampicillin	503.55	474.8	0.2777	470.8	99.2
Cloxacillin	438.45	414.3	0.1320	446.5	107.8
Ampicillin	189.45	178.7	0.1043	176.8	99.0
Cloxacillin	275.25	260.1	0.0796	269.4	103.6
Ampicillin	386.85	364.8	0.2108	357.4	98.0
Cloxacillin	297.45	281.1	0.0681	230.3	82.0
Ampicillin	365.45	344.6	0.1990	337.4	97.9
Cloxacillin	301.65	285.1	0.0896	303.1	106.3
Ampicillin	593.85	560.0	0.3256	552.1	98.6

[a] The ampicillin sample used in the mixtures was recrystallized from water at 70° (*Reference 8*). A purity of 94.3% was obtained by comparing the average change in specific rotation given in Table I to the change in specific rotation for a pure compound given in Table III. The first two mixtures contained sodium cloxacillin, which was recrystallized from water with *n*-butanol, and a purity of 97.3% was assigned by comparing the results in Table II to the value given in Table III. In the remaining six mixtures, the sodium cloxacillin was recrystallized from methanol with ethyl acetate, and a purity of 94.5% was assigned by comparing the results in Table II to the absolute value given in Table III. [b] Activity equals weighed concentration times the purity.

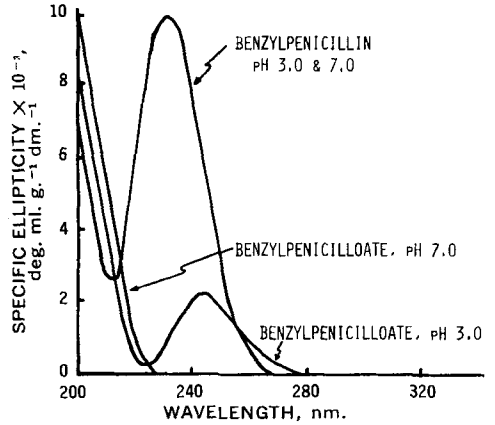


Figure 11—*CD spectra of benzylpenicillin and its penicilloic acid derivative at pH 3.0 and 7.0.*

longer wavelength, but this increases the relative magnitude of the mutarotation; hence, the selection of a proper end-point becomes more critical.

Results from Assaying Benzylpenicillin by CD—A CD phenomenon is associated with any medium that exhibits optical rotation. The CD spectra for benzylpenicillin and its penicilloic acid derivative at pH 7.00 and 3.00 are shown in Fig. 11. The CD spectra for the intact penicillin at these two pH's are essentially the same, showing a maximum around 231 nm., which corresponds to the cotton effect for benzylpenicillin in Fig. 4. However, there is a significant difference in the spectra of benzylpenicilloate at these two pH's. At pH 3.00, where the carboxyl group formed by the cleavage of the β-lactam ring is mostly protonated, a positive CD spectrum is observed between 225 and 260 nm., with a maximum at 243 nm. corresponding to the positive cotton effect in the ORD spectrum in Fig. 4. At pH 7.00, where this carboxyl group is mostly unprotonated, there is no CD spectrum for benzylpenicilloate in this region and, hence, no cotton effect is observed in the ORD spectrum. Therefore, for pH's above 5.50, the selection of an end-point was greatly simplified by the fact that the mutarotation step was not observed in the assay curves. The final reading was constant, and the change in the apparent specific ellipticity was obtained by subtracting the final reading from the initial reading. Below pH 5.50, the mutarotation could be observed with CD but not to the extent observed with optical rotation; thus the end-pcint of $^1/_2t_\alpha$ should be used. Figure 12 shows how the change in specific ellipticity at 231 nm. for benzylpenicillin varies with pH at 27°.

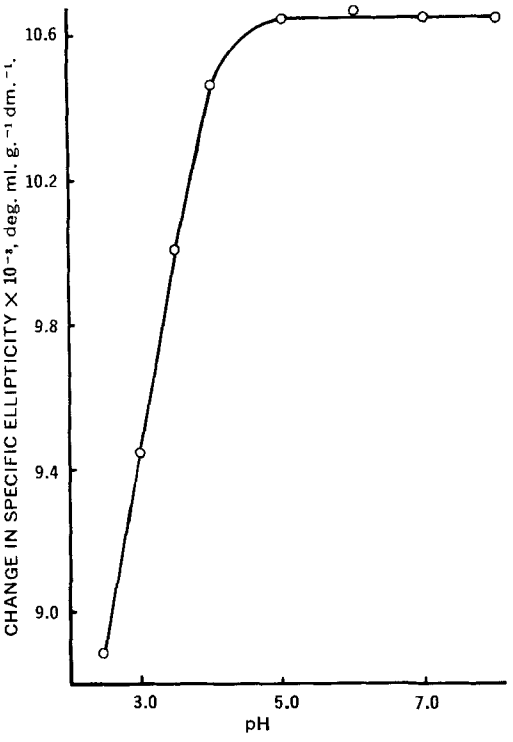


Figure 12—*Plot showing the effect of pH on the change in specific ellipticity at 231 nm. due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase.*

CD appears to be the method of choice when not limited by UV absorption at 231 nm. Since most of the penicillins used today have much greater UV absorption then benzylpenicillin, this procedure is only applicable to a few penicillins.

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