Table I—Half-Lives of Warfarin in Hours Estimated from Terminal Plasma Concentrations[a] Measured by Modified O'Reilly Assays and TLC Assays on Same Samples[b]

Subject	Treatment A (5 5-mg. Tablets) TLC	O'Reilly	Normalized Difference, %[c]	Treatment B (1 25-mg. Tablet) TLC	O'Reilly	Normalized Difference, %
1	33.6	28.8	−15.4	35.1	34.3	−2.3
2	24.6	27.1	9.7	29.9	29.7	−0.7
3	34.8	47.2	30.2	42.7	55.0	25.2
4	26.8	33.7	22.8	29.5	26.2	−11.8
5	40.8	31.1	−27.0	41.4	56.9	31.5
6	—	—	—	33.7	34.5	2.3
6a	49.5	44.2	−11.3	—	—	—
Averages	35.0[d]	35.4[d]	1.5	35.4[e]	39.4[e]	7.4

	TLC	O'Reilly	Normalized Difference, %
Overall averages	35.2[f]	37.4[f]	4.5

[a] Half-lives estimated by obtaining the slope of line, by the method of least squares, when ln C_p plotted *versus* t and dividing the absolute value of the slope into 0.693. Only plasma concentrations corresponding to times equal to or greater than 24 hr. were employed. [b] Data plotted in Fig. 4 of Welling *et al.* (2). [c] Normalized difference = half-life from O'Reilly assays − half-life from TLC assays/average half-life from O'Reilly and TLC assays × 100. [d] Difference in averages is not significant by paired t-test ($t = 0.097$, $p > 0.25$). [e] Difference in averages is not significant by paired t-test ($t = 1.27$, $p > 0.10$). [f] Difference in averages is not significant by paired t-test ($t = 0.95$, $p > 0.25$).

by the least-squares best fit line relating log warfarin concentration to time." The reader should realize that "two of four subjects" are not sufficient to make a decision that one assay leads to different half-lives than another assay, as was clearly shown in Fig. 4 of our original paper (2). Data plotted in that Fig. 4 are detailed in Table I of this communication. The modified O'Reilly assay gave a longer half-life (slower clearance rate) in six trials, but the TLC assay gave a longer half-life in exactly six other trials. The statistics presented in Table I indicate that the difference in average half-lives obtained by the two methods is not significant ($p > 0.25$). These data also show that the particular tablets administered did not affect the half-lives obtained, as would be expected since the half-lives were estimated after absorption ceased. A measured half-life includes assay error effects and is not an "absolute number" as many scientists would like it to be. Table I clearly shows this. One must have a much larger sample than Lewis' "two of four" to imply conclusions such as he did.

Lewis, in commenting on our blank values in the assay, also forgot that a blank is a function of not only the concentration of extraneous materials that absorb at the $\lambda_{max.}$ of the warfarin but also of the pathlength of the cell used. One cannot compare on a microgram equivalent of warfarin per milliliter (C) basis only but must compare on a C/L basis, where L is the pathlength of the cell used. This was done in our original paper (2) when we showed that our blank values were really essentially the same as those reported by O'Reilly *et al.* The average net absorbance of our subjects' zero-hour plasma was reported as 0.216, which is equivalent to 1.18 mcg. warfarin/ml., but our pathlength was 7.5 cm. Hence, our $C/L = 1.18/7.5 = 0.157$. Lewis, in his communication, gave a value of $C/L = 0.15/1 = 0.15$. Hence, the figures are essentially the same.

A key paper cited by Lewis (1) (his Reference 4) is still not published and has not been available to us.

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Received February 16, 1971.
Accepted for publication May 25, 1971.

Kinetic Demonstration of a Metastable Intermediate in Isomerization of Penicillin to Penicillenic Acid in Aqueous Solution

Keyphrases □ Benzylpenicillin methyl ester, isomerization—metastable intermediate kinetics □ Penicillin to penicillenic acid isomerization—metastable intermediate demonstrated

Sir:

Although several kinetic stability studies on penicillins have been performed [*e.g.*, benzylpenicillin (1, 2), phenethicillin (3), methicillin (4), ampicillin (5), and cloxacillin (6)], only a few studies have dealt with the mechanism of the hydrolytic reactions. As a part of a study concerning chemical reactions possibly involved in penicillin allergy, this paper reports preliminary results about the mechanism by which benzylpenicillin methyl ester in aqueous solution isomerizes to methyl benzylpenicillenate.

On the basis of experimental data by Krejci (7), Schwartz (8) showed that the degradation of benzylpenicillin in acidic aqueous solution is characterized by two parallel reactions. The formation of penicillenic acid is thought to be a result of the hydrogen-ion-catalyzed hydrolysis of the penicillinate ion or the kinetically equivalent uncatalyzed rearrangement of undissociated penicillinic acid.

It has now been found that the formation of penicillenic acid from the penicillin molecule goes through a metastable intermediate and that both undissociated and dissociated penicillinic acid are isomerized but to a different extent. In this brief report, only the results obtained with benzylpenicillin methyl ester are presented;

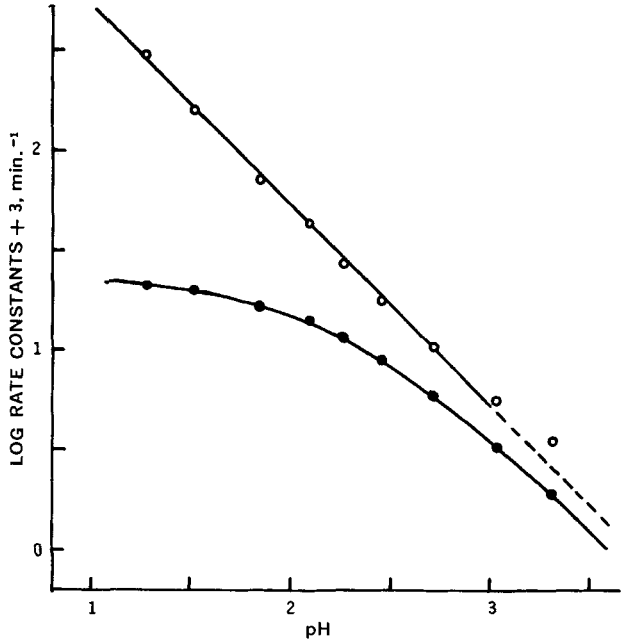


Figure 1—*pH–rate profile of the total degradation of benzylpenicillin methyl ester* (○) *and of the formation of methyl benzylpenicillenate* (●) *at 30°.*

as expected, however, benzylpenicillinic acid was found to give qualitatively similar results.

The hydrolysis of benzylpenicillin methyl ester was carried out at 30° in the pH range 1.28–3.33. The solutions were buffered with 0.02 *M* phosphate and adjusted to an ionic strength of 0.2 with KCl. It has been determined that there is no catalytic effect of the phosphate buffer in this concentration. Mercuric chloride was added to the buffers in a concentration five times as great as the initial benzylpenicillin methyl ester concentration ($\sim 5 \times 10^{-5}$ *M*) to stabilize the very labile methyl benzylpenicillenate (9). It was confirmed (10) that mercuric chloride in the given concentration has no effect upon the rate of methyl benzylpenicillenate formation or the rate of degradation of benzylpenicillin methyl ester. The rates of formation of methyl benzylpenicillenate from benzylpenicillin methyl ester were measured by following the increase in absorbance at 322 nm. as a function of time on a Zeiss PMQ II spectrophotometer, with thermostated cell compartment, connected with a Servogor recorder. Both the rate constants for total degradation of benzylpenicillin methyl ester (K) and for methyl benzylpenicillenate formation (k_{PNCE}) were obtained from the recorded curves by the Guggenheim method and were treated in the manner recently described by Niebergall and Sugita (11). The molar absorptivity of methyl benzylpenicillenate at 322 nm. is 26,600 (12).

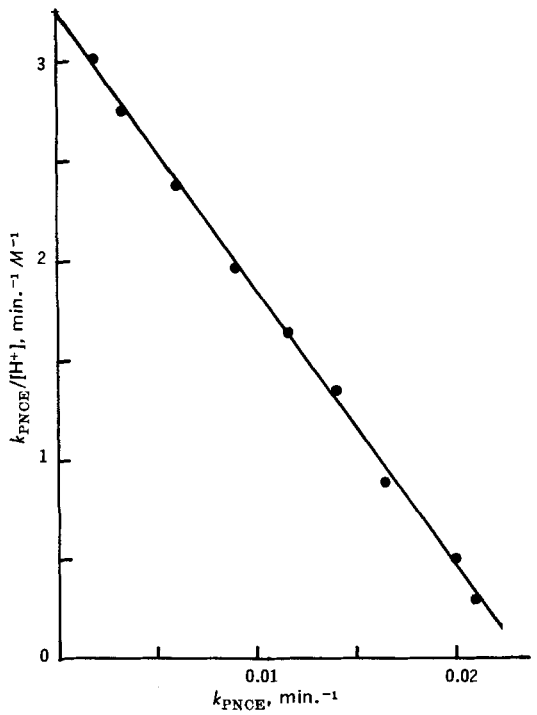


Figure 2—*Plot showing the fulfillment of Eq. 2.*

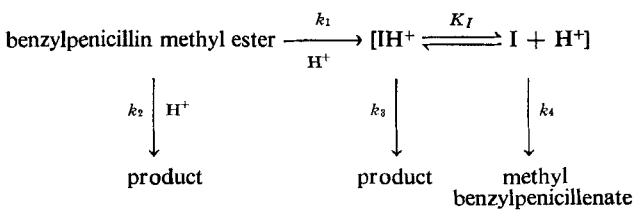
At constant pH, the hydrolysis could be described by pseudo-first-order kinetics. Figure 1 shows a plot of the logarithm of the observed pseudo-first-order rate constants K and k_{PNCE} *versus* pH; the hydrolysis of benzylpenicillin methyl ester at pH < 3 exclusively consists of a specific hydrogen-ion-catalyzed reaction (slope = −1). On the basis of the connection between pH and concentration of hydrogen ion (13) ($\log [H^+] = -pH + 0.12$), an average value of the specific hydrogen-ion-catalytic rate constant, K_{H^+}, was calculated to 4.01. mole^{-1} min.$^{-1}$. However, the value of k_{PNCE} does not increase in a linear fashion when pH is lowered, as anticipated for a reaction undergoing hydrogen-ion catalysis. Instead, as the value of hydrogen-ion concentration increases, the value of k_{PNCE} first increases rapidly and then slowly, becoming an independent function of hydrogen-ion concentration at the higher acid concentrations. Therefore, as the acidity increases, the rate-determining step in the formation of methyl benzylpenicillenate changes from one strongly dependent on acidity to one that is independent. The data were found to fit a curve of the form of Eq. 1:

$$k_{PNCE} = \frac{a \cdot [H^+]}{b + [H^+]} \qquad \text{(Eq. 1)}$$

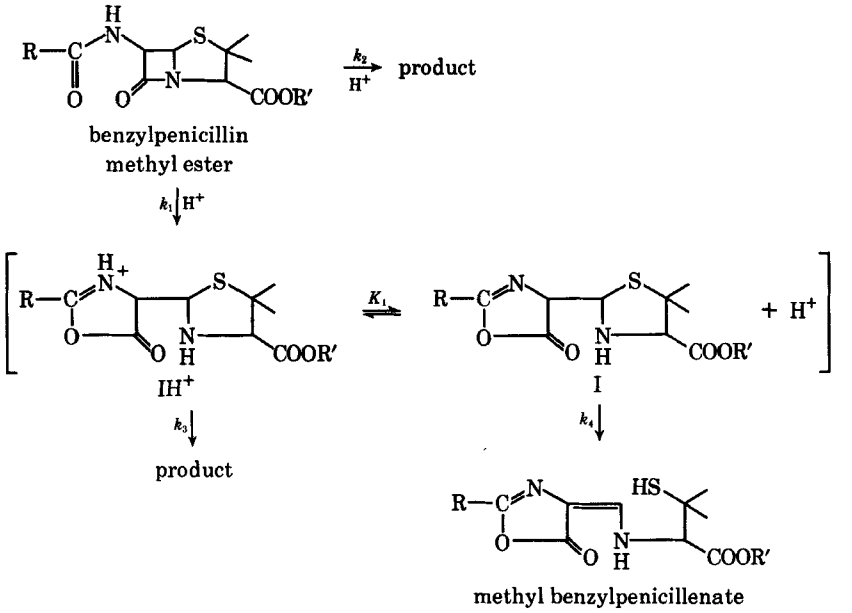
where a and b are constants. Rearrangement of Eq. 1 leads to Eq. 2:

$$\frac{k_{PNCE}}{[H^+]} = -\frac{1}{b} \cdot k_{PNCE} + \frac{a}{b} \qquad \text{(Eq. 2)}$$

As seen in Fig. 2, a plot of $k_{PNCE}/[H^+]$ *versus* k_{PNCE} is linear. From the slope and intercept, the constants a and b were derived: $a = 0.0234$, $b = 0.0072$.



Scheme I



Scheme II—R = $C_6H_5CH_2$—, R′ = CH_3—

The most reasonable mechanism which fits the determined kinetics involves metastable intermediates that are in acid–base equilibrium. Such a mechanism is shown in Scheme I. The IH^+ and I represent intermediates formed from benzylpenicillin methyl ester by a specific hydrogen-ion-catalyzed reaction, K_I represents the dissociation constant of IH^+, and $K = k_1 + k_2$.

Assuming steady state in the metastable intermediates IH^+ and I, the following expression can be derived for the observed rate constant, k_{PNCE}:

$$k_{PNCE} = \frac{[(k_4 \cdot K_I \cdot k^1_{H^+})/k_3] \times [H^+]}{[(k_4 \cdot K_I)/k_3] + [H^+]} \qquad \text{(Eq. 3)}$$

where $k^1_{H^+} = k_1/[H^+]$, since benzylpenicillin methyl ester degrades by a specific hydrogen-ion-catalyzed reaction. This equation possesses the same mathematical form as Eq. 1, showing that the proposed mechanistic scheme fits the experimental data. A fact supporting the metastability of the intermediates is the lack of a lag period in the production of methyl benzylpenicillenate. The proposed mechanism implies that the rate constants k_3 and k_4 are of the same magnitude. If $k_3 = k_4$, the apparent dissociation constant K_I equals $b \sim K_I = 10^{-2.1}$. From Eqs. 2 and 3, the value of $k^1_{H^+}$ is obtained: $k^1_{H^+} = a/b = 3.25$ l. mole^{-1} min.$^{-1}$.

It is suggested that the intermediate is a compound of the oxazolone–thiazolidine structure (Scheme II). Some factors which support this suggestion are: (*a*) compounds of the oxazolone–thiazolidine structure are known to be so unstable that they are capable of only transitory existence (14); (*b*) the β-lactam structure of benzylpenicillin methyl ester is so closely related to the oxazolone–thiazolidine that interconversion is brought about by a simple proton transfer; (*c*) there is a close relation between the oxazolone–thiazolidine structure and methyl benzylpenicillenate; and (*d*) the basicity of the nitrogen atom in the oxazolone is of a size corresponding to a pKa value of about 2 (14). Recently, on theoretical grounds the presumption was made (15) that the oxazolone–thiazolidine structure ("pseudopenicillin") is a probable intermediate in the isomerization of penicillin to penicillenate.

The suggestion previously was made (16) that a reactive structure such as the oxazolone–thiazolidine—if it exists—should be a powerful sensitizer. Further studies are underway to evaluate the significance of the reported findings in the penicillin allergy together with an evaluation of the other degradation products.

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Received February 16, 1971.
Accepted for publication May 5, 1971.