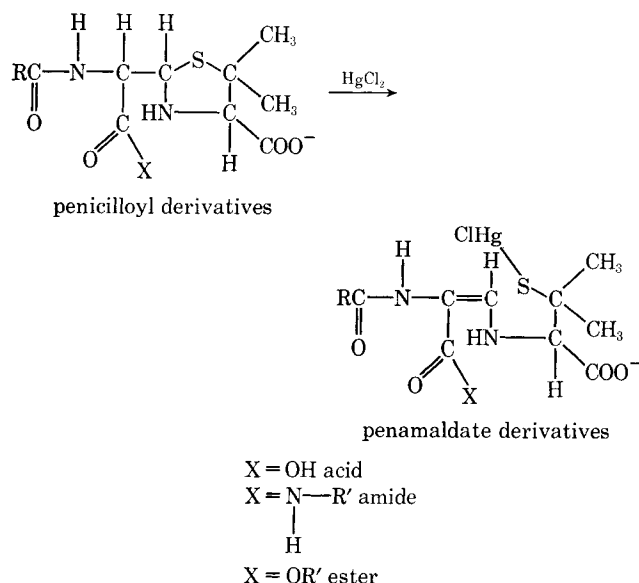# Kinetic Analysis of Penicilloic Acid and Penicilloamides in Combination: Application to Products of Reaction of Penicillin with Tromethamine (Tris) and Poly-L-lysine

MICHAEL A. SCHWARTZ and ANTOINETTE J. DELDUCE

Abstract ☐ Studies of chemical reactions related to penicillin allergy often require an assay for both penicilloic acid and penicilloamides in reaction mixtures. Previous assays were based on conversion of these substances, with HgCl₂, to penamaldates which have an absorption maximum at 282 mμ. Differentiation of the two compounds was accomplished by taking advantage of the lower stability of the penamaldate of penicilloic acid using only the absorbance initially and after 10 min. The present work describes an improved method based on the fact that decay of penamaldate can be resolved kinetically into its two components. Extrapolation to zero time yields very accurate estimates of molar extinction coefficients. The method was applied to products of reaction of tromethamine (Tris) with penicillin which is catalyzed by poly-L-lysine (PLL). In absence of tromethamine both penicilloic acid and penicilloyl-PLL are formed while at high tromethamine concentration the product is almost entirely N-(penicilloyl)-tromethamine. Calculated rates show that PLL catalyzes hydrolysis of penicillin to penicilloic acid as well as aminolysis by tromethamine. These results confirm the previously proposed reaction mechanism.

Keyphrases ☐ Penicilloic acid, penicilloamides, combination—kinetic analysis ☐ Tromethamine-benzylpenicillin-poly-L-lysine reaction products—analysis ☐ Penicillin-penicilloamide-HgCl₂ reaction—kinetics ☐ UV spectrophotometry—analysis

Investigations of chemical reactions involved in penicillin allergy often require a suitable method of assay for the penicilloyl group, which is the principal antigenic determinant found among penicillin hypersensitive patients (1, 2). The penicilloyl group may appear as free penicilloic acid (X = OH), or combined as a penicilloamide (X = NHR'), or penicilloic acid ester (X = OR').

penicilloyl derivatives

penamaldate derivatives

X = OH acid
X = N—R' amide
       |
       H
X = OR' ester

Levine (3) developed a quantitative method for peni-

cilloamides based on their conversion to penamaldate derivatives upon treatment with p-chloromercuribenzoate. The reaction was carried out in pH 9.2 carbonate buffer and the absorbance of the rapidly formed penamaldate read at 285 mμ. Schneider and DeWeck (4) subsequently found that at neutral pH the penamaldate of penicilloic acid was much less stable than that formed from an ester or amide derivative. Using this difference they developed an assay based on the change in absorbance from the time of initial addition of mercuric chloride to 10 min. later. At the latter time, the absorbance of a penicilloic acid solution had decreased to about 25% of its initial value while only about a 4% decrease was noted with an amide derivative. This assay method was applied to mixtures of penicilloic acid and penicilloamide.

The present work represents a further extension of the method applying a kinetic analysis to data obtained when mixtures of a penicillin with a penicilloamide were treated with mercuric chloride. It also reports results of application of the method to the products of reaction between benzylpenicillin and tris(hydroxymethyl)aminomethane (Tris) catalyzed by poly-L-lysine (PLL). The kinetics of the latter reaction have been previously reported (5).

EXPERIMENTAL

Preparation of Penicilloic Acids—(0.001 mole) The penicillin was dissolved in about 5 ml. water in a 1000-ml. volumetric flask. Two milliliters, of 1.0 M NaOH was added and solution allowed to stand 2 hr. Two milliliters of 1.0 M HCl was added and the solution brought to the mark with 0.1 M pH 7 phosphate buffer. Solutions were prepared freshly as needed.

Preparation of Penicilloamides—These were prepared by a similar method to that previously reported by Levine (3): 0.001 mole of the penicillin was dissolved in 75 ml. water and 0.02 mole of amine added. After standing 2 hr. the solution was acidified to pH 2 with concentrated HCl and the precipitated amide collected, washed with water, and dried. Benzylamine was used with phenoxymethylpenicillin while propylamine was used with the other penicillins.

Assay Method—Determination of Mercury Equivalence (4)—This is the amount of HgCl₂ after which a further addition produced no further increase in absorbance. It was determined with a sample from each solution, both standards and unknowns, by titrating the solution with small increments (0.01 ml.) of 1.0 × 10⁻³ M HgCl₂.

Standard Curves—Mixtures of the penicilloic acid and amide of each penicillin were prepared to contain varying concentrations of each with the total concentration 4 × 10⁻⁵ M, in a pH 7.0 phosphate buffer. Mercury equivalence was determined on a 2-ml. sample of solution. Then 2 ml. of solution was placed into a 1-cm. cell, the appropriate amount (found by prior titration) of HgCl₂ solution added, and absorbance recorded at 285 mμ. Zero time was
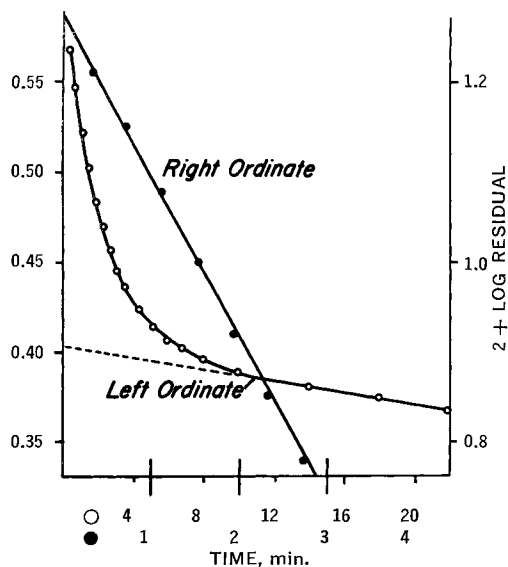
Figure 1.—*Typical absorbance time plot. This particular plot is for a solution containing 2×10^{-5} mole each of benzylpenicilloic acid and N-(benzylpenicilloyl)propylamine.*

taken when $HgCl_2$ was added and it required about 20 sec. to begin recording. The data were treated as described below.

Assays for Products of Reaction of Benzylpenicillin with Tromethamine (Tris) *and PLL*—Solutions were prepared containing benzylpenicillin (0.001 mole), PLL (mol. wt. 95,000, 9×10^{-5} mole) and varying quantities of tromethamine pH 8.8. These were allowed to stand at 35° until the reaction had gone to completion. One sample was assayed as described above to determine penicilloic acid and total penicilloamide. A second sample was dialyzed against large volumes of water for several days and the assay repeated on the solution remaining in the dialysis bag. The latter procedure determined penicilloyl-PLL and the amount of penicilloyl-tromethamine was found by subtraction.

Treatment of Data—A typical example of an absorbance-time plot is shown in Fig. 1, which is for a solution containing 2×10^{-5} mole each of benzylpenicilloic acid and N-(benzylpenicilloyl)propylamine. It was assumed that the degradation of the penamaldate

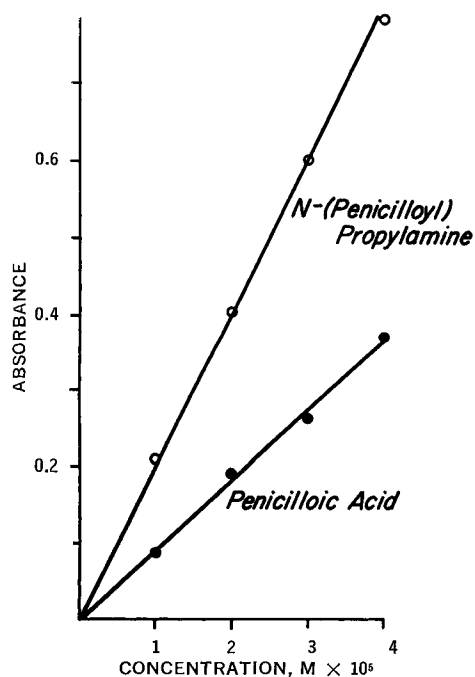**Table I**—Molar Absorptivities

Penicillin	Molar Absorptivities Acid	Amide
Benzyl-	9480	19,625
Phenoxymethyl-	9450	18,650
Oxacillin	7075	12,600
Methicillin	6825	20,250

formed from both the acid and the amide are first-order and mutually independent. The absorbance (A) may then be expressed as follows:

$$A = \epsilon_a c_a + \epsilon_b c_b \qquad (Eq.\ 1)$$

where the subscripts a and b represent acid and amide, respectively, the c's indicate molar concentration, and the ϵ's are the respective molar absorptivities. Since the reactions are both first-order:

$$A = \epsilon_a a_0 e^{-k_a t} + \epsilon_b b_0 e^{-k_b t} \qquad (Eq.\ 2)$$

The rate of degradation of the penamaldate from acid is much faster than that of the amide derivative; hence, $k_a \gg k_b$ and after a short time, when the acid penamaldate has disappeared, only the amide remains. Since its degradation is so slow, extrapolation back to zero time may be made from a linear plot rather than the normal semilog plot. This extrapolated value of A at zero time represents $\epsilon_b b_0$ and thus determines the initial concentration of amide (b_0). The values along the extrapolated line (dashed line in Fig. 1) representing the contribution of amide to the total absorbance are subtracted from that total to leave only the absorbance due to acid. A semi-log plot of the latter values may then be extrapolated to zero time to give $\epsilon_a a_0$ from which a_0 may be obtained. The full mathematical derivation of this treatment has been presented (6).

RESULTS AND DISCUSSION

The calibration curve for benzylpenicilloic acid is shown in Fig. 2, where the slopes represent the molar absorptivities for the respective derivatives. Similar linear plots were obtained with all the penicillins and the extinction coefficients are given in Table I. The value for benzylpenicilloic acid is significantly higher than the previously reported (7) value 8000. This difference reflects the rapid decrease in absorbance of the penamaldate from penicilloic acid which made it difficult to obtain an accurate zero time reading. The present method obviates this difficulty by the extrapolation to
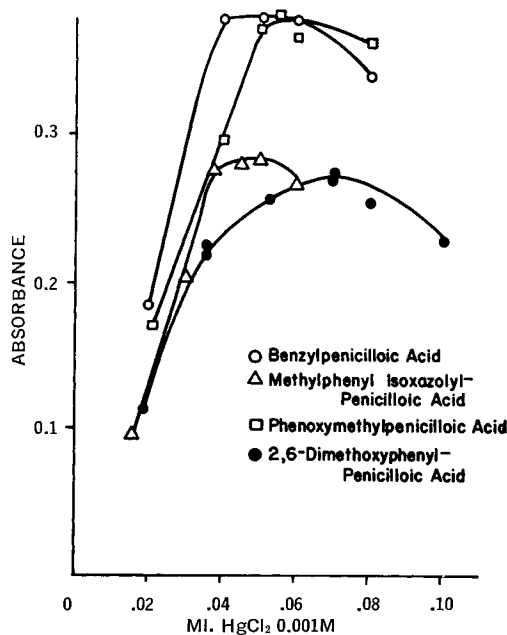

Figure 2—*Calibration curve for benzylpenicilloic acid and N-(benzylpenicilloyl)propylamine.*

Figure 3—*Effect of $HgCl_2$ concentration on absorbance of penamaldates from the penicilloic acids. Concentration of penicilloic acids was 4.0×10^{-5} M.*
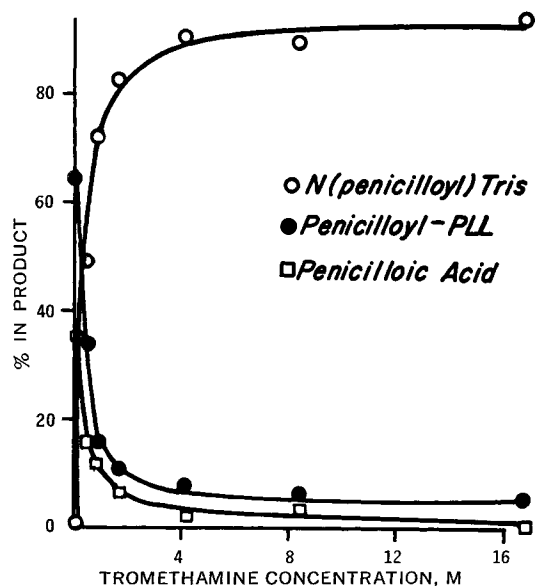
Figure 4—*Results of assays of products of reaction of penicillin with Tris and PLL at pH 8.8.*

zero time providing more accurate determination of penicilloic acid. The extinction values of the amides are lower than previously observed by Levine (3), who found values about 23,000. The decrease may be a result of the difference in pH since Levine's work was done at pH 9.2, and the present study at pH 7.0.

The effect of $HgCl_2$ concentration on absorbance of penamaldate from the penicilloic acids is shown in Fig. 3. It can be seen that, not only are there differences in the maximal absorbance reached with each penicillin but the amount of $HgCl_2$ required to produce this maximum varies. Thus, assay conditions must be established for each penicillin individually.

The results of assay of products of reaction at pH 8.8 of benzylpenicillin with tromethamine and PLL at 35° are shown in Fig. 4. These confirm the previous hypothesis (5) that the penicillin reacts principally with tromethamine and the polymer serves as a catalyst.

The curve for N-(penicilloyl)tromethamine markedly resembles the dependence of rate constant upon tromethamine concentration. One new aspect of the reaction revealed here is that both penicilloic acid and penicilloyl-PLL are formed in the absence of tromethamine, where about 38% of the original penicillin goes to penicilloic acid. The first-order rate constant for loss of penicillin under these conditions was 0.006 min.$^{-1}$. Multiplying by 0.38 gives 0.00228 min.$^{-1}$ as the rate constant for hydrolysis of benzylpenicillin at pH 8.8 in presence of PLL. An approximate value, calculated from data obtained at 31.5° (8), for hydrolysis of benzylpenicillin in absence of PLL is 2×10^{-4} min.$^{-1}$. It thus appears, that PLL is catalyzing the hydrolysis of benzylpenicillin. While the mechanism of this catalysis is unknown, it may be a clue as to one means by which the enzyme penicillinase may exert its catalytic effect.

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Thyroxine Analogs XVI: Synthesis and Activity of 3,5-Dibromo-3'-isopropyl-L-thyronine

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Abstract ☐ 3,5-Dibromo-3'-isopropyl-L-thyronine (V) has been synthesized by two independent routes. Its biological potency, greater than that of L-thyroxine in a number of test systems, demonstrates that iodine is not a required substituent for thyroxine-like effects.

Keyphrases ☐ Thyroxine analogs—synthesis ☐ 3,5-Dibromo-3'-isopropyl-L-thyronine—synthesis ☐ Pharmacological screening—thyroxine analog ☐ Polarimetry—identity ☐ NMR spectroscopy—structure

The importance of iodine in thyroid function has been recognized since the early establishment of its therapeutic value in certain types of goiter. Characterization of the thyroid hormones as iodinated thyronines provided a rationale for the biological importance of the element, but no insight into its functional role or relative importance in the molecule for hormonal actions. Some theories have emphasized a direct participation of iodine, with the rest of the molecule relegated to a lesser role as carrier (1–3).

Iodine on the phenolic ring of the thyroid hormones or their analogs has been replaced by aliphatic, alicyclic, and aromatic residues with retention and even enhancement of hormonal activity (4–10).[1] Methyl groups have

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[1] *Reference 7 is paper XV in this series.*