Kinetics and Mechanism of Hydrolysis of Antimycin A_1 in Solution

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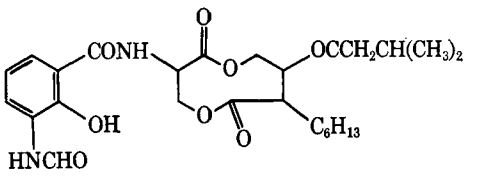
Abstract □ The kinetics of hydrolysis of the antibiotic antimycin A_1 has been determined. Spectrophotometric data obtained in buffer systems indicate that the overall degradation follows the classical consecutive first-order kinetics according to the following scheme: antimycin $A_1 \xrightarrow{k_1}$ blastmycic acid + antimycic lactone $\xrightarrow{k_2}$ fatty acids. An independent check on the above scheme was obtained by determining k_1 and k_2. The pH dependency of both rate constants was determined. The apparent activation energy for k_1 was determined fluorometrically at two different hydroxyl ion concentrations and was found to be 18 kcal./mole.

Keyphrases □ Antimycin A_1 hydrolysis—kinetics, mechanism □ Hydrolysis rate constant, antimycin A_1—pH effect □ Paper chromatography—identity □ UV spectrophotometry—analysis □ Fluorometry—analysis

The streptomyces antibiotic complex, antimycin A, was discovered in the 1940's (1) and has since been isolated by several groups. It is a mixture of several components, mainly antimycin A_1, A_2, A_3, and A_4 with A_1 being the major component (2). These components resemble each other very closely, although they differ somewhat in molecular weight, due to the slight differences in the hydrocarbon side-chain length.



Antimycin A_1

The importance of this antibiotic complex was recognized when it was found to be remarkably effective in inhibiting the hydrogen transport systems of aerobic organisms and consequently has become useful in the study of certain enzyme mechanisms. It has recently been reported to be extremely toxic to fish even at very low concentrations (3, 4) and is being used in fish management.

Although the chemistry of the compound and of its degradative products has been thoroughly investigated by Strong *et al.* (5–8), the kinetics of hydrolysis has not been previously reported.

The purpose of this study was to determine the kinetics and mechanism of hydrolysis of the major component, antimycin A_1, in neutral and alkaline solutions, in order to obtain a better understanding of the rate of degradation when applied to lake water in this pH range.

EXPERIMENTAL

Reagents and Apparatus—Antimycin A_1 100% pure, blastmycic acid, and antimycic acid,[1] and also antimycin lactone,[2] were used.

Buffers and standard sodium hydroxide solutions were prepared using reagent grade materials.

The instruments used in this study were a spectrophotometer,[3] a fluorometer,[4] an atomic spectrophotofluorometer,[5] and a pH meter.[6]

Kinetic Procedures—The hydroxyl and hydrogen ion concentrations of the systems were maintained by using buffer solutions. Buffers used in this study were: phosphate, carbonate, borate, and excess (carbonate free) sodium hydroxide.

The ionic strength of the solutions was adjusted to the desired value by the addition of sodium chloride. All the experiments were conducted at a total ionic strength of 0.5.

Spectrophotometric Determinations of k_1 and k_2—A fresh 2×10^{-2} *M* stock solution of antimycin A_1 in ethanol was prepared. Ten microliters was accurately introduced into a 1-cm. light-path silica cell and exactly 3 ml. of the buffer at the desired pH was injected into the cell and the change in absorbance was followed at 214 mμ. The rate of hydrolysis of the lactone alone, k_2, was similarly determined spectrophotometrically at 214 mμ.

Fluorometric Determinations of k_1—Buffer solutions of the desired pH's were equilibrated in a constant-temperature bath. Approximately 0.2 ml. of a stock solution of antimycin A_1 in methanol was then injected into the buffered solutions to give a final concentration of 5×10^{-6} *M*. Periodically 5-ml. samples were withdrawn and the reaction was quickly quenched with 5 ml. phosphate buffer solution of a desired pH so that the final pH of the quenched reaction is between 7.5 to 8. At this pH range antimycin exhibits its maximum fluorescence intensity (9).

The change in fluorescence of the solutions was then determined in the fluorometer equipped with filter combinations of 7-60 primary and 2A plus 47B secondary (9). This combination corresponds to an activating wavelength of 420 mμ at which the fluorescence peak was observed (10). Figure 1 shows a typical fluorescence concentration-type plot determined at pH 8. (Figure 6 shows fluorescence spectra of equimolar quantities of antimycin A_1 and its degradation products determined on an atomic spectrophotofluorometer.)

Paper Chromatographic Analysis—Three solutions were prepared: one containing 1.25 mg./ml. antimycin A_1 in a 1:1 methanol–0.1 *N* sodium hydroxide solvent mixture and kept for 10 min. at room temperature to insure complete hydrolysis of antimycin; the second containing 1.25 mg./ml. blastmycic acid in the same solvent mixture; and the third containing antimycin A_1 in methanol. Ten microliters of each solution was spotted on a 3 MM chromatography paper. The paper was developed in a descending tank containing a solvent mixture of 62 parts water, 33 parts methanol, and 5 parts glacial acetic acid by volume. Three fluorescent spots were observed on the paper. One at the origin corresponded to that of antimycin A_1 in methanol and two spots near the solvent front corresponded to that of blastmycic acid and of hydrolyzed antimycin. The paper was then air-dried and sprayed with *p*-nitrobenzendiazonium fluoborate solution. The R_f values were calculated and were found to be 0.85 for both blastmycic acid and hydrolyzed antimycin, while antimycin in methanol stayed at the origin.

[1] Furnished by Dr. F. Strong, Biochemistry Department, University of Wisconsin, Madison, Wisc.
[2] Furnished by Dr. C. Orzech, Ayerst Laboratories, Rouses Point, N. Y.
[3] Cary model 14.
[4] Turner model 111.
[5] Baird model SF-1.
[6] Beckman Instruments, Inc., Fullerton, Calif.

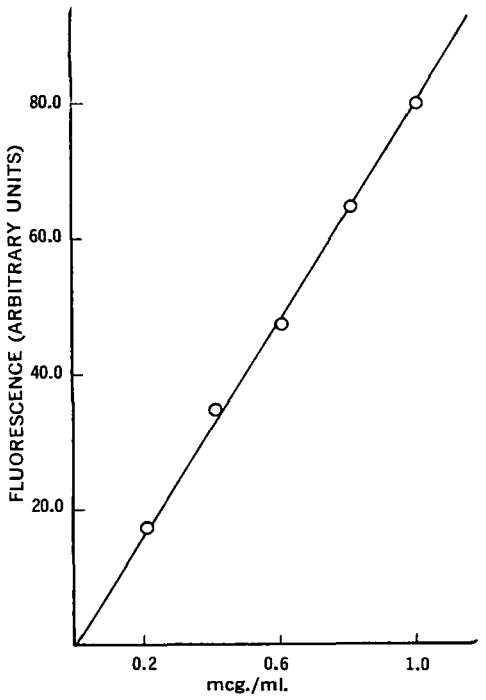


Figure 1—*Standard plot of fluorescent intensity* versus *antimycin A_1 concentration at pH 8.0.*

RESULTS AND DISCUSSION

Spectral Changes Accompanying Degradation of Antimycin A_1—The spectral changes of a freshly prepared aqueous solution of antimycin A_1 can be conveniently followed at 214 mμ. When alcoholic solutions of antimycin A_1 were mixed with aqueous alkaline solutions at 25° directly in a cell,[7] a rapid initial decrease in absorbance at 214 mμ followed by a slower increase was observed. A plot of log of absorbance, of the completed reaction, A_∞, minus the absorbance at time t, A_t, *versus* time gave curves similar to that shown in Fig. 2. As the pH of these solutions was raised, both the initial slope and the final slope of the plots became steeper. The observed spectral changes suggested rapid initial degradation of the compound to form a chemical species which subsequently underwent slower first-order degradation.

It is known qualitatively that mild alkaline hydrolysis of antimycin A_1 yielded blastmycic acid (I) and antimycin lactone (II), while hydrolysis under more severe conditions will yield antimycic acid (III) (5).

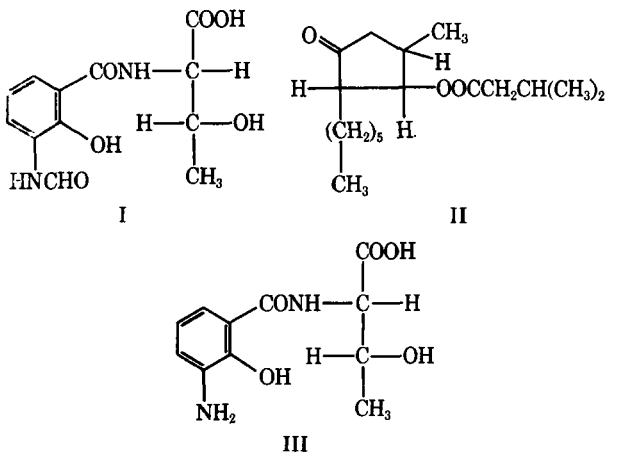


Figure 3 shows the UV spectra of 7.5×10^{-6} *M* solutions of antimycin A_1, blastmycic acid, and antimycic acid at pH 8. Since blastmycic acid was found to be stable under these experimental conditions, while alkaline solutions of antimycin lactone showed a gradual increase in absorbance at 214 mμ, it was assumed that the spectral changes observed upon the addition of alkaline buffers to alcoholic solution of antimycin A_1 are due to the following reaction sequence:

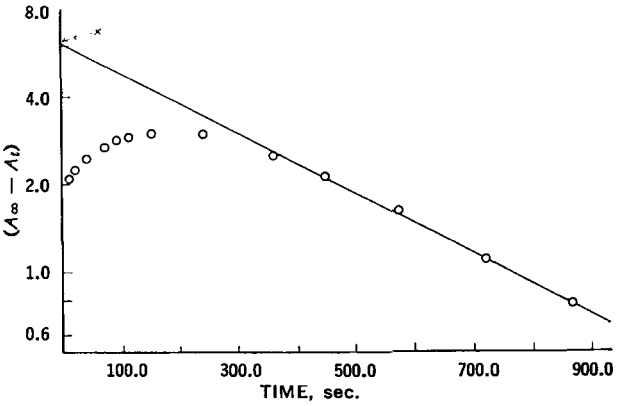


Figure 2—*Semilogarithmic plot of absorbance change at 214 mμ as a function of time in solution containing 6×10^{-5} M antimycin A_1 at pH 11.1 and 25°.*

$$\text{antimycin } A_1 \xrightarrow{k_1} \text{blastmycic acid} + \text{antimycin lactone} \xrightarrow{k_2} \text{hydrolytic products (fatty acids)} \qquad \text{(II)}$$

Determination of Rate of Hydrolysis of Antimycin Lactone, k_2—The rate of hydrolysis of the lactone was followed spectrophotometrically at 214 mμ. Changes in absorbance expressed as $(A_\infty - A_t)$ observed when alcoholic solution of the lactone was mixed with alkaline buffer solution yielded straight-line plots against time on semilog paper, similar to that shown in Fig. 4.

The observed first-order rate constant at any given pH calculated from the slope of these lines was similar to that calculated from the linear slope of plots such as shown in Fig. 2, determined at the same pH.

Spectrophotometric Determination of k_1—If the above system can be treated as two consecutive first-order reactions, then the following standard logarithmic relationship should apply

$$\log\left[e^{-k_2 t} - \frac{(A_\infty - A_t)}{X}\right] - \frac{k_1}{2.303}t \qquad \text{(Eq. 1)}$$

where X is the linear extrapolation of $A_\infty - A_t$ at time zero, as shown in Fig. 2. A plot of $\log [e^{-k_2 t} - (A_\infty - A_t)/X]$ against time should give a straight line with a slope of $-k_1/2.303$. A typical plot of this type for a system containing 6×10^{-5} *M* antimycin A_1 at pH

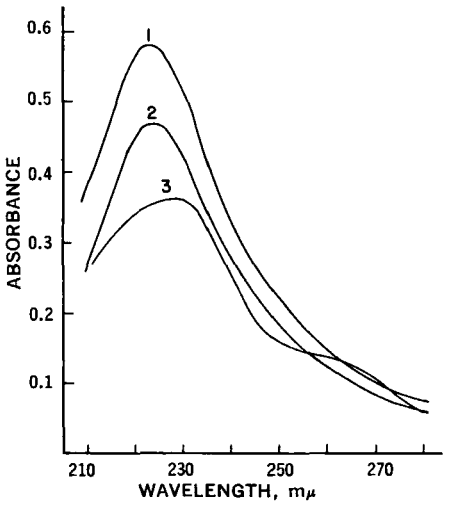


Figure 3—*UV absorption spectra of equimolar quantities (7.5 × 10^{-6} M) of (1) antimycin, A_1; (2) blastmycic acid; and (3) antimycic acid in 0.05 M phosphate buffer at pH 8.0.*

[7] Cary.

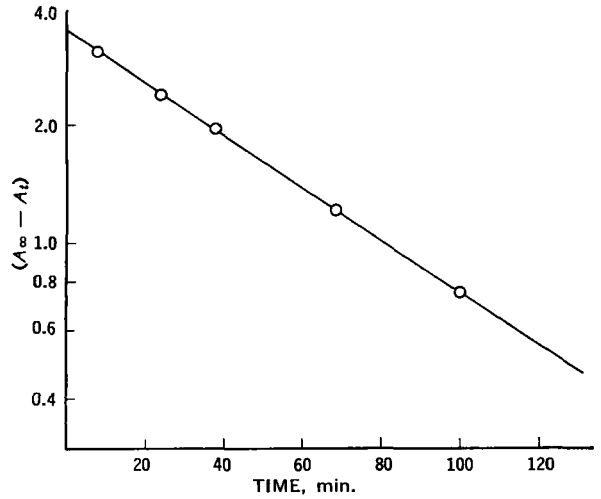


Figure 4—*Semilogarithmic plot of absorbance change at 214 mμ as a function of time in solution containing 6.0 × 10^{-5} M antimycin lactone at pH 10.0 and 25°.*

11.1 and based on the known value of k_2 at this pH is shown in Fig. 5.

Fluorometric Determinations of k_1—In order to obtain an independent check on the spectrophotometric data, the degradation of antimycin was determined following the rate of loss of the compound fluorometrically. Figure 6 shows the fluorescence spectra of equimolar quantities (7.5 × 10^{-6} *M*) of antimycin A_1 and its degradation products, blastmycic and antimycic acid. If the above proposed mechanism is correct, degraded antimycin solutions should exhibit a fluorescence value corresponding to that of the blastmycic acid formed. This was found to be the case and a plot of log fluorescence at time *t*, F_t, minus the fluorescence of the completed reaction, F_∞, *versus* time exhibited a linear relationship, as shown in Fig. 7 for two different hydroxyl ion concentrations. The observed pseudo first-order rate constant calculated from the slope of these lines was essentially the same as those calculated for k_1 from spectrophotometric data.

Furthermore, a paper chromatograph of degraded antimycin solutions showed only one fluorescent spot with an R_f value corresponding to that of blastmycic acid.

These results indicated that antimycin A_1 in aqueous neutral and alkaline solutions decomposes to blastmycic acid and to the antimycin lactone, with the latter undergoing further hydrolysis to the corresponding fatty acids.

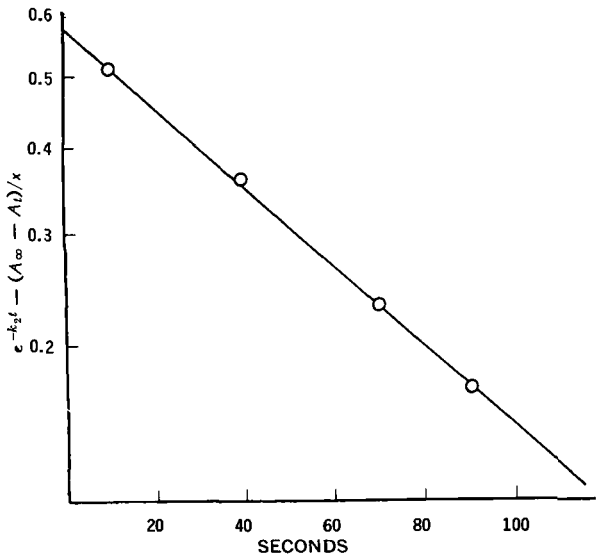


Figure 5—*Semilogarithmic plot showing the linear relationship of* $e^{-k_2t} - (A_\infty - A_t)/X$ *and time for a solution containing 6 × 10^{-5} M antimycin A_1 at pH = 11.1 and 25°.*

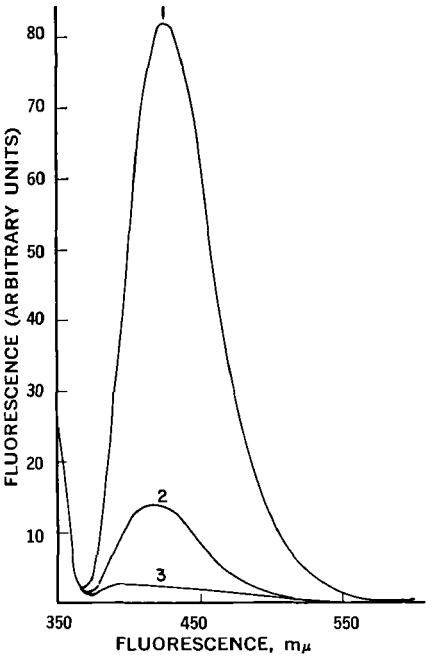


Figure 6—*Fluorescence spectra of equimolar quantities (7.5 × 10^{-6} M) of (1) antimycin A_1; (2) blastmycic acid; and (3) antimycic acid in 0.05 M phosphate buffer at pH = 8.*

One cannot, at the moment, rule out the possibility of ester side chain cleavage and further work is necessary. However, it is known that hydroxyl ion attacks the lactone carbonyl group much faster than it attacks the ester carbonyl group.

Variation of Reaction Rate Constants with pH—The dependency of the observed rate constant for hydrolysis of antimycin A_1 to blastmycic acid and antimycin lactone, $k_{1\ obs.}$, on pH is shown in Fig. 8 where a plot of the log of the pseudo first-order rate constant determined fluorometrically *versus* pH yielded a straight line with slope of one. The points represented as a triangle on the pH profile of $k_{1\ obs.}$ were determined spectrophotometrically and calculated from slopes

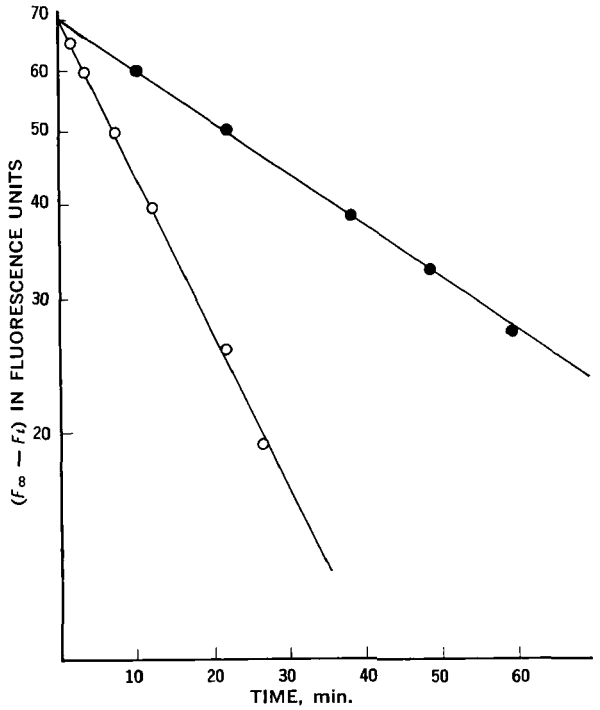


Figure 7—*Semilogarithmic plot of fluorescence change with respect to time for antimycin A_1 solution at pH 9.4 (●) and pH 10.1 (○) at 25°.*

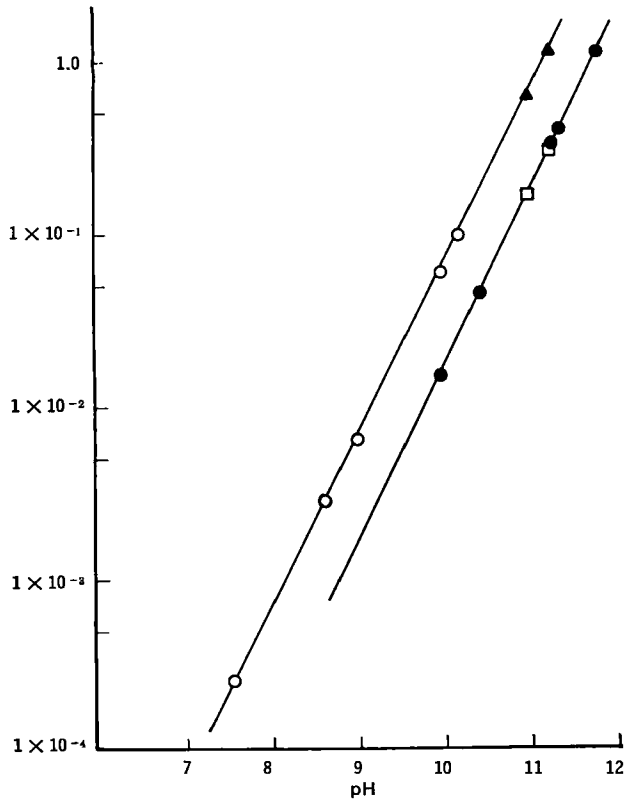


Figure 8—*pH-Rate profile for the degradation of antimycin A_1 and antimycin lactone. Key for* K_1: O, *determined fluorometrically;* ▲, *determined spectrophotometrically. Key for* K_2: ●, *determined directly;* □, *determined from plots such as shown in Fig. 2.*

of lines such as shown in Fig. 5. Since there was no change in the rate constant when the reaction was run at different buffer concentrations of different buffer species (Figs. 9 and 10), it appears that the hydrolysis is a specific hydroxyl ion catalyzed reaction.

The dependency of $k_{2\ obs.}$ on pH of the solutions is shown in the same plot. The points represented by a square were those determined from the final slope, such as shown in Fig. 2.

The values of $k_{1\ obs.}$ and $k_{2\ obs.}$ at 25° at different hydroxyl ion concentrations are given in Table I.

Temperature Dependency of k_1—It was found qualitatively that the effective dose of the antimycin complex for water treatment as a possible pesticide depends on the temperature of the water. It was, therefore, necessary to determine the temperature dependency of k_1. Arrhenius plots for $k_{1\ obs.}$ determined at two pH values are shown in Fig. 11. The apparent activation energy calculated from the slope of these lines was found to be 18 kcal./mole. Since this apparent energy of activation includes the heat of ionization of water (approximately 12 kcal./mole), the energy of activation for the hydroxyl reaction would be approximately 6 kcal./mole. This value appears to be somewhat lower than that for most lactones and esters. For example Hegan *et al.* (11) found 11.3 kcal./mole for the energy of activation

Table I—Observed First-Order Rate Constants, k_1 and k_2, as a Function of pH at 25°C.

pH	$k_{1\ obs.}$ min.$^{-1}$	$k_{2\ obs.}$ min.$^{-1}$
11.85	—	1.1
11.25	1.1	—
11.4	—	0.39
11.3	—	0.2
11.1	0.6	0.16
10.45	—	0.045
10.21	0.098	—
10.0	0.06	0.015
9.0	0.0052	—
8.65	0.0029	—
7.55	0.00025	—

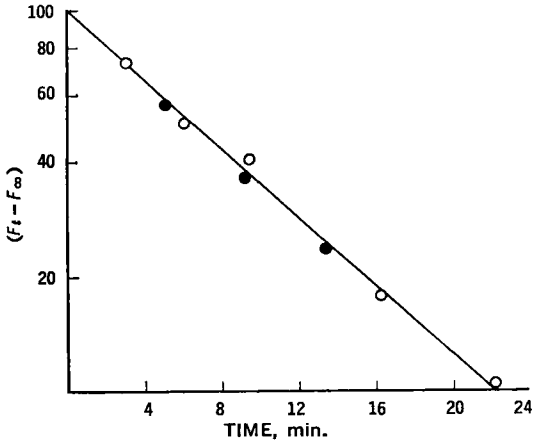
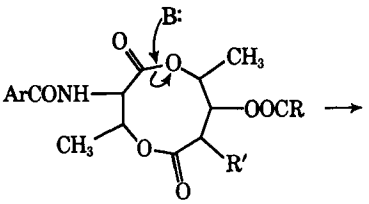
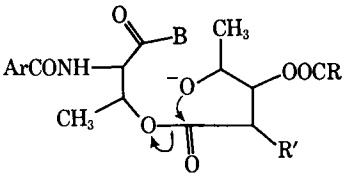


Figure 9—*The effect of change of molarity of phosphate buffer on the rate of hydrolysis of antimycin A_1 at pH 10.2 and ionic strength of 0.5 at 25°. Key:* O, *0.05* M; ●, *0.1* M.

for the alkaline hydrolysis of γ-valerolactone and γ-butyrolactone. However, a low value for the activation energy was anticipated by Strong *et al.* (6), who postulated that hydroxyl ion attack at the lactone carbonyl would release ethereal oxygen for intramolecular attack at the second lactone group as follows:





It is probable that both processes occur almost simultaneously with

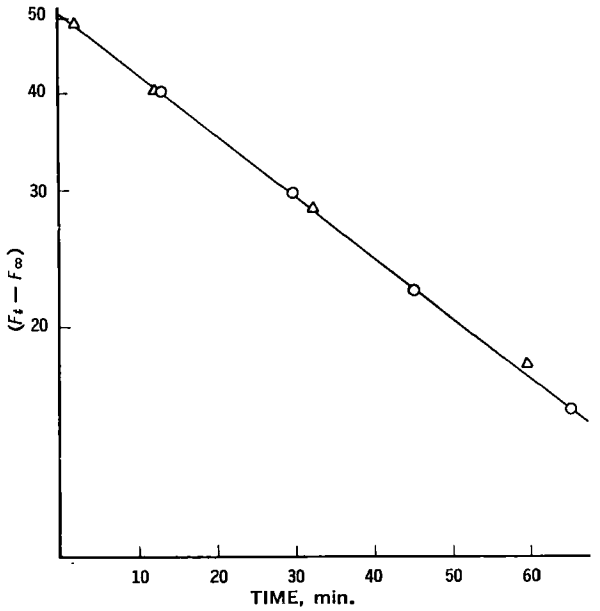


Figure 10—*The effect of change of buffer species on the rate of antimycin A_1 at pH 9.4 and 25°. Key:* O, *phosphate buffer;* △, *borate buffer.*

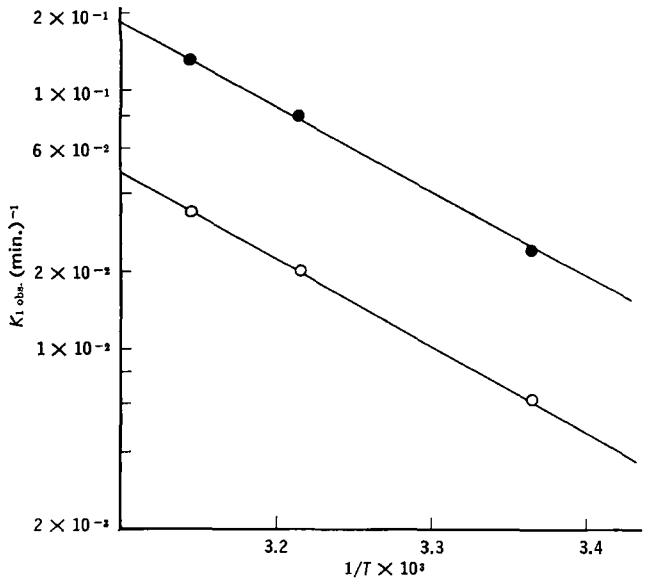


Figure 11—*Arrhenius plots showing the temperature dependency of the hydrolytic reaction of antimycin A_1 (K_1) at pH 9.5 (●) and pH 8.8 (○). These data were determined fluorometrically.*

the result that the activation energy for the overall reaction would be much lower than that for a simple ester hydrolysis.

SUMMARY

The overall degradation of antimycin A_1 in buffer systems appears to follow consecutive first-order kinetics with k_1 being the rate constant for hydrolysis of antimycin A_1 to blastmycic acid and lactone, and k_2 is the rate constant for hydrolysis of the lactone.

The rate of degradation of antimycin A_1 to blastmycic acid and antimycin lactone was found to be first order with respect to hydroxyl-ion concentration. The second-order rate constant of the above reaction at 25° was calculated to be 6×10^2 l. mole^{-1} min.$^{-1}$. The rate of hydrolysis of the lactone was also found to be first order with respect to hydroxyl ion. The apparent activation energy of k_1 was determined at two different hydroxyl-ion concentrations and was found to be 18 kcal./mole.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 6, 1968, from the *Pharmaceutical Development Department, Ayerst Laboratories, Inc., Rouses Point, NY 12979*

Accepted for publication November 4, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author would like to thank Dr. C. Orzech for his helpful discussions and for preparing the antimycin lactone, and Mr. A. Bylow for his assistance in developing the chromatographic system.