Cycloheximide Transformations II

Kinetics and Stability in a Pharmaceutically Useful pH Range

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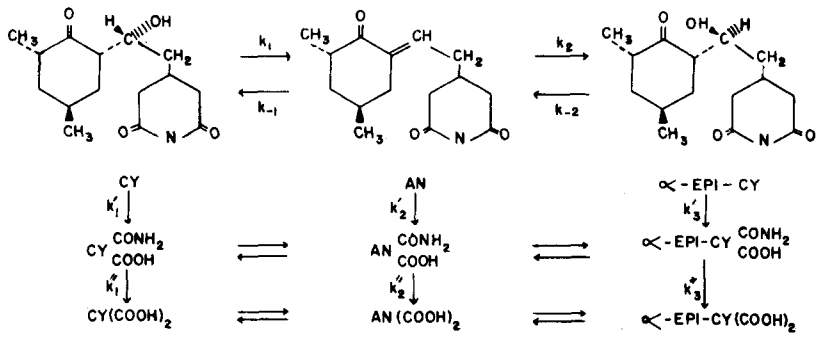
The kinetics of dehydration of the antitumor cycloheximide (CY) to anhydrocycloheximide (AN) are quantified in the pharmaceutically useful acetate buffer region. In addition to specific acid catalysis, solvent, general, and specific base catalysis have been observed and log k-pH profiles constructed below pH 7 where maximal stability exists at pH 4.4. The basic degradative mechanism in the pH region 3–6 is a reversible dehydration and rehydration of the α, β-unsaturated ketone CY $\underset{k_{-1}}{\overset{k_1}{\rightleftarrows}}$ AN, where both k_1 and k_{-1} are specific acid-base, solvent, and general base catalyzed. The specific base solvolysis of the imide function to the monocarboxylic acid is faster than the subsequent hydrolysis to the dicarboxylic acid and is pharmaceutically significant at pH values of 7 or greater.



Scheme I

THE ANTIBIOTIC cycloheximide (CY) is highly active against most yeasts and fungi (1–3) and moderately active against several tumors. Recent investigations have resulted in the discovery of several related antibiotics, of which streptovitacin A and E-73 possess significant antitumor activity (4, 5). The purpose of this series of studies is to characterize completely the kinetics of transformations of these structures, to ascertain any differences in relative ease of alternate pathways which might give some indication concerning the mode of action, and to predict the stability under all formulating conditions.

The previous paper (6) has established the kinetics and mechanisms of cycloheximide transformations in aqueous acid and has quantitatively characterized the specific acid-catalyzed transformation and solvolyses of Scheme I as functions of hydrogen ion activity and temperature. The mechanism of Scheme I is through a reversible dehydration of cycloheximide (CY) to anhydrocycloheximide (AN) with rehydration. An alternate and major product of rehydration may have either the α-epi-cycloheximide or the α-epinaramycin B configuration. Both potential configurations are represented by α-epi-CY in Scheme I. These are the probable assignments of the products that resulted from rehydration of anhydrocycloheximide (AN) and were separable by thin-layer chromatography from AN and the other chromatographically indistinguishable stereoisomers of cycloheximide represented by CY in Scheme I. Hydrolyses of the imides of each of the three cited compounds occur simultaneously but at a slower rate; dehydration and rehydration of solvolyzed products also occurs. The rate-determining step in the acid-catalyzed solvolytic sequences is the hydrolysis to the acid amides, *i.e.*, the k'_i steps.

The purpose of this paper is to evaluate the stability in pharmaceutically useful pH ranges and the mechanisms of these transformations.

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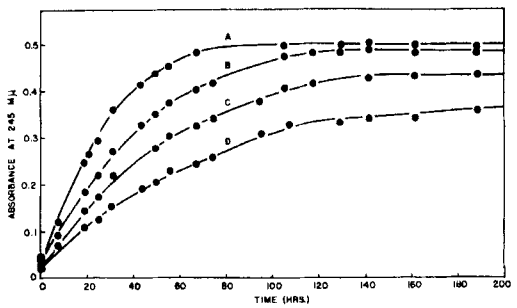

Fig. 1.—Increase in 245 mμ absorbance from the dehydration of cycloheximide ($1.42 \times 10^{-4} M$) in pH 4.63 buffers ($\mu = 0.08$) at 80°. The curves and equimolar acetic acid and acetate ion concentrations are: A, 0.0314; B, 0.0157; C, 0.0079; D, 0.0031.

EXPERIMENTAL

Preparation and Identification of Cycloheximide and Anhydrocycloheximide.—The preparation and identification of the cycloheximide and anhydrocycloheximide used for this study has been described previously (6). The cycloheximide was supplied by Dr. Gerald A. Boyack, The Upjohn Co., Kalamazoo, Mich.

Spectrophotometric Determination of Cycloheximide Dehydration.—The dehydration of cycloheximide can be followed by measuring the appearance of the ultraviolet absorbance due to the formation of the α,β-unsaturated ketone as in the AN structures (Scheme I). This chromophore has an absorption maximum at 245 mμ under the acidic conditions of this study and at 248 mμ under the alkaline conditions. Studies in buffer solutions gave the anticipated increase in absorbance as a function of time (Fig. 1).

Spectral Transformations of Cycloheximide in Acetate Buffer Solutions.—A standard NaOH solution was mixed with standard solutions of acetic acid and KCl to prepare buffers of pH 3 to 5.5 with controlled ionic strength. The buffers were standardized with a Sargent model D recording titrator. The acetic acid concentration of each buffer was established by direct titration with standard NaOH. The acetate concentration was determined by HCl titration of the buffer in ethanol to sharpen the end point. The pH was measured with a Radiometer titrator (Radiometer, Copenhagen, Denmark).

Aliquots of aqueous stock solutions of the compound under study were diluted to appropriate volumes with standard buffer solutions previously equilibrated in constant temperature baths. All solutions were prepared from nitrogen-purged water. Samples were removed as a function of time, cooled immediately, and the absorbance read on a Beckman DU spectrophotometer at 245 mμ with a 0.1-mm. slit width. Complete spectra were obtained as a function of time on a Cary model 15 recording spectrophotometer for several reactions. (See Table I for experimental conditions.)

The reactions of cycloheximide in acetic acid–acetate buffers were monitored by the thin-layer chromatographic methods previously described (6).

Spectral Transformations of Cycloheximide in Alkaline Solutions.—The thermostated cell chamber of a Beckman model DB recording spectrophotometer was sealed with a cover which had an opening for a thermometer.

Thermally equilibrated aqueous solutions of cycloheximide (0.00064 M) and NaOH were mixed. Samples were transferred immediately into the sample cell, and the instrument was turned on. The absorbance at 248 mμ was graphed as a function of time on a Sargent SRL recorder. Complete spectra were obtained at various time intervals, and the temperature in the sample cell was checked before and after each reaction.

Constant pH Hydrolysis of Cycloheximide.—The Radiometer titrator with titrigraph was standardized with pH 7.00 and 10.00 buffers after thermal equilibration of the thermostated titration vessel. A standard NaOH solution was brought to the de-

Table I.—Conditions, Apparent First-Order Rate Constants,[a] Reversible Rate Constants, $CY \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} AN$, and Apparent Equilibrium Constants[b] for the Dehydration of Cycloheximide (CY)

°C.	Observed pH	Buffer Compn. $[HC_2H_3O_2]$	Buffer Compn. $[C_2H_3O_2^-]$	Buffer Compn. [KCl]	$10^6 k$[a]	K[b]	$10^6 k_1$	$10^6 k_{-1}$
80.0	3.16	0.0181	0.0005	0.080	5.19	0.65	2.04	3.15
	4.00	0.0466	0.0093	0.030	6.97	0.63	2.69	4.28
	4.21	0.0419	0.0140	0.025	7.63	0.81	3.41	4.22
	4.21	0.0210	0.0070	0.033	5.35	0.64	2.08	3.27
	4.21	0.0042	0.0014	0.038	3.69	0.52	1.27	2.42
	4.58	0.0335	0.0224	0.017	8.13	0.65	3.20	4.93
	4.62	0.0280	0.0280	0.012	10.6	0.61	4.03	6.57
	4.63	0.0314	0.0316	0.050	11.9	0.77	5.17	6.73
	4.63	0.0157	0.0158	0.065	7.42	0.71	3.09	4.33
	4.63	0.0079	0.0079	0.072	5.51	0.61	2.08	3.43
	4.63	0.0031	0.0031	0.078	3.88	0.56	1.39	2.49
	5.15	0.0100	0.0349	0.003	13.0	0.85	5.97	7.03
	5.15	0.0050	0.0174	0.021	8.48	0.66	3.37	5.11
	5.15	0.0010	0.0035	0.036	4.85	0.55	1.72	3.13
	5.29	0.0089	0.0373	0.000	12.4	0.99	6.16	6.24
	5.29	0.0044	0.0186	0.020	9.38	0.77	4.09	5.29
	5.29	0.0022	0.0093	0.030	7.53	0.67	3.04	4.49
	5.63	0.0037	0.0373	0.000	18.2	0.63	7.00	11.2
50.0	4.06	0.0466	0.0093	0.030	0.456	0.36	0.121	0.335
	4.76	0.0280	0.0280	0.010	0.577	0.43	0.173	0.404

[a] Based on measurements of the rate of achievement of the asymptotic absorbance of anhydrocycloheximide (AN) $k = k_1 + k_{-1}$. [b] $K = [AN]_\infty/[CY]_\infty = k_1/k_{-1}$, where the k_i are in seconds^{-1}.

TABLE II.—APPARENT FIRST-ORDER RATE CONSTANTS (k IN SECONDS^{-1}) FOR THE CONSTANT pH HYDROLYSIS OF CYCLOHEXIMIDE (CY) TO THE CORRESPONDING MONOCARBOXYLIC ACID[a],

$$CY \xrightarrow{k} CY\begin{pmatrix}CONH_2\\COOH\end{pmatrix}$$

10^3 [NaOH]	10^2k 30.2°	34.9°	42.7°
3.13	...	0.702	0.874
4.70	0.864	0.990	1.50
5.37	...	1.14	1.77
5.37	...	1.20	...
6.26	...	1.31	1.87
7.51	...	1.61	2.30
7.51	...	1.63	...
7.51	...	1.67	...

[a] Subsequent hydrolysis to the dicarboxylic acid is extremely slow.

sired temperature in the titration vessel. The pH-stat mechanism was adjusted to maintain the observed pH. An aliquot of a thermally equilibrated aqueous solution of cycloheximide then was introduced into the vessel under constant agitation. The volume of NaOH volumetric solution required to maintain a constant pH was recorded as a function of time by the titrigraph. (See Table II for experimental conditions.)

Alkaline Hydrolysis of Cycloheximide.—Cycloheximide was dissolved in 0.166 *M* NaOH at 25°. One-milliliter aliquots were removed as a function of time and mixed with 20 ml. of 25% ethanol. The samples were adjusted to a pH of 3 with HCl. They were then titrated with 0.166 *M* NaOH using the Radiometer titrator. The first end point was the remaining HCl (6). The concentration of generated carboxyl groups could be calculated from the total standard alkaline titer between the first and second end points. Thus, the per cent hydrolysis of the imide group could be determined as a function of time. From the total standard alkali titer between the second and third end points, the concentration of generated ammonium groups could be calculated. Thus, the per cent hydrolysis of the generated acid amides could be determined as a function of time.

CALCULATIONS AND RESULTS

Cycloheximide Dehydration in Acetate Buffer Region.—The spectrophotometric absorbance at 245 mμ for cycloheximide in acetate buffers increased as a function of time to a maximum value (Fig. 1). The value of the maximum absorbance increased with acetate buffer concentration. No subsequent significant decrease in this absorbance occurred with time. Thin-layer chromatographic monitoring gave spots that could be associated only with anhydrocycloheximide and cycloheximide. No spots were observed that could be assigned to α-epicyclo, the carboxylic acid derivatives of CY, AN, or α-epi-CY (Scheme I), or glutarimide β-acetaldehyde within the time intervals studied.

Observed pseudo first-order rate constants, k, for the achievement of the maximum, A_m, in these buffer solutions were calculated from

$$\ln (A_m - A_t) = -kt + \ln A_m \quad \text{(Eq. 1)}$$

where A_t is the absorbance at time t. These values are given in Table I. The 245 mμ absorbance is associated with the α,β-unsaturated ketone of anhydrocycloheximide (AN) of Scheme I.

The apparent first-order rate constants, k, for the appearance of anhydrocycloheximide may be defined on the assumption of general acid-base catalysis—specifically by acetic acid, HAc, and acetate ions, Ac^-.

$$k = k_{H^+}[H^+] + k_{HAc}[HAc] + k_{Ac^-}[Ac^-] + k_{H_2O}[H_2O] + k_{OH^-}[OH^-] \quad \text{(Eq. 2)}$$

At constant pH, Eq. 2 reduces to

$$k = k_{HAc}[HAc] + k_{Ac^-}[Ac^-] + k_o \quad \text{(Eq. 3)}$$

which may be expressed as

$$k = (k_{HAc}[HAc]/[Ac^-] + k_{Ac^-})[Ac^-] + k_o \quad \text{(Eq. 4)}$$

The observed rate constants, k, at 80° were plotted against acetate ion concentration, $[Ac^-]$, at four different pH values (Fig. 2). The linearity of the plots confirms the expectations (Eq. 2) that the observed pseudo first-order rate constants, k, depend upon acetate ion concentration.

The ionic strength at a given pH was maintained constant. All of the buffers had an ionic strength of 0.04, except those at pH 4.63 and 3.16, where it was 0.08 (Table I). No effect was observed from this change.

The slope, m, of Fig. 2 (Eq. 4) is defined

$$m = k_{HAc}R + k_{Ac^-} \quad \text{(Eq. 5)}$$

where $R = [HAc]/[Ac^-]$ is a fixed ratio for a given pH, and the intercept, k_o, in Fig. 2 is defined by

$$k_o = k_{H^+}[H^+] + k_{H_2O}[H_2O] + k_{OH^-}[OH^-] \quad \text{(Eq. 6)}$$

The slopes, m, of the plots of Fig. 2 and their respective R values permit the estimation (Eq. 5) of the catalytic rate constants. A plot, *m versus R* for 80°, gives an intercept of $k_{Ac} = 2.53 \times 10^{-4}$ (L./mole/second) with a negligible acetic acid catalytic constant, $k_{HAc} = 0.2 \times 10^{-4}$ (L./mole/second).

The intercept, k_o, of the plots for each pH value (Fig. 2) was corrected for hydrogen ion catalysis by subtracting the contribution of $k_{H^+}[H^+]$, where $[H^+] = 10^{-pH}$ and where it has been shown previously (6) that

$$k_{H^+} = 2.55 \times 10^{13} e^{-19{,}000/RT} \quad \text{(Eq. 7)}$$

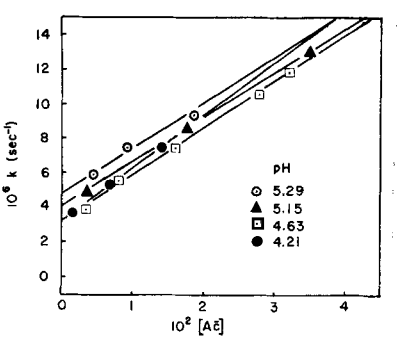

Fig. 2.—Effect of acetate ion concentration on the apparent first-order rate constants at 80° for the achievement of maximal anhydrocycloheximide from the dehydration of cycloheximide.

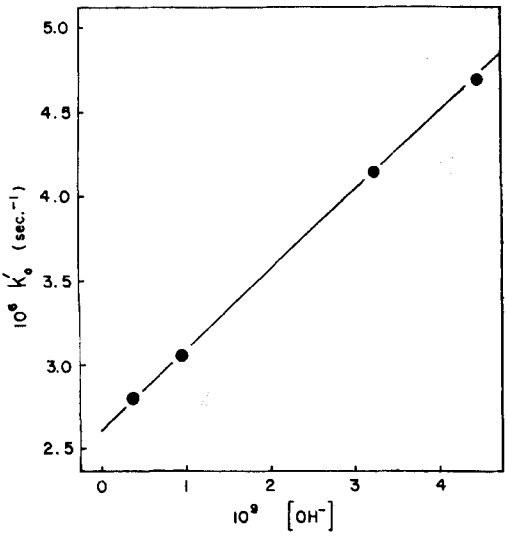

Fig. 3.—The apparent first-order rate constants at 80° for the achievement of maximal anhydrocycloheximide from the dehydration of cycloheximide and corrected for hydrogen ion and buffer catalysis, *i.e.*, k_o' *vs.* hydroxyl ion concentration. The slope is k_{OH^-}, and the intercept is $k_{H_2O}[H_2O]$.

The $k_{H^+}[H^+]$ contribution is negligible in the neutral pH region; at a pH of 4.61, it is 0.11×10^{-6} second^{-1} and less at higher pH values.

From Eq. 6

$$k_o' = k_o - k_{H^+}[H^+] = k_{OH^-}[OH^-] + k_{H_2O}[H_2O] \quad \text{(Eq. 8)}$$

A plot of k_o' *versus* hydroxyl ion concentration, [OH$^-$], was linear with a slope of k_{OH^-} and an intercept of $k_{H_2O}[H_2O]$ (Fig. 3). The hydroxyl ion concentration was calculated from

$$[OH^-] = 10^{-(pK_w - pH)} \quad \text{(Eq. 9)}$$

and the $pK_w = 12.64$ at 80° was obtained from extrapolation of the data in the literature (7). The calculated catalytic rate constants (Eq. 8 and Fig. 3) are $k_{OH^-} = 4.70 \times 10^2$ (L./mole/second) and $k_{H_2O}[H_2O] = 2.62 \times 10^{-6}$ (second^{-1}) at 80°.

Alkaline Dehydration of Cycloheximide.—The rate of conversion of 6.4×10^{-4} *M* cycloheximide to anhydrocycloheximide in alkaline solution was so rapid that reduction of hydroxyl ion concentration (1 to 2×10^{-3} *M*) to that of the cycloheximide concentration was necessary to observe the transformation. The yield of anhydrocycloheximide was of the order of 3% in those cases where the transformation could be observed. Good first-order plots were obtained, but no dependency on hydroxyl ion was determined due to variation of hydroxyl ion concentration during the reaction. The magnitude of the apparent first-order rate constants for the achievement of maximal anhydrocycloheximide absorbance was 10^{-3} and 2×10^{-3} second^{-1} for 28° and 37°, respectively. Titration before and after a typical run showed that cycloheximide consumed a molar equivalent of hydroxide during the time interval required for the spectral change.

Constant hydroxyl ion concentration was maintained by using 10 to 300 times as much sodium hydroxide as cycloheximide. It was impossible to measure the cycloheximide transformation to anhydrocycloheximide under these conditions since the conversion was complete within the few seconds of mixing time. Only the second phase of the reaction, the loss of anhydrocycloheximide, could be measured. The anhydrocycloheximide yields were about 10%, and were maximal at time zero.

Constant pH Hydrolysis of Cycloheximide.—The volume of titer, *V*, necessary to maintain a constant pH during the alkaline hydrolysis of cycloheximide was recorded as a function of time. The initial portions of the curves were linear and constant for different conditions of temperature and hydroxyl ion concentration (Fig. 4A). This apparent zero-order effect was attributed to the reaction speed overcoming the titrator capacity. First-order plots were linear for the subsequent portions of the curve when plotted according to

$$\ln (V_\infty - V_t) = -kt \quad \text{(Eq. 10)}$$

where V_∞ is the asymptotic value, and V_t is the volume at any time subsequent to the zero-order phase (Fig. 4B). Apparent first-order rate constants and experimental conditions are given in Table II. The rate constants reported here are of the same order of magnitude as those of Sircar (8) for various imides hydrolyzed under similar conditions ($k = 5.26 \times 10^{-2}$/second in 0.005 *N* NaOH at 25° for β-ethylglutarimide). Cycloheximide consumed 85–90% of its molar equivalent of NaOH, also in agreement with the literature for similar solvolysis of other imides (8).

The plots of the apparent first-order rate constants (Table II) *versus* hydroxyl ion concentration (Fig. 5) were consistent with the premise that imide hydrolysis is specific hydroxyl ion catalyzed. For a constant [NaOH] = 4.7×10^{-3}, the estimated ΔH_a is 8.42 Kcal./mole, and log *P* is 4.00, as derived from the apparent first-order rate constants.

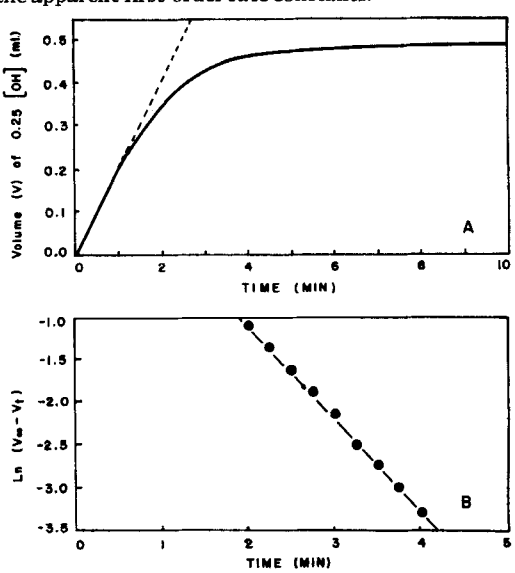

Fig. 4.—Key: A, the volume of standard alkali consumed as a function of time and at a constant pH by a solution of 5.21×10^{-3} *M* cycloheximide in 6.26×10^{-3} *M* NaOH at 42.7°; B, first-order plot for the volume of alkali consumed, *V*, as a function of time for that portion of the curve succeeding the linear phase.

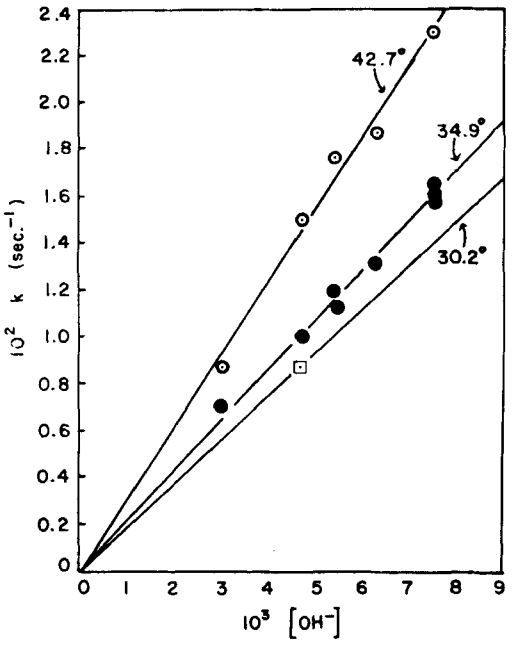

Fig. 5.—Dependence of the apparent first-order rate constants, k, for the constant pH solvolysis of cycloheximide to the corresponding monocarboxylic acid upon hydroxyl ion concentration at various temperatures.

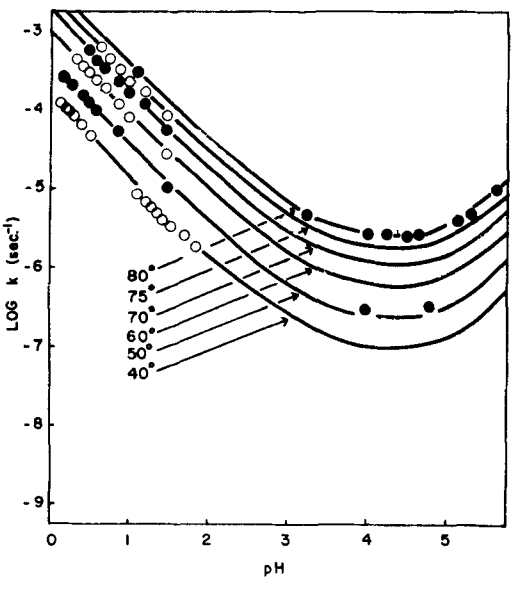

Fig. 6.—The log k-pH profiles for the apparent first-order appearance of anhydrocycloheximide from the dehydration of cycloheximide.

Alkaline Hydrolysis of Cycloheximide.—Titration curves similar to those obtained under acid hydrolysis (6) were obtained for the hydrolysis of cycloheximide in 0.166 M NaOH at 25°. Cycloheximide consumed a molar equivalent of alkali within a few minutes, while subsequent consumption, up to 2 equivalents, was not complete after 24 hours.

DISCUSSION

Prediction of Dehydration of Cycloheximide in Acetate Buffer Region.—The apparent first-order rate constant (k in Eq. 1) for the appearance of anhydrocycloheximide from cycloheximide followed spectrophotometrically in the acetic acid–acetate buffer region can be defined by Eq. 2. The dehydration of cycloheximide is specific acid-base and general base catalyzed and undergoes a water reaction. This latter effect was not observed previously (6) since it was insignificant in the strong acid region.

The log k *versus* pH profiles for the appearance of the α,β-unsaturated ketone of anhydrocycloheximide at various temperatures are given in Fig. 6. The log k values were calculated from Eq. 6 for conditions where buffer catalysts were absent and pH = $-\log [H^+]$. In addition to the values of k_{H+} which can be calculated for any temperature from Eq. 7, the values of $k_{H_2O}[H_2O] = 2.62 \times 10^{-6}$ second^{-1} and $k_{OH^-} = 4.70 \times 10^{-2}$ L./mole/second at 80° and a heat of activation of 20 Kcal./mole were used to predict the curves for lower temperatures (9). The lines of Fig. 6 are such predictions for over-all apparent rate constants at various temperatures. The data obtained at 50° and plotted in Fig. 6 are confirmation of the validity of these predictions. In addition, the ΔH_a and log P values were calculated for comparable studies at 80° and 50° with the same acetic acid–acetate buffer concentrations (Table I). For the k values of the pH 4.0 studies, log P was 7.25 and ΔH_a was 20.1 Kcal./mole. For the k values of the pH 4.7 studies, log P was 8.44, and ΔH_a was 21.8 Kcal./mole.

The observed first-order rate constant is a minimum at pH 4.4. If buffer effects are neglected and only pH and water effects are considered, the over-all rate constant at 20° and pH 4.4 may be estimated from a ΔH_a of 20 Kcal./mole as 1.2×10^{-8} second^{-1} (9). Thus, the time to reach 10% of the anhydrocycloheximide that will eventually form under these conditions is 105 days. At 30°, the time would be 36 days. Acceleration due to buffer components would have to be established for the particular system employed.

Prediction of Stability from Alkaline Degradative Mechanisms.—The alkaline catalyzed hydrolysis of cycloheximide to its acid amide was faster than the subsequent hydrolysis of the acid amide. This is in contrast to the acidic hydrolysis of cycloheximide, where the rate-determining step in the hydrolytic sequence was in the solvolysis of the imide function.

If imide hydrolysis inactivates cycloheximide as it does E-73 (10), then the avoidance of this degradative mechanism is important in maintaining biological activity. The hydroxyl ion catalytic rate constant for imide solvolysis is

$$k_{OH^-} = 2.14 \times 10^6 e^{-8,420/RT} \text{L./mole/second} \quad \text{(Eq. 11)}$$

or 1.12 at 20° and 1.78 at 30°.

The apparent first-order rate constants at constant pH for imide solvolysis can be calculated from

$$k = k_{OH^-} 10^{-(pK_w - pH)} \quad \text{(Eq. 12)}$$

From these derived values the time, $t_{0.9}$, to reach 10% hydrolyzed imide can be calculated (9); and at 20° it is, for the stated pH values: 1.7 minutes, pH 11; 2.6 hours, pH 9; and 11 days, pH 7. Similarly,

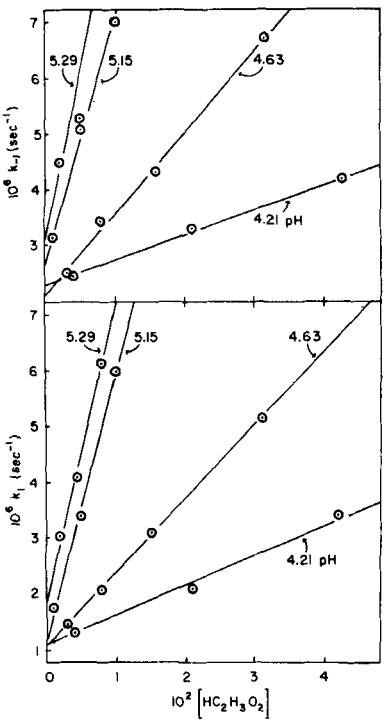

Fig. 7.—Plots of the reversible 80° first-order rate constants, k_1 and k_{-1}, as functions of apparent acetic acid concentrations in acetic acid–acetate ion buffers at various pH values, CY $\underset{k_{-1}}{\overset{k_1}{\rightleftarrows}}$ AN.

the $t_{0.9}$ at 30° is 1.0 minute, pH 11; 1.6 hours, pH 9; and 6.8 days, pH 7.

It is of interest that at 30° and pH 11 imide splitting is of the same magnitude as anhydrocycloheximide formation, *i.e.*, 10^{-3} seconds^{-1}.

Concomitant with the imide splitting in the alkaline region is the extremely fast appearance of the α,β-unsaturated ketone, although in low yield. The fact that only 3 to 10% of the total possible yield of anhydro forms occur in mild alkali indicates that a more significant parallel reaction is proceeding. The parallel reaction is most probably the phenomenon of alkaline catalyzed retroaldolization to the glutarimide β-acetaldehyde and dimethylcyclohexanone, not reported in this paper.

Mechanism in Acetate Buffer pH Region.—The time-achieved absorbance associated with an α,β-unsaturated ketone (Fig. 1) resulted from an apparent first-order process and did not subsequently decrease. Thin-layer chromatographic monitoring of the reaction demonstrated spots consistent with cycloheximide and anhydrocycloheximide as constituents of the reaction mixtures. Previously (6), isolation from these spots had identified CY and AN. Spots attributable to any other carbonyl compounds, *viz.*, α-epi-CY or glutarimide β-acetaldehyde, were absent. Carboxylic acid derivatives which remain at the origin of the TLC plates (6) were negligible in the time intervals studied (Fig. 1).

These facts are consistent with a simplification of the complex system of Scheme I in the acetate buffer pH region

$$\text{CY} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{AN} \qquad \text{(Eq. 13)}$$

where CY includes all cycloheximide forms, and AN includes all anhydrocycloheximide forms that possess the aforementioned physical and chemical properties.

On the basis of the asymptotic absorbances obtained (Fig. 1) and the known absorptivity of anhydrocycloheximide, a value of the equilibrium constant K can be estimated for the various runs (Table I) where

$$K = [\text{AN}]/[\text{CY}] = k_1/k_{-1} \qquad \text{(Eq. 14)}$$

The apparent first-order rate constant, k (Eq. 1), for the achievement of the equilibria characterized by the constant, K, can be defined as the sum of the forward and reverse rate constants

$$k = k_1 + k_{-1} \qquad \text{(Eq. 15)}$$

The k_1 and k_{-1} derived from Eqs. 14 and 15 are given in Table I.

These microscopic rate constants can be analyzed as functions of acetate and acetic acid concentrations in accordance with Eq. 3 which can be expressed

$$k = (k_{\text{HAc}} + k_{\text{Ac}}[\text{Ac}^-]/[\text{HAc}])[\text{HAc}] + k_o \qquad \text{(Eq. 16)}$$

The linearities of the plots of k_1 and k_{-1} against [HAc] in Fig. 7 demonstrate the validity of this equation. The slopes of these plots are

$$m' = k_{\text{HAc}} + k_{\text{Ac}}[\text{Ac}^-]/[\text{HAc}] \qquad \text{(Eq. 17)}$$

and plots of m' against the respective $[\text{Ac}^-]/[\text{HAc}]$

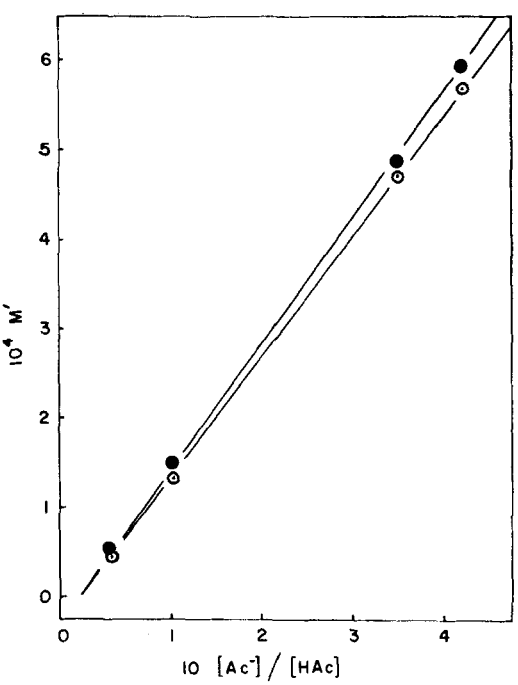

Fig. 8.—Plots of values $m' = k_{\text{HAc}} + k_{\text{Ac}}[\text{Ac}^-]/[\text{HAc}]$ *vs.* the $[\text{Ac}^-]/[\text{HAc}]$ ratios for the k_1 of cycloheximide (CY) dehydration—⊙— and for the k_{-1} of anhydrocycloheximide (AN) rehydration—●— at 80°, CY $\underset{k_{-1}}{\overset{k_1}{\rightleftarrows}}$ AN.

ratios permit the evaluation of $(k_{HAc})_1$ or $(k_{HAc})_{-1}$ as the intercepts and $(k_{Ac})_1$ or $(k_{Ac})_{-1}$ as the slopes. Such plots of Eq. 17 in Fig. 8 demonstrate general base-catalyzed dehydration and rehydration due to acetate ion. General acid catalysis due to acetic acid is negligible since the intercepts are zero. Thus, the apparent first-order rate constant for acetate buffer region dehydration of cycloheximide can be expressed

$$k_1 = (k_{Ac})_1[Ac^-] + (k_o)_1 \qquad \text{(Eq. 18)}$$

where at 80° $(k_{Ac})_1 = 1.36 \times 10^{-3}$ L./mole/second and $(k_o)_1 = 1.0 \times 10^{-6}$ second^{-1} and is largely due to solvent catalysis. An apparent hydroxyl ion-catalyzed dehydration is also indicated from the increasing values of the intercepts in Fig. 7 and should become highly significant at pH values greater than the pH range of the acetate buffer region.

Similarly, the apparent first-order rate constant for acetate buffer region rehydration of anhydrocycloheximide can be expressed

$$k_{-1} = (k_{Ac})_{-1}[Ac^-] + (k_o)_{-1} \qquad \text{(Eq. 19)}$$

where at 80° $(k_{Ac})_{-1} = 1.43 \times 10^{-3}$ L./mole/second and $(k_o)_{-1} = 2.1 \times 10^{-6}$ second^{-1} and is largely due to solvent catalysis. An apparent hydroxyl ion catalyzed rehydration is also indicated from the increasing values of the intercepts in Fig. 7 and should become highly significant at pH values greater than the pH range of the acetate buffer region.

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Solubility Studies on Certain Barbiturates

By RICHARD L. SEDAM, ALFONSO R. GENNARO, and ARTHUR OSOL

The solubilities in water at 25° of five barbiturates were determined by the application of liquid scintillation counting of C^{14}-tagged compounds to the technique of phase solubility analysis. The effects of several added solutes on these solubilities were also determined, and over-all salting-out constants were obtained from graphs based on the empirical Setschenow equation.

THE SOLUBILITIES in water of many of the barbiturates are reported in such general terms as "slightly soluble" or "very slightly soluble" (1, 2). Even when solubilities are defined more precisely, based on quantitative measurements on saturated solutions, the data often differ (3–6). The technique of phase solubility analysis apparently has not been applied to barbiturates.

Solubility data for barbiturates have special utility in preparing liquid pharmaceutical dosage forms; in developing these formulations, a knowledge of the effect of other solutes on barbiturate solubility is highly desirable. In general, the solubility in water of a nonelectrolyte is altered by the addition of an electrolyte. Inorganic electrolytes commonly cause salting-out to occur, although there are exceptions to this general statement. Nonelectrolytes such as sucrose also may cause salting-out. Salting-in is frequently caused by the salts of various organic acids and by organic-substituted ammonium salts. Long and McDevit (7) have summarized the many theories advanced to explain salt effects; unfortunately, none of the theories explains completely the available experimental data. It appears that an empirical or semiempirical approach must be employed at the present time.

In the present investigation, the solubilities in water at 25° of five barbiturates were determined by the application of liquid scintillation counting of C^{14}-tagged compounds to the technique of phase solubility analysis. The effects of four added solutes on these solubilities were determined also.

Phase solubility analysis was described first by Northrop and Kunitz (8) as a criterion of the chemical purity of a substance. These workers also demonstrated that the technique can be used to obtain the exact solubility of the pure substance. This can be accomplished without the

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