metric procedure. From inspection of Table II, it is seen that quantitative recovery was achieved by the extraction and chromatographic techniques. The analyses reported in Table III were carried out in triplicate by the assay method described.

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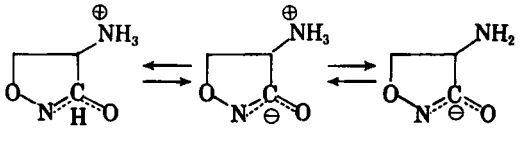
Stability of Cycloserine in Buffered Aqueous Solutions

By LOUIS MALSPEIS and DAVID GOLD

The pH rate profile of the hydrolysis of cycloserine in aqueous buffers is reported. Apparent pseudo first-order plots of the hydrolytic degradation are observed over the entire pH range. Minimal over-all velocity occurs in the vicinity of pH 12. A linear log k-pH plot is found for the protonated molecule and the dipolar ion in the pH ranges 0.4 to 2.3 and 3.0 to 7.0. The hydrolysis is catalyzed by undissociated acetic acid, and the acetic acid-catalyzed reaction appears to be first order with respect to cycloserine, regardless of whether the protonated molecule or the dipolar ion is present in the solution. Primary kinetic salt effects were not detected in hydrolyses involving the protonated, dipolar, or anionic molecules. The kinetics suggest that in the acid-catalyzed hydrolysis the substrate is the protonated cycloserine molecule. An intermolecular electrophilic-nucleophilic mechanism involving this substrate is considered.

THE STRUCTURE of the antibiotic cycloserine[1] has been shown to be D-4-amino-3-isoxazolidone (1, 2); the dipolar ion structure, as deduced from its infrared absorption spectrum and from potentiometric titration in aqueous solution, is II.



I II III

Reports of qualitative observations of the degradation of cycloserine in aqueous solution indicate that the compound is relatively stable in alkaline solution, whereas it is readily hydrolyzed in acid solution. Kuehl *et al.* (1) noted that cycloserine was facilely degraded to serine and hydroxylamine upon acid hydrolysis and that it was relatively stable to alkali. Hidy *et al.* (2) reported that mild acid hydrolysis yielded D-serine and hydroxylamine, while prolonged hydrolysis gave DL-serine and hydroxylamine. In aqueous solution at pH 6, the compound was 63% degraded in 20 hours at room temperature (3). In 15% sodium hydroxide solution at 100°, Ratouis and Behar observed that the compound was 38% degraded in 6 hours (4). Felder and Tiepolo reported that cycloserine is about 2% degraded in 24 hours at physiological conditions (5). The decomposition products were stated to be hydroxylamine, serine, and 3-aminoxyalanine in acidic solution and 2,5-bis(aminoxymethyl)-3,6-dioxopiperazine and 2,5-bismethylene-3,6-dioxopiperazine in neutral solution (5).

Analogous results were reported for 4-benzamido-3-isoxazolidone. This amide was stable to boiling 2 *N* sodium hydroxide, while acid hydrolysis produced ring opening in addition to amide cleavage (6). Ratouis and Behar (4) identified serine and serine amide as the products of the acid-catalyzed hydrolysis.

Inconsistent with these observations is the report of Nishimura and Shimohima (7), who found that the stability point of cycloserine in aqueous solution is at pH 6.0.

Structurally, cycloserine may be viewed as a cyclic *O*-alkyl hydroxamic acid. The observed stability of cycloserine to alkaline hydrolysis indicates that *N*-amide substitution with an oxygen atom results in a compound which is much less susceptible to nucleophilic attack. Simple amides are approximately equally susceptible to nucleophilic and to electrophilic attack (8); the pH rate profile of the hydrolysis of simple amides exhibits a minimal over-all velocity

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[1] Marketed as Seromycin by Eli Lilly and Co., Indianapolis, Ind.

TABLE I.—BUFFER SOLUTIONS

Buffer	Buffer Compn.	Apparent pH[a] 25°	70°	80°	95°
A	0.50 *M* HCl	0.38[b]	...	...	...
B	0.095 *M* HCl 0.005 *M* KCl	1.07	1.14	1.30	1.30
C	0.01 *M* HCl 0.09 *M* KCl	2.10	2.15	2.30	2.40
D	0.106 *M* phthalic acid 0.159 *M* KHphthalate 0.041 *M* NaCl	2.72	2.96	3.11	3.12
E	0.160 *M* CH_3COOH 0.020 *M* CH_3COONa 0.160 *M* NaCl	3.60	3.65	3.77	3.88
F	0.060 *M* CH_3COOH 0.180 *M* CH_3COONa 0.020 *M* NaCl	5.00	5.20	5.35	5.49
G	0.204 *M* H_3BO_3 0.002 *M* $Na_2B_4O_7$	7.13	7.16	7.17	6.95
H	0.04 *M* H_3BO_3 0.09 *M* $Na_2B_4O_7$	9.08	8.90	8.94	8.91
I	0.546 *M* NaOH 0.023 *M* $Na_2B_4O_7$	11.10	11.14	11.10	11.17
J	0.10 *M* NaOH 0.90 *M* NaCl	12.86[c]	...	...	...
K	1.00 *M* NaOH	13.81[c]	...	...	...

[a] pH Values determined at temperature cited with glass calomel electrodes. [b] pH Values calculated from mean activity coefficients (26). [c] pH Values calculated from mean activity coefficients (27).

in the pH range 3 to 4 (9). Accordingly, with the limitation that each region of the pH rate profile must be considered in terms of the degree of protonation of the substrate present in solution, minimal over-all hydrolysis of cycloserine is expected at high pH values.

Relatively few examples of intermolecular general acid catalysis of the hydrolysis of carboxylic acid derivatives are known (10).[2] Recently, the only example of intermolecular general acid catalysis of the hydrolysis of amides was reported by Wyness (11), who demonstrated the acetic acid-catalyzed hydrolysis of *N-n*-butylacetamide. It was observed by Garrett (12, 13) that structural modification of esters which resulted in decreased hydrogen ion-catalyzed hydrolysis promoted intermolecular general base catalysis by acetate ion. Similarly, intermolecular general acid catalysis should be favored in carboxylic acid derivatives exhibiting decreased susceptibility to hydroxyl ion-catalyzed hydrolysis. According to this reasoning, cycloserine appeared to be a favorable system for demonstrating intermolecular general acid catalysis.

With this view, the pH rate profile of the hydrolysis of cycloserine was investigated, and preliminary experiments to obtain evidence for intermolecular general acid-catalyzed hydrolysis were conducted.

EXPERIMENTAL

Materials.—Cycloserine (Lilly, lot No. 501041) recrystallized according to the procedure of Stammer *et al.* (6) and dried *in vacuo*, m.p. 154 to 154.5° dec The reported melting points of this compound include 154–155° (1), 156° (2), and 153–154° (6). All compounds used in the preparation of buffer solutions and in the assay procedure were reagent grade.

Buffer Solutions, pH Measurement, and pK Determination.—The rates of the decomposition of cycloserine as a function of pH and the concentrations of general acids and bases were studied using buffers. The buffer systems employed in the kinetic studies to elucidate the pH rate profile are listed in Table I. The concentrations of the buffer components cited are the values at room temperature.

The Beckman model G pH meter was used to determine the pH of the buffer solutions at room temperature. The instrument was standardized with standard buffer solutions having pH values within two pH units of the solutions measured. At elevated temperatures the pH measurements were performed with the Beckman zeromatic pH meter. The instrument was standardized at the temperature of the solution which was measured with standard phosphate buffer. The pH values of the standard buffer at elevated temperatures were those cited by Britton (14). In all kinetic studies, pH measurements were taken prior and subsequent to the kinetic runs; no change in pH was observed.

The dissociation constants of cycloserine were essentially determined by the method described by Stern (15). The total concentration of dipolar cycloserine and its conjugate acid or base was 0.010 *M*, and the ionic strength of the solution was adjusted to 0.200 with sodium chloride. The pH of the solutions was determined at all temperatures with the Beckman model G pH meter; appropriate temperature compensation corrections were applied.

Kinetic Procedure.—The kinetic measurements of the degradation of cycloserine were conducted at 0.001 *M* in the given buffer solution. Aliquots were sealed in U.S.P. XVI type I ampuls of 10-ml. capacity; all the ampuls for a run were immersed in a liquid constant temperature bath. Time was per-

[2] For an excellent discussion and pertinent references see Bender, M. L., *Chem. Rev.*, **60**, 53(1960).

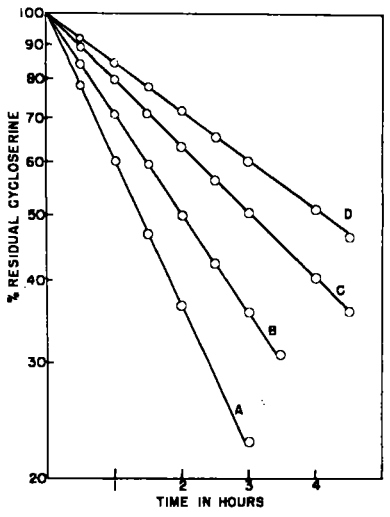


Fig. 1.—Apparent pseudo first-order plots of the hydrolysis of cycloserine at 80° at pH 5.2 in the presence of varying acetic acid concentration. Ionic strength is adjusted to 1.6 with sodium chloride. The following values are given for [CH_3COOH] and [CH_3COONa], respectively. Key: A, 0.064 and 0.136; B, 0.122 and 0.278; C, 0.220 and 0.580; D, 0.385 and 1.215.

mitted for temperature equilibration before removal of the zero-time sample which was considered to contain 100% cycloserine. Individual ampuls were removed at recorded times and immediately immersed in an ice-water bath. The ampuls were stored in the ice-water mixture until completion of the run. The ampuls were then opened, the solutions allowed to warm to room temperature, and the concentration of cycloserine was determined by the colorimetric procedure of Jones (16). The absorbance of each sample was determined against a blank at 625 mμ in a Beckman model DU spectrophotometer. Plots of log (A_t/A_∞) against time, as illustrated by the typical plots shown in Fig. 1, gave good straight lines. The pseudo first-order rate constants, k_1', were obtained by multiplying the slope by 2.303.

For certain runs in the pH region 2 to 0.4, the samples were quenched by chilling, followed by the addition of standard sodium hydroxide to give a pH of 7. The samples were then stored in a refrigerator until the solutions were analyzed.

Thermostats were maintained with ±0.03° at temperatures of 80° or below and within a ±0.06° range at 95°.

Table II.—Observed Rate Constants for the Degradation of 0.001 *M* Cycloserine in Various Buffer Solutions

Buffer	$10^5k_1'$, Sec.$^{-1}$ 50°	60°	70°	80°	95°
A	...	88.8	174	357	...
B	36.9	67.8	136	269	...
C	...	55.0	103	216	502
D	...	44.3	86.9	167	330
E	20.6	...	86.2	169	398
F	...	...	29.6	48.9	103
G	...	...	4.21	9.79	33.5
H	...	...	2.44	6.75	24.1
I	...	...	1.63	3.93	16.0
J	...	...	1.57	4.43	14.7
K	...	...	2.08	5.35	18.0

RESULTS

Dependence of Rate Upon Cycloserine Concentration.—The kinetics of the disappearance of cycloserine from solutions of constant hydronium ion concentration showed the reaction to be first order with respect to the total cycloserine concentration. The observed pseudo first-order rate constants, k_1', were estimated from the semilog plots of the fraction of undegraded cycloserine as a function of time and are listed in Table II. In each run, the reaction was followed to 30% residual cycloserine, and each plot included 7 to 10 points. Typical plots are shown in Fig. 1. At all pH values and irrespective of the buffer components, $-d[C]/dt = k_1'[C]$, where C = cycloserine.

Absence of Salt Effects.—The effect of varying ionic strength on the observed first-order rate constants of the hydronium ion-catalyzed hydrolyses was studied in solutions in which the predominant species of cycloserine is protonated, zwitterionic, or anionic. The results are shown in Table III. No significant change in the rate constant was noted when cycloserine was hydrolyzed at 50° in 0.1 *M* hydrochloric acid solutions adjusted to different ionic strengths with potassium chloride. A salt effect was not observed in the hydrolyses at 80° in boric acid–sodium borate buffer, pH 8.94, over the ionic strength range 0.27 to 0.81. The slight decrease in observed rate with increasing ionic strength seen in the hydrolyses conducted at 80° in biphthalate-phthalate buffer, pH 5.18, over the ionic strength range 0.20 to 0.80, is assumed to be within

Table III.—Hydrolysis of Cycloserine at Constant pH and Varying Ionic Strength

[HCl][a]		[KCl][a]	μ	pH[b]	10^5k_1, Sec.$^{-1}$	Temp., ° C.
0.095		0.005	0.100	1.14	2.69	50
0.095		0.105	0.200		2.79	
0.095		0.305	0.400		2.75	
[KH Phthalate][a]	[K_2 Phthalate][a]	[NaCl][a]				
0.0527	0.0473	...	0.195	4.18	4.19	80
0.0527	0.0473	0.205	0.400		4.11	
0.0527	0.0473	0.605	0.800		4.04	
[H_3BO_3][a]	[$Na_2B_4O_7$][a]	[KCl][a]				
0.04	0.09	...	0.27	8.94	6.75	80
0.04	0.09	0.27	0.54		6.70	
0.04	0.09	0.54	0.81		6.79	

[a] Concentration at 25°. [b] pH Values determined at reaction temperature.

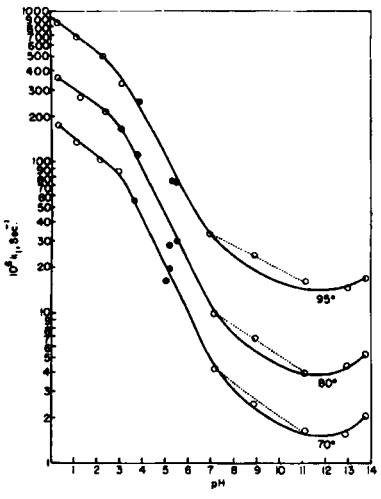


Fig. 2.—The pH rate profile of the hydrolytic degradation of cycloserine. Key: O, observed rates; ●, rates corrected for acetic acid catalysis.

the estimated reproducibility of the first-order constants. It appears that primary kinetic salt effects are not significant, irrespective of the degree of protonation of the substrate. Accordingly, the compositions of the buffer solutions were not maintained at constant ionic strength. It is observed, however, that the ionic strength range of the buffer solutions employed in the acid-catalyzed regions of the pH rate profile is 0.1 to 0.3. It is interesting that primary salt effects were absent in the hydronium ion-catalyzed hydrolysis of L-asparaginylglycine under conditions where the substrate molecule is protonated (17).

Dependence of Rate Upon Hydronium Ion Concentration.—The pH rate profiles of the hydrolysis of cycloserine at 70, 80, and 95° are shown in Fig. 2. It is convenient to examine four regions of the profile. In the pH range 2.3 to 0.4, an apparent linear increase of log k_1' with increasing pH is seen. In the pH range 7.0 to 3.0, the apparently linear dependence of log k_1' on pH exhibits a greater slope than is found in the pH 2.3 to 0.3 region. Between pH 11 and 7 log k_1' decreases with pH; above pH 12, the limited data indicate an increase of log k_1' with pH. The dashed lines in Fig. 2 show linear dependence of log k_1' on pH in the pH range 11 to 7, and the continuous line approximates a catalytic catenary with a minimum in the neighborhood of pH 12. Since the present investigation is concerned with demonstrating general acid-catalyzed hydrolysis, study of the alkaline region of the pH rate profile is deferred. If alkaline degradation of cycloserine above pH 12 is significant, then both acid and base-catalyzed reactions occur simultaneously between pH 12 and 10.

Both linear segments of the pH rate profile in the pH range 7 to 0.4 exhibit apparent fractional slopes. In a study of the degradation of streptozotocin, Garrett (18) attributed such apparent fractional slopes of the log k-pH plot in the acid region to the observed rate due to hydronium ion attack on protonated and nonprotonated substrate molecules in ionic equilibrium with each other. Perhaps the pH rate profile of cycloserine in the acid region presents a comparable situation.

The apparent pKa′ values of cycloserine ±0.01, where the error is the average deviation of three determinations, are given in Table IV. Because of the lability of cycloserine to hydronium ion-catalyzed hydrolysis at elevated temperatures, the experimentally determined pKa′ values are uncertain. However, the pKa′ values do afford a reasonable estimate of the concentrations of protonated, zwitterionic, and anionic cycloserine molecules which are present in each region of the pH rate profile. The classical kinetic expression (12) for the hydronium ion-catalyzed hydrolysis of cycloserine is

$$-dC/dt = -d([C^+] + [C^\pm] + [C^-])/dt \quad \text{(Eq. 1)}$$

$$= -k_{c^+}[H^+][C^+] - k_{c^\pm}[H^+][C^\pm] - k_c[H^+][C^-] - k_{H_2O}[H_2O][C] \quad \text{(Eq. 2)}$$

$$= -k_{H^+}[H^+][C] - k_{H_2O}[H_2O][C] \quad \text{(Eq. 3)}$$

$$= -k_1'[C] \quad \text{(Eq. 4)}$$

Neglecting the contribution of $k_{H_2O}[H_2O]$, where $[H^+]$ = hydronium ion activity, $[C] = [C^+] + [C^\pm] + [C^-]$ = total cycloserine concentration, C^+ = protonated cycloserine, $C^\pm$ = dipolar ion cycloserine, C^- = anion cycloserine.

Inasmuch as the observed rates are correlated with the experimentally measured hydronium ion activity at elevated temperatures, there is considerable uncertainty regarding the 95° pH rate profile. Nevertheless, the large differences in the slopes of the pH rate profile permit interpretation of the hydronium ion dependence. It can be estimated that the second-order rate constant in the region where the molecule is predominantly the dipolar ion is larger than the constant in the region where the principal species is the protonated molecule by a factor of 10^4. One interpretation is the $k_{c^\pm}$ in the above equations is much larger than k_{c^+}, denoting that the hydrolytic lability of the dipolar ion far exceeds that of the protonated molecule, a conclusion which is in accord with the view that the decreased rate is a consequence of the coulombic repulsion of the hydronium ion attack on the protonated molecule. This interpretation of the observed apparent fractional slopes of the pH dependency curve is based upon the assumption that the order of the reaction with respect to hydronium ion is the same in the reactions involving C^+, $C^\pm$, and C^-.

An alternate explanation is required if the order of the reaction with respect to hydronium ion is not the same for the differently protonated substrate molecules. The reaction order with respect to hydronium ion is generally deduced from a linear logarithmic increase of $(k_1' - k_o)$ with pH. The

Table IV.—Apparent Dissociation Constants of Cycloserine at μ = 0.2

Temp., ° C.	pKa₁′	pKa₂′
20.0[a]	4.39	7.39
70.0	4.23	6.83
80.0	4.08	6.76
95.0	3.99	6.64

[a] Reported values at room temperature; pKa₁′, 4.4; pKa₂′, 7.3 (2); pKa₁′, 4.4; pKa₂′, 7.4 (1); pKa₁′, 4.57; pKa₂′, 7.40; $\mu \sim 0.4$ (28).

TABLE V.—HYDROLYSIS OF CYCLOSERINE IN ACETIC ACID–SODIUM ACETATE BUFFERED SOLUTIONS

Part A

[CH_3COOH][a]	[CH_3COONa][a]	[NaCl][a]	pH[b]	Temp., °C.	[CH_3COONa]/[CH_3COOH]	$10^5 k_{obs}$, Sec.$^{-1}$	$10^4 k_{HA}$, L. Mole^{-1} Sec.$^{-1}$
0.048	0.102	0.098	4.04	70	2.13	2.36	
0.064	0.136	0.064				2.63	
0.070	0.149	0.051				2.75	
0.086	0.183	0.017				3.04	1.75[d]
0.032	0.032	0.168	4.67	70	1.00	3.04	
0.064	0.064	0.136				3.59	
0.096	0.096	0.104				4.20	
0.128	0.128	0.072				4.80	1.79[e]

Part B

[CH_3COOH][a]	[CH_3COONa][a]	[NaCl][a]	pH[b]	Temp., °C.	$10^5 k_{obs}$, Sec.$^{-1}$	$10^5 k_1'$,[c] Sec.$^{-1}$	$10^4 k_{HA}$, L. Mole^{-1} Sec.$^{-1}$
0.064	0.136	1.464	5.04	70	2.64	2.64	
0.122	0.278	1.322	5.08		3.64	3.65	
0.220	0.580	1.020	5.01		5.13	5.13	
0.385	1.215	0.385	5.23		7.66	7.68	1.64[f]
0.064	0.136	1.464	5.20	80	4.68	4.68	
0.122	0.278	1.322	5.23		6.31	6.32	
0.220	0.580	1.020	5.29		9.55	9.57	
0.385	1.215	0.385	5.50		14.08	14.14	2.97[g]
0.064	0.136	1.464	5.32	95	11.77	11.77	
0.122	0.278	1.322	5.35		16.04	16.05	
0.220	0.580	1.020	5.41		23.57	23.60	
0.385	1.215	0.385	5.63		35.54	35.63	7.47[h]

[a] Concentration at 25°. [b] pH Values measured with glass calomel electrodes at the temperature cited. [c] Pseudo first-order rates corrected to pH 5.04 at 70°, pH 5.20 at 80°, and pH 5.32 at 95°. [d] $k_o = 1.54 \times 10^{-5}$ sec.$^{-1}$. [e] $k_o = 2.48 \times 10^{-5}$ sec.$^{-1}$. [f] $k_o = 1.59 \times 10^{-5}$ sec.$^{-1}$. [g] $k_o = 2.81 \times 10^{-5}$ sec.$^{-1}$. [h] $k_o = 7.03 \times 10^{-5}$ sec.$^{-1}$.

spontaneous reaction rate, k_o, commonly is small compared to the observed apparent first-order rate constant. If substrate molecules having differing degrees of protonation are present in solution and the slope of the log k_1' *versus* pH plot is not integral, the order of the reaction with respect to hydronium ion activity is equivocal. Moreover, if a plot of $-\log k_1'$ *versus* pH is linear with nonintegral slope, then a plot of $-\log k_1'$ *versus* n pH will also be linear with nonintegral slope. From the reaction mechanism viewpoint, the reaction order with respect to hydronium ion is expected to be an integer, probably 1 or 2, corresponding to a linear plot of $-\log k_1'$ against pH or 2 pH, respectively. Should $-\log k_1'$ for the hydrolysis of C^+ be a linear function of n pH and $-\log k_1'$ for the hydrolysis of $C^\pm$ be a linear function of $2n$ pH, then the observed slopes of the respective plots would prove to be similar. The estimated slope of the pH rate profile (Fig. 2) in the pH range 2.0 to 0.4 is -0.122, and in the pH range 7.0 to 3.0 is -0.313, expressing the observed rate dependence on pH of the hydrolysis of C^+ and $C^\pm$, respectively. A plot of $-\log k_1'$ against 2 pH in the pH range 7.0 to 3.0 is linear with slope -0.157, which indicates that the order with respect to hydronium ion of the hydrolysis rate of $C^\pm$ is approximately twice that of C^+.

Dependence of Rate Upon Acetic Acid Concentration.—Catalysis by buffer components was studied by determining the pseudo first-order hydrolysis rate as a function of the buffer concentration of a series of acetic acid–sodium acetate buffers of constant pH and ionic strength. In accord with the traditional treatment of general acid–general base catalysis (12), at constant pH where both C^+ and $C^\pm$ are present in solution the observed rate constant, k_1', is given by

$$k_1' = k_o + \frac{k_{HA}'[HA] + k_{A^-}'[A^-]}{1 + \frac{Ka_1'}{[H^+]}} + \frac{K_{HA}''[HA] + K_{A^-}''[A^-]}{1 + \frac{[H^+]}{Ka_1'}} \quad \text{(Eq. 5)}$$

$$= k_o + [HA]\left\{\frac{k_{HA}'[H^+] + k_{HA}''Ka_1'}{[H^+] + Ka_1'} + \frac{[A^-]}{[HA]}\left(\frac{k_{A^-}'[H^+] + k_{A^-}'Ka_1'}{[H^+] + Ka_1'}\right)\right\} \quad \text{(Eq. 6)}$$

so that a plot of k_1' *versus* the acetic acid concentration, $[HA]$, is linear. As observed by Wyness (11), constancy of slope for different ratios $[A^-]/[HA]$ indicates that k_{A^-}' and k_{A^-}'' are negligibly small or zero and that catalysis is due to undissociated acetic acid.

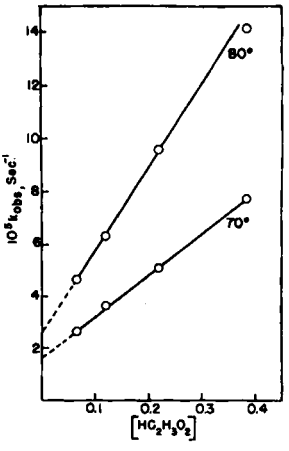


Fig. 3.—Plots showing the catalysis of the hydrolysis of cycloserine by acetic acid at constant pH and ionic strength. Key: 70°, pH 5.04, $\mu = 1.6$; 80°, pH 5.20, $\mu = 1.6$.

Typical plots of the observed rate constants at elevated temperatures as a function of the acetic acid concentration at room temperature (Table V, *Part B*) are shown in Fig. 3. At constant pH and ionic strength, the rates vary linearly with the acetic acid concentration. The apparent second-order rate coefficients, $k_{HA} = k_{obs}/[CH_3COOH]$, for different salt/acid ratios (Table V, *Part A*) are sensibly the same. Hence, catalysis appears to be due to undissociated acetic acid or to both hydronium and acetate ions, but not due to the acetate ion concentration alone. Slight curvature is seen as the acetic acid concentration becomes large.

The fact that the second-order rate coefficients, estimated at different hydronium ion concentrations, are similar indicates that the acetic acid catalytic coefficients k_{HA}' and k_{HA}'' are identical. This conclusion is inconsistent with the generally accepted formulation that k_{HA}' and k_{HA}'' are the catalytic coefficients which apply to C^+ and $C^\pm$, respectively. On the other hand, if the acetic acid-catalyzed hydrolysis of $C^\pm$ depends on the hydronium ion concentration as well as the concentrations of $C^\pm$ and HA, then $k_{HA}' = k_{HA}''$ and $k_{HA} = k_{HA}'(1 + [H^+])$. Accordingly, it would appear that the acetic acid-catalyzed reaction is first order with respect to cycloserine, regardless of whether the protonated molecule or the dipolar ion is present in the solution.

The intercept of the k_1' *versus* CH_3COOH plot, k_o, is a measure of all catalytic effects except acetic acid. Therefore, the intercept rates should fall on the graph of the pH rate profile. Representative intercept values are indicated by the solid circles in Fig. 2, and reasonable fit is observed. The intercept values are tabulated as a footnote in Table V.

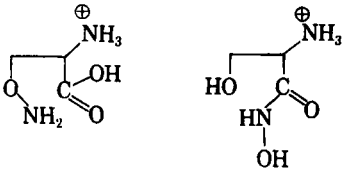
The rate of hydrolysis of cycloserine in phthalic acid–potassium biphthalate buffer solutions of similar pH and ionic strength was studied (Table VI). A fivefold variation in the phthalic acid concentration produced no significant change in rate.

Table VI.—Hydrolysis of Cycloserine in Phthalic Acid–Potassium Biphthalate Buffered Solutions at 80°

[Phthalic Acid]	[KH Phthalate]	[NaCl]	Apparent pH	$10^4k_1'$, Sec.$^{-1}$
0.1060	0.1590	0.0410	3.11	1.67
0.0530	0.0795	0.1205	3.11	1.63
0.0265	0.0398	0.1602	3.09	1.71

DISCUSSION

Intermediates in the Acid-Catalyzed Hydrolysis Reactions.—The end products of the acid-catalyzed hydrolysis of cycloserine have been found to be serine and hydroxylamine (1, 2, 5). In one report, 3-aminoxyalanine was observed to be a product of the acid-catalyzed degradation (5). Inasmuch as carbon-oxygen cleavage is considered unlikely, cleavage of either the carbon-nitrogen bond or the nitrogen-oxygen bond must occur initially during the course of the hydrolysis reaction. Therefore,



the two possible intermediates in the reaction are 3-aminoxy-D-alanine and D-serine hydroxamic acid. The hydroxamate was not identified as an intermediate in the hydrolysis mixture in this laboratory. When ferric ion was included in the hydrolysis mixture, the characteristic red of the ferric hydroxamic acid chelate was not noted. Failure to detect serine hydroxamic acid does not preclude the possibility that it is also an intermediate and that two competitive reactions are taking place, inasmuch as the acid-catalyzed hydrolysis of the hydroxamic acid may occur rapidly.

Justification for the expectation that 3-aminoxyalanine is an intermediate is based upon analogous reactions. The treatment of cycloserine with methanolic hydrogen chloride yields 3-aminoxy-D-alanine methyl ester dihydrochloride (1, 2, 6). Refluxing DL-4-benzamido-3-isoxazolidone in absolute ethanol saturated with hydrogen chloride gives the hydrochloride of 3-aminoxy-DL-alanine ethyl ester (6). The hydrochloride of 5-aminoxymethyl-3-phenylhydantoin is formed from the hydrochloric acid treatment of the reaction product of cycloserine and phenyl isocyanate. In addition, good yields of aminoxyalkanes are obtained from the aqueous acid or alkaline hydrolysis of *O*-alkyl hydroxamic acids and *O*-alkyl urethanes. Thus, heating *O*-butyl benzohydroxamic acid with hydrobromic acid gives aminoxybutane hydrobromide and benzoic acid (19); refluxing ethyl *O*-butylcarbamate with concentrated potassium hydroxide solution yields aminoxybutane (20). Moreover, it is probable that 3-aminoxyalanine is an intermediate in the formation of 2,5-bis(aminoxymethyl)-3,6-dioxopiperazine from cycloserine (21, 22). Therefore, it is reasonable to expect that the carbon-nitrogen bond is preferentially cleaved.

The apparent pKa values of 3-aminoxy-D-alanine methyl ester are 2.3 and 6.9 (1), the former value applying to the aminoxy group. Therefore, in dilute acid solution, the aminoxy group is incompletely protonated. It is possible that serine and hydroxylamine are formed from the aminoxy amino acid by solvent or nucleophilic displacement at either the aminoxy nitrogen or the β carbon atom.

Consideration of the Mechanism of the Hydrolysis of Cycloserine.—The kinetics of the hydrolysis of cycloserine have shown that both the protonated molecule and the dipolar ion are subject to hydronium ion-catalyzed hydrolysis, since the observed rates increase with decreasing pH. The question arises of whether the mechanisms of C^+ and $C^\pm$ differ.

It is not feasible that the rate-determining step in the hydrolysis of the dipolar ion involves proton transfer to the substrate

$$C^\pm + H_3O^+ \rightleftarrows (M^*) \rightarrow C^+ + H_2O \qquad \text{(slow)}$$

$$C^+ + H_2O \rightarrow \text{products} \qquad \text{(fast)}$$

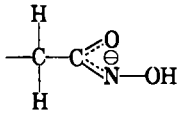
where M^* is the transition state complex in the theory of absolute reaction rates, inasmuch as the hydrolysis of the protonated molecule was not rapid. Pre-equilibration of the dipolar ion and

H_3O^+, followed by the bimolecular reaction between the protonated cycloserine and water

$$C^{\pm} + H_3O^+ \rightleftarrows C^+ + H_2O \quad \text{(fast)}$$

$$C^+ + H_2O \rightleftarrows (M^*) \rightarrow \text{products} \quad \text{(slow)}$$

is similar to the generally accepted mechanism for amide hydrolysis and in this instance corresponds to the solvent-catalyzed reaction of C^+. This mechanism implies that there is present in the C^+ molecule a center which is sufficiently electrophilic to make the water-catalyzed reaction appreciable. Essentially, the mechanism is analogous to the water-catalyzed hydrolysis of a nonprotonated amide, and the rates of such spontaneous reactions are extremely low. If the intermediate product of the hydrolysis reaction is the 3-aminoxy amino acid, the water molecule must attack the carbonyl-carbon; if the product is the hydroxamic acid, the reaction probably occurs at the ring nitrogen. Stammer and co-workers (6) have presented evidence that in hydroxamic acid salts the negative charge resides near the carbonyl-carbon, and it seems likely that there is also a relatively high electron density on the carbonyl-carbon of dipolar cycloserine.



Accordingly, the structure of C^+ is represented as I. It appears unlikely that either the carbonyl-carbon or the ring nitrogen in this molecule have sufficient electrophilic character to make the water-catalyzed hydrolysis of C^+ significant. Therefore, it is more probable that the dipolar molecule must be doubly protonated and that the hydronium ion-catalyzed hydrolysis of $C^{\pm}$ and C^+ proceed *via* the same transition state complex. The resemblance of the energies and entropies of activation in both regions of the pH rate profile (Table VII) supports this contention.

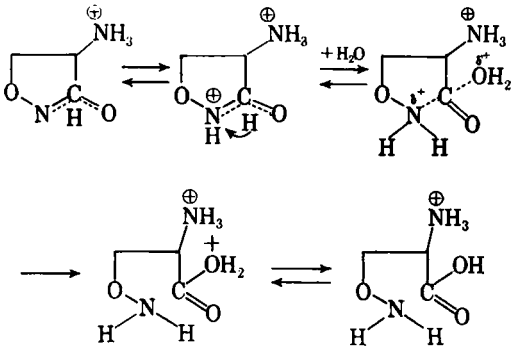
Therefore, in the region of the pH rate profile where the dipolar ion is the principal species, an increase in the hydronium ion concentration causes an increase in the hydrolysis rate as a consequence of the increase in the concentration of diprotonated substrate and $k_1' = k_o + k_{H^+}[H_3O^+]^2$, so that $-\log (k_1' - k_o) = -\log k_{H^+} + 2$ pH. As noted previously, a plot of $-\log k_1'$ *versus* 2 pH in the pH range 7 to 3 is linear, and the slope of this plot approximates the slope of the $-\log k_1'$ *versus* pH plot in the region where the hydronium ion-catalyzed hydrolysis of C^+ takes place. Hence, the steep slope in the pH rate profile is not attributed to the greater hydrolytic lability of $C^{\pm}$ but instead to the premise that the rate increases with the square of the hydronium ion concentration.

TABLE VII.—ACTIVATION ENERGIES AND ENTROPIES FOR THE HYDROLYTIC DEGRADATION OF CYCLOSERINE AT VARIOUS HYDRONIUM ION ACTIVITIES

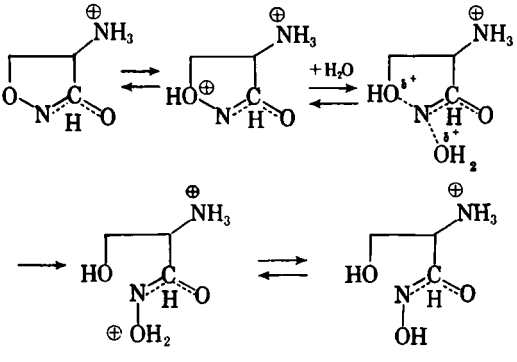
Apparent pH	ΔEa Kcal./Mole	ΔS^+ 80°, e.u.
0.4	16.3	−30.7
1.3	16.1	−31.6
2.3	16.0	−32.3
3.1	15.5	−34.2
3.8	15.9[a]	−33.8[a]
7.2	20.8	−24.9
8.9	23.1	−19.1
11.1	22.8	−21.0
12.9	22.7	−21.1
13.8	21.8	−23.2

[a] Values exclude contribution due to acetic acid catalysis.

A plausible mechanism for the hydrolysis of C^+ is the classical mechanism cited above, in which the transition state results from the attack of a molecule of water on the conjugate acid of the substrate. If the aminoxy amino acid is the intermediate product, the mechanism could be formulated as



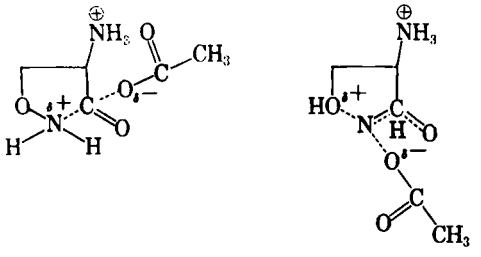
and based upon the hydroxamic acid as the intermediate product, the reaction would proceed by reaction of H_3O on the ring nitrogen. Thus



Before the latter mechanism can be accepted, it will be necessary to preclude the possibility of nucleophilic displacement at the β carbon atom and the possibility of carbonium ion formation at the β carbon atom, in addition to establishing that the hydroxamic acid is an intermediate product.

The kinetics of the hydrolysis of cycloserine in acetic acid–sodium acetate buffers indicates the apparent catalytic dependence of the hydrolysis on undissociated acetic acid. This does not appear to be a case of general acid catalysis, since catalysis by undissociated phthalic acid was not observed. As noted by Wyness (11), Bender's electrophilic-nucleophilic mechanism (23, 24) applied to intermolecular catalysis accommodates the observed kinetic dependence. In essence, the mechanism involves protonation of the substrate by acetic acid, followed by nucleophilic attack by acetate ion on an electrophilic center with concerted anhydride formation and bond cleavage. The postulated transi-

tion states for aminoxyalanine and hydroxamic acid formation are



and the resulting anhydride is then hydrolyzed rapidly. As in the instance of the hydronium ion-catalyzed reaction, it is concluded that the substrate molecule is probably C^+ rather than $C^{\pm}$, since neither the dipolar ion nor the protonated molecule appears to be susceptible to nucleophilic attack by hydroxide ion. The acetic acid-catalyzed hydrolysis of the dipolar ion requires the reaction of acetate ion with a nonprotonated center as the rate-determining step. Based upon the fact that the simple amide is not subject to acetate ion-catalyzed hydrolysis and the premise that the amide has substantially more electrophilic character than is present in the isoxazolidone ring system, such a rate-determining step is highly improbable.

The alternate mechanism (11) for the acetic acid-catalyzed reaction which involves a transition state wherein H_3O attacks the electrophilic center of a hydrogen-bonded complex between acetic acid and cycloserine is not presently considered. Wyness (11) found that the effect of D_2O on the acetic acid-catalyzed hydrolysis of *N-n*-butylacetamide does not support this mechanism.

Above pH 9, the anionic cycloserine molecule III is the only species present in solution. Moreover, at pH values greater than 9, the pH dependency curve has appearance of a hydrolytic catenary, with a minimum in the vicinity of pH 12. The increase in the rate above pH 12 signifies that the degradation of the anionic molecule is base-catalyzed. Considering the broad base of the pH dependency curve, it is probable that the base-catalyzed degradation contributes to the observed rate of the degradation of cycloserine in the pH range 10 to 12. The increase in the observed rate with decreasing pH in the pH range 11 to 9 is indicative of a hydronium ion-catalyzed reaction involving the C^-. Thus, it appears likely that in this region of the pH rate profile the degradation is proceeding by both base and acid-catalyzed reactions. The comparability of the values of the activation parameters in this region with those determined for the base-catalyzed reaction (Table VII) is probably fortuitous.

The extremely low susceptibility of cycloserine to hydroxide ion-catalyzed hydrolysis is noteworthy. Based solely upon inductive effects, it is expected that *N*-amide substitution with an electrophilic oxygen atom should result in enhanced electrophilic character of the carbonyl-carbon and a compound which is much more susceptible to nucleophilic attack. It is evident that the inductive effect does not operate in this way in cycloserine. To a large extent the negligible base-catalyzed hydrolysis in the pH range 6 to 12 is due to the coulombic repulsion of the hydroxide ion by the formal negative charge on the N—C—O system of the zwitterionic and anionic substrate. However, the hydrolysis kinetics in the pH range 4 to 5, a region where the hydroxide ion-catalyzed hydrolysis of simple amides is considerable, indicates that the base-catalyzed hydrolysis of C^+ is unimportant. Thus, the low susceptibility of cycloserine is due to the inherent character of the isoxazolidone ring system, in which the carbonyl-carbon has decreased electrophilic character compared with the carbonyl-carbon of an amide. It is suggested that the *p* orbital on the ring oxygen interacts with those on the adjacent N–C–O system to form a streamer molecular orbital of greater stability than the streamer orbital occurring on the N—C—O system alone. A crystallographic study of cycloserine (25) reveals that the five-membered ring system is nearly planar, so that the steric requirement for such streamer molecular orbital formation is present. A consequence of the delocalization of the π electrons is the apparent decreased electrophilic character of the carbonyl-carbon, so that the isoxazolidone ring is relatively resistant to nucleophilic attack but readily undergoes acid-catalyzed hydrolytic cleavage.

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