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ˣ To whom inquiries should be directed.

RESEARCH ARTICLES

Kinetics and Mechanisms of Degradation of the Antileukemic Agent 5-Azacytidine in Aqueous Solutions

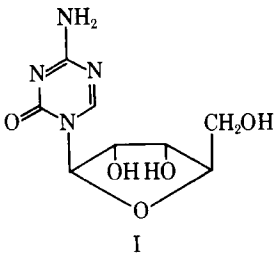
ROBERT E. NOTARI ˣ and JOYCE L. DeYOUNG *

Abstract □ The hydrolytic degradation of 5-azacytidine was studied spectrophotometrically as a function of pH, temperature, and buffer concentration. Loss of drug followed apparent first-order kinetics in the pH region below 3. At pH <1, 5-azacytosine and 5-azauracil were detected; at higher pH values, drug was lost to products which were essentially nonchromophoric if examined in acidic solutions. The apparent first-order rate constants associated with formation of 5-azacytosine and 5-azauracil from 5-azacytidine are reported. Above pH 2.6, first-order plots for drug degradation are biphasic. Apparent first-order rate constants and coefficients for the biexponential equation are given as a function of pH and buffer concentration. A reaction mechanism consistent with the data is discussed together with problems associated with defining the stability of the drug in aqueous solutions. At 50°, the drug exhibited maximum stability at pH 6.5 in dilute phosphate buffer. Similar solutions were stored at 30° to estimate their useful shelflife. Within 80 min, 6×10^{-4} *M* solutions of 5-azacytidine decreased to 90% of original potency based on assumptions related to the proposed mechanisms.

Keyphrases □ 5-Azacytidine—kinetics and mechanisms of degradation in aqueous solutions, effect of pH, temperature, and buffer concentration □ Hydrolysis, 5-azacytidine in aqueous solutions—kinetics and mechanisms, effect of pH, temperature, and buffer concentration □ Antileukemic agents—5-azacytidine, kinetics and mechanisms of degradation in aqueous solutions

The synthesis of 5-azacytidine (I) was reported in 1964 (1). The compound has antimicrobial and antitumor activities (2–4) and is currently being evaluated clinically in the treatment of human leukemias.

The clinical use of this drug makes it desirable to determine kinetic data on its reactivity in aqueous solutions. Such information is especially important in consideration of its relative instability when compared to cytidine itself. Especially notable are the ease with which the triazine ring hydrolyzes and the lability of the sugar–triazine bond (5). Although most hydrolysis products of I have been identified (5), lit-



I

tle has been done concerning the kinetics and mechanism of its degradation.

The purpose of this report is to describe the results of studies on the hydrolysis of I and the related compounds 5-azacytosine (II) and 5-azauracil (III).

EXPERIMENTAL

Analytical Methods—Beer's law plots were constructed for 5-azacytidine, 5-azacytosine, and 5-azauracil in 0.1 *N* HCl using a recording spectrophotometer[1]. Molar absorptivities were 2.36×10^3 for 5-azacytidine at 255 nm; 3.35×10^3, 4.33×10^3, and 5.98×10^3 for 5-azacytosine at 230, 235, and 250 nm, respectively; and 3.67×10^3, 3.80×10^3, and 1.50×10^3 for 5-azauracil at 230, 235, and 250 nm, respectively.

Concentrations of components in mixtures of 5-azacytosine and 5-azauracil were calculated using:

$$10^3[\text{II}] = 0.217A_{250} - 0.088A_{230} \quad \text{(Eq. 1)}$$

$$10^3[\text{III}] = 0.353A_{230} - 0.198A_{250} \quad \text{(Eq. 2)}$$

which were derived from simultaneous equations for total absorbance, *A*, at 230 and 250 nm for mixtures of 5-azacytosine and 5-azauracil in 0.1 *N* HCl. Good agreement was obtained between the results of the assay and the known concentrations in synthetic mixtures.

Mixtures of 5-azacytidine and the two bases were assayed utilizing the differential stability of the components. The absorbance at 255 nm of solutions containing all three components in 0.1 *N* HCl was determined. These solutions were then made 0.05 *N* in sodium hydroxide and allowed to stand at room temperature for 40–50 min. This period was sufficient to degrade 5-azacytidine completely to nonchromophoric products without loss of 5-azacytosine or 5-azauracil. Samples were reacidified with 0.1 *N* HCl, and the absorbances at 235, 250, and 255 nm were determined. The 235- and 250-nm values were used to calculate the concentrations of 5-azacytosine and 5-azauracil:

$$10^3[\text{II}] = 0.234A_{250} - 0.092A_{235} \quad \text{(Eq. 3)}$$

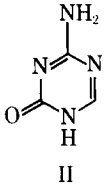
$$10^3[\text{III}] = 0.368A_{235} - 0.267A_{250} \quad \text{(Eq. 4)}$$

After correcting for dilution, the absorbances of the sample before and after treatment with alkali were used to calculate the 5-azacytidine concentration:

$$\text{I} = \Delta A_{255} / \epsilon_{255} \quad \text{(Eq. 5)}$$

This assay method was shown to be satisfactory using synthetic mixtures.

Kinetics of 5-Azacytosine and 5-Azauracil Hydrolysis in Hydrochloric Acid Solutions—Reaction solutions containing 2×10^{-4} *M* 5-azacytosine or 3×10^{-4} *M* 5-azauracil in 0.1 and 0.25 *N* HCl were placed in constant-temperature baths. Samples of the 5-azacytosine reactions were withdrawn as a function of time and assayed for substrate and 5-azauracil as described previously. The spectra of the 5-azauracil reactions decreased with time, with no evidence of formation of a new chromophore. Rate constants for its loss were calculated directly from absorbance data.



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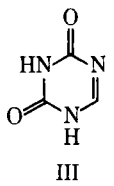


III

[1] Cary 15.

Table I—Apparent First-Order Rate Constants (hour^{-1}) for Hydrolysis in Hydrochloric Acid at 70°

Substrate	[HCl], *N*	k_{obs}	k_{23}	k_{25}	k_{36}
5-Azacytosine[a]	0.10	0.376	0.121	0.255	0.0495[b]
	0.25	0.238	0.119	0.119	0.0636[b]
	[HCl], *N*	k_{obs}	k_{12}	k_{13}	k_{14}
5-Azacytidine[c]	0.10	1.98	0.111	0.110	1.77
	0.25	1.05	0.136	0.101	0.810

[a] See Scheme I. Concentration is 2×10^{-4} *M*. [b] Determined from hydrolysis of 5-azauracil (3×10^{-4} *M*). [c] See Scheme II. Concentration is 2×10^{-4} *M*.

TLC of Reaction Solutions of 5-Azacytosine and 5-Azauracil—Samples of the reactions of approximately 0.01 *M* 5-azacytosine and 0.01 *M* 5-azauracil in 0.10 *N* HCl at 70° were taken after about 50 and 100% of the substrate had reacted. These samples were neutralized and spotted along with authentic samples of 5-azacytosine and 5-azauracil on TLC plates coated with 0.25 mm silica gel GF_{254}. The plates were developed in 1-butanol–acetic acid–water (100:10:30) for a distance of about 10 cm, air dried at room temperature, and examined with a shortwave UV lamp.

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—Reaction solutions containing 5-azacytidine in excess hydrochloric acid were prepared as shown in Tables I and II. The absorbance of reactions in solutions less concentrated than 0.1 *N* HCl was determined by diluting aliquots of the reaction with hydrochloric acid to give a final concentration of 0.25 *N* HCl. In these solutions, the UV absorption spectrum of 5-azacytidine decreased with time without the appearance of any new absorbances. Absorbance data were used directly to calculate the apparent first-order rate constants.

In solutions containing 0.1 *N* or stronger hydrochloric acid, there was evidence that a fraction of the 5-azacytidine decomposed to form 5-azacytosine and 5-azauracil. In these cases, the reactions were assayed using the described differential stability method. At 70°, Eqs. 3, 4, and 5 were used to assay for 5-azacytidine, 5-azacytosine, and 5-azauracil, respectively. In the 50° reactions, the differential absorbance due to 5-azacytidine (Eq. 5) was used to calculate the apparent first-order rate constants for loss of substrate.

Kinetics of 5-Azacytidine Hydrolysis in Buffers—Reaction solutions containing 5-azacytidine in excess buffer were prepared as shown in Table III. Solutions were placed in constant-temperature baths; aliquots were removed at appropriate times, chilled, and diluted with an equal volume of hydrochloric acid to give a final concentration of 0.25 *N* HCl. The absorbance of these solutions was measured at 255, 260, and 265 nm. Since absorbance values decreased with time to become zero when the reaction was complete, absorbance data were used directly in the calculation of

Table II—Apparent First-Order Rate Constants for Hydrolysis of 5-Azacytidine[a] at 50° in Hydrochloric Acid

[HCl], *N*	pH[b]	k_{obs}, hr^{-1}
0.80	0.22	0.0595
0.50	0.44	0.0764
0.25	0.73	0.118
0.097	1.12	0.239
0.0097	2.06	1.12
0.005	2.39	1.39

[a] Reactions in 0.80, 0.50, 0.25, and 0.005 *N* HCl contain 4×10^{-4} *M* substrate. Substrate concentration in 0.097 and 0.0097 *N* HCl reactions is 6×10^{-4} *M*. [b] The pH of hydrochloric acid solutions was calculated using activity coefficient values from the literature (6).

Table III—Experimental Conditions and Apparent First-Order Rate Constants for Loss of 6×10^{-4} *M* 5-Azacytidine in the Presence of Buffers in the 7.6–2.6 pH Range at 50°; $\mu = 0.5$

pH	Buffer			k_a[a], hr^{-1}	k_b[a], hr^{-1}	A[a]	B[a]
	$[NaH_2PO_4]$	$[Na_2HPO_4]$	[NaCl]				
7.59 ± 0.08	0.015	0.15	0.035	15.0	0.74	0.17	0.83
	0.010	0.10	0.19	13.2	0.62	0.17	0.83
	0.005	0.05	0.345	11.2	0.50	0.20	0.80
	0.003	0.03	0.407	10.6	0.44	0.18	0.82
		Unbuffered		9.42[b]	0.37[b]	0.18[c]	0.82[c]
6.52 ± 0.04	0.10	0.10	0.10	5.49	0.29	0.19	0.81
	0.08	0.08	0.18	5.05	0.24	0.21	0.79
	0.05	0.05	0.30	4.38	0.18	0.20	0.79
	0.02	0.02	0.42	3.34	0.12	0.18	0.82
		Unbuffered		2.91[b]	0.076[b]	0.20[c]	0.80[c]
5.42 ± 0.01	0.30	0.03	0.11	5.51	0.41	0.28	0.72
	0.20	0.02	0.24	4.87	0.29	0.25	0.75
	0.10	0.01	0.37	5.19	0.18	0.21	0.79
	0.06	0.006	0.42	5.03	0.13	0.22	0.78
		Unbuffered		5.15[c]	0.065[b]	0.24[c]	0.76[c]
	$[CH_3COOH]$	$[CH_3COONa]$	[NaCl]				
5.60 ± 0.04	0.040	0.40	0.10	4.39	0.15	0.26	0.74
	0.024	0.24	0.26	4.83	0.12	0.23	0.77
	0.016	0.16	0.34	4.72	0.099	0.24	0.76
	0.008	0.08	0.42	4.38	0.080	0.25	0.75
		Unbuffered		4.58[c]	0.064[b]	0.24[c]	0.76[c]
4.62 ± 0.04	0.400	0.400	0.100	2.15	0.48	0.60	0.40
	0.240	0.240	0.260	3.04	0.41	0.47	0.53
	0.144	0.144	0.356	3.70	0.37	0.32	0.68
	0.080	0.080	0.420	4.11	0.27	0.32	0.68
		Unbuffered		4.57[b]	0.25[b]	0.23[b]	0.77[b]
				k_{obs}			
3.58 ± 0.02	0.40	0.040	0.460	1.82[d]			
	0.24	0.024	0.476	1.79[d]			
	0.16	0.016	0.484	1.80[d]			
	0.08	0.008	0.492	1.80[d]			
		Unbuffered		1.80[c]			
	[HCOOH]	[HCOONa]	[NaCl]				
2.58 ± 0.06	0.40	0.040	0.460	1.57[e]			
	0.24	0.024	0.476	1.58[e]			
	0.16	0.016	0.484	1.60[e]			
	0.08	0.008	0.492	1.69[e]			
		Unbuffered		1.61[c]			

[a] See text for explanation of these constants and the method used to calculate them. Average of results for two or three wavelengths. [b] Obtained from intercept of plot of *k versus* buffer concentration. [c] Average of values in presence of buffer. [d] It was not feasible to separate the two phases for analysis. The rate constants given were obtained from the first 17% of the reaction. See text for further details. [e] Reaction appears first order over at least two half-lives.

rate constants. In phosphate buffers of pH 7.6 and 6.5, spectra of nonacidified samples were also obtained. At wavelengths greater than 240 nm, there was eventual loss of the UV absorption spectrum. Absorbance data in this region were used directly for the calculation of rate constants.

In two additional experiments, samples of 5-azacytidine (10^{-2} *M*) were reacted in buffer for a sufficient time to ensure that the initial rapid phase of the reaction was completed. Both reactions were conducted at 70°, one in 0.33 *M* phosphate buffer at pH 7.59 for 10 min and the other in 0.04 *M* phosphate buffer at pH 6.52 for 1 hr. Aliquots from each reaction were added to 10 times their volume of hydrochloric acid at 70° to yield a final reaction condition of 0.1 or 0.25 *N* HCl (Table IV). These reactions were then followed at 70° using the differential assay for 5-azacytidine described earlier (Eq. 5).

Evidence for 5,6-Addition to 5-Azacytidine—The UV spectra of 5-azacytidine and 5-azacytosine were recorded at each of three conditions: 0.1 *N* HCl, 0.001 *N* HCl, and pH 6.5 phosphate buffer. Each solution was then altered to match one of the others so that the sequence represents all possible combinations of the three conditions. Precautions were taken to eliminate any changes in absorbance due to passage of time or dilution of sample. In this way, UV spectra for solutions of authentic samples were compared to those that had been previously prepared and recorded at one or more of the other conditions as described under *Results*.

Solutions of 5-azacytidine (6×10^{-5} *M*) and 5-azacytosine (1.5×10^{-5} *M*) were prepared in 0.025, 0.005, and 0.001 *M* aqueous bisulfite previously adjusted to pH 3.67 ± 0.06. A fourth solution of 5-azacytidine in 0.001 *M* bisulfite containing 0.024 *M* NaCl was also prepared. Immediately after dissolution of the substrate, UV spectra were recorded as a function of time.

Spectrophotometric Determination of pKa Values for 5-Azacytosine—The pH of a solution containing 10^{-4} *M* 5-azacytosine in buffer (0.08 *M* acetic acid–0.008 *M* sodium acetate–0.492 *M* sodium chloride) was measured, and the UV spectrum of the solution was obtained. The pH was then adjusted using hydrochloric acid or sodium hydroxide to obtain a series of spectra ranging from the cationic form of 5-azacytosine to the anionic form. To check for degradation, a sample at high pH was adjusted to a previously obtained lower pH value and the two spectra were compared to show that no irreversible changes had occurred.

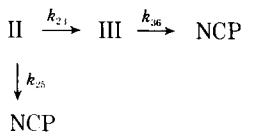
Table IV—Apparent First-Order Rate Constants[a] ($hour^{-1}$) in Hydrochloric Acid at 70° for 5-Azacytidine Compared to Those Obtained when 5-Azacytidine was Previously Reacted in Phosphate Buffer[b]

[HCl], *N*	Unreacted Sample	Phosphate Buffer[b]	
		pH 6.5	pH 7.6
0.10	1.98	1.86	1.95
0.25	1.05	1.07	—

[a] Average of values obtained at two or three wavelengths. [b] See *Experimental* for reaction conditions.

RESULTS

Apparent First-Order Rate Constants in Hydrochloric Acid Solutions—Good first-order plots were obtained when ex-



Scheme I

perimental data were graphed according to:

$$\ln X = \ln X_0 - k_{obs}t \quad \text{(Eq. 6)}$$

where t is time, X is the concentration of unreacted 5-azacytidine or 5-azacytosine, and X_0 is the initial concentration. When appropriate, data were plotted according to:

$$\ln A = \ln A_0 - k_{obs}t \quad \text{(Eq. 7)}$$

where A is the absorbance due to unreacted substrate at 255, 260, or 265 nm for 5-azacytidine or at 230 or 250 nm for 5-azauracil. The apparent first-order rate constants for loss of 5-azacytidine and 5-azacytosine (k_{obs}) and 5-azauracil (k_{36}) are listed in Table I.

TLC of Reaction Solutions of 5-Azacytosine and 5-Azauracil in 0.10 *N* Hydrochloric Acid—The 50 and 100%-reacted samples of 5-azacytosine showed faint spots at the same R_f value as an authentic sample of 5-azauracil. The 100%-reacted sample showed no trace of starting material. The 5-azauracil reaction showed a disappearance of the spot due to the uracil without development of any other compounds absorbing UV light. The R_f values obtained were 0.38 for 5-azacytosine and 0.58 for 5-azauracil.

Kinetics of 5-Azacytosine and 5-Azauracil Hydrolysis in Hydrochloric Acid Solutions—The observed spectral changes during the reactions and the results of the TLC experiments pointed to the applicability of Scheme I to 5-azacytosine hydrolysis. Nonchromophoric products are designated as NCP. A value for k_{36} may be obtained directly from 5-azauracil hydrolysis, while k_{23} and k_{25} were obtained from k_{obs} by:

$$k_{obs} = k_{23} + k_{25} \quad \text{(Eq. 8)}$$

and:

$$k_{23} = f k_{obs} \quad \text{(Eq. 9)}$$

where f is the fraction of 5-azacytosine that deaminates to 5-azauracil. The method used to calculate f is described below. In the case of a sequential reaction with parallel first-order loss, as in Scheme I, the concentration of III as a function of time is given by:

$$[III] = \{k_{23}[II]_0/(k_{36} - k_{23} - k_{25})\}\{e^{-(k_{23}+k_{25})t} - e^{-k_{36}t}\} \quad \text{(Eq. 10)}$$

where $[II]_0$ is the initial concentration of 5-azacytosine. The area under a plot of [III] *versus* time is found by integrating Eq. 10 from zero to infinity:

$$(\text{area III})_1 = f[II]_0/k_{36} \quad \text{(Eq. 11)}$$

where f is the same as in Eq. 9. Similarly, for the simple first-order loss:

$$III \xrightarrow{k_{36}} NCP \quad \text{(Eq. 12)}$$

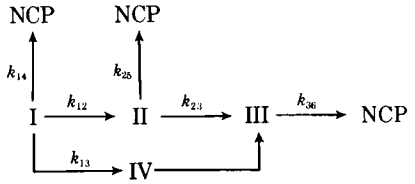
Integrating the equation for III yields:

$$(\text{area III})_2 = [III]_0/k_{36} \quad \text{(Eq. 13)}$$

Application of Eqs. 11 and 13 to experimental data allows the calculation of f. The concentration of 5-azauracil produced from 5-azacytosine was plotted as a function of time. During formation from 5-azacytosine, the area under this curve was estimated by the method of trapezoids. After the 5-azacytosine was exhausted, the remaining area was calculated from:

$$(\text{area})_{t\to\infty} = [III]_t/k_{36} \quad \text{(Eq. 14)}$$

where $[III]_t$ is the concentration of 5-azauracil at time t, and k_{36} is the apparent first-order rate constant for 5-azauracil loss under



Scheme II

identical conditions. The sum of the two areas provides the total area, $(\text{area III})_1$. The area under the concentration *versus* time curve for hydrolysis of an equimolar solution of 5-azauracil, $(\text{area III})_2$, was also calculated from Eq. 14. Since $[II]_0 = [III]_0$, Eqs. 11 and 13 may be combined to yield:

$$(\text{area III})_1/(\text{area III})_2 = f \quad \text{(Eq. 15)}$$

The rate constants obtained are listed in Table I. These were used in an analog computer[2] program to simulate the time course of the various reaction components. A typical plot is shown in Fig. 1, which shows good agreement between observed and computer-generated values.

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—The proposed path for 5-azacytidine hydrolysis in 0.1–0.8 *N* HCl is shown in Scheme II. The apparent first-order rate constants k_{12}, k_{14}, and k_{13} were determined in a manner similar to that used for k_{23} and k_{25}. For example:

$$k_{12} = f(k_{obs}) \quad \text{(Eq. 16)}$$

where f is the fraction of 5-azacytidine that hydrolyzes to 5-azacytosine. This fraction is obtained using Eq. 15, replacing $(\text{area III})_1$ with the area under the concentration curve for 5-azacytosine produced from 5-azacytidine and replacing $(\text{area III})_2$ with the area starting with 5-azacytosine itself.

Since the values for k_{25}, k_{23}, k_{36}, and the time course for II are all known (Scheme II), they can be employed to define the time course for $[III]_{II}$ (the 5-azauracil arising from 5-azacytosine). The difference between the observed values for III as a function of time, [III], and the expected values, $[III]_{II}$, defines the time course for III arising from IV *via* k_{13}:

$$[III]_{IV} = [III] - [III]_{II} \quad \text{(Eq. 17)}$$

The fraction of I converted to III by this route was used to calculate the value for k_{13}; this value, in turn, was employed to calculate k_{14} from:

$$k_{14} = k_{obs} - k_{12} - k_{13} \quad \text{(Eq. 18)}$$

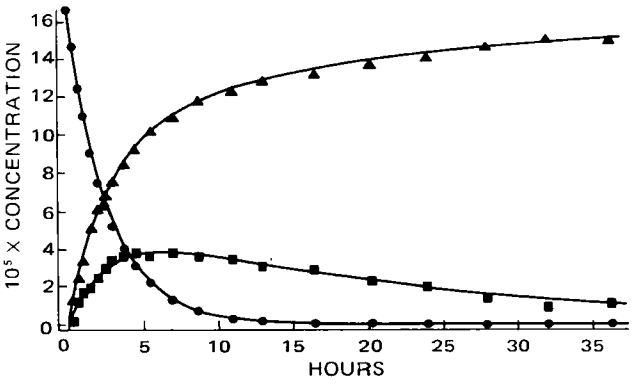


Figure 1—*Time course for 5-azacytosine (●), 5-azauracil (■), and nonchromophoric products (▲) during the hydrolysis of 5-azacytosine in 0.1* N *HCl at 70°. The lines were generated using an analog computer programmed with rate constants listed in Table I.*

[2] EAI, model TR 20.

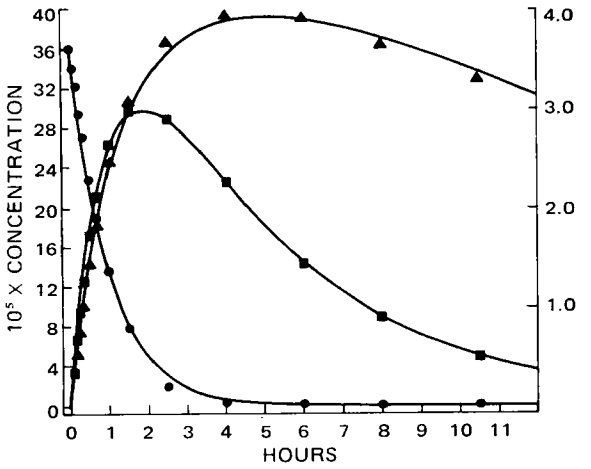


Figure 2—*Time course for 5-azacytidine (●), 5-azacytosine (■), and 5-azauracil (▲) during the hydrolysis of 5-azacytidine in 0.25 N HCl at 70°, showing the agreement between the experimental points and the computer-generated lines. The scale for 5-azacytosine and 5-azauracil is given on the right ordinate.*

These analyses were carried out for the reaction conditions listed in Table I. Typical plots are shown in Fig. 2. The k_{obs} values obtained at 50° are listed in Table II.

Kinetics of 5-Azacytidine Hydrolysis in Buffer Solutions—In buffers at pH 4.6 or higher, the plots of absorbance *versus* time were distinctly biphasic. Acidified samples gave plots similar to Fig. 3, showing a rapid initial decrease in absorbance, followed by a much slower second phase. When the spectra of buffered samples were determined, there was a rapid initial increase in absorbance followed by a slower decrease (Fig. 4).

The biphasic curves were analyzed by feathering the data using commonly employed methods[3]. Treated in this way, each reaction produced two linear first-order plots (Figs. 3 and 4). If the data are normalized relative to the initial absorbance values, a single equation, wherein the algebraic sign for the coefficient A is negative in buffer and positive in acid, may be used to describe the biphasic

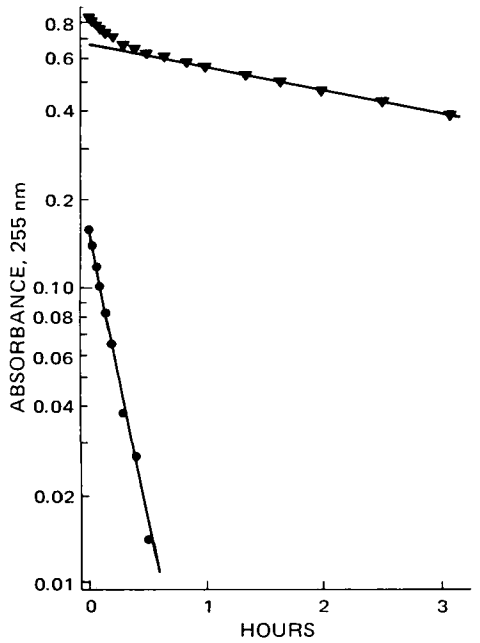


Figure 3—*Absorbance of acidified samples at 255 nm as a function of time (▼) for 5-azacytidine hydrolysis at 50°, pH 6.52, showing lines of slope* k_a *(●) and* k_b *(▼) obtained by feathering.*

[3] For a typical example of this method of data analysis, see Ref. 7.

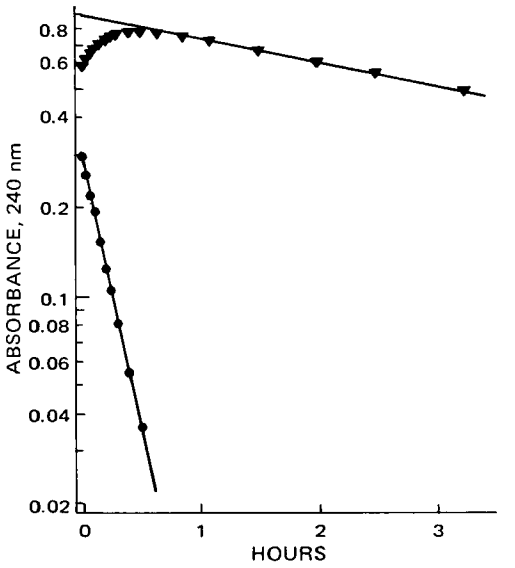


Figure 4—*Absorbance at 240 nm as a function of time (▼) for nonacidified samples of 5-azacytidine at 50°, pH 6.52, showing lines of slope* k_a *(●) and* k_b *(▼) obtained by feathering.*

curves:

$$F(A) = \pm Ae^{-k_a t} + Be^{-k_b t} \qquad \text{(Eq. 19)}$$

where $F(A)$ is the fraction of absorbance remaining; A and $-k_a$ are the intercept and slope of the rapid phase, respectively; and B and $-k_b$ are the intercept and slope of the slow phase, respectively. Rate constants are independent of the pH of analysis. Their values, together with the coefficients in acid, are given in Table III.

Table IV compares first-order rate constants for hydrolysis of 5-azacytidine in hydrochloric acid at 70° to those obtained for 5-azacytidine previously reacted in phosphate buffer. Although the buffer reactions had proceeded into the second phase, the observed rate constants after transfer to hydrochloric acid were the same as those for authentic samples.

At pH 3.58, the plots of ln (A) *versus* time showed definite curvature but were not distinctly biphasic. In fact, the plots were adequately described by a first-order equation over approximately the first half-life. Rate constants, estimated using data representing the first 17% of the reaction, are reported in Table III.

Hydrolysis was apparent first order at pH 2.58, and the rate constants were obtained using Eq. 7 (Table III).

Effect of Buffer on Hydrolysis Rate—Plots of k_a, k_b, or k_{obs}

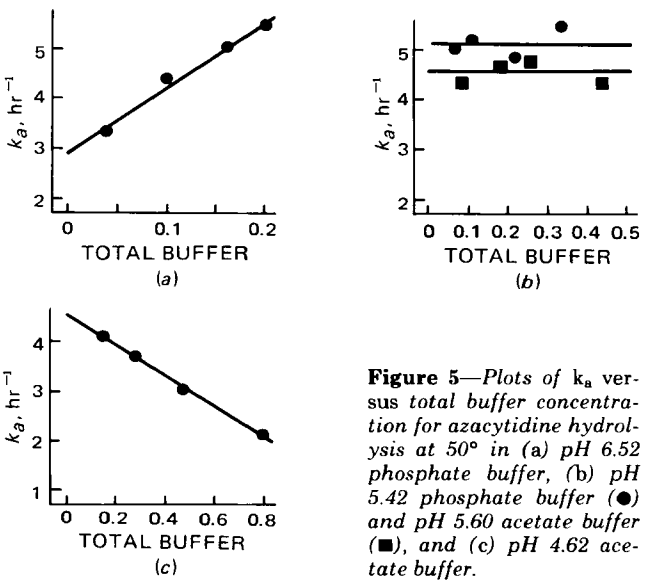


Figure 5—*Plots of* k_a *versus total buffer concentration for azacytidine hydrolysis at 50° in (a) pH 6.52 phosphate buffer, (b) pH 5.42 phosphate buffer (●) and pH 5.60 acetate buffer (■), and (c) pH 4.62 acetate buffer.*

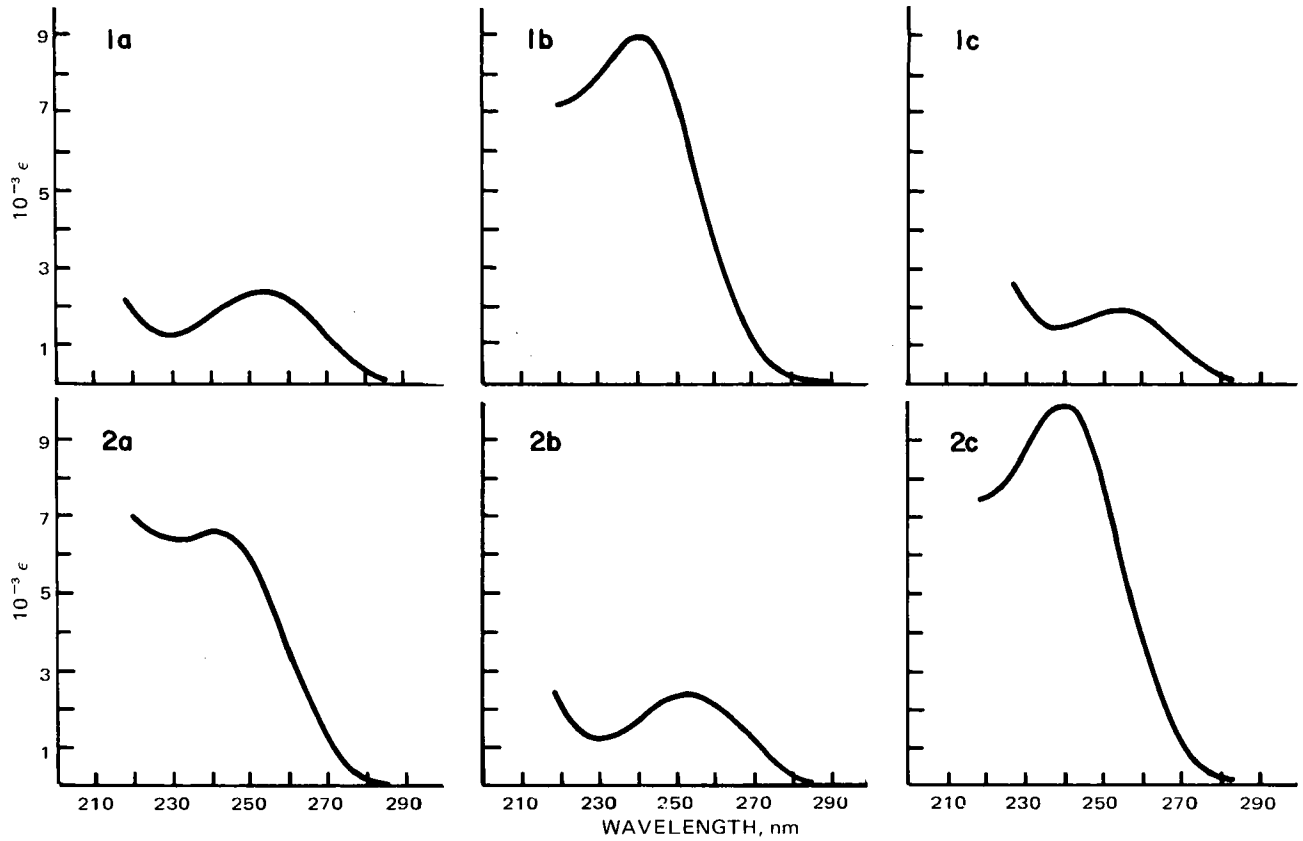


Figure 6—*Plots of apparent molar absorptivity* versus *wavelength for 5-azacytidine in solutions that were originally acid (1a), then adjusted to neutrality (1b), and reacidified (1c), or that were prepared at neutral pH (2a), were acidified (2b), and were reneutralized (2c).*

versus buffer concentration were extrapolated to zero buffer using least-squares linear regression analysis to obtain the values for these constants in the absence of buffer (Table III). In all cases, buffer plots of k_b were linear and showed a positive slope. But plots for k_a had positive slopes at pH 6.52 and 7.59, showed no buffer catalysis at pH 5.60 and 5.42, and indicated inhibition by the buffer at pH 4.62 (Fig. 5). No buffer sensitivity was seen for k_{obs} at pH 3.58 or 2.58.

Evidence for Addition across 5,6-Double Bond of 5-Azacytidine—If the UV spectrum of a 5-azacytidine solution is recorded as a function of pH, that spectrum is dependent on the order of the pH adjustment as well as on the pH itself (Fig. 6). For example, a neutral solution (2*a* in Fig. 6) has a spectrum that is less intense than that of a solution of equal concentration and pH that has been prepared from a previously acidic solution (1*b* in Fig. 6).

Clearly, acid at least partially converts the 5-azacytidine to a form that has a greater absorptivity at neutral pH than the 5-azacytidine itself. However, this rapid conversion does not occur at neutral pH since acidification of an originally neutral sample results in a solution whose spectrum is identical to that of an authentic sample in acid. Furthermore, if the neutral solution that was made acidic is readjusted to neutrality, the spectrum resembles one from an authentic sample in acid adjusted to neutrality (1*b* and 2*c* in Fig. 6).

The degree of difference between a neutralized solution (such as 1*b* in Fig. 6) and a neutral solution (2*a* in Fig. 6) depends upon the acidity of the initial solution. In Fig. 6 the initial acid concentration was 0.1 *N* HCl, and the apparent absorptivity at the absorption maximum for the solution adjusted to pH 6.5 was 9.03×10^3. When the initial acidity was 0.001 *N* HCl, the maximum absorptivity at pH 6.5 was 7.37×10^3. It was consistently observed that solutions made neutral and then reacidified showed decreased maximum absorptivities compared to the original acidic spectra as illustrated for the example in Fig. 6. These changes cannot be attributed to time or dilution factors. This decrease was not observed when solutions were adjusted from 0.1 to 0.001 *N* HCl and back to 0.1 *N*.

When experiments identical to those illustrated in Fig. 6 were carried out using 5-azacytosine, all spectra in 0.1 *N* HCl were identical, as were all spectra at pH 6.5.

Increasing concentrations of bisulfite brought about a progressive loss in intensity of spectra of both 5-azacytidine and 5-azacytosine. When spectra changed with time, initial absorbances were estimated by extrapolating plots of absorbance *versus* time to zero time. When these estimates were used, 5-azacytidine showed apparent molar absorptivities at 240 nm of 5.78×10^3, 3.60×10^3, and 1.38×10^3 in 0.001, 0.005, and 0.025 *M* bisulfite, respectively. Increasing the ionic strength by addition of sodium chloride did not alter the spectrum of the 0.001 *M* solution. In 0.001, 0.005, and 0.025 *M* bisulfite, 5-azacytosine had apparent molar absorptivities of 3.65×10^3, 1.55×10^3, and 0.37×10^3, respectively.

Spectrophotometric Determination of pKa Values for 5-Azacytosine—Absorbances at λ_{max} for the anionic form were plotted *versus* pH. Absorbances were corrected for dilution caused by the pH adjustment using:

$$A_{corr} = A_{exp}(V/V_0) \qquad \text{(Eq. 20)}$$

where V is the volume after addition of acid or base, and V_0 is the original volume. Figure 7 shows the plot obtained. The two pKa values were estimated from the midpoints of the curves and by use of Eqs. 21 and 22 for the pKa's of the conjugate acid and neutral molecule, respectively (8):

$$\text{pKa} = \text{pH} + \log[(A_I - A)/(A - A_N)] \qquad \text{(Eq. 21)}$$

$$\text{pKa} = \text{pH} + \log[(A - A_N)/(A_I - A)] \qquad \text{(Eq. 22)}$$

The absorbance of the solution when all the substrate is ionized is represented by A_I; A_N represents a solution of the neutral molecule; and A is the observed absorbance at the given pH. Two or three pH values within 0.5 pH unit of the pKa's were used. The average pKa values were 2.64 for the protonated molecule and 8.10 for the neutral species.

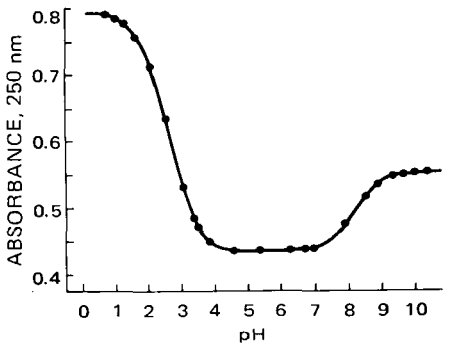


Figure 7—*Absorbance at 250 nm* versus *pH for 1 × 10⁻⁴ M 5-azacytosine.*

DISCUSSION

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—Scheme II outlines the proposed path for 5-azacytidine loss in acid. The formation of 5-azacytosine and 5-azauracil was expected from previously reported results (5) and was confirmed by UV assay. TLC and spectral changes demonstrated that 5-azauracil was produced from the hydrolysis of 5-azacytosine. This was the expected source for the 5-azauracil in the nucleoside hydrolysis.

However, as may be seen in Fig. 2, the data indicate the immediate and rapid production of 5-azauracil. If it were produced solely from 5-azacytosine, a lag phase would be evident. Therefore, it was necessary to postulate the production of 5-azauracil by another, more rapid route. The most logical path involves formation of 5-azauridine as a reactive intermediate. It is proposed to have only a transitory existence and a low concentration and may be said to be in the steady state. This is supported by the absence of a lag time for the formation of 5-azauracil from 5-azacytidine. A lag phase would be expected if 5-azauridine had an appreciable lifetime. Moreover, there was no spectral evidence for the existence of 5-azauridine in the reaction samples.

The steady-state assumption requires that 5-azauridine be very unstable in the reaction system. This assumption is reasonable in view of what is known about the reactivity of the molecule. It is reported to cleave easily in hydrochloric acid at room temperature to form 5-azauracil and ribose. In dilute aqueous ammonia, 1β-D-ribofuranosylbiuret is formed. At pH 5 at room temperature, the UV spectrum of a solution disappeared in less than 1 hr (1).

The absence of 5-azauridine UV absorption was substantiated in the following manner. In neutral solution and as a solid, 5-azauridine actually exists in a form that exhibits no absorbance in the near UV (1). In ~0.1 *N* HCl, solutions have an apparent absorptivity of ~1000 at the λ_{max} of 241 nm (1, 9). Therefore, significant amounts in the reaction would be detected in the UV spectra of the reaction samples. This was tested by using the concentrations calculated for 5-azacytidine, 5-azacytosine, and 5-azauracil at various times during the reaction to generate spectra for the reaction mixtures. These calculated spectra were identical to the actual spectra in the region examined, 235–270 nm. This could not occur in the presence of an appreciable concentration of 5-azauridine unless its spectrum exactly matched that of one of the other components or their absorptivities differed by a constant factor at all wavelengths. Since 5-azacytosine, 5-azauracil, and 5-azacytidine have λ_{max} values of 247, 233, and 255 nm, while 5-azauridine is reported to have its λ_{max} at 241 nm, neither of these conditions can be met.

Since all of the 5-azacytidine loss could not be accounted for by the production of 5-azacytosine and 5-azauracil, it was necessary to include a third path for loss of the substrate to produce nonchromophoric products, probably resulting from fission of the triazine ring (5) (represented by k_{14} in Scheme II). While it is possible that 5-azauridine also contributes to the formation of nonchromophoric products, this pathway would be kinetically equivalent since 5-azauridine is in the steady state.

Data for 5-azacytosine hydrolysis were used to complete Scheme II. Rate constants shown in Scheme II were calculated at 70° (Table I). The observed first-order rate constant for loss of 5-azacytidine decreased at lower pH. This trend was seen also at 50°, as shown in Table II. As the acid concentration was increased, however, a larger fraction of 5-azacytidine formed 5-azacytosine and 5-azauracil (24% in 0.25 *N* HCl as opposed to 11% in 0.1 *N* HCl). 5-Azacytosine showed a similar pH dependency. Only 32% of the 5-azacytosine formed 5-azauracil in 0.1 *N* HCl, and 50% followed this route in 0.25 *N;* the overall rate constant for loss decreased in the more concentrated acid.

Therefore, it appears that increased acid concentration decreases ring opening reactions that produce nonchromophoric products, thereby forcing more substrate to undergo deamination and, in the case of 5-azacytidine, sugar loss. Since these latter two reactions are relatively insensitive to pH changes over the region examined, the overall rate of substrate loss decreased.

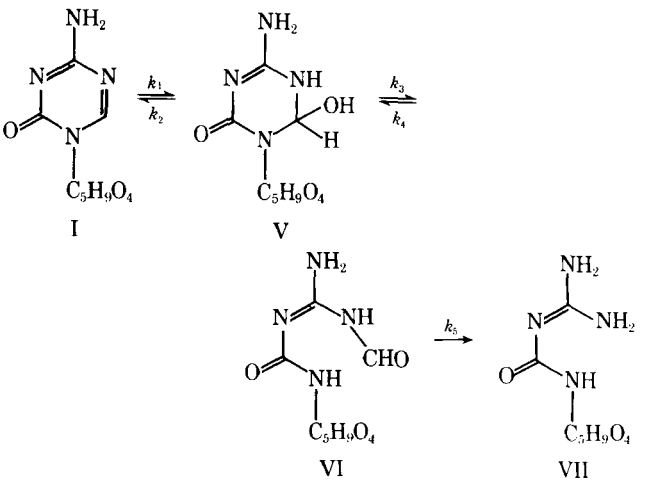
5-Azacytidine Hydrolysis in Presence of Buffers—In formic acid buffer, pH 2.6 and 50°, the loss of absorbance of a 5-azacytidine solution follows first-order kinetics, with the final absorbance being essentially zero. This same behavior is seen in hydrochloric acid solutions more dilute than 0.1 *N*. However, at pH 3.6 the absorbance plots of acidified samples are no longer linear. From pH 4.6 to 7.6, the absorbance plots are biphasic (showing a rapid initial drop in absorbance) and can be separated into two phases by the method of feathering (7) (Fig. 3). Reactions at pH 7.6 and 6.5, followed using nonacidified samples, were also biphasic. In this case the initial change was a rapid increase in absorbance, which was then followed by a slower decrease. An example is shown in Fig. 4. The apparent first-order rate constants obtained by feathering were the same whether acidified or nonacidified samples were used.

Several possible causes of this biphasic behavior were considered: (*a*) the presence of an impurity; (*b*) an A → B → C reaction where the initial phase represents the loss of A to B and the second phase represents the loss of B to C; and (*c*) a reaction with an initial reversible step (or steps) such as A ⇄ B → C (or A ⇄ X ⇄ B → C). The first possibility was eliminated by running a pair of reactions using 5-azacytidine purchased from two suppliers[4]. The absorbance *versus* time plots were identical, making an impurity an unlikely cause for the nonlinear behavior.

Samples taken during the second phase of the reactions, diluted in acid as shown in Table IV, and allowed to react at 70° showed rate constants that agreed with those found for 5-azacytidine. In addition, samples taken throughout the reaction and acidified had spectra that were essentially identical with that of 5-azacytidine at wavelengths longer than 240 nm. Therefore, it appears that 5-azacytidine is present throughout the reaction. This indicates that the formation of B must be reversible, thus eliminating the second reaction scheme.

A pathway with a reversible step is supported by the work of other investigators who reported the reversible formation of the *N*-formyl derivative of ribosylguanylurea (VI in Scheme III) in the decomposition of 5-azacytidine in buffer solutions (5).

By using results of previous work with 5-azacytidine and what is



Scheme III

[4] Schwarz/Mann and Aldrich.

known about the hydrolysis of imines, together with the experimental observations reported here, it is possible to develop a reaction scheme for 5-azacytidine in the presence of buffers as depicted in Scheme III.

Studies of imine hydrolysis (10) indicate that water attack to form a carbinolamine intermediate (V) is fast at low pH since attack proceeds by way of the protonated imine. Loss of free amine (V → VI) becomes the rapid step at higher pH values. This is consistent with the results of pH change experiments, which indicate that formation of an intermediate is rapid in acid but slow at neutral pH, while the reaction of this intermediate to form a third compound is rapid in buffer. This interpretation is also strengthened by the fact that more intermediate appeared to form at pH ~1 (0.1 *N* HCl) than at pH ~3 (0.001 *N* HCl). Based upon what is known about cytosine and its nucleosides (11), the pKa of 5-azacytidine should be little different from the 2.59 found for 5-azacytosine. Therefore, at pH 1, the sample, being nearly all in the protonated form, should contain more hydrate than the pH 3 sample, where more than half of the compound is not protonated.

If water adds across the 5,6-double bond, it seems reasonable that a better nucleophile, such as bisulfite, that is known to attack double bonds of heteroaromatic molecules (12, 13) would also add. Indeed, it was found that with increasing concentrations of bisulfite, the absorbance of 5-azacytidine solutions became progressively less intense, indicating an essentially nonchromophoric product as might be expected from an addition that brings about a loss of conjugation (14).

Although bisulfite adds to 5-azacytosine under the same conditions used for addition to 5-azacytidine, no evidence of hydrate formation was seen when 5-azacytosine was carried through the same pH adjustment experiments as the nucleoside. This finding suggests that the sugar group may be important in the hydration reaction. The 5′-hydroxy could act as a general base, making water a better nucleophile. It is especially well suited to act through a "concerted" mechanism, such as that proposed (15) for the addition of alcohols to phthalimidium cations.

The lack of significant hydrate formation with 5-azacytosine is also demonstrated by the spectral curve for pKa determination (Fig. 7). The protonated form of the molecule has a higher absorptivity than the neutral form. Conversely, 5-azacytidine absorbs less in acid than at neutral pH, presumably due to formation of the nonabsorbing intermediate. This loss of absorbance can be used to estimate the fraction of 5-azacytidine present in the hydrated form in acidic solution. Cytosine nucleosides and bases typically absorb more in the protonated than in the neutral form.

For example, the maximum absorbance of protonated forms of 6-methylcytidine and 6-methylarabinosylcytosine is about 1.7 and 1.6 times greater than that of the neutral molecules at the same wavelength. The protonated form of the base 6-methylcystosine absorbs 1.8 times as strongly as the uncharged form (11). At the wavelength of maximum absorption, protonated 5-azacytosine absorbs 1.6 times more than its neutral form. Without hydration, 5-azacytidine would be expected to exhibit the same pattern. Comparison of the observed absorptivity of 5-azacytidine in acid to that projected from the absorptivity of the unprotonated form (using the typical ratios just described) implies that roughly 70% of the 5-azacytidine exists as the hydrate in 0.1 *N* HCl.

Although the structure for Compound VI in Scheme III was proposed previously, it was neither successfully isolated from the reactions nor synthesized (5). Attempts at synthesizing compounds of this type by formylation of guanylurea led to recovery of either starting material or 5-azacytosine. Formation of 5-azacytosine in this way confirms the reversibility of the ring-opening step. The strong absorbance of analogous compounds such as *N*-formyl-*N*′-cyanoguanide was cited as evidence for formation of a compound like VI, since it could thereby account for the initial increase in absorbance observed in buffered samples (5). The fact that this increase occurs with no apparent lag phase emphasizes that loss of V to VI is rapid compared to formation of V from I.

Pithova *et al.* (5) identified ribosylguanylurea (VII) as a product of the hydrolysis of 5-azacytidine and showed that VII further decomposes to yield guanidine. In the present experiments, a λ_{max} of 221 nm at pH 7.6, observed in spectra for reaction mixtures, was attributed to the formation of VII. First-order plots using absorbance indicated that VII did not show appreciable absorbance at wavelengths longer than 240 nm in acid or buffer.

Table V—Results of Analog Computer Simulation of Scheme III

k_1	k_2	k_3	k_4	k_5	k_a[a]	k_b[a]	k_{obs}[a]
0.9	0.05	5.0	5.0	0.3	0.95	0.14	
					0.83[c]	0.14[c]	
0.7	0.1	7.0	7.0	0.1	0.70	0.044	
					0.72[c]	0.044[c]	
0.7	0.4	7.0	7.0	0.1	0.83	0.037	
					0.84[c]	0.036[c]	
					0.87[b]	0.038[b]	
0.7	0.7	7.0	7.0	0.1	1.01	0.031	
					0.93[c]	0.031[c]	
					1.03[b]	0.031[b]	
7.0	7.0	0.7	0.7	0.1	1.07	0.031	
1.0	7.0	0.7	0.7	0.1	0.86	0.010	
7.0	1.0	0.7	0.7	0.1	1.34	0.044	
7.0	7.0	0.5	0.5	0.5	1.10	0.105	
7.0	7.0	0.2	0.2	0.1	0.42	0.029	
7.0	7.0	0.2	0.2	0.5	0.72	0.066	
7.0	7.0	0.2	0.2	1.0			0.079
7.0	7.0	0.2	0.2	5.0			0.093
7.0	7.0	0.2	0.2	9.0			0.095

[a] All rate constants were obtained from semilog plots of I + V *versus* time unless otherwise noted. Apparent first-order rate constants are expressed as time^{-1}. [b] Obtained from data for I only. [c] Obtained from plots of 0.3 (I + V) + VI.

As stated under *Results*, the values of k_a and k_b obtained experimentally were the same using UV absorption data for the buffered reactions with or without acidification. It was of interest to determine whether Scheme III could provide data that would behave in this manner. Therefore, Scheme III was programmed using an analog computer. The values of the microconstants used are listed in Table V. Various components or combinations of components were studied as a function of time to simulate the spectral changes seen during the reactions.

According to Scheme III, I, V, and VI are present throughout the reaction. Using acidified samples, VI does not absorb at the wavelengths used for kinetic studies. When samples are acidified, I and V equilibrate so that the absorbance obtained actually reflects the sum of their concentrations in the reaction, although V has very little, if any, absorbance. In buffered samples, I and VI are responsible for the largest part of the absorbance, with VI absorbing more strongly than I.

With these considerations in mind, the analog program was adjusted to provide various kinds of data. The time course of I + V *versus* time was used to represent acidified samples. To simulate spectral changes seen with buffered samples, 0.3 (I + V) + VI was generated. The factor 0.3 was arbitrarily chosen to simulate the effect of differences in absorptivity. The time course of I alone was

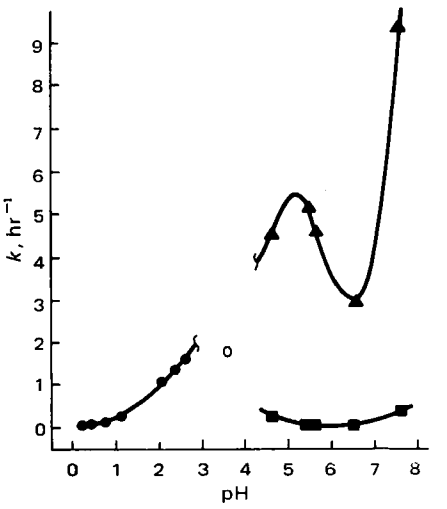
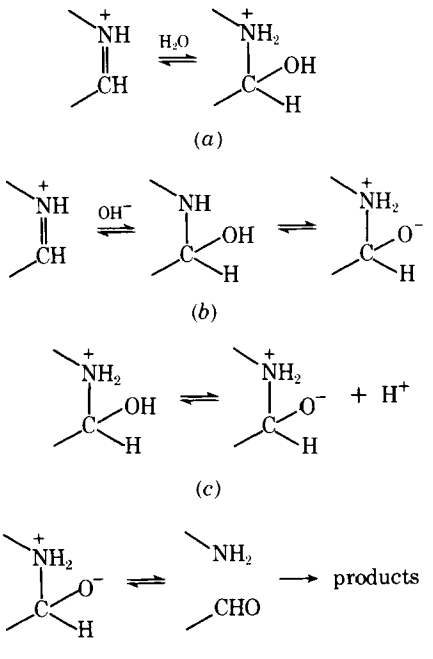


Figure 8—*Apparent first-order rate constants* k_a (▲), k_b (■), *and* k_{obs} (●) *for hydrolysis of 5-azacytidine at 50° as a function of pH. The point at pH 3.58 (○) was obtained as described in the text.*



Scheme IV

also defined. Plotting and feathering the data as described earlier gave estimates for k_a and k_b. These values were the same regardless of which simulation was used (Table V).

Hydrolysis of 5-Azacytidine as a Function of pH—Figure 8 shows a plot of observed first-order rate constants for 5-azacytidine hydrolysis as a function of pH. It is obvious from Scheme III that these are complex kinetic constants composed of several microconstants. This situation precludes an unequivocal interpretation of the pH–rate profile. However, Scheme III can be used in conjunction with present data to suggest mechanisms consistent with observed pH effects.

Both rate constants k_a and k_b are shown for the biphasic region (pH >4) in Fig. 8. Based on Scheme III, k_b may be defined as:

$$k_b = fk_5 \qquad \text{(Eq. 23)}$$

where f is the fraction of (I + V + VI) present as VI. The values for k_5 cannot be calculated from the observed constant k_b, since the value for f is not known. However, it is apparent from Fig. 8 that the product fk_5 is relatively independent of pH. Conversely, k_a is pH dependent. Although it is recognized that k_5 can affect the value of k_a, the observed pH dependency is most likely due to changes in the relative rates of the reversible steps for hydrolysis of I to VI. In the 4.5–6.5 pH region, hydrolysis of the triazine ring may be visualized as a series of steps analogous to those postulated for imine hydrolysis (Scheme IV).

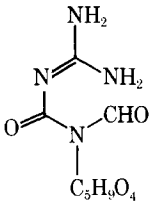
As in imine hydrolysis (10, 16), formation of protonated carbinolamine or neutral carbinolamine (Scheme IV, *a* and *b*) *via* the reactive cationic substrate (IH^+) would be the slowest reversible step at pH 6.5. As the pH decreases, the concentration of IH^+ increases; therefore, the apparent first-order rate constant increases. This increase would be expected to continue until protonation of I was complete, but increasing acidity inhibits formation of the zwitterion (Scheme IV, *c*) so less of it is available to react. Thus, near pH 5 the observed rate constant begins to decrease.

As the pH decreases further, first-order plots become linear instead of biphasic. Although the rate of formation of protonated V (VH^+) from IH^+ becomes faster, the fraction in the reactive form (zwitterion) decreases. The apparent first-order rate constant for loss of IH^+ is now attributed to cleavage of the carbinolamine (Scheme IV, *d*) as described by Scheme V.

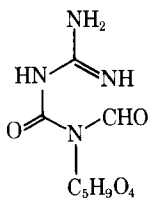
$$IH^+ \underset{\text{fast}}{\rightleftharpoons} VH^+ \underset{\text{slow}}{\rightleftharpoons} VI \underset{\text{fast}}{\longrightarrow} VII$$

Scheme V

Since the hydration of substrate to form VH^+ is fast, the overall loss appears to be simple first order. The observed constant continues to decrease with increasing acidity due to the decreasing fraction of reactive species in the form of zwitterion. Eventually, loss of IH^+ by this path becomes sufficiently slow that formation of 5-azacytosine and 5-azauracil becomes important at a pH of about 1, but k_{obs} continues to decrease in this region, presumably *via* the reduction of zwitterion.



VIII



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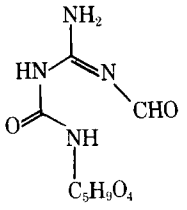

X

The observed increase in k_a at pH 7.6 may result from a pathway involving base-catalyzed water attack on the neutral molecule as observed for certain imines (10).

Scheme IV shows the result of 5,6-addition of water. Cleavage of the ring would then result in Compound VIII. Addition across the 1,6-bond would be necessary to obtain Structure VI, which was suggested previously (5). Such addition appears possible starting from the resonance form of IH^+, which has the positive charge at C-6. Addition of water could then occur across the 5,6- or the 1,6-bond. Since a positive identification of the compound was not made (5) and both VI and VIII yield the same product upon hydrolysis of the amide, both routes are possible. Initial protonation could also occur at the 3-nitrogen, leading to Compounds IX and X, which differ from VI and VIII only in the position of a proton.

Implications for Stability Prediction—This report is the first one to suggest the formation of the proposed 5,6-hydrate of 5-azacytidine. While the hydrate was not isolated, considerable indirect evidence has been presented in support of its existence. Regardless of the exact structure for the proposed intermediate, its facile formation in acidic solutions of 5-azacytidine is readily apparent. The undetermined biological significance of this previously unreported intermediate, along with the complexity of the observed first-order rate constants (as illustrated by the analog simulations), indicates the difficulty in predicting stability of 5-azacytidine potency in solutions.

For example, although the apparent rate constants for loss in acidic solutions are less than the k_a values obtained in buffers, a large fraction (about 0.7) of the drug forms the proposed hydrate immediately upon dissolution in acid. The rate constant observed in acid applies only to the loss of drug after the initial equilibration between drug and hydrate has taken place. Therefore, if the hydrate is not itself biologically active or is not bioreversible to drug, most potency is lost long before predictions based on the observed rate constant would have indicated significant hydrolysis.

Equation 19 describes the absorbance changes for 5-azacytidine in buffer solutions. The rate constant values are the same with or without acidification, but the values of the coefficients A and B differ. Provided that Scheme III applies, it is assumed that neither VI nor VII absorbs in acid at the wavelengths used in this study. It is also assumed that the proposed hydrate (V) does not accumulate to a significant extent at neutral pH. Although these assumptions were not proven, they were justified in previous discussion. By using these assumptions, the absorbance of acidified samples of reactions run in the buffer region may be attributed to 5-azacytidine and Eq. 19 may be written:

$$\text{percent remaining} = 100\,(Ae^{-k_a t} + Be^{-k_b t}) \qquad \text{(Eq. 24)}$$

Table VI—Times to Reach 90, 80, and 70% of Original Concentration of 5-Azacytidine in Buffer Solutions at 50° (Predicted using Eq. 24)

pH	Total Buffer[a]	Minutes		
		90%	80%	70%
	Phosphate			
7.59	0.615	2.5	7	14
	0.033	3.5	10	22
6.52	0.20	6	15	32
	0.04	11	32	81
	(0.04	80	225)[b]	
5.42	0.33	3.5	9	17
	0.066	6	18	52
	Acetate			
5.60	0.44	6	15	34
	0.088	6.5	18	56
4.62	0.80	4	10	16
	0.16	5	11	20

[a] Adjusted to μ = 0.5 with sodium chloride (Table III). [b] At 30°.

This equation may be used to calculate the percent drug remaining at any time by employing data for acidified samples, provided the above conditions are met. Failure of the first condition would lead to inapplicability of the biexponential equation. If the second assumption does not hold, the actual percent remaining will be less than that calculated from Eq. 24.

The percent drug remaining can be calculated using the appropriate constants in Eq. 24, keeping in mind the assumptions involved. For example, the values for A, k_a, B, and k_b listed in Table III were used to estimate the time required to lose 10, 20, and 30% in a variety of buffers at 50°. Results are shown in Table VI, where it can be observed that each succeeding 10% loss requires a longer time. This result is due to the transition from the rapid initial phase to the slower terminal phase. It is apparent that 5-azacytidine is decidedly unstable under these conditions. The maximum time required to lose 10% of the drug is 11 min, and 20% is lost in 32 min under these conditions.

To estimate the loss of potency under potential clinical conditions, a reaction was studied at 30° at the pH of maximum stability observed at 50° (0.02 M Na_2HPO_4–0.02 M NaH_2PO_4–0.42 M NaCl, pH 6.5). Within 30 min, 5% of the drug was lost; after 80 min, 10% was lost; and 20% was gone in 225 min. This apparent instability of 5-azacytidine suggests that it would be prudent to prepare solutions shortly before use to avoid significant degradation. However, it requires more than 2 hr for potency to decrease from 90 to 80% and the decrease from 80 to 70% would take even longer. It is likely that solutions used within 6 hr contain at least 70% of the original drug.

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* Present address: Merrell-National Laboratories, Cincinnati, OH 45215

x To whom inquiries should be directed.