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5-Azacytidine Hydrolysis Kinetics Measured by High-Pressure Liquid Chromatography and ^{13}C-NMR Spectroscopy

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Abstract □ Hydrolysis of 5-azacytidine, an experimental anticancer drug, in aqueous buffers was measured using a high-pressure liquid chromatographic (HPLC) procedure and a ^{13}C-NMR method. The former utilized a 17.5-μm Aminex A-6 strong cation-exchanger column eluted with 0.4 *M*, pH 4.6 ammonium formate buffer at a flow rate of 0.4 ml/min. The hydrolysis sequence as well as the existence of a labile intermediate, *N*-formylguanylribosylurea, was unequivocally established using 6-^{13}C-5-azacytidine and NMR spectral techniques. A reversible ring opening step to the *N*-formylguanylribosylurea with an equilibrium constant of 0.58 ± 0.03 between pH 5.6 and 8.5, followed by an irreversible formation of guanylribosylurea, was found by HPLC. The data confirm previous assumptions on the hydrolytic kinetics. The pH dependency of hydrolysis was examined, and the hydrolysis profile gave a normal V shape with the most stable pH at 7.0. Rather stable intravenous dosage forms can be formulated.

Keyphrases □ 5-Azacytidine—hydrolysis kinetics, aqueous solutions, high-pressure liquid chromatography, ^{13}C-NMR □ Hydrolysis kinetics—5-azacytidine, aqueous solutions □ Antineoplastic agents—5-azacytidine, hydrolysis kinetics, aqueous solutions

A cytidine analog, 5-azacytidine[1] (I), first synthesized in 1964 (1) and subsequently isolated from *Streptoverticillium ladakanus* (2, 3), has shown antitumor activity against several animal neoplasms including L-1210 leukemia (4) and T-4 lymphoma (5). Clinically, it has also demonstrated activity against various solid tumors (6) as well as leukemias (7) and is currently undergoing phase II trials (8).

This compound has long been known to be unstable in aqueous solution. Its lability has been attributed to the facile hydrolytic cleavage across the 5,6-bond (9–11). Consequently, proper formulation has been a problem in its clinical use. In addition, not only has the metabolic evidence indicated that ring cleavage is a major process of its disposition (12–14), the facile process may have a possible relationship to its still unclear overall biological activity. Therefore, the nature and sequence of its hydrolysis must be understood. While hydrolysis of I has been investigated (9–11, 15), the lack of a suitable analytical method has hampered a complete and unequivocal kinetic analysis, although certain information concerning hydrolysis has been obtained through spectroscopic resolution in a complex hydrolytic mixture (10).

Recently, specific information was obtained through TLC, NMR (11), and high-pressure liquid chromatography (HPLC) (15), including the isolation and identification of the labile intermediate; however, a systematic kinetic analysis is still lacking. This paper describes an HPLC method for the simultaneous analysis of I and its labile intermediate, *N*-formylribosylguanylurea (II), which occurs during formation of the hydrolytic product 1-β-D-ribofuranosyl-3-guanylurea (III). Corroborated with Fourier transform ^{13}C-NMR, using a 6-^{13}C-5-azacytidine previously synthesized in this laboratory (14), a detailed kinetic analysis of the hydrolysis of I in aqueous solution is presented.

EXPERIMENTAL

Chemicals and Reagents—All solvents were either analytical or liquid chromatographic grade. 5-Azacytidine was greater than 99% pure by HPLC and was used without further purification. Ninety percent labeled 6-^{13}C-5-azacytidine was synthesized as described previously (14). 1-β-D-Ribofuranosyl-3-guanylurea (III) was synthesized according to

[1] 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2-one, NSC-102816, CAS Reg. No. 320-67-2. Supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20014.

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the procedure of Pithova *et al.* (9). Sodium formate[2] and 90% enriched 1-^{13}C-sodium acetate[3], used as internal standards, were also pure by ^{13}C-NMR.

Analytical Procedure—Methanolic or aqueous solutions of 5-azacytidine were injected into a liquid chromatograph[4,5] *via* a 20-μl high-pressure sample injection valve[5]. The solution was eluted into a 500 × 2-mm i.d. stainless steel column[5] packed with 17.5-μm Aminex A-6[6] strong cation-exchanger with 0.40 *M*, pH 4.6 ammonium formate buffer at a flow rate of 0.4 ml/min. The components emerging from the column were detected *via* a UV detector[5] set at 254 nm. The UV-absorbing components were quantitated using either peak height or peak area as estimated *via* a mechanical disk integrator on a strip-chart recorder[7]. No significant difference was observed between these two quantitative methods.

^{13}C-NMR—NMR studies of 5-azacytidine, 6-^{13}C-5-azacytidine, and guanylribosylurea were recorded in dimethyl sulfoxide or in aqueous buffers at pH 8.0 *via* a 25.2-MHz Fourier transform NMR spectrometer[8]. Typical parameters for the measurements were: acquisition time, 0.8 sec; pulse delay, 1.2 sec; sweep width, 5000 Hz, 8 K data points; and tip angle, 30°.

For kinetic measurements, an aqueous solution of 90% enriched 6-^{13}C-5-azacytidine, 0.1 *M* in pH 8.0, 0.067 *M* phosphate buffer, was placed in the sample tube, which was inserted into the spectrometer probe. The probe temperature was maintained at 38 ± 0.1°. Rapid pulses at a pulse width of 8 were generated, and the appropriate number of transients was accumulated to acquire an adequate signal-to-noise ratio. A timer was started when the 5-azacytidine solution was mixed and placed inside the spectrometer. At the end of each accumulation, the time was noted.

Kinetic Measurements—The hydrolytic degradation of 5-azacytidine was studied quantitatively by HPLC at 25 and 37° in 0.067 *M* sodium phosphate buffer and acetate buffer at pH 4.5–9.1.

All experiments were duplicated. A gas-tight syringe was filled with a solution of 5-azacytidine at either 4.10×10^{-4} or 8.20×10^{-4} *M* in a phosphate buffer of the desired pH. For the 25° runs, the filled syringe was allowed to remain at room temperature in the high-pressure injector valve module. At approximately 10-min intervals, aliquots were pushed into the sample loop and injected immediately. For the 37° runs, the syringe was filled, capped, and placed in a constant-temperature bath. It was then removed at specific intervals to fill the injector loop and quickly replaced in the bath.

The 5-azacytidine concentrations were followed by either the peak height or the peak area method. Stability evaluations were made in Ringer's lactate and in normal saline at room temperature as well as at refrigerated temperature (5°).

RESULTS

Drug Assay—With either methanolic or aqueous solutions of 5-azacytidine kept at ice temperature before injection, a straight line was observed in the $0.041–8.20 \times 10^{-4}$ *M* range when either peak heights or peak areas were plotted against concentrations. The variance of 10 separate injections at 0.20×10^{-4} *M* was less than 1%.

When 5-azacytidine was dissolved in a phosphate buffer, 0.067 *M* at pH 8.0, and chromatographed, in addition to a peak emerging at 3.4 min (peak 1) corresponding to 5-azacytidine, a small, slower running peak emerged at 7.3 min (peak 2) and the intensity of peak 2 appeared to increase with time (Fig. 1). Their peak height ratio (peak 1 to peak 2) appeared to be constant at approximately 3:2 after 150 min. The intensities of these two peaks then diminished with time while maintaining an essentially constant ratio.

Each component was collected into a separate tube at room temperature. Reinjection of peak 1 immediately following collection yielded a single peak corresponding to 5-azacytidine. However, similar treatment of peak 2 gave two components corresponding to peaks 1 and 2, the latter at a higher intensity. After a longer period, repetition of the injection of either peak 1 or 2 revealed two peaks, this time with a peak 1 to peak 2 ratio of approximately 3:2 but with both at much diminished intensities. Collection of peak 2 at ice temperature did not appear to change the reversal of peak 2 to peak 1.

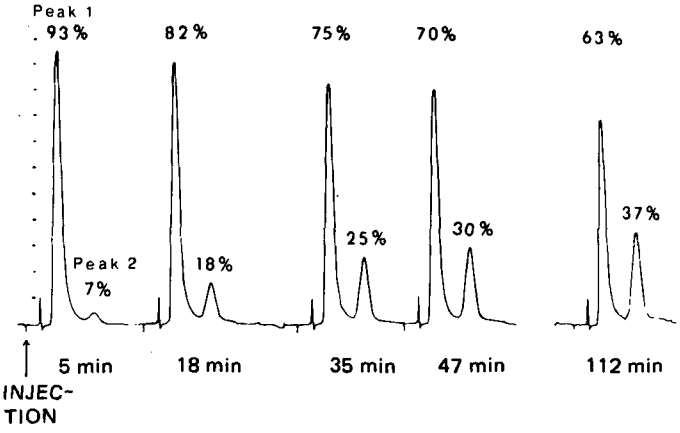


Figure 1—*High-pressure liquid chromatograms of 5-azacytidine, 8.2 × 10^{-4} M in pH 8.0, 0.067 M potassium phosphate at 25°, using 0.4 M, pH 4.6 ammonium formate at 0.4 ml/min.*

Attempts to isolate peak 2 by collecting it into a tube in a dry ice–acetone bath followed by lyophilization failed[9]. Dissolving the residue in water and injecting it into the liquid chromatograph did not give significant amounts of a UV-absorbing peak (254 nm). TLC analysis of the residue using the literature procedure (14) indicated that guanylribosylurea (III) was a major component.

^{13}C-NMR Analysis—The naturally abundant proton-decoupled NMR spectra of 5-azacytidine and guanylribosylurea spiked with formate in aqueous buffer are shown in Figs. 2 and 3, respectively. The chemical shift assignments of 5-azacytidine under the present conditions were referenced from reported values (16), and those for guanylribosylurea were assigned by direct comparison with those of I (Table I). Differences of the chemical shifts of guanylribosylurea from 5-azacytidine were seen in C-2, C-4, and C-1, as expected from the structure.

When a 5-azacytidine solution in pH 8 phosphate buffer was subjected to Fourier transform NMR analysis, new sets of signals not accounted for on the basis of the 5-azacytidine and ribosylguanylurea spectra appeared with time. A new signal appeared at 171.7 ppm, subsequently identified as formate by signal enhancement with the addition of sodium formate. Furthermore, the signal at 157.5 ppm was particularly broadened (Fig. 4).

The kinetic detections of 6-^{13}C-5-azacytidine hydrolysis in a buffered solution are shown in Fig. 5. A known concentration of ^{13}C-sodium acetate was added to the buffered solution in an attempt to quantitate the decomposition kinetics. The signals ratio between the C-6 of 5-azacytidine and the C-1 of sodium acetate was essentially constant until after 17 min. A small signal appeared at 167.8 ppm and increased with time. After 90 min, a second signal at 171.7 ppm appeared. Both new signals increased at the expense of the C-6 of 5-azacytidine, as indicated by slowly decreasing ratios of the signal intensity of the C-6 of 5-azacytidine to the C-1 of acetate. After 270 min, the intensity of the lower field signal exceeded that of the higher field one. The time course of the ratios between the C-6 signal of 5-azacytidine and the C-1 of acetate, an added internal standard, is shown in Fig. 6.

The identity of these new signals was confirmed by off-resonance decoupled NMR measurements, which assess the ^{13}C–H coupling. In this experiment, all signals exhibited a doublet except the signal of acetate (Fig. 5*h*), which indicated the attachment of one proton to each carbon. The chemical shift of the signal at 171.7 ppm coincided with that of the formate. On the basis of the chemical rationale of hydrolysis, UV absorption property *via* HPLC, and C–H coupling *via* the NMR off-resonance decoupling experiments, the signal at 167.8 ppm was assigned as derived from the formyl carbon of the labile intermediate, *N*-formylguanylribosylurea.

Kinetic Analysis—5-Azacytidine hydrolysis was carried out in 0.067 *M* phosphate buffers at pH 4.5–9.1 and at 25 and 37°. Selected hydrolysis profiles as measured by concentrations of 5-azacytidine *versus* times are shown in Fig. 7. Either monophasic or biphasic profiles were observed in all of the pH and temperature studies on semilog plots. At low pH as well as in the vicinity of neutral pH, biphasic profiles were observed, with

[2] Mallinckrodt.
[3] Merck & Co., St. Louis, Mo.
[4] Chromatronix model 3510.
[5] Spectra-Physics, Santa Clara, Calif.
[6] Packed in this laboratory with the resin supplied by Bio-Rad Laboratories, Richmond, Calif.
[7] Varian Aerograph, model 20, Varian Associates, Palo Alto, Calif.
[8] Varian XL-100, Varian Associates, Palo Alto, Calif.

[9] Recently, Beisler (11) was able to isolate and characterize *N*-formylribosylguanylurea by HPLC using a C-18 reversed-phase column and water as the eluant. In the present case, the low pH and high salt content during the lyophilization procedure may have caused the decomposition of this labile intermediate.

Table I—Chemical Shifts [a] of 5-Azacytidine and Guanylribosylurea

Carbon	I	III
C-4	166.4	162.2
C-6	157.5	—
C-2	156.1	165.4
C-1′	91.8	85.5
C-4′	84.6	83.9
C-3′	74.9	74.3
C-2′	69.6	71.2
C-5′	61.1	62.7

[a] Measured in dimethyl sulfoxide and expressed in parts per million with tetramethylsilane as the internal standard.

the most pronounced being at pH 7 and 8. At high pH, *i.e.*, 9.1, the first phase was too rapid to be discernible so that an apparently monophasic profile was observed. Similar biphasic profiles of 5-azacytidine were observed in Ringer's lactate as well as in normal saline, although the terminal phases declined very slowly.

From this evidence for the existence of an initial equilibrium phase between *N*-formylguanylribosylurea and 5-azacytidine, the biphasic behavior of these hydrolysis profiles were attributed to the kinetic scheme of Pithova *et al.* (9) (Path A, Scheme I).

The appropriate rate constants can be solved by making several assumptions: (*a*) the initial process is a rapid equilibrium, (*b*) *N*-formylguanylribosylurea exists at an approximate steady state throughout the hydrolysis, and (*c*) $k_{-1} \gg k_2$. These assumptions were supported by subsequent estimated data, generating a self-consistency. Then k_1 and k_{-1} are solvable by:

$$K_{eq} = \frac{k_1}{k_{-1}} \qquad \text{(Eq. 1)}$$

and (17, 18):

$$\log\left(\frac{A_0 - A_{eq}}{A - A_{eq}}\right) = \frac{k_1 + k_{-1}}{2.303} t \qquad \text{(Eq. 2)}$$

k_2 is solvable by the first-order degradation of the second phase:

$$A = A_0 e^{-Kt} \qquad \text{(Eq. 3)}$$

$$K = K_{eq} k_2 \qquad \text{(Eq. 4)}$$

The estimated rate constants for 5-azacytidine hydrolysis in aqueous buffer solutions at different pH values and at two temperatures are shown in Table II. The stability profile at 25 and 37° as expressed by plotting log k_1 *versus* pH is shown in Fig. 8. The profile at both temperatures followed a typical V shape, with the most stable pH at 7.0.

DISCUSSION

As was first proposed by Pithova *et al.* (9), 5-azacytidine in aqueous solution undergoes hydrolysis according to Scheme I in strong aqueous acid. Glycosidic linkage cleavage to yield 5-azacytosine, 5-azauracil, and D-ribose (Path B) was the major degradative pathway. In neutral and basic media, a facile ring cleavage to yield an instable *N*-formylguanylribosylurea intermediate followed by a loss of formate to form ribosylguanylurea was proposed as the major degradative pathway (Path A). Notari and DeYoung (10) proposed a refined scheme to include the hydration step across the 5,6-double bond as the first step.

In those studies, UV spectral techniques were used to quantify the hydrolysis kinetics of I. Using a TLC and PMR method, Israili *et al.* (15) studied the hydrolysis of 5-azacytidine and found that its degradation in aqueous buffers as well as in human plasma under various conditions followed first-order kinetics, but only monoexponential declines were reported.

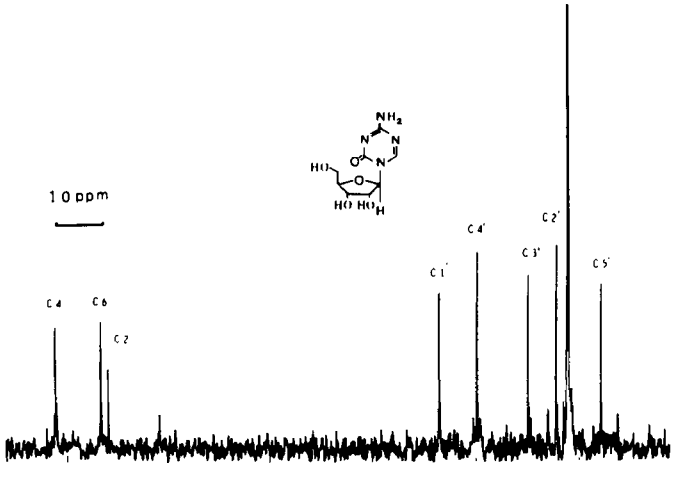


Figure 2—^{13}C-*NMR spectrum of 0.15* M *5-azacytidine at 30°. The solvent was deuterium oxide–water.*

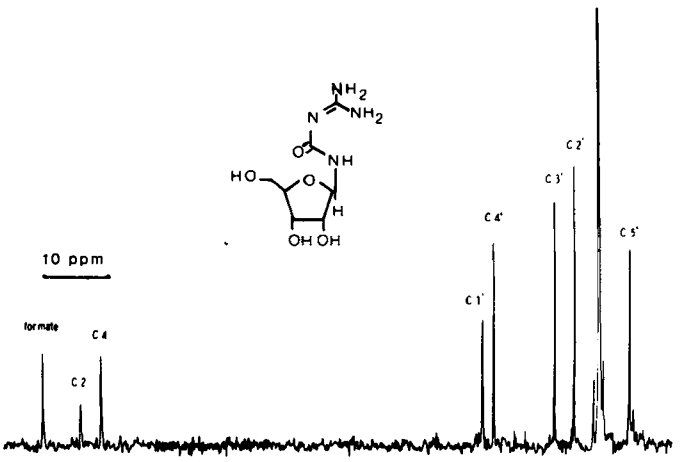
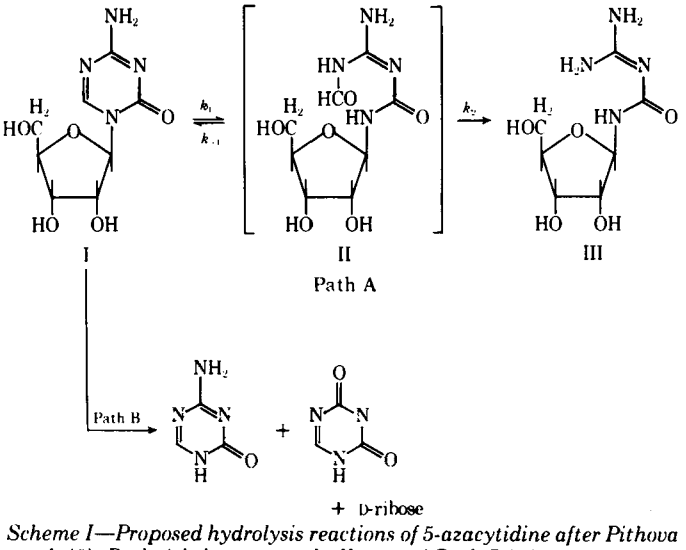


Figure 3—^{13}C-*NMR spectrum of guanylribosylurea (0.15* M*) and sodium formate (0.32* M*) in deuterium oxide–water at pH 10.2 and 30°.*



Scheme I—Proposed hydrolysis reactions of 5-azacytidine after Pithova et al. *(9). Path A is in aqueous buffers, and Path B is in strong acids.*

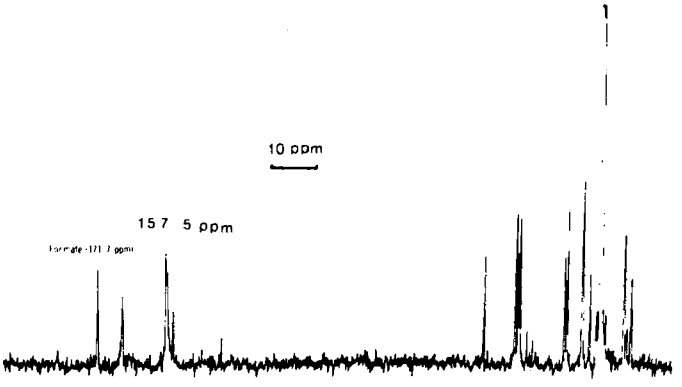


Figure 4—^{13}C-*NMR spectrum of 5-azacytidine in pH 8.0 phosphate buffer (0.15* M*) after 4 hr at 30°.*

Table II—Estimated Rate Constants[a] for 5-Azacytidine Hydrolysis in Aqueous Buffer Solutions

	25°				30°		
pH	$k_1 \times 10^{-3}$ M, min^{-1}	$k_{-1} \times 10^{-3}$ M, min^{-1}	K_{eq}	$k_2 \times 10^{-3}$ M, min^{-1}	$k_1 \times 10^{-3}$ M, min^{-1}	$k_{-1} \times 10^{-3}$ M, min^{-1}	K_{eq}
4.5	7.67 ± 0.37	17.50 ± 0.40	0.44	4.10	15.60 ± 0.40	32.30 ± 1.40	0.48
5.6	6.27 ± 0.37	11.20 ± 1.20	0.57	1.30	11.20 ± 0.60	19.60 ± 1.50	0.58
7.0	3.22 ± 0.02	5.73 ± 0.04	0.56	0.77	7.33 ± 0.00	13.05 ± 0.00	0.56
7.4	4.36 ± 0.04	7.95 ± 0.24	0.55	0.93	12.8 ± 0.50	22.20 ± 0.50	0.58
8.0	9.92 ± 0.03	17.20 ± 0.50	0.58	1.70	35.00 ± 1.80	60.80 ± 1.70	0.58
8.5	24.00 ± 1.10	39.20 ± 1.8	0.61	—	—	—	—
9.1	62.20 ± 3.80	101.0 ± 6.0	0.61	10.80	—	—	—

[a] Calculations were based on the molar concentrations of remaining 5-azacytidine since the *N*-formyl intermediate possesses different extinction coefficients (10, 11). At those times where initial equilibrium was observed and used for calculation, insignificant amounts of irreversible degradation occurred, consistent with the assumptions made, *i.e.*, $k_{-1} \gg k_2$, and with the experimental observations (^{13}C-NMR). This finding is also consistent with the observations in Ref. 11.

None of the previous studies isolated and characterized the *N*-formylguanylribosylurea intermediate. In a preliminary study (19) in this laboratory, the *N*-formyl intermediate was isolated and characterized by ^{13}C-NMR. Recently, using HPLC and PMR, Beisler (11) also isolated and characterized the *N*-formyl intermediate and reported its biological activity. However, no detailed kinetic analysis of the hydrolysis of I was presented.

The aims of this study were to use ^{13}C-NMR coupled with specific stable isotopic labeling to demonstrate unequivocally the existence of the unstable intermediates and the sequence of the degradation and to use an assay method to quantitate the kinetics and hydrolysis of 5-azacytidine as a function of pH and temperature.

^{13}C-NMR—Because of the high sensitivity and speed in data acquisition, Fourier transform ^{13}C-NMR has been used in studying reaction mechanisms (20, 21). In the present studies, the large differences in chemical shifts among the imino carbon of I, the *N*-formyl carbon of II, and the formic acid carbon were utilized for analysis. The C–H coupling (*i.e.*, doublet) should provide a positive identification of their location. Initially, it was anticipated that NMR studies on the basis of naturally abundant C-13 would provide the necessary evidence for the hydrolysis; however, no additional signals at the low field region except the formate carbon have been revealed in the hydrolysate. Subsequently, it was found that, due to the overlapping signal between the C-6 of *N*-formyl carbon and the C-4 and 5-azacytidine, it was not possible to follow the kinetics of the generation of II using naturally abundant ^{13}C-compounds. This problem was circumvented by the use of ^{13}C-5-azacytidine labeled at the C-6 position synthesized previously (14).

Use of 90% labeled 6-^{13}C-5-azacytidine revealed the generation of *N*-formylribosylguanylurea and subsequently formic acid from a solution of 5-azacytidine in pH 8.0 phosphate buffer. The chemical shifts and multiplicity in off-resonance decoupled ^{13}C-NMR and UV absorption characteristics detected from the HPLC studies are all consistent with the assigned *N*-formylguanylribosylurea structure. In addition to the off-resonance decoupled experiment, the formate carbon identity comes from enhancement of signal intensity with addition of sodium formate. On the basis of the timed ^{13}C-NMR studies, the generation sequence of *N*-formylguanylribosylurea and formate was proven unequivocally. Although carbinolamine formation as the first step of hydration across the 5,6-double bond (10) appeared to be reasonable, its detection by ^{13}C-NMR has not yet been accomplished, possibly because of its short life or very low concentration. Thus, this study also demonstrates the use of stable isotope labeling and ^{13}C-NMR in the study of reaction mechanisms.

Since the relaxation mechanism and T_1 of 5-azacytidine and *N*-formylguanylribosylurea are likely to be similar, it was thought that by selecting a reference atom with a similar chemical shift, the degradation kinetics could be followed. Thus, 90% 1-^{13}C-sodium acetate equimolar to 6-^{13}C-5-azacytidine was added to the solution for the kinetic measurement. Decomposition kinetics were followed by a change in the signal ratios between the C-6 of 5-azacytidine and the C-1 of acetate (Fig. 6). However, as shown, the ratio remained relatively constant for the initial several minutes and declined afterwards. The kinetic profile was not consistent with the HPLC data and remained difficult to interpret. The difference was perhaps due to in part to errors in Fourier transform NMR arising from limitations because of the number of data points available in the computer system and from the nonuniformity in numbers of pulses for each time spent. Therefore, steady state may not have been achieved in the experiments with few pulses.

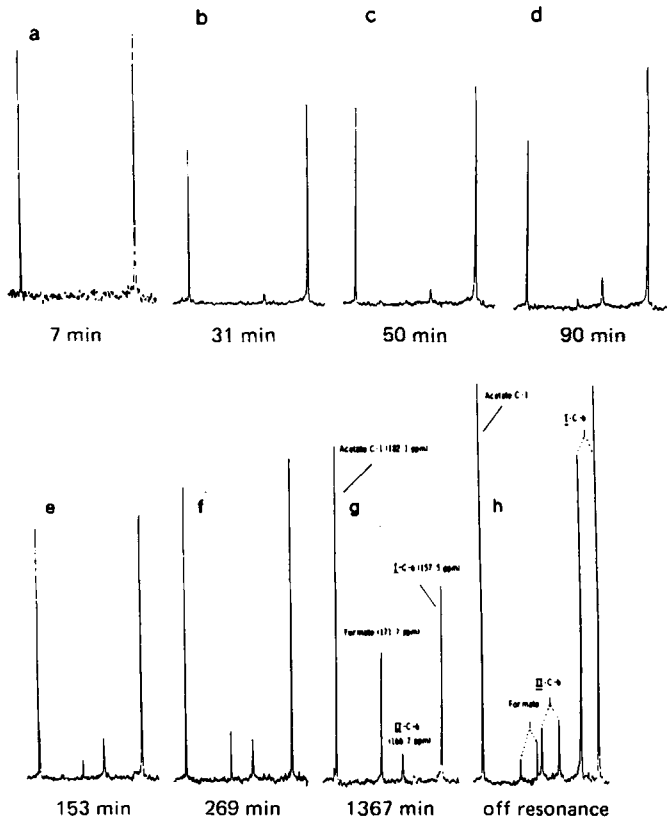


Figure 5—*^{13}C-NMR spectrum of 90% enriched 6-^{13}C-5-azacytidine (0.148 M) in pH 8.0, 0.067 M potassium phosphate buffer containing 30% deuterium oxide and ^{13}C-sodium acetate (0.20 M) at 27°. Figures 5a–5h are spectra at selected times. For complete number of time points, see Fig. 6.*

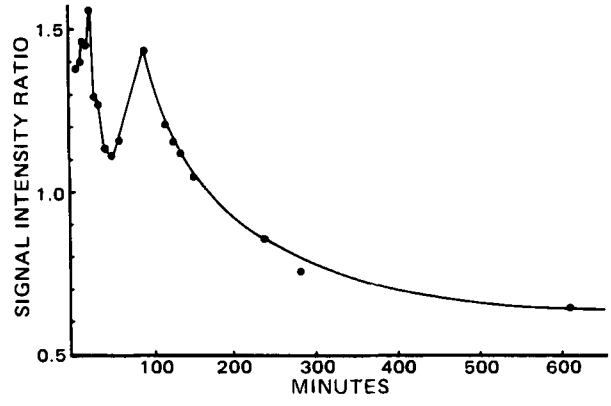


Figure 6—*Kinetic profiles of 90% enriched 6-^{13}C-5-azacytidine (0.158 M) hydrolysis in pH 8.0, 0.067 M potassium phosphate buffer containing 30% deuterium oxide and ^{13}C-sodium acetate (0.20 M) at 27° using ^{13}C-NMR measurements. Vertical axis is signal intensity ratios between 5-azacytidine and sodium acetate.*

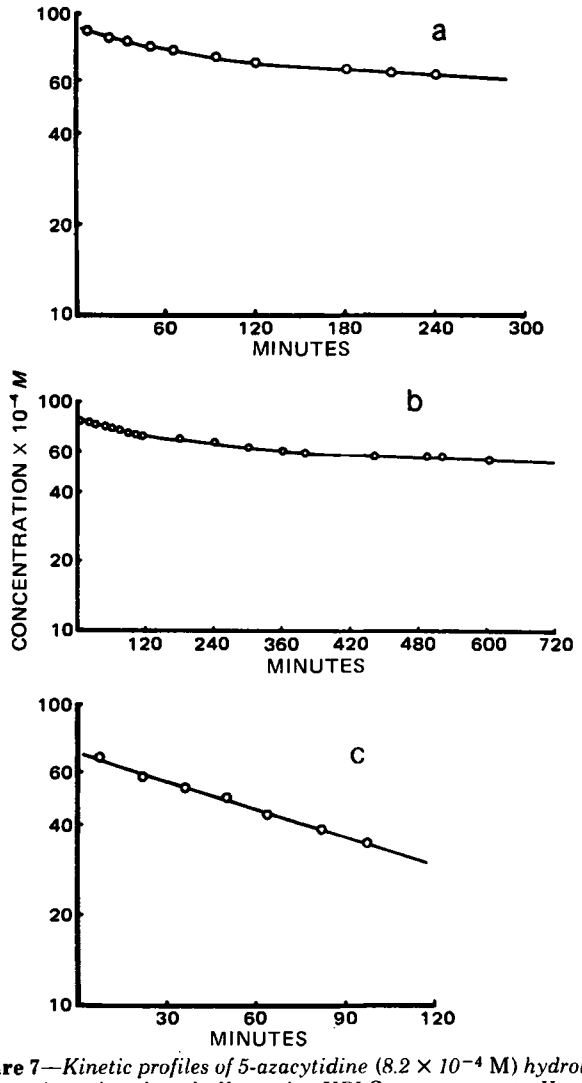


Figure 7—*Kinetic profiles of 5-azacytidine (8.2 × 10^{-4} M) hydrolysis in potassium phosphate buffers using HPLC measurements. Key:* (a), *pH 5.6 and 2.5°;* (b), *pH 7.0 and 25°; and* (c), *pH 9.1 and 25°.*

Kinetics Studies—Using UV measurement at 242 nm, Pithova *et al.* (9) first observed an initial increase followed by a subsequent decline when the extinction coefficient of 5-azacytidine in pH 6.6–7.8 borate buffers was plotted against time. Using similar UV measurements at two different wavelengths, Notari and De Young (10) observed biphasic profiles when 5-azacytidine absorbances in buffer solutions were plotted against time; the decrease or increase with time of the initial phase depended on whether the sample was acidified initially or not. The initial phase decreased with time using initially acidified sample and increased using nonacidified 5-azacytidine.

In the present study (Fig. 7), in buffers from pH 4.5 to 8.5, biphasic declines were observed when 5-azacytidine concentrations were plotted against time. At pH 9.1, the profile appeared to be monophasic and probably resulted from a very rapid equilibrium so that the first phase was not readily discernible. On the basis of the kinetic scheme (Scheme I), the individual rate constants were estimated (Table II). The direct kinetic measurements showed a rapid equilibrium between 5-azacytidine and *N*-formylribosylguanylurea, followed by a slower degradation of the latter to ribosylguanylurea.

As was evident at room temperature and various pH values, the rate constants, k_{-1}, for the ring closure were several times larger than those of the guanylribosylurea formation, k_2, consistent with the assumption made. This observation is reasonable in view of the chemical nature of the cleavages since the latter step requires the breakage of an amide bond. The k_{-1} values were also slightly greater than the k_2 values, the rate constant for the ring opening, yielding an equilibrium constant of 0.58 ± 0.03 between pH 5.6 and 8.5. This value is close to the constant ratio of 0.41 between II and I reported in water (pH unknown) (11). At 37° the equilibrium constants (0.58 ± 0.01) remained essentially unchanged (pH 5.6–8.0). The equilibrium constant decreased slightly at a lower pH (4.5). These observations are qualitatively consistent with the HPLC and PMR observations as well as with reported data (10, 11).

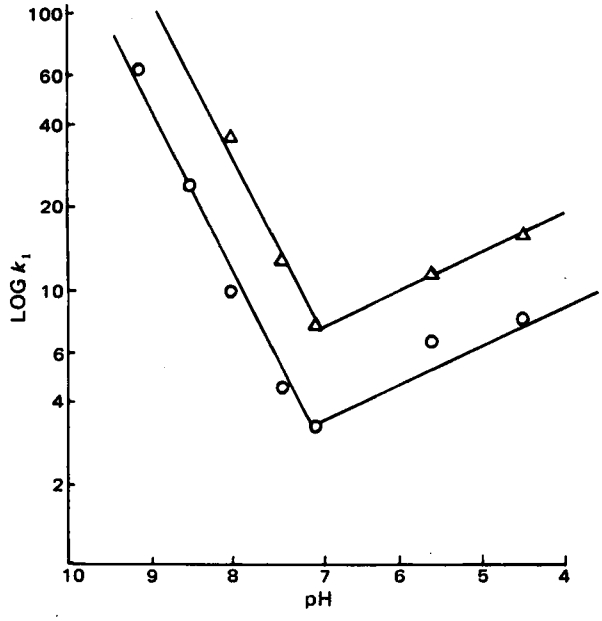


Figure 8—*The pH stability profile of 5-azacytidine (8.2 × 10^{-4} M) in aqueous buffers (potassium phosphate buffer, 0.067 M) at 25° (○) and 37° (△).*

pH-Dependent Hydrolysis—As shown in Table II, the rate constants of the hydrolysis varied with pH. When log k values were plotted against pH at 25 and 37°, a V-shaped curve resulted with both minima at pH 7.0 (Fig. 8), a value slightly different than the pH 6.5 reported (10). The slopes on the basic and acidic portions of the curve were +0.630 and −0.152, respectively. A similar trend was also observed if log k_2 values were plotted against pH. The hydrolysis, therefore, appears to be acid and base catalyzed, similar to the other known examples (22, 23). The deviation of the slopes from +1 and −1 on the basic and acidic pH portions of the curve, respectively, suggests that the hydrolysis may be susceptible to general acid and base catalyses (17). These pH profiles of the hydrolyses were markedly different from those reported by Notari and De Young (10) who described a more complex profile not readily interpretable on the basis of acid and base catalyses.

The hydrolysis kinetics observed were qualitatively similar to those reported using HPLC, PMR, and UV; however, quantitation differences were observed with those reported and may be attributed to differences in experimental conditions as well as methodologies. Under comparable experimental conditions such as those in Ringer's lactate, comparable results were obtained.

Stability Evaluation of 5-Azacytidine in Aqueous Buffers and Other Solutions—Although 5-azacytidine hydrolysis is subject to acid and base catalyses, the rate constants for the reversible formation of *N*-formlyribosylguanylurea as well as for the irreversible formation of ribosylguanylurea are smaller in magnitude than at the basic pH. Since ribosylguanylurea itself does not seem to possess significant cytotoxicity, rapid degradation of 5-azacytidine to this compound will result in a loss of efficacy. Recent biological data suggest that *N*-formylribosylguanylurea also has little cytotoxicity (11), although the evidence was not clearcut. However, other researchers found that aqueous solutions of 5-azacytidine, after long standing at room temperature, resulted in higher cytotoxicity (24, 25). Although formation of *N*-formylribosylguanylurea is reversible, in the absence of more definitive data it is desirable to formulate 5-azacytidine in media where the ring opening reaction is retarded. The pH profile of hydrolysis and kinetic data in Table II show that the rate constants for the hydrolysis in acidic pH are smaller in magnitude than those in basic pH. Therefore, a pH lower than 7.0 will result in slower ring hydrolysis.

In patients, slow infusion of 5-azacytidine in Ringer's lactate has lowered GI toxicity. The present study demonstrated that 5-azacytidine is very stable in this medium (pH 6.4), yielding a terminal $t_{1/2}$ of 4.8 days at room temperature, a value close to the reported one of 4.2 days (15). The $t_{1/2}$ in this medium at refrigerated temperature (0–5°) was considerably longer (31.3 days).

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GI Absorption of β-Lactam Antibiotics III: Kinetic Evidence for *In Situ* Absorption of Ionized Species of Monobasic Penicillins and Cefazolin from the Rat Small Intestine and Structure–Absorption Rate Relationships

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Abstract □ Absorption rates of monobasic β-lactam antibiotics were measured as a function of lumen solution pH between 4 and 9 by utilizing the rat intestinal recirculating method *in situ*. Between pH 6.5 and 9, the absorption rate constants of ionized antibiotics were almost identical; but, at pH 4, the unionized species were highly absorbed, depending on their lipophilicity through the GI membrane lipoidal barrier. The structure-absorption rate relationship was established with the unstirred layer model.

Keyphrases □ Absorption, GI—various monobasic penicillins and cefazolin, effect of ionization, pH, structure–activity relationships □ Penicillins, various—GI absorption, effect of ionization, structure–activity relationships □ Cefazolin—GI absorption, effect of ionization, structure–activity relationships □ Antibiotics—penicillins and cefazolin, GI absorption, effect of ionization, structure–activity relationships

The GI absorption rate of a monobasic penicillin in rats deviated significantly from the pH-partition hypothesis (1). This shift was interpreted successfully by the absorption mechanism of penicillins through the aqueous diffusion layer of the lumen side of the GI membrane, which is restricted by the lipophilicity of the undissociated species (1).

In *in vitro* experiments utilizing an isolated gut technique, the transport of phenoxypenicillin derivatives exhibited a saturable process, but additional study did not produce evidence of active transport (2). A similar *in vitro* study (3) provided evidence for the passive transport of β-lactam antibiotics at pH 7.4. Perrier and Gibaldi (4) attributed the larger absorption of dicloxacillin than of penicillin G and ampicillin from the *in situ* rat intestinal loop to the larger lipophilicity of dicloxacillin. A good correlation was found (5) between the rate of *in situ* intestinal cephalexin and cefazolin absorption in rats and their *in vitro* release rate from liposomes.

Recent studies in this laboratory on the *in situ* absorption kinetics of low lipophilic and completely ionized amphoteric β-lactam antibiotics, such as amoxicillin (6), cyclacillin (7), and cephradine (7), indicated that their intestinal absorption is governed by simple diffusion fol-

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