Table IX—Extent of Complex Formation between 3,4-Dimethylphenol and Ethyl Myristate Determined by Partitioning Study

Total Concentration of 3,4-Dimethylphenol, $\times 10^2 M$	Partition Coefficient	$\frac{[DMP_{comp}]}{[DMP_0]}$
0	1.37	—
1.53	1.73	0.27
3.07	1.96	0.43
6.14	2.61	0.91
9.21	3.31	1.43
15.03	4.57	2.35
30.00	7.94	4.83

for various cosolvent systems, and each showed a straight-line relationship between the two parameters (Fig. 4). The stability constants, $K_{1:1}$ and $K_{1:2}$, for each ester were calculated from Fig. 4 and are shown in Table VII. It is evident from these results that 4-hexylresorcinol forms not only 1:1 but also 1:2 complexes with esters in hexane. The stability constant values obtained for ethyl myristate were somewhat higher than those for the other esters. This result is probably due to the fact that ethyl myristate has a larger hydrocarbon chain, which results in a better interaction with the hydrophobic portion of phenols.

To ascertain whether the formation of 1:2 complexes is due to the involvement of the two hydroxy groups of 4-hexylresorcinol, the partitioning study was repeated with 3,4-dimethylphenol. The data obtained from this study are shown in Tables VIII–X. A plot of $[DMP_{comp}]/[DMP_0][E_T]$ *versus* the total ester concentration is shown in Fig. 5; $[DMP_{comp}]$ is the concentration of 3,4-dimethylphenol in the complex form, and $[DMP_0]$ is the concentration of the free form.

Table X—Extent of Complex Formation between 3,4-Dimethylphenol and Ethyl Pivalate Determined by Partitioning Study

Total Concentration of 3,4-Dimethylphenol, $\times 10^2 M$	Partition Coefficient	$\frac{[DMP_{comp}]}{[DMP_0]}$
0	1.37	—
3.82	1.78	0.30
7.68	2.33	0.70
15.36	3.33	1.44
23.04	4.32	2.17
38.40	6.63	3.86

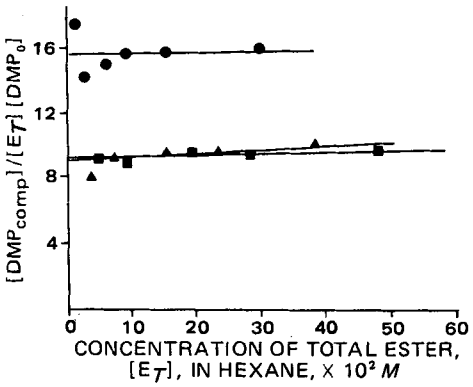

Figure 5—*Plot of $[DMP_{comp}]/[DMP_{free}][E_T]$ as a function of the total ester concentration, $[E_T]$, in hexane; $[DMP_{comp}]$ and $[DMP_{free}]$ represent the concentrations of complexed and free forms of 3,4-dimethylphenol, respectively. ($[DMP_{comp}] = [DMP_T] - [DMP_0]$). Key: ■, ethyl acetate; ●, ethyl myristate; and ▲, ethyl pivalate.*

As seen in Fig. 5, the monohydroxy compound forms only a 1:1 complex with the esters. The stability constants calculated from Fig. 5 are given in Table VII. The results of this study substantiate the conclusion that the diffusion of 4-hexylresorcinol through ethylene–vinyl acetate copolymers involved the formation of 1:1 and 1:2 complexes between the drug and the vinyl acetate portion of the copolymers.

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High-Performance Liquid Chromatographic Analysis of Chemical Stability of 5-Aza-2′-deoxycytidine

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Abstract □ The chemical stability of 5-aza-2′-deoxycytidine (I) in acidic, neutral, and alkaline solutions was analyzed by high-performance liquid chromatography. In alkaline solution, I underwent rapid reversible decomposition to *N*-(formylamidino)-*N*′-β-D-2-deoxyribofuranosylurea (II), which decomposed irreversibly to form 1-β-D-2′-deoxyribofuranosyl-3-guanylurea (III). The pseudo-first-order rate constants for this reaction were determined. The decomposition of I in alkaline solution was identical to that reported previously for the related analog, 5-azacytidine. However, in neutral solution (or water), there was a marked difference in the decomposition of I and 5-azacytidine. The same decomposition products were formed from 5-azacytidine in neutral solution as in alkaline solution. However, in neutral solution, I decomposed to II and three unknown compounds that were chromophoric at 254 nm. Compound I was most stable when stored in neutral solution at low temperature.

Keyphrases □ 5-Aza-2′-deoxycytidine—analysis of chemical stability using high-performance liquid chromatography □ Antileukemic agents—5-aza-2′-deoxycytidine, analysis of chemical stability using high-performance liquid chromatography □ High-performance liquid chromatography—analysis of chemical stability of 5-aza-2′-deoxycytidine

5-Aza-2′-deoxycytidine (I), a nucleoside antimetabolite, is a very active antileukemic agent in mice (1, 2) and a potent cytotoxic agent against neoplastic cells *in vitro* (2, 3). This antimetabolite is related to 5-azacytidine, an agent currently used in the clinical treatment of acute leukemia (4).

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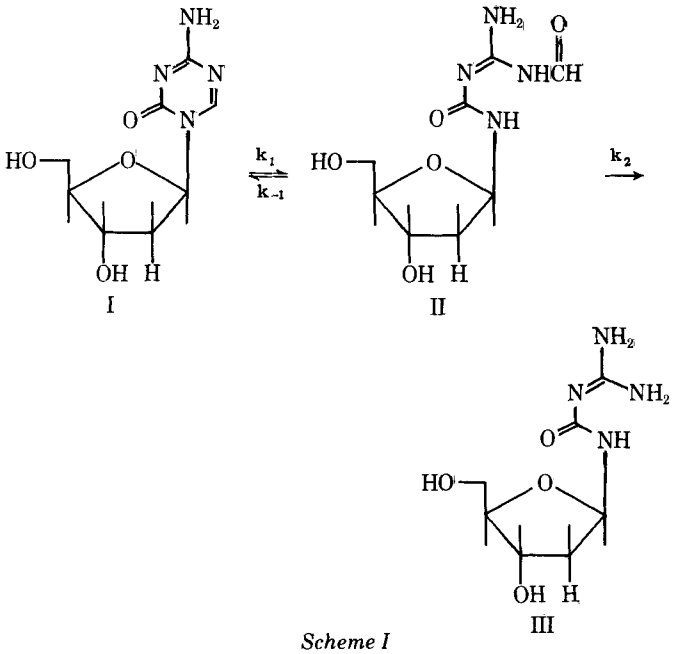

Scheme I

BACKGROUND

One major problem encountered in the clinical formulation of 5-azacytidine is its chemical instability, leading to solutions of decreasing potency on storage. The chemical stability of 5-azacytidine was first studied by Píthová *et al.* (5), who demonstrated that in alkaline solution the triazine ring of 5-azacytidine opens and loses a formyl group to form 1-β-D-ribofuranosyl-3-guanylurea. These workers proposed that the intermediate compound in this reaction was *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea, but they were unable to isolate it using paper chromatography because of its chemical instability. Beisler (6), using high-performance liquid chromatography (HPLC), isolated and identified this intermediate compound and showed that it could be partially converted back to 5-azacytidine.

The chemical decomposition of I at alkaline pH (Scheme I) presumably follows the same reaction steps as described previously for 5-azacytidine. In alkaline solutions, I undergoes a reversible hydrolytic reaction to form *N*-(formylamidino)-*N'*-β-D-2-deoxyribofuranosylurea (II), which, by the irreversible loss of the formyl group, forms 1-β-D-2'-deoxyribofuranosyl-3-guanylurea (III).

The present study investigated the decomposition of I in acidic, neutral, and alkaline solutions and at different temperatures to illustrate the chemical stability of this compound.

EXPERIMENTAL

5-Aza-2'-deoxycytidine (I) was synthesized[1] by modification of an earlier method (7).

Method of Analysis—HPLC[2] was performed with a variable-wavelength detector by monitoring at 220 or 254 nm. Analytical and preparative work was accomplished with a 300 × 3.9-mm i.d. commercially packed octadecylsilane column[3], which was eluted at a flow rate of 2 ml/min with 10 m*M* potassium phosphate buffer (pH 6.8). For analytical and preparative work, 20- and 500-μl injector loops, respectively, were used.

Stock solutions of 10 m*M* I in water were stored at −70°. Aliquots of this solution were thawed quickly and stored at 1–2°. The following buffers were used for the pH stability studies: phosphoric acid, pH 2.2; phosphoric acid–potassium phosphate, pH 3.2; sodium acetate buffer, pH 4.5 and 5.7; potassium phosphate buffer, pH 6.4 and 7.0; and sodium borate, pH 8.5, 9.2, 9.8, and 10.4. Compound II was prepared by addition of 20 μl of 0.5 *M* sodium borate, pH 10.4, to a 1.0-ml solution of 10 m*M* I. The mixture was incubated for 2 min at 24° and then neutralized with 20 μl of 1.0 *M* KH_2PO_4, and a 500-μl sample was injected onto the column for isolation of II.

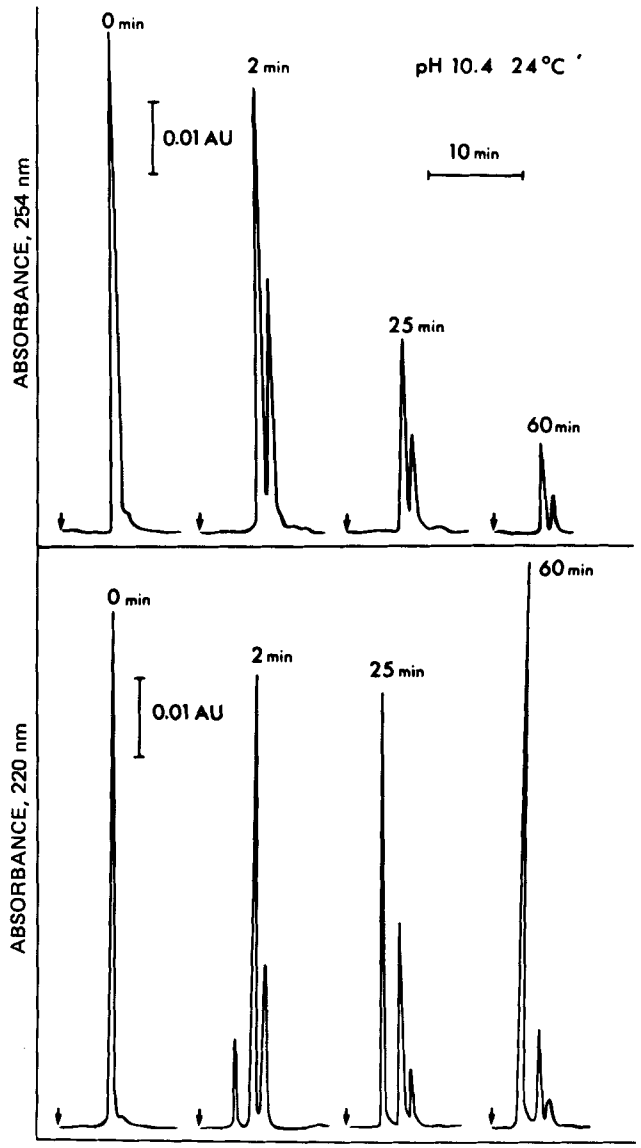

Figure 1—*Decomposition of I at pH 10.4 and 24°. Compound I (1.0 mM) was dissolved in 10 mM borate buffer (pH 10.4). At the indicated times, an aliquot of the solution was neutralized with phosphate buffer and analyzed by HPLC at 254 and 220 nm.*

Kinetic Experiments—A series of tubes containing 10 μl of 0.5 *M* stock sodium borate buffer were incubated at 37° for 10 min. Then 100 μl of isolated II (in 10 m*M* potassium phosphate, pH 6.8) was added to each tube to start the reaction. At timed intervals, an aliquot was withdrawn, and 20 μl was injected onto the column. The absorbance at 220 nm was recorded, and the relative concentration of the observed degradation products was characterized by peak heights. Similar experimental procedures were followed for the kinetic study on 5-azacytidine[4].

RESULTS

The decomposition of I at pH 10.4 and 24° as determined by HPLC is shown in Fig. 1. Measurement of the absorbance of the column eluate at 254 nm initially showed a single major peak (I) with a t_R value of 5.5 min. With time, a second peak (II) appeared with a t_R value of 6.6 min. Both peaks showed a gradual decrease in peak size with time. At 220 nm, the column eluate initially showed a single major peak (I) with a t_R = 5.5 min. With time, peak II (t_R = 6.6 min) and peak III (t_R = 3.5 min) became evident. At 60 min, most of I decomposed to peak III.

[1] By Dr. A. Piskala, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, 16610 Prague 6, Czechoslovakia.

[2] Model 110 pump and Hitachi variable-wavelength detector, Altex Scientific, Berkeley, Calif.

[3] μBondapak C_{18}, Waters Associates, Framingham, Mass.

[4] Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

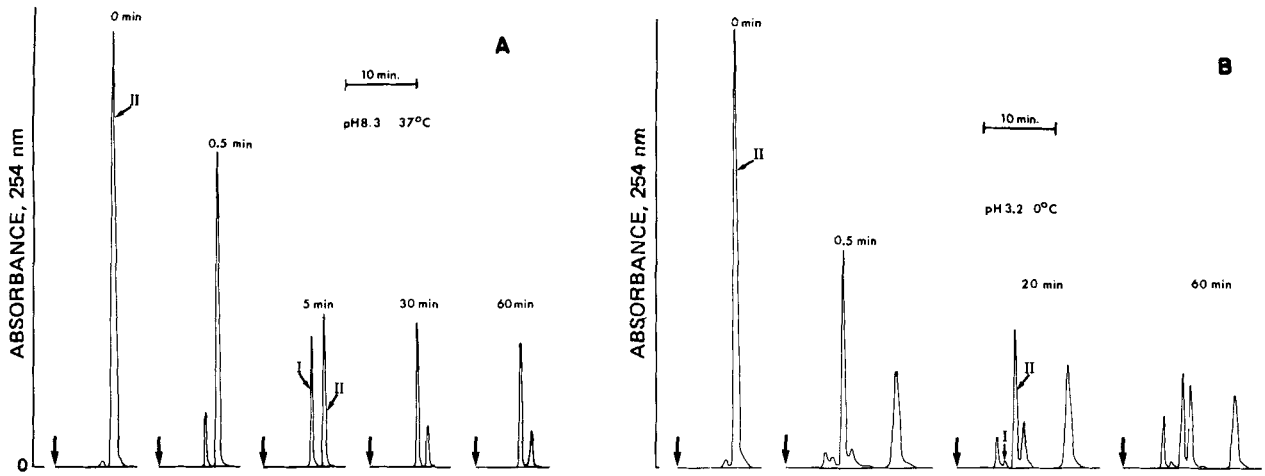

Figure 2—*Conversion of II to I at pH 8.3, 37° (A), and to unidentified compounds at pH 3.2, 0° (B). Peak II was isolated by HPLC and placed in 20 mM potassium phosphate buffer (at final pH 8.3 or 3.2). At the indicated times, aliquots of this solution were analyzed by HPLC.*

When II was isolated and placed at pH 8.3 and 37°, I appeared rapidly, indicating that II could be converted to I (Fig. 2A). However, when II was placed in an acidic solution (pH 3.2), it did not produce I; three unidentified peaks at 254 nm were observed. The latter reaction was very rapid, even at 0° (Fig. 2B).

The decomposition of I and 5-azacytidine when stored in water and in pH 7.4 phosphate buffer at 24° for 24 hr is compared in Fig. 3. Absorbance measurements at 254 nm of the column eluate showed five major peaks for I and two major peaks for 5-azacytidine. At 220 nm, the column eluate showed seven major peaks for I and three major peaks for 5-azacytidine.

The decomposition rates of I in acidic, neutral, and basic solutions at 24 and 37° are shown in Figs. 4A and 4B, respectively. Compound I decomposed more rapidly at pH 2.2, 8.5, and 9.2 than at pH 5.7, 6.4, and 7.0. The decomposition rate of I was the slowest at pH 7.0. At pH 2.2 a peak with the same t_R value (2.3 min) as 5-azacytosine appeared on the chromatogram. The decomposition rate of I was very temperature dependent. For example, at pH 7.0, the decomposition rate was about sevenfold greater at 37 than at 24°.

A kinetic study at pH 8.1 and 9.5 was then carried out with II as the starting substance. According to Scheme I, the pseudo-first-order rate constants k_1, k_{-1}, and k_2 were determined using the following differential

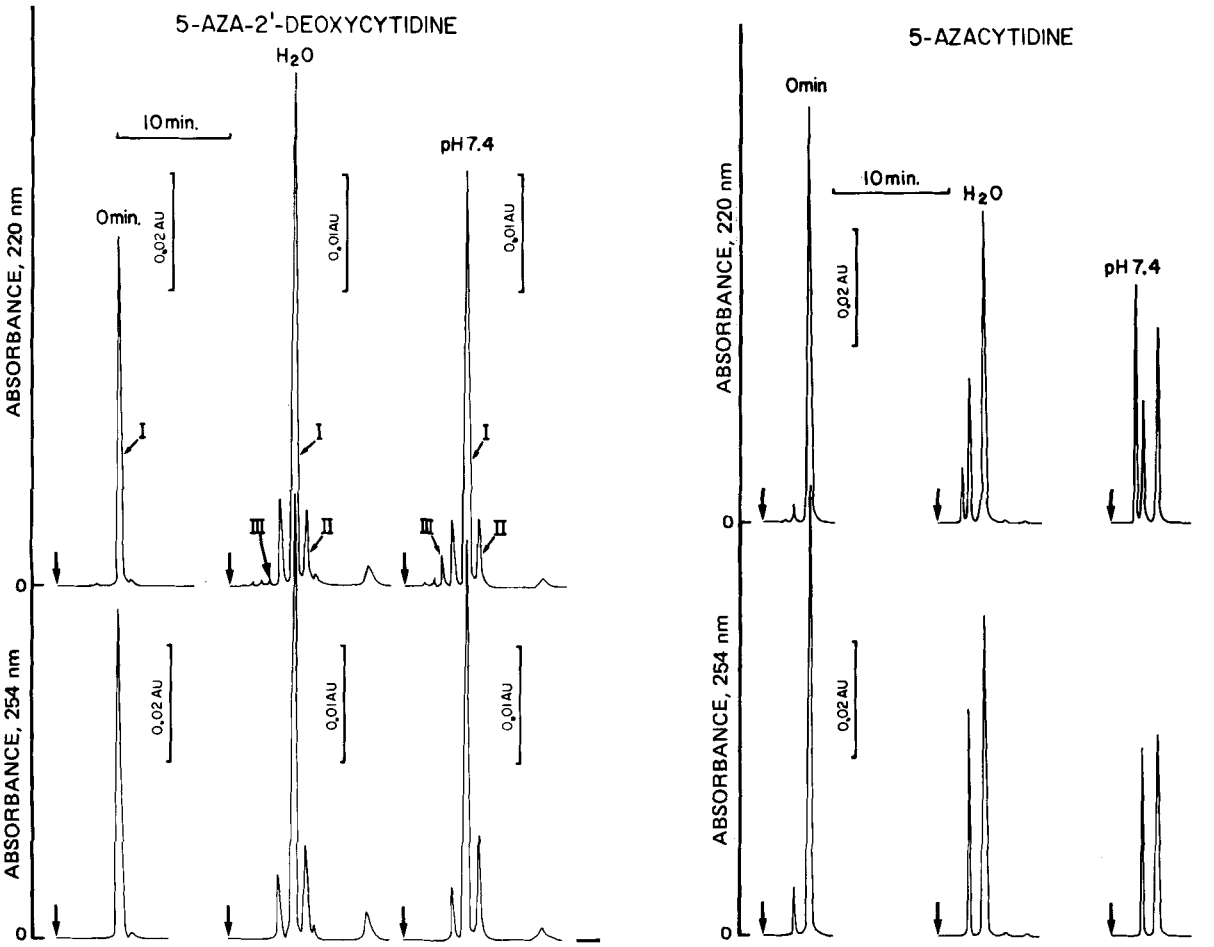

Figure 3—*Decomposition of I and 5-azacytidine in pH 7.4 phosphate buffer and water. 5-Azacytidine (0.5 mM) and I (0.5 mM) were dissolved in 10 mM potassium phosphate (pH 7.4) and water and incubated at 24°. At the indicated times, aliquots of these solutions were analyzed by HPLC at 254 and 220 nm.*

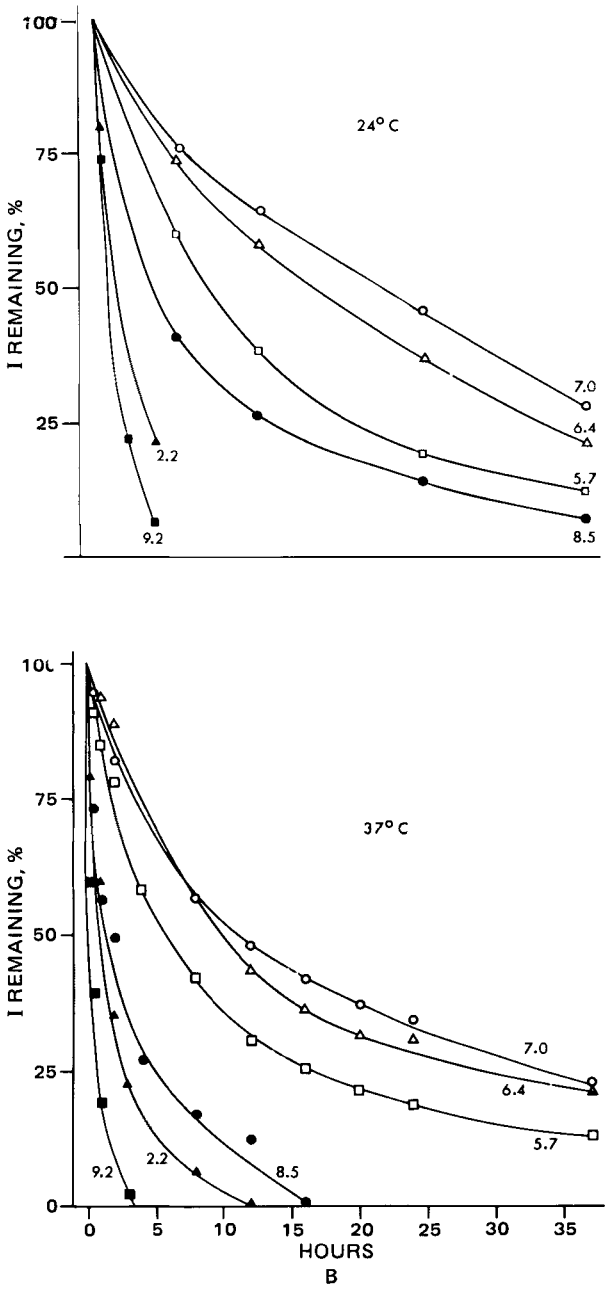

Figure 4—*Effect of pH and temperature on the stability of I. Compound I (1.0 mM) was dissolved in 10 mM of the appropriate buffer with the indicated pH and incubated at either 24° (top) or 37° (bottom). At the indicated times, an aliquot of the solution was neutralized with phosphate buffer and analyzed by HPLC.*

equations (8):

$$I_t = k_{-1} II_0 \left(\frac{1}{B-A} e^{-At} + \frac{1}{A-B} e^{-Bt} \right) \quad \text{(Eq. 1)}$$

$$II_t = II_0 \left(\frac{k_1 - A}{B-A} e^{-At} + \frac{k_1 - B}{A-B} e^{-Bt} \right) \quad \text{(Eq. 2)}$$

$$III_t = II_0 \left(1 - \frac{k_2(k_1 - A)}{A(B-A)} e^{-At} - \frac{k_2(k_1 - B)}{B(A-B)} e^{-Bt} \right) \quad \text{(Eq. 3)}$$

Here A and B are roots of the following quadratic equation taken with the reverse signs:

$$X^2 + X(k_1 + k_2 + k_{-1}) + k_1 k_2 = 0 \quad \text{(Eq. 4)}$$

The rate constants thus obtained are presented in Table I and were used to generate time–concentration profiles for I–III. Normalized experimental data obtained by HPLC were superimposed on these generated time–concentration profiles (Fig. 5). Similar procedures were used for the kinetic study of 5-azacytidine, and the results are shown in Table I and Figs. 6 and 7.

Table I—Rate Constants for the Alkaline Decomposition of I and 5-Azacytidine at 37°

Compound and pH[a]	Pseudo-First-Order Rate Constant, min^{-1}		
	k_1	k_2	k_{-1}
5-Azacytidine, 9.5	2.0×10^{-1}	2.2×10^{-1}	6.0×10^{-1}
5-Azacytidine, 8.1	1.7×10^{-2}	2.0×10^{-2}	5.6×10^{-2}
I, 9.5	2.2×10^{-1}	2.8×10^{-1}	7.5×10^{-1}

[a] The pH value was determined at 37° in a separate tube by mixing one volume of 0.5 *M* stock sodium borate buffer with 10 volumes of 10 m*M* potassium phosphate (pH 6.8).

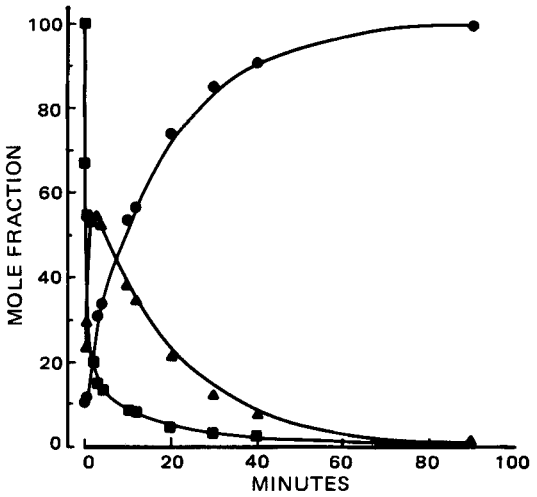

Figure 5—*Time–concentration profile of decomposition for I at pH 9.5 and 37°. Key: ▲, normalized data for I; ■, normalized data for II; and ●, normalized data for III. Solid lines were generated by computer for ▲, ■, and ● using rate constants determined independently.*

DISCUSSION

The hydrolysis of 5-azacytidine in alkaline solution and water results in the opening of the triazine ring between C-6 and N-1 to form *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea in a reversible reaction; when this latter compound loses the formyl group, it irreversibly forms 1-β-D-ribofuranosyl-3-guanylurea (5, 6). By using quantum chemical cal-

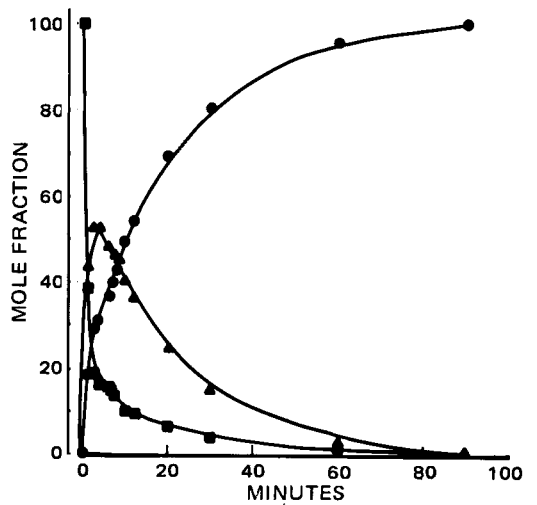

Figure 6—*Time–concentration profile of decomposition for 5-azacytidine at pH 9.5 and 37°. Key: ▲, normalized data for 5-azacytidine; ■, normalized data for N-(formylamidino)-N'-β-D-ribofuranosylurea; and ● normalized data for 1-β-D-ribofuranosyl-3-guanylurea. Solid lines were generated by computer for ▲, ■, and ● using rate constants determined independently.*

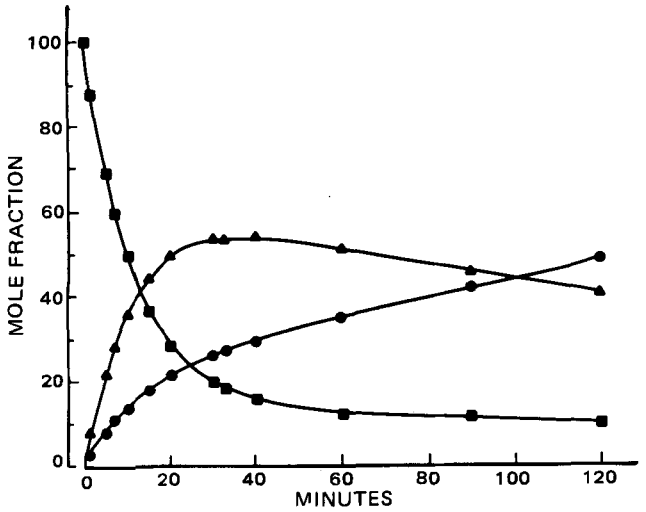

Figure 7—*Time–concentration profile of decomposition for 5-azacytidine at pH 8.1 and 37°. Key: ▲, normalized data for 5-azacytidine; ■, normalized data for* N-*(formylamidino)-*N′-β-D-*ribofuranosylurea; and ●, normalized data for 1-β-D-ribofuranosyl-3-guanylurea. Solid lines were generated by computer for ▲, ■, and ● using rate constants determined independently.*

culations, it was shown (5) that the electron density at C-6 of 5-azacytosine is much lower than cytosine, making this position more susceptible to nucleophilic attack by a hydroxyl ion.

From the structural similarity of 5-azacytidine and I, it would seem that I would decompose in alkaline solution according to Scheme I. This study supports this reaction scheme in alkaline solution. As shown in Fig. 1, the decomposition of peak I (t_R = 5.5 min) at pH 10.4 produced peak II (t_R = 6.6 min) when the column eluate was monitored at 254 nm. Peak III was not observed on this chromatogram because guanylurea derivatives are nonchromophoric at 254 nm (5, 6).

However, at 220 nm, peak III (t_R = 3.5 min) became apparent following the decomposition of II to III. When peak II was isolated, it had the same UV_{max} of 238 nm as reported previously (6) for *N*-(formylamidino)-*N*′-β-ribofuranosylurea. The hydrolysis of I in alkaline solution produced an initial increase in absorbance[5] at 238 nm, supporting the formation of II, since the formylguanylurea derivative has a much higher extinction coefficient than 5-azacytosine derivatives at this wavelength (6). The conversion of II to I (Fig. 2A) indicates that this reaction is reversible, which is in agreement with other observations (5, 6) for 5-azacytidine.

There were marked differences in the decomposition of I and 5-azacytidine in phosphate buffer (pH 7.4) and water (Fig. 3). In these solvents, 5-azacytidine decomposed to form formylguanylurea and guanylurea derivatives, as reported previously (6). However, the decomposition of I in phosphate buffer or water was very different from that of 5-azacytidine as shown by the presence of five (254 nm) and seven (220 nm) peaks on the chromatograms of I. Two of these peaks represent I and II, whereas the other peaks were not identified. These unidentified peaks could also be produced rapidly by placing II at pH 3.2 (Fig. 2B), even at 0°. These unidentified compounds were not the intermediates that eventually formed III, since they were not converted to III when placed in alkaline buffer, as would happen to I and II under the same conditions.

Determination of the pseudo-first-order rate constants for I and 5-azacytidine at pH 9.5 and 37° demonstrated that both compounds decomposed at comparable rates, although the formylguanylurea derivative for ribose appeared to be slightly more stable than that of deoxyribose (Table I). A 22-fold decrease in the hydroxyl-ion concentration (pH 9.5–8.1) reduced all three rate constants for 5-azacytidine equally by about 11-fold. Lowering the pH from 9.5 to 8.1 also greatly reduced the conversion rate of II to I and to III (Figs. 5 and 8). The kinetic data obtained for I at pH 8.1 did not quite fit in Eqs. 1–3 because apparently some of the decomposition did not follow Scheme I precisely due to the formation of minor unidentified peaks. Thus the rate constants were not determined. These unidentified peaks became increasingly prominent as the pH was lowered, and there was virtually no conversion of II to I at pH <4.6. (Fig. 2B).

[5] R. L. Momparler, unpublished data.

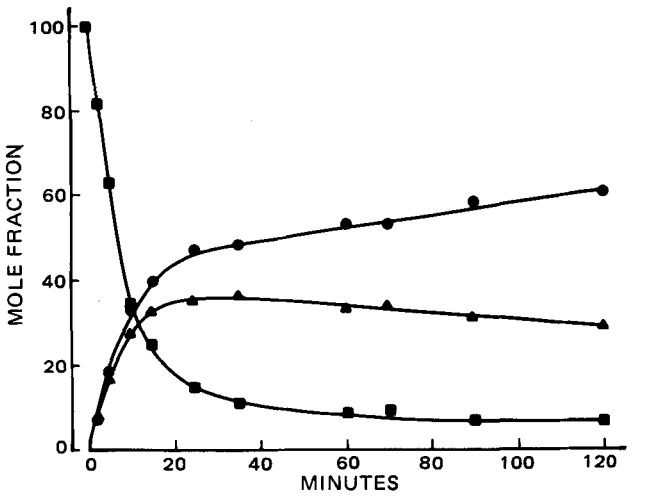

Figure 8—*Time–concentration profile of decomposition for I at pH 8.1 and 37°. Key: ▲, normalized data for I; ■, normalized data for II; and ●, normalized data for III.*

Comparison of the overall stability profile of I in aqueous buffer solution at various pH levels showed that I was most stable at neutral pH and at low temperature (Fig. 4). The instability of I at high pH could be explained on the basis that the opening of the triazine ring (k_1) and the breakage of an amide bond (k_2) is facilitated by a hydroxyl ion. Reduction in pH would slow down both these events and thus stabilize I. However, I became increasing unstable as the pH was gradually reduced below 7.0. This result might be due to the increase in the rate of ring opening as well as to the instability of II in acidic solutions; thus, once ring opening took place and produced II, the latter broke down to the unidentified peaks more rapidly and minimized the reversal back to I (Fig. 2B). In strong acidic solution (with pH <2.2), breakage of the glycolytic bond of I occurred, producing 5-azacytosine[6] as reported previously for I (9) and for 5-azacytidine (5, 10).

The effect of pH and temperature on the chemical stability of 5-azacytidine was studied by Chan *et al.* (11), who observed that this compound is most stable at pH 7.0 and that its rate of decomposition increases in solutions of high or low pH and with temperature.

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[6] Unpublished observations.