Comparative Kinetics of Cytosine Nucleosides. Influence of a 6-Methyl Substituent on Degradation Rates and Pathways in Aqueous Buffers†

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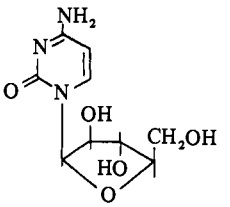
The kinetics of hydrolytic deamination of cytosine has been compared to that of 6-methylcytosine in aqueous acetate buffers at elevated temperatures. As predicted from previously proposed mechanisms, the 6-methyl derivative is 2–5 times more stable to deamination in the absence of buffer catalytic effects (extrapolated values) at pH 3.6 and 4.7, 80 and 90°. Also, as expected, 6-methylcytosine is significantly more stable to general base catalysis. Unlike the simple comparison of the cytosine bases, substituted nucleosides (1-β-D-ribofuranosyl-6-methylcytosine and previously unreported 1-β-D-arabinofuranosyl-6-methylcytosine) were found to undergo reaction pathways that were not observed for their unsubstituted parent compounds which are shown to undergo hydrolytic deamination under identical reaction conditions. Spectrophotometric determinations of pK_a values of the substrates and their reaction products indicate that the 6-methyl nucleosides undergo hydrolysis to yield their corresponding sugars and 6-methylcytosine which then continues to deaminate to 6-methyluracil. *N*-Alkyl cleavage of the pyrimidine–sugar bond has not been previously observed for nucleosides with fully hydroxylated sugars. Possible reasons for the increased rate of this cleavage, which is even faster than 2′-deoxy nucleosides previously reported, are discussed.

1-β-D-Arabinofuranosylcytosine, **1**, is a cytotoxic agent which exerts antitumor and antiviral activity in a variety of



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animal and human neoplasms. It is the most active drug available clinically for the treatment of acute myelogenous leukemia in man.[1,2] The greatest beneficial response in a single disease was observed in acute granulocytic leukemia where 35 of 144 adequately treated patients (or 24%) achieved complete or partial remissions[2,3] and more recently, 21 out of 49 patients (43%) attained complete remission status through rapid drug injection followed by continuous drug infusion to maintain steady-state therapy for 4 hr per day.[4]

This somewhat encouraging remission rate is even more astonishing when one considers the brief duration of bioavailability of this drug. When **1** is injected intravenously in human subjects the resulting blood level data exhibit biphasic first-order plots[5a] that are characteristic of what is called a two-compartment open model in pharmacokinetic analyses.‡ The initial loss from the blood following rapid intravenous injection is extremely fast, has a $t_{1/2}$ of approximately 12 min,[5a–7] and may be attributed to simultaneous distribution and elimination. At the end of the initial phase, over 80% of the drug remaining in blood and urine is in the form of a single metabolite, 1-β-D-arabinofuranosyluracil (**2**), indicating both rapid and extensive deamination during the distribution phase.[5a] The elimination phase (or β phase) has a half-life of approximately 111 min.[5a] Recovery of the metabolite, **2**, in the urine was 86–96% of the total 48-hr recovery in one study[7] and 90% of the total 24-hr recovery (representing 80% of the total dose) in another.[5a] Uptake of **1** by red blood cells is rapid and within 5 min cells attained 60% of the concentration of the plasma.[5a] However the half-life within the cell is only 2–3 min. Thus **1** exhibits a relative short duration of bioavailability and its loss is almost entirely due to deamination to **2** which is apparently inactive as an inhibitor of cell growth.[8]

It is reasonable to expect that an increase in the biological duration of **1** might lead to increased remission rates. Evidence for this hypothesis already exists in the work by Baguley and Falkenhaug[9] who measured plasma $t_{1/2}$ of **1** following 30-min intravenous infusions in leukemic patients. Patients who experienced complete remissions had significantly higher $t_{1/2}$ values than those who did not respond to therapy and this variation was presumably due to variations in deaminase activity. These results suggest that degradation of **1** may cause failure of the drug to produce hematologic remission in certain patients.[9] Further support for this proposal may be taken from the demonstration that the dosage regimen markedly effects its therapeutic index.[4,10,11] Since deamination limits the bioavailability of **1**, we have initiated investigations into methods for decreasing the propensity of cytosines to deamination. This goal is being pursued by synthesizing analogs designed to be more deaminase-stable and determining their relative chemical and enzymatic stability. The ultimate aim of this research is to design derivatives of **1** which will behave as (a) prodrugs, (b) new drugs, or (c) deaminase inhibitors. The latter approach has been examined by other workers,[12,13] but may be least desirable since complete inhibition of deaminase may increase 1-(2′-deoxy-β-D-ribofuranosyl)cytosine plasma levels and reverse the effect of the drug.[8] However one prodrug, 1-β-D-arabinofuranosylcytosine 5′-adamantoate, has been shown to be more active than the parent compound (administered as a single dose or a short course of daily doses) in L1210 leukemic mice where the duration of cytotoxic plasma levels of **1** was greatly increased by administration of the prodrug.[14]

It is well known that cytosine nucleosides undergo hydrolytic deamination to their corresponding uracils and that this may be catalyzed enzymatically[13,15] or chemically.[16–21] The reaction may be represented by Scheme I. However, with the exception of our initial studies,[16,17,19] there are no

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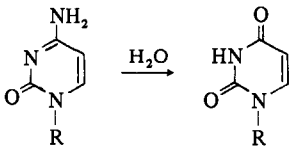
‡For an introduction to the terminology and the principles involved in compartmental analysis see Notari.[5b]

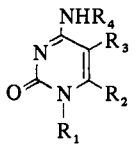
Scheme I



1, R = arabinosyl 2, R = arabinosyl
3, R = ribosyl 4, R = ribosyl
5, R = H 6, R = H

data available showing the effects of substituent groups on the chemical stability of cytosines to hydrolytic deamination and no data examining the possibility that chemical reactivity might be related to the enzyme-catalyzed transformations. Our investigations are designed to provide a systematic study of substituent group effects on the chemical hydrolytic deamination of cytosines and to apply these findings to enzymatic studies with the ultimate goal of extending the biological duration of **1** through one of the three mechanisms listed above.

There are four positions on the cytosine molecule which may be considered for substituent changes without destroying the pyrimidine ring.§ The effect of the sugar (R_1) on deamination has previously been studied by comparing the



7, R_1 = arabinosyl; R_2 = CH_3; R_3 = R_4 = H
8, R_1 = ribosyl; R_2 = CH_3; R_3 = R_4 = H
9, R_1 = H; R_2 = CH_3; R_3 = R_4 = H

deamination kinetics of **1**, **3**, and **5**.[16] Intramolecular general base attack by the 2'-hydroxyl group in **1** to form a 6,2' cyclic intermediate[17] resulted in a 50- to 100-fold increase in deamination rate over **3** and **5**, respectively. This intramolecular catalysis appeared to take the place of general base intermolecular catalysis by buffer in the pH region 3-8 since both **3** and **5** were subject to general acid and general base catalysis whereas general base catalysis for **1** was not significant under similar conditions.[16] Thus, similar mechanisms have been proposed for all three substrates, the only difference being the source of the general base for attack at the C-6 position. Further support for a common deamination pathway is evidenced by the nucleophilic addition of HSO_3^- to C-6 of **1**,[19] as well as to **5**,[20,21] **3**, and 1-(2'-deoxy-β-D-ribofuranosyl)cytosine.[20] Since addition at the C-6 position is involved in the chemical catalysis of deamination, appropriate substitution at that position should hinder catalysis and thereby decrease the deamination rate. In order to test this hypothesis three compounds were synthesized, and the kinetics of their transformations in aqueous buffer were compared to those of the unsubstituted compounds. The derivatives are 1-β-D-arabinofuranosyl-6-methylcytosine (previously unreported) (**7**), 1-β-D-ribofuranosyl-6-methylcytosine (**8**), and 6-methylcytosine (**9**), and the results of the comparisons are reported in this paper.

Experimental Section

Synthesis of 6-Methyl Derivatives. 6-Methylcytosine (**9**) was prepared in 80% yield from 6-methyl-4-methylthio-2-pyrimidone according to the method of Winkley and Robins:[23] mp 365-367° (lit. mp 361-363°).

1-β-D-**Ribofuranosyl**-6-methylcytosine (**8**) was prepared according to Winkley and Robins[23] affording white crystals: mp 230° dec (lit.[23] mp 230-232°).

1-β-D-**Arabinofuranosyl**-6-methylcytosine (**7**). Nucleoside **8** (0.45 g, 1.8×10^{-3} mole) was heated in 10 g of polyphosphoric acid at 80° for 40 hr. To the dark brown homogeneous mixture was added 20 ml of H_2O; this was heated at 100° for 1 hr. After cooling, LiOH solution (10%) was added dropwise to adjust the pH to 9. The precipitate was removed by filtration. To the clear filtrate was added 2 g of $MgCl_2$, 2 ml of 30% NH_4Cl solution, and concentrated NH_4OH to pH 9.5. The precipitate was removed by filtration and 80 mg of crude alkali phosphatase# was added to the filtrate. The solution was incubated under toluene at 37° for 24 hr. The solution was filtered, and the filtrate was evaporated to a small volume to remove excess ammonia. After dilution to 40 ml with H_2O, the solution was placed on Dowex 50W-X8 (H^+, 50-100 mesh, J. T. Baker Chemical Co., Phillipsburg, N. J.) column. The column was washed with 150 ml of H_2O and then with 1 *N* NH_4OH (100 ml). The fractions containing the pentofuranosyl derivatives of cytosine were evaporated to dryness to remove the ammonia; yellow crystals formed. The residual crystals were dissolved in 3.5 ml of 30% MeOH and applied to a Dowex 1-X2 (OH^-, 50-100 mesh)** column previously equilibratedwith 30% MeOH. Upon elution with 30% MeOH (200 ml) followed by evaporation of the eluate to dryness, 80 mg (18%) of white crystals, mp 200-201° dec, was obtained. These crystals of **7** were recrystallized from ethanol with no increase in mp. *Anal.* ($C_{10}H_{15}N_3O_5$) C, H, N.

Comparison of Cytosine and 6-Methylcytosine Deamination Rates. The hydrolytic deamination of **5** to **6** and of **9** to 6-methyluracil (**10**) was studied under pseudo-first-order conditions in the presence of excess buffer at 80 and 90° with constant ionic strength. Experimental details are given in Tables I and II. Reaction solutions were sealed in ampoules, placed in constant-temperature baths, removed, and cooled as a function of time. Aliquots of the reaction were diluted 1:10 with 0.1 *N* HCl, and the uv absorption spectra (Cary Model 15) and/or the absorbance at several wavelengths (Beckman Model DU) was determined.

Equations for assaying **5** in the presence of **6** in 0.1 *N* HCl have been previously developed and tested.[16] Beer's law plots were prepared at 260 and 280 nm using authentic samples of **9** and **10** in 0.1 *N* HCl, and the resultant absorptivities were used to derive similar equations for assay of the 6-methyl derivatives. Equations were tested by analyzing mixtures representing those encountered in a typical reaction. Concentrations were calculated from eq 1 for

$$10^4[C] = 1.37A_{275} - 0.625A_{259} \quad (1)$$

$$10^4[6\text{-MeC}] = 0.981A_{280} - 0.217A_{260} \quad (2)$$

5 and eq 2 for **9**, where *A* is the absorbance at the particular wavelength indicated (in nm).

Kinetics of Deamination of 1-β-D-Ribofuranosylcytosine and 1-β-D-Arabinofuranosylcytosine. Hydrolytic deamination of **3** to **4** and **1** to **2** was studied in the same manner described above for cytosine. Development of the assay for **1** in 0.1 *N* HCl has been previously described[16] and a similar analysis was developed for **3** using the procedure outlined above for **9** and employing authentic samples of **3** and **4**. Concentrations were calculated from eq 3 for **3** and eq 4 for **1** where *A* is absorbance as previously defined. Ex-

$$10^4[\text{Cyd}] = 0.943A_{280} - 0.334A_{260} \quad (3)$$

$$10^4[\text{Ara-C}] = 0.927A_{280} - 0.389A_{260} \quad (4)$$

perimental conditions for **3** are given in Table III and those for **1** are similar.[17]

Kinetics of Spectral Transformations of 1-β-D-Ribofuranosyl-6-methylcytosine and 1-β-D-Arabinofuranosyl-6-methylcytosine. Slower reacting mixtures containing **7** or **8** were sealed in ampoules and treated in the manner described above. Those reactions that were relatively fast (complete in <12 hr) were conducted in glass-stoppered volumetric flasks. These compounds did not give spectrophotometric changes characteristic of deamination to their corresponding uracil products. In both cases a rapid initial decrease in

§ Panzica, *et al.*,[22] have determined that 1-β-D-arabinofuranosylcytosine 3-*N*-oxide is a potential inhibitor of lymphoid leukemia L1210 but no studies on deamination rates of the 3-*N*-oxide have been reported to date.

#Alkali phosphatase activity = 2.6 units/mg. Purchased from Worthington Biochemical Corp., Freehold, N. J.

**Dowex 1-X2 (Cl^-, 50-100 mesh) was converted to the OH form by treating with 1 *N* NaOH (Bio-Rad Laboratories, Richmond, Calif.).

Table I. Experimental Conditions and Apparent First-Order Rate Constants[a] for the Deamination of Cytosine to Uracil

				10^4k, hr^{-1}			
				80°		90°	
pH	[AcOH], *M*	[AcONa], *M*	[NaCl], *M*	Range	Mean	Range	Mean
3.63	0.80	0.080	0.28	11.1 ± 1.0	11.1	31.0 ± 2.6	31.0
	0.48	0.048	0.31	8.91 ± 0.52	8.88	27.2 ± 2.2	25.0
	0.10	0.010	0.35	6.82 ± 0.36	6.90	19.6 ± 1.5	19.5
4.74	0.36	0.36	0.00	12.6 ± 1.1	12.7	29.7 ± 1.5	30.0
	0.18	0.18	0.18	9.45 ± 0.12	9.36	20.2 ± 1.3	20.1
	0.06	0.06	0.30	6.14 ± 0.90	6.14	13.5 ± 0.50	13.5

[a] In each case three values were calculated for the rate constants using: eq 5 with concentration data and eq 6 with absorbance data at 280 and 290 nm.

Table II. Experimental Conditions and Apparent First-Order Rate Constants[a] for the Deamination of 6-Methylcytosine to 6-Methyluracil

				10^4k, hr^{-1}	
pH	[AcOH]	[AcONa]	[NaCl]	80°	90°
3.63	0.80	0.08	0.28	4.92; 4.35	15.1
	0.50	0.05	0.31	2.58	
	0.48	0.048	0.31	2.41	6.68
	0.10	0.01	0.35	1.69; 1.52	4.65
4.74	0.36	0.36	0.00	4.19; 4.52	12.3
	0.18	0.18	0.18	3.19	7.89
	0.10	0.10	0.26	2.69	
	0.06	0.06	0.30	2.48	5.33
	0.05	0.05	0.31	2.37	

[a] Most values are means of constants determined by three methods using: eq 5 with concentration data, and eq 6 with absorbance data at 280 and 290 nm.

Table III. Experimental Conditions and Apparent First-Order Rate Constants for Deamination of 1-β-D-Ribofuranosylcytosine to 1-β-D-Ribofuranosyluracil

	[AcOH],	[AcONa],	[NaCl],	10^4k, hr^{-1}	
pH	*M*	*M*	*M*	70°	80°
3.63	3.15	0.315	0.00	21.0	
	1.80	0.18	0.14	16.8	
	1.35	0.135	0.18		44.0[a]
	0.90	0.09	0.23	13.3	
	0.80	0.08	0.28	13.4	35.8
	0.50	0.05	0.31	11.5	32.2
	0.45	0.045	0.27		31.2[a]
	0.27	0.027	0.29		29.3[a]
	0.10	0.01	0.35	9.55	25.8
	0.09	0.009	0.31	10.5	24.1[a]
4.74	0.36	0.36	0.00	8.01; 7.67[a]	22.4
	0.315	0.315	0.00		18.0[a]
	0.10	0.10	0.26	3.97; 3.90[a]	10.2
	0.09	0.09	0.23		9.92[a]
	0.05	0.05	0.31	3.59; 3.28[a]	9.08
	0.045	0.045	0.27		8.17[a]
	0.005	0.005	0.36	2.66[a]	
	0.0045	0.0045	0.31		6.45[a]

[a] Taken from ref 16.

Table IV. Experimental Conditions and Apparent First-Order Rate Constants for Loss of 1-β-D-Ribofuranosyl-6-methylcytosine Based on Decrease in Ultraviolet Absorption Spectra[a]

	[AcOH],	[AcONa],	[NaCl],	10^2k, hr^{-1}	
pH	*M*	*M*	*M*	80°	90°
3.63	0.80	0.08	0.28	6.52	20.3
	0.48	0.048	0.312	6.38	19.3
	0.10	0.01	0.35	4.58	13.5
4.74	0.36	0.36	0.00	2.10	7.41
	0.18	0.18	0.18	1.99	5.41
	0.06	0.06	0.30	1.77	5.11

[a] Calculated from first-order plots of initial decrease in the 290-nm absorption using eq 6 where the A_∞ value was determined by eq 13.

Table V. Experimental Conditions and Apparent First-Order Rate Constants for Loss of 1-β-D-Arabinofuranosyl-6-methylcytosine Based on Decrease in Ultraviolet Absorption Spectra[a]

	[AcOH],	[AcONa],	[NaCl],	k, hr^{-1}	
pH	*M*	*M*	*M*	80°	90°
3.63	0.80	0.08	0.28	0.94	2.05
	0.48	0.048	0.31	0.82	1.96
	0.10	0.01	0.35	0.78	1.89
4.74	0.36	0.36	0.00	0.51	1.16
	0.18	0.18	0.18	0.50	0.98
	0.06	0.06	0.30	0.34	0.78

[a] Calculated from first-order plots of initial decrease in 290-nm absorption based on eq 6 where the A_∞ value was taken to be the experimentally determined minimum value as described in the text.

the uv-absorbance spectra was observed without a concomitant increase in the 260-nm absorbance that is characteristic of uracil formation. Instead, the spectra rapidly decreased in a relatively uniform manner. The uv-absorbance data were therefore employed directly in the kinetic calculations since any analysis based on the assumption that reaction mixtures contained the starting materials plus their uracil derivatives would be inappropriate. Experimental conditions are found in Tables IV and V.

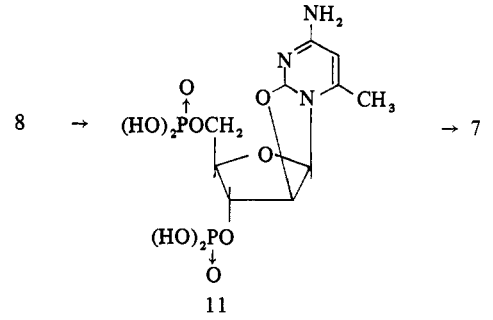
Spectrophotometric Determination of Cytosine Nucleoside pK_a Values. The pK_a of a protonated cytosine derivative in dilute solution can be conveniently determined from its change in uv absorbance as a function of pH. This method was applied to the compounds used in the kinetic studies. The method can be illustrated using 9.

A sample containing 7×10^{-4} *M* **9** in buffer (0.80 *M* AcOH–0.08 *M* AcONa–0.28 *M* NaCl) was diluted 1:10 with distilled water. The pH of the resulting solution was determined (Corning pH Meter, Model 12), and its uv spectrum was obtained (Cary Model 15). The pH of the solution was adjusted using 2 *N* HCl and 2 *N* NaOH in order to obtain a series of spectra for 9 ranging from its protonated form to its unprotonated form. The sample of highest pH was back titrated to a lower pH which had been obtained previously and the spectrum compared to that obtained earlier to show that no degradation had taken place during the titration.

Spectrophotometric pK_a of the 1-β-D-Ribofuranosyl-6-methylcytosine Reaction Mixture after 95 Hr. A solution containing 7×10^{-4} *M* **8** in aqueous buffer (0.80 *M* AcOH–0.08 *M* AcONa–0.28 *M* NaCl) was allowed to react at 90° for 95 hr in a sealed ampoule. The apparent pK_a of the resulting mixture was determined as described above.

Results

Synthesis of 6-Methyl Derivatives. Unknown 1-β-D-arabinofuranosyl-6-methylcytosine (**7**) was prepared from the known ribosyl nucleoside **8** according to a scheme previously described for the preparation of **1**.[23,24] Compounds **8** and **9** were prepared in several steps from **10** according to the published method.[23] Treatment of **8** (λ_{max} 280 nm, pH 1) in polyphosphoric acid at 80° for 40 hr afforded the intermediate phosphorylated 1-β-D-arabinofuranosyl-2,2′-anhydro-6-methylcytosine derivative (**11**). Intermediate **11** was not isolated but was detected in the ultraviolet by a hypsochromic shift from 280 to 260 nm at pH 1. Subse-



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quent hydrolysis with LiOH followed by dephosphorylation with alkali phosphatase afforded a mixture of 7, 8, and **10** which was separated according to the method of Roberts and Dekkar.[24]

Compound 7 was readily characterized by comparing its nmr spectrum to that of nucleoside 8 in D_2O. The H_5 proton resonance in both 7 and 8 appears as a quartet owing to long-range coupling with the 6-methyl group. $J_{CH_3\text{-}H_5} = 0.5$ cps is in agreement with reported methyl to ortho aromatic proton-proton coupling.[25] The chemical shift for the H_5 resonance signal downfield from HOD is 68.5 and 71.5 cps for 7 and 8, respectively. In both compounds 7 and 8 the H_1' proton resonance of the sugar portion appears as a doublet. For 7, the cis coupling constant, $J_{H_1'\text{-}H_2'} = 7.0$ cps; for 8, the trans coupling constant, $J_{H_1'\text{-}H_2'} = 4.0$ cps. However, the H_1' resonance signal, which in 8 appears upfield to H_5 by 10 cps, appears downfield to H_5 in 7 by 21 cps. In 8 the relationship between the 1′-hydrogen and 2′-hydroxyl is cis; the neighboring hydroxyl group is expected to exert a shielding effect on the H_1' resonance signal. A negative Fehling's test eliminated the possibility that 7 and 8 were *O*-glycosides rather than the desired *N*-glycosides.[23,26]

Comparison of Cytosine and 6-Methylcytosine Deamination Rates. Apparent first-order rate constants for deamination of **5** or **9** to their corresponding uracil derivatives were calculated in three ways. Data obtained using eq 1 and 2 were employed in plots based on eq 5, where X is the con-

$$\ln X = -kt + \ln X_0 \quad (5)$$

centration of reactant and $-k$ is the slope of the plot $\ln X$ *vs.* t.

The apparent first-order rate constants were also calculated from the absorbance at 280 and 290 nm using plots based on eq 6, where A_0 is the initial absorbance, A_t is the

$$\ln (A_t - A_\infty) = \ln (A_0 - A_\infty) - kt \quad (6)$$

$$A_\infty = A_0 \epsilon_u / \epsilon_c = A_0 f \quad (7)$$

absorbance at time t, and A_∞ is calculated from eq 7, where ϵ_u is the molar absorptivity of the uracil compound at the appropriate wavelength and ϵ_c is that of the cytosine compound. The value of f at 280 nm is 0.17 for both substrates in 0.1 *N* HCl and the value at 290 nm is approximately 0.02 so that A_∞ was regarded as zero in this case.

Good agreement was obtained between the values calculated by all three methods. This is illustrated in Table I where both the ranges and means are given for the rate constants calculated for cytosine deamination.

Spectrophotometric Determination of Cytosine Nucleoside pK_a Values. Absorbances at λ_{max} for the protonated species were plotted *vs.* pH for the compounds studied, where the absorbances were corrected for dilution brought about by the pH adjustment using eq 8. V is the volume

$$A_{corr} = A_{exp}(V/V_0) \quad (8)$$

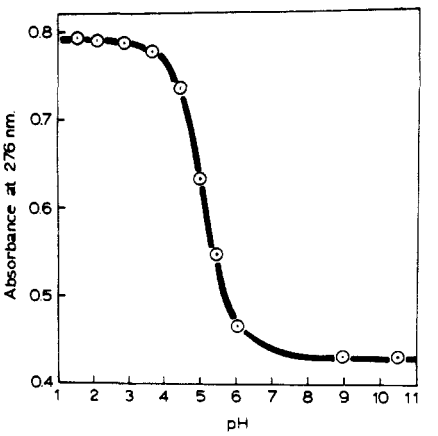


Figure 1. Absorbance at 276 nm *vs.* pH for a 6.5×10^{-5} *M* solution of 6-methylcytosine.

Table VI. Comparison of pK_a Values for Cytosines to Their 6-Methyl Derivatives

Parent compound	Unsubstituted	6-Methyl
Cytosine	4.45[b]	5.13[a]
1-β-D-Ribofuranosylcytosine	4.15[a,c]	4.42[a]
1-β-D-Arabinofuranosylcytosine	4.20[a,d]	4.61[a]
95-hr reaction mixture	5.05[a,e]	

[a] Determined spectrophotometrically in approximately 10^{-5} *M* solutions. [b] Taken from ref 27. [c] Literature values are 4.14[28] and 4.22.[27] [d] Taken from ref 17. [e] See Figure 3.

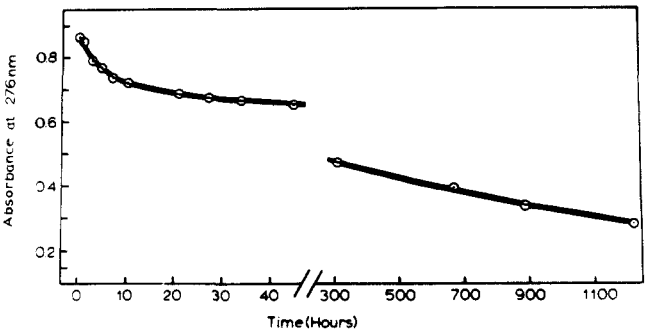


Figure 2. Decrease in 276-nm absorbance for 6.1×10^{-5} *M* 1-β-D-ribofuranosyl-6-methylcytosine at 90° in 0.80 *M* AcOH–0.08 *M* AcONa–0.28 *M* NaCl at pH 3.63. Two time scales are used to illustrate the biphasic curve for decrease of the uv chromophore.

after addition of acid or base, and V_0 is the original volume. A typical plot is shown in Figure 1. The pK_a was read from the midpoint of the curve and Table VI contains the values obtained.

Kinetics of Spectral Transformation of 1-β-D-Ribofuranosyl-6-methylcytosine. Reaction mixtures containing 8 exhibited biphasic curves when the absorbance values at a given wavelength (in the region 260–300 nm) were plotted as a function of time. A typical example is shown in Figure 2 where the absorbance at 276 nm is seen to decrease rapidly at first and then to decrease very slowly by comparison. No increase in 260-nm absorption, characteristic of the formation of 1-β-D-ribofuranosyl-6-methyluracil, was observed during the reaction.

The initial decrease in absorbance was accompanied by a change in the apparent pK_a of the reaction solution. The pK_a of 8 was compared to the apparent pK_a for a reaction mixture after 95 hr at 90° when the initial phase appeared to be complete. The data are shown in Figure 3 and the calculated pK_a values are listed in Table VI. The similarity

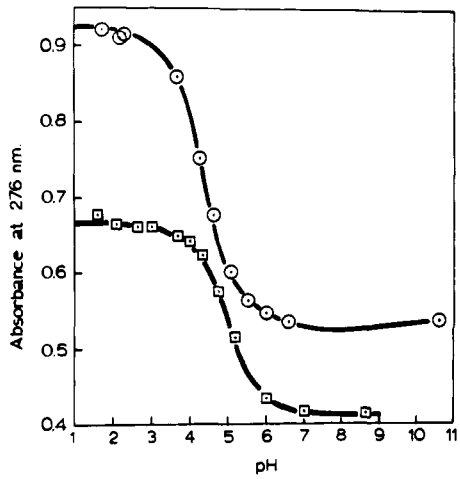


Figure 3. Absorbance at 276 nm *vs.* pH for 6.1×10^{-5} *M* 1-β-D-ribofuranosyl-6-methylcytosine before (⊙) and after (⊡) allowing it to react for 95 hr at 90° in 0.80 *M* AcOH–0.08 *M* AcONa–0.28 *M* NaCl at pH 3.63.

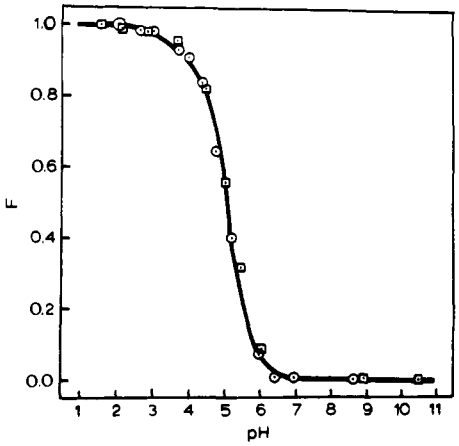


Figure 4. Plots of *F* (eq 9) *vs.* pH for reacted (see Figure 3) 1-β-D-ribofuranosyl-6-methylcytosine (⊙) and 6-methylcytosine (⊡).

of the apparent pK_a value of the 95-hr reaction mixture to that of an authentic sample of **9** suggested the possibility of *N*-alkyl cleavage to yield ribose and **9**. This hypothesis was further tested by comparing several spectral characteristics of the 95-hr reaction mixture to those obtained for an authentic sample of **9**. The calculated ratios A_{260}/A_{276}, A_{280}/A_{276}, and A_{290}/A_{276} for the reaction mixture were in reasonably good agreement with those of the standard. In order to compare the relative absorption values for each compound as a function of pH the fraction, *F*, was defined as

$$F = (A - A_{OH})/(A_H - A_{OH}) \quad (9)$$

where *A* is the absorbance at a given pH, A_{OH} is the absorbance at pH > 8, A_H is the absorbance at pH < 2 and the wavelength is constant at 276 nm. Figure 4 illustrates the results of plotting *F vs.* pH for the mixture and the standard, and the data are seen to be superimposable. Furthermore, the apparent molar absorptivity, ϵ_{app}, in 0.1 *N* HCl was calculated from eq 10, where A_t is the absorbance of

$$\epsilon_{app} = A_t/[6\text{-MeCyd}]_0 \quad (10)$$

the 95-hr reaction mixture at 275 nm and $[6\text{-MeCyd}]_0$ is the starting concentration of 8. The resulting value (11.1×10^3) is in good agreement with the value for **9**, 12.1×10^3. Thus the uv spectral characteristics and pK_a value of a 95-hr reaction mixture of 8 at 90° are in good agreement with those for **9**.

The biphasic curves for loss of **8** are characteristic of consecutive rate processes where the initial step is much faster than the subsequent step. Since the first step appears to be *N*-alkyl cleavage the consecutive rate processes may be written

$$8 \xrightarrow{k_1} 9 \xrightarrow{k_2} 10 \quad (11)$$

The yield of **9** was calculated using uv data after apparent completion of the initial phase and found to represent a quantitative conversion of **8** for each reaction studied. Apparent first-order rate constants for initial loss of uv absorbance of **8** at 276 nm were calculated from plots based on eq 6 where the value for A_∞ was calculated from eq 12

$$A_\infty = \epsilon[6\text{MeCyd}]_0 \quad (12)$$

and ϵ is the molar absorptivity of **9** in 0.1 *N* HCl; $[6\text{-MeCyd}]_0$ is defined as for eq 10. This approach was applied to absorbance values at several wavelengths in the region 260–290 nm with similar results. Rate constants are listed in Table IV.

If **9** (and ribose) is formed in a rapid initial step as shown in eq 11 then deamination of **9** to **10** would be expected to proceed as discussed in the previous section. The values for k_2 were calculated from plots based on eq 6, where A_∞ is defined as in eq 13, ϵ' is the absorptivity of **10** in 0.1 *N* HCl,

$$A_\infty = \epsilon'[6\text{-MeCyd}]_0 \quad (13)$$

and $[6\text{-MeCyd}]_0$ is defined as for eq 10. The values for k_2 at 90° calculated from such plots are 14.0×10^{-4} hr^{-1} (0.80 *M* AcOH–0.08 *M* AcONa–0.28 *M* NaCl) and 11.0×10^{-4} hr^{-1} (0.36 *M* AcOH–0.36 *M* AcONa).

Kinetics of Spectral Transformations of 1-β-D-Arabinofuranosyl-6-methylcytosine. Reaction mixtures containing **7** also exhibited a rapid initial decrease in the uv absorption spectra without evidence for formation of 1-β-D-arabinofuranosyl-6-methyluracil. Figure 5 illustrates the results of a typical experiment. Over the time interval illustrated, the absorbance appears to reach a minimum constant value. The rate constant for the disappearance of **7** was first calculated by using the experimental minimum as the A_∞ value for a log plot drawn from eq 6. This A_∞ value was altered somewhat, if necessary, to achieve a linear plot over at least 2–3 half-lives. The slope of the best plot was used to calculate the rate constant. Plots for data at 278 nm (λ_{max} for **7** in acid) yielded rate constants in good agreement with those using 290-nm data. The rate constants obtained are given in Table V.

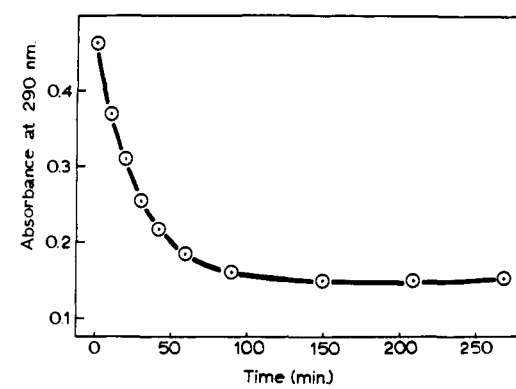


Figure 5. Decrease in absorbance at 290 nm for 6.3×10^{-5} *M* 1-β-D-arabinofuranosyl-6-methylcytosine at 90° in 0.80 *M* AcOH–0.08 *M* AcONa–0.28 *M* NaCl at pH 3.63.

General Acid-Base Catalysis. The apparent first-order rate constants, k, calculated in these studies may be defined *a priori* as

$$k = k_{HA}[AcOH] + k_{A^-}[AcO^-] + k_i \quad (14)$$

where [AcOH] is acetic acid concentration, [AcO^-] is acetate ion concentration, k_{HA} and k_{A^-} their catalytic constants, and k_i is the rate constant in the absence of buffer. The values of k_{HA} and k_{A^-} may be calculated from the slopes of either k *vs.* [AcO^-] or k *vs.* [AcOH] at two or more pH values by methods previously reported.[16] The intercept values of such plots represent k_i which might be defined (*a priori*) as

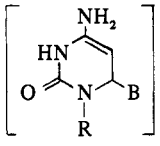
$$k_i = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_s \quad (15)$$

where k_{H^+} and k_{OH^-} are the catalytic constants for hydronium and hydroxyl ion and k_s is the solvolysis constant.††

Table VII gives the results of these calculations, and the intercept values, k_i, are given in Table VIII. These numbers were obtained by means of computerized linear regression analysis.

Discussion

We have previously demonstrated that the kinetics of deamination of **1** is influenced by the β 2′-hydroxyl function; the related ribosyl nucleoside **3**, having an α 2′-hydroxyl group, undergoes deamination at pH ~4, 70-80°, in the absence of buffer at a rate 40 times slower than **1**.[16,17] We have shown **1** to be subject to intramolecular general base type molecular catalysis by the 2′-hydroxyl group. Comparative kinetics studies reveal **1** is subject to intermolecular general acid catalysis whereas both general acid and general base catalysis are required in the deamination of **3** or **9** in the pH region 3-7. In the case of **1** intramolecular general base catalysis is thought to be provided by nucleophilic attack at the 6 position of the pyrimidine ring by the 2′-hydroxyl group, ultimately resulting in formation of biologically inactive **2**. Although there is a significant increase in deamination of **1** due to enhancement of the rate by the 2′-hydroxyl participation, there exists convincing indication that nucleophilic attack at the 6 position is a common step in the catalysis of hydrolytic deamination of cytosines in the pH region below 7.[16-21] A common intermediate in the catalyzed deamination of cytosines might be written



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where B represents the 2′-hydroxyl oxygen in the case of **1** or a general base (by intermolecular addition) in the absence of a 2′-hydroxyl cis to the pyrimidine ring. If this is the case, one would expect that the 6-methyl derivative would hinder nucleophilic attack by general base and result in increased stability to deamination. This appears to be valid for the cytosine base but the nucleosides undergo alternate reaction pathways as a result of 6-methyl substituents. The three cases studied are discussed separately for convenience.

Comparison of Cytosine and 6-Methylcytosine Deamination Kinetics. The hydrolytic deamination of **5** to **6** and **9** to **10** was studied in aqueous acetate buffers under pseudo-first-order conditions. Comparison of the apparent first-order rate constants for deamination at 80 and 90° (Tables I and II) shows that **9** is more stable to deamination than **5** by a factor of 2-4.5 times depending on the conditions chosen for the comparison. Figure 6 illustrates the dependence of the observed first-order rate constants on general acid-base catalysis by buffer. This increased resistance to buffer catalysis is in agreement with the *a priori* hypothesis that substituents in the 6 position would hinder the deamination mechanisms previously proposed.[16-21]

A comparison of the catalytic constants for AcOH and AcO^- is found in Table VII, and the intercept values, k_i, are found in Table VIII. As expected, the 6-methyl derivative is roughly 4-7 times more stable to general base catalysis. Determining the intercept values, k_i, on plots such as those shown in Figure 6 allows direct comparison of the

Table VII. Catalytic Constants

Compound	Temp, °C	$10^4 k_{HA}$, l. mole^{-1} hr^{-1}	$10^4 k_{A^-}$, l. mole^{-1} hr^{-1}
Cytosine	80	4.23	17.4
	90	12.1	42.9
6-Methylcytosine	80	4.01	2.34
	90	13.7	9.67
1-β-D-Ribofuranosyl-cytosine	70	2.41	12.1
	80	11.9	29.3
1-β-D-Arabinofurano-sylcytosine[a]	70	~930	~0
	80	~1500	~0

[a] Taken from ref 16.

Table VIII. Intercept Values ($k_i \times 10^4$ hr^{-1}) from Catalysis Plots

Compound	Temp, °C	pH	k_i
Cytosine	80	3.63	6.21
		4.74	5.08
	90	3.63	17.6
		4.74	10.2
6-Methylcytosine	80	3.63	0.945
		4.74	2.07
	90	3.63	2.08
		4.74	3.84
1-β-D-Ribofuranosylcytosine	70	3.63	9.92
		4.74	2.60
	80	3.63	24.2
		4.74	6.35
1-β-D-Ribofuranosyl-6-methyl-cytosine	80	3.63	452
		4.74	174
	90	3.63	1320
		4.74	439
1-β-D-Arabinofuranosylcytosine[a]	70	3.66	340
		4.71	100
	80	3.66	900
		4.72	180
1-β-D-Arabinofuranosyl-6-methylcytosine	80	3.63	743
		4.74	345
	90	3.63	1860
		4.74	724

[a] Taken from ref 17.

††In the case of 1 (and presumable other arabinosyl nucleosides) participation by the 2′-hydroxyl oxygen in acid pH makes the value for k_i more complex. For 1 it has been proposed that k_i in this region is better defined by the expression: $k_i = k_1k_3H^+/(k_2H^+ + k_3)\cdot(H^+ + K_2H^+ + K_1K_2)$ where K_1 is the dissociation constant for 1, K_2 is the equilibrium constant for decylization of the 6,2′ cyclic intermediate, and k_1, k_2, and k_3 are the rate constants for the steady-state water-adduct intermediate.[17] For the purpose of comparing relative reactivity, in the current paper, it is sufficient to consider k_i simply as the non-buffer-catalyzed rate constant and the possible complexity of its composition will be examined at a later date. In the case of 5 or 3 eq 15 must also be regarded as a first approximation since the observed rate constant appears to reach a maximum value when the substrate becomes completely protonated.[18]

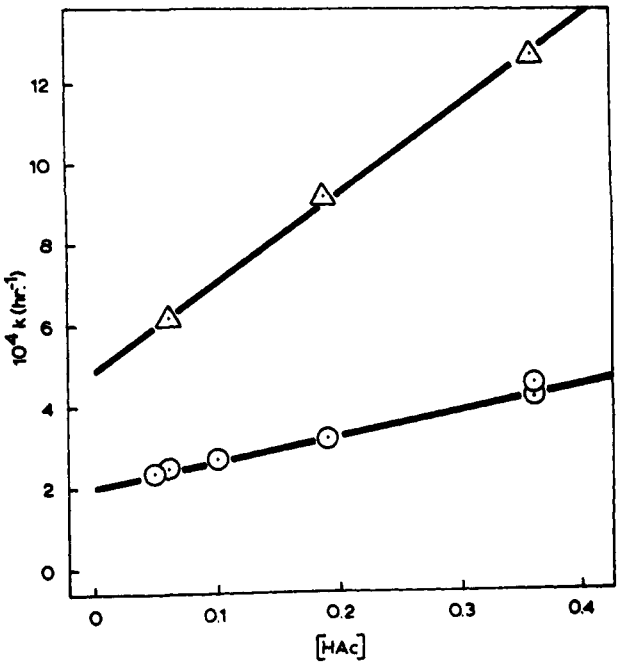


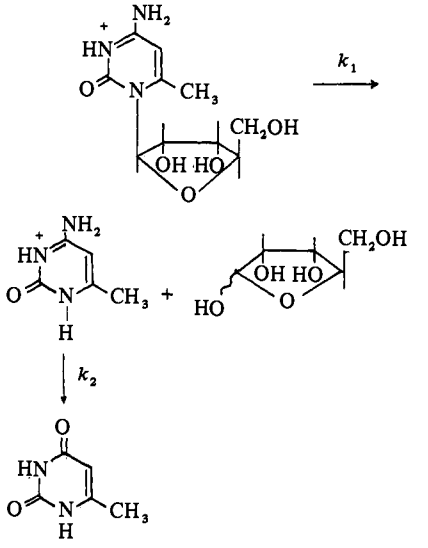
Figure 6. Apparent first-order rate constants for deamination of cytosine (△) and 6-methylcytosine (⊙) *vs.* AcOH concentration at 80°, pH 4.74.

non-buffer-catalyzed instability to deamination. Under the conditions of these studies, **9** is 2–5 times more stable in the absence of buffers at pH 3.6 and 4.7. It has been observed that deamination of **5**[18] and its nucleosides[16-19] increases with protonation of substrate reaching a maximum value at the pH of complete protonation. Since **9** has a pK_a value of 5.13 (Table VI) it would exist to greater extent in the protonated form at pH 3.6 and 4.7 than the unsubstituted **5** with a pK_a of 4.45. Thus the degree of stabilization by the 6-methyl substituent is actually greater than that observed here if one considers the fact that a larger fraction of the substrate is in the protonated or reactive form and this effect is offsetting the rate reduction by the substituent.

It would appear that the reaction route (**5** to **6**) has not been altered by methyl substitution at the 6 position and, as expected, there is a decrease in the rate of deamination in aqueous acetate buffers where both the buffer catalysis has been decreased and the rate in the absence of buffer has also been decreased.

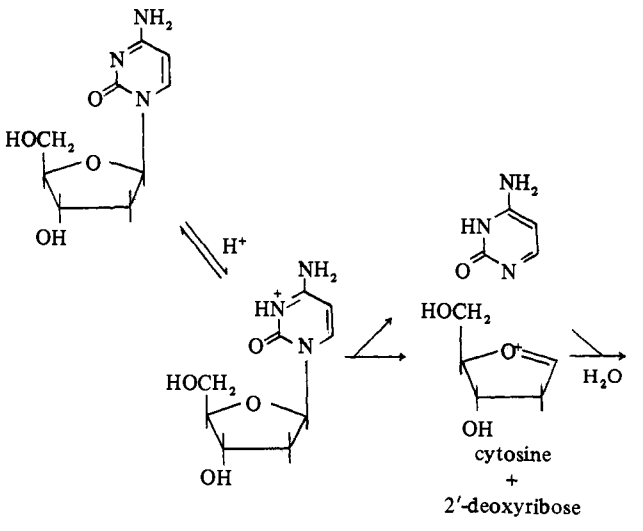
Comparison of Stability of 6-Methyl to Unsubstituted Nucleosides. Unlike the simple case of the cytosine and 6-methylcytosine bases, the nucleosides were found to undergo new reactions as a result of methyl substitution at the 6 position. These compounds may be more stable to deamination than the unsubstituted compounds but it has not been possible to assess this property since spectral changes indicate that a more rapid initial reaction occurs. A typical example of the biphasic decrease in uv absorbance with **8** is shown in Figure 2. The initial decrease in absorbance is not accompanied by any spectrophotometric changes that are characteristic of formation of a uracil derivative. There is a change in the apparent pK_a, determined spectrophotometrically, as illustrated in Figure 3. The apparent pK_a of the **8** reaction mixture at the end of the initial phase agrees with the observed value for **9** under similar conditions. The plots of data points for the mixture and the authentic sample are compared in Figure 4 where it can be observed that a single curve adequately described both sets of data. In addition, the observed uv spectra can be attributed to complete conversion to **9** without material loss to other products. Scheme II is thus compatible with these data. Values calculated for the apparent first-order rate constants associated with the second or slower phase were in good agreement with those for loss of **9** thus supporting the assignment of step 2 in Scheme II. This loss of the sugar moiety must be classed as an unexpected occurrence. In previous studies of nucleoside hydrolysis, Garrett[29] has shown that ribosyl, arabinosyl, and lyxofuranosyl uracils were stable to sugar loss in 1 *N* HCl at 80° regardless of the substituent in the 5 position, except in the case of 1-β-D-ribofuranosyl-5-hydroxyhydroxyuracil. Also, previous cytosine studies have not detected any hydrolysis of the pyrimidine-sugar bond with ribose or arabinose.[16-21] Nucleosides containing deoxy sugars, on the other hand, have been demonstrated to be labile in acid.[29-32]

Scheme II



The mechanism of sugar hydrolysis appears to involve protonation of the heterocycle followed by rate-limiting breakage of the *N*-glycosyl bond (illustrated for 1-(2′-deoxy-β-D-ribofuranosyl)cytosine in Scheme III).[30,31,33]

Scheme III

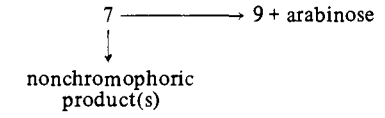


The retardation of hydrolysis noted in compounds containing the 2′-hydroxyl is postulated to be due mainly to an inductive, electron-withdrawing effect which inhibits the formation of the resonance-stabilized oxonium ion. Changes in the nature of the base can markedly effect the

hydrolysis. For example, Shapiro and Danzig[30] have shown that in acidic solution at 75°, 1-(2′-deoxy-β-D-ribofuranosyl)-5-bromocytosine hydrolyzes almost 100 times as rapidly as either 1-(2′-deoxy-β-D-ribofuranosyl)cytosine or 1-(2′-deoxy-β-D-ribofuranosyl)-5-methylcytosine. This rate enhancement is believed to arise from the electron-withdrawing effect of the bromine, which makes the base a better leaving group. However, no studies have been done with compounds substituted at the 6 position of the pyrimidine ring.

A priori, there is no reason to expect the substitution of methyl for hydrogen at carbon 6 to result in a much more reactive compound. While the electron-donating character of the methyl group would favor protonation of the base, this same electronic effect makes the base a somewhat less favorable leaving group, so the effects would tend to offset each other. In addition, it is highly unlikely that the increase in protonation, as reflected by the slight increase in the pK_a's of the 6-methyl derivatives over those of the unsubstituted nucleosides (Table VI), would be sufficient to completely alter the reaction pathway, greatly increasing the reactivity of the *N*-glycosyl linkage. Therefore, any major difference in reactivity between the unsubstituted and the 6-methyl compounds must result from steric factors. Indeed, models of the 6-methyl nucleosides indicate that the methyl group forces the sugar into a position where the 5′-hydroxyl can hydrogen bond with the 2-carbonyl. Such an interaction would tend to bring electrons out of the pyrimidine ring, which would help stabilize the oxonium ion, and could also help make the pyrimidine a better leaving group. The even more rapid loss of the 6-methylarabinosyl compound could then derive at least partly from the fact that the 2′-hydroxyl in the "up" position is also able to hydrogen bond with the carbonyl. However, the rapid initial loss of absorbance in the case of 7 cannot be explained simply in terms of hydrolysis to **9** and arabinose since this would account for only 38% of the initial substrate concentration. Examination of the uv characteristics of the reaction mixture at this point would indicate that other components in the mixture do not contribute significantly to the uv spectrum from 260 to 300 nm. Since the data approach a temporary state where the absorbance is relatively constant with time (Figure 5), it must be concluded that the initial step is complete and since the sugar-pyrimidine hydrolysis is not reversible, the substrate must be expended. This would indicate that loss of 7 is described by Scheme IV. The subsequent spectral transformations are

Scheme IV



not defined at this time. Contrary to 8, the absorbance between 260 and 300 nm increases with time after completion of the first step, with the 260-nm increase being most pronounced. This increase either arises from decomposition of the nonchromophoric products shown in Scheme III or from an alternate reaction scheme that is different from IV but not apparent without isolation of products, which is not possible at this time due to a limited supply of substrate and which will therefore be the subject of a later paper.

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