Deamination of Cytidine by Bisulfite: Mechanism at Neutral pH

S. Slae and R. Shapiro*

Department of Chemistry, Graduate School of Arts and Sciences, New York University, New York, New York 10003

Received March 17, 1978

The kinetics of cytidine deamination in the presence of bisulfite were studied at elevated temperatures at pH 6.55 and 7.30. The reaction was found to be second order in bisulfite ion. The activation enthalpy and entropy were determined. General base catalysis was observed in the presence of added bases. A mechanism is suggested which describes the kinetic properties of this reaction. By extrapolation of our data, the rate of deamination of cytidine by 10^{-4} M bisulfite within a cell, at 37 °C, has been estimated at 4.0×10^{-12} s^{-1}.

The specific deamination of cytosine derivatives to those of uracil by bisulfite[1,2,3] (for example, I → VI, Scheme I) has been used as a synthetic method in nucleoside chemistry and as a means for the chemical modification of nucleic acids.[4] Treatment of viruses and bacteria by bisulfite induces mutations, which have been ascribed to cytosine deamination within DNA.[5]

In an earlier study, we described the principal mechanistic features of the reaction.[6] Our data were primarily collected in acidic solution, in which the optimal rate of deamination is observed. One unresolved point of uncertainty was the nature of the buffer catalysis observed in the deamination step (IV → V, Scheme I). In order to understand this step more fully, we have recently completed a study of the model compound, 1-methyl-5,6-dihydrocytosine.[7]

In our present work, we have conducted additional studies of the deamination of cytidine at neutral pH. With this data, and the conclusions from our model study, we can describe the deamination mechanism fully, and extrapolate the rate to physiological conditions. This information is necessary for an accurate estimate of the possible hazard of bisulfite as an environmental mutagen.[8]

Results

Kinetic Order of the Reaction. The kinetics of the bisulfite-catalyzed deamination of cytidine were measured at pH 6.55, μ = 3.0 and 75 °C under pseudo-first-order conditions at several bisulfite concentrations. The results shown in Table I conform to a rate equation second order in bisulfite ion.[9]

$$\frac{1}{[\mathrm{Sub}]}\frac{\mathrm{d[P]}}{dT} = k_{\mathrm{obsd}}^{\mathrm{SO_3}} = k'_{\mathrm{SO_3}}[\mathrm{HSO_3^-}]_{\mathrm{ST}}^2 \qquad (1)$$

where $k'_{\mathrm{SO_3}} = (2.08 \pm 0.1) \times 10^{-5}$ $\mathrm{mol^{-1}\ s^{-1}}$.

General Base Catalysis. When general-base catalysts were added to solutions of cytidine and bisulfite ion at pH 6.55 and 7.30, μ = 3.0, and 75 °C, an increase in the deamination rate was revealed, the overall reaction rate being described by the rate law

$$\frac{1}{[\mathrm{Sub}]}\frac{\mathrm{d[P]}}{dT} = k_{\mathrm{obsd}} = k'_{\mathrm{SO_3}}[\mathrm{HSO_3^-}]_{\mathrm{ST}}^2 + k'_{\mathrm{B}}[\mathrm{HSO_3^-}]_{\mathrm{ST}}[\mathrm{B}]_{\mathrm{ST}} \qquad (2)$$

When this increase in observed reaction rate,

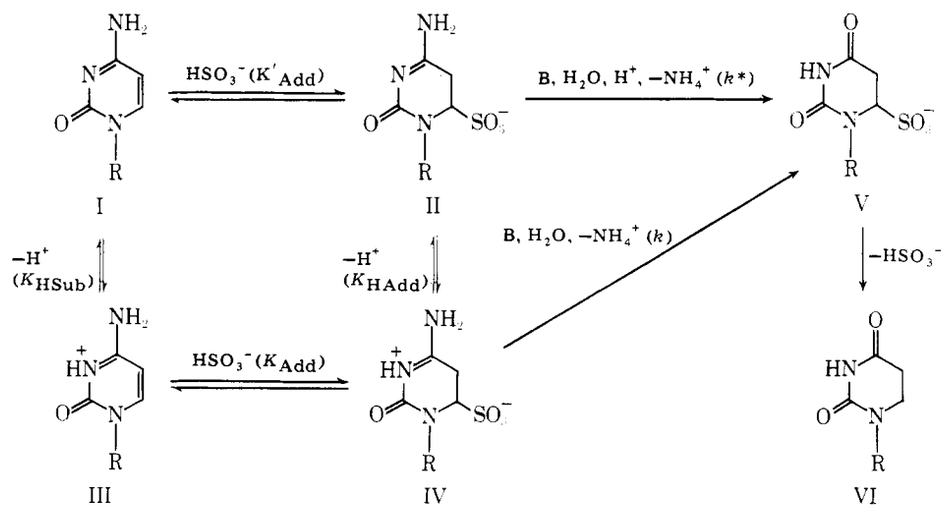
$$\left(\frac{1}{[\mathrm{Sub}]}\frac{\mathrm{d[P]}}{dT} - k'_{\mathrm{SO_3}}[\mathrm{HSO_3^-}]_{\mathrm{ST}}^2\right)/[\mathrm{HSO_3^-}]_{\mathrm{ST}}$$

was combined with the known stoichiometric concentration of base, the catalytic effect of each general-base catalyst, k'_{B}, was obtained.

In solutions of biphosphate and bisulfite ions, the observed "biphosphate" or "sulfite" catalysis is due to the presence of two catalytic species, $H_2PO_4^-$ and HPO_4^{2-}, or HSO_3^- and SO_3^{2-}. An adjustment can be made to the observed rate constants k_{B}, for the minor catalytic effects of $H_2PO_4^-$ and HSO_3^-, so that a good estimate of the k's for HPO_4^{2-} and SO_3^{2-} alone, $(k'_{\mathrm{B}})_{\mathrm{ADJ}}$, can be made.[10] A further correction for the actual concentration of free base present under experimental conditions[11] is made in Table II, where the resulting values of k'_{COR} are compiled.

Activation Parameters. Rate constants for bisulfite-catalyzed deamination of cytidine were measured at elevated temperatures at pH 6.55 and 7.30, and the results are summarized in Table III. The activation parameters derived from

Scheme I. Mechanism of Bisulfite-Catalyzed Deamination of Cytidine



R = β-D-ribofuranosyl

Table I. Observed Catalytic Coefficients in Cytidine Deamination in the Presence of Bisulfite[c]

$[HSO_3^-]_{ST}$	$[B]_{ST}$	$10^5 \times k_{obsd}$, s^{-1} [a]	$10^5 \times k'_B$, $M^{-2}\ s^{-1}$ [b]
		pH 6.55	
0.75		1.03 ± 0.01	
0.93		1.79 ± 0.03	
1.0		2.08 ± 0.02	
0.75	0.56 M imidazole	1.40 ± 0.02	0.66 ± 0.01
1.0	0.25 M $H_2PO_4^-$	2.55 ± 0.02	1.88 ± 0.02
1.0	0.50 M $H_2PO_4^-$	3.15 ± 0.17	2.14 ± 0.10
		pH 7.30	
1.0		0.098 ± 0.01	
1.0	0.40 M $CH_3CO_2^-$	0.141 ± 0.006	0.108 ± 0.006
1.0	0.50 M imidazole	0.134 ± 0.005	0.072 ± 0.005
1.0	0.13 M HPO_4^{2-}	0.109 ± 0.005	0.086 ± 0.06

[a] Estimated uncertainties in values of k are the standard deviations. [b] $k'_B = (k_{obsd} - k_{obsd}^{SO_3})/([HSO_3^-]_{ST}[B]_{ST})$ as explained in the text. [c] μ = 3.0, 75 °C.

this data were: pH 6.55, $\Delta H^{\ddagger}$ = 4.1 ± 0.7 kcal/mol, $\Delta S^{\ddagger}$ = −69 ± 2 eu; pH 7.30, $\Delta H^{\ddagger}$ = 7.0 ± 0.5 kcal/mol, $\Delta S^{\ddagger}$ = −66 ± 2 eu.

Discussion

pH Dependence of Reaction Rates. The cytidine deamination reaction in the presence of bisulfite was shown previously to proceed through either a protonated (IV) or neutral (II) intermediate (see Scheme I), depending on the experimental conditions.[6] In the first case, a rapid equilibration between the species I, II, III, and IV is followed by the rate-determining reaction of the protonated intermediate, IV → V. In the second case, the rate-determining reaction of the intermediate, II → V, follows its rapid preequilibrium formation, I → II. Since either step IV → V or II → V may be catalyzed by a base such as sulfite ion, the observation of second-order rate dependence on the stoichiometric bisulfite concentration as well as general base catalysis is consistent with either case. The lack of nucleophilic catalysis is demonstrated by the small ratio of catalytic coefficients for imidazole compared to monohydrogen phosphate ion,[19] $(k/q)_{im}/(k/q)_{HPO_4^{2-}}$ = 3.42 at pH 6.55.

The observed rate constant for this reaction in the presence of bisulfite alone, $k_{obsd}^{SO_3}$, as well as that for the increase in observed rate constant due to the presence of additional base catalysts, k_{obsd}^{B}, exhibit a calculable dependence on pH. The results of these calculations are compared with experimental measurements in Table IV (see the Supplementary Material for the derivation of these calculations). Under the experimental conditions described here, the mechanism involving the protonated intermediate (IV → V) accurately predicts the pH dependence of $k_{obsd}^{SO_3}$ and k_{obsd}^{B}, while the alternative mechanism (I → II → V) is inconsistent with these results.

Activation Parameters. The enthalpies and entropies of activation measured for the bisulfite-catalyzed cytidine deamination differ significantly from those determined for the deamination of 1-methyl-5,6-dehydrocytosine, MDC[7] ($\Delta H^{\ddagger}$ = 20.7 ± 0.8 kcal/mol, $\Delta S^{\ddagger}$ = −11.0 ± 2.6 eu for the reaction in acidic media). The mechanism proposed here for cytidine deamination can account for these differences.

In the bisulfite-catalyzed cytidine deamination, intermediate IV is formed in a rapid preequilibrium, and it is this species that is the equivalent of the protonated MDC, the reacting species under the conditions studied for MDC (pH 0.4). In the mechanism proposed for cytidine deamination, where IV → V is the slow step, the overall activation enthalpy (and entropy) is the sum of the enthalpies (and entropies) for steps I → II (equilibrium), III → IV (equilibrium), and IV → V (kinetic). Based on earlier measurements[6] of the temperature dependence of the cytidine sulfonate adduct formation, an approximate estimate of $\Delta H°$ = −9.3 ± 4 kcal/mol, and $\Delta S°$ = −60 ± 20 eu can be made for transformation III → IV. The transformation equivalent to I → III for cytidine[18] has $\Delta H°$ = −4.4 kcal/mol and $\Delta S°$ = 5.0 eu. Therefore for the step IV → V, the estimated parameters are $\Delta H^{\ddagger}$ = 18 ± 5 kcal/mol and $\Delta S^{\ddagger}$ = −14 ± 25 eu. When MDC deamination is compared with the kinetically equivalent step of cytidine deamination, the activation parameters are similar.

Brønsted Relationship. The relative rate constants of the general bases compared to sulfite ion can be shown to be equal to the equation

$$\frac{k_{obsd}^{B}/([HSO_3^-]_{ST}[B]_{ST})}{k_{obsd}^{SO_3}/[HSO_3^-]_{ST}^2} = \frac{k_B}{k_{SO_3}}\left(\frac{K_{HB}}{[H^+] + K_{HB}}\right)\left(\frac{[H^+] + K_{HSO_3}}{K_{HSO_3}}\right) \quad (3)$$

The relative catalytic coefficients for the rate-determining step of this mechanism, k_B/k_{SO_3}, can be calculated from the relative observed rate constants (see the Supplementary Material for the derivation). Values so obtained are presented in Table II. The Brønsted relationship[21] for the relative rate constants of the rate-determining step at pH 6.55, μ = 3.0 and 75 °C yields[22]

$$\log (k/q) = (0.35 \pm 0.07)[(pK_a + \log (p/q)] - (8.59 \pm 0.47) \quad (4)$$

Studies of the general-base-catalyzed deamination of 1-methyl-5,6-dihydrocytosine[7] (37 °C, μ = 1.0), which proceeds through a similar rate-determining step, yielded a β of 0.19 ± 0.03 for a series of bases including structural types similar to those used in the present study. The agreement between the values of β is good, considering the differences in experi-

Table II. General-Base Catalysis in Cytidine Deamination in the Presence of 1 M Bisulfite Ion[e]

base	pK_a [a]	$10^6 \times k'_B$, $M^{-1}\ s^{-1}$	$10^6 \times (k'_B)_{adj}$, $M^{-1}\ s^{-1}$ [b]	$K_{HB}/([H^+] + K_{HB})$	$10^6 \times (k'_B)_{cor}$, s^{-1}	$(k'_B)_{cor}$ [c]/$(k'_{SO_3^{2-}})_{cor}$
			pH 6.55			
SO_3^{2-}	6.37	20.8	15.6	0.60	26.0 ± 0.3	1.00
imid	7.12	6.61	6.1	0.21	31.5 ± 0.5	1.21 ± 0.06
HPO_4^{2-}	6.27	20.1	18.1	0.65	27.8 ± 1.8	1.07 ± 0.08
			pH 7.30			
SO_3^{2-}	6.37	0.98	0.93	0.90	1.01 ± 1	1.00
imid	7.12	0.72	0.72	0.60	1.20 ± 0.1	1.20 ± 0.10
HPO_4^{2-}	6.27	0.86	0.84	0.915	0.94 ± 0.7	0.94 ± 0.23
$CH_3CO_2^-$	4.76	1.08	1.08	0.997	1.08 ± 0.08	1.08 ± 0.09

[a] pK estimates for these experimental conditions based on extrapolations of the best literature values,[12-18] estimated error limits are ± 0.05. [b] Adjustment made for the catalytic contribution of conjugate acids, $H_2PO_4^-$ and HSO_3^-, as explained in the text. [c] $(k'_B)_{cor} = (k'_B)_{adj}([H^+] + K_{HB})/K_{HB}$. [d] Average of two measurements, see Table I. [e] μ = 3.0, 75 °C.

Table III. Temperature Dependence of Cytidine Deamination Rate Constants in the Presence of 1 M Bisulfite[a]

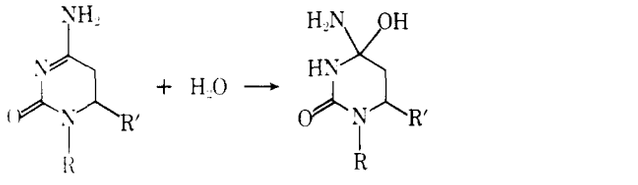
temp, °C	$10^5 \times k_{obsd}$, s^{-1} (pH 6.55)	$10^7 \times k_{obsd}$, s^{-1} (pH 7.30)
55.5	1.31 ± 0.07	5.29 ± 0.30
64.5	1.49 ± 0.04	6.55 ± 0.06
75.0	2.08 ± 0.09	9.80 ± 0.60
86.0	2.42 ± 0.22	14.1 ± 0.30

[a] μ = 3.0.

mental conditions and the uncertainties involved in the estimate of β for cytidine.

Reaction Mechanism. The mechanism presented in Scheme I is consistent with the kinetic order of the reaction, rate dependence on pH, general-base catalysis, absence of nucleophilic catalysis, lack of general-acid catalysis,[6] solvent isotope effect,[6] and activation parameters.

Although the details of transformation IV → V have not been examined, this process probably occurs through a tetrahedral intermediate (**2a**) similar to the mechanism proposed



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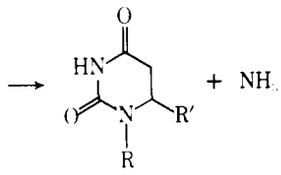

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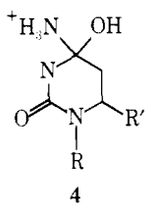
a, R = β-D-ribofuranosyl; R′ = SO_3^-
b, R = CH_3; R′ = H

for the deamination of 1-methyl-5,6-dihydrocytosine (**1b** → **2b** → **3b**) and similar amidines.[7] The rate-determining step of this mechanism is thought to be either the formation of intermediate **2**, or the reaction of the protonated form with which it is in equilibrium under the experimental conditions (**4**). These processes could occur through transition states VII



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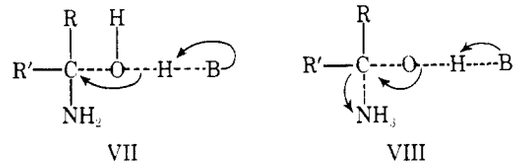
or VIII, respectively.



VII VIII

The Rate of Cytidine Deamination of Bisulfite under Physiological Conditions. It can be shown that in the presence of general base, the rate of cytidine deamination proceeding through intermediate IV can be approximately described by the following rate constant (eq 5)

Table IV. Dependence of Cytidine Deamination Rate Constants on pH in the Presence of 1 M Sulfite and Added Bases

	$[(k'_B)_{6.55}/(k'_B)_{7.30}]$[a]		
	experimental	calculated ratio[b,c]	
base	ratio	case 1	case 2
SO_3^{2-}	17.7 ± 1.1	15.1 ± 2.0	2.6 ± 0.3
imidazole	9.2 ± 0.04	7.7 ± 1.0	1.3 ± 0.2
HPO_4^{2-}	21.5 ± 7.5	16.8 ± 2.0	2.8 ± 0.3

[a] $k'_B = k_{obsd}^{SO_3}/[HSO_3^-]_{ST}^2$ for SO_3^{2-} and $k_{obsd}^B/[HSO_3^-]_{ST}[B]_{ST}$ for imidazole and HPO_4^{2-}. These are the apparent third-order rate constants corrected for pH to reflect true concentrations of reactive forms; 6.55 and 7.30 are the pH values for the respective determination of k'_{obsd}. [b] See Appendix in supplementary material for the basis of these calculations. [c] Case 1 and 2 as described in the text. [d] μ = 3.0, 75 °C.

$$k_{obsd} = [HSO_3^-]_{ST} k_{obsd}^{SO_3}([HSO_3^-]_{ST} + \sum_i [B_i]_{ST}) \quad (5)$$

(see the Supplementary Material for the derivation). Extrapolation from rate data at elevated temperatures yields

$$k_{obsd}^{SO_3} = 2.5 \times 10^{-7}\ mol^{-2}\ s^{-1}$$

for 37 °C, pH 7.30, μ = 3.0.

In order to estimate the rate of deamination within a living cell, we must introduce into eq 5 the total concentration of bases within the cell, $\sum_i [B_i]_{ST}$. This has been estimated as 0.16 M (μ = 0.16).[23-25]

Thus the physiological deamination rate of cytidine by bisulfite in vivo at 10^{-4} M bisulfite can be estimated at $4.0 \times 10^{-12}\ s^{-1}$, while at 10^{-6} M bisulfite it would be $4.0 \times 10^{-14}\ s^{-1}$. The significance of this data for questions of environmental mutagenesis will be discussed elsewhere.

Experimental Section

Materials and Apparatus. Schwartz Mann cytidine and commercially available reagent grade chemicals were used. Imidazole was recrystallized from benzene three times before use (mp 88.5–89.0 °C; lit. 90–91 °C). Water was doubly distilled in Pyrex vessels and deoxygenated with nitrogen. For reactions in the presence of sulfite ion, 10^{-3} M hydroquinine was added as a radical inhibitor.

Apparatus. Ultraviolet absorbance measurements were recorded on a Cary 15 recording spectrophotometer. pH measurements were made at various temperatures on a Radiometer Model 22 pH meter equipped with a glass electrode which had been precalibrated with pH standard solutions at the temperatures of measurement. pH values are ±0.02.

Kinetics of Cytidine Deamination. Ampules (2 mL) sealed with rubber septum caps were filled with reaction mixtures buffered to the desired pH at appropriate temperatures and kept in a constant temperature bath regulated to ±0.1 °C. Temperatures were measured with a thermometer calibrated by the National Bureau of Standards. Samples (50 μL) were removed with a syringe and stored in pH 11.5 buffer solution of 0.2 M Na_2HPO_4 for at least 24 h. The relative concentrations of the reactant (cytidine) and product (uridine) were calculated for each sample from the observed absorbances at 270 and 280 nm by means of the formulas, [Uridine] = $(A_{270} - 1.21A_{280})/3044$; [Cytidine] = $(2.39\ A_{280} - A_{270})/8805$. A plot of log χ_{Cyt} against time was analyzed by the method of least squares on a Hewlett Packard 3000 computer. Estimated errors in k are the standard deviations of the slopes for these kinetic plots. Catalytic coefficients of general bases were obtained by rate measurements in aqueous buffers kept at constant pH. Ionic strength was held constant by the addition of sodium chloride.

Acknowledgment. This research was supported by Grant ES 01033 from the National Institutes of Health, U.S. Public Health Service. R. Shapiro has been a Research Career Development Awardee (GM-50188) of the National Institutes of Health.

Supplementary Material Available: An Appendix containing

the derivations mentioned in the text (6 pages). Ordering information is given on any current masthead page.

Registry No.—Cytidine, 65-46-3; bisulfite, 15181-46-1.

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(9) Symbolism used in this paper includes the following: μ is the ionic strength (in molarity); k_{obsd} indicates the observed rate constant; ST indicates the stoichiometric concentration before correction for equilibration of the different species present; Sub indicates Substrate; "Bisulfite" describes the overall effects of a solution of bisulfite and sulfite ions without identifying the specific species causing that effect.
(10) Assuming that the catalytic properties of general bases are similar for the well-characterized deamination of 1-methyl-5,6-dihydrocytosine,[7] and the rate-determining step of cytidine deamination (See Reaction Mechanism), then $k_{SO_3} = 2k_{HSO_3}$; $k_{HPO_4^{2-}} = 5k_{H_2PO_4^-}$. Under the experimental conditions, the relative concentrations of the basic species present can be calculated to be: $[SO_3^{2-}]/[HSO_3^-] = 1.50$ (pH 6.55), 9.00 (pH 7.30); $[HPO_4^{2-}]/[H_2PO_4^-] = 1.86$ (pH 6.55), 10.8 (pH 7.30). The relative catalytic contribution of each species becomes $(k_{SO_3^{2-}}[SO_3^{2-}])/(k_{HSO_3^-}[HSO_3^-]) = 3.0$ (pH 6.55), 18 (pH 7.30); $(k_{HPO_4^{2-}}[HPO_4^{2-}])/(k_{H_2PO_4^-}[H_2PO_4]) = 9.3$ (pH 6.55), 54 (pH 7.30). Should the rate constant ratio $(k_{SO_3^{2-}}/k_{HSO_3^-})$ equal one (a lower limit), the relative catalytic contribution would become $(k_{SO_3^{2-}}[SO_3^{2-}])/(k_{HSO_3^-}[HSO_3^-]) = 1.50$ (pH 6.55), 9.0 (pH 7.30). The possible error introduced into the calculations by this estimate is not significant enough to modify the final conclusions.
(11) Based on best pK estimates vailable from the literature[12-18] for these conditions.
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Fluorinated Pyrimidine Nucleosides. 2.[1] Reaction of 2,2′-Anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine Hydrochloride with Nitrogen and Sulfur Nucleophiles

Alan F. Cook* and Michael J. Holman

Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

Received March 15, 1978

Reaction of 2,2′-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine hydrochloride (**1**, anhydro-ara-FC) with ammonia gave 1-β-D-arabinofuranosyl-2,4-diamino-5-fluoropyrimidinium chloride (**2**) by attack at C_2 of the pyrimidine ring. Reaction of **1** with methylamine gave the corresponding 2-methylamino derivative **3,** which was rapidly converted into the 2,4-bis(methylamino)arabinoside **4** by amine exchange at C_4. Treatment of **1** with ethylamine or *n*-propylamine similarly produced the corresponding 2,4-bis(alkylamino) derivatives **14** and **15.** Reaction of **1, 2,** or **4** with methylamine for a prolonged reaction period resulted in rearrangement with loss of the sugar moiety to produce 2-amino-5-fluoro-1-methyl-4-methyliminopyrimidine hydrohalide (**9**), the structure of which was confirmed by X-ray crystallography. The reaction of **1** with ^{15}N-enriched ammonia was examined; in addition to C_2 attack and amine exchange at C_4, evidence was found for incorporation of ^{15}N into the pyrimidine ring. Reaction of **1** with sodium hydrosulfide or hydrogen sulfide induced defluorination without cleavage of the anhydro bond to give 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (**21**); the oxazolidinethione **22** was also isolated as a byproduct. Treatment of the corresponding sulfur- and nitrogen-bridged analogues **23** and **26** with sodium hydrosulfide also produced the corresponding defluorinated anhydro nucleosides **25** and **27.**

2,2′-Anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine hydrochloride (**1,** anhydro-ara-FC; Scheme I), a compound first synthesized by Fox et al.,[2] has been shown by Burchenal et al.[3] to be a promising new agent for the treatment of acute myeloblastic leukemia. As part of a synthetic program in the area of fluorinated pyrimidine nucleosides, we have employed anhydro-ara-FC as starting material for the preparation of some 5-fluoropyrimidine nucleosides with potential antitumor activity. The reactions of nitrogen and sulfur nucleophiles form the basis for this report.

Reaction of anhydro-ara-FC (**1**) with methanolic ammonia yielded the highly crystalline 2,4-diamino-5-fluoropyrimidine arabinoside **2** by reaction at C_2 of the pyrimidine ring.[4] This reaction is to be expected since Doerr and Fox have previously shown that the corresponding unfluorinated analogue 1-β-D-arabinofuranosyl-2,4-diaminopyrimidinium chloride was produced by the reaction of 2,2′-anhydro-1-β-D-arabinofuranosylcytosine with ammonia.[5] Although difficulty was experienced by Doerr and Fox in the isolation of the unfluorinated analogue due to the hygroscopic nature of the salt, together with its propensity for recrystallization to the 2,2′-anhydro compound, the hydrochloride salt of **2,** in contrast, was found to be stable indefinitely at room temperature. In aqueous solution, **2** was found to be much less stable; storage of a solution for 4 days at room temperature resulted in almost complete conversion to arabinosyl-5-fluorocytosine. A small amount of a byproduct was isolated from the reaction of **1** with ammonia; this was identified as 2-amino-β-D-arabinofu-

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