of varying shapes is given roughly by eq 4 (from Figure 2) modified by the effects of the differing hydration of the zwitterions. This excludes finally the possibility of folded conformations of the α,ω-amino acids which juxtapose the charged ends of the chain. Thus the folded conformation of 6-aminohexanoic acid would have a shape very similar to that of II (Figure 4) and hence a dielectric increment of about 16, instead of 74.6 as observed. The data also show that substitution of the α-carbon atom by bridging methylene groups markedly affects the δ value, in contrast to the effects of alkyl substitution reported by Wyman.[14]

$$\delta = 0.8R_{max}^2 + 14 \quad (4)$$

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Deamination of Cytosine Derivatives by Bisulfite. Mechanism of the Reaction

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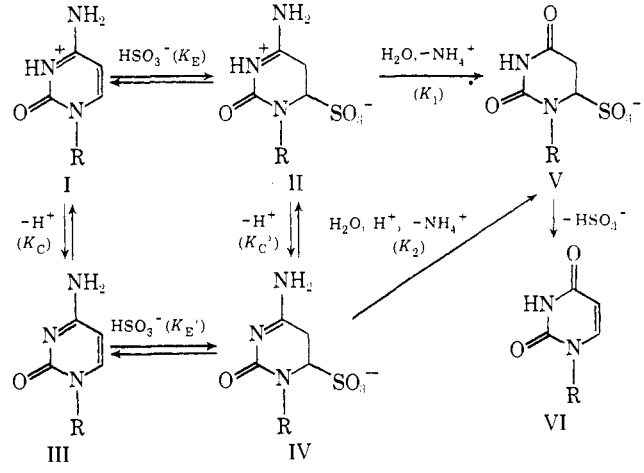
Abstract: The deamination of a cytosine derivative by sodium bisulfite involves the following steps: (1) addition of bisulfite to the 5–6 double bond of cytosine, (2) deamination of the resulting cytosine–bisulfite adduct to give a uracil–bisulfite adduct, and (3) reversal of the latter adduct to give a uracil derivative, by a subsequent alkaline treatment. The equilibrium of the addition reaction for cytidine has been determined at a number of pH values and bisulfite concentrations. The equilibrium is complex, as it involves protonated and unprotonated forms of both the starting cytosine derivative and the adduct. A mathematical expression has been derived to describe the equilibrium, which fits the experimental data well. The amount of adduct present at equilibrium falls as one proceeds from acidic to neutral solution, primarily because of the dissociation of bisulfite to sulfite. The kinetics of the deamination step have been studied with cytidine and deoxycytidine at various pH values and bisulfite concentrations. As a simpler model, the rate of hydrolysis of 1-methyl-5,6-dihydrocytosine has been examined in several buffers. The deamination of both the cytosine nucleosides and the model compound is subject to general base, or nucleophilic, catalysis. Although both protonated and neutral forms of 1-methyl-5,6-dihydrocytosine deaminate, only the protonated form of the cytosine–bisulfite adduct is reactive. The optimal rate of deamination of cytidine or deoxycytidine is at pH 5, in a high bisulfite concentration. These conditions are recommended for the use of the reaction in synthesis, the modification of nucleic acids, and chemical mutagenesis.

The use of sodium bisulfite for the specific deamination of cytosine derivatives was first reported in 1970 by ourselves[2] and by Hayatsu and coworkers.[3] In the intervening few years, the procedure has been widely applied for the chemical modification of nucleic acids and their components.[4] Our initial prediction of the mutagenicity of sodium bisulfite has been confirmed in four microorganisms.[5] These findings have raised the possibility that bisulfite may be an environmental mutagen, as it is used as an additive to foods, beverages, and pharmaceuticals[6] and is also the aqueous form of the common atmospheric pollutant, sulfur dioxide.

An understanding of the mechanism of this reaction is obviously important in determining the best conditions for the synthetic and genetic use of bisulfite, and in evaluating the possible hazard it presents as an environmental mutagen. The separate steps involved (Scheme I) have been given in earlier papers,[2,3] and

Scheme I



represent a specific example of the hydrolytic deamina-

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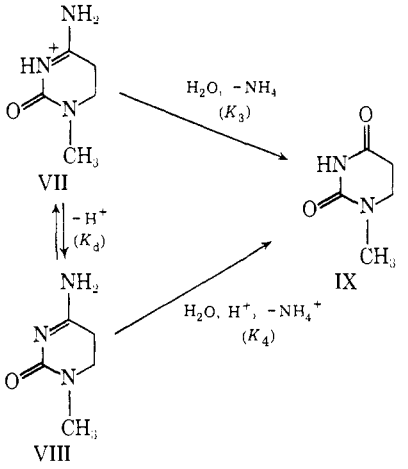
tion of cytidine derivatives, a general class of reaction originally described by us.[7] Additional data have been provided recently on specific features of the various steps,[8] but the mechanisms of the various steps have not been established in detail, nor has any full quantitative treatment of the data been attempted. Furthermore, an apparent contradiction exists between a claim that bisulfite ion catalyzes the deamination step (II → V, Scheme I)[8] and an earlier report that the rate of deamination of a related compound, the photohydrate of cytidine 3′-phosphate, is independent of the nature and concentration of buffer present.[9] In order to resolve this apparent contradiction, and to present a complete picture of the bisulfite-catalyzed reaction, we have conducted additional studies on cytidine, deoxycytidine, and on the model compound, 1-methyldihydrocytosine (VIII, Scheme II).

Scheme II



Experimental Section

Methods and Materials. 1-Methyl-5,6-dihydrocytosine was synthesized by the method of Cheng and Lewis.[10] Water used in kinetic runs was doubly distilled in Pyrex vessels under nitrogen. Measurements of pH were obtained with a Sargent Model DR pH meter equipped with a Thomas combination electrode. Nuclear magnetic resonance (nmr) spectra (100 MHz) were recorded on a Varian XL-100 nmr spectrometer. Ultraviolet absorbance measurements for kinetic experiments were recorded on a Beckman DU or Gilford 240 spectrophotometer. Sodium chloride was used to maintain the stated ionic strength in kinetic runs.

Kinetics of Deamination of 1-Methyl-5,6-dihydrocytosine. The general procedure of Brown and Hewlins[11] was followed. Aliquots of 100 μl of the reaction mixture (maintained at 30.0°) were withdrawn from time to time, and diluted into 10 ml of cold (0°) 0.2 *M* Tris buffer, pH 8.0. The uv absorbance was measured at 245 nm. The slope of the plot of ln $(A - A_\infty)$ *vs.* time was analyzed by the method of least squares on a Univac 1108 computer.

Equilibrium of the Reaction of Cytidine and Bisulfite. Method A. Reaction mixtures, 0.02 *M* in cytidine and 0.5 *M* in sodium bisulfite, were maintained at 24.0°. Aliquots of 100 μl were withdrawn from time to time and added to 10 ml of 0.2 *M* sodium citrate buffer, pH 3.1, at 0°. The absorbance at 280 nm of this solution was measured against a blank, lacking cytidine, treated in the same manner. A plot of ln *A* *vs.* time (ln $A_\infty = 0$) gave a straight line, after the first few minutes of the reaction (see Figure 1, for example). The linear portion of the curve was used to determine the rate of deamination (by the method described for 1-methyl-5,6-dihydrocytosine). The absorbance value obtained by extrapolation of the linear portion of the curve to zero was divided by the initial absorbance to give the fractional amount of cytidine-bisulfite adduct (II + IV, Scheme I) present at equilibrium.

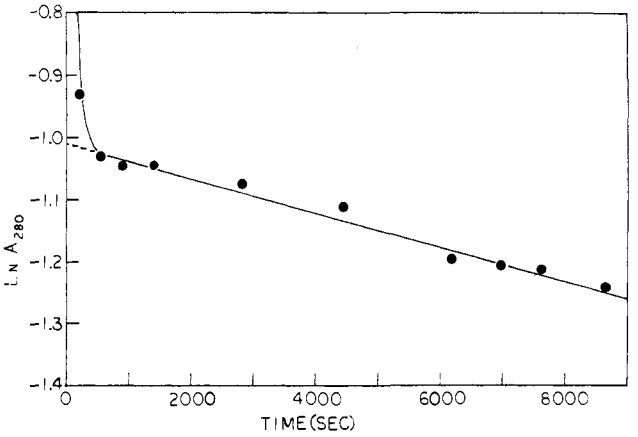


Figure 1. Plot of the change with time of the natural logarithm of the absorbance (280 nm) of aliquots (diluted 1:200) of a reaction mixture. The mixture was 2.6×10^{-2} *M* in cytidine and 0.5 *M* in sodium bisulfite, at pH 7, 24°, $\mu = 2$.

Method B. Solutions 0.01 *M* in cytidine and 0.45 *M* in sodium bisulfite were placed in ultraviolet cells of 1-mm path length within a Beckman DU spectrophotometer. The temperature within the cell compartment was maintained at 24, 37, or 50° using a Beckman thermospacer assembly and a Forma 2095 circulating bath. The absorbance of the reaction was followed at 264.6 nm (the isosbestic point of I and III, ε 8670), against a suitable blank lacking cytidine. In order to correct this reading for the presence of absorption due to IV, it was assumed that the extinction coefficient of IV at 264.6 nm was the same as that of the model compound VIII (ε 2790). The following equation was used to calculate the fractional amount, *X*, of I + III in the reaction mixture: $A = 8670X + 2790K_C' \cdot (1 - X)/(K_C' + [H^+])$, where K_C' represents the dissociation constant for protonated adduct II.

Deamination of Cytidine and Deoxycytidine by Bisulfite. Method A. This method is described in the section on the equilibrium of cytidine and bisulfite.

Method B. Reaction mixtures 0.01 *M* in cytidine and either 0.5 or 2.26 *M* in sodium bisulfite were maintained at the desired pH, or pD, and temperature. Aliquots of 100 μl were withdrawn periodically and added to 10 ml of 0.2 *M* sodium phosphate buffer, pH 11.5. The resulting mixture of cytidine and uridine, or deoxycytidine and deoxyuridine, was analyzed by a direct spectrophotometric method. The details of this method have been described.[12] The absorbance of each solution was determined at 270 and 280 nm against a suitable blank. The formulas used to compute the relative amounts of cytidine and uridine were as follows: [cytidine] $= (2.8A_{280} - A_{270})/10{,}200$ and [uridine] $= (A_{270} - 1.3A_{280})/3070$. In the deoxy series, the corresponding formulas were: [deoxycytidine] $= (3.1A_{280} - A_{270})/13{,}000$ and [deoxyuridine] $= (A_{270} - 1.26A_{280})/3520$. Pseudo-first-order rate constants were determined from these data by the method described above for 1-methyl-5,6-dihydrocytosine.

Determinations of p*K*. The p*K* values for the dissociation of sodium bisulfite was determined to be 6.48, $\mu = 1.0$, 25°, and 6.24, $\mu = 2.0$, 25°, by potentiometric titration. Titration of 2.26 *M* $NaHSO_3$ solution, 37°, gave p*K* = 6.2. The p*K* values for the cations of cytidine (I) and 1-methyldihydrocytosine (VII) were determined spectrophotometrically to be 4.24 and 6.68, respectively, at $\mu = 2.25$, 25°.

The p*K* of 5,6-dihydrocytidine-6-sulfonate cation (II) was determined in D_2O at $\mu = 2.0$ by nmr spectroscopy. A change in the chemical shift of H-5[2] with pD from τ 6.47 at pD 2.3 to 6.77 at pD 7.6 was observed. The pD values were obtained by adding 0.4 to the reading measured on a pH meter.[13] The pD value of the mid-

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point of the transition, 5.3 ± 0.2, was taken to be the same as the pK value. The same method was also applied to 1-methyldihydrocytosine cation. The chemical shift of the H-5 protons varied from τ 6.88 at pD 2.5 to 7.33 at pD 10.8; pK = 7.1. The change in pK associated with the shift from D_2O to H_2O was approximately −0.24 for 1-methyldihydrocytosine cation. The same correction applied to II afforded a value for pK_C' in H_2O of approximately 5.1.

Results

The Equilibrium of Addition of Bisulfite to Cytidine. The reaction of a cytosine derivative in acidic solution leads to an equilibrium involving four species: the protonated and unprotonated cytosine derivative (I and III) and the protonated and unprotonated bisulfite adducts (II and IV) (Scheme I). The relationship between the four species is governed by the dissociation constants of the protonated cytosine (K_C) and the protonated adduct (K_C') as well as the equilibrium constants for the addition of bisulfite to the protonated and unprotonated cytosine (K_E and K_E'). It can be shown readily that these constants are related as described in

$$K_E/K_E' = K_C/K_C' \quad (1)$$

The amount of each substance present under a given set of experimental conditions is determined by the above constants, the pH, and the concentration of bisulfite present in the solution. By manipulation of the equations used to derive the above constants, the following relationships can be derived.

$$\frac{[II] + [IV]}{[I] + [III]} = \frac{K_E([H^+] + K_C')[HSO_3^-]}{[H^+] + K_C} \quad (2)$$

The term on the left represents the ratio of adduct (protonated and unprotonated) to unreacted cytosine derivative (protonated and unprotonated) in the solution. The concentration of bisulfite ion is determined by the initial concentration of bisulfite introduced into the solution, Z, and the dissociation constant of bisulfite (to sulfite and a proton), K_S. By substituting these terms for the bisulfite term in (2), we derive

$$\frac{[II] + [IV]}{[I] + [III]} = \frac{K_E([H^+] + K_C')Z}{([H^+] + K_C)([H^+] + K_S)} \quad (3)$$

Equation 3 does not allow for the consumption of bisulfite in adduct formation, but this is very small in relation to the amounts of bisulfite used in our experiments.

The experimental determination of the amount of bisulfite adduct present under a given set of conditions is difficult. In our initial communication[2] data were obtained by taking the nmr spectra of reactions run in D_2O. Integration of the signals from the 5 and 6 protons of the cytosine derivative and its adduct yielded a rough estimation of the amount of adduct present. In our present study we sought a more accurate ultraviolet spectrophotometric method, applicable in H_2O.

In one approach, measurements were taken directly on the reaction mixture. Bisulfite ion has low ultraviolet absorbance. However, at concentrations above 0.1 M, it is partly converted to strongly absorbing pyrosulfite ion, which contributes considerably to the background.[14,15] Nevertheless, by the use of cells of short path length, we were able to work at bisulfite concentrations up to 0.45 M. The interpretation of the data offered further complexities. To overcome the difficulty introduced by the equilibrium between I and III, readings were taken at the isosbestic point of the two species, 264.6 nm. However, IV was also expected to have a moderate amount of absorbance at that wavelength (the model compound, VIII, Scheme II, absorbs at 264.6 nm) and it was necessary to apply a correction to allow for this factor (see the Experimental Section). In practice, this method was convenient for use at elevated temperatures, but of limited accuracy.

Analysis of the data was simplified when aliquots of the reaction mixture were transferred to acidic solution for analysis. Only I and II are present, and the latter does not absorb in the ultraviolet. The reversal of the reaction on dilution (II → I) was sufficiently slow so that it did not interfere, if readings were taken immediately after dilution. Hayatsu, *et al.*,[3] had diluted reaction aliquots into 0.1 N HCl. In our hands, this method proved to be inaccurate. Solutions containing bisulfite have strong ultraviolet absorbance at pH <3, because of the formation of free H_2SO_3.[14] Good results were obtained, however, when our aliquots were diluted into pH 3 buffer, at 0°. The data from a typical run are given in Figure 1. The initial rapid drop in absorbance of the reaction is due to the rapid formation of the equilibrium shown in Scheme I. The slower decline of A_{280} with time is caused by the deamination of the bisulfite adduct of cytidine to that of uridine, V, which causes further reaction of cytidine, to maintain the equilibrium. The rate of deamination of cytidine to uridine was calculated from the linear portion of the curve. Extrapolation of the line to zero time gave an absorbance reading which allowed the calculation of the extent of adduct formation.

The experimental data obtained were introduced into eq 3. In order to calculate the equilibrium constant, K_E, it was necessary to evaluate K_S, K_C, and K_C'. The first two constants were determined by direct titration: pK_C = 4.24 (K_C = 5.75 × 10^{-5}), pK_S = 6.24 (K_S = 5.75 × 10^{-7}) at 25°, μ = 2. An accurate direct method could not be applied to II, because of its tendency to reverse to I and to deaminate to V. An approximate pK_C' of 5.3 ± 0.2 in D_2O was obtained by studying the pH dependence of the nmr signal of H-5 of the cytidine–bisulfite adduct (II + IV). The model compound, VII (Scheme II), had an acidic dissociation constant, pK_d, 0.2 unit lower in H_2O than in D_2O. The value of pK_C' was thus estimated to be approximately 5.1, but with a considerable degree of uncertainty. In our calculations, we found that the value 4.7 for pK_C' (K_C' = 2.00 × 10^{-5}) gave a constant value 11.2 for K_E, and 4.7 was used in the calculations. We consider the agreement with the experimental determination to be reasonable.

The variation of the per cent of adduct (II + IV) formed with pH in 0.5 M $NaHSO_3$, 25°, is given in Figure 2. The agreement of the experimental data (points) with the values calculated from eq 3 (the curve) is excellent. A value of 11.2 l. mol^{-1} was calculated for K_E. By using eq 1, a value of 3.88 l. mol^{-1} was calculated for K_E'. The decline in the extent of adduct formation with increasing pH is primarily due to the conversion of bisulfite to sulfite.

(14) R. Golding, *J. Chem. Soc.*, 3711 (1960).
(15) E. Hayon, A. Treinin, and J. Wilf, *J. Amer. Chem. Soc.*, **94**, 47 (1972).

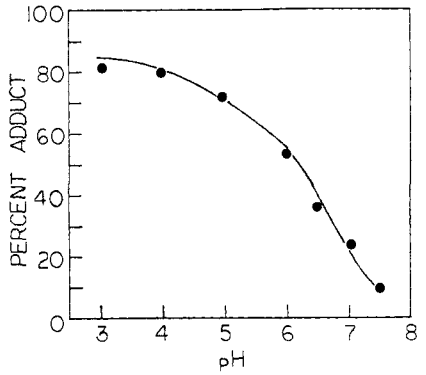


Figure 2. The pH dependence of the extent of bisulfite adduct formation of cytidine in 0.5 *M* sodium bisulfite, 24°, μ = 2.0. The points represent our experimental determinations. The curve was calculated from eq 3.

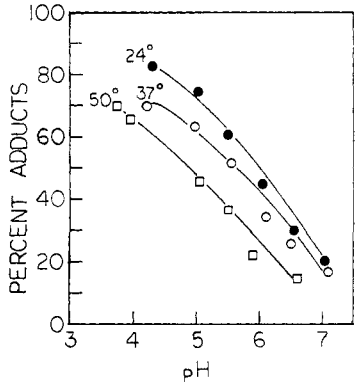


Figure 3. The pH dependence of the extent of bisulfite adduct formation of cytidine in 0.45 *M* sodium bisulfite at the indicated temperatures.

The variation of the extent of bisulfite adduct formation of cytidine with bisulfite concentration (pH 5.0, 25°, μ = 2.0) was also measured. The per cent adduct, 100(II + IV)/(I + II + III + IV), was 74.1 in 0.5 *M* $NaHSO_3$, 82.6 in 0.80 *M* $NaHSO_3$, 85.6 in 1.07 *M* $NaHSO_3$, and 89.7 in 1.60 *M* $NaHSO_3$. From these data, values for K_E and K_E' of 13.7 and 4.74 l. mol^{-1}, respectively, were calculated. These values agree reasonably well with the numbers derived from the pH profile.

The effect of temperature on the equilibrium was examined, using the method of direct ultraviolet readings in short pathlength cells discussed above. The data are presented in Figure 3. An increase in temperature leads to a decrease in the extent of adduct formation at all of the pH values studied.

Deamination of 1-Methyl-5,6-dihydrocytosine. The study of the mechanism of deamination of the bisulfite–cytidine adducts, II and IV, is made difficult by the presence of the high concentrations of bisulfite needed to maintain the concentrations of II and IV. For this reason, we reinvestigated the deamination of a model compound, 1-methyl-5,6-dihydrocytosine (VIII, Scheme II). The earlier studies of Brown and Hewlins[11] made no mention of possible buffer catalysis of the deamination. Buffer catalysis was also denied in the deamination of a related compound, 6-hydroxy-5,6-dihydrocytidine 3′-phosphate.[9] However, recent studies on bisulfite-catalyzed deamination of cytidine 5′-phosphate[8] suggested that the hydrolysis of dihydrocytosines should be catalyzed by both bisulfite ion and ammonium ion. In our studies, we found that the deamination of 1-methyldihydrocytosine cation (VII) is subject to general base (or nucleophilic) catalysis, but not general acid catalysis. The rate of conversion of VII to IX is increased by increasing concentrations of sulfite, acetate, or other buffer anions. It is not affected by increasing concentrations of ammonium ion. The principal role of acid is to convert VIII to the more reactive cation, VII (specific acid catalysis).

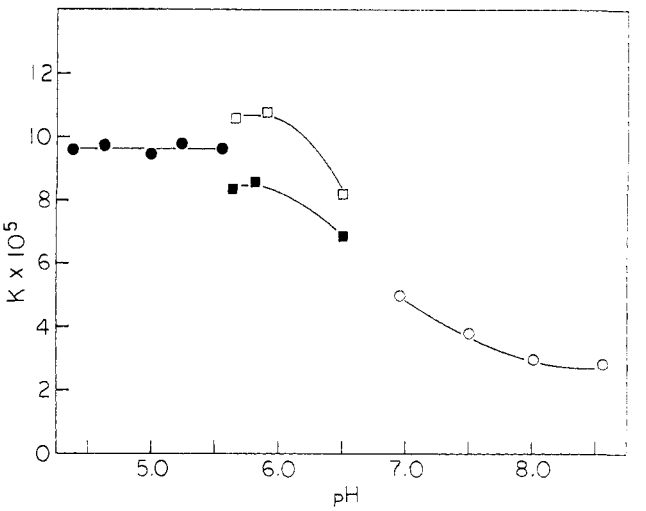


Figure 4. The variation of the rate constants (sec^{-1}) *vs.* pH for the hydrolysis of 1-methyl-5,6-dihydrocytosine at 30°, μ = 1.0, in buffers containing the following concentration of base: 0.149 *M* (sodium) acetate (●); 0.050 *M* (sodium) citrate (dianion + trianion) (■); 0.075 *M* (sodium) citrate (dianion + trianion) (□); 0.0885 *M* Tris (○). In each case, the amount of conjugate acid needed to attain the indicated pH was present.

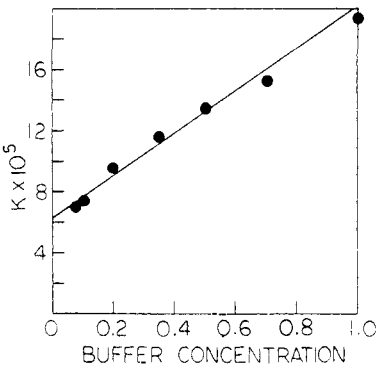


Figure 5. The variation of the rate of hydrolysis of 1-methyl-5,6-dihydrocytosine with the concentration of sodium acetate-acetic acid buffer at pH 5.00, 30°, μ = 1.0.

The variation of the rate of hydrolysis of 1-methyldihydrocytosine with pH, using several buffer systems, is given in Figure 4. In each buffer, the ionic strength and concentration of buffer anion were kept constant. The concentration of buffer acid was varied, to achieve the desired pH. The pH–rate profile generally follows the dissociation curve of the cation, VII, in accord with the observations of Brown and Hewlins.[11] The effect of the buffer ion is illustrated by the experiments using two different concentrations of citrate buffer.

A more extensive study of the effect of buffer anion was performed with various concentrations of sodium acetate buffer, at fixed pH and ionic strength (Figure 5).

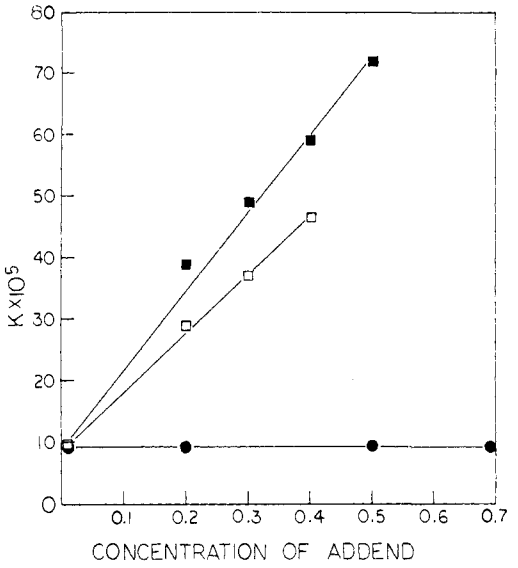


Figure 6. The variation of the rate of hydrolysis of 1-methyl-5,6-dihydrocytosine (at 30°, μ = 1.0, 0.2 *M* acetic acid–sodium acetate buffer) with added: NH_4Cl, pH 5.0 (●), $NaHSO_3$, pH 5.0 (■), and $NaHSO_3$, pH 4.38 (□).

A linear correlation of rate with acetate concentration is observed. The intercept (zero buffer) represents the hydrolysis catalyzed by water. This value (6.2×10^{-5} sec^{-1}) is close to the one observed by us in 1 *N* HCl solution (5.7×10^{-5} sec^{-1}). The effects produced by added bisulfite ion, and ammonium ion, in solutions of constant ionic strength and pH (maintained by a constant amount of sodium acetate buffer and added sodium chloride) are illustrated in Figure 6. No rate enhancement by ammonium ion is indicated. The effect of added bisulfite in enhancing the rate is greater at pH 5.0 than at pH 4.38. In a single experiment performed at pH 5.40, the rate of deamination (0.5 *M* HSO_3^-–0.2 *M* acetate, 30°, μ = 2.0) was observed to be greater than that in the same experiment conducted at pH 5.0 (pH 5.40, $K = 83.5 \times 10^{-5}$ sec^{-1}; pH 5.0, $K = 74.1 \times 10^{-5}$ sec^{-1}). The increase of rate with pH in the above experiments indicated that sulfite ion is an effective catalyst of the deamination.

The apparent pseudo-first-order rate constant for the hydrolysis of VII under the conditions employed by us is thus a composite of several terms (eq 4). In the

$$K = K_{HSO_3^-}\text{-}[HSO_3^-] + K_{SO_3^{2-}}\text{-}[SO_3^{2-}] + K_{OAc^-}\text{-}[OAc^-] + K_{H_2O} \quad (4)$$

above equation, OAc^- represents acetate ion. The catalytic constants in the above equation were evaluated from the data in Figures 5 and 6, and the dissociation constants for bisulfite and acetic acid. The values (l. mol^{-1} sec^{-1}) are $K_{HSO_3^-}$ 7.9×10^{-4}, $K_{SO_3^{2-}}$ 1.7×10^{-2}, K_{OAc^-} 2.7×10^{-4}, and K_{H_2O} 6.2×10^{-5}.

In an additional study, the rate of deamination of 1-methyldihydrocytosine was found to decrease moderately with increasing ionic strength in 0.2 *M* sodium acetate–acetic acid buffer, pH 5.0: μ = 0.2, $K = 11.9 \times 10.5$ sec^{-1}; μ = 1.0, $K = 9.7 \times 10^{-5}$ sec^{-1}; μ = 1.5, $K = 8.8 \times 10^{5}$ sec^{-1}; μ = 2.0, $K = 8.4 \times 10^{-5}$ sec^{-1}.

Deamination of the Bisulfite Adducts (II + IV) of Cytidine and Deoxycytidine. The rate of deamination of cytidine and deoxycytidine was followed by two

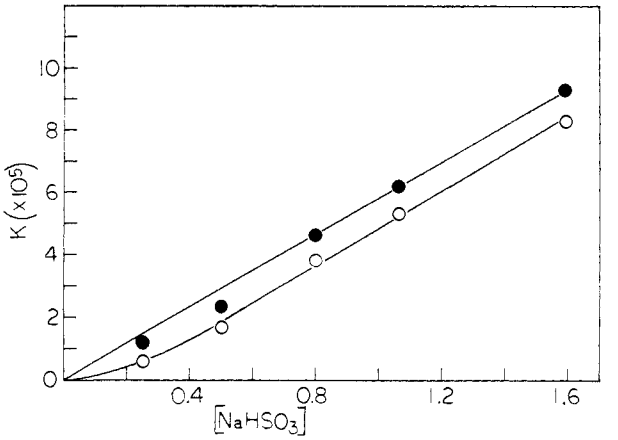


Figure 7. The variation of K_0 (○) and K_1 (●) (for definitions, see the text) with bisulfite concentration in the deamination of cytidine by sodium bisulfite, 25°, μ = 2.0.

alternative procedures. One method, which involves the dilution of aliquots into a pH 3 buffer, has been described above. An alternative was to pipet the aliquot into pH 11 buffer. The resulting mixture of cytidine and uridine or deoxycytidine and deoxyuridine was analyzed, using the readings at two wavelengths in the ultraviolet (see the Experimental Section).

The rate constants thus observed (K_0) apply to the overall rate of deamination of cytidine or deoxycytidine. The rate constants for the direct conversion of II and of IV to V (Scheme I) are represented by K_1 and K_2). The sum, $K_1 + K_2$, is obtained by dividing K_0 by the fractional amount of II + IV in the reaction mixture. As K_2 is very small compared to K_1 (see below), the value of K_1 was generally considered to be the same as $K_1 + K_2$.

The effect of bisulfite concentration upon the rate of deamination of cytidine, pH 5, 25°, is shown in Figure 7. As in the case of 1-methyldihydrocytosine, the rate of deamination increases with buffer concentration. In Figure 7, the change in K_0 reflects the effects of higher bisulfite concentrations in both increasing the equilibrium concentration of the adduct, II, and in catalyzing the deamination step. The plot of K_1 reflects the deamination effect alone, and is linear with bisulfite concentration. An analogous effect was observed by Hayatsu and coworkers[8] in the reaction of cytidine 5′-phosphate with bisulfite. In contrast to the observation of these workers, however, and in conformity with our results with 1-methyl-5,6-dihydrocytosine, no effect by ammonium ion on the rate was observed. Amounts up to 1.25 *M* NH_4Cl were employed, with 0.5 *M* $NaHSO_3$ at constant pH, 5.0, temperature, 25°, and ionic strength, 2. The zero intercept seen in Figure 7 indicates that the catalytic effect of water in this system is also negligible. A slight, but real, catalytic effect by acetate was, however, observed under the same conditions. Rates observed were: 0.137 *M*, K_0 = 1.74, K_1 = 2.30; 0.687 *M*, K_0 = 1.87, K_1 = 2.44; 1.37 *M*, K_0 = 1.95, K_1 = 2.53 (the concentration terms refer to acetate ion, the *K* values are $\times 10^{-5}$ sec^{-1}).

The synthetic or mutagenic applications of bisulfite usually have been performed with concentrated bisulfite solutions. To determine the optimal pH for the use of the reaction, a pH–rate profile was determined for the deamination of cytidine and deoxycytidine in 2.26

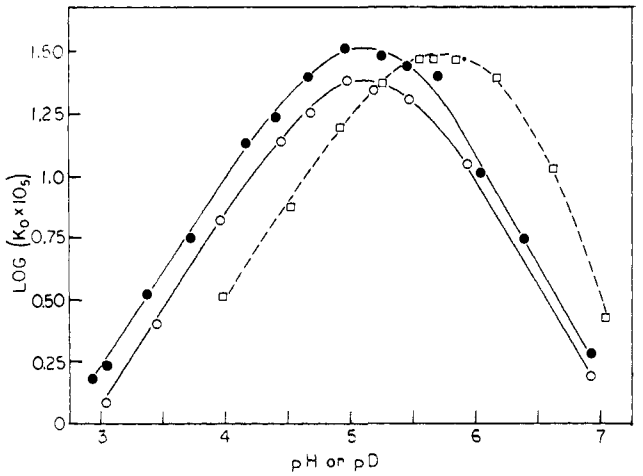


Figure 8. The pH or pD dependence of the logarithms of the rate constants, K_0, for deamination of cytidine (●), and deoxycytidine (○) in H_2O, and deoxycytidine in D_2O (□). All runs were in 2.26 *M* $NaHSO_3$ at 37°. One value, pH 7.48, cytidine, $K_0 = 2 \times 10^{-6}$, log $(10^5K) = -0.7$, is not displayed.

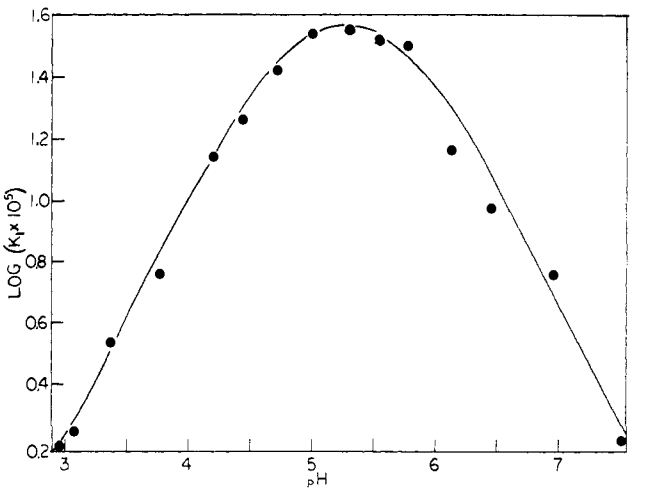


Figure 9. Comparison of the experimental (●) and calculated (–) values for the pH dependence of K_1 (II → V) in the deamination of cytidine by 2.26 *M* $NaHSO_3$, 37°.

M $NaHSO_2$, at 37° (Figure 8). Both compounds exhibited sharp rate maxima near pH 5.0, with the maximum rate 30% greater for cytidine than deoxycytidine. In our initial study[2] a deamination rate maximum for deoxycytidine at pD 5.8 was reported, using a thin-layer chromatographic method of analysis. This was now confirmed by our ultraviolet method (Figure 8). The maximum rate was 22% faster in D_2O than in H_2O. Weak acids are less dissociated in D_2O than in H_2O. Transformations requiring the prior conversion of a reactant to a protonated species therefore proceed more rapidly in D_2O.[16] The rate enhancement we observed in D_2O may thus be due to an increased concentration of species I and II in that solvent.

Rate constants (sec^{-1}) for the deamination of cytidine and deoxycytidine in H_2O (at pH 5.0) and of deoxycytidine in D_2O (pD 5.7) were determined at other temperatures: cytidine, 24°, 1.1×10^{-4}, 50°, 8.1×10^{-4}; deoxycytidine (H_2O), 24°, 7.8×10^{-5}, 50°, 5.6×10^{-4}; deoxycytidine (D_2O), 24°, 9.9×10^{-5}, 50°, 6.8×10^{-4}. The rate of deamination was increased by higher temperatures, despite the adverse effect of temperature on the extent of formation of II and IV.

The observed rate constants for cytidine were divided by the fractional amount of adduct present at each pH to get K_1 (a value of K_E of 8 at 37° was estimated from the data in Figure 3 and used to calculate the fraction of adduct present). The values obtained for K_1 are given as points in Figure 9. Several important facts become clear upon consideration of these data.

(1) The rate above pH 7 is extremely small compared to that at the maximum (compare Figure 9 to Figure 4). This can only be explained if the rate of deamination of IV, K_2, is much smaller than that of II, K_1. At pH values below 7, the rate of deamination of cytidine should then be given by:

$$\text{rate} = [\text{II}](K_{HSO_3^-}[HSO_3^-] + K_{SO_3^{2-}}[SO_3^{2-}]) \quad (5)$$

(2) At pH values less than 4, the concentration of II is relatively pH independent. Plots of K_0 *vs.* the calculated concentration of sulfite ion were linear in that range. From the intercept, a value of 0.6×10^{-5} l. mol^{-1} sec^{-1} was calculated for $K_{HSO_3^-}$. The contribution of bisulfite to the deamination is therefore small for pH values greater than 4, and sulfite is principally responsible for the deamination step.

A calculated curve for the pH dependence of K_1 for cytidine is given in Figure 9 which fits the experimental data quite well. In deriving the curve, values (l. mol^{-1} sec^{-1}) were assigned to $K_{HSO_3^-}$ of 0.6×10^{-5}, and to $K_{SO_3^{2-}}$ of 3.5×10^{-3}. The previously employed values of 4.24 and 4.70 for pK_C and pK_C' were used. From the data for the reactions run at a pH near 7, the relative reactivity of [II] to [IV] was estimated to be approximately 7000:1. In order to derive the rate maximum in Figure 9 at about pH 5.2, however, it was necessary to use a value of 5.8 for pK_s in the calculations. (The use of the titration value of 6.24 for pK_s led to a calculated maximum at 5.5.) The significance of this fact is not clear. Activity coefficients have been neglected in our treatment. It is possible that, in concentrated bisulfite solutions, the effective concentration of sulfite for deamination purposes differs from that revealed by titration. Alternatively, an additional factor which governs the deamination rates may yet await discovery.

Discussion

A consistent picture of the mechanism of deamination of cytosine derivatives by bisulfite has emerged.

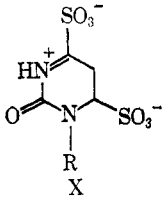
(1) Upon addition of bisulfite, an equilibrium is rapidly set up, involving both the protonated (II) and unprotonated (IV) forms of the cytosine–bisulfite adduct (Scheme I). The extent of adduct formation is controlled by the pH (Figure 2), bisulfite concentration, and temperature (Figure 3). The decline in adduct concentration at equilibrium as the pH is changed from acid to neutral is due to the dissociation of bisulfite to sulfite.

(2) The deamination step proceeds principally through the protonated adduct, II. The relative reactivity of the unprotonated adduct IV is considerably less than 1% of that of II. This situation contrasts with that of the model compound, 1-methyl-5,6-dihydrocytosine, where the reactivity of the neutral molecule, VIII, is approximately one-third that of the pro-

(16) (a) K. Wiberg, *Chem. Rev.*, **55**, 713 (1960); (b) L. Melander, "Isotope Effects on Reaction Rates," Ronald Press, New York, N. Y., 1960, p 126.

tonated species VII. It is possible that the negative charge of the unprotonated cytosine–bisulfite adduct greatly hinders attack by anionic species, slowing its deamination.

(3) The deamination step is catalyzed by basic substances, such as sulfite, bisulfite, and acetate anions. This is most clearly demonstrated in studies with the model compound, 1-methyl-5,6-dihydrocytosine. In moderately concentrated bisulfite solutions, at pH >4, the principal catalytic species is sulfite ion. Our data do not allow us to determine whether the catalytic effect is due to general base catalysis, or nucleophilic catalysis.[17] The latter possibility would involve the formation of X as a transient intermediate in the deamination of cytosine derivatives by bisulfite.



X

We have no direct evidence for the formation of such an intermediate. However, the possibility of attack at C-4 of a cytosine–bisulfite adduct by nucleophiles other than water has been demonstrated.[18] On the other hand, general base catalysis has been reported for other amidine hydrolyses.[19]

(4) Acids influence the deamination reaction by converting the cytosine adduct, IV, to its reactive protonated form, II (specific acid catalysis). At constant pH and ionic strength, there is no rate effect produced by added acidic substances such as ammonium ion.

The optimal pH for the synthetic or mutagenic use of the deamination reaction is 5. The concentration of bisulfite used should be as high as possible. At pH values below 5, the deamination declines sharply (Figure 8) due to the protonation of the most effective catalytic species, sulfite. The rate also declines sharply at pH values above 5. There is an additional increase in the sulfite concentration above pH 5, but this effect is offset by a decline in the extent of adduct formation, and by the conversion of the protonated adduct II, to its unreactive nonprotonated form IV.

The rate of deamination of deoxycytidine measured at physiological pH is about 1% of that observed at the optimum pH. The possibility that ingested bisulfite and inhaled sulfur dioxide may be environmental mutagens remains open. To evaluate this possibility more fully, the following unknowns must be explored: the extent to which bisulfite reaches genetic DNA, the amount of this DNA which exists in single-stranded form (double-stranded DNA is unreactive to bisulfite[20]), and the ability of other cellular nucleophiles to catalyze the vital second step (II → V) of the deamination sequence.

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Communications to the Editor

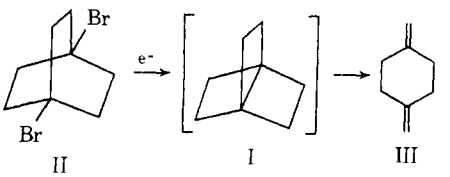
Electrochemical Reduction of 1,4-Dibromobicyclo[2.2.2]octane. Formation of the [2.2.2]Propellane[1]

Sir:

The small ring "propellanes" have proven to be unusually interesting compounds.[2] The [2.2.2]propellane (I) is of particular interest in view of the results of theoretical treatments of its thermal rearrangement to 1,4-dimethylenecyclohexane,[3,4] and in connection with the origin of the low reactivity of cyclobutanes toward electrophiles.[5]

Eaton and Temme[6] have recently reported the synthesis of a derivative of I, but their route does not appear to be a viable source of the hydrocarbon itself. An attempt to obtain I by a dissolving metal reduction of 1,4-dibromobicyclo[2.2.2]octane led to 1,4-dimethylenecyclohexane along with a small amount of bicyclo[2.2.2]octane.[7] The formation of the dimethylenecyclohexane was thought to result from a Grob-type fragmentation.

The observation of Eaton and Temme that their derivative of I underwent rearrangement to a 1,4-dimethylenecyclohexane revived the suggestion that the above reduction may have actually proceeded *via* the propellane as an intermediate and that the latter underwent ring opening under the reaction conditions to give the observed product.



II I III

(1) This investigation was supported by a grant from the National Science Foundation.

(2) For a review, see D. Ginsburg, *Accounts Chem. Res.*, **2**, 121 (1969).

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