Solvolyses of Cytosine and Cytidine

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Abstract □ It has been confirmed by UV spectrophotometry and TLC that cytosine and cytidine deaminate to uracil and uridine, respectively, at all pH values. These products slowly degrade further in strongly alkaline solutions. Cytidine also undergoes a parallel degradation in strongly alkaline solutions to a nonchromophoric compound, which can be assigned to a product of pyrimidine ring cleavage. The kinetic expression for the apparent first-order rate constant for the deamination of cytosine and its derivatives is: $k_1 = (k_{H_2O} + k_{OH}[OH^-])f_{H_2C^+} + (k_{OH})_1[OH^-]f_{HC}$, where $f_{H_2C^+}$ and f_{HC} are the fractions of substrate protonated and nonprotonated, respectively; and log k–pH profiles were prepared for several temperatures. The microscopic rate constants for deamination and their Arrhenius parameters were obtained. Cytosine showed a $pK'a_2$ assignable to a dissociation in highly alkaline solutions, and this anionic form appeared resistant to deamination. No such kinetic $pK'a_2$ was assignable to cytidine. Buffer catalytic constants for deamination were obtained for acetate and carbonate buffer species.

Keyphrases □ Cytosine—solvolysis, deamination mechanisms, pH–rate profiles □ Cytidine—solvolysis, deamination mechanisms, pH–rate profiles □ Solvolysis—cytosine and cytidine, mechanisms □ pH–rate profiles—cytosine and cytidine solvolysis □ UV spectrophotometry—monitoring, cytosine and cytidine deamination □ TLC—identification, deamination products of cytosine and cytidine

This quantitative investigation of the chemical transformations of cytosine and cytidine is a continuation of the series of studies on the solvolytic reactions of pyrimidine and purine nucleosides and their derivatives (1–8). The purpose of this paper is to report on the kinetics and possible mechanisms of the solvolytic degradation of cytosine and cytidine and the determination of pertinent thermodynamic parameters.

Schuster and Schramm (9) found that deamination of the amino groups of the cytosine, adenine, and guanine residues of the nucleic acids could be effected by nitrous acid. Jordan (10) showed that cytosine and guanine could be deaminated under strong acidic conditions; Shapiro and Klein (11) reported that both cytosine and cytidine also underwent deamination in several acidic buffers up to pH 5 at a very high temperature, *e.g.*, 95°, and that the reactions were catalyzed by the species of several buffers. Quantitative data on the buffer-affected deamination of cytosine and of some of its nucleosides were given by Notari (12) and Notari *et al.* (13, 14), who reported that cytosine and these nucleosides underwent deamination in various buffers between pH 3.4 and 7.7. They also demonstrated that the sugar moiety catalyzed the deamination rate and that the change of the configuration of the sugar moiety also affected the deamination rate.

The ready deamination of cytosine derivatives to the corresponding uracil derivatives after the formation of a reversibly hydrolyzing saturated 5,6-double bond adduct with bisulfite ion is of fundamental interest because such a reaction may be responsible for mutagenic action by transforming the cytosine moieties in RNA and DNA (15–19).

Systematic kinetic studies and determination of pertinent Arrhenius parameters are still lacking for acid and alkaline solutions. This paper presents studies that permit the presentation of complete pH–rate profiles for cytosine and cytidine.

EXPERIMENTAL

Materials—Cytosine (analytical grade)[1], cytidine (analytical grade)[1], uridine[2], and uracil[2] were purchased and used without further purification. Other chemicals used were of analytical reagent grade.

Kinetic Procedures—Stock solutions of cytosine (4×10^{-3} *M*) and cytidine (10^{-2} *M*) were prepared in double-distilled and N_2-purged water. The kinetic studies were made on final solutions in the range of $0.8–1.2 \times 10^{-4}$ *M*. For slow reactions, *i.e.*, half-lives in weeks and months, 2–25 ml. of stock buffer solutions and appropriate amounts of 1.00 *N* NaCl to maintain constant ionic strength were added to appropriately diluted volumes of the stock solutions. Amounts of these solutions were sealed in 5-ml. ampuls, which had been cleaned and dried previously. The sealed ampuls were fastened together with stiff, heavy wire and immersed in a constant-temperature bath controlled within 0.5° over periods of months. Samples were taken at appropriate intervals and analyzed spectrophotometrically[3].

For the fast reactions, *i.e.*, half-lives in hours or a few days, 100-ml. volumetric flasks were used instead of ampuls. All reacting solutions and acidified aliquots were followed with time in the spectral range 220–320 nm. The acidified solution was obtained by the addition of 0.20 ml. of concentrated hydrochloric acid to 3.00 ml. of the reacting solution. The same matched spectrophotometric cells (10.00 mm.) were used for all measurements in a single kinetic run.

pH Measurements—The pH values of the sodium hydroxide and hydrochloric acid solutions at the temperatures of study were calculated from extrapolation of activity coefficient data in the literature (20). The pH values of buffer solutions were measured directly at the temperature of the study on a pH meter[4] equipped with a high temperature combination electrode[5], which was standardized with standard buffer solutions at the same temperature.

TLC Studies—Solutions of cytosine (7×10^{-3} *M*) and cytidine (10^{-2} *M*) in 0.25 *N* NaOH were prepared and were maintained at 90° in a constant-temperature bath. Samples were withdrawn at

[1] Calbiochem., Los Angeles, Calif.
[2] Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Cary 15 recording spectrophotometer, Monrovia, Calif.
[4] Direct-reading Beckman pH meter, Beckman Instruments Inc., South Pasadena, Calif.
[5] Corning combination electrode, Sargent-Welch Scientific Co., Birmingham, Ala.

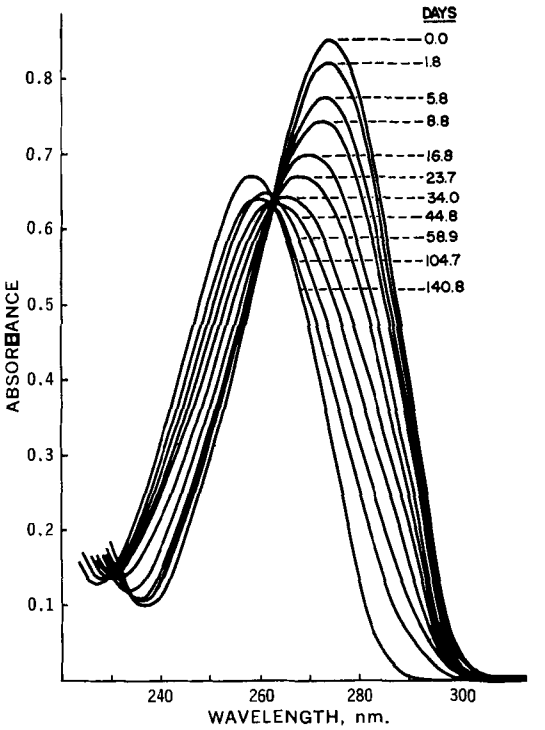


Figure 1—*Typical UV spectral changes of acidified solutions of reacting 10^{-4} M cytosine in solutions more acid than 0.1 N NaOH where the sole product of reaction is uracil as indicated by the maintenance of the isosbestic point at 263 nm. These spectra are for reactions in 0.01 N HCl at 80.0° and are labeled as to the days of reaction.*

suitable time intervals and stored in a refrigerator before TLC studies. Alkaline solutions of cytosine (7×10^{-3} *M*), cytidine (10^{-2} *M*), uridine (10^{-2} *M*), and uracil (10^{-2} *M*) were also prepared as standards for TLC studies. The TLC plates were prepared using silica gel GF_{254}[6]. The chromatograms were spotted with about the same amount (~10 μl.) of sample and developed to about 15 cm. of travel of solvent front in a 1-butanol–water–acetone (50:25:25) solvent system. The R_f values of these compounds were the same for neutral and alkaline solutions. After development of the chromatogram, UV chromophores were located with a short wavelength UV light[7] and also in an iodine chamber.

RESULTS AND DISCUSSION

Spectral Changes—The isosbestic points found in the spectra obtained at the pH of the kinetic study and in the spectra of the acidified solutions for cytosine up to the pH of 0.1 *N* NaOH and for cytidine up to pH 8.2 indicated one-to-one solvolytic transformations. Typical examples are given in Fig. 1. The isosbestic points in the acidified solutions were 263 nm. for cytosine and 265 nm. for cytidine. The use of acidified solutions for the estimation of rate constants was preferred since the difference in λ_{max} values between cytosine and its product, uracil, and between cytidine and its product, uridine, was the greatest in this solution. These products are also highly stable in acid solutions (5).

The spectral changes of the acidified solutions of reacting cytosine followed the same pattern for all solutions more acidic than 0.1 *N* NaOH: a hypsochromic shift in λ_{max} from cytosine (274 nm.) [lit. (21) 276 nm.] to uracil (259 nm.) [lit. (21, 22) 262 nm.]. Similarly, the spectral changes of the acidified solutions of cytidine for reactions up to pH 8.2 had the same pattern: a hypsochromic shift of λ_{max} from cytidine (280 nm.) to uridine (262 nm.). The solvolytic process can be stated to involve deamination of cytosine to uracil up to the pH of a 0.1 *N* NaOH solution and of cytidine to uridine up to pH 8.2. These transformations were shown previously to exist below pH 6 (11–14).

In the more highly alkaline regions, kinetic studies based on spectral studies *in situ* and on acidified solutions did show a shift in λ_{max} from that of cytosine to uracil or from that of cytidine to uridine. However, the lack of isosbestic points in these spectral transformations indicated a more complex sequence of solvolytic degradation. Typical spectral changes of acidified solutions that reacted in these alkaline regions are given as a function of time in Figs. 2 and 3.

The shifts of the λ_{max} concomitant with a loss of absorbance suggest that uracil and uridine degrade further to nonchromophore(s) and/or that cytosine and cytidine also degrade directly to nonchromophoric compounds in addition to their respective deamination to uracil and uridine.

TLC Studies—TLC studies showed that in 0.25 *N* NaOH and at 90°, cytosine (R_f 0.44) degraded to a chromophoric compound which had the same R_f value (0.62) as that of uracil. The amount of this compound initially increased and then slowly decreased until it completely disappeared. Similarly, cytidine (R_f 0.52) degraded to a chromophoric compound with an R_f value (0.64) identical to that of uridine, and this compound degraded further to nonchromophoric compounds. Further proof that the intermediates were uracil and uridine was obtained by eluting the intermediates with water from preparative thick-layer plates. The solutions were centrifuged; the UV spectra of the intermediates in solution, read at various pH values, were the same as those of uracil and uridine (21, 22).

TLC spots of cytosine and uracil were visible in an iodine chamber, whereas the spots of cytidine and uridine were not. The fact that none of these spots appeared in the degradation of cytidine

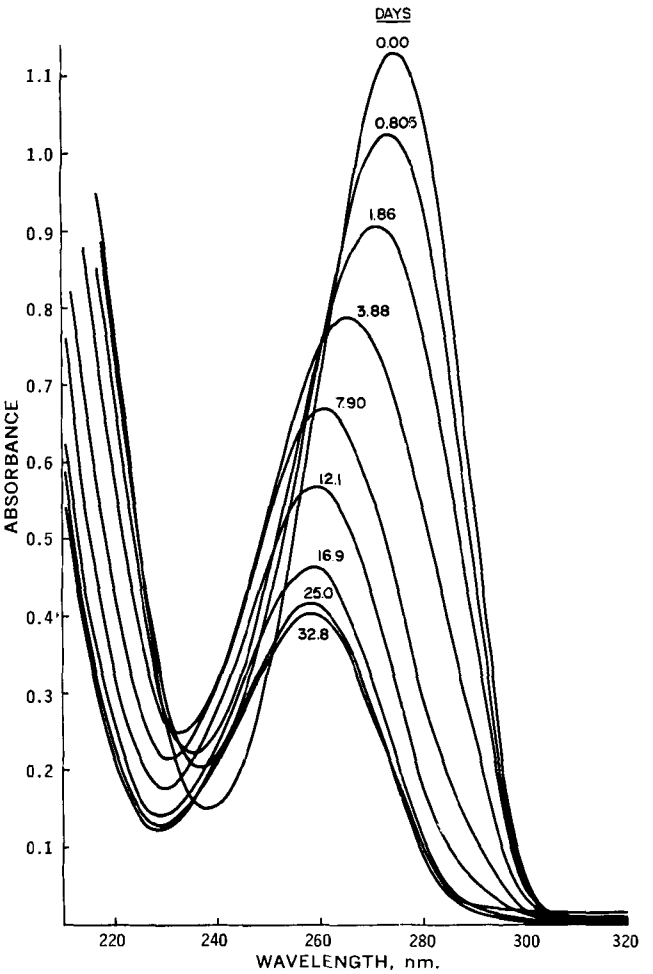


Figure 2—*Typical UV spectral changes of acidified solutions of alkaline reacting 10^{-4} M cytosine. These spectra are for reactions in 0.2 N NaOH at 85.0° and are labeled as to the days of reaction. The lack of maintenance of an isosbestic point is indicative of a parallel and/or sequential reaction to deamination.*

[6] E. Merck AG, Darmstadt, Germany.
[7] Chromato-Vue, Ultra-Violet Products, Inc., San Gabriel, Calif.

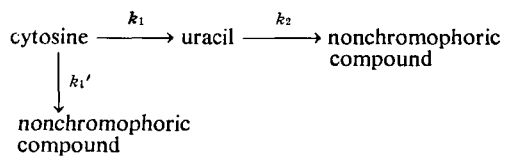
Table I—Apparent First-Order Rate Constants as 10^8k_1, where k_1 is in sec.$^{-1}$, for the Solvolytic Deamination of Cytosine and Cytidine to Uracil and Uridine, Respectively, in the pH Regions where This is the Sole Route of Degradation

Buffer			pH[a]	10^8k_1[b] Cytosine 70.0°	80.0°	85.0°	90.0°	Cytidine 70.0°	80.0°	85.0°	90.0°
[HCl]											
0.99			0.15	—	19.9	—	—	—	—	—	—
0.25			0.74	8.38	25.5	—	61.5	35.7	67.0	—	239
0.20			0.83	—	—	38.1	—	—	—	175	—
0.10			1.11	7.89	25.4	—	—	—	109	—	—
0.05			1.39	—	27.0	—	65.2	35.3	106	—	276
0.02			1.76	—	—	40.0	—	—	—	157	—
0.01			2.05	—	25.4	—	—	—	112	—	—
0.0025			2.63	8.15	—	38.3	—	42.4	—	165	—
[CH_3COOH]	[CH_3COONa]	[NaCl][c]									
0.110	0.015	0.072	3.81	—	21.2	—	—	23.6	63.6	102	—
0.044	0.006	0.149	—	—	19.3	—	—	21.6	59.0	101	—
0.022	0.003	0.175	—	—	18.4	—	—	28.4	54.4	95.2	—
0.0088	0.0012	0.190	—	—	17.2	—	—	26.4	49.2	92.8	—
0.0638	0.0612	0.065	4.58	—	15.3	—	—	—	27.7	—	—
0.0255	0.0245	0.146	—	—	—	—	—	—	21.5	—	—
0.0128	0.0123	0.173	—	—	11.4	—	—	—	20.1	—	—
0.0051	0.0049	0.189	—	—	9.87	—	—	—	18.6	—	—
0.022	0.103	0.074	5.20	—	9.21	—	—	—	11.1	—	—
0.0088	0.0412	0.149	—	—	7.61	—	—	—	8.93	—	—
0.0044	0.0206	0.175	—	—	7.41	—	—	—	8.98	—	—
0.0018	0.0082	0.190	—	—	7.08	—	—	—	8.50	—	—
[KH_2PO_4]	[K_2HPO_4]	[NaCl][c]									
0.042	0.025	0.101	6.53	2.76	8.63	—	22.3	3.52	11.3	—	29.7
0.0168	0.010	0.160	—	2.19	6.45	—	17.5	2.88	8.75	—	25.6
0.0084	0.005	0.180	—	2.30	6.40	—	16.4	2.74	8.51	—	22.4
0.00336	0.002	0.192	—	1.00	6.04	—	15.7	2.78	7.62	—	22.7
0.0083	0.0548	0.042	7.48	1.85	—	—	16.8	3.30	7.85	—	26.4
0.0033	0.0219	0.137	—	2.12	6.93	—	16.7	2.77	7.88	—	23.2
0.0017	0.0110	0.168	—	2.23	6.68	—	16.4	2.66	7.46	—	21.5
0.0007	0.0044	0.187	—	2.03	6.83	—	16.2	2.65	7.44	—	21.8
[H_3BO_3]	[NaOH]	[NaCl][c]									
0.0938	0.0225	0.180	8.18	—	6.84	—	—	—	9.53	—	—
0.0375	0.009	0.192	—	—	6.38	—	9.50	—	8.88	16.2	—
0.0188	0.0045	0.200	—	—	5.71	—	9.28	—	8.48	15.2	—
0.0075	0.0018	0.200	—	—	6.22	—	8.69	—	7.26	15.3	—
[$NaHCO_3$]	[Na_2CO_3]	[NaCl][d]									
0.106	0.050	0.182	9.05	—	9.62	—	—	—	—	—	—
0.0425	0.020	0.313	—	—	8.38	—	—	—	—	—	—
0.0213	0.010	0.356	—	—	7.96	—	—	—	—	—	—
0.0085	0.004	0.383	—	—	7.61	—	—	—	—	—	—
0.075	0.0916	0.108	9.50	—	13.2	—	—	—	—	—	—
0.030	0.0366	0.28	—	—	11.3	—	—	—	—	—	—
0.015	0.0183	0.342	—	—	9.63	—	—	—	—	—	—
0.006	0.0073	0.377	—	—	9.13	—	—	—	—	—	—

[a] The pH values given are only for 80°. The actual pH values at 90, 70, and 85° were not significantly different from these. The pH values of different hydrochloric acid concentrations were calculated from pH = log f[HCl] and activity coefficient, f, data in the literature (20). The pH values of the buffer solutions were measured at the reaction temperature and are given for 80°. [b] Each k_1 value of cytosine is the average of those calculated from the slopes of first-order plots at 275 and 290 nm. Each k_1 value of cytidine is the average of those calculated at 280, 295, and 300 nm. The k_1 values calculated from different wavelengths were not significantly different and agreed within 10%. [c] Ionic strength adjusted to 0.200 *M*. [d] Ionic strength adjusted to 0.400 *M*.

in alkaline solution indicated that cytidine degraded to uridine and that one or both of these compounds degraded further to nonchromophoric compounds other than uracil.

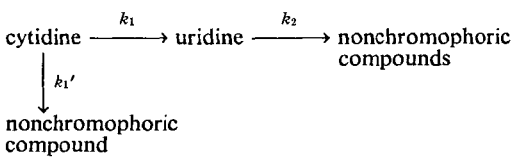
Rate Constants—The reactions in acid and alkaline solutions were followed for a minimum of 1.5 half-lives and frequently to completion ($\geq$7 half-lives). The reactions in buffer solutions were followed up to 211 days. The A_∞ readings of the acidified, completely reacted solutions of the compounds at any pH were zero for cytosine at 290 nm. and for cytidine at 295 and 300 nm. (Figs. 1–3). Plots of ln A *versus* time of the acidified solutions were reasonably linear for all pH values at these wavelengths, whether or not the reactions were followed to completion.



Scheme I

Reasonably linear plots were obtained up to 7 half-lives for ln ($A - A_\infty$) *versus* time plots of the acidified solutions of cytidine (280 nm.) reacting at pH values below that of 0.1 *N* NaOH and of cytosine (275 nm.) below pH 8.2. The apparent first-order rate constants calculated from the linear plots are given in Table I. Reasonable agreements were generally obtained among rate constants calculated from different wavelengths. Typical first-order plots are given in Fig. 4 for cytosine.

Analysis of the spectral curves demonstrated the stoichiometric transformation of cytosine to uracil in solutions more acidic than 0.1 *N* NaOH and of cytidine to uridine in solutions less than pH 8.2. The final absorbances at the uracil and uridine λ_{max} values were



Scheme II

Table II—Apparent First-Order Rate Constants as $10^7 k_i$, where the k_i Values are in sec.$^{-1}$, for the Sequential Solvolytic Degradation of Cytosine[a] in the Alkaline Region

[NaOH]	pH[b]	70.0° k_1	70.0° k_2	80.0° k_1	80.0° k_2	85.0° k_1	85.0° k_2	90.0° k_1	90.0° k_2
0.01	10.55	0.485	—	4.45	—	9.61	—	—	—
0.02	10.84	—	—	—	—	—	—	—	—
0.05	11.20	—	—	10.2	4.30	—	—	25.3	—
0.10	11.49	—	—	11.3	7.67	—	—	—	—
0.20	11.75	5.40	—	—	—[c]	24.1	7.32	—	—
0.25	11.85	—	—	13.8	—	—	—	37.7	8.71

[a] Cytosine $\xrightarrow{k_1}$ uracil $\xrightarrow{k_2}$ nonchromophoric products. [b] Calculated from pH = log pKw − log f [NaOH], where the pKw and activity coefficient values, f, were obtained or extrapolated for the requisite temperatures from the data in the literature (20). The values given here are for the calculated pH values at 80°. [c] Previously, a rough estimate given for uracil degradation at 80° in 0.2 N NaOH was about 4×10^{-7} sec.$^{-1}$ (6).

equivalent to those expected from the initial amounts of cytosine and cytidine, respectively.

In strongly alkaline solutions, the plots of ln $(A - A_\infty)$ of the acidified solutions *versus* time for cytosine at 275 nm. were not linear and indicated a sequential reaction. The obtained absorbance data at 263 nm., the isosbestic point for cytosine and uracil, were fitted as a function of time with an analog computer using the model in Scheme I. The values of $k = k_1 + k_1'$ were obtained from the slopes of the apparent first-order plots of the absorbances of the acidified solutions at 290 nm., a wavelength where uracil (Fig. 1) had no significant absorbance in acid solution. The methods of fitting such data by the analog computer were given previously (6, 7). The values of k_1' were essentially zero and indicated that the only route of cytosine degradation in alkali was through the uracil intermediate. The estimated values of k_1 and k_2 are given in Table II.

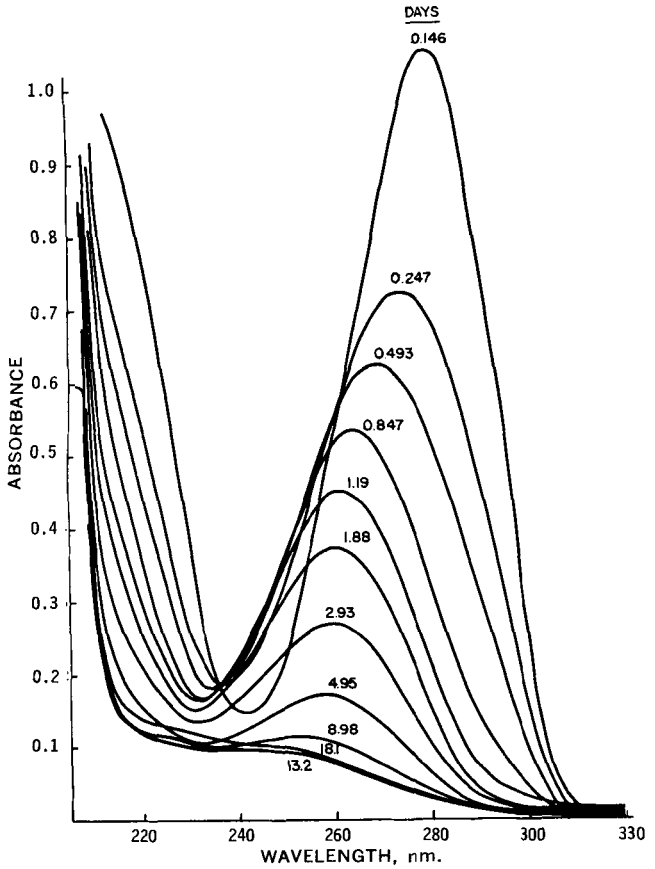


Figure 3—*Typical UV spectral changes of acidified solutions of alkaline reacting 10^{-4} M cytidine. These spectra are for reactions in 0.2 N NaOH at 85.0° and are labeled as to the days of reaction. The lack of maintenance of an isosbestic point is indicative of a parallel and/or sequential reaction to deamination.*

The plots of ln $(A - A_\infty)$ *versus* time for acidified solutions of cytidine above pH 9.0 were also nonlinear; the absorbance data at 265 nm., the isosbestic point of cytidine and uridine, were fitted as a function of time with an analog computer using the model in Scheme II. The values of $k = k_1 + k_1'$ were obtained from the slopes of the apparent first-order plots of the absorbances of the acidified solutions at 295 or 300 nm. The calculated rate constants are given in Table III for the alkaline degradation (pH > 9.0) of cytidine. The kinetic constants, k_2, determined from the computer fitting of the cytidine data were in good agreement with those determined previously for uridine degradation (7). This agreement strongly supported the model in Scheme II. A typical computer-fitted curve for cytidine data is given in Fig. 5.

The low magnitudes of the k_2 values demanded data at much later times of reaction. The formation of cloudy silicate precipitates in highly alkaline solutions interfered with proper spectral readings. These cloudy solutions were centrifuged and/or filtered, and the k_2 rate constants for cytosine and cytidine in alkaline solutions were calculated under the assumption that these procedures would not significantly perturb the true absorbance values.

Buffer Catalysis of Deamination—The apparent first-order rate constant in a buffer solution is the sum of contributions of the hydrogen ion, the hydroxyl ion, the acidic and the basic components of the buffer, and water (23). The overall rate constant for the reaction in a buffer, on the assumption of no water attack, may be expressed (23) by:

$$k = k_{HA}[HA] + k_A[A^-] + k_H[H^+] + k_{OH}[OH^-] \quad \text{(Eq. 1)}$$

or:

$$k = [HA](k_{HA} + k_A \cdot K_a/[H^+]) + k_H[H^+] + k_{OH}[OH^-] \quad \text{(Eq. 2)}$$

or:

$$k = [A^-](k_A + k_{HA} \cdot [H^+]/K_a) + k_H[H^+] + k_{OH}[OH] \quad \text{(Eq. 3)}$$

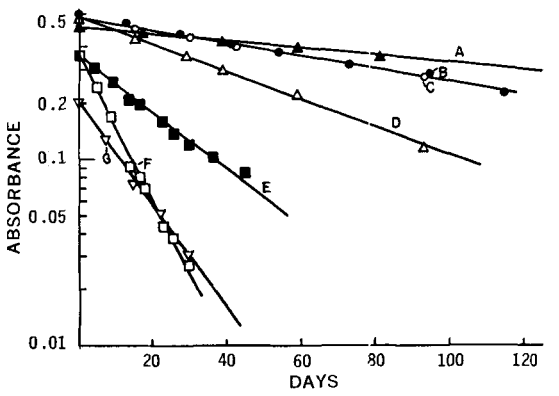


Figure 4—*Typical first-order plots for the spectral monitoring at 290 nm. of the 80.0° degradation of 10^{-4} M cytosine. The curves and the conditions of the reactions were: A, pH 8.2; B, pH 5.2; C, pH 9.5; D, pH 3.8; E, 0.01 N NaOH; F, 0.05 N NaOH; and G, 0.01 N HCl.*

Table III—Apparent First-Order Rate Constants as k_i, where the k_i Values are in sec.$^{-1}$, for the Sequential and Parallel Solvolytic Degradation of Cytidine[a] in the Alkaline Region

Buffer				70.0°			80.0°			85.0°			90.0°		
			pH[b]	10^6k_1	$10^6k_1'$	10^6k_2	10^6k_1	$10^6k_1'$	10^6k_2	10^6k_1	$10^6k_1'$	10^6k_2	10^6k_1	$10^6k_1'$	10^6k_2
[$NaHCO_3$]	[Na_2CO_3]	[NaCl]													
0.106	0.050	0.182	9.05	—	—	—	0.211	0	0.112	—	—	—	—	—	—
0.0425	0.020	0.313	—	—	—	—	0.173	0	0.0861	—	—	—	—	—	—
0.0213	0.010	0.356	—	—	—	—	0.146	0	0.0796	—	—	—	—	—	—
0.0085	0.004	0.383	—	—	—	—	0.127	0	0.0457	—	—	—	—	—	—
0.025	0.140	0.0037	10.21	—	—	—	0.991	0.424	0.431	—	—	—	—	—	—
0.010	0.056	0.231	—	—	—	—	0.811	0.259	0.421	—	—	—	—	—	—
0.005	0.028	0.320	—	—	—	—	0.583	0.162	0.357	—	—	—	—	—	—
0.002	0.0112	0.368	—	—	—	—	0.344	0.652	0.401	—	—	—	—	—	—
	[NaOH]		pH[c]												
	0.02		10.84	—	—	—	—	—	—	5.08	0.440	1.02	—	—	—
	0.05		11.20	2.38	0.287	0.690	8.60	1.88	1.92	—	—	—	15.50	2.35	3.98
	0.10		11.49	—	—	—	13.10	2.73	2.90	—	—	—	—	—	—
	0.20		11.75	—	—	—	—	—	—	20.7	4.9	5.75	—	—	—
	0.25		11.80	9.51	1.59	2.30	32.4	2.84	6.02	—	—	—	74.4	10.1	13.3
	1.00		12.25	—	—	—	135.0	16.3	11.3	—	—	—	—	—	—

[a] Nonchromophoric product $\xleftarrow{k_1'}$ cytidine $\xrightarrow{k_1}$ uridine $\xrightarrow{k_2}$ nonchromophoric product, where the rate constant for the overall loss of cytidine is given as $k = k_1 + k_1'$. [b] Measured at 80°. [c] Calculated from pH = pKw − log f[NaOH], where the pK and activity coefficient values, f, were obtained or extrapolated for the requisite temperature (20). The values given here are for the calculated pH values at 80°.

Thus, a plot of k *versus* [HA] at a constant pH will be linear with intercept $k_H[H^+] + k_{OH}[OH]$ and slope $(k_{HA} + k_AK_a/[H^+])$. A plot of k *versus* [A^-] at a constant pH will give a straight line with intercept $k_H[H^+] + k_{OH}[OH]$ and slope $k_{HA}[H^+]/K_a + k_A$. A plot of k *versus* [HA] or k *versus* [A^-] at two or more pH values will permit calculation of both catalytic constants (20, 21). Both methods were used to calculate the data given in Table IV with consistent results. Typical plots for acetate and carbonate buffer catalytic effects are shown in Figs. 6 and 7 for cytidine and cytosine. Although these data are not of the best, they serve the desired function of permitting reasonable estimates of rate constants at zero buffer concentrations. The data in these plots of apparent first-order rate constants against acetic acid and carbonate-ion concentrations at the several pH values do not permit one to reject the premise of parallelism of these plots and the fact that these species are the probable major catalytic species in the buffers (24, 25). Similar plots against acetate and bicarbonate-ion concentrations for the several pH values were not parallel. The cytidine catalytic constants of acetate buffer at 80° of Notari *et al.* (14) differed from the estimates given in Table IV. This is understandable since the catalytic effects are not too great and the reactions are extremely slow[8].

The experimental results were not accurate enough for the estimation of the catalytic constants of phosphate buffer since, because of the slowness of the reaction, less than 1 half-life was followed. The $t_{1/2}$ of cytosine in phosphate buffer at 70° is ~380 days (14).

The apparent first-order rate constants (Table V) at zero buffer concentration may be obtained from the intercepts of such plots as are given in Figs. 6 and 7 and represent the solvolysis of the substrate by hydrogen- and hydroxyl-ion catalysts (Eqs. 2 and 3).

Log k–pH Profile—The log k_1–pH profiles for the solvolytic deaminations of cytosine and cytidine at zero buffer concentrations are given in Figs. 8 and 9. The rate constants for cytosine and cyti-

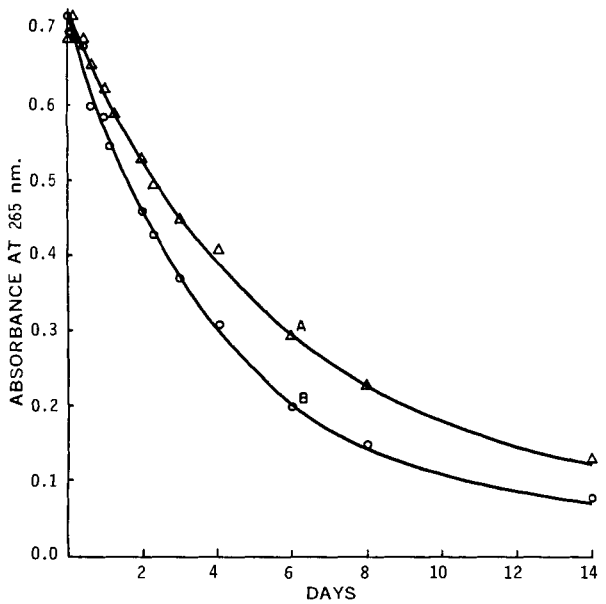


Figure 5—*Analog computer fit of the change in absorbance at 265 nm. against time of 10^{-4} M cytidine at 80.0° in accordance with Scheme II. Curve A is in 0.05 N NaOH. Curve B is in 0.10 N NaOH.*

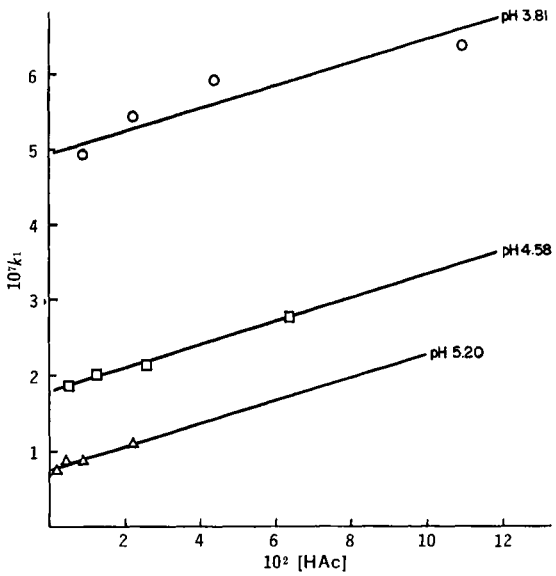


Figure 6—*Plots of apparent first-order rate constants for 10^{-4} M cytidine in various concentrations of acetic acid–acetic buffer systems at constant pH and ionic strength against the acetic acid concentrations.*

[8] In personal correspondence, Dr. Notari has assured us that statistical treatment of many recently repeated studies in his laboratories reconfirm his prior observations of pronounced catalysis of cytidine deamination by acetate ion.

Table IV—Catalytic Constants[a] for Acetate (HAc–Ac$^-$) and Carbonate (HCO_3^-–CO_3^{-2}) Buffers in Deaminations of Cytosine and Cytidine at 80°.

Compound	$10^7 k_{HAc}$	$10^7 k_{Ac^-}$	$10^7 k_{HCO_3^-}$	$10^7 k_{CO_3^{-2}}$
Cytosine	5.90[b]	1.7[b]	0.0	5.35
Cytidine	15.1[c]	0.0[c]	0.0	322

[a] Determined from slopes of the plots of the apparent first-order rate constants for deamination, k, against concentration of the ionic species of the buffers at a constant pH value. The several slopes of $k_A + k_{HA}[H^+]/K_a$ for k *versus* [A$^-$] and $k_{HA} + k_A K_a/[H^+]$ for k *versus* [HA] and the knowledge of the dissociation constant K_a and the hydrogen concentration [H$^+$] permits the estimation of k_A and k_{HA} values. [b] Data at 70° from Notari *et al.* (14) were $k_{HAc} = 1.5 \times 10^{-7}$ and $k_{Ac^-} = 0.83 \times 10^{-7}$. [c] Data at 70° from Notari *et al.* (14) were $k_{HAc} = 3.19 \times 10^{-7}$ and $k_{Ac^-} = 7.3 \times 10^{-7}$.

dine (pK′a values in the range 3.7–3.9 at 80°) are practically independent of pH at pH values below 3 where both compounds are completely protonated. This was qualitatively observed by Shapiro and Klein (11), who attributed it to the fact that water attack on the protonated species was the sole rate-determining step (11, 12). This implies that hydrogen-ion-catalyzed solvolysis of the protonated species is negligible. An alternative explanation is the kinetic equivalent of hydrogen-ion attack on the neutral substrate where the protonated species is stable with respect to deamination.

The decrease of k_1 with increasing pH between pH 2.5 and 6 can be explained by the reduction of the fraction of the protonated species attacked by neutral solvent molecules.

The apparent first-order rate constants of cytosine and cytidine deamination again become pH independent between pH 5 and 9 and indicate either water attack on the neutral species or its kinetic equivalent, hydroxyl-ion attack on the protonated species. Therefore, the log k–pH profiles of cytosine and cytidine at 80° were fitted by (23–25):

$$k_1 = f_{H_2C^+} k_{H_2O} + f_{HC} k'_{H_2O} \qquad \text{(Eq. 4)}$$

where k_{H_2O} and k'_{H_2O} are the apparent first-order rate constants for water attack on the protonated and neutral species, respectively, and are given in Table VI. Also:

$$f_{H_2C^+} = [H^+]/([H^+] + K_{a_1}') \qquad \text{(Eq. 5)}$$

is the fraction of the protonated species, and:

$$f_{HC} = K_a/([H^+] + K_{a_1}') \qquad \text{(Eq. 6)}$$

is the fraction of the neutral species in solution.

The kinetic pK′a$_1$ values (Table VI) were calculated from the log k–pH profile data at 80°. The pK′a$_1$ values of cytosine were determined also by the half-neutralization technique and were 4.55 [lit. (21) 4.45] at room temperature and 3.7 at 80°. The pK′a$_1$ of cytidine was 4.15 [lit. (22) 4.14] at room temperature and 3.61 at 80°. These

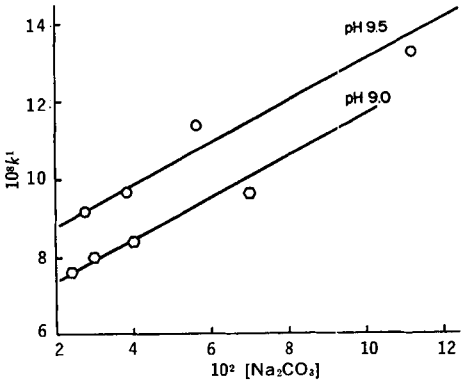


Figure 7—*Plots of apparent first-order rate constants for 10^{-4} M cytosine in various concentrations of carbonate–bicarbonate buffer systems at constant pH and ionic strength against the carbonate-ion concentrations.*

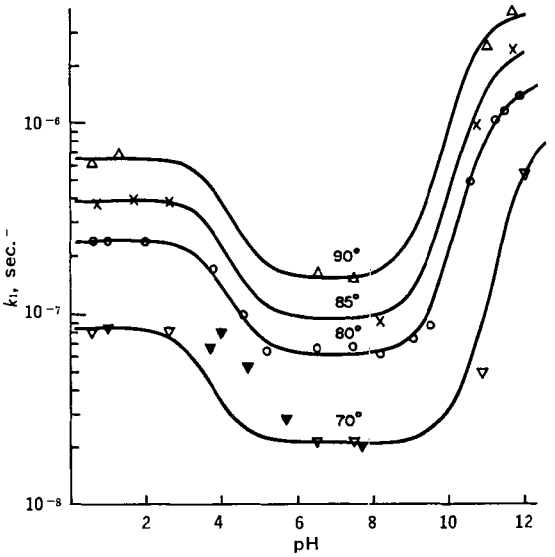


Figure 8—*Log k_1–pH profiles for the deamination of cytosine at several temperatures, where k_1 is the apparent first-order rate constant for deamination to uracil. The solid points at 70° are those of Notari* et al. *(14).*

80° values agreed well with the kinetic pK′a$_1$ values for both cytosine and cytidine (Table VI).

This pK′a$_1$ value at 80° and the experimentally determined pH-independent k_1 values in the pH ranges 0–3 and 6–8 were used to calculate the log k–pH profiles for cytosine and cytidine at several

Table V—Apparent First-Order Rate Constants at Zero Buffer Concentration, k^a, in sec.$^{-1}$, for the Hydrolytic Degradation of Cytosine and Cytidine

		$10^8 k$							
		Cytosine				Cytidine			
Buffer	pH	70.0°	80.0°	85.0°	90.0°	70.0°	80.0°	85.0°	90.0°
Acetate	3.81	—	16.9	—	—	—	49.5	—	—
	3.60	—	—	—	—	—	—	90.7	—
	4.58	—	9.80	—	—	—	18.0	—	—
	5.20	—	6.80	—	—	—	7.60	—	—
Phosphate	6.53	2.10	6.08	—	16.4	2.73	7.50	21.9	—
	7.48	2.11	6.79	—	15.2	2.61	7.39	21.2	—
Borate	8.18	—	6.08	9.30	—	7.60	—	15.4	—
Carbonate	9.05	—	7.5	—	—	—	10.6[b]	—	—
	9.50	—	8.8	—	—	—	58.0[c]	—	—

[a] Obtained from extrapolation to zero buffer concentration of the linear plots of apparent first-order rate constant for nucleoside degradation against the concentration of a buffer species. [b] The apparent rate constant for the uridine intermediate degradation under these conditions was 1.0×10^{-8} sec.$^{-1}$. [c] This overall rate constant for cytidine degradation can be broken down to $k_1 = 52.0 \times 10^{-8}$ sec.$^{-1}$ to uracil and $k_1' = 6.0 \times 10^{-8}$ sec.$^{-1}$ to nonchromophoric products. The apparent rate constant for the uridine intermediate degradation under these conditions was 36.0×10^{-8} sec.$^{-1}$.

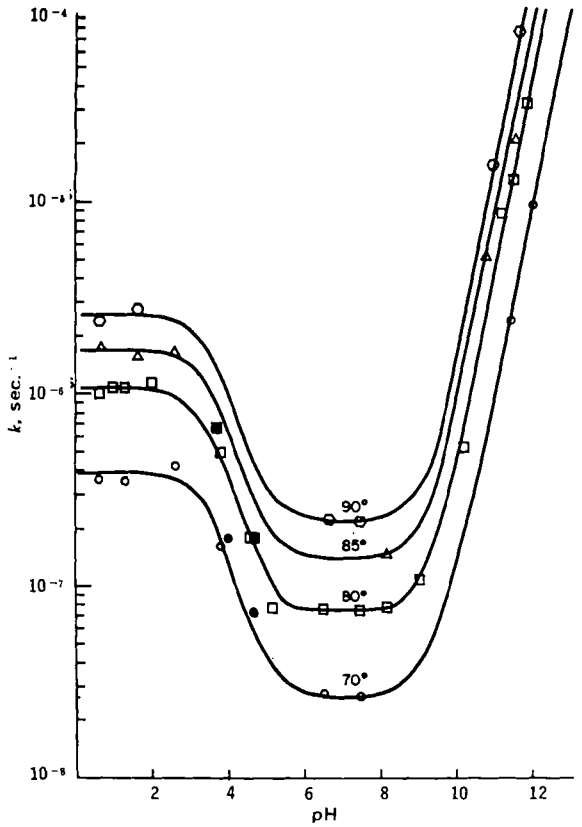


Figure 9—*Log* k_1–*pH profiles for the deamination of cytidine at several temperatures, where* k_1 *is the apparent first-order rate constant for deamination to uridine. The solid points at 70 and 80° are those of Notari* et al. *(14).*

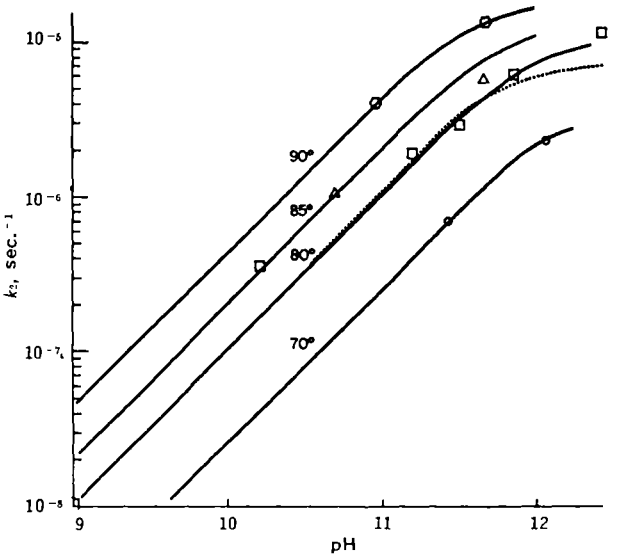


Figure 10—*Log* k_2–*pH profiles for the solvolysis of the first product of cytidine degradation at several temperatures, where* k_2 *is assigned to the apparent first-order rate constant for uridine solvolysis. The dashed line at 80° is based on the data of Garrett and Yakatan (8) for uridine solvolysis.*

other temperatures in the pH range 0–8 (Figs. 8 and 9). The buffer-independent rate constant data of Notari *et al.* (14) in the pH range 4–8, extrapolated from deamination rate constant studies in buffer solutions to zero buffer concentrations, are plotted in Fig. 8 for cytosine deamination at 70° and in Fig. 10 for cytidine deamination at 70 and 80°. The data of Notari *et al.* (14) for that pH range segment are in agreement with the complete log *k*–pH profiles given for cytidine (Fig. 9). However, their data for cytosine deamination in this pH range segment are not consistent with the constructed profile for cytosine deamination at 70° (Fig. 8). A pK′a_1 of approximately 5.0 would have to be postulated for the 70° cytosine deamination data to rationalize this discrepancy.

The deamination rates characterized by the determined rate constants k_1 for both cytosine and cytidine above pH 9 are catalyzed by hydroxyl ions (Figs. 8 and 9). A linear increase of log k_1 with increasing pH at a slope close to unity indicated a specific hydroxyl-ion-catalyzed reaction. The profiles of cytosine bend over at high pH values and indicate that a second acid dissociation constant, K_{a_2}', influences the rate of the reaction. The apparent first-order rate constant for cytosine deamination in alkaline solution may be formulated (1, 23–25) as:

$$k_1 = k_{OH}[OH]f_{HC} + k_{OH}'[OH]f_{C^-} + k'_{H_2O}f_{HC} \quad \text{(Eq. 7)}$$

where f_{HC} is the fraction of uncharged species and f_{C^-} is the fraction of the nucleoside as the anion in the solution. Thus (1, 23–25):

$$k_1 = \frac{k_{OH}K_w}{K_{a_2}' + [H^+]} + \frac{k_{OH}'K_{a_2}'[OH^-]}{K_{a_2}' + [H^+]} + \frac{k'_{H_2O}[H^+]}{K_{a_2}' + [H^+]} \quad \text{(Eq. 8)}$$

The log *k*–pH profiles of cytosine between pH 8 and 12 were fitted by assuming that:

$$k_1 = \frac{k_{OH}K_w}{(K_{a_2}' + [H^+])} + k'_{H_2O} \quad \text{(Eq. 9)}$$

since f_{HC} is approximately unity when k'_{H_2O} makes any significant contribution to the overall rate constant, k_1, for deamination. The bending over of the profiles indicates that the second terms of Eqs. 7 and 8 for hydroxide-ion attack on the nucleoside anions were nonexistent. The k_{OH} and K_{a_2}' were determined from the best

Table VI—Microscopic Rate Constants[a] for the Degradation of Cytosine and Cytidine

Compound	Temperature	$10^6k_{H_2O}$	$10^6k'_{H_2O}$	$10^6(k_{OH})_1$	$10^6(k_{OH}')_1$	$10^6(k_{OH})_2$	pK′a_1	pK′a_2
Cytosine	70.0°	8.50	2.10	4.98			—	11.9[b]
	80.0°	24.0	6.10	70.0			3.90[b], 3.7[c]	11.0[b]
	85.0°	39.0	9.40	167			—	10.8[b]
	90.0°	65.0	15.5	469			—	19.5[b]
				$10^6(k_{OH})_1$	$10^6(k_{OH}')_1$	$10^6(k_{OH})_2$		
Cytidine	70.0°	39.0	2.60	66.7	16.7	8.25	—	—
	80.0°	106	7.40	188	45.5	21.6	3.66[b], 3.61[c]	—
	85.0°	170	13.6	283	61.1	35.6	—	—
	90.0°	260	21.5	445	123	56.1	—	—

[a] Based on the kinetic expression for the overall apparent first-order rate constant for the degradation of cytidine: $k = k_{H_2O}f_{H_2C^+} + k_{H_2O}f_{HC} + [(k_{OH})_1 + (k_{OH}')_1][OH^-]f_{HC}$, where $(k_{OH})_1$ is the bimolecular rate constant for hydroxide-ion-catalyzed deamination of the neutral species and $(k_{OH}')_1$ is for hydroxide-ion-catalyzed cleavage of the cytosine ring. In the case of cytosine, $(k_{OH}')_1 = 0$. The $f_{H_2C^+} = [H^+]/([H^+] + K_{a_1}')$ is the fraction of the cytidine and cytosine of pKa_1′ which is protonated, $f_{HC} = K_{a_1}'/(K_{a_1}' + [H^+])$ in the pH range 0–10 is the fraction of pK′a_1 which is uncharged, and $f_{HC} = [H^+]/(K_{a_2}' + [H^+])$ at pH values >11 for the fraction of cytosine which is undissociated and has a pK′a_2 where the cytosine anion is not susceptible to hydroxide-ion-catalyzed degradation. The $(k_{OH})_2$ is the specific catalytic constant for hydroxide-ion-catalyzed hydrolysis of the uridine product of cytidine deamination. [b] Determined from the fitting of the log *k*–pH data. [c] Determined at 80° as the pH of half-neutralization.

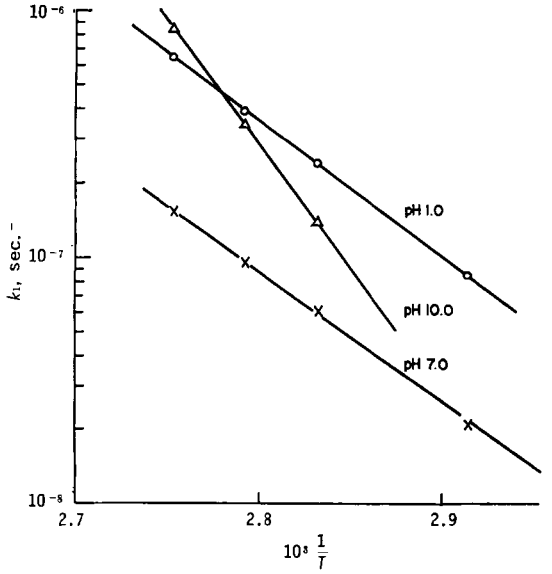


Figure 11—*Arrhenius plots for apparent first-order rate constants for the deamination of cytosine at several pH values.*

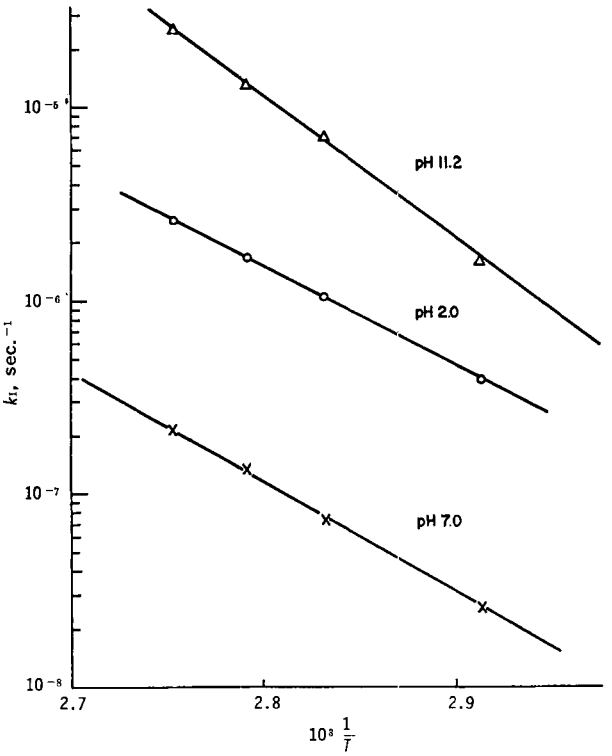


Figure 12—*Arrhenius plots for apparent first-order rate constants for the deamination of cytidine at several pH values.*

fittings of the log k–pH curves and are listed in Table VI. There was no significant degradation of cytosine other than through the uracil intermediate. Thus, $k_1' = 0$. The subsequent degradation of uracil in alkali was an extremely slow process (6), and k_2 for cytosine was difficult to determine accurately.

The log k–pH profiles for cytidine did not bend over at high alkalinities and did not indicate the formation of a nucleoside anion. Thus, the overall rate constant for deamination, k_1, could be characterized by $k_1 = (k_{OH}[OH^-] + k'_{H_2O})f_{HC}$, so that at high alkalinities when k'_{H_2O} is negligible and $f_{HC} = 1$:

$$\log k_1 = \text{pH} - \text{pKw} + \log k_{OH} \qquad \text{(Eq. 10)}$$

The k_{OH} values determined from the application of Eq. 10 to the alkaline branch of the log k–pH profiles of Fig. 9 are given in Table VI.

It was shown previously (8) that the solvolytic degradation of uridine in alkaline solution was specific hydroxide-ion-catalyzed attack on the monoanionic species. The fact that the log k–pH profile for uridine degradation bent over above pH 11.5 indicated a second acid dissociation constant of uridine, most likely in the sugar moiety, which produced a dianion resistant to hydroxide-ion attack. The log k_2–pH profiles for different temperatures are plotted in Fig. 10 and are consistent with this prior observation. The literature data for uridine solvolysis (8) are also plotted in Fig. 10 and show that the evaluated k_2 for the postulated mechanism (Scheme II) of cytidine degradation is consistent with deamination to uridine, with its subsequent alkaline degradation.

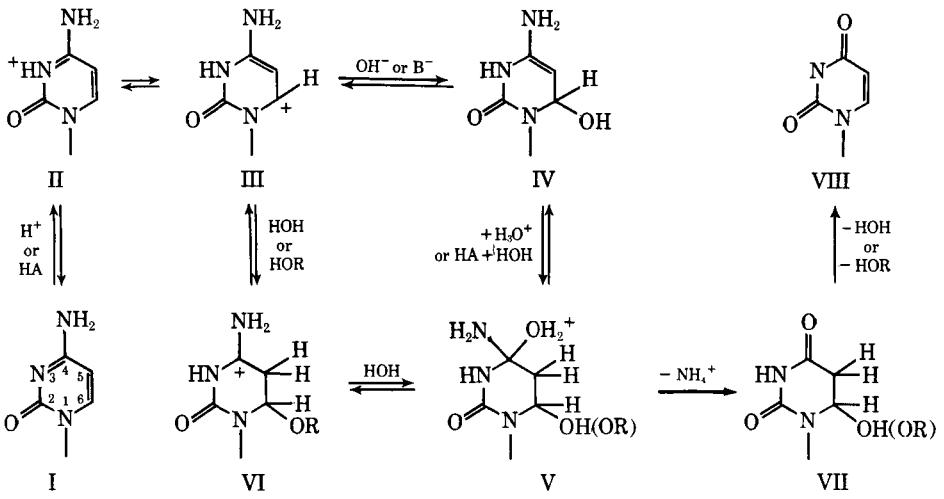
The k_1' value for cytidine was determined with less accuracy. However, the log k_1'–pH profiles at different temperatures could still be constructed and indicate a specific hydroxyl-ion-catalyzed degradation of cytidine to nonchromophoric products, $k_1' = k_{OH}'[OH^-]$, occurring in parallel with its specific hydroxyl-ion-catalyzed deamination to uridine, $k_1 = k_{OH}[OH^-]$.

Rate Dependency on Temperature—Arrhenius parameters for the solvolysis of cytosine and cytidine can be obtained from the slopes and intercepts of plots (Figs. 11 and 12) of log k_i *versus* $1/T$ according to the expression:

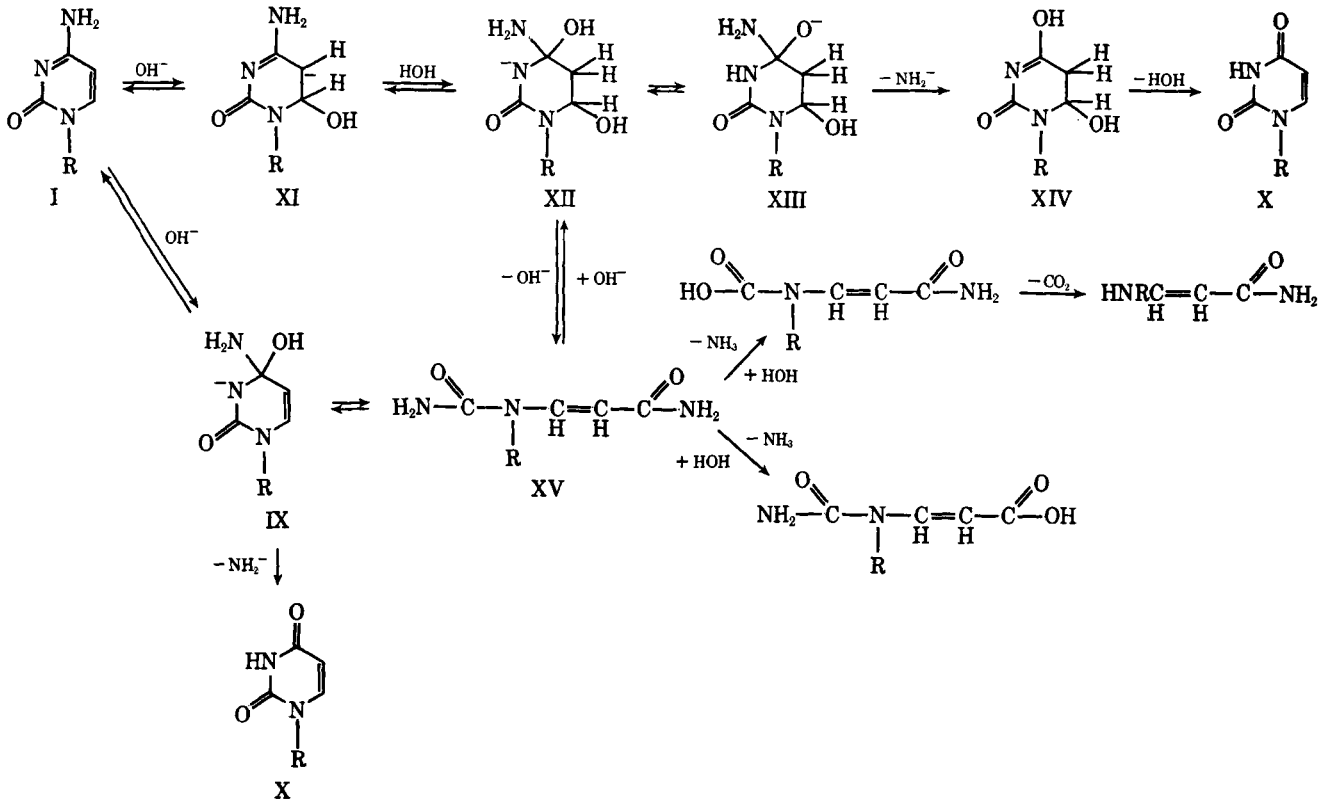
$$\log k = \log P - (E_a/2.303R)1/T \qquad \text{(Eq. 11)}$$

where E_a is the activation energy, P is the frequency factor, and R is 1.987 cal. deg.$^{-1}$ mole^{-1}.

For cytosine, the energies of activation at the several pH values



Scheme III



Scheme IV

were (kcal./mole, pH): 12.7, 1.0; 12.2, 7.0; and 23.2, 9.8. For cytidine, they were: 11.8, 2.0; 13.0, 7.0; and 17.7, 11.0.

Possible Mechanisms for Solvolytic Transformations of Cytosine and Cytidine—Shapiro and Klein (11) stated that there are two possible mechanistic explanations for the specific hydrogen-ion and general acid–base-catalyzed deamination of the cytosine derivatives, I in Scheme III. The one mechanism was analogous to classical amide hydrolysis, wherein water attack on the protonated (in the 3-position) cytosine moiety, II, could be enhanced by general base attack on water to give hydroxyl-ion attack on the protonated species at the 4-position with subsequent loss of ammonia. The proposed alternative method of partial saturation at the 5–6-position, IV, of the ring of the protonated cytosine by the nucleophilic addition of the buffer anion with subsequent deamination by water attack (IV → V → VII) and subsequent loss of the acid conjugate of the buffer anion to give a uracil derivative, VIII, was preferred. This mechanism was considered to be more consistent with the information in the literature showing that hydrolytic deamination was facilitated when cytosine derivatives were saturated in the 5–6-position. Notari (12) and Notari *et al.* (13, 14) also preferred this latter explanation, initially (12) with the additional restriction that the base or anion that attacks the 6-position, III → IV, following the initial protonation at the 3-position, I → II, must have a donatable proton which can saturate the 5-position and promote the nucleophilic attack of water on the positively charged 4-position leading to the loss of ammonium ion and a resultant 5–6-saturated uracil, VII, which can readily form a uracil derivative, VIII, by the elimination of the original attacking anion. This latter explanation was based on the assumption that only negatively charged di- or *n*-polyprotic acids were proper catalytic species when they still contained a donatable proton. Our data, demonstrating that the significant catalytic species in carbonate buffer is carbonate ion (Fig. 7 and Tables III and IV), are not consistent with this hypothesis.

This restriction that the general base catalyst must have a donatable proton was apparently withdrawn since Notari *et al.* (13, 14) admitted acetate buffer-catalyzed deamination of cytosine derivatives in subsequent papers. Both acetate ions and acetic acid were presumed to be implicated in the catalytic deamination of cytidine and cytosine, but only the acetic acid species in the buffer was presumed to deaminate catalytically cytosine arabinoside. The precision of the data in this paper (Fig. 6 and Tables I and IV) does not permit the presumption that acetate ion is a significant catalytic species in acetate buffers for deamination of cytidine[9].

The recent proof (15–19) of the reversible bisulfite addition to the 5,6-double bond of cytosine in its derivatives with subsequent ready deamination of the adduct is potent evidence for the postulated steps of Scheme III.

The HOR attack on III in Scheme III includes the sterically favored intramolecular attack of the 2′-hydroxyl from the sugar moiety of cytosine arabinoside, which was postulated by Notari *et al.* (13, 14) to rationalize the enhanced deamination rate of this compound in contrast to cytidine and cytosine.

If water attack on the protonated species or decomposition of a subsequent adduct is rate determining in the process II → III → VI → V → VII → VIII, this rationalizes the rate constant k_{H_2O} in Eq. 4. The rate-determining hydroxide-ion attack on the protonated species or decomposition of a subsequent adduct in the process III → IV → V → VII → VIII is kinetically equivalent to water attack on the protonated molecule, I, and rationalizes the rate constant k'_{H_2O} in Eq. 4, where the specific ion catalytic rate constant for hydroxide-ion attack on the protonated cytosine derivative is:

$$k_{OH}'' = k'_{H_2O} K_a/K_w \qquad \text{(Eq. 12)}$$

General acid–base catalyses can be rationalized either by accepting the premise that the general acids or bases add to the 5,6-double bond to form an intermediate (as in steps III → IV → V or III → VI → V) which more readily undergoes deamination (V → VII) or that they favor the hydrated form at intermediate pH values.

At higher alkalinities (Figs. 8 and 9 and Tables I–III and VI), there is a significant hydroxide-ion-catalyzed deamination of the uncharged cytosine derivative. This can be formulated (Scheme IV) as a direct hydroxide-ion attack on the 4-carbon of the cytosine moiety (I → IX → X) in analogy with the classical mechanism for amide solvolysis (22). An alternative process can be formulated (Scheme IV) wherein the initial hydroxide attack (I → XI) is at the 6-position in cytosine. This leads to an activated complex (XII or

[9] However, we are willing to accept Dr. Notari's confidence in his data, which he intends to publish, that acetate ion is such a catalytic species.

XIII) similar to IX of the classical mechanism except for saturation of the 5,6-bond, but it may be favored since 6-position attack by hydroxide ions on uracils and pyrimidine nucleosides is well documented (4, 6, 8). The resultant active intermediate (IX or XII) may deaminate (XII → XIII → XIV → X or IX → X) to give a uracil derivative, X, or the cytosine ring may open to give a nonchromophoric compound, IX or XII → XI. The subsequent deamination and/or decarboxylation of this acyclic product makes this pathway irreversible. These postulated mechanisms of Scheme IV readily explain the parallel deamination of cytidine to uridine and its direct loss to a nonchromophoric product in agreement with Scheme II.

REFERENCES

(1) E. R. Garrett, *J. Amer. Chem. Soc.*, **82,** 827(1960).

(2) E. R. Garrett and L. J. Hanka, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 526(1960).

(3) E. R. Garrett, T. Suzuki, and D. J. Weber, *J. Amer. Chem. Soc.*, **86,** 526(1964).

(4) E. R. Garrett, P. B. Chemburkar, and T. Suzuki, *Chem. Pharm. Bull.*, **13,** 1113(1965).

(5) E. R. Garrett, J. K. Seydel, and A. J. Sharpen, *J. Org. Chem.*, **31,** 2219(1966).

(6) E. R. Garrett, J. H. Nestler, and A. Somodi, *ibid.*, **33,** 3460 (1968).

(7) H. J. Nestler and E. R. Garrett, *J. Pharm. Sci.*, **57,** 1117 (1968).

(8) E. R. Garrett and G. J. Yakatan, *ibid.*, **57,** 1478(1968).

(9) H. Schuster and G. Schramm, *Z. Naturforsch.*, **136,** 697 (1958).

(10) D. O. Jordan, "The Chemistry of Nucleic Acids," Butterworths, Washington, D. C., 1960, p. 65.

(11) R. Shapiro and R. S. Klein, *Biochemistry*, **5,** 2358(1966).

(12) R. E. Notari, *J. Pharm. Sci.*, **56,** 804(1967).

(13) R. E. Notari, M. L. Chin, and A. Cardoni, *Tetrahedron Lett.*, **40,** 3449(1969).

(14) R. E. Notari, M. L. Chin, and A. Cardoni, *J. Pharm. Sci.*, **59,** 28(1970).

(15) H. Hayatsu, *J. Amer. Chem. Soc.*, **91,** 5693(1969).

(16) H. Hayatsu and M. Yano, *Tetrahedron Lett.*, **1969,** 755.

(17) R. Shapiro, R. E. Servis, and M. Welcher, *J. Amer. Chem. Soc.*, **92,** 422(1970).

(18) H. Hayatsu, Y. Wataya, and K. Kai, *ibid.*, **92,** 724(1970).

(19) H. A. Hayatsu, Y. Wataya, K. Kai, and S. Iida, *Biochemistry*, **9,** 2858(1970).

(20) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958.

(21) D. Shugar and J. J. Fox, *Biochim. Biophys. Acta*, **9,** 199 (1952).

(22) J. J. Fox and D. Shugar, *ibid.*, **9,** 369(1952)

(23) E. R. Garrett, *Arzneim.-Forsch.*, **17,** 795(1967).

(24) E. R. Garrett, *J. Amer. Chem. Soc.*, **80,** 4049(1958).

(25) E. R. Garrett, *Advan. Pharm. Sci.*, **2,** 1(1967).

(26) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," vol. 1, W. A. Benjamin, New York, N. Y., 1966.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 15, 1971, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601*

Accepted for publication February 9, 1972.

Supported in part by Grants GM-09864 and CA 10738 from the National Cancer Institute and the National Institutes of Health, U. S. Public Health Service.

The authors acknowledge preliminary experiments performed on parts of this work by Dr. Hans J. Nestler and Dr. A. G. Cusimano.

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Drug Absorption VI: Water Flux and Drug Absorption in an *In Situ* Rat Gut Preparation

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Abstract □ The influence of net water transfer on drug absorption occurring in an *in situ* rat intestinal preparation was studied using hypotonic, hypertonic, and isotonic intestinal lumenal solutions composed of sodium phosphate and sodium chloride. It was found that the tonicity of the lumenal solution tended to change to a slightly hypertonic value when the initial solutions were isotonic or hypertonic and to isotonicity when the lumenal solution was initially hypotonic. Net water flux occurred from hypotonic solutions and into hypertonic solution. Water loss from isotonic solutions ranged between 7 and 17% in 3.0 hr. The results indicated that the intestinal water loss or gain which occurred with the different tonicity solutions altered the apparent rate constant for sulfaethidole absorption. It was found that the changes could be accounted for by correcting the rate constant for volume and relative available surface area and that when this was done there were no differences between permeability constants for sulfaethidole from the iso- and hypotonic solutions for absorption. However, the hypertonic solution had a reduced permeability constant.

Keyphrases □ Drug absorption—effect of tonicity and net water transfer, *in situ* rat intestine □ Intestinal absorption, *in situ*, rat—effect of tonicity and net water transfer

In situ rat gut techniques, based on a simple surgical operation and measurement of drug disappearance from the gut perfusion solution, are often used in studies of drug absorption. A single perfusion method (1), a recirculation method (2), and the method of Doluisio *et al.* (3) are examples of *in situ* gut techniques that have been used by various workers to study the dynamics of the drug absorption process. One problem common to all these methods is the loss of water that occurs during experimentation. In our laboratories, we attempted to minimize this factor by limiting experimentation to less than 40 min.; this time is adequate for drugs that are absorbed rapidly, but it is not adequate for drugs that are absorbed slowly. Hayton (4) conducted *in situ* experiments for longer periods and compensated for water loss by periodically adding solvent (isotonic