(54) Theory: L. Radom, J. Paviot, J. A. Pople, and P. v. R. Schleyer, *J. Chem. Soc., Chem. Commun.*, 58 (1974); R. Hoffmann, L. Radom, J. A. Pople, P. v. R. Schleyer, W. J. Hehre, and L. Salem, *J. Am. Chem. Soc.*, **94**, 6221 (1972).
(55) Experiment: A. J. Dobbs, B. C. Gilbert, and R. O. C. Norman, *J. Magn. Reson.*, 11, 100 (1973); K. S. Chen and J. K. Kochi, *J. Am. Chem. Soc.*, **96**, 1383 (1974), and references cited therein.
(56) (a) R. Livingston and H. Zeldes, *J. Am. Chem. Soc.*, **88**, 4333 (1966); (b) C. von Sonntag and E. Thoms, *Z. Naturforsch., B*, **25**, 1405 (1970); (c) C. E. Burchill and K. M. Perron, *Can. J. Chem.*, **49**, 2382 (1971); (d) B. C. Gilbert, J. P. Larkin, and R. O. C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 794 (1972), and earlier papers; (e) K. M. Bansal, M. Gratzel, A. Henglein, and E. Janata, *J. Phys. Chem.*, **77**, 16 (1973); (f) A. Samuni and P. Neta, *Ibid.*, **77**, 2425 (1973); (g) C. Walling and R. A. Johnson, *J. Am. Chem. Soc.*, **97**, 2405 (1975).
(57) (a) T. Foster and P. R. West, *Can. J. Chem.*, **51**, 4009 (1973); (b) *ibid.*, **52**, 3589 (1974); see also ref 56f.
(58) E. Hayon and M. Simic, *Acc. Chem. Res.*, **7**, 114 (1974).
(59) Y. L. Chow, *Acc. Chem. Res.*, **6**, 354 (1973).
(60) T. Mojelsky and Y. L. Chow, *J. Am. Chem. Soc.*, **96**, 4549 (1974).
(61) Cf. the Cu^{2+} oxidation of 1,1-dimethyl-2-hydroxy-2-methoxycyclopropane which gives $CH_2C(CH_3)_2CO_2CH_3$ and $C(CH_3)_2CH_2CO_2CH_3$: S. E. Schaafsma, E. J. F. Molenaar, H. Steinberg, and Th. J. de Boer, *Recl. Trav. Chim. Pays-Bas*, **86**, 1301 (1967).
(62) P. Dowd, M. Shapiro, and K. Kang, *J. Am. Chem. Soc.*, **97**, 4754 (1975).
(63) 6 Å apart according to B. M. Babior, T. H. Moss, W. H. Orme-Johnson, and H. Beinert, *J. Biol. Chem.*, **249**, 4537 (1974); see also K. L. Shepler, W. R. Dunham, R. H. Sands, J. A. Fee, and R. H. Abeles, *Biochim. Biophys. Acta*, **397**, 510 (1975).

Tautomerism in Cytosine and 3-Methylcytosine. A Thermodynamic and Kinetic Study

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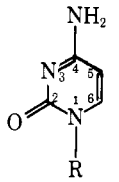
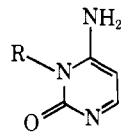
Received December 26, 1975

Abstract: Aqueous cytosine, which exists mainly as the 1(*H*)-aminooxo form (Ia), is shown by temperature-jump spectroscopy to tautomerize slightly to the 3(*H*)-aminooxo form (IIa); the equilibrium constant K = (IIa)/(Ia) is estimated to be (2.5 ± 0.5) × 10^{-3} at 25 °C and the tautomerization enthalpy is 3.1 ± 0.1 kcal M^{-1}. At 10 °C, The interconversion process is catalyzed by water [k_{H_2O} = (1.8 ± 0.3) × 10^3 s^{-1}], by H^+ [k_{H^+} = (1.6 ± 0.3) × 10^{10} M^{-1} s^{-1}], by OH^- [k_{OH^-} = (1.2 ± 0.05) × 10^{10} M^{-1} s^{-1}], and by the cytosinium cation [k_{cation} = (4.4 ± 0.2) × 10^8 M^{-1} s^{-1}]. On the other hand, 3-methylcytosine is shown by uv and ir spectroscopy to exist as a mixture of two tautomeric forms, the aminooxo form IIb and the iminooxo form IIIb; while IIIb is the major form in nonpolar solvents, the amino form IIb largely predominates in water, the equilibrium constant K' = (IIIb)/(IIb) being estimated as ca. 3 × 10^{-2} at 25 °C. The IIb ⇌ IIIb interconversion mechanism was investigated in D_2O solution at 10 °C by temperature-jump spectroscopy. The interconversion is catalyzed by D_2O [k_{D_2O} = (2.9 ± 0.3) × 10^4 s^{-1}], by OD^- [k_{OD^-} = (4.3 ± 0.2) × 10^9 M^{-1} s^{-1}], and by cationic 3-methylcytosine [k_{cation} = (2.2 ± 0.1) × 10^8 M^{-1} s^{-1}]. In both tautomeric equilibria the solvent-catalyzed term in the rate law is shown to be closely related to the basicity of the minor tautomer. The thermodynamics and kinetics of tautomerization of cytosine and cytidine to their rare imino forms are finally tentatively discussed in the light of this work.

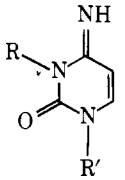
It is now firmly established, by a variety of physical methods,[1] that cytosine exists in solution essentially in the 1(*H*)-aminooxo tautomeric form, Ia.



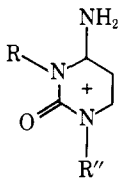
Ia, R = H
b, R = CH_3



IIa, R = H
b, R = CH_3



IIIa, R = R′ = H
b, R = CH_3; R′ = H
c, R = R′ = CH_3



IV

However, as many as six other structures can formally be written for the cytosine molecules, so that small amounts of rare tautomeric forms may exist along with Ia. Much theoretical work has been concerned with the relative stabilities of the various tautomers,[2] but so far, none of the rare forms has been unambiguously observed experimentally. Our present knowledge on the possible occurrence of some of them is based on indirect arguments, such as comparison of the pK's of various nontautomeric methylated derivatives.[3] It has been concluded this way that Ia should predominate over IIa by a factor of about 800 in aqueous solution, while the iminooxo form IIIa would be even less favored, the equilibrium constant K = (IIIa)/(Ia) being about 2 × 10^{-5}. These estimates relied heavily on the assumption that the cations resulting from protonation of cytosine and its methylated derivatives 3-methylcytosine and 1,3-dimethylcytosine all have the common structure IV; another, less severe restriction arises from the hypothesis that the substitution of the labile proton by a methyl group does not modify significantly the pK of the various tautomers.

Despite increasing evidence showing that cytosine and its methylated analogues do form cations of similar structure,[3a,4] there has been a trend in the literature to disregard these conclusions, possibly because they are based on rather indirect arguments. Thus, several claims for a much higher proportion of the iminooxo tautomer IIIa have been published[5-7] and criticized.[8,9] Therefore, there clearly exists a need for a direct characterization of the rare tautomeric forms of cytosine, especially in water. Unfortunately, no experimental technique will, at present, allow the detection of a tautomeric form present in a proportion as low as 2 × 10^{-5}. However, this difficulty could be overcome indirectly by studying separately the two equilibria

$$\text{Ia} \overset{K}{\rightleftharpoons} \text{IIa and IIb} \overset{K'}{\rightleftharpoons} \text{IIIb}$$

This prompted us to undertake an investigation of the tautomerism of cytosine and 3-methylcytosine in water, using the temperature-jump relaxation technique together with uv and ir spectroscopy, and the results of this study are reported here. Temperature-jump relaxation has proved to be well adapted to investigations on tautomerism, since the amplitude of the observed relaxation is a simple function, in most cases, of the equilibrium constants and enthalpies;[10] moreover, this technique will provide valuable information, totally lacking at present, about the kinetics of the tautomerization reaction and, thus, about the lifetimes of rare cytosine tautomers in aqueous solution.

Experimental Section

Materials. Cytosine (Merck) was twice sublimed under vacuum and repeatedly recrystallized from water. 1-Methylcytosine (Cyclochemicals) was recrystallized from water. 3-Methylcytosine hydrochloride (Sigma) was used as such or neutralized to obtain the free base (recrystallized from $CHCl_3$ or aqueous acetone). Deuteration of the exchangeable protons of 3-methylcytosine was achieved by dissolution of the base in D_2O (99.7% D, a product from Spectrometrie Spin et Technique), followed by freeze drying; this procedure was repeated until no -NH stretching band was detectable in the ir spectrum. Small amounts of 1,3-dimethylcytosine were conveniently obtained from 1-methylcytosine according to a procedure used by Brookes and Lawley[11] for the methylation of cytidine; 2,3-dihydro-1*H*-5-oxoimidazo[1,2-*c*]pyrimidine hydrochloride (compound Va below + HCl) and 2,3-dihydro-1-methyl-5-oxoimidazo[1,2-*c*]pyrimidine hydrochloride (Vb, HCl) were prepared according to literature procedures.[12] The free bases were extracted from the salts by neutralization, followed by vacuum sublimation (IIIc and Vb) or chloroform extraction (Va). Uv molar extinction coefficients agreed with previous literature reports.

Solutions for the kinetic runs were prepared by dissolving weighted amounts of bases in 50 ml of a 0.2 M solution of $NaClO_4$ (Merck reagent grade, dried at 130 °C) in distilled water or D_2O. As judged from their uv spectra, the solutions did not undergo any photochemical modification[13] during the kinetic runs, despite prolonged exposure to the powerful uv illuminator of the temperature-jump detection system; furthermore, the kinetic experiments were reproducible regardless of the illuminating conditions used.

Solvents used in this work were spectral grade and were usually used without further purification. However, dioxane (Merck Spectrograde) was refluxed overnight over KOH pellets and distilled over sodium before use; unstabilized chloroform (Sigma, IR Spectrograde) was shaken in the dark with saturated aqueous $NaHCO_3$ for 48 h to remove traces of HCl and then distilled over P_2O_5 immediately before use.

Kinetic Apparatus and Data Processing. Temperature-jump experiments were performed as described elsewhere,[10] except that we used a new detection device from Messanlagen Studiengessellschaft (minimum risetime 100 ns), the output of which is linear in OD scale, irrespective of the magnitude of the OD changes. Whenever the light level made it possible, we used narrow spectral slits (spectral bandwidth 2.5 nm) and a suitable filter to cut off some visible light which is invariably transmitted through the uv monochromator. As described previously, the detection output was fed into a PDP 11 processor (Digital Equipment Corp.) through an analogic/digital converter. The data acquisition process is delayed with respect to the beginning of solution heating, the delay time being usually twice the heating time constant (τ_h) or more. The deviation of the optical density from its final value (y) is then computed as a function of time, and the relaxation time (τ) is obtained from the equation $\ln y = \ln y_0 - t/\tau$.

In our previous paper, the relaxation amplitude (A) was set equal to the value of y extrapolated to zero delay (y_0). However, this procedure is valid only if $\tau_h \ll \tau$. If this does not hold, A should be computed from the complete expression of y as a function of time,[14] viz.

$$y = A\left[\frac{\tau_h}{\tau_h - \tau}\, e^{-t/\tau_h} - \frac{\tau}{\tau_h - \tau}\, e^{-t/\tau}\right]$$

so that A is now given by $y_0\,[(\tau - \tau_h)/\tau]$. The heating time, τ_h, was taken as $RC/2$, where C is the capacity of the discharging capacitor and R is the measured resistance of the sample cell. In practice A was found to differ appreciably from y_0 for $\tau < 50$ μs, viz. for many of the relaxation experiments reported in this work, while the approximation $A = y_0$ was usually justified in our previous paper. The amplitudes quoted in the text are expressed in OD units and refer to a 7-mm optical path cell.

Initial temperature (t_i) and final temperature (t_f) were estimated as in our previous paper;[10] for kinetic measurements, standard experimental conditions were $t_i = 1$ °C, $t_f = 10 \pm 1$ °C, and $\tau_h = 6 \pm 1$ μs (aqueous solutions) or $t_i = 4$ °C, $t_f = 10 \pm 1$ °C, and $\tau_h = 2.7 \pm 0.5$ μs (deuterium oxide solutions). An EA 107 T Metrohm glass electrode, which has a short response time in cold water, was used for pH measurements. pD measurements were made using a pH meter standardized in water, the pD values being deduced from the measured pH by applying the usual[15] correction:

$$\text{pD} = \text{pH} + 0.40$$

Uv Spectra and p*K* Measurements. All uv spectra were obtained using a Cary 15 uv spectrophotometer fitted with thermostated cells. Spectra presented below were run in suitable buffers to ensure that only pure neutral forms of the bases were present.

The p*K*'s (proton gain) of 3-methylcytosine and 1,3-dimethylcytosine were measured spectroscopically as a function of temperature (range 10–60 °C). The p*K* of 3-methylcytosine was found to be close to the pH of the two phosphate primary NBS buffers [KH_2PO_4 (0.025 M) + Na_2HPO_4 (0.025 M), and KH_2PO_4 (0.0087 M) + Na_2HPO_4 (0.0304 M)] while that of 1,3-dimethylcytosine is bracketed by the pH of two other primary NBS buffers, viz. borax buffer ($Na_2B_4O_7$, 0.01 M) and carbonate buffer [$NaHCO_3$ (0.025 M) + Na_2CO_3 (0.025 M)]. Therefore, the spectra of the bases were run in these buffers and compared to the spectra of pure cationic and neutral forms to estimate the p*K* (the Debye-Hückel formula was used to obtain the p*K* at zero ionic strength). Salts purchased from Beckman, identical with primary standards, were used to prepare the buffer solutions. Since the pH of the buffers is known accurately at various temperatures,[16] this procedure allows a reasonably precise determination of ionization enthalpies, especially for 3-methylcytosine for which the plot of p*K* vs. $(1/T)$ showed no curvature. p*K* values quoted in Table I have been measured in this way.

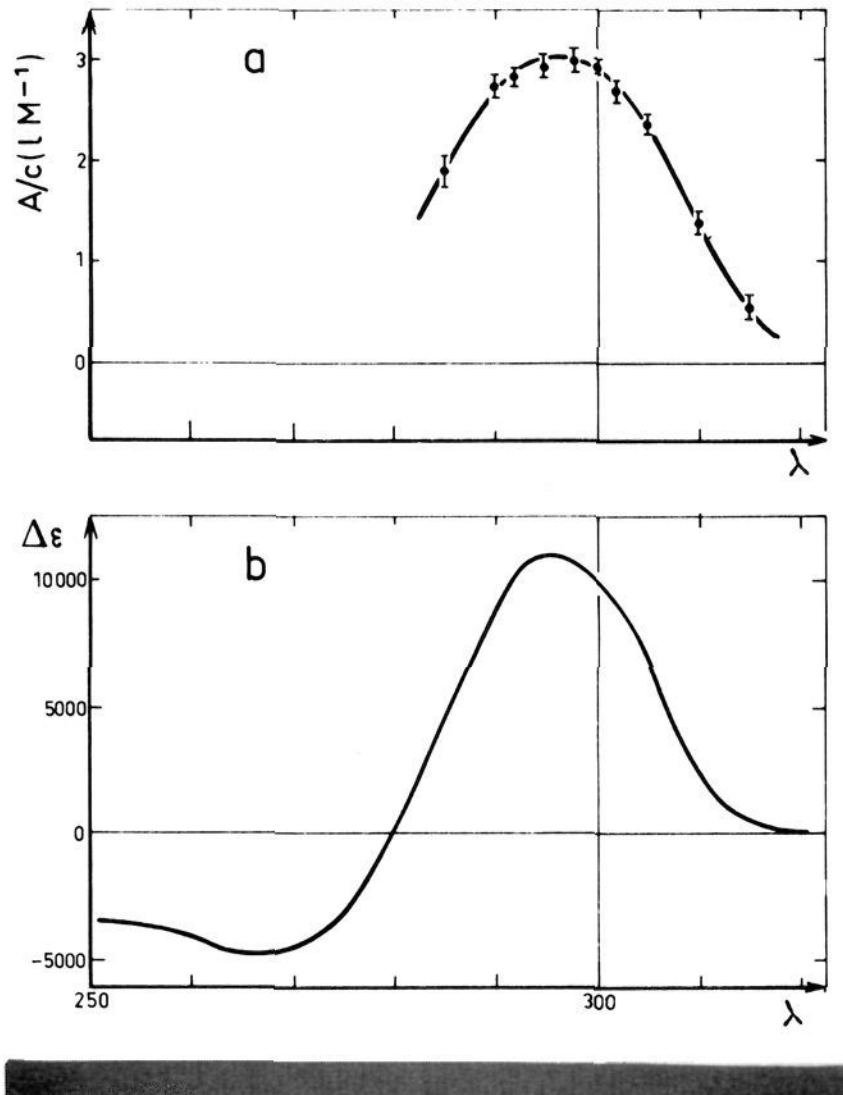
The second p*K* (proton loss) of 3-methylcytosine was determined approximately by recording the spectrum in dilute sodium hydroxide solutions; an extrapolation procedure was used since conversion to the pure anionic form was not complete, even in 1 N NaOH. The p*K*'s (proton gain) of 3-methylcytosine and 1,3-dimethylcytosine in D_2O were estimated by adding 0.5 p*K* unit to the p*K* in water at the same temperature.[17]

Ir Spectroscopy. Ir spectra were recorded on a Perkin-Elmer 225 spectrometer; the optical length was 0.5 cm (KBr windows) in the double bond region or 1 cm (Infrasil cells) in the NH stretching region. 3-Methylcytosine was only slightly soluble in chloroform (ca. 5×10^{-4} M), so that saturated solutions were used to record the spectra. The spectrum of 3-methylcytosine-d_2[18] was run in D_2O-saturated $CHCl_3$ to prevent dedeuteration of the very dilute solute by traces of water present in solution. The solubility of water in chloroform is considered low enough not to alter significantly the solvent properties.

Table I. Thermodynamic Data Used in This Work[a]

Compound	pK_1 (proton gain)	pK_2 (proton loss)
Cytosine	4.58 ± 0.01 (5.14 ± 0.02)[b]	12.15 ± 0.05 (11.5 ± 0.1)[b]
3-Methylcytosine	7.43 ± 0.02 (8.3$_5$ ± 0.1)	14.0 ± 0.1[c]
1,3-Dimethylcytosine	9.23 ± 0.03 (11.5 ± 0.3)	

[a] At 25 °C unless otherwise stated; values in parentheses are dissociation enthalpies (kcal/mol). [b] Reference 23. [c] At 10 °C.



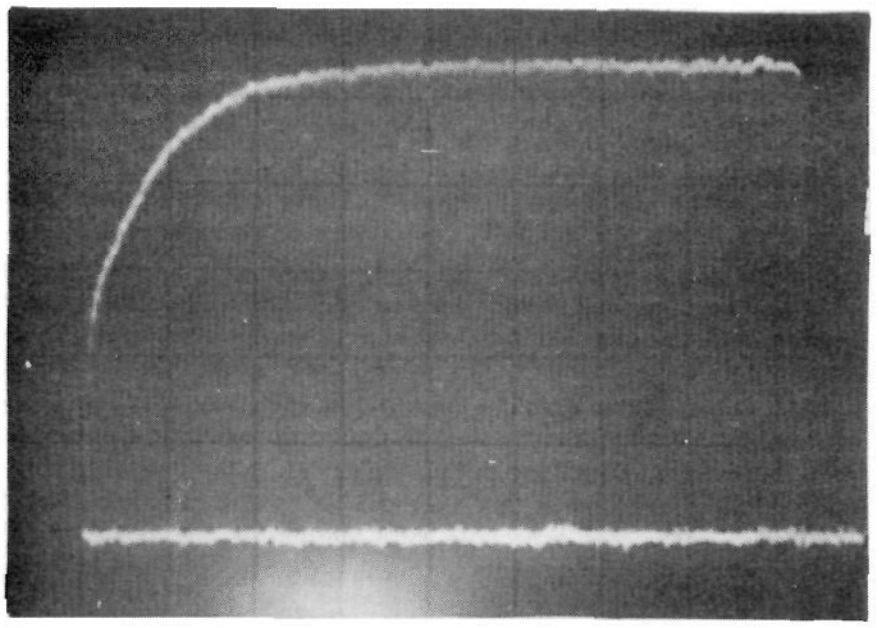


Figure 1. (Upper) (a) Ratio of the relaxation amplitude to the cytosine concentration vs. the wavelength: t_i = 1 °C; t_f = 10 °C; uncertainties are the standard deviations. Positive ordinates correspond to an OD rise with increasing temperature. (b) Plot of the molar extinction coefficient difference ($\epsilon_{3\text{-methylcytosine}} - \epsilon_{1\text{-methylcytosine}}$) as a function of λ at 20 °C. (Lower) Relaxation spectrum of cytosine in water, recorded for a wavelength of 295 nm: initial temperature (t_i) 0 °C; final temperature (t_f) 8 ± 0.5 °C; pH (as measured at t_i) 7.80; cytosine concentration = 2.6×10^{-3} M; vertical scale, 2×10^{-3} OD unit per major division; horizontal scale, 200 μs per major division. The signal corresponds to an increase of the solution OD.

Results

In the following text, unprimed symbols refer to thermodynamic and kinetic constants for the Ia ⇌ IIa equilibrium, while primed symbols are used for the IIb ⇌ IIIb equilibrium; overall dissociation constants corresponding to proton gain and proton loss are denoted pK_1 (or pK_1') and pK_2 (or pK_2'), respectively.

I. Cytosine Relaxation Spectrum. When the optical density of an aqueous solution of cytosine (5×10^{-4} to 4×10^{-3} M), submitted to a fast temperature jump, is recorded as a function of time, in the spectral range 290–310 nm, two successive phenomena are observed: (a) a fast rise in OD with a time constant shorter than that of the heating process; a similar phenomenon is observed with solutions of 1-methylcytosine (Ib) and cytidine (I, R = ribose), as well as with solutions of many pyrimidines and purines, and presumably arises from variation of the extinction coefficients with temperature; and (b) a slower exponential OD rise. This slow relaxation process is not observed with 1-substituted derivatives. We shall limit ourselves to the study of this second relaxation, which is supposed to be related to a chemical change occurring in the cytosine solution.

Table II. Variation of the Relaxation Amplitude A (OD Units) with Temperature (t_i)[a] (Cytosine Solution)

t_i, °K	$A \times 10^3$	σ (A) mult 10^3
274	2.08	0.08
284	2.23	0.10
297	2.68	0.10
303.5	2.70	0.15
313	3.20	0.15

[a] λ = 300 nm; pH is near neutrality; σ is the standard deviation; $C_{\text{cytosine}} = 6.u \times 10^{-4}$ M.

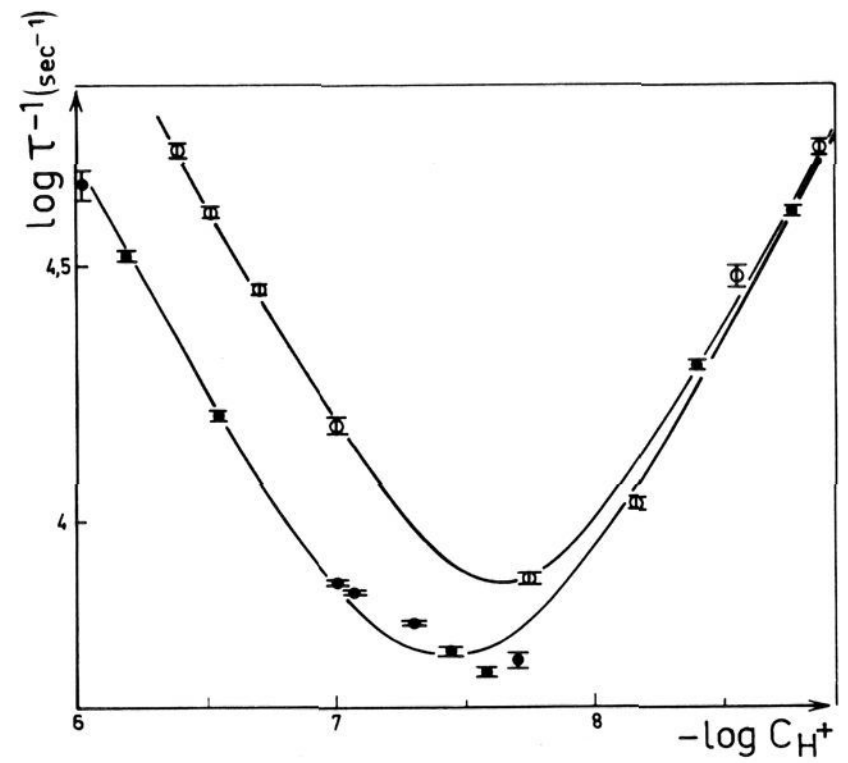


Figure 2. For cytosine, log τ^{-1} as a function of $-\log \bar{C}_{H^+}$. Cytosine concentration, 1.16×10^{-3} M (lower curve) and 3.59×10^{-3} M (upper curve); t_i = 1 °C; t_f = 10 °C; λ = 300 nm.

The relaxation amplitude was found to be pH independent in the pH range 6–9 and proportional to the cytosine concentration. It varies with the wavelength of the analyzing light with a maximum at 297 nm [Figure 1 (a)]. We were unable to study the wavelength dependence of the amplitude below 280 nm because of the unsuitable signal–noise ratio. Increasing the temperature leads to an increase of the amplitude (Table II).

The relaxation time was found to be very sensitive to pH (Table III and Figure 2). It was insensitive to the cytosine concentration for pH >8, but at lower pH, definite evidence for an increase of τ^{-1} was observed when the cytosine concentration was increased (Figure 2). Therefore, the relaxation time was tentatively fitted by the equation

$$\tau^{-1} = k_0 + k_{H^+}\bar{C}_{H^+} + k_{OH^-}\bar{C}_{OH^-} + k_{\text{cation}}\bar{C}_{\text{cation}} \quad (1)$$

where the $\bar{C}_i$ are the equilibrium concentrations corresponding to the final temperature (t_f), computed according to the appendix of ref 10, and $\bar{C}_{\text{cation}}$ stands for the cytosinium ion concentration.

Table III. Variation of the Inverse of the Relaxation Time (τ^{-1}) and of the Amplitude (A) with pH and Cytosine Concentration

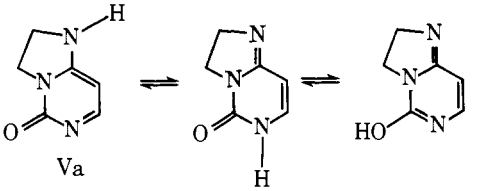
$C_{cytosine}$, M × 10^3	pH[a]	τ^{-1} (exptl), s^{-1} × 10^{-4}	τ^{-1} (calcd),[c] s^{-1} × 10^{-4}	A × 10^3 (OD units)[d]
3.59	6.61	5.32 (0.03)[b]	5.23	10.4 (0.5)[b]
3.59	6.74	4.08 (0.02)	3.96	10.4 (0.2)
3.59	6.92	2.84 (0.02)	2.71	10.5 (0.5)
3.59	7.22	1.58 (0.03)	1.50	10.3 (0.1)
3.59	7.97	0.79 (0.01)	0.77	10.4 (0.1)
3.59	8.46	1.10 (0.02)	1.22	10.2 (0.1)
3.59	8.96	3.1 (0.2)	2.60	10.3 (0.2)
3.59	9.28	5.5 (0.2)	4.85	10.7 (0.3)
2.40	6.49	4.93 (0.02)	4.98	6.2 (0.3)
2.40	6.73	2.73 (0.1)	2.93	6.6 (0.2)
2.40	7.27	1.13 (0.02)	1.06	6.8 (0.1)
2.40	7.67	0.67 (0.01)	0.72	6.8 (0.1)
2.40	7.95	0.66 (0.01)	0.69	6.8 (0.1)
2.40	8.56	1.13 (0.01)	1.31	6.7 (0.2)
2.40	8.87	1.91 (0.02)	2.15	6.4 (0.2)
2.40	9.31	4.7 (0.25)	5.10	6.3 (0.3)
1.16	6.25	4.6 (0.4)	4.80	3.4 (0.2)
1.16	6.42	3.33 (0.05)	3.43	3.25 (0.15)
1.16	6.76	1.62 (0.01)	1.68	3.2 (0.1)
1.16	7.24	0.77 (0.01)	0.75	3.35 (0.05)
1.16	7.30	0.73 (0.01)	0.69	3.15 (0.05)
1.16	7.52	0.64 (0.02)	0.575	3.3 (0.05)
1.16	7.68	0.56 (0.02)	0.55	3.1(0.05)
1.16	7.83	0.51 (0.01)	0.57	3.2 (0.05)
1.16	7.96	0.55 (0.02)	0.62	3.15 (0.05)
1.16	8.81	2.06 (0.02)	1.82	3.3 (0.15)
1.16	9.21	4.10 (0.01)	4.06	3.35 (0.1)
0.53	6.51	1.84 (0.02)	1.82	1.41 (0.14)
0.53	6.86	1.07 (0.04)	0.95	1.57 (0.07)
0.53	7.07	0.83 (0.04)	0.69	1.58 (0.05)
0.53	7.50	0.60 (0.05)	0.48	1.73 (0.05)
0.53	7.79	0.61 (0.04)	0.51	1.64 (0.03)
0.53	8.63	1.10 (0.04)	1.28	1.61 (0.05)
0.53	9.02	2.70 (0.3)	2.60	1.48 (0.05)

[a] As measured at t_i (1 °C) in 0.2 M $NaClO_4$ solutions. [b] Values in parentheses are standard deviations. [c] From least-squares fitting of the data according to eq 1. [d] Recorded at λ = 300 nm with 2.5-nm spectral bandwidth.

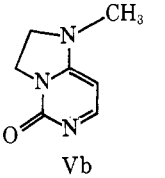
A multilinear least-squares fitting of the data of Table III leads to $k_0 = 1.8 \times 10^3\ s^{-1}$, $k_{H^+} = (1.6 \pm 0.3) \times 10^{10}\ M^{-1}\ s^{-1}$, $k_{OH^-} = (1.2 \pm 0.05) \times 10^{10}\ M^{-1}\ s^{-1}$, and $k_{cation} = (4.4 \pm 0.2) \times 10^8\ M^{-1}\ s^{-1}$. The standard error on k_0 was not available from our least-squares fitting program, but the reproductibility of this value from independent kinetic runs was about ±15%. The existence of a constant term in the rate equation causes the slope of the plot of log τ^{-1} vs. log $\bar{C}_{H^+}$ to be markedly less than unity, even at the extreme pH we investigated (Figure 2).

II. Spectroscopic Evidence for Tautomerism of 3-Methylcytosine in Various Solvents. The uv spectrum of 3-methylcytosine depends markedly on the solvent used. In solvents of high dielectric constant (water, primary and secondary alcohols, acetonitrile), λ_{max} lies in the range 295–305 nm and ϵ_{max} is fairly constant, being about 12 000; by contrast, when recorded in dioxane, chloroform, or ethyl acetate, the spectrum shows a maximum around 265 nm with ϵ_{max} about 5500–6000; *tert*-amyl alcohol or dioxane–water mixtures containing about 5% water behave intermediately in this respect. An isosbestic point is clearly observed in dioxane–water mixtures in the composition range 0–12% water (Figure 3), thereby showing that two species are interconverting. For a higher water concentration the long wavelength component of the spectrum is progressively blue shifted (Figure 3), so that an isosbestic point is no longer observed; a similar blue shift in the position of this band is observed in going from ethanol (λ_{max} = 301 nm) to water (λ_{max} = 294 nm).

The spectrum of compound Va, which is structurally related to 3-methylcytosine and can similarly tautomerize to an imino form, is also markedly blue shifted and reduced in intensity in going from water to dioxane (λ_{max} = 298 nm, ϵ_{max} = 10 400 in water; λ_{max} = 281 nm, ϵ_{max} = 5100 in dioxane). In contrast, the spectrum of the derivative Vb, which cannot exhibit tautomerism, is virtually identical in both classes of solvents (λ_{max} = 306 nm, ϵ_{max} = 12 600 in water; λ_{max} = 310 nm, ϵ_{max} = 12 300 in dioxane).



Va



Vb

III. Relaxation Spectrum of 3-Methylcytosine and Related Compounds. An aqueous solution of 3-methylcytosine, in the concentration range 5×10^{-4} to 4×10^{-3} M, when observed at 250–255 nm and for pH >8.5, shows a relaxation spectrum qualitatively similar to that of cytosine. The relaxation time of the second process is, however, too short ($\tau < 10\ \mu s$) to be accurately measured because of the poor signal–noise ratio of the signal. Replacement of water by D_2O leaves the first signal unchanged, while the relaxation time for the second process

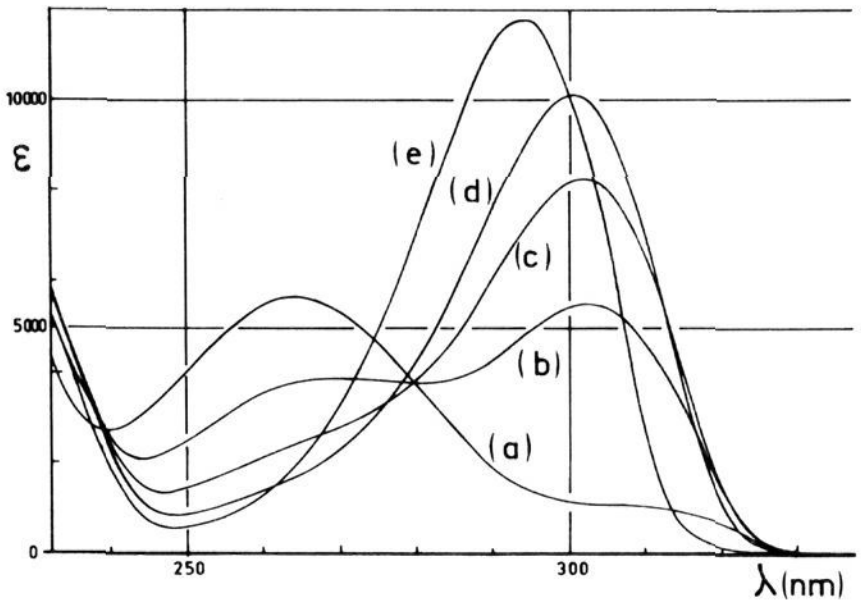


Figure 3. Uv spectra of 3-methylcytosine in water–dioxane mixture at 25 °C. Water content: (a) 0%, (b) 4%, (c) 8%, (d) 16%, (e) 100% (pH 9.18).

increases by a factor 2–3, thereby allowing a quantitative study to be undertaken.

The relaxation time shows the same functional dependence upon pD and concentration as that observed for cytosine (Figure 4 and Table IV). Data from Table IV were fitted by means of eq 1. However, when this was done, it was found that for all pD's and concentrations used, the term $k_{D^+}\bar{C}_{D^+}$ was very small with respect to all other terms in eq 1. Therefore, its numerical value was of little significance and it was dropped from the multilinear correlation. This leads to the following numerical expression

$$\tau^{-1}(s^{-1}) = 2.9 \times 10^4 + (4.3 \pm 0.2) \times 10^9 \bar{C}_{OD^-} + (2.2 \pm 0.1) \times 10^8 \bar{C}_{cation} \quad (2)$$

where $\bar{C}_{cation}$ stands for the concentration of the 3-methylcytosinium ion. The standard error on the pH-independent term was not computed, but its value was reproducible to within ±10% from run to run.

We were unable to study the variation of the relaxation amplitude as a function of wavelength, since the relatively concentrated solutions we used showed large absorbances outside the 250–255-nm range (cf. Figure 5). Furthermore, since no bright light source was available in that spectral region, large spectral slits (spectral bandwidth, 10 nm) have to be used in order to bring the signal–noise ratio to an acceptable level; consequently, the quantitative significance of the amplitude values quoted in Table IV should not be overestimated, since they do not refer to a definite wavelength. However, for pD $\gg$ pK_1' (viz. for pD >9.5), the spectrum of 3-methylcytosine is pD independent, and, therefore, in that pD range, the variation of the amplitude quoted in Table IV should parallel that of the amplitude recorded at a definite wavelength.

Aqueous solutions of compound Va showed, for pH >7.5, a relaxation spectrum qualitatively similar to that of 3-methylcytosine. In that case, however, useful signals were obtained with much more dilute solutions (ca. 10^{-4} M), so that the wavelength dependence of the amplitude could be studied; furthermore, the relaxation times were long enough to be easily measured in water. Although an extensive discussion of the relaxation spectrum of Va is outside the scope of this paper, the two following points are of particular interest here: (a) the amplitude is pH independent provided most of the compound exists in neutral form, viz. for pH >8; and (b) the plot of the amplitude vs. wavelength was found to parallel very closely the modifications observed in the uv spectrum of Va in going from water to dioxane (Figure 6).

In contrast, the nontautomeric compound Vb showed no relaxation spectrum other than the "fast" relaxation effect

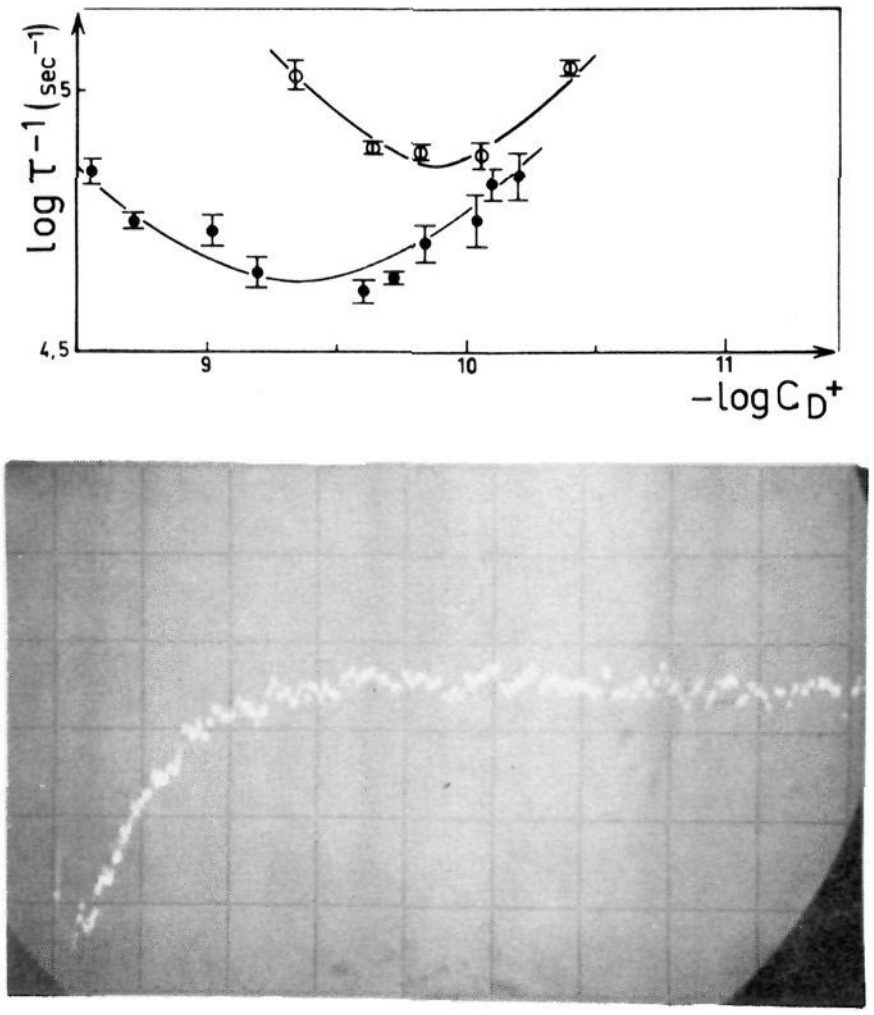


Figure 4. (Upper) For 3-methylcytosine, log τ^{-1} as a function of $-\log C_{D^+}$. 3-Methylcytosine concentration, 4.55×10^{-3} M (upper curve) and 5.34×10^{-4} M (lower curve); t_i = 4 °C; t_f = 10°C; λ = 254 nm. (Lower) Relaxation spectrum of 3-methylcytosine in heavy water, recorded for a wavelength of 254 nm: t_i = 4 °C; t_f = 10 ± 1 °C; pD (as measured at t_i) 9.70; 3-methylcytosine concentration = 2.07×10^{-3} M; vertical scale, 2×10^{-3} OD unit per major division; horizontal scale, 20 μs per major division. The signal corresponds to an increase of the solution OD.

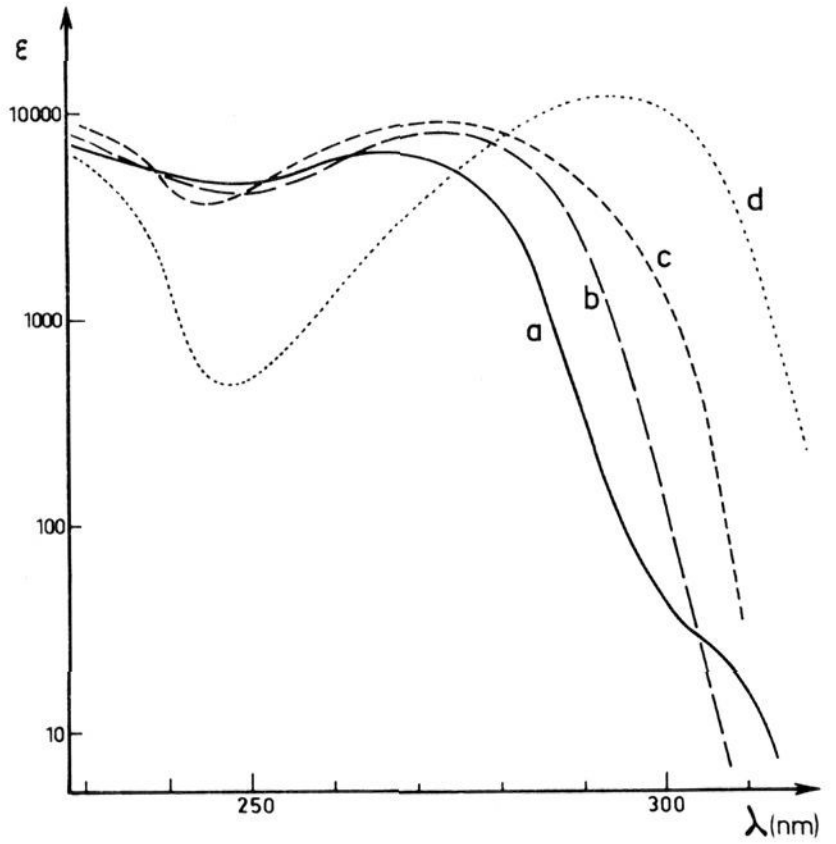


Figure 5. Uv spectra of (a) cytosine; (b) 1-methylcytosine; (c) 1,3-dimethylcytosine; (d) 3-methylcytosine.

caused by temperature variation of the extinction coefficient.

Discussion

I. N(1)H ⇌ N(3)H Equilibrium in Cytosine. A. Assignment of the Relaxation Spectrum. The relaxation phenomenon observed with cytosine solutions must be attributed to a first-

Table IV. Variation of the Inverse of the Relaxation Time (τ^{-1}) and Relaxation Amplitude (A) with pD and 3-Methylcytosine Concentration

$C_{3\text{-methylcytosine}}$, M × 10^3	pD[a]	τ^{-1} (exptl), s^{-1} × 10^{-4}	τ^{-1} (calcd),[c] s^{-1} × 10^{-4}	A × 10^3 (OD units)[d]
4.55	9.64	10.9 (0.9)[b]	10.8	
4.55	9.94	7.8 (0.2)	7.9	
4.55	10.13	7.7 (0.3)	7.3	
4.55	10.38	7.5 (0.5)	7.6	
4.55	10.74	11.0_5 (0.3)	10.5	
2.07	9.06	12.6 (0.7)	13.4	3.6 (0.4)
2.07	9.32	9.1_1 (0.55)	9.6	5.4 (0.4)
2.07	9.66	7.2 (0.7)	6.7	8.7 (1.0)
2.07	10.5	7.4 (0.3)	7.5	8.7 (0.8)
0.92	9.12	7.2_6 (0.8)	7.26	3.6 (0.4)
0.92	9.19	7.6_5 (0.15)	6.8	3.4 (0.1)
0.92	9.41	6.0 (0.15)	5.65	4.5 (0.3)
0.92	9.52	5.4 (0.2)	5.27	4.3 (0.2)
0.92	9.93	4.9 (0.1)	4.86	4.1 (0.2)
0.92	10.38	6.4 (0.3)	6.25	5.0 (0.3)
0.92	10.82	11.0 (0.8)	10.4	5.3 (0.2)
0.53	8.86	7.0 (0.1)	6.74	1.9 (0.1)
0.53	9.02	5.6 (0.2)	5.92	2.7 (0.3)
0.53	9.33	5.4 (0.4)	4.82	2.6 (0.3)
0.53	9.50	4.4 (0.3)	4.3	2.6 (0.3)
0.53	9.93	4.1 (0.2)	4.52	3.1 (0.3)
0.53	10.06	4.3 (0.1)	4.78	3.8 (0.2)
0.53	10.18	5.1 (0.4)	5.14	3.3 (0.3)
0.53	10.40	5.7 (0.7)	6.14	3.4 (0.3)
0.53	10.59	6.9 (0.7)	7.48	3.2_5 (0.4)

[a] Obtained by adding 0.4 unit to the pH measured at t_i.[15] [b] Values in parentheses are standard deviations. [c] From least-squares fitting of the data according to eq 2. [d] Recorded at 254 nm with 10-nm spectral bandwith (see text).

order process involving neutral cytosine itself, since the associated amplitude is pH independent and proportional to the cytosine concentration. Furthermore, no comparable phenomenon was observed with 1-methylcytosine and cytidine solutions; this suggests that it is due to a prototropic equilibrium involving the N(1)H group. The following argument shows that it must be attributed to the Ia ⇌ IIa reequilibration process. The relaxation amplitude (A) associated with the reequilibration of a tautomeric equilibrium after a temperature jump, as monitored by uv spectroscopy, is readily derived[19]

$$A = \frac{1}{RT^2}\,\Delta H\, l \frac{K}{(1+K)^2}\, C\Delta T(\epsilon_1 - \epsilon_2)_\lambda \qquad (3)$$

where ΔH is the reaction enthalpy, K the equilibrium constant, l the optical path, ΔT the magnitude of the temperature jump, C the total concentration, and ϵ_1 and ϵ_2 the extinction coefficients of species 1 and 2 at the wavelength λ chosen for observation. Therefore, if only λ is allowed to vary, the amplitude to concentration ratio will follow $(\epsilon_1 - \epsilon_2)_\lambda$. For cytosine, the plot of the amplitude to concentration ratio vs. λ, at constant T and ΔT, in the wavelength range where it was measurable, was closely similar to the differential spectrum of 3-methylcytosine and 1-methylcytosine (Figure 1). Since these compounds are known to exist mainly as IIb and Ib, respectively, in aqueous solution,[1,12] they should have spectral properties similar to those of IIa and Ia; this strongly supports the assignment of the observed relaxation to the Ia ⇌ IIa reequilibration process. An additional argument, based on the observed interconversion kinetics, will be presented below.

B. Estimate of Thermodynamic Parameters. It is clear from eq 3 that, if the equilibrium constant K = (IIa)/(Ia) is much smaller than unity, and if ΔH and $(\epsilon_1 - \epsilon_2)_\lambda$ can be assumed to be temperature independent, then the product AT^2 will vary as K when the temperature is varied. Thus, plotting ln (AT^2) vs. $(1/T)$ leads to an estimation of ΔH. As an average over six runs, we found $\Delta H = 3.2 \pm 0.6$ kcal M^{-1}. On the other hand,

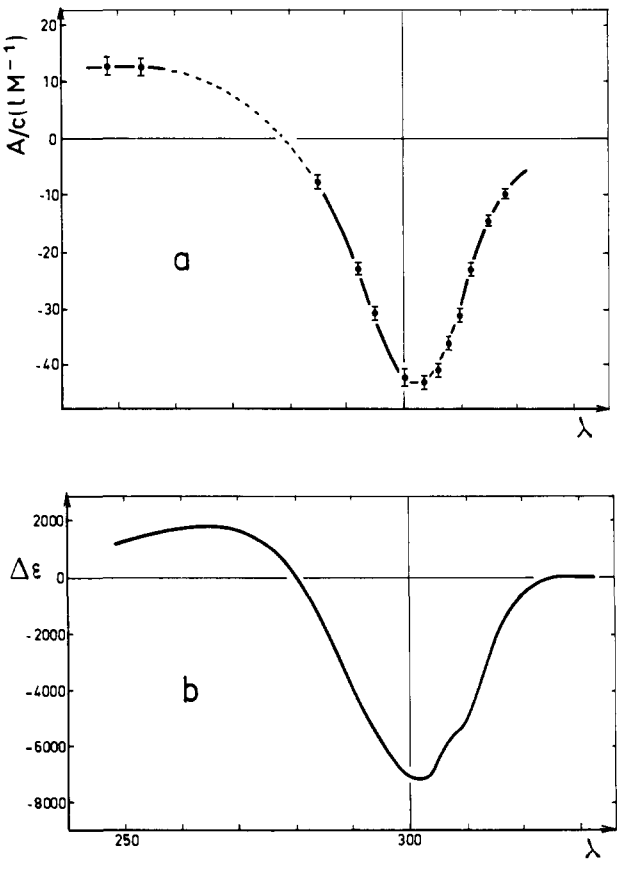


Figure 6. (a) Ratio of the relaxation amplitude to the concentration plotted vs. the wavelength for compound Va: t_i = 2 °C; t_f = 10 °C. Uncertainties are standard deviations. Positive ordinates correspond to a rise in OD when the temperature is raised. (b) Plot of the difference between the molar extinction coefficient of Va in dioxane and in water vs. the wavelength (see text).

K can be determined from eq 3 if ΔH and $(\epsilon_1 - \epsilon_2)_\lambda$ are known; if the assumption is made that the extinction coefficients of IIa and IIb are identical around λ_{max}, the estimated value for ΔH, 3.2 kcal M^{-1}, leads to $K = 2.5 \times 10^{-3}$ at 20 °C.

In some papers devoted to cytosine tautomerism,[5,6] information about the energetics of the tautomerization reaction was tentatively gained from *static* spectral modifications brought about by temperature changes. However, this procedure is generally not valid when one tautomer is strongly favored over the other and/or when the equilibrium enthalpy is close to zero; in such cases, the small OD change due to tautomeric interconversion may be masked by the relatively larger changes caused by the temperature variation of the extinction coefficients. On the other hand, these two contributions to the OD variations are easily distinguishable by temperature-jump spectroscopy, since changes in extinction coefficients cause an immeasurably fast change in optical density (i.e., the "rapid" relaxation of cytosine solutions), while OD changes caused by chemical interconversion usually occur at a measurable rate. In the case of cytosine solutions, we observed that the amplitude of the "rapid" relaxation phenomenon becomes negligible with respect to that of the slower one only for $\lambda > 305$ nm. Therefore, it is only in that wavelength range that the temperature variation of the cytosine extinction coefficient can be attributed to a change in the amount of tautomer IIa. On the other hand, the extinction coefficient of form Ia can probably be assumed negligible with respect to $\epsilon_{cytosine}$ under these conditions.[20] Therefore, for $\lambda > 305$ nm, the tautomeric ratio can be simply calculated from $K = (\epsilon_{cytosine}/\epsilon_{IIa})$,[21] and a plot of ln $(\epsilon_{cytosine})$ vs. $(1/T)$ will lead to an estimate of ΔH. The values thus obtained ($\Delta H = 3.1 \pm 0.1$ kcal M^{-1}; $K \simeq 2.5 \times 10^{-3}$ at 25 °C) are in good agreement with those deduced from amplitude measurements.

Although obtained independently, these two estimates for the tautomeric ratio both rely on the hypothesis that IIa and IIb have identical ϵ_{max}. We feel that the error thus introduced is unlikely to exceed 20%,[22] and, therefore, the value of the tautomeric ratio deduced from pK measurements ($K \simeq 1.4 \times 10^{-3}$ at 25 °C from Table I) is certainly too low. However, it must be kept in mind that this last estimate is derived from the approximate relations

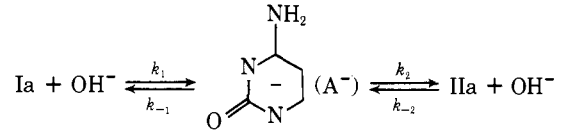
$$K = 10^{(pK_{Ia} - pK_{IIA})} \simeq 10^{(pK_{Ib} - pK_{IIb})} \simeq 10^{(pK_{Ia} - pK_{IIb})} \quad (4)$$

Because of the exponential dependence in eq 4, even a small error in the assumption that the pK difference between the two tautomers is equal to that of the corresponding methylated derivatives can bring a relatively large error in the estimation of K. Nevertheless, we found the method to be much more reliable in predicting tautomerization enthalpies; thus, we measured the ionization enthalpy of 3-methylcytosine to be 8.35 ± 0.1 kcal M^{-1}, while the most reliable value for cytosine (and thus practically for the tautomer Ia) is 5.14 kcal M^{-1}.[23] The difference, 3.2 kcal M^{-1}, provides another estimate of ΔH which compares quite favorable with our direct measurements, although such an excellent agreement is possibly fortuitous.

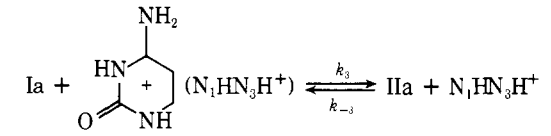
In conclusion, it should be stressed that the detection and spectral characterization of a tautomeric form present in a proportion as low as 0.2% have been possible mainly because of the very favorable spectral properties of the two tautomers; thus, around 290–300 nm, $(\epsilon_1 - \epsilon_2)$ is large ($>10^4$), while the molar extinction coefficient of cytosine is still small (Figure 5), thereby allowing relatively high concentrations to be used.

C. Mechanism of the I ⇌ IIa Interconversion. Cytosine can form a cation in the acidic pH range, presumably with the $N_1HN_3H^+$ structure[3a,4] ($pK_1 = 4.58$ at 25 °C[23]), while under highly alkaline conditions it loses a proton from the pyrimidine ring to form an anion ($pK_2 = 12.15$ at 25 °C[23]). Therefore, the interconversion of Ia and IIa can proceed via a common anion and cation. Under our experimental conditions, the concentration of the cytosinate anion, together with those of the hydroxide and hydronium ions, is much smaller than that of both uncharged tautomers; this does not hold, however, for the cytosinium cation, which is more abundant than IIa up to pH ≃7.5. Nevertheless, it is correct to apply the steady-state approximation to the concentrations of all charged species during the present relaxation mode, which can therefore be considered as a first-order process involving only Ia and IIa; this is first evident experimentally, since the amplitude is found to be pH independent, while this would not hold if a species having a pH-dependent concentration had an appreciable concentration change during this mode. Furthermore, the same conclusion also results from a complete a priori calculation for the relaxation spectrum[24] and can be rationalized in terms of the following simple argument: any change of the cation concentration must be accompanied with changes of similar magnitude of the concentrations of other charged species. Since, around neutrality, these concentrations are very small, changes of cation concentration must also be small and can be assumed negligible during a relaxation mode of appreciable amplitude.

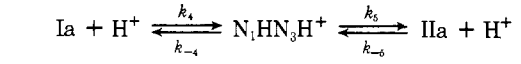
Scheme I. Base Catalysis by OH^-



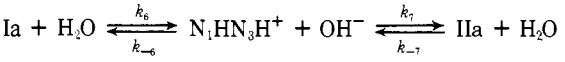
Scheme II. Acid Catalysis by the Cytosinium Cation (Acid Autocatalysis)



Scheme III. Acid Catalysis by H^+



Scheme IV. Acid Catalysis by H_2O



The following interconversion schemes (Schemes I–IV) are proposed to explain the observed dependence of τ^{-1} upon pH and concentration.

When the steady-state approximation is applied to the concentrations of all charged species, it is readily shown that the inverse of the relaxation time can be expressed as $\tau^{-1} = \Sigma_i(k^+_i + k^-_i)$, where k^+_i and k^-_i are first-order rate constants corresponding to the Ia ⇌ IIa interconversion along scheme i. Therefore, the contribution of the different interconversion routes can be considered separately.

(a) Base Catalysis by OH^- (Scheme I). Applying the steady-state approximation to A^- leads to the following expression for the contribution of Scheme I to the relaxation time.

$$(k^+_I + k^-_I) = \frac{k_1 k_2 + k_{-1} k_{-2}}{k_{-1} + k_2} \bar{C}_{OH^-}$$

As shown in ref 10, this expression can be rewritten as

$$(k^+_I + k^-_I) = \frac{k_1 k_{-2}}{k_{av}OH^-} \bar{C}_{OH^-}$$

where

$$k_{av}OH^- = \frac{k_1 \bar{C}_{Ia} + k_{-2} \bar{C}_{IIa}}{\bar{C}_{Ia} + \bar{C}_{IIa}}$$

is the average rate constant for the deprotonation of cytosine by OH^-. Since $\bar{C}_{Ia} \gg \bar{C}_{IIa}$ and since k_1 and k_{-2} are presumably of similar magnitude, being both close to the diffusion limit,[25] $k_{av}^{OH^-}$ differs little from k_1, so that the contribution of Scheme I now becomes simply

$$(k^+_I + k^-_I) = k_{-2}\bar{C}_{OH^-}$$

From eq 1, the experimental value for k_{-2} is 1.2×10^{10} M^{-1} s^{-1}; rate constants of similar magnitude have been reported for the deprotonation by OH^- of other noncharged -NH acids.[10,25,26]

(b) Acid Autocatalysis (Scheme II). The contribution from Scheme II to the inverse of the relaxation time can be expressed as

$$(k^+_{II} + k^-_{II}) = (k_3 + k_{-3})\bar{C}_{N_1HN_3H^+}$$

Therefore, this contribution accounts for the fourth term in eq 1, with $k_{cation} = (k_3 + k_{-3})$. Using our estimate for $K = k_3/k_{-3}$ at the relevant temperature, we obtain $k_3 = 9 \times 10^5$ M^{-1} s^{-1} and $k_{-3} = 4.4 \times 10^8$ M^{-1} s^{-1}. This last value compares well with other kinetic data on thermodynamically favored proton-transfer reactions of similar type.[27]

It should be emphasized that the observation of an efficient autocatalysis by the cytosinium cation, which is known to exist mainly in the $N_1HN_3H^+$ form,[3a,4] demonstrates that this structure is the common protonated form of both tautomers. Therefore, the minor tautomer responsible for the observed relaxation *must* be either IIa or IIIa, the former being clearly favored on spectral grounds (Figures 1 and 5); this illustrates the utility of kinetic arguments in the correct assignment of relaxation spectra.

(c) Acid Catalysis by H^+ (Scheme III). When the steady-state approximation is applied to the concentration of H^+, the following expression for the contribution of Scheme III to the inverse of the relaxation time is obtained,

$$(k^+_{III} + k^-_{III}) = \frac{k_4k_5 + k_{-4}k_{-5}}{(k_{-4} + k_5)}\bar{C}_{H^+}$$

This contribution can be rewritten, analogously to that of Scheme I, as

$$(k^+_{III} + k^-_{III}) = \frac{k_4k_{-5}}{k_{av}^{H^+}}\bar{C}_{H^+}$$

where

$$k_{av}^{H^+} = \frac{k_4\bar{C}_{Ia} + k_{-5}\bar{C}_{IIa}}{(\bar{C}_{Ia} + \bar{C}_{IIa})}$$

is the average rate constant for proton fixation on the cytosine molecule. Since k_4 and k_{-5} must be of the same order of magnitude,[25] $k_{av}^{H^+}$ differs little from k_4, so that the contribution of Scheme III finally becomes

$$(k^+_{III} + k^-_{III}) = k_{-5}\bar{C}_{H^+}$$

From eq 1, the experimental value for k_{-5} is 1.6×10^{10} M^{-1} s^{-1}. Again, this numerical value does not warrant much discussion, being comparable with other rate constants reported in the literature for proton fixation on uncharged nitrogen bases.[10,28]

(d) Acid Catalysis by H_2O. Combining the steady-state equations for the concentration of H^+ and of $N_1HN_3H^+$ (or of OH^- and A^-) allows the contribution of Scheme IV to the relaxation time to be derived.

$$(k^+_{IV} + k^-_{IV}) = \frac{k_6k_7 + k_{-6}k_{-7}}{k_{-6} + k_7}$$

Thus, Scheme IV accounts for the constant term observed in eq 1. This contribution can be further transformed by using the expressions

$$k_6 = k_{-6}\frac{\bar{C}_{OH^-}\bar{C}_{N_1HN_3H^+}}{\bar{C}_{Ia}} = k_{-6}\frac{K_W}{K_1}$$

$$k_{-7} = k_7\frac{\bar{C}_{OH^-}\bar{C}_{N_1HN_3H^+}}{\bar{C}_{IIa}} = k_7\frac{K_W}{KK_1}$$

where K_W stands for the ionic product of water. In deriving these equations, we used the fact that, since $K \ll 1$, the equilibrium constants for proton fixation on Ia and IIa can be approximated by K_1 and KK_1, respectively. The expression for $(k^+_{IV} + k^-_{IV})$ can now be rewritten as

$$(k^+_{IV} + k^-_{IV}) = \left(1 + \frac{1}{K}\right)\frac{K_W}{K_1}\left(\frac{k_{-6}k_7}{k_{-6} + k_7}\right) \simeq \frac{K_W}{KK_1}\left(\frac{k_{-6}k_7}{k_{-6} + k_7}\right)$$

According to eq 1, the experimental value for this constant is 1.8×10^3 s^{-1}; the factor $K_W/(KK_1)$ can be computed from Table I and our estimate for K; this leads to $(k_{-6}k_7)/(k_{-6} + k_7) = 0.85 \times 10^{10}$ M^{-1} s^{-1} at 10 °C. It is then easily shown by simple algebra that the smaller of the two rate constants, k_{-6} and k_7, must lie between 1.7×10^{10} (if k_7 and k_{-6} are assumed equal) and 0.85×10^{10} M^{-1} s^{-1} (if the two constants are very different). However, we feel that the first eventuality is the most likely, for reasons to be discussed now.

Very little is known about the relative neutralization rates of the different acidic centers of a polyacid reacting with OH^-. However, it is well established that the reaction of the substituted ammonium ions with OH^- is controlled by the diffusion of the reactants;[29] consequently, the acidity of the substrate has no influence on the rate constant, and small reactivity differences from one cation to another can be ascribed to variation of the steric factor. The reaction of the cytosinium cation with OH^- is probably diffusion controlled as well; therefore, it can be assumed that the relative reactivity of the two acidic centers, N(1)H and N(3)H, toward OH^-, is governed chiefly by their relative spatial accessibility, which should be rather similar, and not by their relative acidities, which differ markedly. Consequently, we shall assume that, as a first approximation, $k_{-6} = k_7 = 1.7 \times 10^{10}$ M^{-1} s^{-1}. The total rate constant for deprotonation of the cation to yield either Ia or IIa is then found to be 3.4×10^{10} M^{-1} s^{-1}, in good agreement with literature values on ammonium cations,[28,29] thereby strongly supporting Scheme IV as the origin of the constant term observed in the rate law.

It should be finally noted that, since the ratio $(k_{-6}k_7)/(k_{-6} + k_7)$ is not likely to vary much from one system to another, being always close to 10^{10} M^{-1} s^{-1}, the constant term in the rate law is roughly proportional to $K_W/(KK_1)$, viz. to the basicity constant of the minor tautomer; therefore, it can be safely predicted that, in related systems, if the whole tautomeric system is basic enough (small K_1), or if the tautomeric ratio is small (small K), the interconversion rate law will contain a large, predictable, constant term, which will limit the lifetime of the minor species even in the absence of catalyst other than water. Tautomerization of 3-methylcytosine to its very basic imino form IIIb will now provide an illustration of this point.

II. Amino–Imino Tautomerism in 3-Methylcytosine. 3-Methylcytosine has been shown by uv spectroscopy to exist in water mainly as the aminooxo tautomeric form IIb.[12] On the other hand, our spectroscopic results clearly demonstrate that a different tautomeric form predominates in apolar solvents (Figure 3). We shall first discuss the structure of this tautomeric form and attempt to estimate its abundance in water before discussing the assignment of the relaxation spectrum observed with aqueous solutions of 3-methylcytosine.

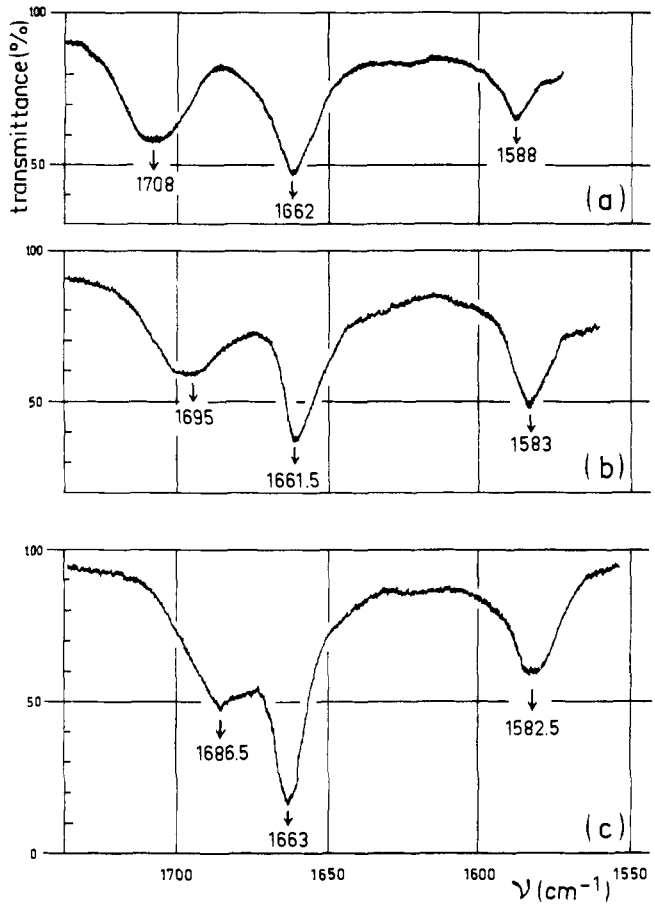
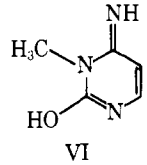


Figure 7. Ir spectra in chloroform (optical length 5 mm): (a) 3-methylcytosine ($\simeq 5 \times 10^{-4}$ M); (b) 3-methylcytosine-d_2[18] ($\simeq 5 \times 10^{-4}$ M); this spectrum is recorded in D_2O-saturated chloroform; (c) 1,3-dimethylcytosine ($\simeq 10^{-3}$ M).

A. Structure of 3-Methylcytosine in Various Solvents. Evidence for a Stable Imino Form. Three tautomeric structure can formally be written for 3-methylcytosine, viz. the aminooxo form IIb, the iminooxo form IIIb, and the iminohydroxy form VI.



VI

Therefore, while the uv spectra demonstrate that the predominant form is no longer IIb in dioxane or chloroform, an ambiguity remains concerning the correct structure in these solvents.

A comparison of the uv spectra of 3-methylcytosine and 1,3-dimethylcytosine (IIIc), which must have the iminooxo structure, is consistent with structure IIIb if allowance is made for spectral shifts due to the methyl substituent;[30] this, however, is not fully conclusive since no "fixed" derivative of structure VI is available for further comparison. The ir spectrum establishes, however, that IIIb is the correct structure of 3-methylcytosine in chloroform solution; thus, no band is observed in the -OH stretch region, thereby ruling out structure VI. On the other hand, the double bond region of the spectrum shows striking similarities with the spectrum of the blocked derivative IIIc, especially when 3-methylcytosine-d_2[18] is considered (Figure 7). The highest frequency band in this spectral region (1708 and 1695 cm^{-1} for 3-methylcytosine and 3-methylcytosine-d_2, respectively) lies in the range usually observed for the nonconjugated carbonyl groups in pyrimidines.[31] By contrast, the spectrum of Vb shows no band in this region above 1665 cm^{-1}, a value typical for a "conjugated" carbonyl group,[31] as expected from its fixed aminooxo structure.

Table V. Dipole Moments Computed from CNDO/II Calculations[a] (μ_{theor}) and Measured Experimentally[b] (μ_{expl}) for Various Structures (Debye Units)

Structure	μ_{theor}	μ_{expl}
Ia	7.9 D	
IIa	8.9 D	
IIIa cis[c]	3.0 D	
IIIa trans	5.4 D	
IIIc[d]		4.4 ± 0.1 D
Vb		7.9 ± 0.1 D

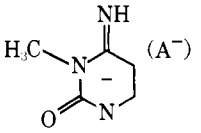
[a] Standard geometries based on x-ray data for similar compounds [cf. J. Donohue, *Arch. Biochem. Biophys.*, **128**, 591 (1968)] were used in the calculations; in any case, the computed moments were found to be quite insensitive to small geometrical changes. [b] Measured in dilute dioxane solutions at 20 °C by P. Bonnet and J. L. Rivail, Laboratoire de Chimie Théorique, Nancy (personal communication). [c] N_3 and the imino hydrogen are cis with respect to the exocyclic C=N bond. [d] 1,3-Dimethylcytosine.

It must therefore be concluded that 3-methylcytosine exists predominantly as the iminooxo tautomer IIIb in nonpolar solvents. The same conclusion presumably holds for the related compound Va which undergoes similar spectral changes in going from water to dioxane solutions. Imino forms are rare in heterocyclic systems capable of tautomerism to amino form,[32] although a change in predominant tautomer with change in solvent has sometimes been observed.[33] Nevertheless, the destabilization of IIb with respect to IIIb in going from polar to apolar solvent, is understandable since its dipole moment is presumably much larger, as shown by dipole moment measurements on the related nontautomeric structures Vb and IIIc, as well as by CNDO/II calculations[34] (Table V). In an effort to settle more quantitatively the dependence of the equilibrium constant upon the polarity of the solvent, we plotted log K' (IIIb/IIb), as evaluated from uv spectra in dioxane-water mixtures, vs. the solvent quantity $(\epsilon - 1)/(2\epsilon + 1)$, where ϵ is the solvent dielectric constant. According to simple electrostatic theory,[35] this plot should be a straight line if the difference in solvation energy of the two tautomers can simply be attributed to differences in their dipole moments, the surrounding solvent being treated as a dielectric continuum. Despite the roughness of these assumptions, an excellent linear correlation is found (Figure 8) showing that the model is basically correct. Presumably the higher order terms in the solvent-solute electrostatic interactions,[36] as well as specific solvation phenomena, which should in principle be included in a complete treatment of the solvent effect, may be neglected here with respect to the solvent-dipole interaction, because of the very large dipole moments involved (Table V). Extrapolation of Figure 8 to pure water leads to $K' = 3.3 \times 10^{-2}$ at 25 °C. On the other hand, a comparison of the pK's of 3-methylcytosine and 1,3-dimethylcytosine leads to $K' \simeq 1.6 \times 10^{-2}$ at 25 °C (Table I). The agreement between these two estimates is considered as good as it can be in view of the limitations of both approaches.

B. Kinetics of the Amino–Imino Interconversion in Water. Straightforward spectral arguments cannot be used as above to assign the relaxation spectrum of 3-methylcytosine to a definite mechanism, since the wavelength dependence of the relaxation amplitude is not available. However, in the case of the related compound Va, this dependence could be studied, and the relaxation amplitude was found to parallel very closely the spectral changes observed in going from water to dioxane (Figure 6). Since these spectral changes have been shown to

be caused by a shift in the predominant tautomer from the aminooxo form (in water) to the iminooxo form (in dioxane), it is clear in that case that the observed relaxation spectrum must be attributed to the interconversion of these two tautomeric forms in water. Analogously, the relaxation spectrum observed with 3-methylcytosine solutions will be assigned to the IIb ⇌ IIIb reequilibration process. Further arguments, based on the observed kinetics, will be presented below in support of this hypothesis.[37]

Along with structures IIb and IIIb, 3-methylcytosine can exist in water in a protonated form, presumably with the structure IV ($N_1HN_3Me^+$)[3a] ($pK_1' = 7.43$ at 25 °C). Furthermore, under highly alkaline conditions, it can lose a proton[12] ($pK_2' = 14.0$ at 10 °C) to form an anion of structure



Therefore, the IIb ⇌ IIIb interconversion can proceed via a common cation and anion. It will be assumed, to a first approximation, that the concentrations of species other than IIb and IIIb change little during the relaxation mode, which can therefore be considered as a first-order process involving only IIb and IIIb. The following interconversion schemes (Schemes V–VII) are proposed to explain the dependence of τ^{-1} upon concentration and pD observed in eq 2.

Scheme V. Base Catalysis

$$\text{IIb} + OD^- \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} A^- \text{(anion)} \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} \text{IIIb} + OD^-$$

Scheme VI. Acid Autocatalysis

$$\text{IIb} + N_1DN_3Me^+ \underset{k_{-3}'}{\overset{k_3'}{\rightleftharpoons}} \text{IIIb} + N_1DN_3Me^+$$

Scheme VII. Acid Catalysis by D_2O

$$\text{IIb} + D_2O \underset{k_{-4}'}{\overset{k_4'}{\rightleftharpoons}} N_1DN_3Me^+ + OD^- \underset{k_{-5}'}{\overset{k_5'}{\rightleftharpoons}} \text{IIIb} + D_2O$$

The D^+ catalyzed interconversion pathway (Scheme III for cytosine) can safely be neglected here since the concentration of D^+ is very small at the pD of the experiments.

Applying the steady-state approximation to the concentrations of A^-, $N_1DN_3Me^+$, and OD^- leads to the following expression for τ^{-1}.

$$\tau^{-1} = k_{-2}'\bar{C}_{OD^-} + (k_3' + k_{-3}')\bar{C}_{N_1DN_3Me^+} + \frac{K_W}{K'K_1'}\frac{k_{-4}'k_5'}{k_{-4}' + k_5'} \quad (5)$$

In deriving eq 5, the following two assumptions have been made: (a) k_1' and k_{-2}' are of the same order of magnitude, being close to the diffusion limit; and (b) IIb largely predominates over IIIb, so that K' can be neglected with respect to unity.

Comparison of eq 5 and 2 shows that the postulated mechanism accounts for the observed dependence of τ^{-1} upon pD and concentration, with $k_{-2}' = 4.3 \times 10^9\ M^{-1}\ s^{-1}$, $(k_3' + k_{-3}') = 2.2 \times 10^8\ M^{-1}\ s^{-1}$, and $(K_W/K'K_1')\ [(k_{-4}'k_5')/(k_{-4}' + k_5')] = 2.9 \times 10^4\ s^{-1}$. Comparatively little is known about the reaction rate of "normal" acids and bases in D_2O;[38] however, the values found for k_{-2}' and $(k_3' + k_{-3}')$ appear reasonable, being somewhat smaller than the corresponding rate constant observed for cytosine in H_2O. Turning now to the third term, the factor $K_W/(K'K_1')$ can be computed from our estimate for the tautomeric ratio, K', and the dissociation constant of 3-methylcytosine in D_2O; the ratio $(k_{-4}'k_5')/(k_{-4}' + k_5')$ is then found to range from 0.3×10^{10} to 0.6×10^{10} $M^{-1}\ s^{-1}$ depending on which of our two estimates for K' is considered. These values compare favorably with that obtained for cytosine ($0.85 \times 10^{10}\ M^{-1}\ s^{-1}$), especially when attention is paid to the fact that diffusion-controlled rate constants should be appreciably lower in D_2O than in H_2O.[39] This leaves little doubt as to the correctness of the attribution, in both cases, of the constant term observed in the rate law to the solvent-catalyzed interconversion scheme. The 16-fold increase in that term observed in going from cytosine (H_2O solution) to 3-methylcytosine (D_2O solution) is mainly ascribable to the increased basicity of the minor tautomer; on the other hand, this term is expected to be even larger for solutions of 3-methylcytosine in ordinary water, since the isotope effect on the dissociation of H_2O $[(pK_W)_{D_2O} - (pK_W)_{H_2O} \simeq 0.9]$ is larger than the isotope effect on the dissociation of a weak acid $[(pK)_{D_2O} - (pK)_{H_2O} \simeq 0.5\text{–}0.6$ if $pK_{H_2O} \sim 9$[17]]. Consequently, the basicity constant for the tautomer IIIb, $(K_W)/(K'K_1')$, will increase by a factor of about 2 on going from D_2O to H_2O. On the other hand, the factor $(k_{-4}'k_5')/(k_{-4}' + k_5')$ will be also somewhat larger in H_2O than in D_2O,[39] so that in H_2O solutions, the constant term in the expression of τ^{-1} is expected to be about $10^5\ s^{-1}$; in agreement with this prediction, we observed that $\tau^{-1} > 10^5\ s^{-1}$ in solutions of 3-methylcytosine in ordinary water.

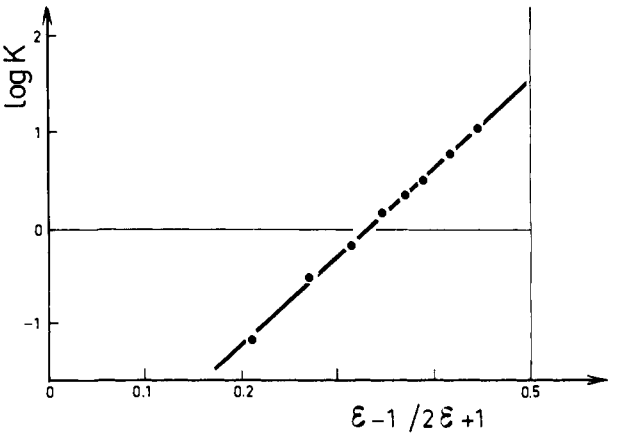


Figure 8. Variation of the equilibrium constant between the amino and imino forms of 3-methylcytosine, K = (amino/imino), in water–dioxane mixtures, vs. the solvent quantity $(\epsilon - 1)/(2\epsilon + 1)$ (see text).

In a study on the tautomerism of 5′-deoxypyridoxal, Ahrens[40] observed in the rate law a pH-independent term which was far too large to be accounted for by water catalysis involving intermediate dissociation to separate ions; therefore, can take place directly from the donor to the acceptor site, without intermediate dissociation. Such a mechanism is obviously unimportant, however, in the tautomeric interconversion discussed here. Further work is in progress to explain this different kinetic behavior.

While the relaxation spectrum of 3-methylcytosine was initially assigned to the IIb ⇌ IIIb interconversion on the basis of the structural relationship between 3-methylcytosine and compound Va, for which the assignment was unambiguous, this attribution is now strongly supported by the following kinetic arguments: (a) the interconversion of tautomers IIb and IIIb is expected to be efficiently catalyzed by their common protonated form $N_1HN_3Me^+$, and experimentally this acid autocatalysis, with a rate constant of the correct order of magnitude, is actually observed; and (b) the magnitude of the pH-independent term in the rate law is consistent with the basicity expected for tautomer IIIb. Furthermore, the smaller amplitude to concentration ratio and shorter relaxation times observed with 3-methylcytosine with respect to Va are easily accounted for by the different basicity of their iminooxo forms; thus, while compound Va and 3-methylcytosine have rather

similar pK values,[12] the pK of the 1-methyl derivative of Va, which must have the iminooxo form, is lower than that of 1,3-dimethylcytosine by more than one pK unit.[12] Therefore, the imino form of Va is predicted from these pK comparisons to be less basic and more abundant than that of 3-methylcytosine, thereby accounting for the slower kinetics and larger amplitudes observed with Va relative to 3-methylcytosine.

The mechanistic model developed here accounts for the observed kinetics. However, the steady-state approximation which was applied above to the concentration of all charged species needs some further justification. Indeed, a complete a priori calculation of the relaxation spectrum, based on reasonable values for the rate constants,[24] shows that this approximation, when applied to the concentrations of $N_1DN_3Me^+$ and OD^-, will progressively break down at the highest pH we used. A refined kinetic model is therefore developed in the Appendix. However, analysis of the data in terms of this more sophisticated approach essentially confirms the kinetic conclusions drawn here.

Conclusion

The thermodynamic data obtained here for tautomerization of cytosine to its 3(H)-aminooxo form, and for tautomerization of 3-methylcytosine to its iminooxo form, agree qualitatively with previous estimates from pK comparisons[3] although quantitatively we found that the amounts of minor forms were somewhat underestimated. Straightforward extension of our results to the amino–imino tautomerism of cytosine and cytidine shows that the equilibrium constant $K_{1,III}$ = (imino/amino) in aqueous solution must be of the same order of magnitude as the product KK' [K = (IIa)/(Ia) and K' = (IIIb)/(IIb)], viz. must lie in the range 10^{-4}–10^{-5} at ordinary temperature. However, it is obvious that solvent changes can have a quite large effect on the equilibrium constant, as shown above for 3-methylcytosine; since the dipole moments of the three tautomeric forms Ia, IIa, and IIIa are predicted to lie in the order $\mu_{IIIa} \ll \mu_{Ia} < \mu_{IIa}$ (Table V), the imino form will be greatly favored in solvents of low dielectric constants. In any case, IIIa will probably become more abundant than IIa in nonpolar environment, as shown by our results on 3-methylcytosine.

Concerning the kinetics of the tautomeric interconversion in aqueous solutions, we found that direct, intramolecular proton transfer without intermediate dissociation to ions is an unimportant interconversion pathway. If this is assumed to hold also for the tautomerization of cytosine and cytidine to their imino forms, then the lifetimes of the very basic imino tautomers will be limited, at neutral pH, mainly by their rapid conversion to the cation through the reaction

$$\text{(imine)} + H_2O \rightleftharpoons \text{(cation)} + OH^-$$

Taking for the reverse rate constant the value obtained above for the Ia $\rightleftharpoons$ IIa equilibrium, viz. 1.7×10^{10} M^{-1} s^{-1}, and estimating the equilibrium constant from the pK of 1,3-dimethylcytosine or 3-methylcytidine,[11] we find the forward rate constant to be 3×10^5 and 10^5 s^{-1} for the imino forms of cytosine and cytidine, respectively, at 20 °C; thus, the lifetimes of the imino forms would be ca. 3×10^{-6} and 10^{-5} s. These estimates should be considered as upper limits, however; thus, in cytosine nucleotides, the phosphate (or polyphosphate) group has a pK intermediate between those of the amino and imino forms of the pyrimidine moiety and thus may well shorten the lifetime of the imino tautomer by catalyzing its conversion to the more stable amino form.

Acknowledgment. We are much indebted to J. C. Fontaine and P. Levoir for their help in maintaining and improving the data acquisition system.

Appendix. A Refined Mechanism for the Relaxation of 3-Methylcytosine

The hypothesis of stationary state applied to the concentration of $N_1DN_3Me^+$ and OD^-, which was made in the text, is presumably not valid at high pD; therefore, the mechanism for the IIb $\rightleftharpoons$ IIIb tautomerization must be rewritten as

$$\begin{cases} \text{IIb} + OD^- \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} A^- \text{(anion)} \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} \text{IIIb} + OD^- \\ \text{IIb} + N_1DN_3Me^+ \underset{k_{-3}'}{\overset{k_3'}{\rightleftharpoons}} \text{IIIb} + N_1DN_3Me^+ \end{cases}$$

$$N_1DN_3Me^+ + OD^- \underset{k_4'}{\overset{k_{-4}'}{\rightleftharpoons}} \text{IIb} + D_2O$$

$$N_1DN_3Me^+ + OD^- \underset{k_{-5}'}{\overset{k_5'}{\rightleftharpoons}} \text{IIIb} + D_2O$$

where the steady-state approximation is now applied to species A^- (anion) only. The first two schemes in this mechanism are therefore equivalent to

$$\text{IIb} \underset{k^-}{\overset{k^+}{\rightleftharpoons}} \text{IIIb}$$

with

$$(k^+ + k^-) = \frac{k_1' k_{-2}'}{k'_{av} \bar{C}_{OD^-}} \bar{C}_{OD^-} + (k_3' + k_{-3}') \bar{C}_{N_1DN_3Me^+}$$

The relaxation spectrum associated with this refined mechanism can be calculated easily by standard algebra;[41] two relaxation times are thus obtained, the slowest of which can be expressed as

$$\tau^{-1} = 0.5 \left[(\tau_I^{-1} + \tau_{II}^{-1} + \tau_{III}^{-1}) - \left[(\tau_{II}^{-1} - \tau_{III}^{-1} + \frac{\tau_I^{-1}}{1+K'} (K'-1))^2 + 4 \left(\tau_{II}^{-1} - k_4' - \frac{\tau_I^{-1}}{1+K'} \right) \times \left(\tau_{III}^{-1} - k_{-5}' - \tau_I^{-1} \frac{K'}{1+K'} \right) \right]^{\frac{1}{2}} \right] \quad \text{(I)}$$

with

$$\tau_I^{-1} = (k^+ + k^-) \simeq k_{-2}' \bar{C}_{OD^-} + k_{-3}' \bar{C}_{N_1DN_3Me^+}$$

$$\tau_{II}^{-1} = k_{-4}' [\bar{C}_{N_1DN_3Me^+} + \bar{C}_{OD^-}] + k_4'$$

$$\tau_{III}^{-1} = k_5' [\bar{C}_{N_1DN_3Me^+} + \bar{C}_{OD^-}] + k_{-5}'$$

The expression of τ^{-1} can be shown to reduce to the simplified expression used above (eq 5 in the text), provided the two following conditions are fulfilled.

$$\tau_I^{-1} \ll \tau_{II}^{-1} \text{ and } \tau_{III}^{-1} \quad \text{(II)}$$

and

$$k_4' \ll \tau_{II}^{-1} \text{ and } k_{-5}' \ll \tau_{III}^{-1} \quad \text{(III)}$$

Assuming that the kinetic parameters derived in the text are to a first approximation valid, then we have $k_{-2}' \gg k_{-3}'$, while the rate constants for neutralization of the cation are presumably even larger than k_{-2}', since the ratio $(k_{-4}'k_5')/(k_{-4}' + k_5')$ was equal 0.3×10^{10} or 0.6×10^{10} M^{-1} s^{-1} depending on the value chosen for K'. Therefore, condition II is clearly satisfied if $\bar{C}_{N_1DN_3Me^+} \gg \bar{C}_{OD^-}$; on the other hand, condition III is fulfilled if

$$(\bar{C}_{N_1DN_3Me^+} + \bar{C}_{OD^-}) \gg K_W/K'K_1 \quad \text{(IV)}$$

Taking into account the value of K' and K_1', we found that these conditions should be satisfied up to pD 10–10.2 even with

the lowest concentrations we used. For higher pD values, however, the complete eq I needs to be considered.

It is apparent from eq I that τ^{-1} can be computed in a straightforward way from the four rate constants k_{-2}', k_{-3}', k_{-4}', and k_5' if K' and K_1' are known. A nonlinear least-squares fitting program was used to fit τ^{-1}_{calcd} obtained from eq I to the experimental values quoted in Table IV. k_{-4}' was taken as a parameter, and k_{-2}', k_{-3}', and k_5' were varied to obtain the best fit. For reasonable values of k_{-4}' (viz. from 4×10^9 to 2×10^{10} M^{-1} s^{-1}) we found that the values of k_{-2}', k_{-3}', and $(k_{-4}'k_5')/(k_{-4}' + k_5')$ thus obtained were within 20% of those deduced in the text; this shows that the kinetic parameters obtained above were significant, even though $\bar{C}_{OD^-}$ and $\bar{C}_{N_1DN_3Me^+}$ cannot be rigorously regarded as constant during the observed relaxation mode, especially at the higher pD values.

References and Notes

(1) For a review of the literature, see (a) J. S. Kwiatkowski and B. Pullman, *Adv. Heterocycl. Chem.*, **18**, 199–335 (1975); (b) A. Albert, *Synth. Proced. Nucleic Acid Chem.*, **2**, 1–122 (1973).

(2) For representative recent work, see (a) J. S. Kwiatkowski and B. Pullman, *Acta Phys. Pol. A*, **45**, 693 (1974); (b) O. M. Sorarrain and E. A. Castro, *Chem. Phys. Lett.*, **19** (3), 422 (1973); (c) D. L. Breen and R. L. Flurry, Jr., *Theor. Chim. Acta*, **23**, 138 (1971); (d) N. Bodor, M. J. S. Dewar, and A. J. Harget, *J. Am. Chem. Soc.*, **92**, 2929 (1970).

(3) (a) A. R. Katritzky and A. J. Waring, *J. Chem. Soc.*, 3046 (1963); (b) B. I. Sukhorukov, A. S. Gukovskaya, L. V. Sukhoruchkina, and G. I. Lavrenova, *Biofizika*, **17**, 5 (1972).

(4) (a) H. T. Miles, *Proc. Natl. Acad. Sci. U.S.A.*, **47**, 791 (1961); (b) O. Jardetzky, P. Pappas, and N. G. Wade, *J. Am. Chem. Soc.*, **85**, 1657 (1963); (c) J. J. Christensen, J. H. Rytting, and R. M. Izatt, *J. Chem. Soc. B*, 1643 (1970).

(5) C. Helene and P. Douzou, *C. R. Hebd. Seances Acad. Sci.*, **259**, 4853 (1964).

(6) H. Morita and S. Nagakura, *Theor. Chim. Acta*, 11, 279 (1968).

(7) G. C. Y. Lee, J. H. Prestegard, and S. I. Chan, *J. Am. Chem. Soc.*, **94**, 951 (1972).

(8) W. C. Johnson, Jr., P. M. Vipond, and J. C. Girod, *Biopolymers*, **10**, 923 (1971).

(9) M. Pieber, P. A. Kroon, J. H. Prestegard, and S. I. Chan, *J. Am. Chem. Soc.*, **95**, 3408 (1973).

(10) M. Dreyfus, G. Dodin, O. Bensaude, and J. E. Dubois, *J. Am. Chem. Soc.*, **97**, 2369 (1975).

(11) P. Brookes and P. D. Lawley, *J. Chem. Soc.*, 1348 (1962).

(12) T. Ueda and J. J. Fox, *J. Am. Chem. Soc.*, **85**, 4024 (1963).

(13) See D. F. Roades and S. Y. Wang, *Biochemistry*, **10**, 4603 (1971), and references cited therein.

(14) M. Eigen and L. de Maeyer, *Tech. Org. Chem.*, **7**(II), 921 (1963).

(15) R. G. Bates, "Determination of pH", Wiley-Interscience, New York, N.Y., 1973, p 375.

(16) Reference 15, p 73.

(17) R. P. Bell, "The Proton in Chemistry", Methuen and Co., London, 1959, Chapter XI.

(18) The two H atoms bonded to nitrogen have been replaced by D.

(19) For analytical expressions of the relaxation amplitudes, see, for instance, (a) D. Thusius, *J. Am. Chem. Soc.*, **94**, 356 (1972), or (b) M. Eigen and L. de Maeyer, *Tech. Org. Chem.*, 7 (II), 973–974 (1963).

(20) Since the amount of IIa is very small at equilibrium, the spectrum of cytosine virtually coincides with that of form Ia, except at long wavelength ($\lambda > 290$ nm) where the contribution of IIa becomes increasingly important. Therefore, it is apparent from Figure 5 that the spectrum of Ia is reduced in intensity and markedly blue shifted (by ca. 7 nm) with respect to that of 1-methylcytosine. If this is assumed to hold also in the wavelength range where the contribution of IIa to the cytosine spectrum cannot be neglected, then, since for $\lambda > 305$ nm $\epsilon^{\lambda}_{cytosine}$ is much larger than $\epsilon^{\lambda+7}_{1\text{-methylcytosine}}$, the relation $\epsilon^{\lambda}_{cytosine} \gg \epsilon^{\lambda}_{Ia}$ must hold.

(21) The extinction coefficient of IIa in that spectral range was obtained from Figure 1 (a), assuming that, at 297 nm, $(\epsilon_{IIa} - \epsilon_{Ia}) \sim \epsilon_{IIa}^{max} \sim \epsilon_{IIb}^{max}$.

(22) Spectra of several 3-substituted cytosines have been reported;[12] the observed ϵ_{max} is found to be rather insensitive to the nature of the 3-substituent.

(23) J. J. Christensen, J. H. Rytting, and R. M. Izatt, *J. Phys. Chem.*, **71**, 2700 (1967).

(24) Cf. appendix of ref 10.

(25) M. Eigen, *Angew. Chem., Int. Ed. Engl.*, **3**, 1 (1964).

(26) (a) H. G. Busse and G. Maass, *Z. Phys. Chem. (Frankfurt am Main)*, **66**, 92 (1969); (b) M. Brennan and K. Kustin, *J. Phys. Chem.*, **76**, 2838 (1972).

(27) See D. Rosenthal and E. Grunwald, *J. Am. Chem. Soc.*, **94**, 5956 (1972), and references cited therein.

(28) M. Eigen, W. Kruse, G. Maass, and L. de Maeyer, *Prog. React. Kinet.*, **2**, 285 (1964).

(29) (a) D. Grimshaw and E. Wyn-Jones, *J. Chem. Soc., Faraday Trans. 2*, **69**, 168 (1973); (b) S. Nishikawa, T. Nakamato, and T. Yasunaga, *Bull. Chem. Soc. Jpn.*, **46**, 324 (1973); (c) M. Eigen, G. Maass, and G. Schwartz, *Z. Phys. Chem. (Frankfurt am Main)*, **74**, 319 (1971).

(30) The spectrum of 3-methylcytosine in dioxane ($\lambda_{max} = 265$ nm, $\epsilon_{max} = 5600$) is blue shifted with respect to that of IIIc ($\lambda_{max} = 273$ nm, $\epsilon_{max} = 7600$). By comparison, 3-methyluracil and 1,3-dimethyluracil, which are structurally related to IIIb and IIIc, respectively, show the following spectral features (in water): 3-methyluracil, $\lambda_{max} = 259$ nm, $\epsilon_{max} = 7250$; and 1,3-dimethyluracil, $\lambda_{max} = 266$ nm, $\epsilon_{max} = 8900$ (from ref 1b, Chapter 2).

(31) (a) A. Psoda, Z. Kazimierczuk, and D. Shugar, *J. Am. Chem. Soc.*, **96**, 6832 (1974); (b) R. C. Lord and G. J. Thomas, Jr., *Spectrochim. Acta, Part A*, **23**, 2551 (1967).

(32) For reviews, see (a) ref 1b, Chapters 1 and 2, and (b) "Physical Methods in Heterocyclic Chemistry", Vol. I, A. R. Katritzky, Ed., Academic Press, New York, N.Y., 1963, pp 27–36.

(33) See D. M. Brown and M. J. E. Hewlins, *J. Chem. Soc. C*, 2050 (1968).

(34) CNDO/II calculations are known to predict reliably the dipole moments of this type of compound; see, for instance, (a) E. D. Bergmann, H. Weiler-Feilchenfeld, and Z. Neiman, *J. Chem. Soc. B*, 1334 (1970; (b) I. Kulakowska, M. Geller, B. Lesyng, and K. L. Wierzchowski, *Biochim. Biophys. Acta*, **361**, 119 (1974).

(35) (a) J. G. Kirkwood, *J. Chem. Phys.*, **2**, 351 (1934); (b) A. R. Martin, *Trans. Faraday Soc.*, **33**, 191 (1937).

(36) For a refined electrostatic approach to the solvent–solute interaction, see R. J. Abraham, H. D. Banks, E. L. Elvel, O. Hofer, and M. K. Kaloustian, *J. Am. Chem. Soc.*, **94**, 1913 (1972).

(37) Alternatively, the observed phenomenon could a priori be attributed to the neutralization of cationic 3-methylcytosine by the base OD^- according to the scheme

$$(\text{cation}) + OD^- \overset{k+}{\rightleftharpoons} (\text{3-methylcytosine}) + D_2O$$

The relaxation time for this mechanism is readily derived as $\tau^{-1} = k^+[\bar{C}_{cation} + \bar{C}_{OD^-} + K_W/K_1']$. Thus, according to this mechanism, the coefficients of $\bar{C}_{OD^-}$ and $\bar{C}_{cation}$ in eq 2 should be equal, while experimentally they differ by a factor of 20. Furthermore, under the experimental conditions we used, $\bar{C}_{OD^-}$ is much smaller than $\bar{C}_{cation}$ and $\bar{C}_{3\text{-methylcytosine}}$, and, therefore, the relaxation amplitude associated with this scheme is predicted[19] to be proportional to $\bar{C}_{OD^-}$ and to be independent of the 3-methylcytosine concentration, which obviously is not observed (Table IV).

(38) See, however, G. Ertl and H. Gerischer, *Z. Elektrochem.*, **66**, 560 (1962), and ref 29a.

(39) According to the Debye formula (cf. ref 25), and assuming the reaction distance and dielectric constant to be equal in D_2O and in H_2O, the rate constant of a diffusion—controlled reaction will vary in going from H_2O to D_2O, as the sum of the diffusion coefficients of the reactants; these coefficients are known to be lowered in D_2O [R. L. Kay and D. F. Evans, *J. Phys. Chem.*, **70**, 2325 (1966)]. More specifically the diffusion coefficient of OD^- in D_2O is about 40% smaller than that of OH^- in H_2O [E. Hetland, *J. Am. Chem. Soc.*, **68**, 2532 (1946)].

(40) M. L. Ahrens, *Biochim. Biophys. Acta*, **320**, 86 (1973).

(41) G. W. Castellan, *Ber. Bunsenges. Phys. Chem.*, **67**, 898 (1963).