Kinetics and Mechanism of Reaction of Hydroxylamine with Methylcytosines

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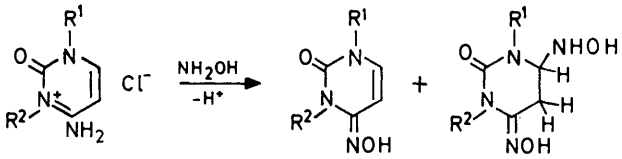
The reaction of hydroxylamine with 1,3-dimethylcytosine in the pH range 5—7 involves terms both first and second order in amine and proceeds entirely *via* the protonated form of the pyrimidine base (1a). The two products of the reaction, *N*(4)-hydroxy-1,3-dimethylcytosine (2a) and 5,6-dihydro-*N*(4)-hydroxy-6-hydroxyamino-1,3-dimethylcytosine (3a) are produced simultaneously in a ratio which varies with pH and with concentration of hydroxylamine. This behaviour, solvent kinetic isotope effects, and partial rates of reaction relative to those for cytosine (1c) and 1-methylcytosine (1b) establish a mechanism in which nucleophilic attack of nitrogen features in the rate-determining step for formation of (2) and is associated with general catalysis of proton transfer in the formation of (3).

GENERAL interest in the reaction of cytosines with hydroxylamine has stemmed from the inital observations of Freese [1,2] and others [3,4] that these reagents are capable of inducing mutations in some T-even bacteriophages at, or near, neutral pH. This effect is accepted as arising from specific modification of cytosine bases in DNA.[5,6] Two products are formed simultaneously: direct substitution of the cytosine amino-group gives *N*(4)-hydroxycytosine derivatives while an alternative process affords 5,6-dihydro-*N*(4)-hydroxy-6-hydroxyaminocytosines.[7,8] Although in principle the mutational event can be attributed to either of these phenomena, it has been argued that the second of these products (3) is that responsible for mutations *in vivo*.[9,10]

There has been much discussion of the mechanism of the Michael-type addition of hydroxylamine to position 6 of cytosines. Considerable clarification has resulted from kinetic and n.m.r. studies, which have established that the transition state is cationic [10] and that the formation of product (3) is associated with predominantly



trans-addition to the 5,6-double bond of (1).[11,12] Hitherto it has not proved possible to diagnose a unique mechanism for this process because of limitations recognised [10] in both these studies. This paper describes the results of a further kinetic investigation of the system which identifies the distribution of charge in the transition state and which, in conjunction with deuterium exchange studies,[13] supports a detailed account of the mechanism of reaction of hydroxylamine with cytosines.

EXPERIMENTAL

Materials.—Reagent grade chemicals were purified immediately before use and glass-distilled water was used throughout. Standard solutions were prepared by using AnalaR grade potassium chloride and Convol potassium hydroxide solution (Hopkin and Williams).

Apparatus.—The apparatus used for spectroscopic and pH measurements has been described previously.[10]

Kinetic Measurements.—All reactions were run in water at 310 ± 0.1 K in the presence of 10^{-3}M-ethylenediaminetetraacetic acid to avoid effects from trace metal ions and at ionic strength 2.0M except where otherwise stated. 1,3-Dimethylcytosine hydrochloride was introduced in aqueous solution (*ca.* 10 μl) into 3 ml of the reaction mixture to give an initial concentration of *ca.* 4×10^{-5}M and the disappearance of starting material was monitored continuously by decrease in optical density at 284 nm. Spectra were recorded from 250 to 330 nm for completed reaction solutions and pH values of reaction solutions were determined at the beginning and end of each run (Table 1).

TABLE 1

Experimental data for the hydroxylaminolysis of methylcytosines at 310 K; ionic strength 2.0M (KCl)

Cytosine (solvent)	pH	No. of runs	$[NH_2OH]_{tot}$/M	% Free base
1,3-Me_2(H_2O)	5.45	5	0.4—2.0	28
	5.60	5	0.4—2.0	33
	5.93	5	0.4—2.0	50
	6.45	5	0.4—2.0	75
	7.00	5	0.4—2.0	92
1,3-Me_2(D_2O)	6.55 [a]	1	2.0	75
1-Me(H_2O)	6.45	6	0.4—2.0	75
1-Me(D_2O) [b]	4.95 [a]	1	1.0	10

[a] pD = (pH +0.40). [b] μ = 1.0M (KCl).

Kinetic data were collected for at least three half-lives and first-order rate constants obtained from plots of $\log(A_t - A_\infty)$ against time. All reactions displayed good first-order behaviour and stable end-points.

Product Analysis.—Reaction solutions of 1,3-dimethylcytosine and hydroxylamine initially showed $\lambda_{max.}$ 283 nm

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[10] G. M. Blackburn, S. Jarvis, M. C. Ryder, and V. C. Solan, *J.C.S. Perkin I*, 1975, 370.
[11] D. M. Brown and P. F. Coe, *Chem. Comm.*, 1970, 568.
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[13] G. M. Blackburn, V. C. Solan, D. M. Brown, and P. F. Coe, *J.C.S. Chem. Comm.*, 1976, 724.

(ε 1.09 × 10^4) but on completion of reaction showed $\lambda_{max.}$ near 290 nm due entirely to *N*(4)-hydroxy-1,3-dimethylcytosine (2a) (ε_{286} 6.75 × 10^3); the second product (3a) is effectively transparent at this wavelength. The mole fraction of (2a) formed from (1a) was calculated from these data and the initial optical densities of reaction solutions at 284 nm.

1,3-*Dimethylcytosine Hydrochloride*.—1-Methylcytosine (1 g) was converted into 1,3-dimethylcytosine hydroiodide, m.p. 524—526 K, as described by Hilbert.[14] This material was dissolved in water and passed down a column of Amberlite 400 (Cl^-) anion-exchange resin (2 × 15 cm) and the eluate was evaporated to dryness. The residue was crystallised repeatedly from ethanol to give the *product*, m.p. 545—547 K (1.2 g, 85%) (Found: C, 41.15; H, 5.8; Cl, 19.95; N, 24.0. $C_6H_{10}ClN_3O$ requires C, 41.05; H, 5.75; Cl, 20.2; N, 23.95%); $\lambda_{max.}$ (H_2O; pH 4.7) 283 (ε 1.09 × 10^4) and 225 nm (9.2 × 10^3); τ (D_2O) 2.04 (d, *J* 7.5 Hz, H-6), 3.60 (d, *J* 7.5 Hz, H-5), 6.32 (s, N·CH_3), and 6.34 (s, N·CH_3).

N(4)-*Hydroxy*-1,3-*dimethylcytosine Hydrochloride*.—This was prepared [15] as crystals (from methanol), m.p. 461—463 K (lit.,[15] 485—488 K) (Found: Cl, 18.35; N, 22.0. Calc. for $C_6H_{10}ClN_3O_2$: Cl, 18.5; N, 21.95%); $\lambda_{max.}$ (H_2O; pH 5.5) 286 nm (ε 6.8 × 10^3); τ (D_2O) 2.78 (d, *J* 8 Hz, H-6), 3.60 (d, *J* 8 Hz, H-5), 6.50 (s, N·CH_3), and 6.62 (s, N·CH_3).

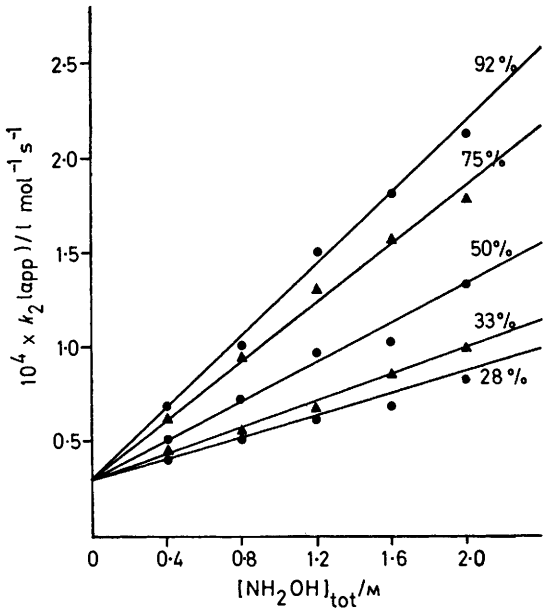


FIGURE 1 Variation of the apparent second-order rate constant for the reaction of hydroxylamine with 1,3-dimethylcytosine hydrochloride as a function of the concentration of hydroxylamine at different buffer ratios; slopes calculated from equation (i) and constants of Table 2

RESULTS

The apparent first-order rate constants for the reaction of hydroxylamine with 1,3-dimethylcytosine hydrochloride show a greater than first-order dependence on the concentration of hydroxylamine and also are independent of pH for constant concentration of free hydroxylamine. The apparent second-order rate constants show a linear dependence on the concentration of hydroxylamine with a constant intercept (Figure 1). Linear regression analysis of these data at each of five pH values provides apparent catalytic rate constants which are directly proportional to the mole fraction of hydroxylamine in the free base form, showing that the catalysis is due solely to the basic component of the buffer acting on the cationic substrate (Figure 2).

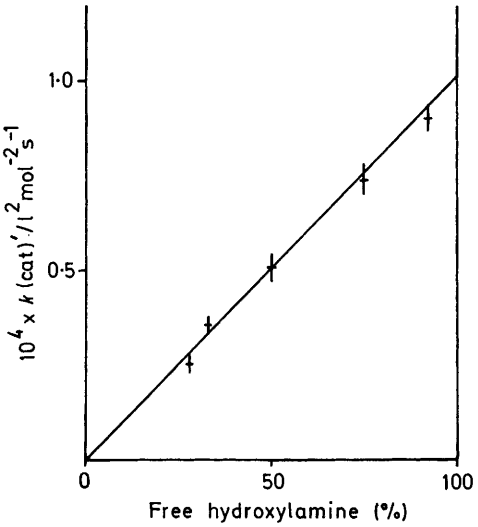


FIGURE 2 Variation of the apparent catalytic rate constant for the reaction of hydroxylamine with 1,3-dimethylcytosine hydrochloride as a function of the composition of hydroxylamine buffer; curve calculated from equation (i) and the value of k_2 in Table 2

Multiple linear regression analysis of the whole set of data provides the rate constants for the water (k_1) and hydroxylamine (k_2) catalysis terms for the reaction between hydroxylamine and protonated 1,3-dimethylcytosine cation according to equation (i), corresponding to the rate and equilibrium constants in Table 2.

TABLE 2

Calculated rate constants for hydroxylaminolysis of cytosines at 310 K; ionic strength 2.0M (KCl)

Cytosine	pK_a	k_1/l mol^{-1} s^{-1}	k_2/l^2 mol^{-2} s^{-1}
1,3-Me_2 (1a)	9.42 ± 0.15	3.05 ± 0.17 × 10^{-5}	1.037 ± 0.022 × 10^{-4}
1-Me (1b)	4.91 ± 0.09	1.71 ± 0.12 × 10^{-4}	1.79 ± 0.18 × 10^{-3}
(1c) [a]	4.57 ± 0.09	6.78 ± 0.45 × 10^{-4}	1.17 ± 0.24 × 10^{-2}

[a] From ref. 10.

$$k_{obs} = \frac{(k_1 + k_2[NH_2OH])[NH_2OH][H^+]}{(K_a + [H^+])} \quad \text{(i)}$$

The rates of reactions of 1-methylcytosine at ionic strength 2.0M and pH 6.45 provided a second-order rate constant which was *ca.* 70% of that previously determined [10] at ionic strength 1.0M.

The products of reaction of 1,3-dimethylcytosine with hydroxylamine were identified by examination of u.v. and ^1H n.m.r. spectra of completed reaction mixtures as

[14] G. E. Hilbert, *J. Amer. Chem. Soc.*, 1934, **56**, 190.
[15] D. M. Brown, M. J. E. Hewlins, and P. L. Schell, *J. Chem. Soc.* (*C*), 1968, 1925.

N(4)-hydroxy-1,3-dimethylcytosine (2a) and 5,6-dihydro-*N*(4)-hydroxy-6-hydroxyamino-1,3-dimethylcytosine (3a). The latter was not sufficiently stable to be obtained in a pure state. It was converted on acidification into (2a), which was isolated and characterised by comparison with an authentic synthetic sample. The ratio of the two products varied with pH and with changes in hydroxylamine buffer concentration and was evaluated by the procedure previously described.[10] The apparent second-order rate constants for the formation of these two products were derived from the product ratios and the gross rate constants for the disappearance of (1a). The second-order rate constant for the formation of (3a) is directly proportional to the concentration of hydroxylamine free base at several pH values and that for (2a) is

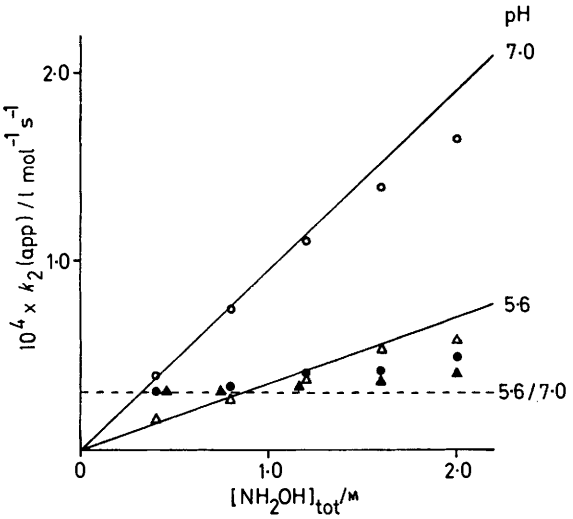


FIGURE 3 Variation in the apparent second-order rate constants for the formation of *N*(4)-hydroxy-1,3-dimethylcytosine [(2a); closed symbols] and 5,6-dihydro-*N*(4)-hydroxy-6-hydroxyamino-1,3-dimethylcytosine [(3a); open symbols] as a function of hydroxylamine concentration at pH 5.6 (▲,△) and pH 7.0 (●,○); slopes calculated from equation (i) and constants in Table 2 for the partial reactions first order (– – – –) and second order (——) in hydroxylamine

almost independent of buffer concentration and of pH (Figure 3).

Hydroxylaminolysis of (1a) in D_2O solution showed a solvent kinetic isotope effect, k_{H_2O}/k_{D_2O} 1.9 in a 25% neutralised hydroxylamine buffer. The corresponding isotope effect for (1b) * was 2.7 in a 90% neutralised hydroxylamine buffer at ionic strength 1.0M.

DISCUSSION

In view of a recent report that the reaction of cytosine (1c) with hydroxylamine is first order with respect to both reactants,[12b] we re-emphasise the fact that for cytosine (1c), 1-methylcytosine (1b), and 1,3-dimethylcytosine (1a), the kinetic data show that at all pH values investigated in this and in previous [10] work the reactions are of an order greater than one with respect to the hydroxylamine buffer (*cf.* Figure 1).

The present results show that the reaction of hydroxylamine with 1,3-dimethylcytosine is subject to general catalysis by hydroxylamine buffers and proceeds *via* a monocationic transition state for the formation of both products (2a) and (3a) (Figures 1 and 2).

The variation in the separate rates of formation of products (2a) and (3a) shows that they do not relate to homocompetitive processes. It appears that the formation of (2a) involving transamination at C-4 is predominantly unimolecular in hydroxylamine and is pH-independent, whereas the formation of (3a) is essentially a pure bimolecular process with respect to free hydroxylamine (Figure 3), in close analogy to the situation previously established for 1-methylcytosine.[10] It can thus be concluded generally that the nucleophilic addition of hydroxylamine to the 5,6-double bond of cytosines requires general acid–base catalysis, whereas transamination at C-4 is predominantly a water-catalysed process. Accordingly, these transformations can be described separately in some detail.

We have already argued [10] that the transition state for transamination at C-4 in cytosines is well represented by (4). The progressive retardation in this process as a result of successive methylation at N(1) and N(3) fully confirms this view. The application of an extended Hammett treatment [16] to the rate constants k_1 with σ values of -0.17 for both methyl substituents on the cytosine ring gives a reaction constant $\rho = +4.0 \pm 0.25$. This compares well with the value (+4.41) found for the aromatic nucleophilic substitution of fluoride by piperidine, a process well known to involve rate-determining formation of an intermediate σ-complex.[17]

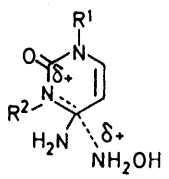
The prime purpose of this study is to resolve the location of the proton in the transition state for the Michael addition of hydroxylamine to simple cytosines and to relate that to the stereochemical features of the reaction. We previously argued [10] that this could be viewed either as a general base catalysis of proton transfer in conjunction with addition of hydroxylamine at C-6 to the cationic substrate or as a general acid catalysis of addition of hydroxylamine to the neutral pyrimidine. These alternatives are related by the equation $k_2 = k_2'K_{NH_2OH}/K_a$ where k_2 is the catalytic rate constant calculated for reaction of the protonated cytosine (Table 2), and k_2' that for reaction of the neutral form with the proton located on the hydroxylamine molecule.

Successive methylation at N(1) and N(3) results in successive diminution of k_2 by factors of 6.5 and 17, respectively (Table 2). Such retardation effects are just those expected for the influence of electron-releasing groups on a nucleophilic addition process at C-6 on the cytosine cation and correspond to a Hammett ρ factor of *ca.* +6. In contrast, the equivalent values for k_2' show that methylation at N(1) retards the reaction by a factor of 3 but subsequent methylation at N(3) enhances the

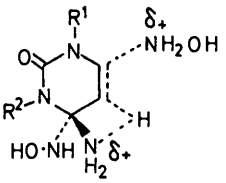
* Correction was made for the state of ionisation of the substrate by using a measured pK_a value for (1b) in D_2O of 5.06. No corresponding correction is necessary for (1a).

[16] H. H. Jaffé, *Chem. Rev.*, 1953, **53**, 191.
[17] C. L. Liotta and D. F. Pinholster, *Chem. Comm.*, 1969, 1243.

rate constant by a factor of 2 000 for the neutral pyrimidine. Since there is no precedent in a concerted addition process for a rate change of that magnitude resulting from the introduction of a methyl substituent, it is clear

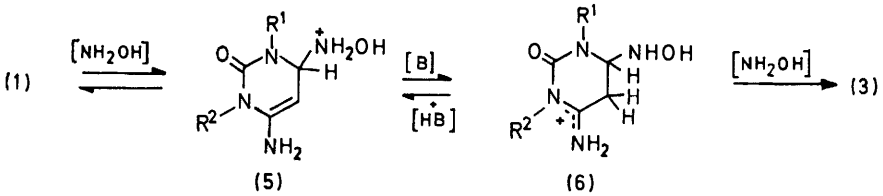


(4)



(7)

that these kinetic data show that the formation of (3) involves general base catalysis of the nucleophilic addition of hydroxylamine to C-6.



(5) (6)

There is evidence that during the course of the formation of (3) from cytosine,[13] 1-methylcytosine,[13,18] and cytidine [19] in deuterium oxide solution, deuterium replaces hydrogen at position 5. This must imply that protonation at C-5 is reversible and is incompletely stereospecific,[13] while the present results show that it is controlled by general acid-base catalysis. We therefore suggest that the transition state for the Michael addition of hydroxylamine to cytosines involves general base-catalysed proton transfer between the hydroxylamine adducts (5) and (6) which is associated with a diffusion-controlled fragmentation of (5) to re-form (1) and a fast hydroxylaminolysis of (6) to give (3), analogous to the rapid reaction between hydroxylamine and other 5,6-dihydrocytosines.[10] We note that the transition state (7) advocated by Schalke and Hall [12b] is indeed compatible with the kinetic scheme which we have established.* However, apart from the improbable geometry it imposes on a 1,3-proton shift, it necessarily demands strict *trans*-stereochemistry for protonation at C-5 relative to the attacking hydroxylamine molecule. Such a restriction is incompatible with the data for deuterium exchange [13,18,19] at position 5 observed for (1), (2), and (3), which will be discussed in detail elsewhere.

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* Notwithstanding the claim [12b] that this process is kinetically first-order in hydroxylamine.

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[19] E. I. Budowsky, E. D. Sverdlov, R. P. Shibaeva, G. S. Monastyrskaya, and N. K. Kochetkov, *Biochim. Biophys. Acta*, 1971, **246**, 300.