Kinetics and Mechanism of Reaction of Hydroxylamine with Cytosine and its Derivatives

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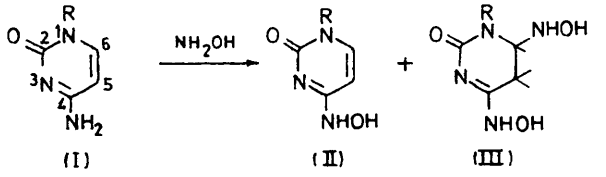
The reactions of hydroxylamine with cytosine, and with 1-methyl-, 5,6-dihydro-, and 5,6-dihydro-1-methylcytosine in the pH range 4—7 involve terms both first- and second-order in amine and proceed predominantly *via* the protonated form of the pyrimidine bases. The sole products of reaction for the dihydrocytosines are the corresponding 5,6-dihydro-N^4-hydroxycytosines. Cytosine and 1-methylcytosine are known to give two products each, namely the N^4-hydroxy- (II) and the 5,6-dihydro-N^4-hydroxy-6-hydroxyamino-cytosines (III). The ratio of these two products is shown to change with changes in pH and in hydroxylamine concentration. This behaviour and the relative rates of reaction of the various substrates supports a mechanism of formation of (IIIb) involving rate-limiting general acid-base catalysis of the Michael addition of hydroxylamine to position 6 of 1-methylcytosine (Ib).

THE mutagenic action of hydroxylamine is generally accepted [1,2] to arise from its reaction with cytosine residues in DNA, for which it exhibits considerable specificity at neutral pH.[3] At high concentrations of hydroxylamine, cytosine and its nucleosides each give two products. Direct substitution of the amino-group by hydroxylamine accounts for the minor reaction [4]



(I) (II) (III)

a; R = H b; R = Me

giving product (II), while an independent, simultaneous process provides the major pathway and gives a 5,6-dihydro-N^4-hydroxy-6-hydroxyaminocytosine [5] (III) In principle, the mutational event can arise from either of these processes since both products exist in tautomeric equilibrium with their imino-forms and this form is presumed to form a base-pair with adenine rather than with guanine in the replication of DNA.[6,7]

The mechanisms by which hydroxylamine gives these two products has been a cause of some discussion. This has been clarified by partial kinetic analysis of the reaction which, however, was restricted to a limited range of conditions.[4,5,8] Additional valuable information is available from stereochemical studies on the reaction of deuteriated hydroxylamine with 1-methylcytosine [9] which shows a specificity in the proton transfer part of the reaction not previously revealed in the kinetic studies.

General acid-base catalysis has been established as an important feature in a variety of reactions involving hydroxylamine and carbonyl groups,[10] many of which can be described in full mechanistic detail. This paper presents the results of a full kinetic analysis of the reactions between hydroxylamine and cytosine and its simple derivatives which support a mechanistic account of these processes.

EXPERIMENTAL

Materials.—Cytosine was obtained commercially and 1-methylcytosine,[11] 5,6-dihydro-1-methylcytosine,[7] and 5,6-dihydrocytosine [12] were prepared by standard pro-

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

Apparatus.—A Radiometer PHM 26 in conjunction with a G2222B glass electrode was used for measurement of pH and coupled to a TTT11 titrator, ABU12 autoburette, and SBR2c titrigraph for pK_a determinations which employed G202C glass and K401 calomel electrodes. Temperature control was maintained by circulation of water from a Haake model Fe thermostat (±0·05 K). N.m.r. spectra were recorded at 100 MHz using a Varian HA 100 machine and u.v. spectral data were collected with Gilford 240 or Unicam SP1800 spectrophotometers.

Kinetic Measurements.—All reactions were run in water in the presence of 10^{-5}M-ethylenediaminetetra-acetic acid to avoid effects from trace metal ions, and at unit ionic strength for 1-methylcytosine and 5,6-dihydro-1-methylcytosine, and at ionic strength 2·0M (KCl) for cytosine and 5,6-dihydrocytosine at 310 ± 0·1 K. The substrate was introduced in aqueous solution (*ca.* 10 µl) into 3 ml of the appropriate reaction mixture to give an initial concentration of 2—5 × 10^{-5}M. The disappearance of starting material was monitored continuously by decrease in optical density at 268 nm for cytosine and 1-methylcytosine, at 240 nm for 5,6-dihydrocytosine, and at 228 nm for 5,6-dihydro-1-methylcytosine, and pH values of reaction solutions were determined at the beginning and end of each run (Table 1).

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 where possible. Slow reactions were analysed by Guggenheim's method [13] but not by initial slopes. All reactions observed displayed good first-order kinetic behaviour and stable end-points.

Product Analysis.—The products of reaction of hydroxylamine with cytosine (Ia) and 1-methylcytosine (Ib) have been fully characterised.[2,5,14] Reaction solutions of (Ib) and hydroxylamine initially showed $\lambda_{max.}$ 275 nm (ε 9·2 × 10^3) but on completion of the reaction showed $\lambda_{max.}$ 282 nm. This was due entirely to the formation of N^4-hydroxy-1-methylcytosine (IIb) (ε_{282} 6·9 × 10^3), since the second product (IIIb) is effectively transparent at this wavelength. The mole fraction of (IIb) formed from (Ib) was calculated using these data and the initial optical density of reaction solutions at 275 nm in conjunction with observed (or, for very slow reactions, computed) terminal optical densities at 282 nm.

The products formed between hydroxylamine and 5,6-dihydrocytosine and its 1-methyl derivative under kinetic conditions were isolated and identified by comparison with authentic samples of 5,6-dihydro-N^4-hydroxy- and 5,6-dihydro-N^4-hydroxy-1-methyl-cytosine respectively.

5,6-*Dihydro*-N^4-*hydroxycytosine.*—Hydroxylamine solution (1M) was prepared from hydroxylamine hydrochloride solution by means of Amberlite IR-400 resin in the hydroxide form. To this solution (8 ml) was added 5,6-dihydrocytosine (0·5 g) and the mixture shaken 5 min at 278 K. The crystalline precipitate gave the *product*, m.p. 501—503 K (from water) (Found: C, 37·45; H, 5·7; N, 32·7. $C_4H_7N_3O_2$ requires C, 37·2; H, 5·45; N, 32·55%); $\lambda_{max.}$ (H_2O, pH 6·5) 219 nm (ε 9·6 × 10^3); τ (CF_3CO_2H) 2·62 (s, N^1H), 6·20 (t, *J* 6·5 Hz, NCH_2CH_2), and 6·80 (t, *J* 6·5 Hz, $CH_2CH_2C{=}$).

TABLE 1

Experimental data for the hydroxylaminolysis of cytosine derivatives at 310 K

Substrate	pH	Number of runs	$[NH_2OH_{tot}]$/M	% Free base
Cytosine (Ia) [a]	4·95	5	0·6—2·0	9
	5·55	6	0·2—2·0	29
	5·91	6	0·2—2·0	50
	6·35	6	0·2—2·0	75
1-Methylcytosine (Ib)	4·86	9	0·3—1·0	10
	5·25	5	0·2—0·9	21
	5·57	7	0·2—1·0	33
	5·91	6	0·2—1·0	52
	6·30	6	0·2—1·0	74
5,6-Dihydrocytosine [a]	5·95	6	0·005—0·1	50
	6·88	6	0·01—0·1	90
	5·17—7·20	8	0·12—0·02	*c*
1-Methyl-5,6-dihydrocytosine [b]	5·07	6	0·01—0·08	15
	5·50	7	0·01—0·06	30
	5·85	6	0·005—0·05	50
	6·23	7	0·01—0·07	70
	6·81	7	0·005—0·06	90

[a] µ = 2·0M (KCl). [b] µ = 1·0M (KCl). [c] $[NH_2OH_{free}]$ = 0·02M.

5,6-*Dihydro*-N^4-*hydroxy*-1-*methylcytosine.*— Hydroxylamine solution (60 ml) and 5,6-dihydro-1-methylcytosine (5·08 g) were shaken at 278 K for 5 min and the *product* had m.p. 511—513 K (from water) (Found: C, 41·75; H, 6·15; N, 29·6. $C_5H_9N_3O_2$ requires C, 41·95; H, 6·35; N, 29·35%); $\lambda_{max.}$ (H_2O, pH 6·5) 223 nm (ε 6·2 × 10^3); τ (CF_3CO_2H) 6·31 (t, *J* 7 Hz, NCH_2CH_2), 6·85 (t, *J* 7 Hz, $CH_2CH_2C{=}$), and 6·87 (s, NCH_3).

RESULTS

The apparent first-order rate constants determined for reactions of all four substrates show a greater than first-order dependence on the concentration of hydroxylamine and a complex variation with the pH of the reaction medium. At a constant concentration of free hydroxylamine, the apparent first-order rate constants for the reactions of 5,6-dihydrocytosine and 5,6-dihydro-1-methylcytosine follow an ionisation curve for which the kinetic pK_a coincides with the pK_a of the substrates measured by potentiometric titration.[15] This is illustrated for the reaction involving 5,6-dihydrocytosine (Figure 1). Similar behaviour is shown by cytosine and 1-methylcytosine, although in these cases all the experiments were carried out at hydrogen ion concentrations at which the pyrimidine was predominantly in the free base form.

All observed rate constants were thus transformed to relate to the reaction of the substrates in their protonated form to facilitate further analysis of the data. The resulting apparent first-order rate constants now show a continuous change with changing buffer ratio at a constant total concentration of that buffer (Figure 2). Plots of the corrected apparent second-order rate constants as a function of hydroxylamine concentration at several pH's revealed that the two dihydrocytosines give products by simultaneous second- and third-order reactions and that the latter simply involves general-base catalysis. For cytosine (Ia) and 1-methylcytosine (Ib) plots of the second-order rate constants against buffer concentration also give a family of curves whose slopes increase with increasing mole

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fraction of hydroxylamine free base and whose intercepts increase with increasing pH (Figure 3). A plot of the

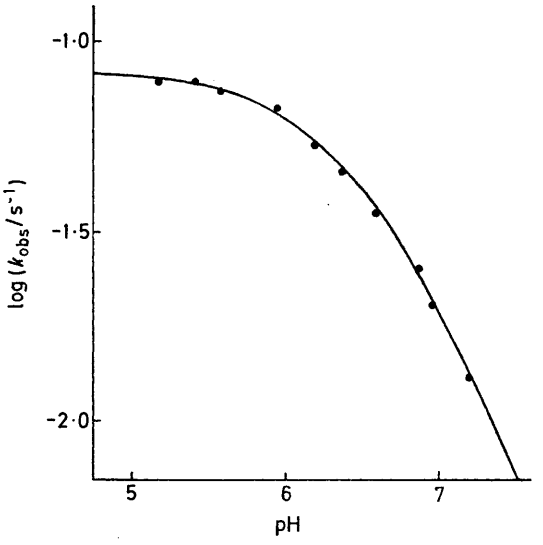


FIGURE 1 Variation of the apparent first-order rate constant for the reaction of 5,6-dihydrocytosine with hydroxylamine as a function of pH at constant concentration (0·02M) of hydroxylamine free base. Curve calculated from equation (2) and constants in Table 2

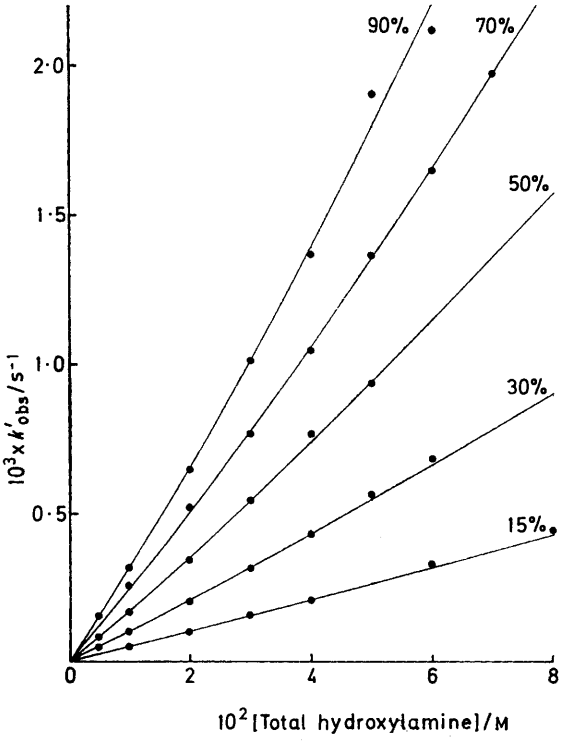


FIGURE 2 Variation of the apparent first-order rate constant for the reaction of hydroxylamine with 5,6-dihydro-1-methylcytosine hydrochloride as a function of hydroxylamine concentration at different buffer ratios, each indicated by the percentage neutralisation of hydroxylamine hydrochloride. Curves calculated from equation (2) and constants in Table 2

gradients of these slopes, computed by linear regression analysis, as a function of the mole fraction of hydroxylamine free base gives a good linear relationship which shows

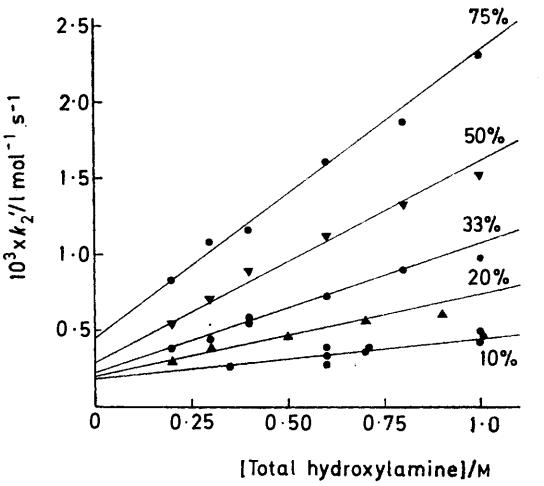


FIGURE 3 Variation of the apparent second-order rate constant for the reaction of hydroxylamine with 1-methylcytosine hydrochloride as a function of the concentration of hydroxylamine at different buffer ratios. Slopes calculated from equation (2) and constants in Table 2

that the catalysis is due only to the basic component of the amine buffer (Figure 4).

Multiple linear regression analysis of the whole data set for each substrate provides the rate constants for the water

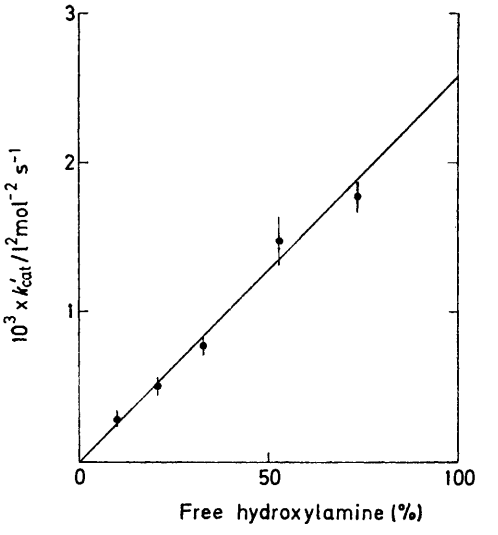


FIGURE 4 Variation of the apparent catalytic rate constant for the reaction of hydroxylamine with 1-methylcytosine hydrochloride as a function of the composition of hydroxylamine buffer. Curve calculated from equation (2) and the value of k_2 in Table 2

(k_1), hydroxylamine (k_2), and apparent hydroxide (k_3') catalysed terms for the reaction of hydroxylamine with protonated substrate according to equation (1).

$$\text{vel.} = (k_1[NH_2OH] + k_2[NH_2OH]^2)[SH^+] + k_3'[NH_2OH][OH^-][SH^+] \quad (1)$$

This equation can be transformed to give the general expression for the reaction of all four substrates with hydroxylamine as in equation (2), corresponding to the rate and equilibrium constants of Table 2.

$$k_{obs} = \{(k_1 + k_2[NH_2OH])[NH_2OH][H^+] + k_3K_a[NH_2OH]\}/(K_a + [H^+]) \quad (2)$$

The products of the reaction of 5,6-dihydrocytosine and 5,6-dihydro-1-methylcytosine with hydroxylamine were identified as 5,6-dihydro-N^4-hydroxycytosine and its 1-methyl derivative respectively by isolation from reaction mixtures and comparison with fully characterised, synthetic materials. All the reactions involving cytosine (Ia) or 1-methylcytosine (Ib) gave mixtures of two products, the former giving (IIa) and (IIIa) and the latter giving (IIb) and (IIIb). The ratio of these dual products varied with changing pH and with changes in the concentration of the hydroxylamine buffer. A detailed analysis of the products given by 1-methylcytosine, based on the absorptivity of (IIb) at 282 nm and the transparency of (IIIb) at that wavelength, showed that the ratio (IIIb) : (IIb) increased with increasing pH and also with increasing buffer concentration, most markedly at high pH. The apparent second-order rate constants for the formation of these two products were derived from the gross rate constants for disappearance of (Ib) and the product ratios (Figure 5). While the second-order rate constant for formation of (IIIb) is directly proportional to the concentration of hydroxylamine free base, that for (IIb) is independent of buffer concentration within the limits of experimental error but displays an increase with increasing pH.

TABLE 2

Calculated rate constants for hydroxylaminolysis of cytosine derivatives at 310 K

Substrate	pK_a	k_1/l mol s^{-1}	k_2/l^2 mol^{-2} s^{-1}	k_3/l mol^{-1} s^{-1}
Cytosine (Ia) [a]	4·57 ± 0·09	6·78 ± 0·45 × 10^{-4}	1·17 ± 0·24 × 10^{-2}	7·40 ± 0·35 × 10^{-5}
1-Methylcytosine (Ib) [b]	4·67 ± 0·02	1·71 ± 0·12 × 10^{-4}	2·58 ± 0·07 × 10^{-3}	7·16 ± 0·45 × 10^{-6}
5,6-Dihydrocytosine [a]	6·47 ± 0·04	4·05 ± 0·10	11 ± 2	
5,6-Dihydro-1-methylcytosine [b]	6·48 ± 0·03	3·44 ± 0·05	12·2 ± 1·5	

pK_a of hydroxylamine = 5·91 ± 0·04;[a] 5·88 ± 0·04 [b]

[a] μ = 2·0M. [b] μ = 1·0M.

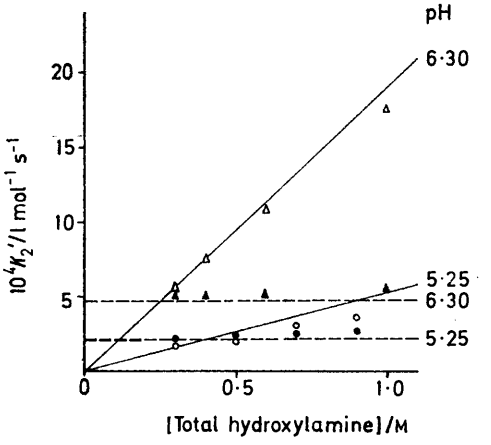


FIGURE 5 Variation in the apparent second-order rate constants for the formation of N^4-hydroxy-1-methylcytosine [(IIb) closed symbols] and 5,6-dihydro-6-hydroxyamino-1-methylcytosine [(IIIb) open symbols] as a function of hydroxylamine concentration at pH 5·25 (●, ○) and pH 6·30 (▲, △). Slopes calculated from equation (2) and constants in Table 2 for the partial reaction first-order (——) and second-order (– – – –) in hydroxylamine

DISCUSSION

These results show that the reaction of hydroxylamine with both cytosines and 5,6-dihydrocytosines is subject to general catalysis by hydroxylamine buffers. In principle this catalysis should also be exhibited by other amines, though this feature was not investigated because of anticipated complications arising from the reaction of the second amine with cytosine. No definition of the nature of this catalysis can precede the identification of the state of ionisation of the substrate in the transition state. The data show that for the 5,6-dihydrocytosines it is the cationic form of the pyrimidine which is reactive (Figure 1) and this is the form favoured under the reaction conditions used. The weaker basicity of cytosine and 1-methylcytosine precluded investigation of the reaction under conditions where they would be fully protonated since at such pH values the concentration of hydroxylamine free base would be too low for the reaction to proceed at an observable rate. However, the nature of the hydrogen ion dependence of reactions of these substrates fully harmonised with treatment of the data based upon reaction of the cationic form of the substrate.

Using this analysis, the buffer effect clearly emerges as a straightforward example of general-base catalysis with no evidence for any contribution to the reaction from general-acid catalysis (Figure 4). This formulation is complemented by the alternative presentation of the reaction as one which involves general-acid catalysis of the reaction of hydroxylamine with cytosine in the free base form,* a general ambiguity to which the discussion will return later.

That part of the reaction which is not subject to buffer catalysis contains two components. The first is best described as the reaction of hydroxylamine free base with the cytosine cation while the latter can be presented either as the reaction of hydroxylamine with cytosine free base [as in equation (2)] or as its reaction with the cytosine cation with specific base catalysis from hydroxide [as in equation (1)]. The former interpretation is adopted both on the principle of greater simplicity and because no hydroxide catalysis is observed for the reactions of the 5,6-dihydrocytosines which, as shown below, react with hydroxylamine by a similar mechanism.

The determination of product ratio (III) : (II) could be made directly at high concentration of hydroxylamine by observation of completed reaction mixtures. However, at low buffer concentration, the end-points

* This ambiguity would, in principle, be resolved by investigation of the pH-dependence of the reaction below the pK_a of cytosine or by the use of a fully methylated analogue, such as 1,3,N^4,N^4-tetramethylcytosinium chloride.

had to be computed by exponential analysis * from kinetic data preferably obtained over at least two half-lives. The calculated apparent second-order rate constants for the separate formation of (II) and (III) clearly show that these products are not formed by homocompetitive processes. When these results are juxtaposed with the calculated curves for the separated bimolecular and apparent termolecular components of the rate equation (2), it is apparent that the production of (II), involving transamination at C-4, is predominantly unimolecular in hydroxylamine. In addition, this reaction proceeds *via* cytosine in the protonated and free base forms (Figure 5). The discrepancy between the calculated curves and the experimental data suggests that some general catalysis may augment the reaction but that it would amount maximally to some 20% of the reaction at molar free hydroxylamine.

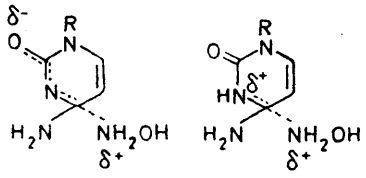
On the other hand, the formation of the second product (III) evidently is a bimolecular process with respect to hydroxylamine free base. This would be, of course, a necessary feature of the reaction were the rate-determining step to involve the transamination at C-4 subsequent to equilibrium addition of hydroxylamine across the 5,6-double bond. If, conversely, the rate limiting step is the Michael addition of hydroxylamine, then this evidence must establish the existence of general catalysis of that process.

These alternatives for the transition state could be distinguished in principle by the partitioning of the presumed intermediate, 5,6-dihydro-6-hydroxyaminocytosine, into cytosine (Ia) and product (IIIa) under identical conditions. Because of the present inaccessibility of this material, recourse was made to a comparison of the rates of reaction of cytosines with those of their 5,6-dihydro-derivatives, an alternative which is acceptable since the addition of hydroxylamine or hydrogen across the olefinic bond can be deemed an exothermic process.

The data obtained (Table 2) show that in both cases the dihydro-compounds are some 10^4 times more reactive than the parent cytosines and this observation applies both to the unimolecular (k_1) and to the bimolecular (k_2) hydroxylamine reactions. Thus it must be concluded that the rate-determining step in the formation of (III) is the Michael addition which is followed by a rapid transaminolysis at C-4. The same conclusion is supported by the differential effects of methylation at N-1 on the reaction of hydroxylamine with cytosine and 5,6-dihydrocytosine (Table 2). For the former, this substitution retards the formation of (III) fourfold and there is a similar effect on the formation of (II). However, there is no such retardation in the reaction of 5,6-dihydrocytosine. Therefore, it follows that this retardation is not shown in the transamination at C-4 if the olefinic bond has been saturated and, consequently, the retardation seen for cytosine must relate to reaction of hydroxylamine with the fully unsaturated pyrimidine ring.

The mechanism for the formation of product (II) can now be described accurately. Jencks has established that breakdown of a symmetrical transition state with two amines bonded to the same carbon involves rupture of the bond to the more weakly basic nitrogen.[16] Since the formation of (II) is the aminolysis of a vinylogous amide, the transition states for transmination at C-4 are depicted with bond formation involving the hydroxylamine unit rather than bond rupture involving ammonia both for the cytosine cation (IV) and for the neutral pyrimidine (V).

The addition of hydroxylamine to 1-methylcytosine to give (IIIb) is a stereospecific process, which has been



(V) (IV)

assigned *trans*-stereochemistry [9] by means of conformational interpretation of n.m.r. data.[17] Similar observations have been made on the addition of bisulphite to 1,3-dimethyluracil, also assigned *trans*-stereochemistry on the basis of n.m.r. data.[17,18] The stereospecificity of both these processes is shown in the reverse direction in which the elimination proceeds under conditions fitting for general base catalysis.[9,19,20] It can therefore be deduced that the addition of the proton to C-5 is concerted with the addition of the hydroxylamine nitrogen to C-6. Thus, the transition state for the formation of (IIIb) must utilise the catalytic hydroxylamine molecule as a proton donor at C-5, as depicted in (VI).

It has already been stated that the kinetic analysis is equally consistent with a transition state in which the proton resides on N-3 of the cytosine ring. This alternative would be analogous to the demonstrated addition of bisulphite to the cytosine cation as well as to the free base form.[21] Such specific base catalysis of the addition to the cation is mechanistically acceptable since the product of that step would be more basic than the reactant and would be largely protonated under the conditions of the reaction. However, such a transition state composition is not compatible with *trans*-stereospecificity, and the catalytic hydroxylamine molecule would need to be involved in a cyclo-addition process with obligatory *cis*-stereochemistry, (VII). The choice

* Computer program kindly supplied by Dr. J. Kinderlerer.

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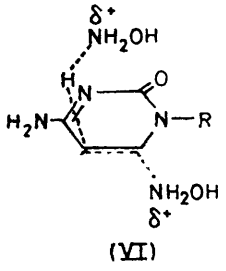
between these alternatives rests on stereochemical and kinetic data, for neither of which is the interpretation



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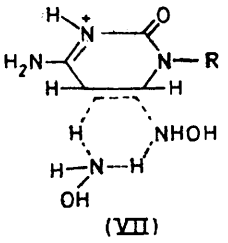

(VII)

incisive. Consequently a rigorous stereochemical assignment or a kinetic study of cytosine derivatives methylated at N-3 is desirable.

Some comments emerge from these conclusions with regard to the use of hydroxylamine for the modification of nucleic acids *in vitro* and *in vivo*. As a mutational agent, it is employed at 0·5—1M concentration [1,22] and can be used in the pH range 6—7. Under these conditions, the product ratio for isolated cytosine molecules would favour its conversion into (IIIa) rather than (IIa) by a factor of four (Figure 5). This inference that (III) is likely to be the mutational species *in vivo*, as suggested by Brown and Phillips,[23] can also be supported by the fact that hydroxylamine is only an effective mutagen at high concentration while at low concentration (0·02M) it acts *in vivo* as an inactivating agent. Moreover, both of the transition states (VI) and (VII) proposed for the formation of (III) are sterically demanding, particularly the former which requires simultaneous approach of hydroxylamine to both faces of the cytosine residue. This is not possible for cytosine residues stacked in a native DNA helical structure and would require denaturation to precede chemical modification, as has been observed for synthetic homopolynucleotides.[15] Finally, this stereochemical requirement is also consistent with the known selectivity of hydroxylamine and methoxylamine for exposed residues in transfer RNA species.[24]

[4/1692 *Received*, 12*th August*, 1974]

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