Deuteriodeprotonation of Methylcytosines by Hydroxylamine in D_2O Solution

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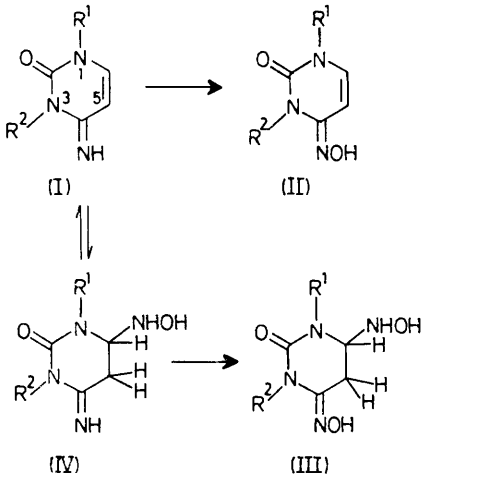
Summary When hydroxylamine reacts with 1-methylcytosine or with 1,3-dimethylcytosine in D_2O solution, partial deuteriodeprotonation is observed at position-5 in both products and to a greater extent in methylcytosines which had not reacted thereby showing that hydroxylamine addition across the pyrimidine 5,6-double bond is reversible and is only partly stereospecific.

HYDROXYLAMINE transforms cytosine (Ia) and its derivatives into two stable products, (IIa) and (IIIa), by independent processes[1] which are mutagenetically significant.[2] The kinetic aspects of these reactions have been adequately explored[1] but contrasting proposals for the mechanism of the reactions have been advanced.[1,3–5] These arise from the apparent *trans*-specificity of addition of hydroxylamine[3,5] to form (III), from conflicting views on the existence[3,5,6] or

the extent[4,7] of deuterium exchange at position-5 in these or in related[8] reactions of cytosines, and from doubt about the role of (IV) as an intermediate in the hydroxylamine reaction.[5] Accordingly, a reinvestigation of this process in deuteriated media appeared essential.

We now present the results of deuterium exchange experiments which establish the reversibility of the formation of (IV) from (I), show that (IV) is an intermediate in the formation of (III), and reveal that the addition of hydroxylamine to (I) is not wholly stereospecific.



a; $R^1 = R^2 = H$ b; $R^1 = Me, R^2 = H$ c; $R^1 = R^2 = Me$

Hydroxylamine reacts with cytosine and 1-methylcytosine predominantly in their cationic form.[1] Since 1,3-dimenthylcytosine, pK_a 9·4, is fully protonated in the pH range under investigation, we examined its reaction with hydroxylamine both kinetically and, in D_2O solution, using ^1H n.m.r. spectroscopy.[7] The former investigation[9] shows that (Ic) is converted into (IIc) and (IIIc) by independent processes which are strictly analogous to those previously established for cytosine and 1-methylcytosine.[1]

When a solution of (Ic) in hydroxylamine (2M) in D_2O at pD 6·9 ($t_{\frac{1}{2}}$ *ca.* 120 min) is monitored by ^1H n.m.r. spectroscopy at 100 MHz, marked deuterium exchange is observed at position-5 both in unchanged (Ic) and in the hydroxylaminolysis product (IIc). This is shown (Figure) by the growth of a singlet inside the AB doublet for H-6 at τ 1·95 (Ic) and 2·8 (IIc). It is very clear that at all times after the commencement of the reaction there is a greater proportion of deuterium in (Ic) than in (IIc). Hydrogen is also replaced by deuterium at position-5 in the second product (IIIc) as shown by the relative intensities of signals at τ 5·24 for H-6 and 6·33 for H-5 in spectra of completed reaction solutions. Moreover, the final extent of that exchange is close to 25% for both (IIc) and (IIIc) under the above conditions.

Since this deuterium exchange is evidently a general acid–base-catalysed function of reaction of the cytosine cation, we examined the behaviour of 1-methylcytosine (Ib) with hydroxylamine (1M) in D_2O solution at pD 4·9. At 310 K this reaction shows a significant solvent kinetic isotope effect, $k_H/k_D = 2·5$. Monitored by ^1H n.m.r. spectroscopy at 100 MHz, the reaction mixture showed slow incorporation of deuterium at position-5 for (Ib) which had not reacted to the extent of some 10% at a point when 93% of (Ia) had been converted into (IIb) and (IIIb). This deuterium exchange was both slower and less extensive in the final product mixture for (IIb) and (IIIb) than for the products from 1,3-dimethylcytosine. Prolonged incubation of completed reaction solutions showed no subsequent incorporation of deuterium into the final products.

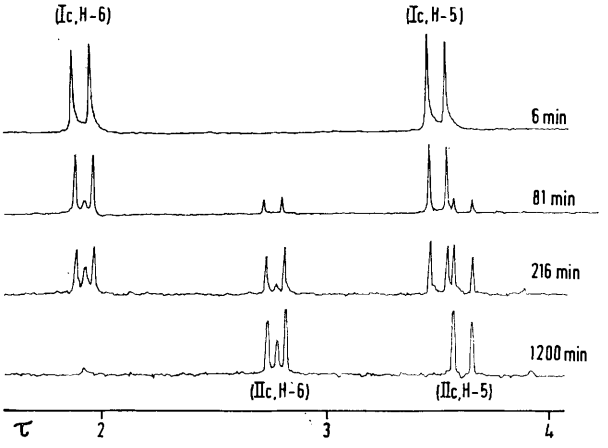
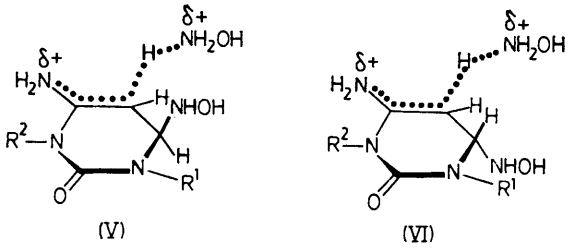


FIGURE. 100 MHz ^1H n.m.r. spectra of a solution of hydroxylamine (1M) and 1,3-dimethylcytosine hydrochloride (0·3M) in D_2O at 310 K and pD 6·9 at stated time intervals after mixing. (Ordinate expansion not necessarily to scale).

Under less acidic conditions, pD 6·9, no deuterium exchange into (Ib) nor into products could be detected.

These data establish the formation of an intermediate which can lose either hydrogen or deuterium from position-5 to reform (I) and which is not on the pathway for the formation of (II). Only (IV) satisfies these requirements and the kinetic evidence[1] and must be formed reversibly as originally suggested by Budowsky *et al.*[4] The transition states for the loss of each of the diastereotopic hydrogens at



position-5 can be represented as (V) and (VI) for the *cis*- and *trans*-geometries respectively. They correspond to the non-concerted forms of the transition states previously presented.[1]

The preponderance of *trans*-addition seen in the formation[3,5] of (III) shows that (VI) is intrinsically of lower energy than (V), which is probably a manifestation of steric opposition of the two hydroxylamine species. However, under conditions of deuterium exchange at position-5, the deprotonation of (IV) to reform (I) will be subject to a primary kinetic isotope effect. This must favour the process of opposite stereochemistry to achieve deprotonation rather than dedeuteriation at position-5.

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