# REACTION OF HYDROXYLAMINE WITH 5-SUBSTITUTED CYTOSINES

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The mutagenic activity of hydroxylamine is well known and, at about neutral pH, is believed to be due largely to its specific reaction with cytosine residues in nucleic acids. The nature of the reaction products has been examined by several observers (Brown & Schell, 1961; Freese, Bautz & Bautz-Freese, 1961; Schuster, 1961; Verwoerd, Kohlhage & Zillig, 1961) and appears to be reasonably well elucidated (Brown & Schell, 1961).

From the known mutagenic action of hydroxylamine on the T-even bacteriophages, it has been inferred that this reagent reacts also with 5-hydroxymethylocytosine (Freese, Bautz-Freese & Bautz, 1961); and Schuster (1961) observed that treatment of phage $T_2$ DNA with hydroxylamine resulted in a partial loss of 5-hydroxymethylcytosine, but the nature of the reaction involved is unknown. It is consequently surprising that 5-methylcytosine has been reported to be unaffected by this reagent (Schuster, 1961; Freese et al., 1961). The increasingly widespread use of hydroxylamine as a mutagenic agent, as well as for possible base sequence studies in nucleic acids (Kochetkov, Budowsky & Simukova, 1962), suggested the advisability of an examination of this apparent inconsistency.

It should be recalled that the experimental technique normally employed for following the reaction of cytosine with hydroxylamine is based on the modification of the UV spectrum of

the former at its principal absorption maximum, resulting from the nucleophilic addition of hydroxylamine to the 5,6 double bond, with consequent saturation of the latter and disappearance of the characteristic absorption maximum (Brown & Schell, 1961; cf. Janion & Shugar, 1960). If, however, 5-methylcytosine is allowed to react with hydroxylamine under similar conditions, and the entire absorption spectrum followed as a function of time, it may easily be seen that a reaction does in fact occur. This is clearly shown

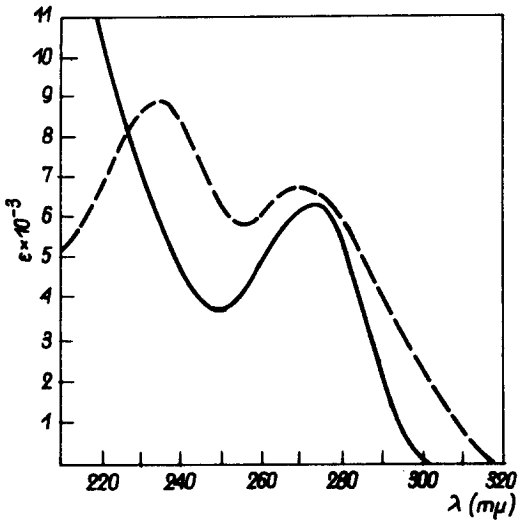


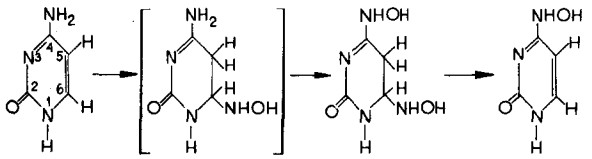

Fig. 1: Conversion of 5-methylcytosine (———) to 2-keto-4-hydroxylamino-5-methylpyrimidine (- - - -) by hydroxylamine at neutral pH; see text for details.

in Fig. 1, which exhibits the modification in absorption spectrum of 5-methylcytosine following 18 hours exposure of a 0.02 M aqueous solution to 2.5 M hydroxylamine at pH 6.6 and 37°.

The reaction product may be readily isolated by precipitation at reduced temperature and differs from 5-methylcytosine by its Rf values in several systems, and by its cationic pK, which is 2.8 as compared to 4.6 for 5-methylcytosine. It has also proven possible to isolate the product in high yield in crystal-

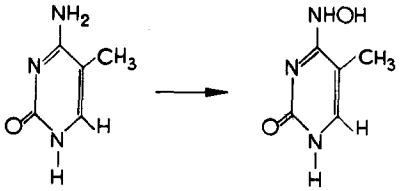
line form, m.p. 241-242°, with an elementary analysis as follows: C - 42.5%; H - 5.6%; N - 29.3%.

The reason for previous failures to observe this reaction is clear from Fig. 1, since the optical density of the principal maximum at 276 mμ remains practically unchanged. With cytosine the initial reaction product is II (via the presumed intermediate I) and this, in acid medium, eliminates one mole hydroxylamine to give III (Brown & Schell, 1961):



(I) (II) (III)

With 5-methylcytosine the absorption spectrum of the product shows that the 5,6 double bond remains unsaturated, so that no product analogous to II is formed. Furthermore the absorption spectrum of the 5-methylcytosine reaction product closely resembles that of III, if the secondary effect of the methyl group is allowed for. The most logical formulation of its structure is therefore the 5-methyl analogue of III, i.e. IV, as follows:



(IV)

This is in accord with the elementary analysis given above, which is to be compared with that expected for IV, viz. C - 42.6%; H - 5.0%; N - 29.8%. The failure of 5-methylcytosine to take up

a molecule of hydroxylamine at the 5,6 bond is in agreement with the fact that UV irradiated cytosine undergoes nucleophilic addition of a water molecule at the 5,6 bond (Sinsheimer, 1957; Shugar & Wierzchowski, 1957), whereas 5-substituted cytosines do not (Wierzchowski & Shugar, 1960).

The foregoing is also in agreement with our observation that, at neutral pH, III will revert slowly to II in the presence of hydroxylamine, but IV is unaffected under these conditions. It is also worth noting that the rate of uptake of hydroxylamine by III is much slower than the rate of formation of II from cytosine; this provides experimental support for the contention of Brown & Schell, (1961) that formation of II from cytosine proceeds <u>via</u> the unstable intermediate I.

Additional evidence for the structure of IV is forthcoming from the reaction with hydroxylamine of 1,5-dimethyl-2-keto-4-ethoxypyrimidine. This reaction is a rapid one and leads to a product which, following crystallization, analyzed for the corresponding 4-hydroxylamino derivative, i.e. to the 1-methyl analogue of IV. The UV spectrum of the latter was in accord with that to be expected for the 1-methyl derivative of IV. It will be shown elsewhere (Janion & Shugar, 1965) that 4-ethoxypyrimidine derivatives provide useful additional model compounds for studying the reaction with hydroxylamine.

Hydroxylamine likewise reacted readily with 5-methylcytidine to give exclusively the riboside of IV. The reaction with 5-hydroxymethylcytosine has also been examined; although the product was not isolated on a preparative scale, its absorption spectrum, measured and compared at a number of pH values with that for IV, was fully consistent with that for a 4-hydroxylamino derivative, i.e. 2-keto-4hydroxylamino-5-hydroxymethylpyrimidine,

the 5,6 double bond being unaffected as for 5-methylcytosine.

It is of some significance that the initial products of reaction with hydroxylamine are basically different for cytosine and 5-substituted cytosines, although both are 4-hydroxylamino derivatives. The former contains a saturated 5,6 bond, which may revert to the original 5,6 double bond, while the latter contains the original 5,6 double bond. It may be anticipated that this will be reflected in the mutagenic action of this agent on nucleic acids containing either unsubstituted or 5-substituted cytosines.

Full details of the foregoing, and of the reaction products with hydroxylamine of other cytosine analogues and of 4-ethoxypyrimidines, will be published elsewhere. We are indepted to Dr. C.B. Reese (Cambridge) and Prof. E. Broda (Vienna) for elementary microanalyses, to Mrs. K. Ziabicka for the preparation of 1,5-dimethyl-2-keto-4-ethoxypyrimidine, to Dr. W. Szer for the 5-methyl cytidine and to Miss K. Wincenciak for excellent technical assistance.

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