SHORT COMMUNICATIONS

The inactivating and mutagenic effect of hydroxylamine on bacteriophage ΦX174

The inactivation of bacteriophage ΦX174 by the mutagenic agents nitrous acid and ultraviolet irradiation proceeds according to a single-hit kinetics[7,8,10]. However, treatment of purified ΦX174 by hydroxylamine (HA) at pH 6 and 25° results in an inactivation that is not strictly exponential. The inactivation curves contain a shoulder, which is easily detectable at a HA concentration of 0.1 M. The ultimate slope of the curves at this concentration is in many cases greater than at 1 M (Fig. 1).

In order to explain the shoulder we imagined a two-step reaction to be responsible for the inactivation, rather than a simple multi-hit event. A number of other possibilities could also be excluded experimentally. The proposed mechanism accounts for the shape of the curves obtained at different concentrations and pH values. Inactivation experiments in which biologically active ΦX174 DNA[4] was used also yielded a shoulder. This implies that the phage protein is not involved in the inactivation mechanism.

The kinetics of mutation induction shows that mutagenesis is due to a single rate-determining reaction (Fig. 2). The mutation frequency was determined by selecting host range mutants of the phage by plating on C/ΦS, a phage resistant strain of *Escherichia coli* C-122 (ref. 9).

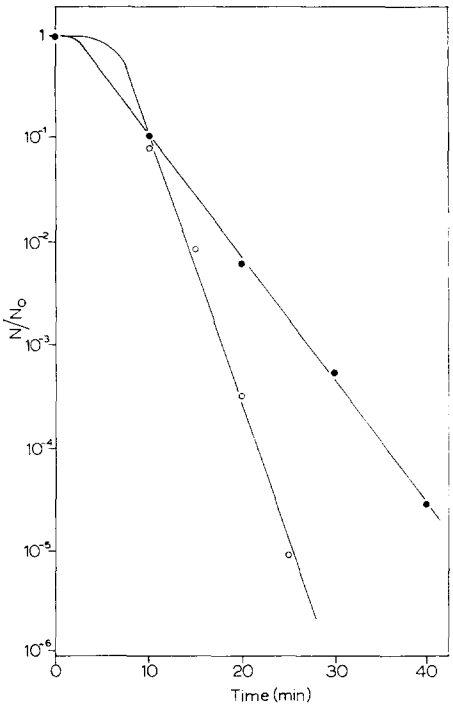
It can be argued (*cf.* ref. 1) that mutation is a rare event with respect to the chemical reaction potentially giving rise to it. Since cytosine is the only base in the DNA that reacts with HA, and no other primary effects on DNA have been reported, it is inferred that inactivation occurs as a result of a two-step reaction with the cytosine residue in the phage DNA. Mutation can then take place when the first step has occurred.

Obviously mutation and inactivation are different processes. This conclusion can also be drawn from our experimental results that the mutation frequency is much higher at 1 M than at 0.1 M HA treatment, and that this frequency increases with decreasing pH, whereas inactivation is found to be optimal at pH 6. It is in agreement, too, with the data of the experiments described below in which methoxyamine was tested and in which the influence of cysteine on the mutagenic and inactivating effect of HA was investigated.

The reaction mechanism of cytidine with HA has been elucidated by VERWOERD *et al.*[11] (see also ref. 3) (Fig. 3).

We put forward the hypothesis that inactivation is due to splitting of the cytosine ring, as was found for uridine[11]. After incubation of cytidine monophosphate with HA for several days and subsequent treatment at pH 1 we observe a decrease of UV-absorbing material, as judged from spectrophotometry (Fig. 4) and paper chromatography. During the acid treatment, carried out for 24 h at room tempera-

Abbreviation: HA, hydroxylamine.



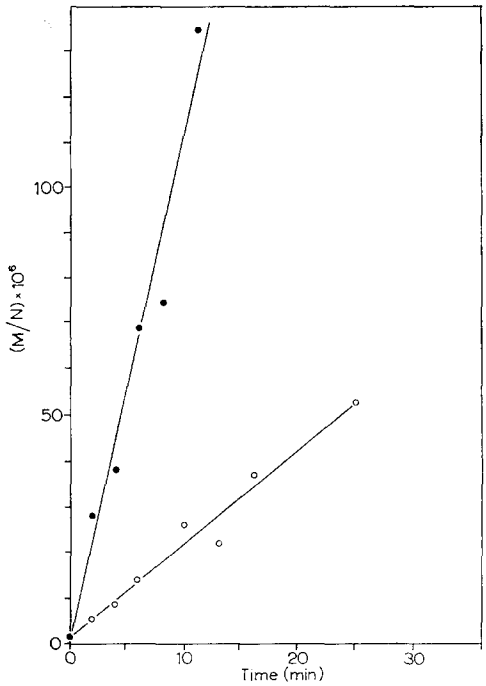


Fig. 1. Inactivation of ΦX174 by 1 M and 0.1 M HA. ●——●, 1 M; ○——○, 0.1 M HA. Survival (N/N_0) is plotted against time of treatment. Inactivation is performed in the presence of 0.03 M phosphate buffer (pH 6.0), 10^{-3} M Mg^{2+}, and about 1 M NaCl, at 25°. The reaction is stopped by dilution into cold 0.05 M Tris (pH 8.0), containing 2% acetone.

Fig. 2. Kinetics of mutation induction by HA. ●——●, 1 M; ○——○, 0.1 M HA. Mutation frequency (the number of host range mutants over the number of survivors: M/N) is plotted against time of treatment. Following HA treatment the surviving particles were allowed to grow for at least one cycle on *E. coli* C, before plating on C/ΦS and C.

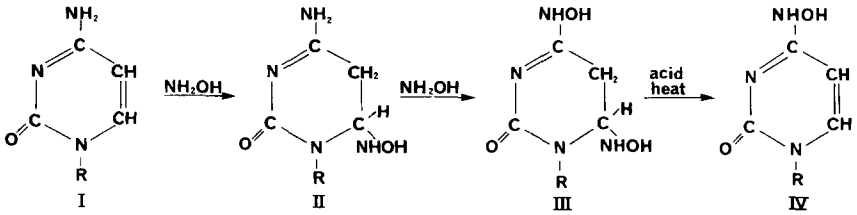


Fig. 3. Reaction scheme of cytidine with HA[11,3]. The reaction mechanism also holds for deoxycytidine[10], and for the phosphate esters. If R = ribose, then: I = cytidine; II = addition product of cytidine (4,5-dihydro-4-hydroxylaminocytidine); III = addition product of uridine 6-oxime (N^6-hydroxy-4,5-dihydro-4-hydroxylaminocytidine); IV = uridine 6-oxime (N^6-hydroxycytidine).

ture[2], or for 30 min at 70°, the addition products II and III are converted into cytidine (I) and uridine 6-oxime (IV). The concentration and pH dependence of the irreversible reaction agrees largely with that of the inactivation of the phage.

KOCHETKOV *et al.*[6] found that methoxyamine reacts with cytidine in the same way as HA, leading to the dimethylderivative of III. In contrast to HA, however,

it did not cause ring splitting with uridine. Our hypothesis implies, by analogy, that methoxyamine will cause mutation but no inactivation. This appears to be the case. Methoxyamine has a strong mutagenic effect, but yields only a slight inactivation. The latter may be ascribed to formation of HA by hydrolysis of methoxyamine[1].

BROWN AND PHILLIPS[2] consider the short-lived addition product of cytosine in the DNA (II in Fig. 3) to be responsible for replication error. Its concentration is too low to account for all mutations, however, especially in ΦX174 DNA, which

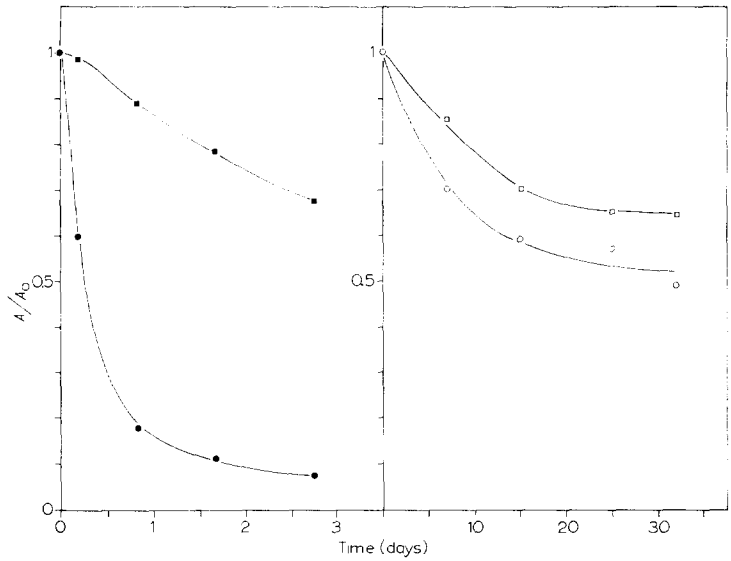


Fig. 4. Decrease of absorbance during incubation of cytidine 2′(3′)-monophosphate with 1 M and 0.1 M HA. ●—·—●, 1 M HA, pH 6.0; ■—·—■, idem, after acid treatment (30 min, 70°) at pH 1; ○—·—○, 0.1 M HA, pH 6.0; □—·—□, idem, after acid treatment (30 min, 70°) at pH 1. The absorbance relative to the absorbance at zero time (A/A_0) was measured at 270 mμ (at pH 6) and 279 mμ (at pH 1) and plotted against time of treatment in HA buffer (see Fig. 1). It should be noted that the HA concentration is not constant, owing to its decomposition (half-life about 20 days). The absorbance of uridine 6-oxime (IV) nearly equals that of cytidine (I)[2,10].

has no ordered secondary structure. We suggest that the great majority of mutations occur as a result of the formation of the more stable addition product of uracil 6-oxime (III in Fig. 3).

Our experiments indicate that some unstable decomposition product of HA is responsible for the inactivating reaction, instead of HA itself. The addition to HA of small amounts of cupric ions (10^{-4} M), which catalyze decomposition, has a strong synergistic effect on the inactivation. This also holds for hydrogen peroxide, a compound giving rise to hydroxyl radicals upon reaction with HA. Since the effect of both compounds is more pronounced at 0.1 M than at 1 M, we feel justified in concluding that the inactivation is due to a compound that reacts rapidly with HA. The concentration of the active product will then be low at high HA concentrations and relatively high at 0.1 M. This might also explain the rapid and variable inactivation rate at the latter concentration of HA without any addition.

Cysteine, in a concentration as low as 0.0025 M, protects the phage almost

completely against inactivation by 0.1 M HA. The mutagenic effect is not obliterated. This result is consistent with the view that cysteine eliminates the product active in the inactivating reaction.

The way in which HA decomposes in alkaline solutions has been partly elucidated by HOLZAPFEL[5]. We found that the ultimate decomposition products, nitrous acid and hyponitrous acid (in concentrations of 0.1 M and 1 M, respectively), did not increase the inactivation rate of HA under standard conditions. Nitroxyl, too, which could be tested indirectly by decomposition of a nitroprussid complex of nickel ($Ni(CN)_3NO^{2-}$), was ineffective up to a concentration of 0.05 M. We therefore conclude that one of the initial decomposition products of HA (*e.g.* OH- or NHOH-radicals, or dihydroxyhydrazine[5]) is the reactive agent in the second step of the inactivating reaction.

A detailed report of this work will be published elsewhere.

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