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Nitrous Acid Damage to Duplex Deoxyribonucleic Acid: Distinction Between Deamination of Cytosine Residues and a Novel Mutational Lesion†

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The rate of nitrous acid deamination of labeled cytosine residues in native *Escherichia coli* deoxyribonucleic acid was monitored in vitro by release of acid-soluble counts after treatment with uracil deoxyribonucleic acid glycosylase. The reaction exhibited a lag and was not stimulated by several agents previously shown to enhance base substitution mutagenesis during nitrous acid treatment of duplex deoxyribonucleic acid. We conclude that a significant proportion of nitrous acid induced mutagenic lesions are novel lesions and not cytosine deaminations.

Deamination of cytosine (C) to uracil (U) is responsible for a significant fraction of spontaneous base substitution mutations at 5-methylcytosine, residues at which sites repair is reduced in repair-proficient *Escherichia coli* strains (3). Mutations due to deamination of C residues are increased and occur at a broad spectrum of sites in Ung$^-$ strains lacking the repair enzyme, uracil DNA glycosylase (B. K. Duncan and J. H. Miller, cited in 3; 6).

Nitrous acid (NA) exhibits mutagenic activity on an array of organisms (28) and is presumed to act by causing deaminations of C to U and of adenine (A) to hypoxanthine (HX). However, Thomas et al. (24) found that NA was strongly mutagenic for phenol-extracted DNA, but was only very weakly mutageneic for carefully prepared duplex *Haemophilus influenzae* DNA. Mutagenicity for this latter native DNA was greatly enhanced by the addition to the reaction mixture of various alcohols, glycols, phenols, and amines. Exposure of NA-treated, intact *E. coli*, or *Salmonella typhimurium* bacteria to polyamines also enhanced base substitution (but not frame-shift) mutagenesis (9, 24). The collective observations led Thomas et al. (24) to suggest that most NA mutagenesis of repair-competent strains was indirect. They proposed that reaction of NA with ubiquitous molecules of low molecular weight first led to the formation of unstable nitrosation products which then served as "delivery vehicles" capable of enhancing the rate of deamination of bases in the DNA and thus exceeding the cellular DNA repair capacities for deaminated bases (cf. 11).

We have tested this hypothesis by observing the rate of uracil production by NA in *E. coli* DNA in vitro both in the presence and in the absence of compounds effective in enhancing NA mutagenesis. [^{3}H]cytosine-labeled DNA was prepared from *E. coli* BD1207 (*thyA36 pyrE*) grown in nutrient broth (Difco Laboratories) to ca. 3×10^8 bacteria per ml and then suspended in minimal E medium (26) containing 6-[^{3}H]uracil at 4 to 5 μCi/ml and including 200 μg of Casamino Acids per ml, 5 μg of thymine per ml, and 10 μg of U per ml. After incubation of bacteria at 37°C for 90 min, DNA was extracted by the method of Marmur (14) and extensively dialyzed against 15 mM sodium citrate buffer (pH 7.4) containing 150 mM NaCl. Analyses by formic acid hydrolysis and descending chromatography in isopropyl alcohol-HCl-water (2) showed that greater than 99% of the label was present in C residues. The final specific activity was 3.8×10^7 cpm/μmol of C.

NA treatment of the DNA (see figure legends) was terminated by the addition of KOH, and the DNA was heated at 95°C for 5 min before dialysis against buffer containing 75 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid KOH (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol. To release U residues, U DNA glycosylase which had been purified to homogeneity (12) was added to excess, namely 10^{-3} units in a final volume of 0.1 ml (1 unit = 1 μmol of U hydrolyzed per min at 37°C). After 30 min at 37°C, unlabeled calf thymus DNA (1 mg/ml) was added, precipitated with trichloroacetic acid, and centrifuged at 5,000 × *g* for 15 min. An aliquot of the supernatant was counted in Triton X-100-toluene fluor in a Packard Tri-Carb model 3320 scintillation spectrophotometer.

Figure 1 shows that NA treatment of native DNA at pH 4.1 to 4.2 failed to engender detectable C → U deaminations for about the first 2 h,

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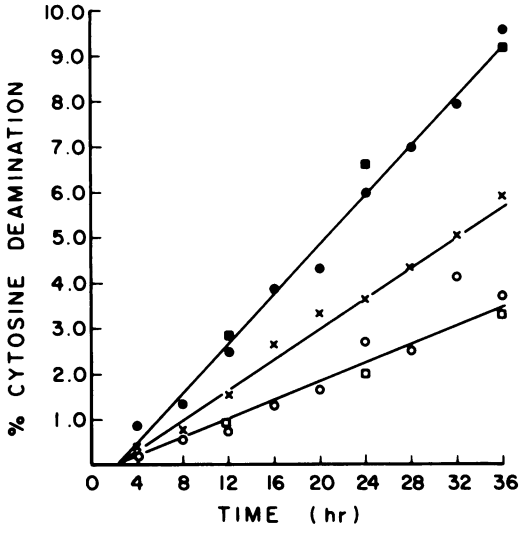

FIG. 1. *U formation as a function of time of DNA treatment in the presence of 100 mM (● and ■ indicate two separate experiments), 50 mM (X), and 25 mM (○ and □ indicate two separate experiments) nitrite. Each reaction mixture contained 26 mM sodium acetate, 52 mM acetic acid (initial pH = 4.1 to 4.2), 156 mM NaCl and 15 μg of [^{3}H]DNA per ml. Freshly prepared 500 mM $NaNO_2$ solution was added to start the reaction and give the final nitrite concentrations shown; incubations were at 37°C.*

after which time the rate of deamination appears linear with time and first-order with respect to nitrite concentration. Our data are not precise enough to define exactly the relationship of NA concentration with the length of the lag period. A lag of similar length occurs in reaction mixtures incubated at pH 4.5 to 4.6 where the subsequent rate of deamination was only about half of that found at pH 4.1 to 4.2 (J. M. Pyper, personal communication). Litman (13), using a completely different methodology, also noted a pronounced lag in deamination of C in native DNA preparations as opposed to no lag for deamination of the free base. Possibly, deamination of C residues in duplex DNA only follows deamination of the opposing G residue (13), deamination of nearby residues, or formation of DNA-distorting cross-links (cf. 1). The relative resistance of C to deamination when in duplex DNA also has been noted recently by others (7, 16). A twofold increase or decrease in the concentration of acetate buffer had no significant effect either on the length of the lag or on the rate of deamination (J. M. Pyper, personal communication). Essentially no increase in the rate of deamination was noted when the nitrite concentration was raised from 100 mM (Fig. 1) to 1 M (data not shown).

In our test system, the rate of deamination of C in denatured DNA, measured under conditions parallel to those shown in Fig. 1, was about two times the rate of deamination of C in native DNA (data not shown). This small difference in rates is in contrast to the over 20-fold protection of A residues observed in duplex DNA (13) and in dAT copolymer (10). Litman (13) observed an approximately fivefold difference between the rates of deamination of free C as opposed to C in duplex DNA (excluding the lag period).

The data in Fig. 2 show that the addition of 0.1 mM (Fig. 2A) or 1.0 mM (Fig. 2B) 1,6-diaminohexane, spermine, or ethanol to NA reaction mixtures containing DNA failed to eliminate the lag in deamination or the rate of deam-

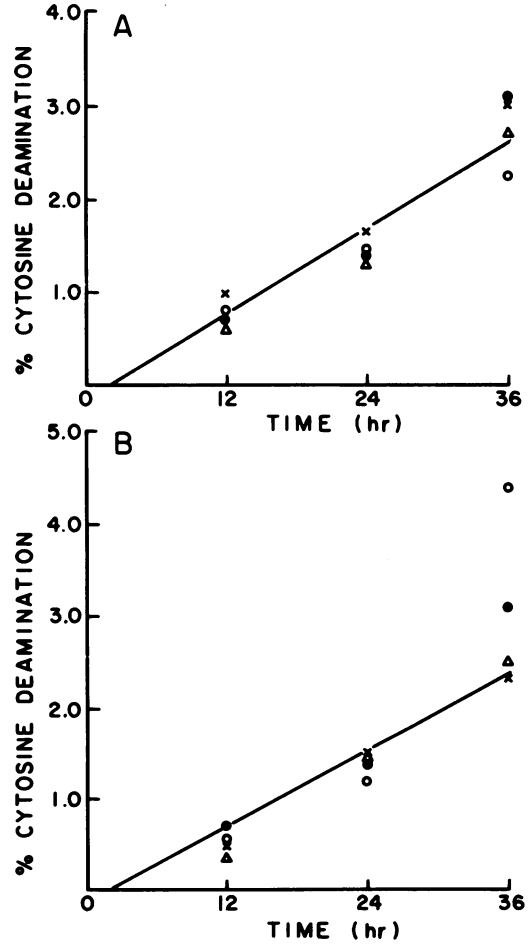

FIG. 2. *U formation as a function of time of DNA treatment in the presence of 30 mM $NaNO_2$ alone (●) and when supplemented with 0.1 mM (A) or 1 mM (B) 1,6-diaminohexane (×), spermine (△), or ethanol (○). Reaction conditions were as described in the legend to Fig. 1.*

ination once in progress. Butylated hydroxytoluene, isoamyl alcohol, ethylene glycol, and phenol at similar molar concentrations also fail to detectably influence the extent of lag or the rate of deamination (data not show). In contrast, each of these seven agents has been shown to effectively promote mutagenesis of *H. influenzae* native DNA in the presence of NA under reaction conditions very similar to those used here (24). Furthermore, mutational lesions appeared in the *H. influenzae* DNA without a perceptible lag period (24). We conclude that the promotion of NA mutagenesis by amines, phenols, glycols, and alcohols is not mediated by an enhancement of C → U deaminations but, rather, has a different molecular mechanism.

Experiments by Murray and co-workers (15; Murphey-Corb and Murray, personal communication) indicate that a mutagenic component in NA plus spermidine reaction mixtures shows enhanced base substitution mutagenesis for Uvr$^-$ *Salmonella* and no detectable mutagenesis for RecA$^-$ *Salmonella*. Their results indicate that much of the base substitution mutagenicity of NA treatments in living organisms possessing duplex DNA may be due to novel lesions that cause helix distortions (Uvr-repairable in *Salmonella*) and, likely, are confined to but one of the two strands of the double helix (i.e., enhanced in Uvr$^-$ *Salmonella*). In contrast, using an *H. influenzae* strain defective in excision of pyrimidine dimers (19) as transformation recipient, Thomas et al. detected no enhancement in mutation frequency over wild-type recipients for DNA treated with a nitrous acid-plus-spermine mixture (24). There are indications, however, that *H. influenzae* carries an error-prone DNA repair pathway with properties distinct from the Rec-Lex type typical of enteric bacteria (8). It may be this latter system in *H. influenzae* which is mutationally responsive to some novel class of lesions induced by nitrous acid in the presence of polyamines. Candidates both for the *Salmonella uvr* and for the *H. influenzae* repair system would be the covalently bound G*pG* and X*pX* dinucleotides detected in NA-treated DNA by Dubelman and Shapiro (4).

Existence of NA-induced DNA lesions other than mere deaminations that cause base substitution mutations could explain mRNA U → C, A → C, and G → U base changes detected in NA-induced revertants of an amber (UAG) *E. coli* mutant (28). In NA-treated yeast, a GC → AT transition was detected at high frequency, but transversion mutations such as AT → TA and AT → CG also were observed (17, 20, 21). In addition, a two-base change was detected (20). Among 100 NA-induced tobacco mosaic virus mutants, a maximum of 88 can be explained by A → G and C → U changes (18, 22); the other 12 are discordant with a stringent theory of exclusive base deamination (5 C → A, 4 A → C, 2 A → U, and 1 A or G → U or C) and appear too frequently to be accounted for merely as spontaneous mutations. Similarly, genetic data indicate that mutations in NA-treated, single-stranded DNA can involve the bases guanine and thymine (23, 25).

We conclude that NA-induced deaminations of C and of A residues to potentially mutagenic lesions in duplex DNA are relatively sluggish processes (10, 13) and that potent repair systems exist for the respective deaminated bases (5, 6, 11, 12). A significant fraction of the base substitution mutagenesis induced by NA treatment of organisms containing duplex DNA may arise from error-prone DNA repair of an array of novel lesions; the production of these novel lesions is enhanced in the presence of ubiquitous molecules which are abundant in living cells and which often are contaminants in cell-free DNA preparations (9, 15, 24).

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