

Molecular Models That May Account for Nitrous Acid Mutagenesis in Organisms Containing Double-Stranded DNA

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Nitrous acid (NA) is often presumed to cause base substitutions in organisms with double-stranded DNA as a direct consequence of oxidative deamination of adenine and of cytosine residues. Here we summarize evidence indicating that other mechanisms are involved in the case of NA-induced G/C→A/T transition mutations. We present several models for pathways of NA mutagenesis that may account for our experimental results and overlapping data noted in the literature. One model proposes that the base substitution mutations observed are due to DNA alkylation damage mediated via nitrosation of polyamines and/or other ubiquitous cellular molecules. Other models assume that pre-

disposing lesions, such as G-to-G cross-links, are first formed. The cross-links are pictured as leading to perturbations in DNA structure that allow subsequent opportunity for NA-induced deaminations of cytosine residues in their immediate vicinity. The deaminations preferentially result in G/C→A/T transition mutations at sites highly dependent on adjoining base sequence context (i.e., in NA "mutational hotspots"). A final model proposes that NA-induced G/C→A/T transition mutations arise mainly from oxidative deamination of guanosine residues and not from deamination of cytosine residues in duplex DNA. © 1994 Wiley-Liss, Inc.

Key words: alkylation, allele-specific oligonucleotides, bacteria, cross-linked bases, deamination, duplex DNA, mutational spectra, nucleotide excision repair, PCR, uracil-DNA glycosylase

INTRODUCTION

Spontaneous deaminations of C→U occur at biologically significant frequencies and are mutagenic unless the U is excised through the action of uracil-DNA glycosylase [Duncan and Miller, 1980; Duncan and Weiss, 1982; reviewed by Lindahl, 1993]. Direct oxidative deamination of bases by nitrous acid (NA) exposure of single-stranded RNA or DNA and of organisms containing these nucleic acids as genetic material also can explain the bulk of NA-induced mutants detected [Zimmermann, 1977; Frankel et al., 1980]. On the other hand, data on NA treatment of duplex DNA in vitro and on mutations obtained after NA treatment of intact organisms containing duplex DNA have been confusing and often not consonant with the simple deamination theory [Zimmermann, 1977; Thomas et al., 1979b; Frankel et al., 1980]. Here we present data on additional tests and formulate models that suggest further experimentation which may help in the synthesis of a unified description of NA mutagenesis of duplex DNA.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli K12 strains matched for concordant background genotypes were of two sets: a) *E. coli* Genetic Stock Center strain CGSC6078 (strain BW310 of B. Weiss) carrying mutation *ung-1* and defective in

uracil-DNA glycosylase [Duncan and Weiss, 1982] and a closely allied strain CGSC259 (formerly strain AB259) which is *ung*⁺, and b) strain AB1157 (see Bachmann [1987] for complete genotype), a DNA repair-proficient strain, and three derivatives, namely AB1885 (*uvrB5*) and AB1886 (*uvrA6*) defective in nucleotide excision repair, and GW5352 (*ada-10 :: TN10*) defective in adaptive response to alkylating agents [Lemotte and Walker, 1985].

Salmonella typhimurium LT2 strains comprised the histidine-requiring mutant *hisG46* that carries a CCC in the mutant codon [Hartman et al., 1986] and its *ΔuvrB*, nucleotide excision repair-defective derivative, strain TA1950 [Maron and Ames, 1983].

Chemicals

Chemicals used were d-biotin, L-histidine free base, putrescine dihydrochloride, rifampicin, spermine free base, spermidine trihydrochloride (Sigma Biochemical Co., St. Louis, MO); sodium nitrite (EM Science, Cherry Hill, NJ); methanol (J.T. Baker Chemical Co., Phillipsburg, NJ). Aqueous solutions of sodium nitrite, spermine, spermidine, and putrescine were made up fresh the day of the experiment.

Media

Complex media comprised Difco nutrient broth and broth supplemented with 1.5% Difco agar when used in Petri dishes (Difco Laboratories,

Received March 12, 1994; revised and accepted May 31, 1994.

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Detroit, MI). Liquid minimal E medium [Vogel and Bonner, 1956] was supplemented with d-biotin (2 µg/ml), L-histidine (20 µg/ml), and 0.2% glucose (pH 7.1). Minimal plus biotin plates contained E medium plus 2 µg/ml d-biotin, 0.2% glucose, and 1.5% Difco agar. Soft agar contained E medium supplemented with 9.6 µg/ml L-histidine, 2 µg/ml d-biotin, 0.2% glucose, and 0.5% agar. Nutrient agar was supplemented with 50 µg/ml rifampicin when used for selection of Rif^r mutants. The rifampicin stock solution was made up in methanol (5 mg/ml).

Nitrous Acid Mutagenesis

Aerated cultures in nutrient broth were grown overnight at 37°C. For log phase cultures, aliquots were diluted 1:10 into fresh nutrient broth and aerated 90 min at 37°C. Bacteria were sedimented at 3,500 rpm for 10 min in a model NSE "Servall" centrifuge (Ivan Sorvall, Inc., Norwalk, CT) and resuspended in 0.5 volume of 0.05 M KH₂PO₄ buffer at pH 4.7. A 500 mM aqueous solution of sodium nitrite was diluted 1:10 into portions of the bacterial suspension in buffer and the mixture incubated at 37°C. Samples were removed at intervals. For assays of viability, dilutions were spread on the surfaces of nutrient agar plates. In experiments with *Salmonella* strains *hisG46* and TA1950, 0.1 ml undiluted samples were added to each of two tubes containing 2.5 ml soft agar and poured onto minimal plus biotin plates to select for His⁺ revertants. Revertant colonies were hand counted on a New Brunswick colony counter after incubation at 37°C for 2 days.

The same protocol was used for *E. coli* strains except for mutant selection. To score for rifampicin-resistant (Rif^r) mutants, 0.1 ml undiluted samples for each time point were spread evenly on each of two nutrient agar plates and incubated at 37°C for 24 hr. This growth allowed phenotypic expression of rifampicin resistance. The resulting bacterial lawn was then replica plated [Lederberg and Lederberg, 1952] onto nutrient agar plus rifampicin plates and incubated for 2 days at 37°C before scoring Rif^r mutants.

Nitrous Acid + Spermidine Mutagenesis

Aerated cultures of TA1950 were grown overnight in minimal medium supplemented with biotin, glucose, and L-histidine. Aqueous solutions of 5 M sodium nitrite and 1 M spermidine were diluted 1:10 into citrate-phosphate buffer, pH 4.2, in the following combinations: control (distilled water only added), nitrite alone, nitrite plus spermidine, and spermidine alone. Reaction mixtures were incubated for 20 min at 37°C and then diluted 1:10 into aliquots of strain TA1950 in minimal medium. After incubation for 10 min at 37°C [Hartman and Hartman, 1987], viability was determined and His⁺ revertants were scored as described under Nitrous Acid Mutagenesis, above. Reaction mixtures containing 500 mM nitrite plus 150, 50, or 16.7 mM spermine or putrescine were similarly tested.

Mutation Analysis

hisG46 revertants from mutagenicity assays were single-colony isolated on Minimal plus biotin plates. Sequence changes in representative populations of spontaneously derived and NA-induced and NA + spermidine-induced *hisG46* revertants were determined using allele-specific colony hybridization [Cebula and Koch, 1990], except that psoralen was not used to cross-link the probe to the immobilized target DNA. Two revertants, not identified by our probing analysis, were characterized by directly sequencing a 187 single-stranded PCR-generated fragment encompassing the *hisG46* locus using previously described primers and amplification conditions [Koch et al., 1994].

RESULTS

Effects of Defects in DNA Repair

Howard-Flanders and Boyce [1966] noted that a strain of *E. coli* K12 (strain AB1886) carrying mutation *uvrA6*, and

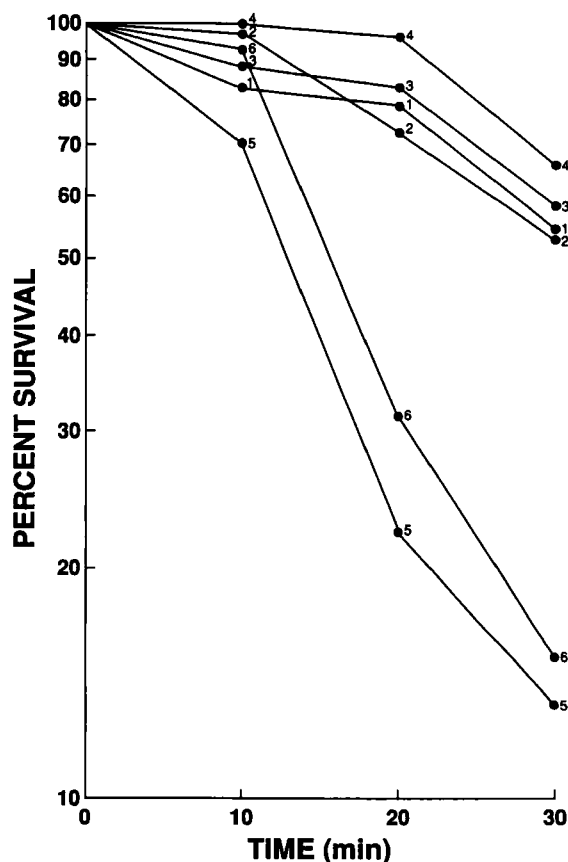


Fig. 1. Inactivation by nitrous acid of relatively isogenic sets of *E. coli* K12 strains. (1) *ung-1*, (2) *ung+*, (3) *ada+ uvr+*, (4) *ada-*, (5) *uvrB5*, (6) *uvrA6*. Only the *Uvr*⁻ strains are strikingly hypersensitive to killing by nitrous acid.

thus defective in nucleotide excision repair, was much more sensitive to killing by NA than its repair-competent parent strain (strain AB1157). Clarke [1970] demonstrated an analogous effect in another pair of *E. coli* strains and showed that mutagenesis by NA also was considerably enhanced in the excision repair-defective strain.

Our data confirm and extend these earlier results. Figure 1 shows killing curves and Figure 2 shows mutation curves for two parental repair-competent *E. coli* K12 strains (#2 and #3) and derivatives defective in correction of alkylated bases (#4 = *ada-*), excision of U's via uracil-DNA glycosylase (#1 = *ung-1*), and the broad-spectrum nucleotide excision repair system (#5 = *uvrB5*; #6 = *uvrA6*). Bacteria were exposed to 50 mM NA, pH 4.7, at 37°C. Only the two *uvr*⁻ strains (#5 and #6) exhibited pronounced sensitivity to killing by NA (Fig. 1). Using a procedure different from that utilized here, Da Roza et al. [1977] indicated a modest increase in sensitivity of *ung*⁻ bacteria to NA. The same two *uvr*⁻ strains also exhibited greatly increased induction of Rif^r by NA (Fig. 2). A surprisingly modest increase in mutagenesis by NA occurred in the case of the uracil-DNA glycosylase-deficient mutant (#1) (Fig. 2).

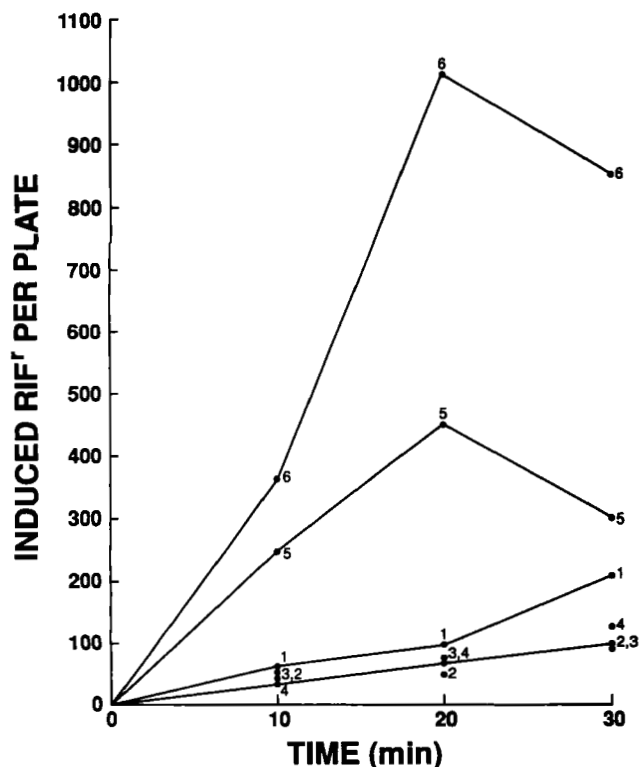


Fig. 2. Induction of mutations to rifampicin resistance (Rif^r) by treatment of relatively isogenic sets of *E. coli* K12 strains with nitrous acid. (1) *ung-1*, (2) *ung*⁺, (3) *ada*⁺ *uvr*⁺, (4) *ada*⁻, (5) *uvrB5*, (6) *uvrA6*. Rif^r mutants are induced very slowly in repair-proficient strains, slightly but significantly more rapidly in the *Ung*⁻ (uracil-DNA glycosylase negative) strain, but much more effectively in the two *Uvr*⁻ (nucleotide excision repair negative) strains. With these two strains a decrease in mutants at long exposure is attributed to enhanced killing of the bacterial population (see Fig. 1).

Spontaneous Rif^r mutants have been shown to arise from base-substitutions (both transitions—especially C → T—and transversions) at a variety of sites in the *rpoB* gene coding for a subunit of RNA polymerase [Jin and Gross, 1988].

From the above, we conclude that the nucleotide excision repair pathway is responsible for removal of an otherwise potentially lethal lesion(s) induced at significant frequency in DNA of bacteria exposed to NA. In addition, persistence of this class of lesion appears critical for a high level of mutagenesis by NA. In contrast, a defect in uracil-DNA glycosylase has strikingly little impact on killing or mutagenesis by NA.

A requirement for the presence of a *uvr* mutation for cell sensitivity to NA and a high-level induced-mutant yield is also found in *Salmonella* (Table I). Bacteria exposed to 50 mM NA, pH 4.7, at 37°C show appreciable killing and reversion of the *hisG46* mutation only in the strain TA1950 that carries a deletion of the *uvrB* gene. The repair-proficient strain, *hisG46*, still failed to exhibit significant NA-induced

reversion even when exposed to 100, 200, 400, and 800 mM NA for up to 36 min, although killing was evident at these higher NA concentrations even after only 6 min incubation (data not shown). In contrast, after 20 min exposure to 50 mM NA, the *uvrB*-defective strain TA1950 exhibited an over 8,000-fold increase in the frequency of revertants per surviving bacterium over the frequency found among spontaneous mutants (Table I). Previously, Murphey-Corb et al. [1980] had noted NA-induced reversion only in *hisG46* strains also carrying a *uvrB* mutation.

Mutational Spectra

Spontaneous revertants of strain TA1950 as well as those present after NA treatment for 5 min (100% survival) and after 20 min (0.26% survival) (Table I) were purified and spectra of reverse mutations to His⁺ determined (upper portion of Table II). The vast majority of NA-induced reversions are due to C → T transition mutations with an approximate twofold preference for the second position of the *hisG46* mutant codon CCC. Such mutations are to be expected if NA deaminates C to U. Transversions (ACC, CAC, GCC, and an A/T → C/G in a suppressor gene = *sup*) are present either at a very low level (e.g., CAC) or are absent in the majority of assays (upper portion of Table II).

Enhancement of NA Mutagenesis by Spermidine

Thomas et al. [1979b] found that purified duplex bacterial transforming DNA is relatively insusceptible to NA mutagenesis in vitro but is readily mutated following denaturation or after addition of various alcohols, glycols, phenols, or amines. Of the three common polyamines, spermine was most effective in enhancing the mutation rate, followed by putrescine and spermidine in that order. Murphey-Corb et al. [1980, 1983] noted comutagenic activity with NA by only a limited number of polyamines in reversion studies of *hisG46* in *Salmonella*; spermidine was an active comutagen whereas putrescine was inactive.

Indeed, spermidine-NA mixtures are highly potent mutagens for *Salmonella* strain TA1950 (Table III; also see Hartman and Hartman [1987]). Similar mixtures containing either putrescine or spermine failed to show a level of mutagenesis above that elicited by NA alone (data not shown). Base changes effecting reversion were analyzed from spontaneous, NA-treated, and NA + spermidine-treated bacteria (lower portion of Table II). As previously found for NA (upper portion of Table II), both NA and NA + spermidine mixtures elicit C → T transition mutations in abundance, again with a decided preference for the base substitution in the second position of the codon (lower portion of Table II).

DISCUSSION

Our data and those in the literature cited in Results clearly demonstrate two key points with regard to NA mutagenesis

TABLE I. Requirement of a *uvrB* Mutation for Expression of Lethal and Mutagenic Actions of NA on Salmonella Strains Bearing *hisG46*

Time of treatment (min)	<i>hisG46</i>		TA1950 = <i>hisG46 uvrB</i>	
	Percent survival	Revertants per plate	Percent survival	Revertants per plate
0	100%	2	100%	12
5	100%	4	100%	140
10	100%	1	—	172
15	100%	3	—	231
20	100%	5	0.26%	263
30	100%	3	0.013%	221

TABLE II. Reversion Spectra of Spontaneous, NA-induced, and NA + Spermidine-induced Revertants of *hisG46* in Strain TA1950

	Revertants per plate	TCC (ser)	CTC (leu)	ACC (thr)	CAC (his)	GCC (ala)	CCC (sup) A/T → C/G ^b
Spontaneous control n = 99 ^a	12	16%	64%	12%	1%	4%	3%
NA (5 min) n = 104	140	31%	64%	0%	4%	0%	1%
NA (20 min) n = 109	263	26%	67%	1%	5%	1%	0%
Spontaneous control n = 101	20	12%	69%	10%	4%	0%	6%
NA n = 112	95	33%	60%	0%	3%	0%	5%
NA + Spermidine n = 105	1158	37%	63%	0%	0%	0%	0%

^an = Number of revertant colonies examined.^b = Kupchella et al. (1994).**TABLE III. Comparison of the Mutagenicity of NA in the Presence and the Absence of Spermidine for Strain TA1950***

	Revertants per plate	
	Experiment 1	Experiment 2
Spontaneous control	20	19
Spermidine alone	20	19
NaNO ₂	95	87
NaNO ₂ + spermidine	1158	553

*Constituents listed were preincubated at pH 4.7 for 20 min at 37°C and an aliquot added to a bacterial culture and incubated at 37°C for an additional 10 min before plating. Experiment I = stationary phase culture; Experiment II = log phase culture.

in bacteria: a) elimination of the nucleotide excision repair (UvrABC) system is requisite for demonstration of a high level of mutagenesis whereas there is little influence by the absence of uracil-DNA glycosylase (Fig. 2; Table I) and b) transition mutations in a highly characteristic spectrum predominate among revertants. Essentially the same spectrum is found when mutagenesis is by NA alone or by NA supplemented with the "comutagen" spermidine (Table II).

The prevalent idea that NA is mutagenic through deamination of C, leading to transition mutations, is consonant with the base changes we observe. However, the lack of impact of a defect in uracil-DNA glycosylase (Fig. 2) is enigmatic. Furthermore, two studies indicate that in NA treatment of duplex DNA there is a very pronounced lag in discernible deamination of C's [Litman, 1961; Frankel et al., 1980]. In fact, NA mutagenesis in duplex DNA can hardly be detected in the absence of comutagens such as polyamines [Thomas et al., 1979b].

Model 1: Mutation via Alkylation

One initially attractive idea would be that NA does not work directly but acts *indirectly* to nitrosate compounds which then lead to formation of DNA base adducts repairable by the Uvr system. Rapid repair of DNA alkylation damage is dependent on nucleotide excision repair [Samson et al., 1988; reviewed by Van Houten, 1990]. Thomas et al. [1979b] observed strong NA mutagenesis of cell-free DNA *in vitro* when any one of several polyamines were present. In *hisG46* bacteria *in vivo*, only spermidine and not spermine

or putrescine acts as a "comutagen" [Murphey-Corb et al., 1980, 1983; this paper]. This could merely indicate more ready cellular uptake of spermidine reaction products, either as free spermidine [Kashiwagi et al., 1993] or via a glutathionyl conjugant [Tabor and Tabor, 1975]. Both spermine and spermidine form a variety of products of nitrosation [Hildrum et al., 1976, 1977; Hotchkiss et al., 1977], a number of which are direct-acting mutagens [Hotchkiss et al., 1979; Thomas et al., 1979a]. A major mutagenic product of spermine nitrosation has a half-life of about 4 min at 37°C in aqueous solution [Thomas et al., 1979b], the same as one of two major mutagenic products of spermidine nitrosation [Murphey-Corb et al., 1980].

Any hypothetical adducts formed by NA reaction products, however, would have to result in the spectrum of mutations described here, a pattern decidedly different from mutational spectra found for DNA methylating agents and for several bulkier adduct-forming mutagens [Koch et al., 1994]. The mutational spectra we observe for NA and NA + spermidine closely mimic those found after treatments, for example with nitroglycerin, that are attributed to deamination of C residues [Wink et al., 1991; Maragos et al., 1993]. While nitric oxide does lead to cytosine deamination in duplex DNA [Wink et al., 1991], it is not firmly established that deamination is a major determinant in mutagenesis by nitric oxide in vivo, although substantial data point in that direction [Routledge et al., 1993, 1994a].

Model 2: Intrastrand Cross-links Facilitate Deamination

A second model that would account for the results described here, earlier advanced by Murphey-Corb et al. [1980], is that NA-induced *intrastrand* cross-links are formed in DNA. Such cross-links, designated pX*pX*, were observed in NA-treated DNA by Dubelman and Shapiro [1977] but not further characterized with respect to their structure, their kinetics of formation, or their possible site-specificity. One could suppose that this cross-linked product, presumed to arise from two adjacent G residues on one DNA strand, would distort the duplex enough to facilitate deamination of C residues on the opposing strand (Fig. 3; Model 2). Thus, deamination effects on mutation would be most striking when the cross-links were left unrepaired by the absence of the Uvr nucleotide excision repair system, allowing time for deaminations to occur. It is established that deamination of C residues in denatured DNA occurs much more rapidly than in double-stranded DNA [Schuster, 1960a,b; Schuster and Vielmetter, 1961; Frankel et al., 1980].

While polyamines do not seem to enhance NA mutagenesis by eliminating the lag or stimulating the overall rate of deamination of C residues in duplex DNA [Frankel et al., 1980], it is possible that they could have impacts at specific sites on modified DNA. Stimulation of cross-link formation

is unlikely since spermine fails to enhance NA inactivation of transforming DNA [Thomas et al., 1979b].

As a variation of this second model, one may hypothetically picture the formation of Uvr-repairable G adducts that would similarly allow enhanced deamination of cytosines in the immediate vicinity when left unrepaired. A possible sequence-specificity in NA-induced mutations is discussed below.

Model 3: Interstrand Cross-Links at Preferred Sites

In Figure 3 (Model 3) we propose a variant of the line of thought used to construct Model 2, but one that has some experimental support. Models 2 and 3 are not mutually exclusive.

G-G *interstrand* cross-links [Shapiro et al., 1977] are known to be formed in vitro in duplex DNA with only a barely perceptible lag following exposure to NA; interstrand cross-links are formed in high yield, namely 1 cross-link per 4 *total* deamination events [Becker et al., 1964; Burnotte and Verly, 1971]. Interestingly, such cross-links (step 1 in Figure 3) are not formed randomly in duplex DNA; rather, interstrand cross-links preferentially occur in specific sequences of which the sequence found at *hisG46* [Hartman et al., 1986], namely 5' CCGG, is considered as an optimal "hotspot" [Kirchner and Hopkins, 1991; Kirchner et al., 1992a,b]. A possible role of polyamines in site-selection of cross-links has not been explored.

Susceptibility of special sequences to NA mutagenesis is consistent with the observation that NA-induced mutations are located non-randomly in genes, i.e., at specific "hotspot" sites in bacteriophage [Benzer, 1961] and *Streptococcus pneumoniae* DNA [Lacks, 1966].

The NA-induced mutations in *S. pneumoniae* DNA so far examined are exclusively G/C → A/T transition mutations [Lacks, 1966; Chen and Lacks, 1991]. One sequenced NA-induced mutation, 582nr, involves the first base pair of a 5' *GCC* / 3' *CGG* sequence [Lacks et al., 1982]. Three mutants in-

duced by other agents [Chen and Lacks, 1991] were found to revert with NA via transition mutations at the middle base

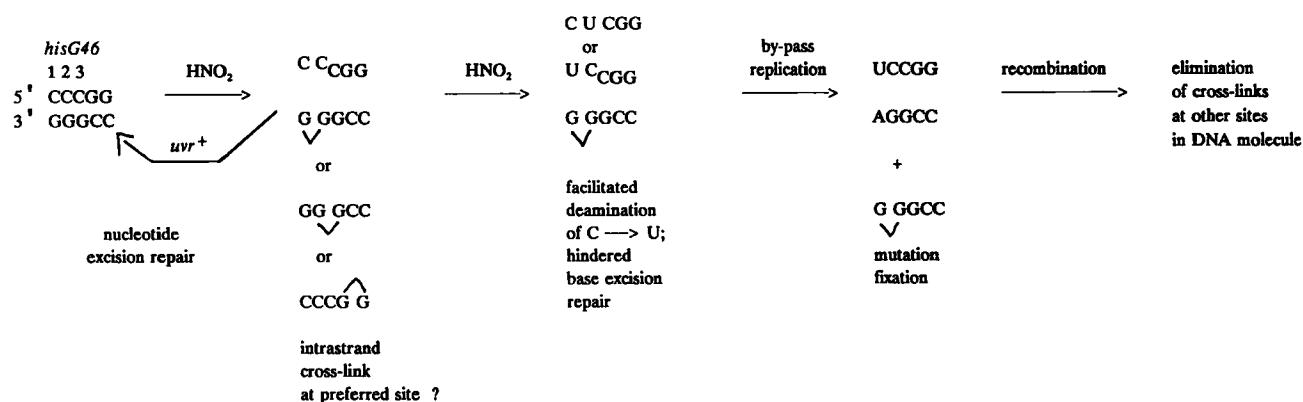
pair of a 5' *GGGCC* / 3' *CCCGG* sequence (M597), at the second base

pair of a 5' *GGCC* / 3' *CCGG* sequence (M532), and at the third base

pair of a 5' *CGG* / 3' *GCC* sequence (M510) (sequences in Lacks et

al. [1982]). All four NA-mutable sites have sequences that would theoretically allow formation of intra- or of interstrand cross-links in close proximity to the base pair susceptible to NA mutagenesis. Since *S. pneumoniae* DNA is 40% G/C, the probability that four NA mutation-prone sites would have such non-random basepair configurations is well below 1%. Model building indicates that G-G inter-

Model 2



Model 3

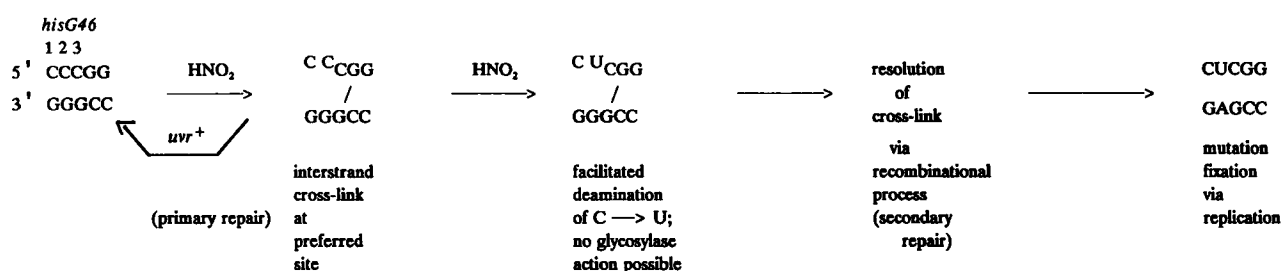


Fig. 3. Model 2: The *Salmonella hisG46* mutant codon, CCC is labeled with respect to its base pairs 1, 2, and 3. Nitrous acid is pictured as forming intrastrand cross-links between G residues as a first step in nitrous acid mutagenesis. This lesion can be repaired by the Uvr nucleotide excision repair system. If unrepaired, DNA structure is distorted, facilitating deamination of one or another C residue. Quite possibly, this cross-link hinders base excision of any U residues formed by deamination of C. By-pass replication would ensure mutation fixation through insertion of an A on the newly synthesized strand opposite the U. Recombination would allow formation of a viable double helix free of cross-links throughout the continuity of the chromosome which would carry a G/C \rightarrow A/T transition mutation at a site "preordained" by the initial intrastrand cross-link.

Model 3: Model 3 is analogous to Model 2 except that the initial event that enhances opportunity for C deamination is a G-G interstrand cross-link preferentially produced at a 5' CG or a 5' GC site. The conformation at this covalent interstrand cross-link, when unrepaired by the Uvr nucleotide excision repair complex, both exposes nearby C residues to deamination and blocks excision of the resulting U's by uracil DNA glycosylase. Resolution of the otherwise lethal interstrand cross-link in Uvr^- bacteria would have to be more complex than that pictured in Model 2. It would have to be initiated by a recombinational process that allows expression of altered bases in close proximity to the cross-link, ultimately resulting in a C \rightarrow T transition mutation at a "nitrous acid hotspot."

strand cross-links perturb DNA structure only slightly, with significant effects extending only a few base pairs away [Kirchner et al., 1992b], perhaps enough to facilitate deamination of C residues (second step in Model 3, Figure 3).

Retention in Uvr^- bacteria of interstrand cross-links would certainly block excision of U's by uracil-DNA glycosylase in the vicinity of the cross-link. As final steps in the process, the interstrand cross-link would need to be resolved and the NA-induced mutation fixed through replication. Wild-type bacteria are apparently able to withstand a large number of cross-links and survive, while much more sensitive, uvr^- bacteria still show multi-hit inactivation kinetics (Fig. 1) [Clarke, 1970]. A *Salmonella* double mutant defective both in nucleotide excision repair (uvr^-) and recombinational repair (recA^-) is ultrasensitive to lethal damage by NA + spermidine mixtures [Murphey-Corb et al., 1980],

and an analogous *E. coli* K12 double mutant is rapidly killed by NA in a kinetically single-hit process [Howard-Flanders and Boyce, 1966]. We conjecture that a slow or late-acting, possibly inducible, recombinational system could be active in allowing mutational expression of the targeted, NA-deaminated bases. NA and NA + spermidine mutagenesis is absent in $\text{uvrB}^- \text{recA}^-$ *Salmonella* double mutants although spontaneous mutation still occurs [Murphey-Corb et al., 1980]. In keeping with this model, recombinational repair of DNA damage is considered to be a very sluggish process [Sinden and Cole, 1978].

The majority, but not all, GC \rightarrow AT transition mutations in the *supF* gene of a NA-treated shuttle vector replicated in transformed human cells [Routledge et al., 1994b] can be accounted for by Models 2 and/or 3. However, in this system, numerous transversion mutations, including GC \rightarrow TA

and GC→CG, were also detected [Routledge et al., 1994b].

Model 4: Xanthine as a Mutagenic Lesion

Model 4 acknowledges a deficiency of cytosine deamination during mild nitrous acid treatment of duplex DNA and the proficiency of guanine deamination in such DNA [Litman, 1961]. Ability of nitrous acid to induce G → A base substitutions, most likely via oxidative deamination of guanosine to xanthosine (X), was clearly demonstrated in NA-treated single-stranded bacteriophage DNA [Vanderbilt and Tessman, 1970]. Kamiya et al. [1992] detected substitution of adenine for X at a precise site in a synthetic duplex DNA c-Ha-ras gene transfected into NIH3T3 cells. In addition, *Drosophila* DNA polymerase α has been shown to incorporate thymine as well as cytosine at high frequency opposite deoxyxanthine in synthetic polymers in vitro [Eritja et al., 1986].

Thus, oxidative deamination of G to X could explain the G/C→A/T mutations reviewed above. This would be particularly applicable if there were hotspots for deamination at particular base sequences noted in the discussion of Model 3. Kinetic experiments on loss of bases in duplex DNA indicate that a minority of G residues, perhaps not all of them involved in cross-links, is very rapidly lost upon initial exposure to NA [Litman, 1961]. Absence of the nucleotide excision repair system in facilitating mutation could result if the UvrABC system actively eliminates T from X:T base pairs. Such X:T base pairs are hypothesized to exist in an equilibrium between two alternate conformations [Eritja et al., 1986; Kamiya et al., 1992]. "Wobble" between conformers might be expected to interfere with base stacking. Base stacking is believed to be the major source of DNA duplex stability. Perturbations in base stacking have been indicated as the predominant signal for recognition by the UvrABC nucleotide excision repair system [Van Houton and Snowden, 1993].

One might additionally conjecture that cells possess a dX glycosylase or analogous activity. In fact, Oeda et al. [1978] detected an enzyme activity in *E. coli* extracts which was active on NA-treated duplex DNA, an activity still present in a uracil DNA glycosylase negative mutant. This activity could well have included a glycosylase for hypoxanthine [Karren and Lindahl, 1978; Dianov and Lindahl, 1991] as well as a xanthine-specific glycosylase active on X:C base-pairs. In contrast, Oeda et al. [1978] observed that partially purified uracil DNA glycosylase failed to exhibit activity on NA-treated duplex DNA.

Future Prospects

Numerous aspects of the models proposed here are subject to critical experimental tests. In fact, a major purpose of this paper is to question current textbook renditions of the mutagenic action of NA on duplex DNA, leading to its reexamination by both in vivo and in vitro experimentation.

ACKNOWLEDGMENTS

We thank Barbara J. Bachmann and Bruce N. Ames for bacterial cultures and Sanford Lacks for guiding us to NA-susceptible sequences in transforming DNA. Rosalie Elespuru, Larry Keefer, and Anthony Dipple provided useful comments on an earlier draft of this paper.

REFERENCES

- Bachmann BJ (1987): Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In Neidhardt FC: "*Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology." Washington, D.C.: American Society for Microbiology, pp 1190-1219.
- Becker EF Jr, Zimmerman BK, Geiduschek EP (1964): Structure and function of cross-linked DNA. I. Reversible denaturation and *Bacillus subtilis* transformation. *J Mol Biol* 8:377-391.
- Benzer S (1961): On the topography of the genetic fine structure. *Proc Natl Acad Sci USA* 47:403-415.
- Burnotte J, Verly WG (1971): A kinetic approach to the mechanism of deoxyribonucleic acid cross-linking by HNO₂. *J Biol Chem* 246: 5914-5918.
- Cebula TA, Koch WH (1990): Analysis of spontaneous and psoralen-induced *Salmonella typhimurium* hisG46 revertants by oligodeoxynucleotide colony hybridization: Use of psoralens to cross-link probes to target sequences. *Mutat Res* 229:79-87.
- Chen J-D, Lacks SA (1991): Role of uracil-DNA glycosylase in mutation avoidance by *Streptococcus pneumoniae*. *J Bacteriol* 173:283-290.
- Clarke CH (1970): Repair systems and nitrous acid mutagenesis in *E. coli* B/r. *Mutat Res* 9:359-368.
- Da Roza R, Friedberg EC, Duncan BK, Warner HR (1977): Repair of nitrous acid damage to DNA in *Escherichia coli*. *Biochemistry* 16:4934-4939.
- Dianov G, Lindahl T (1991): Preferential recognition of I · T base-pairs in the initiation of excision-repair by hypoxanthine-DNA glycosylase. *Nucleic Acids Res* 19:3829-3833.
- Dubelman S, Shapiro R (1977): A method for the isolation of cross-linked nucleosides from DNA: Application to cross-links induced by nitrous acid. *Nucleic Acids Res* 4:1815-1827.
- Duncan BK, Miller JH (1980): Mutagenic deamination of cytosine residues in DNA. *Nature* 287:560-561.
- Duncan BK, Weiss B (1982): Specific mutator effects of ung (uracil-DNA glycosylase) mutations in *Escherichia coli*. *J Bacteriol* 151:750-755.
- Eritja R, Horowitz DM, Walker PA, Ziehler-Martin JP, Boosalis MS, Goodman MF, Itakura K, Kaplan BE (1986): Synthesis and properties of oligonucleotides containing 2'-deoxynebularine and 2'-deoxyxanthosine. *Nucleic Acids Res* 14:8135-8153.
- Frankel AD, Duncan BK, Hartman PE (1980): Nitrous acid damage to duplex deoxyribonucleic acid: distinction between deamination of cytosine residues and a novel mutational lesion. *J Bacteriol* 142: 335-338.
- Hartman Z, Hartman PE (1987): Interception of some direct-acting mutagens by ergothioneine. *Environ Mol Mutagen* 10:3-15.
- Hartman PE, Ames BN, Roth JR, Barnes WM, Levin DE (1986): Target sequences for mutagenesis in *Salmonella* histidine-requiring mutants. *Environ Mutagen* 8:631-641.
- Hildrum KI, Scanlan RA, Libbey LM (1976): Nitrosamines from the nitrosation of spermidine and spermine. In Walker EA, Bogovski P, Griecute L, Davis W: "Environmental N-Nitroso Compounds: Analysis and Formation." IARC Sci Publ #14, Lyon: International Agency for Research on Cancer, pp 205-214.
- Hildrum KI, Scanlan RA, Libbey LM (1977): Formation of volatile, hy-

- droxylated, and chorinated *N*-nitrosamines during the nitrosation of spermidine 3-hydrochloride. *J Agr Food Chem* 25:252–255.
- Hotchkiss JH, Scanlan RA, Libbey LM (1977): Formation of bis(hydroxyalkyl)-*N*-nitrosamines as products of the nitrosation of spermidine. *J Agr Food Chem* 25:1183–1189.
- Hotchkiss JH, Scanlan RA, Lijinsky W, Andrews AW (1979): Mutagenicity of nitrosamines formed from nitrosation of spermidine. *Mutat Res* 68:195–199.
- Howard-Flanders P, Boyce RP (1966): DNA repair and genetic recombination: Studies on mutants of *Escherichia coli* defective in these processes. *Radiation Res [Suppl]* 6:156–184.
- Jin DJ, Gross CA (1988): Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J Mol Biol* 202:45–58.
- Kamiya H, Shimizu M, Suzuki M, Inoue H, Ohtsuka E (1992): Mutation induced by deoxyxanthosine in codon 12 of a synthetic *c-Ha-ras* gene. *Nucleosides Nucleotides* 11:247–260.
- Karren P, Lindahl T (1978): Enzymatic excision of free hypoxanthine from polydeoxynucleotides and DNA containing deoxyinosine monophosphate residues. *J Biol Chem* 253:5877–5879.
- Kashiwagi K, Miyamoto S, Nukui E, Kobayashi H, Igarashi K (1993): Functions of PotA and PotD proteins in spermidine-preferential uptake system in *Escherichia coli*. *J Biol Chem* 268:19358–19363.
- Kirchner JJ, Hopkins PB (1991): Nitrous acid cross-links duplex DNA fragments through deoxyguanosine residues at the sequence 5'-CG. *J Am Chem Soc* 113:4681–4682.
- Kirchner JJ, Sigurdsson ST, Hopkins PB (1992a): Interstrand cross-linking of duplex DNA by nitrous acid: Covalent structure of the dG-to-dG cross-link at the sequence 5'-CG. *J Am Chem Soc* 114:4021–4027.
- Kirchner JJ, Solomon MS, Hopkins PB (1992b): Interstrand cross-linking of duplex DNA by nitrous acid: Determination of the sequence preference at nucleotide resolution. In Sarma RH, Sarma MH: "Structure and Function. I. Nucleic Acids." Albany: Adenine Press, pp 171–182.
- Koch WH, Henrikson EN, Kupchella E, Cebula TA (1994): *Salmonella typhimurium* strain TA100 differentiates several classes of carcinogens and mutagens by base substitution specificity. *Carcinogenesis* 15:79–88.
- Kupchella E, Koch WH, Cebula TA (1994): Mutant alleles of tRNA^{Thr} genes suppress the *hisG46* missense mutation in *Salmonella typhimurium*. *Environ Mol Mutagen* 23:81–88.
- Lacks S (1966): Integration efficiency and genetic recombination in pneumococcal transformation. *Genetics* 53:207–235.
- Lacks SA, Dunn JJ, Greenberg B (1982): Identification of base mismatches recognized by the heteroduplex-DNA-repair system of *Streptococcus pneumoniae*. *Cell* 31:327–336.
- Lederberg J, Lederberg EM (1952): Replica plating and indirect selection of bacterial mutants. *J Bacteriol* 53:399–406.
- Lemotte PK, Walker GC (1985): Induction and autoregulation of *ada*, a positively acting element regulating the response of *Escherichia coli* K-12 to methylating agents. *J Bacteriol* 161:888–895.
- Lindahl T (1993): Instability and decay of the primary structure of DNA. *Nature* 362:709–715.
- Litman RM (1961): Genetic and chemical alterations in the transforming DNA of *Pneumococcus* caused by ultraviolet light and by nitrous acid. *J Chim Phys* 58:997–1003.
- Maragos CM, Andrews AW, Keefer LK, Elespuru RK (1993): Mutagenicity of glyceryl trinitrate (nitroglycerin) in *Salmonella typhimurium*. *Mutat Res* 298:187–195.
- Maron DM, Ames BN (1983): Revised methods for the *Salmonella* mutagenicity test. *Mutat Res* 113:173–215.
- Murphey-Corb M, Kong H-L, Murray ML (1980): Interaction of mutagenic spermidine-nitrous acid reaction products with *uvr*⁻ and *recA*-dependent repair systems in *Salmonella*. *J Bacteriol* 142:191–195.
- Murphey-Corb M, Kong H-L, Murray ML (1983): Mutagenic activity from nitrosation of oligoamines. *Environ Mutagen* 5:101–109.
- Oeda K, Shimizu K, Sekiguchi M (1978): An enzyme activity specific for nitrous acid-treated DNA in *Escherichia coli*. *J Biochem* 84:1165–1169.
- Routledge MN, Wink DA, Keefer LK, Dipple A (1993): Mutations induced by saturated aqueous nitric oxide in the pSP189 *supF* gene in human Ad293 and *E. coli* MBM7070 cells. *Carcinogenesis* 14:1251–1254.
- Routledge MN, Wink DA, Keefer LK, Dipple A (1994a): DNA sequence changes induced by two nitric oxide-donor drugs in the *supF* assay. *Chem Res Toxicol* (in press).
- Routledge MN, Mirsky FJ, Wink DA, Keefer LK, Dipple A (1994b): Nitrite-induced mutations in a forward mutation assay: Influence of nitrite concentration and pH. *Mutat Res* (in press).
- Samson L, Thomale J, Rajewsky MF (1988): Alternative pathways for the in vivo repair of O⁶-alkylguanine and O⁴-alkylthymine in *Escherichia coli*: The adaptive response and nucleotide excision repair. *EMBO J* 7:2261–2267.
- Schuster H (1960a): Die Reaktionsweise der Desoxyribonucleinsäure mit salpetriger Säure. *Z Naturforschg* 15b:298–304.
- Schuster H (1960b): The reaction of nitrous acid with deoxyribonucleic acid. *Biochem Biophys Res Commun* 2:320–323.
- Schuster H, Vielmetter W (1961): Studies on the inactivating and mutagenic effect of nitrous acid and hydroxylamine on viruses. *J Chim Phys* 58:1005–1010.
- Shapiro R, Dubelman S, Feinberg AM, Crain PF, McCloskey JA (1977): Isolation and identification of cross-linked nucleosides from nitrous acid treated deoxyribonucleic acid. *J Am Chem Soc* 99:302–303.
- Sinden RR, Cole RS (1978): Topography and kinetics of genetic recombination in *Escherichia coli* treated with psoralen and light. *Proc Natl Acad Sci USA* 75:2373–2377.
- Tabor H, Tabor CW (1975): Isolation, characterization, and turnover of glutathionylspermidine from *Escherichia coli*. *J Biol Chem* 250:2648–2654.
- Thomas HF, Brown DL, Hartman PE, White EH, Hartman Z (1979a): Aryl-monoalkyl and cyclic triazenes: Direct-acting mutagens. *Mutat Res* 60:25–32.
- Thomas HF, Hartman PE, Mudryj M, Brown DL (1979b): Nitrous acid mutagenesis of duplex DNA as a three-component system. *Mutat Res* 61:129–151.
- Van Houten B (1990): Nucleotide excision repair in *Escherichia coli*. *Microbiol Rev* 54:18–51.
- Van Houten B, Snowden A (1993): Mechanism of action of the *Escherichia coli* UvrABC nuclease: clues to the damage recognition problem. *BioEssays* 15:51–59.
- Vanderbilt AS, Tessman I (1970): Identification of the altered bases in mutated single-stranded DNA. IV. Nitrous acid induction of the transitions guanine to adenine and thymine to cytosine. *Genetics* 66:1–10.
- Vogel HJ, Bonner DM (1956): Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *J Biol Chem* 219:97–106.
- Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS, Keefer LK (1991): DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254:1001–1003.
- Zimmermann FK (1977): Genetic effects of nitrous acid. *Mutat Res* 39:127–148.

Accepted by—
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