JOURNAL OF BACTERIOLOGY, Apr. 1980, p. 191-195
0021-9193/80/04-0191/05$02.00/0

Vol. 142, No. 1

Interaction of Mutagenic Spermidine-Nitrous Acid Reaction Products with *uvr*- and *recA*-Dependent Repair Systems in *Salmonella*

MICHAEL MURPHEY-CORB, HAY-LONG KONG, AND MICHAEL L. MURRAY*

Department of Microbiology and Immunology, Louisiana State University Medical Center, New Orleans, Louisiana 70119

It has been observed previously that the mutagenic action of nitrous acid may be potentiated by polyamines. We examined the cellular response of two deoxyribonucleic acid repair systems to treatment with spermidine-nitrite reaction products. *uvrB*- deficient mutants of *Salmonella typhimurium* LT2 showed enhanced lethal and mutagenic response to the reaction products. Lethal activity was further enhanced in a *uvrB recA* double mutant, whereas mutagenic activity was not detectable. Dependence of mutagenesis on the *recA* gene implicates the action of an error-prone repair system in the fixation of a premutagenic lesion as a mutation. From consideration of the substrate characteristics of the two repair systems studied, it is suggested that the deoxyribonucleic acid lesion formed by the reaction products of spermidine and nitrite is an intrastrand cross-link.

It is known that nitrous acid is mutagenic; there are, however, many puzzling and unresolved aspects of its mutagenic action (23). It has been proposed that some of the base substitution mutageneses observed after treatment of cells with nitrous acid is mediated by the nitrosation of endogenous cellular amines (20). Cellular amines present in the largest quantity are the polyamines; these are known to potentiate the mutagenic action of nitrous acid (12, 20). Polyamines are not themselves mutagenic; on the contrary, they have antimutagenic properties (4). They are, however, suitable substrates for nitrosation (3, 8-11).

To compare the mutagenic action of spermidine-nitrite reaction products with that of other known mutagens, we examined the effect of *uvr* and *recA* repair systems on cells treated with these products. *uvr*-dependent repair has been shown to be error-free, whereas *recA*-dependent repair is error-prone (22). We report that virtually all lesions caused by the reaction products of spermidine and nitrite which revert a missense mutation can be accurately repaired through the product of the *uvrB* gene. Moreover, the occurrence of mutations in response to lesion formation was totally dependent on the action of an *recA*-dependent, error-prone mechanism on cellular DNA. From the known substrate specificity of the *uvr* repair system (14) and the activity of the *recA* gene in the fixation of the lesion as a mutation, we suggest that the lesion produced by spermidine-nitrite reaction products is an intrastrand cross-link.

MATERIALS AND METHODS

Bacterial strains. *Salmonella typhimurium* LT2 strains were obtained from B. N. Ames (1), except for strain GW19, which was obtained from G. C. Walker (21). The genotypes of these strains are listed in Table 1.

Media and reagents. Brain heart infusion broth was obtained from BBL Microbiology Systems (Cockeysville, Md.); agar (Difco Laboratories, Detroit, Mich.) was used as a supplement to minimal E medium when solid medium was desired. All other reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

Preparation of spermidine-nitrite reaction products. A stock solution containing 240 mg of spermidine trihydrochloride per ml and 336 mg of sodium nitrite (molar ratio, 1:5) per ml was dissolved in 0.2 M Sorensen citrate-hydrochloride buffer (pH 4.2) (18) and allowed to react for 20 min at 37°C in a dry heater block. Thereafter, two volumes of dichloromethane were added, and the reaction mixture was further incubated for 1 h at 37°C. The bottom (dichloromethane) layer was then transferred to another tube with a Pasteur pipette and evaporated to dryness under a stream of nitrogen. The residue was then dissolved in the initial volume of citrate buffer (pH 4.2) and retained for analysis of mutagenic potential.

Mutagenesis and viability testing. Mutagenesis assays were adapted from the method of Ames et al. (1). When dose-response analysis was desired, serial twofold dilutions of the extracted mixture described in the previous section were performed by using 0.2 M citrate-hydrochloride (pH 4.2) as the diluent. The *S. typhimurium* strains described in Table 1 were treated by incubating 0.6 ml of the cell suspension (diluted 1:5 in sterile 0.1 M phosphate buffer, pH 7.4) in the presence of 0.020 ml of the reaction mixture at 37°C

for 20 min in a heater block. Except when otherwise noted, the treated cells were sedimented to a pellet (4,000 × *g*, 10 min), the supernatant was decanted, and the cells were suspended in 0.15 M sodium chloride. After another centrifugation and decantation step, the cells were suspended in 0.6 ml of 0.1 M sodium phosphate buffer (pH 7.4). The washed cell suspension was then plated on minimal plates by using a 3-ml agar overlay containing a trace amount of histidine as described by Ames et al. (1). The number of revertant cells was scored after 48 h of incubation at 37°C. Cell viability was scored after mutagenesis by overlaying appropriate dilutions of treated cells on minimal plates in 3 ml of top agar supplemented with 150 μg of histidine per ml. Viability measurements presented here were obtained from samples taken after the 20-min incubation with the spermidine-nitrite mixture but before washing. No differences in viability were observed between these measurements and those obtained with cells which were washed before dilution (data not shown).

RESULTS

Role of the *uvr* repair system. The characteristics of a number of strains with defects in repair-related enzymes and lipopolysaccharide synthesis are presented in Table 1, along with their sensitivity to the mutagenic action of the reacted spermidine and nitrite. These figures represent typical results obtained from repetitive experiments. Missense mutation *hisG46* (7) was reverted to prototrophy, but only in *uvrB recA*$^+$ strains (TA1535, TA100, and TA1950). The *uvrB recA* double mutant GW19 was not sensitive to the mutagenic action of the mixture. The presence of the mutator gene-bearing R factor pKM101 had little effect on mutagenesis, as evidenced by comparing the data obtained from TA100 and TA1535. The frameshift mutations *hisC3076* and *hisD3052* (7) were not sensitive to the spermidine-nitrite reaction products, regardless of the repair capacities of the strains used. Note that nitrite itself was mutagenic for strains TA1535, TA1950, and TA100.

To confirm the results shown in Table 1, we determined the dose response to the mutagenic action of the mixture for the missense mutation *hisG46* in five genetic backgrounds. The mutational response of the five strains (Fig. 1) confirms the pattern of sensitivity seen from Table 1. Only *uvr recA*$^+$ strains (TA1535 and TA1950) were sensitive to mutagenesis; *uvrB*$^+$ strains (*hisG46* and TS24) were resistant. In these and subsequent experiments, a dichloromethane extract of the reaction mixture was used and the treated cells were washed before plating. Tests of the solvent fractions showed that ca. 85% of the mutagenic activity was found in the dichloromethane layer; the balance remained in the aqueous layer (data not shown). No mutagenic activity was found in the dichloromethane layer after extraction of a solution containing nitrite and buffer, but no spermidine (representative data shown with strain TA1950).

Role of *recA*-dependent activity. Strain GW19, a *uvrB recA*$^+$ double mutant (21), was

TABLE 1. *Effect of spermidine-nitrite reaction products on mutagenesis of S. typhimurium strains differing in repair capacities*[a]

Strain	Mutagenic action (no. of revertants per plate)				Genetic characteristic					Presence of pKM101
	Spontaneous (control)	Spd[b] alone	NO_2[c] alone	NO_2 + Spd[d]	*his*	*uvrB*	*recA*	*rfa*	*pol-2*	
hisG46	11	3	5	19	*hisG46*	*uvrB*$^+$	*recA*$^+$	*rfa*$^+$	*pol-2*$^+$	−
TS24	3	3	7	6	*hisG46*	*uvrB*$^+$	*recA*	*rfa*$^+$	*pol-2*$^+$	−
TA1950	40	40	55	451	*hisG46*	*uvrB*	*recA*$^+$	*rfa*$^+$	*pol-2*$^+$	−
GW19	21	25	28	19	*hisG46*	*uvrB*	*recA*	*rfa*$^+$	*pol-2*$^+$	−
TA1535	23	42	105	654	*hisG46*	*uvrB*	*recA*$^+$	*rfa*	*pol-2*$^+$	−
TA2322	4	9	4	22	*hisG46*	*uvrB*$^+$	*recA*$^+$	*rfa*$^+$	*pol-2*$^+$	−
TA100	140	115	140	663	*hisG46*	*uvrB*	*recA*$^+$	*rfa*	*pol-2*	+
TA1975	2	2	2	12	*hisG46*	*uvrB*$^+$	*recA*$^+$	*rfa*	*pol-2*$^+$	−
TA92	33	41	ND[e]	40	*hisG46*	*uvrB*$^+$	*recA*$^+$	*rfa*$^+$	*pol-2*$^+$	+
TA1537	16	5	7	15	*hisC3076*	*uvrB*	*recA*$^+$	*rfa*	*pol-2*$^+$	−
TA1538	15	20	17	25	*hisD3052*	*uvrB*	*recA*$^+$	*rfa*	*pol-2*$^+$	−
TA98	19	23	23	55	*hisD3052*	*uvrB*	*recA*$^+$	*rfa*	*pol-2*$^+$	+
TA1978	8	3	15	10	*hisD3052*	*uvrB*$^+$	*recA*$^+$	*rfa*	*pol-2*$^+$	−

[a] Values for mutagenesis were obtained by plating unwashed cells.
[b] Spd, Spermidine trihydrochloride (150 mM) in reaction buffer.
[c] $NaNO_2$ (30 mM) in reaction buffer.
[d] $NaNO_2$ (30 mM) and spermidine trihydrochloride (150 mM) in reaction buffer.
[e] ND, Not determined.

also insensitive to mutagenesis by the reaction mixture (Table 1, Fig. 1). This result suggests that for a mutation to occur, a lesion must be acted upon by a *recA*-dependent, error-prone process. The percentage of the cells used in Fig. 1 which remained viable after treatment with the reaction mixture is reported in Fig. 2. Most *recA*$^+$ cells, regardless of UvrB phenotype, remained viable after mutagenic treatment, whereas *recA* strains (TS24 and GW19) were sensitive to the lethal action of the reaction mixture.

The product of the *recA* gene has pleiotropic effects; it is implicated in restoration of viability to cells with genetic lesions and with the conversion of those lesions to mutations. Lesions which are fixed as mutations by a *recA*-dependent process may remain unrepaired in strain GW19 and be expressed as lethal events. This is consistent with the observation that the number of revertants obtained was proportional to the number of treated cells for strain TA1950, but not for strain GW19, over a 100-fold range (data not shown). This result indicates that all revertants obtained with strain GW19 occur spontaneously during growth limited by the amount of histidine in the plate medium, whereas most revertants obtained with strain TA1950 are induced by treatment with the reaction mixture.

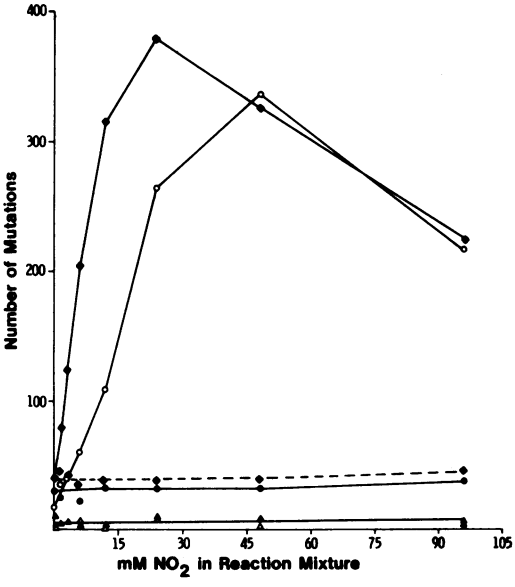

FIG. 1. *Mutagenic response of tester strains with various repair capacities upon exposure to increasing concentrations of spermidine-nitrite reaction products. Symbols: ●, GW19; ◆, TA1950; ▲, TS24; △, hisG46; ○, TA1535. The number of mutations observed per plate is plotted as a function of the concentration of sodium nitrite (millimolar concentration) in the reaction mixture. The data for the sodium nitrite control of strain TA1950 are indicated by the dashed line; these values are representative of those obtained for the other strains examined when assayed as described in the text.*

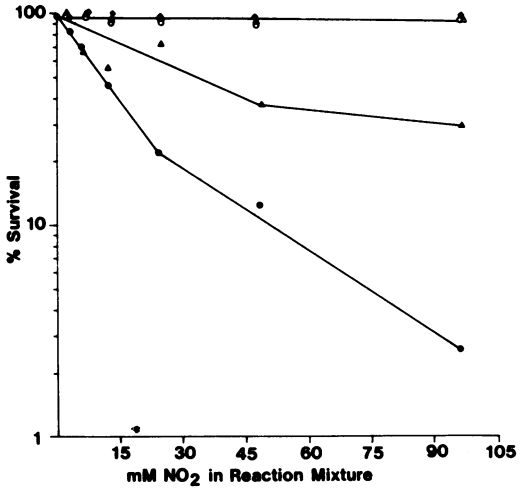

FIG. 2. *Effect of increasing concentration of spermidine-nitrite reaction products on viability of S. typhimurium repair-deficient strains. Symbols: ●, GW19; ◆, TA1950; ▲, TS24; △, hisG46; ○, TA1535. Percent survival is plotted as a function of the concentration of sodium nitrite (millimolar concentration) in the reaction mixture. The dichloromethane extract of a solution containing nitrite but not spermidine had no effect on the survival of any of the strains (data not shown).*

We assume that the *uvrB* gene product acts to maintain viability by the action of an error-free repair process, as manifested by the increased survival of strain TS24 relative to strain GW19, without a corresponding increase in the number of mutations in strain TS24.

Complexity of mutagenic reaction products. The observed mutagenic activity may be due either to a single component or to the combined action of two or more reaction products. This latter possibility is supported by an investigation of the stability of the mutagenic activity at pH 7.4. The mutagenicity of spermidine-nitrite reaction products was assayed at regular intervals after raising the pH of the mixture to 7.4. The data for an experiment performed at 25°C are shown in Fig. 3. No change was evident for the first 4 min; thereafter, the activity decayed with a half-life of 3.7 min. The decline in activity leveled off between 12 and 50 min, at 17 to 35% of the initial amount. An experiment performed at 37°C showed a lag of about 1.5 min and a half-life of 2.5 min (data not shown). The lag observed before the onset of decay of activity may reflect the production at pH 7.4 of one or more mutagenic reactive intermediates which

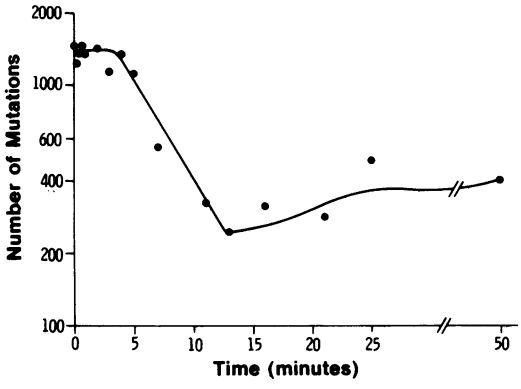

FIG. 3. *Stability of mutagenic activity of spermidine-nitrite reaction products to pH 7.4 at 25°C. Duration of incubation of the mutagen at pH 7.4 before the addition of TA1535 cells is plotted against the number of mutations observed per plate. Least-square analysis of data from 4 to 13 min is consistent with an exponential decay of a mutagenic product with a half-life of 3.7 ± 0.4 (standard deviation) min.*

then evolve to nonmutagenic end products. The multiphasic shape of the curve suggests the presence of at least two mutagenic components, one of which was relatively stable at pH 7.4 and the other inactivated by 10 min at the high pH.

DISCUSSION

Nature of the mutational lesion. Our analysis of the effects of *uvr* repair on the mutagenic activities of spermidine-nitrite reaction products leads us to suggest that the mutational lesions distort the helix. Intrastrand cross-links are one type of mutational lesion known to possess the combination of response to *uvr* repair and enhancement of base substitution mutagenesis (14) that is exhibited by the mutational lesions caused by spermidine-nitrite reaction products. Involvement of a helix-distorting lesion is also consistent with the dependence of mutagenesis on the product of the *recA* gene, since lesions which block replication seem to be effective inducers of error-prone repair.

Formation of mutagens. It is known that the spacing of amino groups in spermidine allows noncovalent bridging of nucleotides to occur (19). Reaction of these amino groups with nitrous acid might produce reactive functional groups with the appropriate spacing to facilitate cross-linkage of DNA. Hildrum and colleagues (3, 8–11) have shown that a nitrosamine is formed at the secondary amino position of spermidine. This group, however, is not likely to be solely responsible for the observed mutagenic activity, since nitrosamines are not generally direct mutagens, and the resulting dialkylnitrosamine would not form cross-links with DNA (13). Indeed, a dialkylnitrosamine was found to be nonmutagenic for cell-free transforming DNA (20). A few nitrosamines are mutagenic without activation by nonbacterial enzymes, and a clue to the nature of the mutagenic compounds formed from the reaction of nitrite with spermidine may be found among them (2, 15–17).

Another possibility, suggested by Thomas et al. (20), involves a transnitrosation mechanism. By this hypothesis, labile nitroso compounds formed from the amines temporarily stabilize reactive nitrous acid species and promote their reaction with DNA. It is known that polyamines do not promote deamination of DNA by nitrous acid (6); however, their effect on the formation of cross-links has not been determined.

Possible role for polyamines in nitrous acid mutagenesis. The results we have obtained are consistent with a role for endogenous cellular polyamines in mutagenesis caused by nitrous acid. Spermidine is abundantly present in gram-negative bacterial cells, and the behavior of the mutational lesions caused by nitrite-spermidine reaction products are consistent with their possible identification as the intrastrand cross-links known to be caused by nitrous acid (5). It remains to be proven, however, whether endogenous polyamines do indeed play such a role in nitrous acid mutagenesis.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service contract No-1CP-53521 and grant 1-R01-CA-26355-01 from the National Cancer Institute, by a grant from the Cancer Association of Greater New Orleans, and by a Public Health Service Biomedical Research Support grant from the National Institutes of Health to the Louisiana State University School of Dentistry.

LITERATURE CITED

1. **Ames, B. N., J. McCann, and E. Yamasaki.** 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. **31**:347–364.
2. **Andrews, A. W., L. H. Thibault, and W. Lijinksy.** 1978. The relationship between mutagenicity and carcinogenicity of some nitrosamines. Mutat. Res. **51**:319–326.
3. **Bills, D. D., K. I. Hildrum, R. A. Scanlan, and L. M. Libbey.** 1973. Potential precursors of N-nitrosopyrrolidine in bacon and other fried foods. J. Agric. Food Chem. **21**:876–877.
4. **Clarke, C. H., and D. M. Shankel.** 1975. Antimutagenesis in microbial systems. Bacteriol. Rev. **39**:33–53.
5. **Dubelman, S., and R. Shapiro.** 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.
6. **Frankel, A. D., B. K. Duncan, and P. E. Hartman.** 1980. Nitrous acid damage to duplex deoxyribonucleic acid: distinction between deamination of cytosine resi-

dues and a novel mutational lesion. J. Bacteriol. **142:** 335–338.

7. **Hartman, P. E., Z. Hartman, R. C. Stahl, and B. N. Ames.** 1971. Classification and mapping of spontaneous and induced mutations in the histidine operon of Salmonella. Adv. Genet. **16:**1–34.
8. **Hildrum, K. I., and R. A. Scanlan.** 1977. Factors influencing the rate of formation of volatile N-nitrosamines during the nitrosation of spermidine. J. Agric. Food Chem. **25:**255–257.
9. **Hildrum, K. A., R. A. Scanlan, and L. M. Libbey.** 1975. Identification of gamma-butenyl-(beta-propenyl) nitrosamine, the principal volatile nitrosamine formed in the nitrosation of spermidine or spermine. J. Agric. Food Chem. **23:**34–37.
10. **Hildrum, K. I., R. A. Scanlan, and L. M. Libbey.** 1977. Formation of volatile, hydroxylated, and chlorinated N-nitrosamines during the nitrosation of spermidine 3-hydrochloride. J. Agric. Food Chem. **25:**252–255.
11. **Hotchkiss, J. H., R. A. Scanlan, and L. M. Libbey.** 1977. Formation of bis(hydroxyalkyl)-N-nitrosamines as products of the nitrosation of spermidine. J. Agric. Food Chem. **25:**1183–1189.
12. **Kokatnur, M. G., M. L. Murray, and P. Correa.** 1978. Mutagenic properties of nitrosated spermidine. Proc. Soc. Exp. Biol. Med. **158:**85–88.
13. **Lawley, P. D.** 1974. Some chemical aspects of dose-response relationships in alkylation mutagenesis. Mutat. Res. **23:**283–295.
14. **Murray, M. L.** 1979. Substrate-specificity of *uvr* excision repair. Environ. Mutagen. **1:**347–352.
15. **Okada, M., E. Suzuki, T. Anjo, and M. Mochizuki.** 1975. Mutagenicity of α-acetoxydialkyl-nitrosamines: model compounds for the ultimate carcinogen. Gann **66:**457–458.
16. **Olajos, E. J., N. Maverakis, and H. H. Cornish.** 1978. Mutagenicity of aliphatic nitrosamines in *Salmonella typhimurium*. Mutat. Res. **56:**219–223.
17. **Rao, T. K., J. A. Young, W. Lijinsky, and J. L. Epler.** 1979. Mutagenicity of aliphatic nitrosamines in *Salmonella typhimurium*. Mutat. Res. **66:**1–7.
18. **Sobre, H. A., and R. A. Harte (ed.).** 1970. Selected data for molecular biology, p. 5234–5237. *In* Handbook of biochemistry. Chemical Rubber Co., Cleveland.
19. **Stevens, L.** 1967. Studies on the interaction of homologues of spermine with deoxyribonucleic acid and with bacterial protoplasts. Biochem. J. **103:**811–815.
20. **Thomas, H. F., P. E. Hartman, M. Mudryj, and D. L. Brown.** 1979. Nitrous acid mutagenesis of duplex DNA as a three-component system. Mutat. Res. **61:**129–151.
21. **Walker, G. C.** 1977. Plasmid (pkM101)-mediated enhancement of repair and mutagenesis: dependence on chromosomal genes in *Escherichia coli* K12. Mol. Gen. Genet. **152:**93–103.
22. **Witkin, E. W.** 1976. Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. Bacteriol. Rev. **40:** 869–907.
23. **Zimmerman, F. K.** 1977. Genetic effects of nitrous acid. Mutat. Res. **39:**127–148.