[CANCER RESEARCH 38, 4630–4633, December 1978]
0008-5472/78/0038-0000$02.00

Mode of Mutagenic Action of Methylnitrosocyanamide, a Potent Carcinogen[1]

Katsuhiko Hidaka, Minoru Ishizawa, Nozomu Otsuji, and Hideya Endo

Cancer Research Institute, Faculty of Medicine [K. H., M. I., H. E.], and Department of Microbiology, Faculty of Pharmaceutical Sciences, [N. O.], Kyushu University, Fukuoka 812, Japan

ABSTRACT

A potent carcinogen, methylnitrosocyanamide was used to induce revertants in a strain of *Escherichia coli* carrying an amber mutation in a gene for tryptophan (*trp*) biosynthesis and an ochre mutation in a gene for alkaline phosphatase biosynthesis. Trp+ revertants were purified and classified into seven categories based on their ability to support the growth of particular nonsense mutants of phage λ and on their content of alkaline phosphatase. About 90% of the Trp+ revertants induced by methylnitrosocyanamide were due to mutations in suppressor genes, and 85% of the suppressor mutations occurred in gene *supE*. Intragenic reversion cannot occur by a GC → AT base substitution mutation, whereas this is the obligate mode of mutation in gene *supE*. We conclude that methylnitrosocyanamide preferentially induces GC → AT transition mutations but that other base substitution mutations are also induced at about 10% of this frequency. *N*-Methyl-*N*-nitrosourea and, particularly, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine also preferentially induce GC → AT transition mutations.

INTRODUCTION

MNC[2] is a powerful carcinogen for the forestomach and esophagus of rats (8). Previously, we reported that MNC was strongly mutagenic for *Salmonella typhimurium* strain TA1535, a tester strain specific for base substitution mutagens (6).

MNC can be formed from nitrite and MG under gastric conditions (6, 7). MG is structurally similar to MNG, which is also easily convertible by nitrosation to MNNG (13), a well-known mutagen (1, 12) and gastric carcinogen (21–23). In addition, MG is similar to methylurea in structure. Methylurea is a precursor of MNU, a brain and gastric carcinogen (3, 25) and base substitution mutagen (11). The structures of MNC, MNNG, and MNU are shown in Chart 1.

For studying the mode of mutagenic action of MNC, MNNG, and MNU, revertants induced by treatment of a strain of *Escherichia coli* carrying nonsense mutations in the tryptophan (*trp*) and alkaline phosphatase (*phoA*) genes were analyzed. All 3 agents cause mutation in a suppressor gene, *supE*, at a high frequency.

MATERIALS AND METHODS

Bacteria and Phage Strains. *E. coli* K12 strains BE1043 ($trp_{am}pho_{am}str^r$) and BE1047 ($trp_{am}pho_{oc}str^r$) were used. Suppressor-sensitive mutants of phage λ (λsusG9, N53, and P3) were supplied by A. Campbell, and R216 was obtained from R. Thomas.

Media. TG medium containing 0.1 M Tris-HCl (pH 7.2), 0.2% glucose, 8×10^{-2} M NaCl, 2×10^{-2} M KCl, 2×10^{-2} M NH_4Cl, 3×10^{-3} M Na_2SO_4, 10^{-3} M $MgCl_2$, 2×10^{-4} M $CaCl_2$, and 2×10^{-6} M $FeCl_3$. To "high-phosphate" medium, 6.4×10^{-4} M KH_2PO_4 was added. To "low-phosphate" medium, 3.2×10^{-5} M KH_2PO_4 was added. Tris buffer-salts consisted of TG medium without glucose (4). Enriched TG medium (ETG) consisted of TG medium with high phosphate, 2.5 g casamino acids, and 20 mg L-tryptophan per liter distilled water. Semienriched TG (STG) agar was low-phosphate TG medium supplemented with 2.5% liquid Difco nutrient broth and solidified with 1.5% agar. Nutrient broth medium (NB) contained 5 g of meat extract and 10 g of polypeptone per liter of distilled water, adjusted to pH 7.2. Peptone medium was used for assaying λ phage and contained 10 g of polypeptone and 2.5 g of NaCl per liter of distilled water.

Mutation Induction. Bacteria grown to log phase (approximately 2×10^8/ml) in TG medium with high phosphate and 20 μg of tryptophan per ml were harvested by centrifugation, washed twice, and resuspended in Tris buffer-salts. Cells at a concentration of about 2×10^9/ml were exposed to various concentrations of chemicals at 37° for 30 min and plated on STG agar to allow maximum phenotypic expression of newly induced Trp+ mutations. Trp+ revertant colonies were visible on the lawn of background growth after 48 hr incubation at 37°. Viable counts were determined by an analogous procedure except that the bacterial suspension was serially diluted before plating.

Test for the Presence and Identification of Suppressors. Suppressors in Trp+ revertants from strain BE1043 (trp_{am}-$pho_{am}str^r$) were detected by the ability of colonies to form alkaline phosphatase. Colonies grown on STG agar were sprayed with α-naphthyl acid phosphate in 0.06% borate (2 mg/ml) and then with 2% *o*-dianisidine (4). Strains carrying suppressors formed alkaline phosphatase and the colonies turned black after several min at room temperature.

Strain BE1047 ($trp_{am}pho_{oc}str^r$) was used to identify genes involved in suppression. In each experiment, more than 50 Trp+ revertants were purified once by single colony isolation on selective STG agar plates. Individual colonies were grown at 37° overnight in 1 ml of NB liquid medium. These

---

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and a grant from the Nissan Science Foundation, Tokyo, Japan.

[2] The abbreviations used are: MNC, methylnitrosocyanamide; MG, methylguanidine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU; *N*-methyl-*N*-nitrosourea. Genetic symbols *arg* and *trp* denote requirements for arginine and tryptophan, respectively; *phoA* and *sup* denote alkaline phosphatase and suppressor, respectively; *lac* denotes the inability to utilize lactose; *am* and *oc* designate amber and ochre mutations, respectively.

Received July 21,1977; accepted May 4, 1978.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/38/12/4630/2860351/cr0380124630.pdf by guest on 08 December 2023

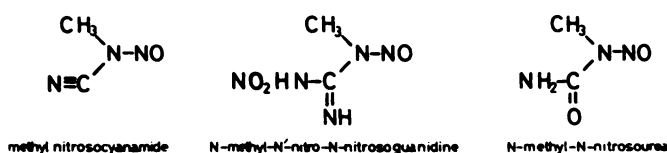

Chart 1. Structures of MNC, MNNG, and MNU.

cultures were used as host cells for growth of λ*sus* mutants and were tested on STG agar for alkaline phosphatase production as described previously. For testing its ability to support the growth of λsus phage, a portion of the NB culture was added to melted soft agar and layered over peptone medium. Drops of λsus phages were spotted and, after about 20 hr at 37°, spots were scored for phage growth. The λsus phages already are characterized to show suppression patterns different from each other for strains carrying *supD*, *supE*, *supF*, *supC*, *sup(ochre)*, and sup^+ (structural gene) of *E. coli* as shown in Table 1 (18). Thus, the Trp^+ revertants could be classified into 7 groups according to their ability to suppress the alkaline phosphatase ochre mutation and to support the growth of various amber mutants of λ phage.

Chemicals. MNC was synthesized by a slight modification (9) of the method described by Mirvish *et al.* (14). MNU was a gift of Dr. M. Nakadate, National Institute for Hygienic Sciences, Tokyo, Japan, and MNNG was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.

RESULTS

For studying the frequency of induction of mutations by MNC, MNNG, and MNU, strain BE1043 ($trp_{am}pho_{am}str^r$) was exposed to various concentrations of these agents and plated on STG agar to allow Trp^+ revertant colonies to develop. The fraction of surviving cells and the induced mutation frequency among survivors are presented as a function of mutagen concentration in Chart 2. The kinetics of mutation induction by MNC and MNNG are very similar, although MNC is a stronger mutagen than is MNNG. Concentrations of MNC and MNNG, which reduced cell survivals to 37%, were 17 and 40 nmol, respectively, and increased mutation frequency was about 4000 and 500 times that seen in the absence of mutagens, respectively. MNU is the weakest mutagen among the 3 compounds; about 10 μmol of MNU per ml reduced cell survival to 37% and increased mutation frequency about 100-fold.

Trp^+ revertant from the strain BE1043 ($trp_{am}pho_{am}str^r$) arises from mutation either in the structural gene or suppressor genes. The number of revertants due to suppressor mutation was determined by counting phosphatase-producing cells by spraying α-naphthyl acid phosphate on the STG selective agar. It was found that more than 90% of the Trp^+ revertants were due to suppressor mutation.

Amber and ochre suppressors result from changes at the anticodons of tRNA molecules (2, 10). Therefore, by classifying suppressor mutation, one can deduce what type of base change has been induced by a particular mutagen (16, 17, 20). For classifying the suppressors, a strain carrying an amber mutation in the *trp* gene and an ochre mutation in the *phoA* gene (strain BE1047) was exposed to MNC, MNNG, and MNU, and Trp^+ revertants were selected. Revertants that supported the growth of particular amber mutants of λ were scored as due to mutations in particular suppressor genes (Table 2), and the rest were scored as due to intragenic reversion in the structural gene. Revertants due to mutation in ochre or amber suppressor genes were differentiated by their ability to synthesize alkaline phosphatase (suppression of *phoA* ochre) (Table 2). Data in Table 2 show that MNC, MNNG, and MNU each induced suppressor mutations at high frequency in strain BE1047. Furthermore, each mutagen predominantly produced class 2 revertants; *i.e.*, MNC, MNNG, and MNU produced them in 77, 77, and 66%, respectively, whereas class 2 revertants occurred spontaneously at a frequency of about 19%.

Table 1

Response of amber mutants of phage to Trp^+ revertants of strain BE1047 ($trp_{am}pho_{oc}str^r$)

Class	Growth of λ*sus* G9	N53	P3	R216	Alkaline phosphatase	Suppressor
1	+[a]	+	+	−	−	Amber (*supD*)
2	+	+	+	+	−	Amber (*supE*)
3	+	+	−	−	−	Amber (*supF*)
4	−	+	+	+	−	Amber (unknown)
5	+	+	−	−	+	Ochre (*supC*)
6	−	−	−	+	+	Ochre (unknown)
7	−	−	−	−	−	sup^+ (structural gene)

[a] +, clear lysis in the area of the λ*sus* spot; −, no lysis.

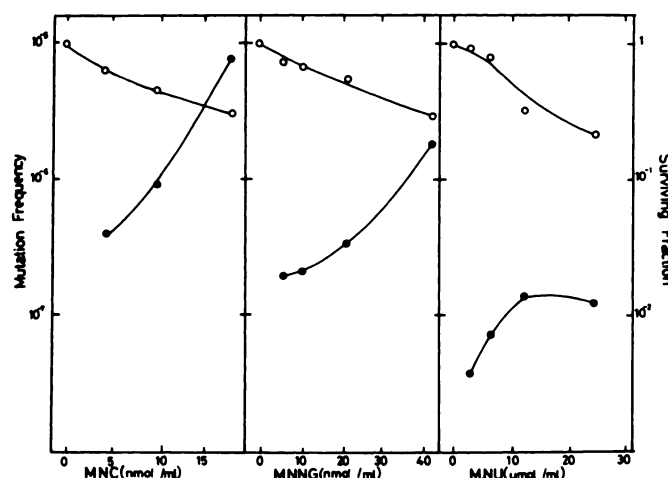

Chart 2. Induced frequency of mutation from *trp* to Trp^+ in strain BE1043 ($trp_{am}pho_{am}str^r$) by MNC, MNNG, and MNU. The bacteria grown to late log phase were treated with various concentrations of chemicals as described in "Materials and Methods." Spontaneous reversion frequencies in MNC, MNNG, and MNU were 2×10^{-9}, 4×10^{-9}, and 1×10^{-9}, respectively. Mutation frequency was expressed as a ratio.

$$\frac{\text{No. of revertants} - \text{No. of spontaneous revertants}}{\text{Surviving cells}}$$

○, surviving fraction; ●, mutation frequency of Trp^+ revertants.

DISCUSSION

About 90% of the Trp^+ revertants induced by MNC are caused by mutations in the suppressor gene (Table 2).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/38/12/4630/2860351/cr0380124630.pdf by guest on 08 December 2023

Table 2

Analysis of mutagen-produced Trp^+ revertants of strain BE1047 ($trp_{am}pho_{oc}str^r$)

Cells were treated with 17 nmol of MNC per ml, 40 nmol of MNNG per ml, and 12 μmol of MNU per ml for 30 min at 37°. Trp^+ revertants were purified by single colony isolation and classified into 7 groups by testing each single colony for synthesis of alkaline phosphatase and for supporting growth of λsus phages.

Total no. of revertants tested		Amber suppressors				Ochre suppressors		Structural gene
		Class 1	Class 2	Class 3	Class 4	Class 5	Class 6	Class 7
MNC	90	1 (1)[a]	69 (77)	0 (0)	6 (7)	4 (4)	1 (1)	9 (10)
MNNG	65	1 (2)	50 (77)	1 (2)	3 (5)	0 (0)	3 (5)	7 (11)
MNU	46	0 (0)	30 (66)	0 (0)	2 (4)	1 (2)	5 (11)	8 (18)
Spontaneous	68	3 (4)	13 (19)	15 (22)	0 (0)	10 (15)	8 (12)	19 (28)

[a] Number in parentheses, percentage.

Reversion by mutation in suppressor genes also predominates with the mutagens MNNG and MNU (Table 2). Furthermore, the most frequently occurring class of revertants is class 2, which arises from mutation in the *supE* gene (Table 2). These results suggest that there is a specificity in the mode of mutation induction by these 3 compounds. However, the question arises whether this specificity is restricted only to a nonsense codon in the *trp* gene of the special strains that we used. This is, however, not the case, since monofunctional mitomycin, which is a stronger mutagen than MNU, induces mutation in the structural gene in the present assay system at higher frequency (63%) than it does in the suppressor gene (37%) (15, 19). In addition, 4-benzoylamido- or 4-acetamido-4-carboxyamido-*n*-(*N*-nitroso)butylcyanamide, which induced mutation in *supE* gene at a high frequency in strains with amber mutation in lactose or arginine cistron, also preferentially induce mutation in *supE* gene in the present assay system (19). These results suggest that specificity of these compounds for induction of suppressor mutation is not restricted to amber mutation in the *trp* cistron of this special strain but in general affects the amber codon in bacterial genes.

We surmise that our classification of revertants into groups of true and suppressor mutations can be used to assess the types of base change preferentially induced by these mutagens. True reversion by base substitution at the nonsense codon in the structural gene can arise from changes in any base in the CTA code, except for a G to A substitution (Table 3). Mutation to amber suppressors arises from base substitution in DNA coding for the tRNA, so that the anticodon becomes CUA (which is complementary to UAG). Similarly, mutation to ochre suppressors arises from changes to TAA in the DNA coding for the anticodon of tRNA's, allowing this codon to become complementary to UAA. The DNA base changes leading to the production of structural gene reversion and of amber and ochre suppressors are shown in Table 3.

Suppressors *supD, supE, supF,* and *supC* insert serine, glutamine, tyrosine, and tyrosine, respectively, into the position of the polypeptide corresponding to the nonsense codon in mRNA. For example, class 2 revertants in which glutamine is inserted by an amber suppressor *supE* arise from the transitions GC → AT. Class 6 revertants are produced by mutation in a structural gene and arise from base substitutions, AT → GC (resulting in a code for tryptophan and glutamine), GC → TA (resulting in a code for tyrosine), AT → CG (resulting in a code for glutamic acid and serine), GC → CG (resulting in a code for tyrosine), and AT → TA (resulting in a code for leucine and lysine). However, the change GC → AT does not produce revertants in the structural gene, since it causes the formation of an alternate nonsense codon, UAA. As shown in Table 2, MNC, MNNG, and MNU induce class 2 revertants at higher frequencies than they induce other classes of revertants, suggesting that these compounds preferentially induce GC → AT transition mutations. Similarly, Osborn *et al.* (17) deduced that ethyl methanesulfonate caused GC → AT transitions.

Table 3

Relation of DNA base changes to amino acid insertions at the site of amber codon

Mutation is assumed to be due to single base substitutions in amber codon leading to structural gene reversion or, in certain tRNA anticodons, to suppressor gene reversion. Mutation to amber or ochre suppressors is assumed to arise from changes in DNA coding for tRNA anticodons, allowing these codons to become the inverse complements of UAG (amber suppressors) or UAA (ochre suppressors).

Base changes	Structural gene reversion	Amber suppressors	Ochre suppressors
GC→AT	Nonsense (UAA)	Gln[a](CAG) Try(UGG)	Gln(CAA) Nonsense (UGA,UAG)
AT→GC	Try(UGG) Gln(CAG)	Nonsense (UAA)	
GC→TA	Tyr(UAU)	Glu(GAG) Ser(UCG)	Glu(GAA) Ser(UCA) Tyr(UAC)
AT→CG	Glu(GAG) Ser(UCG)	Tyr(UAU)	
GC→CG	Tyr(UAC)	Tyr(UAC)	
AT→TA	Leu(UUG) Lys(AAG)	Lys(AAG) Leu(UUG)	Lys(AAA) Leu(UUA) Tyr(UAU)

[a] Gln, glutamine; Try, tryptophan; Tyr, tyrosine; Glu, glutamic acid; Ser, serine; Leu, leucine; Lys, lysine.

The target organs in which MNC, MNNG, and MNU produce cancer differ from each other when the mutagens are administered p.o. to animals (3, 8, 21–23, 25). Our results indicate that this difference should be ascribed to properties of these compounds other than their mode of action on DNA, for example, to differences in their stability in gastric juice or in their ability to penetrate the "blood-brain barrier."

Downloaded from http://aacrjournals.org/cancerres/article-pdf/38/12/4630/2860351/cr0380124630.pdf by guest on 08 December 2023

MNC is a carcinogen that was detected (5, 8) in an attempt to survey naturally occurring compounds related structurally and functionally to the model gastric carcinogen MNNG (24). The present assay system shows that MNC and MNNG to a striking degree share mutagen specificity. As far as base substitution mutagens are concerned, the present system makes it possible to identify qualitatively possible similarities and differences in mode of mutagenic action between model carcinogens and candidate compounds.

ACKNOWLEDGMENTS

The authors express their sincere thanks to Professor P. E. Hartman, Johns Hopkins University, Baltimore, Md., for his critical reading of this manuscript.

REFERENCES

1. Adelberg, E. A., Mandel, M., and Ching Chen, G. C. Optimal Conditions for Mutagenicity by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun., *18:* 788–795, 1965.
2. Altman, S., Brenner, S., and Smith, J. D. Identification of an Ochre Suppressor Anticodon. J. Mol. Biol., *56:* 195–197, 1971.
3. Druckrey, H., Preussmann, R., Schmähl, D., and Müller, M. Chemische Konstitution und carcinogene Wirkung bei Nitrosamine. Naturwissenschaften, *48:* 134–135, 1961.
4. Echols, H., Garen, A., Garen, S., and Torriani, A. Genetic Control of Repression of Alkaline Phosphatase in *E. coli*. J. Mol. Biol., *3:* 425–438, 1961.
5. Endo, H., Ishizawa, M., Endo, T., Takahashi, K., Utsunomiya, T., Kinoshita, N., Hidaka, K., and Baba, T. A Possible Process of Conversion of Food Components to Gastric Carcinogens. Cold Spring Harbor Conf. Cell Proliferation, *4:* 1591–1607, 1977.
6. Endo, H., and Takahashi, K. Identification and Property of the Mutagenic Principle Formed from a Food-Component, Methylguanidine, after Nitrosation in Simulated Gastric Juice. Biochem. Biophys. Res. Commun., *54:* 1384–1392, 1973.
7. Endo, H., and Takahashi, K. Methylguanidine, a Naturally Occurring Compound Showing Mutagenicity after Nitrosation in Gastric Juice. Nature, *245:* 325–326, 1973.
8. Endo, H., Takahashi, K., Kinoshita, N., and Baba, T. Production of Gastric and Esophageal Tumors in Rats by Methylnitrocyanamide, a Possible Candidate of Etiologic Factors for Human Gastric Cancer. Proc. Japan Acad., *50:* 497-502, 1974.
9. Endo, H., Takahashi, K., Kinoshita, N., Utsunomiya, T., and Baba, T. An Approach to the Detection of Possible Etiologic Factors in Human Gastric Cancer. Gann Monograph, *17:* 17-29, 1975.
10. Goodman, H. M., Abelson, J., Landy, A., Zadrazil, S., and Smith, J. D. The Nucleotide Sequence of Tyrosine Transfer RNAs of *Escherichia coli*. European J. Biochem., *13:* 461–483, 1970.
11. Lee, K., Gold, B., and Mirvish, S. S. Mutagenicity of 22 *N*-Nitrosoamides and Related Compounds for *Salmonella typhimurium* TA1535. Mutation Res., *48:* 131-138, 1977.
12. Mandell, J. D., and Greenberg, J. A. A New Chemical Mutagen for Bacteria, 1-Methyl-3-nitro-1-nitrosoguanidine. Biochem. Biophys. Res. Commun., *3:* 575–577, 1960.
13. McKay, A. F., and Wright, G. F. Preparation and Properties of *N*-Methyl-*N*-nitroso-*N'*-nitroguanidine. J. Am. Chem. Soc., *69:* 3028–3030, 1947.
14. Mirvish, S. S., Nagel, D. L., and Sams, J. Methyl and Ethylnitrosocyanamide. Some Properties and Reactions. J. Org. Chem., *38:* 1325–1329, 1973.
15. Murayama, I., and Otsuji, N. Mutation by Mitomycins in the Ultraviolet Light-sensitive Mutant of *Escherichia coli*. Mutation Res., *18:* 117-119, 1973.
16. Osborn, M., and Person, S. Characterization of Revertants of *E. coli* WU36-10 and WP2 Using Amber Mutants and an Ochre Mutant of Bacteriophage T4. Mutation Res., *4:* 504–507, 1967.
17. Osborn, M., Person, S., Philips, S. L., and Funk, F. A Determination of Mutagen Specificity in Bacteria Using Nonsense Mutants of Bacteriophage T4. J. Mol. Biol., *26:* 437–447, 1967.
18. Otsuji, N., and Aono, H. Effect of Mutation to Streptomycin Resistance on Amber Suppressor Genes. J. Bacteriol., *96:* 43–50, 1968.
19. Otsuji, N., and Endo, H. Mode of Mutagenic Action of 4-Benzoylamido- and 4-acetamido-4-carboxamido-*n*(*N*-nitroso)butylcyanamide. Mutation Res., *49:* 9–18, 1978.
20. Person, S., McClosky, J. A., Snipes, W., and Bockrath, R. C. Ultraviolet Mutagenesis and Its Repair in an *Escherichia coli* Strain Containing a Nonsense Codon. Genetics, *78:* 1035–1049, 1974.
21. Sugimura, T., and Fujimura, S. Tumor Production in Glandular Stomach of Rat by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine. Nature, *216:* 943–944, 1967.
22. Sugimura, T., Fujimura, S., and Baba, T. Tumor Production in the Glandular Stomach and Alimentary Tract of the Rat by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine. Cancer Res., *30:* 455–465, 1970.
23. Sugimura, T., Fujimura, S., Kosuge, K., Baba, T., Saito, T., Nagao, M., Hosoi, H., Shimosata, Y., and Yokoshima, T. Production of Adenocarcinomas in Glandular Stomach of Experimental Animals by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Gann Monograph, *8:* 157–196, 1969.
24. Sugimura, T., and Kawachi, T. Experimental Stomach Cancer. Methods Cancer Res., *7:* 245–308, 1973.
25. Thomas, C., Sierra, J. L., and Kersting, G. Hirntumoren bei Ratten nach oraler Gabe von *N*-Nitroso-*N*-methyl-harnstoff. Naturwissenshaften, *54:* 228, 1967.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/38/12/4630/2860351/cr0380124630.pdf by guest on 08 December 2023