

Induction of SOS Functions in *Escherichia coli* and Biosynthesis of Nitrosamine in Rabbits by Nitrogen Dioxide

by Hiroaki Kosaka,* Mitsuro Uozumi,* and Taichi Nakajima*†

Nitrogen dioxide induced SOS functions in *Salmonella typhimurium* and *Escherichia coli* K-12 and was mutagenic in *Escherichia coli* WP2. When a rabbit was administered aminopyrine intravenously and administered nitrogen dioxide by inhalation, *N*-nitrosodimethylamine was detected in its blood. Analysis was conducted with ¹⁵N-nitrosodimethylamine as an internal standard by a combination of capillary gas chromatography and mass spectrometry. Accompanying administration of cystamine increased the blood concentration of *N*-nitrosodimethylamine in the rabbit, suggesting inhibition of its metabolism. Concurrent sulfur trioxide inhalation increased *N*-nitrosodimethylamine formation in the rabbit.

Introduction

Oxides of nitrogen, oxides of sulfur, and ozone occur in the air above the major cities of the world. Nitrogen dioxide (NO₂) is among the most toxic of the nitrogen oxides, but its genetic effects have not been investigated as much as those of ozone. NO₂ has been demonstrated to induce proliferative changes of the terminal bronchiolar and alveolar epithelium. SOS functions, such as enhanced capacity for DNA repair and mutagenesis, inhibition of cell division, and prophage induction (1), were induced by NO₂ but not by NO (2) in *Salmonella typhimurium* (2) and *Escherichia coli* (3).

NO₂ or its dimer (N₂O₄) also reacts with amines to yield diazo and *N*-nitrosamine products even under aqueous neutral and alkaline conditions (4-9). According to Challis (5), nitrosation by gaseous N₂O₃ and N₂O₄ in aqueous solution is a recent finding, probably because both had been expected to undergo rapid hydrolysis at pH > 5 to innocuous nitrite. Hydrolysis does occur, but less rapidly than the nitrosation of many amines. N₂O₃ and N₂O₄ react about 2000 times more rapidly with most amines than with H₂O.

This article briefly reviews our previous investigations on the induction of the SOS functions and mutation by NO₂ (2,3) and nitrosamine formation by NO₂ (7-9).

Materials and Methods

Induction of SOS Functions and Mutation

The plasmid pSK1002 (10), which has a *umuC-lacZ* fusion gene, was introduced into *Escherichia coli* KY700 [Δ (*pro-lac*) *thi ara met srlC300::Tn10*] (11). The gene produces hybrid RecA-LacZ protein when the SOS functions are induced. Beta-galactosidase activity was assayed by Miller's method (12). *Escherichia coli* WP2 (*trpE65*) was used for mutagenesis. Exponentially growing bacteria were used after washing.

Nitrosamine Formation

Air containing NO₂ was prepared with a Standard Gas Dilution System (Model 302, Seitetsu Kagaku Works, Ltd.). A rabbit was tied and treated without anesthesia. A venous blood sample (about 4 mL) was mixed with 1 N KOH (1 mL) and an internal standard [¹⁵N-nitrosodimethylamine (¹⁵N-NDMA)]. Each sample was placed in a dialysis tube and dialyzed against 80 mL of dichloromethane two times for 30 min each with shaking (90 strokes/min). The dialyzates were concentrated to about 5 mL using a K-D evaporator, then left in a water bath (45°C) under a stream of nitrogen for further concentration to 0.1 to 0.3 mL. The 1 μ L aliquot was injected onto a fused silica capillary column (liquid phase PEG 20M, length 25 m, ID 0.25 mm, Nihon Chromato Works, Ltd.), which was mounted in a Hewlett Packard 5710A gas chromatograph with a capillary inlet system 18740B. The carrier gas was helium at a pres-

*Division of Environmental Health Research, Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537, Japan.

†Present address: Department of Environmental Medicine, Kagawa Medical School, Kagawa 761-07, Japan.

sure of 10 psi. The injector and oven temperatures were 200°C and 70°C, respectively. The effluent was directed via an all-glass jet separator (200°C) into the electron ionization ion source (150–200°C) of a Jeol 300 DX mass spectrometer set to monitor the ions at m/z 74 and m/z 75 after peak position adjustment using ^{14}N -NDMA and ^{15}N -NDMA, respectively. The peak height was used for the calculation.

Results

The expression of *umu* operon was examined with the *Escherichia coli* strain K-12 carrying the *umuC-lacZ* fusion plasmid by measuring the levels of β -galactosidase. As shown in Figure 1, 90 $\mu\text{L/L}$ NO_2 induced the expression of *umuC* operon after bubbling of the gas at 100 mL/min for 30 min. The mutagenicity of NO_2 was also investigated. Figure 2 demonstrates that NO_2 increased the induced mutation frequency in a dose-dependent fashion in *Escherichia coli* strain WP2 by reversion to Trp^+ .

Nitrosodimethylamine (NDMA) was detected in the blood of a rabbit administered aminopyrine IV and NO_2 (50 $\mu\text{L/L}$) by inhalation. Interruption of the NO_2 supply led to an immediate decrease in the NDMA level, in agreement with the report that the blood concentration of NDMA declines with a half-life of 11 min (13). Accompanying IV administration of cystamine (200 mg/kg/hr), which is speculated to inhibit NDMA metabolism (14), increased the concentration of NDMA in the blood, as shown in Figure 3. Concurrent sulfur trioxide (SO_3) inhalation accelerated the formation of NDMA in the blood (Fig. 4), where SO_3 was added by passing the gas over a solution (0.3 mL) of 60% fuming sulfuric acid.

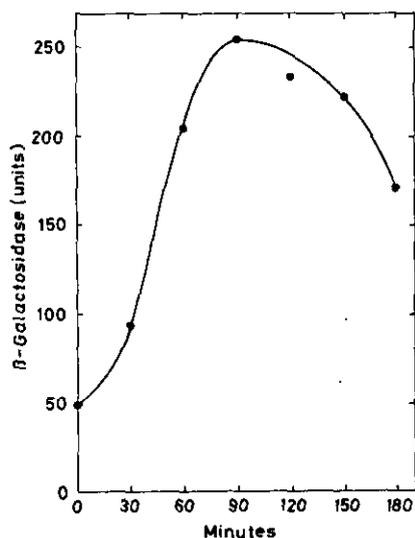


FIGURE 1. Kinetics of induction of β -galactosidase in *Escherichia coli* KY700 carrying a *umuC-lacZ* plasmid, pSK1002. After exponential culture was bubbled with 90 $\mu\text{L/L}$ NO_2 for 30 min at 100 mL/min in M-9 buffer, the culture was grown in K medium.

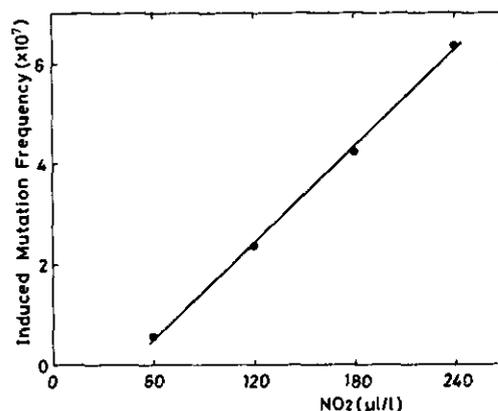


FIGURE 2. Frequency of Trp^+ mutants induced in WP2 by NO_2 . Exponential cultures in buffer were bubbled with NO_2 for 30 min at 100 mL/min before plating. Experiments were carried out five times and were reproducible. The results represent the mean value of two plates of one representative experiment done in parallel.

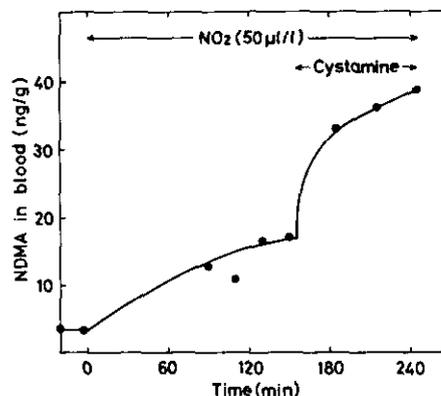


FIGURE 3. Increasing effect of cystamine (200 mg/kg/hr) on NDMA concentration in the blood of a rabbit (2.2 kg) administered aminopyrine IV and 50 $\mu\text{L/L}$ NO_2 by inhalation. Aminopyrine (20 mg/kg/hr) was flowed continuously into a femoral vein of a rabbit through tubing using a peristaltic pump with an IV injection of aminopyrine (30 mg/kg) about 15 min before NO_2 inhalation.

Discussion

NO_2 induced the SOS functions in *Salmonella typhimurium* (2) and *Escherichia coli* K-12 (Fig. 1) and also mutagenesis in *Escherichia coli* WP2 (Fig. 2), although the levels of mutagenesis were low. The weak mutagenesis may have been due to the low level of induction of the SOS functions. Alternatively, NO_2 may be one of the so-called weak mutagens which induce SOS repair activity by arresting DNA replication but do not otherwise induce premutagenic lesions (1). Isomura et al. (15) found that NO_2 induced mutagenicity in *Salmonella typhimurium* TA100 and TA1535, and that mutations and chromosome aberrations were induced in lung cells following *in vivo* exposure of rats to NO_2 . Tsuda et al. (16) also reported that NO_2 could induce chromosome aberrations in cultured Chinese hamster V79-H3 cells.

In the rabbit, IV administration of aminopyrine and

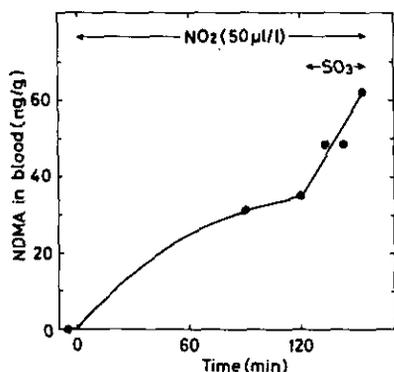


FIGURE 4. Accelerating effect of SO_3 on NDMA formation in a rabbit (2.2 kg) administered aminopyrine IV and $50 \mu\text{L/L}$ NO_2 by inhalation. SO_3 was added by passing the gas (2 L/min) over a solution (0.3 mL) of 60% fuming sulfuric acid. Aminopyrine was administered as described in Fig. 3.

administration of NO_2 by inhalation caused the formation of NDMA in blood. Accompanying SO_3 inhalation accelerated the nitrosation (Fig. 4), suggesting the augment of risk by inhalation of gas, including both NO_2 and SO_3 . Acceleration of nitrosamine formation by SO_3 supports the hypothesis that nitrosamine formation occurs in the lung rather than in circulating blood because of the action of dissolved nitrite. This hypothesis is based on consideration of the capacity of blood to maintain its pH. In addition, nitrite formed from NO_2 dissolved in the blood is transformed to nitrate by oxyhemoglobin (17,18).

The use of ^{15}N -labeled NDMA as an internal standard for NDMA analysis is advantageous, although the Thermal Energy Analyzer for nitrosamine detection also offers excellent specificity. The extract from biomaterial was clean without distillation when a dialysis tube was used. Garland et al. (19) measured NDMA in human plasma by high resolution mass spectrometry with mild chemical ionization using $^{15}\text{N}_2$ -NDMA as an internal standard.

Mammalian nitrate biosynthesis from ammonia has recently been reported (20). It is enhanced by endotoxin treatment, which stimulates a reticuloendothelial system to generate activated oxygens. Oxidation of ammonia to nitrite but not to nitrate by the xanthine oxidase reaction has been reported by Nagano and Fridovich (21). Conversion of nitrite to nitrate by oxyhemoglobin (17,18) is speculated to be responsible for the difference between the nitrite formation *in vitro* and nitrate formation *in vivo*. A fraction of ingested nitrosatable amines or amides (22) may be nitrosated by the biosynthesized nitrite when a reticuloendothelial system is chronically activated.

Cystamine, which has been suggested to inhibit NDMA metabolism (14), increased the concentration of NDMA when concurrently administered (Fig. 3). As ether anesthesia also is reported to inhibit NDMA metabolism in rats (13), experiments have been done with the animal tied down and treated without anesthesia (9). If cytochrome P-450 metabolizes NDMA, amino-

pyrine might inhibit the metabolism of NDMA, as aminopyrine is metabolized by cytochrome P-450, yielding formaldehyde. One observation concerning NDMA metabolism that has puzzled many investigators is that NDMA demethylase activity is not induced by classical inducers and is not significantly inhibited by classical monooxygenase inhibitors, such as SKF-525A and metyrapone, but is inhibited by compounds such as 3-amino-1,2,4-triazole and 2-phenylethylamine. Cytochrome P-450 LM_{3a} isolated from liver microsomes of ethanol-treated rabbits (23) should help resolve these conflicting observations.

This work was supported by a Grant-in-Aid for Environmental Science from the Ministry of Education, Science and Culture of Japan. We thank Takae Nakajima for her technical assistance.

REFERENCES

1. Witkin, E. M. Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Bacteriol. Rev.* 40: 869-907 (1976).
2. Kosaka, H., Oda, Y., and Uozumi, M. Induction of *umuC* gene expression by nitrogen dioxide in *Salmonella typhimurium*. *Mutat. Res.* 142: 99-102 (1985).
3. Kosaka, H., Yamamoto, K., Oda, Y., and Uozumi, M. Induction of SOS functions by nitrogen dioxide in *Escherichia coli* with different DNA repair capacities. *Mutat. Res.* 162: 1-5 (1986).
4. Iqbal, Z. M., Dahl, K., and Epstein, S. S. Role of nitrogen dioxide in the biosynthesis of nitrosamines in mice. *Science* 207: 1475-1477 (1980).
5. Challis, B. C. The chemistry of formation of N-nitroso compounds. In: *Safety Evaluation of Nitrosatable Drugs and Chemicals* (G. G. Gibson and C. Ioannides, Eds.), Taylor and Francis Ltd, London, 1980, pp. 16-55.
6. Gold, A. Stoichiometry of nitrogen dioxide determination in triethanolamine trapping solution. *Anal. Chem.* 49: 1448-1450 (1977).
7. Kusumoto, S., Kimura, T., Nakajima, T., and Nakamura, A. Formation of nitrosodimethylamine by NO_2 exposure in rats pretreated with aminopyrine. In: *Proc. 8th Int. Conf. Occup. Health Chem. Ind.* (N. Takemura and Y. Yamamura, Eds.), Aikawa Shobo Publishing Co., Tokyo, 1981, pp. 48-55.
8. Uozumi, M., Kusumoto, S., Kimura, T., Nakamura, A., and Nakajima, T. Formation of N-nitrosodimethylamine by NO_x exposure in rats and rabbit pretreated with aminopyrine. In: *N-Nitroso Compounds: Occurrence and Biological Effects*. IARC Scientific Publications No. 41 (H. Bartsch, I. K. O'Neill, M. Castegnaro, and M. Okada, Eds.), International Agency for Research on Cancer, Lyon, 1982, pp. 425-432.
9. Kosaka, H., Uozumi, M., and Nakajima, T. Measurement of nitrosodimethylamine by capillary gas chromatography-mass spectrometry with the ^{15}N -labelled compound as an internal standard. *Int. Arch. Occup. Environ. Health* 54: 233-239 (1984).
10. Shinagawa, H., Kato, T., Ise, T., Makino, K., and Nakata, A. Cloning and characterization of the *umu* operon responsible for inducible mutagenesis in *Escherichia coli*. *Gene* 23: 167-174 (1983).
11. Yamamoto, K., Higashikawa, T., Ohta, K., and Oda, Y. A loss of *uvrA* function decreases the SOS functions *recA* and *umuC* induction by mitomycin C in *Escherichia coli*. *Mutat. Res.* 149: 297-302 (1985).
12. Miller, J. H. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.
13. Keefer, L. K., Garland, W. A., Oldfield, N. F., Swagzdis, J. E., and Mico, B. A. Inhibition of N-nitrosodimethylamine metabolism in rats by ether anesthesia. *Cancer Res.* 45: 5457-5460 (1985).
14. Magee, P. N. Interaction of activated intermediates of chemical carcinogens with cellular DNA and its possible prevention. In: *Free Radicals, Lipid Peroxidation and Cancer* (D. C. H. McBrien

- and T. F. Slater, Eds.), Academic Press, London, 1982, pp. 353-376.
15. Isomura, K., Chikahira, M., Teranishi, K., and Hamada, K. Induction of mutations and chromosome aberrations in lung cells following *in vivo* exposure of rats to nitrogen oxides. *Mutat. Res.* 136: 119-125 (1984).
 16. Tsuda, H., Kushi, A., Yoshida, D., and Goto, F. Chromosomal aberrations and sister-chromatid exchanges induced by gaseous nitrogen dioxide in cultured Chinese hamster cells. *Mutat. Res.* 89: 303-309 (1981).
 17. Kosaka, H., Imaizumi, K., Imai, K., and Tyuma, I. Stoichiometry of the reaction of oxyhemoglobin with nitrite. *Biochim. Biophys. Acta* 581: 184-188 (1979).
 18. Kosaka, H., and Uozumi, M. Inhibition by amines indicates involvement of nitrogen dioxide in autocatalytic oxidation of oxyhemoglobin by nitrite. *Biochim. Biophys. Acta* 871: 14-18 (1986).
 19. Garland, W. A., Holowaschenko, H., Kuenzig, W., Norkus, E. P., and Conney, A. H. A high resolution mass spectrometry assay for N-nitrosodimethylamine in human plasma. In: *Nitrosamines and Human Cancer*, Banbury Report No. 12 (P. N. Magee, Ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982, pp. 183-196.
 20. Wagner, D. A., Young, V. R., and Tannenbaum, S. R. Mammalian nitrate biosynthesis: incorporation of $^{15}\text{NH}_3$ into nitrate is enhanced by endotoxin treatment. *Proc. Natl. Acad. Sci. (U.S.)* 80: 4518-4521 (1983).
 21. Nagano, T., and Fridovich, I. The co-oxidation of ammonia to nitrite during the aerobic xanthine oxidase reaction. *Arch. Biochem. Biophys.* 241: 596-601 (1985).
 22. Inoue, K., Shibata, T., Kosaka, H., Uozumi, M., Tsuda, S., and Abe, T. Induction of sister-chromatid exchanges by N-nitrosocimetidine in cultured human lymphocytes and its inhibition by chemical compounds. *Mutat. Res.* 156: 117-121 (1985).
 23. Yang, C. S., Tu, Y. Y., Koop, D. R., and Coon, M. J. Metabolism of nitrosamines by purified rabbit liver cytochrome P-450 isozymes. *Cancer Res.* 45: 1140-1145 (1985).