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Effect of 2-Nitrosonaphthalene on Bacteria and Bacteriophage[1]

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SUMMARY

2-Nitrosonaphthalene (2NON) inactivates the colony-forming ability of *Salmonella typhimurium* Q and its repair-deficient mutants. It inactivates excision-deficient and recombination-deficient (*recA*) mutants rapidly, a DNA polymerase I-deficient mutant moderately, and the wild-type strain slowly. These results suggest that 2NON damages the bacterial genome and that the damaged genome is reparable by the bacterial repair enzymes. 2NON induces phage P221 from its lysogens. Induction of the prophage in an excision-deficient mutant lysogenic for P221 is far more efficient than in the wild-type strain. However, when bacteriophage was directly incubated with 2NON, no phage inactivation was observed. Since 2NON inactivates the bacterial genome but not the phage genome, it was suspected that enzymes in bacterial cells are required to convert 2NON to an activated form before reacting with the bacterial genome. However, we found that 2NON in the presence of NADH and trace amounts of divalent cations, such as copper, zinc, and manganese, can be nonenzymatically converted to a reduced form that inactivates bacteriophage.

INTRODUCTION

Since Leichtenstein's (12) report in 1898, aminonaphthalene has been regarded as a causative agent for human occupational bladder cancer. Belman *et al.* (1) and Boyland *et al.* (2, 3) demonstrated that the *N*-hydroxylated metabolites of aminonaphthalenes, 1-hydroxyaminonaphthalene, and 2-hydroxyaminonaphthalene, are potent carcinogens. From studies of these investigators (1, 3) it has been suggested that the above HAN's[4] may be proximate carcinogens for bladder cancer. HAN's can be further oxidized to nitroso derivatives, 1-nitrosonaphthalene and 2NON (5, 15). Radomski *et al.* (15) found that these nitroso derivatives are carcinogenic and suggested the possibility that the final oxidation forms, the nitroso derivatives, are active carcinogens rather than the *N*-hydroxy derivatives.

The *N*-hydroxylated derivatives of both aminonaphthalenes and 4NQO damage the bacteriophage genome by incubation of phage particles *in vitro* (10, 17, 21, 22). Ishizawa *et al.* (11) and Endo *et al.* (7) reported that 4HAQO, the *N*-hydroxylated derivative of 4NQO, does not interact with bacteriophage genome in the absence of oxygen and suggested that a possible oxidized derivative of 4HAQO, 4NOQO, might be an active form that interacts with genomes. However, 4NOQO has not yet been isolated and synthesized for the study of carcinogenesis because 4NOQO is probably unstable. The availability of the stable nitroso derivative, NON, provided an opportunity to analyze the contribution of a nitroso derivative of an aromatic amine to carcinogenesis.

In this publication we report the interaction of NON and its activated form with phage and bacterial genomes using various bacterial repair-deficient mutants. In addition we describe the mechanism of nonenzymatic activation of NON for its interaction with DNA using bacteriophage.

MATERIALS AND METHODS

Bacterial Strains. Bacterial strains used were *S. typhimurium* Q1 and its repair-deficient mutants; excision-deficient (*hcr*$^-$) and recombination-deficient (*recA*$^-$) mutants (9); and DNA polymerase I-deficient mutant (*pol*$^-$) of Q1 (17). These bacterial strains lysogenic for phage P221 were also used for prophage induction experiments. For assay of phage P221, a streptomycin-resistant and P221-specific assay host, *St/22Sm*^{r}, was used. Q1 and *Escherichia coli* K12 were used for assay of *Salmonella* phage P22 and *E. coli* phage T5. All bacterial strains were cultured by aeration in nutrient broth.

Bacteriophage. *Salmonella* phages P22 and P221 (19, 20) and *E. coli* phage T5 were used. Purified T5 and P22 phage stocks (about 10^{11} PFU/ml) were prepared by differential centrifugation and used for direct interaction with a chemical at the final concentration of about 10^7 PFU/ml.

Media. Nutrient broth consisting of 8 g Difco nutrient broth and 5 g sodium chloride per liter of distilled water was used for making bacterial aeration culture. For phage plating, hard agar contained 23 g Difco nutrient agar and 5 g sodium chloride per liter; and soft nutrient agar contained 7.5 g Difco Bacto-agar, 5 g sodium chloride, and 8 g Difco nutrient broth per liter. Phosphate-buffered saline contained 0.067 M phosphate in 0.1 M NaCl at pH 7.0.

Agents. 2NON was kindly supplied by Dr. Masashi Okada, Tokyo Biochemical Research Institute, Tokyo, Japan. 1- and 2-nitronaphthalene were purchased from K and K Laboratories, Inc., Plainview, N. Y. DMSO (Fisher Scientific Co., Fairlawn, N. J.) was used for solubilizing 2NON and NN.

Enzymes and Proteins. Rat liver cytosol was subfractionated with 50 to 75% saturated ammonium sulfate. Diaphorase was

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[4] The abbreviations used are: HAN, hydroxyaminonaphthalene; 2NON, 2-nitrosonaphthalene; 4-NQO, 4-nitroquinoline 1-oxide; 4HAQO, 4-hydroxyaminoquinoline 1-oxide, 4NOQO, 4-nitrosoquinoline 1-oxide; NON, nitrosonaphthalene; PFU, plaque-forming unit; DMSO, dimethyl sulfoxide, NN, nitronaphthalene.

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purified from this fraction by means of a DEAE-cellulose column (8, 10). A protein fraction containing no diaphorase activity was obtained from a fast-eluted fraction and used as a rat liver protein without diaphorase activity. Other proteins and enzymes used were: bovine serum albumin and bovine pancreatic RNase A (Sigma Chemical Co., St. Louis, Mo.).

Inactivation Kinetics of Bacteria with 2NON. Bacteria (10^7 cells/ml) were treated with 2NON in nutrient broth at 37°. 2NON, 0.01 volume, dissolved in DMSO was added to the bacterial cell suspension. Samples were withdrawn at various intervals, diluted, and assayed for inactivation of colony-forming ability (9, 22).

Prophage Induction of P221 Lysogens (9). Log-phase lysogenic cells (10^7 to 10^8 cells/ml) were treated with 2NON in nutrient broth by the method described above and diluted 100- to 1000-fold in fresh nutrient broth. After 30 min incubation at 37°, 0.1-ml samples were plated on *St/22 Sm*^{r} with agar containing the minimal concentration of dihydrostreptomycin required for growth inhibition of the lysogenic strain (200 μg/ml for a P221 lysogen of the wild-type strain and 25 μg/ml for a P221 lysogen of the *hcr*$^-$ mutant strain). These concentrations of streptomycin kill lysogenic cells on the plates but do not inhibit phage production of cells previously induced (14, 16). Therefore, only cells that are induced by 2NON give P221 infectious centers on *St/22 Sm*^{r}.

Treatment of Phage with 2NON. All manipulations were carried out at room temperature (25°). 2NON, 0.01 volume, dissolved in DMSO was added to T5 or P22 phage suspension (about 10^7 PFU/ml) in phosphate-buffered saline (pH 7.0), and 0.1-ml samples were withdrawn at various intervals and diluted 100-fold or further in phosphate-buffered saline to stop the reaction of the residual agent. Immediately after dilution, 0.25-ml aliquots of each diluted sample were added to 0.1 ml of the nutrient broth culture of indicator bacteria and incubated for 10 min at 37° in a water bath. Then, 2 ml of soft agar were added and poured on nutrient agar plates. The number of plaques following overnight incubation at 37° served as the measure of bacteriophage inactivation.

RESULTS

Effect of 2NON on Bacterial Viability and Prophage Induction. When various bacterial repair mutants were incubated with 0.1 mM 2NON, *hcr*$^-$ *and recA*$^-$ mutants were inactivated rapidly, *pol*$^-$ mutants were inactivated moderately, and wild-type strains were inactivated slowly. Chart 1 shows the typical inactivation kinetics of these bacterial strains. A 7-min exposure gave 64% survivors for the wild type, 18% for *pol*$^-$, 2% for *recA*$^-$, and 0.4% for *hcr*$^-$, respectively. These results suggest that 2NON damages the bacterial genomes and that the damaged genomes are reparable by the bacterial repair enzymes. These inactivation patterns are similar to those caused by UV and 4NQO treatments (13, 19, 21, 22).

Subsequently, prophage inducibility by 2NON in the *hcr*$^-$ mutant and the wild-type strain lysogenic for P221 was tested. As shown in Chart 2, prophage induction in a *hcr*$^-$ lysogen rose sharply (about 200-fold) after 2 min treatment, followed by a sharp decrease after longer treatment. In the wild-type lysogen, however, prophage induction shows only a 15-fold increase at about 7 min. Thus, prophage induction in the *hcr*$^-$ mutant is more efficient than in the wild-type strain, supporting the thesis that 2NON damages the bacterial genome.

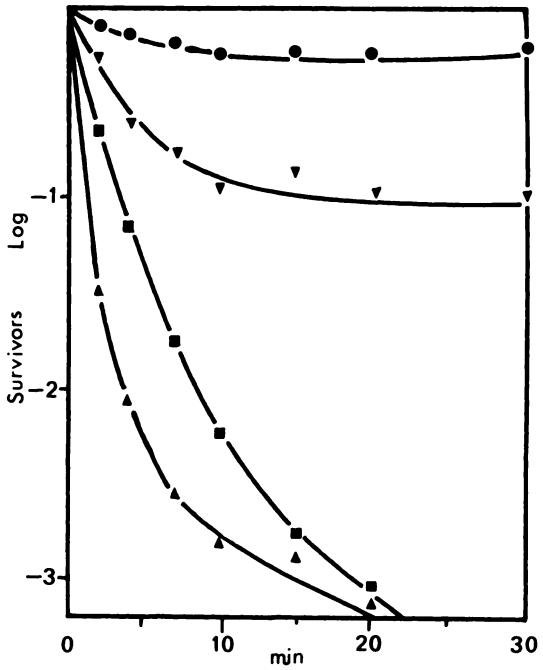

Chart 1. Inactivation kinetics of various repair deficient mutants of *S. typhimurium* by 0.1 mM 2NON. Experimental details are given in the text. ●, wild-type strain; ▲, *hcr*$^-$ mutant; ▼, *pol*$^-$ mutant; ■, *recA* mutant.

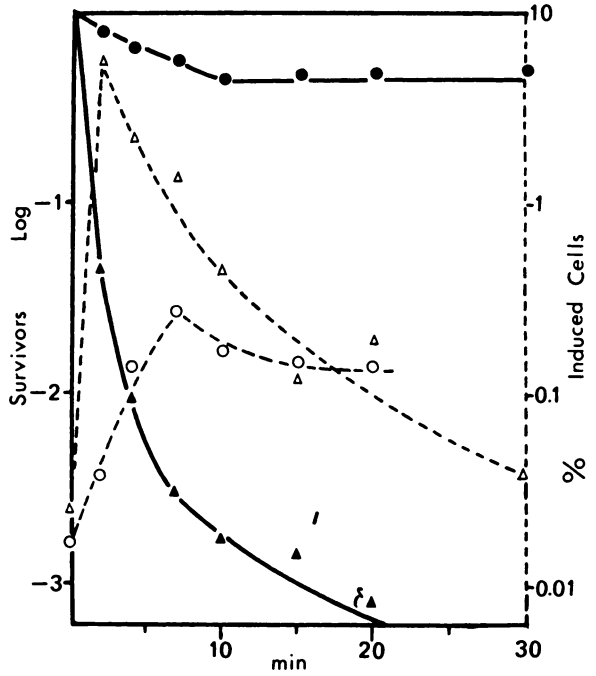

Chart 2. Inactivation of P221 lysogens and their prophage induction by 0.1 mM 2NON. Experimental details are given in the text. ——, survivors; - - - -, prophage induction. ●, ○, wild-type strain; ▲, △, *hcr*$^-$ mutant.

Nonenzymatic Activation of 2NON. Since 2NON inactivates the bacterial genome and induces prophage as did the other carcinogens (9, 21, 22), it was desirable to test its effect on bacteriophage particles as a test for the direct interaction of 2NON with DNA. When T5 or P22 phage particles were incubated with 0.1 mM 2NON for 1 hr, no phage inactivation was observed. Therefore, for bacterial inactivation, it was suspected that 2NON is activated by enzymes in the bacterial cell to become reactive with the bacterial genome.

Bacterial and rat liver diaphorases convert 4NQO to 4HAQO which damages phage genomes (10). It has been suggested that disphorase converts 4NQO to 4NOQO and is further reduced to 4HAQO (7, 11). Therefore, diaphorase may also be able to convert 2NON to a reduced form reactive with the phage genomes. When incubated in the presence of diaphorase and NADH, 2NON (0.1 mM) rapidly inactivated the plaque-forming ability of phage T5. Ten min exposure gave a 10-fold inactivation (Table 1) and 20 min exposure gave an inactivation of about 100-fold. However, a mixture of 2NON and NADH also inactivated phage T5 in the presence of albumin, pancreatic RNase, or rat liver protein fraction containing no diaphorase activity. As shown in Table 1, the levels of inactivation of phage T5 under these conditions are similar to that from 2NON treatment with a mixture of diaphorase and NADH. These observations indicate that not only diaphorase but also the other proteins in the presence of NADH convert 2NON to an active form that inactivates phage T5. However, Table 1 shows that EDTA completely blocked this type of phage inactivation. These results suggest that a divalent cation plays an important role in the activation of 2NON. When the serum albumin was hydrolyzed and tested for its effect on inactivation of the phage by 2NON, hydrolysate was found to retain the original activity of the activation effect of albumin. This activity was also blocked completely by EDTA. These data are summarized in Table 1.

Activation of 2NON by Divalent Cations. Since divalent cations seem to be involved in the activation of 2NON, the effect of various divalent cations on activation of 2NON was studied. As can be seen in Tables 1 and 2, we also occasionally observed substantial inactivation (about 50%) of the phage by 2NON in the presence of NADH without proteins, further suggesting such a role for trace amounts of divalent cations contaminated in the systems.

We previously reported that cupric ion catalytically acts on some reducing agents to yield oxidation products that can inactivate the phage genome (18). To test this possibility, T5 phage particles were incubated for 30 min with NADH in the presence of trace amounts of cupric ion. As shown in Chart 3, no significant phage inactivation was observed. However, when 2NON was added to the above mixture after 30 min incubation, rapid inactivation was observed, at a rate similar to that of a 0-min mixing of 2NON with NADH and cupric ion. Table 2 further shows that 10^{-5} M cupric ion in the presence of NADH converts 2NON to an active form to inactivate phage T5 at a rate of more than 99% in 5 min and that even 10^{-7} M cupric ion causes 95% inactivation. This stimulating effect of cupric ion on phage inactivation by 2NON can be substituted by other divalent cations such as zinc and manganese also in the range of 10^{-5} to 10^{-7} M. Other reducing agents such as mercaptoethanol or cysteine cannot substitute for NADH, however, NADPH is fully as effective as NADH (Table 2). Since these hydrogen donors activate 2NON in the presence of divalent cations, it was desirable to demonstrate the oxidative consumption of NADH under these conditions. When 2 X

Table 1

Activation of 2NON in the presence of NADH and proteins determined by inactivation of bacteriophage T5

Condition[a]	% survivals at 10 min
Control	100
+ 2NON	100
+ 2NN	100
+ 2NON + diaphorase[b] + NADH	10
+ 2NN + diaphorase[b] + NADH	100
+ 2NON + albumin[b] + NADH	5
+ 2NON + RNase[b] + NADH	5–10
+ 2NON + rat liver protein[b] + NADH	15
+ NADH	95–100
+ albumin + NADH	100
+ 2NON + NADH	50–60
+ 2NN + NADH[c]	100
+ 2NON + diaphorase + NADH + EDTA	85
+ 2NON + albumin + NADH + EDTA	85
+ 2NON + albumin hydrolysate + NADH	10
+ 2NOH + albumin hydrolysate + NADH + EDTA	90

[a] Control consists of bacteriophage suspended in phosphate-buffered saline containing 1 mM $MgSO_4$. +, addition to the control. Other experimental details are given in the text. Concentrations used: 2NON, 0.1 mM; 2NN, 0.1 mM; NADH, 0.1 mM; EDTA, 1 mM; diaphorase, 4 μg/ml; bovine serum albumin, 4 μg/ml; RNase, 4 μg/ml; rat liver protein, 4 μg/ml; albumin hydrolysate, 5 μg/ml.

[b] These proteins have no effect on T5 in the presence or absence of NADH.

[c] The same survival was obtained after 30 min or more of incubation.

Table 2
Nonenzymatic activation of 2NON

Condition[a]	% survivors at 5 min
Control	100
+ 2NON + NADH	70–85
+ 2NON + NADH + $CuSO_4$ (10^{-5} M)	<1
+ 2NON + NADH + $CuSO_4$ (10^{-7} M)	5
+ 2NON + NADH + $ZnSO_4$ (10^{-7} M)	5
+ 2NON + NADH + $MnCl_2$ (10^{-5} M)	10
+ 2NON + NADH + $FeSO_4$ (10^{-5} M)	35
+ 2NON + NADH - $MgSO_4$	>95
+ 2NON + cysteine (10^{-4} M) + $CuSO_4$ (10^{-5} M)	70
+ cysteine (10^{-4} M) + $CuSO_4$ (10^{-5} M)	70
+ 2NON + mercaptoethanol (1.4 x 10^{-4} M) + $CuSO_4$ (10^{-5} M)	90
+ mercaptoethanol (1.4 x 10^{-4} M) + $CuSO_4$ (10^{-5} M)	90
+ 2NON + TPNH (10^{-4} M) + $CuSO_4$ (10^{-5} M)	1

[a] Control consists of bacteriophage suspended in phosphate-buffered saline containing 1 mM $MgSO_4$. +, addition to the control; –, ommission from the control. The concentrations of 2NON and NADH used were 0.1 mM. Values in parentheses, final concentration of the chemicals in the reaction mixture. Other experimental details are given in the text.

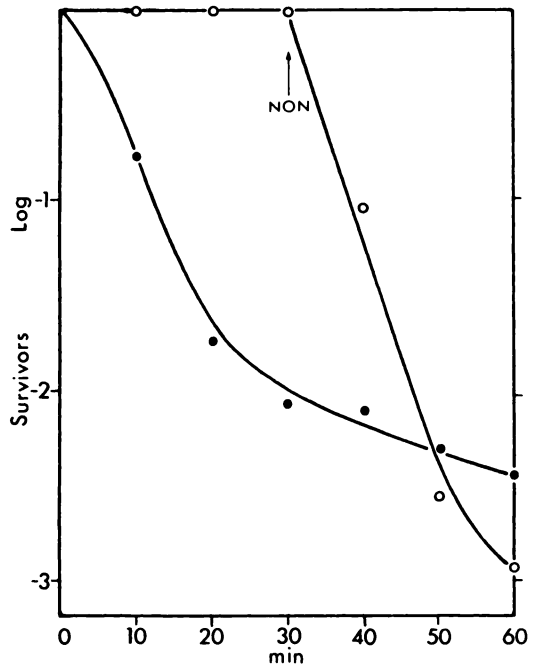

Chart 3. Effect of cupric ion on inactivation of bacteriophage T5 by 2NON in the presence of NADH. Concentrations used were: 2NON, 0.1 mM; NADH, 0.1 mM; and $CuSO_4$, 0.1 mM. •, T5 added to a mixture of 2NON, NADH and $CuSO_4$ at 0 min; ○, T5 added to a mixture of NADH and $CuSO_4$ at 0 min, then at 30 min 2NON added to the above mixture.

10^{-5} M $CuSO_4$ was added to a mixture of 0.3 mM NADH and 0.05 mM 2NON, rapid oxidation of NADH was observed by a decrease in the NADH-specific absorption spectrum at 340 nm; about 70% NADH was oxidized in 7 min. From these observations it was concluded that 2NON must in all likelihood be converted to a reduced form before reacting with the phage genome.

DISCUSSION

2NON can be activated nonenzymatically by NADH in the presence of trace amounts of divalent cations. Since this activation process seems to be reductive, it seems likely that HAN is a possible activated form. However, HAN inactivates phage by a mechanism requiring trace amounts of cupric ion that cannot be substituted by other divalent cations such as zinc or manganese (N. Yamamoto, Y. Tagashira, S. Fukuda, and N. Ushijima, unpublished observations). Therefore, other possible activated forms may be considered for the activation of 2NON.

The mechanism of activation of 2NON by proteins, *e.g.*, serum albumin and RNase, in the presence of NADH could be the same as 2NON activation by divalent cations, since EDTA completely blocks the activation of 2NON by various proteins. This observation suggests that the proteins seem to be contaminated by at least trace amounts of divalent cations. The acid hydrolysate of bovine serum albumin was found to retain the original activity of the activating effect of the protein on activation of 2NON (Table 2). This result can readily be explained by various reports that serum albumin is the major cupric ion transport protein in human, bovine, rat, and dog (6).

The inactivation mechanism of phage by 2NON in the presence of NADH and trace amounts of cupric ion could be due to reaction with phage protein. Therefore, its effect on phage genome was studied by our previously established method for interaction of various carcinogens with phage genome (9, 22). A clear plaque-forming mutant of P22, P22*c*2, was incubated with a mixture of 0.3 mM 2NON, 0.1 mM NADH, and 10^{-6} M $CuSO_4$ for various time intervals and assayed on *S. typhimurium* Q1 and its lysogenic for the wild-type phage P221c^+ carrying turbid plaque phenotype. A greatly increased frequency of turbid (*c*+) plaques of P22 was observed on the lysogenic strain, whereas no *c*+ plaques were found on the nonlysogenic strain. Since no *c*+ plaques were found on the nonlysogenic strain, the high frequency of the

turbid *c*+ plaques on the lysogenic strain must be a consequence of recombination between 2NON-damaged P22*c*2 genome and the prophage P221*c*+. The frequency of *c*+ recombinant formation increases greatly as survivors decrease. The maximum increase of recombination frequency was about 50-fold as compared with untreated controls. It is therefore concluded that the bacteriophage genome is damaged by 2NON in the presence of NADH and trace amounts of cupric ion.

NN is structurally somewhat similar to 4NQO. Their *N*-hydroxylated derivatives, HAN and 4HAQO, have been chemically synthesized by reduction of NN and 4NQO (4, 7). Although *in vivo* metabolism of 4NQO is well established (7), metabolic conversion of NN to NON, HAN, or other reduced metabolites has not been demonstrated. Accordingly, we tested effects of NN on bacteriophage particles with or without the presence of diaphorase and NADH. As shown in Table 1, 2NN has no effect on viability of bacteriophage T5. Similar results with 1NN were also found. Consequently, the possibility that NN might be activated in bacterial cells, as in the case of 4NQO, was considered. When repair-deficient bacterial mutants and the wild-type strain were incubated with NN, no bacterial inactivation was observed. Therefore, it may be concluded that the bacterial cells do not carry an activating enzyme for converting NN to NON or other derivatives that damage the bacterial genome.

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