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Prophage Induction in Lysogenic *Escherichia coli* with *N*-Nitroso Compounds and Derivatives

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Prophage induction in lysogenic *Escherichia coli* W1709 (λ) was determined for 29 *N*-nitroso compounds, 13 of their denitrosated derivatives, and 7 hydroxylamino and hydrazino analogues of nitrosamines. Minimal inducing concentrations of 0.1 to 2.0 μg/ml were demonstrated for eight nitrosamidines, and concentrations of 0.5 to 25.0 μg/ml were shown for six nitrosamides. Weak inducing activities were found with *N*,*N*-diethylhydroxylamine oxalate and *N*-methyl-*N*-phenylhydrazine sulfate, derivatives of inactive *N*-nitrosodiethylamine and *N*-nitrosomethylphenylamine, respectively. Inactive compounds including *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, 11 nitrosamines, 3 *N*,*N*'-dialkyl substituted-*N*-nitrosoureas, 13 denitrosated derivatives, and 5 hydroxylamino and hydrazino analogues of nitrosamines are listed. Since 7 of the 14 prophage-inducing nitrosamidines and nitrosamides reported thus far have carcinostatic activity in rodent tumor systems, it is concluded that the induction test may provide a useful screen for the detection of potential antitumor compounds. The induction test may also be useful for the detection of responsive *N*-nitroso compounds which may be potential toxicological hazards in the environment since, of the six active nitrosamides, five have already been reported to produce mutagenic and carcinogenic effects, four produce chromosome-damaging effects, and two produce teratogenic effects. Use of the prophage induction system for detection of biologically active intermediates formed by *N*-nitroso compounds under physiological conditions is considered.

There appears to be a clearly positive association (12) between the capability of a compound to induce prophage in lysogenic bacteria and its ability to inhibit development of transplanted tumors in rodents. Compounds (12, 13) capable of mutagenic, carcinogenic, and teratogenic effects in various experimental systems are also capable of prophage induction. Potent mutagenic, carcinogenic, carcinostatic, and teratogenic effects produced by *N*-nitroso compounds in experimental systems are documented by an extensive literature (24, 35) which has recently been reviewed. There has been increasing interest in the possibility that various *N*-nitrosamines may have etiological significance in human cancer (22).

These properties suggest that *N*-nitroso compounds might also be capable of prophage induction and, if so, that this simple in vitro test system might prove useful for detecting responsive *N*-nitroso compounds found to be potential hazards in the environment. Only a limited number of *N*-nitroso compounds have been tested thus far for prophage-inducing capability. Induction has been observed with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (1), with both *N*,*N*'-dinitroso-*N*,*N*'-dimethylterephtalamide and *N*,*N*'-dinitroso-*N*,*N*-dimethyloxamide (29), and with streptozotocin (14), an antibiotic chemically related to *N*-nitrosomethyl-urea (17) and produced by *Streptomyces achromogenes*. Both *N*-nitrosodimethylamine (28) and *N*-nitrosodiethylamine (34) were incapable of prophage induction.

The possibility that biologically active *N*-nitroso compounds may occur in various environmental conditions and thus constitute a potential hazard to man and animals has been considered (24). Insofar as I am aware, the occurrence of nitrosamidine derivatives in nature has not been described. *N*-nitrosourea end products of microbial fermentations, such as streptozotocin, for which mutagenic, carcinogenic, carcinostatic, and teratogenic effects have been demonstrated (12), could represent unsuspected environmental and toxicological hazards. There has been interest recently in the potential therapeutic application of *N*-ni-

trosourea compounds as antitumor agents (35). The possible presence of *N*-nitrosamines in tobacco smoke, tobacco, and numerous foodstuffs has been considered (5), and their formation has been demonstrated (39) by reaction of secondary amines and nitrites in the stomach under suitable conditions. *p*-Methyl-nitrosoaminobenzaldehyde, an *N*-nitrosamine, has been identified (18) as a metabolic product of *Clitocybe suavolens*. *N*-nitrosodimethylamine has found industrial application for the manufacture of 1,1-dimethylhydrazine.

In this report, the results of testing 29 *N*-nitroso derivatives and 20 of their denitrosated, hydroxylamino, and hydrazino derivatives for prophage-inducing capability are presented. The minimal inducing concentrations for the effective inducing agents and a list of the inactive compounds are reported. Positive findings in this test system are considered in relation to published results obtained in experimental systems demonstrating mutagenic, chromosome-damaging, carcinogenic, carcinostatic, and teratogenic effects.

MATERIALS AND METHODS

Minimal concentrations of test agent required to induce bacteriophage in streptomycin-dependent *Escherichia coli* W1709 (λ) were determined by use of the technique described in detail by Price et al. (33). Test sample activity is reported in terms of the ratio of the number of plaque-forming λ phage in the test sample (*T*) to that in a control (*C*). All phage present in the control sample are produced spontaneously. An analysis of test results indicates that the minimal concentration of compound having a *T/C* value of 3.0 (three times the spontaneous phage count), or greater, could be considered with some assurance ($P < \lambda$ 0.05) to be effectively inducing the lytic cycle in *E. coli* W1709 (λ) cells.

To minimize changes in the test agent resulting from bacterial metabolism, induction was carried out in the absence of streptomycin, resulting in a transitory starving of the cells during the 1.5-hr induction period at 37 C. Similarly, to minimize chemical reactions, induction was carried out in a synthetic medium at *p*H 7.2.

Preparation of test materials. Compounds were dissolved in deionized water or, when necessary, in solvents just before use in the test to minimize activity losses. The highest final concentrations of solvents that could be employed without effect in the induction test were as follows: ethyl alcohol, 8%; acetone, 8%; and dimethylacetamide, 2%. Agents were tested at final concentrations of as high as 2 mg/ml when they proved to be sufficiently soluble and without toxicity for the lysogenic culture, *E. coli* W1709 (λ).

N-(*n*-butyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-24639) was provided by W. A. Skinner, Stanford Research Institute; *N*-(2-chloroethyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-25959), 2-nitramino-1-nitroso-2-imidazoline (NSC-25958), 2-nitramino-2-imidazoline (NSC-25961), *N*-ethyl-*N*-nitrosourea (NSC-45403), *N*-(2-chloroethyl)-*N*-nitrosourea (NSC-47547), *N,N'-bis*(2-chloroethyl)-*N*-nitrosourea (NSC-409962), 1-nitroso-2-imidazolidone-2 (NSC-73438), and *N*-methyl-*N*-nitrosourethane (NSC-2860) were provided by the Cancer Chemotherapy National Service Center, Washington, D.C.; *N,N'*-dimethyl-*N*-nitrosourea (NSC-48893) and *N*-methyl-*N'*-(2-chloroethyl)-*N*-nitrosourea (NSC-409935) were provided by T. P. Johnston, Southern Research Institute; *N*-methyl-*N'*-nitroguanidine was provided by T. Kawachi, National Cancer Center Research Institute, Tokyo; *N*-nitrosopiperazine, *N*-nitrosomorpholine, *N*-nitrosohexamethyleneimine, and *N*-nitrosomethylcyclohexylamine were provide by W. Lijinsky, University of Nebraska. The remaining test compounds were purchased from commercial sources and were of highest purity available.

RESULTS

The relative capabilities of the 29 *N*-nitroso compounds tested to induce lambda bacteriophage formation in a lysogenic strain, *E. coli* W1709, are shown in Table 1. Inducing activity was observed at minimal concentrations of 0.1 to 2 μg/ml with all eight of the nitrosoamidines tested, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-(2-chloroethyl)-*N'*-nitro-*N*-nitrosoguanidine, *N*-propyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-(*n*-butyl)-*N'*-nitro-*N*-nitrosoguanidine, *N*-(*n*-amyl)-*N'*-nitro-*N*-nitrosoguanidine, *N*-(*iso*-amyl)-*N'*-nitro-*N*-nitrosoguanidine, and 2-nitramino-1-nitroso-2-imidazoline.

Minimal inducing concentrations of 0.5 to 25 μg/ml were obtained with six nitrosamides: *N*-methyl-*N*-nitrosourea, *N*-ethyl-*N*-nitrosourea, *N*-(2-chloroethyl)-*N*-nitrosourea, 1-nitroso-2-imidazolidone-2, *N*-methyl-*N*-nitrosourethane, and *N*-ethyl-*N*-nitrosourethane. It is of interest that three acyl-substituted *N*-nitrosoureas, *N,N'*-methyl-*N*-nitrosourea, *N,N'-bis*(2-chloroethyl)-*N*-nitrosourea, and *N*-methyl-*N'*-(2-chloroethyl)-*N*-nitrosourea, failed to induce prophage. The stability of nitrosoureas is increased considerably (20) by substitution of one or both of the hydrogens on the NH_2 moiety bound to the acyl group. The mutagenic effectiveness of *N*-methylnitrosourea for *Arabidopsis thaliana* was significantly decreased (8) by increasing the number of methyl radicals bound to the NH_2 group.

The 10 *N*-nitrosamines were all found to be inactive, as was the closely related methylazoxymethanol, when tested at their highest nontoxic concentrations up to 2,000 μg/ml.

The relative capabilities of 13 denitrosated analogues and 2 hydroxylamino and 5 hydra-

TABLE 1. *Effectiveness of some N-nitroso compounds as inducers of Escherichia coli W1709 (γ)*

Agent	Minimal inducing concn (μg/ml)	No induction at highest concn tested (μg/ml)
N-nitrosamidines		
N-alkyl-*N'*-nitro-*N*-nitrosoguanidines		
N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NSC-9369)[a]	0.4	
N-ethyl-*N'*-nitro-*N*-nitrosoguanidine (NSC-38191)	0.1	
N-(2-chloroethyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-25959)	2.0	
N-propyl-*N'*-nitro-*N*-nitrosoguanidine (NSC-33674)	0.2	
N-(*n*-butyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-24639)	0.8	
N-(*n*-amyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-34699)	1.5	
N-(*iso*-amyl)-*N'*-nitro-*N*-nitrosoguanidine	1.5	
Imidazolines		
2-Nitramino-1-nitroso-2-imidazoline (NSC-25958)	2.0	
N-nitrosamides		
N-alkyl-*N*-nitrosoureas		
N-methyl-*N*-nitrosourea (NSC-23909)	25.0	
N-ethyl-*N*-nitrosourea (NSC-45403)	10.0	
N-(2-chloroethyl)-*N*-nitrosourea (NSC-47547)	2.0	
N,N'-dialkyl-*N*-nitrosoureas		
N,N'-dimethyl-*N*-nitrosourea (NSC-48893)		200
N,N-bis(2-chloroethyl)-*N*-nitrosourea (NSC-409962)		3.1
N-methyl-*N'*-(2-chloroethyl)-*N*-nitrosourea (NSC-409935)		5.0
Imidazolidones		
N-nitroso-2-imidazolidone (NSC-73438)	20.0	
N-alkyl-*N*-nitrosourethanes		
N-methyl-*N*-nitrosourethane (NSC-2860)	1.0	
N-ethyl-*N*-nitrosourethane (NSC-24890)	0.5	
Miscellaneous		
N-methyl-*N*-nitroso-*p*-toluenesulfonamide		500
N-nitrosamines		
N-nitrosodialkylamines		
N-nitrosodimethylamine		2,000
N-nitrosodiethylamine		2,000
N-nitrosodiarylamines		
N-nitrosodicyclohexylamine		500
N-nitrosoalkylarylamines		
N-nitrosomethylphenylamine		200
N-nitrosomethylcyclohexylamine		250
Cyclic *N*-nitrosamines		
N-nitrosohexamethylenimine		125
N-nitrosopiperidine		1,000
N-nitrosopiperazine		500
N-nitrosomorpholine		2,000
N,N'-dinitrosopiperazine		1,000
Related compounds		
Methylazoxymethanol		100

[a] NSC accession numbers used in this paper were assigned by the Cancer Chemotherapy National Service Center.

zino derivatives of *N*-nitroso compounds to induce prophage in *E. coli* W1709 (λ) are shown in Table 2. All 13 denitrosated analogues were found to be ineffective as inducers when tested at their highest nontoxic concentration. Seven of these analogues were derived from prophage-inducing nitrosamidines and nitrosamides; six of the analogues were from nitrosamines ineffective as prophage inducers. Two of the seven hydroxylamino and hydrazino compounds, derived from nitrosamines ineffective as prophage inducers, were weakly active. *N,N*-diethylhydroxylamine oxalate, derived from *N*-nitrosodiethylamine, induced prophage at 200 μg/ml and *N*-methyl-*N*-phenylhydrazine sulfate, derived from *N*-nitrosomethyl-phenylamine, induced at 250 μg/ml. Four cyclic hydroxylamino and hydrazino de-

TABLE 2. *Inducing capability of denitrosated, hydroxylamino, and hydrazino derivatives of some N-nitroso compounds*

Agent	Minimal inducing concn (μg/ml)	No induction at highest concn tested (μg/ml)
Denitrosated derivatives		
N-methyl-*N'*-nitroguanidine		1,000
2-Nitramino-2-imidazoline		1,000
N-methyl urea		1,000
N-ethyl urea		1,000
2-Imidazolidone		1,000
N-methyl urethane		1,000
N-ethyl urethane		1,000
Dimethylamine hydrochloride		1,000
Diethylamine		1,000
N-methylphenylamine		500
Piperidine		200
Piperazine		1,000
Morpholine		1,000
Hydroxylamino derivatives		
N-hydroxypiperidine		1,000
N,N-diethylhydroxylamine oxalate	200	
Hydrazino derivatives		
N-methyl-*N*-phenylhydrazine sulfate	250	
N-aminopiperidine		200
N-aminomorpholine		2,000
N,N'-diaminopiperazine dihydrochloride		1,000
N,N-dimethyhydrazine		1,000

rivatives and *N,N*-dimethylhydrazine were ineffective inducers.

It is conceivable in those cases in which inducing activity was observed only at 100 μg/ml or greater that induction could be the result of small amounts of undetermined impurities present in the test compound or of conversions produced during preparation of the test solution or during the induction period.

DISCUSSION

Biological activities of interest both from the view of environmental toxicology and from that of human pathology have been observed in experimental systems with a number of the prophage-inducing nitrosamidines and nitrosamides. Mutagenic, chromosome-damaging, carcinogenic, carcinostatic, and teratogenic characteristics of the prophage-inducing agents are assigned in Table 3 on the basis of data obtained thus far in a thorough, but nonexhaustive, search of the literature. No consideration was given to evaluating the reliability of the particular test system employed by investigators to determine these effects.

Mutagenic, chromosome-damaging, carcinogenic, and carcinostatic effects have been demonstrated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Five of the eight nitrosamidines effective as inducers reportedly have carcinostatic activity in mice bearing leukemia L-1210. Sufficient test data regarding their other potential biological effects are not yet available to establish an association between these effects and their prophage-inducing capability.

Of the six prophage-inducing nitrosamides tested thus far, five have produced mutagenic and carcinogenic effects, four have produced chromosome-damaging effects, and two have produced carcinostatic and teratogenic effects.

The finding that the three *N,N'*-dialkyl-*N*-nitrosoureas tested, NSC-48893, 409962, and 409935, were not effective as inducers is of interest. NSC-409935 and 409962 have demonstrated carcinostatic effects (35) in rodent tumors, whereas carcinostatic (35), carcinogenic (3), and mutagenic (7) activities have been assigned to NSC-48893. The deacylated derivatives, NSC-23909 and 47547, are effective prophage inducers. Carcinostatic effects have been observed (Table 3) with both of these deacylated compounds, whereas mutagenic, chromosome-damaging, carcinogenic, and teratogenic effects have been assigned to NSC-23909. These observations suggest that metabolic deacylation may be a preliminary requirement for producing the biological effects of the *N,N'*-dialkyl-substituted *N*-nitrosoureas.

It has been widely suggested, but not yet proven, that the biological effects produced by *N*-nitroso compounds depend upon their chemical or biological conversion, or both, to an active intermediate(s) capable of reacting with critical intracellular targets. It obviously becomes important to determine the chemical nature of such biologically active intermediate(s), if they do indeed exist. Prophage-induction produces an alteration of the bacterial nucleic acid balance in which deoxyribonucleic acid (DNA) synthesis is selectively blocked (15). The observation that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a prophage-inducing agent, inhibits not only the synthesis of DNA but also that of ribonucleic acid and protein (42) suggests the possibility that more than one reaction product may be responsible for the biological effects produced by this agent.

Although it has been widely postulated that intermediates of *N*-nitroso compounds capable of alkylating nucleic acid and protein cell constituents may be responsible for the biological effects observed, no direct chemical evidence

TABLE 3. *Mutagenic, chromosome-damaging, carcinogenic, carcinostatic, and teratogenic properties of N-nitroso compounds detected as inducers of Escherichia coli W1709 (γ)*

Agent	Property studied[a]				
	Mutagenic	Chromosome damaging	Carcinogenic	Carcinostatic	Teratogenic
N-nitrosamidines					
N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NSC-9369)	+ (25)	+ (6)	+ (36)	+ (40)	
N-ethyl-*N'*-nitro-*N*-nitrosoguanidine (NSC-38191)	+ (7)		− (36)	+ (40)	
N-(2-chloroethyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-25959)				+ (40)	
N-propyl-*N'*-nitro-*N*-nitrosoguanidine (NSC-33674)	− (7)			+ (40)	
N-(*n*-butyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-24639)	− (7)			− (40)	
N-(*n*-amyl)-*N'*-nitro-*N*-nitrosoguanidine (NSC-34699)				− (40)	
N-(*iso*-amyl)-*N'*-nitro-*N*-nitrosoguanidine					
2-Nitramino-1-nitroso-2-imidazoline (NSC-25958)				+ (40)	
N-nitrosamides					
N-methyl-*N*-nitrosourea (NSC-23909)	+ (8)	+ (9)	+ (3)	+ (35)	+ (43)
N-ethyl-*N*-nitrosourea (NSC-45403)	+ (7)		+ (3)	− (35)	+ (2)
N-(2-chloroethyl)-*N*-nitrosourea (NSC-47547)				+ (35)	
1-Nitroso-2-imidazolidone-2 (NSC-73438)	+ (26)	+ (9)	+ (3)		
N-methyl-*N*-nitrosourethane (NSC-2860)	+ (26)	+ (10)	+ (3)		
N-ethyl-*N*-nitrosourethane (NSC-24890)	+ (11)	+ (10)	+ (3)		

[a] Symbols: +, active in one or more systems; −, not active in systems tested thus far. Numbers in parentheses are references.

of the production of diazoalkanes under physiological conditions has been presented. Although chemically the imidazoline and the imidazolidone are closely related cyclic analogues of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-ethyl-*N*-nitrosourea, respectively, neither cyclic compound should be convertible to a diazoalkane.

Evidence of the nature of the prophage-inducing agents produced by responsive *N*-nitroso compounds is lacking. Recent studies indicate that the breakdown products of nitrosamides and nitrosamidines may be dependent upon the medium in which the compounds are maintained. Complex mixtures of reaction products may appear under physiological conditions (31, 37, 38), probably as a result of competing or linked reactions. These reaction products may induce significant biological consequences of their own. It has been suggested (19, 23, 37) that activation of the nitrosamidines and nitrosamides may be accomplished through loss of their nitroso group. The nitroso group may be rapidly converted into nitrous acid with the subsequent formation of hydroxylamines and hydrazines which are highly active biologically.

Of the seven denitrosated nitrosamidines and nitrosamides tested (Table 2), *N*-methyl-*N*-nitroguanidine, 2-nitramino-2-imidazoline, *N*-methylurea, *N*-ethylurea, 2-imidazolidone, *N*-methylurethane, and *N*-ethylurethane were incapable of prophage induction, although all of their parent *N*-nitroso derivatives were effective inducers, demonstrating the necessity of the nitroso group for the biological response. Denitrosated derivatives, generally, appear to be biologically inert (11, 27, 40).

Evidence (30) that *N*-hydroxylation may be a relatively general reaction by which carcinogenic, aromatic amines and amides are converted to proximal, carcinogenic metabolites in vivo, has focused attention on the hydroxylamino moiety as a potential carcinogen. In a previous communication (13), hydroxylamine and hydrazine and a number of their analogues for which mutagenicity, carcinogenicity, and teratogenicity data are available were found capable of prophage induction in lysogenic *E. coli* W1709 (λ).

Many of the 11 *N*-nitrosamines tested have exhibited (24) potent mutagenic, chromosome-damaging, carcinogenic, and teratogenic effects, but none was capable of inducing prophage. Their failure to induce could result from inability of the nonmetabolizing lysogenic culture, *E. coli* W1709 (λ), to produce "activating" enzymes capable of converting nitrosamines to derivatives which initiate the biological response. Of the six denitrosated nitrosamines tested (Table 2), dimethylamine hydrochloride, diethylamine, *N*-methylphenylamine, piperidine, piperazine, and morpholine were incapable of prophage induction. *N*-hydroxypiperidine and the four hydrazino derivatives, *N*-aminopiperidine, *N*-aminomorpho-

line, *N*,*N*'-diaminopiperazine dihydrochloride, and *N*,*N*-dimethylhydrazine, were also ineffective prophage inducers. Weak prophage-inducing activities were observed with *N*,*N*-diethylhydroxylamine oxalate, derived from *N*-diethylnitrosamine, and with *N*-methyl-*N*-phenylhydrazine, derived from *N*-nitrosomethylphenylamine. These findings suggest that biological activation of *N*-nitrosamines may be accomplished by formation of simple hydroxylamine and hydrazine derivatives which thus far have received only limited consideration as possible metabolic intermediates. Formation of hydroxylamino (32) and hydrazino (41) derivatives from *N*-nitrosamines incubated with tissue homogenates has been reported.

The prophage induction system has already shown utility (21) when employed to select potential carcinostatic agents present in complex fermentation broths and as an assay procedure for following the extraction and isolation of active agents from such broths. Evidence supporting a relationship between an agent's prophage-inducing capability and its mutagenic and carcinogenic activities has been acquired (4) through study of a series of 16 nitroquinolines and hydroxyaminoquinolines. A bioautographic technique that has proven useful for identification of purified prophage-inducing agents has also been developed (16). It is hoped that the simple in vitro prophage induction system may prove useful for detecting responsive *N*-nitroso compounds in the environment and for assessing the presence and importance of levels of biologically active intermediates of such compounds that have been formed in complicated mixtures of products under physiological conditions.

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