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Phage and Bacterial Inactivation and Prophage Induction by Chemical Carcinogens

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SUMMARY

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Some mammalian carcinogens and their metabolites affect the viability of *Salmonella typhimurium* strains, as indicated by a decrease in colony formation, and also induce prophage. We determined the minimum concentration required for prophage induction and the maximum prophage induction frequency for each carcinogen. The latter value was determined by the ratio of the number of induced phage particles relative to that of spontaneously induced phage particles in the controls. This value is constant for each carcinogen, regardless of its concentration. Since damage of the bacterial genome results in prophage induction, the reactivity of each compound with the genome may be indicated by the minimum concentration required for prophage induction and the maximum frequency of prophage induction. Carcinogens unable to affect bacterial viability are also unable to induce prophage. Failure to induce prophage indicates a requirement for metabolic activation by mammalian enzymes. Interaction of these carcinogens with free phage particles *in vitro* was used as an index of direct interaction of carcinogen with DNA. Among 16 compounds tested, six had a direct effect on the phage genome, resulting in loss of phage viability. Five of these six compounds are hydroxylated compounds, and the other is *N*-acetoxy-2-acetylaminofluorene. From these observations it may be concluded that these six compounds are reactive with genomes without further metabolism.

INTRODUCTION

Chemical carcinogens interact with cellular macromolecules in animals to cause a permanent cellular change, namely, abnormal growth. In many instances abnormal growth results in tumor formation. Because of the permanent change many investigators have suggested that a chemical reaction with DNA is the basis of chemical-induced carcinogenesis. This argument is strengthened by the demonstration that many carcinogens or their activated intermediates are mutagenic for microorganisms. It has been well established that mutagenesis is based upon reactions involving alteration

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of cellular DNA: modification, nicking, degradation, etc. Recently Ames *et al.* (1) demonstrated that the alterations of the DNA by metabolites and derivatives of some carcinogens produces frameshift mutations in *Salmonella typhimurium*. Alteration of cellular DNA can also be demonstrated by prophage induction, and damage and repair of bacterial genomes, using bacterial repair mutants.

Exposure of *Escherichia coli* lysogenic for phage λ to ultraviolet light, X-ray radiation, β-propiolactone, *tert*-butyl peroxide, and 4-nitroquinoline 1-oxide results in prophage induction (2–4). Yamamoto *et al.* (5) reported that 4-nitroquinoline 1-oxide and β-propiolactone reduced the bacterial viability of *S. typhimurium* and that bacterial mutants extremely sensitive to NQO[1] in terms of inactivation of colony-forming ability were isolated and also found to be highly sensitive to both β-propiolactone and ultraviolet light. These bacterial mutants were characterized and subdivided into two groups: bacterial excision-deficient mutants (*hcr*$^-$), lacking repair activity for ultraviolet-damaged bacterial and phage genomes, and recombination deficient mutants (*recA*) (5). Furthermore, NQO and β-propiolactone induced prophage in *hcr*$^-$ mutants more efficiently than in the wild type lysogen (5). These observations provide evidence that the above carcinogens damage the genomes and that the chemically damaged genomes can be restored by the bacterial repair mechanism.

4-Hydroxyaminoquinoline 1-oxide, a reduced metabolite of NQO, damages both bacterial and bacteriophage genomes; NQO, however, damages only the bacterial genome (5). Although both NQO and HAQO produce tumors at their injection sites, HAQO appears to be the active metabolite, since it reacts directly with DNA (6) and bacteriophage genomes (5).

In this communication we report that various metabolites of known mammalian carcinogens cause loss of phage and bacterial viabilities and induce prophage. We also discuss the reactivity of these metabolites with genes.

[1] The abbreviations used are: NQO, 4-nitroquinoline 1-oxide; HAQO, 4-hydroxyaminoquinoline 1-oxide; AAF, 2-acetylaminofluorene; DMSO, dimethyl sulfoxide; HAN, α-hydroxyaminonaphthalene; HU, *N*-hydroxyurethane.

MATERIALS AND METHODS

Bacteriophage. Salmonella phages P22c^+ and P221c^+ were used (7). P22c^+ and P221c^+ are temperate phages which form turbid plaques on *S. typhimurium* strains LT-2 and Q1, as a result of establishment of lysogenic cells.

Bacteria. S. typhimurium Q1 and its repair-deficient mutants lysogenic for P22c^+ and for P221c^+ were used for prophage induction and inactivation kinetic studies of bacterial colony-forming ability. Indicator strains for prophage induction were a tetracycline-resistant *S. typhimurium* Q1 for assay of phage P22 and a streptomycin-resistant mutant of a P22-resistant mutant of *S. typhimurium* LT-2 (*ST/22 Sm*^{r}) for assay of phage P221.

Agents. Hydroxyurethane and alkylnitroso compounds were supplied by the Cancer Chemotherapy National Service Center; α- and β-hydroxyaminonaphthalene, by W. Troll, and also synthesized in our laboratory; and AAF derivatives, by J. Weisburger. Dimethyl sulfoxide was purchased from Fisher Scientific Company and was used for solubilizing carcinogens.

Media. Nutrient broth consisting of 8 g of Difco nutrient broth and 5 g of NaCl per liter of distilled water was used for making phage lysates and bacterial aeration cultures. For phage plating, an agar base containing 23 g of Difco nutrient agar and 5 g of NaCl per liter, and soft nutrient overlay agar containing 7.5 g of Difco Bacto-agar, 5 g of NaCl, and 8 g of Difco nutrient broth per liter, were used. Phosphate-buffered NaCl contained M/15 phosphate in 0.1 M NaCl at pH 7.0.

Inactivation kinetics of bacterial colony-forming ability. S. typhimurium strain Q1 and its lysogenic strain for P221c^+ (10^7 cells/ml) were treated with a chemical carcinogen in nutrient broth at 37° or in buffered NaCl at 25°. Water-insoluble aromatic carcinogens were solubilized in DMSO and tested in a final DMSO concentration of 5% (v/v). Samples were withdrawn at various intervals, diluted, and plated on nutrient agar.

After overnight incubation at 37°, bacterial viability was expressed as the ratio of the number of colonies after exposure to carcinogens relative to the untreated control.

Prophage induction. Logarithmic phase P22c^+ lysogenic cells were harvested by centrifugation, resuspended in buffered NaCl to 10^8 cells/ml, and further diluted 1:10 in buffered NaCl. Then 1.9-ml aliquots were mixed with 0.1 ml of water or DMSO containing increments of the test agent. Aromatic carcinogens were adequately solubilized in a final DMSO concentration of 5% (v/v), which was nontoxic to the bacteria. Phage induction occurred during a 90-min incubation of the cultures in a 37° shaking water bath. After 90 volumes of nutrient broth were added to the induction mixture to dilute the inducing agent, the cultures were incubated for 60 min to permit lysis of the induced cells, then briefly chilled in an ice bath to stop bacterial growth, and diluted in NaCl. Induced phage particles were assayed by plaque formation in 2 ml of soft nutrient overlay agar containing 0.1 ml of diluted sample, 150 μg of tetracycline, and 0.1 ml of an overnight nutrient broth culture of the tetracycline-resistant indicator, *S. typhimurium Q1 TC*. P22 plaques appeared in the poured plates after overnight incubation at 37° because of lysis of the indicator strain. Tetracycline prevents further growth of lysogenic cells and phage release from uninduced lysogens, but does not inhibit plaque formation on the tetracycline-resistant indicator strain.

The kinetics of prophage induction was studied by determining the number of prophage-induced cells. Logarithmic phase P221c^+ lysogenic cells (10^7–10^8 cells/ml) were treated with carcinogenic chemicals in nutrient broth or in buffered NaCl for various time intervals and diluted 100–1000-fold in fresh nutrient broth to dilute extracellular carcinogens. After 30 min of incubation at 37°, samples were plated on *St/22 Sm^r* with agar containing dihydrostreptomycin (200 μg/ml). Streptomycin kills lysogenic cells, but does not inhibit phage production of cells previously induced (4, 5 8). The 30-min incubation of lysogenic cells after treatment with agents is not long enough to lyse and release P221 phage. Therefore only cells that are induced by the agents give P221 plaques (i.e., infectious centers) on *St/22 Sm^r*.

Treatment of phage P22 with carcinogens. All manipulations were carried out at room temperature (25°). One-tenth volume of a chemical solution in phosphate-buffered NaCl (pH 7.0) with DMSO was added to the P22 phage suspension (about 10^7 plaque-forming units/ml), and periodically 0.1-ml samples were withdrawn and diluted 1:100 or further in phosphate-buffered NaCl to stop the reaction of residual agent. Immediately after dilution 0.25-ml diluted samples were added to 0.1 ml of the nutrient broth culture of indicator strain *Q1* and incubated for 10 min at 37° in a water bath. Agents contained in these diluted samples have no influence on either phage or bacterial cells for plaque-forming ability. Then 2 ml of soft agar were added, and the entire mixture was poured on nutrient agar plates. After overnight incubation at 37°, phage viability was expressed as the ratio of the number of plaques after exposure to carcinogens relative to the untreated control.

RESULTS

Loss of bacterial viability by various carcinogens. Inactivation of *S. typhimurium* strain *Q1* by various carcinogens in terms of loss of bacterial viability was studied as described above. Each carcinogen was tested using several concentrations. Among the 16 carcinogens tested, 12 compounds inactivated the bacterial colony-forming ability (Table 1). Typical inactivation kinetics of *Q1* by *N*-methyl-*N*-nitrosourethane at concentrations ranging from 0.5 to 10 mM is shown in Fig. 1. The inactivation rate depends on concentration. At a concentration of 10 mM about a 1000-fold reduction in colony-forming ability was observed by 20 min incubation. At a concentration of 0.5 mM the bacterial survivals decreased to 2-fold after a 10-min lag and reached a plateau at 40 min. Similar inactivation patterns were observed with a few different concentrations of other carcinogens which inactivate bacteria (Table 1). Urethane, β-naphthylamine, 2-acetylaminofluorene, and *N*-hydroxyacetylaminofluorene did not affect bacterial viability.

TABLE 1

Phage and bacterial inactivation and prophage induction by carcinogens

Compound	Bacterial inactivation	Prophage induction[a]	Phage inactivation
Urethane	−	−	−
N-Hydroxyurethane	+	+	+
N-Methyl-*N*-nitrosourea	+	+	−
N-Ethyl-*N*-nitrosourea	+	+	−
N-Methyl-*N*-nitrosourethane	+	+	−
N-Ethyl-*N*-nitrosourethane	+	+	−
β-Naphthylamine	−	−	−
β-Hydroxyaminonaphthalene	+	+	+
α-Hydroxyaminonaphthalene	+	+	+
2-Acetylaminofluorene	−	−	−
N-Hydroxy-AAF	−	−	−
N-Acetoxy-2-AAF	+	+	±
N-Hydroxy-2-aminofluorene	+	+	+
2-Nitrosofluorene	+	+	−
4-Nitroquinoline 1-oxide	+	+	−
4-Hydroxyaminoquinoline 1-oxide	+	+	+

[a] Results with P22 lysogens are always true with P221 lysogens.

+ indicates inactivation of bacterial colony-forming ability (more than 10-fold), prophage induction (at least 5-fold), or inactivation of phage plaque-forming ability (more than 10-fold). − indicates no effect. ± indicates small effect about 4-fold).

If a chemical modifies the bacterial DNA, the bacterial repair functions (e.g., excision, DNA polymerase I, recombination function) restructure the modified bacterial genomes in a stepwise fashion. Therefore interaction of carcinogens with genes can be demonstrated by increased inactivation rates of the bacterial repair-deficient mutants to carcinogens (5, 7). When various repair-deficient mutants were treated with α-hydroxyaminonaphthalene inactivation was rapid for excision-deficient (*hcr*⁻) mutants (Fig. 2) and recombination-deficient (*recA*) mutants (8), moderate for DNA polymerase

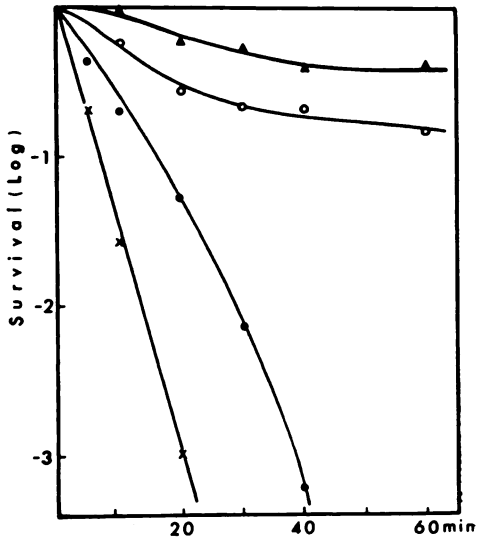

FIG. 1. *Inactivation kinetics of colony-forming ability of S. typhimurium Q1 by various concentrations of N-methyl-N-nitrosourethane*

△, 0.5 mM; ○, 1.0 mM; ●, 3.3 mM; ×, 10 mM.

I mutants (8), and slow for the wild type strain. The implication is that HAN interacts with the bacterial genomes and modifies DNA. Similar results have been found with β-hydroxyaminonaphthalene (9), *N*-acetoxy-2-AAF (10), *N*-hydroxy-2-aminofluorene (10), 2-nitrosofluorene (10), NQO (5), and HAQO (11). However, in our separate study it was found that the *pol*⁻ mutant is far more sensitive than the excision-deficient mutant and wild type strain to some chemicals (9). Different inactivation patterns of various repair mutants for chemicals are dependent on types of DNA damage.

Prophage induction. As reported previously (5, 8), prophage induction increases as the bacterial inactivation proceeds. Therefore prophage induction in the excision-deficient mutant is far more efficient than in the wild type strain (5). An example of prophage induction by HAN at a concentration of 0.1 mM is shown in Fig. 2. Prophage induction in the excision-deficient mutant increased sharply as bacterial inactivation began and reached a maximum value, whereas prophage induction in wild type lysogen increased gradually. Prophage induction in the wild type lysogen has also been reported to reach a maximum induction

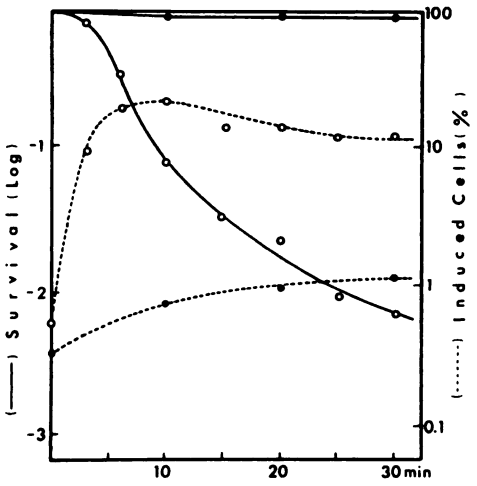

FIG. 2. *Inactivation of colony-forming ability and prophage induction of P221 lysogens of S. typhimurium Q1 and its excision-deficient mutant by 0.1 mM α-Hydroxyaminonaphthalene*

●, wild type lysogen *Q1(P221)*; ○, a P221 lysogen of excision-deficient mutant *QM2(P221)*.

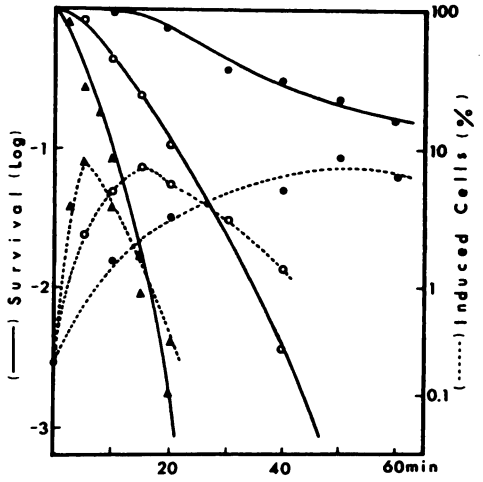

FIG. 3. *Inactivation and prophage induction of S. typhimurium Q1 lysogenic for P221bc+ by various concentrations of N-methyl-N-nitrosourethane*

●, 1 mM; ○, 3.3 mM; △, 10 mM.

frequency (5, 11). Although prophage induction in the excision-deficient mutants is far more efficient than in the wild type strain, the maximum frequencies of prophage induction in lysogens of both the repair-deficient mutants and the wild type are about the same (5, 11). Furthermore, although many carcinogens act more efficiently on excision-deficient mutants than the wild type strain, some do not. Therefore the relative prophage-inducing abilities of carcinogens were studied with lysogens of the wild type strain. Maximal induction frequency could be obtained with a wide concentration range of chemical (Fig. 3). Maximal induction frequencies obtained with three different concentrations of *N*-methyl-*N*-nitrosourethane are about the same. Therefore, although the bacterial inactivation rate increases with carcinogen concentration, the maximal induction frequency should be a constant value for each carcinogen regardless of concentration. Results with carcinogens and their suspected active metabolites are given in Table 2. All the carcinogens which inactivated bacterial colony-forming ability also induced prophage (Table 1). Table 2 also includes the minimum concentration of each carcinogen required for prophage induction, in addition to the maximum induction frequency. The minimum concentration required for prophage induction may indicate the reactivity of the carcinogen with genes. The lower the minimum concentration, the higher is the reactivity of the carcinogen with genes. However, this value can be influenced by stability of the carcinogen. The maximum prophage induction frequency may be a function of (a) reactivity of the carcinogen with genes and (b) its toxic effect on the phage production mechanism in cells.

Chemicals unable to inactivate the bacterial cells are also unable to induce prophage (Table 1). Failure to induce prophage may indicate a requirement for metabolic activation. Hydroxyurethane, a metabolite of urethane (12), induces prophage, although urethane itself is inactive. The *N*-alkyl-*N*-nitrosourethanes and *N*-alkyl-*N*-nitrosoureas, which are carcinogenic in the mouse, rat, and hamster (13–15), are phage inducers. A bladder carcinogen, β-naphthylamine (16, 17), is not prophage-inducing. A metabolite of β-naphthylamine, β-hydroxyaminonaphthalene, induces, as does its isomer, HAN. No phage induction was obtained with *N*-hydroxy-AAF, but *N*-acetoxy-2-AAF, *N*-hydroxy-2-aminofluorene,

TABLE 2

Prophage induction from S. typhimurium Q1 lysogenic for P22 by carcinogens

Compound	Minimum concentration[a]	Ratio: Highest No. of plaques treated/control[b]
	μM	
Urethane	—[c]	
N-Hydroxyurethane[d]	100	20
N-Methyl-*N*-nitrosourea	25	19
N-Ethyl-*N*-nitrosourea	25	40
N-Methyl-*N*-nitrosourethane	5	8
N-Ethyl-*N*-nitrosourethane	1	27
β-Naphthylamine	—	
β-Hydroxyaminonaphthalene[d]	5	ND[e]
α-Hydroxyaminonaphthalene[d]	0.5	50
2-Acetylaminofluorene	—	
N-Hydroxy-2-AAF	—	
N-Acetoxy-2-AAF	ND	95
N-Hydroxy-2-aminofluorene[d]	1.0	23
2-Nitrosofluorene[d]	0.5	40
4-Nitroquinoline 1-oxide	ND	70
4-Hydroxyaminoquinoline 1-oxide[d]	ND	100

[a] Minimum concentration required for at least a 5-fold plaque increase over control.

[b] This ratio gave practically the same values as the ratio of the highest number of induced cells treated to control, because both induced cells and spontaneously induced cells produce about an equal number of phage particles per cell. These ratios are defined as maximum prophage induction frequency.

[c] Dash indicates that the compound failed to induce prophage.

[d] Suspected proximate metabolite.

[e] ND, not determined.

and 2-nitrosofluorene are potent inducing agents (10).

Inactivation of free phage particles. Since those carcinogens which affect bacterial viability were also able to induce the prophage, it was desirable to examine interaction of these carcinogens *in vitro* with free phage particles as a test for direct interaction of carcinogens with DNA. Among the carcinogens tested, six inactivated phage P22 by direct contact of the free phage particles with the carcinogens in buffered NaCl (Table 1). Our separate studies showed that those six carcinogens also greatly increased the frequency of phage recombination about 50-fold, (5, 11).[2] This suggests that these carcinogens interact with the phage genome. One of the six compounds, *N*-acetoxy-AAF, inactivates P22 to a small extent, although it inactivates phage T5 at a faster rate (10). It is known that *N*-acetoxy-AAF binds with DNA and forms a fluorene-DNA complex (18). The other five are hydroxylated compounds. Sugimura *et al.* (6) demonstrated that HAQO produces single-strand scissions in DNA molecules on incubation of DNA *in vitro*. Moreover, our recent studies show that the other four hydroxylated compounds produce single-strand scissions in DNA molecules.[2]

Results of inactivation kinetics of P22 phage by *N*-hydroxyurethane are shown in Fig. 4. Since HU is a hydroxylated form, it may release hydrogen peroxide (19) or be converted to a free radical (20) in the presence of oxygen. To test this possibility, P22 phage was exposed to HU in M/15 phosphate buffer with or without cupric ion, because cupric ion has a catalytic effect on the activity of hydrogen peroxide (21, 22) and the formation of free radicals.[2] Phage inactivation was greatly enhanced by addition of cupric ion (Fig. 4), but the specific active form interacting with the phage genome is unknown.

DISCUSSION

The ability of some known water-soluble carcinogens to induce coliphage has been reported (2). Of the water-soluble and insoluble compounds tested by us, only the suspected proximate carcinogens reduce bacterial colony formation of *S. typhimurium*, induce prophage, and inactivate the plaque-forming ability of *Salmonella* phage. Parental carcinogen activity is dependent on enzymatic or nonenzymatic conversion into proximate carcinogens in

[2] N. Yamamoto, Y. Tagashira, and N. Ushijima, unpublished observations.

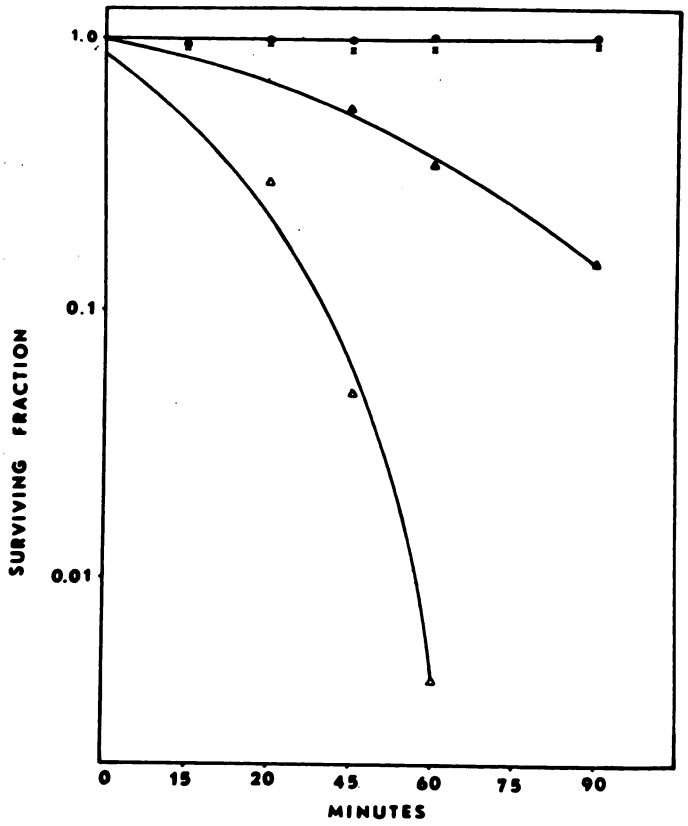

FIG. 4. *Catalytic effect of cupric ion on inactivation of phage P22 by hydroxyurethane*
P22 was treated in phosphate-buffered NaCl (pH 7.0) at 37° and assayed on *S. typhimurium* Q1. ○, control; ×, 10 μM $CuSO_4$; ▲, 10 mM HU; △, 10 mM HU + 10 μM $CuSO_4$.

animals. Bacteria also contain activating enzymes for converting the parent carcinogens to active compounds that interact with genomes (11). Bacterial enzymes reduce a potent carcinogen and prophage inducer, 4-nitroquinoline 1-oxide, to its suspected proximate carcinogen, 4-hydroxyaminoquinoline 1-oxide (5, 11). HAQO inactivates both the plaque-forming ability of the phage and the colony-forming ability of *S. typhimurium* by contact *in vitro* (5, 11). The *N*-alkyl-*N*-nitrosourethanes and *N*-alkyl-*N*-nitrosoureas, which are carcinogenic in the mouse, rat, and hamster (12), are phage inducers. It has been suggested that tumor induction by the alkylnitrosourethanes and alkylnitrosoureas may be due to their spontaneous decomposition, with the generation of the corresponding carbonium ions, which are alkylating agents (23–25). However, these alkylnitrosocompounds do not inactivate the plaque-forming ability of the phage although they destroy bacterial colony-forming ability. Therefore these chemicals must be converted to active metabolites by bacterial enzymes before they can interact with the bacterial genome.

The liver carcinogen AAF is metabolized by susceptible animal species to *N*-hydroxy-AAF, which is deacetylated to *N*-hydroxy-2-aminofluorene and in turn oxidized to 2-nitrosofluorene (26, 27). No phage induction is obtained with AAF or *N*-hydroxy-AAF. *N*-Acetoxy-2-AAF, *N*-hydroxy-2-aminofluorene, and 2-nitrosofluorene were potent prophage-inducing agents.

Many prophage-inducing agents, regardless of form, are known to alter the structure of bacterial DNA (3). Relative reactivities of various carcinogens with the bacterial

genome can be expressed by the following two values: the minimum concentration required for prophage induction and the maximum prophage induction frequency. These values may be influenced by the permeability, stability, and toxicity of the chemicals.

Since bacteria also contain some activating enzymes for carcinogens (11, 28), it became desirable to examine the direct interaction between carcinogens and genes by incubation of the carcinogens with free phage particles *in vitro*. Six of 12 prophage inducers interact with phage genomes without further activation processes. The other six compounds appear to require activation processes in the bacterial cells before interacting with the bacterial genomes. Hydroxyurethane, a metabolite of urethane, inactivates phage, but urethane itself is inactive. The known bladder tumorigenicity of β-naphthylamine suggests its conversion *in vivo* to a carcinogenic urinary metabolite. Of the two naphthylamine metabolites studied, both α-hydroxyaminonaphthalene and β-hydroxyaminonaphthalene inactivated the phage. The production of cancer by both hydroxylamine isomers at the injection site in animals suggests that they are proximate carcinogens (17).

The value of investigation into bacteriophage induction and inactivation to cancer research lies in its capacity to identify proximate carcinogens and to simplify studies of their mode of action on genomes. It is also important to know the mechanism by which the latent viral genome that is integrated in the host chromosomes is activated by chemicals which are local carcinogens in higher species, including man.

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