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Effects of the Carcinogen 2-Acetylaminofluorene and Its Derivatives on Bacteria and Bacteriophages

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SUMMARY

The interaction of various carcinogenic fluorene derivatives with genomes was studied by examining the viability of bacteria and phage and the induction of prophage. Two strains of *Salmonella typhimurium*, wild type (*hcr*$^+$) and an ultraviolet-sensitive mutant (*hcr*$^-$) lysogenic for phage P221, were used for cell viability and prophage induction studies. Free T5 phage particles were employed for phage viability studies; T5 phage was assayed on *Escherichia coli* K-12.

Incubation with 1 mM 2-nitro-, 2-amino-, 2-diacetylamino-, 2-acetylamino-, or *N*-hydroxy-2-acetylaminofluorene or its glucuronide (sodium salt) for 1 hr caused neither changes in bacterial viability nor prophage induction.

N-Hydroxy-2-aminofluorene was very effective in induction of prophage and causing loss of bacterial cell viability. Loss of viability of *hcr*$^-$ cells was more rapid than that of wild type (*hcr*$^+$) cells with this compound when tested at 0.1 and 0.2 mM levels. This compound at 0.1 mM also caused rapid prophage induction in *hcr*$^-$ cells compared to wild type cells. These results suggest that *N*-hydroxy-2-aminofluorene damages the bacterial genome, which is restored by the bacterial repair mechanism. Similarly, nitrosofluorene (0.5 mM) and *N*-acetoxy-2-acetylaminofluorene (1 mM) also caused prophage induction and loss of bacterial cell viability. However, these compounds were not as effective as *N*-hydroxy-2-aminofluorene. In the presence of either rat liver cytosol fraction or guinea pig liver microsomes, *N*-hydroxy-2-acetylaminofluorene effectively induced the prophage and caused loss of *hcr*$^-$ cell viability.

Among the compounds tested at 1 mM levels, only *N*-hydroxy-2-aminofluorene and *N*-acetoxy-2-acetylaminofluorene caused significant loss of viability of free T5 phage particles, the latter being twice as effective as the former.

These data demonstrate a correlation between the carcinogenicity of fluorene compounds and their effects on bacteria and bacteriophages. Such systems might serve as useful tools in testing for active and ultimate carcinogens.

INTRODUCTION

It has been demonstrated that *N*-hydroxylation is the first step of activation not only of 2-acetylaminofluorene but also of several other carcinogenic aromatic amines and amides (1). Esterification of *N*-hydroxy-2-acetylaminofluorene has recently been shown to be the second activation step in the liver carcinogenesis in the rat (1, 2).

Many carcinogens bind to macromolecules, such as proteins, RNA, and DNA, in susceptible tissues (1). In view of the in-

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volvement of DNA binding to carcinogenic chemicals, it has been suggested that mutation may be one of the mechanisms involved in the carcinogenic process (see ref. 1). Several approaches have been taken to examine this problem. Maher *et al.* (3, 4) have reported the mutation and loss of transforming activity of isolated bacterial DNA after reaction with the synthetic sulfate and acetate esters of *N*-hydroxy-2-acetylaminofluorene and other aromatic hydroxamic acids. Similarly, mutations have been studied with several compounds using T4 phage systems (5).

In the present paper we have employed prophage induction and loss of viability of of bacteria and phage to study the interaction of various carcinogenic fluorene derivatives with genomes. The results demonstrate a correlation between the carcinogenicity of fluorene compounds and their effect on bacteria and bacteriophages. Some of these studies were reported in preliminary form (6)

MATERIALS AND METHODS

Chemicals. 2-Nitrofluorene and dimethyl sulfoxide were purchased from Fisher Scientific Company. 2-Diacetylaminofluorene and AF[1] were purchased from K & K Laboratories. AAF was obtained from Mann Research Laboratories. *N*-Hydroxy-AAF (7), *N*-acetoxy-AAF (8) *N*-hydroxy-AF (9), and 2-nitrosofluorene (9) were prepared in this laboratory by procedures described in the references. The sodium salt of the glucuronide of *N*-hydroxy-AAF was generously supplied by Dr. C. C. Irving of the Veterans Administration Hospital, Memphis, Tenn.

Bacteria and bacteriophages. Two strains of *Salmonella typhimurium*—LT-2 [abbreviated as *St*, wild type; host cell reactivation plus (*hcr*$^+$), which is able to reactivate ultraviolet-damaged phage] and the ultraviolet sensitive mutant *hcr*$^-$ (lysogenic for phage P221b)—were used for cell viability and prophage induction studies. Free T5 phage was employed for viability studies. T5 phage was assayed on *Escherichia coli* K-12.

Media. Nutrient broth containing 8 g of Difco nutrient broth and 5 g of NaCl per liter of distilled water was used for the preparation of bacterial aeration cultures and phage lysates.

For phage plating, hard agar, containing 23 g of Difco nutrient agar and 5 g of NaCl per liter, and overlay soft nutrient agar, containing 7.5 g of Difco Bacto-Agar, 5 g of NaCl, and 8 g of Difco nutrient broth per liter, were used.

Studies on loss of viability with bacteria. S. typhimurium (10^7 cells/ml) was suspended in nutrient broth containing 0.14 M NaCl at pH 6.5. To it a fluorene derivative dissolved in dimethyl sulfoxide was added to a final concentration of dimethyl sulfoxide of 10%. This concentration of dimethyl sulfoxide did not affect either the viability of the bacteria and phage or prophage induction. After incubation at 37°, aliquots were withdrawn at various time intervals, diluted 100–10,000-fold with 67 mM phosphate buffer at pH 7.0 containing 0.14 M NaCl, and assayed for loss of viability by determining the colony-forming ability of the bacteria.

Prophage induction of P221b. Lysogenic cells (about 10^7 cells/ml in the logarithmic phase), suspended in nutrient broth containing 0.14 M NaCl at pH 6.5, were treated with fluorene derivatives dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the incubation mixture was 10%. After incubation at 37°, samples were withdrawn at various time intervals, diluted 100–10,000-fold with nutrient broth at pH 6.5, incubated for an additional 30 min at 37°, and plated on agar containing 0.1 ml of a full-grown culture of a streptomycin-resistant mutant of *St* (*St/sm*) and dihydrostreptomycin. The concentrations of dihydrostreptomycin were 200 μg/ml for *hcr*$^+$-(*P221b*) and 25 μg/ml for *hcr*$^-$(*P221b*) cells. Streptomycin kills lysogenic cells but has no inhibitory effect on phage production by previously induced cells (10, 11). Therefore only cells already induced by a fluorene derivative give P221b infectious centers on *St/Sm*.

Treatment of free T5 phage with fluorene derivatives. For studies with free phage, about 10^7 T5 phage particles per milliliter were suspended in 0.18 M Tris buffer, pH 7.5. These particles were then treated with a fluorene derivative dissolved in dimethyl

[1] The abbreviations used are: AF, 2-aminofluorene; AAF, 2-acetylaminofluorene.

sulfoxide. The final concentration of dimethyl sulfoxide in the incubation mixture was 10%. After incubation at 25°, samples were withdrawn at various intervals and diluted, and viable phage particles were assayed on two strains of *E. coli* K-12 (wild type AB1157 and its excision minus mutant, AB1886) (12).

RESULTS

Induction of prophage and loss of viability of S. typhimurium. When several fluorene derivatives were tested for their effect on bacterial viability and prophage induction, it was found that incubation of compounds such as nitrofluorene, AF, AAF, 2-diacetylaminofluorene, *N*-hydroxy-AAF, or its glucuronide at 1 mM levels for 1 hr produced no toxicity to either *hcr*⁻ mutants or wild type cells. These compounds caused neither change in bacterial viability nor prophage induction.

In contrast to *N*-hydroxy-AAF, its deacetylated derivative, *N*-hydroxy-AF, was very effective in induction of prophage and diminishing bacterial cell viability. A typical experiment on bacterial survival in response to *N*-hydroxy-AF is shown in Fig. 1. Loss of viability of *hcr*⁻ cells was more rapid than that of wild type (*hcr*⁺) cells. *N*-Hydroxy-AF at 0.2 mM produced loss of survival of both strains of bacterial cells more rapidly than at 0.1 mM. This compound also caused rapid prophage induction in *hcr*⁻ cells compared to wild type cells (Fig. 2). In three separate experiments the time required to attain maximum prophage induction was always about twice as much for *hcr*⁺ cells as for *hcr*⁻ cells. These results are also in agreement with the data shown in Fig. 1 in terms of time required for the loss of bacterial viability.

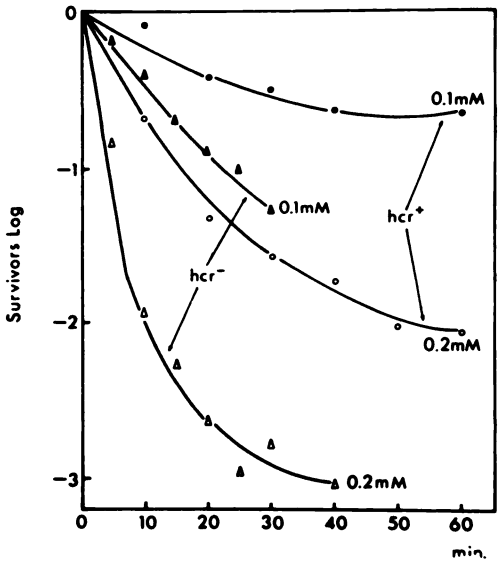

FIG. 1. *Survival of S. typhimurium in the presence of N-hydroxy-AF*

The assay methods are described in MATERIALS AND METHODS. Two concentrations (0.1 and 0.2 mM) of *N*-hydroxy-AF were used in these studies.

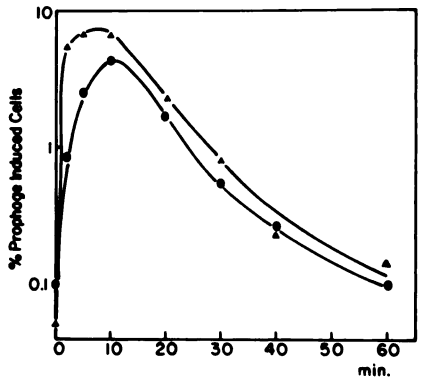

FIG. 2. *Prophage P22lb induction by N-hydroxy-AF (0.1 mM)*

▲——▲, *hcr*⁻ cells; ●——●, *hcr*⁺ cells.

Like *N*-hydroxy-AF, nitrosofluorene also caused prophage induction and loss of bacterial survival (Fig. 3). However, it was not as effective as *N*-hydroxy-AF in producing these changes.

Similarly, *N*-acetoxy-AAF produced more rapid loss of viability of *hcr*⁻ mutants compared to wild type (*hcr*⁺) cells (Fig. 4). This fluorene derivative also produced more rapid prophage induction in *hcr*⁻ cells than in *hcr*⁺ cells.

In some experiments incubation of 1 mM *N*-hydroxy-AAF for 1 hr at 37° in the presence of 25 mM Tris buffer, pH 7.5, with guinea pig liver microsomes equivalent to 1 mg of wet liver caused about 95% loss of bacterial cell (*hcr*⁻) viability. However, either liver microsomes or *N*-hydroxy-AAF alone caused less than 5% loss of bacterial cell survival. In similar studies incubation of

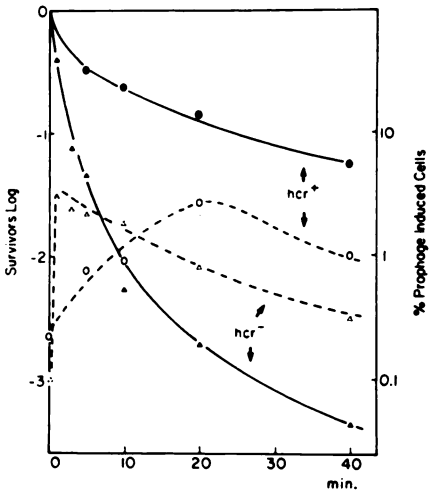

FIG. 3. *Survival of hcr⁺ and hcr⁻ strains of S. typhimurium and induction of prophage P221b by nitrosofluorene (0.5 mM)*

——, bacterial survival; - - -, prophage induction.

1 mM *N*-hydroxy-AAF for 1 hr at 37° in the presence of 25 mM Tris buffer, pH 7.5, with rat liver cytosol equivalent to 50 mg of wet liver caused 97% loss of *hcr⁻* cell survival. Under identical conditions liver cytosol alone did not affect bacterial cell viability. Rat liver cytosol in the presence of *N*-hydroxy-AAF caused 6.9% of the total cells to induce prophage; this induction was about 20 times more than in samples which contained either *N*-hydroxy-AAF or liver cytosol alone.

Recently it has been demonstrated that the *O*-glucuronide of *N*-hydroxy-AF can decrease the transforming activity of bacterial DNA (13). However, unlike *N*-hydroxy-AAF, the glucuronide of *N*-hydroxy-AAF had no effect on bacterial cell (*hcr⁻*) viability after incubation at 1 mM concentration for 1 hr with 25 mM Tris buffer at pH 7.5 and guinea pig liver microsomes equivalent to 1 mg of wet liver.

Loss of phage viability. Since some of the fluorene derivatives tested caused loss of bacterial cell viability and produced prophage induction, it was important to investigate their effects on a free phage system. P22 phage would have been an ideal model for these investigations, since Yamamoto *et al.* (14) established that damage of the P22 genome produces a greatly increased frequency of recombination. However, it was decided to test these fluorene compounds on T5 phage, which is known to be extremely permeable to larger molecules (12).

Incubation of 2-nitrofluorene, 2-nitrosofluorene, AF, AAF, 2-diacetylaminofluorene, and *N*-hydroxy-AAF or its glucuronide at 1 mM concentration for 1 hr at 25° did not affect the survival of free T5 phage particles. *N*-Hydroxy-AAF and its glucuronide had no appreciable effect on phage survival even after 96 hr. Incubation of *N*-hydroxy-AF and *N*-acetoxy-AAF at 1 mM levels for 1 hr, however, caused 52% and 88% losses in

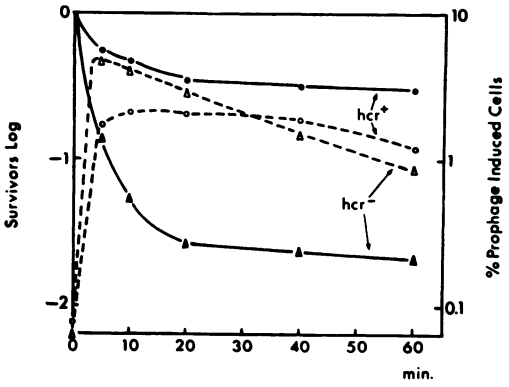

FIG. 4. *Effect of N-acetoxy-AAF (1 mM) on viability and prophage induction of P221b lysogens.*

The assay methods are described in MATERIALS AND METHODS. ——, bacterial survival; - - -, prophage induction.

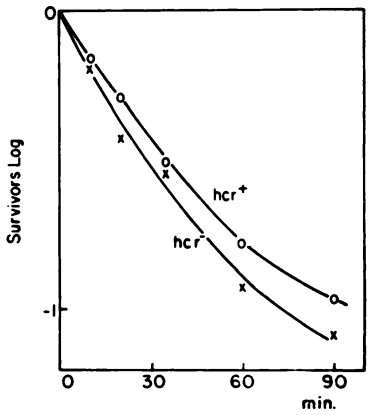

FIG. 5. *Survival of free T5 phage in the presence of N-acetoxy-AAF (1 mM)*

Viable phage particles were assayed on *hcr⁺* and *hcr⁻* strains of *E. coli* as described in the text.

survival of T5 phage particles, respectively. Typical survival data for free T5 phage in the presence of 1 mM *N*-acetoxy-AAF are shown in Fig. 5. There is a small difference in the loss of viability of T5 phage when assayed on *hcr*$^-$ and *hcr*$^+$ *E. coli* cells. This difference may not be as significant as the difference observed between the repair capabilities of *Salmonella* phage P22 damaged by β-propiolactone and other chemicals and those assayed on two strains of *S. typhimurium* (14–16). However, recent studies from our laboratory indicate that, unlike the differences in the reparability of damaged phage P22 by two strains of *S. typhimurium*, the host cell repair mechanism of *E. coli* does not restore T5 phage genome damaged by ultraviolet irradiation and chemicals.[2]

DISCUSSION

It has been shown that carcinogen 4-nitroquinoline 1-oxide is effective in producing loss of viability of *S. typhimurium* cells (14). This has been demonstrated to be due to reduction of the compound by a bacterial enzyme (15). In the present studies 2-nitrofluorene was ineffective in producing loss of bacterial cell viability. This might have been due to inability of the bacterial system to reduce the nitro compound to form effective concentrations of *N*-hydroxy-AF.

The present data indicate that bacteria and bacteriophages may be used as model systems for testing active carcinogens. Thus *N*-acetoxy-AAF, *N*-hydroxy-AF, and nitrosofluorene, which produce sarcomas at the site of subcutaneous injections in male rats (1), also produced loss of viability of bacterial cells and induced prophage production in the present study. In contrast to nitrosofluorene, *N*-hydroxy-AF and *N*-acetoxy-AAF also caused loss of survival of free T5 phage particles. *N*-Acetoxy-AAF is more effective than *N*-hydroxy-AF in producing sarcomas at the site of injection (1). The carcinogenicity data are in agreement with our findings on loss of viability of T5 phage obtained with these two chemicals; however, in some of the present studies *N*-hydroxy-AF was more effective than *N*-acetoxy-AAF in producing loss of bacterial survival. These differences may be due to differences in stability and permeability between the two compounds. Since *N*-hydroxy-AF is active whereas nitrosofluorene is inactive in producing loss of viability of free phage, it is most likely that nitrosofluorene is not an ultimate metabolite. Perhaps its activity requires reduction to *N*-hydroxy-AF.

It has been demonstrated that *N*-hydroxy-AAF does not react nonenzymatically with proteins and nucleic acids *in vitro* at physiological pH (see ref. 1), even though it is carcinogenic in the rat at the site of injection (1). It was therefore suggested that *N*-hydroxy-AAF needs to be metabolized further (1). The present results are in agreement with this hypothesis. Thus, in our studies on loss of viability with bacteria and phage, *N*-hydroxy-AAF was ineffective. In the presence of liver microsomes or cytosol, however, it produced loss of viability of bacterial cells. Deacetylation of *N*-hydroxy-AAF to *N*-hydroxy-AF occurs in the presence of guinea pig liver microsomes (17) and rat liver cytosol (18). Therefore it appears that in the presence of liver microsomes or cytosol the deacetylated product of *N*-hydroxy-AAF was responsible for causing loss of survival of bacteria. It is possible that a similar phenomenon may also occur at the injection site during carcinogenesis by *N*-hydroxy-AAF.

N-Hydroxy-AF was found to be ineffective in diminishing the transforming activity of isolated bacterial DNA (4) and inactivating T4 phage (5). In contrast to these results (4, 5), we found *N*-hydroxy-AF to be very effective in bacterial and phage inactivation. Our data with *N*-acetoxy-AAF are in agreement with those obtained with T4 phage (5) and isolated bacterial DNA (4).

It has been shown previously that *hcr*$^+$ and *hcr*$^-$ cells of *S. typhimurium* lose their viability not only on ultraviolet irradiation (14) but also after treatment with the carcinogens 4-nitroquinoline 1-oxide and its reduced derivative (14, 15) and β-propiolactone (16). It was suggested that the rapid decrease in viability caused by these agents was most probably due to bacterial genome damage which was not reparable in the excision minus mutant (*hcr*$^-$), and hence

[2] N. Yamamoto and S. Fukuda, unpublished observations.

these cells were more sensitive to loss of viability than wild type (*hcr*$^+$) cells. However, such genome damage was easily reparable in the wild type cells by a bacterial repair mechanism (14–16). In analogy with the previous results obtained with ultraviolet irradiation (14) and chemicals (14–16) on prophage induction and loss of bacterial cell viability, both *N*-acetoxy-AAF and *N*-hydroxy-AF may have produced similar changes in the present studies by damage to the bacterial genome. Likewise, it is tempting to speculate that, like the effects of ultraviolet irradiation, β-propiolactone, and 4-nitroquinoline 1-oxide on P22 phage (14, 16), fluorene derivatives may produce loss of viability of T5 phage by damage to the phage genome.

In any event, our present data have demonstrated a correlation between the carcinogenicity of fluorene compounds and their effects on bacteria and bacteriophages. Such systems might serve as useful tools in testing for active and ultimate carcinogens.

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