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Carcinogens as Frameshift Mutagens: Metabolites and Derivatives of 2-Acetylaminofluorene and Other Aromatic Amine Carcinogens

(aromatic nitroso carcinogens/carcinogen detection with *Salmonella*/DNA intercalation)

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ABSTRACT **Several carcinogenic metabolites of the carcinogen 2-acetyl-aminofluorene, especially 2-nitroso-fluorene and *N*-hydroxy-2-aminofluorene, are potent frameshift mutagens for *Salmonella typhimurium*. 2-Nitrosonaphthalene, 2-nitrosophenanthrene, 4-nitroso-*trans*-stilbene, 4-nitrosobiphenyl, and 4-nitrosoazobenzene, all of which are metabolites or likely metabolites of carcinogenic aromatic amines, are also potent frameshift mutagens. These compounds may be frameshift mutagens of the class that intercalates into DNA and then reacts covalently with the DNA; various ultimate carcinogens may be of this type. The utility of a set of bacterial strains for detecting carcinogens as mutagens is shown.**

Various planar polycyclic aromatic compounds, such as the acridines, which can intercalate in the DNA base-pair stack (1–9), are mutagens that cause additions or deletions of nucleotides (10–12). These errors appear to result from stabilization of a shifted pairing in a repetitive sequence in DNA by intercalation of the planar compound and subsequent addition or deletion of base pairs during replication or repair synthesis (11, 13). Mutagens with this property are called frameshift mutagens because of the shift in the reading frame of the mRNA synthesized from the altered DNA template; thus they differ from mutagens that cause base-pair substitutions (11). The potency of an intercalating agent as a frameshift mutagen may increase by 10- to 100-fold if it also contains a side chain that can react covalently with DNA (12, 14–17).

Since it seemed possible that the reactive forms of some carcinogens with planar ring systems could be frameshift mutagens that both intercalate and react with DNA, epoxides of polycyclic hydrocarbons were examined and found to be mutagens of this type (16). In this paper this question is examined further with certain known and potential metabolites of 2-acetylaminofluorene (18). The striking activity of 2-nitrosofluorene as a frameshift mutagen then led to tests of nitroso derivatives related to various other aromatic amine carcinogens. These studies have made use of a set of tester strains (14–16) of *Salmonella typhimurium* that were developed for detection and classification of mutagens.

MATERIALS AND METHODS

Bacterial Test for Mutagens. The mutagenicity test uses four *Salmonella* strains that require histidine (14–16). The test measures the effect of a mutagen on the reversion to growth on a histidine-free medium of these tester strains. Three of the tester strains (TA1531, TA1532, and TA1534) were designed for detecting frameshift mutagens with different specificities; each strain has a frameshift mutation in one of the genes of the histidine operon. The fourth strain (TA1530) has a base-pair change in the histidine *G* gene, and is used to detect mutagens that cause base-pair substitutions. In addition, all four strains lack the excision repair system (because of a deletion through *uvrB*); this makes them much more sensitive to any mutagen that alters the DNA in a way that would normally be repaired by this system (14, 15).

We constructed the tester strain, TA1534, by introducing a *uvrB* mutation (14) into the strain with the frameshift mutation *hisD3052* that was described by Oeschger and Hartman (19). They induced *hisD3052* with the frameshift mutagen ICR-364-OH (12) and showed that it was reverted by ICR-364-OH and ICR-191 (another frameshift mutagen, ref. 12) as well as by hycanthone and 4-nitroquinoline-1-oxide (21); it was not revertible by 2-aminopurine, an agent that causes only base-pair transitions (20).

Chemicals. 2-Aminofluorene (22), *N*-hydroxy-2-acetylaminofluorene (23), *N*-acetoxy-2-acetylaminofluorene (24), *N*-hydroxy-2-aminofluorene (23), and 2-nitrosofluorene (25) were prepared by the methods cited in the references. 2-Acetylaminofluorene (Schwarz–Mann) and 2,7-*bis*-acetylaminofluorene (Schuchardt) were crystallized from ethanol; 2-nitrofluorene (Aldrich) was purified by chromatography on aluminum oxide and crystallization from dichloromethane-hexane. 4-Nitroazobenzene (26) was purified by thick-layer chromatography on silica gel (Merck PF_{254}). 1- and 3-Hydroxy-2-acetylaminofluorene and 5- and 7-hydroxy-2-acetylaminofluorene were generously provided by T. Lloyd Fletcher (University of Washington, Seattle, Wash.) and John H. Weisburger (National Cancer Institute, Bethesda, Md.), respectively. 4-Nitrosobiphenyl, 1-nitrosonaphthalene, 2-nitrosonaphthalene, 4-nitroso-*trans*-stilbene, 2-nitrosophenanthrene, and 4-nitrosoazobenzene were prepared by oxidation of the corresponding hydroxylamines in chloroform with a molar equivalent of diethyl-azodicarboxylate (27, 28). The first green band (or the first red band for 4-nitrosoazobenzene) that emerged on chromatography on aluminum oxide with dichloromethane-hexane (1:1) was collected, and the product was crystallized from cyclohexane or dichloromethane-isopentane (1-nitrosonaphthalene and 4-nitrosoazobenzene). Each of these compounds was homogeneous on thin-layer chromatography and showed in its mass spectrum the appropriate parent ion (m^+) and the characteristic fragment ion ($m^+ - 30$) for loss of the nitroso group. 4-Nitrosoazobenzene showed the parent ion (m^+) and fragments ($m^+ - 105$, $m^+ - 106$) due to cleavage of the azo bond. The preparation contained traces of 4-nitroazobenzene as evidenced by thin-layer chromatography and mass spectroscopy. 2-Nitrosophenanthrene (mp 84–85°) and 4-nitroso-*trans*-stilbene

TABLE 1. *Mutagenicity of various fluorene derivatives and nitroso compounds for S. typhimurium strains*

Compound added	μg per plate	His+ revertants per plate				
		TA1530	TA1531	TA1532	TA1534	*hisD3052*
(control)	0	71;64	4	30	23;27;24	43;40
Fluorene derivatives						
2-amino-	100	41	1	19	22;27	44
2-acetylamino-	100	36	6	29	13;26	33
1-hydroxy-2-acetylamino-	100	56	2	15	29;19	45
3-hydroxy-2-acetylamino-	100	81	4	35	26;21	47
5-hydroxy-2-acetylamino-	100	73	9	26	29;34	35
7-hydroxy-2-acetylamino-	100	*	6	32	24;34	39
2,7-*bis*-acetylamino-	100	*	0	13	703	50
N-hydroxy-2-acetylamino-	100	77	6	20	74	44
N-acetoxy-2-acetylamino-	100	49	8	31	84;67	48
2-nitro-	50	71	0	27	284	38
2-nitroso-	5	54	4	24	10,000	114;160
N-hydroxy-2-amino-	5	72	6	30	858	45;63
4-Nitrosobiphenyl	100	*			153	
	200		3	99	282	35
4-Nitroso-*trans*-stilbene	50	*	2	*	200	28
2-Nitrosonaphthalene	50				97	
	100	*	3	27	270	19
2-Nitrosophenanthrene	5				2108	
	200	*	2	123		97
1-Nitrosonaphthalene	200	*	*	16	32	34
ICR-191	5	94	823	104	36	

Revertant colonies (which do not require histidine) per petri plate. Each number is the result of a count of a separate plate. The underlined numbers are judged to be significantly different from the controls. The procedure used has been described (14). The compounds were added to the molten (45°) top agar: stock solutions (1 mg/ml) were in dimethylsulfoxide (Schwarz–Mann, spectrophotometric grade) except for nitrosofluorene, which was more stable in ethanol. All solutions were sterile. The plates were incubated for 2 days at 37°.

* = negative in qualitative spot tests (about 0.5 mg).

(mp 112–114°) are new compounds. ICR-191 (2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridine dihydrochloride) (12, 17) was generously provided by H. J. Creech (Institute for Cancer Research, Philadelphia, Pa.).

RESULTS

In initial qualitative experiments in which crystals of each of the 12 fluorene derivatives were added in the center of agar plates containing one of the four tester strains, revertants were seen only with TA1534. These qualitative results were confirmed by quantitative studies (Table 1). None of the fluorene derivatives reverted the base substitution-tester strain TA1530 or the two frameshift-tester strains TA1531 and TA1532, but some of the compounds were extraordinarily active in reverting the frameshift-tester strain TA1534. Thus, as has been shown before (14–16), and is apparent from the results with ICR-191 (Table 1), each of the three tester strains for frameshift mutagens is quite specific in its response to various frameshift mutagens.

Fig. 1 shows the proportionality between the amounts of certain of the fluorene metabolites and derivatives and the mutagenic response of strain TA1534. The most active metabolite, 2-nitrosofluorene, has about 20,000 times the activity of the parent compound 2-aminofluorene. The potency of the fluorene derivatives as frameshift mutagens is in the order 2-nitroso > *N*-hydroxy-2-amino ≫ *N*-hydroxy-2-acetylamino > 2-amino. *N*-Acetoxy-2-acetylaminofluorene was also mutagenic and showed similar activity to *N*-hydroxy-2-acetylaminofluorene at a concentration of 100 μg per plate (Table 1). However, *N*-acetoxy-2-acetylaminofluorene caused so much bacterial death that it could not be tested at higher concentrations. The quantitative measurement of the activity of the mutagens presumably reflects their inherent mutagenic activities, but it is possible that the observed activity also is a function of the stabilities of the compounds during the test (especially of the nitroso, *N*-hydroxyamino, and acetoxy derivatives). It is also conceivable that some of these compounds are metabolized to mutagenic or nonmutagenic derivatives, or to both, by the bacteria.

We have recently found that with strain TA1538, a deep rough derivative of strain TA1534, only about one-twelfth as much of the fluorene mutagens is required for the same mutagenic response. Bacteria of this strain lack the normal lipopolysaccharide that coats the surface of bacteria of strain TA1534 and acts as a partial barrier to the passage of compounds to the cell membrane. The construction and advantages of the set of deep rough derivatives of the tester strains will be described separately (ref. 16; Ames and Lee, manuscript in preparation).

There is a general correlation between mutagenic and carcinogenic activity in this series. Thus, 2-nitrosofluorene, *N*-hydroxy-2-aminofluorene, *N*-acetoxy-2-acetylaminofluor-

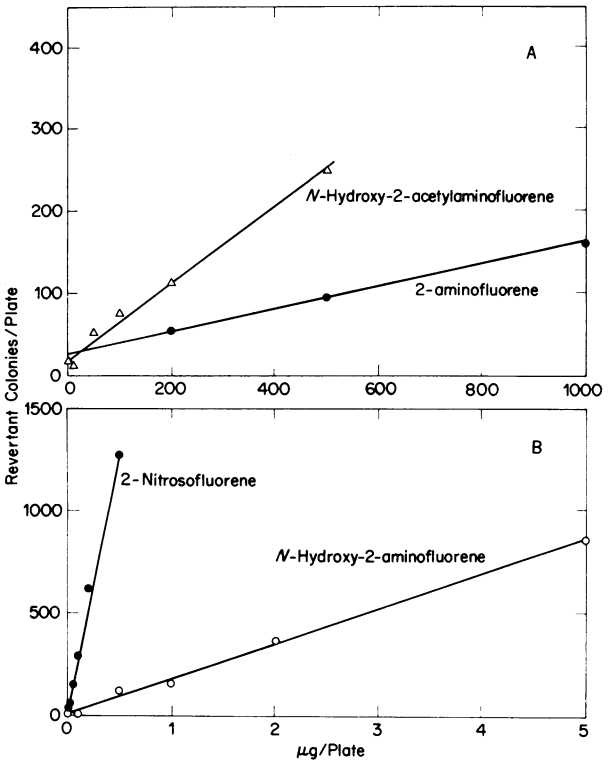

FIG. 1. The number of revertant colonies of strain TA1534 per petri plate as a function of the dose of mutagen.

ene, and *N*-hydroxy-2-acetylaminofluorene are all carcinogenic at the subcutaneous injection site (29) in rats. In contrast, the parent compounds 2-aminofluorene and 2-acetylaminofluorene are not carcinogenic at sites of application but are potent carcinogens in several tissues when administered orally to various rodents, apparently because of their subsequent metabolism (29). The various ring-hydroxy derivatives listed in the table are also known metabolites of 2-acetylaminofluorene in rats, but none of these has shown carcinogenic activity (30–32): these compounds were not mutagenic in any of these tests (Table 1). Two carcinogenic fluorene derivatives, that are not known to be metabolic products, 2-nitrofluorene and 2,7-*bis*-acetylaminofluorene, are also active mutagens (Table 1).

The high mutagenic potency of 2-nitrosofluorene prompted us to study various nitroso derivatives related to other aromatic amine carcinogens. As shown in Table 1, 4-nitrosobiphenyl, 4-nitroso-*trans*-stilbene, 2-nitrosonaphthalene, and 2-nitrosophenanthrene are all quite potent frameshift mutagens for strain TA1534. 4-Nitrosoazobenzene was found to be a weak, but definite, mutagen for strain TA1538, the more sensitive, deep rough derivative of strain TA1534 (about 80 colonies per 100 μg of compound in a dose–response curve as in Fig. 1). 2-Nitrosonaphthalene and *N*-hydroxy-2-aminonaphthalene are known metabolites of the carcinogen 2-aminonaphthalene and are more carcinogenic than the parent compound when injected into newborn mice (33). The carcinogenicity of the other nitroso derivatives has not been studied, but because they are probably derivatives or precursors of carcinogenic aromatic amines and hydroxylamines (34–36), we would expect them to show carcinogenic activity. 1-Nitrosonaphthalene did not show significant mutagenic activity under our test conditions and with our strains; it has been reported to be carcinogenic (33).

We have obtained indirect evidence that the more potent frameshift mutagens are reacting covalently with the bacterial DNA, as well as intercalating, by comparing their mutagenic effect on strains with (*hisD3052*) and without (TA1534 = *hisD3052 uvrB*) excision repair. Intercalating mutagens that can form a covalent bond with DNA, such as the quinacrine-half mustard mutagen ICR 191, are much more active in reverting frameshift mutations in strains without the excision repair system than in strains able to repair damaged DNA (14–16). By contrast, the absence of this repair system has no effect on the potency of simple intercalating mutagens such as quinacrine or 9-aminoacridine or hycanthone (14, 15, 21). This difference in mutation frequency between the $uvrB^+$ and $uvrB^-$ strains (Table 1) is about 100-fold with 2-nitrosofluorene as the mutagen; large differences are also observed with the other active nitroso compounds, *N*-hydroxy-2-aminofluorene, and 2-nitrofluorene. As some frameshift mutations having an added GC pair in a string of GC pairs can revert by frameshift suppression, which might be caused by base-pair substitutions (37), we have analyzed 18 2-nitrosofluorene-induced revertants of strain TA1534: none of the revertants was due to suppressors.

DISCUSSION

Our data presented here and the recent data of Ames, Sims, and Grover (16) on polycyclic hydrocarbon epoxides show that various chemical carcinogens are frameshift mutagens. We show that this is true for certain metabolites of the carcinogen 2-aminofluorene (especially the carcinogens 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene) and nitroso derivatives of five other aromatic amine carcinogens. Their structures and their activities as frameshift mutagens suggest that all of these compounds are members of a particular potent class of frameshift mutagens. Compounds of this class appear not only to intercalate in the DNA base-pair stack, but also to react covalently with the DNA, thus becoming one or more orders of magnitude more potent than simple intercalating frameshift mutagens (12, 14–17). The two characteristics of the reactive frameshift mutagen, the intercalating ring system and the electrophilic side chain, are discussed separately below.

It appears that a frameshift mutation occurs during DNA replication or repair when a mutagen stabilizes a shifted pairing in DNA. A base pair is about 50 Å^2 in area (7), and a planar aromatic ring system, such as an acridine, is about the right size to intercalate between two base pairs, causing a stabilization of the shifted pairing. Various ring systems, many of which are present in known carcinogens, are also present in certain frameshift mutagens, and appear to be of the appropriate aromaticity, planarity, and size for intercalation. The present study identifies derivatives of fluorene, naphthalene, phenanthrene, biphenyl, *trans*-stilbene, and azobenzene as members of this class. Previous work with the set of *S. typhimurium* tester strains has already identified as frameshift mutagens derivatives of acridine (12, 14, 15, 17), azaacridine (12, 14, 15, 17), benzacridine (12, 14, 15, 17), azabenzacridine (12, 14, 15, 17), quinoline (17) (4-nitroquinoline-*N*-oxide, 15, 21), benzanthracene (16), dibenzanthracene (16), carbazole (15), diphenylamine (15), anthracene (Ames, unpublished), and 10-thiaxanthenone (21). The work of Albert (7) is of interest in this context as he investigated various ring systems in an attempt to maximize the anti-

bacterial activity of 9-aminoacridine, which he postulates as due to intercalation in DNA. Some of the above mentioned ring systems and various others intercalate in DNA (9).‡

We propose that certain electrophilic groups that can be produced on these rings by metabolism or can be added synthetically can convert a simple intercalating agent to one that also can react with DNA, thus increasing mutagenic potency by orders of magnitude. The nitroso group and the hydroxylamino group were the most active in our study. These metabolites of 2-aminofluorene and 2-aminonaphthalene are produced by certain mammalian oxidizing systems and are both more carcinogenic than the parent amines under certain conditions (29, 33). One of the criteria we use for suggesting that these compounds react covalently with DNA, as well as intercalate, is the much greater mutagenic activity on the tester strain lacking the excision repair system for DNA. Several other lines of evidence suggest that some of the compounds we find active are reacting with DNA. Slater *et al.* (39) have recently shown that 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene are more inhibitory against a strain of *Escherichia coli* lacking DNA polymerase I, a component of excision repair. Reaction with DNA *in vitro* at neutrality has been shown for *N*-acetoxy-2-acetylaminofluorene and to a much lesser extent for *N*-hydroxy-2-aminofluorene, 2-nitrosofluorene, and *N*-hydroxy-2-acetylaminofluorene (29, 40–46). *N*-Acetoxy-2-acetylaminofluorene reacts primarily with the 8-position of guanine (29, 40–43) and has been shown to cause mutations when DNA from *Bacillus subtilis* is treated *in vitro* and then used in a transformation assay (47, 48). The epoxides of several polycyclic hydrocarbons, produced by a microsomal system, are also frameshift mutagens of this reactive type (16). As indicated with the polycyclic hydrocarbon epoxides (16) and as can be seen with the nitrosonaphthalenes, the specific position of the electrophilic group is important for potent mutagenesis.

Several earlier studies have shown clearly that various fluorene and naphthalene derivatives are mutagenic; however, these studies did not raise the point of specific frameshift mutagenesis by a reactive intercalator. The sulfuric and acetic acid esters of *N*-hydroxy-2-acetylaminofluorene were mutagenic in a *B. subtilis* transformation system (47, 48). *N*-Acetoxy-2-acetylaminofluorene caused mutations in T4 phages (some of which were frameshifts) (49). *N*-Hydroxy-2-aminonaphthalene and *N*-hydroxy-1-aminonaphthalene are mutagenic in *E. coli* (50, 51), and metabolic products produced by oxidizing 1- and 2-aminonaphthalene in a hydroxylating system are mutagens for yeast (52).

The *Salmonella* tester strains have been designed both to detect the various mutagens and to classify them as to type (14–16). We find that 2-nitrosofluorene is quite specific for reverting the frameshift tester strain TA1534 but not the frameshift tester strains TA1531 or TA1532. Strain TA1532 is the one that is reverted well by polycyclic hydrocarbon epoxides (16), and strain TA1531 is reverted very well by the frameshift mutagen ICR-191. None of these three different frameshift mutagens revert the base-pair substitution tester-strain TA1530 at the concentrations that we use in our tests. Any back mutation test, such as the one being used, only detects the reversion of the one particular base pair that is being examined and not other mutations, which of course are occurring also among the 4 million other base pairs in the DNA. Thus, it should be emphasized that mutagens mutate DNA in all the tester strains (and presumably any DNA, as all DNA has the same general structure), even though we only look at one particular base pair in each tester strain. The advantages of using a back-mutation test in mutagen testing have been discussed (15).

Direct evidence as to the type of frameshift mutation induced by 2-nitrosofluorene in strain TA1534 comes from recent work of K. Isono and J. Yourno (personal communication). Yourno and his colleagues (53, 54) have made peptide analyses of altered histidinol dehydrogenases from mutants with frameshift mutations in the *hisD* gene. On extending their investigation to the *hisD3052* mutation (in strain TA1534), Isono and Yourno found that it is probably the result of a single base-pair deletion. Furthermore, they showed that a 2-nitrosofluorene-induced revertant has a second nearby deletion of two adjacent base pairs $\begin{pmatrix}-G-C-\\-C-G-\end{pmatrix}$ from a sequence that was probably $\begin{matrix}-G-C-G-C-G-C-\\-C-G-C-G-C-G-\end{matrix}$ in the wild type; the mutation thus has restored the original frame. It seems quite likely that 2-nitrosofluorene stabilized the four base mispairing in a sequence of GC pairs.

The present study supports the previous suggestion (12, 13) that this set of tester strains of *S. typhimurium* may be very valuable in testing putative mutagens and carcinogens for mutagenic activity. These strains also may be used in procedures designed to test mammalian metabolic products of the test compounds (55–58; Ames *et al.*, in preparation).

In conclusion, the data presented here and the previous data on the epoxides of polycyclic hydrocarbons (16) raise again the old question (59) of the possible mutagenic basis for carcinogenicity. One simple hypothesis, at least for the carcinogens we have examined, is that these chemicals are carcinogenic as a result of a reactive intercalation in DNA. The carcinogenic event could be caused by an addition or deletion mutation produced by the reactive intercalator. This carcinogenic mutation need not necessarily be in the chromosome: intercalating agents can cause a very high frequency of loss of DNA-containing organelles such as mitochondria (60) or kinetoplasts (61); e.g., over 50% of a population of yeast cells showed the *petite* mutation (loss of mitochondrial function) after treatment with oxidation products of 1- and 2-naphthylamine (52). Such mitochondrial loss might be due either to the unwinding of small superhelical DNA, which is known to be caused by intercalating agents (62), or to an addition or deletion mutation.

The hypothesis that the critical target of these carcinogens is DNA is strengthened by the finding that cells from patients with xeroderma pigmentosum, who have a high incidence of UV-induced skin cancer, have subnormal activity of endonuclease required for the repair of UV-induced damage in DNA (63). Cells from these patients are defective in the repair of DNA damaged by certain chemicals, including the reactive frameshift mutagens *N*-acetoxy-2-acetylaminofluorene, polycyclic hydrocarbon epoxides, and 4-nitroquinoline-*N*-oxide (refs. 64–66; Stich, unpublished data). These human mutants are analogous to the *urvB* mutants we have used and the two repair systems seem quite similar.

We suggest that compounds containing the ring systems

‡ In certain cases the interaction of the ring system of the frameshift mutagen with the DNA could be by a stacking interaction that is not a strict intercalation (38).

discussed, or others that are shown to be capable of reactive intercalation in DNA (9), should be treated with caution as possible mutagens and carcinogens for humans.

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