Mutations and Decreases in Density of Transforming DNA Produced by Derivatives of the Carcinogens 2-Acetylaminofluorene and *N*-Methyl-4-aminoazobenzene

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SUMMARY

A series of metabolites and synthetic derivatives of the carcinogens 2-acetylaminofluorene (AAF) and *N*-methyl-4-aminoazobenzene (MAB) was tested at pH 7.5 and room temperature for interaction with biologically active DNA isolated from *Bacillus subtilis*. Previous studies have indicated that AAF and MAB are converted in rat liver, via *N*-hydroxy metabolites, to reactive carcinogenic esters which can attack guanine residues in nucleic acids. In the present work the reactive synthetic esters *N*-acetoxy-AAF, *N*-benzoyloxy-AAF, AAF-*N*-sulfate, and *N*-benzoyloxy-MAB caused: (a) severe reductions in transforming activity, (b) up to 100-fold increases in the frequencies of mutations in transforming DNA, and (c) decreases in the buoyant density of DNA as measured in a CsCl gradient. MAB, AAF, and certain metabolites of AAF less carcinogenic than the reactive esters, viz., *N*-hydroxy-AAF, the glucuronide of *N*-hydroxy-AAF, 2-aminofluorene (AF), and *N*-hydroxy-AF, neither inactivated nor caused mutations or density changes in the transforming DNA. 2-Nitrosofluorene-treated transforming DNA exhibited a marginal increase in mutational frequency. The high carcinogenicities of *N*-acetoxy-AAF, *N*-benzoyloxy-AAF, and *N*-benzoyloxy-MAB, especially at the sites of subcutaneous injection in rats, correlate well with their high reactivities and mutagenicities for transforming DNA. Similarly, the lack of activity of the nonester derivatives in the transforming DNA system is in accord with their lack of reactivity with nucleophiles at neutrality.

In DNA samples reacted with esters of *N*-hydroxy-AAF, the frequencies of induced mutations were directly proportional to the decreases in buoyant density. Furthermore, experiments with *N*-acetoxy-AAF-9-^{14}C demonstrated that the decrease in buoyant density was also directly proportional to the number of fluorene molecules covalently bound to the DNA. The decrease in density appears to result from two opposing effects: binding of the highly buoyant AAF residues and slight denaturation of the reacted DNA. Severely inactivated DNA, after denaturation, showed CsCl and Cs_2SO_4 gradient sedimentation patterns compatible with the occurrence of crosslinks between the complementary strands.

Bacterial strains capable of repairing lesions in DNA caused by ultraviolet radiation restored up to 50% of the transforming ability inactivated by these reactive esters. The induced mutations were spontaneously reversible and thus appeared to be caused by single base-pair changes.

INTRODUCTION

Administration of a variety of chemical carcinogens to experimental animals results in the formation of DNA-, RNA-, and protein-bound derivatives in tissues susceptible to carcinogenesis, and both genetic and epigenetic mechanisms, as well as combinations thereof, have been proposed by which these carcinogen-macromolecule interactions could result in neoplasia (re-

viewed in 1, 2). Though inherently attractive, the concept of somatic mutations as a cause of neoplasia has been difficult to test since, in general, the carcinogenic and mutagenic activities of chemicals cannot be tested in the same target cells. Some alkylating agents are both mutagenic and carcinogenic, but no consistent correlation between these properties has been found for the majority of compounds studied (for reviews, see 3–7). However, at least three situations may account for failures to correlate mutagenicity and carcinogenicity. Thus, many carcinogens are apparently not carcinogenic *per se*, but must be metabolized to active forms (1, 2, 8), which may not be formed under the conditions of mutagenicity tests. Second, some mutagens are so reactive that, on administration to animals, they may decompose or react with noncritical cellular or extracellular components before reaching the critical intracellular targets. Third, permeability barriers may prevent adequate absorption of a mutagen by the target cells in a carcinogenicity assay, or of a carcinogen by the cells utilized in a mutagenicity assay.

The current studies were undertaken to assess the mutagenic activity of metabolic and synthetic derivatives of the carcinogens 2-acetylaminofluorene (AAF)[1] (Fig. 1) and *N*-methyl-4-aminoazobenzene (MAB) (Fig. 2) for transforming DNA in the *Bacillus subtilis* system of Freese and Strack (9). This system was chosen since the reaction with the transforming DNA is performed in the absence of permeability barriers and metabolism of the chemical, and thus permits an assessment of the

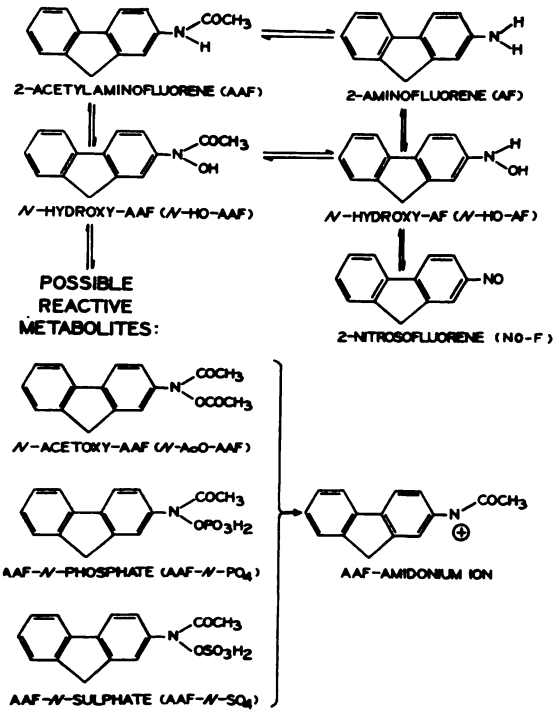


FIG. 1. *Structures of compounds related to the carcinogen 2-acetylaminofluorene (AAF)*

The arrows indicate the known metabolic relationships in the rat (5, 8).

mutagenic activity *per se* of each compound. The compounds tested were chosen on the basis of studies in this laboratory on their metabolism, reactivity, and carcinogenicity. Both AAF and MAB appear to be converted *in vivo* into reactive carcinogenic metabolites by a two-step mechanism of *N*-hydroxylation and esterification

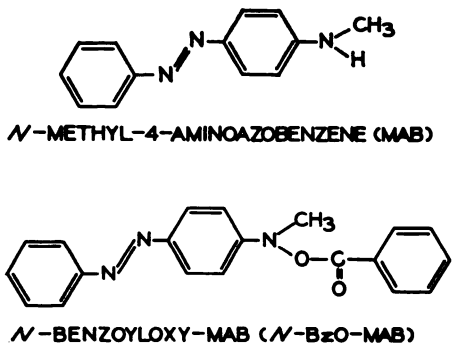


FIG. 2. *Structures of N-methyl-4-aminoazobenzene (MAB) and its N-benzoyloxy derivative (12)*

[1] Abbreviations used in this paper are as follows: AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; *N*-BzO-AAF, *N*-benzoyloxy-AAF; *N*-AcO-AAF, *N*-acetoxy-AAF; *N*-GLO-AAF, glucuronide of *N*-hydroxy-AAF; NO-F, 2-nitrosofluorene; AAF-*N*-SO_4, AAF-*N*-sulfate; MAB, *N*-methyl-4-aminoazobenzene; *N*-BzO-MAB, *N*-benzoyloxy-MAB; SSC, standard saline citrate solution (0.15 M NaCl, 0.02 M trisodium citrate, pH 7.5); DSC, 10-fold diluted SSC, pH 7.5; Poly (U,G), random copolymer of uridylic and guanylic acids.

(8, 10–16). The esters of the *N*-hydroxy metabolites of AAF and MAB are highly reactive in nucleophilic substitution reactions (8, 12–15, 17). In nucleic acids guanine residues are readily substituted at carbon-8 by interaction with esters of *N*-hydroxy-AAF (14, 15); *N*-benzoyloxy-MAB also reacts with nucleic acids, probably on the guanine residues (12).

The following studies were presented in part at a recent meeting of the American Society of Biological Chemists (18).

MATERIALS AND METHODS

Bacterial strains. The DNA donor, *Bacillus subtilis* strain SB 19 prototroph (19), was obtained from Dr. I. Takahashi, McMaster University, Hamilton, Ontario, Canada. The recipient, strain T3, blocked in tryptophan synthesis and unable to grow on indole, was kindly provided by Dr. B. S. Strauss, University of Chicago. The isolation and properties of strain T3 as well as the functions of the genes linked to the tryptophan synthetase genes have been described (9, 20, 21). For enzymatic repair studies the indole-dependent recipient strains 168 *uvr*$^+$, 168 *uvr*$^-$ (22), and 168 *hcr*-9 (23) were kindly provided by Drs. B. S. Strauss and W. R. Romig.

Media. Competence medium. The minimal medium described by Spizizen (24) was supplemented with 0.5% glucose, 0.01% Casamino acids, 0.1% yeast extract, 3×10^{-3} M $CaCl_2$, 2.5×10^{-3} M $MgCl_2$, 5×10^{-4} M spermine tetrahydrochloride (Calbiochem), and 5 μg/ml of adenine, L-tryptophan, and L-histidine (25; and I. Mahler, personal communication).

Selective agar medium. Minimal Vogel-Bonner medium (26) containing 2% Difco agar was supplemented with 0.5% glucose, 0.1% Casamino acids, and indole (1.2 μg/ml). The absence of tryptophan in this agar selects for tryptophan-independent transformants, whereas the presence of indole permits growth of those tryptophan-independent transformants which, along with the functional tryptophan synthetase gene, also acquired DNA with mutagen-induced blocks in the tryptophan pathway prior to indoleglycerol phosphate (see Fig. 3). Indole added at this suboptimal concentration does not interfere with the accumulation of fluorescent intermediates.

Carcinogenic compounds. The names, abbreviations, and chemical structures of the carcinogens are shown in Figs. 1 and 2. These were obtained or synthesized as described in the following references: MAB (27); *N*-BzO-MAB, synthesized by Dr. L. A. Poirier (12); AAF (Mann Biochemicals); *N*-HO-AAF (11, 28); AF (29); *N*-HO-AF (28, 30); NO-F (30); *N*-GLO-AAF,[2] kindly provided by Dr. C. C. Irving, Veterans Administration Hospital, Memphis, Tennessee (31); *N*-AcO-AAF and *N*-BzO-AAF (13).

N-AcO-AAF-9-^{14}C was prepared by dissolving in a 2-ml centrifuge tube 2 mg of *N*-HO-AAF-9-^{14}C (Tracerlab, 7.7 mC/mmole, supplied as a tan powder) in 0.4 ml of fresh 0.5 N NaOH in water and centrifuging to separate dark brown insoluble matter. The clear yellow supernatant was transferred to a 5-ml tube containing 98 mg of pure dry nonradioactive *N*-HO-AAF. The pellet in the first tube was rinsed with 0.45 ml of the NaOH solution; after centrifuging, the solution was added to the second tube. The mixture was stirred until all the hydroxamic acid had dissolved and a heavy suspension of crystals of the sodium salt had formed. Acetic anhydride (50 μl) was delivered into this suspension, and the mixture was stirred until the oil that formed became solid. After the solid was pulverized, the mixture was centrifuged and the supernatant was discarded. The moist solid was dissolved in 2.5 ml of acetone, and 1 ml of water was added. Darco KB activated carbon (25 mg) was added and the suspension was stirred intermittently for 30 min. The suspension was then filtered by suction through a sintered glass disk, and the vacuum was maintained until considerable crystallization of the ester occurred. The crystals were centrifuged, washed with a small amount of cold 1:1 acetone–water, and

[2] *N*-GLO-AAF is of interest since it is a major metabolite of AAF and *N*-HO-AAF and reacts slowly with guanosine at neutrality (17).

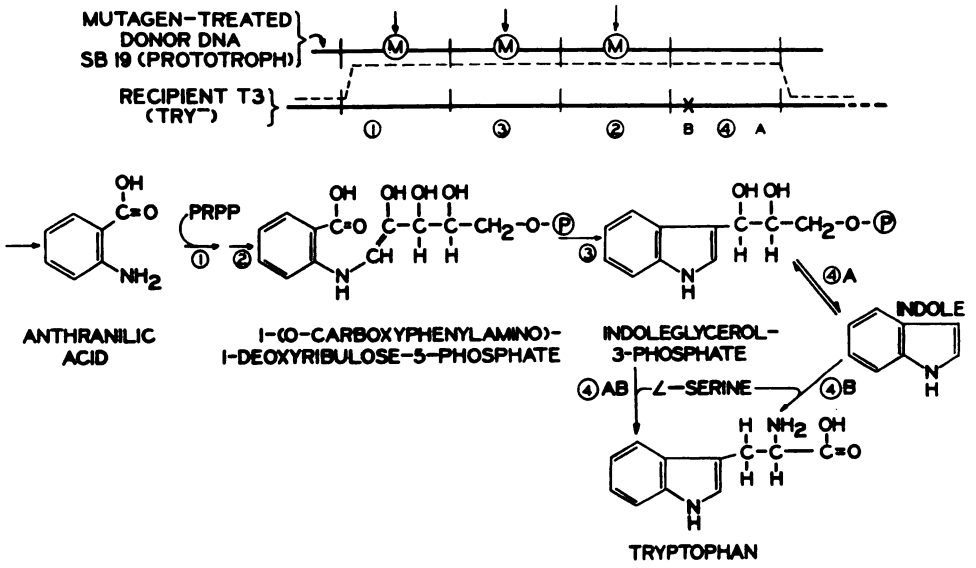


FIG. 3. *Detection of linked mutations in the tryptophan operon of Bacillus subtilis transforming DNA by the method of Freese and Strack (9)*

Symbols *1*, *2*, *3*, and *4* indicate the linked genes (upper portion of the diagram) and the enzymes for which they code (21). Inactivation of region *1* or *2* results in the accumulation of anthranilic acid, while inactivation of region *3* causes the accumulation of 1-(*o*-carboxyphenylamino)-1-deoxyribulose, both of which are fluorescent. In strain T3, region *4*, which codes for tryptophan synthetase, carries an inactivating mutation in gene B. Thus, the growth of the recipient cells (T3) cannot be supported by indole, but requires tryptophan. Transformants of strain T3, which acquire intact region *4B* from the transforming DNA, grow in the absence of tryptophan. If the piece of transforming DNA extends into regions *2*, *3*, or *1* and, by chance, also carries a mutation in these regions (indicated by *M*), the transformed cells will grow only if supplemented with indole. With limited amounts of indole, such cells will accumulate the fluorescent intermediates. The resulting fluorescence distinguishes the colonies of cells mutated at these regions from the other colonies of transformed cells.

dried in the tube over fresh sulfuric acid *in vacuo*. The white dry crystals (48 mg) melted at 111° (Accumelt apparatus, American Instrument Co.) and rearranged at about 130° with a rapid evolution of heat. This was noted in the temperature-time heating curve as a sharp rise to over 200°, followed by an equally sharp fall to about 160°.

AAF-*N*-SO_4 was prepared by addition of pyridine-SO_3 (3.34 g, 21 mmoles) to a solution of *N*-HO-AAF (1.67 g, 7 mmoles) in 35 ml of dry pyridine.[3] This solution was stirred at room temperature for 4 hr, after which 100 ml of anhydrous ethyl ether were added. The precipitate was filtered, washed 3 times with 60-ml volumes of anhydrous ethyl ether, and then dissolved in 135 ml of absolute methanol. After external cooling with ice this solution was treated with a vigorous stream of anhydrous ammonia gas for 3 min. The precipitate was removed by filtration and 2 g of powdered dry potassium acetate was added to the filtrate. The yellowish precipitate was filtered and washed twice with 5 ml of anhydrous methanol and then with anhydrous ethyl ether. The product (1.4 g) was kept cold *in vacuo* over fresh sulfuric acid. The product contained at least 50% of the potassium salt of AAF-*N*-SO_4 on the basis of the yield of *o*-methylmercapto-AAF obtained on mixing the dry product with an excess of methionine in solution (13). Attempts to recrystallize this product from dimethylformamide-ethyl ether mixtures

[3] The synthesis of AAF-*N*-SO_4 was developed in collaboration with Drs. W. Podkoscielny and P. D. Lotlikar.

gave preparations which were less reactive with methionine.

Preparation of transforming DNA. Each of six flasks containing 250 ml of Penassay broth (Difco Antibiotic Medium 3) was inoculated with 0.25 ml of an overnight culture of SB 19 cells grown in Penassay broth from a potato agar spore slant inoculum. Cells were grown in an orbital shaker bath at 37° to a concentration of 6×10^8/ml, centrifuged for 20 min at 2500 *g* (5000 rpm in Lourdes VRA rotor), pooled, resuspended in 15 ml SSC, and lysed by treating for 30–60 min with a freshly prepared solution of lysozyme (100 μg/ml) supplemented with a preincubated solution of Pronase (10 μg/ml). Sodium dodecyl sulfate (2%) was added, and the lysate was deproteinized by 4 extractions with an equal volume of a 4:1 chloroform–butanol mixture. DNA was precipitated by the addition of 1 volume of 95% ethanol and redissolved in 5 ml DSC. Incubation for 30 min at 37° with pancreatic RNase (100 μg/ml) and T_1 RNase (10 units/ml) was followed by 4 additional deproteinizations. The DNA was again precipitated with ethanol, redissolved in 5 ml DSC, and finally reprecipitated by adding 0.5 ml of 3 M sodium acetate followed by 3 ml of isopropanol (32). The DNA was dissolved at 1 mg/ml and stored at −60°. If it was to be stored at 0–4°, the DSC solution was adjusted to the concentration of SSC.

Induction of mutations and chemical inactivation of DNA. Because the compounds tested are poorly soluble in water (with the exception of AAF-*N*-SO_4 and *N*-GLO-AAF) and unstable in solution, they were dissolved in 95% ethanol, and 0.05 ml was immediately added to 0.10 ml of the DNA solution (ca. 1 mg DNA/ml DSC). Various concentrations of the compounds were used, but most commonly the concentrations approached the limits of solubility in ethanol (see Table 1), and some precipitation was obtained on mixing the ethanol solution with the DNA solution. Because of the extremely rapid decomposition of AAF-*N*-SO_4 in solution this compound was pulverized in a test tube, and 0.3 ml of DNA solution (333 μg DNA/ml DSC) was added to the dry powder. *N*-GLO-AAF, on the other hand, is stable in water and far less reactive (17); so one volume of a solution of this compound in water was added to 2 volumes of a DNA solution (1 mg DNA/ml DSC), and the mixture was incubated for 22 hr. In all cases the incubations were at pH 7.5 and room temperature, usually for 1–60 min, and the reaction was stopped by precipitation of the DNA with 95% ethanol. Since the DNA precipitated quantitatively in the form of fibers adhering to the wall of the vial, most of the test compound could be removed by 5–10 rinses with 95% ethanol. The DNA was then dissolved overnight in DSC (100 μg DNA/ml) and repeatedly extracted with DSC-saturated ethyl ether until no further reduction in the optical density in the range of 270–400 mμ was observed.

Assay for loss of transforming activity. Approximately 5×10^6 spores of *B. subtilis*, strain T3, were suspended in saline, heated to 85° for 10 min, and either stored in phosphate-buffered saline (see ref. 33) or directly grown in 10 ml of competence medium for 16 hr at 37° in an orbital shaker bath. The overnight culture was diluted 1:10 into fresh competence medium with 5 μg/ml of arginine replacing adenine. The cells approached maximal competence (0.05–0.1%) after growing for 60 min at 37°. Control DNA was carried through each of the steps described above with the solvent substituted for the solution of the test compound. Treated DNA or control DNA was added to 1- or 2-ml aliquots of competent cells at a final concentration of 0.5 μg DNA/ml. Since concentrations up to 1 μg/ml of DNA gave a linear dose-response curve, the percent survival of transforming activity was a direct function of the proportion of transformant colonies produced by cells receiving the treated DNA as compared to the control DNA. In most cases the percent survival of transforming activity has been expressed in terms of lethal hits calculated from the Poisson distribution equation; i.e., survival levels of 37, 13.5, and 5.0% correspond to 1, 2, and 3 hits, respectively.

Cultures of recipient cells were incubated

with DNA at 37° in a wrist-action shaker for 40 min and diluted in minimal medium (24) so as to obtain no more than 350–400 tryptophan-independent transformant colonies per plate when 0.05–0.15 ml was plated on the selective agar medium. The total number of viable recipient cells, as assayed on nutrient agar, was not influenced by the exposure of the cells to the treated DNA's. Each experiment included a check on the sterility of the DNA preparation and a control in which the T3 recipient cells received no DNA.

To assess the effect of cellular repair mechanisms, inactivation of transforming ability was also assayed in some experiments in the indole-requiring strain 168 (*urv*$^+$) and in two radiation-sensitive derivatives (*uvr*$^-$ and *hcr*-9). In these experiments indole was omitted from the selective agar medium.

Assay for mutagenicity. The selective agar medium ensures that only those recipient T3 cells that have incorporated SB 19 donor DNA with an intact tryptophan gene can produce colonies. However, the presence of a suboptimal concentration of indole (1.2 μg/ml) also permits growth of any tryptophan-independent recipient which has a mutation (spontaneous or induced) in one or more of the genes required for tryptophan synthesis (see Fig. 3), which are physically linked to the tryptophan synthetase genes and which are required for the synthesis of indoleglycerol phosphate. Such mutations cause the accumulation of anthranilic acid and/or 1-(*o*-carboxyphenylamino)-1-deoxyribulose (20, 21), the fluorescence of which can be recognized after incubation for 28–30 hr. Fluorescent transformant colonies were detected and scored under ultraviolet light (254 mμ, Mineralight lamp No. 2537, Ultraviolet Products, Inc., San Gabriel, California) while rotating the plates against a black velvet background (9; and E. Freese, personal communication). The results of the assay are expressed in terms of a mutagenicity index, which is the number of fluorescent colonies per 10^4 transformant colonies (see the legend to Fig. 5 for the derivation of this index).

Density-gradient centrifugations. Samples of native and alkali- or heat-denatured DNA (1 μg DNA/2-degree sector, 12 mm Kel-F cell; see Fig. 8) were subjected to analytical density-gradient centrifugation at 44,770 rpm for 22 hr (CsCl) or at 31,410 rpm for 44 hr (Cs_2SO_4) at 25° under the usual conditions of this laboratory (34). In order to demonstrate crosslinking, samples of DNA (25 μg/ml DSC) were heat-denatured (5 min, 98°) in the presence of 1.1% formaldehyde and centrifuged in Cs_2SO_4 containing 0.22% formaldehyde. Similar samples were heat-denatured (2 min, 98°) in the presence of poly(U,G) (50 μg/ml) (Miles Laboratories, control No. 249) and centrifuged in CsCl. Tracings of the ultraviolet photographs were prepared with a Joyce-Loebl Mark IIIC double-beam microdensitometer equipped with a cylindrical condenser lens using a 4 × 0.04 mm scanning beam. Overlapping bands were resolved using a du Pont 310 Curve Resolver. For preparative centrifugation, DNA in CsCl at a final density of 1.70 g/cm^3 (2.5 ml sample per polyallomer tube, overlayed with 2.5 ml of paraffin oil) was centrifuged at 30,000 rpm for 72 hr at 5° in the SW 39 rotor of the Spinco Model L ultracentrifuge. Fractions of 0.04 ml were collected sequentially from the bottom of the tube (35), and analyzed for OD_{260} in a 2-mm light path, 15-μl microcuvette in the Perkin-Elmer 202 ultraviolet spectrophotometer.

Radioactivity determinations. Following the preparative centrifugation of DNA reacted with *N*-AcO-AAF-9-^{14}C the fractions containing the DNA were diluted 2-fold with distilled water, and precipitated with an equal volume of 95% ethanol. The DNA was redissolved overnight in DSC (150–200 μg/ml), its exact concentration was determined spectrophotometrically (OD_{260}), and 0.1–0.3 ml was mixed with 0.5 ml hydroxide of Hyamine 10-X (Packard). Scintillator fluid (295.2 g naphthalene, 18.48 g 2,5-diphenyloxazole, 0.184 g α-naphthylphenyloxazole, 1400 ml xylene, 1400 ml dioxane, and 840 ml absolute ethanol) was added to a total volume of 10 ml, and

TABLE 1

Inactivation, mutagenesis, and decrease in buoyant density of transforming DNA of Bacillus subtilis by exposure to derivatives of 2-acetylaminofluorene and N-methyl-4-aminoazobenzene

Except for the last column, which presents pooled data from all the experiments, the data in this table are from a representative experiment to show the effects of treatment of transforming DNA with each of the compounds under specified conditions. See Materials and Methods for details of the experiments.

Compound	Moles compound per mole DNA-nucleotide	Reaction time (min)	Inactivation of transforming DNA		Density decrease (mg/cm³)	Fluorescing colonies / Transformant colonies = Frequency of mutants			
			% survival	Hits			(×10⁴)	(all experiments)[a]	(×10⁴)
(Solvent control)	—	30	100	0	0	3/27,000 =	1.1	21/192,000 =	1.1
AAF-*N*-SO_4	6.6	1	0.2	6.2	5.6	14/2,570 =	55	185/55,700 =	33
N-AcO-AAF	8	5	0.4	5.5	4.4	12/4,300 =	46	220/87,400 =	25
N-BzO-AAF	10	5	0.7	5.0	4.4	9/2,000 =	45	75/26,400 =	28
AAF	15	15	96	0	0	0/4,190 =	<2.4	0/24,900 =	<0.4
N-HO-AAF	12	10	90	0.1	0	0/7,660 =	<1.3	3/34,100 =	0.9
N-GLO-AAF	12	1300	94	0	0	0/7,650 =	<1.3	0/9,600 =	<1.0
AF	20	15	94	0	0	1/3,910 =	2.5	1/26,200 =	0.4
N-HO-AF	10	15	79	0.2	0	0/3,450 =	<2.9	1/20,300 =	0.5
NO-F	12	15	97	0	0	3/8,670 =	3.5	27/105,000 =	2.6
N-BzO-MAB	11	15	10	2.3	0.5	4/1,600 =	25	47/23,700 =	20
MAB	18	15	96	0	0	0/2,040 =	<4.9	4/32,400 =	1.2
$NaNO_2$[b]	400	15	5	3	—	7/880 =	81	—	

[a] Includes all experiments with various concentrations of the compound and various times of exposure.
[b] pH 4.5; conditions as described in (36). Included as a positive control.

the radioactivity was determined in a Packard Tri-Carb scintillation counter.

RESULTS

Inactivation of Transforming DNA

As can be seen from Fig. 4 and Table 1, exposure of DNA to any of the four esters, *N*-BzO-MAB, *N*-BzO-AAF, N-AcO-AAF, or AAF-*N*-SO_4, produced a severe loss of the ability to transform the recipient strain T3 to tryptophan-independence. In contrast,

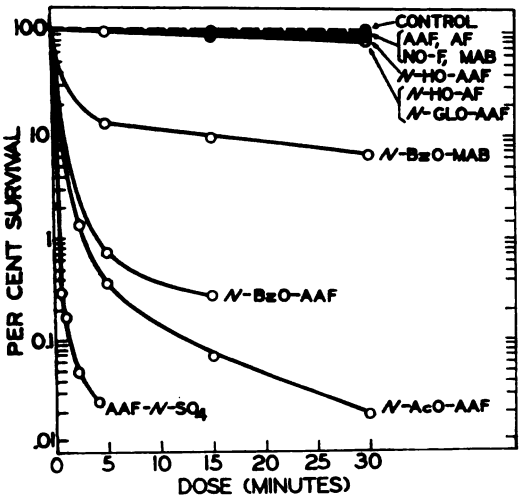


FIG. 4. *Inactivation of Bacillus subtilis SB 19 DNA as assayed by its ability to transform the tryptophan-requiring strain T3*

Transforming DNA was incubated with each of the indicated compounds at pH 7.5 and room temperature in aqueous (AAF-*N*-SO_4 and *N*-GLO-AAF) or 32% ethanolic solution (all other compounds), as described in the Materials and Methods section. The molar ratios of the agents per DNA-nucleotide were as follows: AAF-*N*-SO_4, 6.6; *N*-AcO-AAF, 8; *N*-BzO-AAF, 10; AAF, 15.5; *N*-HO-AAF, 12; *N*-GLO-AAF, 12; AF, 20; *N*-HO-AF, 10; NO-F, 16; *N*-BzO-MAB, 11; and MAB, 18.

exposure of DNA to any of the seven other related nonester compounds at the same or higher levels did not result in an appreciable loss of transforming activity. Higher concentrations of these inactive compounds (excluding *N*-GLO-AAF) were precluded by the limits of their solubilities in ethanol. The departure from exponential kinetics of the survival curves may be explained by the limited stabilities of the esters in aqueous solutions. In the absence of DNA, but with the conditions otherwise equivalent to those under which the reactions with DNA were carried out, the esters had the following approximate half-lives: AAF-*N*-SO_4, less than 1 min; *N*-BzO-MAB, 12 min; *N*-BzO-AAF, 1.6 hr; and *N*-AcO-AAF, 2 hr. The half-lives were determined by the reactivity at various intervals of the residual ester with a 25-fold excess of methionine (17). The half-lives may be shorter in the presence of DNA, since Scribner, Miller, and Miller (manuscript in preparation) found that the removal of the acetoxy group from *N*-AcO-AAF was facilitated by the addition of methionine.

Mutagenic Activity

As shown in Table 1 and Figs. 5 and 6, *N*-BzO-MAB and the three esters of *N*-HO-AAF show strong mutagenic activity toward transforming DNA; up to 100-fold increases in the frequency of fluorescing colonies were observed. NO-F seemed to exhibit a marginal mutagenic effect; the remainder of the compounds tested were inactive (Table 1, last column). Nitrous acid, which is known to be strongly mutagenic toward transforming DNA (36–38), was included as a positive control.

Effects on the Buoyant Density of DNA

The strong mutagenic effect of esters of *N*-HO-AAF is accompanied by a corresponding decrease in the buoyant density of DNA treated with these compounds (Table 1 and Fig. 7). The lines obtained by the method of least squares for the relationship between the mutagenicity index and the inactivation of DNA (Fig. 5) and for the relationship between the decrease in buoyant density and the inactivation of DNA (Fig. 7) both intersect the ordinate axis at about one lethal hit. The interpretation of this intercept is not clear. These data do show, however, that after one lethal hit, there is a direct correlation between the decrease in the buoyant density of the DNA and the number of mutations observed. The relationship between

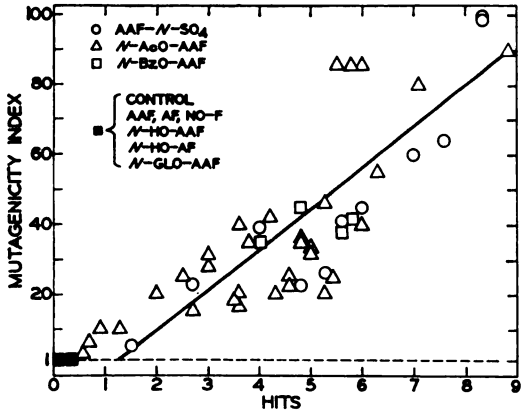


FIG. 5. *Mutagenic effects as a function of the inactivation of the transforming DNA by derivatives of AAF*

Mutations in regions *1*, *2*, or *3* of the tryptophan operon were scored by inspecting the colonies of transformants under ultraviolet light (see the legend to Fig. 3). Among 192,000 colonies derived from cells transformed with control DNA (identical treatment except for the absence of the derivatives of AAF or MAB), 21 fluorescent colonies were found (1.1×10^{-4}), whereas 9 per 147,500 (0.6×10^{-4}) were observed for the transformants produced with DNA treated with apparently nonmutagenic compounds (MAB, AAF, *N*-HO-AAF, *N*-GLO-AAF, AF, and *N*-HO-AF). Thus, the spontaneous mutation frequency under our conditions has been considered to be 1×10^{-4}, and the mutagenicity index has been defined as the frequency of fluorescent colonies among 10^4 colonies of transformants. The line was calculated by the method of least squares. Lethal hits were calculated from the Poisson distribution equation; i.e., survival levels of 37, 13.5, and 5.0% correspond to 1, 2, and 3 hits, respectively.

the extent of inactivation of DNA and the frequency of mutations, as well as the decrease in density, was not altered when the incubation of DNA with *N*-AcO-AAF was carried out under argon in the presence of 0.05 M sodium pyrophosphate, which has been used by Freese and Freese (39) to prevent destruction of transforming activity by free radicals or peroxides. As treatment with *N*-BzO-MAB did not lead to as extensive a loss of transforming activity (Fig. 4) as did reactions with esters of *N*-HO-AAF, only minor decreases were observed in the buoyant density of DNA reacted with this compound (Fig. 6).

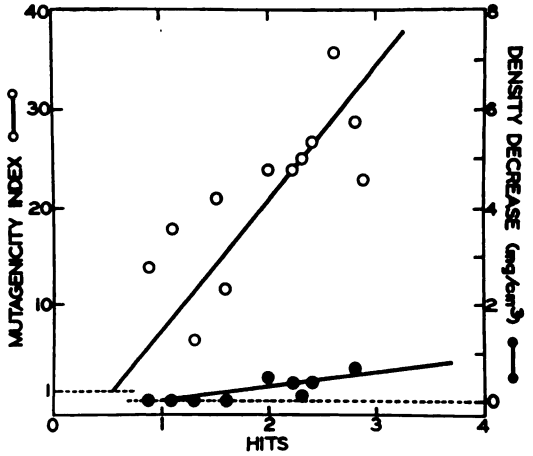


FIG. 6. *Mutagenic effects and decrease in buoyant density of transforming DNA as a function of the inactivation of DNA by N-BzO-MAB*

See caption to Fig. 5 for definition of the mutagenicity index. The density decrease is the difference between the buoyant density of the native treated DNA and the density of untreated bihelical *Bacillus subtilis* DNA (1.7033 g/cm³). The lines were calculated by the method of least squares. Treatment of DNA with MAB caused no change in the mutagenicity index or buoyant density.

Nevertheless, the density shift per lethal hit is only slightly lower than that observed with esters of *N*-HO-AAF.

Crosslinking of DNA Strands

The bonds between DNA and AAF residues are not dependent upon the maintenance of the secondary structure of the DNA molecule since denaturation of *N*-AcO-AAF-treated DNA does not abolish the corresponding density differential between reacted and control DNA's (Fig. 8). However, the CsCl gradient banding patterns obtained after denaturation of the more strongly inactivated samples of native DNA exhibit a bimodal distribution of DNA (Fig. 8, traces *h* and *j*). The peak labeled *dN* has the density expected for the denatured DNA, whereas the shaded peak centers on a density close to that of the native DNA. Similar bimodal banding patterns were observed in Cs_2SO_4 gradients. However, when such DNA was heat-denatured in the presence of formaldehyde and centrifuged in Cs_2SO_4 with formalde-

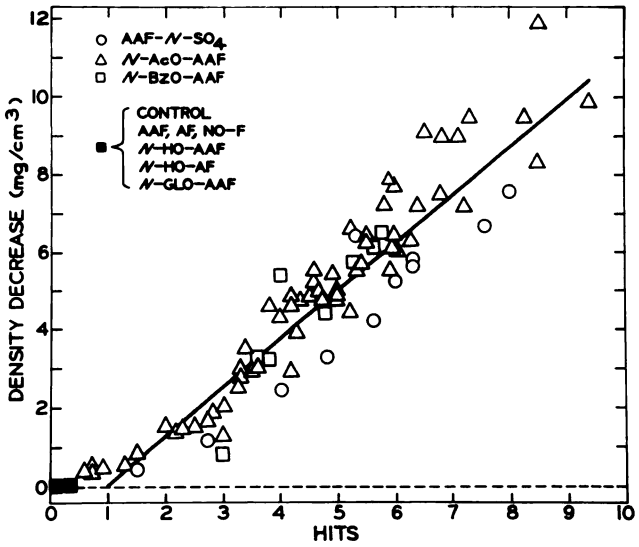


FIG. 7. *Decreases in buoyant density of transforming DNA as a function of its inactivation by derivatives of AAF*

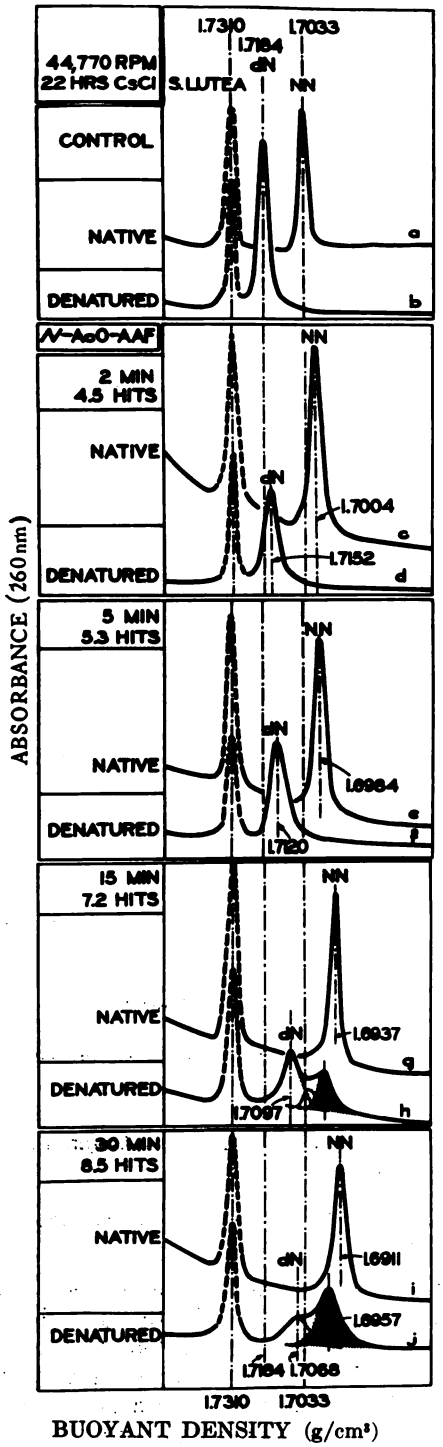
Bacillus subtilis DNA, treated under the conditions specified in the Materials and Methods, was assayed for survival of transforming activity and for buoyant density by analytical CsCl gradient centrifugation. The density decrease is the difference between the buoyant density of the native treated DNA and the density of untreated bihelical *B. subtilis* DNA (1.7033 g/cm³). The line was calculated by the method of least squares.

hyde [in a manner analogous to that described by Opara-Kubinska *et al.* (40) and Iyer and Szybalski (41)], the bimodal distribution was suppressed and the entire sample of DNA exhibited a density close to that expected for denatured treated DNA (42). Spontaneous renaturation of a fraction of DNA and the inhibition of this renaturation by formaldehyde are indicative of covalent crosslinking between the complementary strands in those DNA molecules which band under the shaded peaks (Fig. 8, traces *h* and *j*), as demonstrated previously for DNA crosslinked by ultraviolet irradiation (40) or mitomycin (41, 43).

A further indication of the presence of crosslinked DNA molecules was obtained when treated and untreated samples were heat-denatured in the presence of poly (U,G) before centrifuging in CsCl. The control DNA separated as expected into two equal peaks (density difference, 14.5 mg/cm³) because of the differential binding of the polynucleotide to the two separated strands of *Bacillus subtilis* DNA (44). The treated DNA separated into three peaks, one exhibiting the same density as the treated molecule before denaturation and the other two in the positions predicted for separated strands bearing a specific number of AAF residues. The fraction of molecules which failed to interact with the poly(U,G) behaves as bihelical DNA and is the same as that which showed spontaneous renaturation in earlier experiments (for details, see ref. 42).

Reaction between N-AcO-AAF-9-¹⁴C and DNA and Its Relation to Buoyant Density Decreases

To determine the number of AAF residues bound to DNA and to relate this number to the previously measured parameters, *N*-AcO-AAF-9-¹⁴C was reacted with the transforming DNA. As shown in Table 2, approximately one or two AAF residues were bound per 100 nucleotides in DNA from which the unbound fluorene derivatives had been removed first by ether extraction and finally by CsCl density gradient centrifugation. The latter step was



obligatory, since DNA which was purified only by precipitation with ethanol and exhaustive extraction with ethyl ether retained two or three times more radioactivity than the CsCl gradient-purified DNA (Table 2). A single CsCl gradient purification seems to be sufficient. No firmly bound AAF residues were lost during the preparative CsCl gradient centrifugation, since the buoyant density decreases measured in the analytical centrifuge were not significantly affected by this step. As shown in Table 2, the density decrease in a CsCl gradient which corresponded to one AAF residue bound per 100 nucleotides was 5.5–5.9 mg/cm³ for DNA either lightly or heavily inactivated by exposure to *N*-AcO-AAF. These data support the conclusion drawn from the data of Figs. 7 and 8 that the decrease in density is caused by the covalent binding of AAF residues. The observed decrease (5.5–5.9 mg/cm³) is 10% less than the theoretically predicted decrease of 6.3 mg/cm³. This slight discrepancy indicates some AAF-caused structural changes in the bihelical DNA molecule which affect its buoyant density in a manner similar to the effects of denaturation. Such an indication of slight denaturation and the consequent decrease in hydration is corroborated by the thermal transition

FIG. 8. *Effect of treatment with N-AcO-AAF on the banding pattern of native and denatured DNA in a CsCl gradient*

Bacillus subtilis DNA, which in its native form had been reacted with *N*-AcO-AAF (8 moles/mole DNA-nucleotide) for 0–30 min, was subjected to CsCl equilibrium density gradient centrifugation for 22 hr, in its native or denatured form. The DNA (25 μg/ml DSC) was denatured by 2 min exposure to 0.2 N NaOH, followed by neutralization with 1 M KH_2PO_4. The areas under the peaks for denatured DNA from samples treated for 15 (trace *h*) or 30 min (trace *j*) have been resolved into 3 components with a du Pont 310 Curve Resolver. The DNA fractions represented by shaded peaks retain a density close to that of the corresponding bihelical DNA, and thus appear to be crosslinked. The dashed peaks correspond to native *Sarcina lutea* DNA, which was used as a density marker (1.7310 g/cm³).

TABLE 2

Decrease in buoyant density and transforming activity of DNA as a function of the number of AAF residues bound

N-AcO-AAF-9-^{14}C was reacted with *Bacillus subtilis* DNA (final concentrations, 20 mM *N*-AcO-AAF and 2.1 mM DNA-nucleotide) for 2.5–30 min. The reacted DNA was precipitated with ethanol, dissolved in DSC, extracted extensively with ethyl ether, and finally purified by one or two preparative centrifugations in a CsCl gradient. The DNA recovered from the preparative centrifugation was then assayed for radioactivity and for buoyant density by analytical centrifugation in a CsCl gradient.

				Buoyant density decrease (mg/cm³)			
				Observed[b]			Calculated[c]
				Native		Denatured	Native
Expt. No.	Survival of transforming activity (%)	Number of preparative centrifugation runs in CsCl	AAF residues bound/100 nucleotides[a]	total	per AAF residue/ 100 nucleotides	total	total
1	—	0	(2.10)	2.5	—	2.5	—
		1	0.29	1.6	5.5	1.8	1.9
2	0.10	0	(3.20)	6.3	—	8.0	
		1	1.34	7.7	5.9	—	8.5
3	0.015	0	(5.45)	8.4	—	11.4	
		1	1.46	8.2	5.7	—	9.2
4	0.012	0	(5.72)	9.8	—	8.8–10.1[d]	—
		1	1.76	9.6	5.6	—	11.2
		2	1.70	9.5	5.7	9.6–14.6[d]	10.7

[a] Calculated from the radioactivity of the treated DNA as compared to the specific activity of the *N*-AcO-AAF (275,000 dpm/μmole).

[b] Decrease in buoyant density of native or denatured DNA below that of 1.7033 or 1.7184 g/cm³, respectively.

[c] This is the theoretical decrease in the buoyant density of *B. subtilis* DNA (1.7033 g/cm³) which should result from the simple addition of the observed number of AAF residues. The density of AAF, as determined in this laboratory, is 1.0956 g/cm³. The calculation for Expt. 2, in which 25 μg of CsCl-purified DNA contained 0.23 μg of AAF residues (1.34 per 100 nucleotides), was as follows:

$$\frac{25\ \mu g\ DNA + 0.23\ \mu g\ AAF\ residue}{25\ \mu g/(1.7033\ g/cm^3) + 0.23\ \mu g/(1.0956\ g/cm^3)} = 1.6948\ g/cm^3$$

Calculated density decrease for native DNA = 1.7033 g/cm³ − 1.6948 g/cm³ = 8.5 mg/cm³

[d] Appears to be crosslinked; see Fig. 8, traces *h* and *j*.

profiles of *N*-AcO-AAF-treated DNA[4] and by density measurements in a Cs_2SO_4 gradient (for further development of the data, see ref. 42). The observation of minor denaturation with slight decreased hydration effects caused by the binding of AAF residues contrasts with the strongly increased hydration effects recently reported to result from the binding of nitrogen mustard to DNA (45).

[4] The melting temperature of *N*-AcO-AAF-inactivated DNA (7.1 hits) dissolved in DSC was 68.5° as compared with 72.8° for control native DNA of *B. subtilis*. It appeared also that this treated DNA had a reduced hypochromicity (see 42).

Repair of N-AcO-AAF-Caused Lesions in DNA

As in the studies of Reiter and Strauss (22) and Okubo and Romig (23) with ultraviolet light-inactivated transforming DNA, DNA inactivated by reaction with *N*-AcO-AAF shows higher survival in *uvr*$^+$ recipient cells than in the radiation-sensitive *uvr*$^-$ and *hcr*-9 mutants (Fig. 9). The ratios of the initial slopes for the survival curves were 1.5 and 2.0 for *uvr*$^-$/*uvr*$^+$ and

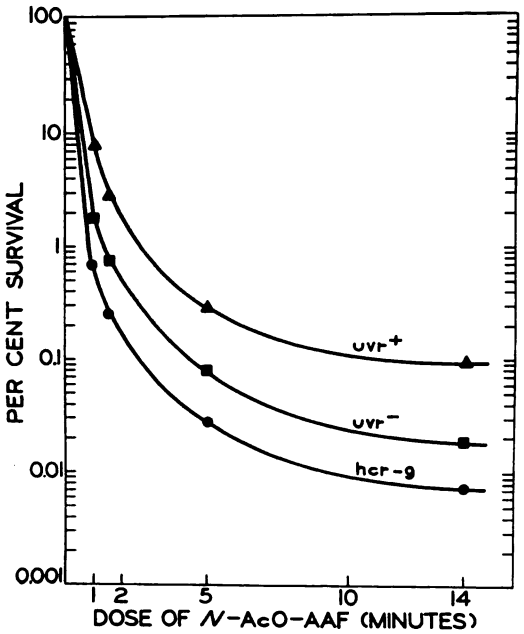


FIG. 9. *Inactivation of Bacillus subtilis SB 19 DNA by N-AcO-AAF as assayed by its ability to transform the indole-requiring radiation-resistant (168 uvr+) and radiation-sensitive (168 uvr− and 168 hcr-9) strains*

Prototrophic SB 19 DNA, reacted with 10 moles of *N*-AcO-AAF/mole of DNA nucleotide for 1–14 min, was assayed for transforming activity in a radiation-resistant strain 168 *uvr*+ and in two radiation-sensitive mutants, *uvr*− and *hcr*-9. The relative initial slopes of the survival curves were 1.0, 1.5, and 2.0 for *uvr*+, *uvr*−, and *hcr*-9, respectively. The relative competencies of strains *uvr*+, *uvr*−, and *hcr*-9 are 1.0, 1.5, and 0.7, respectively.

hcr-9/*uvr*+, respectively. Thus, 35–50% of the *N*-AcO-AAF-induced damage appeared to be repaired in a manner similar to the repair of ultraviolet light-induced lesions.

Frequency of Reverse Mutations among Mutants Induced by N-BzO-MAB and Esters of N-OH-AAF

Seventeen independent cultures were prepared from the individual indole-requiring fluorescent transformant colonies, obtained from the experiments in which tryptophan-dependent T3 recipient cells were transformed with DNA exposed to *N*-BzO-MAB or to esters of *N*-HO-AAF. Cells were grown in Difco nutrient broth (~10^9 cells/ml), centrifuged, washed, resuspended in 1/5 volume of minimal medium (24), and plated (0.05–0.1 ml) on minimal agar selection plates (cf. Materials and Methods) with indole omitted. On this medium, only prototrophic revertants should form colonies. Stable revertants were produced with a frequency of 10^{-5} to 10^{-9} by all the carcinogen-induced mutants tested. Some of these behaved as complete prototrophs, whereas others grew slowly in the absence of indole and still accumulated some fluorescing intermediates. Regardless of the nature of such revertants (back-mutations or suppressor mutations), these data indicate that interaction between DNA and the carcinogenic esters results mainly in point mutations.

DISCUSSION

The major reaction by which AAF residues are bound to DNA appears to be the substitution of guanine residues in the 8-position (Fig. 10); a quantitatively minor reaction pathway may involve the adenine residues. Thus, *N*-AcO-AAF reacts non-

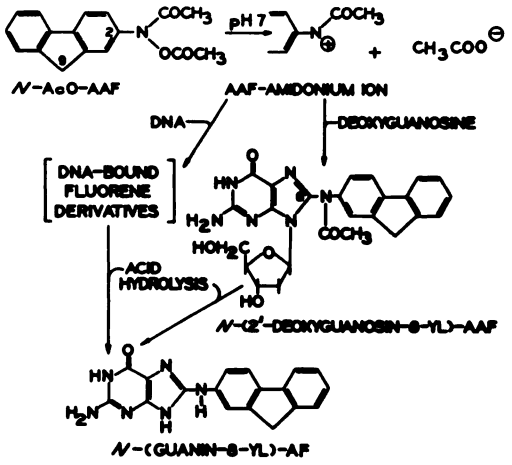


FIG. 10. *The reaction of N-AcO-AAF with deoxyguanosine and DNA (14, 15)*

enzymatically with guanosine or deoxyguanosine in neutral solution to give *N*-(guanosin-8-yl)AAF or N-(deoxyguanosin-8-yl)AAF in high yields (15). Under similar conditions no reaction can be detected with uridine, cytidine, or thymidine, and the reaction with adenosine is only about 2% that with guanosine (8). Like-

wise, reaction of *N*-AcO-AAF with DNA or RNA results in substantial losses of guanine, as determined by base analyses, but no measurable loss of any of the other bases (14). *N*-(Guanin-8-yl)AF has been isolated from *N*-AcO-AAF-reacted DNA after acid hydrolysis (14, 15). The nature of the reaction product of *N*-BzO-MAB with DNA has not yet been established. However, since guanosine and deoxyguanosine are the only nucleotides which have been found to react with *N*-BzO-MAB, guanine is its most likely target in DNA (12).

The arylamidation of deoxyguanosine at carbon-8, in contrast to the alkylation of the 7-nitrogen (46), does not result in labilization of the deoxyribosidic linkage with subsequent depurination and main chain scission, nor does it cause appreciable alteration of the pK_a which might interfere with proper hydrogen bonding (15). Therefore mutagenesis and inactivation through this reaction must depend on some other mechanism, such as the interference by the bulky AAF residues in the large groove of the double helix with the proper approach of DNA polymerase or recombination and repair enzymes. Binding of AAF residues to native DNA seems to be associated with small local denaturation-like[5] changes in the DNA structure since the observed decreases in buoyant density are about 10% lower in CsCl and 50% lower in Cs_2SO_4 gradients than the theoretically computed values (Table 2). In any case, the majority of the mutations apparently involve single base pair changes since all of the seventeen independently isolated fluorescent indole-requiring mutants were found to revert spontaneously.

Crosslinking of the DNA strands in samples reacted extensively with esters of *N*-HO-AAF was an unexpected observation. It seems possible to visualize the reaction as resulting from a formal loss of acetic acid from *N*-AcO-AAF to yield the quinonoid product, *N*-9-dehydro-AAF, which might add to functional groups from two DNA strands at the *N* and 9 positions. However, since the crosslinking could account for only about 0.1% of the AAF residues bound (i.e., 1 crosslink/molecule of DNA with a weight of 20×10^6 daltons), it probably makes little contribution to the biological effects observed.

One can calculate the approximate number of AAF residues bound to the tryptophan synthetase B gene per lethal hit sustained from the data in Table 2 and three assumptions: (a) that the B gene contains 1500 nucleotide pairs (47, 48), (b) that the inactivation of this gene is effected only by the binding of AAF residues, and (c) that the frequency of binding in the B gene is the same as for the molecule as a whole. The DNA of sample 2 (Table 2) contains 1.34 AAF residues per 100 nucleotides or 40 per B gene. This same DNA exhibited inactivation of the B gene equal to 7 hits. However, the inactivation was assayed under conditions permitting repair of approximately 50% of the AAF-induced lesions. Therefore, the number of AAF residues actually bound per lethal hit would appear to be approximately three. Furthermore, since only one strand of DNA is believed to be active in the transformation process, it appears that in the absence of repair the introduction of one or two AAF residues is lethal for this gene.

These data on the mutagenic activities of the esters of *N*-HO-AAF and *N*-HO-MAB indicate that carcinogenesis by AAF and MAB may consist, at least in part, of mechanisms involving mutations.[6] Thus,

[5] The proximity of the bulky C-8 substituents on guanine to the phosphate ester backbone in bihelical DNA results in steric hindrance and local disruption of native DNA structure, as observed for 8-bromoguanine-containing DNA (E. Reich and A. Kapuler, personal communication).

[6] The mutagenicity of *N*-AcO-AAF has also been demonstrated in three other systems. Drs. O. G. Fahmy and M. J. Fahmy of the Chester Beatty Research Institute (personal communication) found that the injection of *N*-AcO-AAF into the hemocoel of adult *Drosophila* males induced small chromosome deletions, resulting in the *minute* phenotype as well as in the initiation of genetic crossing-over. In contrast, this treatment did not result in any recessive visibles or lethals, indicating inactivity with regard to

esters of *N*-HO-AAF and *N*-HO-MAB appear to be the reactive intermediates involved in the carcinogenic activity of AAF and MAB (8), and these compounds are mutagenic in a system in which metabolism of a test compound prior to its reaction with DNA is not possible. On the other hand, the related compounds which were tested (*N*-HO-AAF, *N*-HO-AF, NO-F, AF, AAF, and MAB) and which appear to require conversion to esterified forms as a prerequisite for carcinogenic activity, caused no more than twice the normal frequency of mutations (Table 1). While the lipophilic, uncharged esters *N*-AcO-AAF, *N*-BzO-AAF, and *N*-BzO-MAB were each more carcinogenic than their related derivatives at the subcutaneous site, AAF-*N*-SO_4 has not induced tumors at this site or on cutaneous administration to rats (8). However, this water-soluble, ionic, and very unstable compound may not be able to penetrate the various barriers of mammalian cells fast enough to permit approach to a critical cellular target before it is attacked by another nucleophile. If formed *in vivo*, in proximity to the target site, AAF-*N*-SO_4 may play an important role in the carcinogenic activity of *N*-HO-AAF. Similarly, the activated, reactive form of mitomycin readily reacts with isolated DNA *in vitro*, but is inefficient in a similar crosslinking reaction for DNA embedded in the chromosomes of mammalian cells (43, 50).

The observations in this paper, while suggestive of a role for mutation in carcinogenesis by AAF and MAB, also emphasize the problems inherent in the interpretation of such data on the mutagenic activity of chemical carcinogens. Reactive compounds, such as the alkylating agents and the esters studied above, react not only with DNA, but also with RNA, protein and probably other nucleophilic cellular constituents. Hence, the ability of a carcinogen to induce mutations, even in a system that avoids metabolism of the agent, cannot provide proof that a mutational mechanism is involved in the carcinogenic process it induces. Nevertheless, assays for mutagenesis in nonmetabolizing systems appear to be useful tools in searches for the ultimate biologically reactive forms of chemical carcinogens.

ACKNOWLEDGMENT

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point-mutations. Drs. F. Mukai and W. Troll of New York University Medical Center [see conference summary in (49)] have obtained evidence for substitution of adenine-thymine pairs for guanine-cytosine pairs of the DNA in *Escherichia coli* treated with *N*-AcO-AAF. The mutagenicity of *N*-AcO-AAF and *N*-BzO-AAF for T4 phage has been demonstrated by Mr. T. Corbett, Dr. C. Heidelberger, and Dr. W. F. Dove of the McArdle Laboratory. Preliminary analysis of the forward mutants produced in this system indicates that the primary transitional event is AT → GC, although the occurrence of GC → AT transitions is not excluded by their data.

Further work is required to clarify these divergent results.

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