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Determination of the Mutagenic Activity to Bacteriophage T4 of Carcinogenic and Noncarcinogenic Compounds

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SUMMARY

Forty-one carcinogens and four noncarcinogens were tested for mutagenic activity and the kinds of mutational events produced by the active compounds in bacteriophage T4. Twenty-five carcinogens, including many hydrocarbons, were presumed to have no mutagenic activity because they were not toxic to *Escherichia coli* BB or to T4 phage. Four carcinogenic inorganic salts and five chemical carcinogens (*N*-hydroxy-1-naphthylamine, *N*-hydroxy-2-naphthylamine, *N*-hydroxy-2-aminofluorene, 10-formyl-1,2-benzanthracene, and DL-ethionine) were toxic but not mutagenic to intracellular T4 phage. One compound of definite but low chemical reactivity (the glucuronide of *N*-hydroxy-2-acetylaminofluorene) was not mutagenic by direct treatment of T4 phage. Six chemically more reactive carcinogens (β-propiolactone, propane sultone, *N*-acetoxy-2-acetylaminofluorene and its 7-fluoro derivative, glycidaldehyde, and nitrogen mustard) were mutagenic to T4 phage. The types of mutations induced by each compound were determined. The possible relationship between carcinogenesis and mutagenesis is discussed.

INTRODUCTION

In this laboratory we have long been interested in the cellular and molecular mechanisms of chemical carcinogenesis (1, 2). A hypothesis we and others have considered is that the mechanism of tumor induction involves a mutation as an essential step. However, this hypothesis has remained unresolved since it was introduced by Boveri in 1895 (3).

An approach to the validation of this hypothesis would be to determine the mutational activity, if any, of a series of carcinogenic compounds. A number of such studies have been undertaken with many test systems (*Drosophila*, *Neurospora*, *Vicia faba*, bacteria, bacteriophage) (4–10). Some of these reports must be interpreted with caution on two grounds. First, the induction of gross chromosomal aberrations by a compound is not sufficient proof of mutagenicity unless these aberrations are shown to be heritable. Second, the measurement of reversion frequencies is subject to large clonal fluctuations, so that increases less than 10-fold in magnitude may not be significant.

The understanding of carcinogenesis has been advanced by the realization that many carcinogens are chemically inert and must be metabolically converted in the cell to an "ultimate" carcinogenic form. This ultimate form of the carcinogen is considered by J. A. and E. C. Miller to be chemically reactive and to act as an electrophile (11, 12). Many chemical carcinogens are not metabolized by the organisms used for testing mutagens, and

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thus the possible mutagenic activity of their ultimate carcinogenic metabolite(s) cannot be determined. We have taken advantage of the recent knowledge of the metabolism of carcinogens and have included in our study the highly carcinogenic esters of *N*-hydroxy-2-acetylaminofluorene and several other chemically reactive tumor initiators, which are presumably in an ultimate carcinogenic form.

The usefulness of our study has been greatly increased by the determination of the specific types of mutations induced by these compounds. For this purpose we have employed bacteriophage T4, because mutants are easily detected and the types of mutations induced can be quickly and reliably determined. A dependable determination of the mutational activity of carcinogenic compounds under standard conditions in one organism could add considerably to our understanding of their modes of action toward genetic material.

EXPERIMENTAL PROCEDURE

The T4 Bacteriophage System

A detailed description of the T4rII test system is provided by M. H. Adams (13) and G. S. Stent (14).

The standard (wild type) T4 coliphage contains a segment of DNA called the rII region. This segment is 1.5% of the genome. Replication of T4 within the nonpermissive host *Escherichia coli* KB requires a functional rII region, whereas replication within the permissive host *E. coli* B does not.

Forward mutation. The stock of normal phage is treated with a mutagen. If the mutagen is chemically reactive, the free phages are treated directly. If the mutagen is not chemically reactive, T4-infected *E. coli* B are treated during phage replication. If an alteration in the base sequence is introduced into the rII region such that one of the two rII polypeptides is not functional, then the mutant plaque (lysed area) formed on *E. coli* B will appear large and have a sharp edge. The normal phage produces a smaller, fuzzy-edged plaque. As pointed out above, the rII mutant phage will not give a plaque on *E. coli* KB, whereas the standard phage will. Other large-plaque r mutants occur, but these will grow on *E. coli* KB. Treatment of a normal phage stock with a mutagen will change the frequency of rII mutants from the spontaneous level, near 3×10^{-4}, to over 30×10^{-4}.

Back-mutation. rII point mutants (defined below) can be reverted to the standard state by treatment with the appropriate mutagen. The process corrects or suppresses the error in such a way that rII function is restored. A control stock might have 1 standard phage/10^7 mutants, whereas after treatment it could have 1000 standard phages/10^7 mutants. This would be a 1000-fold increase in the frequency of revertants. Less than a 10-fold increase is not considered significant, because these small frequencies show large fluctuations in stocks of size near 10^9 phages.

Determination of type of rII mutant. In the present work, mutants that show no spontaneous revertants in 5×10^9 phages are assumed to be *extended deletions*. These are deletions of more than one base pair. Mutants that have a single base pair alteration are called *point mutants* and can be divided into base pair transition, frameshift, and nonsense mutants. All point mutants spontaneously give rise to some revertants during growth. The frequency of spontaneous revertants in a stock of such a mutant varies between 10^{-5} and 10^{-8}.

Transition mutants: guanine//cytosine. The mutant has a guanine//cytosine (G//C) base pair at the mutant site, whereas the standard phage has an adenine//thymine (A//T) base pair at the same site. These mutants are revertible by 5-bromouracil, 2-aminopurine, and hydroxylamine (15). Hydroxylamine induces only G//C to A//T base pair transitions (16). 5-Bromouracil induces mainly G//C to A//T base pair transitions (15). 2-Aminopurine causes transitions in both directions, although it slightly favors A//T to G//C conversions (15).

Transition mutants: adenine//thymine. These have an A//T base pair at the mutant site. They are revertible by 2-aminopurine but not by 5-bromouracil or hydroxylamine (15).

Frameshift mutants. These are mutants that have one base pair too few or too many. As a consequence, the reading of the cistron is shifted out of the proper frame. These

mutants are revertible by proflavin (17) but not by 5-bromouracil, 2-aminopurine, or hydroxylamine (15–17).

Nonsense mutants. The codons of nonsense mutants specify termination of the growing polypeptide chain (18). Certain bacterial strains ("suppressor strains") are able to translate these codons and insert an amino acid. Nonsense mutants are characterized by their ability to grow on these bacterial strains but not on others. The DNA sequences for the nonsense mutants are (19): amber, ATC//TAG; ochre, ATT//TAA; UGA, ACT//TGA (sense strand first).

Phage strains. These strains were obtained from the following individuals: S. Brenner, $T4Bo_5$, an osmotic resistant T4 phage; and S. Champe, N-24, an ochre mutant isolated by Benzer and Champe (20) and discussed by Katz (21).

Bacterial strains. These strains were obtained from the following individuals: S. Brenner, B and BB (Berkeley), wild type strains permissive for rII mutants; KB (Benzer), a wild type strain nonpermissive for rII mutants; $CAJ64su^+_{UGA}$, a UGA suppressor (22); J. Weigle, $C600(\lambda)\text{-}su^+_{II}$, an amber suppressor; $CR63(\lambda h)\text{-}su^+_{I}$, an amber suppressor; C. Fuerst, $Y10\text{-}su^+_{II}$, an amber suppressor; and E. Orias, $2320\text{-}R8(\lambda)\text{-}su^+_{ochre}$, an ochre suppressor (23).

Identity of mutants. Two mutants can be proven to be identical or different by the Benzer cross technique (24). *E. coli* B cells are infected simultaneously with the two phage mutants. If there is no increase in the frequency of rII^+ (phage which will grow on *E. coli* KB) in the progeny, the mutants probably are identical. Because of extensive recombination in phage T4, two nonidentical mutants will give rise to many rII^+ phages.

Chemically Reactive Carcinogens

β-Propiolactone treatment of bacteriophage T4. T4 bacteriophages were centrifuged at 27,000 × *g* for 1.5 hr. The supernatant fraction was discarded, and a suspension medium containing 0.07 M NaCl, 0.01 M $MgCl_2$, and 0.05 M Tris buffer, pH 8, was added. The titer was 1×10^{12} phages/ml. Two milligrams of β-propiolactone were added to 1 ml of phage suspension. After 20–40 min at 37°, the phages were diluted 10-fold into a quenching medium containing Tryptone broth, 0.04 M sodium thiosulfate, 1% Casamino acids, and a few drops of chloroform. This standard quenching solution was used for all other chemically reactive carcinogens tested.

The treated stock had an r mutant to normal phage ratio × 10^4 of 50, with 2.9 logs of phage killing (0.2% survival).

Propane sultone. T4 phages were treated at 37° for 1¾ hr with 1.6 mg of propane sultone per milliliter of phage suspension. The phage stock (r:normal × 10^4 = 5.9) was prepared in the same way as in the β-propiolactone experiments. The treated stock had an r mutant to normal phage ratio × 10^4 of 110, with 2.8 logs of killing.

7-Fluoro-N-acetoxy-2-acetylaminofluorene. The phages used for 7-fluoro-*N*-acetoxy-2-acetylaminofluorene, glycidaldehyde, nitrogen mustard, and the glucuronide of *N*-hydroxy-2-acetylaminofluorene were purified by the same procedure used in the β-propiolactone treatment, except that the suspension medium contained 0.006 M Tris buffer, pH 7.2. One milliliter of phage stock (r mutant:normal × 10^4 = 3.7) was treated with 250 μg of 7-fluoro-*N*-acetoxy-2-acetylaminofluorene in 0.05 ml of dimethyl sulfoxide for 2 hr at 37°. The treated stock had an r mutant to normal ratio × 10^4 of 13, with 3.9 logs of phage killing. Under these conditions 500 μg of *N*-acetoxy-2-acetylaminofluorene were needed to produce the same toxicity.

Glycidaldehyde. Since glycidaldehyde tends to polymerize to form an insoluble, amorphous mass, it was impossible to determine the exact concentration of the active compound used. Liquid glycidaldehyde was diluted 300-fold into the phage stock and allowed to react for 30 min at 37°. The treated stock had an r mutant to normal phage ratio × 10^4 of 42, with 3.95 logs of killing.

Nitrogen mustard. The phage stock was treated for 30 min at 37° with 10 μg of nitrogen mustard per milliliter of phage suspension. The treated stock had an r mutant to normal ratio × 10^4 of 17, with 3.08 logs of killing.

Glucuronide of N-hydroxy-2-acetylamino-

fluorene. The phage stock was treated for 24 hr at 37° and pH 8.3 with 5 mg of compound per milliliter of phage suspension. The treated stock had an r mutant to normal ratio $\times 10^4$ of 3.6, with 4.7 logs of killing. The control had an r mutant to normal ratio $\times 10^4$ of 3.7.

Chemically Nonreactive Carcinogens

Toxicity to bacteria. A growth curve of viable bacteria was determined for all chemically nonreactive carcinogens, which were added to *E. coli* BB in M-9 medium (13).

Mutagenesis of intracellular phage. If a compound was found to be toxic to bacteria, it was tested for mutagenic activity toward T4 phage growing in *E. coli* BB (a permissive host) in M-9 medium. The number of infecting phages per bacterium (multiplicity of infection) was between 7 and 10. The bacteria were infected immediately after addition of the compound, and the suspension was incubated at 37° for 25 min in a shaker bath. The suspension was then diluted 20-fold into fresh, warm M-9 medium (without the compound), and growth was continued for an additional 90 min at 37° in the shaker bath. A few drops of chloroform were added to lyse the bacteria, and the sample was centrifuged to remove the bacterial debris. The titer of the phage in the supernatant fraction (number of phages per milliliter) and burst size (number of phages per bacterium) were determined (13).

The size of the burst indicated toxicity. The untreated control had burst sizes of 100–200, whereas a toxic compound added in concentration appropriate for mutagenesis produced burst sizes of 0.01–0.5.

The ratio of r mutants to wild type phage was a measure of mutagenesis. Several thousand plaques were examined to determine this ratio. Each plate was seeded with about 10^8 *E. coli* B cells plus 1000 phages and incubated overnight, and the resulting 1000 plaques were examined. The characteristic large and clear plaque morphology of the rII mutants was easily distinguished from the small and fuzzy-edged wild type plaques.

Dimethyl sulfoxide. Dimethyl sulfoxide was used to solubilize the carcinogens in many cases. *E. coli* in either stationary or growth phase and T4 phage growing in *E. coli* were both unaffected by 4% dimethyl sulfoxide. Free T4 phage was not affected by exposure to 15% dimethyl sulfoxide for 2 hr at 37°.

RESULTS

The forward mutation assay detects agents that cause an inactivation of gene function that is heritable.[3] Since a mutagen causes random lesions throughout the genome, toxicity will occur because of the inactivation of critical genes. If no toxicity is seen in a bacterial assay at the maximum concentration that can be used, the compound is not assayed for mutagenic activity. Twenty-five carcinogens and four noncarcinogens not toxic to *E. coli* BB are 9,10-dimethyl-1,2-benzanthracene; 1,2,5,6-dibenzanthracene; 3-methylcholanthrene; 3,4-benzopyrene; 10-hydroxymethyl-1,2-benzanthracene; 10-methyl-1,2-benzanthracene; 10-methyl-4-fluoro-1,2-benzanthracene; 10-methyl-3-fluoro-1,2-benzanthracene;[4] 1,2,3,4-dibenzanthracene;[4] 9-methyl-1,2-benzacridine;[4] 9-methyl-3,4-benzacridine; 1,2,5,6-dibenzanthracene-9,10-endosuccinate;[4] tricycloquinazoline; 2-methyltricycloquinazoline; aflatoxin B_1; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)hydrochloride (Natulan); *N*-methyl-4-aminoazobenzene; *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene;[5] *N*,*N*-dimethyl-4-aminoazobenzene; 2-aminofluorene; 2-acetylaminofluorene; *N*-hydroxy-2-acetylaminofluorene; *N*-acetoxy-4-acetylaminostilbene; *N*-acetoxy-2-acetylaminophenanthrene; *N*-acetoxy-4-acetylaminobiphenyl;[5] 4-dimethylaminoazobenzene *N*-oxide; 2-naphthylamine; diethylnitrosamine; and dimethylnitrosamine.

Carcinogenic Compounds Toxic But Not Mutagenic to T4 Phage

Four inorganic salts were toxic but not mutagenic to intracellular bacteriophage T4.

[3] Gross genetic lesions such as chromosomal breaks, giant translocations, and chemical cross-linkage are usually lethal (nonheritable) and therefore only represent toxicity. Nitrogen mustard is an example of a chemical that produces mainly nonheritable genetic defects.

[4] Not carcinogenic or very weakly carcinogenic.

[5] Weakly toxic.

These compounds [$NiSO_4$, 300 μg/ml; $Co(NO_3)_2$, 600 μg/ml; $CaCrO_4$, 100 μg/ml; $Pb(CH_3COO^-)_2$, 600 μg/ml] were tested in M-9 medium with various concentrations of Mg^{2+} (0.001 M, 0.0001 M, and no Mg^{2+}) present during the first 25-min exposure. By contrast, using low Mg^{2+} concentrations, Orgel and Orgel (25) and Demerec *et al.* (4) showed that Mn^{2+} is mutagenic for both T4 phage and bacteria. Manganese ion, however, does not produce tumors in birds (26) or in humans (27–29). Lead acetate (30), calcium chromate (31), and cobalt nitrate (32) have been reported to be carcinogenic. Divalent nickel compounds are very carcinogenic (32), although nickel sulfate has not been reported to cause tumors.

Five carcinogenic compounds [*N*-hydroxy-1-naphthylamine, 25 μg/ml; *N*-hydroxy-2-naphthylamine, 50 μg/ml; *N*-hydroxy-2-aminofluorene, 20 μg/ml; 10-formyl-1,2-benzanthracene (33), 1000 μg/ml; and DL-ethionine, 1500 μg/ml] were toxic but not mutagenic to intracellular bacteriophage T4. *N*-Hydroxy-1-naphthylamine (34) and *N*-hydroxy-2-naphthylamine (33, 34) have been reported to cause mutations in bacteria, although we consider that the significance of the mutagenesis data presented in those two reports is marginal (less than a 10-fold increase in the frequency of revertants in back-mutation experiments).

Carcinogenic, Chemically Reactive Compounds Toxic and Mutagenic to T4 Phage

β-Propiolactone. This compound is a monofunctional alkylating agent. Used at high doses in a sensitive strain of mice (35), β-propiolactone is a moderately active tumor initiator. It is also a moderately active mutagen (9). The primary chemical reaction with DNA is an attack on nitrogen 7 of guanosine (35). Figure 1 shows the various consequences of this reaction. Acidic conditions favor depurination (37), whereas basic conditions favor ring opening between carbon 8 and nitrogen 7 (38). There may be a scission of the DNA chain after depurination (39).

β-Propiolactone-treated T4 phage undergoes a time- and pH-dependent loss of viability (storage death) (Fig. 2). After 115 hr at 25° there was a 2.6 log decrease in viability at pH 6, whereas there was a 5.3 log decrease at pH 8. Untreated phage showed no loss of viability on incubation under comparable conditions. Since storage death was accelerated by basic conditions, it is unlikely that depurination alone was the lethal event. Ring opening, which is favored by basic conditions, may have been the cause of storage death. Nitrogen mustard, which also attacks N-7 of guanine, did not cause storage death. Phages treated with propane sultone, glycidaldehyde, and *N*-acetoxy-2-acetylaminofluorene also did not undergo storage death.

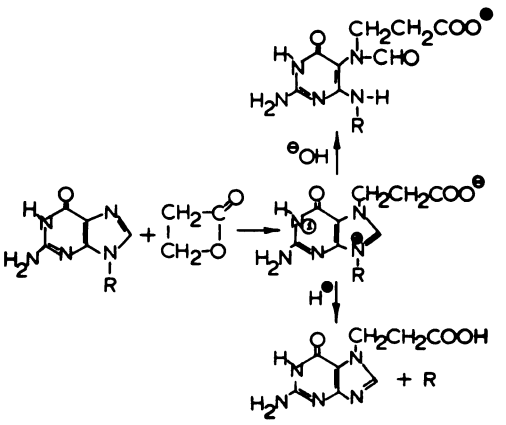

FIG. 1. *Reaction of β-propiolactone with guanosine and derivatives*

7-(2-Carboxyethyl)guanine is the primary product obtained from hydrolysis of mouse skin DNA treated *in vivo* with β-propiolactone (35). An increased ionization at N-1 of 7-(2-carboxyethyl)deoxyguanosine may cause mispairing with thymidine during replication (36). Other consequences of the reaction of β-propiolactone with deoxyguanosine include depurination, which is favored under acidic conditions (37), and ring opening, which is favored under basic conditions (38).

Table 1 shows the types of mutants produced by β-propiolactone under nondepurination and depurination conditions. Sixty-one per cent of the mutants (nondepurination conditions) arose by a G//C to A//T base pair transition. This is in agreement with the primary chemical reaction with DNA guanine (35). Frameshift and large deletion mutations were also produced by this treatment.

A β-propiolactone-treated T4 phage stock

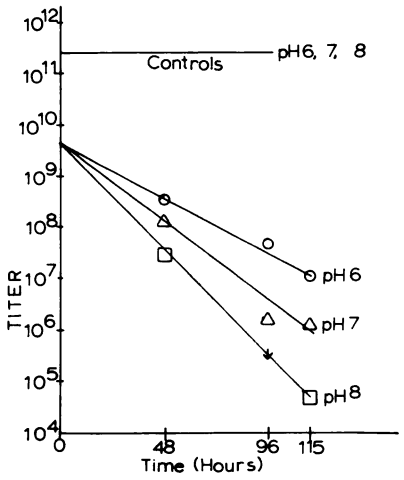

FIG. 2. *Loss of viability of T4 phage treated with β-propiolactone: time and pH dependence (storage death)*

One milliliter of phage suspension was treated with 2 mg of β-propiolactone for 20 min at pH 8 and 37°. The treated phage was diluted in the quenching medium to stop the reaction. After 30 min, samples were adjusted to pH 6, pH 7, and pH 8. These samples were maintained at 25° for 115 hr, and the viability was determined at intervals. Controls were not treated with β-propiolactone.

was maintained for 54 hr at pH 6 and 25° to determine whether depurination would change the mutational spectrum. The results (Table 1) show only a marginal shift in the types of mutations produced. The half-life of the glycosidic bond is not known under these conditions; however, at pH 7.3 and 37° the half-life is 150 hr (39). There was no detectable increase in the ratio of r mutant to normal phage in the treated stock maintained for 54 hr at pH 6. Several other experiments using lower doses of β-propiolactone, designed to detect an increase in the over-all frequency of mutations if due to depurination, failed to show any change.

In order to confirm that β-propiolactone produces G//G to A//T base pair transitions, various G//C mutants were tested for their ability to revert to the wild type with this treatment (Table 2). All G//C mutants tested (five shown in Table 1) reverted significantly. This firmly establishes that β-propiolactone induces guanine//cytosine to adenine//thymine base pair transitions.

It has been postulated (see ref. 14) that one could determine the orientation of a mutant base pair in the rII segment by

TABLE 1

T4rII mutants produced by β-propiolactone

Nondepurination conditions: The r mutant to normal ratio × 10^4 in the untreated stock was 3.3. After treatment with β-propiolactone the r mutant to normal ratio × 10^4 was 50. Therefore less than 8% of the mutants collected were of spontaneous origin. T4 phage (10^{12}/ml) was treated with 2 mg of β-propiolactone per milliliter of phage suspension at pH 8 and 37° for 24 min. The reaction was stopped by dilution of the phage in the standard quenching solution. The treated phages were plated immediately on *E. coli* to minimize depurination and ring opening.

Depurination conditions: The r mutant to normal ratio × 10^4 in the untreated stock was 3.3. After treatment the frequency was 35. Therefore less than 10% of the mutants collected were spontaneous. Conditions for treatment with β-propiolactone were the same as above, except that the treated sample was maintained at pH 6 and 37° for 54 hr before plating on *E. coli* B.

Type of mutation[a]	Nondepurination conditions		Depurination conditions	
	No. of mutants	Per cent of total	No. of mutants	Per cent of total
Frameshift	19	20	16	34
G//C → A//T transition	35	36	12	26
A//T → G//C transition	6	6	1	2
Large deletion	12	13	8	17
UGA (G//C → A//T transition)	13	14	2	4
Amber (G//C → A//T transition)	11	11	7	15
Ochre (G//C → A//T transition)	0	0	1	2
Total	96		47	

[a] See EXPERIMENTAL PROCEDURE for a more detailed explanation of mutation types.

asking whether a base-specific mutagen will produce the revertant phenotype when the treated phage is challenged on the selective host immediately after treatment, or whether an intervening round of growth on the permissive host is required to unmask the revertant phenotype. Were this supposition true, a mutagen which reacts specifically with cytosine should not in general cause immediate reversion of a mutant that immediately is reverted by a guanine-specific mutagen. We have tested this postulate by asking whether rII mutants which revert upon hydroxylamine treatment and immediate challenge also revert upon β-propiolactone treatment and immediate challenge. Our results negate the supposition: all mutants immediately revertible by hydroxylamine were also immediately revertible by β-propiolactone. Thus, this method for determining base pair orientation does not appear to be valid.

TABLE 2

Backward mutagenesis: reversion of frameshift and G//C T4rII mutants by β-propiolactone

The treatment conditions were the same as in Table 1. The frameshift mutants FC0 (+) and FC1 (−) were collected by Crick (40). The other frameshift mutants were produced by treatment with β-propiolactone. The G//C mutants were all mis-sense and were produced by treatment with 2-aminopurine. The logs of killing after treatment indicate the extent of reaction with β-propiolactone. A 10-fold increase in *f*(revertants) over the control value is considered significant backward mutagenesis. For example, this would be 1 normal T4 bacteriophage/10^6 mutants converted to 10 normal bacteriophages/10^6 mutants by the treatment. The reversion of these mutants proves that β-propiolactone causes G//C to A//T base pair transitions.

Mutant	Logs of killing	Increase in *f* (revertants) over control
		-fold
Frameshift mutants		
FC0 (+)	3.78	60
FC1 (−)	3.00	42
No. 104	2.95	50
No. 89	3.70	100
No. 49	3.70	61
G//C mutants		
A-17	3.48	1200
A-5	2.91	370
A-7	2.43	620
A-36	3.48	93
A-8	3.30	64

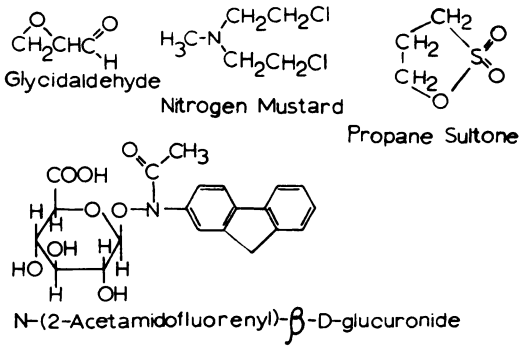

FIG. 3. *Structures of compounds*

All except one of nine frameshift mutants (five shown in Table 2) examined were revertible by β-propiolactone. All these mutants were tested more than once, with the same result. The data indicate that β-propiolactone can revert frameshift mutations.

Propane sultone. Propane sultone (structure in Fig. 3) is a monofunctional alkylating agent. Its half-life in water containing 2.8% dioxane at 20° is 14 hr (41). Propane sultone at a high dose is a potent skin tumor initiator.[6] It also produces sarcomas with single or repeated injection (42). Table 3 shows the types of rII mutants produced by propane sultone. Base pair transitions account for 77% of the mutations (nonsense mutants included). Most of these are G//C to A//T transitions (nonsense mutants probably arise in this way). This compound is a very potent mutagen.

N-Acetoxy-2-acetylaminofluorene and 7-fluoro-N-acetoxy-2-acetylaminofluorene. The structures and interconversions *in vivo* of the aminofluorene derivatives (11, 12) are shown in Fig. 4. The primary chemical reaction of *N*-acetoxy-2-acetylaminofluorene is with carbon 8 of guanine in DNA (11, 12, 43). These esters of 2-acetylaminofluorene are chemically reactive and are strongly carcinogenic

[6] G. T. Bowden and R. K. Boutwell, private communication.

TABLE 3

T4rII mutants induced by propane sultone

The r mutant to normal ratio × 10^4 in the untreated stock was 5.9. After treatment the frequency was 110. Therefore less than 6% of the mutants collected were spontaneous. T4 phage was treated with 1600 μg of propane sultone per milliliter of phage suspension. The treatment was carried out at 37° for 1¾ hr. The reaction was stopped by dilution of the phage into the standard quenching solution.

Type of mutation induced	No. of mutants	Per cent of total
G//C → A//T transition	11	26
A//T → G//C transition	8	19
Frameshift	9	21
UGA (G//C → A//T) transition	10	24
Amber (G//C → A//T) transition	3	7
Large deletions	1	2
Total	42	

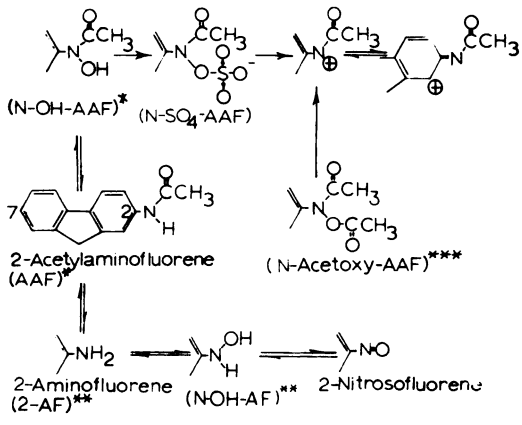

FIG. 4. *Interconversions of aminofluorene derivatives in vivo (11)*

All compounds shown are carcinogenic (11). The most probable reactive form of 2-acetylaminofluorene (AAF) *in vivo* is the *N*-sulfate (11, 12).

* Not toxic in the bacterium-phage system.

** Toxic but not mutagenic to intracellular T4 phage.

*** Toxic and mutagenic to T4 phage.

at local sites of injection (11). They have been found to be mutagenic in bacterial transforming DNA (44). We have investigated the specific types of mutations induced.

The main transitional event was an A//T to G//C conversion (Table 4). This was unexpected, since these compounds react primarily with guanine *in vitro* (11, 12 43). However, an adenine–2-acetylaminofluorene product is also produced in the chemical reaction to 1% of the extent found with guanosine (11).

Since *N*-acetoxy-2-acetylaminofluorene, 7-fluoro-*N*-acetoxy-2-acetylaminofluorene, nitrogen mustard, and glycidaldehyde showed only weak or moderate mutagenic activity, it was decided to cross the carcinogen-induced mutants with spontaneous rII mutants collected from the untreated stock. Mutants can be proved to be identical or different by using the Benzer cross technique (see description of T4 system under EXPERIMENTAL PROCEDURE). The same T4 phage stock was used for the production of rII mutants by 7-fluoro-*N*-acetoxy-2-acetylaminofluorene, glycidaldehyde, and nitrogen mustard. Ten rII mutants were collected from the untreated stock. One was an amber, one was a G//C mis-sense (5-bromouracil-, 2-aminopurine-, and hydroxylamine-revertible), and eight were frameshift mutants. Three of the frameshift mutants were the same.

7-Fluoro-*N*-acetoxy-2-acetylaminofluorene-induced mutants were crossed with the appropriate spontaneous mutants to determine identity. Of the 53 mutants, the one amber mutant and 16 of the 30 frameshift mutants were pre-existing and spontaneous. None of the 2-acetylaminofluorene-induced G//C mutants collected was identical with the one spontaneous G//C mutant. If the data in Table 4 (7-fluoro-*N*-acetoxy-2-acetylaminofluorene) are recalculated, excluding the pre-existing spontaneous mutants, A//G to G//C base pair transitions represent 37%, and frameshift mutants 39%, of the total rII mutants collected.

Examination of various mutants in back-mutation experiments was consistent with the forward mutation data. We also unequivocally established that treatment with 7-fluoro-*N*-acetoxy-2-acetylaminofluorene induced A//T to G//C base pair transitions. We treated an ochre mutant, N-24 (DNA code word, ATT//TAA), and obtained a 70-fold increase over the normal revertants

TABLE 4

T4rII mutants induced by N-acetoxy-2-acetylaminofluorene and its 7-fluoro derivative

7-Fluoro-*N*-acetoxy-2-acetylaminofluorene, 250 μg in 0.1 ml of dimethyl sulfoxide, was added to 0.9 ml of purified T4 bacteriophage stock at pH 7.2. The mixture was incubated at 37° for 45 min. The reaction was stopped by dilution of the suspension 10-fold in the standard quenching solution. Ten rII mutants collected from the untreated stock were crossed with the carcinogen-induced mutants to determine identity. Thirty-two per cent of the mutants collected were of spontaneous origin. For treatment with *N*-acetoxy-2-acetylaminofluorene, the conditions were identical except that the concentration of *N*-acetoxy-2-acetylaminofluorene was 500 μg/ml of phage solution. The phage stocks used in these two experiments were different. The r mutant to normal ratio × 10^4 for the stock used in the *N*-acetoxy-2-acetylaminofluorene experiment was 3.0. After treatment the frequency was 12. Therefore 25% of the mutants collected from the treated stock were spontaneous in origin.

Type of mutation	7-Fluoro-*N*-acetoxy-2-acetylaminofluorine		*N*-Acetoxy-2-acetylaminofluorine	
	No. of mutants	Per cent of total	No. of mutants	Per cent of total
A//T → G//C transition	13	25	8	23
G//C → A//T transition	0	0	0	0
UGA (G//C → A//T transition)	2	4	4	11
Amber (G//C → A//T transition)	1[a]	2	1	3
Large deletion	7	13	5	14
Frameshift	30[b]	47	17	49
Total	53		35	

[a] This amber mutant was spontaneous.

[b] Sixteen of these frameshift mutants were spontaneous.

and, at the same time, a uniformly stimulated transition of the last base pair of the code word of the ochre mutant (T//A to C//G), which was detected because these are amber mutants (DNA code word, ATC//ATG). Belman *et al.* (34) have reported preliminary findings (no data) to indicate that *N*-acetoxy-2-acetylaminofluorene caused G//C to A//T base pair transitions.

The glucuronide of *N*-hydroxy-2-acetylaminofluorene (Fig. 3) was also tested for mutagenic activity.[7] This compound produces chemically the same primary guanosine product as *N*-acetoxy-2-acetylaminofluorene, but to a much smaller extent (45). Another deoxyguanosine product that lost the acetyl group is the major compound formed (45–47). The glucuronide is more reactive under basic conditions than at pH 7. Although ample toxicity was obtained, there was no mutagenesis. Repeated injections of sodium and calcium salts and the triacetylmethyl ester of the glucuronide of *N*-hydroxy-2-acetylaminofluorene produced one, one, and four sarcomas, respectively, in each group of 16 rats (45). The compound is either weakly carcinogenic or noncarcinogenic, and its metabolism and excretion in the rat have been studied (48).

Glycidaldehyde. The types of rII mutants induced by glycidaldehyde, a weak carcinogen (49) (structure, Fig. 3), are shown in Table 5. Ten spontaneous mutants from the untreated stock were crossed with the glycidaldehyde-induced mutants. None of the glycidaldehyde mutants was the same as the 10 spontaneous mutants. This demonstrates that almost all of the mutants collected were induced by the glycidaldehyde treatment.

The major transitional event induced by this mutagen was a A//T to G//C conversion (40%). There was also a significant number of frameshift mutants (44%). This compound requires a much larger phage killing than β-propiolactone to produce the same amount of mutagenesis. With 4 logs of killing, glycidaldehyde treatment produces about the same mutagenesis as 2.9 logs of killing with β-propiolactone.

[7] Sample obtained from C. C. Irving.

TABLE 5

T4rII mutants induced by glycidaldehyde

The r mutant to normal ratio × 10^4 of the untreated stock was 3.7. After treatment the frequency was 42. Therefore the number of spontaneous mutants in the treated stock was less than 9%. None of the mutants was found to be identical with any of 10 spontaneous mutants collected from the untreated stock. Glycidaldehyde was diluted 300-fold into the phage suspension and incubated at 37° for 30 min.

Type of mutant	No. of mutants	Per cent of total
Frameshift	11	44
A//T → G//C transition	10	40
G//C → A//T transition	1	4
Large deletion	2	8
UGA (G//C → A//T transition)	1	4
Total	25	

TABLE 6

T4rII mutants induced by nitrogen mustard

One milliliter of phage solution was treated with 10 μg of nitrogen mustard for 30 min at 37°. The reaction was stopped by dilution of the phage in the standard quenching solution.

Type of mutant	No. of mutants	Per cent of total
Large deletions	8	32
UGA (G//C → A//T transitions)	4	16
G//C → A//T transitions	1	4
Frameshift[a]	12	48
Total	25	

[a] Eight of the frameshift mutants were found to be spontaneous in origin. Therefore 32% of the mutants collected were spontaneous.

Nitrogen mustard. The types of rII mutants induced by nitrogen mustard (structure, Fig. 3) are shown in Table 6. Ten spontaneous mutants from the untreated stock were crossed with the chemically induced mutants. Eight of the frameshift mutants were found to be identical with spontaneous frameshift mutants collected from the untreated stock. None of the other mutants was spontaneous.

The main transitional event was a G//C to A//T conversion. These transitions accounted for 29% of the nonspontaneous mutants. The major mutagenic event was the production of large deletions, which accounted for 47% of the nonspontaneous mutants. Since so many of the frameshift mutants were spontaneous, it was not possible to determine whether nitrogen mustard has this specificity. This compound is a very inefficient mutagen (50). Even with a high extent of reaction (as measured by the logs of phage killing), there was only a very small increase in the number of mutants in the stock. This compound is also a very inefficient carcinogen (51).

DISCUSSION

Twenty-five compounds (including 10 polycyclic hydrocarbons or derivatives) that are toxic and carcinogenic to mammals were not toxic to *E. coli* BB or to bacteriophage T4 and therefore could not be tested for mutagenic activity. This lack of toxicity is probably due either to the absence of enzymes in the bacteria and phage necessary to metabolize the compounds to a chemically reactive form or to the lack of permeability of the bacterial membrane to these compounds. In mammalian cells, many of these carcinogens are changed to a chemically reactive form and become covalently bound to cellular constituents (52–55). Covalent binding with some macromolecule is probably necessary to produce the carcinogenic event (1, 11).

Nine carcinogens were tested that were toxic to intracellular T4 phage but were not mutagens. Four of these compounds were carcinogenic inorganic salts. By contrast, manganese, which is apparently not carcinogenic (26–28), is a potent mutagen in bacteria and phage (4, 25).

Table 7 summarizes the results we obtained with the six chemically reactive carcinogens. These findings have clarified the modes of mutagenic action of several chemically reactive compounds. Brookes and Lawley (36) proposed that the increased ionization of the hydrogen on nitrogen 1 of a 7-alkyldeoxyguanosine might cause it to mispair with thymine during replication. Since β-propiolactone reacts mainly with

TABLE 7

Summary of results obtained with six chemically reactive carcinogens

Compound	Chemical reaction with DNA	Types of mutation induced
β-Propiolactone	Guanine N-7, causing ring opening between N-7 and C-8 and also depurination	G//C → A//T base pair transitions Frameshift
Propane sultone	Unknown	G//C → A//T base pair transitions A//T → G//C base pair transitions Frameshift
N-Acetoxy-2-acetylaminofluorene and its 7-fluoro derivative	C-8 of guanine (major) or adenine (minor)	A//T → G//C base pair transitions Frameshift
Glycidaldehyde	Unknown	A//T → G//C base pair transitions Frameshift
Nitrogen mustard	Guanine N-7 (major) and also cross-linking of guanine residues in DNA	Large deletions G//C → A//T base pair transitions

N-7 of deoxyguanosine in DNA (35) and causes primarily G//C to A//T base pair transitions, this type of mispairing seems the most probable cause of β-propiolactone-induced mutagenesis. Our results with this compound show that depurination neither enhanced the frequency of mutants nor caused a marked shift in the type of mutants collected.

Propane sultone was an effective transition mutagen in both directions. Since propane sultone did not cause storage death, it is unlikely that it reacts extensively with nitrogen 7 of guanine.

Glycidaldehyde, *N*-acetoxy-2-acetylaminofluorene, and 7-fluoro-*N*-acetoxy-2-acetylaminofluorene caused A//T to G//C base pair transitions. Although the 2-acetylaminofluorene derivatives undergo chemical reactions primarily with deoxyguanosine (11), it seems likely from our data that deoxyadenosine is the primary target in DNA for the mutagenesis induced by these compounds.

It is well known that nitrogen mustard induces extensive chromosomal aberrations (10). One must be careful to remember that the only kinds of mutations that can be involved in carcinogenesis are those that alter rather than kill the cell. The observation of chromosomal aberrations immediately after treatment is not sufficient evidence that these aberrations are candidates for carcinogenic mutations.

The frameshift mutants produced by most of these compounds may not be relevant for somatic growth. Frameshift mutations may occur only during recombination (56–58), and mitotic recombination is a rare event in those organisms in which it can be measured.

Our results with the six chemically reactive carcinogens have shown that there is no quantitative correlation between the effectiveness of a compound as a mutagen in this system and its potency as a carcinogen. Propane sultone and β-propiolactone were the most potent mutagens; β-propiolactone is a weak carcinogen. The most carcinogenic of the six are the 2-acetylaminofluorene derivatives, which are very inefficient mutagens. No single characteristic type of mutation was induced by these six compounds. This last finding indicates that if a compound is carcinogenic because it can induce mutations, any type of gene-inactivating mutation is likely to suffice. Yet carcinogens appear to have a great variation in their dose-related specificity for tumor induction. For example, 0.004 μmole of 9,10-dimethyl-1,2-benzanthracene is equivalent in tumor-initiating potency to 240 μmoles of β-propiolactone in mouse skin, although 0.1 μmole of the former compound is bound per mole of DNA phosphorus (52) compared to

400 μmoles of the latter (37). If the binding of the carcinogen to DNA produces a mutation that is responsible for carcinogenesis, it is striking that there is a 4000-fold difference in the amount of these compounds bound to DNA although the carcinogenic response is equivalent.

The results obtained with β-propiolactone, propane sultone, glycidaldehyde, nitrogen mustard, *N*-acetoxy-2-acetylaminofluorene, and its 7-fluoro derivative support the theory that chemically reactive carcinogens have a mutagenic capacity. The glucuronide of *N*-hydroxy-2-acetylaminofluorene, which is chemically reactive, was not mutagenic. Its weak carcinogenic activity, however, could result from breakdown to *N*-hydroxy-2-acetylaminofluorene. It is also clear that although a carcinogenic compound may be inactive in a test system for mutagenesis, its proximal metabolite can be mutagenic, as in the case of the acetylaminofluorene derivatives (44).

Recent work with mammalian cell culture systems has shown that mutations can be produced by chemicals and detected using drug resistance or nutritional requirements as markers (59, 60). Chemical carcinogenesis *in vitro* has been achieved in various mammalian cell culture systems (61–65), and polycyclic aromatic hydrocarbons produce a frequency of colonies of transformed and malignant cells that is proportional to their carcinogenic activities *in vivo* (62, 66, 67). Attempts are now being made in this laboratory to assay chemical mutagenesis and malignant transformation in the same cell line. If successful, these studies could generate a positive or negative correlation between carcinogenesis and mutagenesis in a system considerably more relevant than those presently available. Unfortunately, however, even a completely consistent correlation, whether positive or negative, would not constitute a final proof of whether or not the mechanism of chemical carcinogenesis involves a somatic mutation.

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