Different Patterns of Mutagenicity of Arenediazonium Ions in V79 Cells and *Salmonella typhimurium* TA102: Evidence for Different Mechanisms of Action

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The edible mushroom *Agaricus bisporus* contains several arylhydrazines and arenediazonium ions that are genotoxins. The mechanism whereby arylhydrazines and arenediazonium ions are genotoxic is unknown and may be due to the arenediazonium ion itself or to aryl radicals. The reactions of four arenediazonium ions (p-X$-C_6H_5N_2^+$, X = $-CH_3$, $-CH_2OH$, $-CH_2OCH_3$, $-CO_2H$) with purine bases, their mutagenicity, their ability to cause DNA damage, and their tendency toward free radical formation have been studied to elucidate the genotoxic species. It is suggested that either the arenediazonium or aryl radical can act as the ultimate genotoxin. Which species is dominant is dependent upon the arenediazonium ion reduction potential. This relationship may be useful in designing future studies of arenediazonium ion genotoxicity.

Keywords: *Arenediazonium ion; aryl radical; mutagenicity*

INTRODUCTION

The edible mushroom *Agaricus bisporus* contains several arylhydrazides, arylhydrazines, and arenediazonium ions. Examples include N^2-[L-(+)-glutamyl]-4-(hydroxymethylphenyl)hydrazine (agaritine) (Levenberg, 1961; Kelly et al., 1962), N^2-[L-(+)-glutamyl]-4-(carboxyphenyl)hydrazine (Chauhan and Toth, 1984), p-hydroxymethylphenylhydrazine (Stijve et al., 1986), and 4-(hydroxymethyl)benzenediazonium ion (Levenberg, 1962; Ross, 1982). 4-Methylbenzene diazonium ion may also be present in *A. bisporus* (Toth et al., 1989). With the singular exception of agaritine, all of these compounds and the mushroom itself are mutagenic or produce tumors in mice (Toth and Gannett, 1993). *A. bisporus* also has been identified as a natural carcinogen source (Ames et al., 1987).

The mechanism whereby arylhydrazines and arenediazonium ions, such as those found in the *A. bisporus* mushroom, express their genotoxicity has not been elucidated. It is known that arylhydrazines are oxidatively metabolized to their corresponding arenediazonium ions. We have shown this for the arylhydrazines present in *A. bisporus*, and both cytochrome P-450 and prostaglandin(H)synthase convert them to the corresponding arenediazonium ions (Lawson and Chauhan, 1985; Lawson, 1987). The fate of the arenediazonium ion is less clear. This species may be the ultimate genotoxin. Alternatively, the ultimate genotoxin may be an aryl cation (Malaveille et al., 1982), an aryldiazene or diazenyl radical (Kosower, 1971), or an aryl radical (Galli, 1988) since these species can be formed from arenediazonium ions (Figure 1).

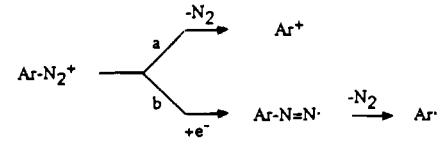

Figure 1. Decomposition pathways for arenediazonium ions leading to (a) aryl cations or (b) diazenyl and aryl radicals. The actual pathway followed depends on both the identity of the diazonium ion and the specific reaction conditions.

Previous studies have suggested the arenediazonium ion as ultimate genotoxin. Chin et al. (1981) and Hung and Stock (1982) investigated the reaction of arenediazonium ions with purines, purine nucleosides, and purine nucleotides. The major product obtained from adenine or adenosine is the C^6-NH_2 triazene. Triazene formation at C^2-NH_2 of guanosine 5′-monophosphate is also observed. These products require that the arenediazonium ion is the reactive intermediate. However, the reaction products of arenediazonium ions were C^8-azo-arylguanine derivatives, and with guanosine, C^8-aryl adducts are the major product (Figure 2). This latter type of product, found by Hung et al. (1982) and also by us (Gannett et al., 1987), suggests that aryl radicals are also formed from arenediazonium ions and therefore may serve as genotoxins, too.

At the DNA level the formation of the triazene adducts with adenine has been indirectly shown. In particular, Koepke et al. (1990) treated DNA with benzene diazonium ion followed by sodium borohydride to reduce the benzene diazonium ion–adenine adduct to aniline and N-aminoadenine. The latter compound was found to be present by HPLC. The formation of C^8-arylguanine adducts was not determined. *In vitro*, arylhydrazines can be oxidized to arenediazonium ions. Their intermediacy has been demonstrated, for example, by trapping with β-naphthol (Lawson and Chauhan, 1985; Lawson, 1987). It is therefore likely that reactions of biological significance result directly from the arenediazonium ion.

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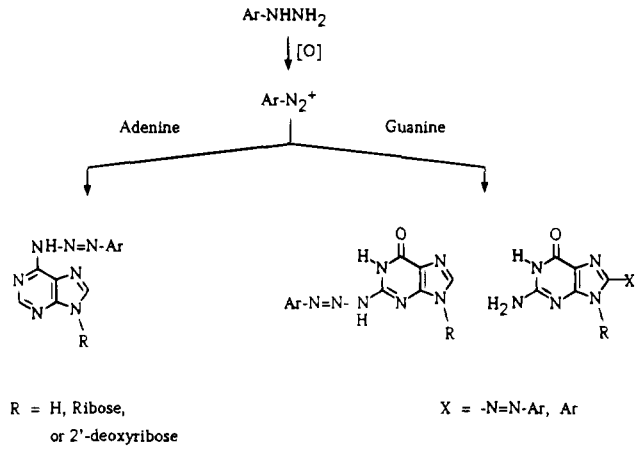
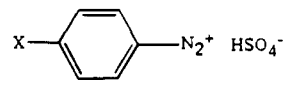


Figure 2. Reactions observed to occur between arenediazonium ions and adenine and guanine and their nucleoside and nucleotide derivatives. [O] refers to chemical or biological oxidation. The specific product formed from guanine and its nucleosides and nucleotides depends on the specific diazonium ion and whether guanine, guanosine, 2′-deoxyguanosine, or 2′-deoxyguanosine 5′-monophosphate is the coreactant.

The chemical generation of aryl radicals from arenediazonium ions is well established and can be accomplished by treating them with reducing agents such as iron(II), ascorbic acid, thiols, and hydroquinones. The chemical reduction of arenediazonium ions first produces a diazenyl radical, which can either abstract hydrogen to form a diazene or lose nitrogen to produce an aryl radical. Since the reducing agents used chemically to produce aryl radicals from the diazonium ions are agents that are also cellular reductants, this process may occur *in vivo*. Two recent studies support this conclusion (Kikugawa et al., 1992; Kato et al., 1992). In particular, the reaction of both *p*- and *o*-hydroxybenzene diazonium ions with DNA nucleosides was investigated. Evidence for the involvement of aryl radicals under a variety of biologically relevant conditions was demonstrated by ESR spin trapping techniques. In addition to the ESR evidence for the intermediacy of aryl radicals from diazonium ions, the formation of C^8-aryl adducts of adenosine and guanosine and DNA single-strand damage were also demonstrated. Thus, aryl radicals are formed from arenediazonium ions, DNA adducts are produced, and DNA damage is caused by them.

Both arenediazonium ions and aryl radicals are formed by metabolism of arylhydrazines. Whether the predominant intermediate is the arenediazonium ion or aryl radical forms determines the type of DNA adducts formed and, perhaps, the observed mutagenicity. The design of studies concerning arylhydrazine genotoxicity will also be impacted. Therefore, it would be useful to be able to predict what the predominant species will be, arenediazonium ion or aryl radical, from a particular arylhydrazine. Since the reduction potential of the arenediazonium ion will control which intermediate forms, a relationship between reduction potential and DNA adduct, DNA damage, or genotoxicity likely exists. We have previously observed differences between biological activity and the substitution of arylhydrazines (Lawson and Chauhan, 1985; Lawson, 1987). Doyle et al. (1985) have shown that both the rate and mechanism of the reaction of arenediazonium ions with hemoglobin showed a dependence on the reduction potential of the arenediazonium ion.

In this study we sought to determine whether or not aryl radicals, in addition to arenediazonium ions, might



X = CH_3	MBD
X = CH_2OH	HMBD
X = CH_2OCH_3	MMBD
X = CO_2H	CBD
X = CHO	FBD

Figure 3. Structure of the diazonium ions examined in this investigation. Of the diazonium ions shown, only HMBD is actually known to be present in *A. bisporus*.

be involved in the genotoxicity of the arenediazonium ions found in *A. bisporus* and related arenediazonium ions (Figure 3). In addition, we have examined the importance of the para substituent. Since the para substituent will affect the ease of arenediazonium ion reduction and hence the ease of aryl radical formation, the para substituent may control the relative importance of the arenediazonium ion and aryl radical. Our studies find that differences in mutagenicity, DNA damage, and aryl radical formation do exist for the arenediazonium ions studied. The observed differences are related to the para substituent and correlate with the reduction potential of the arenediazonium ion and hence the genotoxic potential.

MATERIALS AND METHODS

Safety Note. Arenediazonium ions are genotoxins and should be handled accordingly. Arenediazonium ions, especially when completely dry, are shock sensitive and may detonate.

General Procedures. Chemicals, including *p*-toluidine, *p*-nitrobenzyl alcohol, *p*-aminobenzoic acid, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), platinum dioxide, catechol, sodium cacodylate, ferrous ammonium sulfate, and nitrosyl hydrogen sulfate were purchased from Aldrich. Chelex 100 resin was purchased from Sigma. Solvents were purchase from Fisher Scientific. IR spectra were obtained on a Perkin-Elmer IR 1310. NMR spectra (^1H and ^{13}C NMR) were measured on a Varian broadband Gemini 300. UV spectra were measured on a Hitachi U-2000. Mass spectra were measured on a Finnagan 4021 quadrupole mass spectrometer. ^1H NMR and ^{13}C NMR assignments of protonated carbons were made on the basis of COSY and HETCOR data. Nonprotonated carbons were assigned on the basis of chemical shift correlations and should therefore be considered tentative.

***p*-Aminobenzyl Alcohol.** *p*-Nitrobenzyl alcohol (15.3 g, 0.1 mol) was hydrogenated over platinum oxide (288 mg) in absolute ethanol (200 mL) for 1−2 h at 40−50 psi of H_2 at room temperature. The reaction mixture was filtered through Celite and distilled (bulb to bulb) at 100 °C (0.1 mmHg) to yield 9.46 g of *p*-aminobenzyl alcohol (77%): IR (KBr) cm^{-1} 3376 (NH_2, free), 3230 (NH_2, hydrogen bonded), 3010 (ArH), 2928, 2870 (CH), 1607, 1517 (C=C), 1363, 1261, 1000 (CO); ^1H NMR ($CDCl_3$) δ 3.5 (3H, bs), 4.51 (2H, s), 6.66 (2H, d, J = 8.5 Hz), 7.13 (2H, d, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 65.2 (CH_2-OH), 115.1 ($C^{3,5}$), 128.7 ($C^{2,6}$), 131.1 (C^1), 146.0 (C^4); UV (EtOH) nm (log ϵ) 278 (3.54), 223 (3.35).

Methyl *p*-Aminobenzyl Ether. Methylation of *p*-nitrobenzyl alcohol was accomplished by use of the general procedure of Johnstone and Rose (1979). To a suspension of powdered potassium hydroxide (7.5 g, 0.134 mol, 4.1 equiv) in DMSO (65 mL) was added a solution of *p*-nitrobenzyl alcohol (5 g, 32.7 mmol) and methyl iodide (8.34 mL, 4.1 equiv) in DMSO (15 mL) over a 1 h period while the temperature was maintained below 25 °C. The resulting mixture was stirred for an additional hour, poured into water (600 mL), and extracted with methylene chloride (3 × 100 mL). The combined organic extracts were washed with 10% sodium bisulfite

Table 1. NMR Data on the Isolated Guanine–Arenediazonium Ion Adducts[a]

X[b]	ArH	X[b]	1′	2′	2″	3′	4′	5′
guanine[c]								
CH_3	8.00, 7.40	2.37						
CO_2H	8.18, 8.06							
CH_2OH	8.01, 7.26							
CH_2OCH_3	8.08, 7.50	3.34/4.50						
guanosine								
CH_3	7.74, 7.34	2.40	5.67	5.06		4.07	3.83	3.60, 3.57
CO_2H	7.81, 7.97		5.68	5.05		4.11	3.80	3.66, 3.60
CH_2OH	7.65, 7.45	4.58	5.66	5.03		4.10	3.85	3.68, 3.55
CH_2OCH_3	7.66, 7.41	3.32, 4.59	5.67	5.03		4.09	3.88	3.69, 3.55
2′-deoxyguanosine								
CH_3	7.70, 7.33	2.39	6.08	3.10	2.15	4.30	3.80	3.49, 3.47
CO_2H	7.89, 7.79		6.10	3.20	2.17	4.30	3.85	3.51, 3.47
CH_2OH	7.59, 7.46	4.62	6.09	3.16	2.20	4.35	3.83	3.5
CH_2OCH_3	7.60, 7.45	3.30, 4.51	6.05	3.15	2.21	4.34	3.82	3.48, 3.50

[a] All NMR spectra were recorded in DMSO-d_6 at room temperature and referenced to TMS. [b] X refers to the para substituent of the aryl-substituted purine derived from p-X–$C_6H_4N_2^+$ (see Figure 3). [c] Data reported for the guanine derivatives as their hydrochloride salts.

(100 mL) and water (100 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to yield *p*-methoxymethylnitrobenzene (5.05 g, 92.5%): IR ($CHCl_3$) cm^{-1} 3026 (C=CH), 2931 (CH), 1605, 1523 (C=C), 1328, 1104 (CO); ^1H NMR ($CDCl_3$) δ 3.452 (3H, s, CH_3O), 4.444 (2H, s, CH_2-O), 7.383 (2H, d, J = 8.6 Hz), 8.083 (2H, d, J = 8.6 Hz); ^{13}C NMR ($CDCl_3$) δ 64.800 (CH_3), 79.721 (CH_2), 130.05 ($C^{3,5}$), 134.43 ($C^{2,6}$), 153.40 ($C^{1(4)}$), 154.00 ($C^{4(1)}$); UV (EtOH) nm (log ϵ) 269 (4.34).

This product was taken up in absolute ethanol (70 mL), platinum dioxide (90 mg) was added, and the mixture was hydrogenated at 40–50 psi of H_2 for 1–2 h. The reaction mixture was then filtered and concentrated *in vacuo*: IR ($CHCl_3$) cm^{-1} 3462, 3393 (NH_2), 3004 (C=CH), 2926 (CH), 1623, 1519 (C=C), 1279, 1089 (CO); ^1H NMR ($CDCl_3$) δ 3.344 (3H, s, CH_3O), 3.805 (2H, s, NH_2), 4.339 (2H, s, CH_2O), 6.585 (2H, d, J = 8.6 Hz), 7.126 (2H, d, J = 8.6 Hz); ^{13}C NMR ($CDCl_3$) δ 57.556 (CH_3), 74.736 (CH_2), 114.36 ($C^{3,5}$), 127.50 (C^1), 129.54 ($C^{2,6}$), 146.67 (C^4); UV (EtOH) nm (log ϵ) 290 (3.60), 239 (4.25).

Arenediazonium Ions. All arenediazonium ions were prepared as described by Piercey and Ward (1962). In general, the aryl amine (1 mmol) was dissolved in glacial acetic acid (2 mL) (T < 25 °C) and added to a suspension of $NOHSO_4$ (1.05 mmol) in glacial acetic acid (2 mL) under nitrogen while the temperature was maintained below 20 °C. Following the addition the reaction was stirred for 30 min at room temperature. Diethyl ether (10 mL) was then added and the precipitate isolated by filtration through a sintered glass funnel. The filter cake was resuspended in glacial acetic acid (5 mL) and, after 5 min of stirring, diethyl ether added, the precipitate isolated by filtration, and the filter cake dried *in vacuo*. The proton chemical shifts of the arenediazonium ions in DMSO-d_6 are as follows: MBD 8.37 ($H^{3,5}$), 7.710 ($H^{2,6}$), 2.65 (CH_3); HMBD 8.39 ($H^{3,5}$), 7.74 ($H^{2,6}$), 4.77 (CH_2O); MMBD 8.47 ($H^{3,5}$), 7.82 ($H^{2,6}$), 4.70 (CH_2O), 3.40 (CH_3O); CBD 8.73 ($H^{3,5}$), 8.360 ($H^{2,6}$).

Reaction of Arenediazonium Ions with Nucleosides. A solution of the arenediazonium ion (10 mmol) was prepared in water (20 mL) at 0 °C. This solution was then added, dropwise, to the nucleoside (5 mmol) dissolved in 0.62 N sodium hydroxide (40 mL) while the temperature was maintained at 0 °C. After the addition of the arenediazonium ion was complete, the pH was adjusted to 9–10 and the mixture stirred for 24 h. The pH was periodically tested and maintained within the range of 9–10 during the course of the reaction. The reaction mixture was then filtered, the pH of the reaction mixture was adjusted to 7 by the addition of concentrated hydrochloric acid, and the precipitate that formed was filtered and washed thoroughly with chloroform, water, and methanol. Unreacted guanosine and 2′-deoxyguanosine were removed by heating the filter cake in water and removing, by hot filtration, the insoluble product. The filter cake could be further purified by HPLC (C_{18}, 50% aqueous methanol). Selected ^1H NMR data of the nucleoside adducts are presented in Table 1. Further confirmation of the assigned structure of the guanosine and 2′-deoxyguanosine adducts was obtained by heating at reflux a solution of the nucleoside adduct (1 mmol) in 0.1 M HCl (40 mL) for 1 h. The reaction mixture was then cooled to room temperature and the precipitate isolated by filtration, washed thoroughly with chloroform, water, and methanol, and dried *in vacuo* to yield the C^8-arylguanine derivative. Selected chemical shifts of the C^8-arylguanine adducts are shown in Table 1.

V79 cells (Chinese hamster lung fibroblasts) were maintained in stock culture in Williams medium E (WE). Forty-eight hours prior to the addition of the arenediazonium ion they were subcultured into 75 cm^3 T-flasks so that there would be about 15 × 10^6 cells present when the arenediazonium ions were added. They were cultured in an atmosphere of 5% CO_2/95% air at 37 °C.

Stability of Arenediazonium Ions in WE. Solutions of the four arenediazonium ions (20 mM) were made up in WE and held at 37 °C. After 1, 2, 4, 8, 16, and 32 h, the solutions were analyzed by NMR. Under these conditions, only the corresponding para-substituted phenols were produced. Pseudo-first-order kinetics for all four compounds was observed. After 16 h, the amount of arenediazonium ion remaining decreased in the order HMBD > MBD > MMBD > CBD (29%, 26%, 20%, 14%, respectively).

Incubation Conditions. Immediately prior the addition of the arenediazonium ions, the medium was replaced with fresh WE (20 mL) and the arenediazonium ions were added to the V79 cells (15 × 10^6). The pH of the medium did not change with this addition. The cells were incubated at 37 °C for 16 h in an atmosphere of 5% CO_2/95% air.

Irradiation. One hour before irradiation the T-flasks were transferred to an ice bath and cooled. The cells were irradiated, on ice, with a Picker V9 ^{60}Co γ irradiator at a dose of 200 rad (100 rad/min) (White et al., 1981; Kohn et al., 1981). At this dose, 46.8 ± 1.3% of the DNA from the cells that were irradiated but not treated with the arenediazonium ions remained on the filters at the end of the alkaline elution.

Cell Isolation. The irradiated and nonirradiated cells were harvested following the same procedure. The medium was removed and the cells were washed with phosphate-buffered saline (PBS, 20 mL, pH 7.4). The cells were then incubated with a trypsin solution (2 mL, 0.05% w/v) at 37 °C for 2 min. WE (20 mL) was added and the cells were gently scraped from the flask with a plastic policeman and collected by centrifugation (1000*g*, 10 min). The cells were suspended in PBS (2 mL) for application to the filters for alkaline elution. The concentration of cells was measured with a hemocytometer. About 2 × 10^6 cells were applied to each filter.

Alkaline Elution. DNA single-strand breaks (SSB) were measured by alkaline elution according to the method of White et al. (1981). DNA–DNA cross-links were measured according to the method of Kohn et al. (1981), in which the lysing solution was supplemented with proteinase K (0.5 mg/mL). The apparent frequencies of SSB and cross-links were calculated

Table 2. Mutagenicity of MBD, HMBD, MMBD, and CBD in TA102 and V79 Cells[a]

diazonium ion	10 μM	50 μM	100 μM
	TA102 Mutagenicity[b]		
MBD	56 ± 3	111 ± 5	173 ± 9
HMBD	53 ± 5	73 ± 3	110 ± 4
MMBD	52 ± 5	67 ± 6	90 ± 3
CBD	0	23 ± 7	62 ± 9
	V79 Mutagenicity[c]		
MBD	8 ± 2	14 ± 3	18 ± 2
HMBD	31 ± 3	34 ± 1	61 ± 6
MMBD	14 ± 2	26 ± 3	39 ± 7
CBD	11 ± 1	15 ± 1	31 ± 7

[a] The concentrations noted at the tops of columns 2–4 refer to the initial arenediazonium ion concentration. [b] Values reported as mean ± SD (n = 4) in units of revertants/plate. The negative control was the complete system minus the diazonium ion (revertants/plate = 7 ± 2). The positive control was mitomycin C (revertants/plate = 270 ± 10). [c] Values reported are mutants/10^6 survivors as mean ± SD (n = 8). Mutagenicity was measured as TG resistance. Reported values have been corrected for spontaneous mutants.

Table 3. DNA Damage as SSB and Cross-Links Caused by MBD, HMBD, MMBD, and CBD in V79 Cells[a]

diazonium ion	10 μM	50 μM	100 μM
	SSB[b]		
MBD	0.2	0.9	1.8
HMBD	0.2	0.6	0.7
MMBD	0.1	0.2	0.4
CBD	0.1	0.2	0.7
	DNA Cross-Linking[c]		
MBD	0	0	0
HMBD	0.1	1.6	8.6
MMBD	0.2	0.4	3.0
CBD	0.5	0.5	0.8

[a] Arenediazonium ion/V79 cell incubation time was 16 h for all concentrations. The concentrations noted at the tops of columns 2–4 refer to the initial arenediazonium ion concentration. [b] Values reported are SSB/10^7 bases at the indicated arenediazonium ion concentration. [c] Values reported are the number of DNA–DNA cross-links/10^7 bases at the indicated arenediazonium ion concentration. Cells were incubated for 16 h and then irradiated with ^{60}Co (gamma) source at a rate of 100 rad/min for 2 min.

according to the method of Ewig and Kohn (1978). In both assays elution proceeded at 50 μL/min. Three milliliter fractions were collected.

Mutagenicity. *(i) TA102:* The plate incorporation method using TA102 was used (Maron and Ames, 1983). Plates without added arenediazonium ion served as the negative control. Plates treated with mitomycin C were the positive controls. *(ii) V79 cells:* Mutagenicity was measured in V79 cells at thioguanine (TG) resistance (van Zeeland and Simons, 1976). The background (spontaneous mutants in the absence of diazonium ion) was subtracted from the values reported in Table 2.

ESR spectra were measured on a Varian E-3 spectrometer. Solutions were made up in distilled water that had been treated with Chelex 100 resin. Spectra were measured in cacodylate buffer (20 mM, pH 7.4). A DMPO stock solution was made up as described by Floyd et al. (1984) to remove paramagnetic impurities. The final concentration of the DMPO stock solution was 0.3 M as determined by UV. Stock solutions of the arenediazonium ions were made up in water immediately before use. All ESR spectra were measured within 1 min of mixing. Spectra were followed for at least a 10 min period, after which time the spectra remained constant. Hyperfine coupling constants [a(H) and a(N)] were determined after calibration of the instrument with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Fremy's salt by computer simulation of the observed spectra.

RESULTS

Reactions of Arenediazonium Ions with Adenosine, Guanosine, and 2′-Deoxyguanosine. The reaction of the arenediazonium ions with adenosine produced the C^2-N-triazene (Figure 2). In addition, a second product was obtained from HMBD and MMBD, the triazene that would be formed from the reaction of FBD (Figure 2) and adenosine. In the former case, similar amounts of both products were formed, while in the latter case it was the minor product. In the reaction of HMBD and MMBD with guanosine or 2′-deoxyguanosine, C^8-arylated products were formed (Figure 2). As in the case of adenosine, a second product was formed in the reactions involving HMBD and MMBD, the p-formyl derivative. The ratio of the HMBD to FBD derived products was roughly 2:1, and for MMBD this ratio was about 9:1. Key ^{1}H NMR data for the C^8-arylated products are given in Table 1.

Arylhydrazine Mutagenicity. TA102 and V79 mutagenicity data are shown in Table 2. Mutagenicity in TA102 declined in the order MBD > HMBD > MMBD > CBD. The number of revertants/plate at 100 μM was 173, 110, 90, and 62, respectively. In contrast, the order of mutagenicity based on TG resistance in V79 cells was HMBD > MMBD > CBD > MBD. The number of mutants/10^6 survivors at 100 μM was 61, 39, 31, and 18, respectively.

DNA Damage. SSB frequencies are shown in Table 3. The arenediazonium ions produced SSB in the order MBD > HMBD > CBD > MMBD. The number of SSB/10^7 nucleotides was 1.8, 0.7, 0.7, and 0.4, respectively, at 100 μM. DNA–DNA cross-links were produced from HMBD, MMBD, and CBD and are shown in Table 3 and declined in the order HMBD > MMBD > CBD. The ratio of cross-link frequencies at 100 μM was 8.6:3.0:0.8 for HMBD, MMBD, and CBD, respectively. MBD did not produce DNA–DNA cross-links.

ESR. Spin trapping experiments were conducted on the arenediazonium ions under nonreducing and reducing conditions. The ESR spectra of the DMPO-Ar adducts are shown in Figure 4. Under nonreducing conditions HMBD, MMBD, and CBD produced ESR signals (Figure 4a). For these three arenediazonium ions, two radicals were seen. One of these was due to trapping of the aryl radical formed from the corresponding arenediazonium ion by (i) one-electron reduction and (ii) loss of nitrogen. The second radical observed was a hydroxy radical. Other radical species that could have been spin trapped are the aryl diazenyl radical (ArN=N$^\bullet$) and the diazotate radical (ArN=NO$^\bullet$). However, the a(H) and a(N) values are in agreement with the assigned species. In addition, the signal assigned to DMPO-OH was replaced by DMPO-CO_2Na when sodium formate was added and by DMPO-CH(OH)CH_3 when ethanol was added (data not shown). The intensity of the DMPO-OH signal was also observed to increase with the signal for DMPO-Ar, indicating that the DMPO-OH signal did not arise from being present in the DMPO solution prior to the addition of the arenediazonium ion. These results confirm the presence of the DMPO-OH spin adduct. No signal was detected for MBD under nonreducing conditions. In the presence of a reducing agent [data shown for Fe(II) and similar spectra were obtained for other reductants including ascorbate, dithiothreitol, catechol, and the parent hydrazine], all four arenediazonium ions produced an ESR signal (Figure 4b). The signal intensity was a function

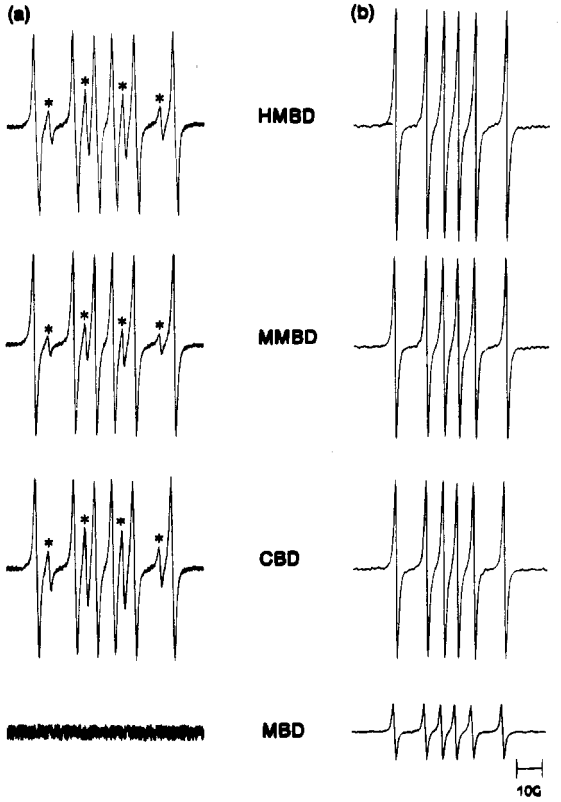


Figure 4. (a) ESR spectra obtained on solutions containing DMPO (60 mM) and the indicated diazonium ion (10 mM) in sodium cacodylate buffer (20 mM, pH 7.4) at 25 °C 4 min after mixing. The instrument settings were gain = 2.5×10^5 (MBD gain 2.5×10^6), time constant = 0.3 s, and sweep width = 200 G. In addition to the DMPO-Ar spin adduct (six lines), the less intense four-line (1:2:2:1) pattern is due to the DMPO-OH spin adduct. The starred peaks are signals due to the spin adduct DMPO-OH. (b) ESR spectra obtained on solutions containing DMPO (60 mM), the indicated diazonium ion (10 mM), and $Fe(NH_4)_2(SO_4)_2$ (2 mM) in sodium cacodylate buffer (20 mM, pH 7.4) at 25 °C 4 min after mixing. The instrument settings were gain = 1.0×10^5, time constant = 0.3 s, and sweep width = 200 G. Identical values for a(H) and a(N) were observed for HMBD, CBD, and MMBD, in the presence or absence of reductant.

Table 4. ESR Coupling Constants (in Gauss) and Signal Intensities (Arbitrary Units) for MBD, HMBD, MMBD, and CBD under Nonreducing and Reducing Conditions[a]

diazonium ion	a(N)[b]	a(H)[b]	$I_{(NR)}$[c]	$I_{(R)}$[d]
MBD	15.5	24.0	ND	108
HMBD	16.0	24.5	500	500
MMBD	15.8	24.5	400	415
CBD	15.3	24.0	400	262

[a] All solutions contained DMPO (60 mM) and the arenediazonium ion (10 mM) in sodium cacodylate (20 mM, pH 7.4). Reactions were initiated by the addition of the arenediazonium ion. [b] a(N) and a(H) were obtained by computer simulation of the ESR spectra. [c] NR, nonreducing conditions. Intensity measured at 4 min with identical spectrometer settings, gain = 2.5×10^5. [d] R, reducing conditions. Reductant was $Fe(NH_4)_2(SO_4)_2$, 2 mM. Intensities were measured at 4 min. Spectra were recorded with a gain setting of 1×10^4 and corrected to 2.5×10^5.

of the arenediazonium ion used and decreased in the order HMBD > MMBD > CBD > MBD. The ESR hyperfine coupling constants and relative intensities of the DMPO-Ar spin adducts are shown in Table 4. The observed values are in agreement with the reported values for DMPO-Ar and DMPO-OH spin adducts (Beuttner, 1987).

DISCUSSION

Arenediazonium ions are formed *in vitro* and *in vivo* from azo dyes (Stiborava et al., 1988a,b), triazenes (Malaveille et al., 1982), and hydrazines and hydrazides (Lawson and Chauhan, 1987; Lawson, 1987). Diazonium ions can react directly with macromolecules such as DNA or may be converted by reduction or decomposition to other reactive and potentially genotoxic species (Figure 1). They can decompose in a manner analogous to that of alkyldiazonium ions. Alternatively, arenediazonium ions can undergo a one-electron reduction to diazenyl radicals, which can lose nitrogen to form aryl radicals. Aryl and diazenyl radicals form adducts with DNA at the C^8 carbon of adenine or guanine (Lister, 1979) (Figure 2).

The reaction of arenediazonium ions with DNA depends on which reactive species is formed. MBD forms 6-[3-(4-methylphenyl)-2-triazen-1-yl]adenine and the corresponding riboside by direct reaction with adenine and adenosine, respectively (Hung and Stock, 1982; Gannett et al., 1987). In contrast, MBD reacts with guanine to give C^8-(4-methylphenylazo)guanine, involving an unknown intermediate. The C^8 position of guanine is sufficiently nucleophilic to react directly with diazonium ions, but it is more reactive to radicals (Lister, 1979) and the diazenyl radical is implicated. MBD forms C^8-(4-methylphenyl)guanosine by reaction with guanosine, and the aryl radical is clearly the intermediate. The nature of the reactive intermediate is determined, in part, by the para substituent on the phenyl ring. It affects the reduction potential: the more electron withdrawing the substituent, the easier it is for reduction to occur.

Reactions of Aryldiazonium Ions with Purine Nucleosides. The consequences of the reaction of an arenediazonium ion with DNA depend on whether it reacts directly or via the aryl cation, aryl radical, or aryldiazenyl radical. It is likely that more than one species forms *in vivo*. The presence of numerous cellular reductants that can reduce aryldiazonium ions makes the reduction of appropriately substituted arenediazonium ions very likely. To determine which intermediates form, we extended our previous studies on the reactions of arenediazonium ions with purine bases and nucleosides (Gannett et al., 1987). Triazenes are formed by reaction with adenine and adenosine, but C^8-azo adducts are formed with guanine. However, with guanosine or 2′-deoxyguanosine C^8-aryl adducts, not C^8-azo adducts, are formed (Figure 1). 4-Hydroxymethylbenzenediazonium ion (Gannett et al., 1987) and 4-sulfonylbenzene diazonium ion (Hoffman and Muller, 1966) also produce C^8-aryl adducts with guanosine. We note that in neither of these studies (Gannett et al., 1987; Hoffmann and Muller, 1966) is there any evidence for the involvement of aryl cations (e.g. O^6-arylated products).

Contrary to our previous report (Gannett et al., 1987), HMBD and MMBD also form C^8-(4-formylphenyl)-guanosine with guanosine and C^8-(4-formylphenyl)-2′-deoxyguanosine with 2′-deoxyguanosine. It is not clear why these were not previously detected, although they could have been lost during the workup of the reaction mixture. In the present study we treated the HMBD–guanosine reaction mixture with HCl to cleave the glycosidic bond. C^8-(4-Formylphenyl)- and C^8-(4-hydroxymethylphenyl)guanine were isolated. We then examined the reaction mixture before HCl treatment by NMR and found C^8-(4-formylphenyl)-2′-deoxygua-

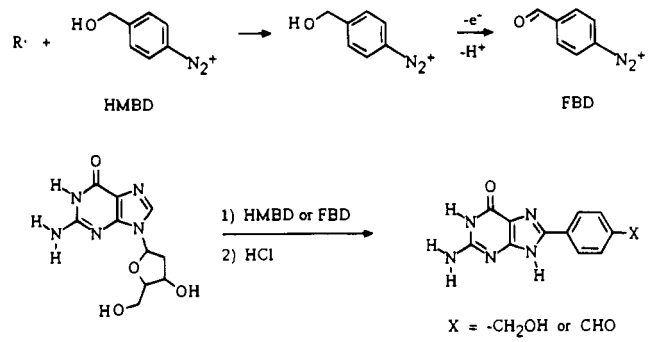


Figure 5. Mechanism for the formation of FBD from HMBD. **R**• = HOC_6H_4, $HOC_6H_4N{=}N$•, $HOC_6H_4N{=}NO$•, HO•, or other radical species. Once FBD is formed, it can be reduced, lose nitrogen, and then arylate guanosine or 2′-deoxyguanosine. The product shown was isolated after treatment of the arylated nucleoside with hydrochloric acid.

nosine and C^8-(4-hydroxymethylphenyl)-2′-deoxyguanosine to be present.

We believe the formyl adduct is formed by hydrogen abstraction by a radical species, which results in the conversion of HMBD into 4-formylbenzene diazonium ion (FBD) (Figure 5). For MMBD, a hydrolysis step is required for FBD formation. The observed adducts would be formed by reaction of FBD with guanosine and 2′-deoxyguanosine.

MBD only produces C^8-(4-methylphenyl)guanosine with guanosine and C^8-(4-methylphenyl)-2′-deoxyguanosine with 2′-deoxyguanosine. Similarly, CBD only produces C^8-(4-carboxyphenyl)guanosine and C^8-(4-carboxyphenyl)-2′-deoxyguanosine. Selected ^{1}H NMR and UV data for the N^6-triazenyladenine, C^8-arylguanosines, and C^8-aryl-2′-deoxyguanosines are shown in Table 1. The data are consistent with the assigned structures.

Arenediazonium Ion Mutagenicity. The multifaceted chemistry of the arenediazonium ions studied here is reflected in the mutagenicity data. The TA102 mutagenicity ranks the arenediazonium ions as MBD > HMBD > MMBD > CBD. In contrast, V79 mutagenicity ranks the arenediazonium ion in the order HMBD > MMBD > CBD > MBD (Table 2). This order differs from that observed in TA102 in that instead of MBD being the most mutagenic, it is the least. The relative order of the remaining three arenediazonium ions remains the same. This result suggests the possibility that different reactive species are responsible for the mutagenic response observed in the two mutagenicity assays. TA102 reversion to prototropy involves an AT → CG transversion (Levin et al., 1987). The arenediazonium ions may be reacting at adenine to form a triazene adduct. The triazene adduct may be unstable and decompose to xanthine. This mechanism has been proposed for the reaction of benzenediazonium ion with adenine (Koepke et al., 1990) (Figure 6). The nature of the mutation produced in V79 cells is not known.

DNA Damage Induced by Arenediazonium Ions. All arenediazonium ions studied caused DNA damage in V79 DNA. The rank order for single-strand breaks (SSB) is MBD > HMBD > CBD > MMBD, similar to that observed for the arenediazonium ion mutagenicity in the Ames assay (MMBD and CBD reversed). In contrast, the rank order toward cross-link formation was HMBD > MMBD > CBD (Table 3). MBD did not produce any detectable DNA cross-links. The observed order for cross-link formation parallels the V79 mutagenicity data. The reactions that produce SSB or

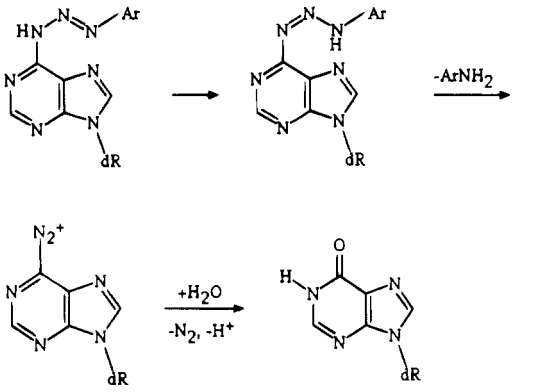


Figure 6. Mechanistic scheme for the deamination of adenine via an aryl triazene.

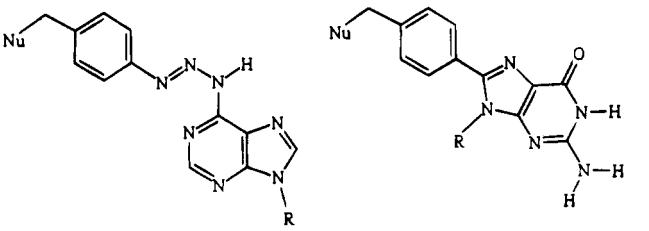


Figure 7. Proposed structures of the DNA–DNA cross-link involving the HMBD–adenine or HMBD–guanine adducts. The cross-link may be either inter- or intrastrand.

cross-linking from arenediazonium ions and DNA are unknown, although possible mechanisms have been proposed (Kato et al., 1992; Levin et al., 1982; Moudrianakis and Beer, 1965). Regardless, the different rank orders seen for SSB and cross-link formation with MBD, HMBD, CBD, and MMBD and the fact that they parallel the mutagenicity data suggest two different intermediates.

DNA cross-linking is usually considered a cytotoxic event. However, following DNA cross-link formation HMBD, MMBD, or CBD could subsequently produce point mutations, too. For example, reaction of HMBD with DNA, at either adenine or guanine, would first produce an N^6-triazenyladenine or a C^8-arylguanine product, respectively. Subseqently, the hydroxyl group of HMBD could be displaced by a nucleophile on the other DNA strand and form the cross-link. A triazenyl cross-link would be unstable and is susceptible to cleavage *in vivo* to hypoxanthine (Figure 6), which is mutagenic (Singer and Kusmierek, 1982). A second point mutation also would be produced on the other DNA strand. In contrast, the cross-link involving the C^8-arylguanine adduct should be relatively stable and resist cleavage. Plausible structures for the cross-link are shown in Figure 7.

Intrastrand DNA cross-links also may form and they, or their decomposition products, may be mutagenic. Finally, the arenediazonium ions may form triazenes at N^2 of guanine. Decomposition of the triazenyl–guanine adduct could result in N^2-diazonium ion derivative, which is known to form DNA cross-links (Kirchner et al., 1992). However, if this pathway was important for the arenediazonium ions examined here, MBD should cross-link DNA and this is not observed.

Cross-link formation is geometrically allowed from either the triazene or C^8-arylguanine adduct. The point of attachment of the aryl group in the triazenyl adduct is on an atom near the axis of the DNA helix. This will also be true for the C^8-arylguanine adducts. Substitution at the C^8-position of guanine causes the conforma-

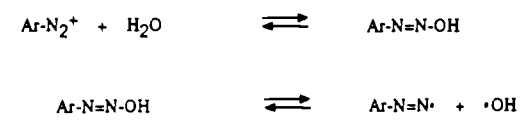


Figure 8. Proposed mechanistic scheme for the generation of hydroxy radical during the decomposition of arenediazonium ions in water.

tion about the glycosidic bond to go from *anti* to *syn* (Stolarski et al., 1980). Therefore, the C^8-aryl group will be directed toward the DNA helical axis. At the nucleoside level, the C^8-arylguanosine and C^8-aryl-2′-deoxyguanosine adducts studied here adopt the *syn* conformation, on the basis of the chemical shift of the H-2′ proton (Stolarski et al., 1980). The affect of the C^8-aryl group in guanine at the nucleoside level should also occur in DNA by analogy to other C^8-substituted guanines that have been studied at both the nucleoside and oligonucleotide levels (Norman et al., 1989; Kouchakdijan et al., 1991).

Aryl Radical Formation from Arenediazonium Ions. MBD, under nonreducing conditions, did not generate an ESR signal in the presence of DMPO. In contrast, HMBD, MMBD, and CBD all produce DMPO-Ar and DMPO-OH adducts (Figure 4a). The identification of the DMPO-OH spin adduct is based on (i) the observed coupling constants [$a(N) \approx a(H) = 14.9$ G] (Buettner, 1987), (ii) replacement of the DMPO-OH signal by that of DMPO−CH(OH)−CH_3 when the reaction mixtures contained ethanol, and (iii) replacement of the DMPO-OH signal by DMPO−CO_2^- when the reaction mixtures contained sodium formate (Buettner, 1987). Also, the intensity of the signal due to the DMPO-OH adduct increased with the signal due to the DMPO-Ar spin adduct signal. Thus, the hydroxy radical and aryl radical are generated simultaneously. Hydroxy radical formation may occur through a process shown in Figure 8 (Dreher et al., 1981).

The formation of a DMPO-Ar spin adduct is observed from all four arenediazonium ions in the presence of reductants (Figure 4b). The relative intensity of the observed signals is HMBD > MMBD > CBD > MBD, the same order as observed for V79 mutagenicity. Formation of the DMPO-OH spin adduct was not observed though under the reaction conditions DMPO-OH radical is stable. This observation supports the mechanistic scheme presented in Figure 8. The added reductant eliminates the need for water to serve as a reducing agent, a process required for hydroxy radical formation.

The ESR data support the idea that the main mutagenic species with MBD is the arenediazonium ion and that aryl radicals are more important from the remaining three arenediazonium ions. The least amount of aryl radical is formed from MBD. Therefore, its direct reaction with adenine to form trizene adducts is more likely to occur than reaction with guanine. In contrast, HMBD, MMBD, and CBD formed aryl radicals much more easily. Therefore, they are more likely to form C^8-arylguanine adducts with guanine than triazenyl adducts with adenine. This may be the source of the different mutagenic responses observed for the diazonium ions in the Ames and V79 mutagenicity assays. Likewise, it may be part of the reason for the differences seen in the DNA damage assays (SSB and cross-linking).

It is of interest that the tendency of the diazonium ions to reduce to aryl radicals is a function of their respective reduction potentials. The reduction potential for arenediazonium ions, as a function of the para substituent, has been studied and can be fit by eq 1 (Elofson and Gadallah, 1969), where σ_p is the substituent constant and $E^\circ_{1/2}$ is the standard reduction potential.

Table 5. Reduction Potentials and σ_p Constants for HMBD, CBD, MMBD, and MBD

compd	*p*-X[a]	σ_p[b]	$E^\circ_{1/2}$[c] (V)	$E^\circ_{1/2}$[d] (V) (exp)
HMBD	$-CH_2OH$	0.00	0.314	
CBD	$-COO^-$	0.00[e]	0.314	0.328
MMBD	$-CH_2OCH_3$	−0.03	0.307	
MBD	$-CH_3$	−0.17	0.275	0.250

[a] *p*-X refers to the para substituent present in the arenediazonium ion *p*-X−$C_6H_4N_2^+$ (see Figure 3). [b] σ_p constants were obtained from Hansch et al. (1973). [c] $E^\circ_{1/2}$ values were calculated from the equation given in the text. [d] The reduction potentials of MBD and CBD have been reported (Elofson and Gadallah, 1969) and are 0.250 and 0.328 V, respectively, in good agreement with the calculated values. [e] At pH 7.4, Ar−CO_2H will mainly be present as Ar−CO_2^- assuming a pK_a of <4. Therefore, the σ_p for $-CO_2^-$ is used for the calculation of $E^\circ_{1/2}$.

$$E^\circ_{1/2}(V) = 0.229\sigma_p + 0.314\ (\pm 0.024) \qquad (1)$$

Using eq 1 and the σ_p values of Hansch et al. (1973), the reduction potentials for the four arenediazonium ions can be calculated, if unknown, and are shown in Table 5. The data shown in Table 5 predict MBD to be the hardest to reduce and the remaining three arenediazonium ions should be comparable with regard to their ease of reduction and hence aryl radical production. A similar dependence of biological activity on reduction potential of arenediazonium ions has been reported (Doyle et al., 1985, 1987).

Arylhydrazines and their oxidative metabolite arenediazonium ions are known genotoxins, although their mechanism of action is unknown. In this study we have examined their reaction with purines, their mutagenicity in both bacterial and mammalian cell assays, and DNA damage as measured by DNA single-strand breaks and DNA−DNA cross-link formation to determine possible structures for the ultimate genotoxin. We find that either the arenediazonium ion itself or an aryl radical may serve as the ultimate genotoxin. These results may be significant for studies aimed at elucidating the mechanism whereby arenediazonium ions and their metabolic precursors express their genotoxicity. For example, studies that explore DNA adduct formation must assay for adenosine and guanosine adducts. Likewise, the interpertation of mutagenicity and DNA damage data must be made in light of the fact that both the arenediazonium ion and aryl radical may be involved. The reduction potential, or its estimation from the σ_p constant, may serve as a guide to determining the relative importance of arenediazonium ions versus its corresponding aryl radical.

ABBREVIATIONS USED

MBD, *p*-methylbenzene diazonium hydrogen sulfate; CBD, *p*-carboxybenzene diazonium hydrogen sulfate; HMBD, *p*-hydroxymethylbenzene diazonium hydrogen sulfate; MMBD, *p*-methoxymethylbenzene diazonium hydrogen sulfate; FBD, *p*-formylbenzene diazonium hydrogen sulfate; TG, thioguanine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SSB, single-strand breaks; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; HGPRT, hypoxanthine−

guanine phosphoribosyltransferase; WE, Williams medium E; PBS, phosphate-buffered saline; a(X), hyperfine coupling constant between the unpaired electron and the nucleus X in gauss (G); COSY, proton–proton correlation spectroscopy; HETCOR, carbon–proton (heteronuclear) correlation spectroscopy.

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Registry No. Provided by the Author: *p*-toluidine, 106-49-0; *p*-nitrobenzyl alcohol, 619-73-8; *p*-aminobenzoic acid, 150-13-0; 5,5-dimethyl-1-pyrroline *N*-oxide, 3317-61-1; platinum dioxide, 1314-15-4; catechol, 120-80-9; nitrosyl hydrogen sulfate, 7782-78-7; thioguanine 2-amino-6-purinethiol, 154-42-7; Fremy's salt potassium nitrosodisulfonate, 14293-70-0.

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