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Comparison of Rates of Enzymatic Oxidation of Aflatoxin B_1, Aflatoxin G_1, and Sterigmatocystin and Activities of the Epoxides in Forming Guanyl-N^7 Adducts and Inducing Different Genetic Responses

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The genotoxicity of the dihydrofurans aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1), and sterigmatocystin (STG) was examined in a bacterial system in which the induction of SOS repair is monitored with the *umu*C gene linked to a *lac*Z reporter gene in plasmid pSK1002. Human liver microsomal cytochrome $P\text{-}450_{NF}$ oxidized the dihydrofurans (in the presence of calf thymus DNA) to give guanyl-N^7 adducts in the order AFB_1 > STG > AFG_1. The order of the *umu* response seen was STG > AFB_1 > AFG_1, when either the dihydrofurans were activated enzymatically or the synthetic epoxides of the dihydrofurans were added directly to the bacteria. Thus, the *umu* response per molecule of guanyl-N^7 DNA adduct follows the order STG > AFB_1 > AFG_1. A similar pattern has been reported in the literature for *Salmonella typhimurium* base substitution dependent *his* reversions, but the pattern AFB_1 > STG > AFG_1 has been found for bacterial frame-shift-dependent mutagenesis and hepatocarcinogenesis. The guanyl-N^7 adduct derived from AFG_1 has considerably less of all of these biological activities per molecule. Neither guanine imidazole ring opening nor apurinic site formation appears to be a factor involved in the differential biological responses seen with the three guanyl-N^7 adducts. These findings indicate that these structurally related guanyl-N^7 DNA adducts have intrinsic differences which give rise to divergent biological responses.

Aflatoxins and related dihydrofurans are of concern to public health because of their widespread occurrence in foodstuffs and their high potential for hepatocarcinogenesis (*1–4*). In addition to practical considerations regarding health issues, aflatoxins are of basic scientific interest because of their high efficiency for mutagenesis and carcinogenesis. Thus, molecular mechanisms underlying the biological effects exerted by these compounds may also be involved in the genotoxic effects of other compounds as well (*5*). However, one of the problems in probing structure–activity relationships among the aflatoxins and related compounds is that AFB_1[1] is the most potent dihydrofuran in most genotoxicity assays and that almost all modifications of the molecule result in decreased activity.

Recently, we obtained evidence that $P\text{-}450_{NF}$ (*6–8*) is the form of P-450 primarily responsible for the activation of AFB_1 in human liver (*9*). The same enzyme also appears to be the predominant one involved in the bioactivation of the analogues AFG_1 and STG (Figure 1). During the course of these investigations we consistently found a greater response for expression of a *umuC′′lac*Z chimeric plasmid (responding to DNA damage and SOS response) (*19–21*) with STG than AFB_1 and AFG_1. Genotoxicity results in the literature have been generated under a variety of different experimental conditions, and we sought to make comparisons on the basis of actual DNA adducts in order to better distinguish among factors such as rates of oxidation, epoxide stability, rate of epoxide–DNA reaction, DNA adduct stability, and biological responses. In addition, the availability of the synthetic epoxides of AFB_1, AFG_1, and STG allowed discernment of some of these contributions. Our results suggest that what appear to be very similar guanyl-N^7 DNA adducts can have rather divergent biological effects.

Experimental Procedures

Chemicals. The epoxide derivatives of AFB_1, AFG_1, and STG were prepared by using dimethyldioxirane according to the procedure of Baertschi et al. (*11*). Details of the characterization will be published elsewhere. Briefly, dimethyldioxirane was prepared as a 0.05–0.1 M solution in acetone (*12, 13*). Approximately 1.5 equiv of dimethyldioxirane was added to the di-

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[1] Abbreviations: P-450, liver microsomal cytochrome P-450; AFB_1, aflatoxin B_1; AFG_1, aflatoxin G_1, STG, sterigmatocystin; AFB_1-N^7-Gua, 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1;[2] AFG_1-N^7-Gua, 9,10-dihydro-9-(N^7-guanyl)-10-hydroxyaflatoxin G_1; STG-N^7-Gua, 1,2-dihydro-2-(N^7-guanyl)-1-hydroxysterigmatocystin; AFB_1 FAPY I, 8,9-dihydro-8-[*N*-formyl-*N*-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl)-amino]-9-hydroxyaflatoxin B_1; AFB_1 FAPY II, 8,9-dihydro-8-[(2-amino-6-formamido-4-oxo-3,4-dihydropyrimidin-5-yl)amino]-9-hydroxyaflatoxin B_1; AFG_1 FAPY I, 9,10-dihydro-9-[*N*-formyl-*N*-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl)amino]-10-hydroxyaflatoxin G_1; AFG_1 FAPY II, 9,10-dihydro-9-[(2-amino-6-formamido-4-oxo-3,4-dihydropyrimidin-5-yl)amino]-10-hydroxyaflatoxin G_1; STG FAPY I, 1,2-dihydro-1-hydroxy-2-[*N*-formyl-*N*-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl)-amino]sterigmatocystin; STG FAPY II, 1,2-dihydro-1-hydroxy-2-[(2-amino-6-formamido-4-oxo-3,4-dihydropyrimidin-5-yl)amino]sterigmatocystin; HPLC, high-performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate.

[2] In the literature the double bond of AFB_1 that undergoes oxidation is referred to as either 8,9 (IUPAC) or 2,3; the IUPAC designation is used here. The IUPAC system also yields the 9,10 designation for the same bond of AFG_1 and 2,1 designation (with C-2 adjacent to the oxygen) for STG (see Figure 1).

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Table I. ^{1}H NMR Resonances of FAPY I Adducts

proton[a]	δ, ppm[b]		
	AFG_1 FAPY I	AFB_1 FAPY I	STG FAPY I
OCH_3	3.91 (s, 3 H)	3.92 (s, 3 H)	3.87 (s, 3 H)
a	6.38, 6.35 (2 s, 1 H)[c]	6.38, 6.36 (2 s, 1 H)[c]	6.25, (s, 1 H)
b	6.50 (d, 1 H, *J* 5.9 Hz)	6.49 (d, 1 H, *J* 5.8 Hz)	6.55 (d, 1 H, *J* 6.3 Hz)
c	nd[d] (under OCH_3)	nd (under OCH_3)	3.97, (d, 1 H, *J* 6.3 Hz)
d	5.07, 4.92 (2 d, 1 H, *J* 4.5 Hz)[c]	5.07, 4.93 (2 d, 1 H, *J* 5.1 Hz)[c]	5.01, 5.14 (2 d, 1 H, *J* 5, 3.5 Hz)[c]
e	6.27, 5.69 (2 s, 1 H)[c]	6.26, 5.68 (2 s, 1 H)[c]	6.33, 5.72 (2 s, 1 H)[c]
f	6.09, 6.07 (2 d, 1 H, *J* 4.6 Hz)[c]	6.07, 6.04 (2 d, 1 H, *J* 5.1 Hz)[c]	6.30, 6.10 (2 d, 1 H, *J* 5, 3.5 Hz)[c]
g	4.32–4.40 (m, 2 H)	nd (under solvent peak)	6.71 (d, 1 H, *J* 8.4 Hz)
h	nd (under solvent peak)	nd (under solvent peak)	7.26 (t, 1 H, *J* 8.4 Hz)
i			6.91 (d, 1 H, *J* 8.4 Hz)
j			13.53 (s, 1 H)
k	9.70, 9.52 (2 s, 1 H)[c]	9.68, 9.51 (2 s, 1 H)[c]	9.28, 9.18 (2 s, 1 H)[c]
l, m[e]	5.95, 5.57 (2 bs)	6.15, 6.00, 5.55, 5.45 (4 bs)	5.51, 5.68, 5.97, 6.08, 6.66, 7.27 (6 bs)
n	8.30, 7.60 (2 s, 1 H)[c]	8.28, 7.57 (2 s, 1 H)[c]	8.27, 7.59 (2 s, 1 H)[c]

[a] See Figure 2. [b] Spectra obtained in $(CH_3)_2SO$-d_6 (δ 2.49). [c] Two peaks, one from each rotamer. [d] nd, not detected [hidden by H_2O and $(CH_3)_2SO$ peaks]. [e] Tentative assignments.

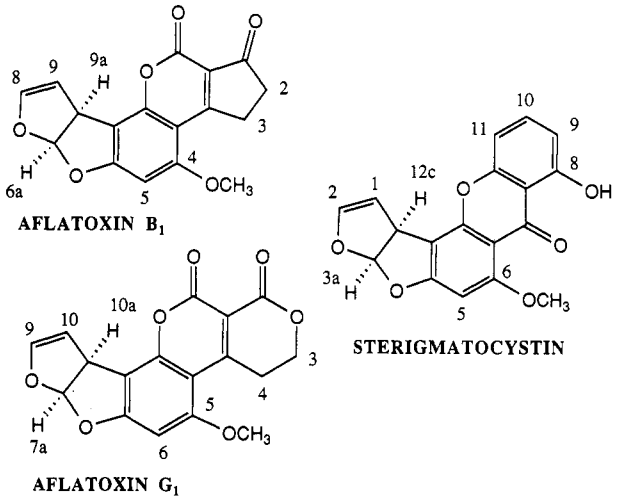

Figure 1. Structures of dihydrofurans used in this work.

hydrofuran in CH_2Cl_2, and the solution was allowed to react for 30 min. The solvent was then evaporated under N_2, the residue was dissolved in acetone-d_6, and ^{1}H NMR spectra were obtained. Yields (as determined by ^{1}H NMR peak areas) were >95% (<5% starting dihydrofuran or dihydrodiol). Solutions of the epoxides were stored in acetone-d_6 at −20 °C and were assayed before use.

Standards of the guanyl-N^7 adducts were prepared by the following procedure: 2-mg aliquots of the individual epoxides (in 1 mL of acetone) were added to 20 mL of 20 mM sodium phosphate buffer (pH 7.0) containing calf thymus DNA (~1 mg mL^{-1}) with stirring. After 1 h the solution was extracted twice with 20 mL of $CHCl_3$/isoamyl alcohol (24:1 v/v). The DNA (in the aqueous phase) was precipitated with ~100 mL of ice-cold C_2H_5OH, spooled onto glass rods, washed with C_2H_5OH, dried, and redissolved in 20 mL of 20 mM sodium phosphate buffer (pH 7.0). The pH was adjusted to 2 with 0.1 N HCl, and each sample was heated at 80 °C for 30 min. After filtration of the solution through a 0.45-μm filter, the guanyl-N^7 adducts were purified by preparative HPLC [Alltech 10-μm octadecylsilyl (C18) semipreparative column, 10 × 250 mm, Alltech Associates, Deerfield, IL]. The solvent consisted of CH_3CN/H_2O (10:90 v/v) until all of the DNA eluted from the column. The CH_3CN concentration was then increased to 40–60% (v/v) to elute the adducts. The purified adducts were lyophilized and then dissolved in $(CH_3)_2SO$-d_6 for ^{1}H NMR analysis. Yields for these reactions were typically ~50% [(mol of adduct recovered/mol of epoxide added) × 100]. For AFB_1 adduct, quantitation was based on UV assay at 364 nm (ε 16 200 M^{-1} cm^{-1}) in 0.1 N HCl (*14*). For quantitation of AFG_1 and STG guanyl-N^7 adducts, a known amount of AFB_1 was added to the sample and ^{1}H NMR spectra were obtained. From peak area measurements, molar ratios were determined and the total amount of adduct was calculated. AFB_1-N^7-Gua: UV λ_{max} 364 nm (0.1 N HCl); ^{1}H NMR [in $(CH_3)_2SO$-d_6] identical to Baertschi et al. (*11*, *15*). AFG_1-N^7-Gua (*16*): UV λ_{max} 365 nm (0.1 N HCl); ^{1}H NMR [in $(CH_3)_2SO$-d_6] δ 4.36 (t, 2 H, *J* = ~6.3 Hz, H3ab), under $(CH_3)_2SO$-d_6 peak (H4ab), 3.88 (s, 3 H, 5-OCH_3), 6.62 (s, 1 H, H6), 6.87 (d, 1 H, *J* = 6.0 Hz, H-7a), 7.32 (s, 1 H, H8-guanine), 6.26 (s, 1 H, H9), 5.20 (d, 1 H, *J* = 4.1 Hz, H-10), 6.39 (d, 1 H, *J* = 4.1 Hz, H10-OH), 4.16 (d, 1 H, *J* = 6.0 Hz, H10a). STG-N^7-Gua (*14*, *17*): UV λ_{max} 329 nm (0.1 N HCl); ^{1}H NMR δ 3.87 (s, 3 H, 6-OCH_3), 4.24 (d, 1 H, *J* = 5.9 Hz, H12c), 5.17 (s, 1 H, H1), 6.32 (s, 1 H, H2), 6.60 (s, 1 H, H5), 6.72 (d, 1 H, *J* = 8.2 Hz, H11), 6.91 (d, 1 H, *J* = 8.3 Hz, H9), 6.86 (d, 1 H, *J* = 5.9 Hz, H3a), 6.40 (br s, 2 H, guanine-NH_2), 7.62 (t, 1 H, *J* = 8.3 Hz, H10), ~13.2 (br s, ~1 H, 8-OH), 7.23 (s, 1 H, H8-guanine).

Standards of the FAPY adducts were prepared by dissolving guanyl-N^7-derivatized DNA (prepared as described above) in 25 mM Na_2CO_3 buffer (pH 9.6) at a concentration of ~1 mg/mL and incubating for 2 h at 37 °C. The solutions were adjusted to pH <2 with 1 N HCl, heated at 95 °C for 20 min, and adjusted to pH 5 by addition of 1 N NaOH. The DNA hydrolysates were then loaded onto Maxi-Clean C18 cartridges (Alltech Associates), and the DNA was eluted with 15 mL of 10% aqueous CH_3OH. The adducts were eluted with 80% CH_3OH, and the solvent was removed in vacuo. The residue was redissolved in 40% aqueous CH_3OH, and the solution was filtered through a 0.45-μm filter and injected onto a preparative HPLC column (vide supra). The solvent system consisted of CH_3OH/H_2O (35:65 v/v) for the AFG_1 FAPY compounds, CH_3OH/H_2O (40:60 v/v) for the AFB_1 FAPY compounds, and CH_3OH/H_2O (60:40 v/v) for the STG FAPY compounds. Retention times under these conditions were 7.1 and 8.4 min for AFG_1 FAPY II and AFG_1 FAPY I, 6.0 and 7.9 min for AFB_1 FAPY II and AFB_1 FAPY I, and 8.6 and 10.1 min for STG FAPY II and STG FAPY I (flow rate 3.0 mL min^{-1}). The adducts were collected after repeated injection, and the solvent was removed in vacuo. The resulting solids were dissolved in $(CH_3)_2SO$-d_6 for ^{1}H NMR analysis. NMR assignments (Table I, Figure 2) were made by comparing the FAPY adduct spectra with those of the guanyl-N^7 adducts as well as the reported spectrum of AFB_1 FAPY I (*18*). UV max: AFG_1 FAPY I (0.1 N HCl) 366 nm, 264 nm; AFB_1 FAPY I (0.1 N HCl) 364 nm, 265 nm; STG FAPY I (0.1 N HCl) 329 nm. The UV spectra of the FAPY II compounds were identical with those of the respective FAPY I products.

AFG_1, AFB_1, and STG dihydrodiol standards were prepared by adding known amounts of the epoxides to aqueous solutions buffered to pH ~3.5 with 20 mM triethylammonium formate. AFG_1 9,10-dihydrodiol: UV λ_{max} (0.1 N HCl) 366 nm, (aqueous NaOH, pH >9) 383 nm; ^{1}H NMR (acetone-d_6) δ 3.49 (m, 2 H) and 4.40 (m, 2 H) (A_2B_2, H-3 and H-4), 3.92 (s, 1 H, 5-OCH_3), 3.95 (d, 1 H, *J* = 6 Hz, H-10a), 4.37 (s, 1 H, H-10), 5.41 (s, 1 H, H-9), 6.45 (s, 1 H, H-6), 6.60 (d, 1 H, *J* = 6 Hz, H-7a). AFB_1 8,9-dihydrodiol: UV λ_{max} (0.1 N HCl) 362 nm, (aqueous NaOH, pH >9) 392 nm; ^{1}H NMR (acetone-d_6) δ 2.50 (m, 2 H) and 3.40 (m, 2 H) (A_2B_2, H-2 and H-3), 3.97 (d, 1 H, *J* = 6 Hz, H-9a), 3.99 (s, 3 H, 4-OCH_3), 4.43 (s, 1 H, H-9), 5.46 (s, 1 H, H-8), 6.47 (s, 1 H, H-5), 6.63 (d, 1 H, *J* = 6 Hz, H-6a). STG 1,2-dihydrodiol: UV λ_{max} (0.1 N HCl) 326 nm, (aqueous NaOH, pH >9) 3.45 nm; ^{1}H

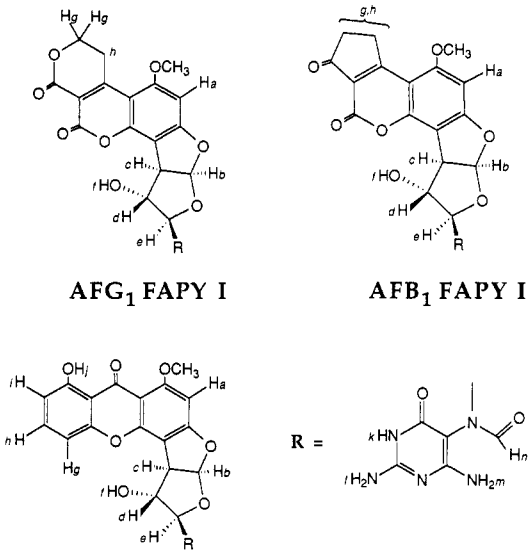

Figure 2. Structures of FAPY adducts.

NMR (acetone-d_6) δ 3.91 (s, 3 H, 6-OCH_3), 4.05 (d, 1 H, J = 6 Hz, H-12c), 4.57 (s, 1 H, H-1), 5.44 (s, 1 H, H-2), 6.44 (s, 1 H, H-5), 6.62 (d, 1 H, J = 6 Hz, H-3a), 6.68 (d, 1 H, J = 8.2 Hz, H-9), 6.97 (d, 1 H, J = 8.3 Hz, H-11), 7.58 (t, 1 H, J = 8.4 Hz, H-10).

Enzyme Preparations. Human liver samples were obtained from organ donors through the Nashville Regional Organ Procurement Agency, and microsomes were prepared (*19*). In this work a sample designated HL 107 was utilized in many of the experiments. Liver microsomes from Sprague-Dawley rats (200 g) were also prepared according to similar procedures.

***umu* Procedure.** The basic method involving *Salmonella typhimurium* TA 1535/pSK1002 is described elsewhere (*20–22*); the bacterial culture containing the plasmid was obtained through Dr. S. Nakamura, Osaka Prefecture Institute of Public Health. Briefly, plasmid pSK1002 contains the regulatory region of the *umuC* gene fused (5′) to the *lacZ* structural gene. When alkylation of bacterial DNA activates the SOS response, *umuC* is one of the bacterial genes involved. In this system, derepression of the chimeric plasmid gene accompanies the cellular response and results in the formation of β-galactosidase, which is measured colorimetrically, and the overall response is termed "*umu* gene activation" in this report. Microsomal samples were added to give a final concentration of 0.005–0.025 μM P-450, with 50 mM potassium phosphate (pH 7.4), 10 μM AFB_1, AFG_1, or STG, an NADPH-generating system (containing final concentrations of 5 mM glucose 6-phosphate, 1 mM $NADP^+$, and 1 IU yeast glucose 6-phosphate mL^{-1}), and an *S. typhimurium* TA 1535/pSK1002 bacterial suspension as previously described (*22*). Incubations were carried out at 37 °C for 120 min, and the induction of *umu* gene expression is presented as units of β-galactosidase activity min^{-1} (nmol of P-450)$^{-1}$ (*23*).

Assay of DNA Adducts. Human liver microsomes (2–4 μM P-450) were incubated with 50 mM potassium HEPES buffer (pH 7.5), the NADPH-generating system described above, calf thymus DNA (1 mg mL^{-1}), and either AFB_1, AFG_1, or STG (50 μM) for 60 min at 37 °C (total incubation volume 1 or 2 mL). In experiments with the synthetic epoxides, similar procedures were used. The reactions were stopped by the addition of sodium dodecyl sulfate to 17 mM and NaCl to 320 mM, extracted twice with an equal volume of a mixture of phenol/$CHCl_3$/isoamyl alcohol (50:50:1 v/v/v), and treated with 10 volumes of cold C_2H_5OH to precipitate DNA, which was spooled onto disposable glass pipettes. When microsomes were not used, the phenol extraction step was omitted. The DNA was washed sequentially with 1–2 mL each of C_2H_5OH, benzene, $CHCl_3$, C_2H_5OH, and $(C_2H_5)_2O$. The dried DNA was dissolved in 2 mL of 50 mM potassium acetate buffer (pH 5.0); insoluble material was removed by centrifugation, and an aliquot of the supernate was diluted (1/50) for estimation of DNA recovery (A_{260}). AFB_1-N^7-Gua, AFG_1-N^7-Gua, and STG-N^7-Gua were released by hydrolysis for 15 min at 95 °C. Adducts were injected onto a 4.6 × 150 mm Zorbax 5-μm octadecylsilyl (C18) HPLC column (MacMod Instruments, Chadds Ford, PA). The elution solvent consisted of 38% CH_3OH (in H_2O, v/v) for AFB_1-N^7-Gua and AFG_1-N^7-Gua and 54% CH_3OH for STG-N^7-Gua with a flow rate of 1.5 mL min^{-1}. Adducts were monitored at a wavelength of 362 nm (AFB_1-N^7-Gua and AFG_1-N^7-Gua) or 325 nm (STG-N^7-Gua). Under these conditions, typical retention times were 4.2, 2.7, and 7.0 min for AFB_1-N^7-Gua, AFG_1-N^7-Gua, and STG-N^7-Gua, respectively. In all HPLC assays, products were estimated by comparison of integrals with those derived from external standards, with appropriate corrections made for recovery of DNA. In some cases involving AFG_1 and STG incubations, AFB_1 was used as a secondary standard.

Measurement of Guanyl-N^7 Adduct Stability. Each of the individual epoxides (100 μg in 200 μL acetone) was added in duplicate to 8 mL of 75 mM potassium HEPES buffer (pH 7.4) containing 7.5 mM sodium citrate, 75 mM NaCl, and 1.1 mg of calf thymus DNA mL^{-1}. The solutions were stirred at room temperature for 5 min, and the DNA was precipitated with 3 volumes of ice-cold C_2H_5OH. The DNA was spooled onto disposable glass pipettes, redissolved in 6 mL of 67 mM sodium phosphate buffer (pH 7.35), and incubated at 37 °C.

Aliquots of the above adducted DNA solutions (500 μL) were removed at 0.75, 6, 12, and 24 h, acidified to pH <2 with 6 N HCl, and heated at 95 °C for 20 min. The hydrolysates were then neutralized with 40% NaOH, filtered through a 0.45-μm filter, and analyzed by analytical HPLC [IBM 5-μm octadecylsilyl (C18), 4.5 × 250 mm] (Nicolet Analytical Division, Madison, WI). Isocratic elution was carried out with CH_3OH and 20 mM aqueous triethylammonium formate (pH 3.2). The ratios of organic to aqueous phase utilized for AFG_1 adducts, AFB_1 adducts, and STG adducts were 28:72, 32:68, and 45:55, respectively. Under these conditions, retention times for AFG_1 FAPY II, AFG_1 FAPY I, AFG_1 9,10-dihydrodiol, and AFG_1-N^7-Gua were 5.4, 7.3, 10.0, and 11.2 min, respectively (flow rate 1.5 mL min^{-1}). Retention times for AFB_1 FAPY II, AFB_1 FAPY I, AFB_1 8,9-dihydrodiol, and AFB_1-N^7-Gua were 6.5, 8.3, 10.4, and 11.8 min, respectively. Retention times for STG FAPY II, STG FAPY I, STG-N^7-Gua, and STG 1,2-dihydrodiol were 8.0, 10.8, 12.0, and 14.0 min, respectively. AFG_1 and AFB_1 adducts were monitored at 362 nm, and quantitation was based upon the response factors of the respective guanyl-N^7 standards. STG adducts were monitored at 325 nm, and quantitation was based upon the response factor of the STG-N^7-Gua standard.

For estimation of the extent of depurination, 200-μL aliquots of the above adducted DNA solutions were removed at 0.75, 6, 12, and 24 h and placed into 1.5 -mL microcentrifuge tubes; DNA was precipitated with 0.8 mL of ice-cold C_2H_5OH. The DNA pelleted upon centrifugation, and the supernatants were collected and evaporated under a stream of N_2. The resulting residues were dissolved in CH_3OH/H_2O (1:1 v/v), filtered through a 0.45-μm filter, and analyzed by analytical HPLC (vide supra).

Results

Preliminary Experiments. Previous work indicated that *umu* gene response to DNA alkylation was highly sensitive to AFB_1 oxidation products (*24*). Good correlations of *umu* gene activation and *S. typhimurium* TA 98 reversion could be made when the degree of AFB_1 metabolic activation was varied by utilizing microsomal fractions prepared from rats treated with various compounds. More recently, the 8,9-oxide derivative of AFB_1 has been synthesized (*11*), and its biological activity was examined directly in the *umu* gene activation system (Figure 3). The cytotoxicity to the bacteria was not sufficient to preclude use in this system. On a molar basis, AFB_1-8,9-oxide was approximately 30% as effective as the direct-acting mutagens 1,3-dinitropyrene and 1,6-dinitropyrene[3] (Figure 4). In other experiments which are not shown, AFB_1-8,9-oxide and (+)-*anti*-7,8-dihydroxy-7,8-dihydro-9,10-oxobenzo-

[3] The high mutagenicity of the nitroaromatics is somewhat misleading because of the ability of the bacteria to reduce these to hydroxylamines.

Table II. Comparisons of Responses to AFB_1, AFG_1, and STG and the Derived Epoxides

	rate or yield measured with each compound[a]		
	AFB_1	AFG_1	STG
Enzymatic Oxidation of Dihydrofurans			
umu response, units min^{-1} (nmol of P-450)$^{-1}$			
rat microsomes[b]	684	86	1540
human microsomes			
$\bar{X}$ (n = 9)[c]	244 ± 13	60 ± 23	574 ± 633
HL 107[d]	115	110	292
guanyl-N^7 adduct formation,[e] pmol min^{-1} (nmol of P-450)$^{-1}$	28 ± 1	12 ± 1	19 ± 4
Reactions of Synthetic Epoxides			
umu response, units (pmol of epoxide)$^{-1}$[e]	2.4	0.4	11.3
guanyl-N^7 adduct formation, nmol (nmol of epoxide)$^{-1}$			
without microsomes	0.73 ± 0.01	0.65 ± 0.01	0.59 ± 0.06
with microsomes	0.31 ± 0.06	0.47 ± 0.01	0.47 ± 0.08

[a] When SD is indicated, results are presented as means of three replicate experiments. Other results are means of two determinations unless indicated otherwise. [b] Prepared from untreated male rats. [c] Means of results obtained with nine different human liver samples. [d] Human liver sample HL 107 microsomes were used for the in vitro alkylation of calf thymus DNA. This sample (HL 107) was utilized in the adduct formation studies (vide infra). [e] The results shown are slopes of lines in Figure 5.

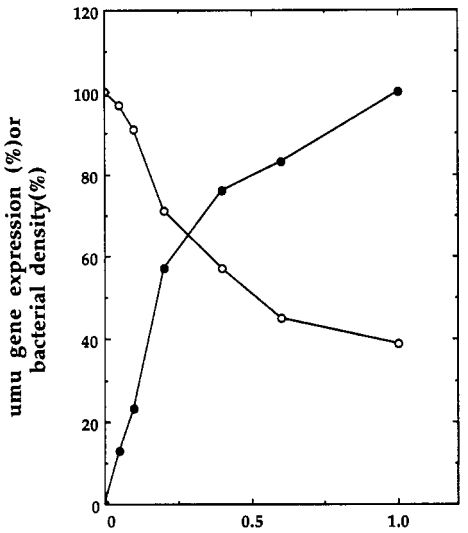

Figure 3. Effect of AFB_1-8,9-oxide on the induction of *umu* gene response and bacterial cell growth. Varying amounts of AFB_1-8,9-oxide (dissolved in 10 μL of acetone) were added to *S. typhimurium* TA 1535/pSK1002 (1.0-mL volume), and incubation proceeded for 120 min at 37 °C; 0.1 mL was diluted for measurement of β-galactosidase activity (*umu* gene activation) (●; 100% = 2000 units mL^{-1}), and the remainder was used to estimate bacterial cell density (○; 100% = A_{600} of 0.85).

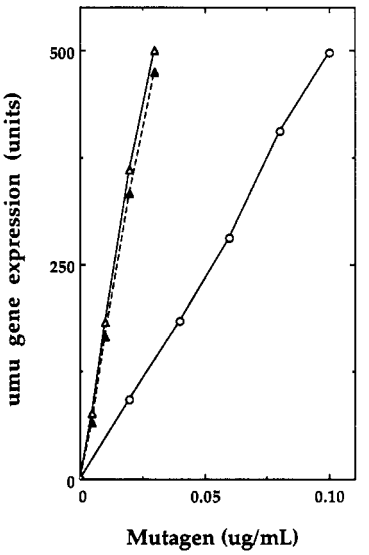

Figure 4. Comparison of *umu* gene response to AFB_1-8,9-oxide and dinitropyrenes in *S. typhimurium* TA 1535/pSK1002. Experiments were as described in Figure 3, and results are shown for 1,3-dinitropyrene (△), 1,6-dinitropyrene (▲), and AFB_1-8,9-oxide (○).

[*a*]pyrene yielded similar *umu* responses (on a molar basis) under these conditions.

Enzymatic Activation of Dihydrofurans. Human liver microsomes were used to activate the dihydrofurans enzymatically, and *umu* gene activation followed the order STG > AFB_1 > AFG_1 (*9*). Further determinations are shown in Table II. A similar pattern was also observed with liver microsomes prepared from untreated rats: STG gave the greatest response. The pattern was rather similar with liver microsomes prepared from rats treated with a number of different chemicals (phenobarbital, 5,6-benzoflavone, isosafrole, pregnenolone-16α-carbonitrile, isoniazid, and ciprofibrate), consistent with earlier findings that AFB_1-induced *umu* gene activation is not particularly enhanced by these treatments (*24*).

These patterns can be compared with those reported by others (Table III) for base-pair-dependent (TA 100) and frame-shift (TA 98, TA 1538) reversion mutations in *S. typhimurium*. However, different rat strains and treatments had been used in the previous literature cited, and

Table III. Previous *S. typhimurium* Responses to AFB_1, AFG_1, and STG

		relative response to each compound		
reference	strain	AFB_1	AFG_1	STG
	Base-Pair Systems			
10	TA 100	27		100
25	TA 100	100		8
26	TA 100	100	22	
	Frame-Shift Systems			
10	TA 98	100		7
25	TA 98	100		13
25	TA 1538[a]	100		5
27	TA 98	100		11

[a] This strain is devoid of the SOS repair response.

direct comparisons of genotoxic activity with DNA adduct formation were sought.

The individual calf thymus DNA guanyl-N^7 adducts derived from in vitro activation of the three dihydrofurans in the presence of human liver microsomes were quantified (Table II). Guanyl-N^7 adducts were found in the order AFB_1 > STG > AFG_1, though the differences were not particularly striking. No evidence for other adducts (re-

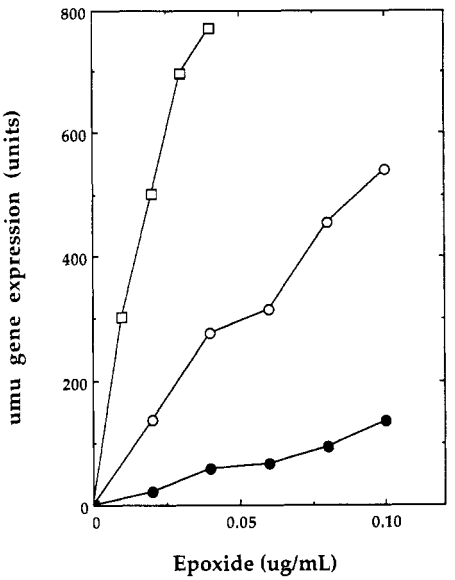

Figure 5. Induction of *umu* gene response by AFB_1-8,9-oxide, AFG_1-9,10-oxide, and STG-1,2-oxide. Experiments were as described in Figure 1, and results are shown for STG-1,2-oxide (□), AFB_1-8,9-oxide (○), and AFG_1-9,10-oxide (●).

leased by pH 5 or pH ≤2 hydrolysis at 95 °C) was obtained with the HPLC systems described here.

Reactions with Synthetic Epoxides. Some inferences as to relative rates of epoxidation and biological response can be drawn from the enzymatic experiments (vide supra). In order to address some of the questions concerning relative reactivities and biological responses, the synthetic epoxides were examined for their effects. All three of the epoxides yielded *umu* gene activation that was directly related to the amount of compound added (Figure 5). The relative order of reactivity was STG > AFB_1 > AFG_1, the same order seen in the microsomal activation experiments, with the effect of STG being more pronounced (Table II).

When the epoxides were mixed with calf thymus DNA in the absence of protein, 50–75% of the epoxide formed the guanyl-N^7 adduct in each case (Table II). When microsomal protein was included to mimic the conditions of the enzymatic activation assays, the yields of DNA adducts were reduced in the case of AFB_1 and the order of adduct formation was altered. In other experiments, attempts were made to estimate the half-lives of the three epoxides (Figure 6). The compounds were mixed with aqueous buffer for varying amounts of time, and then bacteria were added. Under these conditions less than half the *umu* gene activation was seen after a period of 12 s in every case, implying a $t_{1/2}$ of less than 10 s.

Stability and Fate of DNA Adducts. Guanyl-N^7 alkyl adducts in DNA are unstable and can undergo depurination and imidazole ring opening reactions (*18*, *28*). Calf thymus DNA was modified by treatment with either AFB_1-8,9-oxide, AFG_1-9,10-oxide, or STG-1,2-oxide, and the fate of the adducts was examined under conditions resembling physiological systems (Figure 7). In no case were dihydrodiols detected, although the caveat should be presented that the calf thymus DNA used in these experiments contains small amounts of residual protein, which might have reacted with the diols at pH 7.35. In all three cases the loss of guanyl-N^7 adducts occurred at a comparable rate, and the rates of depurination were rather similar. With DNA modified with the AFB_1 and AFG_1, one of the two possible imidazole ring opened derivatives (FAPY I) was formed, but such a residue could not be detected in the case of the STG derivative. Only

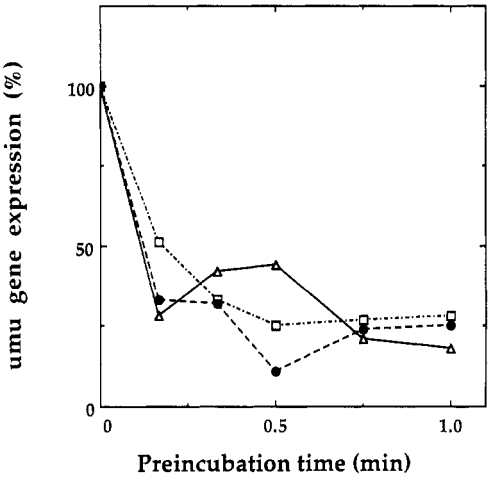

Figure 6. Effects of preincubation time of epoxides of AFB_1, AFG_1, and STG in aqueous buffer on the induction of *umu* gene expression. The general protocol was similar to that described in Figure 1 except that the epoxide was mixed with an aqueous solution of 0.1 M Tris-HCl (pH 7.5) containing 5% (v/v) tetrahydrofuran for the indicated time period before *S. typhimurium* TA 1535/pSK1002 was added. For the zero time points, the epoxide was added directly to the bacterial cells (in the same amount of buffer and acetone). Data are shown for AFB_1-8,9-oxide (△) (100% = 540 β-galactosidase units mL^{-1} with 0.1 μg of epoxide), AFG_1-9,10-oxide (●) (100% = 140 β-galactosidase units mL^{-1} with 0.1 μg of epoxide), and STG-1,2-oxide (□) (100% = 954 β-galactosidase units mL^{-1} with 0.03 μg of epoxide).

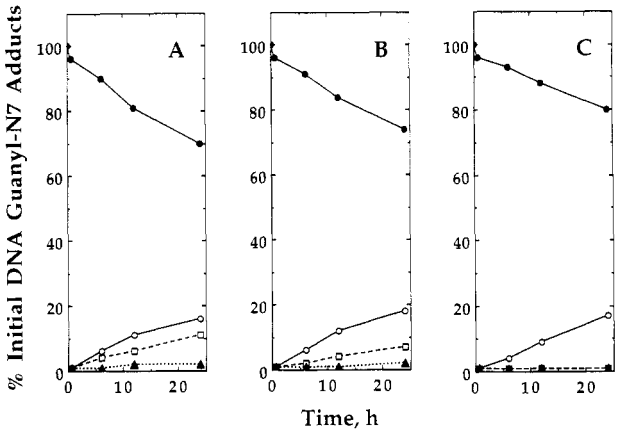

Figure 7. Stability of DNA guanyl-N^7 adducts and formation of specific derivatives. Results are shown for calf thymus DNA modified with (A) AFB_1-8,9-oxide, (B) AFG_1-9,10-oxide, and (C) STG-1,2-oxide. The respective levels of adducts formed in the three cases were 26, 21, and 17 nmol (mg of DNA)$^{-1}$. Modified DNA was dissolved in 67 mM potassium phosphate buffer (pH 7.35) and incubated at 37 °C. At the indicated time points, aliquots were analyzed for residual DNA guanyl-N^7 alkyl residues (●), free guanyl-N^7 derivatives (indicative of abasic site formation) (○), and the imidazole ring-opened derivative of each guanyl-N^7 derivative corresponding to FAPY I (□) and FAPY II (▲). No dihydrodiol could be detected in these assays at any of the time points.

traces of the other imidazole ring opened derivative (FAPY II) were detected in the cases of the AFB_1 and AFG_1 adducts (and none was found in the case of STG) (Figure 7). The results are qualitatively similar to those reported by Groopman et al. (*28*) for the stability and fate of AFB_1-N^7-Gua in calf thymus DNA.

Discussion

The aflatoxins and sterigmatocystin are quite potent in inducing *umu* gene activation, indicative of genotoxicity, in the system under investigation (*9*, *21*, *24*). The synthetic

epoxides of AFB_1, AFG_1, and STG appear to be some of the more potent genotoxins tested in this system. We previously reported that in human liver microsomes the enzyme P-450_{NF} is the principal catalyst involved in the activation of AFB_1, AFG_1, and STG (*9*). In this sense the human liver activation system is experimentally easier to deal with than that derived from rat liver, in which several P-450 enzymes contribute to the activation of each of these dihydrofurans (*24*).[4]

The order of *umu* gene activation seen after activation of dihydrofurans by human P-450_{NF} has been shown to be STG > AFB_1 > AFG_1 (Table II). We considered the hypothesis that *umu* gene activation was a direct reflection of DNA adduct levels. However, the order of guanyl-N^7 adduct formation in the microsomal systems is AFB_1 > STG > AFG_1, though the differences were not very great. [The differences between AFB_1 and AFG_1 are similar to those reported by Garner et al. (*16*).] In order to simplify the experimental system, the synthetic epoxides were added directly to DNA. It should be pointed out that the fraction of epoxide bound to DNA (Table II) is much higher than others have reported for diol epoxide derivatives of polycyclic aromatic hydrocarbons (*29*, *30*). When the labeling experiment was repeated with the synthetic epoxides and DNA in the presence of amounts of microsomal protein used in the enzymatic bioactivation experiments, the yields of guanyl-N^7 adducts were lowered somewhat for the epoxides of AFB_1, but not as much for STG and AFG_1 (Table II). Apparently the proteins sequester the AFB_1-8,9-epoxide and hold it in an aqueous environment where solvolysis is favored over nucleophilic attack by guanine. This situation is in contrast to polycyclic hydrocarbon diol epoxides, where DNA catalyzes hydrolysis and proteins protect against solvolysis (*31*). Lin et al. reported previously that dihydrodiol recovery was reduced in microsomal incubations when DNA was present (*32*). Our interpretation of the results is that DNA binds the AFB_1, AFG_1, and STG epoxides in ways such that covalent adduct formation is favored over hydrolysis, in contrast to the polycyclic hydrocarbon diol epoxides.

The order of *umu* gene activation STG > AFB_1 > AFG_1 was observed in both human and rat liver microsomal activation systems (Table II). This order of genotoxicity was unexpected on the basis of bioassay data, for AFB_1 has been reported to be 1–2 orders of magnitude more hepatocarcinogenic than STG (*4*, *33*). *umu* gene activation was also examined after the direct addition of the epoxide derivatives of AFB_1, AFG_1, and STG to *S. typhimurium*. This experimental protocol circumvented interpretations associated with differences in rates of enzymatic bioactivation; the order of *umu* gene activation was clearly STG > AFB_1 > AFG_1 (Table II, Figure 5). Caveats do need to be considered in making comparisons between in vivo biological response and in vitro DNA adduct formation. For instance, it is conceivable that one of the epoxides might not penetrate the cells very well. However, the in vitro DNA alkylation results obtained in the presence of microsomes would argue against this possibility, if it is assumed that microsomal membranes and bacterial cell markers would react with the epoxides in the same way.

For further consideration of the differential biological effects of the dihydrofurans, description of the bacterial and plasmid systems is useful. The formation of bulky DNA adducts leads to the activation of the protease RecA—such activation is thought to proceed largely from interaction with regions of single-stranded DNA, which accumulate as a result of replication blocks (*34*). The activated protease RecA cleaves the LexA protein, which acts as a repressor of the *umuC*/*umuD* operator, resulting in the increased expression of these genes (*35*). Thus, the plasmid *umuC*′*lacZ* construct is now allowed to produce high levels of β-galactosidase and yield *umu* gene activation. The activated RecA protease also cleaves the UmuD protein directly to produce a fragment that apparently binds to DNA polymerase III and allows replication across the site of the DNA lesion (*36*–*38*) in an "error-prone" manner. This induction of error-prone replication has been shown to increase the extent of both base-pair and frame-shift *S. typhimurium hisG46* mutagenesis by 2 orders of magnitude for both AFB_1 and STG (activated by microsomal preparations derived from Aroclor 1254-treated rats) (*25*). Since induction of the SOS repair response plays such an important role in the *hisG46* mutations that result from adducts generated by the compounds, one might expect to observe similar results for base-pair mutagenesis, frame-shift mutagenesis, and *umu* gene activation and that these parameters should be correlated with levels of DNA adduct formation seen with closely related chemicals.

Previous mutagenesis results regarding AFB_1, AFG_1, and STG are summarized in Table III. Most studies report that AFB_1 is considerably more active than AFG_1 or STG regardless of the system; the report of McCann et al. (*25*) implies that STG has similar activities in base-pair and frame-shift mutagenesis in the systems used. However, Mori et al. (*10*) reported a relatively high level of STG-induced base-pair (but not frame-shift) mutations in *S. typhimurium* TA 98 (relative to AFB_1-induced mutations)—these results appear to be at variance with those of McCann et al. (*25*), although the pattern observed by Mori et al. persisted with several STG derivatives (*10*). In another genotoxicity test system utilizing induction of the *sfi*A gene fused to *lacZ* ("chromotest"), AFB_1 was an order of magnitude more potent than AFG_1 (after microsomal activation) (*37*).

The biological activities of these dihydrofurans can be summarized as follows. AFB_1-derived adducts strongly induce the bacterial SOS response—both base-pair and frame-shift mutations appear to be strongly dependent upon the SOS system. Treatment of (calf thymus) DNA with AFG_1-9,10-oxide gave rise to approximately as many DNA adducts as in the case of AFB_1-8,9-oxide, and with microsomal activation of AFG_1 the adduct level was half of that obtained with AFB_1 (Table II). We presume that a similar ratio of DNA adducts is formed in vivo. AFG_1 adducts lead to weak induction of SOS response (Table II; *39*) and, possibly as a result of this, to only relatively low levels of base-pair or frame-shift mutagenicity (Table III). In the in vitro systems STG–DNA adducts are formed at levels similar to those of AFB_1 and AFG_1 adducts (either after microsomal activation or after direct addition of epoxides), and the SOS repair response, as measured by expression of the artificial *umuC*′*lacZ* plasmid, is even stronger than in the case of AFB_1 (Table II). However, the literature indicates that the resulting frame-shift mutagenesis is weak; base-pair mutagenesis may or may not be as strong as in the case of AFB_1 (*10*, *25*). An explanation for the results can be offered: similar levels of (guanyl-N^7) DNA adducts are formed with AFB_1, AFG_1, and STG. The AFB_1 adducts apparently lead to single-stranded DNA formation because of replication blocks and evoke SOS response, which is a necessary part of bypass replication leading to mutations at the adduct sites. For some reason, AFG_1 adducts may not lead to single-

[4] T. Shimada, M. Iwasaki, M. V. Martin, and F. P. Guengerich, *Cancer Res.* (in press).

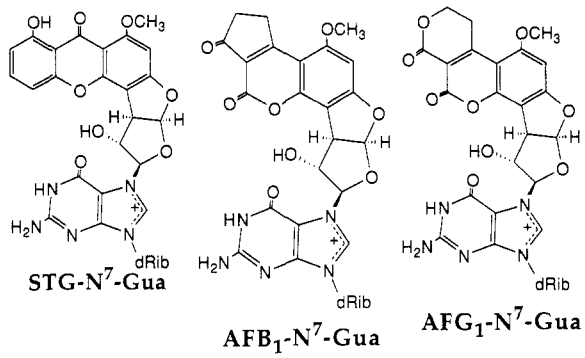

Figure 8. Structures of guanyl-N^7 adducts.

stranded DNA formation and do not evoke the SOS response particularly well. STG adducts are apparently very effective in evoking the SOS response but do not seem to be particularly mutagenic, at least with regard to the *hisG46* site. Some uncertainty exists as to whether base-pair mutagenesis occurs at a high level (*10*, *25*), but what seems to be clear is that the error-prone DNA polymerase III/UmuD peptide complex is not inclined to yield frame-shift mutations at the sites of the STG adducts—whether the basis is continued blockage of the adducted DNA strand or replication without miscoding is still unclear.

To continue discussion of the varying biological effects of the AFB_1–, AFG_1–, and STG–DNA adducts, modifications other than the guanyl-N^7 adducts need to be considered. First, AFB_1 adducts other than AFB_1-N^7-Gua (and its derived products) have not been reported in the literature (*4*, *40*), and attempts to force AFB_1-8,9-oxide reaction with residues in poly(dA·dT) have been unsuccessful.[5] However, it is still very difficult to categorically rule out the possibility that a small population of another structure (other than guanyl-N^7 adducts) may have remarkable biological activity. Of perhaps more concern are the imidazole ring opened products of the guanyl-N^7 adducts derived by base-catalyzed hydrolysis—these products are more persistent than the original AFB_1-N^7-Gua adduct (*4*, *40*). Groopman and Kensler have interpreted the results of studies in which rat tumors were measured as a function of dietary ethoxyquin to mean that the levels of AFB_1-N^7-Gua adducts and not the imidazole ring opened products are most important in determining whether liver tumors will form (*40–42*). In our studies, STG-N^7-Gua adducts did not undergo ring opening; the degree of ring opening did not vary appreciably between the AFB_1- and AFG_1-based adducts, yet the biological effects were quite different. Nonenzymatic action or glycosylase activity can convert guanyl-N^7 adducts to apurinic (abasic) sites, which have been postulated by some to be important in mutagenesis (*43*, *44*). A paradox exists, however, in that a relatively high load of apurinic sites already exists in cells (at least mammalian cells) and the added burden of apurinic sites imposed by strong mutagens and carcinogens does not seem great enough to explain the biological activities of such compounds (*44*). In our studies, we did not see major differences among the guanyl-N^7 adducts derived from AFB_1, AFG_1, and STG in their nonenzymatic rates of apurinic site formation (Figure 7). However, we cannot be sure that the in vivo reactions may not differ. Further, some of the interpretations of mammalian results related to derivatives of the guanyl-N^7 adducts may be irrelevant to bacterial mutagenesis.

Our current working hypothesis is that different conformations of the three guanyl-N^7 dihydrofuran adducts in DNA are the basis of the varying biological effects (Figure 8). Factors related to AFB_1 mutation have been considered by Refolo et al. (*45*). Loechler et al. (*46*, *47*) have proposed a model for major groove binding of AFB_1-8,9-oxide in DNA; our own work favors a model involving intercalation between base pairs, as judged by NMR and plasmid-unwinding studies (*48*, *49*). In principle it should be possible to characterize conformations of all three guanyl-N^7 adducts in oligomeric DNA and nucleotide pairs and relate these structures to biological activities. The point should be made that the bacterial end points considered in this paper may not be equivalent to processes involved in hepatocellular carcinogenesis—the observed mutations contain bias and the induction of bacterial SOS repair and error-prone replication may not have a direct counterpart. Others have found a base-pair substitution in codon 12 of *ras* genes in NIH 3T3 cells transformed by DNA isolated from AFB_1-induced hepatic tumors (*50*, *51*). The etiology of this process and its significance in the entire framework of AFB_1-induced hepatocellular carcinogenesis remain to be established.

This work would suggest that the conformations of AFB_1-N^7-Gua, AFG_1-N^7-Gua, and STG-N^7-Gua may prove to be significantly different in DNA to account for the divergent biological responses. It is of interest to note the 4-fold difference between the enantiomers of aflatoxicol Yourtee et al. reported for base-pair mutagenesis, although the contribution of relative rates of oxidation was not considered (*26*). Differences in the patterns of sequence specificity for DNA modification and mutation spectra might also exist for AFB_1-8,9-oxide, AFG_1-9,10-oxide, and STG-1,2-oxide. Further comparison of the adducts may lead to significant insights as to how these potent carcinogens exert their effects and how the chemistry of DNA adducts is related to genotoxicity and tumorigenesis.

Finally, it should be pointed out that studies with STG are not only of academic interest. STG is a biosynthetic precursor of AFB_1 (*4*) and, although not often detected because of its weak fluorescence, is often found at high levels in the environment. Holzapfel et al. (*33*) reported levels of 0.75 and 1.2 g of STG/kg of meal contaminated with *Aspergillus nidulans* and a *Bipolaris* species, respectively, considerably higher than levels usually reported for AFB_1 (*2*). Davies et al. reported a level of 1% STG (w/w) in the dried mycelia of *Aspergillus versicolor* (52). Although the carcinogenic activity of STG is less than that of AFB_1, much higher levels of this compound (than AFB_1) are often found in foodstuffs, and the toxic potential of STG may be underestimated (*31*).

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[5] K. D. Raney, M. P. Stone, and T. M. Harris, unpublished results. An anecdotal report has been mentioned (*15*) of an adenine–aflatoxin B_1 adduct using a peracid system to activate AFB_1 in the presence of poly(dA).

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