Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 179–183, January 1979
Biochemistry

Sterigmatocystin–DNA interactions: Identification of a major adduct formed after metabolic activation *in vitro*

(myocotoxins/chemical carcinogens/carcinogen–macromolecule interactions/DNA adducts/NMR spectroscopy)

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ABSTRACT **Sterigmatocystin (ST), a potent hepatocarcinogen, was covalently bound to calf thymus DNA by incubation in the presence of phenobarbital-induced rat liver microsomes. Acid hydrolysis of ST-modified DNA liberated a major guanine-containing adduct, present in DNA at an estimated level of 1 ST residue per 100–150 nucleotides. The adduct was isolated by high-pressure liquid chromatography and subjected to structural analysis. Spectral and chemical data identified the adduct as 1,2-dihydro-2-(N^7-guanyl)-1-hydroxysterigmatocystin, the guanine and hydroxyl moieties being in a *trans* configuration. The structure and stereochemistry of this adduct indicated that the exo-ST-1,2-oxide was the metabolite that reacted with DNA, and the quantitative yield of adduct indicated that this metabolite was a major product of the *in vitro* metabolism of ST.**

Sterigmatocystin (ST) is a carcinogenic mycotoxin produced as a secondary metabolite by *Aspergillus*, *Penicillium*, and *Bipolaris* species (1, 2). ST is acutely toxic to the liver of most animals tested (3, 4), and its carcinogenicity has been demonstrated with organ specificity varying with species and route and frequency of administration (5–9). In rats, ST induces hepatocellular carcinomas after oral administration (6) or intraperitoneal injection (5) and squamous cell carcinomas after repeated application to the skin (7). Despite its potent toxic and carcinogenic properties in animals, the importance of ST as a human health hazard is unknown because surveillance programs have detected its presence in foods only infrequently and at low concentrations even though ST-producing fungi are widely distributed (10).

Nonetheless, ST is of interest as a model compound for cancer induction because of its structural similarity to aflatoxin B_1 (AFB_1). In rats and monkeys, the lethal potency of ST is about 1/10th that of AFB_1 (3), and ST is between 1 and 2 orders of magnitude less potent as a hepatocarcinogen for the rat (5, 6). A comparable quantitative difference exists in the toxicities and mutagenicities of ST and AFB_1 in *Salmonella typhimurium* (11, 12).

In contrast to the large literature on AFB_1, little information has been published on the metabolism and biochemical effects of ST. It has been shown that a large portion of the dose administered to monkeys is converted to a ST-glucuronide (13) and that metabolic activation is required for the toxicity and mutagenicity of ST in bacteria and some cultured cells (11, 12, 14–16). Mammalian cells in culture exposed to ST display nucleolar aberrations, inhibited mitosis, inhibited uptake of thymidine and uridine, and stimulated DNA repair synthesis (17–20). ST also has been demonstrated to inhibit RNA synthesis in rat liver (21).

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No data previously existed relating to ST metabolism resulting in covalent binding to nucleic acids and proteins. Most of the biochemical effects of ST, however, are also exhibited by chemicals that interact with cellular macromolecules. Our laboratory is engaged in a series of investigations on the *in vitro* and *in vivo* interactions of chemical carcinogens with informational macromolecules (22–24). Early experiments are emphasizing interactions with DNA, because chemical fixation of damage in this macromolecule could be a prelude to genetic alterations resulting in mutation, cancer, and other diseases resulting from functional alterations of the genetic apparatus. In the present research, the metabolic activation and DNA binding of ST was investigated. The results indicate that ST is metabolically activated to a regiospecifically defined epoxide that binds to DNA to form a major adduct, 1,2-dihydro-2-(N^7-guanyl)-1-hydroxysterigmatocystin (ST-N^7-Gua).§

MATERIALS AND METHODS

ST was metabolically activated in the presence of calf thymus DNA by using phenobarbital-induced rat liver microsomes. The incubation mixture (400 ml; divided into eight 50-ml portions) included approximately 1 mg of microsomal protein per ml, 20 A_{260} units of DNA per ml, an NADPH-generating system, and ST (278 μM dissolved in 8 ml of dimethyl sulfoxide; provided by John Douros, National Cancer Institute). Other details of the system used for the binding of ST to DNA and subsequent formic acid hydrolysis of ST-modified DNA were as described (22). In some experiments the adduct, ST-N^7-Gua, was removed from DNA by heat treatment (100°C, 15 min) in an acidic buffer (0.05 M K acetate, pH 5.3). Subsequently, the heat-denatured DNA was hydrolyzed to mononucleotides with nuclease P1 (10 μg/mg of DNA, 18 hr, 37°C).

High-pressure liquid chromatography (HPLC) of DNA hydrolysates was carried out on a Micromeritics model 7000 chromatograph equipped with a Waters Associates model 440 detector that monitored UV absorbance at 254 and 313 nm. For analytical HPLC, a μBondapak C_{18} column (Waters Associates) was eluted at 53°C and 1 ml/min with 25% ethanol in 10 mM K acetate (pH 5). Preparative HPLC was used to isolate pure ST-N^7-Gua for structure analysis, and it was performed exactly as described (22) for isolation of the major AFB_1–DNA adduct; ST-N^7-Gua eluted 37 min after gradient initiation.

We also used a technique which potentially may find wide application as an adjunct to HPLC in the isolation of nonpolar carcinogen–macromolecule component adducts. Portions of DNA hydrolysates representing up to 100 mg of DNA were

Abbreviations: ST, sterigmatocystin; ST-N^7-Gua, 1,2-dihydro-2-(N^7-guanyl)-1-hydroxysterigmatocystin; HPLC, high-pressure liquid chromatography; AFB_1, aflatoxin B_1.

§ The numbering system for ST is that designated by the *Ninth Collective Index Guide of Chemical Abstracts*.

adjusted to be 5% in methanol and loaded by syringe onto a SepPak C-18 (reversed-phase) cartridge (Waters Associates). This device rapidly performed one of the more time-consuming tasks of the preparative HPLC column—i.e., bulk separation of polar and nonpolar residues. Unmodified DNA components were removed from the cartridge with 45 ml of 10% methanol. The adduct was subsequently eluted with 50 ml of 80% methanol, at a purity of about 90%, which was increased to over 98% by dissolving and reprecipitating ST-N^7-Gua several times with solutions of HCl and KOH, respectively. A total of 3 mg of chromatographically pure ST-N^7-Gua was isolated for structure analysis.

A portion of the purified adduct was treated with perchloric acid (72%) under conditions that cleaved the bond between the ST moiety and the nucleic acid base (100°C, 60 min; ref 22). In a second experiment, a methylated derivative of ST-N^7-Gua was synthesized, and the products of base-methylation were analyzed by cation exchange HPLC (22, 25). In a third experiment, a 6.8-mg portion of ST-modified DNA was treated with alkali in order to convert ST-N^7-Gua to its putative imidazole ring-opened derivative, ST-II. The DNA was dissolved in 0.1 M Na_2CO_3 (pH 9.8) and, after 3 hr, HCl was added to bring the solution to pH 3. The DNA was then heated at 100°C for 30 min to release ST-II.

UV spectra of ST-N^7-Gua were obtained in 0.01 M HCl (pH 2) on a Hitachi Perkin-Elmer model 200-6260 recording spectrophotometer. Fourier-transform NMR spectra were obtained on a Brüker model HX-270 spectrometer. The adduct (approximately 1 mg) was dissolved in either deuterodimethyl sulfoxide or 0.1 M deuterium chloride/deuterium oxide. The spectrum of ST was obtained in deuterochloroform solution. Electron impact mass spectra were recorded on photoplates for high-resolution analysis (model CEC 21-110, duPont), and field desorption mass spectra were recorded on oscillograph paper at low resolution (model 731, Varian MAT).

RESULTS

The covalent binding of ST to DNA (Fig. 1) was brought about by phenobarbital-induced rat liver microsomes. Heat-denatured microsomes were ineffective in producing ST-N^7-Gua in DNA or in incorporating any detectable moieties with ST-like spectral characteristics. The procedure used was identical to the one used, in an earlier adduct characterization study (22), to bind AFB_1 to DNA. In the present investigation, radioactively labeled ST was unavailable, making it impossible to determine precisely the total level of covalent binding to DNA. However, with regard to the adduct ST-N^7-Gua, it was possible to estimate its level of occurrence in DNA at approximately 1 adduct residue per 100–150 nucleotides. This is a conservative estimate, based on quantitative determination of the amount of guanine released by perchloric acid treatment of a portion of adduct obtained from a known amount of DNA. Under identical conditions of metabolic activation, AFB_1 binds to DNA at a level of 1 adduct residue per 60 DNA nucleotides.

Adduct liberation was effected by either partial hydrolysis of DNA with formic acid at ambient temperature or by heat treatment in acidic buffer. The observed acidic lability of the bond that links ST-N^7-Gua to DNA is characteristic of the deoxyglycosidic bond of 7-substituted purines (26, 27).

Analysis of ST–DNA hydrolysates on an analytical reversed-phase HPLC column (Fig. 2) revealed one major and several minor peaks with ST-like absorbance. The largest peak, ST-N^7-Gua, was isolated for structure analysis through the use of preparative HPLC and Sep-Pak C_{18} cartridges.

The structure and stereochemistry of ST-N^7-Gua were deduced from a combination of chemical and spectral data. The

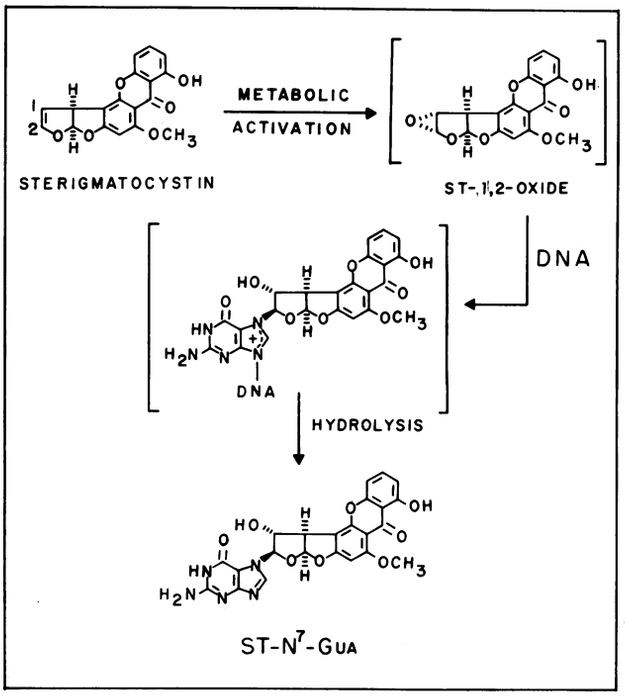


FIG. 1. Metabolic activation and DNA binding of ST.

UV spectrum of the adduct was qualitatively like that of ST, but a more intense absorbance was observed in the 250- to 260-nm region due to the guanyl residue. In acidic solution, the ratios of absorbances at 225 (sh), 248, 283 (sh), and 330 nm were 0.97, 1.00, 0.21, and 0.38, respectively. The small quantity of purified adduct isolated and its hygroscopic nature prevented accurate weighing and, therefore, molar absorptivities could not be calculated.

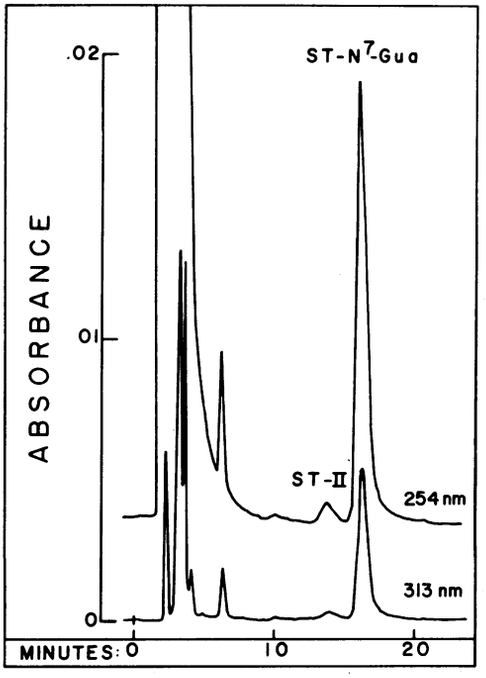


FIG. 2. Analytical HPLC of a ST-bound DNA hydrolysate. A portion of a hydrolysate representing 19 μg of ST–DNA was injected onto an analytical reversed-phase HPLC column and eluted isocratically with 25% ethanol. ST-II is a putative imidazole ring-opened derivative of ST-N^7-Gua.

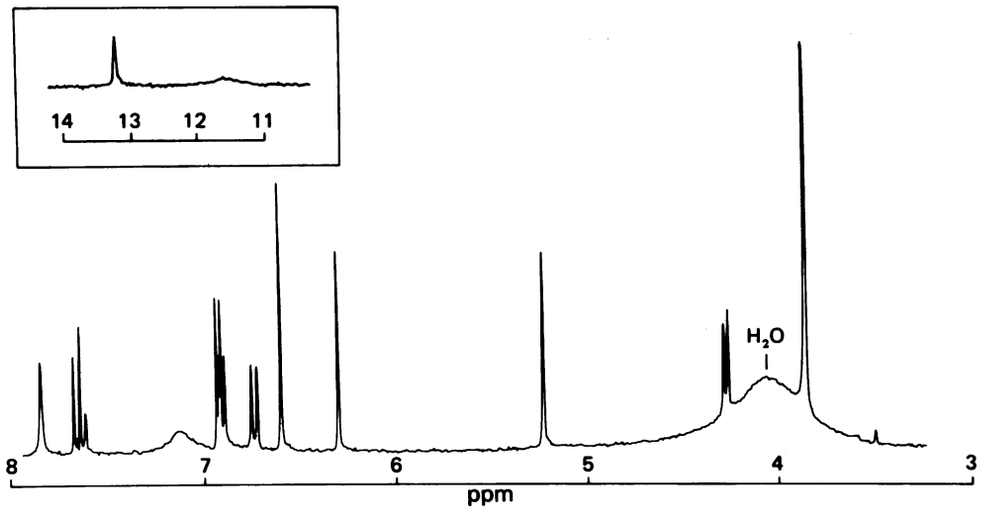


FIG. 3. NMR spectrum (270 MHz) of ST-N^7-Gua in deuterodimethyl sulfoxide.

Perchloric acid treatment of the adduct liberated guanine, as evidenced by its cation-exchange chromatographic behavior and by its UV spectra obtained at acidic and alkaline pH. Methylation of the adduct with dimethyl sulfate yielded two methylated bases after perchloric acid cleavage of the bond linking the ST and guanyl moieties. The major product was 9-methylguanine. The minor product, present at approximately 20% of the level of the major product, was 3-methylguanine. Again, both bases were identified on the basis of their chromatographic and UV spectral characteristics. No evidence of alkylation at the N^7 position was observed—i.e., neither 7-methylguanine nor 7,9-dimethylguanine was detected. These data provide strong evidence that the preferred site of alkylation on guanine, the N^7 atom, was blocked by the ST moiety at the time of methylation. Thus, the bond between the carcinogen and the DNA base links ST to the N^7 atom of guanine.

High-field NMR spectra of the adduct (Fig. 3) displayed resonances similar to those of ST and Gua (Table 1), with additional resonances that were attributed to protons that arise in their ultimate configuration from the *trans*-opening of the precursor ST-1,2-oxide. These latter protons were assigned primarily on the basis of their similarities to comparable protons in the previously identified AFB_1–guanine adduct (22).

The ST-N^7-Gua adduct displayed xanthone system resonances similar to those of ST (Table 1) (28, 29). The one- and two-proton resonances of the guanine amide (H_f) and exocyclic amino group (H_i) were observed at 11.61 and 7.13 δ, respectively. The C-8 guanine proton was observed at 7.84 δ and, as is characteristic of 7-substituted guanine derivatives, this proton shifted downfield to 8.28 δ in acidic medium. Unlike the AFB–guanine adduct (22), the C-1 hydroxyl group proton was not visible in the spectrum of ST-N^7-Gua. However, the resonance of H_c at 5.23 δ indicated its attachment to a carbinol carbon, and its chemical shift was identical to that of the comparable proton in 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1. It is possible that the chemical shift of the hydroxyl proton was the same as or similar to that of H_j and that the hydroxyl proton contributed to the unsymmetrical nature of the triplet attributed to H_j (Fig. 3). This hypothesis is supported by the evidence that the H_j triplet became symmetrical after treatment with deuterium oxide.

The stereochemistry of the adduct was deduced from the NMR data. Spin–spin coupling was observed between H_h and H_b, protons of known *cis* configuration (28). Coupling was not observed between protons H_b and H_c or between protons H_c and H_d; this indicates a *trans-trans* relationship for protons H_b, H_c, and H_d. Thus, the stereochemistry of the adduct is fixed with an exo-1-hydroxyl group *trans* to the guanine base. This also indicates that the most chemically reasonable ST metabolite to have reacted with DNA to give this product was the β (or exo)-ST-1,2-oxide (Fig. 1).

High-resolution mass spectrometry of electron impact-ionized ST-N^7-Gua yielded fragments that fit the composition for guanine (m/e 151) and a hydroxylated sterigmatocystin (m/e 340), the latter being the highest mass detected. Both of these primary fragments exhibited further degradation and rearrangement which was also shown to occur in the AFB_1 adduct of earlier studies (22). One such rearrangement could be accounted for as arising from a methyl group transfer from the sterigmatocystin moiety to guanine, yielding the ions at m/e 165 and another presumably intermolecular methylation of guanine to m/e 179. Loss of the elements H_2O (m/e 322) from the ST moiety (m/e 340) as well as the CH_2 fragment (m/e 312) was expected from the results of earlier studies (22).

The low-energy process of field-desorption ionization indicated a molecular ion at m/e 491. The few additional detectable fragments (Fig. 4) showed masses equal to guanine (m/e 151) and a hydroxylated sterigmatocystin (m/e 340), suggesting that the molecular ion is composed of these two moieties linked by a very labile bond. The other ions in the spectrum can be accounted for by assuming methyl rearrangements or transfer as observed in the electron impact data or both.

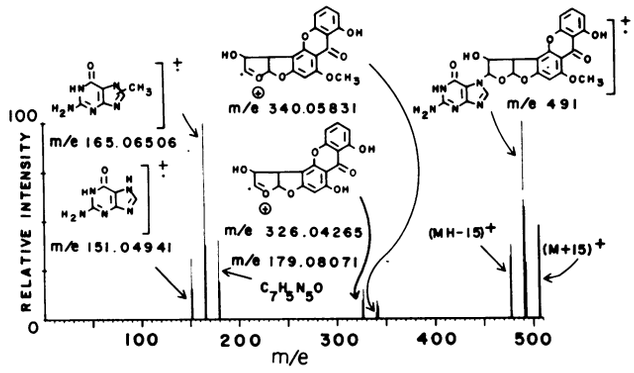
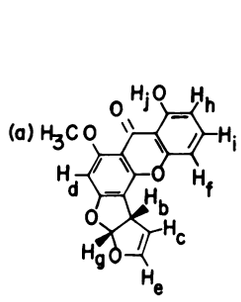
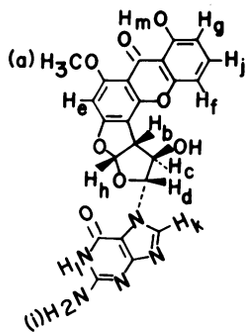


FIG. 4. Mass spectral analysis of ST-N^7-Gua. The spectrum shown was obtained by the field-desorption technique at an anode current of 22 mA. Structures of fragment ions are proposed on the basis of elemental compositions indicated by high-resolution electron-impact mass spectrometry.

Table 1. NMR data for sterigmatocystin and ST-N^7-Gua



STERIGMATOCYSTIN



ST-N^7-Gua

Proton	Chemical shift (δ)*	
a	4.00 (3 H, s)	3.87 (3 H, s)
b	4.82 (1 H, dt, J = 7.0, 2.0)	4.27 (1 H, d, J = 6.0)
c	5.46 (1 H, t, J = 2.4)	5.23 (1 H, s)
d	6.45 (1 H, s)	6.31 (1 H, s)
e	6.51 (1 H, t, J = 2.4)	6.60 (1 H, s)
f	6.77 (1 H, d, J = 8.4)†	6.74 (1 H, d, J = 8.4)
g	6.840 (1 H, d, J = 7.0)	6.922 (1 H, d, J = 8.4)
h	6.843 (1 H, d, J = 8.4)	6.916 (1 H, d, J = 6.0)
i	7.52 (1 H, t, J = 8.4)	7.13 (2 H, bs)‡
j	13.15 (1 H, bs)	7.64 (1 H, t, J = 8.4)
k	— —	7.84 (1 H, s)§
l	— —	11.61 (1 H, vbs)‡
m	— —	13.22 (1 H, bs)‡

* Chemical shifts are given in parts per million downfield from internal tetramethylsilane in deuterodimethyl sulfoxide for ST-N^7-Gua and in deuterochloroform for ST. In parentheses are shown the relative number of protons, multiplicity, and coupling. Abbreviations: s, singlet; d, doublet; t, triplet; dt, doublet of triplets; bs, broad singlet; vbs, very broad singlet; J, coupling constant in hertz.

† The previously reported (28, 29) spectrum of ST in deuterochloroform could not be fully interpreted because of overlapping resonances in the 6–7 δ region. At 270 MHz, it was observed that the nonequivalent xanthone aryl protons constitute a first-order AMX system. Other workers have observed *meta*-coupling of 1–2 Hz between protons H_h and H_f in ST and related compounds (28, 30); this coupling was not observed in either ST or ST-N^7-Gua.

‡ Shift variable in different samples; resonance disappeared upon addition of deuterium oxide.

§ Resonance appeared at 8.28 δ in deuterium chloride/deuterium oxide.

Additional information that supports the spectral data on the proposed adduct structure is the demonstrated alkaline lability of the DNA-bound precursor of ST-N^7-Gua. In alkaline solution, positively charged 7-substituted purine deoxyribonucleosides, including that of AFB_1 (31), characteristically undergo nucleophilic attack by hydroxide at C-8 of guanine, followed by imidazole ring opening (26). The present results indicate that the DNA precursor of ST-N^7-Gua was converted almost quantitatively by alkali to a more polar product, ST-II. Under identical conditions of alkali treatment, the free adduct ST-N^7-Gua was unaffected. A small amount of a component that was chromatographically identical to ST-II was observed consistently in hydrolysates of ST-modified DNA (Fig. 2).

DISCUSSION

Metabolically activated ST reacted with DNA to produce a covalent adduct characterized as 1,2-dihydro-2-(*N*^{7}-guanyl)-1-hydroxysterigmatocystin. Activation was shown to result in the production of the electrophilic exo-ST-1,2-oxide (Fig. 1). Quantitatively, this epoxide was a major product of ST metabolism because, on the basis of its DNA binding level (1 ST-N^7-Gua adduct per 100 to 150 nucleotides), at least 6–9% of the ST in the initial reaction mixture was converted to epoxide and reacted with DNA. This is a conservative estimate of epoxide yield, because no account was taken of ST epoxide that undoubtedly reacted with water molecules to form the ST dihydrodiol or of epoxide that may have reacted with DNA or other nucleophilic compounds in the mixture to form unidentified or undetected adducts.

This project was initiated to investigate the extent to which chemical structure, metabolism, and DNA binding determine similarities and differences in the toxicologies of two chemical carcinogens, ST and AFB_1. Although these compounds differ markedly in certain structural characteristics, both contain the bisfuranomethoxybenzene moiety. With AFB_1, regiospecific epoxidation of the terminal furan ring of this moiety is the principal metabolic event responsible for DNA binding (22, 31), and good evidence exists that this epoxide is the ultimately carcinogenic form of this molecule (23, 31–33). Our data show that the stereochemistry of the ST epoxide is identical to that of AFB_1 and that a major target for both epoxides is the N^7 atom of guanine, which is located in the major groove of DNA in a position apparently readily accessible to attack by bulky chemicals.

Although attempts to relate *in vitro* and *in vivo* metabolism and DNA-binding patterns entail a high degree of uncertainty, it is conceivable that the toxicological similarities and differences between ST and AFB_1 (3–7, 34, 35) may be attributed to their mechanisms of epoxidation and interaction with DNA. A number of reasonable mechanisms can be proposed. In rat liver, for example, the toxic and tumor-producing potency of ST is much lower than that of AFB_1 (3, 5, 6). It is possible that the ultimately carcinogenic form of ST, conceivably the epoxide, is formed in rat liver in lower amounts than that of AFB_1 or that it reacts more readily with other cellular nucleophiles (e.g., glutathione) to produce detoxification products. Alternatively, differences in potency might be attributable to differences in the relative intragenomic distribution of binding by ST and AFB_1, determined perhaps by structural characteristics that might limit access of one or the other to specific areas of the genome. It is also possible that structurally similar DNA adducts of ST and AFB_1 have different efficiencies in initiating carcinogenic events, with AFB_1 generally being more effective than ST in this regard.

These carcinogens may also differ in the kinetic features of adduct removal and repair of their respective modified DNAs, as has been shown to be the case with DNAs modified *in vivo* with alkylating agents (36, 37). Finally, it cannot be overlooked that ST and AFB_1 possibly differ in toxicological properties by virtue of quantitatively minor interactions with DNA, because of interactions with other cellular components by as yet unexplained mechanisms or simply because of differences in the relative distribution of the toxins in various organs after administration.

We gratefully acknowledge the contributions of Professor George Büchi (Department of Chemistry, Massachusetts Institute of Technology) both for his advice on the experimental portion of this work and for his critical review of the manuscript. We also acknowledge the contributions of Professor Klaus Biemann and the staff of the Massachusetts Institute of Technology Mass Spectrometry Facility, which is operated under Grant RR00317 from the Biotechnology Resources Branch, Division of Research Resources of the National Institutes of Health. We are also grateful for the help provided by Dr. Leo Neuringer in obtaining the 270-MHz NMR spectra; the high-field NMR experiments were performed at the NMR Facility located at the F. Bitter National Magnet Laboratory, Massachusetts Institute of Tech-

nology (supported by Grant RR00995 from the Division of Research Resources of the National Institutes of Health and by the National Science Foundation under Contract C-670). Principal financial support for this research was provided by Grants T32 CA 09112 and 5 PO1 ES 00597-06 and Contract N01-CP43265 from the National Institutes of Health.

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