Comparison of the DNA-Alkylating Properties and Mutagenic Responses of a Series of *S*-(2-Haloethyl)-Substituted Cysteine and Glutathione Derivatives†

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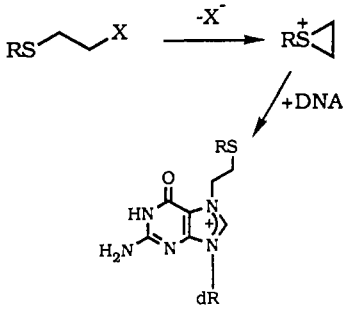
ABSTRACT: The mutagenicity of 1,2-dibromoethane is highly dependent upon its conjugation to glutathione by the enzyme glutathione *S*-transferase. The conjugates thus formed can react with DNA and yield almost exclusively N^7-guanyl adducts. We have synthesized the *S*-haloethyl conjugates of cysteine and glutathione, as well as selected methyl ester and *N*-acetyl derivatives, and compared them for ability to produce N^7-guanyl adducts with calf thymus DNA. The cysteine compounds were found to be more reactive toward calf thymus DNA and yielded higher adduct levels than did the glutathione compounds. Adduct levels tended to be suppressed when there was a net charge on the compound and were not affected by substitution of bromine for chlorine, as expected for a mechanism known to involve an intermediate episulfonium ion. Sequence-selective alkylation of fragments of pBR322 DNA was investigated. The compounds produced qualitatively similar patterns of alkylation, with higher levels of alkylation at runs of guanines. The compounds were also tested for their ability to act as direct mutagens in *Salmonella typhimurium* TA98 and TA100. None of the compounds caused mutations in the TA98 frameshift mutagenesis assay. In the strain TA100, where mutation of a specific guanine by base-pair substitution produces reversion, all compounds were found to produce mutations, but the levels of mutagenicity did not correlate at all with the levels of DNA alkylation. The ratio of mutations to adducts varied at least 14-fold among the various N^7-guanyl adducts examined. *S*-(2-Chloroethyl)glutathione was found to be the most potent mutagen, although it produced only intermediate levels of alkylation, indicating that the guanyl adduct formed in this case seems to be unusually mutagenic. The differences cannot be attributed to error-prone DNA repair mechanisms, since the activation of a *umu*C-containing plasmid (harbored in *S. typhimurium* TA 1535) was weak with all of the compounds examined and the response was in the opposite order of the mutation/adduct ratio. The results indicate that (1) N^7-alkylguanine residues can be quite mutagenic and (2) small differences in the structures of a single such adduct can dramatically alter mutagenicity.

EDB[1] has been shown to be mutagenic in a variety of tester systems (Rannug, 1980; Crepsi et al., 1985; Zoetamelk et al., 1987) and is an animal carcinogen capable of producing tumors in a wide variety of tissue types in experimental animals (Olson et al., 1973; Weisenberg, 1977; Wong et al., 1982; National Toxicology Program, 1982). Although positive epidemiological evidence for human carcinogenicity is lacking (Ramsey et al., 1979; Ott et al., 1980), EDB is acutely toxic and has caused at least two human deaths (Letz et al., 1981).

The bulk of the evidence on the mechanism of EDB and other *vic*-dihaloalkane-induced genotoxicity points toward a GSH-mediated pathway (Rannug, 1980; van Bladeren et al., 1982; Sundheimer et al., 1982; Ozawa & Guengerich, 1983; Inskeep & Guengerich, 1984; Inskeep et al., 1986; Koga et al., 1986). The compounds are first transferred enzymatically to GSH by a GSH *S*-transferase mediated step to produce *S*-(2-haloethyl)GSH conjugates. These compounds can then react with DNA, through an episulfonium ion intermediate (Peterson et al., 1988), to produce almost exclusively N^7-guanyl adducts (Ozawa & Guengerich, 1983; Inskeep et al., 1986; Koga et al., 1986).

Many simple electrophilic species react predominantly at the N^7-position of guanine because this position is highly electronegative and also is exposed to the major groove. These adducts have received relatively little attention because of their inherent instability and the fact that for some short-chain alkylating agents mutagenic activity has been shown to arise from adduction at other positions on guanine or on other bases (Lawley, 1984). In fact, methylating agents that produce only N^7 adducts are very weak mutagens, and N^7-methyl adducts do not block or interfere with DNA replication (Prakash & Strauss, 1970). Notable exceptions to this include the mycotoxins aflatoxin B_1 and sterigmatocystin, which are thought to form exclusively N^7-guanyl adducts as the premutagenic lesion (Busby & Wogan, 1984) and are extremely potent bacterial mutagens in both frameshift and base substitution

Scheme I: Pathway for the Formation of N^7-Guanyl Adducts from *S*-(2-Haloethyl) Derivatives



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[1] Abbreviations: EDB, ethylene dibromide (1,2-dibromoethane); FAB-MS, fast atom bombardment mass spectrometry; GSH, reduced glutathione; HPLC, high-performance liquid chromatography.

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Table I: Spectral Data for Synthetic Materials

compound name	NMR data, δ	mass spectral data, m/z [(peak identification), relative abundance]
S-(2-chloroethyl)GSH	($C^2H_3O^2H$) 2.13 (m, 2 H, Glu β), 2.54 (m, 2 H, Glu γ), 2.81 (dd, 1 H, *J* = 14.0, 9.1, Cys β H_a), 2.89 (m, 2 H, CH_2S), 3.11 (dd, 1 H, *J* = 14.0, 5.0, Cys β H_b), 3.63 (t, 1 H, *J* = 6.2, Glu α), 3.69 (t, 2 H, *J* = 7.8, CH_2Cl), 3.75 (s, 2 H, Gly α), 4.56 (dd, 1 H, *J* = 9.3, 4.7, Cys α)	(−)FAB-MS (3-nitrobenzoic acid) 390, 392 [(M − 2H + Na)$^-$, 25, 12], 368, 370 [(M − H)$^-$, 92, 37]; [$(HOCH_2CH_2)_3N$] 390, 392 [(M − 2H + Na)$^-$, 100, 28], 354 [(M − 2H + Na − HCl)$^-$, 29], 328 [(M − 2H + Na − CH_2CH_2Cl)$^-$, 28]
S-(2-fluoroethyl)GSH	(^2H_2O) 2.21 (m, 2 H, Glu β), 2.57 (m, 2 H, Glu γ), 2.92 (m, 3 H, CH_2S, Cys β H_a), 3.12 (dd, 1 H, *J* = 14.0, 5.3, Cys β H_b), 3.98 (t, 1 H, *J* = 6.5, Glu α), 4.01 (s, 2 H, Gly α), 4.59 (dd, 1 H, *J* = 8.6, 5.3, Cys α), 4.64 (dt, 2 H, *J* = 46.9, 5.7, CH_2F)	(−)FAB-MS (glycerol) 352 [(M − H)$^-$, 78]
S-(2-hydroxyethyl)GSH dimethyl ester	(^2H_2O) 2.23 (m, 2 H, Glu β), 2.58 (m, 2 H, Glu γ), 2.76 (t, 2 H, *J* = 6.5, CH_2S), 2.90 (dd, 1 H, *J* = 14.0, 8.6, Cys β H_a), 3.09 (dd, 1 H, *J* = 14.0, 5.3, Cys β H_b), 3.74 (m, 5 H, CH_2OH, $COOCH_3$), 3.84 (s, 3 H, $COOCH_3$), 4.03 (s, 2 H, Gly α), 4.18 (t, 1 H, Glu α), 4.56 (dd, 1 H, *J* = 8.6, 5.3, Cys α)	(+)FAB-MS (glycerol) 402 [(M + Na)$^+$, 26], 380 [(M + H)$^+$, 59]
S-(2-chloroethyl)cysteine methyl ester	[$(C^2H_3)_2SO$] 2.94 (m, 2 H, CH_2S), 3.12 (d, 2 H, *J* = 5.5, Cys β), 3.76 (s, 3 H, $COOCH_3$), 3.78 (t, 2 H, *J* = 7.3, CH_2Cl), 4.34 (t, 1 H, *J* = 5.7, Cys α)	(+)FAB-MS (3-nitrobenzoic acid) 198, 200 [(M + H)$^+$, 44, 17]
S-[2-(*N*^{7}-guanyl)ethyl]GSH dimethyl ester	(^2H_2O) 1.96 (m, 2 H, Glu β), 2.36 (m, 2 H, Glu γ), 2.65 (dd, 1 H, *J* = 14.0, 8.7, Cys β H_a), 2.84 (dd, 1 H, *J* = 14.0, 5.1, Cys β H_b), 3.07 (m, 2 H, CH_2S), 3.57 (t, 1 H, Glu α), 3.72 (s, 6 H, $2COOCH_3$), 3.99 (s, 2 H, Gly α), 4.44 (m, 3 H, Cys α, CH_2N), 8.00 (s, 1 H, C^8H)	(−)FAB-MS (glycerol) 513 [(M − H)$^-$, 10]
S-[2-(*N*^{7}-guanyl)ethyl]cysteine methyl ester	(^2H_2O) 2.67 (dd, 1 H, *J* = 14.0, 5.1, Cys β H_a), 2.75 (dd, 1 H, *J* = 14.0, 7.0, Cys β H_b), 3.07 (m, 2 H, CH_2S), 3.76 (s, 3 H, $COOCH_3$), 3.85 (m, 1 H, Cys α), 4.49 (m, 2 H, CH_2N), 8.04 (s, 1 H, C^8H)	(−)FAB-MS (glycerol) 311 [(M − H)$^-$, 19]

reversion assays (McCann et al., 1975). However, caveats concerning the formation of imidazole ring opened products must be considered [see Groopman et al., (1981)].

In this paper the formation of N^7-guanyl adducts by a series of *S*-(2-haloethyl) derivatives of GSH and cysteine (Scheme I) was examined in terms of their alkylating ability and their base sequence selectivity. The compounds were tested for their ability to elicit the bacterial SOS response by monitoring the expression of the *umuC* gene. Finally, the direct mutagenicity of these derivatives was monitored in a base mispair dependent reversion assay (*Salmonella typhimurium* TA100).

Materials and Methods

General. Cysteine, *N*-acetylcysteine, GSH, and guanosine were all purchased from Sigma Chemical Co. (St. Louis, MO). 1-Bromo-2-chloroethane and EDB were obtained from Aldrich Chemical Co. (Milwaukee, WI). NMR spectra were recorded on a IBM NR-300 spectrometer. Chemical shifts are reported in ppm; either tetramethylsilane or 2,2-dimethyl-2-silapentane-5-sulfonate was used as an internal standard. Mass spectra were recorded in the Vanderbilt University Mass Spectrometry Laboratory by Dr. B. Sweetman and B. Nobes on a VG 70-250 system having an extended geometry, a standard VG FAB-MS ion source, a standard Ion-Tech saddle field FAB-MS gun producing xenon atoms of 8-kV energy, and a VG 11/250 data system. The spectra were recorded without matrix subtraction. The sample matrix materials are listed in Table I.

4-(*p*-Nitrobenzyl)pyridine assays were done to standardize the levels of the half-mustards ($RSCH_2CH_2X$). Briefly, each compound was dissolved in 10% aqueous CH_3OH, and then an aliquot (50 μL) was added to 0.5 mL of a solution of 50 mM 4-(*p*-nitrobenzyl)pyridine [in a mixture of acetone/0.1 M *N*-(2-acetamido)-2-iminodiacetic acid (pH 6.5)/ethylene glycol (1:2:4 v/v/v)]. After the samples were heated for 10 min at 100 °C, 0.5 mL of an acetone/$(C_2H_5)_3N$ mixture (1:1 v/v) was added and the A_{560} was measured. This procedure was used to determine an alkylation potential relative to *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester for each compound. An ϵ_{560} of 5.7 mM^{-1} cm^{-1} was determined for this compound and, because of the nature of the compounds, is presumed for all of the half-mustard derivatives used here. This method was necessary because the GSH derivatives were not completely pure compounds, containing some salt and residual GSH (vide infra). After the extent of alkylation of 4-(*p*-nitrobenzyl)pyridine had been determined, the amount of compound used in each assay was adjusted accordingly.

Syntheses (*Table I*). The *S*-(hydroxyethyl) derivatives of cysteine and *N*-acetylcysteine were synthesized by published procedures (van Bladeren et al., 1980) and converted to their respective chlorides by known methods (Carson & Wong, 1964).[2] *N*-Acetyl-*S*-(2-bromoethyl)cysteine methyl ester was synthesized according to van Bladeren et al. (1981).[2] The

[2] NMR and mass spectral data for these compounds are as follows: *S*-(2-chloroethyl)cysteine, ^1H NMR [$(C^2H_3)_2SO$] δ 2.96 (m, 2 H, CH_2S), 3.10 (m, 2 H, Cys β), 3.79 (t, 2 H, *J* = 7.4, CH_2Cl), 4.19 (m, 1 H, Cys α), (−)FAB-MS (thioglycerol) 182, 184 (M − H)$^-$, 146 (M − H − HCl)$^-$; *N*-acetyl-*S*-(2-chloroethyl)cysteine, ^1H NMR [$(C^2H_3)_2SO$] δ 1.88 (s, 3 H, $NHCOCH_3$), 2.78 (dd, 1 H, *J* = 13.7, 8.3, Cys β H_a), 2.90 (t, 2 H, *J* = 7.3, CH_2S), 2.97 (dd, 1 H, *J* = 13.7, 4.8 Cys β H_b), 3.74 (t, 2 H, *J* = 7.4, CH_2Cl), 4.38 (m, 1 H, Cys α), 8.30 (d, 1 H, *J* = 7.9, $NHCOCH_3$), (−)FAB-MS (glycerol) 224, 226 (M − H)$^-$; *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester, ^1H NMR [$(C^2H_3)_2SO$] δ 1.86 (s, 3 H, $NHCOCH_3$), 2.81 (dd, 1 H, *J* = 13.8, 8.4, Cys β H_a), 2.95 (m, 3 H, Cys β H_b + CH_2S), 3.62 (t, 2 H, *J* = 7.7, CH_2Cl), 3.64 (s, 3 H, $COOCH_3$), 4.45 (m, 1 H, Cys α), 8.43 (d, 1 H, *J* = 7.9, $NHCOCH_3$), mp 48–50 °C (lit. mp 47–49 °C).

N^7-guanyl derivatives of GSH and *N*-acetylcysteine were synthesized from N^7-(2-bromoethyl)guanosine coupling with the appropriate thiolate according to the procedure outlined by Peterson et al. (1988). The compounds had identical HPLC and NMR characteristics as those reported previously (Peterson et al., 1988; Kim & Guengerich, 1989). The N^7-guanyl derivative of cysteine was synthesized by mixing guanosine with an excess of *S*-(2-chloroethyl)cysteine in a $(CH_3)_2SO/H_2O$ solution (1:1 v/v) and allowing the reaction to proceed for 24 h at 37 °C, followed by neutral thermal hydrolysis (100 °C, 30 min). The compound was then purified by reverse-phase HPLC (octadecylsilyl, Beckman Ultrasphere-ODS, 5 μm, 10 × 250 mm, Beckman Instruments, San Ramon, CA): the column was eluted with 1% CH_3OH in 20 mM $NH_4CH_3CO_2$ (pH 4.5) for 5 min followed by a linear gradient to 30% CH_3OH over 20 min at a flow rate of 4 mL min^{-1}. *S*-[2-(N^7-Guanyl)ethyl]cysteine eluted at 8 min under these conditions. NMR and mass spectral data correspond to previously reported values (Foureman & Reed, 1987). The methylated GSH and cysteine N^7-guanyl derivatives were made by dissolving the appropriate compound in 2 N methanolic HCl and stirring for 2 h, followed by reverse-phase HPLC purification (octadecylsilyl, Beckman Ultrasphere-ODS, 5 μm, 10 × 250 mm): the column was eluted with 5% CH_3OH in 20 mM $NH_4CH_3CO_2$ (pH 4.5) for 5 min followed by a linear gradient to 30% CH_3OH over 20 min at a flow rate of 4 mL min^{-1}. *S*-[2-(N^7-Guanyl)ethyl]cysteine methyl ester eluted at 13 min and *S*-[2-(N^7-guanyl)ethyl]GSH dimethyl ester eluted at 25 min under these conditions.

S-(2-Chloroethyl)GSH was synthesized by dissolving 1.0 g (3.2 mmol) of GSH in 25 mL of freshly distilled CH_3OH in which 0.24 g (10.8 mmol, 3.3 equiv) of Na had just been dissolved. After the GSH had completely dissolved, the solution was added dropwise to a stirred solution of 4.6 g (32 mmol) of 1-bromo-2-chloroethane in 25 mL of CH_3OH. The reaction was stirred for 1 h and then 0.56 mL of glacial CH_3CO_2H was added; the resulting precipitate was collected by centrifugation. After drying, 0.4 g of white solid was recovered and could be stored—the material was stable when kept at 4 °C in a desiccator. The actual content of *S*-(2-chloroethyl)GSH was determined by hydrolyzing a portion of the material and then isolating *S*-(2-hydroxyethyl)GSH by reverse-phase HPLC. This assay showed that 60% of the isolated material was actually product, the remainder being sodium salts. This number is in good agreement with that found with the 4-(*p*-nitrobenzyl)pyridine assay.

S-(2-Fluoroethyl)GSH was synthesized in the same manner as the chloride except that the reaction mixture was dried by rotary evaporation after the addition of CH_3CO_2H. The resulting white solid was then purified by reverse-phase HPLC (octadecylsilyl, Beckman Ultrasphere-ODS, 5 μm, 10 × 250 mm). The column was eluted with 1% CH_3CN in H_2O (both solutions containing 0.1% CF_3CO_2H) for 2 min followed by a linear gradient to 20% CH_3CN over 13 min at a flow rate of 4 mL min^{-1}. The compound eluted at 13 min under these conditions. The appropriate fractions were lyophilized to give a white powder.

S-(2-Chloroethyl)GSH dimethyl ester was prepared by esterification of *S*-(2-chloroethyl)GSH, which was accomplished by stirring the compound in HCl-saturated CH_3OH for 2 h followed by solvent evaporation. Because of sample instability, characterization of this compound was done after hydrolysis to *S*-(2-hydroxyethyl)GSH dimethyl ester.

S-(2-Chloroethyl)cysteine methyl ester was synthesized by stirring *S*-(2-chloroethyl)cysteine in HCl-saturated CH_3OH for 2 h followed by solvent evaporation.

Alkylation of Calf Thymus DNA. The compound of interest was dissolved in 0.5 M Tris-HCl buffer (pH 7.7) containing 10% CH_3OH (v/v) to final concentrations of 1.4, 7, or 35 mM, added to a solution of calf thymus DNA in the same buffer, and incubated at 37 °C for 30 min. The concentration of DNA was 2.0 mg mL^{-1}, and the final volume of each reaction was 250 μL. After incubation, the samples were heated for 30 min at 100 °C to release the N^7-guanyl adducts. The DNA was precipitated by the addition of 2.5 volumes of cold C_2H_5OH, and levels of adducts in the concentrated supernatants were quantified by measuring fluorescence intensities of HPLC eluates (reverse phase, for conditions see next section) with a Kratos FS900 Spectrofluro monitor (λ excitation 290 nm, λ emission 370 nm). Quantitation was done by comparison to external standards as in Peterson et al. (1988). The adduct formed from *S*-(2-chloroethyl)cysteine methyl ester alkylation was found to completely hydrolyze under the conditions used for depurination and was thus detected at a retention time corresponding to the nonesterified compound. Also, the adduct formed from *S*-(2-chloroethyl)GSH dimethyl ester alkylation hydrolyzed but gave a mixture of monoester and unesterified products, so adduct levels were determined as the sum of the two peak areas corresponding to these products.

Alkylation of S. typhimurium TA100 DNA. Bacteria were grown in nutrient broth (Difco Laboratories, Detroit, MI) for 12 h at 37 °C. At this point the *S*-(2-haloethyl) derivatives (dissolved in 10% aqueous CH_3OH) were added to 500-mL cell suspensions to give final concentrations of 0.2, 0.5, or 1.0 mM. After a 30-min incubation, cells were collected by centrifugation and the pellets were washed with 10 mM Tris-HCl (pH 7.4) buffer containing 25 mM EDTA. The suspensions (15 mL) were made up to 1 mg of proteinase K mL^{-1} and 1% sodium dodecyl sulfate (w/v) and then were incubated at 37 °C for 1 h. An equal volume of a $CHCl_3$/isoamyl alcohol/phenol mixture (25:1:24) was used to extract the solution, and the DNA (remaining in the aqueous phase) was precipitated by the addition of 2.5 volumes of C_2H_5OH. Samples were then treated with RNase A (100 μg mL^{-1}) to digest remaining RNA. DNA adducts were released by neutral thermal hydrolysis (100 °C, 30 min). Again, the esterified products formed after alkylation with *S*-(2-chloroethyl)cysteine methyl ester and *S*-(2-chloroethyl)GSH dimethyl ester were almost completely hydrolyzed, so adduct levels were determined by measuring peak areas corresponding to the appropriate nonesterified compound. Fluorescent quantitation was done for each adduct after chromatography by using either condition C or D listed below. In this case, clear separation of adducts required two chromatographic steps for each adduct, and four different types of HPLC separation systems were employed. The HPLC systems used were as follows: system A, aminopropyl (Altex Ultrasil-NH_2, 10 μm, 4.6 × 250 mm) column eluted with a gradient of 8–760 mM $NH_4CH_3CO_2$ (pH 7.0, in 80% CH_3OH) over 40 min at 1.0 mL min^{-1}; system B, octadecylsilyl (C18, Whatman Partisil 5 ODS-3, 5 μm, 4.6 × 250 mm, Phenomenex, Torrance, CA) column eluted for 5 min with 5% CH_3OH in 50 mM $NH_4CH_3CO_2$ (pH 5.1) followed by a linear gradient to 30% CH_3OH over 30 min at 1.0 mL min^{-1}; system C, same column as in B eluted for 5 min with 5% CH_3OH in 50 mM $NH_4H_2PO_4$ (pH 2.5) followed by a linear gradient increasing to 30% CH_3OH over 25 min at 1.0 mL min^{-1}; system D, strong cation-exchange (Nucleosil SA, 5 μm, 4.6 × 250 mm, Jones Chromatography, Littleton, CO) column eluted with 3%

CH_3CN in 0.5 M $NH_4H_2PO_4$ (pH 2.5) at 1.3 mL min^{-1}. Retention times for the adducts were as follows: *S*-[2-(N^7-guanyl)ethyl)cysteine, 11 min (system B), 7 min (system C); *N*-acetyl-*S*-[2-(N^7-guanyl)ethyl]cysteine, 20 min (system B), 19 min (system C), 5 min (system D); *S*-[2-(N^7-guanyl)-ethyl]GSH, 22 min (system A), 15 min (system C); *S*-[2-(N^7-guanyl)ethyl]GSH monomethyl ester, 22 min (system A), 25 min (system C).

Sequence-Specific Alkylation of pBR322 DNA. 5′-^{32}P-End-labeled fragments were generated by digestion of pBR322 DNA with the restriction endonuclease *Bam*HI, removal of 5′-phosphate termini with bacterial alkaline phosphatase, end-labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase, and restriction endonuclease digestion with *Hin*dIII and/or *Sal*I, generating 346- and 275-base fragments, respectively [as in Maxam and Gilbert (1980)]. The fragments were purified on a DNA PLUS column (4.6 × 250 mm, MacModd, Chadds-Ford, PA), monitored by UV absorbance at 254 nm. The initial column eluent consisted of 20% CH_3CN in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM tetrapropylammonium bromide (flow rate 1 mL min^{-1}). A gradient was used for fragment elution: from 20% to 40% CH_3CN over 30 min, then from 40% to 44% over the next 15 min, and finally from 44% to 53% over the next 5 min. The fragments of interest eluted at approximately 45 min under these conditions. A more shallow gradient was employed when both *Bam*HI/*Sal*I and *Bam*HI/*Hin*dIII fragments were separated in the same run. The peak(s) of interest was collected, 5 μg of calf thymus DNA was added as carrier, and the DNA was precipitated by adding 2.5 volumes of C_2H_5OH and 0.1 volume of 2.5 mM sodium acetate (pH 5.2). Low molecular weight species were removed from the samples by using Sephadex G-50 spin columns (Maniatis et al., 1982).

The DNA was resuspended in 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA and aliquoted for further use. Additional calf thymus DNA was added to each aliquot to give a final DNA concentration of 0.03 mg mL^{-1}. The half-mustards were quickly dissolved in the same buffer containing 10% CH_3OH (v/v) and added to the DNA. Several concentrations of the half-mustards were added in order to achieve an alkylation level of no more than one modification per fragment. Samples were incubated at 37 °C for 20 min, and reaction byproducts were removed by Sephadex G-50 spin-column chromatography. A control treatment was done by adding only buffer solution to the DNA.

All samples were treated with piperidine and prepared for electrophoresis on 6% polyacrylamide gels (Maxam & Gilbert, 1980). Standard guanine- and (guanine + adenine)-specific cleavage reactions were carried out for sequence comparison with dimethyl sulfate and formic acid, respectively. Two sample loadings were made at times approximately 3.5 h apart. Autoradiography was performed by using Kodak XAR film with intensifying screens at −70 °C. Band intensities were quantitated by using a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). Multiple scans of each lane were recorded to obtain averaged peak areas.

Biological Assays. The *umu* assay was performed according to procedures outlined elsewhere (Oda et al., 1985; Shimada & Nakumura, 1987; Shimada et al., 1989). *S. typhimurium* TA100 and TA98 assays were performed essentially by the methods outlined by Maron and Ames (1983).

Results

Synthesis. Although *S*-(2-chloroethyl)GSH has been reported in the literature (Reed & Foureman, 1986), efforts to use this synthetic procedure were not practical. The solubility of GSH thiolate in CH_3OH and the lack of reaction of the half-mustard in that solvent allowed the use of the simple synthetic procedure described here. The synthesis of *S*-(2-chloroethyl)GSH has allowed direct comparisons of this compound with other *S*-(2-haloethyl) compounds. *S*-(2-Bromoethyl)GSH could also be prepared by this method. The bromide was not as stable to the reaction conditions and hence could not be isolated in as pure a form; thus it was only used in initial experiments. The synthesis of *S*-(2-chloroethyl)GSH can be carried out on a large scale and produces a relatively pure compound. Purification steps are not very practical because the measured half-life for hydrolysis at 37 °C is 3–4 min and the compound is only sparingly soluble in organic solvents. Also, the only impurities present are small amounts of *S*-(2-hydroxyethyl)GSH and unreacted GSH, neither of which interfere in the experiments described here. A third haloethyl derivative of GSH, the fluoroethyl compound, was synthesized because it could be made in an analytically pure form and used for comparison with the Cl and Br compounds.

Table II: N^7-Guanyl Adduct Levels Formed in Calf Thymus DNA[a]

compound	adduct levels [nmol (mg of DNA)$^{-1}$] at compound concn (mM)		
	1.4	7.0	35.0
S-(2-fluoroethyl)GSH	0.6	3.2	15.7
S-(2-chloroethyl)GSH	0.7	3.0	11.0
S-(2-bromoethyl)GSH	0.7	2.8	10.6
S-(2-chloroethyl)GSH dimethyl ester	0.3	1.5	13.0
S-(2-chloroethyl)cysteine	0.8	3.0	21.2
S-(2-bromoethyl)cysteine	1.1	6.1	24.1
N-acetyl-*S*-(2-chloroethyl)cysteine	0.2	0.4	2.2
S-(2-chloroethyl)cysteine methyl ester	0.5	2.0	11.0
N-acetyl-*S*-(2-bromoethyl)cysteine methyl ester	1.2	3.9	22.5

[a] Calf thymus DNA (0.5 mg) was mixed with the indicated amount of each compound. All compounds have been standardized on the basis of reaction in the 4-(*p*-nitrobenzyl)pyridine assay (see Materials and Methods). Each value is the average of at least two determinations.

Alkylation of Calf Thymus DNA. Alkylation levels were measured at three different concentrations of half-mustard and the levels of the N^7-guanyl adducts show a reasonably linear concentration dependence (Table II). The chloro derivatives were found in most cases to be more accessible synthetically than the bromo derivatives, so experiments were done to measure the relative efficiencies of alkylation by these halides. In the two cases examined there was no measurable difference in the observed level of N^7-guanyl adduct. This result allowed the use of either of the halo-substituted compounds, whichever was synthetically available, in further experiments. The fluoro derivative also gave very similar results, although the half-life of this compound is greatly increased and the incubation times are far longer (the fluoro derivative was incubated for 72 h, approximately 6 half-lives). This result demonstrates that even though the chloro compound is partially contaminated with salts and residual GSH (vide supra), it is reasonable to assume the contaminates will not interfere with the experiments described and to use the compound as is. In general, levels of N^7-guanyl adducts were greater for the cysteine compounds than for the GSH analogues. Within the set of cysteine compounds there is a rough inverse correlation between net charge of the compound and the observed degree of alkylation. The net neutral compounds were the best alkylating agents, and the compound with a net charge of −1 is the poorest. However, the methyl ester does not follow the trend, showing

Table III: DNA N^7-Guanyl Adduct Levels and Revertant Numbers in *S. typhimurium* TA100

compound	$RSCH_2CH_2X$ concn (mM)	DNA adduct (pmol mg^{-1})	revertants/μmol[a]	relative revertants/adduct[b]
S-(2-chloroethyl)GSH	0.2	24		
	0.5	76	7740	14
	1.0	210		
S-(2-chloroethyl)GSH dimethyl ester	0.5	391	3030	1.0
S-(2-chloroethyl)cysteine	0.2	17		
	0.5	31	440	1.8
	1.0	42		
N-acetyl-*S*-(2-bromoethyl)cysteine methyl ester	0.5	185	3010	2.0
N-acetyl-*S*-(2-chloroethyl)cysteine	0.5	93	1010	1.4
S-(2-chloroethyl)cysteine methyl ester	0.5	17	880	6.5

[a] Values from Figure 4 at a half-mustard concentration of 0.5 mM (1 μmol). [b] Ratio of values in columns 4 and 3 (the two preceding columns).

an intermediate level of adduct formation. The observation that the GSH compounds showed the lowest alkylation and that covering of various charged groups on these molecules did not adversely effect binding suggests that specific H-bond or charge–charge interactions between the GSH conjugate and DNA do not play a major role in the gross alkylation of DNA guanyl groups.

Alkylation of S. typhimurium DNA. Levels of N^7-guanyl adducts formed in bacteria were measured to determine if the results from the calf thymus DNA alkylation experiments present a valid estimate of the amount of DNA alkylation produced in *S. typhimurium* (Table III). These experiments were done on a much larger scale than the individual mutation assays to allow adduct detection, but concentrations of the compounds were identical in both assays. The measurement of the aflatoxin B_1 adduct has been done by using the same approach, and the large scale used for the adduct detection did not influence mutation efficiency or cell viability (Stark et al., 1979). For two of the compounds, *S*-(2-chloroethyl)-GSH and *S*-(2-chloroethyl)cysteine, the effect of changing the concentration was examined and the adduct level shows a linear response. The compound that showed the highest level of alkylation in this case was the dimethyl ester of *S*-(2-chloroethyl)GSH. Again, as in the calf thymus experiments, *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester gave a very high value, followed by *N*-acetyl-*S*-(2-chloroethyl)cysteine and *S*-(2-chloroethyl)GSH. Very low alkylation levels were observed for *S*-(2-chloroethyl)cysteine and its methyl ester.

Sequence Selectivity of DNA Binding. Several of the half-mustards were examined to determine if any sequence selectivity was seen in the binding to DNA. A 275 bp *Bam*HI/*Sal*I restriction fragment of pBR322 DNA was alkylated with each half-mustard, and a modified sequencing reaction was carried out (Maxam & Gilbert, 1980) using the N^7-guanine adducts as sites for strand cleavage. The results of these experiments using *S*-(2-chloroethyl)GSH, *S*-(2-chloroethyl)cysteine, and *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester are shown in Figure 1. As seen, the compounds produced breaks at all guanines to some extent. The level of DNA modification was greater than one alkylation per fragment for the reactions in lanes 4 and 6, resulting in the increased intensity of the lower molecular weight bands. Most alkylating agents that show a preference for N^7-guanyl alkylation, especially positively charged ones, show a pattern of stronger alkylation at adjacent guanines or runs of guanines (Kohn et al., 1987; Wurdeman et al., 1989), and such a pattern was observed with all the compounds examined [in addition to the compounds from Figure 1, *N*-acetyl-*S*-(2-chloroethyl)cysteine and *S*-(2-bromoethyl)GSH were used]. The difference between the highest and lowest levels of modification at different guanyl sites was about 10-fold; the difference between the highest level and the mean was about 3-fold (Figure 2; because of several runs of guanines this particular sequence gave a mean alkylation level somewhat higher than that seen elsewhere). Similar results were obtained with the 346 bp *Bam*HI/*Hin*dIII restriction fragment (not shown).

umu Assay. Three compounds of the series that were tested in the *umu* gene activation assay showed weak activity. This assay is based on the incorporation of the pSK1002 plasmid containing the *umuC* gene into *S. typhimurium* TA1535, a *S. typhimurium* strain devoid of a majority of the elements involved in the SOS response. Induction of *umuC* gene expression is indicative of the SOS pathway being activated. Thus, because the compounds under investigation are rather weak inducers of *umuC* gene expression, they are also weak inducers of the SOS response [cf. Baertschi et al. (1989)]. *N*-Acetyl-*S*-(2-bromoethyl)cysteine methyl ester proved to be strongest of the three agents in terms of induction of *umu* gene expression, and *S*-(2-chloroethyl)cysteine and *S*-(2-chloroethyl)GSH were significantly less active (Figure 3).

S. typhimurium Reversion Assays. None of the compounds tested were active in reverting *S. typhimurium* TA98, where frameshift mutations are needed to restore prototrophy. All compounds showed positive responses in the base-pair substitution dependent strain TA100, and *S*-(2-chloroethyl)GSH was by far the most potent compound of the set (Figure 4). The concentration-dependent plateau observed for this compound is most likely due to toxicity at the higher dose levels. The next most active derivatives were *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester and *S*-(2-chloroethyl)GSH dimethyl ester. The remaining compounds all produced low revertant levels. Compounds were also tested in the TA1535 strain, which does not have the pKM101 plasmid sensitive to the SOS response, and the results found were found to be very similar (results not shown).

The numbers of revertants and N^7-guanyl adduct levels were determined at the same concentration of each compound (Table III). The ratios of revertants to adduct vary considerably, with the *S*-(2-chloroethyl)GSH-derived adduct giving the highest ratio by far.

Discussion

The synthesis of what is postulated to be the ultimate carcinogenic form of EDB has allowed a careful study of this and several related compounds in terms of their DNA-alkylating abilities and their mutagenic properties to be carried out. All of the compounds examined reacted with calf thymus DNA to form N^7-guanyl adducts, although there was a large range in the number of adducts formed. The measurement of the alkylation levels in *S. typhimurium* gave very different values than those measured with calf thymus DNA. The apparent discrepancy is probably due to differential

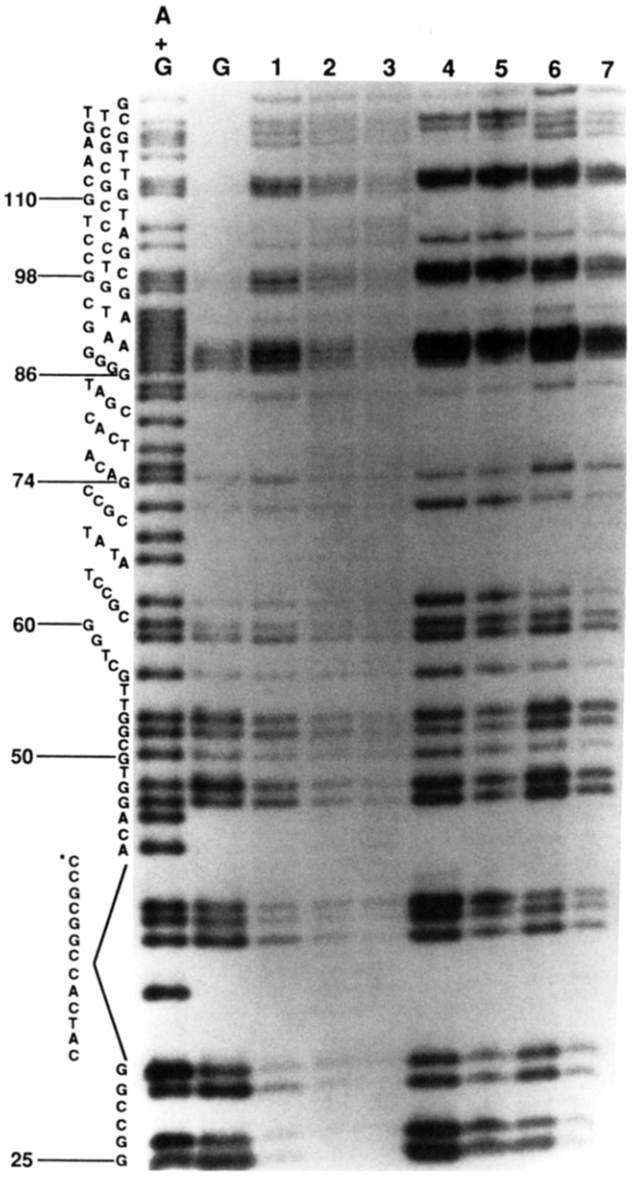

FIGURE 1: Sequence-dependent alkylation of the *Bam*HI/*Sal*I restriction fragment of pBR322 DNA. The guanine- and (guanine + adenine)-specific lanes are standard Maxam and Gilbert sequencing reactions. The half-mustards were added to the DNA at the following concentrations: *S*-(2-chloroethyl)GSH at 500 μg mL^{-1} (lane 1), 100 μg mL^{-1} (lane 2), and 20 μg mL^{-1} (lane 3); *S*-(2-chloroethyl)cysteine at 50 μg μL^{-1} (lane 4) and 10 μg mL^{-1} (lane 5); and *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester at 40 μg mL^{-1} (lane 6) and 8 μg mL^{-1} (lane 7). The sequence and corresponding fragment lengths are shown. The asterisk indicates a region in which a compression zone occurred on the gel.

transport—although these bacteria are permeable to most substances, *Escherichia coli* are known to have specific di- and oligopeptide uptake systems (Sussman & Gilvarg, 1971) and *Salmonella* are thought to contain very similar transport properties (Ames et al., 1973). The amino acid derivative histidinol phosphate ester was not able to traverse the *S. typhimurium* membrane but was taken up when linked with a dipeptide, presumably due to the oligopeptide permease activity (Ames et al., 1973). In any case, it seems that there may be some type of facilitated transport for the GSH compounds. The results of the current work emphasize the importance of actually measuring adduct levels in bacteria if accurate assessments of DNA binding are to be related to biological responses.

It was of interest, knowing that all these compounds formed N^7-guanyl adducts, to investigate whether they did so in a sequence-specific manner. Experiments showed that there is not a particularly dramatic sequence specificity seen with these compounds, at least for plasmid DNA [cf. Warpehoski and Hurley (1988)]. The preferential alkylation at runs of guanines is probably due to sequence-dependent changes in the electrostatic component of the stacking energy (Aida & Nagata, 1986). Calculations have shown that the electrostatic potential of the N^7-position of guanine is increased whenever there is a guanine at the 5′-position (Pullman & Pullman, 1981).

The biological response seen in the *umu* assay correlates fairly well with the adduct level measured (the *S. typhimurium* TA100 and TA1535 strains used are very similar, so there should be little if any difference in adduct levels). These compounds give a very weak response in this assay, especially when compared to aflatoxin B_1 8,9-epoxide (Baertschi et al., 1989). The steric bulk and intercalative modes of binding seen with the aflatoxin may produce replication blocks. The compounds also gave very similar results in the *S. typhimurium* TA100 and TA1535 assays (containing and devoid of the SOS response, respectively), and the rank of the compounds in terms of biological potency is almost completely reversed when the reversion results are compared with the *umu* assay results. This observation, along with the low response in the *umu* assay, argues that the primary pathway by which these compound produce mutagenic lesions does not involve induction of the SOS response and its error-prone polymerase activity (Little & Mount, 1982).

The ratio of revertants to N^7-guanyl DNA adducts varies considerably (Table III), with *S*-(2-chloroethyl)GSH giving a value that is at least twice that found with any other compound tested. It is very surprising that there is so much variation in these numbers, because all of the adducts might be expected to have similar effects on the adducte l guanine (Lawley, 1984). Further, the apparent 14-fold difference in the mutant/adduct ratio among the derivatives is a very conservative estimate, in that the response seen to *S*-[2-(N^7-guanyl)ethyl]GSH is not very linear (Figure 4) and use of the initial slope of the curve could raise the variation to 50-fold or more. It should be noted that the approach is applicable here because (1) only a base-pair substitution at a target guanine is capable of producing a mutant in the assay system used and (2) essentially only one type of guanine adduct is formed.[3]

The availability of data on both levels of adduct formation and values for *S. typhimurium* TA100 revertants allows an estimate of the mutation frequency to be made. At a treatment level of 0.5 mM, *S*-(2-chloroethyl)GSH produced 7700 revertants and the measured adduct level was 75 pmol (mg of DNA)$^{-1}$ or, by using 618 as the average molecular weight of a base pair, 1 adduct per 2.2×10^4 bp. The size of the bacterial genome is 4.5×10^6 bp (Drake, 1969), so a value of 210 adducts per cell can then be calculated. If all guanines are modified equally, then the total number of guanines,[4] (4.5×10^6 bp)/4, divided by 210 gives the number of cells that need to be modified before there is an equal probability of modifying each guanine once [the single target guanine in the *hisG46* operon is the modification of interest (position 2 of codon 69)]. Thus, the probability is that 1 cell in 5400 will have the target

[3] Recently *S*-[2-(N^1-adenyl)ethyl]GSH has been identified as a minor DNA adduct formed from EDB in vitro and in rat liver (Kim et al., 1990). This adduct cannot play a role in the mutations seen in the *S. typhimurium* TA100 screen.

[4] *S. typhimurium* DNA is slightly GC rich, but not >60% GC (Shapiro, 1970) and not enough to affect this determination.

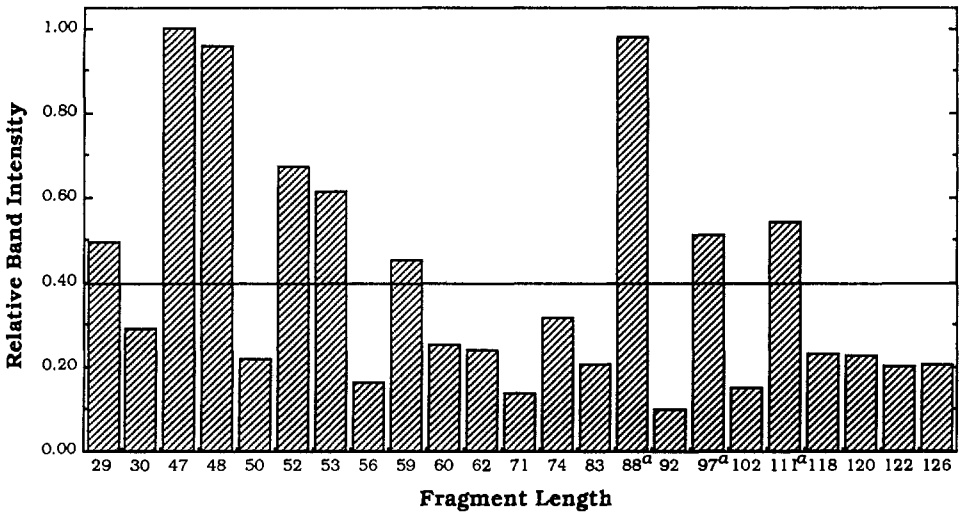

FIGURE 2: Densitometric quantitation of alkylation-induced strand breaks caused by *S*-(2-chloroethyl)GSH. Lane 1 of Figure 1 was scanned as described under Materials and Methods. The data were normalized to the value found for the band of fragment length 47. The straight line indicates the average band intensity for this sequence. The letter "a" indicates that band intensities were averaged over these runs of guanines because individual bands could not be accurately resolved and quantitated.

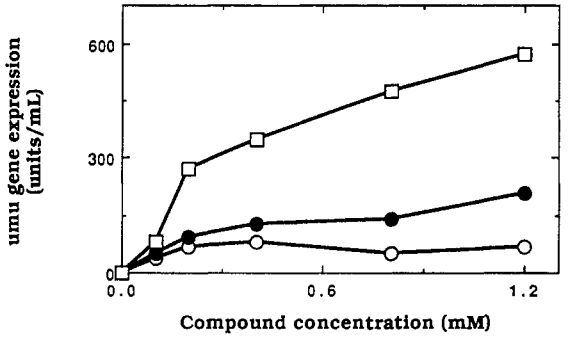

FIGURE 3: Response of *umu* gene expression to *S*-(2-haloethyl)-substituted GSH and cysteine derivatives. The indicated amounts of *S*-(2-chloroethyl)cysteine (●), *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester (□), or *S*-(2-chloroethyl)GSH (○) were incubated with the *S. typhimurium* TA1535/pSK 1002 test system for 120 min at 37 °C.

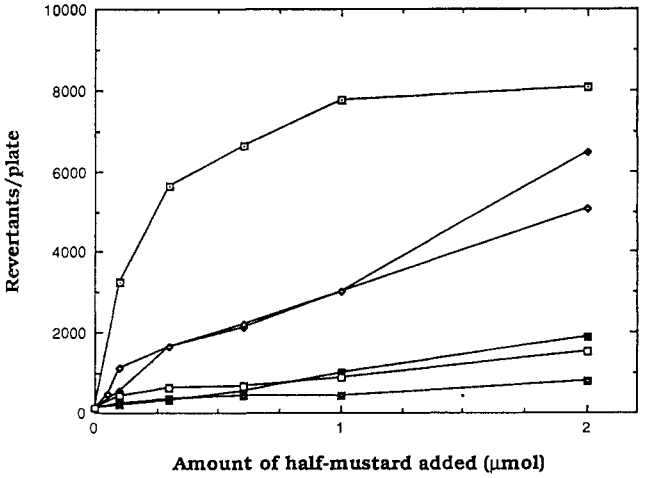

FIGURE 4: Histidine-independence reversion assay of mutations caused by *S*-(2-haloethyl)-substituted GSH or cysteine derivatives. The indicated amounts of *S*-(2-chloroethyl)cysteine (solid square with white dot), *S*-(2-chloroethyl)cysteine methyl ester (□), *N*-acetyl-*S*-(2-chloroethyl)cysteine (■), *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester (◆), *S*-(2-chloroethyl)GSH (⊡), and *S*-(2-chloroethyl)GSH dimethyl ester (◇) were mixed with *S. typhimurium* TA100, and the number of colonies were counted after 48 h. Each point is the average of either duplicate or triplicate determinations.

guanine modified. The measured number of revertants is (7.7 × 10^3)/10^8 cells, or 1 cell in 1.3 × 10^4. Dividing the two probabilities [(5.4 × 10^3)/(1.3 × 10^4)] gives a 42% chance that a cell with the target guanine modified will become a revertant.

This calculation is based on an assumption of uniform alkylation of each guanine in the genome. The sequence of the target region is CTAGGGCCA (Carlomagno et al., 1988); and although there is no reason to think the region would be especially prone to modification, it is a GGG triplet, and the middle guanine of a triplet would be expected to be modified two or three times as heavily as the average guanine (Figure 2). Thus, the estimated mutational frequency should be attenuated to the 14–20% level. The assumption was also made that only the middle guanine can be a mutagenic target, which would give a mutation to CXC [in the wild type X = T, but a mutation to CAC is also a known reversion (Eisenstadt et al., 1989)]. However, evidence exists that mispairng across from the first G of the codon can produce revertants (Eisenstadt et al., 1989), so the target may be slightly larger.

This extremely high mutational frequency allows the definitive conclusion that the mutations observed in the case of *S*-(2-chloroethyl)GSH are due to *S*-[2-(*N*^{7}-guanyl)ethyl]GSH adducts and not a minor guanyl adduct which might be present in quantitites too small to detect. The adduct in this case would have to be present at least 14% as often as the *N*^{7}-guanyl adduct and have a 100% mutational frequency to be responsible for the mutations seen. Even if the frequency is only one-tenth of that calculated, this level of another adduct would be easily detectable. In in vitro assays and in vivo experiments using both bacteria and mammals, no other adducts have been detected at levels >3% those of *S*-[2-(*N*^{7}-guanyl)ethyl]GSH (Ozawa & Guengerich, 1983; Koga et al., 1986; Inskeep et al., 1986).[3] Further, we have recently synthesized the imidazole ring opened form of *S*-[2-(*N*^{7}-guanyl)ethyl]GSH and determined that this is not formed in isolated DNA or in rat liver (Kim et al., 1990).

Another very important point to be made is that when the same experiments were done with aflatoxin B_1 8,9-epoxide and the *N*^{7}-guanyl adduct was measured in this laboratory, the estimated mutational frequency was only 7%,[5] 6 times less than that seen for the *S*-[2-(*N*^{7}-guanyl)ethyl]GSH adduct. Aflatoxin 8,9-epoxide is more effective at alkylating DNA, for in

[5] D.-H. Kim, K. D. Raney, T. M. Harris, and F. P. Guengerich, unpublished results.

these assays 300 times more *S*-(2-chloroethyl)GSH than aflatoxin B_1 8,9-epoxide was used to achieve similar levels of alkylation—however, once the adducts are formed, the glutathionyl adduct seems to be considerably more mutagenic.

Mutational frequencies for point mutations produced by random alkylation have not been estimated very often. Lawley and Martin (1975) used a bacteriophage T4rII AP72 reversion assay and ethylating agents and estimated a reversion frequency of 36% (this study assumed that all mutations were the result of O^6-ethylguanine adducts). Frequencies have been calculated in forward mutation assays, but estimates are much more difficult to make because of ill-defined target regions. Stark et al. (1979) calculated a frequency of 3–4% for aflatoxin B_1 in an *S. typhimurium* TM677 8-azaguanine resistance assay.[6] There are many more examples of mutational frequencies calculated after site-specific DNA adduct incorporation. In these types of experiments, frequencies as high as 75% have been found for O^6-methylguanine adducts (Bhanot & Ray, 1986). For (acetylamino)fluorene-derived C^8-guanyl adducts the calculated values range from 1% to 7% (Moriya et al., 1988; Gupta et al., 1989). Of the adducts considered here and elsewhere only the O^6-alkylguanines, which directly affect the base pairing portion of the base, have frequencies on the order of the *S*-(2-chloroethyl)GSH-derived adduct *S*-[2-(N^7-guanyl)ethyl]GSH.

There are several mechanisms by which N^7-guanyl adducts could product base mispairing: (1) A misinsertion could occur opposite an apurinic site generated upon adduct depurination. (2) The electronic configuration of the adducted guanine could be sufficiently perturbed so that another base pair might be favored [the pK_a of the N^1-position of guanine drops from 9 to 7 upon N^7 adduct formation (Lawley & Brookes, 1961)]. (3) The geometry of the base may be altered such that it could not form a normal Watson–Crick type pair. Although the exact molecular mechanism by which these N^7-guanyl adducts cause base mispairing is at present unknown, we believe the premutagenic lesion is the N^7-guanyl adduct and are attempting to define the exact mechanism. Because of the apparent lack of mutagenicity of (N^7-guanyl)methyl adducts (Lawley, 1984) the role of depurination can probably be disregarded, unless there is an adduct-induced polymerase pause followed by a timed depurination [as suggested by Loeb (1985)]. However, such a mechanism would be expected to induce error-prone misincorporation by the SOS system (Loeb, 1985), and the system studied here definitely shows characteristics that are inconsistent with such a hypothesis (e.g., lack of SOS response—Figure 3 and comparisons of reversion frequencies in *S. typhimurium* TA100 and TA1535, vide supra). The second possibility, that of electron perturbation, seems unlikely because these adducts as well as other N^7-alkyl adducts would be expected to be very similar electronically. Indeed, we have found that some model N^7-guanyl derivatives related to these do not have pK_a values (e.g., O^6) differing from those of the corresponding methyl compounds.[7] The electronic change could, however, become important if it were coupled with an adduct-specific alteration of base geometry. Whatever the mechanism turns out to be, it will have to account for the high base mispairing caused by the *S*-[2-(N^7-guanyl)ethyl]-GSH adduct and lesser mutagenicity of the analogues.

Many of the structure–activity comparisons can be rationalized. The cysteine compounds are much more avid DNA-alkylating agents but have the disadvantage of poor bacterial transport. The cysteine adducts are more effective in inducing the SOS response than are the GSH adducts, but they do not seem to be nearly as mutagenic in terms of generating base mispairing. A compound that displays rather distinct behavior is *S*-(2-chloroethyl)GSH dimethyl ester [which gives rise to *S*-[2-(N^7-guanyl)ethyl]GSH dimethyl ester]. It forms a large number of adducts in bacteria, possibly as a result of facilitated transport, but the adducts do not appear to be highly mutagenic. The physical basis for the apparent difference in the mutagenicity of the *S*-[2-(N^7-guanyl)ethyl]GSH and its dimethyl ester derivative warrants further investigation.

[6] This value is rather close to that estimated in our system.

[7] L. A. Peterson and F. P. Guengerich, unpublished results.

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Registry No. GSH, 70-18-8; *S*-(2-chloroethyl)GSH, 75607-61-3; *S*-(2-chloroethyl)GSH dimethyl ester, 129541-01-1; *S*-(2-chloroethyl)cysteine methyl ester, 88169-60-2; *S*-(2-fluoroethyl)GSH, 129541-02-2; *S*-(2-chloroethyl)cysteine, 28361-96-8; *N*-acetyl-*S*-(2-chloroethyl)cysteine, 58337-49-8; *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester, 77109-49-0; *S*-(2-bromoethyl)GSH, 81907-45-1; *S*-(2-bromoethyl)cysteine, 88169-61-3; *S*-[2-(N^7-guanyl)ethyl]cysteine, 106947-24-4; *S*-[2-(N^7-guanyl)ethyl]cysteine methyl ester, 129570-24-7; *S*-[2-(N^7-guanyl)ethyl]GSH dimethyl ester, 129570-25-8; guanosine, 118-00-3; 1-bromo-2-chloroethane, 107-04-0; *S*-ethylGSH, 24425-52-3; *S*-ethylcysteine, 2629-59-6.

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