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# *In Vivo* Roles of Conjugation with Glutathione and $O^6$-Alkylguanine DNA-Alkyltransferase in the Mutagenicity of the *bis*-Electrophiles 1,2-Dibromoethane and 1,2,3,4-Diepoxybutane in Mice

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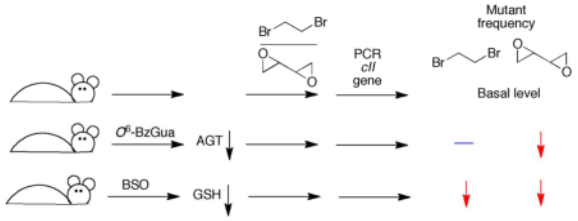
## Abstract




Several studies with bacteria and *in vitro* mammalian systems have provided evidence for the roles of two thiol-based conjugation systems, glutathione (GSH) transferase and $O^6$-alkylguanine DNA-alkyltransferase (AGT), in the bioactivation of the *bis*-electrophiles 1,2-dibromoethane and 1,2,3,4-diepoxybutane (DEB), the latter an oxidation product of 1,3-butadiene. The *in vivo* relevance of these conjugation reactions to biological activity in mammals has not been addressed, particularly with DEB. In the present work we used transgenic Big Blue® mice, utilizing the *cII* gene, to examine the effects of manipulation of conjugation pathways on liver mutations arising from dibromoethane and DEB *in vivo*. Treatment of the mice with butathionine sulfoxime (BSO) prior to dibromoethane lowered hepatic GSH levels, dibromoethane-GSH DNA adducts ($N^7$-guanyl), and *cII* mutation frequency. Administration of $O^6$-benzylguanine ($O^6$-BzGua), an inhibitor of AGT, did not change the mutation frequency. Depletion of GSH (BSO) and AGT ($O^6$-BzGua) both lowered the mutation frequency induced by DEB, and BSO lowered the levels of GSH-DEB $N^7$-guanyl and $N^6$-adenyl DNA adducts. Our results provide evidence that the GSH conjugation pathway is a major *in vivo* factor in dibromoethane genotoxicity; both GSH and AGT conjugation are major factors in the genotoxicity of DEB. The latter findings are considered to be of relevance to the carcinogenicity of 1,3-butadiene.

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SUPPORTING INFORMATION
Measurements of GSH levels in livers of B6C3F1 and Big Blue® transgenic mice, gel electrophoresis imaging of AGT activity measurements, AGT activities in livers of B6C3F1 and Big Blue® transgenic mice at 6 and 24 h after treatment, quantitative analysis of DNA adducts in livers of Big Blue® transgenic mice, and independent mutations in the liver *cII* gene of Big Blue® transgenic mice. This material is available free of charge via the Internet at http://pubs.acs.org.

## INTRODUCTION

1,2-Dibromoethane (ethylene dibromide) has been used extensively as a pesticide, but its industrial use was curtailed after demonstration of carcinogenicity.[1–4] In rodents, dibromoethane produces mammary gland, spleen, adrenal, liver, kidney, and subcutaneous tissue tumors.[1,2] This compound is classified as "Probably carcinogenic to humans" by the International Agency for Cancer Research (IARC).[5]

1,3-Butadiene is used in the synthetic rubber industry and its annual use in the United States is ~ $2 \times 10^9$ kg.[6,7] It is carcinogenic in rodents (much more in mice than rats) and has been classified as "Carcinogenic to humans" by the IARC.[8] There is also concern about exposure to humans from other sources, e.g. cigarette smoke.[9,10]

The mechanisms of action of both dibromoethane and 1,3-butadiene are both generally accepted to be genotoxic and involve metabolism. Dibromoethane is conjugated with glutathione (GSH) by GSH transferase (GST) and the resulting half-mustard ($GSCH_2CH_2Br$) reacts with DNA via the intermediacy of an episulfonium ion (Scheme 1).[14–18]1,3-Butadiene is oxidized by P450s (P450 2E1, 2A6)[19,20] to butadiene monoepoxide[21] and then to 1,2,3,4-diepoxybutane (DEB). Of the known oxidative metabolites, DEB is the most toxic and mutagenic.[22,23] The much higher level of DEB found in mice compared to rats is thought to explain the much greater carcinogenicity in mice relative to rats.[24–28]

The major DNA adduct formed from dibromoethane is *S*-[2-($N^7$-guanyl)ethyl]GSH.[11,16,29] Minor adducts (formed *in vitro*) include *S*-[2-($N^2$-guanyl)ethyl]GSH, *S*-[2-($O^6$-guanyl)ethyl]GSH, and *S*-[2-($N^1$-adenyl)ethyl]GSH.[11] Of these, the $N^7$-guanyl adduct has been found *in vivo*.[18,30] Limited studies have been done on misincorporation opposite these adducts with DNA polymerases.[31]

A myriad of DNA adducts have been identified from reactions of DEB with nucleosides and DNA, including those in Scheme 2.[32–44] Only some of these have been identified in *in vivo* settings.[12,32, 45–48] Four of these have been incorporated into oligonucleotides and found to be miscoding under some conditions: $N^1$-(2-hydroxy-3-buten-1-yl)deoxyinosine,[49] a DEB $N^6$-$N^6$-adenine-adenine cross-link,[49] $N^3$-(2-hydroxy-3-buten-1-yl)deoxycytidine,[42] and 1,$N^6$-(2-hydroxy-3-hydroxymethyl-2-deoxyadenosine.[50] However, none of these studies provides direct evidence of *in vivo* mutagenicity or a role in carcinogenicity.

With dibromoethane, a strong case for the role of GSH conjugation can be made in toxicity. Bacterial mutagenesis of dibromoethane is highly dependent upon GST activity.[51] Disulfiram increases both tumor incidence[1,52] and *in vivo* levels of the DNA adduct *S*-[2-($N^7$-guanyl)ethyl]GSH,[18] apparently by blocking the oxidative detoxication of dibromoethane (to 2-bromoacetaldehyde)[53] (1,2-dichloroethane was used in ref.[52]).

With 1,3-butadiene and DEB, a role for GSH conjugation in mutagenicity and carcinogenesis has been proposed.[54] Although DEB is a direct-acting mutagen, its bacterial mutagenicity is strongly enhanced by GSTs.[54–56] The GSH-DEB conjugate *S*-(2-hydroxy-3,4-epoxybutyl)GSH is 20-fold more mutagenic than DEB in the *Salmonella typhimurium* TA1535 base pair tester system.[56,57] In *Escherichia coli* TGR8, GST also increased the mutagenicity of DEB and for systems in which 1,3-butadiene was oxidized by P450s.[57] In this test strain, the mutation spectra of GSH-enhanced systems differed from that obtained with DEB.[57] The DNA adduct *S*-[4-*(*$N^6$-deoxyadenosinyl)-2,3-dihydroxybutyl]GSH was miscoding with human DNA polymerase κ (and bacteriophage DNA polymerase T7 exonuclease$^-$) but not with other DNA polymerases.[58] The DNA

adducts *S*-[4-(*N*$^6$-deoxyadenosinyl)-2,3-dihydroxybutyl]GSH and *S*-[4-(*N*$^7$-guanyl)-2,3-dihydroxybutyl]GSH have been detected and quantitated *in vivo* in livers of rats and mice.[12]

Another conjugation system that activates *bis*-electrophiles is the DNA repair protein *O*$^6$-alkylguanine DNA-alkyltransferase (AGT). Expression of bacterial or human AGT in bacterial or human cells enhances the toxicity and the mutagenicity of DEB.[59–63] The reactivity of AGT is related to the low p$K_a$ (4–5) of AGT Cys-145, rendering it a thiolate anion at physiological pH.[64] The chemistry of the reaction is presumed to be similar to GSH activation (Scheme 1).[65] The putative AGT-Cys-145-$CH_2CH_2$Br half-mustard reacts with DNA to form adducts at the guanine N7,[65] N1, N2, and O6 atoms and the adenine N6 atom.[13] The sites of DNA modification by the putative AGT Cys-145-DEB conjugate have not been determined.

All of the previous work established a case for thiol conjugation in the bioactivation of both dibromoethane and DEB. However, the *in vivo* biological relevance has not been established. In the present work we used transgenic Big Blue® mice, utilizing the *cII* gene, to examine the effects of manipulation of conjugation pathways on *in vivo* mutations arising from dibromoethane and DEB. Our results provide evidence that the GSH conjugation pathway is a major *in vivo* factor in dibromoethane genotoxicity, and both GSH and AGT conjugation are major factors in the genotoxicity of DEB and probably 1,3-butadiene.

## EXPERIMENTAL PROCEDURES

### Materials

1,2-Dibromoethane, (racemic) DEB, butathionine-*S*,*R*-sulfoximine (BSO), *O*$^6$-benzylguanine (*O*$^6$-BzGua), 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), piperidine, polyethylene glycol 400, and enzymes for digestion were purchased from SigmaAldrich (St. Louis, MO). Phusion High-Fidelity DNA polymerase and uracil DNA glycosylase (UDG) were obtained from New England Biolabs (Ipswich, MA). The oligonucleotides were purchased from Midland Certified Reagents (Midland, TX) for AGT assays or from Integrated DNA Technologies (Coralville, IA) for sequence analysis of the *cII* mutants and were purified by the manufacturers using HPLC.

The three major DNA adducts formed by GSH conjugation with dibromoethane—*S*-[2-(*N*$^2$-guanyl)ethyl]GSH, *S*-[2-(*O*$^6$-guanyl)ethyl]GSH), and *S*-[2-(*N*$^6$-adenyl)ethyl]GSH—and the internal standard *S*-[2-(*N*$^2$-guanyl) [$^2H_4$]-ethyl]GSH were synthesized and purified as described previously.[11,66] The six major DNA adducts induced by GSH conjugation with DEB—*S*-[4-(*N*$^3$-adenyl)2,3-dihydroxybutyl)GSH (N$^3$A-$(OH)_2$butyl-GSH), *S*-[4-(*N*$^6$-deoxyadenosinyl)2,3-dihydroxybutyl)GSH (N$^6$dA-$(OH)_2$butyl-GSH), *S*-[4-(*N*$^7$-guanyl)2,3-dihydroxybutyl)GSH (N$^7$G-$(OH)_2$butyl-GSH), *S*-[4-(*N*$^1$-deoxyguanosinyl)2,3-dihydroxybutyl)GSH (N$^1$dG-$(OH)_2$butyl-GSH), *S*-[4-(*N*$^4$-deoxycytidinyl)2,3-dihydroxybutyl)GSH (N$^4$dC-$(OH)_2$butyl-GSH), *S*-[4-(*N*$^3$-thymidinyl)2,3-dihydroxybutyl)GSH (N$^3$dT-$(OH)_2$butyl-GSH)—and internal standards (*N*$^6$dA-$(OH)_2$butyl-[*glycine*-$^{13}C_2$,$^{15}$N]-GSH, *N*$^7$G-$(OH)_2$butyl-[*glycine*-$^{13}C_2$,$^{15}$N]-GSH) were also synthesized and purified as described previously.[12]

### Animals and Treatments

Seventy male Big Blue® transgenic mice (five mice per group × 7 treatment groups × 2 time points), age 8 weeks (Agilent/Taconic) were housed in plastic cages (with bedding), according to U. S. National Institutes of Health guidelines. All procedures involving the use of animals were approved by the LRRI Institutional Animal Care and Use Committee. Following an acclimation period of 7 days, Big Blue® transgenic mice were treated with

vehicle (corn oil ($n$ = 4), saline ($n$ = 4), and 40% polyethylene glycol 400 in phosphate-buffered saline ($n$ = 2); total $n$ = 10), dibromoethane (30 mg/kg, ip, in corn oil) ($n$ = 10), BSO (8 mg/kg, ip, in saline)/dibromoethane (30 mg/kg, ip, in corn oil) ($n$ = 10), $O^6$-BzGua (80 mg/kg, ip, in 40% polyethylene glycol 400 in phosphate-buffered saline (PBS, 10 mM potassium phosphate, pH 7.4, containing 0.9% NaCl, w/v)/dibromoethane (30 mg/kg, ip, in corn oil) ($n$ = 10), DEB (25 mg/kg, ip, in corn oil) ($n$ = 10), BSO (8 mg/kg, ip, in saline)/DEB (25 mg/kg, ip, in corn oil) ($n$ = 10), or $O^6$-BzGua (80 mg/kg, ip, in 40% polyethylene glycol 400 (v/v) in PBS)/DEB (25 mg/kg, ip, in corn oil) ($n$ = 10). $O^6$-BzGua was administered 1 h prior to treatment with dibromoethane or DEB and BSO was administered 2 h prior to treatment with dibromoethane or DEB. Mice were killed 6 h (five mice per group) or 24 h later (five mice per group). Livers were isolated, frozen, and stored at −80 °C until further analysis.

## GSH Assay

The GSH assay was performed as previously described.[67] Liver (100 mg) was homogenized in 8 mL of 20 mM EDTA, and trichloroacetic acid was added to a final concentration of 5% (w/v). GSH levels from the mouse liver were measured spectrophotometrically after the incubation of the trichloroacetic acid supernatant with DTNB.

## AGT Assay

Mouse liver was homogenized in cold 70 mM HEPES buffer (pH 7.8) containing 1.6 mM EDTA, 1 mM dithiothreitol (DTT), and 5% glycerol (v/v). The homogenate was sonicated and centrifuged at 4 °C for 2 min (12,000 × $g$). The resulting supernatants were used for the AGT assay.

AGT assays were performed based on a previously described method[68] by measuring the transfer of an alkyl group from DNA ($O^6$-methylguanine ($O^6$-MeGua)) to AGT using a 30-/36-mer duplex DNA substrate (5′-GCCTCGAGCCAGCCGCAGACGCAGCGAGGA-3′, 3′-CGGAGCTCGGTCGGCGTCTGCGUCXCTCCTGCGGCT-$^{32}$P-5′ (X: $O^6$-MeGua), with the 36-mer (with $O^6$-MeGua) 5′-end-labeled with $^{32}$P[58,68]). Tissue extracts (0.5 mg of protein) were incubated with 60 nM DNA substrate in 50 mM Tris-HCl buffer (pH 7.5) containing 1.6 mM EDTA (to block nuclease action), 1.0 mM DTT, and 5% glycerol (v/v) at 37 °C for 1 h. The reactions were quenched with 0.3 M NaOH, followed by neutralization with 0.3 M HCl. DNA substrate/product was isolated by spin column separations. The resulting product was incubated with 3 units of UDG in 50 mM Tris-HCl buffer (pH 7.5) containing 1.0 mM DTT at 37 °C for 30 min, followed by 0.20 M piperidine treatment at 95 °C for 30 min. The resulting product was dried by lyophilization and redissolved in a mixture of $H_2O$ and formamide (1:3, v/v). Products were separated using 16% acrylamide (w/v) electrophoresis gels, and results were visualized using a phosphorimaging system (Bio-Rad Molecular Imager FX, BioRad, Hercules, CA).

## Measurement of DNA Adducts

DNA from mouse liver was isolated as previously described,[18] followed by thermal or enzymatic digestion.[12] The reactions were filtered through MWCO Centricon filters (3 kDa cut-off, Millipore, Billerica, MA) and spiked with synthetic $N^6$dA-$(OH)_2$butyl-[*glycine*-$^{13}C_2$,$^{15}N$]-GSH, $N^7$G-$(OH)_2$butyl-[*glycine*-$^{13}C_2$,$^{15}N$]-GSH, and *S*-[2-($N^2$-guanyl)[$^2H_4$]-ethyl]GSH. The resulting reactions were analyzed by LC-MS/MS as previously described.[12] LC-MS/MS analysis was performed using a Waters Acquity UPLC system (Waters, Milford, MA) interfaced to a Thermo-Finnigan LTQ mass spectrometer (ThermoElectron, Sunnyvale, CA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved with a Waters Acquity UPLC BEH $C_{18}$ octadecylsilane column (2.1 mm × 100 mm, 1.7 μm). LC conditions were as follows:

Solvent A was 0.1% $CH_3CO_2H$ in $H_2O$ (v/v) and solvent B was 0.1% $CH_3CO_2H$ in $CH_3CN$ (v/v). The following gradient program (v/v) was used with a flow rate of 300 μL $min^{-1}$: the gradient started with 5% B (v/v), increased to 15% B (v/v) at 2 min, to 30% B (v/v) at 6 min, and held at 30% B (v/v) for 1 min. The column was re-equilibrated for 3 min with 5% B (v/v). The temperature of the column was maintained at 40 °C. The MS conditions were as follows: positive ion mode; ion spray voltage, 4.5 kV; capillary voltage, 20 V; capillary temperature, 350 °C; tube lens voltage, 40 V.

### *cII* Mutation Assay

High molecular weight genomic DNA was extracted from mouse liver using a RecoverEase DNA Isolation Kit (Agilent/Stratagene, La Jolla, CA). The packaging of the phage, plating the packaged DNA samples, and determination of mutation frequencies were performed according to the manufacturer's instructions for the λ Select-*cII* Mutation Detection System for Big Blue Rodents (Agilent/Stratagene).

### Sequence Analysis of the *cII* Mutants

Single, well-isolated *cII* plaques were picked and suspended in 100 μL of sterile distilled $H_2O$. These suspensions were heated at 100 °C for 5 min and centrifuged at 12,000 × *g* for 3 min. The supernatant (10 μL) was used as the DNA template in PCR. The *cII* gene was amplified by PCR using 5′-CCACACCTATGGTGTATG-3′ (forward primer), 5′-CCTCTGCCGAAGTTGAGTAT-3′ (reverse primer), and Phusion High-Fidelity DNA polymerase. The PCR cycling conditions were as follows: initial melting (95 °C, 3 min), 35 cycles of denaturation (95 °C, 30 s), annealing (60 °C, 1 min), and extension (72 °C, 1 min) followed by a last extension step at 72 °C for 10 min. The PCR products were purified using a QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and submitted to the Vanderbilt DNA Sequencing Facility for nucleotide sequence analysis.

### Statistical Analysis

All GSH, AGT, DNA adduct, and mutation frequency results are expressed as means ± SD, with five mice per group (except $n = 10$ in control (vehicle) group). Statistical significance was determined by Student's *t*-test and Fisher's exact test, with significance levels indicated in the figures and tables.

## RESULTS

### Rationale

The object of this work was to determine if the GSH and AGT conjugation pathways are important *in vivo* for the biological effects of two *bis*-electrophiles, dibromoethane and DEB, for which there was considerable *in vitro* evidence. Cell culture models could be considered but these are not *in vivo* systems, and manipulation of GSH and AGT levels might not be relevant to *in vivo* situations. A mouse model with a reporter transgene was selected for ease in analysis of mutants. In principle, one approach would be to use animals in which a GST was deleted. However, previous studies showed that several GSTs are active toward dibromoethane[51,69] and DEB.[12] GSH depletion with BSO, an inhibitor of the γ-glutamylcysteine synthetase reaction in the synthesis of GSH, had previously been demonstrated to lower the level of dibromoethane-GSH-DNA adducts in rats and mice.[18] Although AGT knockout mice ($mgmt^{(-/-)}$) have been characterized, use of the established Big Blue® mutation system would require breeding. The AGT inhibitor $O^6$-BzGua, in clinical development,[70] has been shown to be a selective inhibitor of AGT.[71] We utilized *in vivo* mutations as a biomarker for cancer. An actual chronic cancer bioassay might be difficult to interpret in the context of long term GSH depletion, which would be expected to

have pleiotropic effects in light of the current understanding of the importance of thiol/redox regulatory systems.

### Attenuation of GSH and AGT Levels

Preliminary trials were done with B6C3F1 strain mice (because of the expense of the Big Blue® animals) to define conditions that might be applicable for the transgenic animals (Supporting Information Figure S1). The results obtained in the studies with Big Blue® mice showed decreased liver GSH levels (to 30% basal) 6 h following treatment (Supporting Information Figure S2) with BSO and either DEB or dibromoethane (BSO administered 2 h prior to other chemicals). The level of GSH returned to ~ 70% of the basal level after 24 h.

Treatment with $O^6$-BzGua (1 h prior to other chemicals) lowered the level of AGT activity to ~ 10% after 6 h (Supporting Information Figures S3, S4). After 24 h the activity returned to 30% of the basal level. Because AGT doe not recycle in the reaction, the results are representative of the amount of active AGT.

### Effects of GSH and AGT Depletion on DNA Adducts

We had previously shown the effect of BSO treatment/GSH depletion on the levels of liver S-[2-($N^7$-guanyl)ethyl]GSH DNA adducts in B6C3F1, ICR, and A/J mice and in three strains of rats.[18] This effect was observed again in the Big Blue® mice, with the adduct level attenuated to 45% at 6 h after treatment (Supporting Information Table S1). The level remained this low at 24 h. No $N^1$- or $N^6$-adenyl adducts were detected.

GSH-containing DEB DNA adducts were analyzed following treatment with BSO (Figure 1, Supporting Information Table S2). A similar pattern (~50% decrease) was seen with BSO treatment for the two adducts[12] that were detected, the $N^7$-guanyl and $N^6$-adenyl GSH-containing adducts. These were the only GSH-containing DNA adducts detected in previous *in vivo* work in mice and rats.[12] The levels also remained attenuated after 24 h.

### Effects of GSH and AGT Depletion on Mutations

Dibromoethane treatment increased the *cII* mutant frequency ~ 6-fold at 6 h after treatment (Figure 2A). DEB also increased the mutant frequency, although only ~ ½ as much as dibromoethane did, at the 6 h time point (Figure 2B). At 6 h, BSO treatment (GSH depletion) decreased the dibromoethane-induced mutations by 70%, and both BSO and $O^6$-BzGua treatment (AGT depletion) decreased the DEB-induced mutations by 50% (Figure 2A, 2B). The same patterns were seen 24 h after treatment (Figure 2C, 2D), although the mutation frequencies were attenuated less. However, the BSO and $O^6$-BzGua inhibition patterns were similar.

### Mutation Spectra

Mutants (37 to 42 independent mutants in each case) were selected from each set obtained from livers of treated animals and subjected to nucleotide sequence analysis (Tables 1, 2 and Figures 3, 4). Details of the individual mutants are listed in the Supporting Information, Tables S3 and S4. The spontaneous (“vehicle”) mutants were characterized by the prominent GC to AT transitions (Figures 3A, 4A). Treatment with dibromoethane led to a statistically significant increase in GC to TA transversions ($P < 0.001$), which was suppressed by treatment with BSO ($P < 0.001$) but not $O^6$-BzGua. Treatment with DEB yielded increased AT to GC transitions ($P < 0.0001$), which were suppressed by either BSO ($P < 0.02$)or $O^6$-BzGua ($P < 0.05$) (Figure 4).

## DISCUSSION

Mouse studies were done to address the *in vivo* roles of GSH and AGT conjugation in the genotoxicity of dibromoethane and DEB. The results of the attenuation of the levels of each lead to the conclusion that GSH conjugation is a major factor in the *in vivo* genotoxicity of dibromoethane and that both GSH and AGT conjugation are major mechanisms of genotoxicity of DEB *in vivo*. These findings support *in vitro* findings with bacterial and mammalian cells and purified enzyme systems.[14,51,54–58,72–74] The literature supports genotoxic mechanisms for the carcinogenicity of dihaloalkanes and DEB, and we propose that the conjugation pathways considered here are important in the carcinogenicity of both the industrial chemicals dibromoethane and 1,3-butadiene.

Several points are in order regarding the mutation analyses. First, the mutation spectra were dominated by single base pair mutations (~ 90% in all cases, Tables 1 and 2) as opposed to frameshifts and tandem mutations. This *in vivo* pattern differs from that reported in DEB-treated human lymphoblasts[23] and splenic T cells isolated from mice treated with DEB.[22,] The discrepancy may be due to differences in concentrations, to the presence of conjugating enzymes (i.e., GST) *in vivo* (and in spleen T cells vs. liver), or to the nature of the phenotypic selection inherent in mutations in the *cII* vs. the *hprt* and *tk* loci.

Racemic DEB was used in this study, and the possibility can be considered that the individual stereoisomers of DEB may differ in their metabolism and genotoxicity. However, our own studies with the three isomers of DEB showed only ~ a 2-fold difference in the efficiency of GSH conjugation by individual GSTs or the genotoxicity of the GSH conjugates prepared from the three DEB isomers.[12] Other work has shown only ~ 2-fold differences among the stereoisomers of DEB with regard to their crosslinking ability[43] or genotoxicity in a yeast-based system.[75] On the basis of these prior studies, we conclude that the use of racemic DEB is justified and that the conclusions are applicable to all three isomeric oxidation products (DEB) of 1,3-butadiene.

We cannot discount roles of some of the individual DNA adducts formed directly by reaction of dibromoethane, butadiene monoepoxide, or DEB (Scheme 2) with DNA.[33–42] The literature shows that very little mutagenicity is seen with dibromoethane in the absence of GST.[14,51,72,73] The mutagenicity of *S*-(2-hydroxy-3,4-epoxybutyl)GSH is ~ 20-fold greater than that of DEB.[56,57] In the work presented here, *in vivo* depletion of 70% of the GSH (6 h time point, Supporting Information Figures S1, S2) was associated with a 70% decrease in mutant frequency (Figure 1A) for dibromoethane. Apparently AGT conjugation is not an important contributor to the *in vivo* mutagenicity of dibromoethane, in that 90% depletion of AGT (6 h, Supporting Information Figure S4) was not associated with a statistically significant change in the mutant frequency (Figure 2A). Conjugation reactions appear to play a major role in the *in vivo* genotoxicity of DEB, in that the 70% decrease in GSH and 90% decrease in AGT (Supporting Information Figures S2 and 3) were associated with 60 and 50% decreases in DEB genotoxicity (Figure 2B). Together these two pathways, if additive, can account for all of the *in vivo* genotoxicity of DEB, within the limits of statistical error.

One caveat about the role of GSH conjugation in the carcinogenicity of 1,3-butadiene is that monoepoxides (butadiene monoepoxide and 1,2-epoxy-3,4-dihydrobutane, the respective immediate oxidation product of 1,3-butadiene and the immediate hydrolysis product of DEB) can be conjugated with GSH and these pathways would be involved in detoxication. These two epoxides have low genotoxicity, however.[56] Only the monoepoxide is an issue, because the diol epoxide would derive from DEB (which the mice were treated with). We

did not measure the levels of adducts derived from the monoepoxide (Scheme 2) in our studies.

The mutation spectra arising from dibromoethane treatment showed an increase in GC to AT transitions (Figure 3). This finding is consistent with our previous work in bacteriophage M13mp18[11] and yeast/human *p53*[76] systems treated with *S*-(2-chloroethyl)GSH, the halogen analog of the dibromoethane half-mustard (Scheme 1). The results are probably not attributable to depurination, which would be expected to favor GC to TA transversions due to the "A Rule" for misinformational mispairing.[77] The GC to AT transitions seen in the AGT-dibromoethane pathway in *E. coli* TGR8 cells[65] were not apparently changed in mouse liver, but this result is consistent with the lack of an effect of $O^6$-BzGua (i.e., lack of a significant role for the AGT pathway with dibromoethane in liver).

The increased AT to GC transitions seen with DEB (and attenuation by treatment with BSO or $O^6$-BzGua) (Figure 4) are consistent with our finding that human DNA polymerase κ inserted dCTP opposite *S*-[4-(*N*$^6$-deoxyadenosinyl)-2,3-dihydroxybutyl]GSH.[58] This finding (Figure 4) is also consistent with our previous report of enhanced AT to GC transitions in the *rpoB* gene of *E. coli* TRG8 cells treated with the DEB-GSH conjugate *S*-(2-hydroxy-3,4-epoxybutyl)GSH[57] and the predominant A to G transitions in *H-ras* codon 61 in Harderian gland tumors of B6C3F1 mice treated with 1,3-butadiene by inhalation.[78] However, increased AT to TA transversions were reported in the *lacF* gene in bone marrow of B6C3F1 mice treated with 1,3-butadiene.[79] The incidence of this latter mutation was low in all of the DEB-derived mouse liver samples in our study (Figure 4). In all of the mutation studies, the contribution of phenotypic bias should not be discounted.

In summary, our results demonstrated that attenuation of GSH levels decreased hepatic levels of the major dibromoethane DNA adduct ($N^7$-guanyl) and *cII* mutations in Big Blue® mice. We conclude that the GSH conjugation pathway has a major role in dibromoethane mutagenicity (and probably carcinogenicity) *in vivo*. AGT conjugation does not appear to play a major role. The results of similar attenuation studies lead to the conclusion that *both* the GSH and AGT conjugation pathways have major roles in the *in vivo* mutagenicity of DEB. The latter findings are considered to be of relevance to the carcinogenicity of 1,3-butadiene.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

| | |
|---|---|
| **AGT** | $O^6$-alkylguanine DNA-alkyltransferase |
| ***O*$^6$-BzGua** | $O^6$-benzylguanine |
| **BSO** | butathionine-*S*,*R*-sulfoximine |

| | |
|---|---|
| **DEB** | (1,2,3,4-diepoxybutane |
| **DEB-GSH conjugate** | *S*-(2-hydroxy-3,4-epoxybutyl)GSH |
| **dibromoethane** | 1,2-dibromoethane |
| **DTNB** | 5,5′-dithio-*bis*-(2-nitrobenzoic acid |
| **DTT** | dithiothreitol |
| **ESI** | electrospray ionization |
| **GSH** | glutathione |
| **GST** | GSH transferase |
| **$N^3$A-$(OH)_2$butyl-GSH** | *S*-[4-($N^3$-adenyl)2,3-dihydroxybutyl)GSH |
| **IARC** | International Agency for Cancer Research |
| **$N^6$dA-$(OH)_2$butyl-GSH** | *S*-[4-($N^6$-deoxyadenosinyl)2,3-dihydroxybutyl)GSH |
| **$N^7$G-$(OH)_2$butyl-GSH** | *S*-[4-($N^7$-guanyl)2,3-dihydroxybutyl)GSH |
| **$N^1$dG-$(OH)_2$butyl-GSH** | *S*-[4-($N^1$-deoxyguanosinyl)2,3-dihydroxybutyl)GSH |
| **$N^4$dC-$(OH)_2$butyl-GSH** | *S*-[4-($N^4$-deoxycytidinyl)2,3-dihydroxybutyl)GSH |
| **$N^3$dT-$(OH)_2$butyl-GSH** | *S*-[4-($N^3$-thymidinyl)2,3-dihydroxybutyl)GSH |
| **$N^7$G-DEB** | $N^7$-(2,3,4-trihydroxybutyl)guanine |
| **$N^3$A-DEB** | $N^3$-(2,3,4-trihydroxybutyl)adenine |
| **$N^6$A-DEB** | $N^6$-(2,3,4-trihydroxybutyl)adenine |
| ***$O^6$*-MeGua** | $O^6$-methylguanine |
| **PBS** | phosphate-buffered saline (10 mM potassium phosphate buffer, pH 7.4, containing 0.9% NaCl, w/v) |

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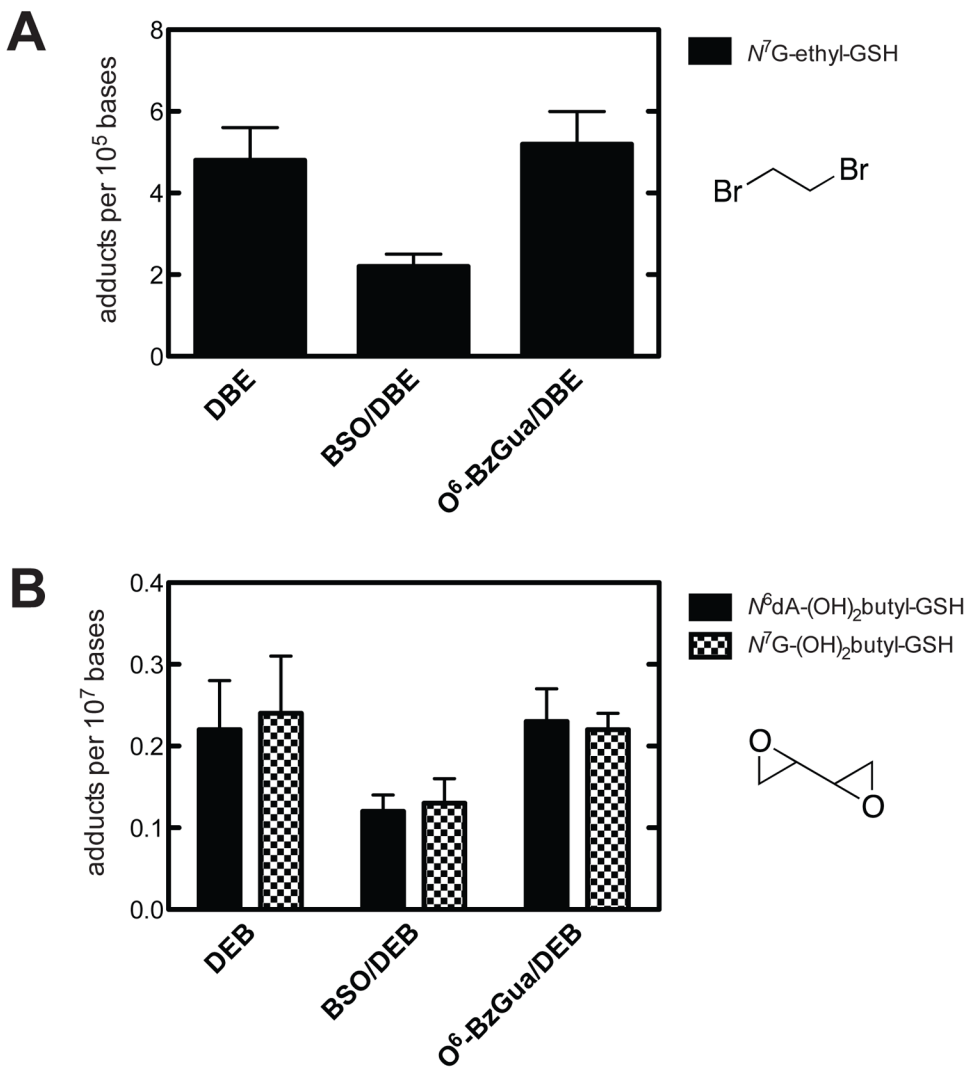



**Figure 1.**

Quantitative analysis of DNA adducts in livers of Big Blue® transgenic mice 6 h after treatment with dibromoethane (DBE), BSO/dibromoethane, or $O^6$-BzGua/dibromoethane (A) and DEB, BSO/DEB, or $O^6$-BzGua/DEB (B). $O^6$-BzGua (80 mg/kg, ip) was administered 1 h prior to treatment with dibromoethane (30 mg/kg, ip) or DEB (25 mg/kg, ip), and BSO (8 mg/kg, ip) was administered 2 h prior to treatment with dibromoethane (30 mg/kg, ip) or DEB (25 mg/kg, ip).

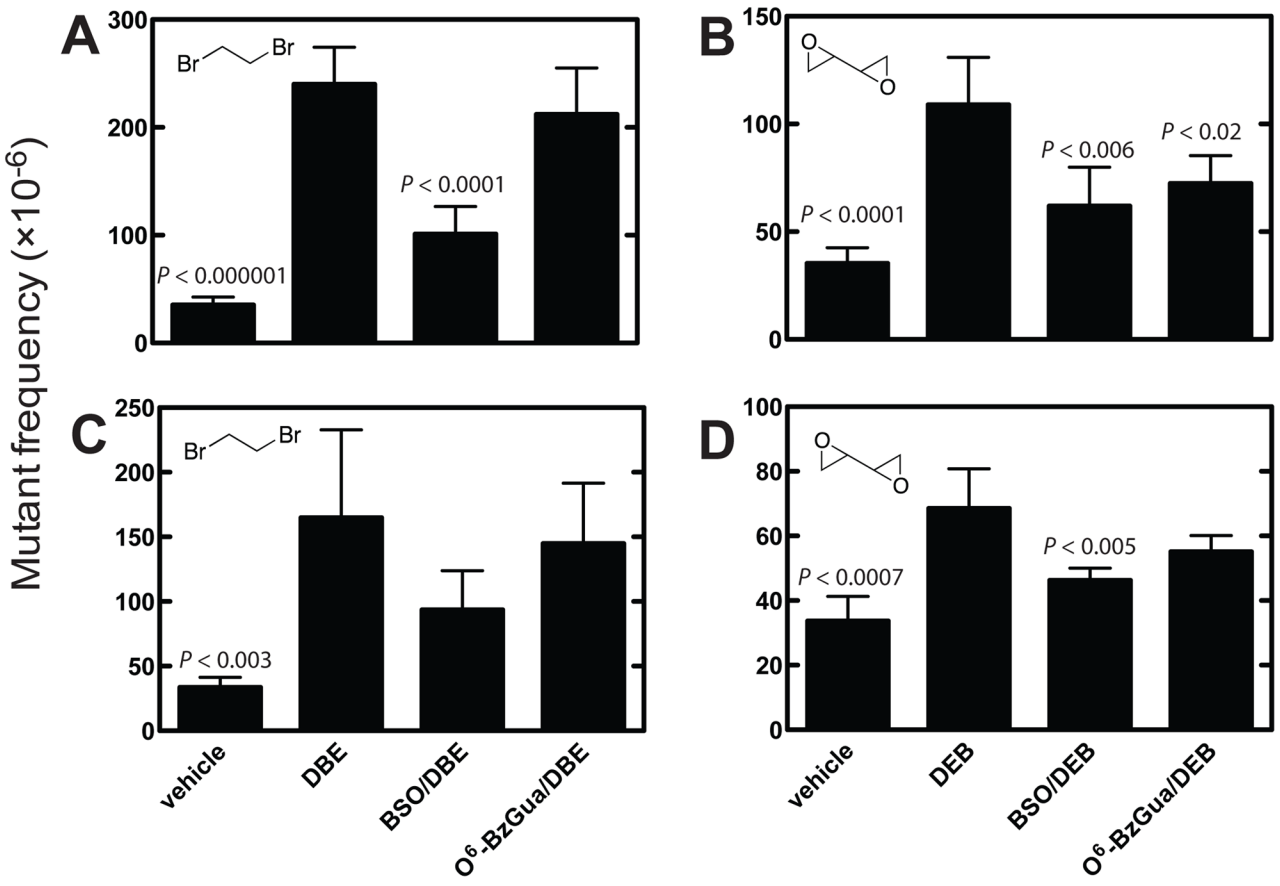



**Figure 2.**

*cII* mutant frequencies in liver of Big Blue® transgenic mice at 6 (A, B) and 24 h (C, D) after treatment with vehicle, dibromoethane (DBE), BSO/dibromoethane, or $O^6$-BzGua/dibromoethane (A, C) and vehicle, DEB, BSO/DEB, or $O^6$-BzGua/DEB (B, D). $O^6$-BzGua (80 mg/kg, ip) was administered 1 h prior to treatment with dibromoethane (30 mg/kg, ip) or DEB (25 mg/kg, ip) and BSO (8 mg/kg, ip) was administered 2 h prior to treatment with dibromoethane (30 mg/kg, ip) or DEB (25 mg/kg, ip). The statistical significance values were from compareisons with the *cII* mutant frequencies induced by dibromoethane or DEB and evaluated using Student's *t*-test (shown in figure). In Part A, the relative mutations frequencies (compared to the highest value, with dibromethane treatment set at 100%) were 15% for vehicle, 42% for +BSO, and 88% for +$O^6$-BzGua. In Part B, the relative mutations frequencies (compared to the highest value, with dibromethane treatment set at 100%) were 20% for vehicle, 43% for +BSO, and 88% for +$O^6$-BzGua. In Part C, the relative mutations frequencies (compared to the highest value, with DEB treatment set at 100%) were 32% for vehicle, 57% for +BSO, and 67% for +$O^6$-BzGua. In Part D, the relative mutations frequencies (compared to the highest value, with DEB treatment set at 100%) were 49% for vehicle, 68% for +BSO, and 80% for +$O^6$-BzGua.

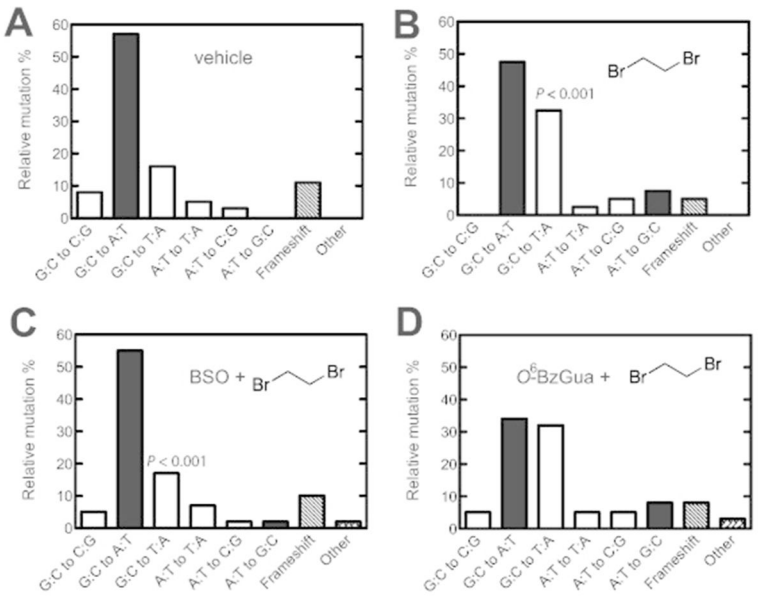



**Figure 3.**

Relative independent mutations induced by vehicle (A), dibromoethane (DBE) (B), BSO/dibromoethane (C), or $O^6$-BzGua/dibromoethane (D) in liver of Big Blue® transgenic mice. $O^6$-BzGua (80 mg/kg, ip) was administered 1 h prior to treatment with dibromoethane (30 mg/kg, ip) and BSO (8 mg/kg, ip) was administered 2 h prior to treatment with dibromoethane (30 mg/kg, ip). Transition mutations (■); transversion mutations (□); frameshifts and others (diagonal shading). The P values are for comparison of the GC to AT transversions between Parts A and B ($P < 0.001$) and between Parts B and C ($P < 0.001$).

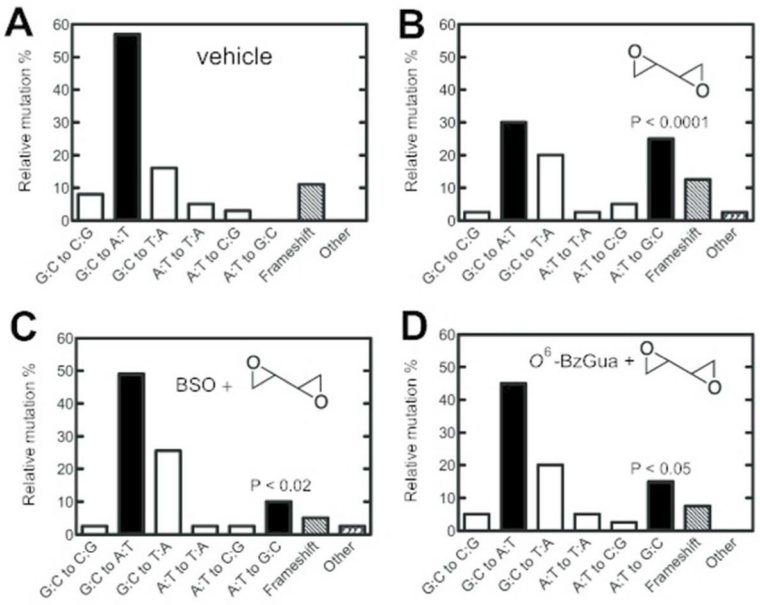
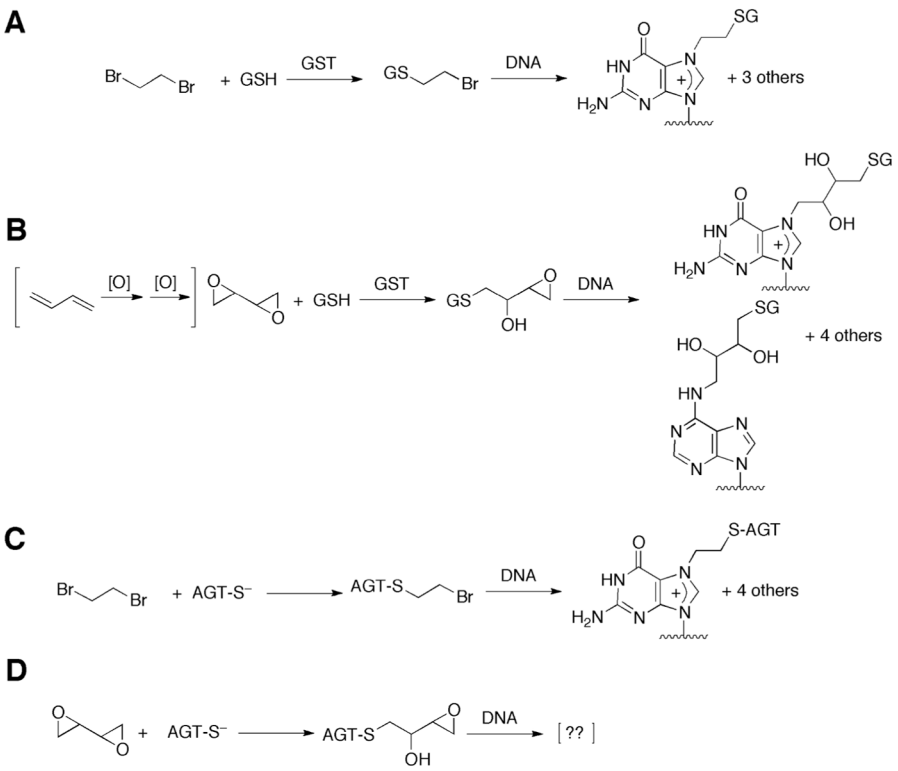


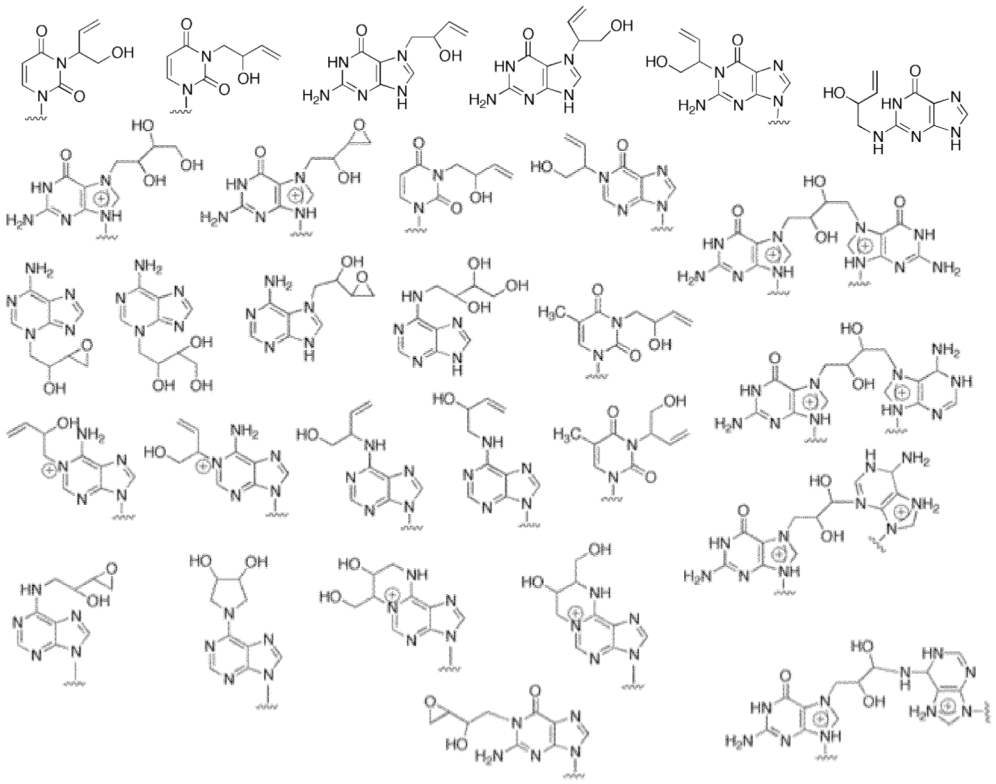

**Figure 4.**

Relative independent mutations induced by vehicle (A), DEB (B), BSO/DEB (C), or $O^6$-BzGua/DEB (D) in livers of Big Blue® transgenic mice. $O^6$-BzGua (80 mg/kg, ip) was administered 1 h prior to treatment with DEB (25 mg/kg, ip) and BSO (8 mg/kg, ip) was administered 2 h prior to treatment with DEB (25 mg/kg, ip). Transition mutations (■); transversion mutations (□); frameshifts and others (diagonal shading). The P values are for comparison of the AT to GC transversions between Parts A and B ($P < 0.0001$), between Parts B ad C (($P < 0.02$) and between Parts B and C ($P < 0.05$).




**Scheme 1. GSH (A, B) and AGT (C, D) Conjugation Pathways for Activation of Dibromoethane (A, C) and DEB (B,D)**

For the identities of the other DNA adducts of dibromoethane (GSH),[11] DEB (GSH),[12] and dibromoethane (AGT)[13] see the indicated references.



**Scheme 2. DNA Adducts from Reaction of Oxidized Products of 1,3-Butadiene**

See the references.[33–42] (Known stereoisomers of several of the adducts are not considered here.)

**Table 1**

Summary of Independent Mutations in the *cII* Gene of Livers of Big Blue® Transgenic Mice Treated with Vehicle, Dibromoethane, BSO/Dibromoethane, or $O^6$-BzGua/ Dibromoethane

| | number of independent mutations (%) | | | |
|---|---|---|---|---|
| **type of mutation** | **vehicle** | **dibromoethane** | **BSO/dibromoethane** | ***O*$^6$BzGua/ dibromoethane** |
| G:C to C:G | 3 (8) | 0 (0) | 2 (5) | 2 (5) |
| G:C to A:T | 21 (57) | 19 (47.5) | 23 (55) | 13 (34) |
| G:C to T:A | 6 (16) | 13 (32.5) | 7 (17) | 12 (32) |
| A:T to T:A | 2 (5) | 1 (2.5) | 3 (7) | 2 (5) |
| A:T to C:G | 1 (3) | 2 (5) | 1 (2) | 2 (5) |
| A:T to G:C | 0 (0) | 3 (7.5) | 1 (2) | 3 (8) |
| frameshift | 4 (11) | 2 (5) | 4 (10) | 3 (8) |
| other (tandem base substitution) | 0 (0) | 0 (0) | 1 (2) | 1 (3) |
| total mutants screened | 37 (100) | 40 (100) | 42 (100) | 38 (100) |

## Table 2

Summary of Independent Mutations in the *cII* Gene of Livers of Big Blue® Transgenic Mice Treated with Vehicle, DEB, BSO/DEB, or $O^6$-BzGua/DEB

| | number of independent mutations (%) | | | |
|---|---|---|---|---|
| **type of mutation** | **vehicle** | **DEB** | **BSO/DEB** | **$O^6$-BzGua/DEB** |
| G:C to C:G | 3 (8) | 1 (2.5) | 1 (2.6) | 2 (5) |
| G:C to A:T | 21 (57) | 12 (30) | 19 (49) | 18 (45) |
| G:C to T:A | 6 (16) | 8 (20) | 10 (25.6) | 8 (20) |
| A:T to T:A | 2 (5) | 1 (2.5) | 1 (2.6) | 2 (5) |
| A:T to C:G | 1 (3) | 2 (5) | 1 (2.6) | 1 (2.5) |
| A:T to G:C | 0 (0) | 10 (25) | 4 (10) | 6 (15) |
| frameshift | 4 (11) | 5 (12.5) | 2 (5) | 3 (7.5) |
| other (tandem base substitution) | 0 (0) | 1 (2.5) | 1 (2.6) | 0 (0) |
| total mutants screened | 37 (100) | 40 (100) | 39 (100) | 40 (100) |