# Conjugation of Haloalkanes by Bacterial and Mammalian Glutathione Transferases: Mono- and Vicinal Dihaloethanes

James B. Wheeler, Nina V. Stourman,† Richard N. Armstrong,† and F. Peter Guengerich*,†

*Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146*

*Received January 26, 2001*

Glutathione (GSH) transferases are generally involved in the detoxication of xenobiotic chemicals. However, conjugation can also activate compounds and result in DNA modification. Activation of 1,2-dihaloethanes ($BrCH_2CH_2Br$, $BrCH_2CH_2Cl$, and $ClCH_2CH_2Cl$) was investigated using two mammalian theta class GSH transferases (rat GST 5-5 and human GST T1) and a bacterial dichloromethane dehalogenase (DM11). Although the literature suggests that the bacterial dehalogenase does not catalyze reactions with $CH_3Cl$, $ClCH_2CH_2Cl$, or $CH_3CHCl_2$, we found a higher enzyme efficiency for DM11 than for the mammalian GSH transferases in conjugating $CH_3Cl$, $CH_3CH_2Cl$, and $CH_3CH_2Br$. Enzymatic rates of activation of 1,2-dihaloethanes were determined in vitro by measuring *S*,*S*-ethylene-bis-GSH, the major product trapped by nonenzymatic reaction with the substrate GSH. *Salmonella typhimurium* TA 1535 systems expressing each of these GSH transferases were used to determine mutagenicity. Rates of formation of *S*,*S*-ethylene-bis-GSH by the GSH transferases correlated with the mutagenicity determined in the reversion assays for the three 1,2-dihaloethanes, consistent with the view that half-mustards are the mutagenic products of the GSH transferase reactions. Half-mustards [*S*-(2-haloethyl)GSH] containing either F, Cl, or Br (as the leaving group) were tested for their abilities to induce revertants in *S. typhimurium*, and rates of hydrolysis were also determined. GSH transferases do not appear to be involved in the breakdown of the half-mustard intermediates. A halide order (Br > Cl) was observed for both GSH transferase-catalyzed mutagenicity and *S*,*S*-ethylene-bis-GSH formation from 1,2-dihaloethanes, with the single exception (both assays) of $BrCH_2CH_2Cl$ reaction with DM11, which was unexpectedly high. The lack of substrate saturation seen for conjugation of dihalomethanes with GSTs 5-5 and T1 was also observed with the mono- and 1,2-dihaloethanes [Wheeler, J. B., Stourman, N. V., Thier, R., Dommermuth, A., Vuilleumier, S., Rose, J. A., Armstrong, R. N., and Guengerich, F. P. (2001) *Chem. Res. Toxicol.* **14**, 1118−1127], indicative of an inherent difference in the catalytic mechanisms of the bacterial and mammalian GSH transferases.

## Introduction

1,2-Dihaloethanes (vicinal dihalides) have been the subject of considerable interest because of their high volume use and potential toxicity. $BrCH_2CH_2Br$ was formerly used as a fumigant and an antiknock agent in leaded gasoline. Deaths have occurred in humans exposed to very high concentrations (*1*−*4*). The compound produces tumors at a variety of sites in rats (*5*−*7*), and primarily because of this issue, the industrial and agricultural use of $BrCH_2CH_2Br$ was curtailed (*8*). $ClCH_2CH_2Cl$ is considerably less toxic than $BrCH_2CH_2Br$; this compound is used in very high volume [$\sim 8 \times 10^9$ kg/year in U.S. (*9*)] as a precursor of vinyl chloride. Other vicinal dihalides of interest include 1,2,3-trichloropropane (*10*, *11*) and 1,2-dibromo-3-chloropropane (*12*−*14*), which have issues of reproductive toxicity.

Metabolism of 1,2-dihaloethanes is generally agreed to be critical for their toxicity, except possibly at very high concentrations (*2*, *3*). The metabolism of 1,2-dihaloethanes involves two major routes. Hydroxylation by P450 enzymes yields a *gem*-halohydrin that dehydrohalogenates to form a 2-haloacetaldelhyde. van Bladeren and Breimer showed that ∼80% of the metabolism of $BrCH_2CH_2Br$ occurred via this route in rats (*15*). The other route involves conjugation by GSH transferases (GSTs)[1] (Scheme 1). The initial conjugation product is a half-mustard, which undergoes nonenzymatic dehalogenation to yield an episulfonium ion (*16*, *17*). The episulfonium ion reacts rapidly with $H_2O$ or other nucleophiles.

Both metabolic pathways yield electrophilic species. Rannug (*18*, *19*) first demonstrated the importance of the GSH-dependent pathway in the activation of 1,2-dihaloethanes to genotoxic products. The half-mustards (*20*) and 2-haloacetaldehydes (*21*, *22*) are capable of reacting

* To whom correspondence should be addressed. Phone: (615) 322-2261. Fax: (615) 322-3141. E-mail: guengerich@toxicology.mc.vanderbilt.edu.

† Vanderbilt University School of Medicine.

[1] Abbreviations: GST, GSH transferase (designating a specific enzyme); GST T1h, human GST T1 with C-terminal pentahistidine tag; IPTG, isopropyl β-D-thiogalactoside; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); DTT, dithiothreitol; PCR, polymerase chain reaction; TTBS, 20 mM Tris-HCl buffer (pH 7.6) containing 0.05% Tween 20 (v/v) and 0.5 M NaCl.

10.1021/tx0100183 CCC: $20.00 
Published on Web 07/18/2001

with DNA to generate miscoding adducts. However, the rates of reaction of the haloacetaldehydes with DNA are rather slow (*23*, *24*). In addition, both dehydrogenases and GSH transferases are capable of inactivating the haolacetealdehyde intermediate (*24*). In previous work, we have studied the DNA adducts derived from the episulfonium ion pathway (*16*, *20*, *25*−*37*). *S*-[2-(*N*$^7$-Guanyl)ethyl]GSH is the major adduct (*20*, *26*, *27*) but the *N*$^2$- and *O*$^6$-guanyl adducts (*35*−*37*) may also contribute to explain the predominance of GC:AT transitions in several organisms (*35*, *38*−*40*). An unexpected and yet unexplained observation is the increased bacterial mutagenicity seen with expression of *O*$^6$-alkyguanine transferase (*41*−*43*), which does not appear to be related to the GSH conjugates.[2]

Several mammalian GSH transferases can catalyze the conjugation of GSH with haloalkanes (*30*, *44*, *45*); theta-class GSH transferases are most active (*45*). Human GST T1 is polymorphic in humans and, depending on race, the lack of the GST T1 gene can vary from 20 to 80%. Caucasians show ∼20% null phenotype (homozygotes) whereas Asians are reportedly ∼80% null phenotype. Individuals have either the null allele (nonconjugators), one allele (conjugators), or both alleles (high conjugators) based on their ability to conjugate $CH_3Cl$ (*46*−*50*). Epidemiological studies have suggested an association of the presence or absence of GST T1 with incidence of certain forms of cancer, e.g., oral (*51*, *52*), bladder (*48*, *53*), and brain (*54*−*56*), although the involvement of individual chemicals in the etiology is unknown.

The involvement of specific mammalian GSH transferases in the metabolism and activation of dihaloalkanes is not fully understood. Interestingly, there exist bacterial enzymes that efficiently metabolize haloalkanes. For example, some bacteria (e.g., *Methylophilus* sp.) can use $CH_2Cl_2$ as a carbon source because of the presence of a specific GSH transferase. One of these GSH transferases (DM11) exhibits 26% sequence identity with mammalian theta class GSH transferases, including active site residues involved in the activation of GSH. For example, the amino acid Ser12 of DM11 aligns with Ser10 of rat GST 5-5 suggesting a common function in both enzymes (*57*). The side-chain hydroxyl group of Ser12 is believed to be involved in the catalytic mechanism, as demonstrated by site-directed mutagenesis (*58*).

A number of issues still exist regarding the activation of 1,2-dihaloethanes via the GSH-dependent pathway. The ability of the bacterial enzyme (DM11) isolated from *Methylophilus sp* strain DM11 (*59*) to conjugate 1,2-dihaloethanes was investigated along with the mammalian class theta enzymes in order to better understand the mechanisms of mammalian activation of diahloalkanes to mutagenic species. Another issue of interest is the halide reaction order in both the conjugation and the mutagenicity of the 1,2-dihaloethanes. The studies with different 1,2-dihaloethanes and GSH transferases are considered in their relevance to other haloalkanes, particularly mono- and dihalomethanes; also, see the following paper in this issue (*60*).

## Experimental Procedures

**Reagents and Chemicals.** 2,4-Dinitro-1-fluorobenzene, dihaloethanes, $CH_3CH_2Br$, and $CH_3CH_2Cl$ (both gases), and all other reagent-grade chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Agar, nutrient broth, and other Ames test reagents were obtained from VWR (South Plainfield, NJ). AccQFluor reagents were purchased from Waters (Milford, MA) and used according to the manufacturer's directions. DEAE Fast-Flow Sepharose and hydroxylapatite were purchased from Amersham/Pharmacia (Piscataway, NJ) and Bio-Rad (Hercules, CA), respectively. $Ni^{2+}$-nitrilotriacetic acid agarose was obtained from Qiagen (Valencia, CA). Chemiluminescent substrates were obtained from Amersham/Pharmacia (Piscataway, NJ).

Pfu polymerase and other reagents used for cloning and DNA manipulation were purchased from Promega (Madison, WI).

Rabbit anti-rat GST 5-5 was a gift from Thomas Schultz (Göttingen, Germany). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from ICN (Irvine, CA).

**Synthesis of *S*-HaloethylGSH Compounds.** The halide half-mustards ($GSCH_2CH_2X$) were prepared by reaction of trisodium GSH with the appropriate haloethyl bromide in dry $CH_3OH$ and the amounts of product were estimated using a 4-(4-nitrobenzyl)-pyridine assay, with $\epsilon_{560} = 5700\ mM^{-1}\ cm^{-1}$ (*16*, *31*, *61*).

*S*-(2-Acetoxyethyl)GSH was prepared by displacement of Br from 2-bromoethyl acetate (Aldrich) by sodium GSH thiolate in $CH_3OH$ using the general procedure described previously (*31*). When the compound was subjected to electrospray MS (direct infusion, capillary temperature 180−200 °C), the $MH^+$ of *S*-(2-hydroxyethyl)GSH was observed at *m*/*z* 394 (plus adducts with 1, 2, and 3 $Na^+$). The compound formed a typical purple adduct with 4-(4-nitrobenzyl)pyridine when heated and treated with $(C_2H_5)_3N$ (*16*, *31*). $^1H$ NMR ($D_2O$) indicated a singlet at $\delta$ 2.02, assigned to the acetyl $CH_3$ group and distinct from the $\delta$ 2.10 signal observed for $CH_3CO_2H$ added to the sample. Other peaks were those expected for GSH adducts (*26*).

**GSH Transferase Expression Constructs.** *(1) GST 5-5.* The GST 5-5 cDNA (*44*, *62*) was expressed using the vector pKK233-2 as described previously (*44*).

*(2) Human GST T1 with C-Terminal Pentahistidine Tag (GST T1h).* Modification of human GST T1 cDNA (*63*) to add a C-terminal $(His)_5$ tag was done by polymerase chain reaction (PCR) methods using one oligonucleotide primer (CAATTTCACACAGGAAACAG) within pKK233-2, upstream of the 5′ end of the GST T1 start sequence, and a reverse primer (GCGAAGCTTTATTAATGGTGATGGTGATGCCGGATCATGGCTAGCACCCAG) containing a portion of the 3′ end of the GST T1 cDNA and additional sequence encoding for the $(His)_5$ region, a stop codon, and a *Hin*dIII restriction site. The PCR product was digested with the restriction enzymes *Nco*I and *Hin*dIII, purified, and subcloned into pET24d, obtained from Novagen (Madison, WI) (which had also been digested with *Nco*I and *Hin*dIII and purified). The plasmid was used to transform *Escherichia coli* HMS174 DE3. The presence of the GST T1h insert was confirmed by restriction digest mapping and nucleotide sequence analysis.

*(3) DM11. Methylophilus* sp. strain DM11 was obtained from S. Vuilleumier (ETH-Zentrum, Zürich) (*57*).

**DM11 Expression in *S. typhimurium*.** DM11 was subcloned from pE 1962 into pTrc99A (Pharmacia, Piscataway, NJ). The original ATG start codon was located within an *Nde*I restriction site, which was mutated by PCR methodology to an *Nco*I site for introduction into pTrc99A. As a result of this mutation, the amino acid sequence was changed from Met-Ser-Thr to Met-Gly-Thr. PCR of the DM11 gene was done using one primer (ATAACCATGGGTACTAAACTACGATATCTAC) containing the *Nco*I restriction site and a reverse primer (ACGAAGCTTATCCGTGTTGAGTAACGAATG) that contained a *Hin*dIII restriction site. The PCR product was digested with the restriction enzymes *Nco*I and *Hin*dIII, purified, and subcloned into pTrc99A which had also been digested with *Nco*I and *Hin*dIII and purified. The plasmid was transformed into *Salmonella typhimurium* TA1535, and the presence of the DM11 gene was confirmed by restriction digest mapping and nucleotide

[2] Liu, L., Pegg, A. E., and Guengerich, F. P., unpublished results.

sequence analysis (done in the Vanderbilt Cancer Center facility, USPHS P30 CA68485).

**Protein Expression and Purification.** *(1) Rat GST 5-5.* Rat GST 5-5 was expressed in *E. coli* DH5α F′IQ (Gibco-BRL). The cells were transformed with the plasmid pKK233-2 containing the GST 5-5 cDNA (*44*). The cells were grown to an $OD_{600}$ of 0.4−0.6 and induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). The culture was incubated for 18 h at 28 °C and the cells were centrifuged at 3000*g* for 15 min. Bacteria were resuspended in lysis buffer (75 mM Tris-HCl, pH 8.0, containing 0.25 M sucrose, 0.25 mM EDTA, and 20 μg of lysozyme /mL) for 30 min at 4 °C. Lysed cells were centrifuged at 14000*g* for 15 min at 4 °C and the pellet was resuspended in sonication buffer (20 mM sodium phosphate, pH 7.5, containing 25 μM phenylmethanesulfonyl fluoride, 1 μg of leupeptin/mL, 20% glycerol (v/v), and 2 mM 2-mercaptoethanol). Sonication was performed for 4 × 15 s intervals, with 30 s intermittent periods. The material was centrifuged at $10^5$ × g for 45 min and the supernatant was collected for purification.

Cytosol (50 mL) was loaded onto an 80 mL DEAE Sepharose column equilibrated with 30 mM Tris-HCl buffer (pH 8.5) containing 10% glycerol (v/v) and 2 mM 2-mercaptoethanol (buffer A), at a flow rate of 4 mL/min. GST 5-5 was eluted from the column with a 100 mM NaCl stepwise gradient in buffer A (100 mL). Fractions containing the highest GSH transferase activity based on the 1,2-epoxy-3-(*p*-nitropheoxy)propane conjugation assay (*44*) were pooled and concentrated to a total volume of ∼2 mL using a PM10 Amicon filter ($M_r$ $10^4$ cutoff) (Millipore-Amicon, Lexington, MA).

The pooled and concentrated DEAE eluant was diluted with 10 mM potassium phosphate (pH 7.5) containing 10% glycerol (v/v) and 2 mM 2-mercaptoethanol (buffer B) and loaded onto a 5 mL hydroxyapatite column equilibrated with buffer B at a flow rate of 1 mL/min. The column was washed with 5 column volumes of buffer B (at pH 6.9) and the GSH S-transferase was eluted with 20 mL of 80 mM potassium phosphate buffer (pH 6.9) containing 10% glycerol (v/v) and 2 mM 2-mercaptoethanol. The fractions containing the greatest GSH S-transferase activity were pooled and immediately dialyzed twice against 2 L of 30 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol (v/v) and 2 mM 2-mercaptoethanol for 2 h each time. The purified GST 5-5 was frozen in aliquots and stored at −20 °C.

GST 5-5 enzyme concentrations were determined by quantitative amino acid analysis in the Vanderbilt facility. Highly purified protein was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (*64*) and the proteins were transferred to polyvinylidiene fluoride membranes. The membrane was stained with Coomassie brilliant blue R-250 and the appropriate band corresponding to GST 5-5 was cut from the membrane for amino acid analysis. Transfer efficiency was estimated from a standard sample of bovine serum albumin carried through the same procedure simultaneously.

*(2) Human GST T1h.* Human GST T1h was expressed in *E. coli* HMS174 (DE3) cells. The cells were transformed with the expression plasmid pET24d (Novagen) containing the GST T1h cDNA. The expression conditions were the same as those described for GST 5-5. All purification steps were done at 4 °C.

Cytosol (40 mL, prepared as with GST 5-5) was loaded onto a 3 mL $Ni^{2+}$-nitrilotriacetic acid column equilibrated with 20 mM sodium phosphate (pH 8.0) containing, 2 mM 2-mercaptoethanol, 20% glycerol (v/v), and 5 mM imidazole (buffer C) at a flow rate of 2 mL/min. The column was washed with buffer C containing 50 mM imidazole and adjusted to pH 7.0 until a steady baseline at 280 nm was achieved. GST T1h was eluted with buffer C containing 200 mM imidazole (pH 7.0). Fractions were assayed using the 1,2-epoxy-3-(*p*-nitrophenoxy)propane assay and those fractions containing the greatest activity were pooled and dialyzed twice against 2 L of 30 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol (v/v) and 2 mM 2-mercaptoethanol for 2 h each time. The purified and dialyzed material was frozen and stored in aliquots at −20 °C. The protein concentration was determined using $\epsilon_{280} = 40\ 300\ M^{-1}\ cm^{-1}$ based on the algorithm amino acid sequence biopolymer calculator (http://paris.chem.yale.edu/).

*(3) DM11. E. coli* BL21(DE3)pLysS cells were transformed with the expression plasmid pE 1962 (or dcmA). Transformed cells were grown in Luria-Bertani media containing ampicillin (100 mg/L) and chloramphenicol (30 mg/L) at 26 °C to an $OD_{600}$ of 1.0. Protein expression was induced with 0.3 mM IPTG. The culture was incubated for 16 h at 26 °C. Cells were harvested by centrifugation at 6000*g* for 15 min, resuspended in ice-cold 100 mM potassium *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer (pH 7.0) containing 20% glycerol (v/v), 1 mM EDTA, and 4 mM dithiothreitol (DTT) and disrupted by sonication (4 × with 3 min intervals). Cell debris was removed by centrifugation at 37000*g* for 40 min. $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation and the suspension was centrifuged at 49000*g* for 50 min; the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 75% saturation. The pellet recovered following centrifugation at 49000*g* for 75 min was dissolved and dialyzed twice against 4 L of 20 mM HEPES buffer (pH 7.0) containing 10% glycerol (v/v), 1 mM EDTA, and 4 mM DTT for 16 h each time.

The dialyzed protein was loaded on to a DEAE-cellulose column (3 × 15 cm) equilibrated with 20 mM HEPES buffer (pH 7.0) containing 10% glycerol (v/v), 1 mM EDTA, and 4 mM DTT. DM11 was eluted with a linear gradient of 0 to 400 mM NaCl in 20 mM HEPES buffer (pH 7.0) containing 10% glycerol, 1 mM EDTA, and 4 mM DTT. Fractions containing $CH_2Cl_2$ dehalogenase activity (i.e., formation of HCHO, Nash assay) (*65*, *66*) were combined, dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol (v/v), 1 mM EDTA, and 4 mM DTT, concentrated, flash frozen, and stored at −80 °C.

**$GSCH_2CH_3$ Formation Assays (Scheme 1).** A typical 100 μL reaction included 100 mM sodium borate (pH 8.0), 5 mM GSH, 0.5−5 μM enzyme, and 0−20 mM haloethane. Reactions were started by adding the haloethane (preincubated at 37 °C) to a mixed (37 °C preincubated) solution of enzyme, GSH, and buffer. Reactions were stopped after 2 or 5 min with the addition of 10 μL of 1 N HCl. After ≥5 min, each reaction was neutralized with 10 μL of 1 N NaOH. Iodoacetate (7.5 μL of a 160 mM solution) and a Val internal standard (15 μL of a 2 mM solution) were added to a final volume of 0.15 mL, and the reactions were incubated at room temperature for 1 h. A 10 μL aliquot of the reaction was added to 80 μL of AccQFluor reagent buffer. Derivatization of *S*-carboxymethylGSH, $GSCH_2CH_3$, and Val was done by adding 10 μL of AccQFluor reagent. Samples were analyzed in 1.5 mL autosampler vials containing volume-reducing inserts; the amount of sample injected onto the HPLC column was 10 μL. HPLC analysis was carried out using an octadecylsilane ($C_{18}$) column (YMC-ODS AQ, YMC, Wilmington, NC). Elution was done using the following schedule (solvent A, 0.15 M $NH_4CH_3CO_2$, pH 5.05; solvent B, 100% $CH_3CN$): 0 to 15 min 15−35% gradient solvent B (v/v), 16−20 min, 90% solvent B (v/v), 21−24 min, 15% solvent B (v/v). A standard curve consisting of varying concentrations of $GSCH_2CH_3$ and the derivatized Val internal standard was used to determine the concentration in the enzyme reactions. Injections were made by a Hitachi 7250 autosampler and products were detected using a McPherson FL-750 HPLC fluorimeter ($\lambda_{excitation}$ 250 nm, $\lambda_{emission}$ 389 nm cutoff filter). Quantitation and analysis were done using Hitachi model D7000 Chromatography Data Station Software (Hitachi, San Jose, CA).

**Hydrolysis of Half-Mustards.** The experiments were performed as described in Peterson et al. (*16*). 4-(4-Nitrobenzyl)-pyridine was used to quantify the half-mustard remaining at each time point.

***S,S*-Ethylene-bis-GSH Assay.** Enzymatic reactions were carried out in amber 1.5 mL autosampler vials containing volume-reducing inserts. A typical 100 μL reaction included 50 mM potassium phosphate (pH 8.0), 5 mM GSH, 0.5−5 μM enzyme, and 0−3 mM dihaloethane. Dihaloethanes were added

to a 40% $C_2H_5OH$ (v/v) solution to make 50 mM stock concentrations, and $C_2H_5OH$ was added to the reactions to adjust the final concentration to 2.5% (v/v) in all cases. Reactions were started by adding the 1,2-dihaloethane (preincubated at 37 °C) to a mixed (37 °C-preincubated) solution of enzyme, GSH, and buffer. Reactions were stopped after 15 min by the addition of 10 $\mu$L of 0.5 N HCl. After at least 5 min, each reaction was neutralized with 10 $\mu$L of 0.5 N NaOH. Iodoacetate (10 mM, buffered with 50 mM $NaHCO_3$) was added and the vials were incubated at room temperature for 1 h. Derivatization of *S*-carboxymethylGSH, oxidized GSH, and *S,S′*-ethylene-bis-GSH was performed by the addition of 66 $\mu$L of 39 mM 2,4-dinitro-1-fluorobenzene in $C_2H_5OH$. The amount of sample used for each analysis was 10 $\mu$L.

HPLC analysis was carried out with an octadecylsilane ($C_{18}$) column (YMC-ODS AQ, YMC, Wilmington, NC) using the following gradient with $CF_3CO_2H$ in $H_2O$ (v/v), solvent A, 0.1% $CF_3CO_2H$; solvent B, 100% $CH_3CN$: 0 to 10 min, 40% solvent B; 11 to 15 min, 90% solvent B; 16 to 22 min, 40% solvent B (all v/v). An external standard curve prepared using varying concentrations of oxidized GSH (GSSG) was used to estimate the concentration of *S,S′*-ethylene-bis-GSH. Oxidized GSH and *S,S′*-ethylene-bis-GSH eluted at $t_R$ 8.5 and 9.9 min, respectively. Injections were made with a Hitachi 7250 autosampler and products were detected at 350 nm using a Spectromonitor 3100 UV detector (Milton Roy, Riviera Beach, FL). Quantitation and analysis were done using Hitachi model D7000 Chromatography Data Station software (Hitachi, San Jose, CA).

**Antibody Production.** Rabbits were immunized by multiple (*5*−*7*) dorsal injections (subcutaneously) twice with 0.5 nmol of purified protein mixed with Titermax (CytRx Corp, Norcross, GA) at 8 day intervals. A third set of (intramuscular) injections was carried out 1 month after the second immunization with 0.1 nmol of purified protein in 12 mM phosphate buffer (pH 7.4) containing 140 mM NaCl and 3 mM KCl. Rabbits were bled one week after the last injection, and the serum was isolated and tested by immunoblot analysis (vide infra) for sensitivity and specificity. All rabbits immunized with DM11 protein produced a similar antigenic response as judged by immunoblot analysis.

One rabbit immunized with GST T1h was injected subcutaneously and intramuscularly 3 months after the third injection of the above schedule with 10 nmol of purified GST T1h mixed with Titermax Gold (CytRx). The rabbit was bled one week later and the serum isolated was subsequently used for all quantitative experiments.

Sensitivity of the antibodies was determined by titrating purified protein until a signal was no longer detected by immunoblot analysis. Specificity was determined by immunoblot analysis, comparing *S. typhimurium* cell lysates containing purified protein (GST T1h, DM11, or GST 5-5) against *S. typhimurium* cell lysates alone.

**Quantitative Immunoblot Analysis of GSH Transferases.** Prior to performing genotoxicity tests, 0.6 mL of *S. typhimurium* culture was centrifuged (12000*g*, 3 min) and the media was aspirated, frozen, and stored at −20 °C. Frozen cells were resuspended in 150 $\mu$L of sodium dodecyl sulfate electrophoresis sample buffer (*64, 66*) and incubated at 95 °C for 20 min. Aliquots of the whole cell lysates (1−15 $\mu$L) were loaded onto a 12% (w/v) polyacrylamide gel for electrophoresis (*66*). A standard curve of the appropriate purified enzyme was loaded into separate lanes of the same gel. Proteins were transferred to nitrocellulose (*66*). Nitrocellulose membranes were blocked with 20 mM Tris-HCl buffer (pH 7.6) containing 0.05% Tween 20 (v/v), 0.5 M NaCl (TTBS), and 3% bovine serum albumin (w/v) for 1 h with shaking. Rabbit sera containing the primary antibody (anti-DM11, anti-GST 5-5, or anti-GST T1) were diluted 1:2000 (v/v) in TTBS-3% bovine serum albumin (w/v) and incubated at 25 °C overnight with shaking. Membranes were washed 3× with TTBS and incubated with goat anti-rabbit immunoglobulin G (heavy and light chains, ICN catalog no. 67-4371) diluted 1:60000 (v/v) in TTBS. The blots were developed using a chemiluminescence method (Amersham/Pharmacia kit no. RPN2209) and imaged using a Bio-Rad Fluor-S Multiimager; quantitation was done with MaxS image quantitation software (Bio-Rad, Hercules, CA).

**Table 1. Enzyme Efficiencies for Conjugation of Monohaloethanes by GST 5-5 and GST T1h**

| substrate | catalytic efficiency $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$)[a] GST 5-5 | GST T1h | ratio of efficiency GST 5-5:GST T1h |
|---|---|---|---|
| $CH_3CH_2Br$ | 190 ± 10 | 31 ± 2 | 6.2 |
| $CH_3CH_2Cl$ | 7.7 ± 0.2 | 1.35 ± 0.02 | 5.7 |

[a] (±) SE from linear regression.

**Mutagenicity Assays.** *S. typhimurium* TA1535 cells were transformed with either the vector pKK233-2 expressing GST 5-5 or GST T1 (*44*) or pTrc99A expressing DM11. Control cells contained either pKK233-2 or pTrc99A for the appropriate experiment. *S. typhimurium* cells expressing either GST 5-5 or GST T1 were grown in nutrient broth overnight at 37 °C prior to mutagenesis experiments. *S. typhimurium* expressing DM11 were grown at 37 °C to an $OD_{600}$ of 0.4 and induced with 0.1 mM IPTG. The cells were then incubated for 5.5 h at 27 °C before mutagenesis experiments were performed. For experiments in which untransformed *S. typhimurium* TA1535 were used, the cells were grown overnight in nutrient broth in the absence of antibiotics.

Mutagenesis tests were performed as previously described (*67*). *S. typhimurium* culture (100 $\mu$L) was mixed with 100 $\mu$L of the mutagen diluted in 0.2 M sodium phosphate buffer (pH 7.4) for 5 min at 37 °C in sealed 3 mL polypropylene tubes. Dihaloethanes were dissolved in 40% $Me_2SO$ (v/v) at a concentration of 50 mM and subsequently diluted in 0.2 M sodium phosphate buffer (pH 7.4) for the mutagenesis tests. *S*-(2-Haloethyl)GSH compounds and *S*-(2-acetoxyethyl)GSH were incubated with the bacteria for 2 min at 22 °C prior to mixing with top agar.

**Analysis.** Linear and nonlinear regression analysis were calculated using Prism 3 (Graphpad Software, San Diego, CA).

## Results

**Kinetics of $GSCH_2CH_3$ Formation.** All of the GSH transferases tested (DM11, rat GST 5-5, and human GST T1h) accelerated the reaction of GSH with $CH_3CH_2Br$ at pH 8.0. A substantial nonenzymatic rate of reaction was also observed and was subtracted from the rates in the presence of enzyme. The nonenzymatic contribution in the reaction with $CH_3CH_2Cl$ was negligible.

Both rat GST 5-5 and human GST T1h showed nonsaturating first-order kinetics with respect to the concentration of either haloethane (Figure 1, panels A and B). The catalytic efficiencies ($k_{cat}/K_m$) of the enzymes for each of the compounds (based on the slopes of the linear regression fits for the data) are shown in Table 1. GST 5-5 was approximately 6-fold more efficient than GST T1h in conjugating both of the compounds. The ratio of $k_{cat}/K_m$ for conjugation of $CH_3CH_2Br$ was approximately 25-fold more efficient than for $CH_3CH_2Cl$ in both enzyme systems. DM11 exhibited typical Michaelis−Menten (hyperbolic) kinetics with both compounds (Figure 1C). Apparent $k_{cat}$ and $K_m$ values and catalytic efficiencies are shown in Table 2. The $k_{cat}$ values for the substrates were similar and the $K_m$ for $CH_3CH_2Br$ was about 6-fold less than for $CH_3CH_2Cl$.

**Product Inhibition.** The enzymatic formation of $GSCH_2CH_3$ from $CH_3CH_2Cl$ was carried out in the presence of varying concentrations of $Cl^-$ ion (0, 5, 10,

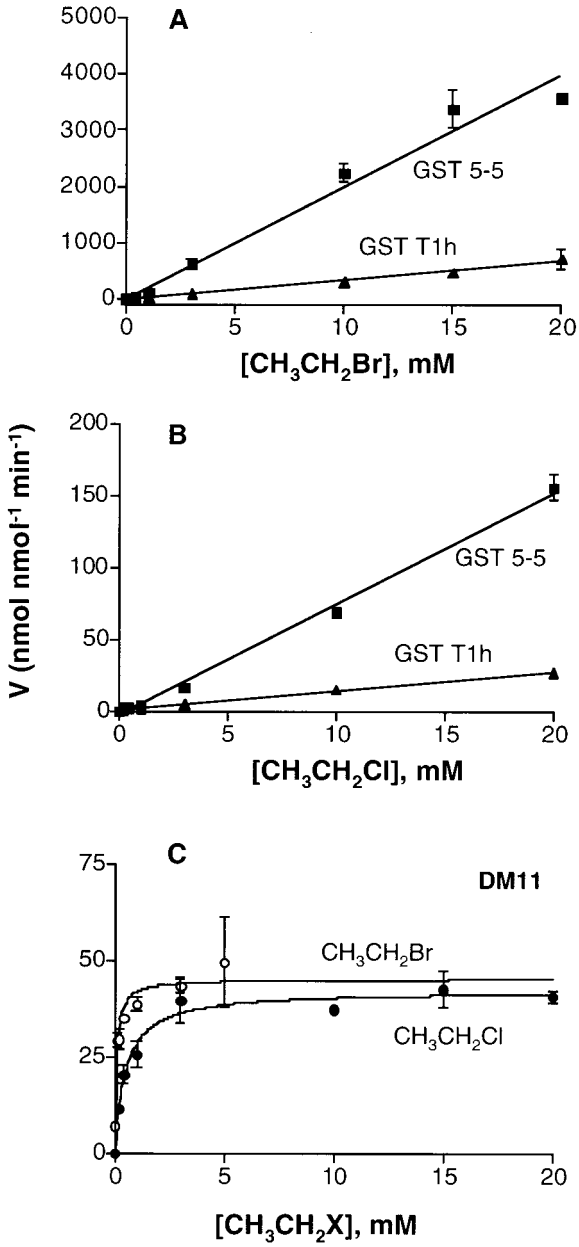



**Figure 1.** Conjugation of $CH_3CH_2Br$ and $CH_3CH_2Cl$ as a function of substrate concentration. Rates of $GSCH_2CH_3$ formation by GST 5-5 (■) and GST T1h (▲) were determined for (A) $CH_3CH_2Br$ and (B) $CH_3CH_2Cl$. (C) Rates of DM11 $GSCH_2CH_3$ formation with $CH_3CH_2Br$ (○) and $CH_3CH_2Cl$ (●). Reactions were carried out at 37 °C for 2 and 5 min for $CH_3CH_2Br$ and $CH_3CH_2Cl$, respectively. The background rate of $GSCH_2CH_3$ formation from $CH_3CH_2Br$ was subtracted from the rates ($CH_3CH_2Cl$ did not produce a measurable blank rate). Each point represents the mean of results of duplicate reactions.

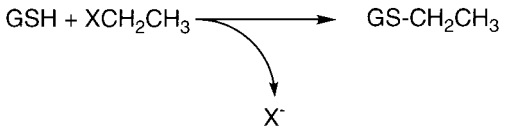
**Table 2. Steady-State Rate Constants for Conjugation of Monohaloethanes by DM11**

| | parameters for $GSCH_2CH_3$ formation[a] | | |
|---|---|---|---|
| substrate | $k_{cat}$ ($min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) |
| $CH_3CH_2Br$ | 45 ± 3 | 0.081 ± 0.03 | 560 ± 210 |
| $CH_3CH_2Cl$ | 42 ± 2 | 0.49 ± 0.10 | 86 ± 18 |

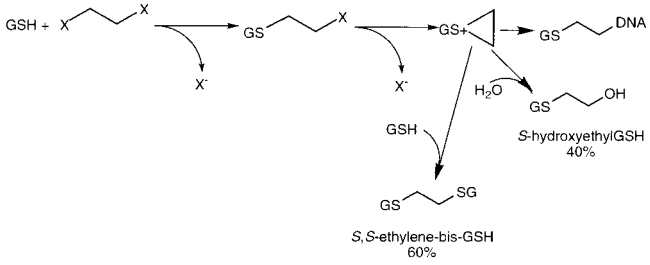
[a] (±) SE from nonlinear regression.

50, and 100 $\mu$M) or $GSCH_3$ (0, 5, 10, 50, and 100 $\mu$M). Neither GST 5-5 nor DM11 showed any detectable inhibition from $GSCH_3$ or $Cl^-$ (results not shown).

**Scheme 1. Pathway for Conjugation of $CH_3CH_2X$ with GSH**




**Scheme 2. Pathway for Conjugation of $XCH_2CH_2X$ with GSH and Subsequent Nucleophilic Interactions**




**Hydrolysis of Half-Mustards.** The reaction mechanism for the formation of DNA adducts involves an *S*-(2-haloethyl)GSH intermediate as the reaction product that leads to interactions with DNA (Scheme 2) (*16*, *35*). The stability of the intermediate half-mustard may be important in determining the potential to form DNA adducts, particularly if the half-mustard is generated outside the cells. To determine the half-life of the *S*-(2-haloethyl)GSH compounds, each was dissolved in phosphate buffer and the rate of hydrolysis was determined, using a previously described 4-(4-nitrobenzyl)pyridine assay (*16*) (the same buffer was used as described in the subsequently describe reversion assays). The half-lives of the *S*-(2-haloethyl)GSH compounds followed the expected leaving group order, with the longest half-life for *S*-(2-fluoroethyl)GSH and the shortest half-life for *S*-(2-bromoethyl)GSH (Figure 2A). The stabilities of these GSH half-mustards are somewhat greater than for any of the Cys half-mustard analogues we examined earlier (*16*).

In the mechanism proposed in Scheme 2, GSH transferases only catalyze the formation of the *S*-(2-haloethyl)-GSH intermediate and the enzyme does not participate in the removal of the second halogen. To test this hypothesis, the rate of hydrolysis of *S*-(2-chloroethyl)GSH was examined in the presence and absence of either DM11 or rat GST 5-5. No significant difference between the alkylation rates was detected (Figure 2B), indicating that the GSH transferases are not involved in the formation of the episulfonium ion (Scheme 2).[3]

Acetate is generally a poorer leaving group than any of the halides, even F, in aliphatic displacement reactions (*69*). *S*-(2-Acetoxyethyl)GSH was prepared from 2-bro-

[3] An estimate of the limit of the contribution of GSH transferases to this step can be made by calculating an enzymatic rate constant when the low limit of a 95% confidence interval (0.00181−0.00257 $s^{-1}$) of the known rate of $GSCH_2CH_2Cl$ decomposition is used (in combination with a diffusion-limited rate for the formation of an ES complex, E = GSH transferase, S = $GSCH_2CH_2Cl$, and P = $GSCH_2CH_2OH$). The mechanism S → P ($k_1$), E + S → ES ($k_2$), ES → P ($k_3$), was set up in DynaFit (*68*) with $k_1 = 0.001\,81\ s^{-1}$ (Figure 1B), $k_2 = 2.7 \times 10^7\ M^{-1}\ s^{-1}$ ($10^4\ s^{-1}$ at 2.7 mM, irreversible), [E] = 1 $\mu$M, and [S] = 2700 $\mu$M, fitting $k_3$ in plots of ([S] + [ES]) vs *t*. The calculated value of $k_3$ was $0.0375\ s^{-1}$ ($2.3\ min^{-1}$), which can be interpreted as an upper limit of any contribution of GST 5-5 or DM11 to removal of $Cl^-$ from $GSCH_2CH_2Cl$. DM11 conjugates $BrCH_2CH_2Cl$ to *S*,*S*-ethylene-bis-GSH at a rate of at least $4.6\ min^{-1}$, suggesting that DM11 does not catalyze $Cl^-$ release as part of the enzymatic mechanism.

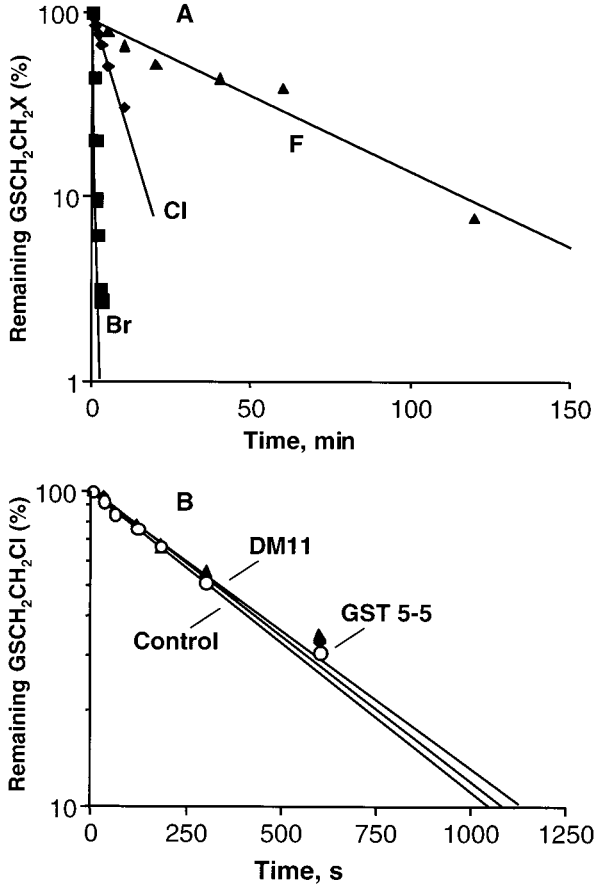



**Figure 2.** Rates of hydrolysis of *S*-(2-haloethyl)GSH compounds. *S*-(2-Haloethyl)GSH compounds were dissolved in 0.2 M sodium phosphate buffer (pH 7.4) at 22 °C and aliquots were removed at various time points and assayed for remaining *S*-(2-haloethyl)GSH using 4-(4-nitrobenzyl)pyridine (*16*). (A) Hydrolysis rates of *S*-(2-bromoethyl)GSH (■), *S*-(2-chloroethyl)GSH (◆), and *S*-(2-fluoroethyl)GSH (▲) were estimated to be 1.6, 0.13, and 0.019 $min^{-1}$, respectively (with $t_{1/2}$ = 0.44, 5.3, and 37 min). (B) *S*-(2-Chloroethyl)GSH hydrolysis in the absence or the presence of either 1 $\mu$M DM11 or GST 5-5. The initial concentration of *S*-(2-chloroethyl)GSH was 2.7 mM. The hydrolysis rate was not enhanced in the presence of either enzyme.

moethyl acetate and characterized. This half-mustard reacted with 4-(4-nitrobenzyl)pyridine under the usual conditions (100 °C for 10 min, followed by addition of base) to yield a purple adduct, but the apparent $\epsilon_{560}$ was only 1500 $M^{-1}$ $cm^{-1}$, ~25% that determined for other GSH and Cys half-mustards (*31*). The compound was extremely stable in $H_2O$; the intensity of the colored product in the 4-(4-nitrobenzyl)pyridine reaction did not decrease following incubation of *S*-(2-acetoxylethyl)GSH at 23 °C over 30 h.

**Reversion Assays with Half-Mustards.** To examine the relative mutagenicity of the half-mustards, *S. typhimurium* TA 1535 cells (without expressed GSH transferases) were incubated with *S*-(2-haloethyl)GSH compounds containing either F, Cl, or Br (Figure 3A). At the lower concentrations tested, there was an apparent relationship between the leaving group and the number of revertants. The order (from most to least revertants) observed was *S*-(2-fluoroethyl)GSH > *S*-(2-chloroethyl)-GSH > *S*-(2-bromoethyl)GSH. In contrast to the other half-mustards, *S*-(2-acetoxyethyl)GSH did not cause a significant increase in revertants with *S. typhimurium* TA 1535 except at concentrations of >100 mM (Figure 3B).

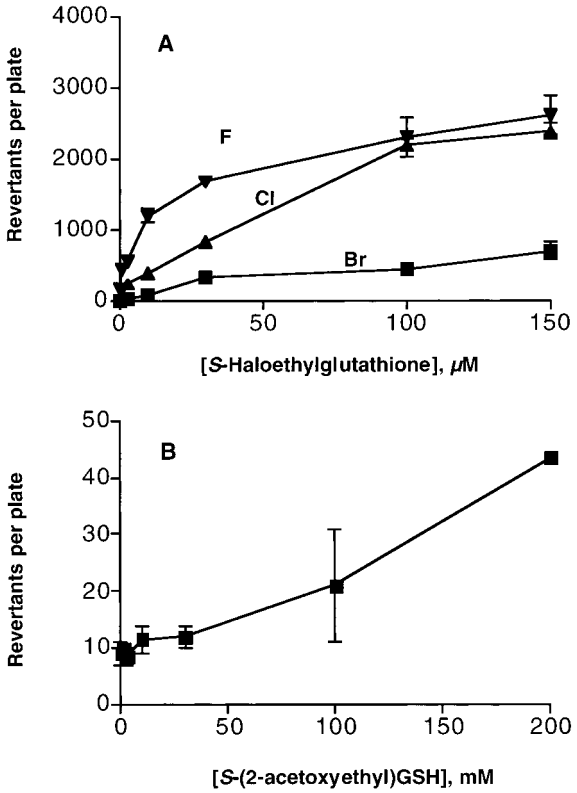



**Figure 3.** Reversion tests with (A) *S*-(2-haloethyl)GSH compounds and (B) *S*-(2-acetoxyethyl)GSH. The dry *S*-(2-haloethyl)-GSH compounds were dissolved in cold $H_2O$, diluted immediately into cold 0.20 M sodium phosphate buffer (pH 7.4), and mixed with *S. typhimurium* TA 1535 before the top agar was added. The process was performed as rapidly as possible to minimize hydrolysis. The typical time elapsed before addition to the bacteria was 2 min.

**Mutagenicity Assays with 1,2-Dihaloethanes.** GSH transferases were expressed within *S. typhimurium* TA 1535 and compared for their ability to produce revertants in the Ames test. Antibodies were used to estimate the GSH transferase expression levels within each set of bacteria for normalization. For each GSH transferase enzyme, the expression levels were relatively similar between experiments. However, expression levels of the different GSH transferases in *S. typhimurium* TA1535 varied greatly, i.e., DM11 was expressed in the 1−2 $\mu$M range whereas GST 5-5 was expressed in the 60−250 nM range. GST T1 had very low expression, in the 2−7 nM range. The specificity of the rabbit serum and the relative abundance of the GSH transferases in *S. typhimurium* are shown in Figure 4.

For all of the dihaloethane compounds, a concentration-dependent increase of revertants was observed with each of the enzymes (Figure 5, Table 3). Bacteria that contained only the expression plasmid pKK233-2 (Figure 4D) did not show any increase in revertants over the range of concentrations tested, indicating that GSH transferase expression is required for activation of the 1,2-dihaloethanes. At low concentrations of dihaloethanes, there was a clear difference among the compounds. From most to least revertants, the human and rat GSH transferases produced a similar order for mutation rates, with $BrCH_2CH_2Br$ > $BrCH_2CH_2Cl$ > $ClCH_2CH_2Cl$ (Figure 5, panels B and C). The bacterial enzyme DM11 (Figure 5A) differed in that $BrCH_2CH_2Cl$ produced more revertants than $BrCH_2CH_2Br$ at the lower concentrations. At higher

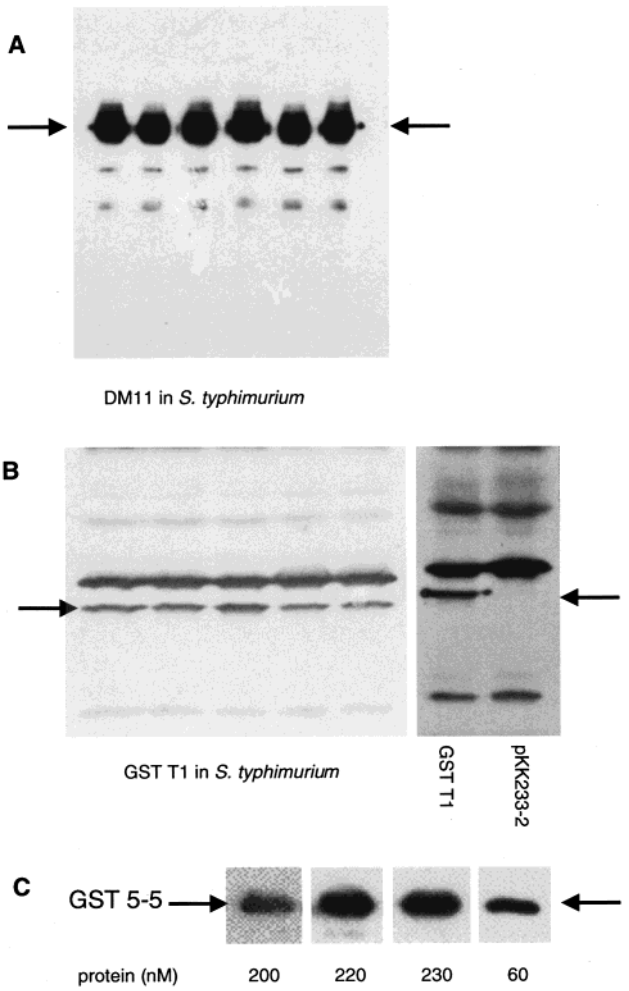



**Figure 4.** Immunoblots of *S. typhimurium* whole cell lysates expressing GSH transferases. Whole cell lysates of *S. typhimurium* expressing DM11, GST T1, or GST 5-5 were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with rabbit serum raised against (A) DM11, (B) GST T1h, or (C) GST 5-5. The rabbit serum raised against GST T1h reacted with several other proteins in *S. typhimurium*. The two lanes on the right side represent a comparison of whole cell lysates of *S. typhimurium* expressing GST T1 or the plasmid pKK233-2. The arrows point to the GSH transferases. An example of the relative amounts of GST 5-5 expressed in *S. typhimurium* is shown in part C.

concentrations, there was no significant difference between $BrCH_2CH_2Cl$ and $BrCH_2CH_2Br$ with DM11.

**Kinetics of *S,S*-ethylene-bis-GSH Formation.** GSH, dihaloethane, and enzyme were combined, and the rate of *S,S*-ethylene-bis-GSH formation (Scheme 2) was determined following derivatization with 2,4-dinitro-1-fluorobenzene (*70*) and HPLC. Because the dihaloethanes are poorly soluble in $H_2O$, an organic compound was used as a cosolvent. Of the cosolvents tested [$C_2H_5OH$, $Me_2SO$, *N,N*-dimethylformamide, and acetone, 2.5% (v/v)], only $Me_2SO$ exhibited any inhibition (results not shown). $C_2H_5OH$ was chosen as a cosolvent (final concentration did not exceed 2.5% (v/v) in any of the experiments). The upper limit of concentrations was ≤3 mM for each 1,2-dihaloethane. In previous work with a radiometric assay (*30*), we established that under these conditions ~60% of the episulfonium ion is trapped as *S,S*-ethylene-bis-GSH and the remainder appears to react with $H_2O$ to form *S*-hydroxyethylGSH.

With both GST 5-5 and GST T1h, the amount of *S,S*-ethylene-bis-GSH generated from $ClCH_2CH_2Cl$ was insufficient to be detected in the assay system. DM11 showed Michaelis−Menten kinetics at the higher concentrations of $ClCH_2CH_2Cl$ (Figure 6A). The order of $k_{cat}/K_m$ was $BrCH_2CH_2Cl$ > $BrCH_2CH_2Br$ > $ClCH_2CH_2Cl$ (Table 4). In contrast, GST 5-5 and GST T1h showed first-order kinetics in dihaloethane and did not saturate (Figure 5, panels B and C); $BrCH_2CH_2Br$ was the 1,2-dihaloethane that was conjugated at the fastest rate. Efficiencies (based on the slopes from the regression lines) indicated that GST 5-5 efficiencies were ~5-fold greater than for GST T1h (Table 5).

## Discussion

The analysis of products generated from monohaloethanes by the GSH transferases DM11, 5-5, and T1h was simplified in that only the direct conjugation products are measured, avoiding postenzymatic chemical reactions that complicate measurements of the initial products. The relative catalytic efficiencies ($k_{cat}/K_m$) of the three enzymes toward $CH_3CH_2Br$ and $CH_3CH_2Cl$ generally reflect the expected halide order for leaving groups from alkyl compounds (Br > Cl). Thus, GSTs 5-5 and T1 exhibit a ~24-fold higher efficiency toward $CH_3CH_2Br$ > $CH_3CH_2Cl$. The difference with the DM11 enzyme is somewhat less (~6-fold). The $k_{cat}/K_m$ values for the two substrates reflect, at least in part, the chemical reactivity of the compounds, which is not surprising because the rate constant $k_{cat}/K_m$ is influenced by the barriers on the reaction coordinate up to and including the first irreversible step. The first irreversible step is almost certainly the formation of the GSH-ethyl halide conjugate.

The $k_{cat}$ values for the mammalian enzymes could not be accurately measured because saturation kinetics were not observed, up to the solubility limits of the substrates. However, the DM11 enzyme exhibited identical values of $k_{cat}$ toward the two haloethane substrates. This result suggests that the rate of catalytic turnover of the DM11 enzyme is not limited by the chemical reaction of GSH with the alkyl halide but by another step in the reaction such as product release, substrate binding, thiolate formation, or an associated conformational transition of the protein. Product release is unlikely to be rate limiting because neither the halide ion nor $GSCH_2CH_3$ was a potent product inhibitor. The consequence of nonsaturating kinetics is that the enzyme is never in a state of maximum velocity. The lack of inhibition byproducts indicates that catalytic turnover will not be hampered by excess product, allowing time for further metabolism of products by other enzymes or transport across a membrane.

The accepted mechanism of activation and mutagenesis of 1,2-dihaloethanes is shown in Scheme 1 (*16*). Protein adducts can also be formed (e.g., Cys) (*71*). Because the strongest nucleophile present in the enzyme assay is GSH, the major product is *S,S*-ethylene-bis-GSH; however, a significant amount of *S*-(2-hydroxyethyl)GSH is also formed (*30*). The role of the transferase in the reaction was determined by examining the decomposition of half-mustards in the presence or absence of one of the GSH transferases. Because there was no appreciable change in the rate of decomposition of *S*-chlorethylGSH in the presence of DM11 or GST 5-5, the GSH transferases appear not to be involved in the removal of the second halogen, and the process is considered to be nonenzymatic.[3]

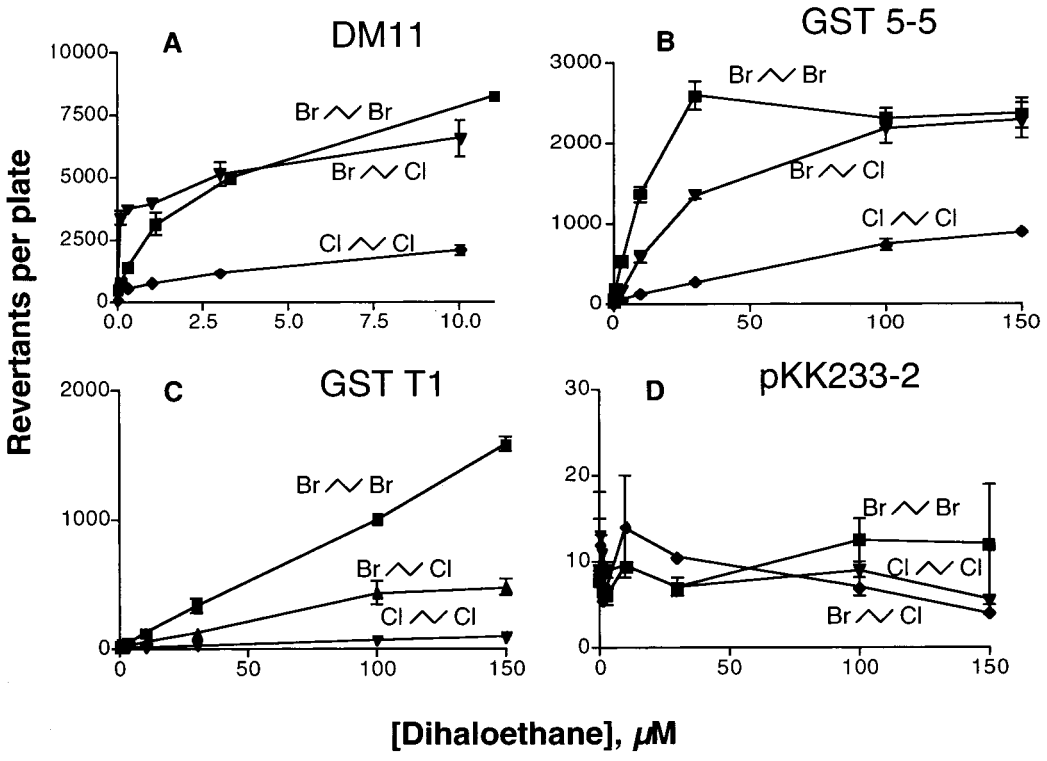



**Figure 5.** Reversion assays with 1,2-dihaloethanes. *S. typhimurium* TA 1535 transformed with either (A) DM11, (B) rat GST 5-5, (C) human GST T1, or (D) control plasmid pKK233-2 were incubated with varying concentrations of $BrCH_2CH_2Br$ (■), $ClCH_2CH_2Cl$ (▼), or $BrCH_2CH_2Cl$ (◆) for 5 min at 37 °C prior to plating. Each point represents the mean of duplicate assays. The results were normalized for enzyme concentration, determined from quantitative immunoblots for each enzyme and each assay.

**Table 3. Mutagenicity Observed with GSH Transferases in *S. typhimurium***

| | mutation rate (revertants $\mu M^{-1}$ per plate)[a,b] | | | |
|---|---|---|---|---|
| substrate | GST 5-5 | GST T1 | DM11 | pKK |
| $BrCH_2CH_2Cl$ | 45 ± 2 | 3.3 ± 0.3 | 2200 ± 1100 | <0.1 |
| $CH_3CH_2Br$ | 85 ± 6 | 10.3 ± 0.2 | 1300 ± 140 | <0.1 |
| $ClCH_2CH_2Cl$ | 7.1 ± 0.3 | 0.5 ± 0.04 | 170 ± 20 | <0.1 |

[a] Calculated from linear regression of linear portion of data points from normalized reversion test plots. [b] (±) SE from linear regression.

The relationship between the decomposition rates for each of the half-mustards and the mutations seen in the reversion assays suggests that the first leaving group is the main factor governing reversion rates and decomposition rates. The relationship followed the halogen order (from slowest to fastest decomposition) F > Cl > Br with a slower decomposition rate corresponding to a higher reversion rate in the mutagenicity assay. In previous work (*31*), we established that these different *S*-(2-haloethyl)GSH half-mustards yield equivalent amounts of adducts when mixed directly with DNA (*31*). All of these half-mustards generate the same episulfonium ion, which then partitions between reaction with $H_2O$ and any nucleophiles. Therefore, the stability of the half-mustards must be a significant issue in determining their abilities to enter cells and cause mutations (Figures 1 and 2). However, *S*-(2-acetoxyethyl)GSH was very stable but only weakly mutagenic (Figure 2B), consistent with its extremely low reactivity. Thus, of the half-mustards considered here, *S*-(2-fluoroethyl)GSH is optimized for stability and reactivity and is most mutagenic.

The conjugation and decomposition of 1,2-dihaloethanes is more complex than the conjugation of monohaloalkanes. The reaction rates estimated with the monohalogenated ethanes are significantly greater than any of the corresponding dihaloethanes.[4] The decreased rates due to the presence of a vicinal halide group may be the result of an altered inductive effect in the substrates. Alternatively, a decreased affinity for the GST may be a possibility and cannot be dismissed in the absence of more information about the catalytic mechanisms of the enzymes. Conjugation of $ClCH_2CH_2Cl$ was detected (in vitro) with DM11 but not with the mammalian theta GSH transferases. However, the GST 5-5-mediated reaction is progressing below the (*S*,*S*-ethylene-bis-GSH) assay detection limit, because reversion assays show mutations in the presence of $ClCH_2CH_2Cl$, although at significantly lower levels than for $BrCH_2CH_2Br$ and $BrCH_2CH_2Cl$.

GST 5-5 and GST T1 have similar, nonsaturating reaction rate patterns (v vs S) for each of the dihaloethanes (Figure 6), which are similar to the results obtained for monohaloethanes (Figure 1) and dihalomethanes (*60*, *72*). For DM11, $BrCH_2CH_2Cl$ was the most rapidly conjugated substrate, rather than $BrCH_2CH_2Br$, which differs from the situation observed with the mammalian theta class GSH transferases. As with the monohaloethanes (Figure 1), DM11 exhibited saturating kinetics for the 1,2-dihaloethanes [$K_m$ values were significantly higher for dihalomethanes than for monohaloethanes (*60*)]. For each of the enzymes, the substrate that produced the highest conjugation rates in vitro produced the highest reversion rates in the Ames test, suggesting that the rate-limiting step in mutagenesis is the initial GSH conjugation reaction carried out by the

[4] The lower rates of conjugation for the 1,2-dihaloethanes are not the result of inefficient trapping of the episulfonium ion by GSH (Scheme 1), because previous work indicated that 60% of the product was trapped as *S*,*S*-ethylene-bis-GSH under similar conditions (*30*).

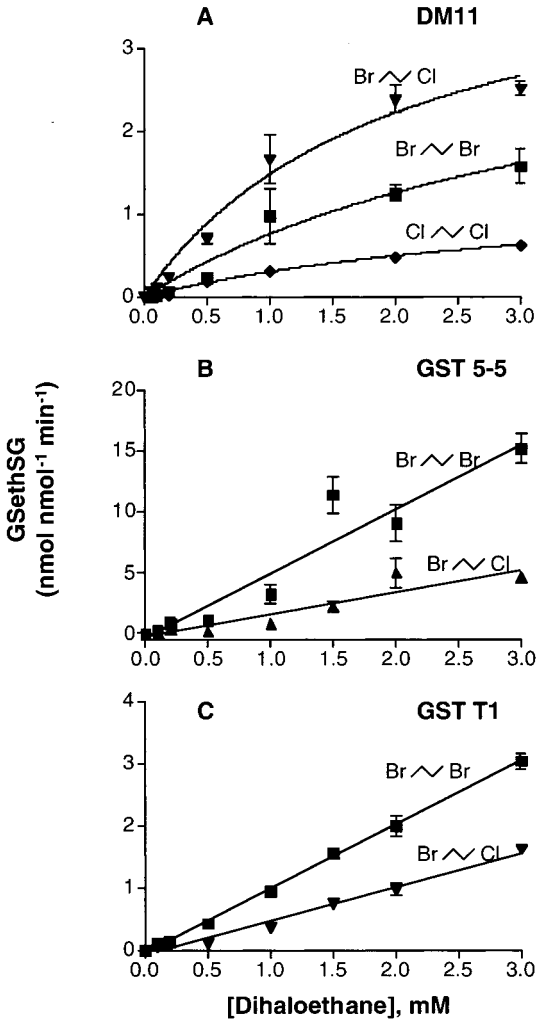



**Figure 6.** Conjugation of 1,2-dihaloethanes as a function of substrate concentration. Rates of *S*,*S*-ethylene-bis-GSH formation were determined for (A) DM11, (B) GST 5-5, and (C) GST T1h for $BrCH_2CH_2Br$ (■), $BrCH_2CH_2Cl$ (▼), and $ClCH_2CH_2Cl$ (◆). Reactions were carried out at 37 °C for 15 min. Each point represents the mean of duplicate reactions. Neither GST 5-5 nor GST T1h produced sufficient *S*,*S*-ethylene-bis-GSH from $ClCH_2CH_2Cl$ to be detected in this assay.

**Table 4. Steady-State Kinetic Constants for Conjugation of Dihaloethanes by DM11**

| | *S*,*S*-ethylene-*bis*-GSH formation[a] | | |
|---|---|---|---|
| substrate | $k_{cat}$ ($min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) |
| $BrCH_2CH_2Cl$ | 4.5 ± 0.7 | 2.0 ± 0.6 | 2.2 ± 0.8 |
| $CH_3CH_2Br$ | 3.7 ± 1.5 | 3.8 ± 2.5 | 1.0 ± 0.7 |
| $ClCH_2CH_2Cl$ | 1.3 ± 0.2 | 3.3 ± 1.0 | 0.4 ± 0.1 |

[a] (±) SE from nonlinear regression.

**Table 5. Enzyme Efficiencies for Conjugation of Dihaloethanes by GSTs 5-5 and T1h**

| | catalytic efficiency $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$)[a] | |
|---|---|---|
| substrate | GST 5-5 | GST T1h |
| $CH_3CH_2Br$ | 5.3 ± 0.5 | 1.0 ± 0.02 |
| $BrCH_2CH_2Cl$ | 1.8 ± 0.2 | 0.54 ± 0.02 |

[a] (±) SE from linear regression.

enzyme. This is in contrast to the half-mustard mutation assays in which overall stability of the half-mustard determined the number of mutations. These results imply that degradation of the mustards occurs during transport to the intracellular target (DNA) and also suggest that episulfonium ion formation occurs at a rate faster than half-mustard formation (Figure 2). The in vitro assays were carried out over a time course of 15 min, i.e., at least three half-lives for *S*-(2-chloroethyl)GSH (an additional 10 min was added after the reactions were stopped to allow complete formation of *S*,*S*-ethylene-bis-GSH).

DM11 did not follow the same substrate reaction rate order as GST 5-5 and GST T1h. This result indicates a difference between DM11 and mammalian GSH transferase reaction mechanisms, as noted with monohaloalkane conjugation (*60*). DM11 had equivalent $k_{cat}$ values for $CH_3CH_2Cl$ and $CH_3CH_2Br$, suggesting that the rate of displacement of the halogen atom is not rate-limiting. With the 1,2-dihaloethanes, the reactions are much slower and there is a difference in $k_{cat}$ values for each of the compounds. The higher rate observed with $BrCH_2CH_2Cl$ than $BrCH_2CH_2Br$ in the GSH trapping assay (Table 3) is also observed in the reversion assays (Figure 5). One possible explanation is that $BrCH_2CH_2Cl$ simply fits better into the DM11 active site than $BrCH_2CH_2Br$.

With the exception of the anomaly regarding DM11 and $BrCH_2CH_2Cl$, the results can all be rationalized in terms of halide order. In contrast, the halide order is more complex in the conjugation of dihalomethanes. The conjugation and mutagenicity of a set of dihalomethanes is considered in the following paper in this issue (*60*).

**Acknowledgment.** This work was supported in part by U.S. Public Health Service (USPHS) Grants R35 CA44353, R01 ES10546 (F.P.G.), R01 GM 30910 (R.N.A.), and P30 ES00267 (F.P.G., R.N.A.). J.B.W. is the recipient of USPHS postdoctoral fellowship F32 CA80376. We thank K.M. Williams for acquiring the NMR spectra.

## References

(1) Hueper, W. C., and Smith, C. (1935) Fatal ethylene dichlorid poisoning. *Am. J. Med. Sci.* **189**, 778−784.
(2) Garrison, S. C., and Leadingham, R. S. (1954) A fatal case of ethylene dichloride poisoning in an occupational therapy department of a neuropsychiatric hospital. *Phys. Med.* **33**, 230−237.
(3) Nouchi, T., Miura, H., Kanayama, M., Mizuguchi, O., and Takano, T. (1984) Fatal intoxication by 1,2-dichloroethane—a case report. *Int. Arch. Occup. Environ. Health* **54**, 111−113.
(4) Letz, G. A., Pond, S. M., Osterloh, J. D., Wade, R. L., and Becker, C. E. (1984) Two fatalities after acute occupational exposure to ethylene dibromide. *J. Am. Med. Assoc.* **252**, 2428−2431.
(5) Huff, J. E. (1983) 1,2-dibromoethane (ethylene dibromide). *Environ. Health Perspect.* **47**, 359−363.
(6) Wong, L. C. K., Winston, J. M., Hong, C. B., and Plotnick, H. (1982) Carcinogenicity and toxicity of 1,2-dibromoethane in the rat. *Toxicol. Appl. Pharmacol.* **63**, 155−165.
(7) *NTP Technical Report on the Carcinogenesis Bioassay of 1,2-Dibromoethane in F344 Rats and B6C3F1 Mice (Inhalation Study)* (1982) pp 1−163, U.S. Government Printing Office, Washington, DC.
(8) Ethylene dibromide: worker exposure, use restricted. (1983) *Chem. Eng. News* **61**, 4.
(9) Top 50 chemicals: increases in output for past two years well above average for past decade. (1989) *Chem. Eng. News* **67**, 39.
(10) La, D. K., Lilly, P. D., Anderegg, R. J., and Swenberg, J. A. (1995) DNA adduct formation in B6C3F1 mice and Fischer-344 rats exposed to 1,2,3-trichloropropane. *Carcinogenesis* **16**, 1419−1424.
(11) Weber, G. L., and Sipes, I. G. (1990) Covalent interactions of 1,2,3-trichloropropane with hepatic macromolecules: studies in the male F-344 rat. *Toxicol. Appl. Pharmacol.* **104**, 395−402.
(12) Pesticide suit hits Dow: six workers awarded $5 million. (1983) *Chem. Eng. News* **61**, 4.
(13) Omichinski, J. G., Brunborg, G., Søderlund, E. J., Dahl, J. E., Bausano, J. A., Holme, J. A., Nelson, S. D., and Dybing, E. (1987) Renal necrosis and DNA damage caused by selectively deuterated and methylated analogs of 1,2-dibromo-3-chloropropane in the rat. *Toxicol. Appl. Pharmacol.* **91**, 358−370.

(14) Pearson, P. G., Søderlund, E. J., Dybing, E., and Nelson, S. D. (1990) Metabolic activation of 1,2-dibromo-3-chloropropane: evidence for the formation of reactive episulfonium ion intermediates. *Biochemistry* **29**, 4971−4981.

(15) van Bladeren, P. J., Breimer, D. D., van Huijgevoort, J. A. T. C. M., Vermeulen, N. P. E., and van der Gen, A. (1981) The metabolic formation of *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine from tetradeutero-1,2-dibromoethane. Relative importance of oxidation and glutathione conjugation *in vivo*. *Biochem. Pharmacol.* **30**, 2499−2502.

(16) Peterson, L. A., Harris, T. M., and Guengerich, F. P. (1988) Evidence for an episulfonium ion intermediate in the formation of *S*-[2-($N^7$-guanyl)ethyl]glutathione in DNA. *J. Am. Chem. Soc.* **110**, 3284−3291.

(17) Webb, W. W., Elfarra, A. A., Webster, K. D., Thom, R. E., and Anders, M. W. (1987) Role for an episulfonium ion in *S*-(2-chloroethyl)-DL-cysteine-induced cytotoxicity and its reaction with glutathione. *Biochemistry* **26**, 3017−3023.

(18) Rannug, U., Sundvall, A., and Ramel, C. (1978) The mutagenic effect of 1,2-dichloroethane on *Salmonella typhimurium*. I. Activation through conjugation with glutathione in vitro. *Chem.-Biol. Interact.* **20**, 1−16.

(19) Rannug, U., and Beije, B. (1979) The mutagenic effect of 1,2-dichloroethane on *Salmonella typhimurium*. II. Activation by the isolated perfused rat liver. *Chem.-Biol. Interact.* **24**, 265−285.

(20) Ozawa, N., and Guengerich, F. P. (1983) Evidence for formation of an *S*-[2-($N^7$-guanyl)ethyl]glutathione adduct in glutathione-mediated binding of 1,2-dibromoethane to DNA. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5266−5270.

(21) Barrio, J. R., Secrist, J. A., III, and Leonard, N. J. (1972) Fluorescent adenosine and cytidine derivatives. *Biochem. Biophys. Res. Commun.* **46**, 597−604.

(22) Guengerich, F. P., and Persmark, M. (1994) Mechanism of formation of ethenoguanine adducts from 2-haloacetaldehydes: $^{13}$C labeling patterns of 2-bromoacetaldehyde. *Chem. Res. Toxicol.* **7**, 205−208.

(23) Guengerich, F. P., Mason, P. S., Stott, W. T., Fox, T. R., and Watanabe, P. G. (1981) Roles of 2-haloethylene oxides and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. *Cancer Res.* **41**, 4391−4398.

(24) Guengerich, F. P. (1992) Roles of the vinyl chloride oxidation products 1-chlorooxirane and 2-chloroacetaldehyde in the *in vitro* formation of etheno adducts of nucleic acid bases. *Chem. Res. Toxicol.* **5**, 2−5.

(25) Inskeep, P. B., and Guengerich, F. P. (1984) Glutathione-mediated binding of dibromoalkanes to DNA: specificity of rat glutathione *S*-transferases and dibromoalkane structure. *Carcinogenesis* **5**, 805−808.

(26) Koga, N., Inskeep, P. B., Harris, T. M., and Guengerich, F. P. (1986) *S*-[2-($N^7$-Guanyl)ethyl]glutathione, the major DNA adduct formed from 1,2-dibromoethane. *Biochemistry* **25**, 2192−2198.

(27) Inskeep, P. B., Koga, N., Cmarik, J. L., and Guengerich, F. P. (1986) Covalent binding of 1,2-dihaloalkanes to DNA and stability of the major DNA adduct, *S*-[2-($N^7$-guanyl)ethyl]glutathione. *Cancer Res.* **46**, 2839−2844.

(28) Kim, D. H., and Guengerich, F. P. (1989) Excretion of the mercapturic acid *S*-[2-($N^7$-guanyl)ethyl]-*N*-acetylcysteine in urine following administration of ethylene dibromide to rats. *Cancer Res.* **49**, 5843−5851.

(29) Kim, D.-H., and Guengerich, F. P. (1990) Formation of the DNA adduct *S*-[2-($N^7$-guanyl)ethyl]glutathione from ethylene dibromide: effects of modulation of glutathione and glutathione *S*-transferase levels and the lack of a role for sulfation. *Carcinogenesis* **11**, 419−424.

(30) Cmarik, J. L., Inskeep, P. B., Meyer, D. J., Meredith, M. J., Ketterer, B., and Guengerich, F. P. (1990) Selectivity of rat and human glutathione S-transferases in activation of ethylene dibromide by glutathione conjugation and DNA binding and induction of unscheduled DNA synthesis in human hepatocytes. *Cancer Res.* **50**, 2747−2752.

(31) Humphreys, W. G., Kim, D.-H., Cmarik, J. L., Shimada, T., and Guengerich, F. P. (1990) Comparison of the DNA alkylating properties and mutagenic responses caused by a series of *S*-(2-haloethyl)-substituted cysteine and glutathione derivatives. *Biochemistry* **29**, 10342−10350.

(32) Kim, D.-H., Humphreys, W. G., and Guengerich, F. P. (1990) Characterization of *S*-[2-($N^1$-adenyl)ethyl]glutathione formed in DNA and RNA from 1,2-dibromoethane. *Chem. Res. Toxicol.* **3**, 587−594.

(33) Humphreys, W. G., Kim, D. H., and Guengerich, F. P. (1991) Isolation and characterization of $N^7$-guanyl adducts derived from 1,2-dibromo-3-chloropropane. *Chem. Res. Toxicol.* **4**, 445−453.

(34) Oida, T., Humphreys, W. G., and Guengerich, F. P. (1991) Preparation and characterization of oligonucleotides containing *S*-[2-($N^7$-guanyl)ethyl]glutathione. *Biochemistry* **30**, 10513−10522.

(35) Cmarik, J. L., Humphreys, W. G., Bruner, K. L., Lloyd, R. S., Tibbetts, C., and Guengerich, F. P. (1992) Mutation spectrum and sequence alkylation selectivity resulting from modification of bacteriophage M13mp18 with *S*-(2-chloroethyl)glutathione. Evidence for a role of *S*-[2-($N^7$-guanyl)ethyl]glutathione as a mutagenic lesion formed from ethylene dibromide. *J. Biol. Chem.* **267**, 6672−6679.

(36) Kim, M.-S., and Guengerich, F. P. (1997) Synthesis of oligonucleotides containing the ethylene dibromide-derived DNA adducts *S*-[2-($N^7$-guanyl)ethyl]glutathione, *S*-[2-($N^2$-guanyl)ethyl]glutathione, and *S*-[2-($O^6$-guanyl)ethyl]glutathione at a single site. *Chem. Res. Toxicol.* **10**, 1133−1143.

(37) Kim, M.-S., and Guengerich, F. P. (1998) Polymerase blockage and misincorporation of dNTPs opposite the ethylene dibromide-derived DNA adducts *S*-[2-($N^7$-guanyl)ethyl]glutathione, *S*-[2-($N^2$-guanyl)ethyl]glutathione, and *S*-[2-($O^6$-guanyl)ethyl]glutathione. *Chem. Res. Toxicol.* **11**, 311−316.

(38) Foster, P. L., Wilkinson, W. G., Miller, J. K., Sullivan, A. D., and Barnes, W. M. (1988) An analysis of the mutagenicity of 1,2-dibromoethane to *Escherichia coli*: influence of DNA repair activities and metabolic pathways. *Mutat. Res.* **194**, 171−181.

(39) Ballering, L. A. P., Nivard, M. J. M., and Vogel, E. W. (1994) Mutation spectra of 1,2-dibromoethane, 1,2-dichloroethane and 1-bromo-2-chloroethane in excision repair proficient and repair deficient strains of *Drosophila melanogaster*. *Carcinogenesis* **15**, 869−875.

(40) Fossett, N. G., Byrne, B. J., Tucker, A. B., Arbour-Reily, P., Chang, S., and Lee, W. R. (1995) Mutation spectrum of 2-chloroethyl methanesulfonate in *Drosophila melanogaster* premeiotic germ cells. *Mutat. Res.* **331**, 213−224.

(41) Abril, N., Luqueromero, F. L., Prieto-Alamo, M. J., Margison, G. P., and Pueyo, C. (1995) *ogt* alkyltransferase enhances dibromoalkane mutagenicity in excision repair-deficient *Escherichia coli* K-12. *Mol. Carcinogen.* **12**, 110−117.

(42) Abril, N., Ferrezuelo, F., Prieto-Alamo, M. J., Rafferty, J. A., Margison, G. P., and Pueyo, C. (1996) Contribution of *ogt*-encoded alkyltransferase to resistance to chloroethylnitrosoureas in nucleotide excision repair-deficient *Escherichia coli*. *Carcinogenesis* **17**, 1609−1614.

(43) Abril, N., Luque-Romero, F. L., Prieto-Alamo, M.-J., Rafferty, J. A., Margison, G. P., and Pueyo, C. (1997) Bacterial and mammalian DNA alkyltransferases sensitize *Escherichia coli* to the lethal and mutagenic effects of dibromoalkanes. *Carcinogenesis* **18**, 1883−1888.

(44) Thier, R., Pemble, S. E., Taylor, J. B., Humphreys, W. G., Persmark, M., Ketterer, B., and Guengerich, F. P. (1993) Expression of mammalian glutathione *S*-transferase 5-5 in *Salmonella typhimurium* TA1535 leads to base-pair mutations upon exposure to dihalomethanes. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8576−8580.

(45) Ploemen, J. H. T. M., Wormhoudt, L. W., van Ommen, B., Commandeur, J. N. M., Vermeulen, N. P. E., and van Bladeren, P. J. (1995) Polymorphism in the glutathione conjugation activity of human erythrocytes towards ethylene dibromide and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane. *Biochim. Biophys. Acta* **1243**, 469−476.

(46) Pemble, S., Schroeder, K. R., Spencer, S. R., Meyer, D. J., Hallier, E., Bolt, H. M., Ketterer, B., and Taylor, J. B. (1994) Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochem. J.* **300**, 271−276.

(47) Warholm, M., Rane, A., Alexandrie, A. K., Monaghan, G., and Rannug, A. (1995) Genotypic and phenotypic determination of polymorphic glutathione transferase T1 in a Swedish population. *Pharmacogenetics* **5**, 252−254.

(48) Kempkes, M., Golka, K., Reich, S., Reckwitz, T., and Bolt, H. M. (1996) Glutathione S-transferase GSTM1 and GSTT1 null genotypes as potential risk factors for urothelial cancer of the bladder. *Arch. Toxicol.* **71**, 123−126.

(49) Nelson, H. H., Wiencke, J. K., Christiani, D. C., Cheng, T. J., Zuo, Z.-F., Schwartz, B. S., Lee, B.-K., Spitz, M. R., Wang, M., Xu, X., and Kelsey, K. T. (1995) Ethnic differences in the prevalence of the homozygous deleted genotype of glutathione S-transferase theta. *Carcinogenesis* **16**, 1243−1254.

(50) Peter, H., Deutschmann, S., Reichel, C., and Hallier, E. (1989) Metabolism of methyl chloride by human erythrocytes. *Arch. Toxicol.* **63**, 351−355.

(51) Hung, H.-C., Chuang, J., Chien, Y.-C., Chern, H.-D., Chiang, C.-P., Kuo, Y.-S., Hildesheim, A., and Chen, C.-J. (1997) Genetic

polymorphisms of CYP2E1, GSTM1, and GSTT1: environmental factors and risk of oral cancer. *Cancer Epidem. Biomarker. Prev.* **6**, 901−905.
(52) Nair, U. J., Nair, J., Mathew, B., and Bartsch, H. (1999) Glutathione S-transferase M1 and T1 null genotypes as risk factors for oral leukoplakia in ethnic Indian betel quid/tobacco chewers. *Carcinogenesis* **20**, 743−748.
(53) Brockmöller, J., Cascorbi, I., Kerb, R., and Roots, I. (1996) Combined analysis of inherited polymorphisms in arylamine *N*-acetyltransferase 2, glutathione *S*-transferases M1 and T1, microsomal epoxide hydrolase, and cytochrome P450 enzymes as modulators of bladder cancer risk. *Cancer Res.* **56**, 3915−3925.
(54) Hand, P. A., Inskip, A., Gilford, J., Alldersea, J., Elexpuru-Camiruaga, J., Hayes, J. D., Jones, P. W., Strange, R. C., and Fryer, A. A. (1996) Allelism at the glutathione S-transferase GSTM3 locus: interactions with GSTM1 and GSTT1 as risk factors for astrocytoma. *Carcinogenesis* **17**, 1919−1922.
(55) Elexpuru-Camiruaga, J., Buxton, N., Kandula, V., Dias, P. S., Campbell, D., McIntosh, J., Broome, J., Jones, P., Inskip, A., Alldersea, J., Fryer, A. A., and Strange, R. C. (1995) Susceptibility to astrocytoma and meningioma: influence of allelism at glutathione S-transferase (GSTT1 and GSTM1) and cytochrome P-450 (CYP2D6) loci. *Cancer Res.* **55**, 4237−4239.
(56) Stroombergen, M. C. M. J., and Waring, R. H. (1999) Determination of glutathione *S*-transferase $\mu$ and $\theta$ polymorphisms in neurological disease. *Hum. Exp. Toxicol.* **18**, 141−145.
(57) Bader, R., and Leisinger, T. (1994) Isolation and characterization of the *Methylophilus* sp. strain DM11 gene encoding dichloromethane dehalogenase/glutlathione S-transferase. *J. Bacteriol.* **176**, 3466−3473.
(58) Vuilleumier, S., and Leisinger, T. (1996) Protein engineering studies of dichloromethane dehalogenase/glutathione *S*-transferase from *Methylophilus* sp. strain DM11. Ser12 but not Tyr6 is required for enzyme activity. *Eur. J. Biochem.* **239**, 410−417.
(59) Scholtz, R., Wackett, L. P., Egli, C., Cook, A. M., and Leisinger, T. (1988) Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane-utilizing bacterium. *J. Bacteriol.* **170**, 5698−5704.
(60) Wheeler, J. B., Stourman, N. V., Thier, R., Dommermuth, A., Vuilleumier, S., Rose, J. A., Armstrong, R. N., and Guengerich, F. P. (2001) Conjugation of haloalkanes by bacterial and mammalian glutathione transferases: mono- and dihalomethanes. *Chem. Res. Toxicol.* **14**, 1118−1127.
(61) Epstein, J., Rosenthal, R. W., and Ess, R. J. (1955) Use of *p*-(4-nitrobenzyl)pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem.* **27**, 1435−1439.
(62) Pemble, S. E., and Taylor, J. B. (1992) An evolutionary perspective on glutathione transferases inferred from class-Theta glutathione transferase cDNA sequences. *Biochem. J.* **287**, 957−963.
(63) Thier, R., Pemble, S. E., Kramer, H., Taylor, J. B., Guengerich, F. P., and Ketterer, B. (1996) Human glutathione S-transferase T1−1 enhances mutagenicity of 1,2-dibromoethane and 1,2,3,4-diepoxybutane in the *Salmonella typhimurium* mutagenicity assay. In *Proceedings, Fifth International Sympos. Biological Reactive Intermediates*, 4−8 January, Munich.
(64) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. *Nature* **227**, 680−685.
(65) Nash, T. (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**, 416−421.
(66) Guengerich, F. P. (1994) Analysis and characterization of enzymes. In *Principles and Methods of Toxicology*, 3rd ed. (Hayes, A. W., Ed.) pp 1259−1313, Raven Press: New York.
(67) Maron, D. M., and Ames, B. N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutation Res.* **113**, 173-215.
(68) Kuzmic, P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV protease. *Anal. Biochem.* **237**, 260−273.
(69) March, J. (1992) *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 4th ed., p 357, Wiley, New York.
(70) Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1999) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.* **106**, 55−62.
(71) Erve, J. C. L., Barofsky, E., Barofsky, D. F., Deinzer, M. L., and Reed, D. J. (1995) Alkylation of *Escherichia coli* thioredoxin by *S*-(2-chloroethyl)glutathione and identification of the adduct on the active site cysteine-32 by mass spectrometry. *Chem. Res. Toxicol.* **8**, 934−941.
(72) Reitz, R. H., Mendrala, A., and Guengerich, F. P. (1989) In vitro metabolism of methylene chloride in human and animal tissues: use in physiologically based pharmacokinetic models. *Toxicol. Appl. Pharmacol.* **97**, 230−246.

TX0100183