Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8576–8580, September 1993
Biochemistry

Expression of mammalian glutathione *S*-transferase 5-5 in *Salmonella typhimurium* TA1535 leads to base-pair mutations upon exposure to dihalomethanes

(glutathione/chemical carcinogens/mutagenicity)

RICARDA THIER*, JOHN B. TAYLOR†, SALLY E. PEMBLE†, W. GRIFFITH HUMPHREYS*‡, MAGNUS PERSMARK*, BRIAN KETTERER†, AND F. PETER GUENGERICH*§

*Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232-0146; and †Cancer Research Campaign Molecular Toxicology Group, Middlesex School of Medicine, Cleveland Street, London, W1P 6DB, United Kingdom

Communicated by Allan H. Conney, June 15, 1993 (received for review March 23, 1993)

ABSTRACT **Dihalomethanes can produce liver tumors in mice but not in rats, and concern exists about the risk of these compounds to humans. Glutathione (GSH) conjugation of dihalomethanes has been considered to be a critical event in the bioactivation process, and risk assessment is based upon this premise; however, there is little experimental support for this view or information about the basis of genotoxicity. A plasmid vector containing rat GSH *S*-transferase 5-5 was transfected into the *Salmonella typhimurium* tester strain TA1535, which then produced active enzyme. The transfected bacteria produced base-pair revertants in the presence of ethylene dihalides or dihalomethanes, in the order CH_2Br_2 > CH_2BrCl > CH_2Cl_2. However, revertants were not seen when cells were exposed to GSH, CH_2Br_2, and an amount of purified GSH *S*-transferase 5-5 (20-fold excess in amount of that expressed within the cells). HCHO, which is an end product of the reaction of GSH with dihalomethanes, also did not produce mutations. *S*-(1-Acetoxymethyl)GSH was prepared as an analog of the putative *S*-(1-halomethyl)GSH reactive intermediates. This analog did not produce revertants, consistent with the view that activation of dihalomethanes must occur within the bacteria to cause genetic damage, presenting a model to be considered in studies with mammalian cells. *S*-(1-Acetoxymethyl)GSH reacted with 2′-deoxyguanosine to yield a major adduct, identified as *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH. Demonstration of the activation of dihalomethanes by this mammalian GSH *S*-transferase theta class enzyme should be of use in evaluating the risk of these chemicals, particularly in light of reports of the polymorphic expression of a similar activity in humans.**

Dihalomethanes, particularly CH_2Cl_2, are of interest because of their demonstrated tumorigenicity in mouse liver and lung (1) and the widespread use of these chemicals (2). However, rats and hamsters form neither liver nor lung tumors (3, 4). Dihalomethanes are transformed by two routes, oxidation (by cytochrome P450) and glutathione (GSH) conjugation (Fig. 1) (6). The GSH conjugation pathway has been considered relevant to tumor formation, largely because oxidation is a low-K_m reaction, whereas both *in vitro* GSH conjugation and tumorigenesis continue to increase with higher CH_2Cl_2 levels (10). Rates of GSH conjugation of CH_2Cl_2 are considerably lower in rats and humans than in mice, and this information—along with physiologically based pharmacokinetic models—has been used to predict that humans, like rats, will be considerably less likely than mice to develop tumors from CH_2Cl_2 exposure (7, 10, 11).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

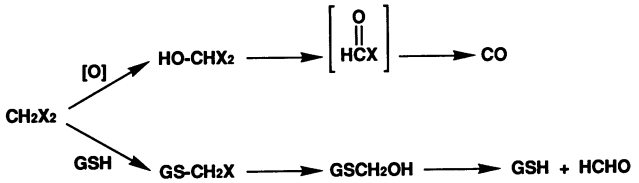

FIG. 1. Scheme for biotransformation of dihalomethanes by oxidation (5) and GSH conjugation (6). The oxidation is catalyzed primarily by cytochrome P-450 2E1 (7, 8), and the conjugation is catalyzed primarily by GSH *S*-transferase 5-5 and related theta-class enzymes (9).

Although there is a compelling case for GSH-dependent genotoxicity of 1,2-dihaloethanes (12), the basis for such a view with the dihalomethanes is lacking. The literature presents conflicting reports on the role of microsomal and cytosolic rodent fractions and GSH in the bioactivation of dihalomethanes (13–16), and we have been unable to demonstrate a consistent response of CH_2Br_2-induced base-pair mutations in *Salmonella typhimurium* tester strains in the presence of GSH or mouse liver cytosol, which would be expected to be considerably more active than rat liver cytosol (7). Enzymes of the GSH *S*-transferase theta class, especially rat GSH *S*-transferase 5-5, have been shown to be proficient in the conjugation of CH_2Cl_2 (9). *Methylobacterium* DM4 has been demonstrated to have a high level of a theta class enzyme that can be effective in conjugating CH_2Cl_2 (17), and the possibility exists that *S. typhimurium* contains such an enzyme for activating dihalomethanes and 1,2-dihaloethanes (17, 18). Our efforts to demonstrate the GSH-dependent conjugation through depletion of GSH within the cells were not particularly successful, and we also considered the possibility that the putative *S*-(1-halomethyl)GSH products might be unable to cross cell membranes. Therefore, we introduced a plasmid expression vector containing a cDNA clone for rat GSH *S*-transferase 5-5 into a standard tester bacterium, *S. typhimurium* TA1535.

MATERIALS AND METHODS

Chemicals. All dihalides were purchased from Aldrich and used without further purification. 1,2-Epoxy-3-(4′-nitrophenoxy)propane was from Eastman Kodak. [N^{2}-^{15}N]dGuo, where dGuo is 2′-deoxyguanosine, was from B. DeCorte (of this center) [prepared by treatment of 2-fluorodeoxyinosine (19) with $^{15}NH_4OH$]. *S*-(1-Acetoxymethyl)GSH was pre-

Abbreviations: GSH, glutathione; dGuo, 2′-deoxyguanosine; dCyd, 2′-deoxycytidine; FAB, fast atom bombardment.

‡Present address: Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543.
§To whom reprint requests should be addressed.

pared by adding bromomethyl acetate (Aldrich, 250 mg, 1.63 mmol) dropwise to a 7-ml solution of GSH (272 mg, 0.89 mmol) and Na° (67 mg, 2.9 mmol, 3 equivalents) in dry CH_3OH; the product precipitated immediately and was recovered by centrifugation ($3 \times 10^3 \times g$, 10 min), dried under N_2, and stored in a dessicator at −20°C. Attempts to record NMR and mass spectra were unsuccessful, primarily due to lack of solubility in nonaqueous solvents; however, analysis (20) indicated a 70% yield of HCHO after hydrolysis at pH 7.4 (70°C, 20 min).

This product (50 mg) was added directly to 8 mg of dGuo [in 1.0 ml of 0.10 M potassium phosphate buffer, pH 7.4, containing 20% (vol/vol) $(CH_3)_2NCHO$] or 2′-deoxycytidine (dCyd) [12 mg in 0.5 ml of the above buffer but lacking $(CH_3)_2NCHO$]. After 2 hr at 23°C, each reaction was analyzed by HPLC [10 × 250 mm octadecylsilane column, Beckman Ultrasphere—5 μm, 4.5–45% linear gradient of CH_3OH (vol/vol) in 50 mM NH_4HCO_2 (pH 5.5), increasing over 30 min]. In both cases a new A_{260} peak (10–20% yield) eluted ≈1 min after the starting material (dGuo or dCyd) and was collected and recovered by lyophilization, rechromatography, and repeated lyophilization to remove salt.

GSH *S*-Transferase 5-5 Plasmid Expression Constructs. Expression of GSH *S*-transferase 5-5 in *Escherichia coli* was achieved by using a combination of PCR, a full-length transferase subunit-5 cDNA clone (21) as PCR template, oligonucleotide primers each containing a recognition site for *Bsa* I (a class IIS restriction endonuclease that cleaves outside its recognition sequence—GGTCTC—which does not occur in the cDNA), and the expression vector pKK233-2 (Pharmacia). The N-terminal primer (GGGGTCTCCC*ATGGTGCTGGAGCTCTAT*) hybridized to the first six codons (italicized region coding for MVLELY), whereas the 3′ antisense primer (GGGGTCTCCCATGTCA*TCACTGGATCATGGTCAG*) hybridized to the last six codons (italicized region coding for LTMIQstop). To ensure correct translational termination, a second in-frame stop codon (TCA) was included adjacent to the six codons. The PCR product, when restricted with *Bsa* I, yielded a 727-bp fragment,

```
CATGGTGCTGGAGCTCTAT.....CTGACCATGATCCAGTGATGA
    CACGACCTCGAGATA.....GACTGGTACTAGGTCACTACTGTAC,
```

which has complementary ends to *Nco* I-digested pKK233-2. Digestion of pKK233-2 with *Nco* I exposes the translation start site of this vector; thus, ligation with the 727-bp fragment resulted in equal numbers of sense [designated (+)GST 5-5] and antisense [designated (−)GST 5-5] clones on transfection into *E. coli* JM105. Fidelity of the PCR products was confirmed by nucleotide-sequence analysis (22). Transfection of *S. typhimurium* strain TA1535 was done according to Lederberg and Cohen (23) with heat shock at 37°C, using plasmid purified from the *E. coli* strains.

Mutation Assays. The mutation assays were done according to Maron and Ames (24) with a preincubation time of 5 min at room temperature (23°C). All test compounds were dissolved in 200 mM sodium phosphate buffer, pH 7.4, except for ethylene dibromide, which was diluted in C_2H_5OH.

The rate of spontanous mutations of the designed strains *S. typhimurium* (+)GST 5-5 and (−)GST 5-5 was ≈15 revertants per plate, similar to that seen with *S. typhimurium* TA1535. These strains were also tested for histidine auxotrophy, *uvrB* deletion, *rfa* mutation, and ampicillin resistance; they exhibited the same characteristics as *S. typhimurium* TA1535, except for the ampicillin resistance acquired as a result of the pKK233-2 plasmid.

Growth and Purification of GSH *S*-Transferase 5-5 in *E. coli*. A 500-ml overnight culture of *E. coli* JM105 containing the pKK233-2 plasmid with the GSH *S*-transferase 5-5 construct [in LB broth (25) containing 100 μg of ampicillin·ml^{-1} and 2.0 mM isopropyl α-D-thiogalactoside] was cooled on ice before centrifugation at $5 \times 10^3 \times g$ (4°C). The cell pellet was resuspended in 20 ml of 10 mM potassium phosphate buffer (pH 7.0)/0.20 mM dithiothreitol/25 μM phenylmethanesulfonyl fluoride, sonicated for 30 s, cooled on ice, and sonicated for another 30 s. The sonicate was centrifuged at $10^5 \times g$ for 30 min at 4°C. DNase I (1 mg, 2000 units) was added to the supernatant before dialysis at 4°C against 1 liter of sonication buffer (containing 3 mM EDTA) for 4 days; buffer was changed twice each day. The dialysate was added to a Matrex gel orange A column, and the enzyme was eluted as described by Meyer *et al.* (9).

Assays for GSH *S*-Transferase 5-5. Cytosol of the *Salmonella* strains used to determine GSH *S*-transferase 5-5 enzyme activity and $NaDodSO_4$/PAGE (26) was prepared as for *E. coli* but without dialysis. Protein content was assayed with the bicinchonic acid reagent (Pierce). The enzyme-activity assay (27) measures the absorbance change from the conjugation of GSH to 1,2-epoxy-3-(4′-nitrophenoxy)propane ($\Delta\varepsilon_{360} = 500\ M^{-1}\cdot cm^{-1}$). The epoxide (16 μl of 30 mM stock solution in C_2H_5OH) and 20 μl of 250 mM GSH were added to 1.0 ml of 100 mM potassium phosphate buffer (pH 6.5, 37°C) in a glass cuvette with the aid of a manual mixing device, in a modified Cary 14 spectrophotometer (OLIS, Bogart, GA).

RESULTS

Initial Studies with *S. typhimurium*. Studies were done with CH_2Br_2 as a prototypic dihalomethane, expected to be more active than the dichloride. The compound could produce revertants in *S. typhimurium* strains sensitive to base-pair (e.g., TA100) or frameshift mutations (TA98) at high concentrations. However, we experienced poor and irreproducible success in showing the dependence of revertants upon addition of either mouse or rat liver cytosol or GSH (added to dialyzed cytosol).

Expression of Rat GSH *S*-Transferase 5-5 in *S. typhimurium*. A construct of the pKK233-2 vector containing the rat GSH *S*-transferase 5-5 cDNA (21) was used to transfect *S. typhimurium* TA1535, using the ampicillin resistance marker (*S. typhimurium* TA100 already has an ampicillin resistance marker and could not be directly used). The vector containing the same cDNA inserted in the opposite direction was also transfected into *S. typhimurium* TA1535 for use as an appropriate control [(−)GST 5-5]. The *S. typhimurium* TA1535 treated with (+)GST 5-5 construct expressed GSH *S*-transferase 5-5 (Fig. 2); the level of expression was estimated at 2–5% of the total cellular protein, as judged by electrophoresis or comparison of enzymatic activity with that of the purified enzyme (9). Although the pKK233-2 vector used is inducible by isopropyl α-D-thiogalactoside, we found considerable expression without addition of inducer, apparently due to the strength of the promoter and did not add this inducer in subsequent studies.

Production of Revertants in *S. typhimurium* TA1535 Expressing GSH *S*-Transferase 5-5. CH_2Cl_2, CH_2BrCl, CH_2Br_2, and ethylene dibromide all produced revertants in a dose-dependent manner in the *S. typhimurium* TA1535 expressing GSH *S*-transferase 5-5—(+)GST 5-5—but did not produce revertants in those bacteria with the control construct, (−)GST 5-5 (Fig. 3). The order of sensitivity to mutations was Br > Cl, as expected from the halogen order for aliphatic substitution reactions (Fig. 1). Ethylene dibromide is known to cause mutations through GSH *S*-transferase-mediated GSH conjugation (29), but although other GSH *S*-transferases, such as rat 2-2 and 3-3, are known to catalyze this reaction, GSH *S*-transferase 5-5 had not yet been assayed (30).

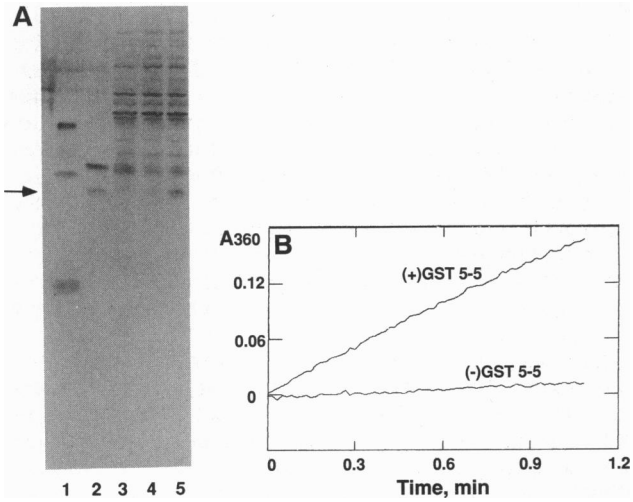

FIG. 2. (*A*) NaDodSO$_4$/PAGE (26) of a mixture of standard proteins (lane 1), including bovine β-lactoglobulin (18 kDa), human carbonic anhydrase (29 kDa), and chicken ovalbumin (45 kDa); GSH *S*-transferase 5-5 partially purified from *E. coli* (lane 2); and extracts of *S. typhimurium* TA1535 (lane 3) and *S. typhimurium* TA1535 derivatives (−)GST 5-5 and (+)GST 5-5 (lanes 4 and 5, respectively). The gel was stained with silver and NH$_4$OH (28). The arrow shows 27-kDa GSH *S*-transferase 5-5. (*B*) Conjugation of 1,2-epoxy-3-(4′-nitrophenoxy)propane by (+)GST 5-5 and (−)GST 5-5 derivatives of *S. typhimurium* TA1535. Equivalent amounts of cell lysate (0.3 mg of protein) were used.

Parental *S. typhimurium* TA1535 (i.e., no transfected plasmid) was exposed to CH_2Br_2 in the presence of partially purified GSH *S*-transferase 5-5 (produced in *E. coli*; Fig. 2*A*), which was in 20-fold excess of that expressed within the *S. typhimurium* (+)GST 5-5 strain. The frequency of revertants did not detectably increase. The reaction of GSH with dihalomethanes produces HCHO (Fig. 1), and we considered whether this product might be responsible for the mutations. HCHO was rather toxic to the cells but did not produce any revertants in the *S. typhimurium* TA1535 (+)GST 5-5 cells (Fig. 4*A*).

S-(1-Acetoxymethyl)GSH was synthesized as a model for the putative reactive *S*-(1-halomethyl)GSH intermediates

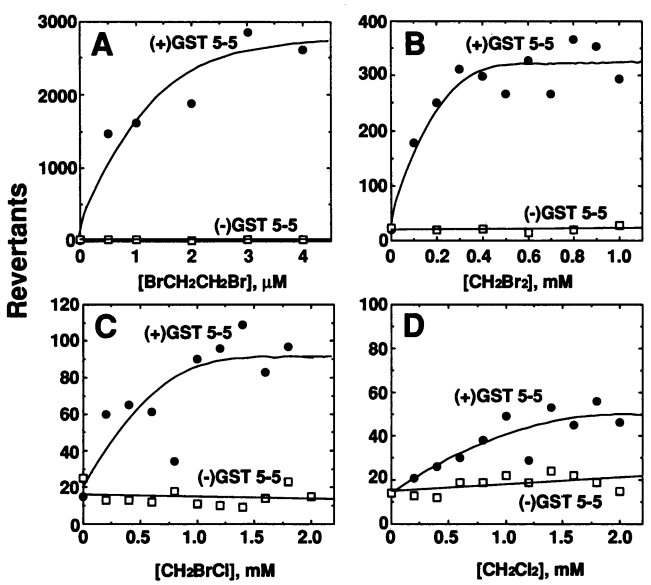

FIG. 3. Revertants produced in *S. typhimurium* TA1535 (+)GST 5-5 and (−)GST 5-5 with test chemicals. (*A*) Ethylene dibromide. (*B*) CH_2Br_2. (*C*) CH_2BrCl. (*D*) CH_2Cl_2.

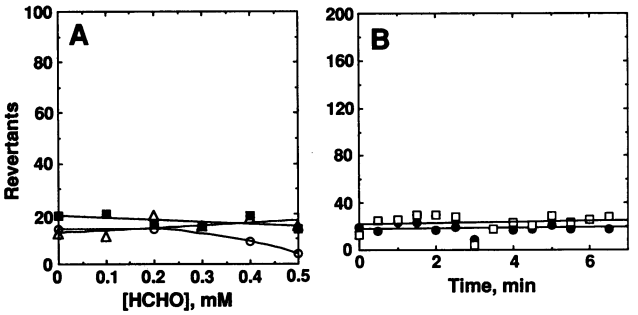

FIG. 4. Lack of revertants in *S. typhimurium* TA1535 (+)GST 5-5 in the presence of HCHO or *S*-(1-acetoxymethyl)GSH. (*A*) HCHO: ○, TA1535; ■, TA1535 (+)GST 5-5; △, TA1535 (−)GST 5-5. (*B*) Dry *S*-(1-acetoxymethyl)GSH was added to a final concentration of 0.2 mM directly to a cell mixture and plated at the indicated times after addition. In other experiments no revertants were seen when the level of *S*-(1-acetoxymethyl)GSH was 0.5 mM or when the compound was quickly dissolved in H_2O before addition. □, *S. typhimurium* TA1535; ●, TA1535 (+)GST 5-5.

(31). This compound was prepared in CH_3OH and was stable when kept dry. It resisted spectral characterization, but a 70% yield of HCHO was obtained upon hydrolysis, and the adducts formed in the reaction with dGuo (*vide infra*) argue for its identity. This compound did not produce revertants, even when added dry to a bacterial suspension to minimize decomposition before reaching the cells (Fig. 4*B*).

Products of Reaction of *S*-(1-acetoxymethyl)GSH with dGuo and dCyd. The production of revertants in *S. typhimurium* TA1535 is most consistent with a lesion at a guanine or cytosine residue (32, 33). Reaction of *S*-(1-acetoxymethyl)GSH with dGuo yielded a major product identified as *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH by its spectra: UV (H_2O)—λ_{max} (pH 1) 260 nm, (pH 7) 257 nm, (pH 13) 255 nm; fast atom bombardment (FAB)$^-$ MS *m/z* 585 (8, [M-H]$^-$), 607 (M+Na); FAB$^+$ MS *m/z* 587 (3, [M+H]$^+$) (Fig. 5). The high-resolution NMR spectra of the adduct are shown in Fig. 6. All signals could be identified as belonging to the dGuo or

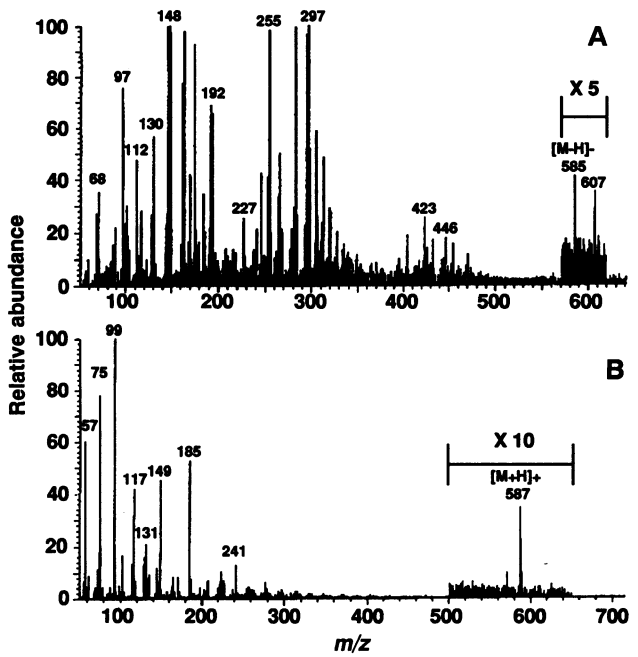

FIG. 5. Mass spectra of *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH. (*A*) FAB$^-$ MS [in glycerol/$(CH_3)_2SO$]. (*B*) FAB$^+$ MS (in glycerol/CF_3CO_2H/HCl). Spectra were recorded using a Kratos Concept IIHH instrument (Kratos Analytical Instruments).

the GSH moiety, on the basis of the literature (34–36), except the added methylene linking the dGuo and GSH. Attachment at the N2 atom was indicated by the lack of UV spectral properties associated with adducts formed at other sites on the guanine moiety and confirmed by the ^{15}N splitting of the -CH_2-protons [derived from the methylene moiety of *S*-(1-acetoxymethyl)GSH] and especially the ^{13}C signals in the product obtained when the reaction was done using [N^2-^{15}N]dGuo (Fig. 6). All assignments were confirmed with the use of two-dimensional ^1H-^{13}C correlation spectroscopy and are presented in the figure legend (data not shown).

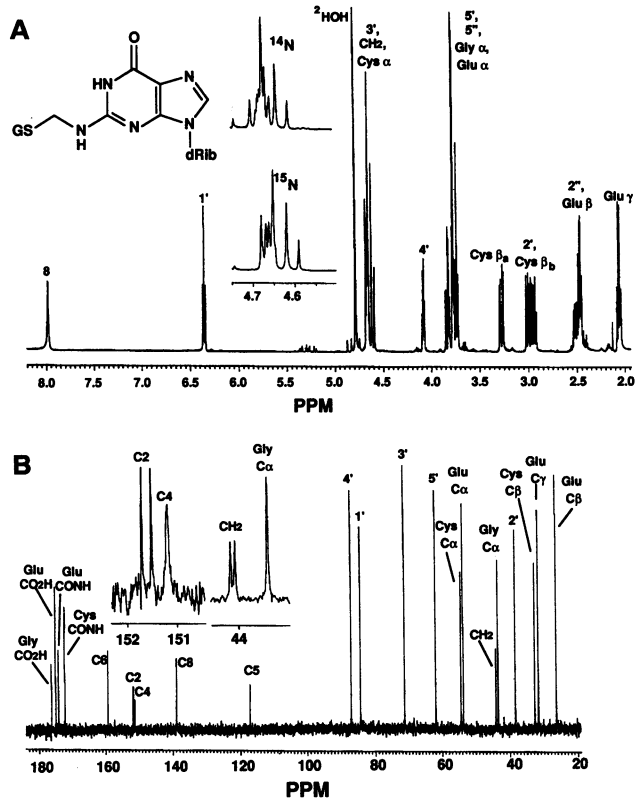

FIG. 6. (*A*) ^1H NMR spectra of *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH, recorded in ^2H_2O with a Bruker AMX-500 instrument (Billerica, MA). The full spectrum is shown for the adduct formed from [N^2-^{15}N]dGuo. (*Insets*) Assigned structure and the only portion of the spectra that differed between the ^{14}N- and ^{15}N-containing materials. Assignments of regions of the spectrum are indicated; specifically they are as follows: δ 2.057 (m, 2H, Glu β-CH_2-, $J_{\beta1,\beta2}$ = 14.2 Hz, $J_{\beta,\alpha}$ = 7.5 Hz), 2.47 (m, 2H, Glu γ-CH_2-), 2.51 (m, 1H, H-2'), 2.94 (m, 1H, H-2"), 2.997 (m, 1H, Cys $_{\beta1}$-CH_2-, $J_{\beta1,\beta2}$ = 14.3 Hz), 3.274 (m, 1H, Cys $_{\beta2}$-CH_2-, $J_{\beta2,\alpha}$ = 5.1 Hz), 3.730 (t, 1H, Glu α-H, $J_{\alpha,\beta}$ = 7.4 Hz), 3.746 (d, 1H, Gly α_1-H, $J_{\alpha1,\alpha2}$ = 18.4 Hz), 3.762 (m, 1H, H-5'), 3.788 (d, 1H, Gly α_2-H), 3.832 (m, 1H, H-5", J = 6.6 Hz, J = 12.5 Hz), 4.079 (m, 1H, H-4'), 4.605 (d, 1H, $CH_{2\,a}$, J = 14.3 Hz), 4.659 (m, 1H, H-3'), 4.666 (m, 1H, Cys α-H), 4.668 (d, 1H, $CH_{2\,b}$), 6.354 (dd, 1H, H-1', J = 6.8 Hz), 7.979 (s, 1H, H-8). (*B*) Natural abundance ^{13}C NMR spectrum of *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH, recorded in ^2H_2O (Bruker AMX-500). The spectrum was obtained with the adduct derived from [N^2-^{15}N]dGuo. (*Inset*) Expansion of regions where splitting of signals was seen (-CH_2- and C2 of the guanine). The indicated assignments were made on the basis of literature precedents (34–37) and two-dimensional proton correlation spectroscopy: 26.39 (Glu β), 31.67 (Glu γ), 32.74 (Cys β), 38.40 (2'), 43.45 (Gly α), 44.07 and 44.15 (CH_2, J_{CN} = 10.5 Hz), 53.66 (Glu α), 54.36 (Cys α), 61.91 (5'), 71.25 (3'), 84.35 (1'), 87.04 (4'), 117.1 (C5), 138.9 (C8), 151.2 (C4), 151.5 and 157.7 (C2, J_{CN} = 24.0 Hz), 159.2 (C6), 172.3 (Cys CONH), 174.1 (Glu CONH), 175.0 (Glu CO_2H), 176.2 (Gly CO_2H). In both *A* and *B*, CH_2 denotes the methylene linking the guanine and GSH moieties, the prime indicates deoxyribose signals, GSH atoms are indicated by the amino acid designations, and other atoms are in the guanine moiety.

The product of the dCyd reaction was not as stable; the structure *S*-[1-(N^4-deoxycytidyl)methyl]GSH is tentatively assigned: UV (H_2O)—λ_{max} (pH 1) 285 nm, (pH 7) 273 nm, (pH 13) 270 nm {similar to dCyd but not to N^3-methyldCyd and *S*-[2-(N^3-deoxycytidyl)ethyl]GSH at neutral pH (28)}. FAB$^{+/-}$ MS was unsuccessful. The ^1H NMR spectrum of a sample purified by HPLC (pH 5.5) and treated by repeated lyophilization showed the presence of both dCyd and GSH peaks: (^2H_2O) δ 2.14 (m, 2H, Glu β-CH_2), 2.36 (m, 2H, Glu γ-CH_2), 2.44 (m, 1H, H-2'), 2.53 (m, 1H, H-2"), 3.00 (m, 1H, Cys β_a-H), 3.23 (m, 1H, Cys β_b-H), 3.77 (m, 4H, Gly α-H, Glu α-H, H-5', and H-5"), 4.09 (H-4'), 4.80 (^{2}HOH, H-3' presumably obscured), 6.06 (d, 1H, H-5, $J_{5,6}$ = 7.3 Hz), 6.28 (t, 1H, H-1', $J_{1',2'}$ = 6.4 Hz), 7.83 (d, 1H, H-6, $J_{5,6}$ = 7.6 Hz). The spectrum also showed a peak at δ 8.45 that can be attributed to HCHO (s, 1H); analysis of the sample by HPLC after recording the NMR spectra showed that the sample had completely degraded to dCyd. However, the adduct clearly had a GSH moiety attached, as shown by the NMR, before its degradation. Subsequent work showed ≈70% degradation even after a single lyophilization step (pH 5.5).

DISCUSSION

The GSH conjugation of dihalomethanes has been clearly demonstrated to result in genotoxicity. This result cannot be attributed to the production of HCHO (Fig. 4*A*). An analog of the postulated reactive product has been used to derive a stable dGuo adduct, *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH, which was characterized here (Figs. 5 and 6).

Many activated chemicals readily diffuse into cells to react with DNA and produce their genotoxic effects. However, in this case it appears that the reactive product may not be stable enough to enter the bacteria and induce genotoxicity, as judged by the lack of revertants produced with externally added GSH *S*-transferase 5-5 or *S*-(1-acetoxymethyl)GSH. This situation is probably not unique, and it points out an advantage of expressing enzymes involved in biotransformation within the target cells used in analysis of genotoxicity. Although a number of the enzymes involved in such bioactivation reactions have been used in mammalian cells (38), the only previous report of the expression of a mammalian enzyme in a simple bacterial tester system appears to be the work of Grant *et al.* (39), who expressed human *N*-acetyltransferase in *S. typhimurium* TA98. Recent developments in the heterologous expression of cytochrome P450 enzymes in bacteria (40) suggest that it may be possible to develop other simple bacterial genotoxicity test systems containing specific enzymes involved in bioactivation and detoxication. Also, it should be possible to insert such constructs into other bacterial systems, such as *S. typhimurium* TA1535/pSK1002 and its derivatives, which utilize the induction of the *umu* response by many genotoxins and facilitate the rapid screening of chemicals (41).

The expression of GSH *S*-transferase 5-5 in *S. typhimurium* renders the bacteria sensitive to mutation with dihalomethanes but may be protective against mutations and genetic damage caused by other carcinogens. To date we have examined several other compounds for differential mutagenicity in the (+)- and (−)GST 5-5 *S. typhimurium* strains but found that the chemicals were either not very mutagenic at all in this system (CH_3Cl, CH_3I, styrene oxide) or that there was no difference between the strains (NaN_3, propylene oxide). However, the mutagenicity of the GSH *S*-transferase 5-5 substrate 1,2-epoxy-3-(4'-nitrophenoxy)propane (9) (Fig. 2*B*) was attenuated in the (+)GST 5-5 strain (data not shown). As with other enzymes involved in transformation of xenobiotic chemicals, the presence of GSH *S*-transferase 5-5 can be beneficial or detrimental, depending upon the situation encountered. These studies with GSH *S*-transferase 5-5 are of

particular interest in light of the recently demonstrated human polymorphism of a theta class GSH *S*-transferase, in which ≈30% of Caucasians are devoid of the enzyme (42). Further, Wiencke and his associates (43) have shown that sister-chromatid exchange is seen in blood lymphocytes of some individuals when these cells are exposed to 1,3-butadiene diepoxide; this polymorphism could be due to a theta class GSH *S*-transferase. A test system such as the bacteria described here should be very useful in evaluating the particular hazard that deficient individuals may encounter from various chemicals.

The demonstration of revertants in *S. typhimurium* TA1535 strongly suggests that lesions on guanine or cytosine are involved in mutations. Apparently the HCHO generated by hydrolysis of the initial reaction product cannot be responsible for this genotoxic action. The major adduct derived from the model *S*-(1-acetoxymethyl)GSH with dGuo was characterized as *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH. Interestingly, the similar N^2-guanyl *ethyl* adduct is a minor product in the reaction of an *S*-(2-haloethyl)GSH, the conjugate of ethylene dibromide (28). Although the N^7 adduct is dominant with the 2-haloethyl compounds, the proposed methylene species seems to prefer the exocyclic amino positions [as for adducts derived from HCHO (44)]. Further studies will be required to determine whether this adduct is formed in DNA from dihalomethanes *in vivo* and to evaluate its role in mutagenesis and more complex genotoxic events. Preliminary attempts to isolate *S*-[1-(N^2-guanyl)methyl]GSH from DNA were unsuccessful due to the product instability under the acidic conditions used. We have not seen evidence for an N^7-guanyl adduct of the type identified with ethylene dibromide (37) in our chemical studies thus far.

Finally, the point is made again that this work provides evidence that the genotoxic effects of dihalomethanes are attributable to GSH *S*-transferase-catalyzed conjugation. These experiments lend credence to schemes of relative risk estimation made on this premise (7). The results suggest that CH_2Cl_2 is orders of magnitude less genotoxic than CH_2Br_2 and ethylene dibromide (Fig. 3), at least in this system. The relevance of this model to mammalian situations may be assessed through similar transfection studies and searches for individual DNA adducts.

We thank G. Chenery and Drs. D.-H. Kim and K.-L. Park for participating in some of the preliminary studies. We also thank Prof. B. N. Ames for providing the *S. typhimurium* strains and for his pioneering work in the development of such systems for use in mutagenicity testing. This research was supported by U.S. Public Health Service Grants CA44353 and ES00267 (F.P.G.) and by the Cancer Research Campaign (S.E.P., J.B.T., B.K.). R.T. is the recipient of a fellowship from the Deutsche Forschungsgemeinschaft, and W.G.H. and M.P. are the recipients of U.S. Public Health Service Awards ES05473 and ES05592, respectively.

1. National Toxicology Program (1985) *NTP Technical Report on the Toxicology and Carcinogenesis Studies of Dichloromethane in F-344/N Rats and B6C3F1 Mice (Inhalation Studies)*, NTP-TR-306 (National Institute of Environmental Health Sciences, Research Triangle Park, NC).
2. Reich, M. S. (1993) *Chem. Eng. News* **71**, 10–13.
3. Burek, J. D., Nitschke, K. D., Bell, T. J., Wackerle, D. L., Childs, R. C., Beyer, J. D., Dittenber, D. A., Rampy, L. W. & McKenna, M. J. (1984) *Fundam. Appl. Toxicol.* **4**, 30–47.
4. Nitschke, K. D., Burek, J. D., Bell, T. J., Kociba, R. J., Rampy, L. W. & McKenna, M. J. (1988) *Fundam. Appl. Toxicol.* **11**, 48–59.
5. Kubic, V. L. & Anders, M. W. (1978) *Biochem. Pharmacol.* **27**, 2349–2355.
6. Ahmed, A. E. & Anders, M. W. (1976) *Drug Metab. Dispos.* **4**, 357–361.
7. Reitz, R. H., Mendrala, A. & Guengerich, F. P. (1989) *Toxicol. Appl. Pharmacol.* **97**, 230–246.
8. Guengerich, F. P., Kim, D.-H. & Iwasaki, M. (1991) *Chem. Res. Toxicol.* **4**, 168–179.
9. Meyer, D. J., Coles, B., Pemble, S. E., Gilmore, K. S., Fraser, G. M. & Ketterer, B. (1991) *Biochem. J.* **274**, 409–414.
10. Andersen, M. E., Clewell, H. J., III, Gargas, M. L., Smith, F. A. & Reitz, R. H. (1987) *Toxicol. Appl. Pharmacol.* **87**, 185–205.
11. Hanson, D. (1991) *Chem. Eng. News* **69**, 8.
12. Humphreys, W. G., Kim, D.-H., Cmarik, J. L., Shimada, T. & Guengerich, F. P. (1990) *Biochemistry* **29**, 10342–10350.
13. Jongen, W. M. F., Alink, G. M. & Koeman, J. H. (1978) *Mutat. Res.* **56**, 245–248.
14. van Bladeren, P. J., Breimer, D. D., Rotteveel-Smijs, G. M. T. & Mohn, G. R. (1980) *Mutat. Res.* **74**, 341–346.
15. Green, T. (1983) *Mutat. Res.* **118**, 277–288.
16. Osterman-Golkar, S., Hussain, S., Walles, S., Anderstam, B. & Sigvardsson, K. (1983) *Chem.-Biol. Interact.* **46**, 121–130.
17. La Roche, S. D. & Leisinger, T. (1990) *J. Bacteriol.* **172**, 164–171.
18. Summer, K. H., Göggelmann, W. & Greim, H. (1980) *Mutat. Res.* **70**, 269–278.
19. Gerster, J. F. & Robins, R. K. (1966) *J. Org. Chem.* **31**, 3258–3262.
20. Nash, T. (1953) *Biochem. J.* **55**, 416–421.
21. Pemble, S. E. & Taylor, J. B. (1992) *Biochem. J.* **287**, 957–963.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
23. Lederberg, E. M. & Cohen, S. N. (1974) *J. Bacteriol.* **119**, 1072–1074.
24. Maron, D. M. & Ames, B. N. (1983) *Mutat. Res.* **113**, 173–215.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY).
26. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
27. Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139.
28. Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203.
29. Cmarik, J. L., Humphreys, W. G., Bruner, K. L., Lloyd, R. S., Tibbetts, C. & Guengerich, F. P. (1992) *J. Biol. Chem.* **267**, 6672–6679.
30. Cmarik, J. L., Inskeep, P. B., Meyer, D. J., Meredith, M. J., Ketterer, B. & Guengerich, F. P. (1990) *Cancer Res.* **50**, 2747–2752.
31. Böhme, V. H., Fischer, H. & Frank, R. (1949) *J. Liebig's Annalen der Chemie* **563**, 54–72.
32. Carlomagno, M. S., Chiariotti, L., Alifano, P., Nappo, A. G. & Bruni, C. B. (1988) *J. Mol. Biol.* **203**, 585–606.
33. Eisenstadt, E., Miller, J. K., Kahng, L.-S. & Barnes, W. M. (1989) *Mutat. Res.* **210**, 113–125.
34. Jones, A. J., Grant, D. M., Winkley, M. W. & Robins, R. K. (1970) *J. Am. Chem. Soc.* **92**, 4079–4087.
35. Jung, G., Breitmaier, E. & Voelter, W. (1972) *Eur. J. Biochem.* **24**, 438–445.
36. Breitmaier, E. & Voelter, W. (1987) *Carbon-13 NMR Spectroscopy: High-Resolution Methods and Applications in Organic Chemistry and Biochemistry* (VCH, New York).
37. Koga, N., Inskeep, P. B., Harris, T. M. & Guengerich, F. P. (1986) *Biochemistry* **25**, 2192–2198.
38. Gonzalez, F. J., Crespi, C. L. & Gelboin, H. V. (1991) *Mutat. Res.* **247**, 113–127.
39. Grant, D. M., Josephy, P. D., Lord, H. L. & Morrison, L. D. (1992) *Cancer Res.* **52**, 3961–3964.
40. Barnes, H. J., Arlotto, M. P. & Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5597–5601.
41. Nakamura, S., Oda, Y., Shimada, T., Oki, I. & Sugimoto, K. (1987) *Mutat. Res.* **192**, 239–246.
42. Schröder, K. R., Hallier, E., Peter, H. & Bolt, H. M. (1992) *Biochem. Pharmacol.* **43**, 1671–1674.
43. Wiencke, J. K., Christiani, D. C. & Kelsey, K. T. (1991) *Mutat. Res.* **248**, 17–26.
44. Huang, H., Solomon, M. S. & Hopkins, P. B. (1992) *J. Am. Chem. Soc.* **114**, 9240–9241.