Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5266–5270, September 1983
Biochemistry

Evidence for formation of an *S*-[2-(N^7-guanyl)ethyl]glutathione adduct in glutathione-mediated binding of the carcinogen 1,2-dibromoethane to DNA

(bioactivation/DNA adducts/glutathione *S*-transferase/N^7-ethylguanine)

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Communicated by James A. Miller, May 25, 1983

ABSTRACT **The carcinogen 1,2-dibromoethane and reduced glutathione (GSH) were irreversibly bound to calf thymus DNA in equimolar amounts when *in vitro* incubations were carried out in the presence of GSH *S*-transferase. In studies carried out with isolated hepatocytes, equimolar amounts of 1,2-dibromoethane and endogenous GSH were also bound to intracellular DNA and RNA and extracellular DNA. These findings support the hypothesis that the major interaction of 1,2-dibromoethane with DNA involves covalent modification by a preformed complex of the carcinogen and GSH—i.e., *S*-(2-bromoethyl)GSH or the resulting episulfonium ion. Enzymatic hydrolysis of calf thymus DNA labeled with 1,2-dibromoethane in the presence of GSH and GSH *S*-transferase and subsequent high-performance liquid chromatography of the residues yielded a major fraction, which also was found to contain radiolabel derived from GSH. The fraction thus isolated was reductively desulfurized to yield N^7-ethylguanine, which was isolated and identified by comparison with authentic material in two other high-performance liquid chromatography systems and by UV and mass spectrometry. Therefore, the structure of the undesulfurized adduct is assigned as *S*-[2-(N^7-guanyl)ethyl]GSH. This adduct is unusual in that it is involved in a situation in which GSH plays a role in the bioactivation of a chemical carcinogen, as opposed to the more typical detoxication reactions. Further, a chemical carcinogen has been shown to cross-link DNA with a small physiological peptide.**

1,2-Dibromoethane is used as a soil, grain, and fruit fumigant, as an industrial solvent, and as a lead scavenger in gasoline. The annual production in the United States is on the order of 10^8 kg (1). 1,2-Dibromoethane has been found to be a potent mutagen in several systems and is capable of producing liver, lung, stomach, mammary, adrenal, spleen, skin, and kidney tumors (2–5).

1,2-Dibromoethane appears to require metabolic activation to elicit its mutagenic and carcinogenic properties. Two pathways can be considered for bioactivation. Mixed function oxidation produces a putative *gem*-halohydrin, which dehydrobrominates to form 2-bromoacetaldehyde (6). 2-Bromoacetaldehyde can react rapidly with reduced glutathione (GSH) or protein thiols and also can react with DNA, although the kinetics of DNA binding are rather slow (7). 1,2-Dibromoethane also can react directly with GSH, in a reaction catalyzed by GSH *S*-transferase, to form *S*-(2-bromoethyl)GSH (Fig. 1). This derivative then can form *S*-(2-hydroxyethyl)GSH or *S*,*S*′-ethylenebis(GSH) and derived sulfoxides (8), or can release ethylene (9). *S*-(2-Bromoethyl)GSH also may undergo an internal dehydrobromination to form a putative ethylGSH episulfonium (thiiranium) ion, which might react with DNA. Several studies indicate that both the oxidative and conjugative pathways are operative in rats and that the conjugative pathway is probably more closely related to DNA binding and mutagenesis (4, 10–13). Our previous studies on 1,2-dichloroethane activation also support this view (14).

Further chemical support is needed for the postulated GSH episulfonium pathway, as the episulfonium ion has not been directly demonstrated. If GSH-mediated binding of 1,2-dibromoethane occurs by the attack of nucleophilic moieties of DNA on methylene carbons of the GSH-ethylene episulfonium ion, then GSH should become bound to DNA through an ethylene bridge. Further, chemical characterization of the principal DNA adducts is necessary before subsequent studies can be appropriately done on their roles in tumor initiation, rates of repair, etc. We now provide evidence for the linkage of GSH to DNA by ethylene bridges during GSH-mediated bioactivation of 1,2-dibromoethane and assign the structure of the major adduct formed as *S*-[2-(N^7-guanyl)ethyl]GSH.

MATERIALS AND METHODS

Chemicals. GSH was purchased from Sigma and was used without further purification. 1,2-Dibromoethane, 1,2-dichloroethane, and 1-bromo-2-chloroethane were purchased from Aldrich and used without further purification. 1,2-Dibromo-[1,2-^{14}C]ethane, [^{35}S]GSH, [*glycine*-^{3}H]GSH, and [^{35}S]methionine were purchased from New England Nuclear.

Ethyldeoxyguanosine (EtdG) and ethylguanine (EtGua) derivatives were synthesized by methods described elsewhere (15). The UV spectra of N^1-EtdG, N^7-EtdG, O^6-EtdG, N^1-EtGua, N^2-EtGua, N^3-EtGua, N^7-EtGua, N^9-EtGua, O^6-EtGua, and N^7-ethyladenine (EtAde), which were recorded between 220 and 300 nm in H_2O, 0.1 M HCl, and 0.1 M KOH, were all consistent with the literature (15).

Enzymes and Cellular Preparations. Rat liver GSH *S*-transferase (a mixture of isozymes), DNase I, *Escherichia coli* alkaline phosphatase, and nuclease P1 were purchased from Sigma. GSH *S*-transferase was passed through a 1.2 × 50 cm Bio-Gel P-10 column (Bio-Rad) in 0.1 M potassium phosphate buffer (pH 7.7) at 4°C to remove residual GSH before use in metabolic incubations (14). When the GSH *S*-transferase preparation was subjected to polyacrylamide gel electrophoresis (10% acrylamide gel) in the presence of $NaDodSO_4$, densitometric measurements indicated that >98% of the staining was present in two bands migrating with apparent monomeric M_rs of 27,000 and 29,000, the accepted values of the Ya and Yc subunits (16).

Hepatocytes were isolated from male, 200-g Sprague–Daw-

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Abbreviations: GSH, (reduced) glutathione; EtdG, ethyldeoxyguanosine; EtGua, ethylguanine; EtAde, ethyladenine.

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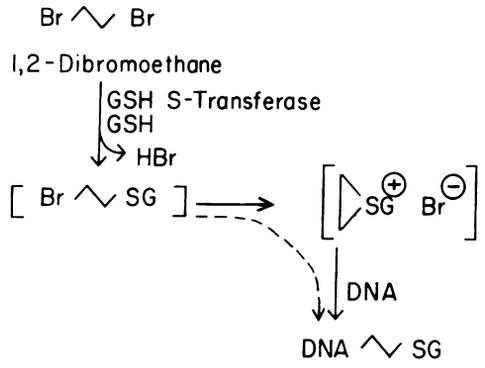

FIG. 1. Postulated pathway of activation of 1,2-dibromoethane by GSH *S*-transferase.

ley rats (Harlan Industries, Indianapolis, IN) with a collagenase perfusion procedure described elsewhere (17). The fraction of viable cells was estimated (by trypan blue exclusion) to be 89%.

RESULTS

Stoichiometry of 1,2-Dibromoethane and GSH Binding to DNA in the Presence of GSH *S*-Transferase. Previous work had established that irreversible binding of 1,2-dichloroethane (14) and 1,2-dibromoethane (11) to DNA occurs more readily in the presence of rat liver cytosolic fractions containing GSH than in NADPH-fortified microsomal fractions. The 1,2-dibromo[1,2-^{14}C]ethane binding data (Table 1) show that binding of the carcinogen to DNA was dependent upon the presence of both GSH and GSH *S*-transferase in the system used here. Incubation of radioactive GSH (^{35}S or ^{3}H labeled) with DNA yielded a finite level of radioactivity in the recovered DNA fraction. However, the level of radioactivity was increased when unlabeled 1,2-dibromoethane was added (in the presence of GSH *S*-transferase). The net binding of GSH was nearly equimolar with that of 1,2-dibromoethane when either form of labeled GSH was used. Bilirubin, an inhibitor of GSH *S*-transferase (19), inhibited the binding of 1,2-dibromo[1,2-^{14}C]ethane to DNA under these conditions by 25, 36, and 75% at concentrations of 12.5, 125, and 375 μM, respectively.

1-Bromo-2-chloroethane also increased the level of GSH label recovered in the DNA fraction, but only to the extent of one-fourth of that observed with 1,2-dibromoethane. 1,2-Dichloroethane did not significantly increase the level of [^{35}S]-GSH recovered in the DNA fraction under these conditions, although under prolonged conditions GSH-dependent binding of 1,2-dichloro[1,2-^{14}C]ethane has been shown (14).

Stoichiometry of 1,2-Dibromoethane and GSH Binding to Nucleic Acids in Isolated Hepatocytes. Isolated rat hepatocytes were incubated with [^{35}S]methionine, a precursor of GSH, for 3 hr to label intracellular GSH. The cells were washed and then incubated with 1,2-dibromoethane for an additional 2 hr, with calf thymus DNA added to the hepatocyte medium. In parallel experiments, portions of the hepatocyte preparation were incubated without labeled methionine and then with 1,2-dibromo[1,2-^{14}C]ethane under the same conditions. Analysis of the isolated nucleic acids indicated that approximately equimolar binding of the labels from GSH and 1,2-dibromoethane occurred with the intracellular RNA, intracellular DNA, and extracellular DNA (Table 2). The total binding of the 1,2-di-

Table 1. Binding of GSH and 1,2-dihaloethanes to DNA in the presence of GSH *S*-transferase

Additions to DNA	Labeled material bound to DNA, pmol/mg DNA	
	Total	Net
Experiment I		
GSH, transferase, $^{14}CH_2Br^{14}CH_2Br$	676 ± 17	656
GSH, $^{14}CH_2Br^{14}CH_2Br$	20 ± 1	10
Transferase, $^{14}CH_2Br^{14}CH_2Br$	10 ± 4	—
$^{14}CH_2Br^{14}CH_2Br$	11 ± 5	1
[^{35}S]GSH, transferase, CH_2BrCH_2Br	618 ± 45	556
[^{35}S]GSH, transferase, CH_2BrCH_2Cl	217 ± 19	155
[^{35}S]GSH, transferase	62 ± 4	—
[^{35}S]GSH	74 ± 14	12
Experiment II		
[*glycine*-^{3}H]GSH, transferase, CH_2BrCH_2Br	1,251 ± 47	804
[*glycine*-^{3}H]GSH, transferase, CH_2BrCH_2Cl	674 ± 66	227
[*glycine*-^{3}H]GSH, transferase	447 ± 8	—
[*glycine*-^{3}H]GSH	455 ± 3	8
GSH, transferase, $^{14}CH_2Br^{14}CH_2Br$	862 ± 9	849
Transferase, $^{14}CH_2Br^{14}CH_2Br$	13 ± 4	—
$^{14}CH_2Br^{14}CH_2Br$	15 ± 4	2

1,2-Dibromoethane or 1,2-dibromo[1,2-^{14}C]ethane (5 mM; 2.0 mCi/mmol; 1 Ci = 3.7×10^{10} Bq), GSH or [^{35}S]GSH or [*glycine*-^{3}H]GSH (2 mM; 1.0 mCi/mmol), and calf thymus DNA (2 mg/ml) were incubated in the presence of GSH *S*-transferase (0.2 mg/ml) in 0.1 M Tris·HCl buffer (pH 7.7) for 60 min at 37°C. $NaDodSO_4$ was added to 1% (wt/vol), and each sample was diluted 10-fold with 50 mM Tris·HCl buffer (pH 7.7). DNA was recovered as a pellet after centrifugation at 105,000 × *g* for 18 hr (25°C) and further purified (18). Except in the case of incubations that included [*glycine*-^{3}H]GSH, the DNA samples were also subjected to treatment with proteinase K. The recovered DNA was quantified by using A_{260} measurements ($E^{1\%}_{260}$ = 214 cm^{-1}), and radioactivity was determined with liquid scintillation spectrometry. Net binding involves subtraction of the value obtained with a component (unlabeled GSH or 1,2-dibromoethane) of the complete system absent. Results are presented as means ± SD of triplicate incubations.

Table 2. Binding of GSH and 1,2-dibromoethane to DNA and RNA in isolated hepatocytes

Source of label	Binding to nucleic acids		
	Extracellular DNA, nmol/mg DNA	Intracellular DNA, nmol/mg DNA	Intracellular RNA, nmol/mg RNA
$^{14}CH_2Br^{14}CH_2Br$	0.14 ± 0.04	4.45 ± 0.24	3.14 ± 0.21
[^{35}S]GSH	0.18 ± 0.01	3.96 ± 0.60	2.72 ± 0.10

Rat liver hepatocytes were isolated from the liver of a 200-g male Sprague–Dawley rat and divided into two batches [20 ml each in Joklik media (17)]. The first batch was incubated with 1 nmol (1 mCi) of L-[^{35}S]methionine for 3 hr at 37°C. GSH was separated from a trichloroacetic acid extract by TLC [Whatman K2 cellulose, *n*-butanol/CH_3CO_2H/H_2O, 4:1:2 (vol/vol); silica gel G, $CH_3CH_2CH_2OH$/28% aqueous NH_4OH, 7:3 (vol/vol)], and radioactivity was determined; GSH was estimated fluorimetrically (20). The specific activity of the GSH was determined to be 5.8 mCi/mmol. To confirm the level of radioactivity, an aliquot of the hepatocyte extract was allowed to react with excess acrylonitrile (21), and the resulting *S*-(2-cyanoethyl)GSH was separated in the silica TLC system used above and counted. The cells were resuspended (in the original volume) immediately, after washing briefly six times, in fresh media containing 0.5 mM 1,2-dibromoethane and 0.5 mg of calf thymus DNA per ml and were incubated for 2 hr at 37°C. The second batch of cells was incubated for 3 hr in the absence of labeled methionine, washed six times, and incubated in medium containing 0.5 mM 1,2-dibromo[1,2-^{14}C]ethane (5.0 mCi/mmol) and 0.5 mg of calf thymus DNA per ml for 2 hr at 37°C. The cells (which were >83% viable at this point) were precipitated by centrifugation. The extracellular DNA was isolated by using the procedures described under Table 1, and the DNA and RNA in the hepatocytes were separated as described elsewhere (18). Nucleic acids were estimated by A_{260} measurements, and radioactivity was estimated by liquid scintillation spectrometry. Results are presented as means ± SD of triplicate incubations carried out with portions of the hepatocyte preparations.

bromo[1,2-^{14}C]ethane label was calculated to consist of 10 nmol bound to intracellular RNA, 4 nmol bound to intracellular DNA, and 0.7 nmol bound to extracellular DNA under these conditions, when hepatocytes equivalent to 3 g of liver were used. In addition, 1.5 nmol were recovered as S,S-ethylenebis(GSH) and 9 nmol were recovered as S-(2-hydroxyethyl)GSH, as determined after separation of the adducts by TLC [silica gel G; *n*-butanol/CH_3CO_2H/H_2O, 4:1:2 (vol/vol); $CH_3CH_2CH_2OH$/28% aqueous NH_4OH, 7:3 (vol/vol)].

Identity of the GSH-1,2-Dibromoethane-DNA Adduct. The above experiments indicated that irreversible binding of 1,2-dibromoethane and GSH to DNA occurred in a stoichiometric manner, suggesting that GSH and a 1,2-dibromoethane metabolite were linked together in an adduct with DNA, as might be predicted in the episulfonium hypothesis (Fig. 1). We proceeded to determine the chemical identity of the major adducts that were formed.

Calf thymus DNA was labeled by incubation with GSH and 1,2-dibromo[1,2-^{14}C]ethane in the presence of GSH S-transferase, and the labeled DNA was enzymatically digested to deoxyribonucleosides. HPLC of the deoxyribonucleosides yielded radiochromatograms such as shown in Fig. 2, with the radioactivity recovered in four peaks. The first peak was eluted in the void volume and subsequently was found to consist of S-(2-hydroxyethyl)GSH and S,S-ethylenebis(GSH) by means of TLC (see above). Two small peaks of variable intensity were eluted at 4 and 8 min. The last peak, which appears to be the major adduct, was eluted at nearly the same retention time as deoxyguanosine. The same radioactive peaks were found when the DNA was labeled with [^{35}S]GSH (in the presence of unlabeled 1,2-dibromoethane), with relatively more label recovered in the void volume fraction. The material (unlabeled deoxyguanosine and the radioactive adduct) present in the major peak was treated with nickel and $NaBH_4$ to reduce the putative thioether bond of the adduct. Rechromatography of this material (recovered in 30% yield) yielded a single major peak, which migrated with the same retention time as N^7-EtGua (Fig. 2).

The sample obtained after desulfurization and HPLC was rechromatographed in a second HPLC system involving ion-paired reverse-phase chromatography, and the radioactivity migrated in the same position as authentic N^7-EtGua (Fig. 3). In other experiments, which are not presented, the radioactivity of the desulfurized sample and authentic N^7-EtGua comigrated (retention time, 3.3 min) in another HPLC system, which involved a Whatman Partisil SCX column and a 0.005 M NH_4OH (pH 9.7) mobile phase.

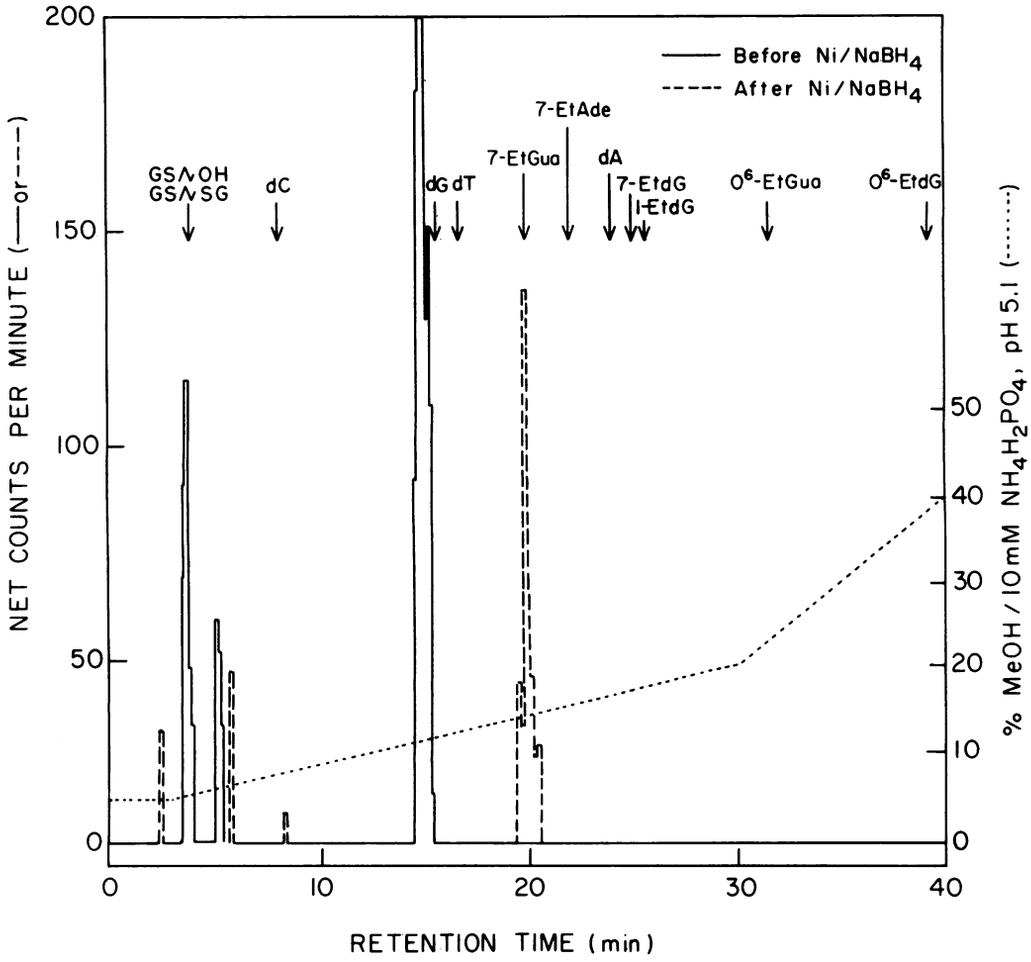

FIG. 2. HPLC profiles of 1,2-dibromo[1,2-^{14}C]ethane-DNA adducts after enzymatic digestion. Calf thymus DNA (2 mg/ml) was mixed with 5 mM 1,2-dibromo[1,2-^{14}C]ethane (1.0 mCi/mmol) in the presence of 10 mM GSH and 0.2 mg of GSH transferase per ml in 150 ml of 0.1 M Tris·HCl buffer (pH 7.7) for 2 hr at 37°C. The DNA was precipitated by centrifugation in $NaDodSO_4$ (Table 1) and hydrolyzed by sequential treatment with DNase I, nuclease P1, and *Escherichia coli* alkaline phosphatase (22). The residue was chromatographed on a 1 × 25 cm Altex-Ultrasphere ODS column by using an increasing CH_3OH gradient in 10 mM ammonium phosphate buffer (pH 5.1). The flow rate was 4.0 ml min^{-1}. —, The radioactivity profile monitored with a flow liquid scintillation counter in line with the UV detector. The large peak, which eluted at 15 min in this system, was collected and desulfurized with nickel and $NaBH_4$ (23). ----, Radioactivity profile upon rechromatography in the original system. Arrows denote the elution positions of standard compounds {*S*-(2-hydroxyethyl)GSH, GS ∧ OH; *S,S'*-ethylenebis(GSH), GS ∧ SG}.

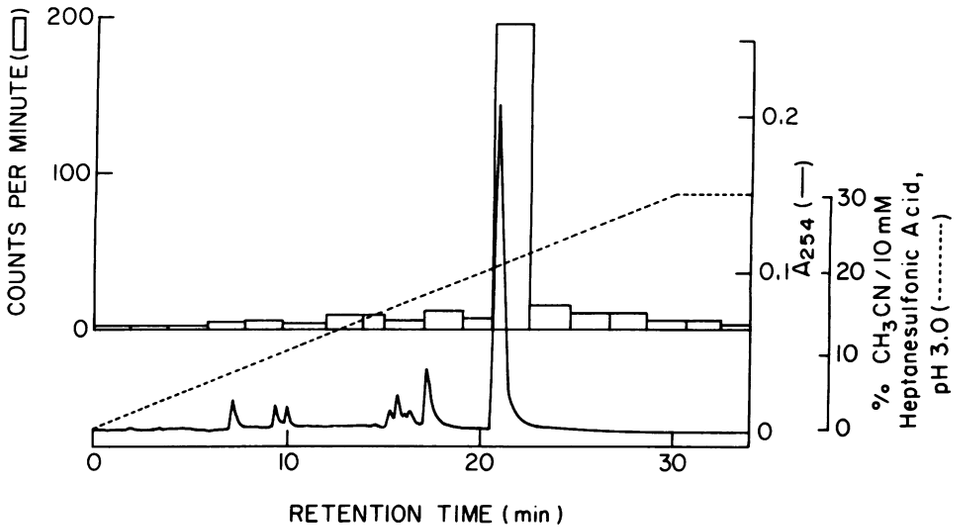

FIG. 3. HPLC of the desulfurized 1,2-dibromoethane-GSH-DNA adduct and N^7-EtGua. The adduct, prepared by desulfurization of the major adduct shown in Fig. 2, was chromatographed on the same column with an ion-paired system consisting of a CH_3CN gradient in 10 mM heptanesulfonic acid (pH 3.0). The radioactivity profile is shown in the upper trace for the ^{14}C-labeled adduct (fractions were collected from the column, and radioactivity was estimated by liquid scintillation spectrometry). The lower trace shows the A_{254} profile of a sample of authentic N^7-EtGua in the same chromatographic system.

UV spectroscopy of the derivative formed by desulfurization yielded spectra that were very similar to those of authentic N^7-EtGua under neutral, acidic, and alkaline conditions (Fig. 4). The spectra were clearly distinguished from those of N^1-EtGua, O^6-EtGua, N^2-EtGua, N^3-EtGua, and N^9-EtGua obtained under identical conditions (15).

Electron-impact mass spectrometry of the desulfurized adducts yielded a spectrum that was nearly identical to that obtained with authentic N^7-EtGua (Fig. 5). The material was clearly distinguished from N^1-EtGua, N^9-EtGua, and O^6-EtGua (all of which lacked a significant m/z 164 peak) and N^2-EtGua (which had a strong m/z 164 peak but showed a base peak at m/z 135), which were analyzed under identical conditions at the same time.

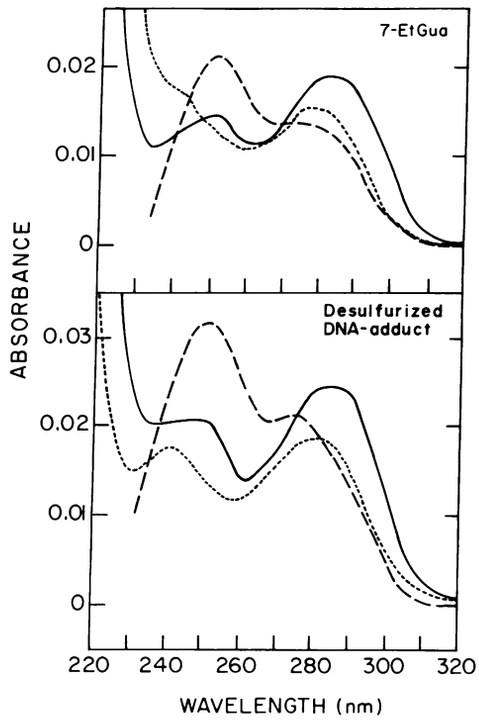

FIG. 4. UV spectra of the desulfurized 1,2-dibromoethane-GSH-DNA adduct (*Lower*) and authentic N^7-EtGua (*Upper*). Both materials were purified by using the HPLC techniques described in Fig. 2. The collected fractions were taken to dryness under N_2 and dissolved in H_2O (----), 0.1 M HCl (·····), or 0.1 M KOH (—) before spectra were recorded with a Cary 219 spectrophotometer. The concentration of authentic N^7-EtGua was 17 μM.

DISCUSSION

The major DNA adduct formed with 1,2-dibromoethane in the presence of GSH was assigned the structure *S*-[2-(N^7-guanyl)ethyl]GSH (Fig. 6). Incubation of DNA with GSH and 1,2-dibromoethane yielded equal amounts of radioactivity from the two chemicals irreversibly bound to DNA. Enzymatic hydrolysis of DNA modified with GSH and 1,2-dibromo[1,2-^{14}C]-ethane yielded a major peak upon HPLC, and the same radioactive peak could be derived from [^{35}S]GSH. Although attempts to completely purify the intact molecule by several HPLC

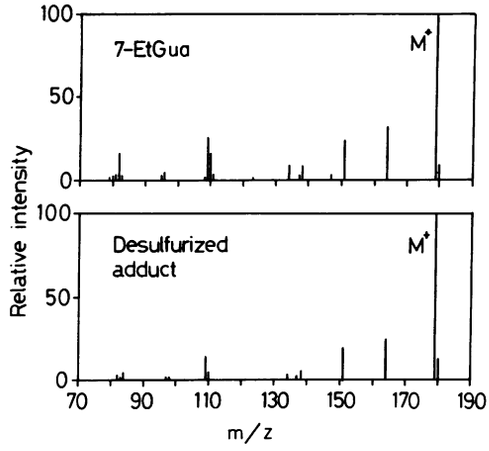

FIG. 5. Mass spectra of the desulfurized 1,2-dibromoethane-GSH-DNA adduct (*Lower*) and authentic N^7-EtGua (*Upper*). Spectra were obtained by using a Ribermag R-10-10 instrument in the electron-impact mode (70 eV) with a solid probe. The desulfurized 1,2-dibromoethane-GSH-DNA adduct was prepared and purified as described in Fig. 2. Spectra were also obtained for authentic N^7-EtGua. The spectra of O^6-EtGua, N^1-EtGua, N^2-EtGua, and N^9-EtGua (not shown) were also obtained and did not match the spectra shown.

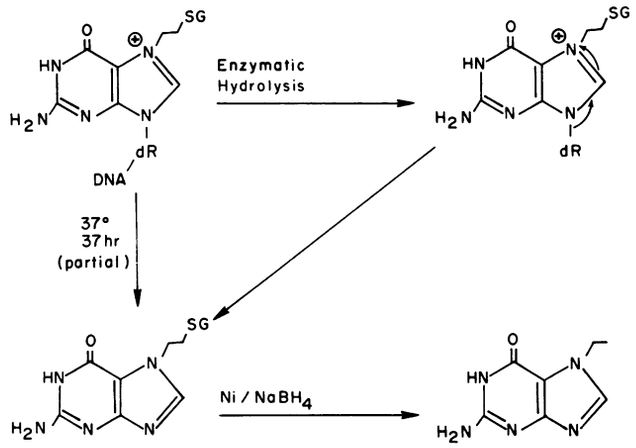

FIG. 6. Degradation of the putative 1,2-dibromoethane-GSH-DNA adduct. The DNA-bound adduct is postulated to be enzymatically degraded to the N^7-substituted deoxyguanosine derivative which, because of the charge at N^7, spontaneously deglycosylates to form S-[2-(N^7-guanyl)ethyl]GSH. The derivatized DNA also slowly releases this compound nonenzymatically. S-[2-(N^7-guanyl)ethyl]GSH can be reductively desulfurized to form N^7-EtGua.

methods for direct analysis have not been successful to date, reductive desulfurization of partially purified material and further chromatography yielded N^7-EtGua, identified by cochromatography in three HPLC systems, UV spectroscopy in acidic, basic, and neutral media, and electron-impact mass spectrometry. Unless an unusual rearrangement occurred during the analysis, the only logical assignment of the structure of the adduct is S-[2-(N^7-guanyl)ethyl]GSH. Apparently deglycosylation of the deoxyribose moiety of the nucleoside (Fig. 6) occurs readily because of the positive charge at N^7, as reported in other cases (24). The undigested DNA was also found to slowly release S-[2-(N^7-guanyl)ethyl]GSH (identified by HPLC) upon standing in buffer at 37°C (20% of the adduct was released after 37 hr).

This type of adduct is unusual in that GSH appears to play a role in the bioactivation of 1,2-dibromoethane. In general, GSH participates in the detoxication of chemical carcinogens and other toxic materials (19). The adduct is also unusual in the sense that it involves the crosslinking of a physiological oligopeptide to DNA by a chemical carcinogen.

The adduct we have characterized here would appear to be the major one involved in the reaction of 1,2-dibromoethane with DNA. 1,2-Dihaloethanes are metabolized to products that bind to DNA (and are mutagenic) much more rapidly by GSH-containing cytosolic liver fractions than by NADPH-fortified microsomal fractions (11, 14, 25). The oxidative metabolism of 1,2-dibromoethane yields 2-bromoacetaldehyde (6). This α-halocarbonyl compound binds rapidly to protein but the kinetics of binding to DNA are very slow (7). When 1,2-dibromoethane was incubated with isolated rat hepatocytes, nearly equivalent levels of GSH and 1,2-dibromoethane were bound to DNA and RNA. If GSH-independent pathways were involved to a large degree, the ratio should have been quite different.

When binding of 1,2-dibromoethane to DNA located outside of hepatocytes was examined, we found that a maximum of 5% of the putative S-(2-bromoethyl)-GSH could have migrated through the plasma membrane. This percentage (which could be attributed to broken cells) is lower than that observed in the case of several carcinogens that are oxidatively bioactivated (7, 17, 26, 27), presumably due to differences in reactivity of the ultimate electrophiles. The fraction (60%) of 1,2-dibromo[1,2-^{14}C]ethane metabolites that became irreversibly attached to nucleic acids in the hepatocytes was very high (see above). Further studies will be required to determine whether the N^7-guanyl nitrogen attacks S-(2-bromoethyl)GSH in a concerted manner (S_N2 mechanism) or reacts with an intermediate episulfonium ion (S_N1 mechanism) (Fig. 1) (28). The latter possibility seems more likely because of the enhanced electrophilicity of the methylene carbons.

Finally, the fate of the S-[2-(N^7-guanyl)ethyl]GSH is a matter of speculation at present. Cleavage of the glycosidic bond in DNA, which occurs spontaneously at a low rate (Fig. 6), would produce an apurinic site, which also could cause distorted reading. Enzymatic repair is another possibility. After S-[2-(N^7-guanyl)ethyl]GSH groups are released, the possibility exists that they may be degraded to 2-(N^7-guanyl)ethylmercapturic acid derivatives, which may be excreted in urine and possibly serve as markers of damage.

We thank A. Slaughter for the mass spectral analyses. This research was supported in part by U.S. Public Health Service Grants ES 01590 and ES 00267. F.P.G. is the recipient of U.S. Public Health Service Research Career Development Award ES 00041 and is a Burroughs Wellcome Scholar in Toxicology.

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