ABSTRACT

The major DNA adduct formed from the carcinogen ethylene dibromide (1,2-dibromoethane, EDB) is S-[2-(N'-guanyl)ethyl]glutathione, resulting from the reaction of guanyl residues with the half-mustard S-[2-bromomethyl]glutathione, which is generated by glutathione S-transferase-catalyzed conjugation of EDB with glutathione. The half-life of the alkylating species [putative S-(2-bromomethyl)]glutathione or the derived episulfonium ion] was estimated to be <10 s. However, the stability was enough for approximately half of the alkylating metabolites to leave isolated rat hepatocytes before reacting with nucleic acids. Treatment of isolated rat hepatocytes with diethylmaleate decreased covalent binding of EDB to DNA, but treatment with 1-phenylimidazole did not, consistent with the view that conjugative metabolism is of greater importance than oxidation with regard to DNA binding. When EDB was administered to experimental animals, only one major adduct, S-[2-(N'-guanyl)ethyl]glutathione, was formed in liver or kidney. S-[2-(N'-guanyl)ethyl]glutathione was found in liver and kidney DNA of rats treated with 1,2-dichloroethane, but other adducts were also present. The β-galutathionyl transeptidase inhibitor AT-125 [L-(α-SSS)-α-amino-S-chloro-4,5-dihydro-5-isoxazoleacetic acid] did not affect the level of EDB bound to DNA by glutathione-fortified rat kidney homogenates or bound to liver or kidney DNA in vivo. The in vitro half-life of S-[2-(N'-guanyl)ethyl]glutathione in calf thymus DNA was 150 h; the half-life of the adduct in rat liver, kidney, stomach, and lung was between 70 and 100 h. Isolated S-[2-(N'-guanyl)ethyl]glutathione did not react with DNA to form new adducts. These results provide a further basis for understanding the carcinogenic action of 1,2-dihaloethanes.

INTRODUCTION

EDB is a mutagen (1–4) and carcinogen (1, 2, 4, 5) in experimental animals. Few reports have dealt with the potential carcinogenicity of EDB in humans. Two epidemiology studies on incipient liver cancer exposed to relative high levels of EDB were inconclusive (6, 7). EDB is also acutely toxic, and deaths due to ethylene dichloride also bind to DNA to give S-[2-(N'-guanyl)ethyl]glutathione, and show that, once formed, S-[2-(N'-guanyl)ethyl]glutathione does not react further with DNA.

MATERIALS AND METHODS

Chemicals. Calf thymus DNA and hydroxyapatite were purchased from Calbiochem-Behring (San Diego, CA). Rat liver GSH S-transferase (EC 2.5.1.18), GSH, and collagenase (type 4) were purchased from Sigma (St. Louis, MO). [1,2-14C]EDB was obtained from Amersham-Searle (Arlington Heights, IL). [1,2-14C]EDC was obtained from Pathfinder Laboratories (St. Louis, MO). Fischer’s medium for leukemic mouse and calf serum were obtained from Grand Island Biological Co. (Grand Island, NY). AT-125 was a gift of the Upjohn Co. (Kalamazoo, MI). All other reagents used were of the highest quality commercially available.

Tissue Sources. Male Sprague-Dawley rats (200–250 g) were purchased from Harlan Industries (Indianapolis, IN) and housed under conventional procedures without further treatment before experiments or use as enzyme sources. Hepatocytes were isolated using procedures described elsewhere (11).

Isolation of DNA. Following in vitro incubations with calf thymus DNA, DNA was purified by centrifugation in buffer containing 1% (w/v) sodium dodecyl sulfate and subsequent hydroxyapatite chromatography as described previously (12).

In the in vitro work, tissues were washed, suspended in 8–10 volumes (w/v) 4-aminosalicylate (pH 7.0), minced with scissors, and homogenized in a glass-Teflon homogenizer for 20–30 s at a low speed to prevent excessive foaming. The homogenate was extracted 3 times with an equal volume of a mixture of chloroform:isoamyl alcohol:phenol (24:1:25, v/v/v). The samples were then washed 3 times with equal volumes of ethyl acetate and twice with ether. DNA was precipitated by the addition of 2.5 volumes of cold (−20°C) ethanol. The samples were applied to 1.5-g hydroxyapatite columns, and the DNA was eluted as described previously (12).

DNA from rat hepatocytes was isolated in the manner described for rat tissues, after extracellular radioactivity was removed from the cell suspensions by centrifugation (10' × g, 10 min). In appropriate experiments, extracellular DNA was precipitated with 2 volumes of cold ethanol, dissolved in buffer, and purified by hydroxyapatite chromatography as described above.

Isolation of DNA Adducts. DNA adducts were released by a neutral thermal hydrolysis procedure (15). The DNA-containing fraction recovered after hydroxyapatite chromatography (in 300 mM sodium phosphate buffer, pH 6.8) was heated at 95°C for 30 min. During this treatment, which favors release of N'-guanyl adducts, >97% of EDB-derivatively associated with the DNA was rendered ethanol soluble. After the samples were cooled, 2.5 volumes of ethanol were added to precipitate apurinic/apyrimidinic acid and phosphate. The supernatants were concentrated in vacuo (23°C) and dissolved in the initial HPLC mobile phase for further purification. The HPLC used in this work utilized the reverse-phase octadecylsilyl procedure described elsewhere (14).

Determination of Chemical and Biological Half-Lives of EDB-DNA Adducts. To determine the chemical stability of the EDB-DNA adduct, calf thymus DNA was incubated as previously described (12) with 5 mM [1,2-14C]EDB (5 mCi/mmol), 2 mM GSH, and GSH S-transferase (0.2 mg/ml) for 120 min at 37°C. The labeled DNA was isolated as purified GSH S-transferase and in incubations of rat hepatocytes with EDB (11, 12).

In this paper we report on the chemical stability and biological half-life of S-[2-(N'-guanyl)ethyl]glutathione, provide evidence that the EDB-GSH conjugate can leave the cell in which it is formed and bind to extracellular DNA, show that ethylene dichloride also binds to DNA to give S-[2-(N'-guanyl)ethyl] glutathione, and show that, once formed, S-[2-(N'-guanyl)ethyl] glutathione does not react further with DNA.

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5 Adducts were isolated from EDB, ethylene dibromide (1,2-dibromoethane); EDC, ethylene dichloride (1,2-dichloroethane); GSH, glutathione; DEM, diethylmaleate; HPLC, high performance liquid chromatography; AT-125, L-(α-SSS)-α-amino-S-chloro-4,5-dihydro-5-isoxazoleacetic acid (Acivicin).
1,2-DIHALOALKANE/DNA ADDUCTS

![Diagram]

Fig. 1. Scheme showing formation of the major DNA adduct from EDB and EDC and the breakdown of the adduct.

Described above and incubated in 50 mM sodium phosphate buffer containing 0.02% (w/v) NaN₃ at 37°C. Separate incubations were carried out at pH 6.0 and pH 7.0. Aliquots were removed at various times, and the DNA was isolated by hydroxylapatite chromatography. After determination of the amount of radioactivity bound to the DNA, the remainder of the DNA fractions were hydrolyzed to obtain adducts. These adducts were isolated by HPLC (both ion-exchange and reverse phase) as described elsewhere (14).

To determine the half-life of the DNA adduct in vivo, rats were given injections i.p. of 37 mg of EDB (5 mCi/mmol) per kg of body weight (in 0.4 ml of dimethyl sulfoxide). Animals were killed at various intervals; the livers, kidneys, lungs, and stomachs were removed; and DNA was isolated from these tissues as described. For the livers, separate tissue samples were prepared for HPLC isolation of the adduct as described (14).

Reaction of S-[2-(N'-guanylyl)ethyl]glutathione with Calf Thymus DNA. In experiments designed to determine if S-[2-(N'-guanylyl)ethyl]glutathione can dissociate and form new DNA adducts, 10 mg of calf thymus DNA were incubated with 3 μM S-[2-(N'-guanylyl)1,2-14C]ethylglutathione (0.11 mCi/mmol) for 1 h or 1 μM S-[N'-guanylyl]1,2-14C]ethylglutathione (0.64 mCi/mmol) for 3 h in 20 ml of 50 mM Tris-HCl buffer (pH 7.35) at 37°C with shaking. DNA was isolated from the incubations as described above.

RESULTS

Stability of Alkylating Species. In order to gain some insight into the stability and reactivity of the DNA-alkylating species formed from EDB, we set up an experiment in which a steady-state concentration of alkylating agent would be produced, with the idea that further production of the active metabolite could be quenched. Triphenyltin chloride has been reported to be an effective inhibitor of other GSH S-transferase reactions (16), and we found that a 1 mM concentration of this compound also serves as an effective inhibitor of other GSH S-transferase reactions (16), and we found that a 1 mM concentration of this compound also blocked the formation of DNA adducts from EDB (Fig. 2A). This concentration of inhibitor was added to an incubation mixture containing [1,2-14C]EDB, GSH, and GSH S-transferase to block further production of alkylating species. Aliquots of the mixture were then mixed with DNA (for 60 min); the formation of DNA adducts was measured and compared to the level formed when the original incubation mixture had been mixed with DNA immediately prior to the addition of triphenyltin chloride (Fig. 2B). At even the first time point measured (30 s), no more than 10% of the control level of DNA binding was detected. Thus, we estimate that the half-life of the alkylating species under these conditions is <10 s.

Activation of EDB and Migration of Reactive Metabolites in Isolated Hepatocytes. In our earlier work (11), we incubated EDB with rat hepatocytes in medium containing calf thymus DNA and measured the formation of extracellular DNA adducts. At that time we concluded that alkylating species were not readily able to leave the hepatocytes. This apparent result was surprising, since many other reactive metabolites are extruded from hepatocytes in such experiments (for review, see Ref. 17). We repeated these experiments and again found that the relative levels of adducts formed with intracellular DNA and RNA were greater than with the extracellular DNA (Table 1). However, when the total nucleic acid adducts are considered, about one-half was found outside the cells, consistent with the view that the stability of the alkylating species is great enough for it to leave these cells.

In confirmation of the work of Sundheimer et al. (10), we found that the addition of 0.19 mM DEM to isolated rat hepatocytes decreased intracellular binding of EDB to 59% of the control value (2-h incubation). In the presence of 0.50 mM...
considered (19). S-[2-(N'-guanyl)ethyl]glutathione was the only major adduct formed in vivo in rat liver (1.3 nmol/mg of DNA) or kidney (0.95 nmol/mg of DNA) 8 h after i.p. administration of a single dose of EDB (Fig. 3) (>97% of the bound radioactivity was released from the DNA by neutral thermal hydrolysis).

When rats were administered a single dose of [1,2-14C]EDB, DNA adducts in vivo and the liver was analyzed 8 h later, 78% of the DNA adducts (nominal 0.25 nmol/mg of DNA) could be released by the neutral thermal hydrolysis method used for EDB-DNA adducts. HPLC analysis (Fig. 4A) showed the presence of a single major peak (c), although minor peaks were also present (a and b). Kidney DNA was also labeled in the rats treated with [1,2-14C]EDC (nominal 0.35 nmol/mg of DNA). Only 57% of the bound radioactivity was released by neutral thermal hydrolysis, and HPLC indicated that the released radioactivity was distributed among roughly 5 different fractions (Fig. 4B, a to e), only one of which (c) appeared to be S-[2-(N'-guanyl)ethyl]glutathione. In both the liver and kidney chromatograms, Peak b appeared to correspond in retention time to the product of covalent processing on the reactivity of S-(2-bromoethyl)-

Role of γ-glutamyl Transpeptidase in Formation of DNA Adducts. In order to further address the possible role of enzymatic processing on the reactivity of S-(2-bromoethyl)glutathione, we examined covalent binding of [1,2-14C]EDB to calf thymus DNA in the presence of GSH and rat kidney homogenates, which contain γ-glutamyl transpeptidase and other enzymes that can further process GSH conjugates. The addition of AT-125 inhibited γ-glutamyl transpeptidase activity (measured using GSH) but had no effect on the level of formation of DNA adducts [S-(2-(N'-guanyl)ethyl]glutathione or any other adducts (Fig. 5A).

Rats were treated with i.v. doses of 0–1.5 mg of AT-125 (per rat) and then, after 1 h, with [1,2-14C]EDB (i.p., 37 mg/kg) for 4 h. The level of kidney homogenate γ-glutamyl transpeptidase activity varied in the treated rats, but the formation of kidney DNA adducts did not appear to be related to the level of γ-glutamyl transpeptidase activity (Fig. 5B). Administration of 1.5 mg of AT-125 (i.v.) per rat, which almost completely abolished γ-glutamyl transpeptidase activity in these experiments, did not affect the formation of hepatic EDB-DNA adducts in similar experiments [1.82 ± 0.27 nmol of adducts/mg of DNA in untreated rats (n = 3); 1.58 ± 0.03 nmol of DNA adducts/mg of DNA (n = 3) in AT-125-treated rats].

Stability of DNA Adducts in Vivo and in Vitro. Calf thymus DNA containing adducts formed from [1,2-14C]EDB in vitro was incubated at 37°C in phosphate buffer at pH 6.0 or pH 7.0. Radioactivity bound to the DNA decreased with a half-life of 1.50 h (Fig. 6). In addition, the radioactivity recovered in the ethanolic supernatant fractions coeluted with S-[2-(N'-guanyl)ethyl]glutathione in reverse-phase HPLC (14).

Radiolabeled S-[2-(N'-guanyl)ethyl]glutathione was incubated with calf thymus DNA, and the DNA was reisolated. The radioactivity measured in the DNA-containing fractions eluted from the hydroxylapatite columns was not statistically greater than in background fractions recovered from the column. The limit of detection of formation of new DNA adducts in these experiments was 3 fmol per mg of DNA, corresponding to <0.3% of the S-[2-(N'-guanyl)ethyl]glutathione bound to DNA in 3 h under the conditions used.

DNA was isolated from the livers, kidneys, lungs, and stomachs of rats given injections of [1,2-14C]EDB and [1,2-14C]EDC (2841 mg/kg). Half-lives (Fig. 7) were similar for all tissues (70–100 h), although initial binding of radioactivity to DNA of liver and kidney was considerably higher than the binding of lung and stomach. For all tissues, the adduct half-lives were much shorter than the in vitro half-life measured with calf thymus DNA.
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Fig. 5. Lack of effect of AT-125 on formation of DNA adducts from EDB. In A, in vitro: rat kidney homogenates (made with 4 volumes of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1 M KCl per g tissue) were incubated with 5 mM GSH, 2 mM [1,2-14C]EDB, and calf thymus DNA (1 mg/ml) for 60 min in the presence of the indicated levels of AT-125. In each case the rate of disappearance of GSH had been determined by HPLC (20) and, in this experiment, GSH was added every 5 min to maintain the level of GSH at 5 mM. DNA was isolated by hydroxylapatite chromatography, and DNA and bound radioactivity were determined. γ-Glutamyl transpeptidase activity was determined using the method of Meister et al. (19) (activity expressed in those units). In B, in vivo: rats were given injections i.v. of 0–1.5 mg of AT-125. Sixty min later, [1,2-14C]EDB was administered (i.p., 37 mg/kg in 0.4 ml dimethyl sulfoxide), and the rats were killed 4 h later. Kidney DNA was isolated, and DNA and bound radioactivity were estimated. γ-Glutamyl transpeptidase activity was also estimated in kidney homogenates using the method of Meister et al. (19).

Fig. 6. Chemical stability of S-[2-(N'-guanyl)ethyl]glutathione in calf thymus DNA during incubation at 37°C in 50 mM sodium phosphate buffer (•, pH 7.0; ■, pH 6.0). The half-life of radioactivity bound to DNA was 150 h.

After neutral thermal hydrolysis and HPLC of the DNA isolated from the livers (all time points), >95% of the radioactivity was recovered in the fraction corresponding to S-[2-(N'-guanyl)ethyl]glutathione (cf. Fig. 3), and the data obtained from total DNA adduct and S-[2-(N'-guany|)ethyl]glutathione measurements were superimposable (Fig. 8).

DISCUSSION

While the toxicity and carcinogenicity of 1,2-dihaloalkanes have been recognized for a number of years, the mode of action has been seriously addressed only recently. While an observation that EDB did not induce γ-glutamyl transpeptidase-positive foci has been interpreted to mean that the oncogenic activity is more related to promotion than initiation (21), several other lines of investigation show a high potential for DNA damage and mutagenesis (1–5, 22). α,ω-Dihaloalkanes are capable of directly producing DNA strand breaks when used at high concentrations (23), but the bulk of evidence indicates that bioac-
tivation of EDB is necessary for these effects on mammalian DNA.

Early work had suggested that GSH was involved in the overall metabolism of EDB to urinary metabolites (24). EDB is metabolized by initial hydroxylation or conjugation (of EDB) with GSH; the ratio of the two pathways in rats is about 4:1 (25). Rannug (2) first reported that mutagenic compounds could be generated from the reaction of GSH with 1,2-dihaloalkanes. While some DNA damage can be produced via the oxidative pathway under in vitro conditions (26—28), a number of lines of investigation indicate that the GSH-conjugation pathway is more important than the hydroxylation pathway in causing DNA damage (2, 10, 28). The latter pathway can produce copious amounts of 2-haloacetalddehydes, which readily bind to protein and nonprotein thiol (29, 30). We have recently characterized the chemistry of the major DNA adduct formed from EDB and reported the structure to be that of S-[2-(N'-guanyl)ethyl]glutathione, formed from EDB by conjugation with GSH and reaction of the putative episulfonium ion with guanine (Fig. 1) (11, 12, 14). In this paper, we report some of the biochemical features regarding the formation and fate of this adduct.

We used an experiment in which the enzyme-generated steady-state level of the reaction metabolite was perturbed by stopping its synthesis (Fig. 2). This experiment indicates a half-life of <10 s for the reactive intermediate (at pH 7.7). Schastene and Reed (31) have reported a half-life of 40 s for (2-bromoethyl)cysteine at pH 8.0 (27°C) and a half-life of 70 s at pH 6.0, 37°C for the alkyllylation of 4-(p-nitrobenzyl)pyridine by that compound. We postulate that S-(2-bromoethyl)glutathione (or, more likely, the derived episulfonium ion) is the reactive intermediate. One might expect its half-life to be longer on the basis of the reported data (31); however, the experiment reported here was more complex and also involved the presence of a high concentration of GSH, which also reacts with S-(2-bromoethyl)glutathione to form S,S-ethylenebisglutathione (11, 24). Despite this short half-life, the reactive alkylating agent formed from EDB is still stable enough to cross the plasma membrane and leave hepatocytes (Table 1).

Experiments with rat hepatocytes showed that treatment with DEM (i.e., GSH depletion) decreased the formation of DNA adducts, but treatment with the relatively nonspecific cystochrome P-450 inhibitor 1-phenylimidazole did not. These findings are consonant with those of Sundheimer et al. (10), who found that DEM inhibited the formation of in vivo DNA binding, but SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate) did not.

The only major adduct formed from EDB in DNA in vitro or in vivo in rat liver or kidney is S-[2-(N'-guanylyethyl)glutathione. This observation is consistent only with the conjugation pathway shown in Fig. 1. Thus, other potential modes of oxidative and reductive metabolism (27, 32, 33) and direct DNA binding (23) are probably irrelevant in considering genetic damage. In addition, further metabolic processing of the half-life of guanyl imidazole ring opening to formamidopyrimidine adducts.

Other experiments (14) indicate that this persistence is not due to guanyl imidazole ring opening to formamidopyrimidine adducts. The possibility exists that S-[2-(N'-guanyl)ethyl]glutathione adducts might decompose to reform the putative episulfonium ion with guanine serving as a leaving group. If such a reaction occurred, then adducts lost by depurination might present a further risk to DNA in the same and other cells. Such a reaction would also need to be considered in terms of risks to humans from bound residues in foods. However, our in vitro studies showed no detectable DNA adducts formed when S-[2-(N'-

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*M. J. Meredith and F. P. Guengerich, unpublished results.
guanyl)ethylglutathione was incubated with DNA, and we conclude that this reaction does not occur readily.

REFERENCES


Covalent Binding of 1,2-Dihaloalkanes to DNA and Stability of the Major DNA Adduct, S-[2-(N'-Guanyl)ethyl]glutathione

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