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BIOACTIVATION OF DIBROMOETHANE BY CONJUGATION WITH GLUTATHIONE

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ABSTRACT: *The pesticide and carcinogen ethylene dibromide(EDB) is metabolized both by cytosolic GSH S-transferase and by microsomal mixed function oxygenase. Cytochrome P-450 IIE1 appears to be major enzyme to metabolize EDB. EDB is activated to a mutagen by enzymatic conjugation with glutathione (GSH). Such activation is an exception to the general mode of detoxification via GSH S-transferase action. The primary DNA adduct (≥95) is S-[2-(N^7-guanyl)ethyl] GSH and a minor adduct is S-[2-(N^7-guanyl)ethyl]cysteine, which is excreted in the urine and may serve as a biomarker of damage. In rat liver the level of the major DNA adduct is decreased by GSH depletion and elevated by either induction of GSH S-transferase or inhibition of the cytochrome P-450 oxidation of EDB by disulfiram. The unstable conjugate GS-CH_2CH_2Cl and other cysteine or GSH derivatives have been synthesized and found to be a potent mutagen producing bacterial base pair mutation as does EDB.*

INTRODUCTION

The important step in chemical carcinogenesis for most known carcinogens identified to date is thought to be the modification of DNA(Miller and Miller, 1981; Weisburger 1982). The DNA modifications are translated in some way into tumor initiation, although this process is far from being understood.

Although its use is now regulated, ethylene dibromide(EDB) is still an extensively used agricultural and industrial chemical. The main uses for EDB have been as an anti-knock agent in leaded gasoline and as an agricultural fumigant, although recent public concern has led to a ban of its use as a pesticide. EDB is acutely toxic and two human deaths can be attributed to high exposures (Ramsey *et al.*, 1979: Litz *et al.*, 1981). Epidemiological surveys have proved inconclusive with regards to cancer risk to humans (Ott *et al.*, 1980).

The manner in which EDB produces mutagenic and carcinogenic effects is of interest. EDB has some inherent chemical reactivity, but several lines of evidence indicate that its effects are primarily due to the metabolites. The work of van

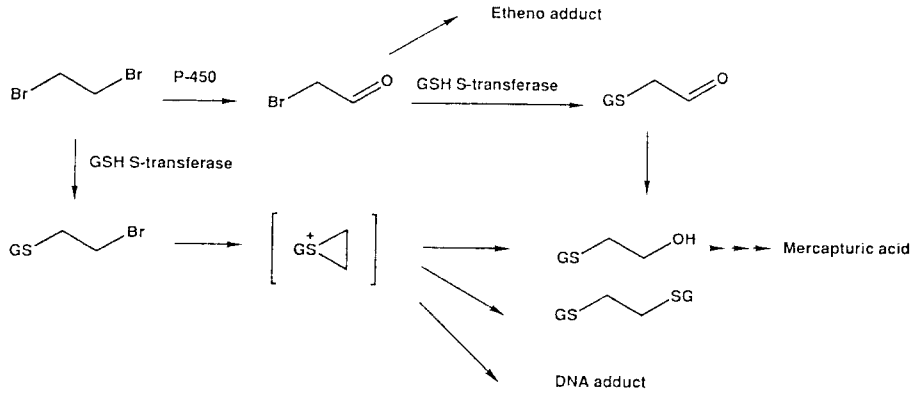

Fig. 1. General scheme dipicting reactions involved in the biotransformation of ethylene dibromide.

Bladeren *et al.* (1981) demonstrated that the major portion of metabolism is oxidative, yielding 2-bromoacetaldehyde. 2-Bromoacetaldehyde is capable of reacting with DNA, but the rate is very slow (Guengerich *et al.*, 1980).

EDB has also been postulated to be conjugated with GSH. Experiments by Rannug *et al.* (1978) suggested that a mechanism for producing mutation might involve the conjugation of EDB with GSH to half-mustards and subsequent nucleophilic attack on DNA (via episulfonium ion intermediate). Further evidence has been provided.

Metabolic Activation of EDB

The principal metabolic routes for EDB involve both oxidative and conjugative transformation (Fig. 1). EDB is primarily transformed by microsomal oxidation. van Bladeren *et al.* (1981) demonstrated that about 80% of EDB administered to rats is oxidized through microsomal mixed function oxygenase by using deutrium-substituted EDB. The oxidative metabolites, bromoacetaldehyde can react with cellular macromolecules, mainly cellular proteins.

The major cytochrome P-450 involved in oxidation of EDB appears to be P-450 IIE1. van Bladren *et al.* (1981) observed an 80% decrease in the urinary levels of N-acetyl-S-(2-hydroxylethyl)cysteine following treatment of rats with the P-450 inhibitor disufiram which was proved to be P-450 IIE1 specific. This was clearly demonstrated by immunoinhibition experiment, correlationship between the amount of P-450 IIE1 in microsome and transformation of EDB, use of P-450 IIE1 specific inhibitor diethyldithiocarbamate, and reconstitued P-450 system (Guengerich *et al.*, 1991). However, several lines of evidence suggest that GSH-S-transferase-catalyzed conjugation is the principal route leading genotoxicity (Rannug, 1978; Buijs *et al.*, 1984). Conjugation of EDB with GSH is catalized by GSH S-transferase and presumably yields S-(2-haloethyl)glutathiones. These conjugates are very reactive and can react with cellular DNA to produce DNA adducts. The irreversible binding of EDB to DNA occurs more readily in the

presence of rat liver cytosolic fractions containing GSH than in NADPH-fortified microsomal fractions. Incubation of radioactive GSH(^{3}S or ^{3}H labeled) with DNA in the presence of GSH S-transferase yielded a finite level of radioactivily in the recovered DNA. The net binding of ^{14}C-labeled EDB in the same incubation system was nearly equimolar with that of GSH (Ozawa and Guengerich, 1985).

Structures and Disposition of Adducts Derived from EDB

The intial work of Rannug indicating that EDB mutations were GSH dependent suggested that an adduct derived from DNA contains a DNA base, GSH, and an ethylene moiety. Such stoichometry was established in *in vitro* radiolabeling experiments as mentioned above.

Further, the major adduct formed *in vitro* was partially purified and then reduced with Raney Ni to form ^{7}N-ethylguanine, which was characterized and postulated to arise from the adduct S-[2-(N^7-guanyl)ethyl] GSH. (Ozawa and Guergerich, 1983). When neutral thermal hydrolysate of the DNA isolated from rat liver after administration of ^{14}C-EDB was analyzed on reverse-phase HPLC column, single major peak appeared (>95% of total DNA binding). This adduct was characterized as S-[2-(N^7 -guanyl)ethyl]GSH based on FAB mass spectometry and NMR spectrum (Fig. 2)(Koga *et al.*, 1986). We further characterized the nature of remaining DNA binding portion(about 4-5% of total binding). HPLC analysis of acid hydrolysate of DNA produced 4 peak(Fig. 3). Peak a was unambigously identified as S-[2-(N^1 − adenyl)ethyl]GSH on the basis of UV, ^{1}H-NMR, and Mass spectrometry(Kim *et al.*, 1990). This adduct exists in the tautomer. Peak *c* was a major adduct S-[2-(N^7-guanyl)ethyl]GSH and peak *d* was proved to be a degradation product of major adduct during acid hydrolysis (100℃, 1 hr 0.1N HCl). No evidence for the formation of an N^6-adenyl adduct and a O^6-guanyl adduct could be found(all were synthesized). The structure of very minor DNA(peak *b*) adducts remain undefined but apparently do not contain purines(Kim *et al.*, 1990).

The half-lives of all the DNA-adducts are about 100 h in rat liver(Kim *et al.*,

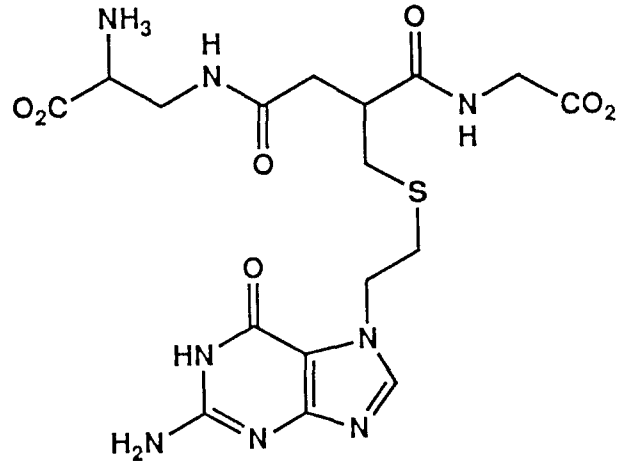

Fig. 2. Structure of major DNA adduct S-[2-(N^7-guanyl)ethyl]GSH derived from ethylene dibromide.

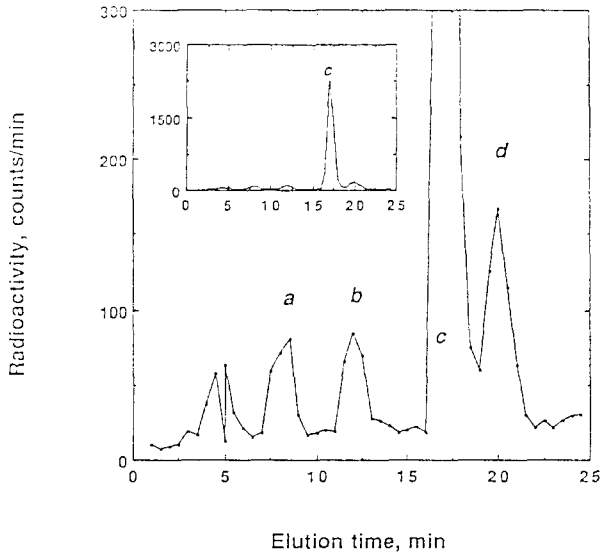

Fig. 3. HPLC of adducts released by mild acid hydrolysis from RNA modified by incubation with GSH, rat liver cytosol, and 1,2-dibromo[1,2-^{14}C]ethane. Yeast-soluble RNA was modified by incubation with rat liver cytosol, GSH, and 1,2-dibromo[1,2-^{14}C] ethane as described under Experimental Section, and an aliquot of the acid-hydrolyzed sample was analyzed by HPLC using a 4.6×250 mm Beckman C_{18} column with a gradient of 5-25% CH_3OH(in 50 mM $NH_4CH_3CO_2$, pH 5.1) applied over 25 min (flow rate 1.0 mL min $^{-1}$). Subsequent work showed that peak a is S[2-(N^1-adenyl)ethyl]GSH, peak c is S-[2-(N^7-guanyl)-ethyl]GSH, peak d is S-[2-(N^7-guanyl)ethyl]cysteinylglycine.

1990). Repair of DNA adduct S-[2-(N^7-guanyl)ethyl]GSH appears to be proceeded primarily by excision repair in prokaryotes and eukaryotes(Cmarik *et al.*, 1990). Once this adduct is released from DNA *in vivo*, it is presumably excereted in urine after further processing to the mercapturic acid derivative. The non-invasive estimation of urinary mercapturic acid would provide excellent approach to human risk assessment related to EDB exposure.

The urinary mercapturic acid S-[2-(N^7-guanyl)ethyl]-N-acetylasteine derived from DNA adduct was characterized by various chemicl approach and a good correlation was found between the excretion of mercapturic acid and the (*in vivo*) formation of DNA adducts in liver and kidney DNA(Fig. 4)(Kim and Guengeich 1989).

Modulation of Adduct-levels and Lack of a Role for Sulfation

The formation of hepatic DNA adduct was higher in rats than in mice while no difference was noted among strains within each species(Kim and Guengerich 1990).

The level of S-[2-(N^7-gunyl)ethyl]GSH in rat liver could be decreased by depletion of GSH, and the level of the adduct could be increased by either (a)

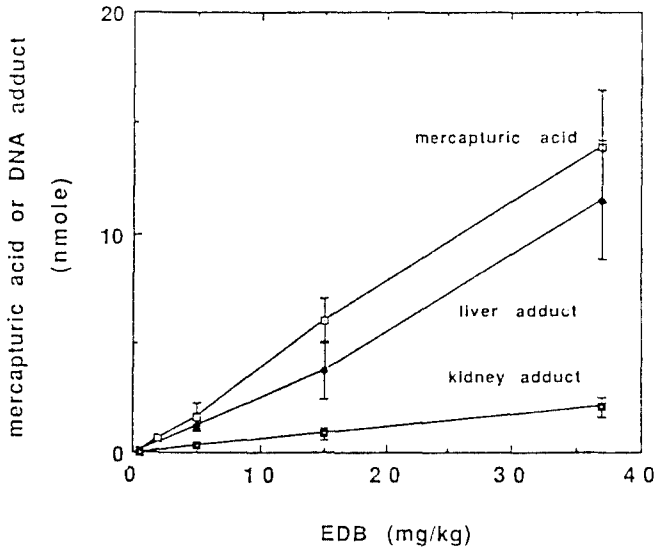

Fig. 4. Comparison of dose responses for the formation of the DNA adduct S-[2-(N^7-guanyl)ethyl]GSH in hepatic and kidney DNA and the excretion of mercapturic acids in the urine of rats administered with various doses of EDB. The data for DNA adduct represent the amount present in DNA 24 h after injection. Total amounts of DNA adducts were calculated on the basis of DNA content: 1.75±0.18 mg/g liver, 2.03±0.33 mg/g kidney (four animals, x±SE). The levels of urinary mercapturic acid are those excreted within 25 h after injection.

Table 1. GSH S-transferase activities in liver cytosol and the formation of DNA S-[2-(N^7-guanyl) ethyl]GSH after treatment of rats with various inducers[a]

Treatment	GSH S-transferase activity (nmol/min/mg protein)			DNA adduct (pmol/mg DNA)
	CDNB	DCNB	EDB	
None	790±94	38.6±2.4	6.86±1.00	360±47
Phenobarbital	1193±72*	45.6±1.6*	8.93±1.03*	246[b]
β-Naphthoflavone	1056±72*	30.7±2.6	8.14±1.13	341±75
Butylated hydroxytoluene	1567±53*	50.5±2.5*	10.70±1.05*	510±33*
Disulfiram	815±45	40.1±2.3	7.46±0.19	673±140*
Phenobarbital plus disulfiram	1301±191*	46.1±3.9*	7.86±0.42	679±103*

[a] Sprague-Dawley rats, in groups of seven, were treated as follows: phenobarbital, 80 mg/kg i.p. daily for 3 days; β-naphthoflavone, 40 mg/kg i.p. daily for 3 days; butylated hydroxytoluene, 0.4% in diet for 7 days; disulfiram, 0.15% in diet for 14 days. EDB was administered i.p. (37 mg/kg body weight) to rats in groups of three 24 h after the last treatment and determination of DNA adducts was done 4 h after EDB injection. Cytosols were prepared from the remaining rats in each group.
[b] Value represents mean of two animals because of a death during treatment (individual values were 272 and 210).
*Values significantly different from control group ($p<0.05$).

induction of GSH S-transferase or (b) inhibition of the cytochrome P-450 IIE1-catalyzed oxidation of EDB by disulfiram(Table 1), which is known to dramatically increase the level of EDB-induced hepatocarcinogenesis (Wang *et al.*, 1982. van Baderen *et al.*, 1981). The possibility of sulfation in DNA adduct formation was also investigated. The ultimate oxidative products S-(2-hydroxyethyl)GSH is about 80% of total metabolites. If sulfation occurs, then the putative episulfonium ion might be regenerated with sulfate serving as a leaving group and the DNA adduct could be formed. No DNA binding was observed through this pathway (Kim and Guengerich, 1990).

Mechanism of Mutagenesis

EDB produced mutagenic effect when incubated with cytosolic fraction in Ames test(Ranug *et al.*, 1978). The approach of site-specific mutagenesis has not been applied in the case of EDB derived adduct because the inherent instability of N^7-alkylguanine adduct to depurination. An alternative strategy was utilized in studies on the mechanism of mutagenesis, where base-pair mutations were examined in *Salmonella typhimurium* TA 100 at the two adjacent guanines known to lead to reversion. A series of half-mustard analogs of S-(2-bromoethyl)GSH was examined with regard to mutagenicity as a function of dose. Generally GSH derivatives produce more mutation compared to cysteine derivatives. Bacteria were treated with a single concentration of each half-mustard and the N^7-alkylguanine adducts were released from bacterial DNA and quantified by HPLC with fluorescence detection, with reference to synthetic standards that were prepared for all adducts. The levels of alkylation varied considerably. These values differ from those estimated for the *in vitro* alkyltion of calf thymus DNA for the same compounds, apparently being influenced by bacterial membrane permeability and transport (Humphreys *et al.*, 1990). The ratio of mutations/adducts could be calculated with assumption about bacterial DNA concentration, and indicate that 1 in every 3 S-[2-(N^7-guanyl)ethyl]GSH adducts give rise to a mutation. Even though sequence selectivity in the alkylation(alkylation occurs selectively in runs of guanines), the mutation frequency of an S-[2-(N^7-guanyl-ethyl]GSH adduct should still be regarded as being >5% in this model, even with adjustment for alkylation specificity. This level of mutation can probably only be explained in terms of S-[2-(N^7-guanyl)ethyl]GSH, since no evidence for other guanyl adducts exists, even at levels 1/10 of the mutation frequency. Finally, the mutations are blocked by the presence of an excision repair system but are not dependent upon the SOS response. Mutations were observed at similar levels in *S. Typhimurium* TA 1535, devoid of the SOS response; and no positive response to these half-mustards was seen in an *S. Typhimurium* TA 1535/PSK 1002 system, where a plasmid containing the *umu C* regulatory gene is fused to a *lacZ* structural reporter gene. The lack of SOS response argues against the involvement of apurinic sites as a factor in the mutagenesis seen with these compound. No frame-shift mutation have been seen.

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