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THE INFLUENCE OF DISULFIRAM AND OTHER INHIBITORS OF OXIDATIVE METABOLISM ON THE FORMATION OF 2-HYDROXYETHYL-MERCAPTURIC ACID FROM 1,2-DIBROMOETHANE BY THE RAT

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Abstract—The mercapturic acid derivative, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine, is a major metabolite of 1,2-dibromoethane *in vivo*. This compound can be formed via two pathways, both involving a potentially dangerous reactive intermediate. One way involves the intermediacy of bromoacetaldehyde, formed by microsomal oxidation, followed by loss of hydrogen bromide. The second pathway, direct conjugation of 1,2-dibromoethane with glutathione, gives rise to *S*-2-bromoethyl glutathione. Using several inhibitors of microsomal mixed function oxidases, it was found that under these conditions about 10% of the mercapturic acid derivative formed via direct conjugation.

Disulfiram, an inhibitor of aldehyde dehydrogenases, but also of microsomal oxidation, also markedly inhibits the excretion of the mercapturic acid, after administration of a single high dose (1 g/kg) or upon chronic treatment with a low dose (50 mg/kg). The inhibitory effect is maximal after 10 days of chronic treatment. Administration of large amounts of 1,2-dibromoethane (> 0.20 nmole/rat) following a single lower dose of disulfiram (125 mg/kg) also leads to a lower excretion of mercapturic acid metabolite a phenomenon associated with a decrease in cytochrome P-450 levels. From these results it is concluded that the enhanced carcinogenic effect of the combination disulfiram (chronic)/1,2-dibromoethane is not caused by bromoacetaldehyde, since its formation is completely inhibited under these conditions, but by *S*-2-bromoethyl-glutathione, although a role for 1,2-dibromoethane itself cannot be excluded.

1,2-Dibromoethane (ethylene dibromide) is a widely used industrial chemical [1]. It is also a well known mutagen [2, 3] and carcinogen [4, 5]. Its biotransformation has been studied extensively, both *in vivo* and *in vitro*. A major metabolite *in vivo* is the mercapturic acid derivative, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine. In rats, up to 55% of the dose administered was excreted in urine in the form of this mercapturic acid [3].

Two different pathways are followed for the formation of this metabolite, both involving the intermediacy of a reactive species. Microsomal oxidation of 1,2-dibromoethane, followed by loss of hydrogen bromide, leads to the formation of bromoacetaldehyde, a highly reactive substance which can bind covalently to cellular macromolecules [6, 7]. Direct conjugation of 1,2-dibromoethane to glutathione, catalysed by the glutathione transferases, leads to *S*-2-bromoethyl-glutathione, a substance in which the halogen atom is highly activated by the neighbouring sulphur atom (sulphur–mustard effect) (Fig. 1). Recent evidence indicates that this reactive conjugate is responsible for the mutagenic effects of 1,2-dibromoethane towards *Salmonella typhimurium* TA100 [3] and *Escherichia coli* 342/113/uvrB [8]. Conjugation of bromoacetaldehyde with glutathione, followed by reduction of the aldehyde moiety to the alcohol, and reaction of *S*-2-bromoethyl-glutathione with water lead to the same conjugate (Fig. 1) and, after several enzymatic steps, to the same mercapturic acid derivative which is ultimately excreted in the urine.

Recently it was reported that the carcinogenic potential of 1,2-dibromoethane is increased substantially by concomitant administration of disulfiram [5], although disulfiram itself is non-carcinogenic to both rats and mice, and even inhibits carcinogenesis induced by benzo(a)pyrene and dimethylhydrazine [9]. It has been suggested [27] that disulfiram treatment results in a higher amount of bromoacetalde-

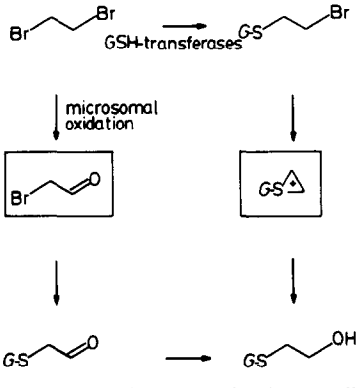

Fig. 1. Involvement of two reactive intermediates in the metabolism of 1,2-dibromoethane. The hydroxyethyl-glutathione conjugate can be formed via both intermediates.

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Table 1. Excretion of *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine by rats after administration of 0.10 mmole of 1,2-dibromoethane (94 mg/kg) and pretreatment with different inhibitors of drug metabolism

Treatment	Mercapturic acid* (mmole)	Percentage of control value
Control	0.051 ± 0.001 (4)	100%
Disulfiram†, 25 mg	0.054 ± 0.004 (6)	105%
Disulfiram†, 200 mg	0.012 ± 0.002 (6)	23.5%
Disulfiram†, 10 mg on 8 consecutive days	0.010 ± 0.001 (3)	19.6%
1-Phenylimidazole‡, 10 mg	0.009 ± 0.004 (5)	17.6%
6-Nitro-1,2,3-benzothiadiazole‡, 10 mg	0.006 ± 0.003 (4)	11.8%
4-Chlorophenyl-4(5)-imidazole‡, 10 mg	0.014 ± 0.003 (4)	27.5%

* Total mercapturic acid was excreted in 24 hr in all cases. Mean values ± S.E.M. are given. The numbers in brackets give the number of determinations.

† Dissolved in 1 ml of propylene glycol, and administered orally by stomach tube.

‡ Dissolved in 0.2 ml of dimethylsulfoxide, and administered intraperitoneally.

hyde present in the rat, through inhibition of the aldehyde dehydrogenases. On the other hand, disulfiram is a known inhibitor of cytochrome P-450 dependent reaction and might completely inhibit formation of bromoacetaldehyde, leaving only *S*-2-bromoethyl-glutathione as a reactive intermediate in the metabolism of 1,2-dibromoethane.

The present studies were performed to study the degree of occurrence of the two possible reactive intermediates *in vivo* in the rat. To this end the excretion of the major metabolite, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine was used as a tool. The influence of pretreatment of rats with different doses of disulfiram and with inhibitors of microsomal oxidation, notably 1-phenylimidazole [12], on the excretion of this metabolite was studied.

MATERIALS AND METHODS

Chemicals. 1,2-Dibromoethane was obtained from Baker Chemicals and purified by preparative g.l.c. to 99.9+% purity. *N*-Acetyl-*S*-2-hydroxyethyl-L-cysteine was synthesised as described previously [3]. 1-Phenylimidazole was synthesised according to Johnson *et al.* [10]. 6-Nitro-1,2,3-benzothiadiazole and 4-chlorophenyl-4(5)-imidazole were gifts from Dr. C. F. Wilkinson (Ithaca, New York). Disulfiram was obtained from Aldrich Chemical Co. (Gillingham, U.K.).

Animals and treatment. Male rats of the laboratory-bred SPF Wistar strain, weighing about 200 g, were used. They were kept in stainless steel metabolism cages and urine was collected for 24 hr. During, but not before, the experiment the animals were fasted, and they had free access to water. 1,2-Dibromoethane was administered orally, by stomach tube, as 1 ml of a solution in arachis oil. (For dose levels see figure legends, range 47–282 mg/kg). Disulfiram was administered orally, using a stomach tube, as a solution in propylene glycol (1 ml) or intraperitoneally as a solution in dimethylsulfoxide (0.2 ml), 24 hr before administration of 1,2-dibromoethane. (For dose levels see figure legends; 25, 50, 125 and 1000 mg/kg were

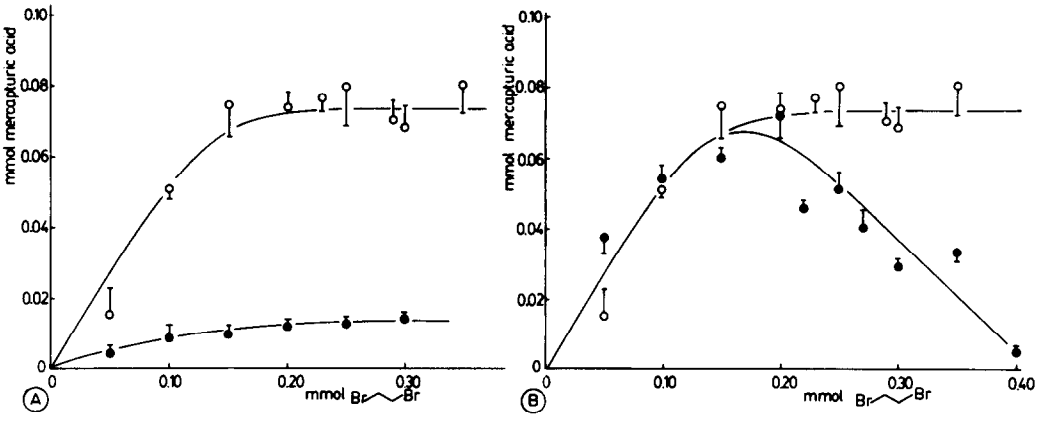

Fig. 2.(A) Influence of 1-phenylimidazole on the excretion of the mercapturic acid, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine, by rats as a function of the dose of 1,2-dibromoethane adminstered. The mean values of at least 5 rats are given (± S.E.M.). ○ = Untreated rats. ● = Rats pretreated with 10 mg of the cytochrome P-450 inhibitor 1-phenylimidazole (50 mg/kg), dissolved in 0.2 ml dimethylsulfoxide, intraperitoneally, 10 min before administration of 1,2-dibromoethane. (B) Influence of a single dose of disulfiram on the excretion of the mercapturic acid, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine, by rats as a function of the dose of 1,2-dibromoethane administered. Each point represents the mean result of at least 5 rats, ± S.E.M. ○ = Untreated rats. ● = Rats pretreated with a single dose of 25 mg (125 mg/kg) disulfiram per rat, as a suspension in 1 ml of propylene glycol, orally, 24 hr before the administration of 1,2-dibromoethane.

used). 1-Phenylimidazole, 6-nitro-1,2,3-benzothiadiazole and 4-chloro-4(5)-imidazole were administered as 0.2 ml of a solution in dimethylsulfoxide intraperitoneally, 10 min before the administration of 1,2-dibromoethane. Control animals were treated with the vehicle alone.

Assays. Quantitative determination of *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine was performed by g.l.c., after freeze-drying the urine samples and methylating the mercapturic acids in the residue, by a procedure described previously [3]. Preparation of microsomes and 100,000 *g* supernatant and measurements of the glutathione transferase activity in livers of treated rats towards 1,2-dibromoethane were described earlier [3]. Cytochrome P-450 contents of microsomes from livers of treated rats was measured according to Omura and Sato [11].

RESULTS

In Table 1 the excretion of *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine after administration of 0.10 mmole of 1,2-dibromoethane by control rats and by rats receiving different pretreatment regimens is presented. The administration of 25 mg disulfiram per rat does not result in a significant change in the amount of mercapturic acid derivative excreted. A much higher dose, (200 mg) however, gives rise to a decrease of 76.5%. A similar effect is obtained by dosing the rats with the well-known cytochrome P-450 inhibitors 1-phenylimidazole, 6-nitro-1,2,3-thiadiazole or 4-chloro-4(5)-imidazole [12, 13]. Interestingly, the same decrease in excreted mercapturic acid derivative is obtained when the rats were treated with a low dose of disulfiram for 8 consecutive days.

In Fig. 2A the influence of dose of 1,2-dibromoethane on *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine excretion is shown. Up to a dose of about 0.15 mmole of 1,2-dibromoethane there is a proportional increase of mercapturic acid derivative excretion with dose. At higher dose levels a plateau is reached. Pretreatment of rats with 1-phenylimidazole resulted in a strong decrease in mercapturic acid metabolite excretion over this whole dose range.

In Fig. 2B the amount of mercapturic acid derivative excreted by untreated rats is compared to the amount excreted after pretreatment with a relatively low dose of disulfiram (25 mg), upon administration of different doses of 1,2-dibromoethane. Although at doses below 0.20 mmole there is no difference between the two experiments, at higher dose levels the amount of mercapturic acid derivative excretion decreases almost proportionally with dose. In order to study the cause of this observation, livers were isolated from some of the animals after the experiment and 100,000 *g* supernatant and microsomes were prepared. In the 100,000 *g* supernatant the specific activity of the glutathione transferases towards 1,2-dibromoethane was measured and found to be not different from control values (28.6 nmole/min/mg protein) neither at a dose of 0.10 mmole 1,2-dibromoethane (30.3 nmole/min/mg protein), nor at 0.30 mmole 1,2-dibromoethane (28.1 nmole/min/mg protein). On the other hand the cytochrome P-450 content of the microsomes was more than 6-fold lower than the control value of

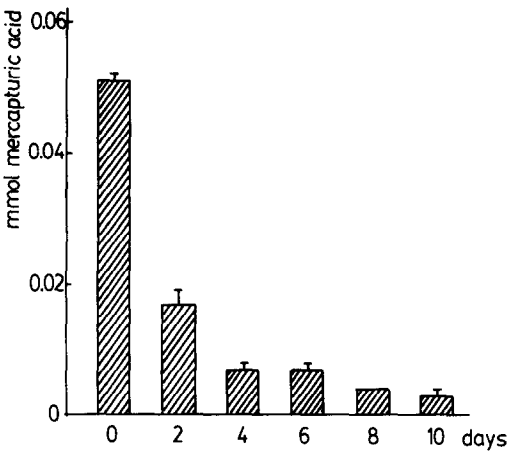

Fig. 3. Time dependence of the decrease of mercapturic acid excretion by chronic treatment with 10 mg disulfiram dissolved in 0.2 ml of dimethylsulfoxide (50 mg/kg). A group of rats was administered disulfiram daily, at different times three of them were treated with 0.10 mmole 1,2-dibromoethane (94 mg/kg) and the excretion of *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine was determined. The mean results ± S.E.M. are depicted.

1.006 nmole/mg protein (0.159 nmole/mg protein instead) for rats given 0.30 mmole of 1,2-dibromoethane. With the dose of 0.10 mmole 1,2-dibromoethane cytochrome P-450 content was only slightly lower than the control value (0.927 nmole/mg protein).

It was already pointed to the fact that chronic treatment of rats with a low dose of disulfiram results in the same decrease in excretion of mercapturic acid derivative as a single high dose (Table 1). In Fig. 3 the time course of this decrease is depicted. Rats were administered 5 mg of disulfiram in 0.2 ml of dimethylsulfoxide i.p. each day. At different time intervals the rats were treated with 0.10 mmole 1,2-dibromoethane. The effect was most pronounced during the first four days, whereas a slight gradual decrease occurred until the tenth day, when the excretion is down to only 10% of the normal value. The disulfiram was given intraperitoneally in DMSO in this case. Compared to suspensions in propylene glycol administered orally this mode of administration results in a greater effect at lower concentrations (compare Table 1).

DISCUSSION

The most abundant ultimate metabolite in the metabolism of 1,2-dibromoethane *in vivo* is the mercapturic acid, *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine, comprising up to 55% of the dose [3]. The total amount of 1,2-dibromoethane conjugated to glutathione will be even higher, since a number of related metabolites have been identified: *S*-2-hydroxyethyl-L-cysteine [14], *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine-*S*-oxide [15], *S*-carboxymethyl-L-cysteine and thiodiglycolic acid [16]. In Fig. 4 the two metabolic pathways that lead to the formation of the major mercapturic acid have been depicted. Enzymatic conjugation with glutathione

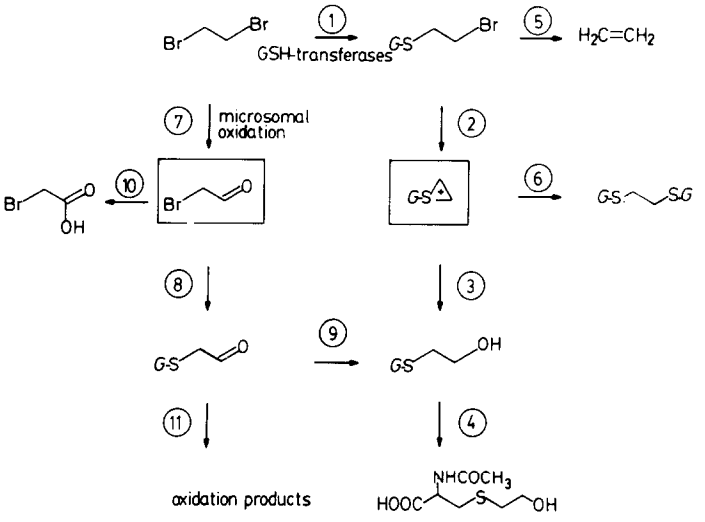

Fig. 4. The possible metabolic pathways of 1,2-dibromoethane. Via both primary reactions, microsomal oxidation and direct conjugation with glutathione, the same mercapturic acid is formed. The different steps are described in the text.

(step 1), which has been shown to occur *in vitro* [3, 6, 17], gives rise to the reactive intermediate, *S*-2-bromoethyl-glutathione. Intramolecular attack of the sulphur atom lone pairs on the carbon atom bearing the halogen substituent leads to a thiiranium ion (step 2) [18], which is responsible for the mutagenic effect of 1,2-dibromoethane in bacteria [3, 8]. Attack of a second molecule of glutathione on the sulphur atom of the glutathione conjugate leads to formation of ethylene (step 5) [19], attack on the carbon atoms of the thiiranium ion gives rise to a double conjugate (step 6), which has only been identified *in vitro* [17]. Reaction of the thiiranium ion with water followed by enzymatic breakdown and acetylation of the glutathione conjugate leads to the mercapturic acid derivative (steps 3 and 4). On the other hand, microsomal oxidation of 1,2-dibromoethane gives rise to bromoacetaldehyde, identified by Hill *et al.* [6] and is responsible for covalent binding of 1,2-dibromoethane to cell macromolecules [6, 7]. This aldehyde is probably formed via an intermediate bromohydrin or a bromoso compound, as has been established for 1,2-dichloroethane [20] (step 7). Conjugation of bromoacetaldehyde with glutathione (step 8) followed by reduction of the aldehyde moiety leads to the same glutathione conjugate as is formed through direct conjugation of 1,2-dibromoethane (step 9) and ultimately to the same mercapturic acid derivative (step 4). Oxidation of the aldehyde in the glutathione conjugate would lead to *S*-carboxymethyl-L-cysteine and thiodiglycolic acid, while bromoacetaldehyde similarly would give bromoacetic acid.

Although via both primary reactions, glutathione conjugation and microsomal oxidation, several final products can be formed, the major metabolite *N*-acetyl-*S*-2-hydroxyethyl-L-cysteine is formed through both. Changes in the relative importance of the two routes would be reflected in the amount of this mercapturic acid derivative formed.

1- and 4(5)-Phenylimidazoles and benzothiadiazoles are well-known inhibitors of cytochrome P-450 dependent reactions [12, 13], presumably by binding to the substrate and the oxygen binding sites [21]. When rats were pretreated with either of these compounds, a considerable reduction (70–90%) in the amount of mercapturic acid derivative excreted occurs, indicating that up to 90% of mercapturic acid derivative is formed via a cytochrome P-450 dependent reaction sequence (Fig. 4, steps 7, 8, 9 and 4). The remaining 10–30% are probably formed via conjugation to glutathione (steps 1, 2, 3 and 4). This implies that the reactive glutathione conjugate intermediate plays indeed an important role *in vivo*. The ratio of about 8:2 between the two pathways is in agreement with studies on the metabolism of fully deuterated 1,2-dibromoethane, where it was found that *ca.* 25% of the mercapturic acid derivative excreted had retained four deuterium atoms, which is only compatible with direct conjugation with glutathione, a pathway in which no C—H bonds are broken (Van Bladeren and Van Huygevoort, unpublished).

Disulfiram (tetraethylthiuram disulphide) is used in the treatment of alcoholism because it inhibits aldehyde dehydrogenase activity in the liver [22]. This compound is also known to inhibit other enzymic reactions [23], notably cytochrome P-450 dependent oxidation reactions [24]. For instance, the metabolism of antipyrine in humans is considerably slower when disulfiram is administered [25]. On the other hand, disulfiram induces glutathione transferases *in vivo* [26]. Possibly because of this inhibition of oxidative biotransformation it was found that disulfiram has a protective effect against carcinogenesis induced by certain chemicals in rats and mice [9]. In contrast it was recently found that disulfiram potentiates the carcinogenicity of 1,2-dibromoethane to a considerable extent [5].

From Fig. 4 it can be seen that apart from a direct alkylation of DNA, two reactive intermediates may be involved in the carcinogenic effects of 1,2-

dibromoethane: bromoacetaldehyde and *S*-2-bromoethyl-glutathione. As suggested by Plotnick *et al.* [27] the enhanced carcinogenicity of the combination disulfiram/1,2-dibromoethane might be due to inhibition of the oxidation of bromoacetaldehyde to bromoacetic acid (step 10), thus causing an increased concentration of the aldehyde. If this were the case one would expect an unchanged or even increased formation of mercapturic acids by rats pretreated with disulfiram. Indeed, when a single low dose of disulfiram was administered 24 hr before treatment with 1,2-dibromoethane excretion of mercapturic acids at low doses of 1,2-dibromoethane (below 0.20 mmole) remained constant or even showed a slight increase. The dose-dependent decrease found at higher levels of 1,2-dibromoethane can be explained by toxicity to the cells, as witnessed by the observed destruction of the cytochrome P-450 at these dose levels, by the large amounts of bromoacetaldehyde formed.

On the other hand, inhibition of cytochrome P-450 dependent oxidation reactions would lead, in analogy with the results obtained with 1-phenylimidazole and the other inhibitors, to a considerable decrease in mercapturic acid excreted. This decrease was indeed found, both after a single high dose and chronic treatment with low doses of disulfiram. Moreover, the effect of disulfiram was additive, and had reached its maximal effect only after a number of days. Plotnick *et al.* [27] also found a decrease in urinary metabolite excretion using radioactive [^{14}C]1,2-dibromoethane after chronic treatment of rats with disulfiram. From these facts it can be concluded that the chronic disulfiram treatment used in the carcinogenicity assay of the combination 1,2-dibromoethane/disulfiram [5, 27] completely inhibited the microsomal oxidation of 1,2-dibromoethane. Since the agent responsible for the enhanced carcinogenicity found for this combination cannot have been 2-bromoacetaldehyde, only two alternatives are left: either 1,2-dibromoethane itself, or *S*-2-bromoethyl-glutathione. Both compounds might be available in higher concentrations due to the inhibition of the main biotransformation pathway. An important phenomenon in this respect is the observed induction by disulfiram of the glutathione transferases *in vivo* [26], which will favour formation of the reactive glutathione conjugate. The special role of this glutathione adduct is stressed by the fact that it has been shown to be responsible for the enhanced effect of 1,2-dibromoethane towards bacteria by metabolic activation [3, 8]. The extent to which 1,2-dibromoethane itself contributes to the carcinogenic effect is not clear at this moment.

In conclusion it can be stated that, although the greater part of the mercapturic acid derivative is formed via oxidation, this pathway is not responsible for the enhanced carcinogenic effect of 1,2-dibromoethane in rats treated chronically with disulfiram. The present study has shown that the primary glutathione conjugate, *S*-2-bromoethyl-glutathione plays an important role in the carcinogenicity of 1,2-dibromoethane.

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