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DEPLETION BY 1,2-DIBROMOETHANE, 1,2-DIBROMO-3-CHLOROPROPANE, TRIS(2,3-DIBROMOPROPYL)PHOSPHATE, AND HEXACHLORO-1,3-BUTADIENE OF REDUCED NON-PROTEIN SULFHYDRYL GROUPS IN TARGET AND NON-TARGET ORGANS*

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Abstract—The abilities of several aliphatic organohalide compounds to deplete reduced non-protein sulfhydryl (NPS) concentrations in rodent kidney, liver, lung, stomach, and testis were measured. The sites of NPS loss were compared to the sites of tissue injury as documented in previous reports. Single intraperitoneal (i.p.) injections of 1,2-dibromo-3-chloropropane (DBCP), 1,2-dibromoethane (ethylene dibromide, EDB), or hexachloro-1,3-butadiene (HCBD) decreased hepatic and renal NPS concentrations in mice in a dose-related manner. Small NPS losses were produced in lung, testis and stomach, but only at high organohalide doses. Administration of tris(2,3-dibromopropyl)phosphate (TRIS) decreased NPS content in liver only. Pretreatments with the enzyme inducer polybrominated biphenyls (PBB) or the enzyme inhibitor piperonyl butoxide quantitatively decreased the renal and hepatic NPS-depleting actions of DBCP or EDB *in vivo*, but the enzyme inhibitor β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) was without demonstrable effect. The acute, i.p. LD_{50} value of DBCP was increased by prior treatment of mice with PBB. The LD_{50} values of DBCP and EDB, however, were unaffected by prior treatment with piperonyl butoxide. DBCP or EDB enhanced the loss of reduced NPS groups from rat hepatic homogenates *in vitro*; the reaction was time dependent and appeared to be enzyme mediated. Although the primary target organs for DBCP or TRIS toxicity are reported to be the kidney and the testis, the major site of NPS depletion was found to be the liver. Also, HCBD depleted renal NPS in mice only (not rats), though HCBD is nephrotoxic in rats as well as in mice. The correlations between tissue depletion of NPS and sites of organohalide injury, therefore, were poor.

Halogenated aliphatic hydrocarbons have important applications in agriculture and industry as pesticides, chemical intermediates, and solvents. Many of these compounds are mutagenic in bacterial systems [1], carcinogenic in rodent bioassays [2], and acutely toxic to mammalian soft-tissue organs [3]. It has been shown that some, particularly the chlorinated and brominated hydrocarbons, are readily metabolized to reactive, electrophilic products that can interact with critical cellular molecules such as DNA and membrane proteins [4–7]. Alkylation of macromolecules has been proposed as a mechanism of chemical-induced mutagenesis, carcinogenesis, and acute tissue injury [8–10]. Glutathione, a nucleophilic cellular tripeptide, in the reduced form (GSH) can combine with electrophilic substrates via a reaction catalyzed by the glutathione-*S*-transferases [11]. The product of the reaction is further metabolized and then excreted as a mercapturic acid. Several investigators have hypothesized, therefore, that conjugation with GSH can be a detoxification mechanism as well as an excretory pathway [11–13]. Moreover, it has been shown for certain toxicants that the minimum dose needed to produce acute tissue injury is coincident with that for depletion of GSH in the target tissue [13, 14]. Thus, dose thresholds may exist for the toxicities of haloalkane compounds based on the capacities of detoxification systems such as glutathione conjugation.

The effects of single treatments with the dibromoalkane hydrocarbons 1,2-dibromoethane (EDB), 1,2-dibromo-3-chloropropane (DBCP) and tris(2,3-dibromopropyl)phosphate (TRIS) on reduced non-protein sulfhydryl (NPS; largely GSH) contents in target and non-target organs were measured to assess the relationship between dose-dependent NPS depletion and sensitivities to tissue injuries. All three of these compounds are bacterial mutagens [1, 15, 16] and rodent carcinogens [17, 18], and they produce nephrosis and testicular atrophy in a variety of mammalian species [19–21]. In addition, mercapturic acid derivatives of EDB and DBCP have been isolated from the urine of rats treated with these agents [22, 23], and the mutagenicities of EDB and

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TRIS are altered by addition of GSH to the assay system [24, 25]. Thus, evidence exists both for *in vivo* metabolism of these compounds and for their reaction with glutathione. The effects of a non-brominated haloalkane compound, hexachloro-1,3-butadiene (HCBD), on tissue reduced NPS concentrations were also determined. HCBD is a carcinogen and a potent nephrotoxicant in rodents [26, 27].

METHODS

Animals. Male, adult (15–20 g) ICR mice and male, adult (150 g) F344 rats were purchased from Harlan Industries (Indianapolis, IN). The animals were housed in a temperature-, light-cycle- and humidity controlled room and allowed at least 1 week to become acclimated before use.

Depletion of reduced NPS in vivo. Mice and rats were given a single intraperitoneal (i.p.) injection of EDB (> 99 percent pure, Aldrich Chemical Co., Milwaukee, WI), DBCP (99.3 percent pure, Dow Chemical Co., Midland, MI), TRIS (high purity grade, Firemaster LVT23P, Velsicol Chemical Co., Ann Arbor, MI), HCBD (> 99 percent pure, Aldrich), or an appropriate vehicle and were killed 2 hr later. (Preliminary experiments conducted 1, 2, 3, 4, 6 and 8 hr after chemical treatment indicated that maximum depletion of NPS was achieved by 2 hr.) All chemicals were dissolved in corn oil and administered in a total volume of 5 (mice) or 2 (rats) ml/kg, except for the highest dose of TRIS (10,000 mg/kg), which was used undiluted. All animals were killed at 11 a.m. to minimize the effects of diurnal variation on tissue GSH content. Both kidneys, the whole lung, one lobe of liver, both testes, and the entire stomach were rapidly excised, rinsed in ice-cold 0.9% NaCl (the stomach was inverted prior to washing), weighed, and homogenized in 9 vol. of ice-cold 3% trichloroacetic acid–1 mM EDTA with a motor-driven Potter–Elvehjem tissue grinder (0.01 mm clearance). The denatured protein was removed by centrifugation, and the NPS (largely glutathione) concentration in the supernatant fraction was measured with Ellman's reagent [28].

Effects of anesthesia and ischemia on reduced NPS concentrations. Mice were anesthetized with i.p. injections of sodium pentobarbital (70 mg/kg) or urethane (150 mg/kg) or by intermittent exposure to ether vapors. The animals were maintained in a comatose state for 2 hr and then killed. Livers and kidneys were removed and analyzed for NPS content as before.

Mice in another group were anesthetized lightly with ether, the left kidney was exposed through an incision in the back and a ligature was placed tightly across the renal artery and vein at the hilus. The right kidney was exposed and manipulated similarly, but no ligature was applied. The incisions were closed with stainless steel wound clips. The mice were killed 2 hr later, and the left (ischemic) and right (sham) kidneys were removed and analyzed for NPS content as before.

Depletion of reduced NPS in vitro. Twenty-five percent (wet tissue weight/volume) homogenates of liver or kidney in 20 mM Tris (tris[hydroxymethyl]aminomethane)–1.15% KCl buffer (pH 7.40) were prepared at 4° with a Potter–Elvehjem tissue grinder. Incubations consisting of 3 mM concentrations of EDB or DBCP in the crude homogenates were conducted in sealed 25-ml Erlenmeyer flasks (air atmosphere) at 37° in a Dubnoff shaking incubator (120 oscillations/min) for up to 30 min. The reactions were stopped by the addition of ice-cold 3% trichloroacetic acid–1 mM EDTA, and the flask contents were then centrifuged and the supernatant fractions analyzed for NPS contents as before.

Effects of β-diethylaminoethyl diphenylpropylacetate (SKF 525-A), piperonyl butoxide and polybrominated biphenyls on depletion of reduced NPS and on the LD_{50} *values of EDB and DBCP.* Mice were given a subcutaneous injection of the enzyme inhibitor SKF 525-A (Smith Kline & French Laboratories, Philadelphia, PA), (75 mg/kg) or of piperonyl butoxide, the methylenedioxyphenyl-derivative enzyme inhibitor, (K & K Laboratories, Plainview, NY), (600 mg/kg) 90 min prior to an i.p. injection of EDB or DBCP (250 mg/kg). Another group of mice was fed a diet containing 100 ppm of polybrominated biphenyls (PBB, Firemaster BP-6, Michigan Chemical Co., St. Louis, MI) for 18 days and then given an i.p. injection of EDB or DBCP (100 mg/kg). All mice were killed 2 hr after treatment with EDB or DBCP and the reduced NPS concentrations in kidney and liver were measured as before.

LD_{50} Values were determined by i.p. injection of EDB or DBCP into control (naive) mice, mice treated with 75 mg/kg SKF 525-A or 600 mg/kg piperonyl butoxide 90 min prior to treatment with EDB or DBCP, and mice fed a diet containing 100 ppm PBB for 18 days. Each treatment group consisted of eight animals. The mice were observed for 14 days after treatment; most deaths occurred within 24 hr.

Statistics. LD_{50} Values were computed by the method of Litchfield and Wilcoxin [29]. All other data were analyzed by a one-way analysis of variance and treatment means were compared with the Student–Newman–Keuls test [30], with the exception of NPS concentrations following ischemia, which were analyzed with a paired *t*-test [30]. In all cases, $P < 0.05$ was used as the criterion of significance.

RESULTS

NPS depletion in vivo. All four experimental compounds (DBCP, EDB, HCBD, and TRIS) decreased hepatic NPS concentrations in mice in a dose-related manner (Fig. 1), with the exception of the highest dose of TRIS. This was administered undiluted and, upon examination of the carcasses, did not appear to be well absorbed from the site of injection. Treatments with DBCP, EDB and HCBD also decreased renal NPS concentrations in mice in a dose-related manner (Fig. 1). In contrast, renal NPS concentration was unaffected by TRIS. NPS concentrations in murine lung, testis and stomach were decreased by DBCP, EDB and HCBD, but the degrees of depletion, in general, were not as great as in kidney and liver and often occurred only at the higher doses (Fig. 1). Treatment with TRIS had no effect on NPS

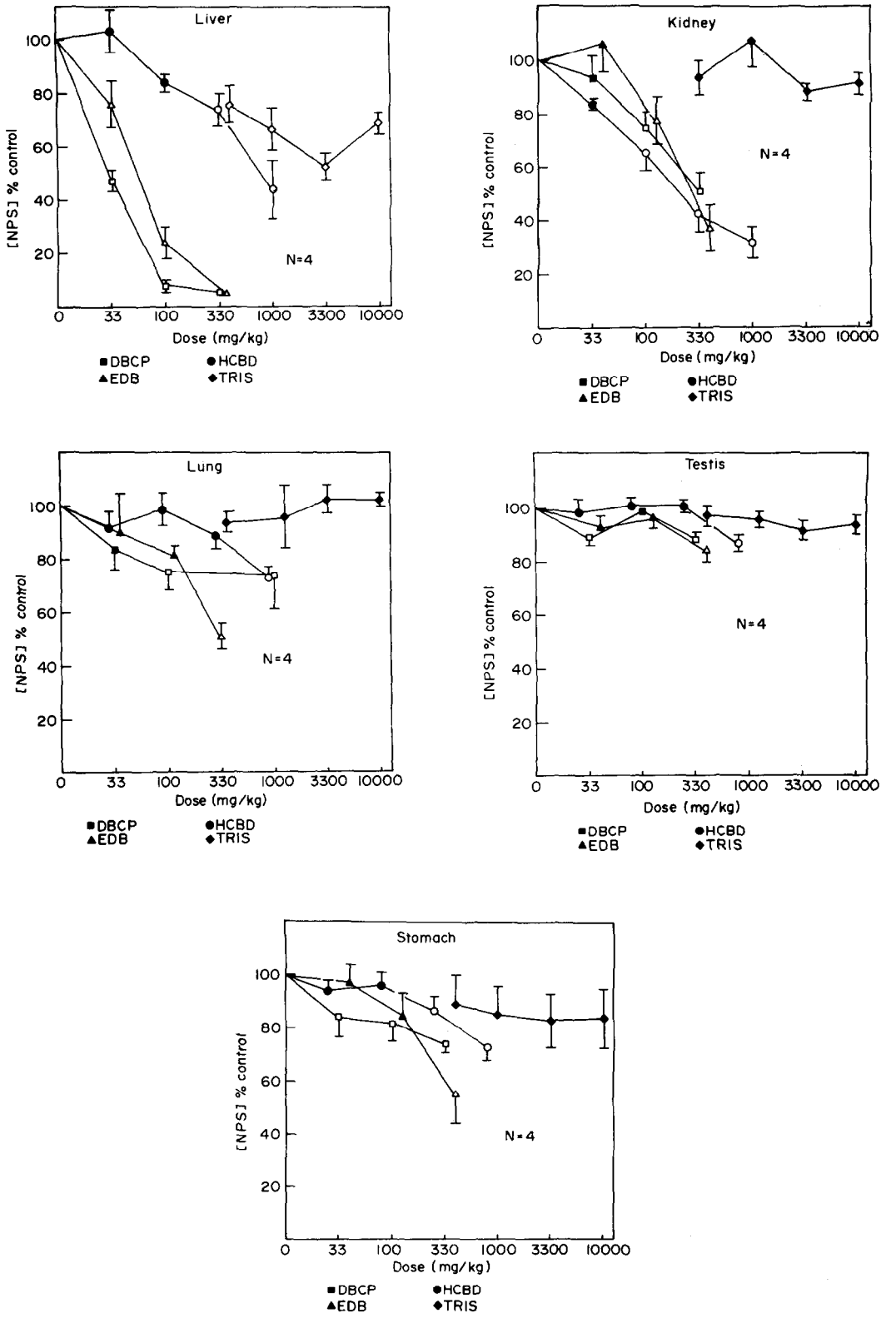

Fig. 1. Depletion of reduced NPS in liver, kidney, lung, testis and stomach. The mice were killed 2 hr after i.p. administration of DBCP, EDB, HCBD, TRIS or vehicle. Each symbol is the mean ± S.E. of six mice. The administered doses (abscissa) are plotted on a log scale. Open symbols are significantly different from vehicle-treated controls, $P < 0.05$. Concentrations of reduced NPS in control animals were: 7.16 ± 0.38 μmoles/g liver, 3.57 ± 0.42 μmoles/g kidney, 2.78 ± 0.54 μmoles/g lung, 2.65 ± ± 0.42 μmoles/g stomach, and 2.11 ± 0.39 μmoles/g testis, using reduced GSH as standard.

Table 1. Effects of HCBD on renal NPS concentrations in mice and rats*

Dose (mg/kg)	[NPS] (μmoles/g) Mice	Rats
0 (vehicle control)	3.57 ± 0.42	2.36 ± 0.33
100	2.36 ± 0.18†	2.49 ± 0.41
250		2.29 ± 0.38
330	1.57 ± 0.25†	2.18 ± 0.37
1000	1.28 ± 0.24†	‡

* All animals were killed 2 hr after HCBD treatment. Each value is the mean ± S.E. of six animals.
† Significant loss of NPS, $P < 0.05$.
‡ All rats died at this dose.

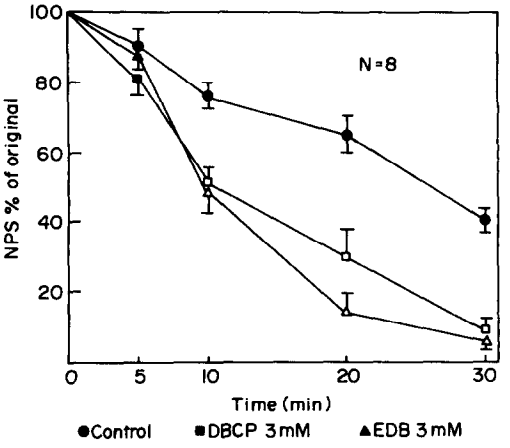

Fig. 2. Loss of reduced NPS from rat hepatic homogenates *in vitro*. NPS concentrations were measured after 0, 5, 10, 20 or 30 min of incubation in the presence of DBCP, EDB or the appropriate vehicle. Each symbol is the mean ± S.E. of eight incubations. Open symbols denote values significantly lower than controls at the same incubation time, $P < 0.05$. The reduced NPS concentration at the start of the incubations was approximately 1.7 mM.

concentrations in lung, testis and stomach. Similar effects were produced by DBCP, EDB, HCBD and TRIS in rats, except that HCBD did not deplete renal NPS (Table 1). The inability of HCBD to deplete GSH from the rat kidney has been reported previously by Lock and Ishmael [31].

Doses of 100 mg/kg, and larger, of DBCP and EDB (and, to a lesser extent, of HCBD) produced severe narcosis and may have interrupted the nutrient supply to the tissues used. Therefore, the effects of 2 hr of deep anesthesia on reduced NPS concentrations in mice as well as the effects of 2 hr of complete renal ischema on renal NPS content were determined. As shown in Table 2, NPS concentrations in all five tissues were unaffected by anesthesia. Renal NPS concentration, however, was decreased to 60 percent of normal by complete ischemia.

NPS depletion in vitro. Incubation of crude kidney and liver homogenates at 37° resulted in a time-dependent loss of NPS even without the addition of EDB or DBCP. Only with rat hepatic homogenates were the effects of 3 mM concentrations of EDB and DBCP on NPS concentration distinguishable from that of incubation time alone. As shown in Fig. 2, reduced NPS concentration was decreased to 50 percent of control after 10 min of incubation with EDB or DBCP (in comparison to 80 percent of control without EDB or DBCP) and to 5–10 percent of control after 30 min of incubation. The EDB- and DBCP-dependent losses of NPS appeared to be enzyme catalyzed, since EDB and DBCP did not reduce NPS concentrations when incubated with 1, 3 or 5 mM GSH alone, or in the presence of boiled homogenate and GSH (data not shown).

Effects of PBB, SKF 525-A, and piperonyl butoxide on NPS depletion. Treatment of mice with 100 mg/kg of EDB or 250 mg/kg of DBCP decreased renal and hepatic NPS concentrations in a manner consistent with the data in Fig. 1. Pretreatment with the microsomal enzyme inducer PBB decreased the NPS-depleting effects of 100 mg/kg EDB and DBCP (Table 3). EDB- and DBCP-induced losses of NPS were also less after prior treatment with the enzyme inhibitor piperonyl butoxide, but the inhibitor SKF 525-A had no demonstrable effect.

Effects of PBB and piperonyl butoxide on the LD_{50} *values of EDB and DBCP.* Single dose LD_{50} values for i.p. administered EDB and DBCP are shown in Table 4. PBB pretreatment significantly increased the LD_{50} of DBCP in mice, but it was without significant effect on the LD_{50} of EDB (although the numerical value of the LD_{50} was increased slightly). Piperonyl butoxide pretreatment did not alter the LD_{50} values of EDB or DBCP in mice (Table 4).

Table 2. Effects of anesthesia and ischemia on reduced NPS concentrations in mice*

Anesthetic	NPS (% of control) Liver	Kidney	Lung	Testis	Stomach
Pentobarbital	96 ± 5	98 ± 5	92 ± 13	86 ± 16	99 ± 7
Urethane	83 ± 7	101 ± 2	86 ± 12	106 ± 14	112 ± 5
Ether	107 ± 6	87 ± 6	81 ± 12	99 ± 4	103 ± 15
Ischemia†		60 ± 2‡			

* Each value is the mean ± S.E.; N = 5.
† NPS content in the ligated kidney expressed as a percentage of that in the sham kidney.
‡ Significant loss of NPS, $P < 0.05$.

Table 3. Effects of PBB, SKF 525-A, and piperonyl butoxide on EDB- and DBCP-induced losses of reduced NPS in mice*

Treatment	NPS (% of non-treated)	
	Kidney	Liver
EDB (100 mg/kg)	79 ± 7	38 ± 4
+PBB	98 ± 5†	81 ± 7†
EDB (250 mg/kg)	33 ± 4	14 ± 2
+SKF 525-A	42 ± 5	11 ± 2
+ piperonyl butoxide	82 ± 6†	38 ± 4†
DBCP (100 mg/kg)	67 ± 6	23 ± 5
+PBB	89 ± 6†	64 ± 10†
DBCP (250 mg/kg)	48 ± 4	9 ± 2
+ SKF 525-A	50 ± 4	16 ± 4
+ piperonyl butoxide	92 ± 4†	45 ± 4†

* Control (non-treated) values for renal NPS were 3.18 ± 0.22 μmoles/g in the EDB experiments and 3.32 ± 0.19 μmoles/g in the DBCP experiment. SKF 525-A, piperonyl butoxide, or PBB treatments alone had no significant effect on hepatic or renal NPS concentrations. Each value is the mean ± S.E.; N = 6.

† Significantly different from EDB or DBCP alone (no prior treatment), $P < 0.05$.

DISCUSSION

The rank order for depletion of NPS by DBCP or EDB in mice was liver> kidney> lung, stomach> testis. Organ-specific NPS depletion by HCBD in mice followed a similar pattern, except that the kidney was more susceptible than the liver. In contrast, TRIS decreased NPS concentration only in the liver. These effects may have been due to the initial tissue distributions of the experimental compounds. Lipophilic chemicals administered intraperitoneally must traverse the liver before reaching the systemic circulation, thus ensuring high exposure of the liver. Similarly, the kidney is perfused at a higher rate ($ml \cdot min^{-1} \cdot g^{-1}$) than the testis and stomach and would be exposed to relatively greater amounts of systemically absorbed toxicants. The initial distributions of radioactivity following oral treatment with [^{14}C]DBCP and [^{14}C]EDB, highest in kidney and liver [23, 32], also indicate an affinity of these agents for kidney and liver. Thus, the organ-specific depletion of NPS in mice may have been a function of toxicant distribution.

DBCP, EDB and HCBD all produced narcosis at high doses. Since an altered nutrient supply could conceivably affect GSH biosynthesis, analyses were made of the effects of ischemia and the comatose state on NPS concentrations. The failure of deep, sustained (2 hr) anesthesia with pentobarbital, urethane or diethyl ether to significantly decrease tissue NPS concentrations (Table 2) suggests that the quantitatively large losses from liver and kidney were not caused solely by a diminution of vascular nutrient supply. Even 2 hr of total ischemia produced less renal NPS loss than did treatments with DBCP, EDB or HCBD (Table 2, Fig. 1). However, the relatively small organohalide-induced changes in NPS concentrations in lung, testis and stomach (Fig. 1) were within the range of those produced by anesthesia (Table 2) and could conceivably have resulted from a decrease in nutrient supply. Elucidation of the mechanisms of loss of NPS from lung, testis and stomach will require further experimentation.

Additions of EDB or DBCP to rat hepatic homogenates enhanced the rate of disappearance of NPS, though EDB and DBCP did not combine with GSH at an appreciable rate in the absence of the liver homogenate or in the presence of denatured (boiled) homogenate. Thus, depletion of NPS by EDB and DBCP *in vitro* appears to be an enzymatic process, possibly involving the activation and subsequent conjugation of these compounds to endogenous GSH. The activities of glutathione-*S*-transferases are highest in liver and kidney, but they are present also in other tissues [33–36]. The patterns of organ depletion of NPS, therefore, may also reflect the catalytic activities of these tissues for organohalide metabolism and for conjugation with GSH. The decreased EDB- and DBCP-induced losses of NPS after treatment with piperonyl butoxide also suggest enzyme catalysis of the overall reactions of EDB and DBCP with tissue NPS. Piperonyl butoxide is believed to inhibit enzyme activity by combining with and inactivating cytochrome P-450 [37–39]; however, microsomal oxidations of EDB or DBCP (to epoxides, for example) may be necessary for subsequent conjugation reactions. PBB, an inducer of cytochrome P-450 and many microsomal enzymes [40–42], also decreased the NPS-depleting effects of EDB and DBCP in mice, indicating the existence of a microsomal pathway of metabolism that leads to products that do not react with tissue NPS. The exact effects of PBB and piperonyl butoxide on the metabolism of EDB and DBCP remain to be elucidated.

In general, there was a poor correlation between reported organ sensitivities to EDB, DBCP, HCBD

Table 4. Effects of PBB and piperonyl butoxide on the LD_{50} values for EDB and DBCP in mice

Pretreatment	LD_{50} and 95% confidence interval (mg/kg)	
	EDB	DBCP
None	205 (187–223)	175 (158–192)
PBB	220 (207–233)	230 (201–259)†
Piperonyl butoxide	205 (182–228)	180 (159–201)

† Significantly different from non-pretreated $P < 0.05$.

and TRIS toxicities and acute tissue-specific depletion of NPS. For example, testicular atrophy (seminiferous tubular degeneration) is a major toxic effect of DBCP and TRIS [20, 21], yet only a small loss of testicular NPS was produced by DBCP and none by TRIS (Fig. 1). Similarly, the potent nephrotoxicant HCBD [27, 43] failed to decrease renal NPS concentrations in rats, though it causes renal tubular necrosis at these and lower doses [43]. DBCP was a potent depletor of hepatic NPS in rats and mice (Fig. 1), but produced only minor liver injury when administered acutely or repeatedly to rats at near lethal doses [20; W. M. Kluwe, unpublished information]. Repeated oral administrations of DBCP and EDB produced squamous cell carcinomas in rodent forestomach [17] and, in fact, decreased NPS concentrations in the stomach when administered i.p. (Fig. 1). The effects on stomach NPS contents, however, were relatively small in comparison to those on liver and kidney. EDB, DBCP and HCBD also caused small losses of pulmonary NPS, though the lung has not been reported to be a direct target organ for these toxicants [19, 20, 26, 27]. Thus, acute depletions of tissue NPS by EDB, DBCP, HCBD and TRIS do not correlate well with reported sites of tissue injury. Rather, organohalide-induced tissue NPS depletions appeared to correlate well with the initial organ distributions of the experimental compounds [23, 32] and with tissue capacities for GSH conjugation [11, 12]: greatest in liver, and higher in kidney than in lung, stomach or testis.

No correlation was observed between the effects of piperonyl butoxide on the acute, i.p. LD_{50} values of DBCP and EDB and depletion of NPS. However, the mechanisms of the acute, lethal effects of these two agents are not known. In this study, most of the animals died within 24 hr of treatment; death may have resulted from a deep narcosis rather than from injury to the liver, kidney or another specific tissue. PBB treatment significantly increased the LD_{50} of DBCP in mice (Table 4) and decreased depletion of NPS (Table 3). Kato *et al.* [23] reported that an appreciable amount (18 percent of an orally administered dose of 20 mg/kg) of DBCP is converted to CO_2 in rats. Thus, it is possible that a non-conjugative pathway of DBCP catabolism exists. PBB treatment may stimulate the non-conjugative metabolism of DBCP and thereby decrease depletion of NPS while simultaneously hastening DBCP catabolism and reducing its lethal potency.

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