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STUDIES ON THE DISTRIBUTION AND COVALENT BINDING OF 1,1-DICHLOROETHYLENE IN THE MOUSE

EFFECT OF VARIOUS PRETREATMENTS ON COVALENT BINDING *IN VIVO*

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Abstract—The distribution and covalent binding of a single dose of [1,2-^{14}C]1,1-dichloroethylene (DCE; 125 mg/kg, i.p.) was studied in male C57Bl/6N mice. Total radioactivity was distributed in whole homogenates of all tissues studied, with peak levels occurring within 6 hr. Covalent binding of radioactive material peaked at 6–12 hr in all tissues, and highest levels were found in kidney, liver, and lung with smaller amounts in skeletal muscle, heart, spleen, and gut. Covalent binding in kidney, liver, and lung fell to 50% of peak levels in about 4 days. Between 12 hr and 4 days after DCE administration, 70–100% of total radioactivity present in homogenates of kidney, liver, and lung was covalently bound. The three tissues showed a similar spread in total radioactivity in subcellular fractions 24 hr after exposure to DCE; most of the radioactivity was covalently bound (60–100%) and distributed fairly uniformly with a slight tendency to concentrate in the mitochondrial fraction. Phenobarbital (PB) and 3-methylcholanthrene (3-MC) pretreatments increased the covalent binding in the liver and lung but had no effect in the kidney. Piperonyl butoxide and SKF-525A decreased the covalent binding in liver and lung, but the latter increased binding in the kidney while the former decreased it. Diethylmaleate administration increased the covalent binding (2- to 3-fold) in all three tissues as well as increasing lethal toxicity. These results are consistent with the view that DCE is metabolized to some reactive intermediate(s) which may be detoxified by conjugation with glutathione.

1,1-Dichloroethylene (DCE) is widely used as a monomeric intermediate in the production of plastics. Thus, workers in the plastic industry are likely to be exposed to this volatile compound by inhalation and ingestion [1]. DCE has been identified as a contaminant of drinking water and has been ranked highly among the hazardous chloro-organic compounds of drinking water in the U.S. today [2] because of its potential mutagenic and carcinogenic properties [3–6].

Toxicity studies in rats have indicated that DCE causes damage to both liver [7–9] and kidney [10]. Although there are no reports of pulmonary toxicity of DCE in rats, administration of a single dose of DCE (125 mg/kg, i.p.) to C57Bl/6J mice causes extensive necrosis of the Clara cells of the bronchiolar epithelium, 24 hr after exposure, without any morphological changes in the liver and kidney [11]; this is accompanied by reduction in pulmonary microsomal cytochrome P-450 and its dependent monooxygenase activities without any effect on the liver enzymes [11]. By contrast, renal microsomal O-deethylation of 7-ethoxyresorufin and 7-ethoxycoumarin are induced (6- to 7-fold) within 1–2 days and return to control values by day 4 [12]. However, recent studies with ddY strain mice with a similar dose of DCE demonstrated severe renal tubular necrosis and marked reductions in reduced glutathione (GSH) levels but less severe hepatic damage and inconsistent changes in GSH [13]. Species differences in toxicity of DCE between rats and mice have been attributed to greater production of reactive metabolites in the mouse [14, 15].

Studies of the effects of inducers and inhibitors of the microsomal monooxygenases on the toxicity of DCE have produced equivocal results; pretreatment of rats with phenobarbital or 3-methylcholanthrene has no effect [16] or protects against the hepatotoxic effects of DCE. On the other hand, SKF-525A and disulfiram (inhibitors of monooxygenases) increase, decrease, or have no effect on DCE toxicity in rats or mice [17–19]. Fasting or pretreatment of rats with diethylmaleate increases DCE toxicity as a result of decreased tissue reduced glutathione (GSH) levels [20, 21]. Metabolic studies indicate that conjugation of DCE or its metabolites with GSH is an essential step in its detoxification [22, 23].

Thus, although work has been done on some aspects of DCE metabolism and toxicity, particularly in the rat, very little is known about its distribution and covalent binding in tissues and subcellular fractions of the mouse, the species thought to be most susceptible to its toxic effects. Furthermore, there are no comparative data on the effects of various pretreatments with inducers and inhibitors of microsomal monooxygenases on the covalent binding in mouse tissues.

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MATERIALS AND METHODS

Chemicals

1,1-Dichloroethylene (DCE) and diethylmaleate were purchased from the Aldrich Chemical Co. (Milwaukee, WI). [1,2-^{14}C]DCE in mineral oil containing 0.1% *p*-methoxyphenol (sp. act. 0.272 mCi/mmole, 99% radiochemical purity), protosol tissue solubilizer and Aquasol liquid scintillation solution were obtained from New England Nuclear (Boston, MA). Piperonyl butoxide and 3-methylcholanthrene were purchased from ICN Pharmaceuticals Inc. (Plainview, NY) and the Eastman Kodak Co. (Rochester, NY) respectively. SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate) was a gift from Smith Kline and French Laboratories (Philadelphia, PA). Benzoyl peroxide was purchased from Fluka AG (Buchs, Switzerland). Phenobarbital (sodium salt) and all other chemicals used were obtained from the Fisher Scientific Co. (Silver Spring, MD) and were the highest purity available.

Animals

Male C57Bl/6N mice (20–25 g body wt) obtained from Frederick Cancer Research Facility (Frederick, MD) were used. They were allowed free access to standard laboratory chow and water and acclimatization to our animal facility for 5–7 days.

Treatment of animals

All test animals received approximately 4 μCi [1,2-^{14}C]DCE (125 mg/kg, i.p.) and were killed 24 hr later, unless indicated otherwise. The dosing solution was prepared on the day of use by mixing non-radioactive DCE and olive oil with 106 μCi of radiolabeled DCE contained in 0.35 ml mineral oil such that 0.1 ml/animal gave the required dose. Control animals each received 0.1 ml olive oil.

Animals receiving pretreatments were dosed at various times prior to [1,2-^{14}C]DCE administration as follows: phenobarbital (100 mg/kg/day, i.p., in saline for 4 days) or 3-methylcholanthrene (40 mg/kg/day, i.p., in olive oil for 2 days) plus [1,2-^{14}C]-DCE 24 hr after last dose; piperonyl butoxide (1200 mg/kg, s.c., in olive oil) or SKF-525A (50 mg/kg, i.p., in saline) plus [1,2-^{14}C]DCE 1 hr later; and diethylmaleate (125 mg/kg, i.p., in propylene glycol) plus [1,2-^{14}C]DCE 2 hr later. Control animals received equivalent volumes of pretreatment test vehicle (0.1 ml/20 g body wt) prior to [1,2-^{14}C]DCE administration. In all cases, regardless of pretreatment, animals were killed 24 hr after [1,2-^{14}C]DCE administration.

Tissue preparation

Animals were killed by cervical dislocation. Blood samples were collected, and tissues were immediately removed and placed on dry ice. Tissue homogenates (10–20%, w/v) in 150 mM KCl–50 mM Tris–HCl, pH 7.4, composed of samples pooled from three to eight animals, were prepared using a motor-driven teflon–glass homogenizer; 0.2 ml of whole homogenate and 0.1 ml of blood were digested with 1 N NaOH (1:4, v/v) at 60°, and aliquots (10–20 μl) were used for protein assay [24] with bovine serum albumin as standard.

Radioactivity determinations

Total radioactivity. To a 0.5-ml aliquot of tissue homogenate in a scintillation vial was added 1.0 ml of protosol tissue solubilizer, and the mixture was digested at 60° for 6 hr. In the case of blood, 1.0 ml of protosol–ethanol (1:2, v/v) was added to 0.1 ml of blood and kept at 60° for 1 hr. Then 0.25 ml of benzoyl peroxide (25%, w/v) in toluene was added to each digest and reheated for 1 hr to remove color in digests. Samples were allowed to cool at room temperature, and 10 ml of aquasol liquid scintillation fluid was added and mixed thoroughly. The samples were then counted in a Packard Tricarb liquid scintillation counter model 460 CD.

Covalent binding. Duplicate samples (1.5 ml each) of tissue homogenates in stoppered tubes were first treated with 3 ml of 20% (w/v) trichloroacetic acid (TCA) and vortex-mixed for 10 sec, with the aid of a glass rod. The samples were kept in ice for 45 min and centrifuged at 3000 *g* for 5 min. Further, extraction was done with 4 ml of 15% (w/v) TCA and then with 5 ml methanol–ether (4:1, v/v) until no further radioactity was detectable in the wash (this normally occurred after six to eight organic solvent extractions). The pellets were heated at 60° to evaporate any organic solvent remaining, and then each sample pellet was resuspended in 3 ml NaOH (1 N). The samples were heated in screw-capped tubes at 60° overnight, and aliquots were taken for measurements of radioactivity (0.5 ml) and protein (0.02 ml).

Subcellular fractionation. Tissue homogenates (20%, w/v) in 150 mM KCl–50 mM Tris–HCl buffer, pH 7.4, were fractionated as previously described [25]. Fractionate pellets were resuspended in 2–5 ml

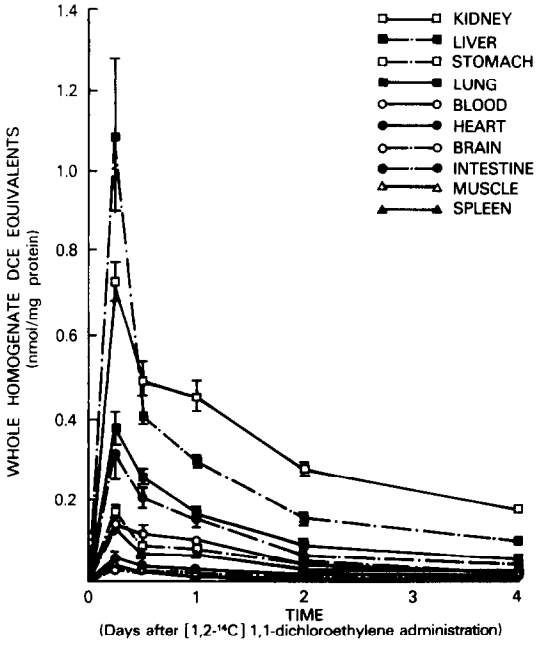

Fig. 1. Time course of the distribution of total radioactivity in tissue homogenates of the mouse after a single i.p. dose of [1,2-^{14}C]DCE (~4 μCi, 125 mg/kg). Values shown are the means ± S.E.M. of four determinations. (Tissues pooled from three to six animals were used for each determination.)

buffer, and aliquots of the suspensions and the cytosolic fraction were taken, as previously, for total radioactivity, protein assay, and covalent binding studies.

RESULTS

Tissue distribution of [^{14}C]*DCE-derived radioactivity*

Figure 1 presents the levels of total radioactivity in various tissues of the mouse at times from 6 hr to 4 days after i.p. administration of [^{14}C]DCE. Total radioactivity was widely distributed in all the tissues studied, but particularly in the liver, kidney, and lung, with peak levels being reached within 6 hr. The highest level of total radioactivity was found in the liver at 6 hr and was overtaken by the kidney 6 hr later. The lowest levels of total radioactivity were found in the brain, heart, and muscle where levels remained relatively constant over the period of study. The stomach, intestine, and spleen showed identical patterns of distribution with their levels of total radioactivity being intermediate between the former two sets of three organs. Blood levels remained consistently and markedly lower than the kidney, liver, and lung over the 4-day period with the mean tissue/blood ratio of total radioactivity being about 5, 4, and 2, respectively, as compared to 0.3–1.4 in the other tissues, suggesting preferential accumulation in these three tissues.

Covalent binding of [^{14}C]*DCE-derived radioactivity*

As was found for radioactivity, total covalent binding showed decided tissue selectivity, the degree of covalent binding being kidney > liver > lung. Tem-

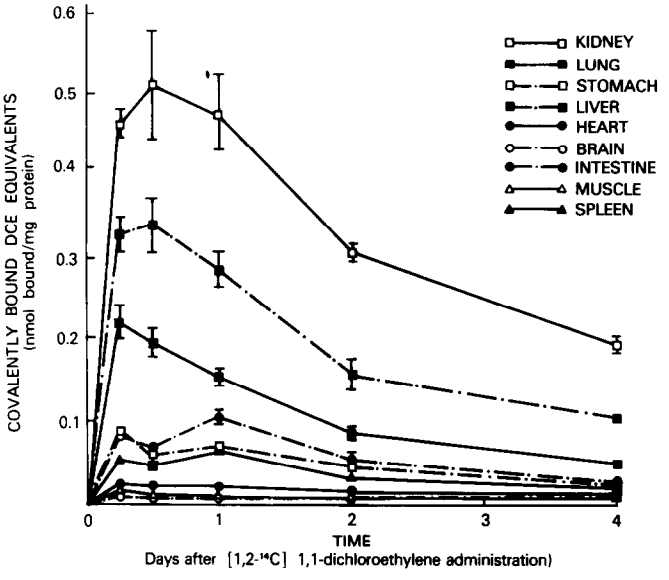

Fig. 2. Time course of the covalent binding of DCE-derived radioactivity in tissue homogenates of the mouse. Animal treatment and values are expressed as in Fig. 1.

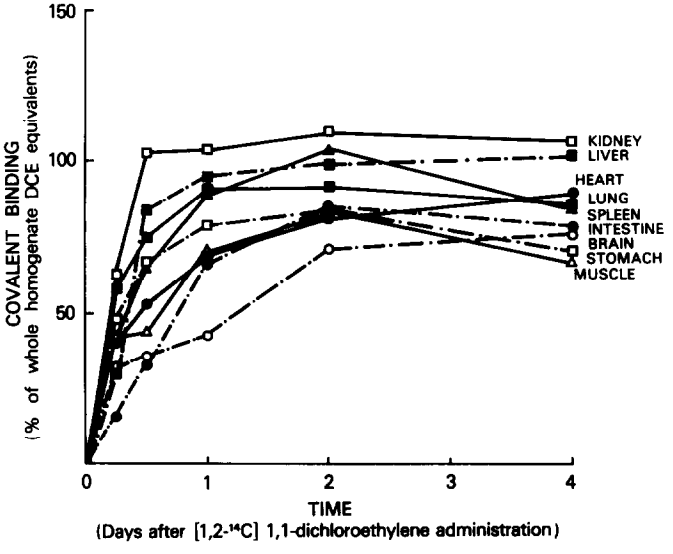

Fig. 3. Time course of the covalent binding of DCE-derived radioactivity expressed as a percentage of whole homogenate radioactivity.

porally, binding peaked in all three organs at 6–12 hr (Fig. 2). It is important to note that the amount of covalently bound material relative to total radioactivity in all tissues except kidney increased gradually to a maximum of 70–100% at 48 hr (Fig. 3). In the kidney, however, covalent binding reached 100% at 12 hr and remained constant at this level over the 4-day period.

Subcellular distribution and covalent binding of DCE-derived radioactivity

The subcellular distribution and covalent binding of radiolabel in kidney, liver, and lung 24 hr after administration of [^{14}C]DCE to mice are shown (Figs. 4 and 5). The radioactivity was non-selectively distributed across the five subcellular fractions in all the tissues; covalently bound material represented 70–100% of total radioactivity. Furthermore, the levels of covalently bound material relative to total radioactivity were nearly identical in the subcellular fractions of the liver while they were uneven in the kidney and lung (Fig. 6). The radioactivity was predominant in the mitochondrial fraction, particularly in the kidney and lung.

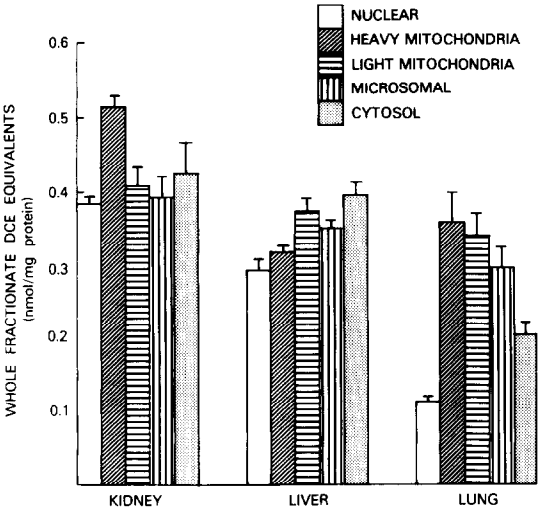

Fig. 4. Distribution of total radioactivity in subcellular fractions of mouse organs 24 hr after a single i.p. dose of [1,2-^{14}C]DCE (~4 μCi, 125 mg/kg). Values shown are the means ± S.E.M. of three determinations. (Tissues pooled from four to eight animals were used for each fractionation.)

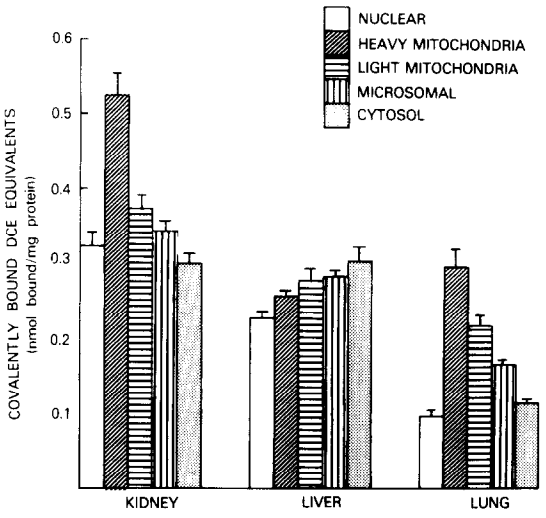

Fig. 5. Covalent binding of DCE-derived radioactivity in subcellular fractions of mouse organs. Animal treatment and values are expressed as in Fig. 4.

Effects of various pretreatments on the covalent binding in vivo *of radioactivity derived from* [^{14}C]*DCE*

Figure 7 presents the effects of various pretreatments on the *in vivo* covalent binding of DCE-derived material in kidney, liver, and lung. Pretreatment of mice with the inducers of microsomal monooxygenases, phenobarbital (PB) and 3-methylcholanthrene (3-MC), produced insignificant

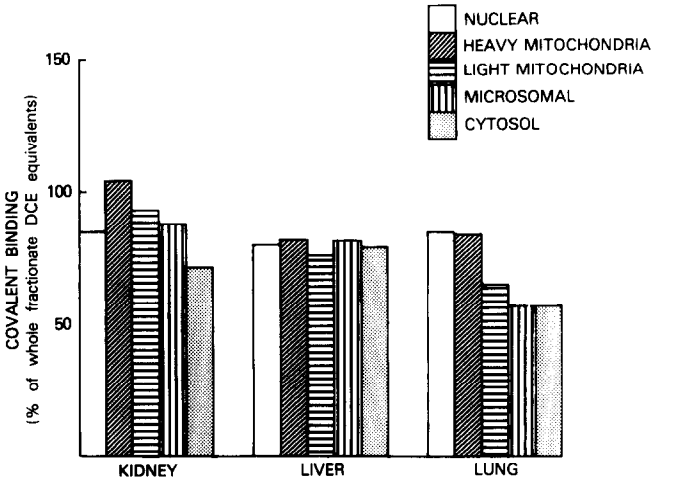

Fig. 6. Covalent binding of DCE-derived radioactivity in subcellular fractions of mouse organs expressed as a percentage of whole fractionate radioactivity.

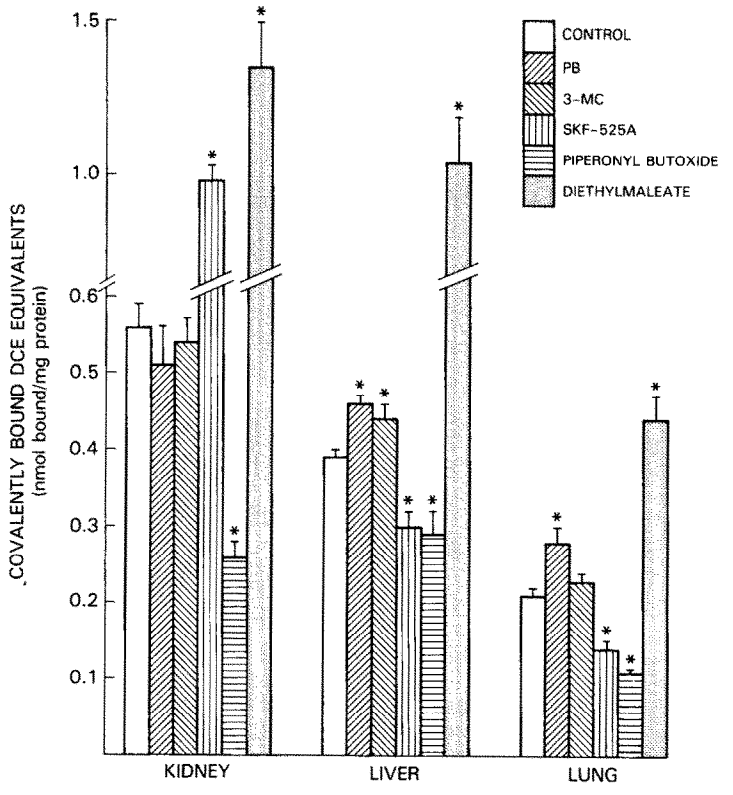

Fig. 7. Effects of inducers (PB and 3-MC) and inhibitors (SKF-525A and piperonyl butoxide) of the monooxygenases, and the GSH depleting agent (diethylmaleate) on the *in vivo* covalent binding of DCE-derived radioactivity in various tissues of the mouse. Animal treatments are as described in Materials and Methods. Values shown are the means ± S.E.M. of four to eight determinations. (Tissues pooled from two to three animals were used for each determination.) (*) Key: significantly different from control, $P < 0.01$ (Student's *t*-test).

changes in the covalent binding of DCE-derived material in kidney. However, PB significantly increased the covalent binding in both the liver and lung, whereas 3-MC significantly increased the covalent binding in the liver but not in the lung. Pretreatment with SKF-525A or piperonyl butoxide, inhibitors of microsomal monooxygenases, significantly reduced covalent binding in the liver and lung. However, in the kidney, SKF-525A significantly increased, whereas piperonyl butoxide decreased, the covalent binding. Diethylmaleate (125 mg/kg, i.p.) given 2 hr prior to [1,2-^{14}C]DCE significantly increased the covalent binding (2- to 3-fold) in all three tissues. The LD_{50} of diethylmaleate was ≥650 mg/kg, and the maximum tolerable dose of diethylmaleate when given in combination with DCE (125 mg/kg, i.p.) was 150 mg/kg, i.p., given 2 hr prior to DCE administration (results not shown). Thus, diethylmaleate has the potential of markedly increasing the toxicity of DCE.

DISCUSSION

Metabolic studies in animals have suggested that the toxicity of DCE is mediated by reactive metabolites produced by the microsomal monooxygenase system [21, 26–28]. The unstable DCE epoxide and other products, like chloroacetyl chloride and monochloracetic acid, have been suggested as possible toxic metabolites of DCE [15, 26, 27]. Recent studies have indicated that the DCE epoxide is a minor metabolite and is also not an obligatory intermediate in the formation of other DCE metabolites [29, 30]. Furthermore, the half-life of the epoxide in aqueous mediums at different pH values is ≤2 sec [29]. Conjugation of the proposed reactive metabolites with GSH is considered as the major detoxification pathway [22, 23]. DCE is known to reduce tissue GSH levels [13, 31–33], and its toxicity is potentiated by fasting or pretreatment with diethylmaleate [20, 21] which reduces tissue GSH levels.

Our studies indicated that DCE-derived radioactivity was widely distributed in all tissues studied but particularly in the kidney, liver, and lung. Peak levels of total radiolabel in these three tissues occurred within 6 hr with the liver showing the highest level of total radioactivity, while covalently bound radioactivity at this same point was highest in the kidney. Furthermore, only 25% of the DCE-derived radioactivity in the liver was covalently bound as compared to 60–65% in the kidney and lung 6 hr after administration (Fig. 3). The *in vitro* rates of microsomal metabolism and covalent binding of DCE in the kidney and lung, organs whose microsomal monooxygenase activities are 6–400 times lower than the liver [11, 12], were negligible or 4 times lower than the liver respectively (unpublished results). These observations suggest the liver to be

the primary site of DCE metabolism from which metabolites are transported to target organs like the kidney and lung and become covalently bound.

From the foregoing, one would expect the liver to show the highest level of covalently bound material. That this was not so may be explained by the extent of detoxification of the metabolites possibly through GSH conjugation and, thus, depends on tissue GSH levels. Hence, the consistently low levels of GSH in the kidney as compared to the liver over a 24-hr period of DCE exposure in mice [13] may explain the higher covalent binding in the kidney. That GSH may be essential for the detoxification of DCE or its metabolites is indicated by the markedly increased covalent binding (2- to 3-fold) in all three tissues on pretreatment of mice with diethylmaleate (Fig. 7). This further suggests that DCE or its metabolites may bind to protein thiol groups. Possible modification of some proteins (DNA replication protein regulators) resulting from such bindings may lead to tumor formation (epigenetic carcinogenesis). More extensive studies on the role of GSH in DCE metabolism and toxicity are presently underway in our laboratory.

Maximum covalent binding of DCE-derived radioactivity in the three tissues occurred within 6–12 hr and preceded any toxic effects reported 24 hr after exposure. This suggests a possible correlation between irreversible covalent binding and toxicity, particularly in the lung where extensive necrosis of the Clara cells of the bronchiolar epithelium coupled with a reduction in pulmonary monooxygenases have been observed at 24 hr [11]. The lack of toxic effects in the kidney and liver, even though they showed higher levels of covalent binding than the lung (2- to 3-fold), suggests a threshold level of covalently bound material for the expression of toxicity. This is supported by the observation that pretreatment with SKF-525A increased the covalent binding in the kidney (2-fold), and this was accompanied by an observed change in color (pale appearance) and increase in size of the kidney which have been reported in respect to a toxic response to DCE only, in ddY strain mice [13]. This variation in response to DCE may be due to strain differences in metabolism; kidney microsomes of ddY strain mice appear to metabolize DCE [13] while those of C57Bl/6N mice do not (our unpublished results). Furthermore, although the lung showed less covalent binding than liver and kidney, its cells may be more susceptible to the toxic insult of DCE. This is in sharp contrast to 4-ipomeanol, a pulmonary toxin, which preferentially accumulates only in lung tissue [34].

Subcellular distribution studies 24 hr after exposure to [1,2-^{14}C]DCE indicated that DCE-derived radioactivity was rather uniformly and nonspecifically distributed across all the fractions mostly in the form of covalently bound material. The association of covalently bound radioactivity with the mitochondrial fraction suggests that it may be a possible primary target of the DCE toxic insult as has been proposed in the rat where initial morphological changes involve damage to the mitochondria with the asssociated increase in hepatic citric acid levels [8, 19]. The fact that the rat showed similar patterns of subcellular distribution and covalent binding [19] but is less susceptible to DCE toxicity may support earlier suggestions that the mouse produces higher levels of DCE toxic metabolites than the rat [14, 15].

The effects of inhibitors or inducers of microsomal monooxygenases on the toxicity of a compound that is metabolically activated *in vivo* are not easily predictable because of possible effects on both toxification and detoxification pathways. For example, although cobaltous chloride inhibits monooxygenase activities, it also enhances tissue GSH levels [35]. PB is known to preferentially induce a specific cytochrome P-450 in liver (form 2) and its dependent monooxygenase activities without any effect on kidney and lung hemoproteins [36, 37], whereas 3-MC selectively induces a different P-450 species (form 4) and its dependent monooxygenase activities in all three tissues [38, 39]. PB pretreatment of rats increases *in vitro* metabolism of DCE by liver microsomes, whereas pretreatment with β-naphthoflavone, a form 4-type P-450 inducer, is without effect [28]. The fact that values for covalent binding in liver of mice pretreated with PB and 3-MC were similar may be explained by an increase in liver glutathione *S*-transferace or epoxide hydrolase activities as a result of PB pretreatment [40].

The decrease in covalent binding of DCE-derived material in the liver and lung after pretreatment of mice with SKF-525A and piperonyl butoxide suggests that metabolism of DCE to an active metabolite is essential for covalent binding and hence DCE toxicity. However, the anomalous increase in covalent binding in kidney as a result of SKF-525A pretreatment as opposed to a decrease by piperonyl butoxide may explain the lethality of DCE observed in the rat [17] and the mouse [18] on SKF-525A pretreatment, and indicates that this may be due to nephrotoxicity. This observation lends support to the view that a second microsomal system, possibly epoxide hydrolase, which converts the toxic epoxide intermediate to less toxic metabolites may be inhibited by SKF-525A [17]. It has been shown that pretreatment of rats with styrene oxide, an inhibitor of epoxide hydrolase, greatly enhances the toxicity of DCE [41].

In conclusion, it may be said that DCE may affect its own metabolism through its effect on tissue GSH levels. Its toxicity may be dependent on the metabolism and covalent binding of metabolites produced predominantly in the liver and transmitted to target tissues, particularly the kidney and lung, with threshold levels of covalently bound metabolites being required for the expression of toxicity. The latter may explain the animal species and strain differences in toxic response to DCE. However, the relation of these findings to human toxicity is unclear. Studies on the metabolism of DCE *in vitro* and the factors affecting it are presently in progress.

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