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METABOLISM OF BROMOBENZENE TO GLUTATHIONE ADDUCTS IN LUNG SLICES FROM MICE TREATED WITH PNEUMOTOXICANTS*

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Abstract—Recent studies showing that the bronchiolar Clara cell and alveolar Type II cell are major loci of cytochrome P-450 monooxygenases in the lung suggested that measurement of xenobiotic metabolizing enzyme activity might provide a useful and sensitive index of injury to these cell types. Accordingly, an assay has been developed for quantitating the rate of formation of reactive bromobenzene metabolites in lung slices which is based upon measuring the rate of formation of bromobenzene glutathione adducts. To demonstrate that monitoring adduct formation would yield quantitatively similar data to the traditional covalent binding assay for measuring the formation of reactive bromobenzene intermediates, covalent binding and conjugate formation were assayed in incubations of phenobarbital-induced hepatic microsomes conducted in the presence of various cytochrome P-450 monooxygenase inhibitors. Incubation conditions which decreased the rate of covalent binding (incubations done in the absence of glutathione) resulted in similar decreases in conjugate formation (incubations done in the presence of glutathione). In lung slices, the metabolism of bromobenzene to glutathione conjugates was linear for 20 min and continued to increase with time over the entire 160 min of the study. The formation of bromobenzene glutathione adducts in lung slices from piperonyl butoxide-treated animals occurred at a significantly lower rate than control. Likewise, lung slices from animals treated with butylated hydroxytoluene or carbon tetrachloride, agents known to injure alveolar epithelial cells, metabolized bromobenzene to glutathione conjugates at significantly slower rates than control. In contrast, treatment with naphthalene or dichloroethylene, agents which damage the bronchiolar epithelial cells, had little or no effect on conjugate formation. Similarly, there were no significant differences in the rate of bromobenzene glutathione conjugate formation between lungs of air- and ozone-exposed (1.0 ppm × 4 hr) mice killed 2, 24, 48, 72, or 120 hr after exposure. These studies suggest that monitoring the rate of bromobenzene glutathione conjugate formation in lung slices may be a useful and sensitive biochemical index of injury to certain cells of the lung but that severe damage to the nonciliated bronchiolar epithelial cells has little effect on the rate of metabolic activation of this aromatic hydrocarbon.

Recent attempts to correlate biochemical and morphologic alterations in rodent lungs after exposure to pulmonary toxicants have been directed at developing a rapid and sensitive method for monitoring the extent of lung damage caused by exposure to both blood-borne and air-borne agents [1–3]. However, massive doses of toxicant are often required before changes in the biochemical status of the lung are observed. In part, this is due to the cellular heterogeneity of the lung and the fact that lung damage by many pulmonary toxicants is highly localized. Use of biochemical measurements as a correlative index of the extent of lung injury has been most successful in experiments that monitor a biochemical function highly localized in those cell types injured by the agent under study. For example, administration of monocrotalin or oxygen, agents which damage capillary endothelial cells (for review see Ref. 4), markedly alters the rate of serotonin uptake [5–7], a process thought to occur exclusively in the capillary endothelial cell.

Reports showing that a major portion of the pulmonary cytochrome P-450 monooxygenase activity is highly localized in Clara and Type II cells [8–10] suggested that damage to bronchiolar or alveolar cell populations might be reflected by a decrease in pulmonary monooxygenase activity. Indeed, recent studies by Boyd *et al.* [11], Tong *et al.* [12, 13] and Krijgsheld *et al.* [14] have shown that administration of pulmonary toxic doses of CCl_4, naphthalene or dichloroethylene decreases cytochrome P-450 as well as the rate of metabolism of several substrates including benzphetamine, 4-ipomeanol and benzo[*a*]pyrene in lung microsomes. However, with the exception of 4-ipomeanol, there is little evidence to suggest that the metabolism of any of these substrates occurs preferentially in Clara vs Type II cells of the mouse.

Previous studies demonstrating that intraperitoneal administration of bromobenzene results in dose-dependent pulmonary bronchiolar necrosis and that radioactivity from [^{14}C]bromobenzene is preferentially bound to bronchiolar epithelial cells [15] suggested that the metabolism of this aromatic

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hydrocarbon to reactive intermediates might occur primarily in the bronchiolar epithelial cells and might, therefore, offer a convenient method of monitoring monooxygenase activity in these cells. However, since methods for measuring pulmonary cytochrome P-450-dependent formation of reactive metabolites from agents like bromobenzene are based on the quantitation of radiolabel bound covalently to protein in microsomal incubations and since lung microsomal yields are low thereby requiring the use of several animals per determination, the studies reported here were done in an effort to develop a convenient method for measuring the pulmonary metabolism of bromobenzene to reactive intermediates in lung slices. The method is based on trapping the reactive bromobenzene-3,4-epoxide with glutathione and measuring the resulting adducts [16]. To determine whether measuring the metabolic activation of bromobenzene in lung slices would provide a sensitive biochemical monitor of injury to bronchiolar or alveolar epithelial cells, we have examined the *in vitro* metabolism of bromobenzene in lungs of animals treated with a variety of pneumotoxic agents whose effects on pulmonary morphology have been studied extensively. We report here that administration of agents which result in extensive necrosis and sloughing of bronchiolar epithelial cells and which have been reported to cause decreases in pulmonary microsomal xenobiotic metabolism has no effect on the formation of bromobenzene glutathione adducts in lung slices while administration of those capable of injuring alveolar epithelial cells results in markedly decreased rates of bromobenzene adduct formation.

MATERIALS AND METHODS

Animals. Male Swiss–Webster mice (20–25 g) were obtained from Charles River Breeding Laboratories, Wilmington, MA. They were housed over hardwood bedding in the UCI vivarium for at least 5 days prior to use and were allowed food and water *ad lib.*

Radiochemical. Bromobenzene[U-^{14}C] was purchased from Amersham Searle, Arlington Heights, IL, with a specific activity of 19.8 mCi/mmole. This material was shown to be greater than 99% radiochemically pure by high pressure liquid chromatography (HPLC) on a C_{18} μBondapak column in 70% methanol/water. [^{14}C]Bromobenzene was diluted with unlabeled compound (Mallinckrodt) to achieve final specific activities of 800–1200 dpm/nmole.

Chemicals. Reduced glutathione, NADP and glucose-6-phosphate were purchased from Calbiochem, La Jolla, CA. Glucose-6-phosphate dehydrogenase and butylated hydroxytoluene were purchased from the Sigma Chemical Co., St. Louis, MO. Dichloroethylene and naphthalene were from Mallinckrodt, St. Louis, MO.

Animal pretreatments. Hepatic microsomes were prepared from animals given 0.1% phenobarbital in the drinking water for 5 days. Twenty-four hours before sacrifice, phenobarbital was replaced with distilled water. All other drugs were diluted in corn oil, and 0.1 ml was administered i.p. per 10 g body weight. Piperonyl butoxide (1600 mg/kg) was administered 1 hr before cervical dislocation. Butylated hydroxytoluene, naphthalene, carbon tetrachloride and dichloroethylene were administered at the doses and times specified.

Microsomal/cytosolic enzyme preparation and incubations. Liver microsomes and cytosolic enzymes containing the glutathione transferases were prepared as described previously [17]. Incubations were prepared on ice in a total volume of 2 ml of 0.1 M sodium phosphate buffer, pH 7.4. They contained: [^{14}C]bromobenzene (0.5 mM) added in 10 μl of acetonitrile, $MgCl_2$ (7.5 mM), cytosolic enzymes (2 mg), microsomes (6 mg), NADPH-generating system [NADP (0.21 mM), glucose-6-phosphate (4.7 mM), glucose-6-phosphate dehydrogenase (0.12 I.U.)], and glutathione (5.0 mM). Inhibitors of the cytochrome P-450 monooxygenases were added prior to incubation as indicated: piperonyl butoxide (0.5 mM in 10 μl of methanol) or SKF-525A (0.5 mM in 100 μl of pH 7.4 sodium phosphate buffer). Substrate was added last, and the incubation vessels were capped and transferred to a shaking incubator at 37° for 30 min. The reaction was stopped by the addition of 2 ml of ice-cold methanol.

Lung slice incubations. Mice were killed by cervical dislocation and the lungs were perfused via the pulmonary artery with 3 ml of ice-cold heparinized saline. The lung was removed, weighed, sliced with a razor blade in 0.5 to 1 mm cubes, and placed in an incubation vessel on ice containing 0.02 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered balanced salt solution, pH 7.4 (NaCl, 140 mM; KCl, 5 mM; KH_2PO_4, 0.4 mM; Na_2HPO_4, 0.3 mM; $CaCl_2 \cdot 2H_2O$, 1 mM; $MgSO_4 \cdot 7H_2O$, 1.0 mM; glucose, 5.5 mM; NaOH, 5 mM). Glutathione (5 mM), the NADPH-generating system and substrate were added, and the vessels were capped and incubated at 37° for the indicated times. The incubation vessels were transferred to an ice bath, the contents of each were homogenized, and 2 ml of ice-cold methanol was added to precipitate the protein.

Covalent binding. Covalently bound radioactivity was assayed in the precipitated protein by procedures similar to those described previously [18]. When no further radioactivity could be detected in the wash, the protein pellet was dissolved with 1 N NaOH in a hot water bath at 60°, an aliquot was added to 5 ml ACS (Amersham Searle), and the samples were counted for 20 min in a Beckman LS-3150T liquid scintillation counter. Quench corrections were made by internal standardization with [^{14}C]toluene. Protein was determined on an additional aliquot [19], and the results were expressed as nmoles bound/mg protein.

Quantitation of bromobenzene glutathione adducts. This method is similar to that reported recently by Monks *et al.* [16]. An aliquot of methanol/water supernatant fraction from the incubations was extracted with 2 vol. of trimethylpentane to remove unmetabolized [^{14}C]bromobenzene. The remaining methanol/water phase was filtered, evaporated to dryness under vacuum, and reconstituted in mobile phase for HPLC analysis. All samples were chromatographed using a Waters Associates M440 UV detector (254 nm), M6000A pump and U6K injector. Samples were chromatographed on either a C_{18}

μBondapak column (0.39 × 30 cm) in a mobile phase of 15% methanol/1% glacial acetic acid/84% water at 1.5 ml/min or on a radial pak (μBondapak) column (0.8 × 10 cm) in 20% methanol/1% glacial acetic acid/79% water at 2.5 ml/min. Column eluant was collected into scintillation vials and radioactivity was determined as described previously. The two bromobenzene glutathione adducts were not well resolved on either system and were collected into a single scintillation vial. The results reported in this manuscript are for total adduct formation.

Ozone exposure. Mice were placed in cages partitioned with wire screens, transferred to a stainless steel, 1 m^3, hexagonal cross-section Rochester-type exposure chamber, and exposed to 1.00 ± 0.05 ppm of ozone for 4 hr at a relative humidity of 39.1 ± 0.4% (mean ± S.E. for ozone concentrations taken at 10-min intervals during the exposure). Ozone was generated from medical grade oxygen by a Sander Type II ozonizer, filtered, and passed into the exposure chamber. Controls were placed in similar cages in a separate chamber and exposed to filtered air.

Morphologic confirmation of pulmonary damage. To confirm reports by others of pulmonary injury by dichloroethylene [14, 20] and carbon tetrachloride [11, 21–23], groups of four mice each were treated with dichloroethylene (100 or 200 mg/kg, i.p.) or carbon tetrachloride (1.25 or 2.5 ml/kg, p.o.) and animals were killed 24 hr later by pentobarbital overdose. Lungs were fixed by tracheal infusion of Karnovsky's fixative and were embedded in paraffin, cut at 5–6 μm, and stained with hematoxylin and eosin. Dose-dependent bronchiolar injury was observed in all animals treated with dichloroethylene or carbon tetrachloride but not in animals treated with vehicle alone. Naphthalene-induced pulmonary bronchiolar injury has been studied extensively by us [24, 25] and by others [12, 13, 26]. In the male Swiss–Webster mouse, a dose of 150 mg/kg naphthalene does not result in detectable lung damage while the 300 mg/kg dose consistently causes extensive exfoliation and necrosis of bronchiolar epithelial cells. Type I alveolar epithelial cell injury illicited by butylated hydroxytoluene has been reported numerous times [27–31]. Lung wet weight 3 days after butylated hydroxytoluene was nearly twice that of controls. Moreover, light microscopic examination of fixed lungs revealed no apparent damage to bronchiolar epithelial cells.

RESULTS

Effect of cytochrome P-450 inhibitors on conjugate formation and covalent binding. To determine whether measuring the rate of conjugate formation (in the presence of glutathione) would provide results quantitatively similar to the traditional covalent binding assay (in the absence of glutathione) for measuring the rate of metabolic activation of bromobenzene, hepatic microsomes from phenobarbital-induced mice were incubated in the presence of several cytochrome P-450 monooxygenase inhibitors. The data in Fig. 1 show that addition of the cytochrome P-450 monooxygenase inhibitors, piperonyl butoxide or SKF-525A, markedly decreased the rate of glutathione conjugate formation (A) and covalent binding (B). Likewise, microsomes incubated in a nitrogen atmosphere or prepared from uninduced animals catalyzed the formation of glutathione adducts and covalently bound metabolites at decreased rates compared to phenobarbital-induced microsomes incubated in air. These results indicate that incubation conditions which result in a decrease in the rate of covalent binding also show a decrease in the rate of conjugate formation.

Time course formation of bromobenzene conjugates in lung slice incubations. The data in Fig. 2 show that the metabolism of bromobenzene to glutathione adducts by mouse lung slices was linear for at least the first 20 min of incubation after which it continued to increase for the entire 160-min incubation period. In identical incubations prepared with 0.1 M sodium phosphate buffer, pH 7.4, conjugate formation was linear for 10 min and proceeded at approximately one-half the rate of incubations conducted in HEPES-buffered salt solution. Covalent binding in lung slice incubations was low and variable and showed only a slight increase with incubation time, thus indicating that a major portion of reactive bromobenzene metabolites were trapped by glutathione in lung slice incubations (data not shown).

Effect of in vivo *treatment with piperonyl butoxide on conjugate formation in lung slices.* Mice were treated with piperonyl butoxide (1600 mg/kg, i.p., 1 hr prior to killing) to determine whether inhibition of the pulmonary cytochrome P-450 monooxygenases would affect the rate of metabolism of bromobenzene to glutathione conjugates in lung slices. The formation of bromobenzene glutathione conjugates in lung slices prepared from piperonyl butoxide-pretreated animals occurred at only 32% of the rate of vehicle-treated controls (control = 10.5 ± 0.5 nmoles per lung per 20 min, mean ± S.E., N = 3).

Effect of in vivo *treatment with various pulmonary toxicants on the rate of conjugate formation in lung slices.* To determine whether lung injury induced by blood-borne agents would alter the rate of metabolism of bromobenzene to glutathione conjugates in lung slices, mice were treated with toxicants previously shown to cause reproducible alveolar or bronchiolar damage in mouse lung. Administration of 200 or 400 mg/kg butylated hydroxytoluene (doses previously reported to cause necrosis of Type I alveolar cells followed by Type II epithelial cell proliferation [27–31]) 72 hr prior to sacrifice resulted in a marked increase in lung wet weight (control = 153 ± 6 vs BHT-400 = 281 ± 13 vs BHT-200 = 214 ± 22 mg/lung means ± S.E., N = 3–5) and a decrease in the rate of bromobenzene conjugate formation in comparison to vehicle-treated animals (Fig. 3). Likewise, treatment with carbon tetrachloride (1.25 or 2.5 ml/kg, 24 hr prior to sacrifice), a compound that injures both Type II and Clara cells [11, 21–23; these studies], resulted in dose-dependent decreases in the rate of formation of bromobenzene glutathione conjugates. In contrast, prior administration of dichloroethylene, at doses which cause necrosis and exfoliation of bronchiolar epithelial cells [14, 20; these studies], resulted in slight but not significant alterations in the rates of conjugate formation. Similarly, metabolism of bromobenzene in lung slices of animals treated with either

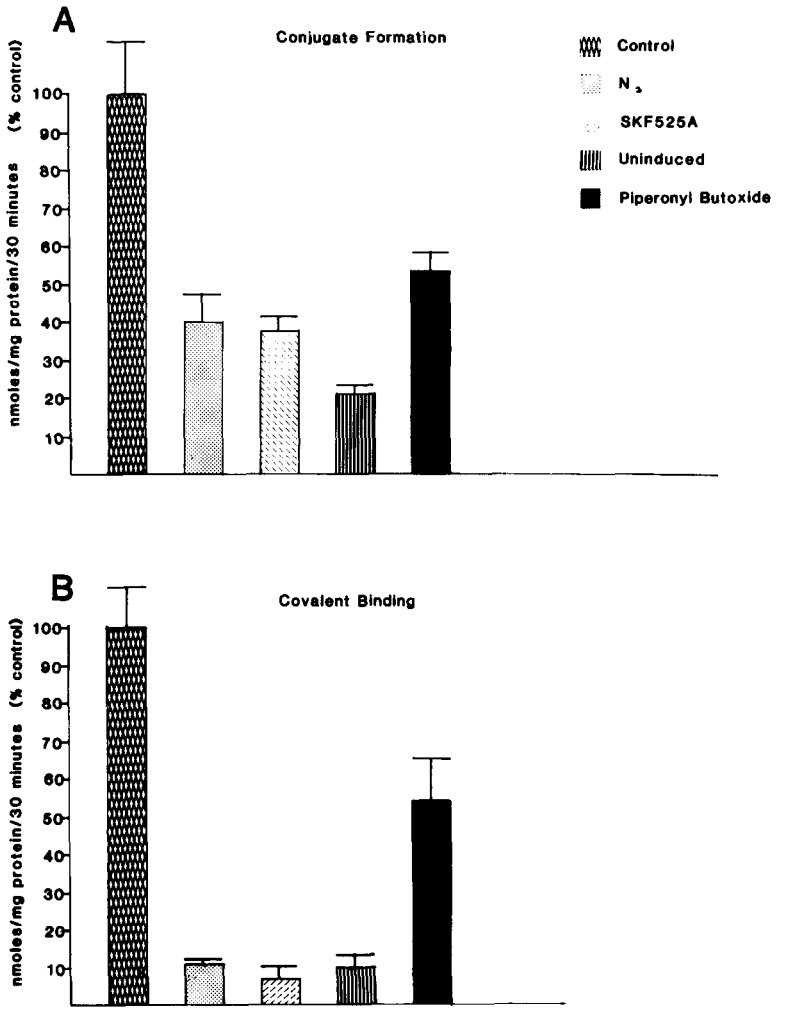

Fig. 1. Effects of various incubation conditions on covalent binding (absence of glutathione) and glutathione conjugate formation (presence of glutathione). Phenobarbital-induced liver microsomes were incubated as described in Materials and Methods. Values for covalent binding or GSH conjugate formation, expressed as percent control, are means ± S.E. of three incubations. Control values for covalent binding were 8.29 ± 0.02 nmoles bound per mg protein per 30 min and for conjugate formation were 8.00 ± 1.12 nmoles conjugate formed per mg protein per 30 min. Separate incubations were performed to determine the effect of piperonyl butoxide, and control values were 5.22 ± 0.57 nmoles per mg protein per 30 min for covalent binding and 8.50 ± 0.22 nmoles per mg protein per 30 min for conjugate formation.

lung toxic or nontoxic doses of naphthalene 24 hr prior to sacrifice was slightly, but not significantly, decreased in comparison to control.

Metabolism of bromobenzene to glutathione conjugates in lung slices at varying times after the administration of naphthalene or butylated hydroxytoluene. To determine if the lack of effect of naphthalene on enzyme mediated bromobenzene glutathione conjugate formation in lung slices was due to the time of animal sacrifice, groups of three mice each were treated with naphthalene (300 mg/kg) and were killed 24, 48 or 120 hr later. The time course effects of butylated hydroxytoluene administration were examined as well. The results shown in Fig. 4 indicate that the rate of bromobenzene glutathione conjugate formation was not affected by pretreatment with naphthalene 24 hr or 48 hr before sacrifice. However, lungs from mice treated with naphthalene 5 days prior to sacrifice showed a slight increase in conjugate formation over the vehicle-treated controls (131% of control). Consistent with the previous studies on butylated hydroxytoluene (Fig. 3), administration of toxic doses of this agent produced a marked decrease in bromobenzene metabolism in lung slices. Butylated hydroxytoluene-induced decreases in bromobenzene glutathione adduct formation were maximal between 24 and 72 hr after toxicant administration and returned to 80% of the control level at 5 days.

Effect of exposure to ozone on the rate of bromobenzene glutathione conjugate formation. Earlier studies with both mice and rats have shown that

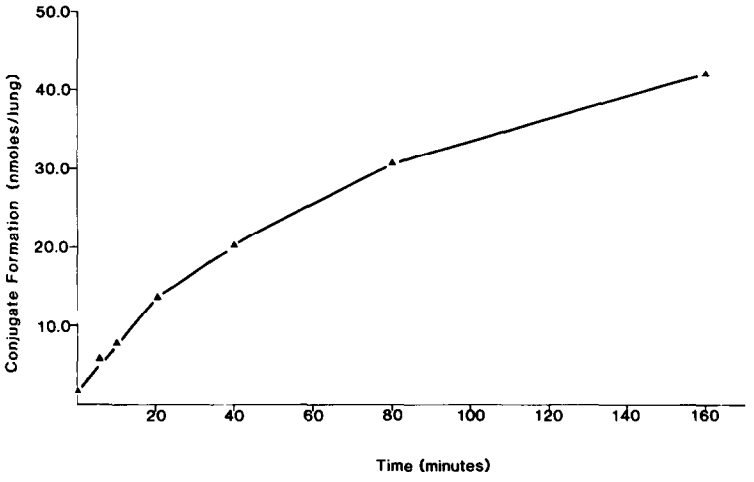

Fig. 2. Time course of bromobenzene glutathione conjugate formation in lung slice incubations containing: [^{14}C]bromobenzene (1 mM), 1 mM glutathione, and an NADPH-generating system. Conjugate formation was determined by HPLC, and the values are expressed as the means for two animals.

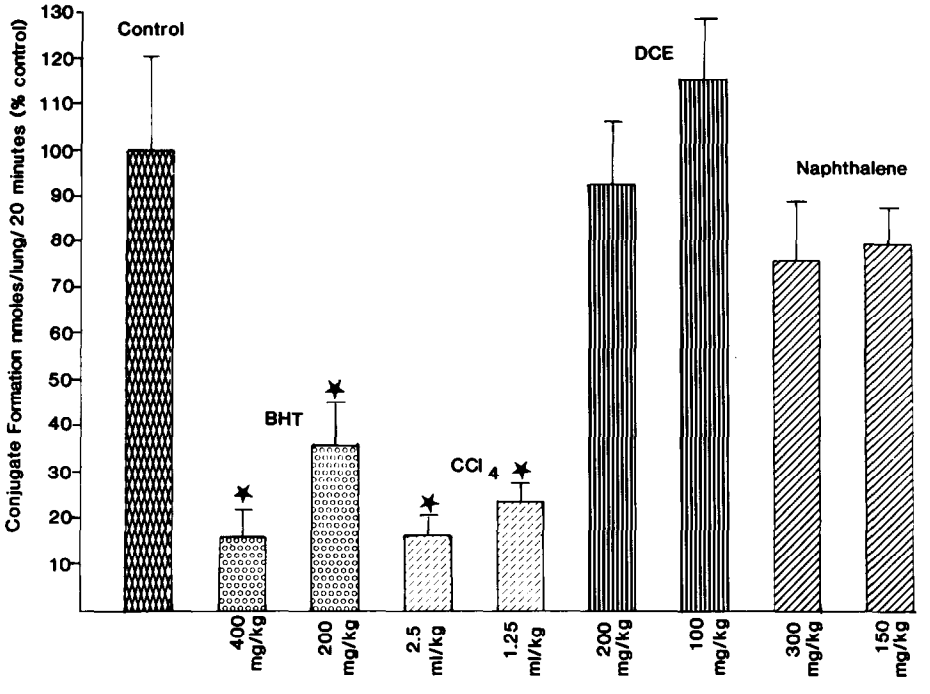

Fig. 3. Effects of prior treatment with various pneumotoxicants 24 hr [carbon tetrachloride (CCl_4), dichloroethylene (DCE) or naphthalene] or 72 hr [butylated hydroxytoluene (BHT)] before sacrifice on the rate of bromobenzene-GSH conjugate formation in lung slice incubations. Results are expressed as percent control and are means ± S.E. for three animals. The control value (nmoles conjugate formed per lung per 20 min) for mice treated with butylated hydroxytoluene, carbon tetrachloride or naphthalene was 15.5 ± 1.6 (mean ± S.E., N = 5) while that for the dichloroethylene-treated animals was 19.1 ± 4.0 (mean ± S.E., N = 3). Stars indicate a significant difference from control ($P < 0.05$, Student's two-tailed t-test).

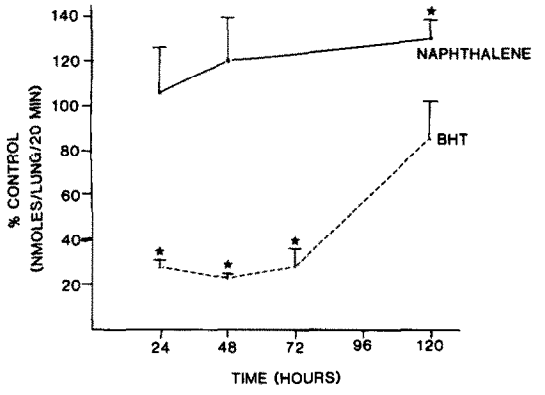

Fig. 4. Formation of bromobenzene glutathione adducts in lung slices from mice treated at varying times before sacrifice with 300 mg/kg naphthalene or 400 mg/kg butylated hydroxytoluene. Values are the mean ± S.E. for three incubations and are expressed as a percent of control. Stars indicate a significant difference from control ($P < 0.05$, Student's two-tailed t-test).

exposure to ozone causes morphological alterations of the nonciliated bronchiolar epithelial cells and, after long exposure, results in hyperplastic foci of this cell type [32–34]. To determine whether exposure to an airborne pulmonary toxicant like ozone would alter the metabolism of bromobenzene at varying times after exposure, the rate of bromobenzene glutathione conjugate formation was assessed in lungs of mice killed at intervals ranging from 2 to 120 hr following exposure. The results (Fig. 5) show that exposure to 1 ppm ozone for 4 hr had no significant effect on the rate of metabolism of bromobenzene to glutathione conjugates in comparison to air-exposed controls at any time point studied.

DISCUSSION

Immunofluorescence studies with antibodies prepared to purified cytochrome P-450 [9] and cytochrome c reductase [35], in conjunction with xenobiotic metabolism studies in lung cell fractions highly enriched in Clara or Type II cells, indicated that a major portion of the lung's capacity to metabolize xenobiotics resides within these cell types [10, 36, 37]. The high degree of localization of cytochrome P-450 monooxygenase activity in Clara cells is consistent with both the vulnerability of this cell type to cytotoxic [8] and carcinogenic [37] chemicals requiring metabolic activation and with the observed decreases in pulmonary cytochrome P-450 monooxygenase activity which occur as a result of chemical-induced necrosis and exfoliation of Clara cells [11–14]. Therefore, it appeared that measurement of the metabolism of a substrate with high affinity for monooxygenases within the Clara cell could serve as a highly sensitive biochemical marker of toxic insult to nonciliated bronchiolar cells. It seemed likely that bromobenzene was being metabolized by Clara cell cytochrome P-450 since bronchiolar epithelial cells had been shown to be target cells for the covalent binding of reactive bromobenzene metabolites and for bromobenzene-induced cytotoxicity [15]. An advantage to bromobenzene over agents like 4-ipomeanol for use as a marker substrate for Clara cell monooxygenases is that radiolabeled bromobenzene is commercially available.

As an alternative to measuring the quantity of radioactivity covalently bound in microsomal incubations, the rate of metabolic activation of bromobenzene was assayed by trapping the reactive intermediate with glutathione and measuring the adducts formed by high pressure liquid chromatography. That this method yields quantitatively similar results to the covalent binding assay was supported by the data showing that covalent binding levels in liver microsomal incubations without glutathione consistently paralleled the rate of glutathione conjugate formation in identical incubations with glutathione (Fig. 1). The advantage to measuring glutathione adduct formation as a means of monitoring the formation of reactive bromobenzene interme-

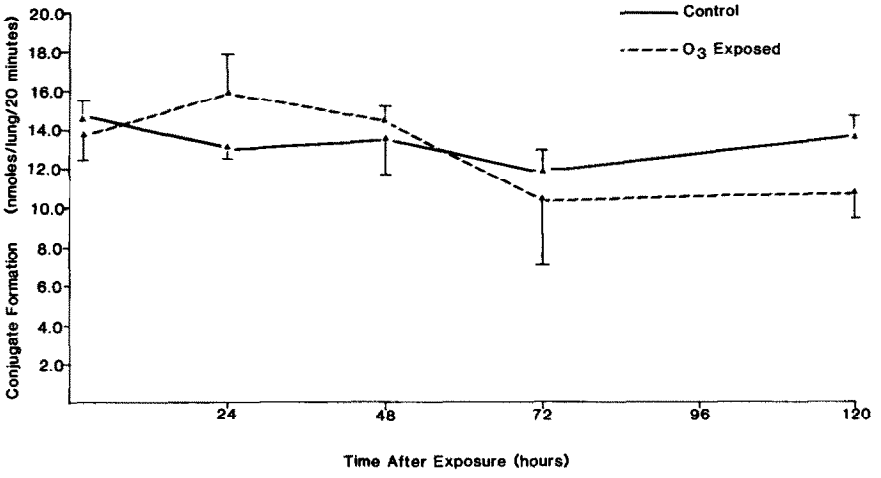

Fig. 5. Effect of a 4 hr × 1 ppm ozone exposure on the rate of metabolism of bromobenzene to glutathione adducts in lung slices. Values are the mean ± S.E. for three incubations of air-exposed (control) and ozone-exposed mice. No significant differences were observed in the rate of bromobenzene metabolism in lungs of air-exposed and ozone-exposed mice ($P < 0.05$, Student's two-tailed t-test).

diates is that the metabolism of this aromatic hydrocarbon can be measured in sliced tissue, thereby enabling determinations to be done on a single mouse lung. For most substrates, lungs from at least five mice would be required for a single measurement of the rates of microsomal metabolism. The data obtained using lung slices is reasonably consistent; standard errors for determinations in three to five animals rarely exceeded 15% of their respective means. The rate of adduct formation in lung slices was decreased substantially by pretreatment with piperonyl butoxide, thus providing further evidence that this assay is measuring monooxygenase activity.

The results of the present studies are in marked contrast to those of Tong *et al.* [12, 13] who have observed substantial decreases in pulmonary cytochrome P-450 levels and in microsomal 7-ethoxyresorufin *O*-deethylase, aryl hydrocarbon hydroxylase and benzphetamine *N*-demethylase after treatment with lung toxic doses of naphthalene. Similar studies by this group also have demonstrated substantial diminutions in lung microsomal metabolism of benzo[a]pyrene and benzphetamine following treatment with dichloroethylene [14]. Neither naphthalene nor dichloroethylene administered to mice at doses which cause extensive necrosis and exfoliation of bronchiolar epithelial cells had any discernible effect on bromobenzene glutathione adduct formation in lung slices. These studies were repeated on several occasions with identical results. These data are surprising, especially in light of the observation that the cells of the bronchiolar epithelium are the target cells for cytotoxicity and covalent binding by bromobenzene metabolites.

In contrast to the results with naphthalene and dichloroethylene, substantial decreases in bromobenzene adduct formation were observed after treatment with butylated hydroxytoluene or carbon tetrachloride (Figs. 3 and 4), toxicants which injure alveolar epithelial cells [20, 22, 23, 27–31]. The observation that carbon tetrachloride treatment results in markedly decreased rates of bromobenzene metabolism is consistent with earlier studies demonstrating marked decreases in pulmonary cytochrome P-450 [19] and 4-ipomeanol metabolism [8] after administration of this agent. The findings with naphthalene, dichloroethylene and butylated hydroxytoluene suggest that carbon tetrachloride-induced inhibition of bromobenzene metabolism in lung slices may be a result of the alveolar injury by this agent rather than the damage to the bronchiolar epithelium.

Although the alterations in bromobenzene glutathione adduct formation resulting from prior treatment with butylated hydroxytoluene or carbon tetrachloride have been attributed to the cytotoxic effects of these two agents, other intrepretations of this data must be considered. The direct interaction of either butylated hydroxytoluene or carbon tetrachloride with the cytochrome P-450 monooxygenases could result in decreases in bromobenzene metabolism which are independent of the cytotoxic effects produced by these compounds in the lung. The addition of butylated hydroxytoluene to the diet (0.5% for 10–15 days) has been shown to result in a marked increase in mouse liver microsomal aniline hydroxylase and a slight decrease in benzo[*a*]pyrene monooxygenase activities [38]. Dietary butylated hydroxytoluene also has been shown to markedly alter epoxide hydrolase activities in mouse liver [38]. However, recent studies indicate that, while butylated hydroxyanisole (an antioxidant producing effects on xenobiotic metabolism similar to those of butylated hydroxytoluene) produced a 3-fold increase in the rate of pulmonary microsomal metabolism of naphthalene to naphthalene dihydrodiol, there was little alteration in the rate of naphthalene glutathione adduct formation*. Whether large doses of butylated hydroxytoluene such as those given in the present study could produce direct inhibition of the metabolism of bromobenzene which is unrelated to the cytotoxic effects of this compound cannot be ruled out by the data in this study.

If the effects of carbon tetrachloride and butylated hydroxytoluene on bromobenzene glutathione adduct formation are associated with the cytotoxic effects of these compounds, the data reported here raise the possibility that reactive intermediates from bromobenzene may not be formed in those cells intoxicated by the compound and to which reactive metabolites are bound but may arise via metabolism in alveolar cell populations. Lau *et al.* [39] have presented evidence recently that bromobenzene-3,4-oxide, the putative toxic intermediate from bromobenzene, can diffuse across intact membranes of the hepatocyte. Likewise, *in vivo* studies have suggested that reactive metabolites of naphthalene formed in the liver can efflux from the site of origin and become bound covalently in extrahepatic tissues [25]. Thus, it is not inconceivable that reactive metabolites of bromobenzene formed in alveolar cells diffuse to bronchiolar cells where they preferentially bind covalently. It is apparent from studies with naphthalene and dichloroethylene [12–14] that significant cytochrome P-450 monooxygenase activity remains in the lung even after almost complete loss of Clara cells. Thus, even though the activity of monooxygenases within other cell types such as the Type II cell may be relatively lower than that in Clara cells, their overall contribution to pulmonary cytochrome P-450 may be significant. The reasons for the vulnerability of bronchiolar cells to toxic metabolites(s) apparently formed in other lung cell types cannot be answered with the currently available information on the biochemistry of alveolar vs bronchiolar cells. However, it is interesting to note that i.p. administration of 2-methylnaphthalene [40, 41] or certain organometallic compounds [42] causes necrosis of Clara cells which does not appear to be mediated by cytochrome P-450 monooxygenase-dependent metabolism of these compounds. The underlying basis for the sensitivity of Clara cells to chemical-induced cytotoxicity, therefore, appears to depend not only upon the high degree of P-450 localization in these cells but upon other factors as well.

Since both alveolar and terminal bronchiolar regions of the lung are target areas for the inhaled toxicants ozone and NO_2 [32, 33] and because mul-

* A. R. Buckpitt, L. S. Bahnson and R. B. Franklin, manuscript submitted for publication.

tiple exposures to such agents cause hyperplasia of bronchiolar cells [34], it seemed likely that exposure to these agents might alter pulmonary monooxygenase activities. Indeed, earlier studies [43, 44] have demonstrated that exposure to high concentrations of ozone over relatively long time periods results in significant decreases in benzo[*a*]pyrene hydroxylase and benzphetamine *N*-demethylase activities respectively. The data presented in Fig. 5 indicate that 4-hr exposures to 1 ppm ozone had no significant effects on the rate of pulmonary monooxygenase-mediated formation of bromobenzene glutathione conjugates. This is interesting in light of recent studies by Graham *et al.* [45, 46] who have demonstrated that 1 ppm × 5 hr ozone exposures result in significant increases in pentobarbital-induced sleeping times in mice. Thus, the effects of ozone on monooxygenase activities in tissues other than the lung may be more significant than the effects in the lung itself.

In summary, the studies reported here present a rapid method for determining cytochrome P-450-dependent xenobiotic metabolism in lung tissue from a single mouse. The rate of metabolism of bromobenzene to reactive intermediates which have a putative role in bronchiolar cytotoxicity and arylation by this agent is not affected by treatment with agents that severely injure bronchiolar epithelium yet is markedly decreased by alveolar epithelial toxicants. Additional work is needed to determine the precise reasons for the vulnerability of the Clara cell to chemical-induced toxic insult.

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