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Bromobenzene Metabolism in Isolated Rat Hepatocytes

$^{18}O_2$ Incorporation Studies

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SUMMARY

Bromobenzene metabolites have been determined in incubations of hepatocytes isolated from untreated, phenobarbital-treated, and β-naphthoflavone-treated rats. The total formation of bromobenzene metabolites was increased 9-fold in incubations with hepatocytes isolated from phenobarbital-treated rats, and the percentage of total metabolites recovered as bromobenzene-3,4-dihydrodiol and 4-bromocatechol was more than doubled, compared to incubations using hepatocytes from untreated rats. The formation of 2-bromophenol and bromobenzene-2,3-dihydrodiol was increased more than 10-fold in incubations of hepatocytes from β-naphthoflavone-treated rats, as compared to those of hepatocytes from untreated rats, but recovery of 4-bromocatechol was unchanged. The mechanism of 4-bromocatechol formation from bromobenzene was investigated by examining the incorporation of ^{18}O from $^{18}O_2$ and $H_2{}^{18}O$ into 4-bromocatechol during incubations of bromobenzene with hepatocytes from untreated and phenobarbital-treated rats. Potential metabolic precursor molecules of 4-bromocatechol were also incubated individually with isolated hepatocytes, in order to clarify their roles in 4-bromocatechol formation. The results of these studies show that 4-bromocatechol is formed in intact cells almost exclusively from bromobenzene-3,4-dihydrodiol, rather than from the bromophenols. The bromophenols are, instead, mostly conjugated. The rapid and extensive conjugation of the bromophenols by intact cells may restrict their role as precursors of 4-bromocatechol, while bromobenzene 3,4-dihydrodiol is well converted into 4-bromocatechol by hepatocytes.

INTRODUCTION

Bromobenzene is a hepatotoxic chemical which causes centrilobular necrosis in laboratory animals (1). Its toxicity has been shown to depend upon bioactivation to a reactive metabolite or metabolites (2, 3). Bromobenzene metabolism has therefore been widely investigated, both as a prototypic halogenated aromatic hydrocarbon, and as a model hepatotoxin.

The oxidative metabolism of bromobenzene is shown in Fig. 1. The initial oxidation of bromobenzene by liver microsomal cytochrome P-450 presumably forms either the 2,3- or 3,4-bromobenzene oxide. Several competing metabolic pathways are then available to the epoxides. They may act as electrophiles, binding to glutathione or to tissue macromolecules; they may rearrange to yield bromophenols; or they may be hydrolyzed by epoxide hydrolase to form dihydrodiols. Bromobenzene-3,4-oxide has long been postulated to be the primary reactive metabolite produced by bromobenzene (4). Formation of bromobenzene-2,3-oxide is considered to be an inactivating pathway of bromobenzene metabolism (5). This is based largely on the observation that 3-methylcholanthrene pretreatment of rats selectively increases formation of 2-bromophenol (presumably via the 2,3-oxide) but decreases bromobenzene hepatotoxicity. Phenobarbital pretreatment, on the other hand, both increases 4-bromophenol formation and potentiates bromobenzene toxicity (4).

From the primary metabolites, i.e., phenols and dihydrodiols, secondary metabolites are formed. For example, CAT[1] is a known metabolite of bromobenzene (6) and it can, theoretically, be formed via either 3,4-DHD or the phenols (Fig. 1). Oxidation of dihydrodiols to catechols has been reported to occur with benzene (7) and the

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[1] The abbreviations used are: CAT, 4-bromocatechol; 2-OH, 2-bromophenol; 3-OH, 3-bromophenol; 4-OH, 4-bromophenol; 2,3-DHD, bromobenzene-2,3-dihydrodiol; 3,4-DHD, bromobenzene-3,4-dihydrodiol; MET-CAT, methoxy-4-bromocatechol; PB, phenobarbital; BNF, β-naphthoflavone; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMS, trimethylsilyl.

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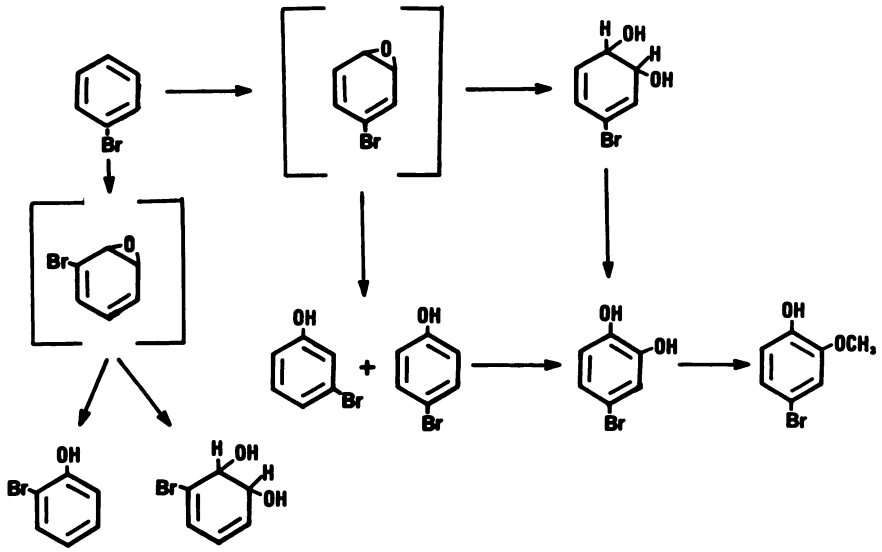

FIG. 1. *Theoretical metabolic pathways of the phase I metabolism of bromobenzene*
The bromobenzene metabolites shown in brackets have not been directly observed; their existence is inferred from the detection of their subsequent products. For simplicity, both 4-OH and 3-OH are shown as products of the 3,4-oxide, although a direct insertion mechanism has not been disproven and chlorobenzene-3,4-oxide is known to rearrange primarily to 4-OH (26).

enzyme responsible, dihydrodiol dehydrogenase, has been isolated from hepatic cytosol in the rat (8) and the mouse (9). The substrate specificity of this enzyme for dihydrodiol substrates in general has, however, not been described. In addition, its role in the overall metabolism in intact cells of aromatic compounds to catechols has not been established.

The present study was undertaken to determine the mechanism of catechol formation from bromobenzene in intact hepatocytes. That is, to elucidate whether 3,4-DHD or the phenol is the precursor of CAT in intact cells. This question is important to understanding the hepatotoxicity of bromobenzene because catechols are readily converted to semiquinones and quinones, which are in general, sufficiently electrophilic to be regarded as suspect toxic agents (10, 11). Indeed, Lau *et al.* (12) very recently concluded that a portion of the radioactivity covalently bound to protein upon incubation of ^{14}C-labeled bromobenzene with rat liver microsomes is due to CAT formation. In addition, Hesse *et al.* (13) reported that [^{14}C]bromophenol (4-OH) is converted to "catechol-like" metabolites which bind covalently to microsomal protein.

The approach used in the present studies to investigate the mechanism of CAT formation was to measure the incorporation of ^{18}O into metabolically formed CAT when bromobenzene was incubated under an $^{18}O_2$ atmosphere or in the presence of $H_2{}^{18}O$. This technique has been previously used to study catechol formation from butamoxane (14) and biphenyl (15). Under these conditions, hydroxylation by cytochrome P-450 is expected to incorporate atmospheric ^{18}O into the molecule, while hydrolysis of epoxides is expected to incorporate ^{16}O from the aqueous medium. Thus, CAT formed by two successive P-450 hydroxylations will contain two atoms of ^{18}O, while CAT formed by aromatization of dihydrodiols will contain one ^{18}O atom from arene oxide formation, and one ^{16}O atom from hydrolysis of the epoxide. The incorporation of ^{18}O into bromobenzene metabolites was monitored by GC-mass spectrometry.

Preliminary studies using this technique showed that CAT formed in isolated hepatocytes from PB-treated rats occurs primarily via oxidation of dihydrodiol (16). However, an important aspect of the studies was to also determine what factors influence catechol formation from bromobenzene by one route or the other. To accomplish all these objectives it was necessary to establish an analytical method to determine all of the phase I (non-conjugated) metabolites of bromobenzene and to determine individually the roles of those metabolites in CAT formation with a variety of conditions. Extensive experiments on bromobenzene toxicity in isolated hepatocytes have been previously reported by Thor *et al.* (17–20), but in these studies the formation of individual bromobenzene metabolites was not measured. During the preparation of this manuscript, Monks *et al.* (21) described the metabolism of bromobenzene in isolated hepatocytes of PB-treated rats using an HPLC method, and showed that significant diffusion of the 3,4-oxide out of the hepatocytes occurs. The present paper describes the metabolic profile of bromobenzene in hepatocytes isolated from untreated, PB-treated, and BNF-treated rats, and shows that CAT is formed in these cells almost entirely via the dihydrodiol, rather than by two successive cytochrome P-450-mediated hydroxylations.

MATERIALS AND METHODS

Chemicals. Bromobenzene and the 2,3- and 4-bromophenols were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. ^{14}C-labeled bromobenzene (20 mCi/mmol, 96% radiochemical purity) was purchased from Amersham Corp., Arlington Heights, IL. CAT was the generous gift of Dr. John W. Daly, Department of Health and Human Services, National Institutes of Health, Bethesda, MD. 3,4-DHD was isolated from incubations of bromobenzene with isolated hepatocytes by using reverse-phase HPLC; the mobile phase was 26% methanol/

0.1% acetic acid. Collagenase (type IV), PB, BNF, bovine serum albumin (type V), and D(+)-galactosamine HCl were purchased from Sigma Chemical Co., St. Louis, MO. Glusulase (β-glucuronidase-arylsulfatase) was purchased from Endo Laboratories, Garden City, NY.

$^{18}O_2$ was purchased from Monsanto Research Corp., Mound Facility, Miamisburg, OH. The isotope composition of the gas, as assayed by the supplier, was as follows: ^{16}O, 2.7%; ^{17}O, 2.1%; ^{18}O, 95.2%. $H_2{}^{18}O$, 99 atom %, was purchased from Bio-Rad Laboratories, Richmond, CA. Regisil (bis(trimethylsilyl)trifluoroacetamide) was purchased from Regis Chemical Co., Morton Grove, IL. All other chemicals used were of reagent grade or better.

Experimental animals. Male Sprague-Dawley rats, 250–350 g were used, and they were purchased from TIMCO Breeding Laboratories, Houston, TX. In some experiments, rats were given PB in the drinking water (0.5%) for at least 1 week prior to preparation of hepatocytes. BNF was dissolved in corn oil and given at a dose of 80 mg/kg intraperitoneally 2 days prior to hepatocyte preparation.

Hepatocyte preparation and incubation. Rat hepatocytes were isolated by perfusing the liver with Krebs-Henseleit bicarbonate buffer containing collagenase as previously described (22). The isolated hepatocytes were suspended in Krebs-Henseleit bicarbonate buffer, pH 7.6, containing 2% bovine serum albumin. Sulfate-free medium was prepared, when required, by omitting magnesium sulfate from the medium and substituting magnesium chloride. The cells were counted using a hemacytometer and light microscope, and their viability was estimated by trypan blue exclusion. Only preparations with viability greater than 85% were used. Unless otherwise stated, hepatocytes were suspended at a concentration of 2×10^6 cells/ml. In some preparations, bromobenzene toxicity was assessed by determination of the release of lactate dehydrogenase activity into the incubation medium. The proportion of total lactate dehydrogenase activity in the medium was determined by comparison to the lactate dehydrogenase concentration of homogenized hepatocytes.

Five-ml aliquots of the hepatocyte suspension were incubated in 50-ml Erlenmeyer flasks. The cells were equilibrated with 95% O_2:5% CO_2 for 15 min prior to addition of the substrate. The flasks were tightly sealed with Teflon-wrapped ground-glass stoppers, and the substrate was added in methanol. The final methanol concentration was 0.25% in all flasks. The incubations were conducted in an orbital shaking water bath (Lab-Line) set at 37° and 85 oscillations/min.

Metabolite analysis. The incubations were terminated by the addition of 0.5 ml of 1 M sodium acetate buffer, pH 5. The samples were then incubated overnight at 37° with 40 μl of Glusulase (3,600 units of β-glucuronidase and 400 units of arylsulfatase) in order to hydrolyze conjugated metabolites. Following hydrolysis, cellular debris was removed by centrifugation, the pellet was washed with water, and the combined supernatant fractions were saturated with NaCl. Bromobenzene metabolites were extracted using three extractions with a total volume of 18 ml of diethyl ether. 4-Hydroxybiphenyl, dissolved in 100 μl of pyridine, was added to the ether extract as an internal standard. The extraction efficiency was determined to be in excess of 98% for the three bromophenols and CAT.

The ether extracts were partially concentrated by heating at 37° and then dried over anhydrous $MgSO_4$. The dried ether extracts were concentrated to approximately 100 μl under reduced pressure at 30–35°, using a Buchler Evapo-Mix equipped with Teflon test tube adaptors. Care was taken that the extracts never went to complete dryness. Fifty-μl aliquots of the concentrated extracts were derivatized by heating for 15 min at 50° with 100 μl of Regisil. The metabolites were analyzed by gas-liquid chromatography using a Hewlett-Packard 5830 A gas chromatograph equipped with an 8-foot × 2 mm glass column packed with 3% SE30 on Gas Chrom Q. The metabolites were separated using a 3°/min temperature gradient which started at 80°, and they were detected by flame ionization.

Incubations of hepatocytes with $^{18}O_2$. Incubations with $^{18}O_2$ required a nonvolatile buffer. Therefore, bicarbonate was omitted from the Krebs-Henseleit buffer, and 25 mM Hepes (pH 7.4) was used instead. These incubations were carried out in specially designed 25-ml flasks equipped with side arms, and Thunberg-type glass stoppers at the top. The top stoppers were connected to glass joints, which allowed the flasks to be connected to a vacuum manifold. The flasks could then be sealed by rotating the top stopper, breaking the connection to the manifold. A 5-ml aliquot of the cell suspension was placed into a flask which was then chilled in an ice bath. Bromobenzene (dissolved in methanol) was placed into the side arm of the flask, and the flask was stoppered. The flask was evacuated to less than 10 torr and then quickly filled with N_2 to restore atmospheric pressure. The procedure of evacuation and flushing was repeated two more times; the flask was evacuated a fourth time, and $^{18}O_2$ was added to atmospheric pressure. All evacuations were conducted at approximately 5°, and pressures inside the flask were monitored by an attached mercury manometer. The flask was then sealed, and the reaction was started by tilting the flask to add the bromobenzene to the cell suspension. The incubations were carried out at 37° for 2 hr, and the reactions were terminated and the metabolites extracted and derivatized as described above. Incubations with $^{16}O_2$ followed the same procedure, using compressed oxygen gas ($^{16}O_2$), rather than $^{18}O_2$.

Mass spectrometry. Gas chromatographic/mass spectrometric analyses were performed on a 5-μl aliquot of the trimethylsilylated samples using a Finnigan 3300 GC/MS/INCOS data system (Finnigan MAT, San Jose, CA). Chromatographic separation was achieved using a glass column (1.8 m × 2 mm i.d.) packed with 3% OV-1 on 100/120 mesh Chromosorb W/HP (Applied Science, State College, PA). The temperature was programmed from 80–270° at 4°/min. Ionization was accomplished by electron impact at 70 eV, and selected ion monitoring was performed on the ions of interest using the MID program of the INCOS data system.

The per cent incorporation of ^{18}O into the metabolites was calculated from areas under the peaks of the selected ion chromatograms. For each metabolite, molecular ions were monitored and the areas under the chromatograms were determined by computer-assisted integration. Per cent incorporation of ^{18}O is expressed as the fraction (×100) of the total area (^{16}O + ^{18}O) represented by the product that was labeled with one or two ^{18}O atoms.

RESULTS

Metabolite profile in isolated hepatocytes. A typical GLC tracing of bromobenzene metabolites is shown in Fig. 2. Seven bromobenzene metabolites were routinely observed in incubations using hepatocytes from PB-treated rats. The phenols (2-OH, 3-OH, and 4-OH) and CAT were identified by comparison of their chromatographic retention times and of their mass spectra to those of known standards. CAT was partially methylated in the cells, and the MET-CAT was identified by its mass spectrum and by comparison with a standard biosynthesized by incubation of CAT with partially purified catechol-*O*-methyltransferase and *S*-adenosylmethionine. Whether the MET-CAT found was 3-methoxy or 4-methoxy was not determined. Bromobenzene-2,3- and 3,4-dihydrodiols were tentatively identified on the basis of their mass spectra (described later). The identifications of the dihydrodiols were confirmed by incubating 2,3-DHD and 3,4-DHD overnight with 1 N HCl at 60°. This procedure converted the metabolites, as expected, into 2-OH and 4-OH, respectively. This behavior is consistent with that expected from dihydrodiols.

The percentage of the various bromobenzene metabolites found to be "free" (not conjugated with glucuronic acid or sulfate) following 120-min incubations was determined to be as follows: 2-OH, 9 ± 1; 3-OH, 8 ± 2; 4-OH, 12 ± 3; 2,3-DHD, 85 ± 8; 3,4-DHD, 87 ± 7; and CAT, 16

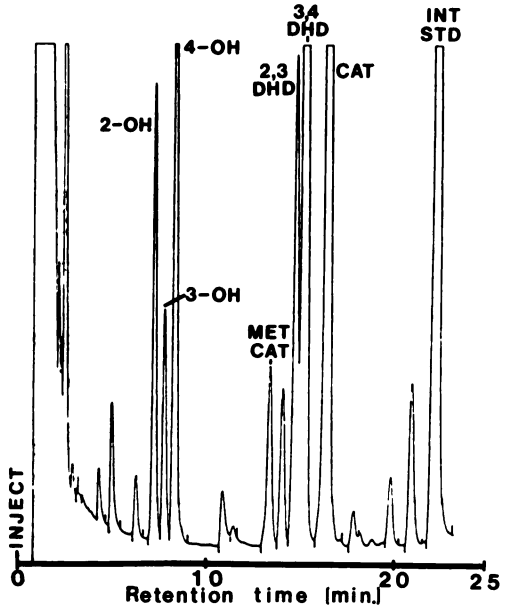

FIG. 2. *A typical gas chromatographic analysis of trimethylsilyl ethers of bromobenzene metabolites*

Hepatocytes from PB-treated rats were incubated for 2 hr with 3 mM bromobenzene. The GC tracing shows flame ionization detector response to the derivatized bromobenzene metabolites and to the 4-hydroxybiphenyl internal standard (*INTSTD*). Peaks not identified represent endogenous compounds also present in extracts of hepatocytes incubated without bromobenzene.

± 2. These results represent mean ± standard error in experiments using three separate hepatocyte preparations. Glutathione conjugates of bromobenzene were estimated by measuring non-ether-extractable radioactivity remaining in the aqueous phase, following enzymatic hydrolysis and ether extraction. Incubations using 3 mM ^{14}C-labeled bromobenzene incubated for 120 min with hepatocytes from PB-treated rats showed that 15–24% of the total bromobenzene metabolites formed were non-ether-extractable. These were presumed to be largely glutathione conjugates.

Characterization of incubation parameters. A preliminary experiment (data not shown) showed that the formation of the various bromobenzene metabolites, except for CAT, was linear with cell concentrations between 1 × 10^6 and 5 × 10^6 cells/ml in hepatocytes isolated from PB-pretreated rats. The proportion of CAT in the total bromobenzene metabolite profile was increased by increasing the cell concentration. This result is consistent with what is expected for the formation of a secondary metabolite. A cell concentration of 2 × 10^6 hepatocytes/ml was used in all subsequent incubations because this concentration produced sufficient quantities of CAT and it was well within the linear range for the formation of the other metabolites.

The time course of bromobenzene metabolism in hepatocytes isolated from PB-treated rats is shown in Fig. 3. Using 3 mM bromobenzene and 10^7 hepatocytes, 3,4-DHD was the major bromobenzene metabolite observed at any of the time intervals examined. Bromobenzene metabolism was found to be linear for 60 min, with total

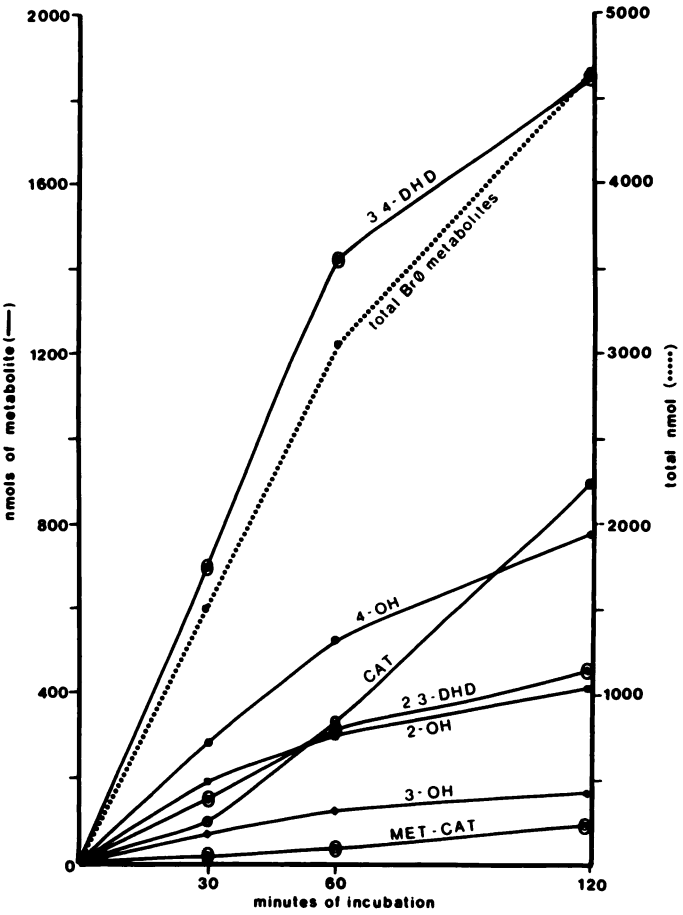

FIG. 3. *The time course of bromobenzene metabolite formation*

Hepatocytes isolated from PB-treated rats were incubated for 30, 60, or 120 min with 3 mM bromobenzene. The results shown represent total nanomoles recovered (mean ± standard error) of each metabolite per 10^7 cells per 5 ml, in three separate experiments.

recoverable metabolites being formed at a rate of 5.0 nmol/min/10^6 cells.

Effect of PB and BNF on metabolic profile. The profile of bromobenzene metabolites was determined in hepatocytes isolated from untreated, PB-treated, and BNF-treated rats incubated with various concentrations of bromobenzene for 120 min. These data are shown in Table 1. In hepatocytes from untreated rats, overall bromobenzene metabolism was maximal at 5 mM bromobenzene, although the increase over metabolism at 3 mM bromobenzene was very slight. Bromophenols were the major bromobenzene metabolites produced by hepatocytes from untreated rats; the combined dihydrodiol and catechol metabolites comprised less than 25% of the total bromobenzene metabolites produced at any of the three bromobenzene concentrations. Bromobenzene dihydrodiol and catechol metabolites were increased greatly by PB pretreatment. Overall bromobenzene metabolism was 9-fold greater in hepatocytes from PB-treated rats than in hepatocytes from untreated rats, and the proportion of the total bromobenzene metabolites isolated as dihydrodiols and catechols was increased to 67%. Bromobenzene metabolism in hepatocytes isolated from BNF-pretreated rats led to the formation of large amounts of 2-OH and 2,3-DHD, as expected. The in-

TABLE 1

Effects of bromobenzene concentration and PB and BNF pretreatment on the bromobenzene metabolite profile

Hepatocytes isolated from untreated, PB-treated, or BNF-treated rats were incubated at 37° for 120 min with 1, 3, or 5 mM bromobenzene. Phenobarbital, 0.5%, was given in drinking water, for at least 7 days prior to hepatocyte isolation. BNF, 80 mg/kg, was injected intraperitoneally 2 days prior to hepatocyte isolation. The data are expressed as nanomoles of product (mean ± SE) recovered with 10^7 cells in a 2-hr incubation. The numbers of separate hepatocyte preparations used were: untreated and PB-treated rats, $n = 5$; BNF-treated rats, $n = 2$.

Substrate	Treatment	2-OH	3-OH	4-OH	MET-Cat	2,3-DHD	3,4-DHD	CAT	Total
1 mM	None	75 ± 12	49 ± 6	118 ± 15	ND[a]	38 ± 6	21 ± 4	16 ± 3	317
	PB	268 ± 30	147 ± 12	659 ± 96	22 ± 13	306 ± 31	1285 ± 180	650 ± 102	3337
	BNF	1210 ± 172	58 ± 10	218 ± 27	ND	429 ± 71	28 ± 1	24 ± 1	1967
3 mM	None	121 ± 14	72 ± 7	175 ± 16	ND	53 ± 6	25 ± 4	18 ± 2	464
	PB	368 ± 35	150 ± 18	834 ± 88	65 ± 25	454 ± 30	1890 ± 297	613 ± 112	4374
	BNF	1603 ± 88	74 ± 5	313 ± 22	ND	682 ± 142	40 ± 4	25 ± 2	2737
5 mM	None	122 ± 26	64 ± 14	171 ± 28	ND	68 ± 18	34 ± 8	16 ± 5	475
	PB	274 ± 45	88 ± 19	430 ± 67	66 ± 23	331 ± 48	1109 ± 149	297 ± 94	2595
	BNF	1167 ± 19	51 ± 5	247 ± 4	25 ± 11	685 ± 247	56 ± 13	17 ± 1	2248

[a] ND not detected. Limit of detection was approximately 5 nmol/incubation flask.

crease in the formation of these metabolites did not lead to significantly increased formation of recoverable catechols.

Bromobenzene at the highest concentration (5 mM) was toxic in cells from all treatment groups. Greater than 90% leakage of lactate dehydrogenase was observed in incubations using hepatocytes from untreated, PB-treated, or BNF-treated rats. One mM bromobenzene was essentially nontoxic in any of the hepatocyte preparations, while 3 mM bromobenzene was moderately toxic to hepatocytes isolated from PB-treated rats and nontoxic in the other hepatocyte preparations. Nontoxic concentrations of bromobenzene were used for all ^{18}O incorporation experiments.

^{18}O incorporation into bromobenzene metabolites. The mass spectra of the TMS derivatives of 4-OH and CAT (Fig. 4) which were formed from bromobenzene in cells were identical to the spectra of authentic standards and gave strong molecular ions which were used for quantitation of ^{18}O incorporation. This was also true for the spectra of 2-OH and 3-OH (data not shown). Fig. 4 also shows the mass spectrum of the TMS-derivatized bromobenzene metabolite which had been tentatively identified as 3,4-DHD. This mass spectrum is entirely consistent with that structure assignment. Strong doublet molecular ions (M^+) at 334 and 336 were observed, due to the approximately equal natural abundance of ^{79}Br and ^{81}Br. The most convincing evidence that this metabolite is indeed 3,4-DHD is the abundant ion at m/z 191. This fragment is characteristic of TMS-derivatized vicinal diol structures, such as dihydrodiol (23), and is attributed to $[(CH_3)_3SiOCHOSi(CH_3)_3]^+$. The GC peak attributed to 2,3-DHD gave a mass spectrum (not shown) similar to the spectrum of the 3,4-DHD. That is, the expected molecular ions were observed at m/z 334 and 336, as was the ion at m/z 191. This spectrum was virtually identical to that recently reported by Monks *et al.* (24).

Table 2 shows the results of single ion chromatography of bromobenzene metabolites formed by hepatocytes incubated with $^{16}O_2$ and $^{18}O_2$. Incorporation of ^{18}O into 4-OH gave the expected results: the mass to charge ratio of the molecular ions of 4-OH was increased by two mass units. The slight alteration observed in the relative intensities of the doublet molecular ions reflects the presence of approximately 3% ^{16}O in the $^{18}O_2$-labeled atmosphere used for these incubations. The results shown in Table 2 are consistent, as expected, with incorporation of one atmospheric oxygen atom into each 4-OH molecule formed from bromobenzene.

As shown in Table 2, incorporation of ^{18}O into 3,4-DHD also yielded the expected increase by two mass units of the molecular ions. These results are consistent with the incorporation of one ^{18}O atom from the atmosphere, and one ^{16}O atom from the aqueous medium, into each 3,4-DHD molecule. The 21% incorporation of ^{18}O into 3,4-DHD in incubations using water enriched 21% with $H_2{}^{18}O$ confirmed that one of the 3,4-DHD oxygens was derived from water.

As shown in Table 2, metabolic incorporation of ^{18}O from $^{18}O_2$ into CAT shifted the doublet molecular ions two mass units from m/z 332,334 to m/z 334,336. This is consistent with the incorporation of one ^{18}O atom into CAT. To determine the percentage of CAT molecules which contained two atoms of ^{18}O, the ratio of the intensities of the M^+ and $M^+ + 2$ ions (m/z 334 and 336, respectively) of unlabeled CAT was compared with the corresponding ratio in CAT formed under an $^{18}O_2$ atmosphere. The increase in this ratio from 5.53% in CAT formed in $^{16}O_2$ to 7.47% in CAT formed in $^{18}O_2$ (Table 2) shows no more than 2% of the CAT formed incorporated two ^{18}O molecules when it was formed in incubations under an $^{18}O_2$ atmosphere. The 21% incorporation of ^{18}O into CAT in incubations using water enriched 21% with $H_2{}^{18}O$ confirmed that one of the CAT oxygen atoms was derived from water.

Incubation of catechol precursor metabolites. The conversion of 3-OH, 4-OH, and 3,4-DHD to CAT in hepatocytes isolated from PB-treated rats is shown in Table 3. Incubation of 250 μM 3-OH or 4-OH with hepatocytes from PB-treated rats did not result in the formation of significant amounts of CAT. The maximum amount of

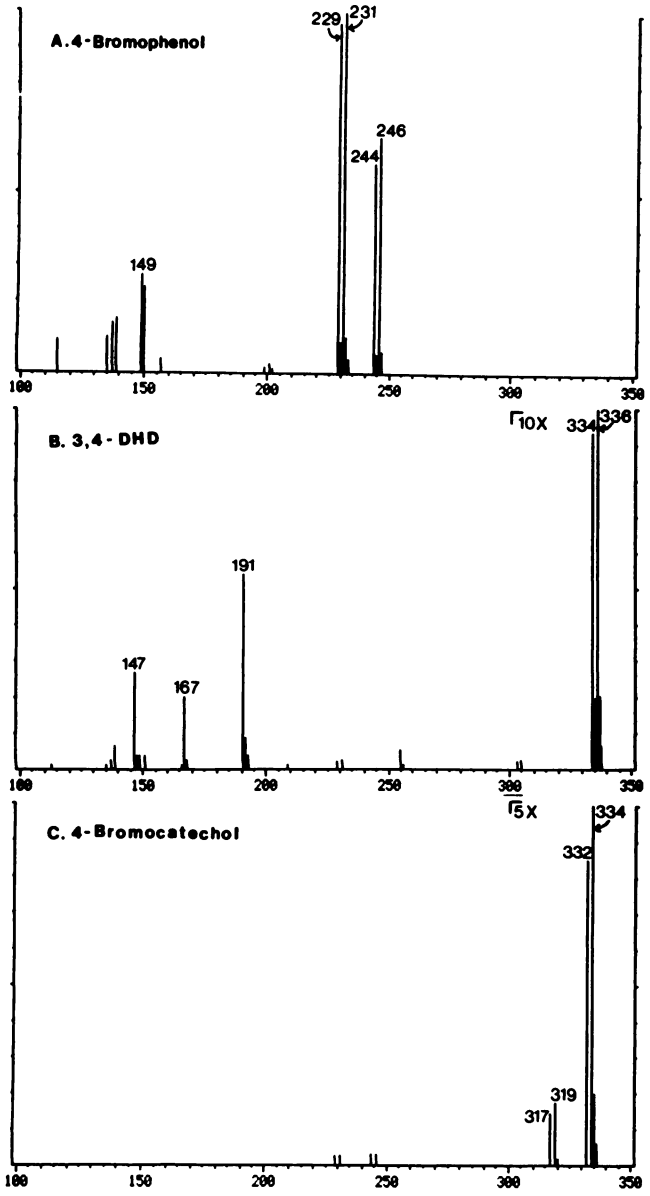

FIG. 4. *The mass spectra of the trimethylsilyl ethers of selected bromobenzene metabolites*

Hepatocytes from PB-treated rats were incubated with 1 mM bromobenzene for 2 hr, using 10^7 cells per 5 ml. The results shown are the mass spectra of the trimethylsilyl ethers of the following bromobenzene metabolites: A, 4-OH; B, 3,4-DHD; C, CAT. The mass spectra shown are for incubations using Hepes-buffered Krebs-Henseleit buffer, pH 7.4, with a standard O_2 atmosphere.

CAT produced in these experiments (23.3 nmol) is less then 4% of the amount of CAT formed by hepatocytes from 1–3 mM bromobenzene. The 250 μM concentration of the bromophenols used in this experiment represents 1.25 μmol/5-ml incubation with 10^7 cells, which is substantially more bromophenol than these cells produce from bromobenzene during a 2-hr incubation (Table 1). In contrast, 3,4-DHD (125 μM) was extensively converted by these cells to CAT, as shown in Table 3.

As shown in Fig. 5, 4-OH, 500 μM, was rapidly conjugated in incubations with hepatocytes from PB-treated rats. Lower concentrations of 4-OH were also nearly completely conjugated, and this was true even in the presence of galactosamine (3 mM), and a sulfate-free medium (data not shown). Galactosamine inhibits glucuronidation by interference with UDP-glucuronic acid synthesis (25). Conjugation of 4-OH at higher concentrations (1 mM) was reduced by incubation with this medium (Fig. 5). Only in this incubation with a very high concentration of 4-OH, 1 mM, in the presence of 3 mM galactosamine, was CAT produced in amounts comparable to those produced from bromobenzene by these cells.

TABLE 2

Incorporation of ^{18}O into bromobenzene metabolites

Hepatocytes isolated from untreated or PB-treated rats were incubated with bromobenzene at 37° for 120 min in Hepes-buffered (pH 7.4) Krebs-Henseleit buffer. $^{18}O_2$ incubations were performed with 97% $^{18}O_2$ atmospheres. Incubations with ^{18}O-enriched water were performed using a final concentration of 21% $H_2{}^{18}O$ in Hepes-buffered (pH 7.4) Krebs-Henseleit buffer. The results shown are the results of selected ion monitoring at the *m/z* of the ions shown. The results are expressed as the relative ion intensities, based upon setting at 100% the area of the most abundant of the ions shown.

	m/z			
	244	246	248	
A. 4-Bromophenol				
$^{16}O_2{}^a$	95.54	100	2.33	
$^{18}O_2{}^a$	3.05	100	95.75	
$^{18}O_2{}^b$	0.49	100	98.08	
	334	336	338	340
B. 3,4-DHD				
$^{16}O_2{}^a$	93.28	100	5.04	0.07
$^{18}O_2{}^a$	3.11	97.48	100	6.57
$^{18}O_2{}^b$		93.18	100	2.59
$H_2{}^{18}O^a$	81	100	21	
	332	334	336	338
C. 4-Bromocatechol				
$^{16}O_2{}^a$	92.38	100	5.53	0.11
$^{18}O_2{}^a$	3.23	96.59	100	7.47
$^{18}O_2{}^b$	0.36	94.29	100	1.50
$H_2{}^{18}O^a$	76	100	21	

[a] Indicates that the incubation was performed using hepatocytes from PB-pretreated rats.

[b] Indicates that the incubations were performed using hepatocytes from untreated rats.

TABLE 3

Formation of CAT from bromobenzene metabolites

Hepatocytes isolated from PB-treated rats were incubated with 3-OH, 4-OH, or 3,4-DHD for 2 hr, using 10^7 cells in a 5-ml total volume. The results are expressed as total nanomoles of CAT recovered per 10^7 cells per 2 hr. These results were obtained from two separate experiments using different hepatocyte preparations. ND, none detected.

Substrate	Concentration	CAT recovered
	μM	nmol
3-OH	250	ND
3-OH	250	Trace
4-OH	250	23.3
4-OH	250	6.0
3,4-DHD	125	259
3,4-DHD	125	327

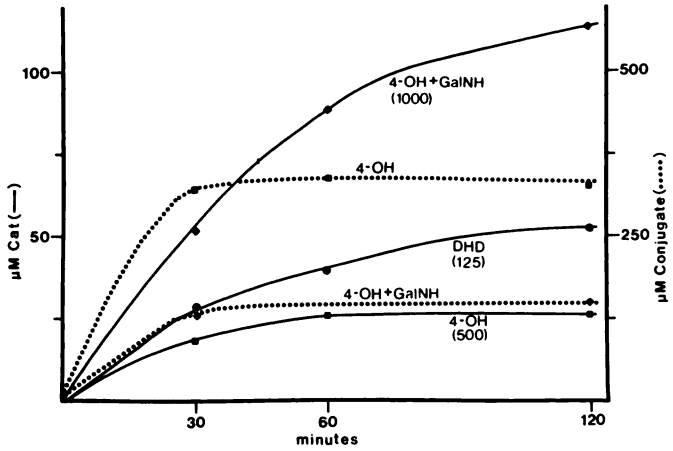

FIG. 5. *Formation of CAT and 4-OH conjugates in incubations of 3,4-DHD and 4-OH with isolated hepatocytes*

Heptocytes isolated from PB-treated rat were incubated for 30, 60, or 120 min with 125 μM 3,4-DHD, 500 μM, 4-OH, or 1.0 mM, 4-OH. All incubations were conducted in sulfate-free Krebs-Henseleit bicarbonate buffer, pH 7.6, in the presence or absence of 3 mM D(+)-galactosamine HCl. The results shown represent the final concentrations in the incubation flask of the metabolites listed.

DISCUSSION

The effects of bromobenzene concentration and PB and BNF treatment on the bromobenzene metabolite profile (Table 1) suggest that the isolated hepatocyte system is indeed appropriate for the study of the mechanism of bromobenzene metabolite formation. The overall rate of bromobenzene metabolism in hepatocytes from PB-treated rats was 5.0 nmol/10^6 cells/min (Fig. 3). This rate compares reasonably closely with the rate of 3.7 nmol/10^6 cells/min reported by Thor *et al.* (18), but is approximately 9-fold greater than that reported by Monks *et al.* (21). The metabolic profile of bromobenzene reported in this paper, using 30-min incubations with hepatocytes isolated from PB-treated rats (Fig. 3) was similar to that reported by Monks *et al.* (21). The bromobenzene metabolite profile showed relatively little change with bromobenzene concentrations ranging from nontoxic to highly toxic although the toxicity was correlated with apparent formation of the 3,4-oxide as reported by Thor *et al.* (19). The reduced formation of metabolites with 5 mM bromobenzene is probably due to loss of cell viability.

The bromobenzene metabolite profile was greatly altered by pretreatments (PB and BNF) known to alter bromobenzene metabolism *in vivo* (4, 6). As shown in Table 1, the recoveries of 4-OH and 3,4-DHD were dramatically increased by PB, presumably due to increased formation of the bromobenzene 3,4-oxide. Analogously, recoveries of 2-OH and 2,3-DHD were increased by BNF via increased formation of bromobenzene-2,3-oxide. However, increased formation of bromobenzene-3,4-oxide appeared to yield largely the dihydrodiol metabolite, while increased formation of bromobenzene-2,3-oxide gave largely the phenol (Table 1). These results suggest that either bromobenzene-2,3-oxide rearranges more rapidly to the phenol than does bromobenzene-3,4-oxide, or, less likely, that the 2,3-oxide is either a less suitable substrate for epoxide hydrolase or a better substrate for glutathione transferase than the 3,4-oxide.

The overall ratio of phenolic metabolites to dihydrodiols was very different in cells from untreated and PB-treated rats (Table 1). Bromophenols constituted more than 75% of the total recoverable bromobenzene metabolites in incubations with cells from untreated rats, but no more than 32% in incubations with cells from PB-treated rats (data from Table 1). Bromobenzene dihydrodiols, on the other hand, represented 21% or less of the total with cells from untreated rats, but 47% or more of the total in incubations with cells from PB-treated rats (data from Table 1). The variation in the proportions of these metabolites provided an appropriate system for investigating the mechanism of CAT formation under widely varying conditions.

Incubation of bromobenzene with hepatocytes under an $^{18}O_2$ atmosphere led, as expected, to very high levels of incorporation of ^{18}O into the bromophenols, while incubations with ^{18}O-enriched water did not. Incubations of bromobenzene with hepatocytes from either untreated or PB-treated rats led to the incorporation of one oxygen atom from the atmosphere, and one oxygen atom from water, into both 3,4-DHD and CAT (Table 2). These results clearly demonstrate that CAT formation in isolated hepatocytes proceeds via the dihydrodiol in hepatocytes from either untreated or PB-treated rats. They further show that 3-OH and 4-OH are not metabolized to CAT to a significant degree in intact cells.

The incubation of potential CAT precursor metabolites (3-OH, 4-OH, and 3,4-DHD) not only serves to support the above conclusion regarding the mechanism of CAT formation, but to suggest why bromophenols are not major CAT precursors in hepatocytes. The results in Table 3 show that neither 3-OH nor 4-OH is well converted into CAT by hepatocytes from PB-treated rats at concentrations somewhat greater than those in which they are formed by cells from bromobenzene. 3,4-DHD, on the other hand, is well converted to CAT even at a lower concentration (Table 3). The rapid and extensive conjugation of 4-OH (Fig. 5) apparently limits its role as a CAT precursor, under normal circumstances. Significant quantities of CAT were produced from 4-OH only under conditions which reduced 4-OH conjugation: high 4-OH concentration (1 mM), the use of sulfate-free medium, and the presence of 3 mM galactosamine. These results suggest that although substantial quantities of CAT may result from microsomal incubations (which are lacking in cofactors for phase II reactions) with 4-OH (12, 13), these experiments do not reflect the dominant route of CAT formation in hepatocytes exposed to bromobenzene.

Bromobenzene dihydrodiol and catechol metabolites have long been known to be excreted in the urine of rats treated with bromobenzene, though in relatively small amounts compared to the bromophenols. For example, Zampaglione *et al.* (6) reported that the relative amounts of 4-OH, dihydrodiols, and CAT in the urine of normal rats treated with bromobenzene are 37, 4, and 6% of the total metabolism, respectively, versus 36, 7, and 9%, respectively, in the urine of PB-treated rats. The results

of the present study of bromobenzene metabolism by hepatocytes isolated from untreated rats were surprisingly similar to the above *in vivo* data: the percentages of total metabolites represented by 4-OH, 3,4-DHD, and CAT were 38, 5, and 4, respectively (data from Table 1, untreated rat, 3 mM bromobenzene). However, the present study found these metabolites to represent 20, 39, and 19%, respectively, of the total bromobenzene metabolites isolated from incubations with hepatocytes from PB-treated rats (data from Table 1, 1 mM bromobenzene).

One possibility for the high yields of CAT and dihydrodiols from bromobenzene in hepatocytes compared to urine is that the 3,4-DHD and/or 3,4-oxide extensively diffuse out of the hepatocytes. *In vivo*, their further metabolism would be limited by their transit time through the liver. In contrast, they would be expected to diffuse readily in and out of the hepatocytes in suspension and to be more extensively metabolized. This suggestion is supported by the data of Monks *et al.* (21), from which they concluded that a large percentage of the 3,4-oxide diffuses from heptocytes. This efflux would limit formation of both 3,4-DHD and CAT and would account for the lower yield of these metabolites *in vivo*.

It is likely, however, that CAT would be formed *in vivo* from 3,4-DHD rather than the phenols, despite these perfusion limitations. This is suggested by our data with hepatocytes from untreated rats, in which 3,4-DHD is a minor metabolite relative to the phenols, but it is essentially the exclusive precursor of CAT under these conditions. The data presented in this paper show that the phenols are not significantly converted to CAT in intact cells, probably because they are rapidly and extensively conjugated (Table 3 and Fig. 5). This is also expected to be true in the *in vivo* situation. Interestingly, Selander *et al.* (26) have concluded that chlorobenzene is converted to 4-chlorocatechol *in vivo* in rabbits via the dihydrodiol rather than the phenol. This conclusion is based on the absence of deuterium in 4-chlorocatechol formed from 4-deuteriochlorobenzene, whereas the 4-chlorophenol retained 54% of the deuterium label (27). Catechol formed from the phenol would be expected to retain at least 50% of the deuterium present in the phenol.

Finally, the relatively low amounts of the dihydrodiol and catechol metabolites found in urine (6) may partially reflect their more extensive conversion to reactive metabolites *in vivo*. Hesse *et al.* (13) have suggested that the incubation of CAT with microsomes leads to the formation of reactive semiquinones or quinones, which are involved in the irreversible binding of bromobenzene in microsomes. During the preparation of this manuscript, Lau *et al.* (12) reported that the addition of 3 mM ascorbate (which reduces semiquinones and quinones to catechols) to microsomal incubations with bromobenzene both doubles the recovery of CAT and decreases by 50% the bromobenzene covalent binding in the microsomal systems. These results, taken together, clearly imply that the formation of reactive metabolites of CAT in microsomes significantly reduces the recovery of CAT in this system. Whether this is also true *in vivo* remains to be examined.

A clear understanding of the mechanism of CAT formation from bromobenzene in hepatocytes may be crucial to understanding the role of CAT in the overall formation of reactive metabolites from bromobenzene. Certainly, the substantial quantities of CAT produced by incubation of bromobenzene with isolated hepatocytes suggest that CAT and its quinone and semiquinone oxidation products should be thoroughly examined for their contribution to bromobenzene covalent binding and toxicity.

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