MOLECULAR PHARMACOLOGY, 27:574–577

Mechanism of Microsomal Metabolism of Benzene to Phenol

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Received November 28, 1984; Accepted February 20, 1985

SUMMARY

The mechanism of microsomal hydroxylation of benzene to phenol has been studied by examining the microsomal metabolism of the specifically deuterated derivative 1,3,5-[$^2H_3$]benzene. Evidence for the formation of the following four products was obtained: 2,3,5-[$^2H_3$]phenol, 3,5-[$^2H_2$]phenol, 2,4,6-[$^2H_3$]phenol, and 2,4-[$^2H_2$]phenol. The presence of 2,3,5-[$^2H_3$]phenol and 2,4-[$^2H_2$]phenol shows that, in the microsomal metabolism of benzene to phenol, a NIH shift had occurred. A deuterium isotope effect (k_H/k_D) of approximately 4 was detected in both the *meta*- and *para*-deuterated phenols. This finding indicates that cyclohexadienone, formed either by isomerization of the epoxide or directly from the enzyme-substrate complex, is a major intermediate in the metabolism of benzene to phenol.

INTRODUCTION

Although benzene is one of the most important compounds in our industrial society, neither the mechanism of metabolism nor the relationship of metabolism to its toxicity is well understood (1, 2). It is commonly assumed that the microsomal cytochrome P-450-catalyzed metabolism of benzene to phenol is via an intermediate epoxide; however, definitive evidence for this intermediate is not available (3, 4). Since migration of a substituent such as deuterium from the site of hydroxylation to the adjacent carbon (NIH shift) (3) is commonly assumed to indicate an intermediate epoxide, we have examined the microsomal metabolism of 1,3,5-[$^2H_3$]benzene for products indicative of substituent migration.

MATERIALS AND METHODS

Materials. 1,3,5-[$^2H_3$]Benzene was synthesized using previously described procedures (5) by Merck Isotope, Inc. (Montreal, Canada). Mass spectral analysis revealed the sample to be 98.4% [$^2H_3$]benzene. Hepatic microsomes were obtained from untreated male CD rats (280 g). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP were products of Sigma Chemical Co.

Incubations and product isolation. In a preliminary experiment, [$^2H_3$]benzene (150 μmol) was incubated with male CD rat liver microsomes (150 mg), EDTA (50 μmol), a NADPH-generating system (6), and sodium phosphate (pH 7.4, 10 mmol) for 0.5 hr. Subsequently, the phenols were extracted into ether and analyzed directly by GC-MS.[1] Even though the data indicated approximately three-fourths of the phenol metabolites contained three deuteriums and approximately one-fourth contained two deuteriums, there were not enough phenol metabolites for a thorough characterization. Subsequently, larger incubations were run for longer periods of time to obtain adequate phenol for a more rigorous analysis. [$^2H_3$]Benzene (600 μmol) was incubated in triplicate at 37° with male CD rat liver microsomes (600 mg), EDTA (200 μmol), a NADPH-generating system (6), and sodium phosphate buffer (pH 7.4, 10 mmol) in a total volume of 200 ml. After 0.5 hr, an additional 600 μmol of [$^2H_3$]benzene was added neat to each incubation mixture and the incubations continued another 0.5 hr. The reactions were terminated by addition of acetone (200 ml). The protein was removed by filtration and the acetone was evaporated under reduced pressure. Subsequently, the phenols were extracted from the aqueous phases with ether (200 ml). The volumes of the ether phases were evaporated to approximately 1 ml under reduced pressure and the phenols were purified by HPLC (Waters Instrument Co.) using a μBondapak C_{18} 7.8 mm × 30 cm column (100% water to 100% methanol in 20 min at a flow rate of 3 ml/min; retention time, 14 min). Approximately 300 μg of the product was isolated from each incubation mixture. This material was used for derivatization and mass spectral analysis. For NMR analysis, the samples were combined and repurified using the same HPLC system. One-half of the repurified phenols (180 μg) was analyzed in CD_2Cl_2 and the other half in $CDCl_3$.

Spectral analyses. Mass spectra were obtained on a Finnigan 4023 GC-MS using temperature programming on a 1.5% OV-17 plus 1.95% OV-210 capillary column (source temperature = 270°; ionization energy = 70 V), a system where separation of the various deuterated phenols was not observed. Analysis of the deuterium content was calculated by a computer program using the mass spectra of the deuterated and nondeuterated phenols. ^1H NMR spectra were obtained at 500 MHz with a Bruker WM 500 spectrometer. Based upon signal to noise, the estimated accuracy for the reported analyses was ±2%.

RESULTS AND DISCUSSION

The phenols from each incubation mixture were analyzed by GC-MS and the deuterium content was determined by processing the data using a computer program to correct for natural isotope abundance (Table 1). Greater than 98% of the phenol was a mixture of [$^2H_2$]phenol and [$^2H_3$]phenol and of this amount, 76% was [$^2H_3$]phenol and 24% was [$^2H_2$]phenol.

To obtain evidence for a NIH shift in the metabolism of 1,3,5-[$^2H_3$]benzene, a small amount of the phenol samples (30 μg/ml in CCl_4) from each incubation mixture

[1] The abbreviations used are: GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography.

0026-895X/85/050574-04$02.00/0

TABLE 1

Mass spectral analysis of deuterium content of phenol metabolite

Each phenol sample was isolated from a separate microsomal incubation mixture. The phenol metabolites were purified by ether extraction followed by HPLC and were further separated and analyzed by GC-MS as described in *Materials and Methods*. The data are presented as per cent abundance of the masses of phenol containing from 0 to 5 deuterium atoms.

Incubation	*m/z*					
	94	95	96	97	98	99
1	1	0	23	75	1	0
2	1	0	23	75	1	0
3	1	0	24	75	1	0

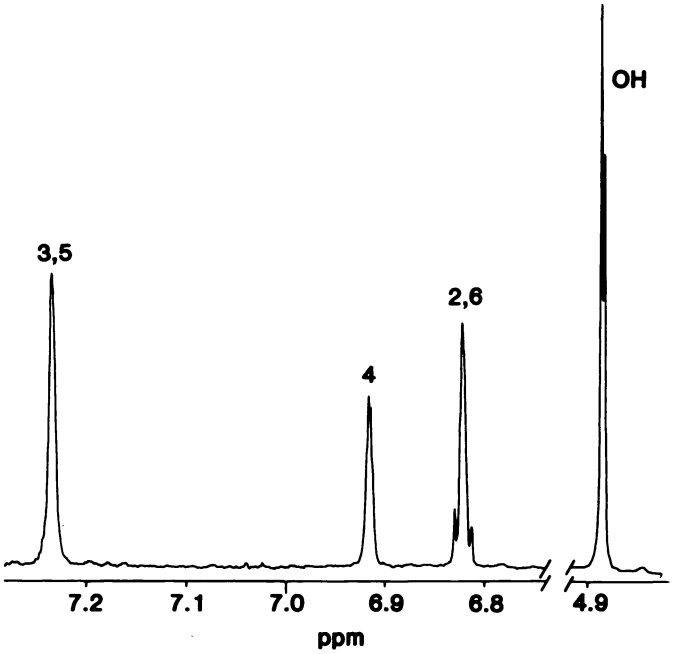

FIG. 1. *500 MHz NMR spectrum of deuterated phenol metabolites*

^1H NMR data were obtained at 500 MHz with a Bruker WM 500 under the following data acquisition conditions: pulse width, 80°; relaxation delay, 100 sec; sweep width, 6K; filter width, 9K; data size, 32K; number of scans, 500. The metabolites (180 μg) were purified as described in the text and the spectrum was run in CD_2Cl_2.

was brominated by addition of an equivalent amount of Br_2 in CCl_4. Separation and analysis of the products by GC-MS revealed that the deuterated *p*-bromophenol was present as a mixture of [$^2H_1$]*p*-bromophenol, [$^2H_2$]*p*-bromophenol, and [$^2H_3$]*p*-bromophenol. Since *p*-bromination of 2,4-[$^2H_2$]phenol (see Fig. 2, *IV*) and 2,3,5-[$^2H_3$]phenol (*I*) would result in [$^2H_1$]*p*-bromophenol and [$^2H_3$]*p*-bromophenol, respectively, the finding of [$^2H_1$]*p*-bromophenol and [$^2H_3$]*p*-bromophenol indicates the presence of these metabolites in the deuterated phenols. Thus, a NIH shift occurred in the metabolism of benzene to phenol. The [$^2H_2$]*p*-bromophenol may be formed by *p*-bromination of either 3,5-[$^2H_2$]phenol (*II*) or 2,4,6-[$^2H_3$]phenol (*III*). Since an isotope effect occurred during bromination (residual phenol after bromination was greater than 76% [$^2H_3$]phenol), these data could be used only for qualitative information.

Quantitative information on product distribution was

TABLE 2

Quantitation of deuterated phenol metabolites

The amount (%) of each compound (*I–IV*) was determined from ^1H NMR spectroscopy peak area measurements and solution of simultaneous equations. Spectra were obtained in CD_2Cl_2 and $CDCl_3$ as described in the legend to Fig. 1. The baseline between the *ortho* doublet and the *ortho* singlet of an enlarged spectrum was estimated using a French curve and all peak areas were measured using a cut and weigh technique. The sum of one-half the *meta* plus the *para* peak areas was equal to 99% of the hydroxyl proton peak area in the spectrum run in CD_2Cl_2. The *meta* proton peak was obscured in the spectrum run in $CDCl_3$. The equations used for quantitation from peak areas were as follows: In CD_2Cl_2: *IV* = *ortho* doublet + (*para* + *meta*/2); *III* + *IV* = *meta*/2 + (*para* + *meta*/2); *I* + *II* = *para* + (*para* + *meta*/2); *I* + 2*II* = *ortho* + (*para* + *meta* + 2). In $CDCl_3$: *IV* = *ortho* doublet + hydroxyl; *I* + *II* = *para* + hydroxyl; *I* + 2*II* = *ortho* + hydroxyl; and *III* + *IV* = 1 − (*I* + *II*).

Metabolite	Solvent	
	CD_2Cl_2	$CDCl_3$
	%	
I. 2,3,5-[$^2H_3$]Phenol	41	42
II. 3,5-[$^2H_3$]Phenol	11	11
III. 2,4,6-[$^2H_3$]Phenol	39	39
IV. 2,4-[$^2H_2$]Phenol	9	8

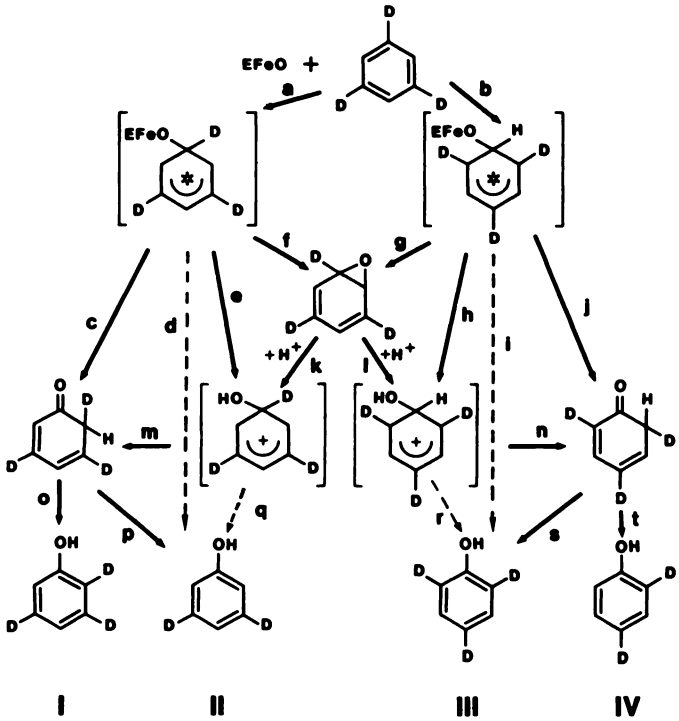

FIG. 2. *Mechanism of metabolism of 1,3,5-[$^2H_3$]benzene*

Pathways *a* and *b* from cytochrome P-450 (*EFeO*) enzyme-substrate complexes. The asterisk indicates a reactive intermediate. Pathways *f* and *g* produce benzene epoxide. Pathways *c*, *j*, *m*, and *n* form cyclohexadienones. The phenol metabolites are: *I* = 2,3,5-[$^2H_3$]phenol, *II* = 3,5-[$^2H_2$]phenol, *III* = 2,4,6-[$^2H_3$]phenol, and *IV* = 2,4-[$^2H_2$]phenol. The data indicate pathways *d*, *i*, *q*, and *r* (dashed lines) are not significant pathways. Adapted from Hanzlik *et al.* (9).

obtained by 500 MHz ^{1}H NMR of the combined samples, a technique which quantitates only the protons. Fig. 1 is the NMR spectrum of this mixture in deuterated methylene chloride. The *ortho*-[2,6], *para*-[4], *meta*-[3,5], and hydroxyl proton chemical shifts were 6.82, 6.91, 7.23, and 4.89 ppm, respectively. The relative intensities were: *ortho* singlet, 0.62; *ortho* doublet, 0.09; *para*, 0.51; *meta*, 0.95; and hydroxyl, 1.00. The area under the *para* proton peak plus one-half of the area under the *meta* proton peak were essentially equal to the area under the hydroxyl proton peak. These data are consistent with the formation of products *I–IV* (Table 2). In a decoupling experiment, the *meta* protons were irradiated and the *ortho* doublet disappeared. Thus, the *ortho* doublet (Fig. 1) was assumed to be exclusively from 2,4-[$^2H_2$]phenol (*IV*). By utilizing the equations shown in the legend of Table 2 and the areas under the peaks, the relative contribution of each metabolite was determined: $I = 41\%$, $II = 11\%$, $III = 39\%$, and $IV = 9\%$ (Table 2).

A number of mechanisms have been postulated whereby aromatic compounds may be hydroxylated (7–10). These mechanisms are: 1) an initial abstraction of hydrogen, either as a hydrogen atom or a hydride ion, followed by reaction with hydroxyl radical or hydroxide ion, respectively, to form phenol; 2) a direct insertion of an oxygen atom across the carbon-hydrogen bond to yield phenol; 3) the addition of activated oxygen to form a tetrahedral intermediate (Fig. 2, *a* and *b*) which rearranges either by a stepwise (Fig. 2, $e + m$ and $h + n$) or concerted mechanism (Fig. 2, *c* and *j*) to the cyclohexadienone which subsequently isomerizes to phenol (Fig. 2, *o*, *p* and *s*, *t*) (addition-rearrangement); and 4) the formation of an epoxide (Fig. 2, $a + f$ and $b + g$) which also isomerizes (Fig. 2, $k + m$ and $l + n$) via the cyclohexadienone to form phenol. If benzene were metabolized by mechanisms 1 and 2, only 3,5-[$^2H_2$]phenol (*II*) and 2,4,6-[$^2H_3$]phenol (*III*) would have been expected to be formed as benzene metabolites. The finding that 2,3,5-[$^2H_3$]phenol (*I*) and 2,4-[$^2H_2$]phenol (*IV*) are significant metabolites (Table 2) indicates that benzene is metabolized by epoxidation (mechanism 4) and/or addition-rearrangement (mechanism 3). Since Tunek *et al.* (4) found a radiolabeled metabolite of [^{14}C]benzene with the thin layer chromatographic properties of the dihydrodiol when excess epoxide hydratase was added to a microsomal incubation mixture, it seems likely that benzene is metabolized to phenol at least in part via the intermediate epoxide.

A deuterium isotope effect was observed in the microsomal metabolism of 1,3,5-[$^2H_3$]benzene ($^2H_3/^2H_2 > 1$). This effect is believed to be derived from isomerization of the cyclohexadienone to the phenol (Fig. 2, *o*, *p* and *s*, *t*). The isotope effect arises from a lower zero point energy of the carbon-deuterium bond versus the carbon-hydrogen bond in the ground state which becomes more equivalent in the transition state. This isotope effect would be classified as a type of primary isotope effect (11). Surprisingly, the retention of deuterium in the isomerization of the two cyclohexadienones (Fig. 2, *o* and *p* versus *s* and *t*) (*I/II* versus *III/IV*) was not significantly different (k_H/k_D or $^2H_3/^2H_2 = 4.0 \pm 0.3$). These data indicate that cyclohexadienone formation is apparently the major pathway in benzene metabolism. If pathways *d*, *i* or *q*, *r* (Fig. 2, dashed lines) or mechanisms 1 or 2 were significant, the ratio of *I/II* would be very different from the ratio of *III/IV*. This primary deuterium isotope effect ($k_H/k_D = 4.0 \pm 0.3$) is very similar to the value ($k_H/k_D = 4.05 \pm 0.2$) reported by Hanzlik *et al.* (9) for a number of substrates for the isotope effect governing retention or loss of deuterium in reactions occurring via NIH shift mechanisms and for the isomerization of 1-[^{2}H]- and 2-[^{2}H]naphthalene oxide to 1-naphthol ($k_H/k_D = 4$) (12).

Not only was the above discussed isotope effect observed in the metabolism of 1,3,5-[$^2H_3$]benzene, but the data (Table 2) suggest preferential hydroxylation at the carbon-deuterium bond ($[III + IV]/[I + II] < 1$). Preferential hydroxylation of $I + II$ versus $III + IV$ is consistent with an inverse isotope effect on formation of the tetrahedral intermediate from sp^2-hybridized aromatic rings. However, the difference between the mass spectral determination of [$^2H_3$]phenol (76%) versus the NMR determination of [$^2H_3$]phenol (80%) indicates there is at least a ±2% error in quantitation and this finding is not significant. This observation is consistent with the previous report that there are not significant differences between the rate of microsomal metabolism of deuterated versus nondeuterated benzene (7) or that deuterium substitution does not significantly alter the rate of isomerization of benzene epoxide to phenol (13).

It is commonly accepted that the toxicity induced by benzene is a result of a reactive metabolic intermediate such as an epoxide, a quinone, or a semiquinone (2, 14–16). Since benzene is oxidatively metabolized to phenol and phenol was found to be noncarcinogenic in the National Cancer Institute bioassay (17), it seems reasonable that benzene epoxide may be the ultimate carcinogenic metabolite. Epoxides of various polycyclic aromatic hydrocarbons are believed to be ultimate carcinogenic metabolites (18). However, the finding by Jung *et al.* (19) that benzene epoxide is not genotoxic, at least in the *Salmonella* assay, indicates further work is required for a more complete understanding of the toxicity of benzene.

ACKNOWLEDGMENTS

The authors thank Dr. Walter Korfmacher for the mass spectral analysis of some samples and Drs. L. Fishbein, F. Kadlubar, F. Beland, and J. McDonald for review of the manuscript.

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