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Microsomal Biphenyl Hydroxylation: the Formation of 3-Hydroxybiphenyl and Biphenyl Catechol

RUTH E. BILLINGS[1] AND ROBERT E. MCMAHON

Lilly Research Laboratories, Indianapolis, Indiana 46206

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SUMMARY

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3-Hydroxybiphenyl has been identified as a metabolite of biphenyl incubated with liver microsomes. Liver microsomes from hamster, mouse, and rabbit form 3-hydroxybiphenyl as well as 2-hydroxybiphenyl and 4-hydroxybiphenyl. The ratio of 2-hydroxybiphenyl to 3-hydroxybiphenyl is about 2:1 with hamster and rabbit microsomes and 1:1 with mouse microsomes. The major metabolite in all three species is 4-hydroxybiphenyl, but its relative amount also depends upon the species. Control rat liver microsomal hydroxylation of biphenyl yields 4-hydroxybiphenyl almost exclusively. 3-Methylcholanthrene or β-napthoflavone treatment of rats preferentially induces 2-hydroxybiphenyl formation, whereas increased amounts of 3- and 4-hydroxybiphenyl are formed after administration of phenobarbital. These results indicate that 3-hydroxybiphenyl is formed by a pathway different from that of either 2- or 4-hydroxybiphenyl. The existence of isotope effects for 3-hydroxybiphenyl formation but not for 2- or 4-hydroxybiphenyl formation from perdeuterobiphenyl suggests that this hydroxylation occurs at least partially via a direct hydroxylation pathway. In addition to the monohydroxylated products of biphenyl, the microsomal oxidation of biphenyl yields the catechol, 3,4-dihydroxybiphenyl. This same catechol is produced by the hydroxylation of either 3- or 4-hydroxybiphenyl. Studies with ^{18}O suggest that 3,4-dihydroxybiphenyl is formed from biphenyl via two consecutive hydroxylations.

INTRODUCTION

The metabolism of biphenyl by hepatic oxidases *in vitro* has been studied in considerable detail (1-5). These studies have shown that the principal routes of the metabolism of biphenyl *in vitro* are hydroxylations to 4-hydroxybiphenyl (major route) and 2-hydroxybiphenyl (minor route). These studies were all performed using fluorescence technology or thin-layer chromatography for detection and quantitation. In a 1976 preliminary communication from this laboratory it was reported that by the use of a gas-liquid chromatographic assay, 3-hydroxybiphenyl could also be detected (6). Raig *et al.* (7) have also recently reported the presence of 3-hydroxybiphenyl when biphenyl is incubated with liver fractions from several species. Studies *in vivo* in several species have shown that 2-, 3-, and 4-hydroxybiphenyl are excreted as conjugates after biphenyl administration (8-10). Preliminary studies *in vitro* of species differences and the effects of inducers and inhibitors suggested that the 3-hy-

[1] Present address: The Toxicology Center, Department of Pharmacology, The University of Iowa, Iowa City, Iowa 52242.

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droxylation of biphenyl might involve a mechanism different from that of 2- and 4-hydroxylation (6). It is generally accepted that the formation of phenolic metabolites from aromatic compounds by hepatic microsomal oxidases proceeds via an arene oxide pathway and that this pathway does not lead to significant isotope effects, since carbon-hydrogen bonds are not broken in the oxidation step (11, 12). However, studies of the deuterium isotope effect on a large number of aromatic substrates (11) have shown that the *m*-hydroxylation *in vivo* of a very limited number of substrates does result in significant isotope effects. It has been proposed that these *m*-hydroxylations proceed by direct oxygen insertion (11–13) in a manner much like that of aliphatic hydroxylation (14). Although studies *in vivo* of deuterium isotope effects in the *m*-hydroxylation of biphenyl were negative (11), these studies have now been done with hepatic microsomes in the light of our previously reported results (6). In addition to the investigation of the monohydroxylation of biphenyl, the dihydroxylation of biphenyl has been investigated with respect to product identification and reaction mechanisms. In particular, catechol formation (3,4-dihydroxybiphenyl) is shown to proceed via successive monohydroxylation.

MATERIALS AND METHODS

Chemicals. Biphenyl, 2-hydroxybiphenyl, and 4-hydroxybiphenyl were obtained from Aldrich Chemical Company. 3-Hydroxybiphenyl was a generous gift from Dr. Russell A. Prough, University of Texas. 3,4-Dihydroxybiphenyl and 3-methoxy-4-hydroxybiphenyl were synthesized by Dr. Frederick Marshall of the Lilly Research Laboratories according to published methods (15). All biochemicals were purchased from Sigma Chemical Company except for *S*-adenosyl[*methyl*-^{14}C]methionine, which was supplied by New England Nuclear. Biphenyl-d_{10} was obtained from Merck Sharp & Dohme. Its isotopic purity was determined by field ionization mass spectrometry to be greater than 95% d_{10}. $^{18}O_2$ was purchased from Miles Laboratories. Pregnenolone 16α-carbonitrile was a gift from Dr. Aziz Karim of Searle. All other chemicals were of analytical quality and were purchased from commercial suppliers.

Animals. Male Sprague-Dawley rats (180–220 g) and white Swiss mice (25–30 g), obtained from Laboratory Supply, Indianapolis, were used throughout these studies. Other species used included Golden Syrian hamsters (80–110 g) and New Zealand rabbits (2–2.5 kg).

Incubations. Livers were removed from animals that had been fed ad libitum and were homogenized in 4 volumes of 1.15% KCl containing 0.01 M phosphate buffer, pH 7.4. The 9000 × *g* supernatant fraction or microsomes were prepared by differential centrifugation. For incubation the microsomal pellet was resuspended in the original volume of 0.1 M phosphate buffer, pH 7.4. These homogenate fractions were used on the day of their preparation.

Each incubation (5 ml) consisted of 2.5 ml of 9000 × *g* supernatant fraction or microsomes, 250 μmoles of phosphate buffer (pH 7.4), 4 mM $MgCl_2$, an NADPH-generating system, and 1 mM biphenyl in 25 μl of dimethyl sulfoxide. This amount of dimethyl sulfoxide did not affect either the rate of biphenyl hydroxylation or the ratio of hydroxy isomers formed. With microsomes, the NADPH-generating system consisted of 0.5 mM $NADP^+$, 10 mM sodium DL-isocitrate, and 0.1 mg of isocitrate dehydrogenase (pig heart, 2 units/mg, Boehringer/Mannheim). When the 9000 × *g* supernatant fraction was employed, the NAPDH-generating system consisted of 0.5 mM $NADP^+$ and 2 mM glucose 6-phosphate.

Incubations were conducted at 37° in a water bath shaking at 120 oscillations/min. The formation of all three biphenyl hydroxy isomers was linear with time up to 15 min, and it was linear with enzyme concentrations when up to 1 g of liver was used. Microsomes from 1 g of liver contained about 35 mg of protein (biuret method). The same rates of hydroxylation and isomer ratio were obtained with the 9000 × *g* supernatant fraction and microsomes. All incubations were conducted in duplicate.

Analysis. Incubations were terminated by the addition of 0.25 ml of 5 N HCl. The precipitated protein was removed by centrifugation, then the solution was extracted twice with 7 ml of distilled *n*-butyl chloride. The combined extracts were concentrated under vacuum to 1 ml and then extracted with 5 ml of 0.1 N NaOH. The NaOH was acidified with 0.6 ml of 5 N HCl and extracted twice with 4 ml of *n*-butyl chloride. The combined extracts were then evaporated to dryness under vacuum. The back-extraction into NaOH and re-extraction from acid were necessary to eliminate a contaminant that interfered with the determination of 3-hydroxybiphenyl. However, these steps were not necessary when biphenyl catechol was to be assayed, and they were omitted.

The dried *n*-butyl chloride extracts were dissolved in 0.1 ml of Regisil [bis(trimethylsilyl)trifluoroacetamide, Regis]. After standing at room temperature for 1 hr the solutions were analyzed by gas chromatography, using a Hewlett-Packard model 402B gas chromatograph equipped with dual flame ionization detectors. A 4-foot column packed with 3% OV-1 on Gas-Chrom Q (Applied Science Laboratories) was used. The flame detector and flash heater were maintained at 230°. The helium flow rate was 60 ml/min. The oven temperature was raised from 160° to 200° at a rate of 10°/min, starting 2 min after the sample was injected. 2-, 3-, and 4-Hydroxybiphenyl formed in the incubations were quantified from a standard curve constructed by adding authentic compounds to incubation mixtures and carrying these samples through the entire analytical procedure.

Mass spectra were obtained using an LKB 9000 combined gas chromatograph–mass spectrometer equipped with a similar gas chromatograph column.

Thin-layer chromatography was done using coated silica gel 60 F254 plates (Merck). The plates were developed with a benzene–ethyl acetate (9:1) solvent system.

Mass spectral analysis of deuterated hydroxybiphenyls. To determine the existence of isotope effects in the hydroxylation of biphenyl, a 1:1 mixture of unlabeled (d_0) and deuterium (d_{10})-labeled biphenyl was incubated for 10 min with microsomes. The isotopic ratio of the hydroxylated metabolites was then determined in extracts from these incubations. For this purpose, mass fragmentography was done using an LKB 9000 combined gas chromatograph-mass spectrometer equipped with an accelerating voltage alternator. This methodology was necessary since deuterated hydroxybiphenyl isomers were found to have a considerably shorter retention time than the unlabeled compounds. This phenomenon led to substantial inaccuracy in determination of the isotopic composition of deuterated and unlabeled compounds when the entire spectrum was recorded. For the mass fragmentographic analysis, the accelerating voltage alternator was adjusted to monitor the *m/e* 242 and *m/e* 251 ions, which are the molecular ions of the trimethylsilyl ether of unlabeled and d_9-labeled hydroxybiphenyl. The frequency rate of monitoring each ion was set at 3/sec.

A standard curve was constructed by measuring *m/e* 242:251 ratios of solutions containing known amounts of unlabeled and d_9-labeled hydroxybiphenyls. From this curve, it was determined that a 1:1 mixture gave an *m/e* 242:251 ratio of 1.09 ± 0.03. This ratio was the same for all three hydroxybiphenyl isomers. The difference between molar ratio and peak height ratio probably reflects differences between hydroxybiphenyl and hydroxybiphenyl-d_9 in the yield of the M^+ ion in the mass spectrometer. This systematic error has been corrected for by use of the standard curve to calculate the ratio of unlabeled to d_9-labeled hydroxybiphenyl formed in the incubations.

RESULTS AND DISCUSSION

Figure 1 shows the gas chromatographic analysis of a silylated extract from incubation of biphenyl with mouse liver 9000 × *g* supernatant fraction. The three peaks in this chromatogram correspond to authentic samples of 2-, 3-, and 4-hydroxybiphenyl. The identities of the peaks were further confirmed by gas chromato-

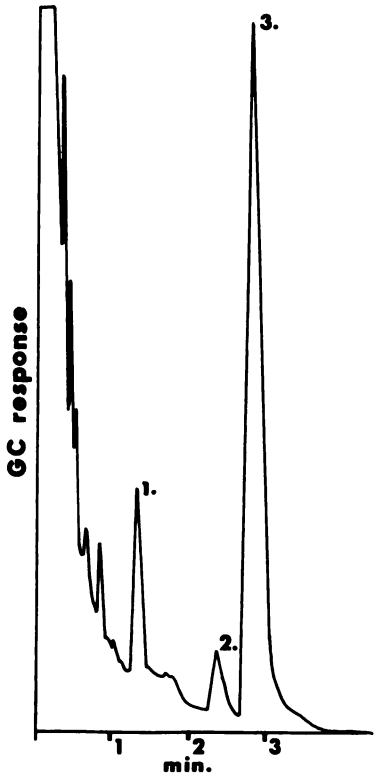

FIG. 1. *Gas chromatographic (GC) analysis of trimethylsilyl ethers of hydroxybiphenyl isomers formed by liver microsomal hydroxylation of biphenyl*

Biphenyl was incubated for 10 min with the 9000 × *g* supernatant fraction from 500 mg of mouse liver. Peaks 1, 2, and 3 correspond, respectively, to authentic samples of 2-, 3-, and 4-hydroxybiphenyl.

graphic-mass spectrometric analysis.

3-HO-BP[1] was formed from biphenyl with the hepatic 9000 × *g* supernatant fraction from rabbit, hamster, and mouse (Fig. 2). With the rat liver 9000 × *g* supernatant fraction, the 3-isomer was not detectable under the conditions reported in Fig. 2. However, when the 9000 × *g* supernatant fraction from 10 g of liver was incubated with biphenyl for 30 min, 2.1 μg of 3-HO-BP were formed along with 3.0 μg of 2-HO-BP and 160 μg of 4-HO-BP. The results shown in Fig. 2 confirm the observations of others that 4-HO-BP is the major microsomal metabolite of biphenyl in all species that have been studied (1-4). In addition, the 2-HO-BP:4-HO-BP ratio is known to be species-dependent. Our results indicate that the 2-HO-BP:3-HO-BP and 3-HO-BP:4-HO-BP ratios are also dependent upon the species.

Characteristics of biphenyl 3-hydroxylation. Biphenyl 3-hydroxylation as well as the formation of 2-HO-BP and 4-HO-BP requires NADPH (Table 1). However, addition of NADH stimulated the hydroxylations.

The formation of all three hydroxy iso-

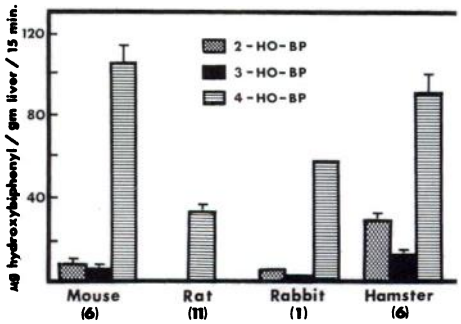

FIG. 2. *Species differences in hydroxylation of biphenyl*

The 9000 × *g* supernatant fraction from 500 mg of liver was incubated for 15 min with 1 mM biphenyl. The number of experiments is shown in parentheses.

TABLE 1

Cofactor requirement for hydroxylation of biphenyl with mouse liver microsomes

Microsomes from 500 mg of mouse liver were incubated for 10 min with 1 mM biphenyl. The concentration of all cofactors was 0.25 mM. NADPH was generated in the incubations from $NADP^+$ and isocitrate dehydrogenase.

Cofactor	2-HO-BP[a]	3-HO-BP[a]	4-HO-BP
	μg/500 mg liver/10 min		
None	0	0	0.38
NADH	0	0	2.66
NAD^+	0	0	0.50
$NADP^+$	0	0	1.54
NADPH[b]	2.46	1.46	31.30
NADPH + NADH	3.52	2.70	54.34

[a] The minimum amount detectable was 0.25 μg.

[b] There was no increase in the hydroxylation rates when the concentration of NADPH was increased to 0.5 mM.

[1] The abbreviations used are: 2-, 3-, and 4-HO-BP, 2-, 3-, and 4-hydroxybiphenyl; SKF 525-A, diethylaminoethyl diphenylpropylacetate; DPEA, 2,4-dichloro-6-phenylphenoxyethylamine; 7,8-BF, 7,8-benzoflavone (α-naphthoflavone).

mers was inhibited by typical microsomal monooxygenase inhibitors such as SKF 525-A and DPEA (Table 2). The involvement of the cytochrome P-450-mediated monooxygenase system is also suggested by the observation that carbon monoxide inhibited the three hydroxylations. 7,8-Benzoflavone was a weak inhibitor of 3- and 4-hydroxylation but stimulated 2-hydroxylation in microsomes from untreated mice (Table 3). However, 7,8-BF inhibited 2-hydroxylation in microsomes from β-naphthoflavone (5,6-benzoflavone)-treated mice and in hamster liver microsomes. These results are consistent with the idea that 7,8-BF inhibits the polycyclic hydrocarbon-inducible form of the monooxygenase system, but not other forms (16–18). The ability of 7,8-BF to inhibit microsomal hydroxylation may depend upon the relative amounts of different forms of this enzyme system. In support of this hypothesis are the observations of Wiebel and Gelboin (18), which suggest that rat liver contains at least two types of aryl hydroxylating enzyme systems that vary in their relative amounts depending upon the age, sex, and nutritional state of the rat as well as on their exposure to specific inducers. For example, they found that 7,8-BF stimulated 3,4-benzpyrene hydroxylation in liver microsomes from immature rats but inhibited it in microsomes from adult rats.

The ability of 7,8-BF to stimulate 2-hydroxylation of biphenyl in mouse liver microsomes prompted us to investigate the ability of other compounds to stimulate this hydroxylation reaction. McPherson *et al.* (19) reported that 3,4-benzpyrene, 3-methylcholanthrene, and β-naphthylamine are among several carcinogenic compounds that stimulate biphenyl 2-hydroxylation in rat liver microsomes. 3,4-Benzpyrene was also found to stimulate this hydroxylation in hamster liver microsomes (20). However, we observed that under the conditions of our experiments, these carcinogenic compounds inhibited 2-hydroxylation of biphenyl (Table 3). The reasons for these discrepancies are not immediately clear.

Table 4 shows the effects of cytochrome P-450 inducers on biphenyl hydroxylation in rat liver microsomes. Phenobarbital treatment of rats selectively stimulated 3- and 4-hydroxylation, whereas 3-methylcholanthrene and β-naphthoflavone preferentially stimulated 2-hydroxylation. The effect of phenobarbital and 3-methylcholanthrene induction on 2- and 4-hydroxylation has been reported previously (2–5). Pregnenolone 16α-carbonitrile has been observed to increase preferentially some phenobarbital-inducible microsomal monooxygenase activities (21). However, it had little effect on biphenyl 3- and 4-hydroxylation and only moderately induced 2-hydroxylation. Phenobarbital and β-naphthoflavone treatment had essentially the same effect on biphenyl 2- and 4-hydroxylation in mouse microsomes as

TABLE 2

Effects of microsomal monooxygenase inhibitors on biphenyl hydroxylation

The 9000 × *g* supernatant fraction from 500 mg of mouse liver was incubated for 10 min with 1 mM biphenyl. SKF 525-A and DPEA were added to the incubation mixture 1 min before the addition of substrate. Each result is the average of three experiments.

Inhibitor	Concentration	Activity		
		2-HO-BP	3-HO-BP	4-HO-BP
	μM		% control	
SKF 525-A	50	54	67	73
	500	43	29	36
DPEA	5	37	72	56
	50	15	23	15
CO[a]	50% CO–10% O_2–40% N_2	11	0	3
	20% CO–16% O_2–64% N_2	42	10	14

[a] Microsomes were used instead of the 9000 × *g* supernatant fraction.

TABLE 3

Effects of carcinogenic compounds and 7,8-benzoflavone on microsomal biphenyl hydroxylation

Microsomes from 500 mg of liver were incubated for 10 min with 7,8-benzoflavone or the carcinogens before addition of biphenyl (1 mM). 7,8-Benzoflavone was added in 10 μl of dimethyl sulfoxide, and the carcinogenic compounds were added in 10–100 μl of acetone. The incubations were then continued for 5 min.

Species	Compound added	Concentration	Activity		
			2-HO-BP	3-HO-BP	4-HO-BP
		μM		*% control*	
Mouse	7,8-Benzoflavone	100	150	78	77
		500	194	49	53
	3,4-Benzpyrene	10	36	95	250
		500	37	110	212
	3-Methylcholanthrene	100	59	93	92
		500	46	71	70
	β-Naphthylamine	100	55	45	91
		500	21	30	46
Hamster	7,8-Benzoflavone	500	70	30	33
	3,4-Benzpyrene	1	82	99	97
		50	48	78	81
		500	37	65	83
	β-Naphthylamine	50	106	114	124
		500	67	73	61
β-Naphthoflavone-treated mouse[a]	7,8-Benzoflavone	100	62	105	104
		500	95	58	61

[a] Mice were treated with β-naphthoflavone (80 mg/kg intraperitoneally) 48 hr before death.

TABLE 4

Effect of cytochrome P-450 induction on biphenyl hydroxylation in hepatic 9000 × g supernatant fraction

The hepatic 9000 × *g* supernatant fraction was prepared from rats or mice previously treated with cytochrome P-450 inducers. Phenobarbital (50 mg/kg intraperitoneally) was given once daily for 4 days. Pregnenolone 16α-carbonitrile (50 mg/kg intraperitoneally) was given once daily for 3 days. Animals were killed 24 hr after the last dose. Animals were treated with β-naphthoflavone (80 mg/kg intraperitoneally) or 3-methylcholanthrene (20 mg/kg intraperitoneally) 48 hr before death. Control animals received no treatment. The 9000 × *g* supernatant fraction from 500 mg of liver was incubated for 10 min with 1 mM biphenyl. The number of experiments is shown in parentheses. Results are averages ± standard errors.

Treatment of animals	2-HO-BP	3-HO-BP	4-HO-BP
	μg/500 mg liver/10 min		
Rat			
None (7)	<0.25	<0.25	9.2 ± 1.0
Phenobarbital (6)	0.80 ± 0.09	6.4 ± 0.78	44 ± 5.9
β-Naphthoflavone (8)	6.2 ± 0.77	2.0 ± 0.31	16 ± 3.3
3-Methylcholanthrene (10)	6.6 ± 1.1	1.6 ± 0.27	16 ± 2.8
Pregnenolone 16α-carbonitrile (4)	1.4 ± 0.21	0.25 ± 0.10	5.6 ± 0.67
Mouse			
None (3)	1.9 ± 0.24	1.5 ± 0.12	23 ± 2.1
Phenobarbital (3)	2.0 ± 0.38	2.4 ± 0.34	48 ± 10
β-Naphthoflavone (3)	6.9 ± 0.78	2.5 ± 0.17	34 ± 2.7

in rat microsomes, but 3-hydroxylation in mouse microsomes was not induced by phenobarbital.

Mechanism of biphenyl 3-hydroxylation. The results obtained in this study show that the extent of 3-hydroxylation of biphenyl varies with the species, the exposure of the animals to inducers, and the presence of monooxygenase inhibitors. Furthermore, 3-hydroxylation varies in-

TABLE 5

Deuterium isotope effects during biphenyl hydroxylation by liver microsomes

Microsomes from 500 mg of liver were incubated for 10 min with a 1:1 mixture of biphenyl and biphenyl-d_{10}. K_H/K_D was determined by measuring the ratio of hydroxybiphenyl to hydroxybiphenyl-d_9 by mass fragmentography. Results are the averages ± standard errors of three experiments.

Species	K_H/K_D		
	2-HO-BP	3-HO-BP	4-HO-BP
Mouse	1.08 ± 0.009	1.29 ± 0.009	1.15 ± 0.009
Hamster	1.11 ± 0.032	1.43 ± 0.069	1.16 ± 0.003
Phenobarbital-treated rats[a]	1.08 ± 0.047	1.27 ± 0.027	1.14 ± 0.009

[a] Rats were injected with phenobarbital (50 mg/kg intraperitoneally) once daily for 4 days before preparation of microsomes.

dependently of the variation in 2- or 4-hydroxylation. Although these observations are consistent with the idea that 3-hydroxylation of biphenyl proceeds via a pathway other than through a 2,3-epoxide or a 3,4-epoxide intermediate, other interpretations of these data are possible. For example, if the metabolism of the postulated epoxides varies with the species, etc., the extent of 3-hydroxylation could vary independently of the extent of 2- or 4-hydroxylation. More conclusive evidence that 3-HO-BP is formed by an alternative hydroxylation pathway was obtained from the observation of isotope effects during its formation (Table 5). The values of K_H/K_D for 2-hydroxylation ranged from 1.08 to 1.11 with a standard error of 0.009–0.047. This ratio is not significantly different from 1. In contrast, K_H/K_D was found to be 1.27–1.45 (±0.009–0.069) for 3-hydroxylation. There was not a significant difference in the isotope effects observed with microsomes from mice, hamsters, or phenobarbital-treated rats. While K_H/K_D for 4-hydroxylation is significantly greater than 1.0 (1.14–1.16 ± 0.003–0.009), it is generally considered that K_H/K_D must be greater than 1.2 in order to be a significant isotope effect not attributable to systematic errors (11). Thus we have concluded that both the 2- and 4-hydroxylations of biphenyl proceed without significant isotope effects. The magnitude of the isotope effect of 3-hydroxylation is similar to those reported by Tomaszewski *et al.* (11) for the *m*-hydroxylation *in vivo* of nitrobenzene (1.40) and for *m*-hydroxylation of methylphenylsulfone (1.75).

Formation of 3,4-dihydroxybiphenyl. West *et al.* (22), Raig and Ammon (8), and, more recently, Meyer, Scheline, and co-workers (9, 10) have reported that 3,4-dihydroxybiphenyl is a metabolite *in vivo* of biphenyl. Our present studies with liver microsomes, which indicate that 3-hydroxylation occurs independently of 4-hydroxylation, suggest that 3,4-biphenyl catechol may be formed by two consecutive hydroxylations rather than via the dihydrodiol-epoxide pathway. In order to assess the formation of catechols, the method of Daly *et al.* was employed (23). In this method, the 9000 × *g* supernatant fraction incubations included *S*-adenosyl[*methyl*-^{14}C]-methionine. When a catechol is formed it is readily methylated by catechol *O*-methyltransferase and the [^{14}C]methylated product can be quantified by radioactivity content. When biphenyl, 3-HO-BP, or 4-HO-BP was incubated with mouse liver 9000 × *g* supernatant fraction, NADPH, and *S*-adenosyl[^{14}C]methionine, a large amount of radioactivity was incorporated into extractable metabolites. When these extracts were analyzed by gas chromatography, a new metabolite peak was readily apparent. The metabolite had a retention time (4 min) identical with that of authentic 3-methoxy-4-hydroxybiphenyl. In addition, the mass spectrum of this metabolite was identical with that of the authentic compound. Thin-layer chromatographic analysis of the extracts showed that the major radioactive metabolite (more than 60% of the extractable radioactivity) co-chromatographed with 3-methoxy-4-hydroxybiphenyl (R_F 0.65). Minor radioactive metabolites of biphenyl and 3- and 4-hydroxybiphenyl were apparently formed

by further metabolism of the catechol or the methylated catechol, since the same metabolites were found when these substituted biphenyls served as substrates. Thus the formation of 3,4-dihydroxybiphenyl could be quantified by analysis of the extracts for radioactivity. Results are shown in Table 6.

TABLE 6

Formation of 3-methoxy-4-hydroxybiphenyl from biphenyl and 3- and 4-hydroxybiphenyl by mouse liver 9000 × g supernatant fraction

The 9000 × *g* supernatant fraction from 500 mg of mouse liver was incubated for 30 min with 1 mM substrate. Incubations also contained 0.5 mM $NADP^+$, 2 mM glucose 6-phosphate, and 0.125 mM *S*-adenosyl[*methyl*-^{14}C]methionine (specific activity, 3510 dpm/nmol). Incubation mixtures were extracted as described in MATERIALS AND METHODS. The amount of radioactivity in the extract was determined, and the amount of 3-methoxy-4-hydroxybiphenyl was calculated from the specific activity of the *S*-adenosylmethionine. Each result represents one experiment.

Substrate	3-Methoxy-4-hydroxybiphenyl	
	Complete system	Without NADPH-generating system
	nmoles	
None	0.03	
Biphenyl	157, 141	0.4
4-Hydroxybiphenyl	221, 222	11
3-Hydroxybiphenyl[a]	225	

[a] Before incubation, 3-hydroxybiphenyl was purified by high-pressure liquid chromatography [25 cm × 4.6 mm Partisil column (Reeve Angel) eluted with chloroform–heptane (6.5:3.5)] in order to remove a small amount of 4-hydroxybiphenyl.

In order to gain further evidence that biphenyl 3,4-catechol is formed by consecutive hydroxylation rather than via the intermediate formation of a dihydrodiol or an endoperoxide, the method of Murphy *et al.* (24) was employed. The method involves incubation of substrates in an atmosphere of $^{18}O_2$ or a 1:1 mixture of $^{18}O_2$–$^{16}O_2$. Isotope incorporation was determined by mass spectral analysis of the trimethylsilyl derivative of the methylated catechol or the phenol. The molecular ion (*m/e* 272 or 242, respectively) was used for this analysis. The results of these experiments are shown in Table 7. As discussed by Murphy *et al.* (24), if the catechol is formed from biphenyl via a dihydrodiol intermediate, in the presence of $^{18}O_2$ the catechol should contain 1 atom of ^{18}O from the atmosphere and 1 atom of ^{16}O from H_2O. If the catechol is formed by consecutive hydroxylations, both atoms will contain ^{18}O from the atmosphere. The high percentage of ^{18}O incorporation into the phenols indicates that they are formed by direct oxidation. In order to distinguish the peroxide pathway from that of consecutive hydroxylation, the gas phase was a 1:1 mixture of $^{18}O_2$–$^{16}O_2$. If consecutive

TABLE 7

Incorporation of $^{18}O_2$ into hydroxylated metabolites of biphenyl

The 9000 × *g* supernatant fraction from 1 g of mouse liver was incubated in sealed, 50-ml, round-bottomed flasks with 1 mM biphenyl, 0.5 mM $NADP^+$, 2 mM glucose 6-phosphate, 500 μmoles of phosphate buffer (pH 7.4), 4 mM $MgCl_2$, and 0.125 mM *S*-adenosylmethionine. The incubation volume was 10 ml, and the incubation was conducted for 30 min. The appropriate gas phase (25 ml) was achieved as described by Murphy *et al.* (24). Isotope incorporation into the metabolites was determined by analysis of their mass spectra.

Substrate	Gas phase	Isotope distribution			
		2-HO-BP	3-HO-BP	4-HO-BP	3-Methoxy-4-hydroxybiphenyl
	% $^{18}O_2$	% ^{18}O	% ^{18}O	% ^{18}O	% ^{18}O
Biphenyl	94.8	91	89	91	83
4-Hydroxybiphenyl	94.8				89
Biphenyl	47[a]	43	42	45	33 ^{16}O–^{16}O
					46 ^{16}O–^{18}O
					21 ^{18}O–^{18}O

[a] If 3-methoxy-4-hydroxybiphenyl is formed by consecutive hydroxylations, the calculated isotope distribution under 47% $^{18}O_2$ is 28% ^{16}O–^{16}O, 50%, ^{16}O–^{18}O, and 22% ^{18}O–^{18}O.

hydroxylation were the pathway, 50% of the catechol molecules would have 1 atom of ^{18}O and 1 atom of ^{16}O rather than 2 atoms of either ^{18}O or ^{16}O. The results shown in Table 7 clearly indicate that 3,4-dihydroxybiphenyl is formed by consecutive hydroxylations.

COMMENT

The observation that the hydroxylation of biphenyl in hepatic microsomes produces *m*-hydroxylation as well as *o*- and *p*-hydroxylation is not surprising. In most cases in which *o*- and *p*-hydroxylation have been observed, *m*-hydroxylation can also be demonstrated to occur to a minor extent when careful analytical procedures are used. The observation that the extent of 3-hydroxylation appeared to vary independently of either 2- or 4-hydroxylation when the effects of species differences, oxygenase inhibitors, or oxygenase inducers were studied indicated that 3-hydroxylation might occur at least in part by a different mechanism than does 2- or 4-hydroxylation. In addition, Dr. Paul Servé, working in Dr. Donald Jerina's laboratory at the National Institutes of Health, has observed that the isomerization of biphenyl 3,4-oxide yields exclusively the 4-hydroxy isomer,[2] a result which indicates that this epoxide is not an intermediate in the formation of 3-HO-BP. The observation that 3-hydroxylation occurs with a deuterium isotope effect while 2- and 4-hydroxylations do not also suggests that 3-hydroxylation is unique. An attractive possibility is that 3-hydroxylation does not proceed via an arene oxide mechanism, which occurs without an isotope effect, since a carbon-hydrogen bond is not broken. Rather, 3-hydroxylation may occur at least in part by direct oxygen insertion, a mechanism expected to elicit an isotope effect. While the isotope effect for 3-hydroxylation, 1.3–1.5, is not as large as that of a typical aliphatic hydroxylation, 1.8 (14), it nevertheless supports the conclusion that a carbon-hydrogen bond is broken in a rate-limiting step. An unlikely alternative is that there is a unique secondary isotope effect for 3-hydroxylation but not for 2- or 4-hydroxylation. The lack of an isotope effect for 2- and 4-hydroxylation is not direct proof of an arene oxide mechanism, but the results are supportive of this mechanism. Thus the monohydroxylation of biphenyl by monooxygenase enzymes occurs at three positions, each with its own characteristics. For example, 2-hydroxylation appears to respond to typical cytochrome P-448 inducers, while hydroxylation at position 4 responds to typical cytochrome P-450 inducers. Hydroxylation at position 3, on the other hand, reacts to inducers in a mixed fashion and appears to occur at least in part via a different mechanism. Whether or not the three hydroxylations are catalyzed by different monooxygenases with different mechanisms, or perhaps by several monooxygenases, cannot be answered from the present experiments. However, one practical consequence of these observations is that biphenyl appears to be an ideal substrate for use in assessing the over-all capabilities of the standard microsomal preparations used in many standardized toxicology tests, such as bacterial mutagen screens (25).

The results of the studies on the mechanism for the formation of biphenyl catechol represent another example of the formation of catechols via successive monohydroxylations. Although catechol formation via enzymatic dehydrogenation by oxidoreductases is an attractive mechanism, it has been demonstrated in only one case, that of dihydroxydihydrobenzene (26). Only further work with other substrates will show which of these two mechanisms is the general mechanism for catechol formation.

[2] D. Jerina, personal communication.

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REFERENCES

1. Creaven, P. J., Parke, D. V. & Williams, R. T. (1965) *Biochem. J.*, **96**, 879–885.
2. Creaven, P. J. & Parke, D. V. (1966) *Biochem. Pharmacol.*, **15**, 7–18.
3. Burke, M. D. & Bridges, J. W. (1975) *Xenobiotica*, **5**, 357–376.
4. Burke, M. D. & Prough, R. A. (1976) *Biochem. Pharmacol.*, **25**, 2187–2195.
5. Prough, R. A. & Burke, M. D. (1975) *Arch. Biochem. Biophys.*, **170**, 160–168.
6. Billings, R. E. & McMahon, R. E. (1976) *Pharmacologist*, **18**, 155.
7. Raig, V. P., Beschorner, J. & Ammon, R. (1976) *Arzneim.-Forsch.*, **26**, 2178–2182.
8. Raig, P. & Ammon, R. (1972) *Arzneim.-Forsch.*, **22**, 1399–1404.
9. Meyer, T. & Scheline, R. R. (1976) *Acta Pharmacol. Toxicol.*, **39**, 419–432.
10. Meyer, T., Larsen, J. C., Hansen, E. V. & Scheline, R. R. (1976) *Acta Pharmacol. Toxicol.*, **39**, 433–441.
11. Tomaszewski, J. E., Jerina, D. M. & Daly, J. W. (1975) *Biochemistry*, **14**, 2024–2031.
12. Smith, J. R. L., Jerina, D. M., Kaufman, S. & Milstein, S. (1975) *J. Chem. Soc., Chem. Commun.*, 881–882.
13. Reinhold, V. N. & Brum, R. J. (1976) *Biomed. Mass Spectrom.*, **3**, 335–339.
14. McMahon, R. E., Sullivan, H. R., Craig, J. C. & Pereua, W. E. (1969) *Arch. Biochem. Biophys.*, **132**, 575–577.
15. London, J. D. & Scott, J. A. (1953) *J. Chem. Soc.*, 265–269.
16. Wiebel, F. J., Leutz, J. C., Diamond, L. & Gelboin, H. V. (1971) *Arch. Biochem. Biophys.*, **144**, 78–86.
17. Goujon, F. M., Nebert, D. W. & Gielen, J. E. (1972) *Mol. Pharmacol.*, **8**, 667–680.
18. Wiebel, F. J. & Gelboin, H. V. (1975) *Biochem. Pharmacol.*, **24**, 1511–1515.
19. McPherson, F., Bridges, J. W. & Parke, D. V. (1974) *Nature*, **252**, 488–489.
20. McPherson, F. J., Bridges, J. W. & Parke, D. V. (1976) *Biochem. Pharmacol.*, **25**, 1345–1350.
21. Lu, A. Y. H., Somogyi, A., West, S., Kuntzman, R. & Conney, A. H. (1972) *Arch. Biochem. Biophys.*, **152**, 457–462.
22. West, H. D., Lawson, J. R., Miller, I. H. & Mathura, G. H. (1956) *Arch. Biochem. Biophys.*, **60**, 14–20.
23. Daly, J., Inscoe, J. K. & Axelrod, J. (1965) *J. Med. Chem.*, **8**, 153–157.
24. Murphy, P. J., Bernstein, J. R. & McMahon, R. E. (1974) *Mol. Pharmacol.*, **10**, 634–639.
25. Cline, J. C. & McMahon, R. E. (1977) *Res. Commun. Chem. Pathol. Pharmacol.*, **16**, 523–533.
26. Ayengar, P. K., Hayaishi, O., Nakajima, M. & Tomida, I. (1959) *Biochim. Biophys. Acta*, **33**, 111–119.