Biochemical Pharmacology, Vol. 27, pp. 1245–1249.

0006-2952/78/0415-1245$02.00/0

METABOLISM OF BROMOBIPHENYLS

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(*Received* 19 *May* 1977; *accepted* 7 *September* 1977)

Abstract—The metabolism *in vivo* of 2-, 3- and 4-bromobiphenyl in the rabbit gave a series of mono- and dihydroxylated metabolic products. The nuclear magnetic resonance spectra of the two major 4-bromobiphenyl metabolites confirmed their structures as 4′-bromo-4-biphenylol and 4′-bromo-3,4-biphenyldiol. The metabolism of 4′[^{2}H]4-bromobiphenyl gave the corresponding monohydroxy compound in which 61 per cent of the deuterium had been retained whereas the deuterium content of the catechol was 19 per cent. The NIH shift of deuterium is consistent with metabolism via an arene oxide which rearranges to the phenol and undergoes hydration/dehydrogenation to give the catechol. The retention of 19 per cent of the deuterium in the catechol also indicated that some of the diol is formed via direct hydroxylation, as observed in the metabolism of 4-chlorobiphenyl. The metabolism *in vitro* of [^{3}H]4-bromobiphenyl with hepatic microsomes gives polar metabolic products and the radioactivity is also incorporated into the protein component of the microsomal fraction. 4-Bromobiphenyl is highly mutagenic to the *Salmonella typhimurium* TA 1538 mutant and metabolic activation with a liver microsomal enzyme fraction is required for this activity.

Polybrominated biphenyls (PBB) are industrial compounds which have been used as flame retardants in diverse plastic products for the electrical industry and in synthetic fibres. Their chemical stability and flame retardant properties are typical of many widely used halogenated aromatic compounds [1]. FireMaster BP-6 is a commercial formulation of PBB which contains a complex mixture of isomers, one of which has been identified as 2,2′,4,4′,5,5′-hexabromobiphenyl [2]. In 1973, several bags of FireMaster BP-6 were accidentally shipped as a cattle food supplement and the PBB was mixed with feed which was distributed to farmers throughout the State of Michigan. The extent of the damage to the farm animals has not been fully evaluated yet, although thousands of these animals have been quarantined and destroyed [3]. Many farmers and their families and consumers in Michigan have eaten PBB-contaminated meat and this has resulted in the bioaccumulation of PBB in this population [4]. This parallels the uptake and storage of other well known and more ubiquitous halogenated aromatic pollutants [e.g. dichloro diphenyltrichloroethane (DDT) and the polychlorinated biphenyls (PCB)] which accumulate in higher trophic levels of the food chain [5].

The biological properties of PBB show a marked similarity to those reported for the structurally related PCB. Both PBB and PCB induce symptoms such as hair loss, chloracne, enlarged livers and central nervous system damage in test animals [3, 6–8]. PBB and PCB are both metabolized to give their corresponding hydroxylated metabolites [11–14].

Previous results have shown that the rate of metabolism of halogenated biphenyls decreases with increasing halogen content of the biphenyl nucleus; however, in all cases analogous hydroxylated aromatic metabolites have been identified [14–16]. This study reports the metabolism *in vivo* in rabbits of the isomeric monobromobiphenyls as model PBB substrates, the precise structural identification of the metabolites and the mechanism of the metabolic process. The metabolism *in vitro* of [^{3}H]4-bromobiphenyl to give hydroxylated metabolites and a covalently bound fraction is also reported along with the bacterial mutagenicity of 4-bromobiphenyl to the *Salmonella typhimurium* strain TA 1538.

EXPERIMENTAL

The 2-, 3- and 4-bromobiphenyl substrates were obtained from commercial sources (Aldrich and Eastman Chemicals). 4′[^{2}H]4-bromobiphenyl was prepared by reduction of 4-4′-dibromobiphenyl (1.0 g) with lithium aluminium deuteride (Merck, 0.25 g) in dry tetrahydrofuran (25 ml) as described [13]. The product (300 mg) was separated from unchanged starting material and biphenyl by preparative thin-layer chromatographic (t.l.c.) using Silica gel Hf_{254} (Merck) and hexane as solvent. The bromobiphenyl substrates (400 mg) were dissolved in a minimum volume of corn oil (*ca.* 4–5 ml) and administered by intraperitoneal injection to male albino rabbits (2.5 to 5 kg). The animals were housed in metabolic cages and the urine and feces were collected for 7 days and stored at 0° prior to analysis.

The unconjugated urinary metabolites were extracted from the rabbit urine as described [13], and the urine was acidified with sulfuric acid to give a 2 N solution and refluxed for 90 min on a steam bath. The solution was cooled and extracted with ether (2 × 200 ml) to give a second lipophilic extract. The metabolites were isolated from the ethereal urine extracts by repeated preparative t.l.c. as described [13], and the molecular identities of the t.l.c. bands were continually monitored by mass spectrometry. In some cases the metabolite could only be purified by forming the acetate derivative using acetic anhydride/sodium acetate. When suffi-

cient purified metabolite could be isolated the structures were confirmed by mass spectrometric analysis and their nuclear magnetic resonance spectra. Analysis of the ether extract obtained after acid hydrolysis revealed only trace levels of bromobiphenyl metabolites.

The purity of the metabolite fractions was monitored by gas–liquid chromatography (g.l.c.) on a Hewlett–Packard model 5710 chromatograph equipped with a flame ionization detector. A glass column (0.3 cm × 2 cm) packed with 3% OV 225 on Gas Chrom Q was used and operating conditions were: helium carrier gas flow, 30 ml/min; hydrogen flow, 60 ml/min; air flow, 200 ml/min; and flame ionization detector and injection port temperatures, 290°.

The 220 MHz nuclear magnetic resonance spectra were recorded on a Varian HR 220 instrument using deuterochloroform as solvent and tetramethylsilane as the internal standard. Mass spectra were recorded on a Varian MAT CH-7 spectrometer equipped with electrical detection and a direct insertion probe.

Tritiated 4-bromobiphenyl was prepared by palladium-catalyzed tritium exchange in trifluoroacetic acid/tritiated water (New England Nuclear) and purified by t.l.c. prior to use. An untreated New Zealand male albino rabbit (2.5 kg) was sacrificed by cervical dislocation and the liver microsomes were obtained as described [17] with the following modifications: the 0.25 M sucrose solutions were supplemented with 0.1 mM EDTA, and the 12,000 *g* supernatant was centrifuged at 100,000 *g* onto a 1.6 mM EDTA cushion (4 ml) to facilitate recovery and resuspension of the microsomes. The microsomes were resuspended in 0.25 M sucrose–0.1 mM EDTA (6 mg/ml) and used immediately. The incubation mixtures consisted of Tris hydrochloride (50 μmoles, pH 7.6), NADPH (1 μmole), [^{3}H]4-bromobiphenyl (0.24 μmole, 18.6 mCi/m-mole) suspended by sonication with a Biosonic microprobe in dimethyl sulfoxide–Tween 80–water (10:1:20, 0.1 ml) and the liver microsomal enzyme suspension (1.0 ml). The mixture (3.0 ml total volume) was then incubated at 37° for 30 min.

The bromobiphenyl substrate and metabolites were isolated by diluting the incubation mixture with 0.2 M acetate buffer (3.0 ml, pH 4.7) and repeated extraction with diethyl ether (3 × 6 ml). Metabolites were separated by t.l.c. and the radioactivity in the various bands was determined [18]. The macromolecular fraction was purified of RNA by phenol extraction and then protein was precipitated from the aqueous phase with 0.1 vol. of 2 M acetate buffer (pH 4.7) and ethanol (3 vol.). The protein precipitate was purified by repeated methanol washes (7 × 6 ml) at 60°. Protein was solubilized with NCS (tissue solubilizer, BDH Chemicals) and 3 mg counted in toluene/PPO* at 32 per cent efficiency. The incorporation of radioactivity into the protein fraction required air and NADPH and incubation of the [^{3}H]4-bromobiphenyl with denatured microsomal protein for 30 min resulted in the binding of 7 *p*moles substrate/mg of protein. This value was *ca.* 5 per cent of the binding observed with intact microsomes.

The Ames bacterial test for mutagenesis using the *S. typhimurium* mutant strain TA 1538 was carried out as described [19].

RESULTS

The urinary metabolites were first purified by t.l.c. and converted into their acetates. The acetates in turn were purified by t.l.c. All fluorescent polar bands were monitored by mass spectrometry to detect the presence of the bromobiphenyl metabolites. This assay procedure proved to be very useful since bromine-containing chemicals typically exhibit predictable ion multiplicity due to the ^{79}Br and ^{81}Br isotopes. A summary of the metabolism results is given in Table 1.

The 2-bromobiphenyl gave two polar metabolites and the mass spectral analyses of their corresponding acetates indicated the formation of two isomeric monohydroxylated products. The n.m.r. spectrum of the major product exhibited two proton doublet signals at 7.17 and 7.45 ppm with coupling constants of 8.2 Hz. This pattern is typical of an AA′ BB′ system which is consistent only with ring hydroxylation at the 4 position of the unsubstituted ring. The n.m.r. spectrum of the minor metabolite was complex and the precise position of the hydroxyl group could not be assigned.

The 3-bromobiphenyl metabolites were also separated by t.l.c. and the n.m.r. spectrum of the major monohydroxylated product did not exhibit a recognizable AA′ BB′ pattern, thus indicating hydroxylation of the bromine substituted ring. The quartet and doublet resonances at 7.07 (J = 8.2 and 2.0 Hz) and 7.26 (J = 2.0 Hz) ppm, respectively, are typical of the B and X protons of an ABX system where the B and X hydrogens are meta-coupled and the B proton is also ortho-coupled to the A hydrogen. Hydroxylation at positions 4 or 6 in the substituted ring would both result in an ABX pattern; therefore, 3-bromo-4-biphenylol and 5-bromo-2-biphenylol are two possible structures for this metabolite. A second minor dihydroxylated product was detected by mass spectrometry; however, insufficient material was isolated to establish the structure by n.m.r. analysis.

The metabolism of 4-bromobiphenyl gave two metabolites (isolated as acetates) indicating both mono- and dihydroxylation. The spectrum of the monohydroxylated product exhibited two AA′ BB′ patterns and this clearly supported the structure of 4′-bromo-4-biphenylol as the major metabolite. The n.m.r. spectrum of the diacetate was analogous to that observed for 4′-chloro-3,4-biphenylyl diacetate [13]; two proton doublets at 7.26 and 7.57 ppm corresponded to the AA′ BB′ pattern for the bromophenyl ring; H_2 gave a meta-coupled doublet at 7.37 ppm (J = 2.0 Hz) and H_6 exhibited a quartet at 7.43 ppm due to meta-coupling with H_2 and ortho-coupling with H_5; H_5 appeared as an ortho-coupled doublet (J = 8.2 Hz) at 7.43 ppm.

Preparation of 4′[^{2}H]4-bromobiphenyl via controlled lithium aluminum deuteride reduction of 4,4′-dibromobiphenyl gave a 73 per cent isotopically

* PPO = 2,5-diphenyloxazole.

Table 1. Summary of bromobiphenyl rabbit urinary metabolites

Substrate	Metabolite (R_t* and % yield)	Spectral data
2-Bromobiphenyl	2'-Bromo-4-biphenylyl acetate‡ (2.7 min/220°, 1%)	M^+ = 290; δ (ppm), 7.17 (d, 2 H, J = 8.2 Hz), 7.37, 7.69 (m, 4 H), 7.45 (d, 2 H, J = 8.2 Hz)
	Bromobiphenylyl acetate‡ (2.3 min/220°, traces)	
3-Bromobiphenyl	3-Bromo-4-biphenylol or 5-bromo-2-biphenylol (12.4 min/180°, 4%)	M^+ = 248; δ (ppm), 7.07 (q, 1 H, J = 8.2, 2.0 Hz), 7.26 (d, 1 H, J = 2.0 Hz), 7.30–7.58 (m, 6 H)
	Bromobiphenylyl diacetate (6.4 min/250°, < 1%)	M^+ = 348
4-Bromobiphenyl	4'-Bromo-4-biphenylyl acetate‡ (5.3 min/220°, 2%)	M^+ = 290; δ (ppm), 7.22 (d, 2 H, J = 8.2 Hz), 7.35 (d, 2H, J = 8.2 Hz), 7.41 (d, 2H, J = 8.2 Hz), 7.41 (d, 2H, J = 8.2 Hz)
	4'-Bromo-3,4-biphenylyl diacetate‡ (6.4 min/250°, 1.5%)	M^+ = 348; δ (ppm), 7.26 (d, 2H, J = 8.2 Hz), 7.37 (1H, d, J = 2.0 Hz), 7.41 (d, 1H, J = 8.2 Hz), 7.43 (q, 1H, J = 8.0, 2.2 Hz), 7.57 (d, 2H, J = 8.2 Hz)
4'[^{2}H]4-bromobiphenyl	[^{2}H]4'-bromo-4-biphenylyl acetate	M^+ = 290/291
	[^{2}H]4'-bromo-3,4-biphenylyl diacetate‡	M^+ = 348/349

* Gas–liquid chromatography retention time/column temperature.
† Based on the amount of substrate administered to the animal.
‡ Purified and isolated as the acetate derivative.

pure isomer as determined by mass spectrometry. The 4'[^{2}H]4-bromobiphenyl metabolites were isolated and their deuterium content was determined by mass spectrometric analysis and corrected for the isotopic purity of the synthetic substrate. The results showed that the 4'-bromo-4-biphenylyl acetate retained 61 per cent of the deuterium and this is consistent with the arene oxide mechanism as shown in Fig. 1, which involves the 1,2-migration of deuterium from the site of hydroxylation to the adjacent carbon atom (NIH shift). The diol retained only 19 per cent of the original deuterium atom.

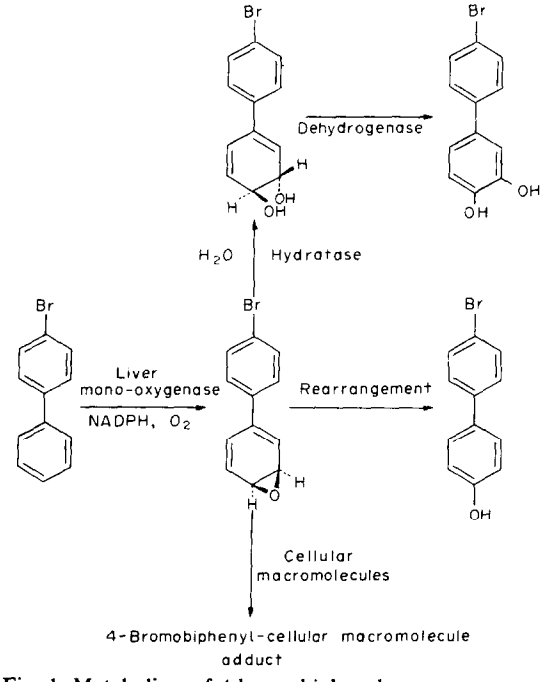

Fig. 1. Metabolism of 4-bromobiphenyl: a summary.

Incubation of [^{3}H]4-bromobiphenyl with rabbit liver microsomes followed by repeated extraction with ether gave two major degradation products. Examination of the radioactive metabolites by t.l.c. (Fig. 2) clearly indicated chromatographic behavior which was identical to that of the metabolites *in vivo*, 4'-bromo-4-biphenylol and 4'-bromo-3,4-biphenyldiol. Precipitation of the macromolecular fraction and repeated extraction of this precipitate

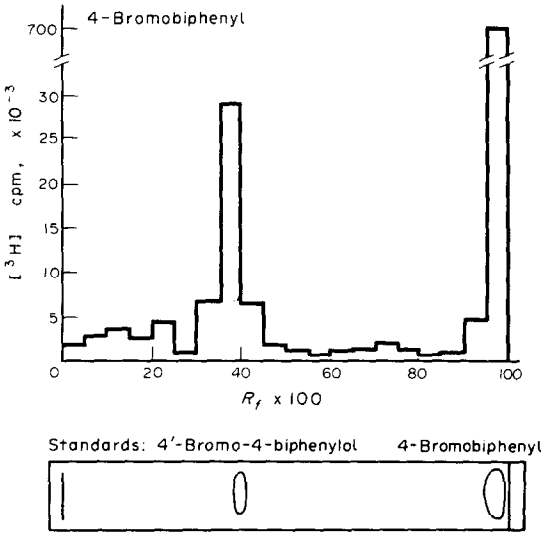

Fig. 2. Thin-layer chromatograms indicating the radioactivity of the polar metabolic products formed in the incubation *in vitro* of tritiated 4-bromobiphenyl.

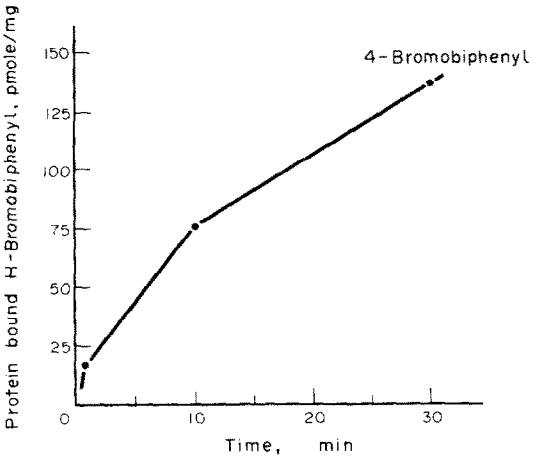

Fig. 3. Uptake of radioactivity in the macromolecular fraction after incubation of tritiated PBB with hepatic microsomes from a non-induced rabbit.

with hot methanol to remove possible glutathione conjugates also indicated that [^{3}H]4-bromobiphenyl formed an adduct with the protein component of the microsomal enzyme homogenate (Fig. 3).

The mutagenicity of 4-bromobiphenyl to the *S. typhimurium* TA 1538 mutant strain was dependent on the presence of the Aroclor 1254-induced rat microsomal enzyme preparation (i.e. S-9 fraction) in the incubation system [18, 19]. In the absence of this fraction, the number of revertant colonies per plate was identical to that of the control values. The number of revertant colonies observed at concentrations of 50, 100 and 150 μg/plate was 1000, 5050 and 3950 respectively. The mutagenic response falls off at the highest concentration due to the lethal mutations induced by the 4-bromobiphenyl substrate. The number of revertant colonies on the control plates varied from 30 to 40/plate.

DISCUSSION

The metabolism *in vivo* of the model PBB substrates, 2-, 3- and 4-bromobiphenyl, all proceeded with the formation of mono- and dihydroxylated products. The major metabolites of 4-bromobiphenyl and 4-chlorobiphenyl were identical with respect to the sites of hydroxylation, namely at the 4 and 3,4 positions of the unsubstituted ring. This similarity in metabolic pathways was also observed for the corresponding 4-4′-dichloro- and 4,4′-dibromobiphenyl isomers [12]. Clearly metabolic degradation of PBB represents an important pathway for the detoxication of these chemicals via urinary excretion. However, by analogy with PCB this can also pose additional problems since the PBB metabolites presumably can then be bioaccumulated, and recent analytical data have shown that hydroxylated PCB are indeed present as residues in wildlife samples [20]. The biological activity of both PCB and PBB metabolites is unknown and it is possible that these halogenated aromatic pollutant metabolites may also exert some adverse biological effects.

The metabolism *in vivo* of 4′[^{2}H]4-bromobiphenyl resulted in the formation of the expected mono- and dihydroxylated products. Mass spectral analysis of the phenolic metabolite gave a molecular ion cluster at m/e 290 and 291 and the results showed 61 per cent retention of deuterium in this product. This indicated that the biohydroxylation is accompanied by a substituent (i.e. ^{2}H) migration and is consistent with the intermediacy of an arene oxide which can then rearrange to give a phenol [21]. A similar result was also observed in the metabolism of 4′[^{2}H]4-chlorobiphenyl in which 79 per cent of the 4′-deuterium atom was retained [13]. The mass spectrum of 4′-bromo-3,4-biphenyldiol exhibited molecular ions at m/e 348 and 349 with the calculated retention of 19 per cent of the original deuterium. The two most likely pathways for the genesis of this metabolite are indicated in Fig. 1, namely (a) hydration of the arene oxide to form the transient dihydrodiol which is rapidly dehydrogenated to give the catechol and (b) direct hydroxylation of the phenolic metabolite to yield the catechol. Both of these metabolic routes are prominent in the metabolism of aromatics [21]; however, the former would result in the complete loss of the deuterium label whereas the catechol formation via a second hydroxylation would retain *ca.* one-half the deuterium present in the phenol. This latter process was observed in the hydroxylation of tyrosine to give 3,4-dihydroxyphenylalanine [22] and 4′-chloro-4-biphenylol to 4′-chloro-3,4-biphenyldiol [13] and does not involve an arene oxide [21]. The 19 per cent retention of deuterium in 4′-bromo-3,4-biphenyldiol suggests that both pathways are involved in the formation of this metabolite.

The incubation *in vitro* of [^{3}H]4-bromobiphenyl with a non-induced rabbit hepatic microsomal enzyme fraction also gave polar metabolites with chromatographic behavior identical to the corresponding hydroxylation products *in vivo*. The intermediacy of a 4-bromobiphenyl arene oxide was supported by the isolation of a 4-bromobiphenyl macromolecule (protein) fraction, as shown in Fig. 3. The alkylation of cellular macromolecules has been reported for diverse metabolically activated aromatic molecules such as benzo(a)pyrene and related chemical carcinogens [21], and this process is associated with the induction of cancer by these chemicals [23]. As a preliminary screening for mutagenicity, 4-bromobiphenyl was shown to be highly mutagenic to the *S. typhimurium* mutant TA 1538 [19] which is sensitive to frameshift mutations. This *in vivo* result complements the metabolic data, and research on the metabolism and carcinogenicity of diverse PBB isomers is now in progress.

Acknowledgements—The financial assistance of the National Research Council of Canada is gratefully acknowledged; the assistance of Dr. A. Grey and the 220 MHz NMR Centre was appreciated.

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