Identification of *in Vitro* Metabolites of 2,4,6,2',4',6'-Hexachlorobiphenyl from Phenobarbital-Treated Dog Liver Microsomes

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We studied *in vitro* metabolites of 2,4,6,2',4',6'-hexachlorobiphenyl (HCB, IUPAC PCB No. 155) produced by liver microsomes of a phenobarbital (PB)-treated beagle dog. The major metabolites were 3-hydroxy-2,4,6,2',4',6'-HCB (M-1), 4-hydroxy-2,6,2',4',6'-pentachlorobiphenyl (PenCB, M-2) and 3,4-dihydroxy-2,6,2',4',6'-PenCB (M-3). Furthermore, 4-hydroxy-2,3,6,2',4',6'-HCB (M-4), which could be formed *via* the 3,4-epoxidation and the subsequent NIH-shift of the chlorine from the 4 to the 3 position, was also detected. We found that M-3 is a common secondary metabolite of the two major monohydroxy metabolites, M-1 and M-2. These results indicate that the dog seems to metabolize and eliminate this congener not only by a mechanism involving direct insertion of a hydroxyl group but also *via* an arene oxide intermediate.

Keywords 2,4,6,2',4',6'-hexachlorobiphenyl; *in vitro* metabolism; dog liver microsome; arene oxide intermediate; secondary metabolism; polychlorinated biphenyl

Polychlorinated biphenyls (PCBs) are well known as widespread environmental pollutants and one of the causal agents of Yusho disease. It is known that they are mainly metabolized by hepatic mixed function oxygenase systems. Although many metabolic pathways have been reported to date,¹⁻⁴ the hydroxylation reaction seems to be the major mechanism.

It is well known that PCB congeners without adjacent unsubstituted carbon atoms are difficult to metabolize in most animals. Matthews and Tuey suggested that in such PCB congeners an unoccupied *meta* position may offer a site for enzymatic attack on the biphenyl rings in the rat.⁵ One of these congeners, 2,4,5,2',4',5'-hexachlorobiphenyl (2,4,5,2',4',5'-HCB), was reported to be rapidly excreted only in the dog.⁶ Schnellmann *et al.* assumed that an unsubstituted *meta*-position particularly facilitates PCB elimination in the dog.⁷ In fact, they observed that 2,4,6,2',4',6'-HCB (IUPAC PCB No. 155) was eliminated about twice as fast as 2,4,5,2',4',5'-HCB in the dog. In addition, we have reported that the dog can metabolize 2,4,5,2',4',5'-HCB by a novel mechanism *via* a 2,3-arene oxide intermediate.⁸

In the present study, we aimed to elucidate the metabolic pathways of 2,4,6,2',4',6'-HCB by using liver microsomes from a phenobarbital (PB)-treated dog in order to investigate whether this congener can be metabolized by mechanisms similar to 2,4,5,2',4',5'-HCB in the dog.

Materials and Methods

Chemicals 2,4,6,2',4',6'-HCB was synthesized from 2,4,6-trichloriodobenzene by the method of Ullmann with some modifications reported by Kornblum and Kendall.⁹ 2,4,6-Trichloriodobenzene was synthesized from 2,4,6-trichloroaniline by a standard procedure.¹⁰ 2,4,6-Trichloroaniline, 2,4,6-trichlorophenol, 3,5-dichlorophenol, 2,4-dichlorophenol and 2,3,5-trichlorophenol were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other reagents and materials used were of the highest quality commercially available.

Synthesis of Authentic Samples Figure 1 shows the synthetic methods used to obtain the possible monohydroxy metabolites of 2,4,6,2',4',6'-HCB. Among them, 3-hydroxy-2,4,6,2',4',6'-HCB (S-1) was synthesized by the method of Cadogan (reaction A)¹¹ and all others were by the method of Colbert and Lacy (reaction B, C and D)¹² with some modifications.

Although S-1 could also be obtained in the same yield by the second

method, a large amount of by-product was also produced. In contrast, other sample metabolites could not be obtained by this Cadogan reaction, but were easily obtainable by the Colbert reaction. All the synthesized samples were methylated by the method described previously,¹³ and purified using a silica gel column and/or preparative thin-layer chromatography (TLC).⁸ Spectral data of the authentic samples synthesized in this study are summarized in Table I.

Animal Treatment and Preparation of Liver Microsomes An adult male beagle dog (8.1 kg b.w.) was purchased from Hazelton Research Animals, Inc. (Cumberland, U.S.A.) and housed in a safety assessment laboratory, Panapharm Laboratories Co. (Uto Kumamoto, Japan). Phenobarbital-Na (PB) was administered orally to the dog by the method of Duignan *et al.*¹⁴ The animal was starved 24 h after the final treatment and sacrificed under pentobarbital-anesthesia. The liver was perfused with physiological saline, and the microsomes were prepared using standard procedures and resuspended in a quantity of 10 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 0.1 mM EDTA, equivalent to the original liver weight. The resulting suspensions were stored at -80 °C until use.

Incubation of 2,4,6,2',4',6'-HCB with Liver Microsomes 2,4,6,2',4',6'-HCB (20 μ M) dissolved in dimethylsulfoxide (0.8% of the final incubation mixture) was preincubated for 5 min at 37 °C with microsomes (1.0 mg protein/ml incubation mixture), 6 mM MgCl₂, 0.8 mg/ml of BSA as a PCB carrier, and 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate (HEPES) buffer (pH 7.5) in a final volume of 5 ml. The reaction was initiated by addition of a NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase) and terminated by addition of an equal volume of chloroform-methanol (2:1, v/v) after 20 min incubation.

Extraction of Metabolites After shaking the incubation mixture with chloroform-methanol (2:1, v/v) for 10 min, two volumes of *n*-hexane were added and the unchanged parent compound and metabolites were extracted by shaking for 10 min. Unchanged HCB and most metabolites were extracted into the chloroform-methanol (2:1, v/v) fraction. A small amount of M-2 and M-3 were further extracted as follows: the aqueous layer was shaken with an equal volume of dichloromethane and extracted with two volumes of *n*-hexane, followed by further shaking with chloroform and extracting with *n*-hexane as described above. These organic phases were pooled and evaporated to dryness *in vacuo*.

Analytical Methods Gas chromatography (GC) was performed using a Shimadzu GC-3BE gas chromatograph equipped with an electron-capture detector under the following conditions: 3 mm $\times$ 2.1 m glass column packed with 1.5% OV-17 on Chromosorb W (AW-DMCS); carrier gas, N₂, 40 ml/min; injection port temp., 270 °C; column temp., 235 °C or 250 °C. GC-mass spectrometry (MS) was carried out on a Shimadzu QP-1000 gas chromatograph connected to a mass spectrometer. The carrier gas used was He, 38 ml/min. The other conditions were: 3.3 mm $\times$ 1.1 m glass column packed with 2.0% OV-17 on Chromosorb W (AW-DMCS); column temp., 220 °C. Electron-impact (EI) mass spectra (70 eV) were recorded over the mass range *m/z* 150—400. 270 MHz ¹H-nuclear magnet-

Fig. 1. Synthesis of Possible Monohydroxy-Metabolites of 2,4,6,2',4',6'-HCB

TABLE I. Spectral Data of Authentic Samples Synthesized in This Study

Compound	NMR spectrum ^{a)}	Mass spectrum ^{b)}			
	δ in ppm	[M	-15	-43	-50] ⁺
2,4,6,2',4',6'-HCB	7.47 (s, H ₃ , H ₅ , H _{3'} , H _{5'})	358 (100)	—	—	—
3-Methoxy-2,4,6,2',4',6'-HCB (S-1)	3.93 (s, OCH ₃), 7.47 (s, H ₃ , H ₅), 7.50 (s, H _{3'} , H _{5'})	388 (100)	373 (55)	345 (25)	338 (9)
2-Methoxy-4,6,2',4',6'-PenCB (S-2a)	3.76 (s, OCH ₃), 6.91 (d, $J=1.81$ Hz, H ₃), 7.16 (d, $J=1.81$ Hz, H ₅), 7.43 (s, H _{3'} , H _{5'})	354 (100)	—	—	304 (40)
4-Methoxy-2,6,2',4',6'-PenCB (S-2b)	3.86 (s, OCH ₃), 7.00 (s, H ₃ , H ₅), 7.45 (s, H _{3'} , H _{5'})	354 (100)	—	311 (11)	—
3-Methoxy-4,6,2',4',6'-PenCB (S-2c)	3.89 (s, OCH ₃), 6.72 (s, H ₂), 7.45 (s, H ₃ , H ₅), 7.53 (s, H _{3'} , H _{5'})	354 (100)	339 (24)	311 (22)	304 (9)
3-Methoxy-2,6,2',4',6'-PenCB (S-2d)	3.95 (s, OCH ₃), 6.98 (d, $J=8.93$ Hz, H ₄), 7.40 (d, $J=8.93$ Hz, H ₂), 7.46 (s, H ₃ , H ₅)	354 (100)	339 (23)	311 (30)	304 (9)
2-Methoxy-3,4,6,2',4',6'-HCB (S-4a)	3.67 (s, OCH ₃), 7.46 (s, H ₃), 7.47 (s, H _{3'} , H _{5'})	388 (100)	—	—	338 (62)
4-Methoxy-2,3,6,2',4',6'-HCB (S-4b)	3.98 (s, OCH ₃), 7.03 (s, H ₃), 7.46 (s, H _{3'} , H _{5'})	388 (100)	—	345 (30)	—

a) Multiplicity, coupling constant and assignment of signals are shown in parentheses: s, singlet; d, doublet. b) Relative peak height expressed as a percentage of the molecular ion peak are shown in parentheses.

ic resonance (NMR) spectra were measured with a JEOL GX-270 spectrometer using tetramethylsilane as an internal standard and chloroform-*d*₁ (CDCl₃) as a solvent.

Results

Detection of Metabolites As shown in Fig. 2, at least four major metabolites were detected in the methylated extract of the complete incubation mixture, but none in the control (minus the NADPH-generating system). Figure 3 shows the mass spectra of these metabolites.

The methylated derivatives of M-1 and M-4 showed a molecular ion peak at m/z 388 and diagnostic isotope peaks, attributable to the six chlorine atoms in the molecule, indicating that M-1 and M-4 are monohydroxy-HCB. Methylated M-2 gave a molecular ion peak at m/z 354 and typical isotope peaks indicating five chlorine substituents in the molecule. Therefore, M-2 was probably a dechlorinated metabolite, monohydroxy-pentachlorobiphenyl (PenCB). Methylated M-3 exhibited a molecular ion peak at m/z 384 with diagnostic isotope peaks due to five chlorine atoms in the molecule. Therefore, this metabolite

Fig. 2. Gas Chromatogram of Methylated Derivatives of 2,4,6,2',4',6'-HCB Metabolites Formed

—, test; ----, control.

was thought to be a dihydroxy-PenCB.

Identification of M-1 and M-2 Identification of these two metabolites was performed by comparing the retention times (t_R) on GC of their methylated and/or trimethylsililated derivatives and diagnostic fragmentation patterns of mass spectra with those of the corresponding authentic samples.

Metabolic studies of 2,4,6,2',4',6'-HCB have been reported only in rats, and one metabolite, 3-hydroxy-2,4,6,2',4',6'-HCB has been identified so far.^{15,16} As described in Materials and Methods, this compound (S-1) was obtained by the reaction A in Fig. 1. The structure of S-1 should be 3-hydroxy-2,4,6,2',4',6'-HCB from the synthetic method used, but this was further confirmed by the $^1\text{H-NMR}$ spectrum of the methylated derivative which is shown in Table I.

In this spectrum, the chemical shift of a singlet corresponding to one proton (H_5) unexpectedly shifted by 0.036 ppm to a lower magnetic field than a signal attributable to the equivalent two protons (H_3 and H_5). Hydroxy- and/or methoxy-substituted aromatic ring protons are known to exhibit signals at a higher magnetic field than the corresponding unsubstituted ring protons in general.¹⁷ In order to clarify this conflicting result, that the shifted signal was due to the H_5 proton, $^1\text{H-NMR}$ spectra of 1,3,5-trichlorobenzene and 2,4,6-trichloroanisole were measured. In contrast to the above description, the signal of the equivalent two protons (H_3 and H_5) in 2,4,6-trichloroanisole was shifted by 0.034 ppm to a lower mag-

netic field than that of the equivalent three protons (H_2 , H_4 and H_6) in 1,3,5-trichlorobenzene (data not shown). Based on these results, the singlet signal of methylated S-1, located at a lower magnetic field, was assigned to the H_5 proton.

Both methylated and trimethylsililated derivatives of M-1 possessed the same t_R as those of S-1 (data not shown). In addition, since the mass spectra of the methylated M-1 and S-1 were identical, M-1 was confirmed to be 3-hydroxy-2,4,6,2',4',6'-HCB.

Dechlorinated metabolites of PCBs have been identified in some metabolic studies^{8,13,18,19} and it has been assumed that these metabolites are derived from an arene oxide intermediate. M-2 was determined to be such a metabolite as follows. Since 2,3- and 3,4-arene oxides have been shown to rearrange exclusively to *ortho* and *para* phenols, respectively,^{20,21} the two expected metabolites in Fig. 4 were synthesized by reaction B in Fig. 1.

On the other hand, according to the hypotheses of Kato *et al.*¹⁶ and Schnellmann *et al.*,⁷ 3-hydroxy-4,6,2',4',6'-PenCB and 3-hydroxy-2,6,2',4',6'-PenCB were also expected to be produced *via* 2,3- and 3,4-arene oxides, respectively. These compounds were synthesized by reaction C (Fig. 1 and Table I). Among these four compounds, S-2b showed a t_R on GC identical to that of M-2. In addition, the mass spectrum of methylated S-2b gave the same pattern as that of methylated M-2. In the $^1\text{H-NMR}$ spectrum (Table I) of methylated S-2b, two singlets, each of which corresponded to two protons, were observed at 7.00 ppm and 7.45 ppm and this is good evidence that the structure of M-2 is 4-hydroxy-2,6,2',4',6'-PenCB.

In the mass spectrum, Jansson and Sundström suggested that PCBs possessing a methoxyl group at the *para* (4 and/or 4') position afford a fragment ion peak of $[\text{M}-15]^+$ with relatively high intensity, which is due to loss of a methyl group.²² However, methylated S-2b had no $[\text{M}-15]^+$ fragment ion peak. The same phenomenon was observed in our previous study.¹³ Thus, M-2 was identified as 4-hydroxy-2,6,2',4',6'-PenCB and assumed to be derived from the 3,4-epoxy-2,4,6,2',4',6'-HCB intermediate.

Identification of M-4 If 2,4,6,2',4',6'-HCB is metabolized by direct hydroxylation, only one product, 3-hydroxy-2,4,6,2',4',6'-HCB, should be formed, because all four unsubstituted positions of 2,4,6,2',4',6'-HCB are equivalent. Since M-1 was already identified as 3-hydroxy-2,4,6,2',4',6'-HCB, M-4 could be any other compound, a chlorine-shifted metabolite. It was speculated above that the dechlorinated metabolite, 4-hydroxy-2,6,2',4',6'-PenCB (M-2) might be produced *via* the 3,4-epoxy-2,4,6,2',4',6'-HCB intermediate. This intermediate could also be transformed to 4-hydroxy-

Fig. 3. Mass Spectra of Four Methylated Metabolites

Fig. 4. Possible Metabolic Pathways for M-2 Formation Using Microsomes of PB-Treated Dog

Fig. 5. Mass Spectra (a, c) and ^1H -NMR Spectra (b, d) of the Methylated Derivative of a Further Metabolite of M-1 and M-2, Respectively

2,3,6,2',4',6'-HCB, a candidate for M-4, as shown in Fig. 6. An attempt to synthesize this postulated NIH-shift metabolite was made by reaction D in Fig. 1. In this reaction, 2-hydroxy-3,4,6,2',4',6'-HCB was also expected to be obtained, and this compound could also be produced via the 2,3-epoxy-2,4,6,2',4',6'-HCB intermediate by an NIH-shift. We reported previously that the microsomal mixed-function oxygenase system in PB-treated dog liver could metabolize 2,4,5,2',4',5'-HCB (IUPAC PCB No. 153) via a 2,3-arene oxide intermediate.⁸⁾ Therefore, 2-hydroxy-3,4,6,2',4',6'-HCB was also synthesized to ascertain whether 2,4,6,2',4',6'-HCB could be metabolized by the same mechanism.

Reaction D gave two products as expected. After removal of unreacted trichlorophenol *in vacuo*, the residue was dissolved in a small amount of acetone and methylated with dimethyl sulfate in the presence of 0.5 g of K_2CO_3 . Methylated derivatives of two compounds were then isolated by preparative TLC and designated methylated S-4a and S-4b in decreasing order of their R_f values. The mass spectra and ^1H -NMR spectra of these compounds are shown in Table I. It is known that the *ortho* proton of phenol exhibits its signal at a higher magnetic field than does the *para* proton.¹⁷⁾ Furthermore, methylated S-4a showed a diagnostic fragment ion peak at $[\text{M} - 50]^+$, being due to a loss of a methyl group and a chlorine atom from the molecular ion. Such a peak indicated the existence of an *ortho* [2(2') or 6(6')] methoxy group.²²⁾ Thus, S-4a and S-4b were concluded to be 2-hydroxy-3,4,6,2',4',6'-HCB and 4-hydroxy-2,3,6,2',4',6'-HCB, respectively.

The retention time on GC and the mass spectrum of methylated M-4 were identical to those of methylated S-4b. Therefore, we concluded that the structure of M-4 was 4-hydroxy-2,3,6,2',4',6'-HCB. 2-Hydroxy-3,4,6,2',4',6'-HCB could not, however, be found in the incubation mixture.

Identification of M-3 As described above, M-3 was

identified a dihydroxy-PenCB and could be a secondary metabolite of either M-1 or M-2. To examine this possibility, authentic 3-hydroxy-2,4,6,2',4',6'-HCB, corresponding to M-1, was incubated with liver microsomes under the conditions described in Materials and Methods. As a result, only one major metabolite was formed and the retention time on GC and the mass spectrum of its methylated derivative were identical to those of methylated M-3. Then, we collected the metabolite to measure its ^1H -NMR spectrum. Figure 5 shows the mass (a) and ^1H -NMR (b) spectra of the metabolite of M-1. Based on this ^1H -NMR spectrum, which shows two singlet signals corresponding to one and two protons, respectively, at 6.99 and 7.45 ppm, it is evident that the two hydroxyl groups of this molecule are located on one phenyl ring.

To further confirm the structure of M-3, metabolism of M-2 was examined in the same way described above by use of the authentic sample (4-hydroxy-2,6,2',4',6'-PenCB) as a substrate. Interestingly, only one major metabolite was again obtained and the retention time on GC and the mass spectrum (Fig. 5c) of its methylated derivative were the same as those of methylated M-3. TLC analysis of both metabolites from M-1 and M-2 demonstrated the same R_f value (data not shown). Moreover, we measured the ^1H -NMR spectrum (Fig. 5d) of the metabolite of M-2.

As shown in Fig. 5d, the spectrum is identical to that of the metabolite of M-1 (Fig. 5b). From these results M-3 was concluded to be a common secondary metabolite of both M-1 and M-2, and the structure was thus confirmed as 3,4-dihydroxy-2,6,2',4',6'-PenCB.

Discussion

In the present study, we were able to find at least four metabolites of 2,4,6,2',4',6'-HCB, using PB-treated dog liver microsomes. The structures of these metabolites were 3-hydroxy-2,4,6,2',4',6'-HCB (M-1), 4-hydroxy-2,6,2',4',6'-

Fig. 6. Proposed Metabolic Pathways *in Vitro* of 2,4,6,2',4',6'-HCB in the Dog

PenCB (M-2), 3,4-dihydroxy-2,4,6,2',4',6'-PenCB (M-3) and 4-hydroxy-2,4,6,2',4',6'-HCB (M-4).

3-Hydroxy-2,4,6,2',4',6'-HCB is the only metabolite identified in feces of rats treated with 2,4,6,2',4',6'-HCB.^{15,16} With regard to the mechanism for biotransformation of this congener in the rat, Kato *et al.* assumed a direct hydroxylation mechanism, since there was no evidence of a chlorine-shift or dechlorination.¹⁶ In contrast, we found a chlorine-shifted metabolite (M-4) and a dechlorinated metabolite (M-2), together with 3-hydroxy-2,4,6,2',4',6'-HCB, in this study. This evidence suggests that the dog metabolizes this congener not only by a direct hydroxylation mechanism but also *via* an arene oxide intermediate.

It is interesting to note that M-3 was obtained as a common secondary metabolite from M-1 and M-2. Therefore, M-3 could be formed from M-1 by a dechlorination-hydroxylation mechanism and/or from M-2 by a direct hydroxylation mechanism. In our other investigation, reported elsewhere, M-3 was also detected in the feces of a dog treated with 2,4,6,2',4',6'-HCB,²³ but it is still not known whether M-3 was derived predominantly from either M-1 or M-2 *in vivo*. In the case that M-3 is produced from M-1, a mechanism involving a 4,5-arene oxide intermediate is shown in Fig. 6.

Although a hypothetical chlorine-shifted metabolite, 3,4-dihydroxy-2,5,6,2',4',6'-HCB, was not found in this study, a chlorine-shift does not always seem to occur simultaneously with dechlorination.^{24,25}

We reported that 2,4,5,2',4',5'-HCB could be metabolized by a mechanism *via* a 2,3-arene oxide intermediate using microsomes of PB-treated dog.⁸ On the other hand, the

present study has made clear that 2,4,6,2',4',6'-HCB can be metabolized *via* a 3,4-, but not a 2,3-arene oxide with the same source of microsomes. This apparent discrepancy could be explained in part by the fact that different cytochrome P-450 isozymes were involved in the metabolism of these two congeners. Our preliminary study showed that the metabolism of 2,4,5,2',4',5'-HCB increased greatly after PB-treatment in the dog, while the metabolism of 2,4,6,2',4',6'-HCB could occur appreciably even with untreated dog microsomes and was not increased as much as that of the former congener by PB-treatment. In addition, the metabolism of 2,4,5,2',4',5'-HCB was highly inhibited by chloramphenicol, which is reported to be a selective inactivator of the major PB-inducible P-450 isozyme of dog liver, compared with the inhibition of 2,4,6,2',4',6'-HCB metabolism using PB-treated dog microsomes.²³

Sipes *et al.* supposed that the dog may possess a higher ability than other species for direct insertion of a hydroxyl group at *meta* positions [3(3') or 5(5')] on the biphenyl ring.²⁶ However, our data strongly suggest that the dog possesses unique ability for the formation of arene oxide intermediates even in PCBs without two adjacent unsubstituted carbon atoms.

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