Incubation of phenobarbital-induced rat liver microsomes with 2,2',5,5'-tetrachlorobiphenyl (TCB) yielded about 30% of the substrate as 3-hydroxy-TCB, 3,4-dihydroxy-TCB, 3,3'-dihydroxy-TCB (tentative identification), and 3,4-dihydro-3,4-dihydroxy-TCB in relative amounts of about 1:1:0.05:0.05. Under identical conditions, 2,2',5,5'-tetrachlorobiphenyl 3,4-oxide (TCBO) yielded about 45% of the substrate as the above products in an approximate ratio of 0.1:1:0.01:1, as well as 10% as TCB. Omission of NADPH from incubations of TCBO decreased the yields of 3-hydroxy-TCB, both dihydroxy-TCBs, and TCB by 75–100%, increased the yield of 3,4-dihydro-3,4-dihydroxy-TCB 4-fold, and permitted the recovery of small amounts (0.5% yield) of 4-hydroxy-TCB. 3-Hydroxy-TCB and 4-hydroxy-TCB were both extensively metabolized to 3,4-dihydroxy-TCB; 3-hydroxy-TCB was also metabolized to the presumed 3,3'-dihydroxy-TCB. The metabolism of TCBO to 3,4-dihydro-3,4-dihydroxy-TCB was inhibited by 1,1,1-trichloropropene 2,3-oxide. These data indicate that the majority (>90%) of the primary oxidation of TCB occurred via 3-hydroxylation mechanisms not involving TCBO and that only a small fraction occurred via 3,4-epoxidation. The formation of 3-hydroxy-TCB and TCBO from TCB via different pathways was consistent with the observation that TCB-treated rats excreted 6-fold higher levels of 3-hydroxy-TCB in their feces than did TCBO-treated rats.

The oxidative metabolism of many aromatic hydrocarbons and their derivatives has been established to occur primarily via addition of oxygen atoms to aromatic double bonds to form arene oxides which can then isomerize to phenols (1). Based on deuterium isotope effects, non-arene oxide mechanisms of aromatic hydroxylation have also been implicated in the meta-hydroxylation of nitrobenzene and methylphenyl sulfone by the rat (2) and in the meta-hydroxylation of biphenyl by hepatic microsomes from mice, hamsters, and PB'-induced rats (9). Similarly, non-arene oxide mechanisms of oxidation have been suggested to play a role in the metabolism of ellipticine (4) and 2,2',4,4',6'-hexachlorobiphenyl (5) in the rat, but for these substrates this conclusion was inferred from the absence of a detectable NIH shift. The interpretation of these data is complicated by the fact that substrates with ionizable substituents para to the site of hydroxylation also undergo minimal NIH shift during metabolism via arene oxide mechanisms of oxidation (6). The most direct data for non-arene oxide mechanisms of aromatic hydroxylation have been provided by Selander and co-workers (7, 8) who observed that meta-chlorophenol was formed as a minor metabolite from chlorobenzene in the rat and by rat liver microsomes but was not formed via isomerization of either 3- or 4-chlorobenzene oxide. Tomaszewski et al. (2) suggested that direct insertion of an oxygen atom into a carbon-hydrogen bond is the most likely mechanism for non-arene oxide hydroxylation, but they noted that abstraction and addition-rearrangement mechanisms of oxidation have not been experimentally excluded.

Recent investigations on the metabolism of TCB (9-12), a major component of the polychlorinated biphenyl mixture Aroclor 1254, have shown that in the rat this halogenated aromatic hydrocarbon is rapidly metabolized and excreted in the feces and bile as conjugates of 3-OH-TCB (major metabolite), 3,4-dihydriodiol-TCB (minor metabolite), and 4-OH-TCB (trace metabolite). TCB is also rapidly metabolized by PB-induced rat liver microsomes to form 3-OH-TCB as a major metabolite, 3,4-dihydriodiol-TCB as a minor metabolite, and two di-OH-TCB metabolites (13). Involvement of TCBO in the formation of 3,4-dihydriodiol-TCB has recently been demonstrated by the isolation of [3H]TCBO from microsomal incubations of [3H]TCB in which the formation of the dihydriodiol was blocked by TCPO (14, 15). The role of an arene

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† Address after June 1, 1983, Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, School of Medicine, University of Washington, Seattle, WA 98195.

§ To whom correspondence should be addressed.

1 The abbreviations used are: PB, phenobarbital; NIH shift, aromatic carbon hydroxylation with simultaneous migration of a ring substituent; TCB, 2,2',5,5'-tetrachlorobiphenyl; OH-TCB, hydroxy-2,2',5,5'-tetrachlorobiphenyl; 3,4-dihydriodiol-TCB, 3,4-dihydriodiol-2,2',5,5'-tetrachlorobiphenyl; di-OH-TCB, dihydroxy-2,2',5,5'-tetrachlorobiphenyl; TCBO, 2,2',5,5'-tetrachlorobiphenyl 3,4-oxide; TCPO, 1,1,1-trichloropropene 2,3-oxide; HPLC, high performance liquid chromatography; GC/MS, gas-liquid chromatography/mass spectrometry; [3H]TCB, 2,2',5,5'-tetrachloro[3H]biphenyl; 3,4-di-OH-biphenyl; 3,4-dihydriodiolbiphenyl; 3-MC, 3-methylcholanthrene; 7,8-BF, 7,8-benzoflavone; MeSO, dimethyl sulfoxide; BSTFA/TMCS, N,O-bistrimethylsilyl trifluoroacetamide with 1% trimethylchlorosilane; SKF-525A, 2-diethylaminooethyl-2,2-diphenylvalerate·HCl; metyrapone, 2-methyl-2-hydroxy(3-pyridyl)·1-propa-
oxide in the formation of the major metabolite 3-OH-TCB is unclear. TCB is a remarkably stable arene oxide (half-life in methanol at 23 °C = 20 days) which undergoes nonenzymatic isomerization to yield predominantly 4-OH-TCB (80%) and smaller amounts of 3-OH-TCB (20%) (16, 17).

The present paper compares the metabolism of TCB, TCB, 3-OH-TCB, and 4-OH-TCB by rat liver microsomes and the metabolism of TCB and TCB by rat liver microsomes. Direct evidence is presented that 3,4-dihydroxylation and 3-hydroxylation occur via different pathways, both of which are catalyzed by PB-inducible cytochrome P-450 isozymes. This appears to be the first example of cytochrome P-450-mediated ring hydroxylation of an aromatic substrate which occurred almost exclusively (>90%) via a non-arene oxide mechanism.

MATERIALS AND METHODS

Chemicals—TCBO (99% pure by HPLC) and 4-OH-TCB (95% pure with 5% 3-OH-TCB by GC/MS) were prepared by Dr. I. L. Reich (Department of Chemistry, University of Wisconsin) according to a modified procedure of Reich and co-workers (13). The elution was monitored at 1 ml/min with a 50-min linear gradient ranging from 50 to 100% methanol in water (v/v). The elution was monitored at 254 nm, and 1-min fractions were collected directly into scintillation vials and mixed with 10 ml of Aquasolve (New England Nuclear, Boston, MA) for determinations of H in a Nuclear-Chicago model pds/3-Isocap/300 liquid scintillation counter (Des Plaines, IL). The counts/min were converted to disintegrations/min by use of an external standard (1.4×107 dpm/ml). 3,4-Dihydroxy-TCB and the metabolite tentatively identified as 3,3'-dihydroxy-TCB co-eluted from the HPLC column with a retention time of 29 min. 3-OH-TCB and TCB eluted at 43 and 49 min, respectively, while the metabolite identified as 5,4-dihydroxy-TCB eluted as a broad peak centered under the TCB peak.

Microsome Preparation—Male outbred albino Sprague-Dawley rats (200-250 g) were obtained from ARS/Sprague-Dawley (Madison, WI) and housed in suspended wire cages at 23 °C with a 12-h light/12-h dark cycle for 1 week prior to use. Control rat chow No. 5012 (Ralston-Purina Co., St. Louis, MO) and tap water were provided ad libitum. Microsomal induction was achieved by daily intraperitoneal injection of PB in water (80 mg/kg body weight/day for 4 days) or 3-MC in corn oil (30 mg/kg body weight/day for 4 days). Untreated animals served as controls. The diet was removed after the last injection, and all animals were killed by decapitation 24 h thereafter. The livers were removed immediately after decapitation, the hepatic microsomal fraction was isolated as previously described (13) and stored at −70 °C. The metabolite yields were not significantly affected by storage in this manner. Microsomal protein concentrations were determined by the procedure of Lowry et al. (19) as adapted by Cooper (20). Microsomal Incubations—The incubations were carried out in un-stopped Erlenmeyer flasks for 15-60 min at 37 °C in a shaking metabolic incubator. Each flask contained, in a total volume of 3.4 ml, the substrate (4-70 μM), sodium phosphate buffer (44 mM, pH 7.4), MgCl2 (3.3 mM), glucose-6-phosphate (2.9 mM), glucose-6-phosphate dehydrogenase (0.7 unit/ml), NADP (1.5 mM), and rat liver microsomes (1.3 mg of protein/ml). Where indicated, 7,8-β-F, metyrapone, or SKF-525A (1 mM) were added in acetone (0.5%, v/v); these inhibitors were added immediately prior to the addition of substrate. The incubations were started by the addition of substrate in methanol (40 μl) to each flask. The methanol was evaporated, and the incubation mixtures were extracted four additional times with equal volumes of ethyl acetate, and the pooled extracts were analyzed by GC/MS or HPLC as described above or by Sephadex LH-20 column chromatography as previously reported (13). Under these conditions, the elution time of TCB (60 μM) proceeded at a constant rate for approximately 30 min (as judged by the appearance of substrate). The relative proportions of TCB metabolites formed were not significantly affected by the solvent vehicles (methanol, Me2SO, and acetone) present in the incubation mixtures.

Catalytic Dechlorination of Hydroxy-TCBs—Dechlorination of hydroxy-TCB metabolites was achieved by reaction with Raney nickel (Pfaltz and Bauer, Inc., Stamford, CT) in aqueous alkali as previously described (13). The dechlorinated products of interest were not detected by GC/MS in the diethyl ether extracts of the reaction mixture. To facilitate the recovery of catalyst-bound products, the Raney nickel was filtered from the reaction mixture, and the filtrate was evaporated to dryness. The filtrate was then reacted with 20 ml of 2 N HCl at room temperature with frequent stirring until H2 gas was no longer released (approximately 5 h). The 2 N HCl suspension of the catalyst was then extracted two times with 20 ml of benzene and five times with 20 ml of diethyl ether. The benzene and diethyl ether extracts were pooled and dried under reduced pressure at 37 °C, and the residue was dissolved in 50 μl of methylene chloride and reacted with an equal volume of BSTFA/TMCS prior to GC/MS analysis.

Isolation of Rat Fecal Metabolites—Male outbred albino Sprague-Dawley rats (100-200 g) were randomly divided into two groups (5 rats/group) and housed in suspended wire cages (1 rat/cage) as described above. Wayne Breeder Box (Wayne Vet Food Division, Continental Grain Co., Chicago, IL) and water were provided ad libitum. The animals of each group were injected intraperitoneally with either TCB or TCBO (170 μmol/kg body weight) in 0.4-0.5 ml of “90+”% tricaprylin (Sigma Chemical Co., St. Louis, MO). The

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Addition of TCBO to a microsomal incubation mixture and by the difference in the levels of TCB phenols before and derivatized diOH-TCB metabolites which were previously isolated (13). Twenty-one to 25% of the substrate was recovered as unmodified TCB and (6) its mass spectrum which exhibited minimal effluent at 344 m/e + showed no GC peaks with an isotope abundance of about 96% of the substrate as TCBO, 2% as 4-OH-TCB, and the other diOH-TCB metabolite (Fig. 1A). The compound eluting in peak 1 was unambiguously identified as Me3Si-derivatized 3-OH-TCB (70 μm); B, TCBO (70 μM); C, TCBO (70 μM) in the absence of NADP; D, TCBO (70 μM) plus TCPO (700 μM) in the absence of NADP; E, 3-OH-TCBO (14 μM) plus 4-OH-TCBO (56 μM) from the acid-catalyzed isomerization of TCB; F, 4-OH-TCBO (70 μM); and G, 3-OH-TCBO (4 μM). Incubations were conducted at 37 °C for 15 min in a 10.2-ml total volume containing substrate, PB-induced rat liver microsomes, and an NADPH-generating system (with and without NADP) as described under "Materials and Methods." All of the incubations were started by the addition of the substrate in 90 μl of methanol. The ethyl acetate extracts from each incubation (10.2 ml, 5 times) were pooled and dried at 37 °C under reduced pressure. The residue was dissolved in 0.5 ml of methylene chloride, and the resultant yellow residue was dissolved in 1 ml of toluene and mixed with authentic TCB and 3-OH-TCB; a 0.1-ml aliquot was analyzed by HPLC. The mass spectra of the Me3Si-derivatized diOH-TCB metabolites. The feces were dried under reduced pressure at room temperature, pulverized, and extracted four times with 10 ml of toluene. The toluene was removed under reduced pressure at 37 °C, and the resultant yellow residue was dissolved in 1 ml of toluene and applied to a silica gel (5% deactivated) column (6 × 0.6 cm). The column was eluted with 10 ml of toluene and the eluent was dried under reduced pressure at 37 °C. The residue was dissolved in 50 μl of dry methylene chloride and reacted with 50 μl of BSTFA/TMCS; 2-μl aliquots of the methylene chloride:BSTFA/TMCS solution were analyzed by GC/MS.

RESULTS

Microsal Monocyclic Metabolism of TCB, TCBO, 4-OH-TCB, and 3-OH-TCB to Ring-hydroxy Metabolites—GC/MS analyses of Me3Si-derivatized ethyl acetate extracts from 15-min incubations of PB-induced microsomes with TCB confirmed that TCB was converted to the four previously isolated hydroxy metabolites (13), viz. 3-OH-TCB, 3,4-dihydrodiol-TCB, and two diOH-TCBs (Fig. 1A). No 4-OH-TCB or 6-OH-TCB was detected (retention time of authentic Me3Si-derivatized 6-OH-TCB = 8.1 min; limit of detection = 0.05%). Seventy-one to 74% of the substrate was recovered as unmodified TCB (retention time = 3.7 min). Monitoring of the GC column effluent at 344 m/e* showed no GC peaks with an isotope abundance or fragmentation pattern characteristic of a Me3Si-derivatized hydroxytrichlorobiphenyl.

Incubations of TCBO, conducted under identical conditions, yielded primarily 3,4-dihydrodiol-TCB and one of the diOH-TCB metabolites, as well as small quantities of 3-OH-TCB and the other diOH-TCB metabolite (Fig. 1B). The compound eluting in peak 1 was ambiguously identified as Me3Si-derivatized 3-OH-TCB and distinguished from the closely eluting Me3Si-derivatized 4-OH-TCB by (a) its co-chromatography with authentic Me3Si-derivatized 3-OH-TCB and (b) its mass spectrum which exhibited minimal fragmentation to [M − Me − HCO2] ion (22% relative intensity) at the isotopic peak of Me3Si-derivatized 4-OH-TCB (13). There was no detectable 4-OH-TCB. After 15 min of incubation, only 9–10% of the substrate was recovered unchanged, as determined by the difference in the levels of TCB phenols before and after treatment of the extracts with concentrated H2SO4. Addition of TCBO to a microsomal incubation mixture and immediate extraction with ethyl acetate resulted in the recovery of about 98% of the substrate as TCB, 2% as 4-OH-TCB, 1% as 3-OH-TCB, 1% as 3,4-dihydrodiol-TCB, and <0.05% as each of the diOH-TCBs (data not shown).

Incubations of TCBO (15 min) conducted in the absence of NADP resulted in high yields of 3,4-dihydrodiol-TCB and small amounts of 3-OH-TCB and 4-OH-TCB (Fig. 1C). There was no detectable formation of either diOH-TCB metabolite. Twenty-one to 25% of the substrate was recovered unchanged.

FIG. 1. GC/MS analyses of Me3Si-derivatized ethyl acetate extracts from PB-induced microsomal incubations. A, TCB (70 μM); B, TCBO (70 μM); C, TCBO (70 μM) in the absence of NADP; D, TCBO (70 μM) plus TCPO (700 μM) in the absence of NADP; E, 3-OH-TCB (14 μM) plus 4-OH-TCBO (56 μM) from the acid-catalyzed isomerization of TCB; F, 4-OH-TCBO (70 μM); and G, 3-OH-TCBO (4 μM). Incubations were conducted at 37 °C for 15 min in a 10.2-ml total volume containing substrate, PB-induced rat liver microsomes, and an NADPH-generating system (with and without NADP) as described under "Materials and Methods." All of the incubations were started by the addition of the substrate in 90 μl of methanol. The ethyl acetate extracts from each incubation (10.2 ml, 5 times) were pooled and dried at 37 °C under reduced pressure. The residue was dissolved in 0.5 ml of methylene chloride, and the resultant yellow residue was dissolved in 1 ml of toluene and mixed with authentic TCB and 3-OH-TCB; a 0.1-ml aliquot was analyzed by HPLC.

The mass spectra of the Me3Si-derivatized diOH-TCB metabolites in peaks 1 and 2 were identical with those of the Me3Si-derivatized diOH-TCB metabolites "Hb" and "Hc," respectively, which were previously isolated (13).
The microsomal incubations of TCBO (70 μM) were conducted as described in Fig. 1. The ethyl acetate extracts from each incubation (10 ml, 5 times) were pooled and dried at 37 °C under reduced pressure. The residue was dissolved in 0.5 ml of methylene chloride, and 0.1 ml aliquots were mixed with 1 drop of concentrated H₂SO₄; μl of the methylene chloride phase was analyzed by GC/MS. Quantitation was achieved by comparing the total-ion current peak areas obtained for the TCBO metabolite by GC with those obtained for known amounts of authentic TCB. The data are for two experiments carried out at different times on the same pooled, frozen microsomes.

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>TCBO recovered as TCB (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>15</td>
<td>9.5, 9.8</td>
</tr>
<tr>
<td>15 (no NADP)</td>
<td>0.2, 0.2</td>
</tr>
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</table>

Addition of TCPO (700 μM) to incubations of TCBO conducted in the absence of NAPD completely inhibited the formation of 3,4-dihydriodiol-TCB and enhanced the yields of both 3-OH-TCB and 4-OH-TCB (Fig. 1D). Only 27–32% of the substrate was recovered unchanged.

The observation that 4-OH-TCB was recovered from incubations of TCBO only in the absence of NAPD (Figs. 1, B–D) suggested that 4-OH-TCB might be undergoing secondary metabolism. Incubations of a mixture of 56 μM 4-OH-TCB and 14 μM 3-0H-TCB (prepared via acid-catalyzed isomerization of TCBO, Ref. 15) yielded the dioxH-TCB eluting as a Me₃Si derivative in peak 5 as a minor metabolite and the dioxH-TCB eluting as a Me₃Si derivative in peak 4 as a minor metabolite (Fig. 1F). The 5:1 ratio of 4-OH-TCB:3-OH-TCB recovered after 15 min of incubation indicated that 3-OH-TCB might also be undergoing secondary metabolism. Incubations of 4-OH-TCB (70 μM) alone gave high yields of the dioxH-TCB eluting as a Me₃Si derivative in peak 5 and no detectable formation of the dioxH-TCB eluting as a Me₃Si derivative in peak 4 (Fig. 1F). Both dioxH-TCB metabolites were formed from 3-OH-TCB (4 μM) (Fig. 1G).

Microsomal Reduction of TCBO to TCB—GC/MS determinations of TCBO formed during the microsomal metabolism of TCBO were confounded by the fact that TCBO undergoes partial (60%) reduction to TCB under the GC conditions used. To avoid this complication, quantitation of TCBO in the presence of TCBO was performed by treating the incubation extracts with concentrated H₂SO₄ prior to GC/MS analysis; this treatment catalyzed the quantitative isomerization of TCBO to 3- and 4-OH-TCB without modifying TCB. With this analysis, PB-induced microsomes were observed to catalyze the reduction of TCBO to TCB (Table I). Negligible amounts of TCBO were obtained from incubations that lacked NADP or from uninoculated reaction mixtures (Table I).

Identification of 3,4-dioH-TCB—The observation that the dioxH-TCB eluting as a Me₃Si derivative in peak 5 was a metabolite of both 4-OH-TCB (Fig. 1F) and 3-OH-TCB (Fig. 1G) suggested that the hydroxyl groups were at C-3 and C-4 or at C-3 and C-4'. The mass spectrum of this Me₃Si-derivatized dioxH-TCB exhibited an ion at 147 m/z (5% of M⁺); this finding suggested the formation of a [Me₃Si=O-SiMe₃]⁺ ion, which results from the conjugation of two silyl ether residues on proximal carbon atoms (22). Fragments at 147 m/z were also observed in the spectra of Me₃Si-derivatized 3,4-dioH-biphenyl and Me₃Si-derivatized 3,4-dihydriodiol-TCB, but they were not seen in the spectrum of the Me₃Si-derivatized dioxH-TCB eluting in peak 4. Moreover, the mass spectral fragmentation patterns of the Me₃Si-derivatized dioxH-TCB in peak 5 and authentic Me₃Si-derivatized 3,4-dioH-biphenyl were nearly identical. Both molecules exhibited base peaks at 73 m/z (consistent with [Me₃Si⁺]), strong molecular ions, and fragmentation predominantly to ions consistent with [M − Me₃]*, [M − OSiMe₂ + 1]*, [M − OSiMe₂ + 2 − Me]*, and [M − OSiMe₂ + 1 − 2Me]*. These data strongly suggested that this dioH-TCB was the 3,4-dioH-biphenyl. This structure was confirmed by dechlorination of the ethyl acetate extracts from a microsomal incubation of 4-OH-TCB and comparison of the dechlorinated products with authentic 3,4-dioH-biphenyl. Dechlorination of extracts which contained 50 μg of 4-OH-TCB and 160 μg of the dioH-TCB yielded a product (0.06%) yield which, after Me₃Si derivatization, exhibited a GC retention time and mass spectral fragmentation pattern identical with those of authentic Me₃Si-derivatized 3,4-dioH-biphenyl (Fig. 1). Another product (0.4% yield) with a GC retention time (2.1 min) and mass spectral fragmentation pattern (m/z+ (relative intensity) = 242 (100), 227 (77), 211 (30)) identical with those of authentic Me₃Si-derivatized 4-hydroxybiphenyl was also identified in the dechlorinated, Me₃Si-derivatized extracts.

Tentative Identification of 3,3'-dioH-TCB—The observation that the dioH-TCB eluting as a Me₃Si derivative in peak 4 was a metabolite of 3-OH-TCB suggested that the hydroxyl groups were at C-5 and C-5'. The mass spectrum of this Me₃Si-derivatized dioH-TCB exhibited an ion at 171 m/z (5% of M⁺); this finding suggested the formation of a [Me₃Si=O-SiMe₃]⁺ ion, which results from the conjugation of two silyl ether residues on proximal carbon atoms (22). Fragments at 171 m/z were also observed in the spectra of Me₃Si-derivatized 3,3'-dioH-biphenyl and Me₃Si-derivatized 3,4-dihydriodiol-TCB, but they were not seen in the spectrum of the Me₃Si-derivatized dioH-TCB eluting in peak 4. Moreover, the mass spectral fragmentation patterns of the Me₃Si-derivatized dioH-TCB in peak 5 and authentic Me₃Si-derivatized 3,4-dioH-biphenyl were nearly identical. Both molecules exhibited base peaks at 73 m/z (consistent with [Me₃Si⁺]), strong molecular ions, and fragmentation predominantly to ions consistent with [M − Me₃]*, [M − OSiMe₂ + 1]*, [M − OSiMe₂ + 2 − Me]*, and [M − OSiMe₂ + 1 − 2Me]*. These data strongly suggested that this dioH-TCB was the 3,4-dioH-biphenyl. This structure was confirmed by dechlorination of the ethyl acetate extracts from a microsomal incubation of 4-OH-TCB and comparison of the dechlorinated products with authentic 3,4-dioH-biphenyl. Dechlorination of extracts which contained 50 μg of 4-OH-TCB and 160 μg of the dioH-TCB yielded a product (0.06%) yield which, after Me₃Si derivatization, exhibited a GC retention time and mass spectral fragmentation pattern identical with those of authentic Me₃Si-derivatized 3,4-dioH-biphenyl (Fig. 2). Another product (0.4% yield) with a GC retention time (2.1 min) and mass spectral fragmentation pattern (m/z+ (relative intensity) = 242 (100), 227 (77), 211 (30)) identical with those of authentic Me₃Si-derivatized 4-hydroxybiphenyl was also identified in the dechlorinated, Me₃Si-derivatized extracts.

![Fig. 2. GC/MS analyses of Me₃Si-derivatized (A) authentic 3,4-dioH-biphenyl (100 ng) and (B) dechlorinated ethyl acetate extracts from an incubation of 4-OH-TCB with PB-induced microsomes.](http://www.jbc.org/...)

The limited quantities of pure 3-OH-TCB available precluded conducting incubations at a 70 μM substrate concentration.
ylation at C-4' appeared less likely than hydroxylation at C-3' because: 1) the diOH-TCB was not formed from 4-OH-TCB, and 2) 3-OH-TCB contains a 1-phenyl-2,5-dichlorobenzene moiety which, as evidenced by TCB, is preferentially hydroxylated at C-3. Based on these arguments, the diOH-TCB eluting as a MeSi derivative in peak 4 was tentatively assigned the structure of 3,3'-diOH-TCB.

Effects of Microsomal Induction on [\(^3\H\)]TCB Metabolism—
Fractionation of the ethyl acetate extracts from 60-min microsomal incubations of [\(^3\H\)]TCB (60 \(\mu\)M, 15 mCi/mmol) on a Sephadex LH-20 column (13) capable of separating unmetabolized TCB from 3-OH-TCB (peak 1) and from 3,4-dihydriodiol-TCB, 3,4-dioH-TCB, and the presumed 3,3'-diOH-TCB (co-eluting in peak 2) revealed significant differences in the metabolite yields from control, PB-induced, and 3-MC-induced microsomes. Control microsomes converted about 1% of the TCB substrate to peak 1 and 2% to peak 2 metabolites. 3-MC induction of microsomes had no significant effect on the yields of peak 1 (1%) or peak 2 (3%) metabolites, while PB induction, as previously reported (13), greatly increased the yields of metabolites in both peaks 1 (20%) and 2 (7%). HPLC analysis of the peak 2 metabolites generated by PB-induced microsomes showed that the \(^3\)H in this LH-20 column fraction was distributed between 3,4-dihydriodiol-TCB plus 3,3'-dioH-TCB (about 30%; not resolved) and 3,4-dioH-TCB (10%). Approximately 20% of the \(^3\)H in peak 2 was associated with three unidentified products, while 40% eluted from the reverse-phase HPLC column very slowly upon exhaustive elution with 100% methanol. Greater than 80% of the \(^3\)H in the peak 1 fraction from the LH-20 column was shown by HPLC to elute with 3-OH-TCB.

Effects of Enzyme Inhibitors on the Microsomal Metabolism of [\(^3\H\)]TCB—
Incubations of PB-induced microsomes with [\(^3\H\)]TCB (70 \(\mu\)M; 2.4 mCi/mmol) were conducted with and without 1 \(\mu\)M 7,8-BF, metyrapone, or SKF-525A for 15 min under conditions where the metabolism proceeded at a constant rate for 30 min. In the absence of an inhibitor, the rates of formation of 3-OH-TCB and of 3,4-dihydriodiol-TCB plus the presumed 3,3'-dioH-TCB (not resolved) were about 400 and 50 pmol/min/mg of protein, respectively, as determined by HPLC. Metyrapone and SKF-525A inhibited the formation of these three metabolites by >90%, 7.8, BF, on the other hand, did not inhibit the formation of 3-OH-TCB and inhibited the formation of 3,4-dihydriodiol-TCB and/or the presumed 3,3'-dioH-TCB by about 30%. The rates of 3,4-dioH-TCB formation were not determined, because this metabolite eluted as a broad peak centered under the TCB peak.

Fecal Excretion of TCB and TCBO Metabolites in the Rat—
GC/MS analyses of MeSi-derivatized toluene extracts of feces from rats treated with TCB or TCBO showed two peaks with ions at \(m/e^+\) 378 \((\text{relative intensity}) = 100\), 290 (40), 255 (40), 220 (15), 185 (21)). The cumulative excretion (days 1–6) of TCB from TCB-treated rats was 0.035 \pm 0.005 \(\mu\)mol/kg body weight, while that from TCBO-treated rats was 0.017 \pm 0.006 \(\mu\)mol/kg body weight (\(p < 0.1\)). GC/MS analysis of a reaction mixture of authentic TCB plus BSTFA/TMCS yielded a 40:20:15:25 mixture of TCB (retention time = 6.4 min; mass spectrum \(m/e^+\) (relative intensity) = 306 (100), 290 (9), 271 (15), 255 (5), 243 (22), 236 (56), 220 (13), 207 (32), its reduction product TCB (retention time = 4.4 min), non-MeSi-derivatized 3-OH-TCB (retention time = 6.9 min; mass spectrum \(m/e^+\) (relative intensity) = 306 (81), 271 (18), 243 (26), 236 (100), 207 (76)) and non-MeSi-derivatized 4-OH-TCB (retention...
Non-arene Oxide Hydroxylation of 2,2',5,5'-Tetrachlorobiphenyl

D. Preston, J. A. Miller, and E. C. Miller, manuscript in preparation.

Biliary Excretion of \(^3\)H/\(^7\)Cl/TCB Metabolites in the Rat—Approximately 30% of the \(^3\)H from injected \(^3\)H/\(^7\)Cl/TCB was excreted in the bile during the first 24 h after treatment. HPLC analysis of toluene extracts of bile showed that about 2% of the \(^3\)H/\(^7\)Cl/TCB dose co-eluted with 3-OH-TCB, 0.03% co-eluted with TCB, and 0.03% had a retention time identical with those of 3,4-dihydrodiol-TCB and the presumed 3,3'-diOH-TCB. Sulfatase/glucuronidase treatment of the aqueous phase permitted the recovery of an additional 12% of the \(^3\)H/\(^7\)Cl/TCB dose as material which co-eluted with 3-OH-TCB and 3% which had a retention time identical with those of 3,4-dihydrodiol-TCB and the presumed 3,3'-diOH-TCB; an additional 3.9% was distributed between two unidentified peaks with retention times of 36 and 39 min. The relative proportions of material which eluted as 3-OH-TCB and as 3,4-dihydrodiol-TCB were very similar to the proportions of these metabolites generated by incubations of PB-induced rat liver microsomes with \(^3\)H/\(^7\)Cl/TCB. About 10% of the 3-OH-TCB excreted via the bile was toluene-extractable and therefore presumably unconjugated, while about 90% was conjugated with sulfate and/or glucuronide. Cannulation of the bile duct reduced the fecal level of toluene-extractable 3-OH-TCB by approximately 99% when compared to noncannulated rats.

**DISCUSSION**

The finding that the conversion of TCB to 3-OH-TCB in vivo and by PB-induced rat liver microsomes proceeded with 6- and 8-fold greater efficiency, respectively, than did the conversion of TCBO to 3-OH-TCB strongly suggested that 3-hydroxylation and 3,4-epoxidation of TCB occurred, at least in part, via different pathways. A primary role of hepatic cytochrome P-450 isozymes b and/or e (23, 24) in both of these pathways is indicated by the observations that: 1) PB, but not 3-MC, induction of microsomes greatly increased the yields of both 3-OH-TCB and 3,4-dihydrodiol-TCB; and 2) the cytochrome P-450 inhibitors metyrapone (24, 25) and SKF-525A (26) strongly inhibited the formation of these metabolites, while the cytochrome P-448 inhibitor 7,8-BF (27) had no effect on 3-OH-TCB formation and only slightly inhibited the formation of 3,4-dihydrodiol-TCB plus the presumed 3,3'-diOH-TCB. These data are consistent with reports that ortho-, ortho'-substituted polychlorinated biphenyls are good substrates for cytochrome P-450 enzymes and relatively poor substrates for cytochrome P-448 enzymes (28, 29).

Based on the patterns of TCBO isomerization observed in organic solvents (16, 17), it was anticipated that very slow isomerization of TCBO during microsomal incubations would yield predominantly 4-OH-TCB. In contrast, microsomal incubations of TCBO yielded 3-OH-TCB as well as 3,4-dihydrodiol-TCB, 3,4-dioH-TCB, the presumed 3,3'-dioH-TCB, and TCB, but no detectable 4-OH-TCB. In the absence of NADPH (omission of NADP), the yields of 3-OH-TCB, TCB, and both dioH-TCBs were decreased by 75–100%; the yield of 3,4-dihydrodiol-TCB increased about 4-fold and small amounts of 4-OH-TCB were recovered. Thus, no more than 25% of the 3-OH-TCB formed during the metabolism of TCBO could have been formed via nonenzymatic isomerization of TCBO, while at least 75% arose via NADPH-dependent pathways. A pathway involving an initial NADPH-dependent reduction of TCBO to TCB via an epoxide reductase (30, 31) and subsequent NADPH-dependent 3-hydroxylation of TCBO by a mechanism not involving TCBO (Fig. 5) is consistent with our data (herein and Ref. 13). The excretion of TCB and of 3.5-fold greater amounts of 3-OH-TCB than 4-OH-TCB in the feces of TCBO-treated rats is consistent with this mechanism.

The recovery of small amounts of 4-OH-TCB only from TCBO incubations conducted in the absence of NADPH suggested that NADPH-dependent metabolism of 4-OH-TCB also occurred. Facile metabolic oxidation of 4-OH-TCB to 3,4-dioH-TCB was observed. However, the amounts of 3,4-dioH-TCB formed from TCBO in the presence of NADPH (approximately 25% yield) were much greater than those expected from the amounts of 4-OH-TCB formed from TCB in the absence of NADPH (approximately 0.5% yield). Further studies showed that 3-OH-TCB was also a good substrate for the formation of 3,4-dioH-TCB as well as the presumed 3,3'-dioH-TCB. These data indicate that the dioH-TCBs formed during the metabolism of TCBO arose primarily (>95%) by secondary metabolism of 3-OH-TCB and thus originated via a circuitous pathway involving reduction of TCBO to TCB, 3-hydroxylation of TCB by a mechanism not involving TCBO, and secondary metabolism of 3-OH-TCB (Fig. 5). In addition to its apparently nonenzymatic isomerization to phenols and enzymatic reduction to TCB, TCBO was hydrolyzed to 3,4-dihydrodiol-TCB. The conversion of TCBO to the dihydrodiol proceeded at a rate of approximately 3 nmol/min/mg of protein in the absence of NAPDH and was strongly inhibited by TCPO. Thus, as compared to other arene oxides (32), TCBO is a relatively poor substrate for PB-induced rat liver epoxide hydroxylase. The low recoveries of TCBO plus TCBO metabolites from microsomal incubations of TCBO indicate that other modifications of TCBO also occurred during the incubation. Recent data from our laboratory* show that TCBO reacts with S-containing amino acids.
acids and may thus be involved in the metabolism-dependent binding of TCB to macromolecules (33).

Taken together, these data indicate that, of the TCBO formed by the microsomal metabolism of TCB, approximately 35% was reduced back to TCB, 20% was hydrated to 3,4-dihydriodiol-TCB, 10% was not further metabolized, and about 35% was not recovered. Only 1% of the TCBO underwent nonenzymatic isomerization to an about 1:4:1 mixture of 4-OH-TCB:3-OH-TCB during a 15-min incubation. Based on this distribution of TCBO metabolites and assuming that the 3,4-dihydriodiol-TCB formed during TCB metabolism arose solely from TCBO, it is estimated that the majority (>90%) of the primary metabolic oxidation of TCB occurred via 3-hydroxylation mechanisms not involving TCBO and only a small fraction (<10%) occurred via 3,4-epoxidation.

Alternative pathways for the formation of 3-OH-TCB include isomerization of TCB 2,3-oxide or non-arene oxide mechanisms (2). Two arguments suggest that metabolism via TCB 2,3-oxide is unlikely. First, C-2 of TCB would presumably be a poor target for enzymatic epoxidation because of the steric hindrance imposed by the chlorine atom at C-2 and the aromatic ring ortho to C-2 (1). Although sterically hindered arene oxides can be formed enzymatically (5, 34-42), their rates of formation are very low (5, 41, 42). Second, electron resonance stabilization of a 2-OH-3-carbocation formed during isomerization of TCB 2,3-oxide would be expected to be far greater than for a 3-OH-2-carbocation, since the 2-OH-3-carbocation should disperse its charge throughout both biphenyl ring systems at physiological temperatures where inter-ring rotation of ortho, ortho'-substituted biphenyls occurs quite rapidly (43). Thus, TCB 2,3-oxide would be expected to undergo epoxide ring opening in a manner similar to that of biphenyl 2,3-oxide (44) to yield 2-hydroxy-2',3,5,5'-tetrachlorobiphenyl (via an NIH shift of a chlorine) and/or 2-hydroxy-2',5,5'-trichlorobiphenyl (via an NIH shift and dechlorination) as the major products and only small amounts of 3-OH-TCB. The data presented here and those in the literature (9-11, 13-15) provide no evidence for the formation of TCB metabolites that result from NIH shifts of chlorine or dechlorination. Although a pathway involving site-selective, enzymatic isomerization of TCB 2,3-oxide to 3-OH-TCB cannot be excluded, this type of reaction is apparently a rare phenomenon (45). Therefore, our data strongly indicate that the formation of 3-OH-TCB from TCB by PB-induced rat liver microsomes occurs primarily by one or more of the non-arene oxide mechanisms proposed by Tomaszewski et al. (2).

Although, as detailed in the Introduction, non-arene oxide mechanisms of aromatic ring hydroxylation have been implicated in the metabolism of a number of compounds (2-5, 7, 8), the most direct evidence has been reported in the case of meta-hydroxylation of chlorobenzene (7, 8). In this case, meta-hydroxylation comprised only about 10–20% of the total metabolism of the substrate; the major metabolites were formed via arene oxide pathways. Our data appear to provide the first example of an aromatic substrate which undergoes ring hydroxylation almost exclusively by a non-arene oxide pathway. TCB may be a useful substrate for detailed examination of non-arene oxide mechanisms of oxidation.

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Non-arene Oxide Hydroxylation of 2,2',5,5'-Tetrachlorobiphenyl


Non-arene oxide aromatic ring hydroxylation of 2,2',5,5'-tetrachlorobiphenyl as the major metabolic pathway catalyzed by phenobarbital-induced rat liver microsomes.
B D Preston, J A Miller and E C Miller


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