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DIRECT EVIDENCE THAT AN ARENE OXIDE IS A METABOLIC INTERMEDIATE OF 2,2',5,5'-TETRACHLOROBIPHENYL

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Summary: Radiolabeled arene oxide was recovered from incubations containing [^{3}H]-2,2',5,5'-tetrachlorobiphenyl (^{3}H-TCB), unlabeled 2,2',5,5'-tetrachlorobiphenyl-3,4-oxide (TCBAO), 3,3,3-trichloropropene-1,2-oxide (TCPO), NADPH, and liver microsomes from phenobarbital-induced rats. No labeled arene oxide was generated in the absence of NADPH, nor during the metabolism of unlabeled TCB in the presence of [^{3}H]-H_2O. The recovered oxide (radiolabeled and carrier) was characterized by mobility on silica gel and by conversion to 3- and 4-hydroxy-TCB. Formation of a dihydrodiol metabolite was apparently blocked by inhibition of epoxide hydrase. These data provide the first direct evidence that arene oxides are intermediates of halogenated biphenyl metabolism.

Formation of arene oxides, catalyzed by hepatic cytochrome P-450 monooxygenases, is most probably an initial oxidative event in the metabolism of halogenated benzenes and biphenyls with vicinal unsubstituted carbon atoms (8, 23, 27). Observed NIH shifts and phenolic metabolites may be attributed to highly characteristic, spontaneous rearrangement of the epoxide ring (8, 26). The reported *trans*-dihydrodiol metabolites probably arise exclusively via epoxide hydrase mediated hydrolysis of arene oxide intermediates (8). Arene oxides react with intracellular nucleophiles such as glutathione, cysteine and purine bases. The occurence of NIH shifts and the formation of *trans*-dihydrodiols and thio conjugates are indirect evidence and established criteria for the intermediacy of arene oxides (8, 16, 17, 26).

Abbreviations: ^{3}H-TCB, [^{3}H]-2,2',5,5'-tetrachlorobiphenyl; TCBAO, 2,2',5,5'-tetrachlorobiphenyl-3,4-oxide; Br-AO, 2,5-dibromo-2',5'-dichlorobiphenyl-3,4-oxide; TCPO, 3,3,3-trichloropropene-1,2-oxide; PCB, Polychlorinated biphenyl; GC, gas chromatography, GC-MS, gas chromatography-mass spectroscopy; INC#1, etc., incubations as defined in text; E-INC#1, etc., material eluted from silica gel plates as defined in text.

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Polychlorinated biphenyls (PCB's) are environmental toxicants of great concern due to their widespread contamination of animals and humans (28). PCB metabolism likely occurs by direct hydroxylation as well as by epoxidations (8, 13, 27). Reported metabolism of PCB congeners that are not chlorinated in both *para* positions is limited to reactions at positions *meta* and *para* to the biphenyl bridge. PCB's with vicinal unsubstituted carbon atoms are metabolized and excreted much more rapidly than other isomers, a fact that may derive from the importance of this structural feature in epoxide ring formation (8, 13, 27). Metabolites of TCB and 2,2',4,5,5'-pentachlorobiphenyl have been identified as *trans*-dihydrodiols (1, 2, 5). Thioether and methylsulfone derivatives of tri-, tetra-, and pentachlorobiphenyls are known (15-17). NIH shifts (3, 22, 27, 31) occur during the metabolism of 4-chloro- and 4,4'-dichlorobiphenyl.

Establishment of the existence of arene oxides as intermediates in the mammalian metabolism of PCB's is a necessity to verify proposed metabolic schemes and is a prerequisite of studies of the role of these electrophiles in PCB toxicity and carcinogenicity (8, 14, 29, 30). Reich *et al.* (21) recently accomplished the synthesis of TCBAO, the expected intermediate of TCB metabolism. TCBAO is remarkably stable to spontaneous rearrangement. Metabolic production of an oxide intermediate would likely be enhanced by phenobarbital induction, known (20) to greatly increase the rate of TCB hydroxylation. It was anticipated that measurable quantities of an oxide intermediate could be recovered from a microsomal incubation if epoxide hydrase was blocked by TCPO and if unidentified degradative pathways were inhibited by exogenous TCBAO.

MATERIALS and METHODS

^{3}H-TCB and unlabeled TCB were synthesized as described by Hutzinger and Safe (6). Platinum catalyzed tritium exchange between the TCB precursor 2,2',5,5'-tetrachlorobenzidine and [^{3}H]-trichloroacetic acid was performed by New England Nuclear (Boston, MA). The final purification of the ^{3}H-TCB was by chromatography on silica gel G (Analtech, Newark, DE) using hexane-ether (9:1). This material was radiochemically pure on the basis of rechromatography in the same system. GC analysis revealed a single peak (Hewlett-Packard 7620A;

3% SE-30 on Gaschrom Q, 100-200 mesh; Argon-Methane, 95:5, apx. 40 ml/min; 1/8 inch x 6 feet glass column; ^{63}Ni electron capture detector). TCBAO and 2,5-dibromo-2',5'-dichlorobiphenyl-3,4-oxide (Br-AO) and 3- and 4-hydroxy-TCB were synthesized by the procedure of Reich *et al.* (21).

$^{3}H_2O$ was obtained from New England Nuclear. TCPO and *bis*-(trimethylsilyl)-trifluoroacetamide were obtained from Aldrich (Milwaukee, WI). Sodium $NADP^+$, NADH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO). Phenobarbital and Sephadex LH-20 were obtained from Mallinckrodt (St. Louis, MO) and Pharmacia (Piscataway, NJ) respectively.

Liver microsomes were prepared from male Sprague-Dawley rats (apx. 250 g) that had received injections (i.p.) of 80 mg/kg body weight phenobarbital on each of four consecutive days. Livers were homogenized in ice-cold buffer (240 mM Tris-HCl at pH 7.5; 1 mM EDTA, 0.2% w/v niacinamide) in a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 16,000 x g (average) for 10 min; the resultant supernatant was centrifuged at 16,000 x g for 20 min, and the second supernatant was centrifuged at 100,000 x g for 60 min. The microsomal pellet was resuspended in 1.15% KCl, 5 mM Tris-HCl at pH 7.4 and again centrifuged at 100,000 x g for 60 min. The microsomes were resuspended in 30% v/v glycerol, divided into aliquots and stored at -70°C. Protein was measured by the procedure of Lowry *et al.* (12).

Incubations of 40 ml, which were designated INC#1 to #4 were performed in a Dubnoff shaker bath for 30 min. INC#1 contained 25 µg/ml TCBAO (added in 120 µl acetone), 25 µg/ml ^{3}H-TCB (16.4 Ci/mole, 120 µl acetone), 0.4 mM TCPO (40 µl acetone), 0.9 mg/ml microsomal protein, 5 mM $MgCl_2$, 0.3 mM NADH, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and 50 mM potassium phosphate at pH 7.4. NADH and the NADPH regenerating system were omitted from INC#2. An equal weight of Br-AO replaced TCBAO in INC#3, which was otherwise the same as INC#1. ^{3}H-TCB was replaced by an equivalent amount of unlabeled TCB in INC#4, which also contained 70 µCi[^{3}H]-H_2O in addition to the other materials listed for INC#1. Incubations were terminated by extraction (5X) with two volumes of ethyl acetate.

The combined ethyl acetate extracts of a given incubation were reduced to dryness in a Roto-vap. The residue was dissolved in solvent A (CH_2Cl_2, hexane, methanol; 65:35:1) and applied to a 1 x 25 cm Sephadex LH-20 column that was developed first with this solvent and subsequently with solvent B (65:35:5). An aliquot of each 3.2 ml column fraction was added to 10 ml toluene-Triton X-100 (2:1), which contained 3.33 g/L Omnifluor (New England Nuclear), and the radioactivity was measured in a Packard 3255 scintillation counter. A portion of combined fractions 4-6 was applied 2 cm from the end of 20 cm flexible, thin-layer silica gel plates (Baker-Flex 1B, Phillipsburg, NJ) that were developed with hexane-ether (9:1, trace NH_4OH). Spots were visualized with 254 nm light. The plates were cut into 4 mm sections that were immersed in 10 ml scintillation fluid and counted. Alternatively, material that migrated as TCBAO (R_f=0.40) was recovered by hexane elution of sections 15-16. Portions of the eluted material were rechromatographed on silica gel. Other portions were treated with *p*-toluenesulfonic acid in CH_2Cl_2 and rechromatographed after extraction (5X) of the acid with water. Radioactivity in 4 mm sections of these plates was measured.

GC-MS analyses of INC#1 metabolites were performed using a Hewlett-Packard 5710A gas chromatograph - 5980A mass spectrometer as detailed by Preston and Allen (20). All GC-MS samples were converted to the trimethylsilyl derivatives by reaction (apx. 30 min, 25°C) with excess *bis*-(trimethylsilyl)-trifluoroacetamide in CH_2Cl_2. Portions of combined LH-20 column peak II fractions were injected onto a 1% SE-30 column that was run with a temperature program (170°C for 2 min increasing to 220° at 8°/min). The effluant

was monitored on the basis of total ion or single ion (m^+/e=466, m^+/e=468) current. GC-MS analysis of the material (E-INC#1) eluted from silica gel sections 15-16 and treated with acid utilized a 1.5% OV-17/1.95% SP-2401 column operated at 190°C.

RESULTS

^{3}H-TCB was incubated for 30 min with PB-induced microsomes in the presence of carrier TCBAO, an NADPH regenerating system and TCPO. An ethyl acetate extract of this incubation (INC#1) was chromatographed on Sephadex LH-20 (Figure 1). TCB and TCBAO standards elute in fractions 4-6. Preston and Allen (20) have shown that peak I corresponds to 3-hydroxy-TCB, and that peak II fractions contain two different dihydroxy-TCB's (designated IIb and IIc) as well as 3,4-dihydro-3,4-dihydroxy-TCB (dihydrodiol); the latter present in the greatest amount. Peak II fractions were pooled and a portion was analyzed by GC-MS. Metabolite IIc was readily detected (full scale deflection) and identified from its retention time on the 1% SE-30 column and its mass spectrum (20). Neither the dihydrodiol nor metabolite IIb were detectable by either single ion (m^+/e=468 or m^+/e=466) or total ion current.

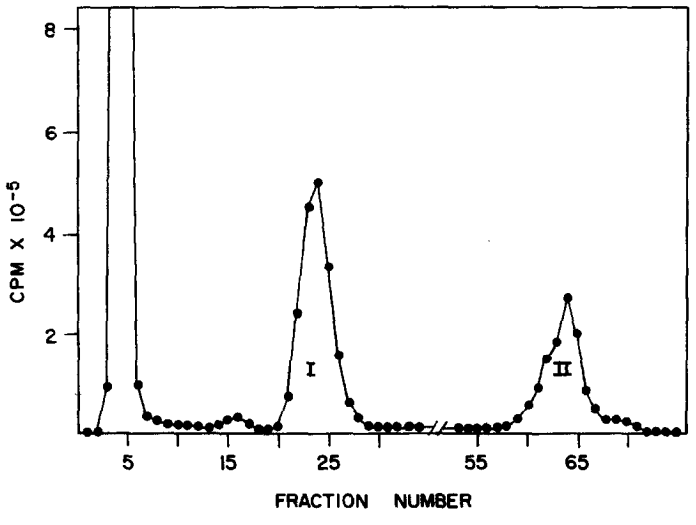

Figure 1. Sephadex LH-20 chromatography of ethyl acetate extract of INC#1. The developing solvent was changed from solvent A to B at fraction 45. Radioactivity in 50 µl portions of each fraction was measured. Values presented are total radioactivity calculated for each fraction. Fractions 4 and 5 contained 33.3 and 3.2 x 10^6 cpm respectively.

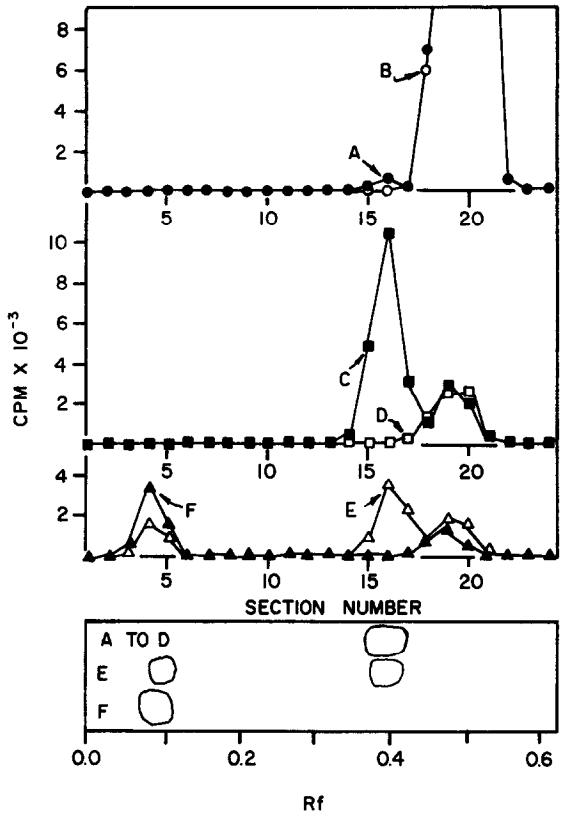

Figure 2. Silica gel radiochromatograms. Radioactivity in 4 mm sections is presented. Section 1 contains the origin. Chromatography was terminated when the solvent front was 15 cm from the origin. The appearance of the plates under UV (254 nm) light is represented in the bottom panel.

A. (●). LH-20 column fractions 4-6 from INC#1 were pooled and apx. 180,000 cpm were applied.

B. (○). LH-20 column fractions 4-6 from INC#2 were pooled and apx. 180,000 cpm were applied.

C. (■). Rechromatography of material (E-INC#1, apx. 25,000 cpm) eluted from sections 15-16 of chromatograms as in "A".

D. (□). Rechromatography of material (E-INC#2, apx. 6,500 cpm) eluted from sections 15-16 of chromatograms as in "B".

E. (△). Partial conversion of E-INC#1 (apx. 12,000 cpm).

F. (▲). Complete conversion of E-INC#1 (apx. 8,000 cpm).

A small radioactive peak (R_f=0.40) was resolved from the ^{3}H-TCB peak (R_f=0.49) on silica gel chromatograms of material that was eluted in column fractions 4-6 (Figure 2, curve A). The only spot on the plate that was detected with UV (254 nm) light comigrated with authentic TCBAO and coincided with the small peak. When material (designated E-INC#1) that was eluted from sections 15-16 of additional plates was chromatographed a second time, most of the radiolabel migrated as TCBAO (curve C). In contrast, only the ^{3}H-TCB

peak was observed on radiochromatograms of fraction 4-6 material from an incubation (INC#2) in which NADPH was omitted (curve B). Material (E-INC#2) that was eluted from sections 15-16 of additional plates was rechromatographed. All E-INC#2 that was radiolabeled migrated as ^{3}H-TCB (curve D). The oxide region spots of the carrier TCBAO on INC#2 chromatograms were always larger than those of INC#1 when the same amount of radioactivity was applied to each.

The radiolabeled material comigrating with TCBAO was labile. After two days at 25°C or after exposure to 254 nm light for a few minutes portions of E-INC#1 migrated (R_f=0.08) on silica gel as standard hydroxy-TCB (curve E). Acid treatment resulted in complete conversion (curve F). The presence of 3- and 4-hydroxy-TCB (1:2.5) in acidified E-INC#1 was adduced from comparison of GC retention time and mass spectra with those (20) of hydroxy-TCB standards.[1] Acidification of either E-INC#1 or E-INC#2 was associated with a diminution in size, or disappearance of the UV-detectable spot in the oxide region and a compensatory increase in size of the hydroxy-TCB region spot. However, radiochromatograms of acidified E-INC#2 always appeared as curve D.

Detectable radioactivity was not incorporated into Br-AO when this brominated biphenyl arene oxide replaced TCBAO in the incubation (INC#3). The ^{3}H-TCB peak was the only one present on radiochromatograms of column fractions 4-6 material. Br-AO comigrates with TCBAO on the silica gel plates. Material (E-INC#3) that was eluted from sections 15-16 of additional plates was again chromatographed on silica gel. No oxide region peak was observed. The presence of Br-AO in E-INC#3 was verified by the presence of oxide region spots on the plates and by GC analyses (retention time on the 3% SE-30 column was 1.69 relative to TCB).

No radiolabeled TCB, TCBAO, or other TCB derivative could be recovered from an incubation (INC#4) identical to INC#1 except for the addition of unlabeled TCB and [^{3}H]-H_2O. No radioactivity was eluted from Sephadex LH-20 with either solvent A or B.

[1] The presence of TCBAO was verified in this manner because TCBAO appears to be reduced to TCB under GC conditions.

DISCUSSION

These results indicate that radiolabeled arene oxide was generated, in an NADPH-dependent manner, as a result of ^{3}H-TCB metabolism by phenobarbital-induced microsomes. The labeled oxide likely is TCBAO since it elutes from Sephadex LH-20 with TCBAO and comigrates with the carrier on silica gel. If the specific radioactivities of the labeled oxide and ^{3}H-TCB are equal, then about 3 μg were recovered from INC#1. Measurable tritium exchange involving carrier TCBAO is considered extremely unlikely on a chemical basis. An NADPH-dependent exchange between TCBAO and a ^{3}H-TCB metabolism product that does not exchange with H_2O or Br-AO cannot be rigorously excluded.

The existence of TCBAO as an intermediate of TCB metabolism is further substantiated by the fact that production of the dihydrodiol was blocked in the presence of a potent uncompetitive inhibitor (19) of microsomal epoxide hydrase. The *trans*-3,4-dihydrodiol, which has been identified as a TCB metabolite in rabbits (2), monkeys (5), and rats (probably *trans*) (18, 20), would almost certainly arise via this enzyme (8). Dihydroxy-TCB metabolites could arise from subsequent dehydrogenation of the dihydrodiol (1, 9), or as a result of sequential direct hydroxylations. Since only one of the two characterized dihydroxy metabolites was detected, it is suggested that TCBAO is a precursor of one dihydroxy-TCB.

Arene oxides are most likely involved in the metabolism-dependent covalent modification of cellular proteins and nucleic acids (4, 24, 25, 29-31). It is suspected that metabolism of PCB's to strongly electrophilic oxides is analogous to the metabolic activation of carcinogens such as benzo[a]pyrene (10).

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