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METABOLISM OF THE ARYL HYDROCARBON RECEPTOR AGONIST 3,3′,4,4′-TETRACHLOROBIPHENYL BY THE MARINE FISH SCUP (*STENOTOMUS CHRYSOPS*) *IN VIVO* AND *IN VITRO*

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ABSTRACT:

The metabolism of the polychlorinated biphenyl congener 3,3′,4,4′-tetrachlorobiphenyl (TCB) was examined *in vitro* and *in vivo* in the marine fish scup (*Stenotomus chrysops*). Untreated scup liver microsomes catalyzed metabolism of TCB with an estimated K_M of 0.7 μM, at a rate 0.13 pmol/min/mg. Metabolism was NADPH-dependent and inhibited by cytochrome *c* and CO, indicating cytochrome P450 (CYP) involvement. α-Naphthoflavone strongly inhibited microsomal TCB metabolism, and treatment of fish with CYP1A inducers increased the rates by ~2-fold, suggesting involvement of CYP1A. Scup were injected intraperitoneally with 0.1 or 5 mg TCB/kg and sampled on days 1–16 after treatment (after 3 days without food at each sampling). Concentrations of unmetabolized TCB in liver peaked on day 5 in low dose fish and on day 12 in high dose fish. In both groups the TCB content in liver had declined 60% or more by day 16, suggesting depuration or redistribution from the liver. GC and MS revealed TCB and TCB metabolites in bile within 24 hr of treatment. The concentrations of TCB and metabolites in bile peaked at the same time that TCB concentrations peaked in the liver. The major metabolites were 5-hydroxy-3,3′,4,4′-TCB (5-OH-TCB) and 4-hydroxy-3,3′,5,4′-TCB (4-OH-TCB); 2-hydroxy-3,3′,4,4′-TCB and 6-hydroxy-3,3′,4,4′-TCB were minor metabolites. Animals given the high dose had much less 5-OH-TCB and much more parent TCB in bile than did fish given the low dose. Amounts of 4-OH-TCB in bile did not differ between doses. The reduced excretion of 5-OH-TCB coincided with a suppression of CYP1A in fish given the high dose, that did not occur in low dose fish, consistent with an involvement of CYP1A in TCB metabolism and particularly in formation of 5-OH-TCB. This study provides the first direct demonstration of 3,3′,4,4′-TCB metabolism by fish. Data also indicate that these fish are able to eliminate TCB both as parent compound and as metabolites, despite a very slow rate of metabolism *in vitro*.

PCBs[1] are ubiquitous contaminants in the global environment (1, 2). Some PCB congeners have been linked to deleterious health effects in both animals and humans (3, 4). Coplanar PCBs, including TCB (IUPAC 77), are AhR agonists that elicit a suite of toxic and biochemical effects in mammals, similar to those elicited by the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5). PCBs in the environment are distributed ultimately to aquatic systems (1, 6). Marine fish accumulate PCBs in tissues (7, 8) and are vectors of PCBs to humans (9). The fates of PCBs in fish may include retention in fatty tissues; elimination of parent (unmetabolized) compound *via* the gills, the bile, or in gametes at spawning (10–12); or metabolism and subsequent elimination. Although a large body of work addresses PCB uptake and elimination by fish, little is known about the pathways or enzymes involved in PCB metabolism by these animals.

In the earliest studies with fish, investigators did not detect metabolism of the PCB congeners 4-chlorobiphenyl, 4,4′-dichlorobiphenyl or the noncoplanar congener 2,2′,5,5′-TCB (13). In later studies, low levels of metabolites of noncoplanar PCB congeners were detected in bile or tissues of fish exposed to these compounds (14–18). PCB metabolism by fish also has been inferred from the apparent depletion of certain congeners from tissues of animals contaminated by PCB mixtures. Depletion from fish tissues of the coplanar congeners 3,3′,4,4′-TCB and 3,3′,4,4′,5-PCB (7, 19), and of noncoplanar congeners—including 2,2′,5,5′-TCB and 2,2′,4,5,5′-PCB (20, 21)—has been described. Yet, even in recent studies with fish, investigators have been unable to detect metabolism of the coplanar 3,3′,4,4′-TCB *in vitro* (22).

The oxidative metabolism of chlorobiphenyls in mammals is catalyzed principally by CYPs. The position of halogen substituents may determine which CYP enzyme catalyzes the transformation. In rat, CYP1A1 induced by Ah receptor agonists can metabolize congeners with adjacent, unsubstituted *ortho-meta* carbons (23). Halogen substituents at one or both *para* positions seem to favor metabolism by

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[1] Abbreviations used are: PCB, polychlorinated biphenyl; TCB, 3,3′,4,4′-tetrachlorobiphenyl; AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; Ah, aromatic hydrocarbon; P450, cytochrome P450; ECD, electron capture detection; 2-MeO-TCB, 2-methoxy-3,3′,4,4′-tetrachlorobiphenyl; 5-MeO-TCB, 5-methoxy-3,3′,4,4′-tetrachlorobiphenyl; 4-MeO-TCB, 4-methoxy-3,3′,4,4′-tetrachlorobiphenyl; 6-MeO-TCB, 6-methoxy-3,3′,4,4′-tetrachlorobiphenyl; MeO-TCB, methoxy-3,3′, 4,4′-tetrachlorobiphenyl; MC, 3-methylcholanthrene; TCDF, 2,3,7,8-tetrachlorodibenzofuran; DBOFB, 4,4,′-dibromooctafluorobiphenyl; AOAC, Association of Official Analytical Chemists; MSD, mass selective detector; RF, response factor; EROD, ethoxyresorufin *O*-deethylase; CYP1A, cytochrome P450 IA; IgG, immunoglobulin G; P450E, cytochrome P450E; DMSO, dimethylsulfoxide; dpm, disintegrations per minute; ANF, α-naphthoflavone; N_2, nitrogen; CO, carbon monoxide; O_2, oxygen; 5-OH-TCB, 5-hydroxy-3,3′,4,4′-tetrachlorobiphenyl; 4-OH-TCB, 4-hydroxy-3,3′,4′, 5-tetrachlorobiphenyl; 6-OH-TCB, 6-hydroxy-3,3′,4,4′-tetraclorobiphenyl.

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CYP1A1, whereas an *ortho* halogen often impedes that metabolism. In contrast, phenobarbital-inducible rat CYP2B forms (CYP2B1 and CYP2B2) preferentially metabolize PCB congeners with adjacent, unsubstituted *meta-para* carbons, and at least one *ortho*-halogen seems to favor that metabolism (23–25). One might deduce the involvement of CYP1A-like and CYP2B-like activities in PCB metabolism in fish from the relative abundance of PCB congeners in tissues of contaminated animals (7, 19–21, 26); but, a role for piscine CYP in PCB metabolism has not been established directly. We investigated both *in vitro* and *in vivo* metabolism of 3,3′,4,4′-TCB, a rat CYP1A1 substrate, in the marine fish scup (*Stenotomus chrysops*). The results provide the first directly measured rates for coplanar PCB metabolism in fish and indicate a role for P450 and ostensibly for CYP1A in this metabolism.

Materials and Methods

Chemicals. 3,3′,4,4′-TCB [U-^{14}C] (52.1 mCi/mmol) was purchased from Pathfinder (St. Louis, MO). Analysis by GC/ECD and GC/MS revealed no hydrophobic contaminants (>99% pure); small amounts of polar contaminants were removed by dissolving the TCB in hexane and extracting twice with NaOH. Unlabeled TCB was obtained from Pathfinder. Purity of the TCB was >99% by GC/ECD and GC/MS analysis. Ethoxyresorufin was from Molecular Probes (Eugene, OR). Other chemicals used were the highest grades available from commercial suppliers.

Methylated monohydroxy metabolites of 3,3′,4,4′-TCB—2-MeO-TCB, 5-MeO-TCB, 4-MeO-TCB, and 6-MeO-TCB—were a gift from the National Institute of Environmental Health Sciences (Research Triangle Park, NC). The methylated standards were synthesized according to the methods of Darnerud *et al.* (27) by the Cadogan diaryl coupling reaction. We confirmed the characteristics of the MeO-TCB standards used herein, by full-scan GC/MS. The fragmentation patterns of the standards matched those reported by Darnerud *et al.* (27) and Koga *et al.* (28). Likewise, the GC retention times of each standard on a DB5 capillary column matched those reported by Klasson Wehler *et al.* (29).

Animals and Treatment. Scup used in all studies were caught by angling in Vineyard Sound, MA, during the summer months (ambient temperature ~20°C). Some fish used for *in vitro* studies were killed immediately upon capture and are referred to as "feral" fish. Livers of feral fish were stored in liquid nitrogen until use. Livers from 20 to 70 fish were pooled for microsome preparation. Other fish used for *in vitro* studies were maintained at 20°C for 1 year in a flowing sea water system that draws directly from Vineyard Sound to allow depuration of environmental chemical residues. Prior studies in our laboratory have shown that fish maintained under this regimen have little or no detectable CYP1A, suggesting that environmental inducers are depurated from their tissues. Fish were fed Purina trout chow and frozen squid. At the time of treatment, experimental animals (mixed sex) were gonadally regressed and weighed 76–276 g.

Depurated fish were injected intraperitoneally with corn oil ("control" fish), MC in corn oil (20 mg/kg), or TCDF in corn oil (3 μg/kg) and killed 5 days later by cervical transection. Livers were removed, and microsomes were prepared as previously described (30). Protein content was measured as before (31).

For *in vivo* studies, individuals were injected intraperitoneally with either corn oil (carrier controls) or with TCB dissolved in corn oil, at either 0.1 or 5 mg TCB/kg body weight. At each time point, 4 or 5 animals from each treatment group were killed by cervical transection and dissected immediately. A group of uninjected fish was used as a "zero" point in the time course. Fish were not fed for 3 days before killing. Gallbladders were removed immediately, and then liver was sampled. Muscle was sampled from the side of the fish, midway between the head and tail, and ventral to the lateral line. Sections of liver and muscle were rinsed in methanol, and these and gallbladders were stored in acid-washed vials at −20°C until analysis.

Analysis of Tissue and Bile for TCB and Metabolites. Equally sized pieces of liver or muscle from all fish within a treatment group were pooled, fortified with the surrogate internal standard (DBOFB) and extracted after AOAC method 983.21 (32). Samples were mixed with sodium sulfate and petroleum ether, homogenized, and serially extracted with petroleum ether. The combined extracts were evaporated to dryness under nitrogen, then redissolved in hexane. Bile samples were collected by puncturing the gallbladder and rinsing the interior of the bladder with water to collect the bile. The water/bile mixture was fortified with an internal standard (2,3′,4,4′-TCB) and hydrolyzed in 1 M NaOH at 70°C for 3 hr (29). This mixture was acidified with 4 M HCl, extracted with hexane, dried with sodium sulfate, and concentrated under nitrogen. The hexane extracts of muscle, liver, and bile then were treated and analyzed in the same manner. Extracts were methylated with diazomethane (1 hr) and coextracted lipids removed by washing with concentrated sulfuric acid. Extracts were cleaned on a 2 g Florasil column that was eluted with hexane and then concentrated to 100 μl under nitrogen. An internal standard 2,4,5,6-tetrachloro-*m*-xylene was added to each extract just before analysis.

Sample extracts were analyzed with GC/ECD and GC/MS. The GC/ECD was an HP 5890 Series II; the GC/MS was an HP 5890 Series II with an HP 5972 MSD. Both instruments were interfaced to separate HP ChemStation data systems. Both GCs were equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, J & W Scientific, Folsom, CA), and injections were made in the splitless mode at 220°C. Both GCs used the same temperature program: 80°C for 2 min, ramp at 20°C/min to 200°C, ramp at 2°C/min to 270°C, and hold for 20 min. Hydrogen was used as the carrier gas for the GC/ECD and helium for the GC/MS. The MSD interface temperature was 280°C, the ion source was 140°C, the ion source pressure was 1.5×10^{-5} mm Hg, and the ionization energy was 70 eV. The MSD was operated in full-scan mode for initial standard characterization, but concentrations in actual samples were too low for full scan mode. Molecular ions were selected from the full mass spectra of the standards for use in selected ion monitoring of actual samples. Additional ions were monitored during a separate analysis to identify the presence of dimethoxy TCB metabolites (350, 352, and 354) and methylsulfonyl TCB metabolites (368, 370, and 372). Chromatographic peaks in samples were identified based on retention times compared with those of the standards (±0.05 min) for GC/ECD and GC/MS, and a match between unknown and standard in the fragmentation patterns determined by GC/MS (relative abundance of molecular ions, ±15%).

Response factors for both instruments were generated using a 4-point calibration for DBOFB, 2,3′,4,4′-TCB (#66), parent 3,3′,4,4′-TCB, 2-MeO-TCB, 4-MeO-TCB, 5-MeO-TCB, and 6-MeO-TCB. The relative standard deviation of the RFs was within 10% for each analyte. A calibration standard was analyzed after every five samples and the RFs always were within 10% of the initial calibration. The average recoveries of DBOFB and PCB 66 were 76% and 98%, respectively. Concentrations of parent PCB were corrected for DBOFB recoveries; concentrations of TCB metabolites were corrected for PCB 66 recoveries. The average recoveries for duplicate matrix spikes were as follows: parent 3,3′,4,4′-TCB (110%), 2-MeO-TCB (92%), 4-MeO-TCB (85%), 5-MeO-TCB (94%), and 6-MeO-TCB (90%). The precision of duplicate analysis for all analytes was within 4%.

Characterization of Monooxygenase Systems. Microsomal CYP content was determined using dithionite difference spectra, as previously described (33, 34), with an extinction coefficient of 91 mM^{-1} cm^{-1} (33). EROD activity was quantified with a fluorometric kinetic assay, using a Cytofluor 2300 fluorescence plate reader (Millipore, Bedford, MA), as previously described (35). Immunoblot analysis was performed as previously described (36). Monoclonal antibody 1-12-3 raised against scup cytochrome P450E (CYP1A) (37) was the primary antibody, and alkaline phosphatase-linked goat-anti-mouse IgG was the secondary antibody. Hepatic microsomal content of CYP1A was quantified by densitometric scanning of antibody-reactive bands with a video imaging system (Masterscan, Scanalytics/CSPI, Billerica, MA) and comparison to samples with known P450E content.

TCB/P450 Binding Spectrum. Hepatic microsomes from feral scup were diluted to a concentration of 1 mg/ml in a Tris buffer (see herein) and divided evenly between two cuvettes. 3,3′,4,4′-TCB was dissolved in DMSO to a concentration of 0.5 mM, and added to the microsomal suspension in the sample cuvette at final concentrations of 1–10 μM (0.2–2% DMSO in the sample). Equivalent volumes of DMSO were added to the reference cuvette. A UV/visible difference spectrum of the microsome/TCB suspension was obtained by scanning over a wavelength range of 500 to 350 nm on a Shimadzu UV-260 spectrophotometer.

TCB Metabolism Assay. The assay of Schnellman *et al.* (38) was used,

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with modifications. Incubations were performed at 30°C under air in a shaking incubator. This temperature has been demonstrated to be optimal for hepatic microsomal monooxygenase activities in scup (39). Reaction mixtures were 0.5 ml and contained 1 mg/ml microsomal protein (unless stated otherwise), 50 mM Tris (pH 7.6), 0.1 mM EDTA, and 2 μM ^{14}C-labeled TCB that had been dissolved in acetone. Acetone concentration in the mixtures was ~1–3%. Substrate saturation profiles indicated that the 2 μM concentration was optimal in scup. After 2-min preincubation, reactions were initiated by adding 1 mM NADPH and run for 60 min (unless stated otherwise). Reactions were terminated by adding 0.75 ml of 0.5 N NaOH, and then heated at 70°C for 10 min to aid in microsome solubilization (38). To remove parent TCB, the samples were extracted 3 times with 2.5 ml aliquots of *n*-hexane:100% ethanol (19:1). Five hundred microliters of the aqueous/NaOH sample was neutralized with HCl and combined with 6 ml Scintiverse II (Fisher Scientific, Fair Lawn, NJ) and ^{14}C radioactivity was quantified by liquid scintillation counting (Beckman LS5000TD). Amounts of radioactivity extracted from incubations containing either boiled microsomes or no microsomes (both controls gave similar results) were subtracted from that of complete incubations and dividing by specific activity of the substrate to convert dpm to pmol PCB metabolites. The limit of resolution of the assay was ~20 dpm, giving an approximate detection limit of 0.03 pmol/min/mg microsomal protein.

Substrate saturation was determined by varying the TCB concentration from 0.05 to 10 μM. In some assays 1 mM NADH was substituted for NADPH. Horse heart cytochrome *c* (Sigma Chemical Co., St. Louis, MO) dissolved in assay buffer was added to some reaction mixtures at 100 μM. ANF in methanol was added to some assays at 1, 10, or 100 μM (10 μl methanol per 500 μl reaction mixture); blank reactions for comparison included 10 μl methanol alone. Some reactions were conducted in controlled atmospheres, according to the methods of Binder (40), with modifications. Microsomes, buffer, and substrate were combined, and the mixture was bubbled gently with either N_2 or CO for 60 sec. Tubes were immediately sealed with air-tight serum stoppers. The gas phase in the tube above the reaction mixture was then adjusted to contain either N_2 or CO:O_2 (80:20). The sealed tubes were connected to a manifold with capillary tubing, and a syringe needle attached to the tubing was passed through the serum stopper into the gas space. The tubes were repeatedly evacuated and gassed with either N_2 or CO. After several cycles, the tubes were disconnected from the manifold. In the CO tubes, the total volume of gas was adjusted to 8 ml with a syringe, and 2 ml of O_2 was injected through the serum stopper. Reactions were initiated by injection of NADPH through the serum stoppers and incubated as described.

Statistics. Significant differences between treatment groups were evaluated with an ANOVA using Statview SE+ Graphics, v.1.03 (Abacus Concepts, Berkeley, CA) for Macintosh. Significant differences were assessed with a Dunnett's one-tailed test.

Results

TCB Disposition *In Vivo*. After injection with either 0.1 or 5 mg TCB/kg, the TCB in muscle reached a maximal concentration within 24 hr (Fig. 1). When expressed on a wet weight basis (fig. 1, legend), the TCB concentrations in muscle were similar to the doses given. Thus, fish given 0.1 mg/TCB/kg body weight had 0.1 mg TCB/kg of muscle and fish given 5.0 mg TCB/kg body weight had 2.2 mg TCB/kg of muscle. After the first day, the TCB concentrations in the liver (and probably other organs) increased; a maximal concentration in liver was seen on day 5 in the low dose fish and day 12 in the high dose fish. TCB concentrations in liver declined substantially by day 12 at the low dose (60% decline from maximum), and by day 16 at the high dose (80% decline from maximum), suggesting that the bulk of the TCB was depurated from liver by the end of the study.

At both doses of TCB, parent (unmetabolized) TCB was detected in bile sampled throughout the experiment (table 1). The concentrations of parent compound in bile peaked at the same times when concentrations peaked in liver. The amounts of parent TCB were as much as 100 times greater in bile of fish given the high dose than in bile of fish given the low dose of TCB. However, the amounts of parent compound plus metabolites in bile, as a proportion of the dose administered, were less in the high dose than in the low dose fish.

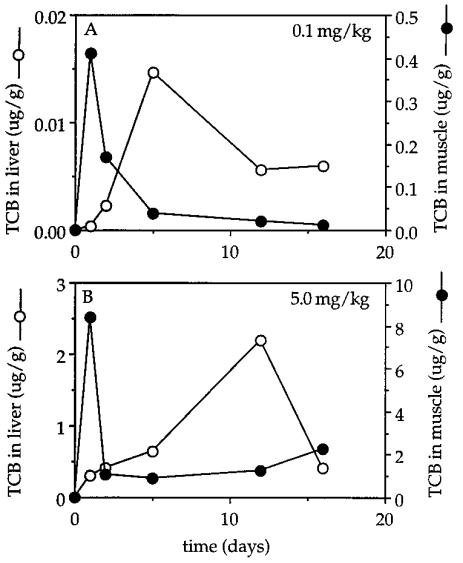

FIG. 1. *Concentration of TCB residues in liver and muscle of scup (Stenotomus chrysops).*

(*A*) Scup treated with 0.1 mg/kg TCB. (*B*) Scup treated with 5 mg/kg TCB. X-axis is number of days subsequent to TCB injection. Y-axis is TCB concentration in μg TCB/g tissue dry weight for muscle (*left axis*) and liver (*right axis*); note the difference in scale. Each point is a measurement on a single sample, consisting of pooled tissue sections from 4 or 5 fish in each treatment group. (●) Liver. (○) Muscle. Values may be converted from a dry weight basis to a wet weight basis as follows: liver dry weight is 25% of wet weight, muscle dry weight is 26.3% of wet weight. Data for the TCB concentrations in liver are from White *et al.* (45) and are included herein for comparison. No TCB was detected in tissues of corn oil-treated fish or untreated fish (limits of detection were 5 ng/g liver and 1 ng/g muscle).

TCB Metabolites. In fish treated with either TCB dose, the major metabolites found in bile were 5-OH-TCB and 4-OH-TCB (table 1). Minor metabolites found in bile of fish treated with 0.1 mg/kg TCB included 6-OH-TCB, 2-OH-TCB, a dihydroxy-tetrachlorobiphenyl and a dihydroxy-trichlorobiphenyl. The identification of the monohydroxy-TCB metabolites was based on nearly identical GC retention times (within 0.05 min) (table 2) and on mass fragmentation patterns of methylated derivatives of the metabolites (table 2), compared with fragmentation patterns of methylated monohydroxy standards. The relative abundance of molecular ions differed <15% between the unknowns and standards. Similarities between standard and sample fragmentation patterns are shown in fig. 2 for the major metabolites 4-MeO-TCB and 5-MeO-TCB.

The four monohydroxy metabolites in table 1 also were detected in liver of TCB-treated fish, although these were not measured quantitatively. No metabolites were detected in muscle tissue of TCB-treated fish (limits of detection: 2 ng/g muscle). We monitored the three primary molecular ions for methylsulfonyl TCB metabolites (*m/z* 368, 370, and 372) and found no evidence (<1 ng/g) for these in liver or in bile.

The extent and patterns of TCB metabolism differed between fish given the different doses of TCB. At the low dose, the ratio of 5-OH-TCB to 4-OH-TCB in bile was ~6:1 or greater, at each of five sampling times (table 1). In contrast, in bile of fish given the high dose of TCB, the ratio of 5-OH-TCB to 4-OH-TCB was close to 1:1 or favored the 4-OH-TCB, at the 4 times when metabolites were detected (table 1). The total amounts of TCB metabolites were 4- to 20-fold

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TABLE 1

Concentrations of TCB and metabolites in bile of scup[a]

Day after Treatment	Parent TCB	2-OH-TCB	4-OH-TCB	5-OH-TCB	6-OH-TCB	Total OH-TCBs
0.1 mg/kg						
0	0	0	0	0	0	0
1	0	0	0.5	16	0	16.5
2	0.8	0.9	7.3	70	0	78.2
5	3.2	2.2	19.0	124	0.9	146.1
12	1.1	0.6	11.6	82	0.8	95.0
16	1.2	0.8	8.1	55	0.6	64.5
5 mg/kg						
0	0	0	0	0	0	0
1	12	0	0	0	0	0
2	14	0	4	0	0	4
5	43	0	6	0	0	6
12	420	0	12	14	0	26
16	125	0	9	9	0	18

[a] Fish (*Stenotomus chrysops*) were treated with 0.1 or 5 mg/kg TCB and sampled as described in *Materials and Methods*. Four or five gallbladders were pooled from each treatment group for analysis of bile residues. Concentrations are given in ng of TCB or TCB metabolites/g of gallbladder. All parent TCB and metabolite concentrations are based on the GC/ECD quantification, after confirmation by GC/MS. Although the MSD was run in the selected ion monitoring mode, a sufficient number of ions were monitored to distinguish these four metabolites from most interferences. The fragmentation pattern could not be used at concentrations below ~5 ng/g gallbladder. In those cases, only the retention time was used to identify the metabolite. TCB and TCB metabolites were not detected in the bile of corn oil-treated fish or untreated fish (limit of detection: 0.5 ng/g gallbladder). Analytes were identified as their methylated (MeO) derivatives, but are referred to as the unmethylated hydroxylated structures.

greater in bile of fish given the low dose than in fish given the high dose (table 1). The ratio of total metabolites to parent compound also was greater in fish-given the low dose, ~45:1, than in those given the high dose, in which the ratio of metabolites:parent was 1:16. These results indicate that TCB was metabolized to a much lesser extent in the fish given the high dose.

TCB Metabolism *In Vitro*. Hepatic microsomes prepared from freshly caught (feral) scup were used to characterize TCB metabolism *in vitro*. The formation of aqueous-soluble TCB metabolites was dependent on native protein and on NADPH (fig. 3). The rates of formation were linear with incubation time up to 60 min and increased with increasing content of microsomal protein (not shown). Under optimal conditions, the maximal rate of TCB metabolism in these samples was ~0.13 pmol/min/mg microsomal protein. Analysis of substrate saturation profiles gave an estimated K_M of 0.7 μM TCB, although nonspecific binding of TCB to microsomes may make this a high estimate.

Rates of TCB metabolism with NADH were 60% less than with NADPH, and in fact were not different from the rates without NADPH (fig. 3). Addition of 100 μM cytochrome *c* reduced the activity rate by ~50% (fig. 3). Addition of ANF, a competitive inhibitor of EROD (CYP1A) in scup (41), inhibited TCB metabolism in a concentration-dependent manner, with complete inhibition at 100 μM ANF (fig. 3). Addition of CO (80% atmosphere) reduced the rate of metabolism by 42% (fig. 3). These data indicate that the metabolism of 3,3′,4,4′-TCB is catalyzed by a P450 enzyme in scup liver microsomes.

Bubbling of reaction mixtures with N_2 and incubation under an N_2 atmosphere, which should inhibit P450 activity by limiting the availability of O_2, reduced TCB metabolism only by 7% (fig. 3). This result may be related to the very slow rate of TCB metabolism *in vitro* and thus to the small oxygen requirement. If we assume a low coupling efficiency (moles of NADPH or O_2 consumed per mole of substrate metabolized) of 1% for scup CYP1A (41), then metabolism of 1 mol of TCB would consume 100 mol of O_2. Under those conditions, 1.2 nmol (~10^{-9} mol) of O_2 would be consumed in the metabolism of TCB by 1 mg of microsomal protein for 1 hr, at a rate of 0.2 pmol/min/mg. Before evacuation and gassing the 10 ml headspace in the reaction contained ~10^{-4} mol O_2, based on a conversion factor of 0.209 liters O_2 in 1 liter of air. The methods used might not have decreased the O_2 content in the headspace enough to limit the transformation of TCB. Reaction mixtures rendered free of O_2 would be necessary to confirm an O_2 requirement for this catalytic activity.

Rates of TCB metabolism were determined with liver microsomes from feral (freshly caught) scup, control (depurated) scup, and scup that were depurated and then treated with CYP1A inducers. Contents of hepatic microsomal P450 and CYP1A and rates of CYP1A-catalyzed EROD activity were greater in all groups, compared with controls (table 3). TCB metabolism rates with hepatic microsomes of fish treated with the CYP1A inducers MC or TCDF were between 0.2 and 0.3 pmol/min/mg, 2- to 3-fold greater than the rates in depurated fish (table 3).

Binding Spectrum. Binding spectra were obtained to assess further whether the metabolism of 3,3′,4,4′-TCB in scup hepatic microsomes involved a direct interaction with P450. The combination of TCB and microsomes produced a type I binding spectrum, with a peak at 390 nm and a trough at 420 nm (fig. 4), indicative of a low spin to high spin transition of the heme iron that is associated with substrate binding to the P450 active site (42). Although spectral changes associated with TCB binding were small, these changes were clearly distinguishable from baseline, were found to increase in magnitude with increasing concentrations of TCB added, and were obtained reproducibly in repeated experiments. Due to the very small absorbance changes measured, a spectral dissociation constant could not be determined accurately from these data.

TABLE 2

GC/MS data for TCB and methyl derivatives of monohydroxy-TCB metabolites from scup bile

Compound	Retention Time[a] (min)	Mass Spectrum, *m/z* (Relative Abundance %)[b]
Parent TCB	19.14	M: 295(55), 292(100), 290(80); M-70: 222(26), 220(40)
2-MeO-TCB	20.59	M: 324(25), 322(60), 320(52); M-50: 274(40), 272(90), 270(100)
4-MeO-TCB	23.08	M: 324(60), 322(100), 320(74); M-15: 309(40), 307(77), 305(63); M-43: 281(23), 279(50), 277(38)
6-MeO-TCB	23.35	M: 324(47), 322(100), 320(81); M-50: 274(29), 272(87), 270(96)
5-MeO-TCB	26.20	M: 324(50), 322(100), 320(79); M-43: 281(18), 279(31), 277(30)

[a] Absolute retention time of parent TCB and methylated metabolites from GC/MS.

[b] Three molecular ions are listed from the isotopic cluster (M+4, M+2, M)+ for M, M-15 (M-CH3), M-43 (M-COCH3), M-50 (M-CH3Cl), and M-70 (M-2Cl).

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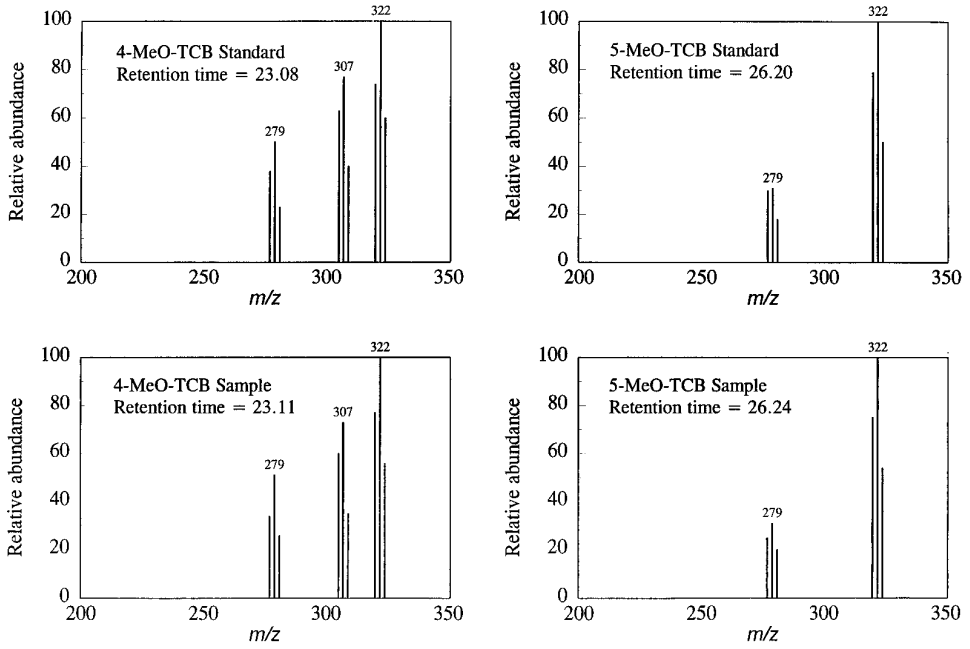

FIG. 2. *Mass fragmentation patterns of TCB metabolites and standards.*

Electron-impact mass fragmentation patterns were reconstructed from GC/MS selected ion monitoring data (see *Materials and Methods*). Bile sample extract from day 5 (0.1 mg/kg treatment) is compared with the 4-MeO-TCB and 5-MeO-TCB standards. Retention times (min) are listed for the peaks that correspond to each fragmentogram and the M+2 molecular ion is indicated for each isotopic cluster. Although the analytes were identified as their methylated (MeO) derivatives, the text refers to all TCB metabolites as unmethylated hydroxy metabolites.

Discussion

The results herein demonstrate the metabolism of 3,3′,4,4′-TCB *in vivo* in TCB-treated marine fish (scup) and describe a sensitive assay used to accomplish the first direct measurement of 3,3′,4,4′-TCB metabolism by fish liver microsomes. *In vitro* studies indicate the involvement of P450 and ostensibly of scup CYP1A in TCB metabolism, but *in vivo* data indicate the possible involvement of other enzyme(s) as well. Studies from 1972 to 1994 have sought but failed to directly demonstrate *in vitro* metabolism of coplanar PCB congeners, including 3,3′,4,4′-TCB, by fish (13,22). The rate of microsomal metabolism of TCB determined herein was exceedingly slow, <0.3 pmol/min/mg under optimal and induced conditions. If rates of TCB metabolism are similarly slow in other fish species, this could explain the previous inability to detect this activity.

TCB Disposition *In Vivo*. TCB concentrations in muscle tissue peaked on day 1 after treatment and declined sharply thereafter. The reason for this early peak in TCB content in muscle is not known. TCB content in muscle was generally greater than that in liver throughout the time course, when expressed in microgram per gram tissue dry weight (fig. 1). Muscle tissue from these scup had a greater lipid content (11.7% of wet weight) than did liver (3.4% of wet weight). PCBs have been shown to partition into lipid (43). When TCB concentrations in tissues were normalized to tissue lipid content, the TCB concentrations in liver at times exceeded those in muscle.

Concentrations of TCB in the liver peaked by day 5 in low dose fish, but not until day 12 in the high dose fish. Pharmacokinetic theory suggests that the time required to reach steady-state concentration depends on the rate of elimination from an organ (44). Thus, data are consistent with a slower elimination of TCB from fish treated with the high TCB dose. The content of TCB metabolites and the ratio of metabolites to parent compound in bile were less (4- to 20-fold) in the high dose fish than in the low dose fish, at each sampling time (table 1). Thus, the extent of TCB metabolism was much less in the high dose than in the low dose fish. CYP1A1 has been implicated in the metabolism of 3,3′,4,4′-TCB in rats (23). Hepatic CYP1A content and activity were strongly suppressed in the scup given the high dose of

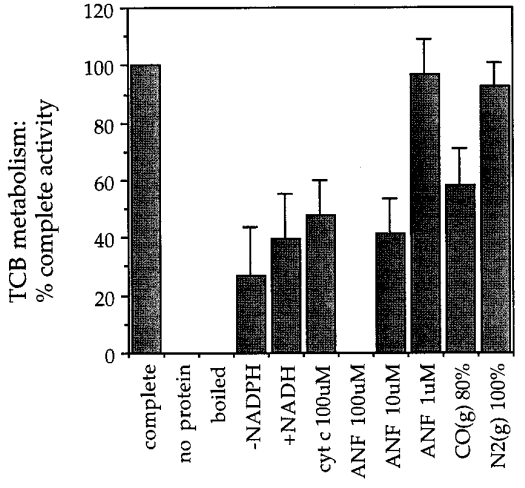

FIG. 3. *Metabolism of TCB by hepatic microsomes of feral scup (Stenotomus chrysops).*

The *bar at the left* represents the rate of metabolism in a complete reaction mixture assayed as in *Materials and Methods* and assigned a value of 100% (actual rates were as listed in table 2). Subsequent bars indicate activities relative to that in the complete reaction. Reactions from *left* contained: complete reaction, no microsomal protein, boiled microsomes, no NADPH, NADH substituted for NADPH, 100 μM cytochrome *c* (cyt c), 100 μM ANF, 10 μM ANF, 1 μM ANF, CO (80% atmosphere), and N_2 (near 100% atmosphere). The aqueous radioactivity in assays containing boiled microsomes was equal to that in assays containing no microsomal protein, and these were used as subtractive blanks and assigned a rate of 0. *Bars* represent means ±SD of 3–9 measurements at each condition, using a single pool of microsomes from livers of 20 fish.

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TABLE 3

Hepatic microsomal P450, CYP1A, and enzyme activities in control, feral, and treated scup[a]

Treatment (N = 3)	Total CYP	EROD	CYP1A1	TCB Metabolism
	nmol/mg	*nmol/min/mg*	*pmol/mg*	*pmol/min/mg*
Control (depurated)	0.13 ± 0.02[b]	0.105 ± 0.064	0.4 ± 0.7	0.09 ± 0.10
Feral (freshly caught)	0.33 ± 0.13	0.715 ± 0.391	67 ± 39	0.13 ± 0.07
MC (20 mg/kg)	0.45 ± 0.11*	4.054 ± 2.461**	219 ± 58**	0.21 ± 0.02*
TCDF (3 μg/kg)	0.68 ± 0.21**	5.011 ± 1.764**	288 ± 119**	0.23 ± 0.02*

[a]Animals were depurated of environmental residues and treated with either corn oil (control) or with the CYP1A inducers MC or TCDF. Three feral samples were prepared from pooled livers of freshly caught animals, with 20–70 fish/pool.

[b] Values are means ± SD.

p values are as follows—Sample groups are significantly greater than control at a level: * $p < 0.05$; ** $p < 0.01$.

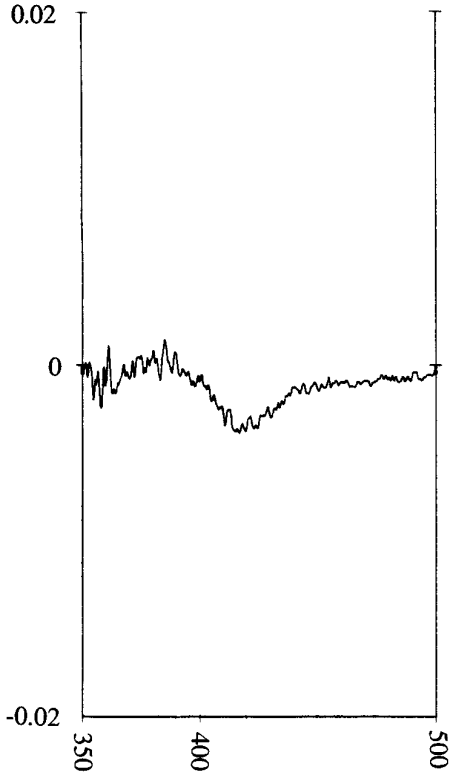

FIG. 4. *TCB binding spectrum.*

10 μM TCB was added to a 1 mg/ml suspension of hepatic microsomes from feral scup, and UV/visible difference spectrum was obtained. Plotted is absorbance (y) *vs.* wavelength in nm (x). The trace shows a typical type I binding spectrum, with a peak at 390 nm and a trough at 420 nm.

TCB but strongly induced in fish given the low dose (45). That the negative effect of high dose TCB on CYP1A expression was associated with the lesser extent of TCB metabolism implies that CYP1A in scup is involved in the metabolism of 3,3′,4,4′-TCB. Despite the slow rate of TCB metabolism in fish given the high dose, they still were able to eliminate TCB, largely as parent compound.

Metabolite Profiles. In bile of scup given 0.1 mg TCB/kg, 5-OH-TCB was 85% of total metabolites, 4-OH-TCB was 13%, and 2-OH-TCB and 6-OH-TCB were 1% each. A similar metabolite profile was seen in bile of chick embryos injected as eggs with 0.3 mg TCB/kg; 5-OH-TCB was 83% of total metabolites, 4-OH-TCB was 16%, and 2-OH-TCB was 1% on day 6 after treatment (46). In contrast, in bile of scup treated with 5 mg TCB/kg, the amounts of 4-OH-TCB were similar to or greater than amounts of 5-OH-TCB. In mice given 10 mg TCB/kg, there also were equivalent amounts of 4-OH-TCB and 5-OH-TCB in feces and urine at 1–2 days after treatment, and the proportion

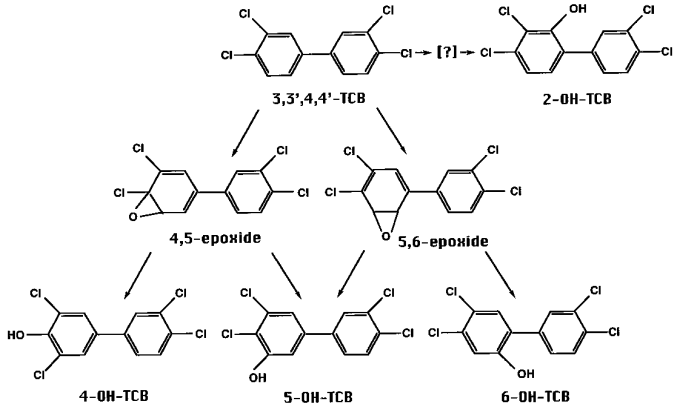

FIG. 5. *Proposed pathways for the metabolism of TCB in scup (Stenotomus chrysops).*

The hydroxylated metabolites were identified in bile of fish injected intraperitoneally with TCB. The epoxides are postulated intermediates.

that was 5-OH-TCB continued to decline over time (29). Likewise, 4-OH-TCB and 5-OH-TCB occurred in nearly equal amounts in feces of rats 1–4 days after being given 150 mg TCB/kg (orally) (47). Metabolite profiles and ratios may be a function of species, route of exposure, and sampling time. However, data suggest that dose may be a particularly important determinant of metabolic pathways, with lower TCB doses (<1 mg/kg) yielding a predominance of 5-OH-TCB and doses of 5–150 mg TCB/kg resulting in the appearance of nearly equal amounts of 4-OH-TCB and 5-OH-TCB. In the case of scup, dose also determined the total amount of TCB metabolites produced; greater quantities occurred in the bile of fish treated with a lower dose of TCB.

The 4-OH-TCB, 5-OH-TCB, and 6-OH-TCB could be rearrangement products of 4,5- and 5,6-epoxides (fig. 5), as postulated earlier for the mouse (29). 6-OH-TCB was not detected, and the amount of 5-OH-TCB was greatly reduced in bile of scup treated with 5 mg TCB/kg, compared with scup treated with 0.1 mg/kg TCB. Yet, the amounts of 4-OH-TCB were comparable in bile of the two dose groups (table 1). This is consistent with a hypothesis that formation of the 5,6-epoxide, but not the 4,5-epoxide, is suppressed at the high dose of TCB, and implies involvement of more than one catalyst in TCB metabolism in scup. CYP1A could metabolize TCB to the 5,6-oxide, whereas other CYP may produce the 4,5-oxide. Unlike CYP1A, which was suppressed in the high dose fish, other CYP occurred at similar levels in liver of scup treated with 0.1 or 5 mg TCB/kg (45).

Role of CYP in TCB Metabolism. TCB elicited a slight but highly reproducible type I binding spectrum when added to scup liver microsomes, indicating that TCB binds to the active site of P450. The *in*

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vitro metabolism of TCB was inhibited by cytochrome *c* and CO, consistent with a P450-catalyzed activity. P450 involvement is indicated also by inhibition of TCB metabolism by ANF. ANF strongly inhibits scup CYP1A (48). However, evidence (49, 50) indicates that ANF can inhibit other CYP also. Thus, the ANF inhibition of TCB metabolism indicates a role for CYP including but perhaps not limited to CYP1A. Treatment of scup with CYP1A inducers (MC and TCDF) increased the content of CYP1A and the rates of TCB metabolism *in vitro* (table 2). TCB metabolism was not induced to the same degree as was EROD activity, possibly due to inhibition of CYP1A by TCB (see herein). Data nevertheless indicate that scup CYP1A can contribute to TCB metabolism *in vitro*. Because the *in vitro* rates of this activity were so slow, other systematic approaches in our laboratory to establish involvement of a particular CYP isozyme (51) have been inconclusive.

In support of a role for teleost CYP1A in the metabolism of TCB, Gooch *et al.* (52) found that TCB is a competitive inhibitor of CYP1A-catalyzed EROD activity in scup hepatic microsomes, with a K_i of 0.3 μM. The K_M for TCB metabolism estimated here in was of a similar magnitude, 0.7 μM. More recently, using lower protein concentrations, a K_i as low as 0.02 μM was estimated for TCB inhibition of scup liver EROD activity.[2] These data indicate a high affinity active-site binding by TCB. Polyclonal antibodies raised against scup CYP1A were able to immunoprecipitate specifically a CYP1A protein-TCB complex[3] further indicating a strong binding of TCB to scup CYP1A.

Rates of TCB Metabolism. The inability of previous investigators to detect 3,3′4,4′-TCB metabolism in fish may be due to the very slow rate of this activity. The rates that we measured *in vitro* were only 3–10 times higher than the detection limit of the assay. Such slow metabolism *in vitro* of this substrate that apparently binds avidly to the CYP1A active site could result from steric hindrance by chlorine substituents, which may interfere with the oxidation of TCB in the P450 active site, whereas not preventing the enzyme-substrate binding. In both fish and mammals, rates of TCB metabolism are far slower than rates of metabolism of some other CYP1A substrates. Rates of metabolism of benzo[*a*]pyrene in hepatic microsomes from induced scup are 250–964 pmol/min/mg (48), 2000–4000 times higher than rates of TCB metabolism in comparable samples examined herein (table 3). Similarly, in liver microsomes from MC-treated adult male rats, rates of TCB metabolism range from 18 to 50 pmol/min/mg (23), whereas rates of benzo[*a*]pyrene metabolism range from 1.6 to 11 nmol/min/mg (see for example, ref. 53).

Slow rates of metabolism *in vitro* suggest that metabolism might contribute little to the elimination of TCB from the tissues of fish. Yet, appreciable quantities of hydroxylated TCB metabolites were found in the bile of TCB-treated scup. If *in vitro* rates like those reported herein (*e.g.*0.1 pmol/min/mg) are extrapolated to a full day for a 100 g fish (assuming a microsomal yield of 10 mg/g liver and a liver weight of 1 g), then TCB may be metabolized at a rate of 1.4 nmol/day, or 420 ng/day. At such a rate, the 0.1 mg/kg TCB dose could be metabolized completely in 24 days, if it all resided in the liver. This estimate agrees reasonably well with the data describing the disposition of TCB *in vivo* (fig. 1), which indicate that much of the TCB dose had been depurated from liver of low dose fish by day 16. Thus, even if metabolism of PCB congeners occurs as slowly *in vivo* as it does *in vitro*, metabolism does seem to contribute to PCB depuration over time *in vivo*. The pharmacokinetics would be more complex than indicated by this calculation, which does not address partitioning to and metabolism in extrahepatic tissues.

The direct demonstration of TCB metabolism in scup supports suggestions based on analysis of PCB residues in fish tissues, that metabolism may enhance elimination of some PCB congeners from fish. Brown (26) analyzed PCB residues in tissues of 32 teleost species, and based on relative "depletion" of selected congeners, inferred CYP1A-like PCB metabolism in 18 species and CYP2B-like PCB metabolism in four different species. Others also have noted selective loss from fish tissues of coplanar congeners (7, 19), suggesting CYP1A-like activity, or of congeners with adjacent, unsubstituted *meta-para* positions (20, 21), thus suggesting CYP2B-like activity. The evidence herein indicates a role for teleost CYP1A in coplanar PCB metabolism; the possibility of CYP2B-like metabolism of *ortho*-substituted PCBs in fish is under investigation.

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[2] M. Hahn, unpublished observations.

[3] ?? Schlezinger and J. J. Stegeman, unpublished data.

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