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β-Naphthoflavone- and self-induced metabolism of 3,3′,4,4′-tetrachlorobiphenyl in hepatic microsomes of the male, pregnant female and foetal rat

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1. The *in vitro* metabolism of 3,3′,4,4′-tetrachloro-[^{14}C]-biphenyl ([^{14}C]-TCB) by hepatic microsomes from the Wistar rat was investigated with liver microsomes from the male, pregnant female and foetus.

2. Three hydroxylated metabolites (4-OH-3,3′,4,5′-tetrachlorobiphenyl, 5-OH-3,3′,4,4′-tetrachlorobiphenyl, and 6-OH-3,3′,4,4′-tetrachlorobiphenyl) were identified by hplc and gc–ms after incubations of liver microsomes from the β-naphthoflavone-pretreated male rat and TCB-treated pregnant rat. No metabolites of [^{14}C]-TCB were found after incubation with foetal liver microsomes from dams pretreated with [^{14}C]-TCB. The results indicate that the *in vivo* accumulation of 4-OH-tetraCB in the foetal compartment is probably due to transplacental transport rather than the formation of this metabolite in the foetus.

3. Pretreatment of the male rat with β-naphthoflavone substantially induced the formation of hydroxylated metabolites, but pretreatment with phenobarbital and dexamethasone was without effect. Based on *in vitro* incubations of liver microsomes from the β-naphthoflavone pretreated male rat, an apparent K_m and V_{max} of 4·5 μM and 240 pmol/mg protein/min respectively was determined for the metabolism of [^{14}C]-TCB. The formation of phenolic metabolites of [^{14}C]-TCB was most likely dependent on P4501A induction.

Introduction

The wide range of biological activity exhibited by polychlorinated biphenyls (PCBs) may be caused by a variety of mechanisms involving the interaction of the parent compound or a metabolite thereof with a specific receptor protein (Safe 1994). The most toxic PCB congeners, 3,3′,4,4′-tetrachlorobiphenyl (TCB), 3,3′,4,4′,5-pentachlorobiphenyl, and 3,3′,4,4′,5,5′-hexachlorobiphenyl have a coplanar structure and have a high affinity for the Ah (arylhydrocarbon) receptor (Bandiera *et al.* 1982), which mediates most of the toxic effects of these coplanar PCBs (Safe 1994). The binding of coplanar PCBs to the Ah receptor can result in the induction of P4501A enzymes and the associated ethoxyresorufin-*O*-deethylase (EROD) activity in microsomes of animal tissues (Safe 1994).

Several reports indicate that the P4501A1 isoenzyme may catalyse the hydroxylation of PCBs, including TCB (Mills *et al.* 1985, Ishida *et al.* 1991). TCB

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is readily metabolized to phenolic metabolites *in vivo* as well as *in vitro* (Yoshimura *et al.* 1987, Klasson Wehler *et al.* 1989, Ishida *et al.* 1991). In contrast, 3,3′,4,4′,5-pentachlorobiphenyl and 3,3′,4,4′,5,5′-hexachlorobiphenyl are hardly metabolized in animals (Mills *et al.* 1985, Koga *et al.* 1990). Other non-planar, *ortho*-substituted PCBs may also be metabolized, resulting in the formation of hydroxylated (Bergman *et al.* 1994) or methylsulphonated compounds (Mitzutani *et al.* 1978, Bergman *et al.* 1992). These metabolites have a wide range of biological activity, including interactions of hydroxylated PCBs with thyroid hormone-binding proteins (Lans *et al.* 1993) and the oeostrogenic activity of hydroxylated PCBs (Korach *et al.* 1988, Jansen *et al.* 1993) or the binding of methylsulphonyl PCBs to $\alpha_{2\mu}$-globulin (Larsen *et al.* 1990). Moreover, the induction of several P450 isoenzymes by methylsulphone derivatives of PCBs has been demonstrated (Kato *et al.* 1993).

Both mouse and rat rapidly metabolize TCB, resulting in the formation of three major metabolites, namely the 1,2-shift metabolite, 4-OH-3,3′,4′,5-tetrachlorobiphenyl (4-OH-tetraCB), 5-OH-3,3′,4,4′-tetrachlorobiphenyl (5-OH-TCB), and 6-OH-3,3′,4,4′-tetrachlorobiphenyl (6-OH-TCB) (Yoshimura *et al.* 1987, Klasson Wehler *et al.* 1989, Morse *et al.* 1995). Interestingly, only 4-OH-tetraCB accumulates in both mouse and rat plasma (Klasson Wehler 1989, Brouwer *et al.* 1990, Morse *et al.* 1995), whereas 5-OH-TCB is excreted in greater amounts than 4-OH-tetraCB in faeces (Yoshimura *et al.* 1987). A re-evaluation of a previously published study that demonstrated the accumulation of phenolic metabolites of TCB in the foetal mouse (Darnerud *et al.* 1986) revealed that the major hydroxylated metabolite of TCB was 4-OH-tetraCB (E. Klasson Wehler, personal communication). Recent research from our laboratory has confirmed that 4-OH-tetraCB is the major compound accumulating in the late gestational rat foetus following maternal TCB exposure (Morse *et al.* 1995). The selective retention of 4-OH-tetraCB in rat plasma is explained by competitive binding to transthyretin (TTR), the major plasma thyroid hormone-binding protein in the rat and mouse (Brouwer and Van den Berg 1986, Savu *et al.* 1989, Vranckx *et al.* 1990), as a result of the structural resemblance of 4-OH-tetraCB to thyroxine (T4) (Lans *et al.* 1993). The accumulation of 4-OH-tetraCB in the foetal plasma results in a decrease of the binding capacity of TTR for T4 (P. O. Darnerud and D. C. Morse, unpublished data) and consequently in decreases in plasma T4 levels (Morse *et al.* 1995). However, it was not clear from the *in vivo* studies above if 4-OH-tetraCB in the foetus is maternally derived or formed in the foetus itself.

The aim was to enable the use of [^{14}C]-TCB as a model compound to investigate the metabolic capacity of adult, foetal, and maternal tissues. Moreover, it was considered to be of interest to determine which pretreatment with inducers of hepatic mono-oxygenases resulted in the greatest induction of [^{14}C]-TCB metabolism. β-Naphthoflavone and phenobarbital (inducers of PCB metabolism; Mills *et al.* 1985) and dexamethasone (inducer of chlorinated benzene metabolism; den Besten *et al.* 1991) as well as TCB itself were used as pretreatments *in vivo*. The reaction conditions for the incubation of [^{14}C]-TCB with hepatic microsomes from the male rat were validated using hplc. Gc-ms was used to confirm the identity of the extractable radioactive products. The hplc technique was then used to determine the source of phenolic metabolites found in the foetus *in vivo* by examining [^{14}C]-TCB metabolism with hepatic microsomes obtained from the TCB-exposed pregnant rat and its foetus.

Materials and methods

Materials

3,3',4,4'-Tetrachloro-[^{14}C]-biphenyl (37·1 μCi/μmol, radiochemical purity 95%) and bovine serum albumin (96–99% pure) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Unlabelled 3,3',4,4'-tetrachlorobiphenyl (>99% pure) was obtained from Promochem (Wesel, Germany). 2-OH-3,3',4,4'-tetrachlorobiphenyl, 4-OH-3,3',4',5-tetrachlorobiphenyl, 5-OH-3,3',4,4'-tetrachlorobiphenyl, and 6-OH-3,3',4,4'-tetrachlorobiphenyl (chemical purity >99%) were synthesized according to Klasson Wehler *et al.* (1990). The following chemicals were purchased from Merck (Darmstadt, Germany): Tris, NaOH, Folin–Ciocalteu phenol reagent, $MgCl_2$, acetone, diisopropyl either, methanol, ethanol, ethyl acetate, and diethyl ether (all solvents were analytical grade). Methanol (hplc quality) was purchased from Janssen Chimica (Tillburg, The Netherlands). Glucose 6-phosphate, glucose 6-phosphate-dehydrogenase, $NADP^+$ and NADPH were obtained from Boeringer Mannheim (Almere, The Netherlands).

Methods

Hepatic microsomes were prepared from Wistar rats from two different experimental situations.

Experiment 1. Male Wistar WU rats (300 g) were treated with dexamethasone (DEX; four consecutive daily oral administrations of 300 mg/kg body weight dissolved in 2% Tween 80), β-naphthoflavone (β-NF; three daily intraperitoneal (i.p.) injections of 30 mg/kg dissolved on cornoil), or phenobarbital (PB; 0·1% w/v, in drinking water for 7 days). Control rats were of a similar weight but were untreated. One day after the last treatment the rats were killed under ether anaesthesia and the livers removed. The livers of three rats per treatment group were pooled. Microsomes were prepared as previously described and stored at −80°C (Morse *et al.* 1993). Two different batches of β-NF microsomes were used for the experiments and are designated as β-NF(1) and (2). The specific enzyme activity (ethoxyresorufin-*O*-deethylase) of both batches are presented in the Results.

Experiment 2. Pregnant female Wistar WU rats (16 weeks old) were treated with 25 μmol 3,3',4,4'-tetrachlorobiphenyl per kg body wt dissolved in corn oil (2 ml/kg body wt) on day 13 of gestation. On day 20 of gestation the pregnant rats were killed under ether anaesthesia and both foetal and maternal livers removed. Foetal livers were pooled from each dam. Microsomes were prepared and stored as above.

Purification of [^{14}C]-3,3',4,4'-tetrachlorobiphenyl (TCB)

[^{14}C]-TCB was purified using semipreparative hplc. Of [^{14}C]-TCB, 50 μCi was dissolved in 400 μl methanol and injected in 200-μl aliquots on a Zorbax ODS reverse-phase column (Dupont, 4·6 mm i.d. × 25 cm) and eluted with a mixture of 85% methanol (v/v) and 15% water at a flow rate of 2 ml/min. Fractions were collected every 1 min for 30 min and UV absorbance monitored at 262 nm. A 100-μl aliquot of each fraction was counted by liquid scintillation counting (LSC) using Ultima Gold (Packard) as the scintillation fluid and the main fractions containing [^{14}C]-TCB radioactivity were pooled. The recovery of total radioactivity from the column was >99%. The column had previously been calibrated using unlabelled TCB as a standard.

The water content of the pooled hplc fractions was adjusted to 50% and a final volume of 90 ml. The methanol:water (1:1) phase was then extracted four times with 10 ml hexane by thorough mixing in a volumetric flask and removing the hexane after the phases had separated. Extraction efficiency for [^{14}C]-TCB in this latter case was >99%. The hexane extract was evaporated under nitrogen, redissolved in 2 ml toluene and stored at 4°C. In order to check the purification, 1 μCi of the purified extract was redissolved in 100 μl methanol and 50 μl was injected on the same column using the same mobile phase conditions as described above. Fractions were collected every 30 s and when counted by LSC the radiochemical purity was >99·8%. No contaminating peaks were found by monitoring the absorbance at 262 nm.

Incubation of [^{14}C]-TCB with hepatic microsomes

Initial experiments were carried out with β-NF(1) microsomes because previously published research indicated that inducers of P4501A activity resulted in an induction of TCB metabolism in rat hepatic microsomes (Ishida *et al.* 1991). Incubations were carried out in glass tubes at 37°C in a shaking water bath with 0·5 μCi [^{14}C]-TCB per tube in a total volume of 2 ml with a 0·1 M (final concentration) Tris–HCl buffer, pH 7·5. Microsomal protein concentrations were varied between 0·1 and 2·0 mg protein/ml. The final concentration of TCB used varied between 1 and 50 μM. Unlabelled TCB was added to [^{14}C]-TCB to obtain the desired stock solution, which was dissolved in acetone and added to the pre-incubation mixture containing the microsomes and buffer (25 μl acetone/ml incubation mixture) and vortexed for 1 min. NADPH was used as a cofactor in concentrations of 1 and 2 mM. An NADPH regenerating system was also used that consisted of 0·3 mM $NADP^+$, 10 mM glucose 6-phosphate, 0·25 units glucose 6-phosphate dehydrogenase, and 7·5 mM $MgCl_2$ (final concentrations). The incubation mixtures were prepared in tubes placed in an ice water bath. Incubations were pre-incubated for 2 min at 37°C and the

reaction initiated by addition of NADPH, or in the case of the NADPH-regenerating system with glucose 6-phosphate. The reaction time was varied between 0 and 30 min. In order to investigate the effect of glucuronidation on the appearance of phenolic metabolites in the incubation mixture, β-NF(2) microsomes (1 mg/ml) were incubated for 15 min with 10 μM [^{14}C]-TCB, 1 mM NADPH, with and without 1 mM uridine-diphosphoglucuronic acid.

Reactions were stopped by the addition of 2 ml ice-cold methanol to the incubation mixture and vortexed for 30 s. After the addition of methanol, the incubation mixtures were extracted three times with 4 ml diisopropyl ether by vortexing for 30 s, centrifuged at 1000*g* for 5 min, and the diisopropyl ether was removed. Blanks were carried out by performing identical incubation with heat-inactivated microsomes (boiling water bath for 10 min). The ether extracts were pooled and dried under nitrogen and stored at 4°C until analysis. The lower phase containing the microsomal pellet and the water/methanol mixture was also stored at 4°C until further analysis. For hplc analysis of the water/methanol phase it was first centrifuged at 1000*g* for 5 min, the supernatant removed, evaporated under nitrogen and resuspended in 100 μl water:methanol (1:1).

Hplc analysis of extractable radioactivity

Metabolite analysis was conducted using a Gilson 300 series hplc system fitted with a Perkin–Elmer 3 μm C-18 column (4·6 mm i.d. × 83 mm). The UV absorbance was monitored at 262 nm, which yielded the best mean response of the standards used according to a wavelength scan (200–400 nm) in 78% methanol/22% water with a Beckman DU-64 spectrophotometer. The diisopropyl ether extracts were redissolved in 100 μl methanol containing 2·5 μg of the following standards: 2-OH-3,4,3′,4′-tetrachlorobiphenyl, 4-OH-3,3′,4′,5-tetrachlorobiphenyl, 5-OH-3,4,3′,4′-tetrachlorbiphenyl, and 6-OH-3,4,3′,4′-tetrachlorobiphenyl. Of the ether extract or the concentrated water/methanol phase, 20 μl was injected on the column. The mobile phase was 78% (v/v) methanol, 22% water (containing 0·01% w/v Na-azide) for 20 min, followed by a gradient to 100% methanol in 5 min, and 100% methanol for another 10 min. The flow rate was 1 ml/min. The column was allowed to equilibrate at 78% v/v methanol, 22% water (0·01% w/v Na-azide) for at least 10 min between injections. Fractions were collected every 0·4 min for 35 min with a Redifrac fraction collector (Pharmacia). Of scintillation fluid (Ultima Gold, Packard), 5 ml was added to each fraction and was then counted in a liquid scintillation counter (Tri-carb 1600, Packard). Identification of the metabolites was based on the coelution of radioactivity with authentic standards.

Gc-ms analysis of extractable metabolites

Once the pattern of the metabolites formed had been firmly established on the basis of coelution with authentic standards using hplc analysis, further confirmation of the identity of the metabolites formed in the *in Vitro* incubations of hepatic microsomes with [^{14}C]-TCB was provided by gc with electron-capture detection (gc-ECD) and gc-ms. The ether extracts from three incubations were pooled. The incubation conditions were: 2 ml final volume with 10 μM [^{14}C]-TCB, 1 mg microsomal protein (β-NF(2)) per ml, 1 mM NADPH in 1 mM Tris-HCl buffer, pH 7·5, and a 10-min incubation time. The pooled extracts were resuspended in 200 μl methanol and 20 μl was injected on the C-18 column that was routinely used for analysis of the extracts. Fractions (1 ml) were collected in glass tubes and 50-μl of each fraction was counted using LSC as above. The fractions containing the radioactivity were pooled separately for each peak and the solvent was evaporated under nitrogen. The dried residue was redissolved in 1 ml *n*-hexane and methylated with diazomethane. Unreacted diazomethane and hexane was then evaporated under nitrogen.

Gc-ms was performed on an ITS40 instrument (Finnigan). The GC (Varain 3400) was equipped with a fused silica capillary column (DB5, 30 m × 0·25 mm i.d.; J&W Scientific Inc., CA, USA) and a split-splitless injector. The temperature program was 80°C for 2 min, then 10°C/min to 240°C, and maintained at 240°C for 20 min. The injector temperature was 260°C and the injections were made in the splitless mode using an autosampler (CTC A200S, Finnigan). The mass spectrometer was operated in the electron impact mode and the manifold temperature was 220°C.

Gc-ECD analyses were performed with a Varian 3400 gc equipped with a DB5^{+} fused silica column (30 m × 0·25 m i.d.; J&W Scientific), a split-splitless injector operated at 250°C, and a ^{63}Ni ECD operated at 300°C. The samples were injected in the splitless mode and the column temperature was programmed as follows: initial temperature 80°C for 2 min, followed by 10°C/min up to 300°C.

Covalent binding to microsomal protein

Covalent binding of metabolites to microsomal protein was determined as previously described (den Besten *et al.* 1991) after diisopropyl ether extraction of the incubation mixture. The water/methanol phase containing the microsomal proteins was centrifuged at 1000*g* for 5 min and the supernatant removed. The pellet was resuspended and washed successively with 2 ml solvent of declining polarity (water, methanol, ethanol, ethyl acetate). The microsomal pellet was recovered by centrifugation at 1000*g* for 5 min and removal of the solvent. After the final wash with ethyl acetate, the pellet was resuspended in 1 ml 1 N NaOH. Radioactivity was determined in the wash solvents and 500 μl of the resuspended pellet

by LSC. The remaining 500 μl of the resuspended pellet was used for a protein determination with bovine serum albumin as a standard (Lowry *et al.* 1951).

The radioactivity in the water/methanol phase was analysed for the presence of glucuronic acid conjugates and phenolic metabolites by incubation of 50-μl aliquots of the water:methanol phase (after concentration to 200 μl) with and without 150 μl β-glucuronidase solution (1000 units/ml 0·1 potassium phosphate buffer, pH 6·8) for 30min at 37°C (Shen *et al.* 1991) or 4 N HCl at 100°C for 1 h, followed by diisopropyl ether extraction and hplc analysis as described above.

Ethoxyresorufin-O-deethylase (EROD) activity

EROD activity was determined using a final concentration of 1 μM ethoxyresorufin, 0·1 mM NADPH, and 100 μg microsomal protein per ml at 37°C (Burke *et al.* 1977). To detect the deethylation product resorufin the excitation wavelength of the fluorimeter was 530 nm and the emission wavelength was 580 nm. Initial reaction velocities were used to calculate EROD activity.

Results

Optimization of incubation conditions

Incubations were first optimized for cofactor requirements, length of incubation, microsomal protein concentration, and TCB concentration by examining the total amount of extractable metabolites formed with hplc. Calculations of total extractable metabolites formed were based on the percentage of the total radioactivity in the hplc radiochromatogram eluting in the metabolite fractions, adjusted for the total amount of TCB (unlabelled and labelled) present in the incubation. Recoveries in the diisopropyl ether extracts were always > 95% for 5-min incubations.

In order to determine whether the addition of NADPH or an NADPH regenerating system yielded the highest activity, β-NF(1) microsomes (1 mg/ml) were incubated for 15 and 30 min with 1 or 2 mM NADPH and an NADPH regenerating system with 1·7 μM [^{14}C]-TCB. Since the amount of extractable metabolites formed at both time points was highest with 1 mM NADPH (data not shown), this concentration was therefore used for further experiments. Because the reaction was nonlinear over 30 min, a second experiment was conducted with 1 mM NADPH, 1 mg microsomal protein/ml and 1·5 μM [^{14}C]-TCB and the amount of extractable metabolites formed was determined at 1, 5, 10, 15 and 30 min, and revealed that the reaction was linear for the first 5 min at this TCB concentration (data not shown).

In order to determine the effect of substrate concentration on metabolite production, incubations were performed with 1–50 μM [^{14}C]-TCB, 1 mg microsomal protein/ml and 1 mM NADPH for 5 min. At 50 μM [^{14}C]-TCB the solubility of the substrate was poor, yielding large variations in the amount of extractable metabolites formed. The poor solubility of 50-μM [^{14}C]-TCB was confirmed by the finding that there were large variations in the amount of radioactivity recovered when five 100-μl aliquots of the incubation mixture were quantified with LSC prior to extraction, and therefore the data from the incubations with 50-μM [^{14}C]-TCB were not used for the Lineweaver–Burke plot. Saturing concentrations of [^{14}C]-TCB were not completely achieved by 32 μM (figure 1 (*a*)). From the Lineweaver–Burke plot (figure 1 (*b*)) the apparent K_m and V_{max} were determined to be 4·5 μM and 240 pmol/mg protein/min respectively as calculated from the amount of diisopropyl ether-extractable metabolites formed.

The effect of microsomal protein concentration on the formation of extractable metabolites was examined at 2·5 and 10 μM [^{14}C]-TCB, 1 mM NADPH and an incubation time of 5 min (data not shown). At 10 μM [^{14}C]-TCB, the formation of extractable metabolites (pmol/min) increased linearly with the protein concentration up to 1 mg microsomal protein/ml. At the lower substrate concentration used

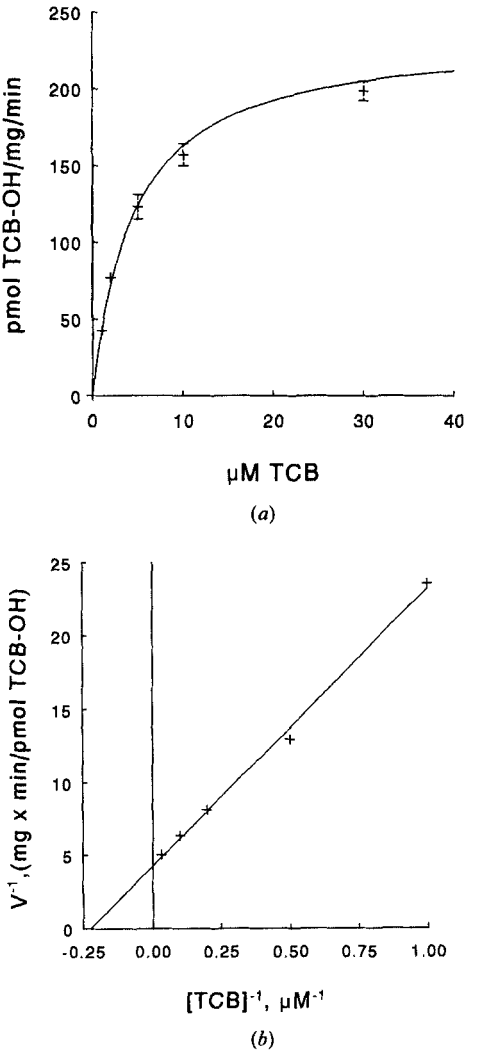


Figure 1. Influence of substrate concentration on the formation of diisopropyl ether-extractable metabolites (TCB-OH) of [^{14}C]-TCB with microsomes from the β-naphthoflavone-treated rat (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5 min. Results (mean ± range) are the mean of duplicate incubations. If no error bar is visible it is smaller than the data point. The curve is fitted by computer for first-order kinetics with an apparent K_m and V_{max} of 4·5 μM and 240 pmol/mg protein/min respectively using the program Slidewrite 5.0. (*b*) Lineweaver–Burke plot of the mean data from (*a*).

(2·5 μM) the formation of extractable metabolites was not a linear function of the protein concentration.

Identification of metabolites

Hplc fractionation followed by LSC of the ether-extractable radioactivity revealed four radioactive metabolite peaks. A typical histogram of a reverse-phase hplc fractionation of the ether extractable radioactivity following the incubation of [^{14}C]-TCB with β-NF microsomes is shown in figure 2. The first three radioactive peaks coeluted with the metabolite standards 4-OH-tetraCB, 5-OH-TCB, and

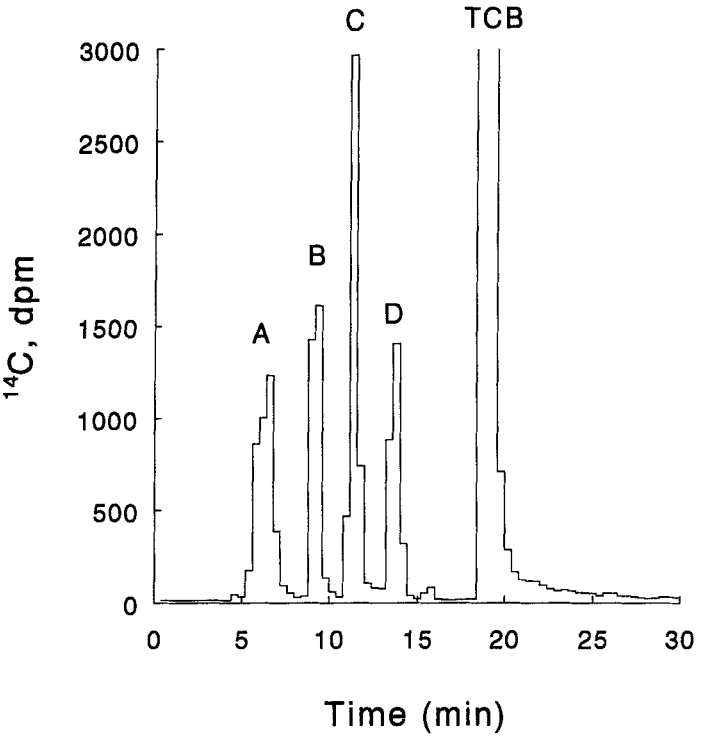


Figure 2. A typical histogram of reversed-phase hplc analysis of diisopropyl ether-extractable metabolites of 10 μM [^{14}C]-TCB with microsomes from the β-naphthoflavone-treated rat (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5 min. (A) 4-OH-3,3′,4′,5-tetrachlorobiphenyl; (B) 5-OH-3,3′,4,4′-tetrachlorobiphenyl; (C) 6-OH-3,3′,4,4′-tetrachlorobiphenyl; and (D) unidentified peak in the hplc radiochromatogram.

6-OH-TCB respectively. The fourth radioactive peak did not coelute with any of the authentic metabolite standards and no radioactive peak coeluted with 2-OH-TCB.

Gc-ECD and gc-ms analysis of the methylated fractionated peaks after hplc separation of pooled incubation extracts confirmed the identity of the radioactive peaks that coeluted with the authentic standards for 4-OH-tetraCB, 5-OH-TCB, and 6-OH-TCB, both the retention time and mass spectra of the isolated metabolites after methylation corresponded to the methylated authentic standards (data not shown). No 2-OH-TCB was detected by gc-ECD as its methylated derivative in the hplc fraction collected where the authentic standard would elute or in an unfractionated diisopropyl ether extract of incubation with β-NF microsomes (data not shown). The radioactive peak that did not coelute with any of the authentic standards (figure 2, peak D) yielded two compounds after methylation when analysed with gc-ms with molecular ions of 320 and 306, both with an isotropic cluster corresponding to four chlorine ions (data not shown).

Extracting the incubations three times with diisopropyl ether effectively removed all phenolic metabolites and TCB from the aqueous lower phase. When the lower phase was evaporated and resuspended in methanol, followed by reversed-phase hplc analysis, only one radioactive peak was observed that eluted close to the front of the chromatogram and comprised approximately 10% of the total metabolites

formed. Treatment of the lower phase with β-glucuronidase or 4 N HCl did not change the position of this peak in the radiochromatogram (data not shown).

The formation of ether-extractable, polar and covalently bound metabolites was followed over a period of 15 min in an incubation of β-NF(2) microsomes (1 mg/ml) with 10 μM [^{14}C]-TCB and 1 mM NADPH. Over 15 min the rate of formation of 4-OH-tetraCB (17·1 ± 0·5pmol/mg protein/min) was 50% greater than that of the other two identified phenolic metabolites, 5-OH-TCB and 6-OH-TCB (11·4 ± 0·6 and 12·5 ± 0·5 pmol/mg protein/min; figure 3(*a*)). The radioactive peak containing unidentified extractable metabolites was formed at a rate similar to that of 5-OH-TCB and 6-OH-TCB (13·3pmol/mg protein/min).

Of the metabolites that were non-extractable in ether, the largest amount formed was in the form of one or more water soluble compounds (23·4 ± 0·1 pmol/mg protein/min) during the first 5 min (figure 3 (*b*)). The formation rate of water-soluble compounds was even higher during the second 5 min of incubation (36·4 ± 5·3 pmol/mg protein/min). However, between 10 and 15 min the concentration of water soluble compounds present in the incubation mixture decreased from 150 ± 2·6 to 106 ± 22·3 pmol/ml. Although we were unable to demonstrate the presence of phenolic metabolites in the water/methanol phase by acid or enzymatic hydrolysis, the addition of 1 mM UDGPA to the incubation mixture significantly reduced the amount of 5-OH-TCB in the incubation mixture after 15 min from 86 ± 4·4 to 59 ± 7·3 pmol/ml ($p < 0·05$, Student's t-test, $n = 3$). The levels of the other phenolic metabolites in the incubation mixture were unaffected by the addition of 1 mM UDPGA (data not shown).

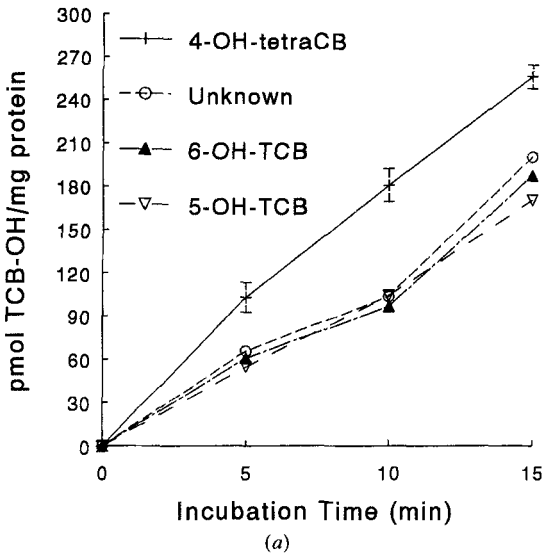
Covalently bound metabolites were formed over the first 5 min of the incubation at a low rate (4·3 ± 0·9 pmol/mg protein/min), which increased to 9·3 ± 3·9 pmol/mg protein/min in the second 5 min of the incubation, and remained at the same rate (9·3 ± 0·4 pmol/mg protein/min) during the third 5 min.

The production of metabolites covalently bound to microsomal proteins was investigated in relation to substrate concentration and protein concentration. After 5-min incubations of 1–32 μM [^{14}C]-TCB with 1 mg microsomal protein (β-NF1)/ml and 1 mM NADPH, the amount of covalently bound metabolites formed increased in a nonlinear fashion with the [^{14}C]-TCB concentration (figure 4 (*a*)). When the microsomal pellet increased linearly between 0·5 and 2·0 mg protein/ml (figure 4 (*b*)). However, at a lower substrate concentration (2·5 μM [^{14}C]-TCB) the amount of covalently bound metabolites did not increase in a linear fashion (figure 4 (*b*)).

Influence of pretreatment and development

The induction of TCB metabolism by various inducers of hepatic mono-oxygenases was investigated in the male Wistar rat. The incubation conditions were 1 mg microsomal protein/ml, 10 μM [^{14}C]-TCB, 1 mM NADPH and an incubation time of 5 min. The only pretreatment that resulted in the induction of TCB metabolism (formation of extractable metabolites) was β-naphthoflavone (table 1). β-NF treatment also resulted in the greatest induction of EROD activity relative to the untreated rat. Although pretreatment with phenobarbital induced EROD levels four-fold relative to controls, neither treatment with phenobarbital not dexamethasone had any effect on hepatic microsomal TCB metabolism.

The capacity of foetal and maternal rat microsomes from day 20 of gestation to metabolize [^{14}C]-TCB or ethoxyresorufin after pretreatment on day 13 of gestation with either unlabelled TCB (25 μmol/kg) or the vehicle alone (corn oil, 2 ml/kg) was



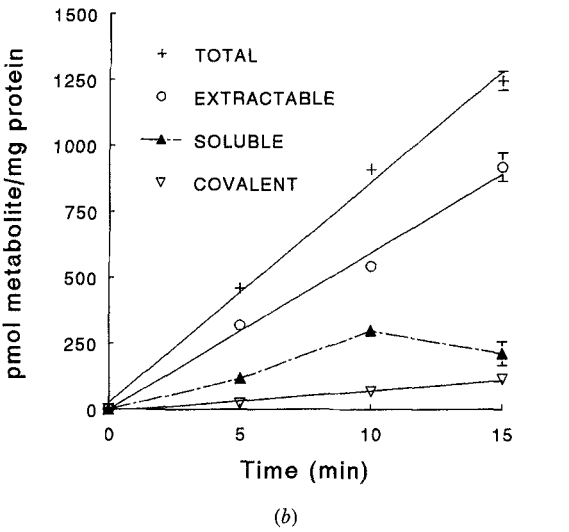


Figure 3. *Formation of individual diisopropyl ether-extractable metabolites of 10 μM [^{14}C]-TCB with microsomes from the β-naphthoflavone-treated rat (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5, 10 and 15 min. Results (mean ± SD) are the mean of three incubations. For clarity, error bars are presented only for 4-OH-tetraCB. 4-OH-tetraCB, 4-OH-3,3',4',5-tetrachlorobiphenyl; 5-OH-TCB, 5-OH-3,3',4,4'-tetrachlorobiphenyl; 6-OH-TCB, 6-OH-3,3',4,4'-tetrachlorobiphenyl; and unknown, unidentified peak in the hplc radiochromatogram. (b) Formation of diisopropyl ether-extractable, water soluble and covalently bound and total metabolites of 10 μM [^{14}C]-TCB with microsomes from the β-naphthoflavone-treated rat (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5, 10 and 15 min. Results (mean ± SD) are the mean of three incubations. If no error bar is visible it is smaller than the data point.*

also investigated. The incubation conditions were 1 mg microsomal protein/ml, 10 μM [^{14}C]-TCB, 1 mM NADPH and an incubation time of 5 min. There was no detectable formation of extractable metabolites in incubations with microsomes from the corn oil-treated dam and foetus or with microsomes from the TCB-exposed foetus. Only incubations of [^{14}C]-TCB with microsomes from the TCB-treated dam

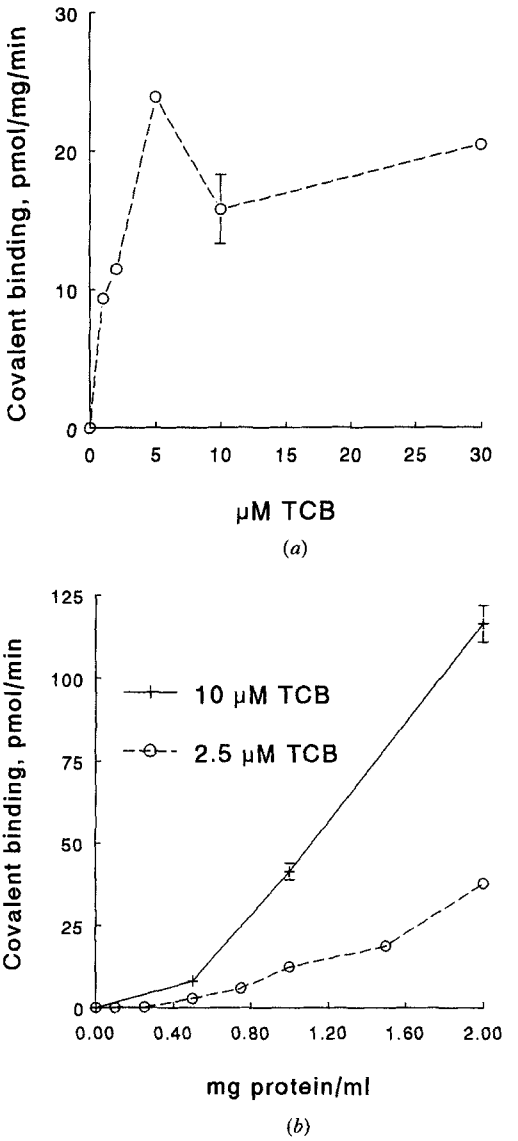


Figure 4. (*a*) Influence of substrate concentration on the formation of covalently bound metabolites of [^{14}C]-TCB with microsomes from the β-naphthoflavone-treated rat (1 mg protein/ml) with 1 mM NADPH and an incubation time of 5 min. Results (mean ± range) are the mean of duplicate incubations. (*b*) Influence of microsomal protein concentration on the formation of covalently bound metabolites of 2·5 and 10μM [^{14}C]-TCB with microsomes from the β-naphthoflavone-treated rat with 1 mM NADPH and an incubation time of 5 min. Results (mean ± range) are the mean of duplicate incubations. If no error bar is visible, it is smaller than the data point.

resulted in the production of ether-extractable metabolites (table 2). The level of EROD activity with microsomes from the TCB-treated dam was 100-fold higher than that of the corn oil-treated dam, and foetal EROD activity was undetectable irrespective of the pretreatment. EROD activity was also three-fold lower with microsomes from the corn oil-treated dam (17 pmol/mg protein/min) than the untreated male rat (53 pmol/mg protein/min). The relative amounts of phenolic PCB

Table 1. Microsomal metabolite production from [^{14}C]-TCB after different pretreatments of the male Wistar rat.

Treatment	TCB metabolism (pmol OH-TCB/mg protein/min)	EROD activity (nmol RR/mg protein/min)
Control	3·0 ± 0·4	0·05 ± 0·01
Phenobarbital	3·6 ± 0·7	0·20 ± 0·05
Dexamethasone	2·1 ± 0·4	0·06 ± 0·01
β-Naphthoflavone:		
(1)	157 ± 7·2	6·13 ± 0·60
(2)	62·9 ± 3·8	2·29 ± 0·24

Incubation conditions were: 10 μM [^{14}C]-TCB, 1 mg microsomal protein/ml, 1 mM NADPH, 37°C for 5 min. The rate of metabolism is based on the amount of ether-extractable metabolites formed in the incubations. Results are the mean ± SD of triplicate incubations. EROD, ethoxyresorufin-*O*-deethylase; and RR, resorufin.

Table 2. Microsomal metabolite production from [^{14}C]-TCB in the control and TCB-treated pregnant Wistar rat and foetus.

Treatment	TCB metabolism (pmol OH-TCB/mg protein/min)	EROD activity (nmol RR/mg protein/min)
Control dam	nd	0·02 ± 0·00
Control foetus	nd	nd
TCB dam	43·9 ± 8·5	1·70 ± 0·43
TCB foetus	nd	nd

Incubation conditions were: 10 μM [^{14}C]-TCB, 1 mg microsomal protein/ml, 1 mM NADPH, 37°C for 5 min. The rate of metabolism is based on the amount of ether-extractable metabolites formed in the incubations. Results are the mean ± SD of duplicate incubations of microsomes from four individual dams or their foetuses. nd, not detected; EROD, ethoxyresorufin-*O*-deethylase; and RR, resorufin.

Table 3. Comparison of the production of phenolic metabolites of [^{14}C]-TCB by microsomes from the β-NF-treated male and TCB-treated pregnant female Wistar rat.

Metabolite	β-NF(2)-treated male	TCB-treated dam
4-OH-tetraCB	13·5 ± 2·3	20·7 ± 2·0
5-OH-TCB	7·1 ± 1·1	10·9 ± 0·7
6-OH-TCB	8·7 ± 1·3	12·1 ± 0·6
Unknown	6·1 ± 1·1	13·1 ± 1·4

Values are pmol OH-TCB/mg protein/min.

Incubation conditions were: 10 μM [^{14}C]-TCB, 1 mg microsomal protein/ml. 1 mM NADPH, 37°C for 5 min. The rate of metabolism is based on the amount of ether-extractable metabolites formed in the incubations. Results are the mean ± SD of duplicate incubations of microsomes from four individual dams or triplicate incubations of β-NF(2) microsomes. β-NF, β-naphthoflavone.

metabolites and the extractable unknown compound(s) formed were nearly identical in incubations of [^{14}C]-TCB with microsomes from the β-NF-treated male Wistar rat and TCB-treated pregnant female Wistar rat (table 3).

Discussion

The major phenolic metabolites of TCB formed in the incubation mixture (4-OH-3,3′,4′,5-tetraCB, 5-OH-TCB, 6-OH-TCB) were resolved by hplc and their identity confirmed by coelution with authentic standards on a reverse-phase hplc

column. Definitive confirmation of the identity of the phenolic compounds in the hplc fractions was given by gc-ECD and gc-ms analysis after methylation of the fractions. The reaction conditions were partially optimized for cofactor requirements, substrate and microsomal protein concentration. Ideally, a substrate concentration should be chosen that will result in V_{max} conditions, however at 30 μM [^{14}C]-TCB the V_{max} conditions were not attained and at 50 μM [^{14}C]-TCB the solubility of the substrate in the incubation mixture was poor at a microsomal protein concentration of 1 mg/ml. The use of 10 μM [^{14}C]-TCB forms a reasonable compromise as the production of metabolites was a linear function of time (up to 15 min) at 10 μM [^{14}C]-TCB using highly induced microsomes (1 mg protein/ml).

In the male rat only treatment with the P4501A inducer, β-NF, resulted in a significant induction of [^{14}C]-TCB metabolism relative to control microsomes. This is in agreement with previous studies (Mills *et al.* 1985, Ishida *et al.* 1991) that demonstrated the P4501A1 dependence of TCB metabolism. Phenobarbital treatment has been shown to induce the metabolism of 2,2',5,5'-tetrachlorobiphenyl, but not 3,3',4,4'-tetrachlorobiphenyl (Ishida *et al.* 1991). In rat, dexamethasone is an extensive inducer of P4503A1 (Wrighton *et al.* 1985) and the hydroxylation of various chlorinated benzenes such as hexachlorobenzene (Van Ommen 1987), pentachlorobenzene, and 1,2,4-trichlorobenzene (den Besten *et al.* 1991), 1,2-dichlorobenzene and 1,4-dichlorobenzene (den Besten *et al.* 1992). However, in the current study the pretreatment of rat with dexamethasone did not result in the increased hepatic microsomal metabolism of [^{14}C]-TCB, indicating that the presence of a phenyl ring in place of a chlorine (as in 1,2,4-trichlorobenzene) effectively inhibits the hydroxylation of [^{14}C]-TCB by P4503A1.

In the current study, three phenolic metabolites, 4-OH-3,5,3',4'-tetraCB, 5-OH-TCB, and 6-OH-TCB, were positively identified in the ether extracts. A fourth radioactive peak in the hplc fractions of the ether-extracts appeared to contain two compounds upon gc-ms analysis, although we were unable to identify the structure of these latter compounds.

Interestingly, two *in vivo* studies (Yoshimura *et al.* 1987, Koga *et al.* 1989), and one *in vitro* study (Ishida *et al.* 1991), all using the male Wistar rat or microsomes, did not find any faecal excretion or *in vitro* production of 6-OH-TCB, despite the use of an authentic standard. 6-OH-TCB is one of the most lipophillic of the phenolic metabolites, as judged by its relatively long retention time on a C-18 hplc column relative to the other phenolic metabolites formed, and may thus be poorly excreted into the faeces following its formation. This hypothesis is supported by the report that when the C57BL mouse was treated with [^{14}C]-TCB only trace amounts of 6-OH-TCB were detected in the faeces, but 6-OH-TCB comprised 16% of total radioactivity in the adipose tissue (Klasson Wehler 1989). It is unclear why the production of 6-OH-TCB was not detected in the study using microsomes from the 3-methylcholanthrene-treated Wistar rat (Ishida *et al.* 1991). One significant difference in the analytical methods is that in this study high resolution capillary gc-ECD and gc-ms was used and in the previous study (Ishida *et al.* 1991) packed column gc was used, which could result in a difference in the resolution of the metabolites on the column.

One minor metabolite previously identified *in vivo* in the chicken embryo and mouse urine, 2-OH-TCB (Klasson Wehler 1989, Klasson Wehler *et al.* 1990), was not found in the ether extracts of incubations with highly induced microsomes, either by hplc or gc-ms analysis. Similarly, 2-OH-TCB was also not found in the faeces

of the Wistar rat treated with TCB (Yoshimura *et al.* 1987), which may indicate species specific metabolism of TCB. In the current study the nature and relative amounts of the ether-extractable metabolites formed in incubations with hepatic microsomes from the TCB-treated dam were identical to those formed in incubations with microsomes from the male rat pretreated with β-NF. 4-OH-tetraCB and 5-OH-TCB have also been found in the liver of the pregnant rat treated with TCB (Morse *et al* 1995).

When we examined the metabolism of [^{14}C]-TCB with microsomes from the TCB-treated pregnant rat and its foetus, the formation of phenolic metabolites was observed only in incubations of maternal microsomes. Even when the incubation time was extended to 30 min no phenolic metabolite production was observed in incubations with foetal microsomes. In addition, no induction of foetal hepatic microsomal EROD activity was observed following maternal TCB treatment. These data support the hypothesis that 4-OH-tetraCB and other phenolic metabolites are produced in the maternal liver, only 4-OH-tetraCB is selectively retained in the maternal plasma bound to TTR, then crosses the placenta and accumulates in the foetal compartment. A recent study has shown that the accumulation of 4-OH-tetraCB in the foetal rat is associated with decreases in foetal plasma TT4 levels in the absence of effects on maternal TT4 levels at the same time of gestation (Morse *et al.* 1995). This finding suggests that maternally produced metabolites may affect foetal thyroid hormone status, although there are no obvious effects on maternal thyroid status.

Although TCB is only a minor component of commercial PCB mixtures and environmental extracts, other PCBs may be metabolized to hydroxylated metabolites, of which a limited number, mainly with an 1,2-shift hydroxy-substitution in the *para*-position, are selectively retained in the blood of rat and environmentally exposed animals (Bergman *et al.* 1994). Recent research has shown that, analogous to the foetal accumulation of 4-OH-tetraCB in the rat, the major metabolite (4-OH-2,3,5,3′,4′-pentachlorobiphenyl) found in adult rat, mouse and mink plasma following Aroclor 1254 exposure (Klasson Wehler *et al.* 1993, Bergman *et al.* 1994) also accumulates in foetal rat plasma following Aroclor 1254 exposure to the pregnant rat (E. Klasson Wehler, personal communication).

We were unable to identify the nature of the water-soluble metabolites formed in incubations of [^{14}C]-TCB with β-NF microsomes. Although no compounds coeluting with the metabolite standards were produced by acid hydrolysis of β-glucuronidase treatment of the lower phase, the addition of UDPGA to the incubation mixture selectively reduced the amount of 5-OH-TCB present in the ether extracts, which may indicate the formation of glucuronic acid conjugates. The presence of conjugated metabolites of TCB has been indicated in the urine of mouse after an oral dose of TCB (Klasson Wehler *et al.* 1989).

The covalent binding of [^{14}C]-TCB metabolites to microsomal or erythrocyte membrane proteins has been previously demonstrated in *in vitro* studies (Shimada and Sawabe 1983, Shimada *et al.* 1985). Metabolites of [^{14}C]-TCB also bind to cellular macromolecules in several organs of the rat *in vivo* (Shimada and Sawabe 1984), although the significance of this covalent binding for toxicity is not clear. It has been suggested that the high retention of [^{14}C]-TCB-derived radioactivity in the blood (Shimada *et al.* 1985) is due to covalent binding to blood components and that the retention in the blood is related to the high toxicity of TCB. However, in a recent study it has been demonstrated that there is a selective accumulation of one

metabolite, 4-OH-3,5,3′,4′-tetrachlorobiphenyl in the plasma of rat following [^{14}C]-TCB exposure (Morse *et al.* 1995), which is due to competitive binding of this metabolite to TTR, the major thyroid hormone transport protein in rat plasma (Brouwer *et al.* 1986, 1990).

Although the metabolism of TCB appears to be largely similar between rat and mouse and is related to the induction of P4501A enzymes, this may not be the case for some other species. A qualitative interspecies variation in the production of phenolic metabolites of TCB by hepatic microsomes from mammals and birds has recently been observed (Murk *et al.* 1994). The most notable result was that environmentally exposed marine mammals (seal and porpoise) showed a very similar metabolite pattern to the β-NF treated rat, whereas eider duck microsomes (exposed to either Clophen A50 or TCB) produced almost exclusively 5-OH-TCB.

In conclusion, we have developed a technique for the hplc analysis of ether-extracts of hepatic microsomes with [^{14}C]-TCB, which can provide qualitative and quantitative information on the formation of hydroxylated metabolites of TCB. The reaction kinetics of [^{14}C]-TCB with hepatic microsomes have been partially characterized to allow for optimal incubation conditions. The technique is useful for the investigation of the metabolic capacity in different developmental stages, following various pretreatments (this study), or in different animal species (Murk *et al.* 1994).

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