Toxicology and Applied Pharmacology **180,** 157–163 (2002)
doi:10.1006/taap.2002.9395, available online at http://www.idealibrary.com on IDEAL®

In Vitro Estrogen Receptor Binding of PCBs: Measured Activity and Detection of Hydroxylated Metabolites in a Recombinant Yeast Assay

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Received November 12, 2001; accepted February 25, 2002

In Vitro **Estrogen Receptor Binding of PCBs: Measured Activity and Detection of Hydroxylated Metabolites in a Recombinant Yeast Assay. Layton, A. C., Sanseverino, J., Gregory, B. W., Easter, J. P., Sayler, G. S., and Schultz, T. W. (2002).** *Toxicol. Appl. Pharmacol.* **180, 157–163.**

The estrogenic activities of 17β-estradiol, biphenyl, chlorinated biphenyls, and Aroclor mixtures 1221, 1242, and 1248 were measured with a modified recombinant yeast estrogen assay (i.e., a *Saccharomyces cerevisiae***-based** *lac***-Z (β-galactosidase) reporter assay). Modifications of the assay included the use of glass vials instead of plastic microtiter plates and the addition of the medium and yeast before the test substrate. ^{14}C-labeled compounds were used to follow improvements in the assay procedures. ^{14}C-17β-estradiol recovery from plastic microtiter plates and glass vials using the standard or the modified procedure was approximately 89%. However, ^{14}C-4-CB (4-chlorobiphenyl) recovery was considerably less, ranging from 3% in plastic microtiter plates using the standard procedure to 26% in vials using the modified procedure. These results suggest that the toxicity of strongly hydrophobic chemicals may be underestimated. Using the modified yeast estrogen assay, full agonist activity was observed for 4-CB, 2,4,6-CB, and 2,5-CB while each of the Aroclor mixtures were only partial agonists. The equivalent EC_{50} values in ppm were in environmentally relevant concentrations for biphenyl (19 ppm), 4-CB (4.5 ppm), 2,5-CB (21 ppm), 2,4,6-CB (0.8 ppm), Aroclor 1221 (2.9 ppm), Aroclor 1242 (0.65 ppm), and Aroclor 1248 (2.3 ppm). Estrogen receptor binding for the individual PCB congeners was 25- to 650-fold less than the reported estrogen binding for the corresponding hydroxylated PCB metabolite. Gas chromatographic/mass spectrometric analysis of yeast extracts indicated that** *S. cerevisiae* **hydroxylated the individual PCB congeners in the ppb range. With the exception of biphenyl, the concentration of hydroxylated metabolites obtained from incubation of** *S. cerevisiae* **with PCB congeners was consistent with the concentration necessary to elicit a positive estrogen receptor-binding response. This work provides evidence that** *S. cerevisiae* **are capable of metabolic transformation of PCBs and that estrogen receptor binding of PCBs is mediated through the hydroxylated metabolite rather than through the direct interaction of the PCB congeners with the estrogen receptor.** © 2002 Elsevier Science (USA)

Key Words: **estrogen activity; polychlorinated biphenyls; aroclors;** *Saccharomyces cerevisiae***.**

Polychlorinated biphenyls (PCBs)[2] are man-made contaminants, which have been identified in a variety of ecosystems. Commercial formulations of PCBs are mixtures of biphenyl compounds differing in the degree and location of chlorination. The biochemical and toxicological responses elicited by individual PCB congeners are diverse and complex and affect several systems, including the estrogen and thyroid hormone systems (Brouwer *et al.*, 1999). Although PCBs are extremely persistent compounds, many PCB congeners are metabolized *in vivo* to more polar derivatives (Sundstrom *et al.*, 1976; Sipes and Schnellmann, 1987). In mammals, hydroxylated biphenyls are found as metabolites of the parent halogenated biphenyls (Kato *et al.*, 1980; Bergman *et al.*, 1994). PCBs containing chlorines in the *meta* and *para* positions are less susceptible to biotransformation than those lacking chlorines in those positions (Borlakoglu and Wilkins, 1993).

Environmental endocrine disrupters can be grouped into one of four categories: direct-acting agonists, direct-acting antagonists, indirect-acting agonists, and indirect-acting antagonists (Cooper and Kavlock, 1997). For direct-acting compounds, competitive binding is a required condition and affinity can be measured using ligand-binding assays. In the case of the estrogen receptor, ligand binding may result in either an estrogenic (agonists) or an antiestrogenic (antagonists) response. In contrast, gene activation assays such as the yeast estrogen reporter system can segregate agonists from antagonists and are more rapid than whole animal assays. Thus, it is a useful screening tool for the detection of potentially estrogenic chemicals. Fang *et al.* (2000) have quantitatively compared the three

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[2] Abbreviations used: 4-CB, 4-chlorobiphenyl; 4-CB-4′-OH, 4-chloro-4′-biphenylol; CPRG, chlorophenol red-β-D-galactopyranoside; MOP, modified operating procedures; PCBs, polychorinated biphenyls; SAS, probit analysis of Statistical Analysis System; SIM, selective ion monitoring mode; SOP, standard operating procedure; YES, yeast estrogenic system.

0041-008X/02 $35.00

major *in vitro* assays for estrogenic activity and found the recombinant yeast-based reporter assay was as good as the estrogen receptor competitive binding and the MCF-7 cell proliferation assays in identifying xenoestrogens.

While it is still unresolved which, if any, PCB congeners are directly estrogenic without transformation (Soto *et al.*, 1995; Nesaretnam *et al.*, 1996; Fielden *et al.*, 1997; Andersson *et al.*, 1999; Kramer and Giesy, 1999; Vakharia and Gierthy, 1999, 2000), hydroxy-substituted PCBs have been linked to estrogenicity (Korach *et al.*, 1988; McKinney and Waller, 1994; Gierthy *et al.*, 1997; Schultz *et al.*, 1998; Kramer and Giesy, 1999) and antiestrogenicity (Kramer *et al.*, 1997). This is based in large part to the fact that hydroxylated derivatives of PCBs are structurally similar to the polarized phenolic A-ring of 17β-estradiol, a structural alert for xenoestrogens (Shi *et al.*, 2002).

Previous work in this laboratory found nonhydroxylated PCBs were nonestrogenic, and it was hypothesized that this was due to the inability of the yeast to hydroxylate PCBs in the time frame of the assay (Schultz *et al.*, 1998). However, recent studies by Beresford *et al.* (2000) and Schultz *et al.* (2000) stimulated a rethinking of the standard procedure of the yeast assay, specifically, with regards to the use of plastic 96-well flat-bottom plates when evaluating highly hydrophobic nonpolar toxicants due to potential nonreversible binding of these compounds to the plastics. The aims of this study were to evaluate the effect of using plastic plates on the binding of biphenyls, develop an alternative protocol for the yeast estrogen assay using glass vials, and demonstrate that yeast can metabolize chlorinated biphenyls into hydroxylated derivatives. This modified yeast estrogen assay was used to measure the estrogen receptor-binding activity for individual PCB congeners and Aroclor mixtures. Gas chromatographic/mass spectrometric analysis of derivatized products was used to identify hydroxylated metabolites in the yeast extracts.

METHODS

Binding of 17β-estradiol and 4-chlorobiophenyl in plastic and glass. ^{14}C-C$_4$-labeled-17β-estradiol (specific activity 52 mCi/mmol, NEN, Dupont, DE) and ^{14}C-UL-4-chlorobiphenyl (specific activity 7.2 mCi/mmol, Sigma Chemical, Co., St. Louis, MO) were used to determine the portion of each compound that bound to glass vials or plastic microtiter plates. Radioactivity was measured in 10-ml Beckman Ready-Safe scintillation fluid (Beckman Instruments, Inc., Fullerton, CA). ^{14}C-labeled compounds (10 μl) were added in triplicate to microtiter wells or glass vials with glass syringes, dried, and 200 μl of yeast assay medium was added (standard operating procedure (SOP)). Alternatively, 200 μl of yeast assay medium was added to triplicate microtiter plates or glass vials followed by the addition of 2 μl ^{14}C-labeled compounds (modified operating procedure (MOP)). The plates and vials were incubated for 24 h. Total volumes in the microtiter wells or glass vials were removed and added to 10 ml scintillation fluid. The wells and glass vials were rinsed twice with 100 μl dH$_2$O, and the rinse fluid was added to the scintillation fluid. A liquid scintillation counter (Beckman Model LS3801) was used to determine radioactivity in all samples. The H# method was used for automatic quench compensation and dpm conversion from cpm was based on quench curves using ^{14}C standards.

Estrogenic analysis. The Glaxo Welcome-derived *Saccharomyces cerevisiae* strain is a molecular toxicology assay, which measures β-galactosidase activity as a surrogate for estrogen receptor binding. With this assay, chemicals that induce estrogen receptor-mediated transcriptional activity can be identified (Routledge and Sumpter, 1996). This assay, referred to as the yeast estrogen assay (YES assay), has a 0.08 nM detection limit, 10,000-fold responsiveness, short duration, and low cost. The strain constitutively expresses the gene for the human estrogen receptor integrated into the yeast genome. On a separate expression plasmid, the estrogen-responsive sequences control the expression of the *lac*-Z reporter gene. Natural or xenoestrogens interact with the bound receptor on the hybrid promoter to activate transcription of the genes resulting in the production of β-galactosidase. The β-galactosidase activity is measured colorimetrically using the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). In the medium, β-galactosidase transforms the initially yellow chromogen into a red product that is measured by absorbance at 540 nm.

Biphenyl, 4-chlorobiphenyl (4-CB), 2,5-chlorobiphenyl (2,5-CB), 2,4,6-chlorobiphenyl (2,4,6-CB), 4-chloro-4′-biphenylol (4-CB-4′-OH), and selected polychlorinated biphenyl mixtures (i.e., Aroclors) were tested in a modified yeast estrogen assay. Into 4-ml sterile glass vials, 200 μl assay medium containing yeast and 0.1 mg/ml CPRG (Routledge and Sumpter, 1996) was added. Twelve 1:2 serial dilutions of PCB and 17β-estradiol were made in ethanol in sterile glass tubes using sterile glass pipettes. Ethanol volumes were reduced from 5% (10 μl/200 μl) to 1% (2 μl/200 μl) to reduce ethanol toxicity to the yeast. Two microliters of each dilution were transferred using a glass syringe to the vials containing medium, yeast, and CPRG. Negative controls consisted of sterile HPLC-grade water serially diluted in ethanol. The glass vials were incubated at 30°C for 3 days. On the third day, the contents of the vials were transferred to microtiter plates for reading the absorbance (OD_{540}, OD_{620}). The microtiter plates were incubated for 4 more days, and absorbance measurements were taken daily. Serial dilutions of 17β-estradiol and test compounds were tested in triplicate, and serial dilutions of negative controls were tested in duplicate. All compounds were tested on two separate days.

The EC50 (the concentration eliciting an activity equal to 50% of the positive control 17β-estradiol) was determined for each compound and each time point by probit analysis of Statistical Analysis System (SAS) software (SAS Institute, 1989). The dependent variable was the absorbency normalized as a percentage of control. The independent variable was the toxicant concentration in g/liter or mol/liter. The EC50 values are reported in molar units or ppm.

Extraction of hydroxylated PCBs from S. cerevisiae cultures. *S. cerevisiae* cultures (15–25 ml) were incubated in assay medium containing CPRG and 5–10 ppm of individual PCB congeners or 50 ppm for Aroclor 1221 for 2 to 5 days until the medium was visibly red ($OD_{540} > 0.6$). The cultures were acidified to pH 2.0 with concentrated H_2SO_4. Aliquots (15–20 ml) of cultures were extracted with an equal volume of pesticide grade ethyl ether (Fisher Scientific International, Pittsburgh, PA) by horizontal shaking for 1 h. Ether extracts (10 ml) were evaporated under a gentle stream of nitrogen. The dried extract was resuspended with 450 μl of dimethylformamide (Pierce, Rockford, IL), followed by addition of 50 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce) to derivatize the estrogen compounds. The derivatization mixture was allowed to stabilize overnight at 25°C before being analyzed by GC/MS. Derivatized samples were analyzed on an HP 6890 Series gas chromatograph with a DB-5MS capillary column (J&W Scientific; 30 m $\times$ 0.25 mm i.d.; 0.25-μm film thickness) using an HP 5973 mass selective detector. Helium was the carrier gas; a flow rate of 1 ml/min was used. A splitless injection method with a 260°C injector port was used. The time/temperature program started at 240°C for 2 min and then increased at 10°/min to 300°C, where it was held for 10 min. Trimethylsylil-derivatized samples were identified in selective ion monitoring (SIM) mode by retention time and primary molecular weight ions compared to known standards. The primary ions selected for quantification were for the individual hydroxylated congeners were 242, 276, 310, and 342 *m/z* for 4-biphenylol, 4-CB-4′-OH, 2,5-chloro-4′-biphenylol

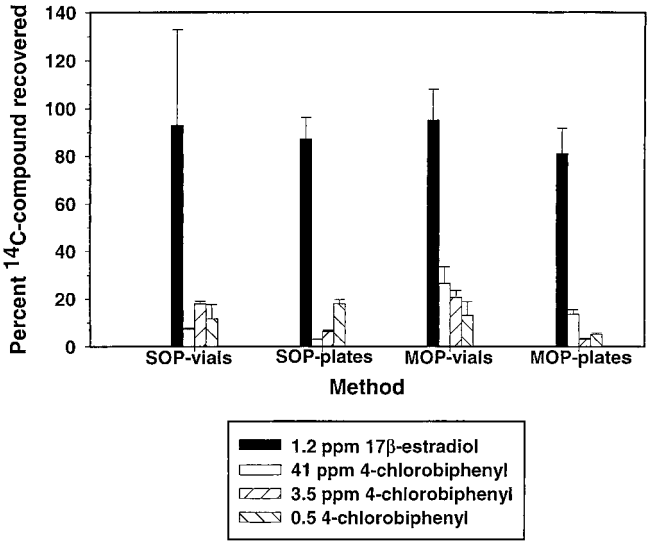

FIG. 1. Percentage recoveries of ^{14}C-4-chlorobiphenyl from treatments in glass vials and plastic microtiter plates using SOP and MOP.

(2,5-CB-4′-OH), and 2,4,6-chloro-4′-biphenylol (2,4,6-CB-4′-OH), respectively. Standard curves ranging from 0.5 to 5 ppm were used for calculation of concentrations. The extraction efficiency of yeast cultures incubated with 250 ppb of 4-CB-4′-OH was 96 ± 5%.

RESULTS

Binding of 4-chlorobiphenyl and 17β-estradiol to plastic microtiter plates and glass vials. The testing of Aroclor mixtures and individual PCB congeners in the YES assay using plastic microtiter plates usually resulted in no color development, although occasional color responses were detected for 4-CB. ^{14}C-labeled compounds were used to determine whether the lack of activity was due to a lack of bioavailability of PCBs to the yeast due to the binding of the chlorinated biphenyls to the plastic. When 1.2 ppm ^{14}C-17β-estradiol was added to plastic microtiter plates or vials using either the SOP or the MOP, equivalent percentages of ^{14}C-17β-estradiol (approximately 89%) were recovered from the medium after 24 h (Fig. 1). However, the percentage of ^{14}C-4-CB recovered from glass vials or plates after 24 h was considerably less (3 to 26%), with the highest recoveries in vials using the MOP (Fig. 1). In addition, there is no clear relationship between the concentration of the 4-CB and percentage recovery from the plastic and glass in all treatments (Fig. 1). These results indicated that a modified assay using glass vials and compounds added to the yeast assay medium would approximately double the availability of PCBs to the yeast.

Measurement of estrogenic activity of Aroclor mixtures in the modified YES assay. When the YES assay was performed in glass vials using the modified YES assay, estrogenic responses to 4-CB occurred (Fig. 2). Individual chlorinated biphenyls showed similar estrogenic activity to 4-CB (Table 1) and were 25- to 650-fold less estrogenic than the corresponding hydroxylated metabolites. The compound 4-CB-4′-OH was tested in both the standard and modified YES protocols, as a control to determine whether data collected for the hydroxylated chlorinated biphenyls in plastic microtiter plates were comparable to data collected in glass vials. Although color development was about 1 day faster in the glass vials, the relative estrogenic activities were approximately the same (9.5×10^{-4} for glass vials versus 1.2×10^{-3} for plastic plates). Therefore, data were normalized to 17β-estradiol to provide relative estrogenic values (Table 1).

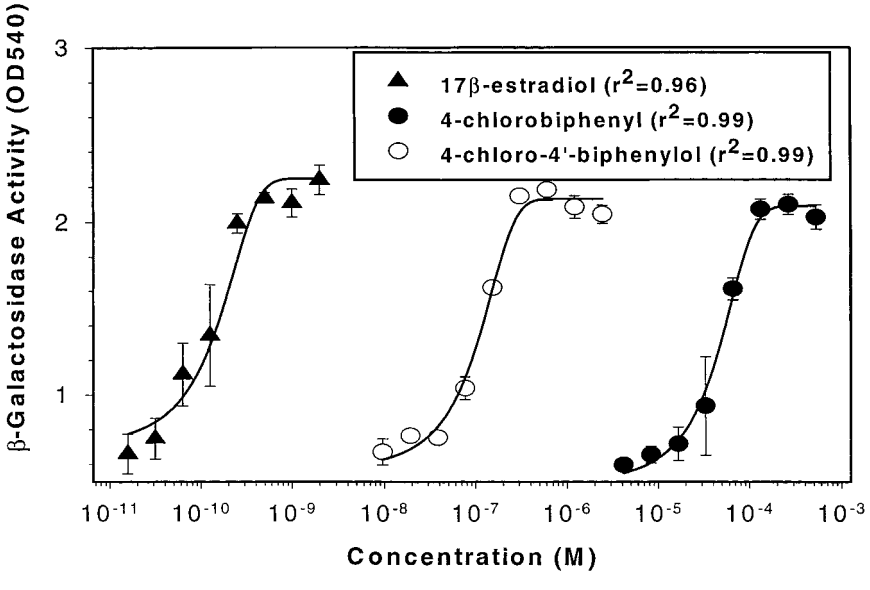

FIG. 2. Estrogen receptor-binding activity of 4-chlorobiphenyl and 4-chloro-4′-biphenylol after 4 days of incubation in the YES assay.

TABLE 1
Relative Estrogenic Activities (EC50s) for Biphenyl, Chlorinated Biphenyls, and Aroclor Mixtures in the YES Assay

Compound	Estrogenic activity EC50 (ppm)	Relative estrogenic activity[a]	Relative estrogenic activity of corresponding hydroxylated metabolite[a]
Biphenyl	19	1.2×10^{-6}	3.4×10^{-5}
4-Chlorobiphenyl	4.5	5.2×10^{-6}	6.5×10^{-4}
2,5-Chlorobiphenyl	21	2.0×10^{-6}	1.3×10^{-3}
2,4,6-Chlorobiphenyl	0.8	9.3×10^{-5}	3.0×10^{-2}
Aroclor 1221	2.9	8.2×10^{-6}	ND[b]
Aroclor 1242	0.65	5.2×10^{-5}	ND
Aroclor 1248	2.3	1.6×10^{-5}	ND
Aroclor 1254	NE[c]	NE	ND

[a] Relative estrogenic activity = EC50 (17-β-estradiol)/EC50 (test compound).
[b] ND, not determined.
[c] NE, no measurable estrogenic activity.

Full agonist activity was observed for 4-CB and 4-CB-4′-OH, while each of the Aroclor mixtures showed only partial agonist activity (Fig. 3). The relative estrogenic activity measurements for four Aroclor mixtures ranged from EC50s of 5.2×10^{-5} for Aroclor 1242 to not detectable for Aroclor 1254 (Table 1).

Detection of hydroxylated PCB metabolites in S. cerevisiae extracts. *S. cerevisiae* cell cultures (15–25 ml) were incubated with biphenyl, chlorobiphenyls, and Aroclor 1221 (Fig. 4, Table 2). In all cultures, red color resulting from β-galactosidase activity, indicating activation of the estrogen receptor, was detected. After ether extraction and derivatization, ppb concentrations of hydroxylated metabolites were detected in the cultures incubated with PCBs but not with 17β-estradiol (Fig. 5, Table 2). The concentrations of 2,5-CB-4′-OH and 2,4,6 CB-4′-OH detected in the *S. cerevisiae* extracts were higher than the EC50 necessary to produce an estrogenic response (Table 2). The concentration of 4-CB-4′-OH was slightly lower than the reported EC50 value but was still high enough to produce an estrogenic response. The concentration of 4-biphenylol extracted was approximately 200-fold less than that necessary to produce an estrogenic response. This result may be due to the formation of another metabolite, such as 4,4′-biphenylol, which has a lower EC50 than 4-biphenylol, metabolism of 4-biphenylol by *S. cerevisiae*, or inefficient extraction or derivatization of the metabolite. For analysis of metabolites from Aroclor 1221, only three potential metabolites were quantified due to the lack of available standards (Aroclor 1221 does not contain 2,4,6-CB) (Table 2). Both 4-CB-4′-OH and 2,5-CB-4′-OH were present in sufficient concentrations to elicit estrogenic responses. GC/MS analysis also suggested that other unidentified metabolites could be present.

DISCUSSION

In previous studies, the lack of estrogenicity of PCB congeners in the YES assay was attributed to the inability of *S. cerevisiae* to hydroxylate PCBs (Coldham *et al.*, 1997; Schultz *et al.*, 1998). Modifications of the YES assay, including the use of glass vials and pipettes and the addition of medium before the test compound, resulted in estrogen receptor-binding activity for biphenyl, three individual PCB congeners, and three Aroclor mixtures. The ^{14}C-binding experiments suggested that the lack of estrogenic activity previously reported was due to irreversible binding of the PCBs to the plastic microtiter plates.

Estrogenicity of PCBs is attributed to the formation of hydroxylated metabolites (Korach *et al.*, 1988). Hydroxylated PCBs that interact directly with the receptor produce estrogenic effects at very low concentrations in rainbow trout hepatocytes (Andersson *et al.*, 1996; Blom *et al.*, 1998). Hydroxylated PCBs have been found in the plasma of humans (Bergman *et al.*, 1994) and polar bears (Sandau and Norstrom, 1996). Although many hydroxylated PCBs are readily excreted (Bergman *et al.*, 1994), hydroxylated congeners with the hydroxyl moiety in the *para* position and chlorine atoms in the adjacent positions such as 3,5-chloro and 4-hydroxyl substituted are retained due to plasma binding (Bergman *et al.*, 1994; Conner *et al.*, 1997). This pattern resembles thyroxine, a natural hormone and ligand to the thyroxine-transporting protein transthyretin (Letcher *et al.*, 2000).

Metabolic transformation of PCBs is by formation of arene oxide intermediates catalyzed by cytochrome P450 isoenzymes, e.g., CYP1A, CYP2B, and possibly CYP2C and CYP3A (Sundstrom *et al.*, 1976; Bergman *et al.*, 1994; Letcher *et al.*, 2000) or by direct insertion of a hydroxy group (Koga *et al.*, 1992). In the case of arene oxide formation, the intermediate is subsequently rearranged to a hydroxylated PCB or further metabolized to a diol via secondary hydroxylation (Ariyoshi *et al.*, 1997). The arene oxides are commonly formed

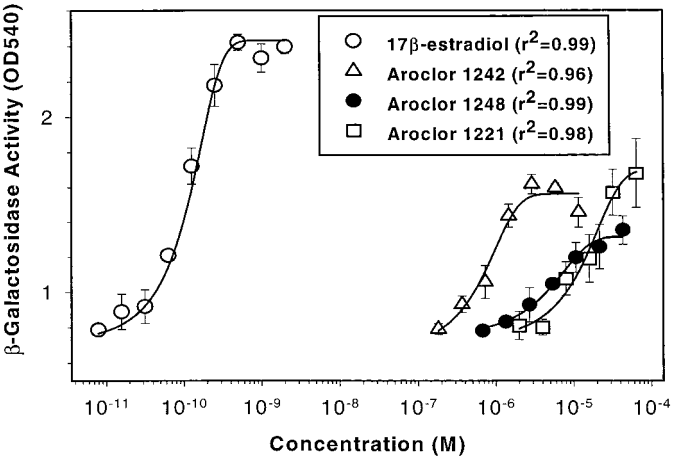

FIG. 3. Estrogen receptor-binding activity of Aroclor mixtures after 5 days of incubation in the YES assay.

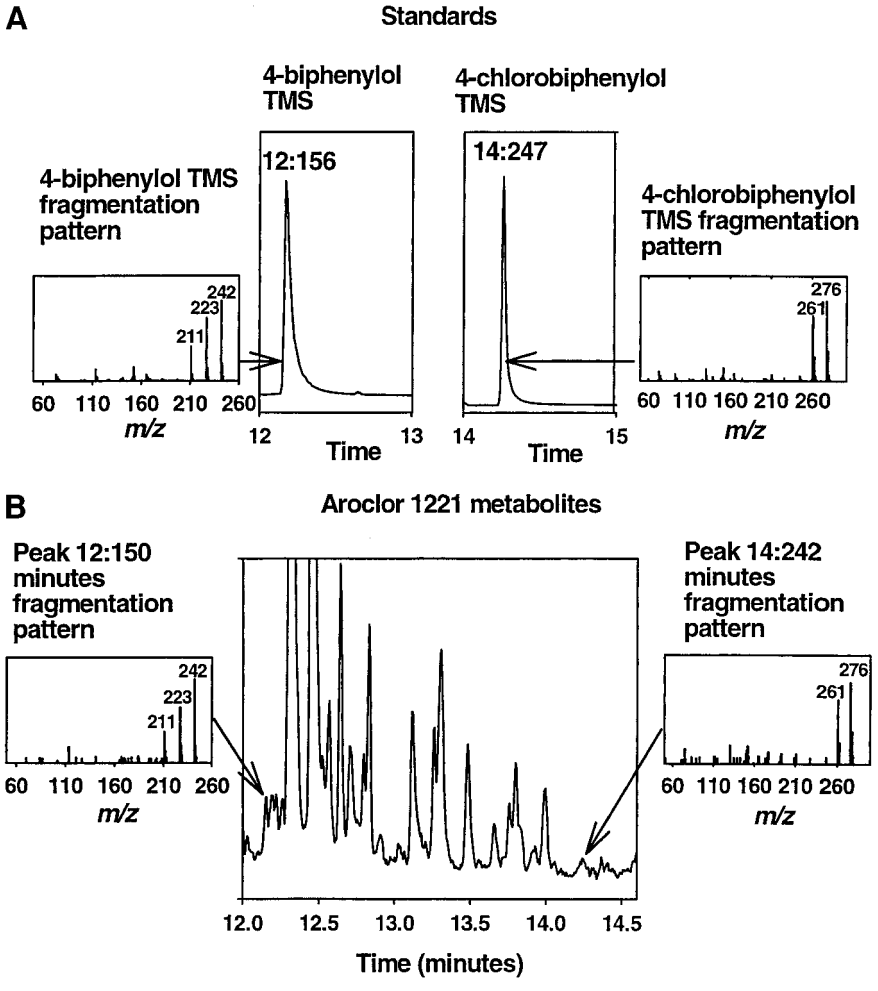

FIG. 4. Identification of the hydroxylated metabolites 4-biphenylol and 4-chloro-4′-biphneylol using GC/MS scan mode and associated fragmentation patterns. The percent match of 4-biphenylol fragmentation pattern with the standard was 81% and the percent match of the 4-chloro-4′-biphenylol fragmentation pattern with the standard was 93%.

in the *meta* or *para* positions (Letcher *et al.*, 2000). Because *S. cerevisiae* contain NADPH-cytochrome P450 reductase (Murakami *et al.*, 1990), it is likely that hydroxylation of the PCB congeners in this study was via a CYP.

Gas chromatographic/mass spectrometric analysis indicated that *S. cerevisiae* were able to hydroxylate chlorinated congeners. The metabolites obtained in the yeast assay were similar to the results obtained using a combined assay consisting of PCB metabolites generated with human liver microsomes followed by an ER-binding assay (Vakharia and Gierthy, 1999, 2000). In addition, the concentrations of PCB metabolites in the yeast extracts were similar to previously published concentrations, which elicited estrogenic responses. The amount of 4-biphenylol obtained from incubation of *S. cerevisiae* with biphenyl was considerably less than that expected to produce an estrogenic response. This may be due to formation of a metabolite not investigated, such as 4,4′ biphenylol, or the metabolism of 4-biphenylol by *S. cerevisiae*. The measured estrogen receptor activity of the Aroclor mixtures is also likely

TABLE 2
Conversion of Biphenyl, Chlorobiphenyls, and Aroclor 1221 to Hydroxylated Metabolites by Yeast

Compound (starting concentration, ppm)	Metabolite detected (measured concentration, ppb)	EC50 of compound[a] (ppb)
Biphenyl (10)	4-Biphenylol (1.7 ± 0.4)	205
4-Chlorobiphenyl (10)	4-Chloro-4′-biphenylol (3.1 ± 1.0)	12.4
2,5-Chlorobiphenyl (10)	2,5-Chloro-4′-biphenylol (15.5 ± 7.7)	7.3
2,4,6-Chlorobiphenyl (5)	2,4,6-Chloro-4′-biphenylol (1.2 ± 0.6)	0.36
Aroclor 1221 (50)	4-Biphenylol (61 ± 14)	205
	4-Chloro-4′-biphenylol (9.0 ± 1.0)	12.4
	2,5-Chloro-4′-biphenylol (13 ± 4.0)	7.3

[a] EC50 ppb values were calculated from previously published EC50 values in the YES assay (Schultz *et al.*, 1998).

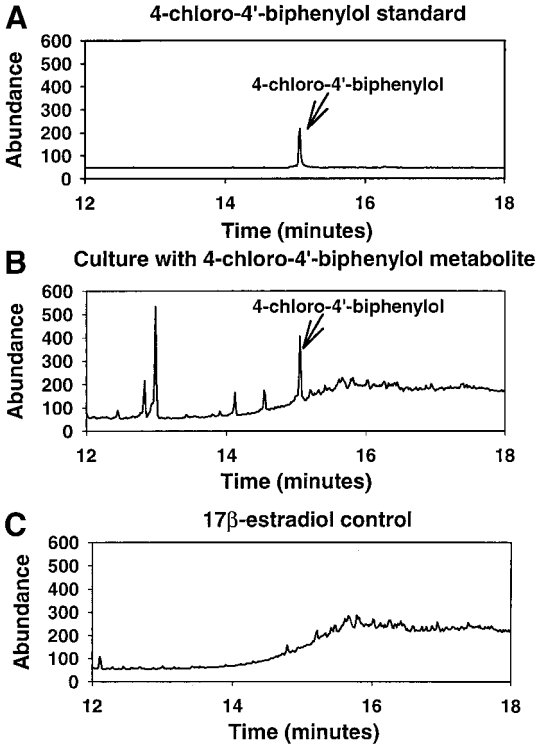

FIG. 5. Identification of the hydroxylated metabolite 4-chloro-4′-biphenylol using GC/MS analysis in yeast cell cultures incubated with 4-chlorobiphenyl using SIM mode. The retention time of 4-chloro-4′-biphenylol retention time is 15.06 min.

to be dependent on hydroxylation of the parent compound by *S. cerevisiae* because hydroxylated metabolites were detected in yeast extracts after incubation with Aroclor 1221.

Modification of the YES assay allowed measurement and confirmation of the estrogenic activity of Aroclor mixtures. However, the estrogenic binding of the individual PCB congeners ranged from 25- to 650-fold less than the corresponding hydroxylated PCB congeners. These differences may result from the combination of several congener-specific factors. First, the hydrophobic PCB compounds may not be completely bioavailable to the yeast. Even in the modified assay, approximately 75% of the ^{14}C-4-CB was not recovered compared to almost 90% recovery of ^{14}C-17β-estradiol. Another recent study indicates that high amounts of PCB bind to glass, and the amount of PCBs binding to glass is dependent on the degree of chlorination (Lung *et al.*, 2000b). The partitioning effect of PCBs to glass and yeast is difficult to extrapolate over the 5-day assay because yeast also serves as a potential sink for the hydrophobic PCBs. PCBs may desorb from the glass over time and become bioavailable to the yeast. Second, in *S. cerevisiae*, only a fraction of the PCBs are transformed to the hydroxylated compounds and the amount of transformation may be congener specific. Additional research may be needed to determine whether the rates of hydroxylation in *S. cerevisiae* are similar to vertebrates. Last, the general toxicity of the PCB congeners may have an impact on basic metabolic processes in *S. cerevisiae*. Because transcription and translation must be completed before β-galactosidase activity can be detected, any toxic effects impairing these processes will hinder β-galactosidase activity.

In conclusion, these results suggest that Aroclor mixtures are potentially estrogenic at environmentally relevant concentrations in the low parts per million range. In addition, PCBs may still not be completely bioavailable in these assays, therefore, estrogen receptor binding may be underestimated. Treatments of the glass vials with recently reported coatings (Lung *et al.*, 2000a) may enhance bioavailability and provide more accurate estrogen-binding activity responses. The potential for binding of other hydrophobic compounds, such as high-molecular-weight PAHs, to plastic microtiter plates may also need to be considered when performing toxicity testing in plastic microtiter plates.

ACKNOWLEDGMENTS

This study was supported in part by the United States Department of Energy Grant DE-FG02-97ER62350 and in part by the Waste Management Research and Education Institute at The University of Tennessee.

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