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Halogenated Aromatic Hydrocarbon-Mediated Porphyrin Accumulation and Induction of Cytochrome P4501A in Chicken Embryo Hepatocytes

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ABSTRACT. Concentration-dependent induction of cytochrome P4501A (CYP1A) and intracellular porphyrin accumulation were observed following treatment of chicken embryo hepatocyte (CEH) cultures with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77, IUPAC nomenclature), 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 169), and a commercial mixture of PCBs (Aroclor 1254). For these halogenated aromatic hydrocarbons (HAHs), or mixture, maximal CYP1A activity [measured as ethoxyresorufin-O-deethylase (EROD) activity] and immunodetectable protein were observed at concentrations just prior to, or coincident with, the concentrations at which porphyrin accumulation became evident. Both immunodetectable CYP1A protein and catalytic activity decreased at high concentrations of these compounds, but the rate and extent of decrease of immunodetectable CYP1A protein varied. Time–course studies with PCB 77 indicated a decrease in potency and an increase in maximal CYP1A induction between 24 and 48 hr of exposure which may indicate *in vitro* metabolism of this HAH. Intracellular accumulation of total porphyrins without CYP1A induction, was observed for 2,2′,5,5′-tetrachlorobiphenyl (PCB 52), 2,2′,6,6′-tetrachlorobiphenyl (PCB 54), 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95), 2,2′,4,5,5′-pentachlorobiphenyl (PCB 101), 2,2′,3,3′,6,6′-hexachlorobiphenyl (PCB 136), and 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153). Overall, these results are consistent with a role for CYP1A induction and/or Ah receptor activation in porphyrin accumulation mediated by HAHs with a planar configuration, whereas those that are not planar may mediate porphyrin accumulation by a mechanism not involving induction of CYP1A. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:373–384, 1997.

KEY WORDS. P4501A; CYP1A; porphyrin; halogenated aromatic hydrocarbon; chicken embryo hepatocytes; avian

HAHs§ are widespread environmental contaminants that have been shown to elicit a wide range of toxic and biochemical effects in both laboratory animals and wildlife [1–6]. To assess indirectly the toxicological potency of these compounds and their mixtures, the biochemical endpoint that has received the most attention is measurement of CYP1A catalytic activity. Although CYP1A induction is not a toxic endpoint *per se* [3, 7, 8], there have been many studies that have shown a strong correlation between CYP1A induction and the appearance of HAH-mediated toxic effects (reviewed in Ref. 9).

Although CYP1A induction is the biochemical endpoint most commonly measured to assess the toxic potency of HAHs, deregulation of the heme biosynthetic pathway has also been associated with HAH exposure [10]. Recent studies with rodents have suggested the involvement of CYP1A2 in HAH-mediated porphyrin accumulation [11–14]. Multiple CYP1A forms have also been identified in chickens [15, 16], and evidence suggests that, similar to rodents, one or more of these forms plays a role in HAH-mediated porphyrin accumulation [17]. Studies to date in our laboratory (unpublished results) indicate that methoxyresorufin-O-deethylase activity, which is associated with CYP1A2 induction in rodents, was not elevated in CEH cultures treated with porphyrinogenic concentrations of TCDD. Despite this inability to specifically detect CYP1A2 catalytic activity, CEH cultures are sensitive to disruption of the heme biosynthetic pathway, resulting in

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§ *Abbreviations:* HAHs, halogenated aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; PCB, polychlorinated biphenyl; AHH, aryl hydrocarbon hydroxylase; CEH, chicken embryo hepatocyte; EROD, ethoxyresorufin-O-deethylase; MAb, monoclonal antibody; ALA, δ-aminolevulinic acid; ALAS, δ-aminolevulinic acid synthetase; and PB, phenobarbital.

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measurable porphyrin accumulation. In contrast, rat and human hepatocytes and established mammalian liver cell lines appear to be refractory to porphyrin accumulation after HAH exposure *in vitro* [12, 18]. Thus, with the CEH culture system, it is possible to investigate the interrelationships between HAH-mediated CYP1A induction and porphyrin accumulation.

Studies by Kawanishi *et al.* [19] and Sassa *et al.* [20] suggested that coplanarity, in conjunction with *para* and *meta* chlorine substitution, were necessary for PCB-mediated porphyrinogenic activity in cultured CEHs. The coplanar PCBs, 77 and 169, were found to be the most active, while non-coplanar PCBs, such as 52, 54, 136, and 153, which have 2,2′-*ortho* chlorine substituents were not effective in stimulating porphyrin accumulation. Although structural properties were correlated with the abilities of the various PCBs tested to mediate porphyrin accumulation, these results were based on single PCB concentrations of either 2 μg/mL medium [19] or 0.5 μg/mL medium [20].

Although both EROD and porphyrin concentration–response data were obtained for ten tetra- to hexachlorinated PCB congeners, including PCBs 52, 77, 101, 126, 153, and 169, in a study by Rodman *et al.* [21], a limited number of concentrations were used, and in many cases full concentration–response data were probably not obtained. In addition, they found that maximal EROD and porphyrin responses occurred at similar concentrations for PCBs 77, 126, and 169. This differs from earlier studies conducted in our laboratory where maximal EROD activity generally coincided with the *onset* of porphyrin accumulation [22]. Furthermore, the CEHs used in the study by Rodman and co-workers were cultured under different conditions, and exogenous ALA (heme precursor) was added to the medium.

The present study was undertaken to thoroughly compare the CYP1A induction and porphyrinogenic properties of structurally different HAHs, and to relate these data to proposed mechanisms of action. Complete concentration–response data were obtained for EROD activity, immunodetectable CYP1A protein, and intracellular porphyrin accumulation. Previous studies in avian hepatocyte cultures have not characterized HAH-mediated CYP1A induction in terms of immunodetectable CYP1A protein, and, in most cases, exogenous ALA was added to the cell culture medium. In the present study, ALA was not added to the culture medium and the accumulated porphyrins were further characterized in terms of their specific types and ratios after 24- and 48-hr exposure periods. Finally, because some HAHs, such as PCB 77, are readily metabolized *in vivo*, studies were conducted to evaluate the time–course of both CYP1A induction and porphyrin accumulation for PCB 77 in CEH cultures.

MATERIALS AND METHODS

Materials

PCBs and TCDF were from Ultra Scientific (Kingstown, RI, U.S.A) and were stated to be at least 99% pure by the supplier. TCDD was provided by Dr. J. J. Ryan (Health Canada, Ottawa, Ontario, Canada). Postmitochondrial supernatants from livers of rats treated with Aroclor 1254 were obtained from Molecular Toxicology (Annapolis, MD, U.S.A.) and were used to prepare microsome standards for western blot experiments. Mouse MAb 1-12-3 prepared against scup (*Stenotomus chrysops*) cytochrome CYP1A1 was a gift from Dr. J. J. Stegeman (Woods Hole Oceanographic Institution). All other reagents were from sources described previously [23].

Cell Culture, EROD, Porphyrin and Protein Assays

Preparation, culture, HAH treatment, EROD, and total intracellular porphyrin assays of CEHs were carried out as described previously [23]. Briefly, after 19 days of egg incubation, CEH cultures were prepared in 48-well plates. The following day, the culture medium was replaced and the CEH cultures were treated with serial dilutions of each HAH or mixture for 24 or 48 hr (as indicated) without further medium replacement or HAH treatment. After the exposure period, the medium was removed, and the plates were rinsed and frozen at −80°. After thawing, EROD assays were carried out in the cell culture plates, and the reaction product (resorufin) was measured with a fluorescence plate reader (Cytofluor 2300, Millipore Ltd., Bedford, MA, U.S.A.). Following the EROD assay, porphyrins were extracted from the CEH cultures with 3 N HCl, and total porphyrins were measured with a fluorescence plate reader. Mean total cellular protein per well was determined on separate untreated plates using the fluorescamine protein assay modified for fluorescence plate readers [24]. For time-course experiments, cells were exposed to PCB 77 for various periods of time ranging from 2 to 48 hr prior to medium removal and freezing of the plates. Separate plates were prepared for HPLC analysis of porphyrin patterns as described previously [25]. Briefly, CEH cultures were treated with porphyrinogenic concentrations of each HAH or mixture (6 wells/concentration) for 24 or 48 hr (as indicated). The culture medium was discarded, and the plates were rinsed and frozen at −80°. After thawing, each well received 0.5 mL of 1:1 acetonitrile:HCl, and the cell suspension was sonicated for 2–3 sec/well. The solubilized cells for each treatment were pooled, brought to 25 mL with distilled water, and mixed. The samples were then loaded onto tC_{18} Sep-Paks (Millipore, Ltd.), eluted with 2.5 mL acetonitrile, and brought to dryness under nitrogen. The dried samples were resuspended in 100 μL of concentrated HCl, mixed, and allowed to sit for 10 min. Then distilled water (900 μL) was added to each sample, followed by mixing and filtering through 0.45 μm filters. The samples were analyzed by HPLC with fluorescence detection using chromatographic conditions similar to those described previously [26].

Immunoblotting

Previously treated and frozen CEH cultures (6 wells/treatment) were solubilized and pooled on ice in sample treat-

ment buffer [0.25 M Tris–HCl, pH 6.8, 40% (v/v) glycerol, 4% sodium dodecyl sulfate, 0.008% bromophenol blue, and 5% (v/v) β-mercaptoethanol] to a final concentration of 1.5 μg total protein/μL based on the mean total protein concentration determined for plates of untreated cells. Then the samples were boiled for 5 min to ensure complete solubilization and to inactivate proteases. Based on the original western blot method developed by Towbin *et al.* [27], 10 μL of the solubilized cells was separated on sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE, 9% acrylamide) and electrophoretically transferred to Rad-Free membranes (Schleicher & Schuell, Keene, NH, U.S.A.). Aliquots of the same preparation of Aroclor 1254-induced rat liver microsomes, solubilized in sample treatment buffer as per CEH samples, were used as standards for all blots. Immunodetection of CYP1A was performed using MAb 1-12-3, which recognizes CYP1A in all taxonomic groups of vertebrates examined so far [28, 29]. The secondary antibody was a goat anti-mouse IgG linked to alkaline phosphatase. Immunoreactive proteins were detected by chemiluminescence (Rad-Free, Schleicher & Schuell), and the light intensities of the immunoreactive protein bands were quantified by video imaging densitometry (UVP Gel Documentation System 7500, San Gabriel, CA, U.S.A.).

RESULTS AND DISCUSSION

In mammals and birds, previous studies suggest that regulation of CYP1A induction by HAHs is mediated by binding of these compounds to an intracellular protein, the Ah (Aromatic hydrocarbon) receptor [3, 30]. The planar HAHs, TCDD and TCDF, bind with higher affinities to Ah receptor than the non-*ortho* substituted PCBs. In mammals, the *ortho* substituted PCBs are much less, if at all, effective at receptor binding.

Based on these structural requirements, the HAHs studied can be divided into three groups, as indicated in Figs. 1, 2, and 3. The first group consists of the classical Ah receptor ligands, TCDD and TCDF (Fig. 1). These two compounds were the most potent inducers of EROD activity tested, with 70 and 34% increases in enzyme activity above basal activities observed at 0.001 nM concentrations of TCDD and TCDF, respectively (Table 1). At the same concentration, immunodetectable CYP1A protein concentrations were increased by 61 and 86% above basal levels for TCDD and TCDF, respectively. Porphyrin accumulations exceeding 50 pmol/mg protein were observed in hepatocytes dosed with 30 nM or greater TCDD and 10 nM or greater TCDF (Table 1). HPLC analysis revealed that uroporphyrin and heptacarboxylporphyrin accounted for >95% of the total porphyrins produced in hepatocytes treated with a 1000 nM concentration of these HAHs after either 24- or 48-hr exposure periods (Fig. 4, top row).

Data for the second group of HAHs—the non-*ortho* substituted PCBs, PCB 118, and Aroclor 1254—are shown in Fig. 2. PCB 118, which has an *ortho* chlorine substituent, has been included in this group since a weak affinity for the Ah receptor has been demonstrated in rat hepatic cytosol

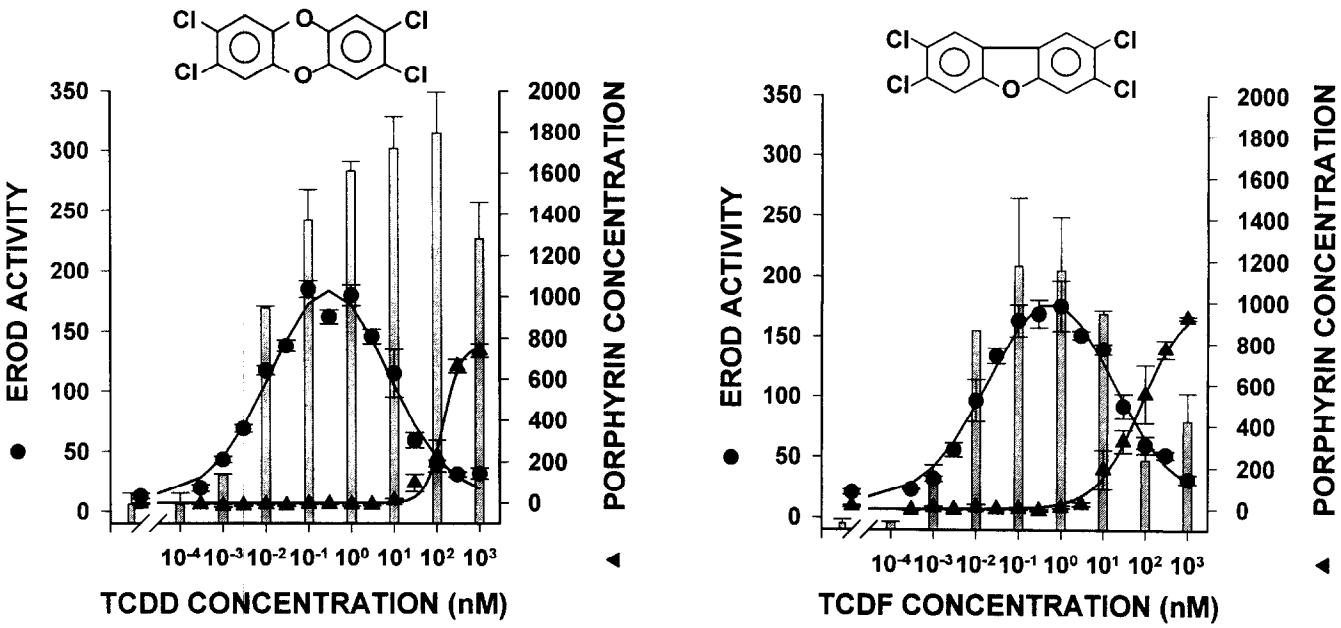
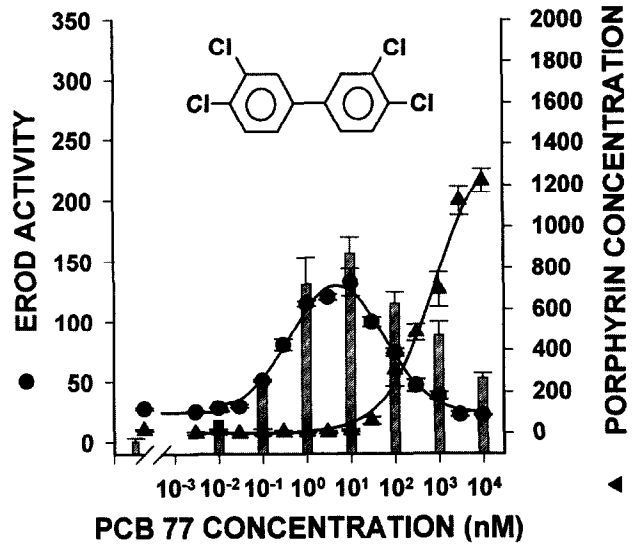
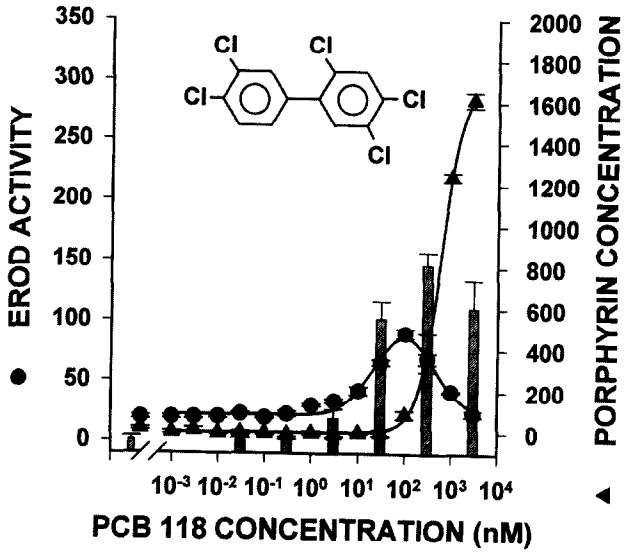
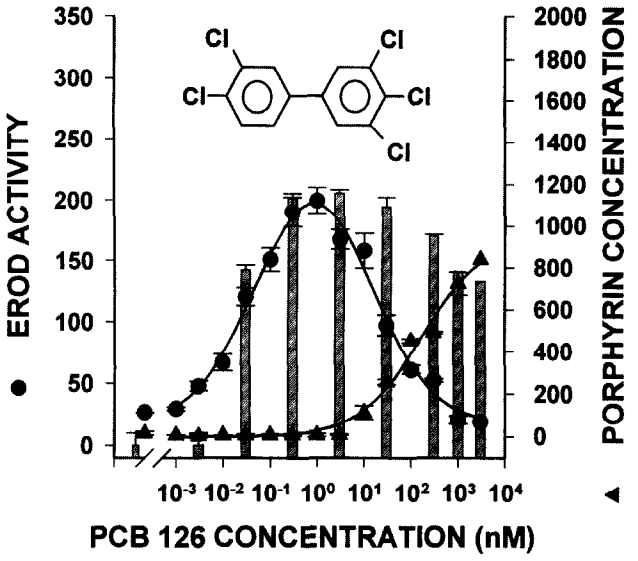
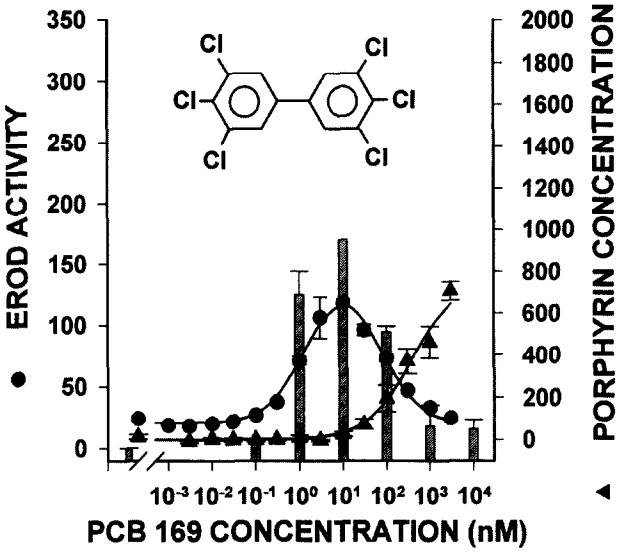


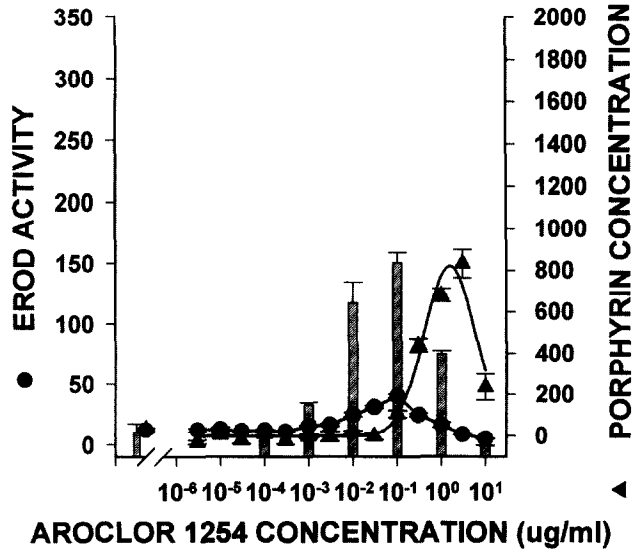
FIG. 1. Concentration–response data for intracellular EROD activity (●, pmol resorufin/min/mg protein), total porphyrin accumulation (▲, pmol/mg protein), and immunodetectable CYP1A protein (bars, sample intensity/standard intensity, same relative scale for all plots and figures—not shown) for the planar HAHs, TCDD and TCDF. CEH cultures were exposed for a period of 24 hr to either TCDD or TCDF prior to biochemical analysis. For EROD and porphyrin data, each symbol represents the mean response of 3 wells, and for CYP1A protein, each bar represents the mean concentration of at least 2 determinations of the same sample (pool of 6 wells). Data prior to axis break indicate responses observed for control (DMSO-treated) hepatocytes and bars indicate standard error.











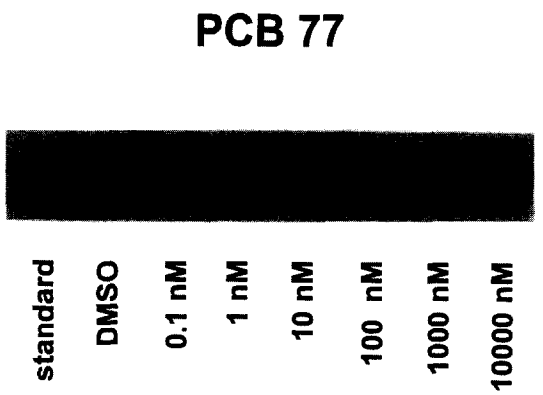
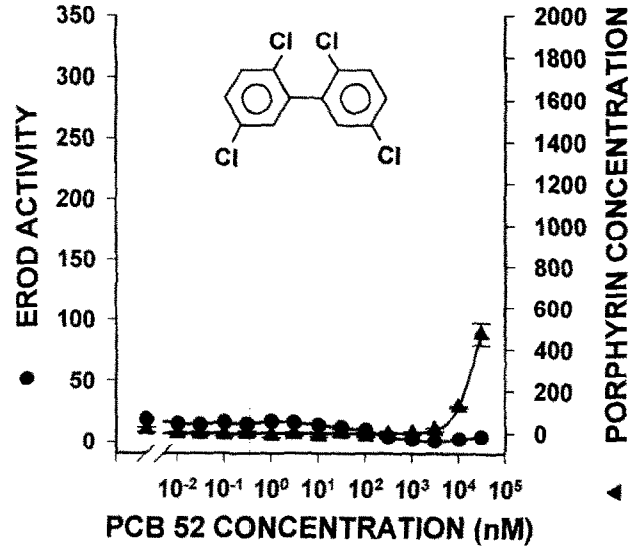
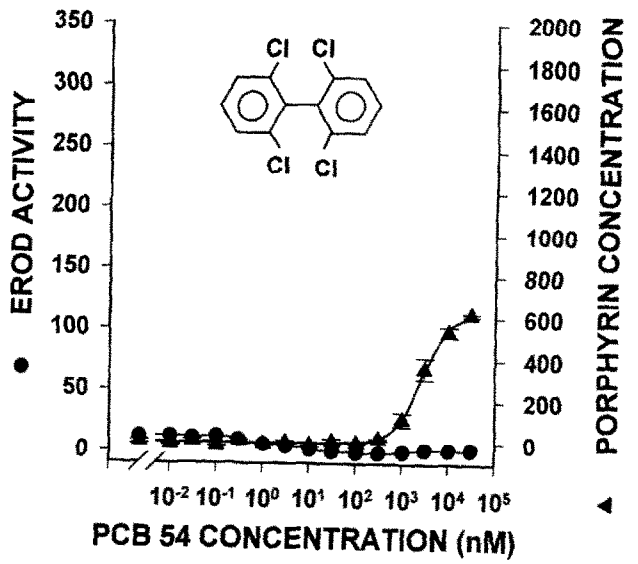
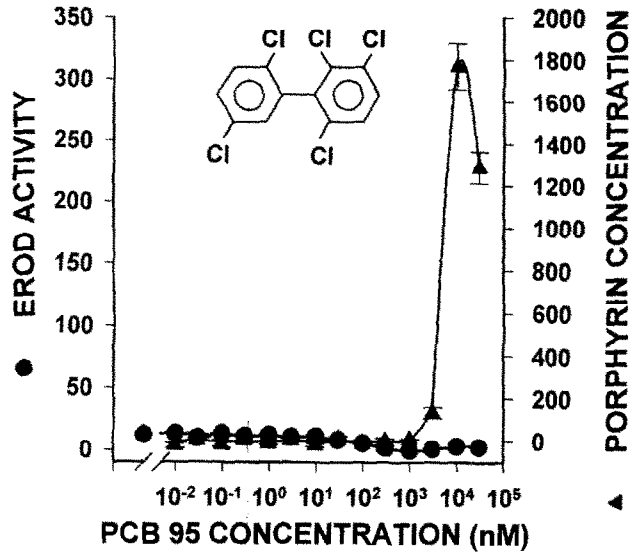
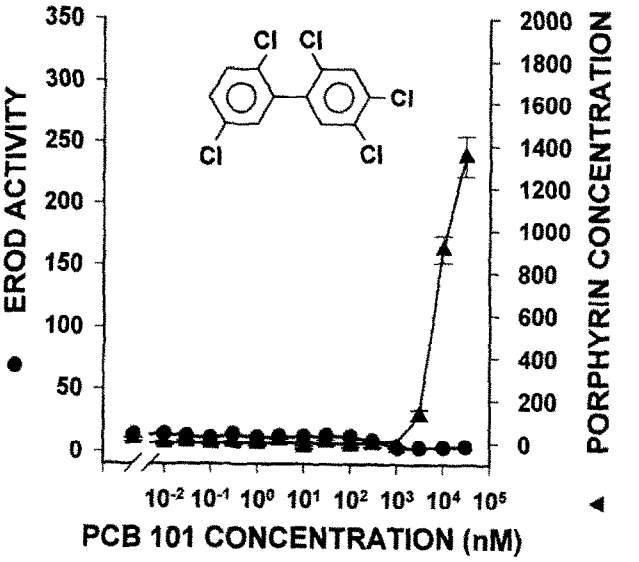


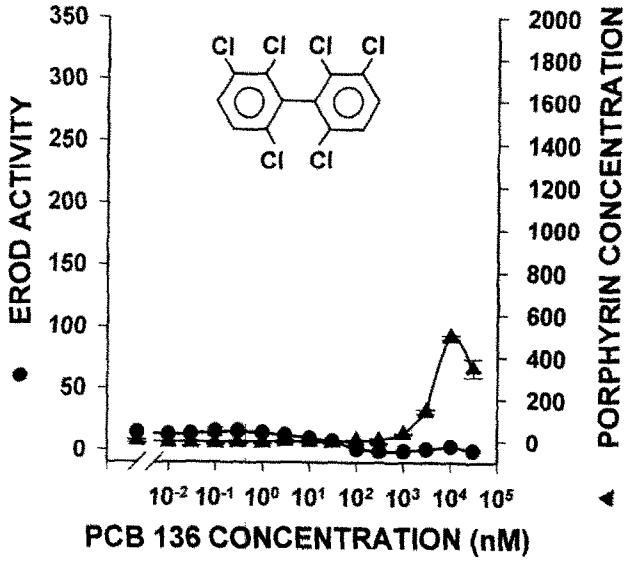
FIG. 2. **Concentration–response data for non-*ortho* substituted PCBs 77, 126 and 169, PCB 118, and Aroclor 1254.** CEH cultures were exposed for a period of 24 hr to the HAHs or mixture shown prior to biochemical analysis. A typical western blot for samples prepared from PCB 77-treated CEH cultures and probed with MAb 1-12-3 is also shown (bottom, right). Symbols, units, and scale for immunodetectable CYP1A protein are as described in the legend of Fig. 1.











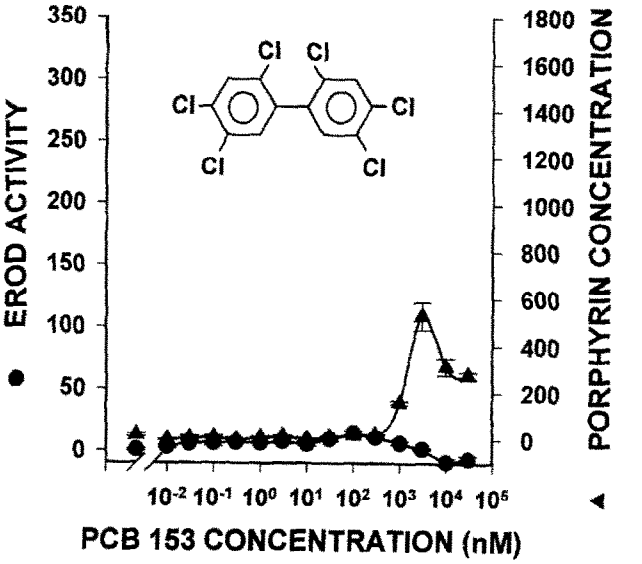


FIG. 3. Concentration–response data for *ortho* substituted PCBs. CEH cultures were exposed for a period of 48 hr to the HAHs shown prior to biochemical analysis. Symbols and units are as described in the legend of Fig. 1, except that data for immunodetectable CYP1A are not shown (no response).

TABLE 1. Summary and comparison of the CYP1A induction and porphyrinogenic potencies of the HAHs and HAH mixture

HAH	Lowest concentration resulting in EROD activity induction	Lowest concentration resulting in CYP1A protein induction	Lowest concentration resulting in porphyrin accumulation	Lowest concentration resulting in inhibition of induced or basal EROD activity
Planar HAHs: 24-hr exposure period (Fig. 1)				
TCDD	0.001 nM	0.001 nM	30 nM	3 nM
TCDF	0.001 nM	0.001 nM	10 nM	3 nM
Non-ortho substituted PCBs, PCB 118 and Aroclor 1254: 24-hr exposure period (Fig. 2)				
PCB 77	0.1 nM	0.01 nM	30 nM	30 nM
PCB 118	3 nM	3 nM	100 nM	300 nM
PCB 126	0.003 nM	0.03 nM	10 nM	3 nM
PCB 169	0.1 nM	1 nM	30 nM	30 nM
Aroclor 1254	0.01 μg/mL	0.001 μg/mL	0.1 μg/mL	0.3 μg/mL
Ortho substituted PCBs: 48-hr exposure period (Fig. 3)				
PCB 52	NR	NR	10,000 nM	100 nM
PCB 54	NR	NR	1,000 nM	3 nM
PCB 95	NR	NR	3,000 nM	300 nM
PCB 101	NR	NR	3,000 nM	300 nM
PCB 136	NR	NR	3,000 nM	30 nM
PCB 153	NR	NR	1,000 nM	3,000 nM
PCB 77: Time–Course (Fig. 5)				
PCB 77: 2 hr	NR	NR	NR	NR
PCB 77: 6 hr	0.1 nM	1 nM	NR	100 nM
PCB 77: 17 hr	0.1 nM	≤0.1 nM	30 nM	30 nM
PCB 77:24 hr	0.1 nM	≤0.1 nM	30 nM	100 nM
PCB 77: 48 hr	0.3 nM	0.1 nM	10 nM	300 nM

The lowest experimental concentrations resulting in EROD activity induction, immunodetectable CYP1A protein induction, intracellular porphyrin accumulation, and inhibition of induced or basal EROD activity are shown for each HAH or mixture tested. The HAHs are grouped according to structure and/or CEH culture exposure period, as indicated. Concentrations were estimated to the nearest data point with the following criteria: the lowest concentration resulting in EROD activity induction showed at least a 25% increase in activity above the basal activity; the lowest concentration resulting in CYP1A protein induction showed at least a 50% increase in CYP1A protein above the basal level; the lowest concentration resulting in porphyrin accumulation showed at least a 50 pmol/mg protein increase in total porphyrin concentration above basal level; and the lowest concentration resulting in inhibition of induced or basal EROD activity showed at least a 15% decrease in activity from peak or basal EROD activity.

NR = no response.

[31]. Aroclor 1254, a commercial mixture of PCBs, includes both non-*ortho* and *ortho* substituted congeners. Similar to the responses observed for TCDD and TCDF, all of the PCBs and the PCB mixture in the second group induced EROD activity and immunodetectable CYP1A protein. Typical concentration–response data for immunodetectable CYP1A in CEH cultures exposed to PCB 77 for 24 hr are shown in Fig. 2 (bottom, right). PCB 126 was the most potent inducer of EROD activity (Table 1). Porphyrin accumulation exceeding 50 pmol/mg protein was observed for HAHs in the second group at concentrations equal to or exceeding 10 nM and was maintained at the highest concentration of all PCBs, but declined at the highest concentration of Aroclor 1254. The cause of this decline is not known. Treatment of CEH cultures with PCBs 77 or 118 consistently resulted in the accumulation of the highest concentrations of total intracellular porphyrins for this group of HAHs. As with TCDD and TCDF, uroporphyrin and heptacarboxylporphyrin accounted for >95% of the total porphyrins that accumulated in CEH cultures treated with 3000 nM of the PCBs, or 3 μg/mL Aroclor 1254, for either 24- or 48-hr exposure periods (Fig. 4, middle row). The pattern of porphyrin accumulation observed for PCB 77 and Aroclor 1254 is in agreement with previous data obtained for these compounds using similar cell culture conditions [32].

The final group of HAHs consists of the *ortho* substituted PCBs (Fig. 3). PCBs 52, 54, 95, 101, 136, and 153 elicited intracellular porphyrin accumulation in the absence of elevated EROD activity after treatment of cultured CEHs for a 24-hr exposure period (data not shown). These results differ from those of Rodman *et al.* [21] who reported PCB 153-mediated EROD induction and a lack of intracellular porphyrin accumulation for PCB 52 after CEH cultures were exposed for 18–20 hr to these PCBs. The graphs shown in Fig. 3 illustrate the EROD activity and porphyrin accumulation responses for the *ortho* substituted PCBs after a 48-hr exposure period. Even after 48 hr, EROD activity was not elevated above basal levels and tended to decrease below basal levels at high PCB concentrations (Table 1).

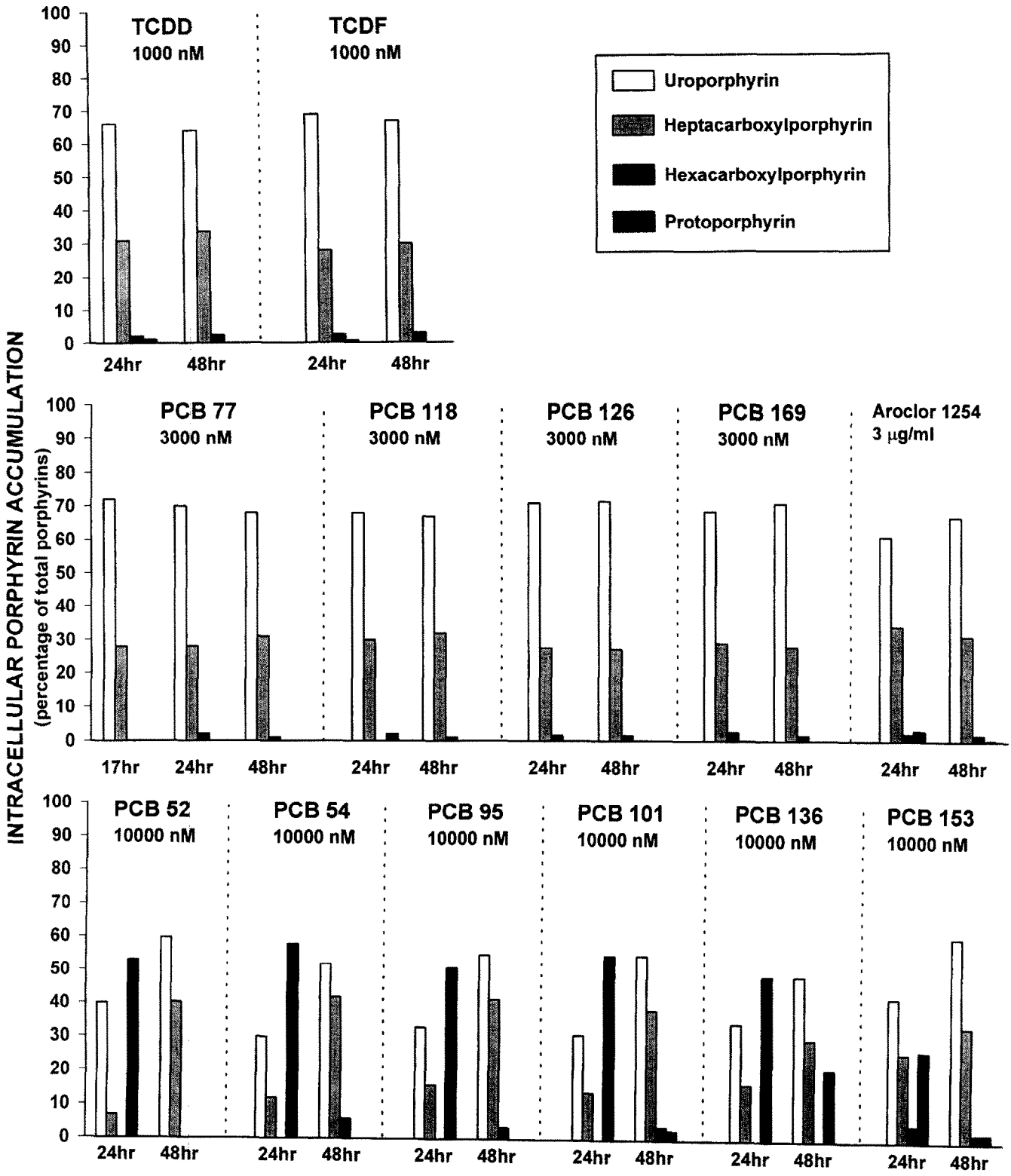


FIG. 4. HPLC porphyrin profile data for CEH cultures exposed to various HAHs. Porphyrins were extracted from CEH as described in Materials and Methods after either 17 (PCB 77 only), 24, or 48 hr of exposure to the indicated concentration of each of the HAHs or mixture tested. Data for each porphyrin are expressed as a percentage of the total porphyrins extracted.

Furthermore, no induction of immunodetectable CYP1A protein was observed after either 24- or 48-hr exposure times (data not shown). Although total intracellular porphyrin accumulation was apparent after a 24-hr exposure period (data not shown), the magnitude of this response was amplified 3- to 30-fold after the 48-hr exposure period (Fig. 3). Porphyrin accumulation occurred at PCB concentrations equal to or exceeding 1000 nM—at least 10-fold

higher than those that resulted in porphyrin accumulation in the first two groups (Table 1). A decrease in accumulation, similar to that observed for Aroclor 1254, was observed at the highest concentrations of PCBs 95, 136, and 153.

HPLC analysis revealed that after a 24-hr exposure period, 10,000 nM concentrations of each of the *ortho* substituted PCBs, except PCB 153, resulted in a porphyrin profile dominated by protoporphyrin (Fig. 4, bottom row). However, after a 48-hr exposure period, uroporphyrin and heptacarboxylporphyrin accounted for >75% of the total accumulated porphyrins, with heptacarboxylporphyrin contributing a higher percentage relative to uroporphyrin than was observed for the first two groups of HAHs and PCB mixture. The observed decrease in protoporphyrin accumulation between the 24- and 48-hr exposure periods (Fig. 4, bottom row) can be possibly explained by the tendency of this porphyrin to aggregate at a specific rate constant into non-fluorescent dimers in aqueous solutions [33]. Because the culture medium was serum-free, it is unlikely that protoporphyrin was released into the medium [34]. This was confirmed by HPLC analysis (data not shown). Treatment of CEH with PCB 153 resulted in a porphyrin profile that was intermediate between that observed for the other *ortho* substituted PCBs and the first two groups of HAHs and PCB mixture (Fig. 4, bottom row). Previous studies with the *ortho* substituted PCBs, 2,2′,3,3′,4,4′-hexachlorobiphenyl (PCB 128), 2,2′,4,4′,6,6′-hexachlorobiphenyl (PCB 155), and PCB 153 in CEH cultures, using culture conditions similar to those reported here, indicated that the porphyrin profiles observed for these compounds were not substantially different from those obtained for PCB 77 after a 24-hr exposure period [32]. Reasons for this discrepancy are not known.

Time–course data for PCB 77 are shown in Fig. 5 and Table 1. CEH cultures were maintained in PCB 77-treated medium for various periods of time and assayed for EROD activity, immunodetectable CYP1A protein, porphyrin accumulation, and porphyrin composition as described in Materials and Methods. EROD activity and immunodetectable CYP1A protein were detected as early as 6 hr after PCB 77 treatment, whereas porphyrin accumulation was first observed at 17 hr after PCB 77 treatment. HPLC analysis of extracts prepared from PCB 77-treated CEH cultures indicated that uroporphyrin and heptacarboxylporphyrin accounted for >98% of the total porphyrins produced in an approximate 70%:30% ratio, respectively, for exposure time periods of 17, 24, and 48 hr (Fig. 4). Maximal responses for EROD activity, immunodetectable CYP1A, and total porphyrin accumulation increased with increasing time of PCB 77 exposure. For both EROD activity and immunodetectable CYP1A data, the lowest experimental concentrations required to elicit a response were higher at 48 hr than at 24 hr of PCB 77 exposure (Table 1). In contrast, the lowest experimental concentration required for porphyrin accumulation was lower at 48 hr than at 24 hr (Table 1). This shift suggests that as time of exposure of CEHs to PCB 77 increased, the potency of this HAH to induce CYP1A decreased. Alternatively, the CEH cultures may have become less sensitive to the CYP1A-inducing effects of PCB 77. This shift also coincides with the greatest change in maximal EROD activity and immunodetectable CYP1A protein.

One possible explanation for this "curve-shift" phenomenon is intracellular metabolism of PCB 77. The graph in Fig. 5 (bottom, right) illustrates the change in immunodetectable CYP1A concentration as a function of PCB 77 concentration between 24 and 48 hr of exposure. At a concentration of 0.1 nM PCB 77, there was a 64% decrease in the amount of immunodetectable CYP1A protein over this 24-hr time period. This would correspond to a CEH CYP1A half-life of approximately 17 hr, which is considerably shorter than the 38-hr half-life reported for CYP1A in Aroclor 1254-treated rat liver [35, 36]. Experiments to directly measure the turnover rates for CEH CYP1A protein will be needed to confirm the short half-life suggested by these data. Nevertheless, the decline in CYP1A protein at the 0.1 nM concentration, combined with the observation that at PCB 77 concentrations greater than 0.1 nM the content of immunodetectable CYP1A protein continued to increase throughout the 48-hr period, suggests that substantial metabolism of PCB 77 was occurring at the lower concentration. At the higher concentrations, the metabolic capacity may have been exceeded, allowing the retention of inducer in amounts sufficient for continued activation of CYP1A transcription. Direct evidence for PCB 77 metabolism by cultured CEHs has been documented by Lambrecht *et al.* [37]. They observed that 29% of PCB 77 was converted to water-soluble metabolites following a 24-hr incubation with CEH cultures (initial concentration of PCB 77: 34 or 100 nM). Studies to further clarify the mechanisms underlying the time-dependent changes in CYP1A concentration–response curves are currently underway in our laboratory. At this time, it is important to note that the relative potency of an HAH may depend on the duration of exposure.

Another interesting feature of the EROD time–course study for PCB 77 was the observation that high concentrations of this PCB did not induce EROD activity at any time point. The lack of EROD induction at high concentrations was apparent as early as 6 hr after dosing, the same time when induction at the lower concentrations was first observed (Fig. 5). To date, in this laboratory, all PCBs, dioxins, furans, and extracts that have EROD-inducing capabilities have shown a decrease in EROD activity at high concentrations of inducer, such that a bell-shaped concentration–response curve is observed [22, 38, 39]. Although the mechanism resulting in decreased EROD activity is not understood completely, this phenomenon has been documented previously in both *in vivo* and *in vitro* systems [21, 40]. Determination of esterase activities of CEH cultures suggested that the viability of the hepatocytes treated with

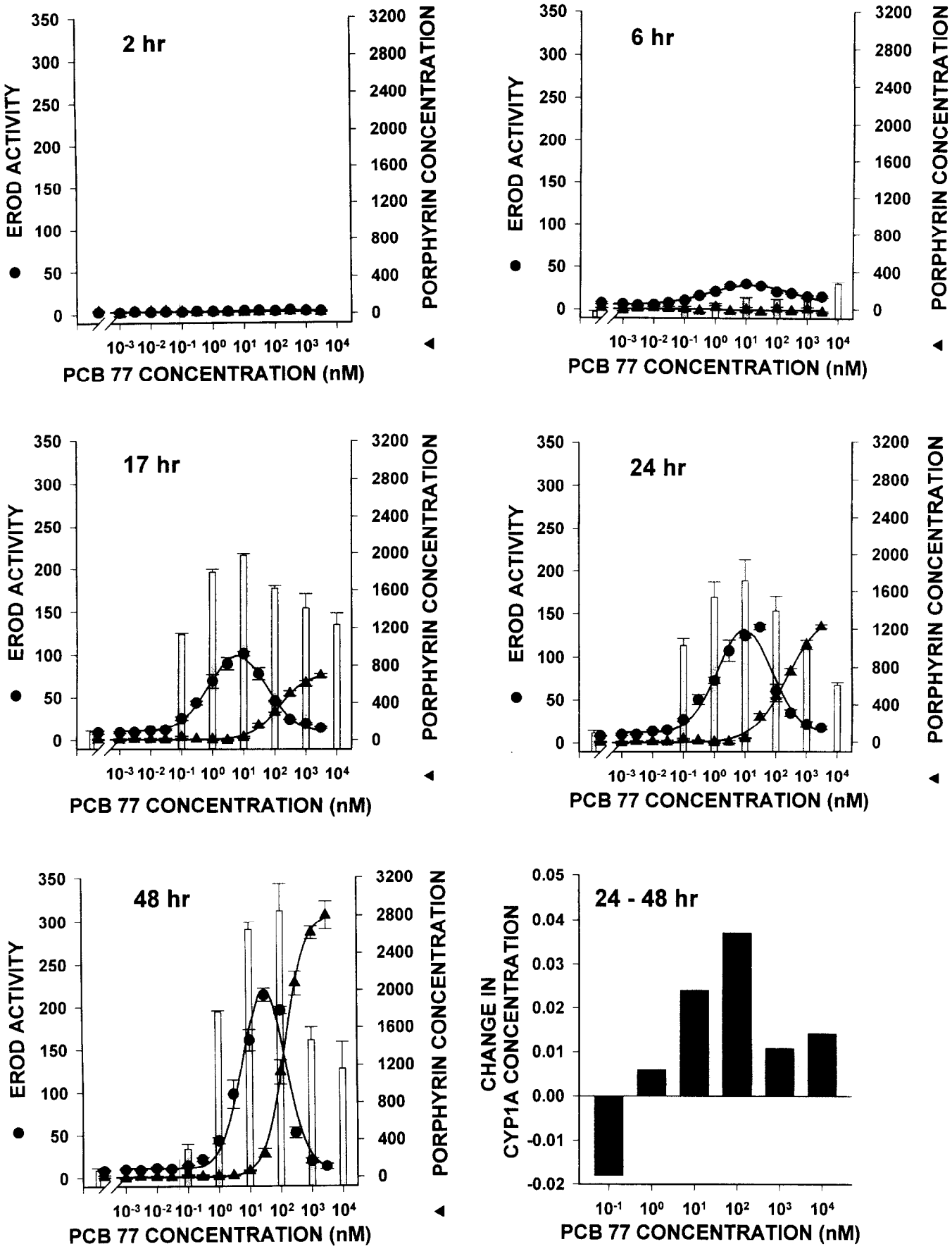


FIG. 5. Time–course data for PCB 77. Symbols, units, and scale for immunodetectable CYP1A protein are as described in the legend of Fig. 1. Duration of exposure of CEH cultures to PCB 77 is indicated in the top left-hand corner of each panel. The bottom, right panel indicates the change in immunodetectable CYP1A concentration as a function of PCB 77 concentration between 24 and 48 hr of exposure of CEH cultures to PCB 77.

high concentrations of TCDD, TCDF, or other HAHs was not compromised (data not shown). As demonstrated to some extent in the present study and by others [37, 41, 42], decreased CYP1A protein may contribute to the observed decrease in the catalytic activity of this enzyme at high concentrations of inducers. Interestingly, in the present study and in a study by Hahn and Chandran [43], the extent of the decrease of immunodetectable CYP1A protein may be congener-specific. For example, in the present study, the EROD concentration–response curves for TCDF and PCB 169 had a pattern that was very similar to that obtained for immunodetectable CYP1A protein for these HAHs (Figs. 1 and 2). For TCDD and PCB 126, immunodetectable CYP1A remained elevated while EROD activity declined at high concentrations (Figs. 1 and 2). For other HAHs, such as PCBs 118 and 77, there was some decline in immunodetectable CYP1A protein at high concentrations, but not to the same extent as the decrease in EROD activity observed at the same concentrations (Fig. 2). In the study by Hahn and Chandran [43] using a fish hepatoma cell line, decreases in EROD activity were observed at high concentrations of TCDD, TCDF, and various PCBs tested, but a coincident decline in immunodetectable CYP1A was observed only for TCDD and TCDF. These data suggest multiple mechanisms for the decline in EROD activity at high concentrations of inducers.

Not only did the rate and extent of decrease of immunodetectable CYP1A protein vary with the specific HAH tested, but maximal EROD activity or immunodetectable CYP1A also appeared to be congener-specific (Figs. 1 and 2). The mechanism(s) responsible for establishing congener-specific maximal EROD activity or immunodetectable CYP1A protein is not understood. Furthermore, for risk assessment studies, variable maxima are problematic, as reliable estimates of the relative potencies of the various HAHs cannot be obtained using standard statistical methods.

The concentrations of TCDD, TCDF, non-*ortho* substituted PCBs, PCB 118 and Aroclor 1254 that resulted in *inhibition* of EROD activity were very similar to the concentrations that resulted in the *onset* of porphyrin accumulation (Table 1), consistent with a possible relationship between porphyrin accumulation and CYP1A induction and/or inhibition. Although there are likely three phenomena at work, namely (a) induction of CYP1A, (b) interaction of HAHs with induced CYP1A, and (c) induction of ALAS, several reports have suggested an important role for CYP1A in porphyrin accumulation after exposure to HAHs. In rodents, studies by Jacobs *et al.* [11] and Sinclair *et al.* [12] indicated that CYP1A2, rather than CYP1A1, is associated with uroporphyinogen oxidation and probably has a role in HAH-mediated porphyria. In contrast to mammals, the CYP1A subfamily in birds has not been as well characterized. Early work by Sinclair *et al.* [17] showed that oxidation of uroporphyrinogen (and hence uroporphyrin accumulation) involved a 3-methylcholanthrene-inducible form of P450 in chick embryo liver extracts. Recently, two TCDD-inducible CYP1A forms have been identified in chickens [15, 16]. The catalytic activities (AHH, EROD) and tissue-specific expression (liver, kidney, heart) of form "$TCDD_{AHH}$" are like those of mammalian CYP1A1. Catalytically and immunochemically, the other chick form, "$TCDD_{AA}$" (Rifkind's nomenclature) resembles mammalian CYP1A2 more than CYP1A1 [15]. However, these apparent relationships are complicated by recent evidence suggesting that these two chick CYP1A forms are not exclusively orthologous to either of the mammalian CYP1A forms [44]. More to the point, it is not yet known whether one or both of these chick CYP1A forms can catalyze uroporphyrinogen oxidation. With regard to the identity of the form(s) measured in the present study, the N-terminal sequence of Rifkind's $TCDD_{AHH}$ is identical to Sinclair's "P450MC" [45], which is recognized by MAb 1-12-3 [29]. The ability of MAb 1-12-3 to recognize the $TCDD_{AA}$ form has not been tested. Thus, it is likely that the EROD and western blot assays used in this study both detected $TCDD_{AHH}$; whether $TCDD_{AA}$ was also being measured in the western blots ($TCDD_{AHH}$ and $TCDD_{AA}$ have nearly the same molecular weight) is unknown.

The mechanism by which HAHs interact with CYP1A2 to cause porphyrin accumulation in rodents is not clear. Similar to the mechanism of induction for CYP1A1, the Ah receptor is believed to play a role in induction of CYP1A2. Studies by Hahn *et al.* [46] showed that the porphyrinogenic HAH, hexaclorobenzene, bound to the Ah receptor and induced both CYP1A1 and CYP1A2 in the rat. A study by Voorman and Aust [14] demonstrated that certain porphyrinogenic HAHs with the capacity to induce CYP1A2 also bind to the cytochrome and inhibit its catalytic activity. This selective binding was observed for various HAHs including TCDD, PCB 77, and PCB 126. Further studies are required to establish an association between porphyrin accumulation and ligand-CYP1A2 interactions.

The PCB 77 time–course study indicated that total intracellular porphyrin accumulation lagged behind the onset of EROD induction by about 11 hr, such that porphyrin accumulation first became evident after approximately 17 hr of PCB 77 exposure. Previous reports have suggested that the induction of CYP1A2 lags behind the time required for CYP1A1 induction. For example, Sinclair *et al.* [12] found that the maximum amount of immunodetectable CYP1A2 protein was not observed until 3 days after maximal CYP1A1 protein and EROD activity were observed in mouse hepatocytes treated with PCB 169. Similar data were obtained by Hahn *et al.* [46] in experiments conducted on hexachlorobenzene-treated rats. While both CYP1A1 and CYP1A2 were induced after 12 or more hours of treatment, CYP1A1 reached maximal levels after 1–7 days, whereas CYP1A2 reached maximal levels after 5–7 days.

Although previous studies and the present work indicate a biochemical association between CYP1A induction and porphyrin accumulation in CEH cultures exposed to TCDD, TCDF, non-*ortho* substituted PCBs, PCB 118 and

Aroclor 1254, it is less clear what mechanism results in porphyrin accumulation in CEH treated with *ortho* substituted PCBs that do not induce CYP1A. Previous studies have shown that PB, 2-propyl-2-isopropylacetamide [18], and (−)-enantiomers of two *ortho* substituted PCBs [47] also cause uroporphyrin accumulation in the absence of EROD induction. These investigators suggested that a mechanism involving induction of a cytochrome(s) P450 different than CYP1A2 may be involved. Treatment of rats with *ortho* substituted PCBs results in the induction of various types of cytochromes P450 and often is dominated by PB-type induction (reviewed in Ref. 48). In cultured CEHs, PB-type compounds induce cytochromes CYP2H1/2, which are immunochemically related to CYP2B1/2—the two major forms of P450 induced by PB in the rat (reviewed in Ref. 48). Although a review by Sinclair and Sinclair [45] states that nonplanar halogenated biphenyls are CYP2H1/2 inducers in cultured CEHs, this conclusion was based on data for a single compound, 2,3′,4,4′,5-pentabromobiphenyl (the brominated analogue of PCB 118), which induced not only a 50-kDa protein with associated benzphetamine demethylase (PB-type) activity, but also a 57-kDa protein with associated EROD activity [42]. The combination of these limited data with the observation in the present study that the composition and relative proportion of the various porphyrins produced after treatment of CEH cultures with *ortho* substituted PCBs were different than those observed after treatment of the hepatocyte cultures with TCDD, TCDF, non-*ortho* substituted PCBs, PCB 118, or Aroclor 1254 provides further evidence that a different mechanism is responsible for porphyrin accumulation mediated by *ortho* substituted PCBs. However, the concentrations of the *ortho* substituted PCBs required to elicit intracellular porphyrin accumulation were, in all cases, greater than the concentrations required for inhibition of basal EROD activity by these compounds, with the exception of PCB 153 (Table 1). Thus, an alternative possible mechanism for *ortho* substituted PCB-mediated porphyrin accumulation may involve interaction of these PCBs with constitutively expressed (basal) CYP1A. Studies are underway in our laboratory using specific P450 inhibitors to further evaluate the possible involvement of CYP2H1/2 induction and/or constitutive CYP1A in *ortho* substituted PCB-mediated porphyrin accumulation in CEH cultures.

In conclusion, our results are consistent with a role for CYP1A induction and/or Ah receptor activation in porphyrin accumulation mediated by HAHs with a planar configuration, whereas those that are not planar may mediate porphyrin accumulation by a mechanism not involving induction of CYP1A. Furthermore, because CEH cultures are responsive to both P450 induction and disruption of the heme biosynthetic pathway, this system provides an ideal model for mechanistic studies to further evaluate the interrelationships of these HAH-sensitive biochemical endpoints.

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References

1. Parkinson A and Safe S, Mammalian biologic and toxic effects of PCBs. In: *Polychlorinated Biphenyls (PCBs): Mammalian and Environmental Toxicology* (Eds. Safe S and Hutzinger O), pp. 49–75. Springer, Berlin, 1987.
2. Safe S, Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* **51:** 51–88,1990.
3. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22:** 517–554, 1982.
4. Giesy JP, Ludwig JP and Tillitt DE, Deformities in birds of the Great Lakes region: Assigning causality. *Environ Sci Technol* **28:** 128–135, 1994.
5. Gilbertson M, Kubiak T, Ludwig J and Fox G, Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDS) in colonial fish-eating birds: Similarity to chick-edema disease. *J Toxicol Environ Health* **33:** 455–520, 1991.
6. Fox GA, Kennedy SW, Norstrom RJ and Wigfield DC, Porphyria in herring gulls: A biochemical response to chemical contamination of Great Lakes food chains. *Environ Toxicol Chem* **7:** 831–839, 1988.
7. Bradlaw JA and Casterline JL Jr, Induction of enzyme activity in cell culture: A rapid screen for detection of planar polychlorinated organic compounds. *J Assoc Off Anal Chem* **62:** 904–916, 1979.
8. Whitlock JP Jr, Mechanistic aspects of dioxin action. *Chem Res Toxicol* **6:** 754–763, 1993.
9. Safe S, Development of bioassays and approaches for the risk assessment of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. *Environ Health Perspect Suppl* **101:** 317–325, 1993.
10. Marks GS, Exposure to toxic agents: The heme biosynthesis pathway and hemoproteins as indicator. *Crit Rev Toxicol* **15:** 151–179, 1985.
11. Jacobs JM, Sinclair PR, Bement WJ, Lambrecht RW, Sinclair JF and Goldstein JA, Oxidation of uroporphyrinogen by methylcholanthrene-induced cytochrome P-450. *Biochem J* **258:** 247–253, 1989.
12. Sinclair PR, Bement WJ, Lambrecht RW, Gorman N and Sinclair JF, Chlorinated biphenyls induce cytochrome P4501A2 and uroporphyrin accumulation in cultures of mouse hepatocytes. *Arch Biochem Biophys* **281:** 225–232, 1990.
13. Hahn ME, Gasiewicz TA, Linko P and Goldstein JA, The role of the *Ah* locus in hexachlorobenzene-induced porphyria. Studies in congenic C57BL/6J mice. *Biochem J* **254:** 245–254, 1988.
14. Voorman R and Aust SD, Specific binding of polyhalogenated aromatic hydrocarbon inducers of cytochrome P-450d to the cytochrome and inhibition of its estradiol 2-hydroxylase activity. *Toxicol Appl Pharmacol* **90:** 69–78, 1987.
15. Rifkind AB, Kanetoshi A, Orlinick J, Capdevila JH and Lee C, Purification and biochemical characterization of two major cytochrome P-450 isoforms induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in chick embryo liver. *J Biol Chem* **269:** 3387–3396, 1994.
16. Paroli L, Lee C and Rifkind AB, Identification of hepatocytes as the major locus of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1-related P450s, $TCDD_{AA}$ and $TCDD_{AHH}$, in chick embryo liver. *Drug Metab Dispos* **22:** 962–968, 1994.

17. Sinclair P, Lambrecht R and Sinclair J, Evidence for cytochrome P450-mediated oxidation of uroporphyrinogen by cell-free liver extracts from chick embryos treated with 3-methylcholanthrene. *Biochem Biophys Res Commun* **146:** 1324–1329, 1987.
18. DeMatteis F, Harvey C, Reed C and Hempenius R, Increased oxidation of uroprophyrinogen by an inducible liver microsomal system. Possible relevance to drug-induced uroporphyria. *Biochem J* **250:** 161–169, 1988.
19. Kawanishi S, Mizutani M and Sano S, Induction of porphyrin synthesis in chick embryo liver cell culture by synthetic polychlorobiphenyl isomers. *Biochim Biophys Acta* **540:** 83–92, 1978.
20. Sassa S, Sugita O, Ohnuma N, Imajo S, Okumura T, Noguchi T and Kappas A, Studies of the influence of chloro-substituent sites and conformational energy in polychlorinated biphenyls on uroporphyrin formation in chick-embryo liver cell cultures. *Biochem J* **235:** 291–296, 1986.
21. Rodman LE, Shedlofsky SI, Swim AT and Robertson LW, Effects of polychlorinated biphenyls on cytochrome P450 induction in the chick embryo hepatocyte culture. *Arch Biochem Biophys* **275:** 252–262, 1989.
22. Kennedy SW, Jones SP and Bastien LJ, Efficient analysis of cytochrome P4501A catalytic activity, porphyrins and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Anal Biochem* **226:** 362–370, 1995.
23. Kennedy SW, Lorenzen A, James CA and Collins BT, Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. *Anal Biochem* **211:** 102–112, 1993.
24. Lorenzen A and Kennedy SW, A fluorescence-based protein assay for use with a microplate reader. *Anal Biochem* **214:** 346–348, 1993.
25. Kennedy SW and James CA, Improved method to extract and concentrate porphyrins from liver tissue for analysis by high performance liquid chromatography. *J Chromatogr Biomed Appl* **619:** 127–132, 1993.
26. Kennedy SW, Wigfield DC and Fox GA, Tissue porphyrin pattern determination by high-speed high-performance liquid chromatography. *Anal Biochem* **157:** 1–7, 1986.
27. Towbin H, Staehlin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354, 1979.
28. Park SS, Miller H, Klotz AV, Kloepper-Sams PJ, Stegeman JJ and Gelboin HV, Monoclonal antibodies to liver microsomal cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): Cross reactivity with 3-methylcholanthrene induced rat cytochrome P-450. *Arch Biochem Biophys* **249:** 339–350, 1986.
29. Stegeman JJ, Cytochrome P450 forms in fish: Catalytic, immunological and sequence similarities. *Xenobiotica* **19:** 1093–1110, 1989.
30. Safe SH, Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu Rev Pharmacol Toxicol* **26:** 371–399, 1986.
31. Safe S, Bandiera S, Sawyer T, Robertson L, Safe L, Parkinson A, Thomas PE, Ryan DE, Reik LM, Levin W, Denomme MA and Fujita T, PCBs: Structure–function relationships and mechanism of action. *Environ Health Perspect* **60:** 47–56, 1985.
32. Marks GS, Powles J, Lyon M, McCluskey S, Sutherland E and Zelt D, Patterns of porphyrin accumulation in response to xenobiotics. In: *Mechanisms of Chemical-Induced Porphyrinopathies* (Eds. Silbergeld EK and Fowler BA), pp. 113–127. New York Academy of Sciences, New York, 1987.
33. Margalit R, Shaklai N and Cohen S, Fluorometric studies on the dimerization equilibrium of protoporphyrin IX and its haemato derivative. *Biochem J* **209:** 547–552, 1983.
34. Granick S, Sinclair P, Sassa S and Grieninger G, Effects by heme, insulin, and serum albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and a spectrofluorometric assay for porphyrin composition. *J Biol Chem* **250:** 9215–9225, 1975.
35. Parkinson A, Thomas PE, Ryan DE and Levin W, The *in vivo* turnover of rat liver microsomal epoxide hydrolase and both the apoprotein and heme moieties of specific cytochrome P-450 isozymes. *Arch Biochem Biophys* **225:** 216–236, 1983.
36. Shiraki H and Guengerich FP, Turnover of membrane proteins: Kinetics of induction and degradation of seven forms of rat liver microsomal cytochrome P-450, NADPH-cytochrome P-450 reductase, and epoxide hydrolase. *Arch Biochem Biophys* **235:** 86–96, 1984.
37. Lambrecht RW, Sinclair PR, Bement WJ and Sinclair JF, Uroporphyrin accumulation in cultured chick embryo hepatocytes: Comparison of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3,3′,4,4′-tetrachlorobiphenyl. *Toxicol Appl Pharmacol* **96:** 507–516, 1988.
38. Lorenzen A, James CA and Kennedy SW, Effects of UV irradiation of cell culture medium on PCB-mediated porphyrin accumulation and EROD induction in chick embryo hepatocytes. *Toxicol* In Vitro **7:** 159–166, 1993.
39. Kennedy SW, Lorenzen A and Norstrom RJ, Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. *Environ Sci Technol* **30:** 706–715, 1996.
40. Hahn ME, Lamb TM, Schultz ME, Smolowitz RM and Stegeman JJ, Cytochrome P4501A induction and inhibition by 3,3′,4,4′-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquat Toxicol* **26:** 185–208, 1993.
41. Gooch JW, Elskus AA, Kloepper-Sams PJ, Hahn ME and Stegeman JJ, Effects of *ortho-* and non-*ortho*-substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicol Appl Pharmacol* **98:** 422–433, 1989.
42. Sinclair PR, Frezza J, Sinclair JF, Bement WJ, Haugen S, Healey J and Bonkovsky H, Immunochemical detection of different isozymes of cytochrome P-450 induced in chick hepatocyte cultures. *Biochem J* **258:** 237–245, 1989.
43. Hahn ME and Chandran K, Uroporphyrin accumulation associated with cytochrome P4501A1 induction in fish hepatoma cells exposed to Ah receptor agonists, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and planar chlorobiphenyls. *Arch Biochem Biophys* **329:** 163–174, 1996.
44. Gilday D, Yutzey K, Bader D and Rifkind AB, Molecular cloning of two TCDD-induced chicken CYP1A related enzymes distinct from mammalian CYP1A1 and 1A2. *FASEB J* **10:** A283, 1996.
45. Sinclair JF and Sinclair PR, Avian cytochrome P450. In: *Cytochrome P450* (Eds. Schenkman JB and Greim H), pp. 259–277. Springer, New York, 1993.
46. Hahn ME, Goldstein JA, Linko P and Gasiewicz TA, Interaction of hexachlorobenzene with the receptor for 2,3,7,8-tetrachlordibenzo-*p*-dioxin *in vitro* and *in vivo*. *Arch Biochem Biophys* **270:** 344–355, 1989.
47. Rodman LE, Shedlofsky SI, Mannschreck A, Puttmann M, Swim AT and Robertson LW, Differential potency of atropisomers of polychlorinated biphenyls on cytochrome P450 induction and porphyrin accumulation in the chick embryo hepatocyte culture. *Biochem Pharmacol* **41:** 915–922, 1991.
48. Safe S, Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, toxicology and mechanism of action. *Crit Rev Toxicol* **13:** 319–395, 1984.