

Serum Alters the Uptake and Relative Potencies of Halogenated Aromatic Hydrocarbons in Cell Culture Bioassays

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The effects of many chemicals on cellular processes are governed by their ability to enter the cell, which is in turn a function of the composition of the cell's external environment. To examine this relationship, the effect of serum in cell culture medium on the bioavailability of cytochrome P450 1A (CYP1A)-inducing compounds was determined in PLHC-1 (*Poeciliopsis lucida* hepatocellular carcinoma) cells. The presence of 10% calf serum in the medium increased the EC₅₀ (effective concentration to achieve 50% maximal response) for induction of ethoxyresorufin *O*-deethylase (EROD) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) 20-fold as compared to treatment in serum-free medium. Measurement of [³H]TCDD uptake and Ah receptor binding indicated that the apparent difference in potencies was a result of decreased bioavailability in the presence of serum, effectively reducing the concentration of TCDD within the cells. Induction of EROD and CYP1A protein in response to treatment with each of three coplanar polychlorinated biphenyls (PCB congeners 77, 126, and 169) was similarly affected by serum, although the magnitude varied among inducers and assays. Relative potencies (calculated as EC₅₀_{TCDD} / EC₅₀_{PCB}) for EROD induction by the three PCBs were significantly higher in the absence of serum. However, serum showed no significant effect on the relative potencies for CYP1A protein induction. These results demonstrate that measured inducing potencies, and relative potencies for EROD induction, by halogenated aromatic hydrocarbons are strongly dependent on the composition of culture medium, which can lead to artificial differences in comparisons among cell types.

Key Words: bioassay; bioavailability; dioxin; PCB; serum; TEF.

Cells in culture, both primary cultures and established cell lines, have become important systems for investigating toxic mechanisms and evaluating the potential toxicity of previously unstudied compounds. Establishing accurate concentration-response relationships in such systems is critical. The impact of artifacts introduced by cultured cell assays on the ability to compare responses among organisms, cell types, and individual compounds is poorly understood. One potential source of error introduced in such assays is the presence of serum in the

culture medium. Serum is poorly defined, comes from diverse donor animals, and has substantial lot-to-lot variability in composition. In addition, cell lines have differing serum requirements, which introduce further variability when comparing responses among cell lines. Serum factors affect the proliferation rates of cells as well as a host of other metabolic processes.

The induction of cytochrome P450 1A (CYP1A) protein and catalytic activity in cultured cells is being used with increasing frequency to compare the sensitivities of a variety of organisms to the effects of halogenated aromatic hydrocarbons (HAH). Cells from mammals (Safe, 1987; Sawyer and Safe, 1982; Tillitt *et al.*, 1991), birds (Kennedy *et al.*, 1996a; Kennedy *et al.*, 1996b), and fish (Clemons *et al.*, 1996; Hahn *et al.*, 1996) have been used to study the mechanisms of HAH toxicity and, in the absence of *in vivo* data, to establish taxon-specific toxic equivalency factors (TEFs) for these compounds (van den Berg *et al.*, 1998). Comparisons of these results can reveal mechanistic differences in the induction pathway of CYP1A among taxa. However, differences in CYP1A induction among cell culture systems can also reflect the culture conditions of the cells.

Serum is known to impact the effects of CYP1A inducers and CYP levels in cultured cells. For example, the presence of 10% fetal calf serum reduces the potency of TCDD and PCB126 for inhibiting aromatase (CYP19) activity in JEG-3 human choriocarcinoma cells (Drenth *et al.*, 1998). Serum and other medium components can also alter the detectable levels of cytochromes P450 in rat hepatocytes (Hammond and Fry, 1992; Turner and Pitot, 1989) and HepG2 cells (Doostdar *et al.*, 1991; Doostdar *et al.*, 1988). Despite these provocative findings, there has been no quantitative study of the effect of serum on CYP1A induction by HAH. Because these compounds are very hydrophobic and have limited aqueous solubilities, it can be expected that serum components, such as proteins and lipids, would have a significant effect on the bioavailability of HAH for cell uptake. Because entry into the cell is the first step in the toxic mechanism of these compounds, effects at this stage will be propagated (and perhaps multiplied) through subsequent cell responses.

Following entry of an inducer into the cell, CYP1A induc-

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tion is controlled by the ligand-activated transcription factor aryl hydrocarbon receptor (AHR). Binding of HAH to the AHR activates transcription of CYP1A and mediates the toxicity of the inducer. A compound's potency for CYP1A induction *in vivo* or in cultured cells is a strong predictor of its toxicity (Safe, 1984). Use of cell culture systems for rapid analysis of the potential toxicity of individual compounds and environmental samples has increased with refinements in CYP1A measurement techniques. Levels of both the CYP1A protein (Bruschweiler *et al.*, 1996; Hahn *et al.*, 1996) and its ethoxyresorufin *O*-deethylase (EROD) activity (Kennedy *et al.*, 1993) can be measured directly in the same multiwell plates used for growth of the cells and exposure to HAH.

The toxic equivalency approach utilizes these induction data to assess the toxic potential of individual compounds or mixtures relative to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The potency of a compound for eliciting a response can be compared to the potency of TCDD for the same response by calculating the ratio of their respective EC50s (concentration eliciting a 50% maximal effect). Such relative potencies from several systems, including cultured cells, are then used to determine TEFs for individual taxa (van den Berg *et al.*, 1998).

In previous reports we have established the conditions and methods for measuring EROD activity and CYP1A induction in PLHC-1 cells (Hahn and Chandran, 1996; Hahn *et al.*, 1993; Hahn *et al.*, 1996), which are derived from a hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* (Hightower and Renfro, 1988). Here we make use of those findings to examine the role of serum in uptake of HAH from the culture medium by cells. This represents a first step in examining the complex interaction between these cells and their chemical milieu. The results provide compelling evidence that serum affects the potency of AHR ligands, and likely other hydrophobic compounds, in cells in culture.

MATERIALS AND METHODS

Chemicals and solutions. The 2,3,7,8-tetrachloro[1,6-³H]dibenzo-*p*-dioxin ([³H]TCDD, stated purity $\geq 97\%$, specific activity 27 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS). Its radiochemical purity was $> 91\%$ as determined by HPLC immediately prior to use for uptake experiments and $> 96\%$ for specific binding determination. Unlabeled TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF), PCB 77 (3,3',4,4'-tetrachlorobiphenyl), PCB 126 (3,3',4,4',5-pentachlorobiphenyl), and PCB 169 (3,3',4,4',5,5'-hexachlorobiphenyl) were all obtained from Ultra Scientific (Kingstown, RI). Resorufin, ethoxyresorufin, and Amplex Red were obtained from Molecular Probes (Eugene, OR). Peroxidase conjugated goat anti-mouse antibody was from Pierce (Rockford, IL). All other reagents were obtained from Sigma (St. Louis, MO).

Phosphate-buffered saline (PBS) is 0.8% NaCl, 0.115% Na₂HPO₄, 0.02% KCl, 0.02% KH₂PO₄, pH 7.4. Phosphate buffer is 50 mM Na₂HPO₄ with pH adjusted to 8.0 with 50 mM NaH₂PO₄. TCDD, TCDF, and PCB solutions were prepared in dimethyl sulfoxide (DMSO) as described previously (Hahn *et al.*, 1996). Concentrations of [³H]TCDD solutions were verified by liquid scintillation counting (LSC) on a Beckman LS5000TD.

Growth and treatment of cells. PLHC-1 cells (Hightower and Renfro, 1988) were grown at 30°C in minimum essential medium (MEM) containing Earle's salts, nonessential amino acids, L-glutamine and 10% calf serum (Sigma C6278, lot 106H4628), as described previously (Hahn *et al.*, 1993). One day prior to dosing, cells were suspended to 0.5 to 1 × 10⁶ per ml and seeded into 48- or 96-well plates (Costar; Cambridge, MA) at 0.5 or 0.2 ml per well, respectively. One day later the medium was removed and replaced with fresh medium. Media used in the experiments include MEM without serum, with 5% serum, with 10% serum, with 10% delipidated, charcoal-stripped calf serum (Sigma C1696), and with 10% fetal bovine serum (FBS; Gibco; Grand Island, NY). Serum-free MEM supplemented with bovine serum albumin (BSA) was also used. The cells were then treated by addition of solutions dissolved in DMSO or DMSO alone (2.5 or 1.0 μl/well). DMSO concentrations were $\leq 0.5\%$ (v/v) in all treatments. Following treatment, plates were incubated at 30°C for 24 h unless otherwise indicated. For TCDD-specific binding experiments, cells were seeded into 24-well plates (Corning; Corning, NY) at 2 × 10⁶ cells in 1 ml culture medium per well. With the exception of the delipidated serum and FBS, all serum used was from a single lot. None of the media or HAH treatments reduced cell viability, as assessed by Trypan blue exclusion.

EROD and protein assays. EROD activity was measured using a multiwell fluorescence plate reader by a modification of the method of Kennedy *et al.*, (1995). Cells were rinsed once with 0.5 ml room-temperature PBS, and the EROD reaction was then initiated with the addition of 2 μM 7-ethoxyresorufin in phosphate buffer (200 μl/well). The reaction was stopped after 8 min (resorufin production is linear with respect to time over this period; Hahn *et al.*, 1996) with the addition of 150 μl ice-cold fluorescamine solution (0.15 mg/ml in acetonitrile). After a 15-min incubation, resorufin and fluorescamine fluorescence was measured. Resorufin and protein concentrations were determined from standard curves prepared in the same plate. BSA was used for the protein standard curve. In some experiments, the EROD reaction was followed kinetically over 8 min, as described previously (Hahn *et al.*, 1996). Protein was measured using fluorescamine as described above.

TCDD uptake. PLHC-1 cells were seeded in 48-well plates, grown for 1 day, and then fed media as indicated in figure legends. They were treated with [³H]TCDD in DMSO as above and incubated at 30°C. At 0.5, 1, 2, 7, and 24 h post-treatment, the culture medium was transferred from each well to a separate vial. Cells were removed by sequential incubation with two 0.2-ml aliquots of 0.05% (w/v) trypsin, which were then combined in a single vial. Cell removal was verified by microscopy. TCDD retained on well surfaces was extracted with a single 1-ml aliquot of hexane. TCDD associated with each fraction (medium, cells, and well) was determined by LSC. Protein concentrations were determined using fluorescamine in duplicate wells fed each medium and treated with DMSO alone.

TCDD binding. Specific binding of [³H]TCDD in PLHC-1 cells was measured by a whole-cell filtration assay (Dold and Greenlee, 1990). One day after seeding in 24-well plates, the cells were fed 0.5 ml of the indicated media. Cells were treated with 0.18 nM [³H]TCDD in the presence or absence of 40 nM TCDF and incubated 2 h at 30°C. This time was determined to be sufficient to achieve a steady state of bound radioligand (not shown). Following the incubation, medium was removed, cells were rinsed with 0.5 ml ice-cold PBS, then detached with 0.5 ml trypsin. The trypsin was inactivated by the addition of 0.5 ml ice-cold culture medium (with 10% serum), and cells from each well were collected under vacuum on a 25-mm Whatman GF/F filter that had been prewashed with PBS. Filters were then washed four times with 2.5 ml acetone that had been precooled to -80°C. Replicates were processed in batches of 12 on a Millipore 1225 filter manifold. Radioactivity remaining on the filter was quantified by LSC. Specific binding was measured in triplicate as the difference of each of three total binding (without TCDF) replicates and the average of three nonspecific binding (with TCDF) replicates in each medium. Protein concentrations were determined in duplicate wells fed each medium and treated with DMSO alone.

ELISA assay. Enzyme-linked immunosorbence assays to detect CYP1A were performed essentially as described (Bruschweiler *et al.*, 1996). One day

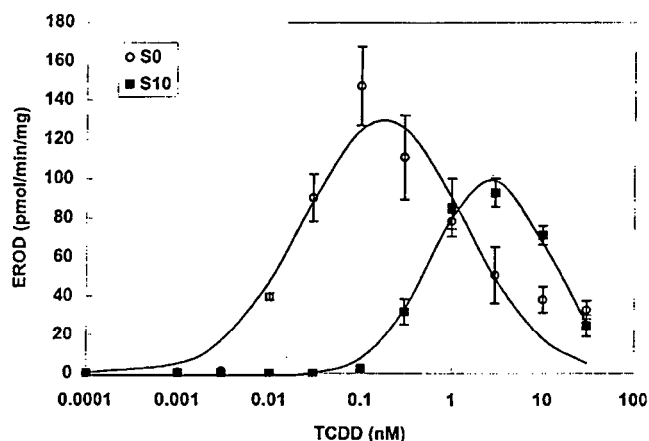


FIG. 1. Effect of serum on potency of EROD induction by TCDD. Cells were fed culture medium with (S_{10}) or without (S_0) 10% calf serum immediately prior to treatment with TCDD. EROD activity (picomoles of resorufin formed per min per milligram of cellular protein) was measured 24 h later. The 0.0001 nM TCDD concentration represents treatment with DMSO alone. Points are means \pm SE of four wells. The modified Gaussian fits to these data are plotted.

after treatment in 96-well plates, cells were fixed in 50% ethanol 15 min, in 75% ethanol 15 min, and in 95% ethanol 30 min. After washing three times with PBS, nonspecific antibody binding was blocked with 10% fetal bovine serum and 2% BSA in PBS for 1 h. The primary antibody, mouse anti-scup CYP1A monoclonal antibody 1-12-3 (10 μ g/ml; Park *et al.*, 1986), was then added in 100 μ l blocking solution for 1 h. After three washing steps with PBS, 100 μ l secondary antibody, peroxidase conjugated goat anti-mouse (1:1000 in blocking solution), was added for 1 h. After another three washing steps with PBS, 100 μ l substrate solution (100 μ M Amplex Red, 100 μ M H_2O_2 in phosphate buffer, pH = 7.0) was added for 30 min. All incubations were performed at room temperature. Resorufin formation was measured in the fluorescence plate reader. For each treatment the background fluorescence, defined as the fluorescence detected in untreated cells, was subtracted, and all values were normalized to the maximum response measured. The assay was also performed on wells without cells or without the addition of primary antibody, and these controls yielded fluorescence values nearly identical to those in untreated cells, consistent with our earlier results detecting no CYP1A protein or EROD activity in untreated cells (Hahn *et al.*, 1996).

Curve fitting and statistical analysis. For determination of dose-response relationships, EROD data were fit to a modified Gaussian function, and CYP1A induction data were fit to a logistic function. The rationale for use of these functions has been described previously (Hahn *et al.*, 1996; Kennedy *et al.*, 1993). The Gaussian function properly reflects the biphasic nature of EROD induction, while a logistic function forms a plateau at higher inducer concentrations, consistent with CYP1A protein induction in these cells. Statistical analyses were performed with the aid of Excel (Microsoft; Redmond, WA) and JMP IN (SAS Institute, Inc.; Cary, NC) software.

RESULTS

Culture Medium Composition Affects TCDD Uptake

While investigating the effects of culture media composition on responses to TCDD in PLHC-1 cells, we found that TCDD was more potent in eliciting an EROD response when added to cells in serum-free medium (S_0) than in medium with 10% calf

serum (S_{10} ; Fig. 1). In this experiment, cells were grown in S_{10} that was replaced by either S_0 or S_{10} immediately prior to treatment with TCDD. CYP1A-catalyzed EROD activity was measured 24 h later. The dose of TCDD required to elicit a 50% maximal induction of EROD was about 20-fold less in cells treated in S_0 than in cells treated in S_{10} (Table 1). A separate comparison of EROD induction in medium supplemented with 10% FBS showed that the EC50 was about 4-fold lower than in S_{10} and 5-fold higher than in S_0 (not shown). Consistent with earlier results (Hahn *et al.*, 1996), there was no basal EROD activity regardless of medium used.

In order to determine whether the culture medium affected partitioning of TCDD within the environment of a well, we measured the uptake of [3H]TCDD. Four different serum treatments were compared: S_0 , S_{10} , 10% delipidated serum (S_{DL}), and 5% calf serum (S_5). After growth in S_{10} , PLHC-1 cells were treated with 1 nM [3H]TCDD in each of these media and sampled at subsequent times to determine the timing of TCDD uptake by the cells (Fig. 2A). The TCDD associated with cells declined steadily from an early maximum and reached steady state between 2 and 7 h post-treatment. TCDD was added directly to the medium overlying the cells in a DMSO solution, and the higher density of that solution accounts for the large early values of cell-associated TCDD in the adherent PLHC-1 cells. Once a steady state had been achieved, cell-associated TCDD was 2- to 3-fold greater in cells treated in S_0 or S_{DL} than in S_5 or S_{10} .

Having established the timing of TCDD uptake, it was possible to determine its partitioning in wells at different concentrations. Using the same four media, the fraction of total TCDD added that was associated with the cells was determined 24 h post-treatment for four different concentrations of TCDD (Table 2 and Figure 2B). Again, the fraction of TCDD associated with the cells was highest in the cells treated in S_0 and lowest in those treated in S_{10} . The fraction of TCDD associated with cells was nearly constant between 0.01 nM and 1 nM within each medium treatment, but declined at 10 nM (Figure 2B). The fraction of TCDD in the overlying medium was similar within each medium treatment at all concentrations of TCDD; at 10 nM a greater fraction of TCDD was found associated with the polystyrene walls of the wells (Table 2).

TABLE 1
Effect of Medium on Dose-Response Relationship Parameters for EROD Rates in PLHC-1 Cells Treated with TCDD

Medium	EC50 (nM) ^a	EC100 (nM) ^a
S_0	0.022	0.138
S_{10}	0.474	2.590

Note. Modified Gaussian functions were fit to data from Figure 1 (S_0 and S_{10}) to obtain these values.

^a EC50 and EC100 are nominal TCDD concentrations producing 50% and 100% of maximal EROD induction, respectively.

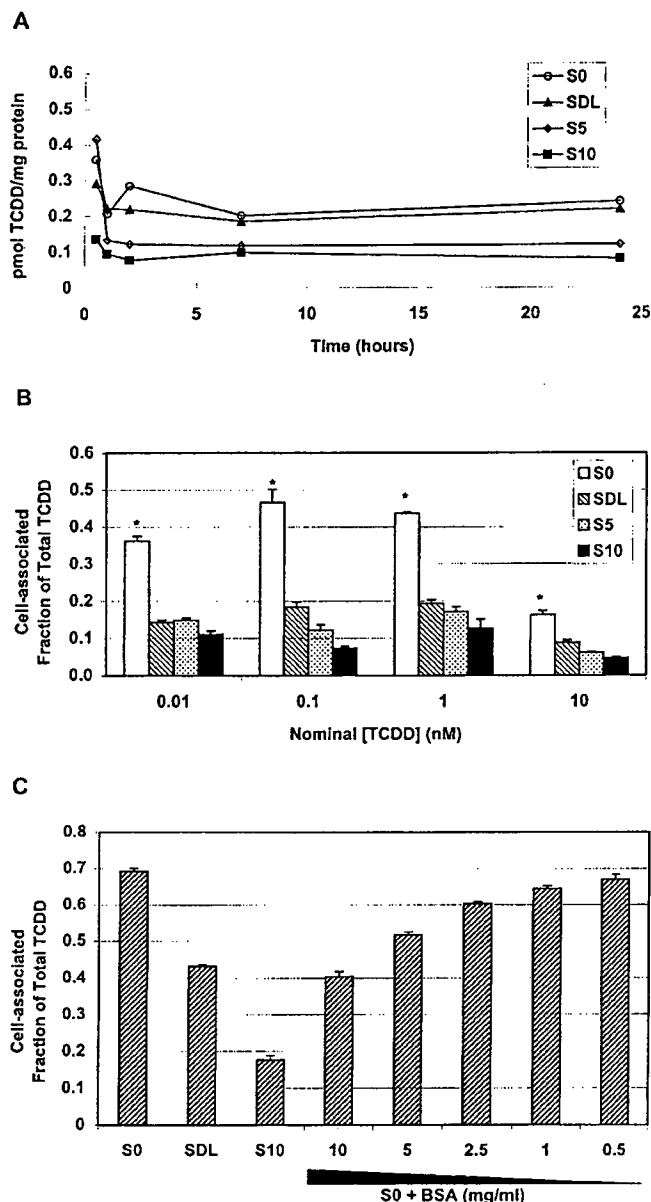


FIG. 2. Effect of serum on TCDD uptake in PLHC-1 cells. Cells were fed culture medium with no serum, 10% delipidated calf serum (S_{DL}), 5% calf serum (S₅), 10% calf serum, or increasing concentrations of BSA (numbers in milligrams per milliliter). (A) Cells were treated with 1 nM [³H]TCDD and sampled at the indicated times to determine the amount of TCDD associated with the cells. Cell-associated TCDD was normalized to protein content to account for differences in cell number among the treatments. Points are means of duplicate wells. (B) Cells were treated with the indicated concentrations of [³H]TCDD and collected after 24 h. The ordinate represents the fraction of the total amount of TCDD recovered that was associated with the cells. *Indicates significantly different from all other medium treatments ($p < 0.05$, ANOVA). (C) As (B), except that S₀ supplemented with 10, 5, 2.5, 1, and 0.5 mg/ml BSA were also compared, and treatment was with 1 nM TCDD. Points are means \pm SE of three wells.

Because the delipidation process may remove several serum constituents, it was necessary to determine which component(s) of serum was responsible for retaining TCDD in the medium. The effect of protein concentration on TCDD uptake was investigated. Cells were treated with 1 nM TCDD as above, except that additional treatments consisting of S₀ supplemented with increasing concentrations of BSA were added (Figure 2C). Protein at concentrations near those in S₁₀ or S_{DL} (5–8 mg/ml according to the supplier) produced uptake identical to that in S_{DL}, indicating that protein and lipid both contribute to reduced uptake in the presence of serum.

Differences in Uptake Affect TCDD Binding by the AH Receptor

The effect of medium composition on specific binding of TCDD by AHR in PLHC-1 cells was measured. Cells were grown in S₁₀, fed one of the four media, treated with 0.18 nM [³H]TCDD in the presence or absence of 40 nM TCDF, and incubated for 2 h. This concentration of TCDD was selected because it is near the value at which cells treated in S₀ or S₁₀ showed the greatest difference in EROD response (Figure 1). Binding of TCDD to the AHR was measured by a whole-cell filtration assay (Dold and Greenlee, 1990). The amount of TCDD bound was 3- to 4-fold higher in cells treated in S₀ and showed the same relationship among medium treatments as the fraction of TCDD associated with the cells, i.e., S₀ >> S_{DL} > S₅ > S₁₀ (Fig. 3). Thus, the differences in specific binding reflect the differing concentrations of TCDD within the cells among the treatments, as shown in Figure 2.

Differences in EROD Response Are Due to Differences in TCDD Uptake

PLHC-1 cells were fed the four media and treated with 0.01, 0.1, 1, or 10 nM [³H]TCDD exactly as for the uptake experiment, except that after 24 h the levels of CYP1A-catalyzed EROD activity were measured (Figure 4A). As expected from the results shown in Figures 1 and 3, a greater amount of TCDD bound by the AHR in S₀ led to a greater induction of EROD even though nominal TCDD concentrations were the same. For example, at 0.1 nM TCDD, the magnitude of the EROD response among the medium treatments showed the same rank order as the magnitude of TCDD-specific binding. Though the use of fewer concentrations of TCDD makes determination of a dose-response relationship less precise, the induction potencies in this assay were similar to those seen in the initial experiment (Fig. 1).

When the dose-response curves are expressed, in terms of cell-associated TCDD rather than nominal concentration in the medium, the points from the individual treatments align into a single biphasic induction curve typical of EROD induction by TCDD in PLHC-1 cells (Fig. 4B). This relationship suggests that the difference in induction potency among media used for treatment of PLHC-1 cells is due solely to differential parti-

TABLE 2
Fractions of TCDD Associated with Culture Medium, PLHC-1 Cells, and Well Surfaces

Medium treatment	Nominal TCDD concentration (nM)	Fraction of total TCDD associated with		
		Medium	Cells ^a	Wells
S ₀	0.01	37%	36% (1.4)	27%
	0.1	33%	47% (15)	21%
	1	33%	44% (160)	23%
	10	28%	16% (460)	56%
S _{DL}	0.01	72%	14% (0.75)	14%
	0.1	71%	18% (5.7)	10%
	1	71%	19% (78)	9%
	10	59%	9% (261)	33%
S ₅	0.01	74%	15% (0.67)	11%
	0.1	83%	12% (3.6)	5%
	1	78%	17% (61)	5%
	10	74%	6% (180)	20%
S ₁₀	0.01	79%	11% (0.56)	10%
	0.1	90%	7% (2.4)	3%
	1	86%	12% (45)	2%
	10	79%	5% (170)	16%

Note. PLHC-1 cells were grown in S₁₀ for 24 h after subculture, fed the indicated medium, and treated with the indicated nominal concentration of [³H]TCDD. Partitioning of the [³H]TCDD was determined after 24 h as described in Materials and Methods.

^a Average pmol of [³H]TCDD associated with the cells in a well for each medium treatment and [³H]TCDD concentration are indicated in parenthesis.

tioning of TCDD between the media and the cells. The concentrations of TCDD necessary to induce given EROD responses can be expressed in terms of picomoles of TCDD per milligram of cellular protein for comparison across all treatments (EC₅₀ = 0.025 pmol/mg, EC₁₀₀ = 0.135 pmol/mg). Because protein content of the wells is linear with respect to cell number (Hahn *et al.*, 1996), TCDD doses shown are proportional to the dose per individual cell.

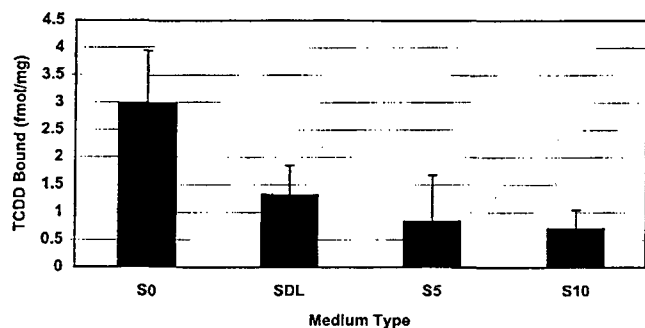


FIG. 3. Effect of serum on specific binding of TCDD in PLHC-1 cells. Cells were fed the indicated medium immediately prior to treatment with 0.18 nM [³H]TCDD in the presence or absence of 200-fold molar excess TCDF. Specific binding was determined by the whole-cell filtration assay of Dold and Greenlee (1990). Specific binding of TCDD is reported as femtomoles of TCDD per milligram of total cellular protein. Points are means \pm SE of three specific binding determinations.

Culture Medium Composition Alters the Relative Potency of HAH

We wished to determine if serum reduces the uptake of other HAH to the same degree that it does TCDD. PLHC-1 cells were exposed to each of three coplanar PCBs in medium with or without serum, and EROD activity was assayed 24 h later (Fig. 5). The EC₅₀ values for the responses are shown in Table 3. For each compound, the EC₅₀ in S₀ was lower than that in S₁₀. The differences ranged from about 20-fold for TCDD to about 2000-fold for PCB 77, although there is substantial uncertainty in the latter value, because a precise EC₅₀ is difficult to obtain for this compound in S₁₀ (e.g., Hahn *et al.*, 1996).

As the potency of EROD induction by PCB 77 in PLHC-1 cells is quite variable and the efficacy of EROD induction was much lower for both PCBs 77 and 169, levels of CYP1A protein were analyzed more directly using an ELISA. Cells were treated as for the EROD assay, but were fixed and analyzed for CYP1A content using the monoclonal antibody 1-12-3, as described in Materials and Methods (Fig. 6).

As with EROD induction, EC₅₀ values for ELISA-measured CYP1A induction (Table 3) were consistently higher in S₁₀. However, the magnitudes of the increases (the ratio in the final column of Table 3) were 5- to 10-fold smaller with PCBs 77 and 169 for CYP1A protein as compared to EROD. The EC₅₀ for induction of CYP1A protein was greater than the EC₅₀ for EROD induction in all treatments, in agreement with our previous results (Hahn *et al.*, 1996).

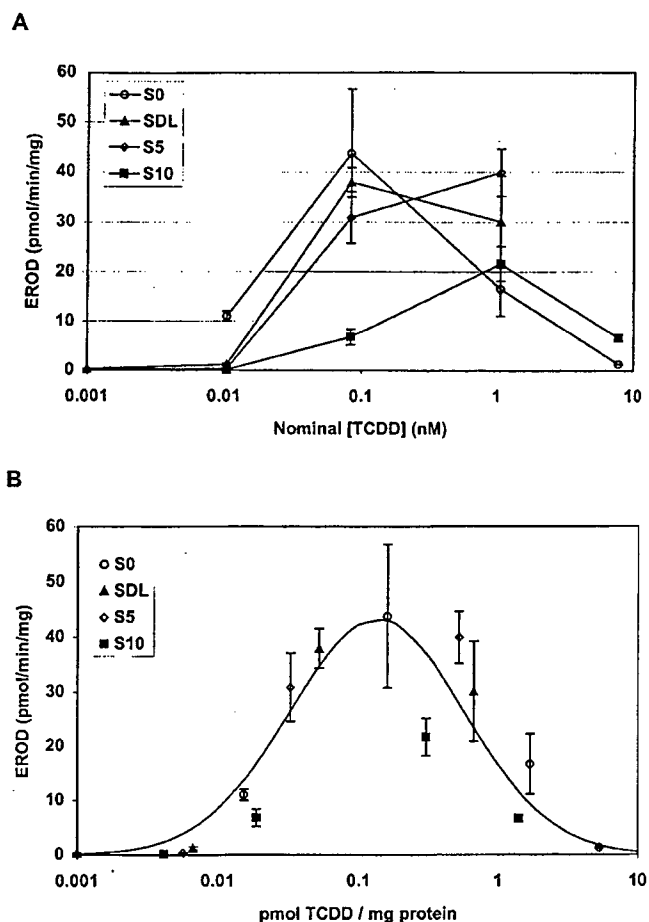


FIG. 4. TCDD uptake and EROD induction. (A) EROD rates versus nominal TCDD concentration in medium. Cells were fed and treated as in Figure 2B. EROD activity was measured 24 h later. The 0.001 nM nominal TCDD concentration represents treatment with DMSO alone. Points are means \pm SE of four wells. (B) The EROD rates in (A) plotted against cell-associated TCDD determined as in Figure 2B. TCDD concentrations are expressed as picomoles of cell-associated TCDD per average milligram cellular protein for each treatment. Average cellular protein contents for the medium treatments were 92, 116, 116 and 134 μ g per well for S_0 , S_{DL} , S_5 , and S_{10} , respectively. The modified Gaussian fit to the EROD data is plotted ($EC_{50} = 0.025$ pmol/mg, $EC_{100} = 0.135$ pmol/mg).

The ELISA as performed provides only a relative measure of CYP1A protein content, but the range of values produced and the pattern of induction by TCDD in S_{10} closely parallel those previously obtained by Western blot (Hahn *et al.*, 1996). This indicates that the response as measured in this assay can be correlated with the values from a more quantitative approach. Furthermore, maximal levels of detected fluorescence from the ELISA assay were similar among all the treatments, indicating that the maximally induced level of CYP1A is similar among the four compounds, regardless of medium used.

The EC_{50} s for the EROD and ELISA assays were used to calculate relative potencies for the four compounds within each medium treatment (Table 4). Relative potencies as determined by

EROD assay for the three PCBs were significantly lower in S_{10} than in S_0 . In contrast, when CYP1A induction was measured by ELISA, there were neither consistent nor significant differences in relative potencies determined with cells in the two media.

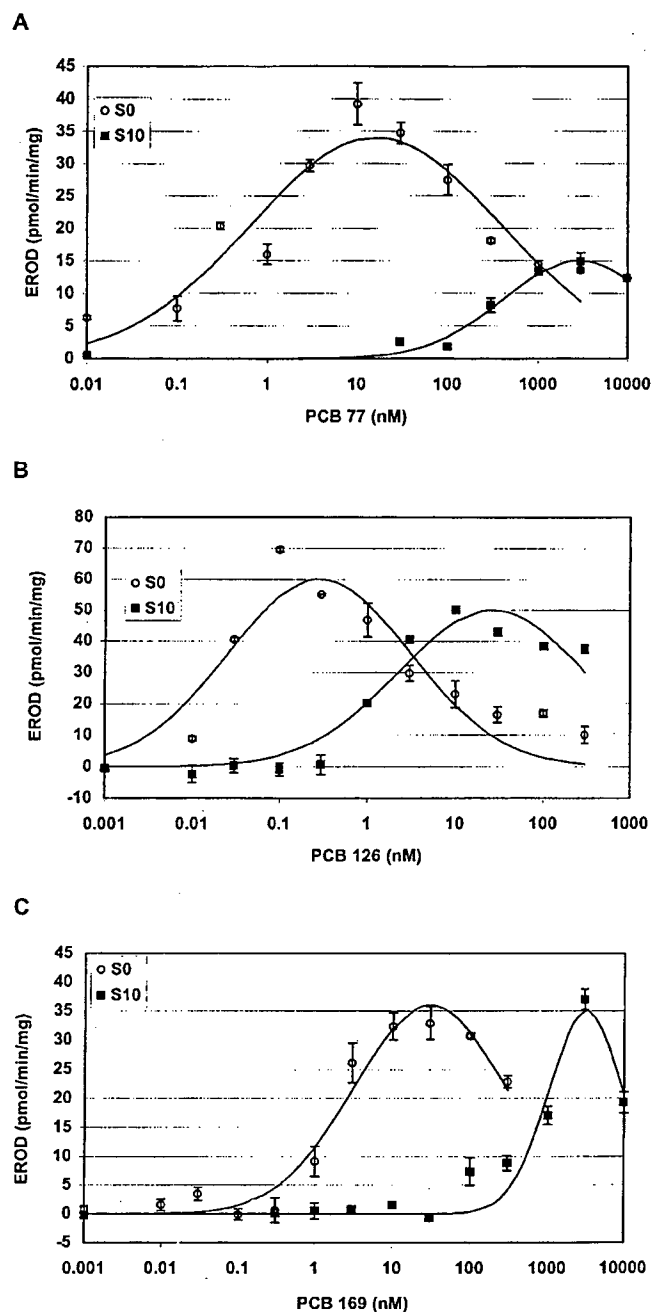


FIG. 5. Effect of serum on potency for EROD induction by coplanar PCBs. Cells were treated and assayed as in Figure 1, except that treatment was with (A) PCB 77, (B) PCB 126, or (C) PCB 169. The lowest concentration in each panel represents treatment with DMSO alone. Points are means \pm SE of four wells. The modified Gaussian fits to these data are plotted.

TABLE 3
Effect of Serum on CYP1A Induction EC50s Measured by EROD and ELISA for TCDD and Three Coplanar PCBs

	EROD EC50 (nM) ^a			ELISA EC50 (nM) ^a		
	S ₀	S ₁₀	Ratio of EC50s ^b	S ₀	S ₁₀	Ratio of EC50s ^b
TCDD	0.016 ± 0.004	0.33 ± 0.10	21	0.021 ± 0.003	1.2 ± 0.3	57
PCB 169	1.58 ± 0.43	246 ± 102	160	38 ± 5	1400 ± 148	37
PCB 126	0.029 ± 0.004	0.99 ± 0.18	35	0.24 ± 0.12	4.4 ± 2.7	19
PCB 77	0.73 ± 0.30	1500 ± 550	2000	13 ± 5	2200 ± 82	170

^a EC50s were determined from modified Gaussian functions for EROD dose responses and from logistic functions for ELISAs. Values are means ± SE of three or four replicate determinations, such as those shown in Figures 1, 5, and 6.

^b Ratio = EC50(S₁₀) / EC50(S₀) for each compound and assay.

DISCUSSION

This series of experiments demonstrates a reduction in HAH uptake by PLHC-1 cells when bovine serum is included in the culture medium. This in turn leads to decreased occupancy of the AHR and an apparent decrease in the CYP1A induction

potency of the compounds. Furthermore, the magnitude of this decrease is not the same among the HAH studied; this may lead to changes in relative potencies for EROD and CYP1A induction among the compounds. Serum will likely have a similar effect on the uptake of other hydrophobic chemicals.

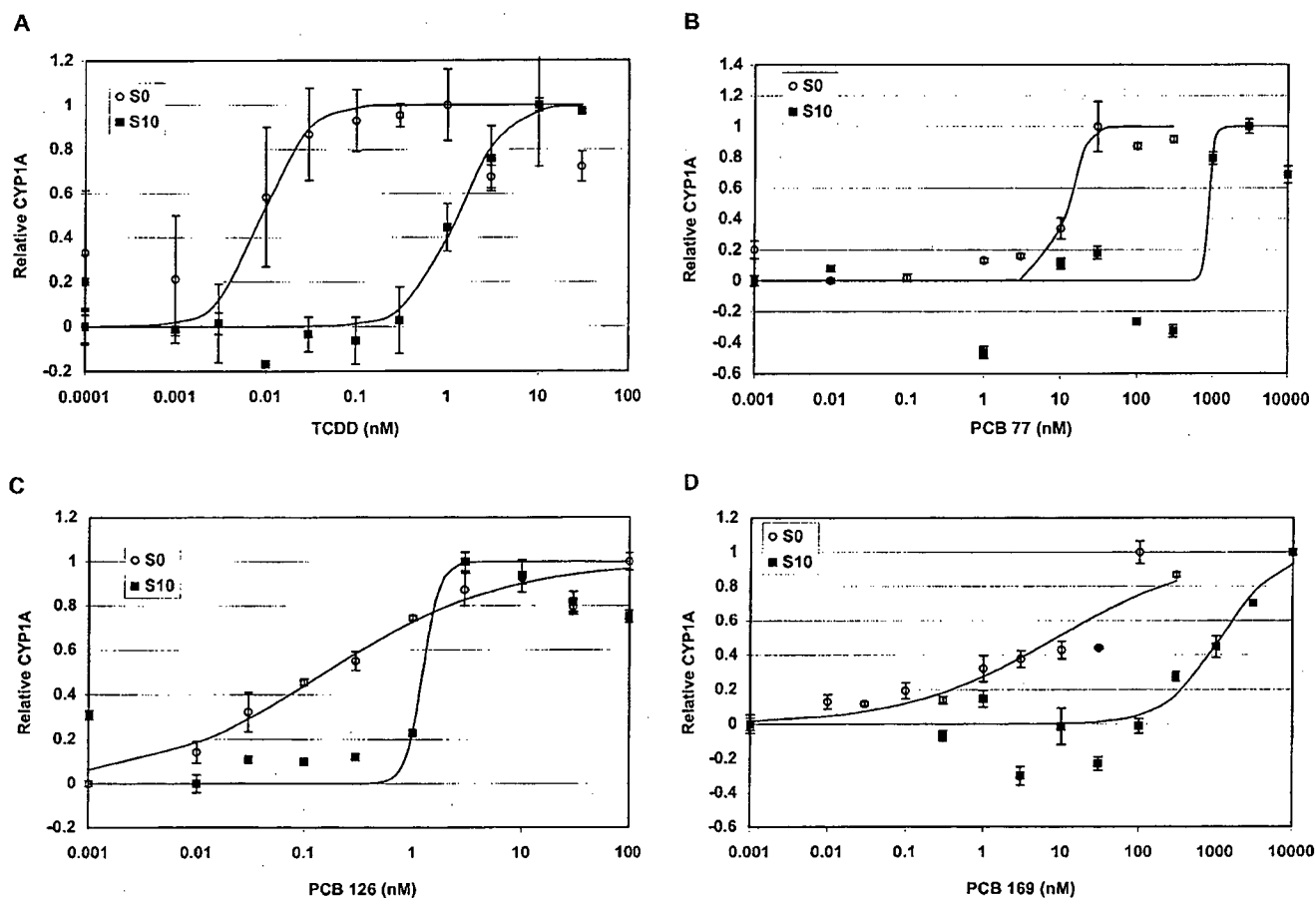


FIG. 6. Effect of serum on potency of CYP1A induction by HAH. Cells were treated as in Figures 1 and 5. CYP1A content was measured 24 h later by ELISA. The compounds used were (A) TCDD, (B) PCB 77, (C) PCB 126, or (D) PCB 169. The lowest concentration in each panel represents treatment with DMSO alone. Points are means ± SE of four wells. The logistic fits to these data are plotted.

TABLE 4
Effect of Serum on Determination of Induction Relative Potencies

	EROD Relative Potency ^a			ELISA Relative Potency ^a		
	S ₀	S ₁₀	Ratio ^b	S ₀	S ₁₀	Ratio ^b
TCDD	1	1	1	1	1	1
PCB 169	0.0075 ± 0.0018	0.0016 ± 0.0010	0.21 ^c	0.0016 ± 0.0008	0.00049 ± 0.00009	0.31
PCB 126	0.67 ± 0.18	0.12 ± 0.02	0.18 ^c	0.031 ± 0.004	0.075 ± 0.041	2.4
PCB 77	0.017 ± 0.005	0.00040 ± 0.00013	0.02 ^c	0.0016 ± 0.0008	0.00054 ± 0.00012	0.34

^a Relative potencies were determined for each medium treatment and assay by dividing the EC50 for TCDD by the EC50 for each PCB. Values are means ± SE of three or four replicate determinations.

^b Ratio = relative potency (S₁₀) / relative potency (S₀) for each compound and assay.

^c Indicates relative potency in 10% serum is significantly lower than relative potency in 0% serum ($p < 0.05$) by one-tailed paired *t*-test. Relative potencies from each experiment were paired for the analysis.

HAH Partitioning in a Multiwell Plate

Our measurements of TCDD partitioning demonstrate that the majority of the compound remains in the medium when serum is present. Thus, small changes in medium composition could have significant effects on the amount of compound that enters the cells. The fraction of total TCDD associated with the polystyrene wells was approximately equal to that found in the cells, suggesting that the composition of the chamber used for treatment also could affect the amount of compound that reaches the cells.

The percentage of total TCDD associated with the PLHC-1 cells was lowest at the highest nominal concentration of TCDD (10 nM), regardless of the medium used for treatment (Table 2). Also, at 10 nM TCDD, the fraction associated with the well walls increased, perhaps because at this concentration the cells were saturated with TCDD, and the compound was diffusing through the basal membrane of the cells to the floor of the well. Reduced diffusion at low TCDD concentrations is consistent with the finding of Yu *et al.*, (1997) that H4IIE cells apparently reduced sorption of PCB 77 to the floor of culture plates. That same study also found that a majority of PCB 77 (~75%) remained in the medium, which was supplemented with 15% FBS. They found no effect of carrier (isooctane vs. DMSO) on the fraction of the compound associated with the cells, which was at most 5%. Uptake studies with radiolabeled PCB77 have demonstrated similarly low levels associated with PLHC-1 cells (A. Patel and M. E. Hahn, unpublished results), suggesting that HAH partitioning is consistent between these two cell types and their media. In contrast, Schirmer *et al.*, (1997) found that the presence of 10% FBS in culture medium greatly altered the solubility of fluoranthene but did not significantly change the amount of that compound associated with cells from two fish lines.

AHR Occupancy and CYP1A Induction

The magnitude of the effect of serum on AHR occupancy was nearly identical to the difference in uptake of TCDD by

cells (compare Fig. 3 and the 0.1 nM TCDD group in Fig. 2B). This supports a direct relationship between the amount of compound associated with the cells and the amount bound by the AHR when the concentration of TCDD is sufficiently below the amount required for receptor saturation. The latter condition is satisfied here, as the concentration used was less than the K_D for TCDD binding to the AHR ($K_D = 0.8$ nM in S₀; Hestermann *et al.*, in preparation).

However, comparison of receptor occupancy and induction of EROD or CYP1A does not reveal a direct relationship like that occurring between TCDD uptake and receptor occupancy. There was a 4-fold increase in receptor occupancy in cells in S₀ rather than S₁₀ medium, but a much larger increase in CYP1A content (compare Fig. 3 with the 0.1 nM nominal TCDD concentration in Figs. 1 and 6A). This is most likely the result of a nonlinear occupancy-response relationship (also known as "spare receptors" or "receptor reserve") (Kenakin, 1999) for TCDD and the AHR in these cells. Under such conditions, submaximal receptor occupancy will produce maximal cell response, so that small changes in occupancy would produce much larger changes in downstream responses. We are pursuing the precise nature of this relationship in the PLHC-1 cell line.

Relative potencies of the three coplanar PCBs determined in S₀ were significantly higher than those determined in S₁₀ for EROD response but not CYP1A protein induction. This suggests that the presence of serum has an effect on CYP1A catalytic activity that is separate from its effect on induction via the AHR. The biphasic dose-response relationships typical of EROD induction are a result of the balance between CYP1A induction and competitive inhibition of catalytic activity by the inducer at higher concentrations (Gooch *et al.*, 1989; Hahn *et al.*, 1993; Petrusis and Bunce, 1999). Inhibition lowers EROD induction EC50s relative to EC50s for induction of CYP1A protein, and thereby increases the apparent relative potency for the EROD response (Hahn *et al.*, 1996). It therefore seems likely that serum influences the inhibitory effect of the induc-

ing compounds. Alternatively, there may be serum components that alter EROD activity in PLHC-1 cells by another mechanism.

Implications of Reduced Uptake

Perhaps the greatest potential for error in interpretation of *in vitro* bioassay data suggested by our results is in comparison of induction EC₅₀s and relative potencies among cell lines. Cell lines vary widely in culture medium contents. Serum may be absent or present at concentrations of up to 20%, and may come from a variety of animals and different developmental stages. Based on our results, such variations in media composition will affect cellular uptake of HAH and thus measured CYP1A induction potencies. Differences in potencies thus might incorrectly be attributed to mechanistic differences in CYP1A induction among the cell types and lead to false conclusions about relative sensitivities of the cells to the HAH in question.

One solution to this potential problem is to treat different cells in a single medium. Serum-free medium is the best candidate, as variations in composition among the chemically defined basal media (e.g., MEM, DMEM, RPMI-1640, F12) should have a negligible effect on bioavailability. Using serum-free medium also allows the greatest sensitivity in response to inducing compounds. The ability of each cell type to respond to HAH in serum-free medium should be determined, because serum withdrawal greatly reduces AHR content in Swiss 3T3 cells (Vaziri *et al.*, 1996) and can abrogate CYP1A induction in PLHC-1 cells after 48 h (Hestermann *et al.*, unpublished data). As noted previously, the presence of serum also affects the levels of cytochromes P450 in some cultured cells (Doostdar *et al.*, 1991; Doostdar *et al.*, 1988; Hammond and Fry, 1992; Turner and Pitot, 1989).

PLHC-1 cells have recently been adapted to long-term culture in media with serum replacements (Ultra-Culture, CPSR-1, and TurboDoma; Ackermann and Fent, 1998), providing promise for their future use in a chemically defined medium. Such media should reduce the problems with lot-to-lot variability that can be encountered with serum; however, the serum replacements used still have a high protein and/or lipid content, which can be expected to reduce bioavailability as serum does. In addition, the ability of cells grown in these media to respond to HAH exposure has not been determined.

The effect of serum on bioavailability is also a concern for other assays involving uptake of hydrophobic compounds. The reduction in specific TCDD binding in the presence of 10% serum shown here is an example of such an assay. Serum composition also affects bioavailability of estrogenic compounds (Arnold *et al.*, 1996; Nagel *et al.*, 1997). This suggests that the effect of serum is a general one, and its magnitude should be determined for individual compounds. Comparisons of apparently anomalous results among assays performed in different laboratories or cell lines should take this factor into

account, and previous conclusions regarding extrapolation from cultured cells may require reexamination.

This report continues our work of establishing the utility and optimal conditions for use of PLHC-1 cells in studying the mechanisms of HAH action. It also establishes a framework for measuring other effects of culture medium composition on AHR signal transduction in these cells. Through continued use of this model we hope to gain a better understanding of the molecular mechanisms that ultimately result in HAH toxicity. By comparing the shared and distinct features of AHR signal transduction in a variety of taxa, we can also better approach questions of AHR function and evolution.

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REFERENCES

- Ackermann, G. E., and Fent, K. (1998). The adaptation of the permanent fish cell lines PLHC-1 and RTG-2 to FCS-free media results in similar growth rates compared to FCS-containing conditions. *Mar. Environ. Res.* **46**, 363-367.
- Arnold, S. F., Collins, B. M., Robinson, M. K., Guillette, L. J., Jr., and McLachlan, J. A. (1996). Differential interaction of natural and synthetic estrogens with extracellular binding proteins in a yeast estrogen screen. *Steroids* **61**, 642-646.
- Bruschweiler, B. J., Wurgler, F. E., and Fent, K. (1996). An ELISA assay for cytochrome P4501A in fish liver cells. *Environ. Toxicol. Chem.* **15**, 592-596.
- Clemons, J. H., Lee, L. E. J., Myers, C. R., Dixon, D. G., and Bols, N. C. (1996). Cytochrome P4501A1 induction by polychlorinated biphenyls (PCBs) in liver cell lines from rat and trout and the derivation of toxic equivalency factors (TEFs). *Can. J. Fish. Aquat. Sci.* **53**, 1177-1185.
- Dold, K. M., and Greenlee, W. F. (1990). Filtration assay for quantitation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) specific binding to whole cells in culture. *Anal. Biochem.* **184**, 67-73.
- Doostdar, H., Burke, M. D., Melvin, W. T., and Grant, M. H. (1991). The effects of dimethylsulphoxide and 5-aminolaevulinic acid on the activities of cytochrome P450-dependent mixed function oxidase and UDP-glucuronosyl transferase activities in human Hep G2 hepatoma cells. *Biochem. Pharmacol.* **42**, 1307-1313.
- Doostdar, H., Duthie, S. J., Burke, M. D., Melvin, W. T., and Grant, M. H. (1988). The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived Hep G2 cell line. *FEBS Lett.* **241**, 15-18.
- Drenth, H. J., Bouwman, C. A., Seinen, W., and Van den Berg, M. (1998). Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3. *Toxicol. Appl. Pharmacol.* **148**, 50-55.

- Gooch, J. W., Elskus, A. A., Kloepper-Sams, P. J., Hahn, M. E., and Stegeman, J. J. (1989). 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.
- Hahn, M. E., and Chandran, K. (1996). Uroporphyrin accumulation associated with cytochrome P4501A 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.
- Hahn, M. E., Lamb, T. M., Schultz, M. E., Smolowitz, R. M., and Stegeman, J. J. (1993). 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.
- Hahn, M. E., Woodward, B. L., Stegeman, J. J., and Kennedy, S. W. (1996). Rapid assessment of induced cytochrome P4501A (CYP1A) protein and catalytic activity in fish hepatoma cells grown in multi-well plates: Response to TCDD, TCDF, and two planar PCBs. *Environ. Toxicol. Chem.* **15**, 582-591.
- Hammond, A. H., and Fry, J. R. (1992). Effect of serum-free medium on cytochrome P450-dependent metabolism and toxicity in rat cultured hepatocytes. *Biochem. Pharmacol.* **44**, 1461-1464.
- Hightower, L. E., and Renfro, J. L. (1988). Recent applications of fish cell culture to biomedical research. *J. Exp. Zool.* **248**, 290-302.
- Kenakin, T. (1999). *Pharmacologic Analysis of Drug-Receptor Interactions*. CRC/Raven Press, New York.
- Kennedy, S. W., Jones, S. P., and Bastien, L. J. (1995). 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.
- Kennedy, S. W., Lorenzen, A., James, C. A., and Collins, B. T. (1993). Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multi-well plate reader. *Anal. Biochem.* **211**, 102-112.
- Kennedy, S. W., Lorenzen, A., Jones, S. P., Hahn, M. E., and Stegeman, J. J. (1996a). Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **141**, 214-230.
- Kennedy, S. W., Lorenzen, A., and Norstrom, R. J. (1996b). 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.
- Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M., and Welshons, W. V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ. Health Perspect.* **105**, 70-76.
- Park, S. S., Miller, H., Klotz, A. V., Kloepper-Sams, P. J., Stegeman, J. J., and Gelboin, H. V. (1986). 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.
- Petruelis, J. R., and Bunce, N. J. (1999). Competitive inhibition by inducer as a confounding factor in the use of the ethoxyresorufin-O-deethylase (EROD) assay to estimate exposure to dioxin-like compounds. *Toxicol. Lett.* **105**, 251-60.
- Safe, S. (1984). Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. *CRC Crit. Rev. Toxicol.* **13**, 319-395.
- Safe, S. (1987). Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. *Chemosphere* **16**, 791-802.
- Sawyer, T., and Safe, S. (1982). PCB isomers and congeners: induction of aryl hydrocarbon hydroxylase and ethoxyresorufin O-deethylase enzyme activities in rat hepatoma cells. *Toxicol. Lett.* **13**, 87-94.
- Schirmer, K., Chan, A. G. J., Greenberg, B. M., Dixon, D. G., and Bols, N. C. (1997). Methodology for demonstrating and measuring the phototoxicity of fluoranthene to fish cells in culture. *Toxicol. In Vitro* **11**, 107-119.
- Tillitt, D. E., Giesy, J. P., and Ankley, G. T. (1991). Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ. Sci. Technol.* **25**, 87-92.
- Turner, N. A., and Pitot, H. C. (1989). Dependence of the induction of cytochrome P-450 by phenobarbital in primary cultures of adult rat hepatocytes on the composition of the culture medium. *Biochem. Pharmacol.* **38**, 2247-2251.
- van den Berg, M., Birnbaum, L., Bosveld, A. T. C., Brunström, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X. R. v., Liem, A. K. D., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998). Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, and PCDFs for humans and wildlife. *Environ. Health Perspect.* **106**, 775-792.
- Vaziri, C., Schneider, A., Sherr, D. H., and Faller, D. V. (1996). Expression of the aryl hydrocarbon receptor is regulated by serum and mitogenic growth factors in murine 3T3 fibroblasts. *J. Biol. Chem.* **271**, 25921-25927.
- Yu, K. O., Fisher, J. W., Burton, G. A., Jr., and Tillitt, D. E. (1997). Carrier effects of dosing the H4IIE cells with 3,3',4,4'-tetrachlorobiphenyl (PCB77) in dimethylsulfoxide or isoctane. *Chemosphere* **35**, 895-904.