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 (Poecilopsis 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 \[^{3}H\]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 $EC_{50,\text{ serum}} / EC_{50,\text{no serum}}$ 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 (Clemens 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 (Drent 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 Piotot, 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-
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 multwell 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 ([^1]H)TCDD, stated purity ≥97%, specific activity 27 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS). Its radiolabeled 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-tetrachlorodibenzo furan (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, 0.015% Na2HPO4, 0.025% 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[^1]HJTCDD 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 × 10^6 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 (FPS; 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 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 × 10^5 cells in 1 ml culture medium per well. With the exception of the delipidated serum and BFS, 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 time to over this period; Hahn et al., 1996) with the addition of 150 µg 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[^1]H]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[^1]H]TCDD in PLHC-1 cells was measured by a whole-cell filtration assay (Gold 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[^1]H]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 prewetted 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 Milipore 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
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 μg/ml; Park et al., 1986), was then added in 100 μl blocking solution for 1 h. After three washing steps with PBS, 100 μl 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 μl substrate solution (100 μM Amplex Red, 100 μM H2O2 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 (S0) than in medium with 10% calf serum (S10; Fig. 1). In this experiment, cells were grown in S10 that was replaced by either S0 or S10 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 S0 than in cells treated in S10 (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 S10 and 5-fold higher than in S0 (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: S0, S10, 10% delipidated serum (SDL), and 5% calf serum (S2). After growth in S0, 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 S0 or SDL than in S2 or S10.

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 S0 and lowest in those treated in S2. 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).

![Figure 1](https://example.com/figure1.png)

**FIG. 1.** Effect of serum on potency of EROD induction by TCDD. Cells were fed culture medium with (S0) or without (S2) 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 ± SE of four wells. The modified Gaussian fits to these data are plotted.

### Table 1

<table>
<thead>
<tr>
<th>Medium</th>
<th>EC50 (nM)*</th>
<th>EC100 (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>0.022</td>
<td>0.138</td>
</tr>
<tr>
<td>S10</td>
<td>0.474</td>
<td>2.590</td>
</tr>
</tbody>
</table>

*EC50 and EC100 are nominal TCDD concentrations producing 50% and 100% of maximal EROD induction, respectively.
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 S_0 supplemented with increasing concentrations of BSA were added (Figure 2C). Protein at concentrations near those in S_0 or S_10 (5–8 mg/ml according to the supplier) produced uptake identical to that in S_0, 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 S_0, 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 S_0 or S_10 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 S_0 and showed the same relationship among medium treatments as the fraction of TCDD associated with the cells, i.e., S_0 >> S_10 >> S_0 >> S_10. (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 S_0 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

<table>
<thead>
<tr>
<th>Medium treatment</th>
<th>Nominal TCDD concentration (nM)</th>
<th>Fraction of total TCDD associated with Medium</th>
<th>Cells*</th>
<th>Wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_0$</td>
<td>0.01</td>
<td>37%</td>
<td>36% (1.4)</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>33%</td>
<td>47% (15)</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33%</td>
<td>44% (160)</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>28%</td>
<td>16% (460)</td>
<td>56%</td>
</tr>
<tr>
<td>$S_{10}$</td>
<td>0.01</td>
<td>72%</td>
<td>14% (0.75)</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>71%</td>
<td>18% (5.7)</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>71%</td>
<td>19% (78)</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>59%</td>
<td>9% (261)</td>
<td>33%</td>
</tr>
<tr>
<td>$S_1$</td>
<td>0.01</td>
<td>74%</td>
<td>15% (0.67)</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>83%</td>
<td>12% (3.6)</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>78%</td>
<td>17% (61)</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>74%</td>
<td>6% (180)</td>
<td>20%</td>
</tr>
<tr>
<td>$S_{10}$</td>
<td>0.01</td>
<td>79%</td>
<td>11% (0.56)</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>90%</td>
<td>7% (2.4)</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>86%</td>
<td>12% (45)</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>79%</td>
<td>5% (170)</td>
<td>16%</td>
</tr>
</tbody>
</table>

Note. PLHC-1 cells were grown in $S_0$ 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.

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

Partitioning 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 ($EC_{50} = 0.025$ pmol/mg, $EC_{100} = 0.135$ 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.

Cultivation 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 $S_0$ was lower than that in $S_{10}$. 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 $S_{10}$ (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 $S_{10}$. 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. 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 ± SE of three specific binding determinations.](image-url)
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. 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 ± 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 \( \mu \)g per well for \( S_0, S_5, S_5 \), and \( S_{10} \), respectively. The modified Gaussian fit to the EROD data is plotted (EC50 = 0.025 pmol/mg, EC100 = 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 paralleled 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 EC50s 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

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 ± SE of four wells. The modified Gaussian fits to these data are plotted.
TABLE 3
Effect of Serum on CYPlA Induction EC50s Measured by EROD and ELISA for TCDD and Three Coplanar PCBs

<table>
<thead>
<tr>
<th>Compound</th>
<th>EROD EC50 (nM)</th>
<th>ELISA EC50 (nM)</th>
<th>Ratio of EC50s&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EROD EC50 (nM)</th>
<th>ELISA EC50 (nM)</th>
<th>Ratio of EC50s&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>0.016 ± 0.004</td>
<td>0.33 ± 0.10</td>
<td>21</td>
<td>0.021 ± 0.003</td>
<td>1.2 ± 0.3</td>
<td>57</td>
</tr>
<tr>
<td>PCB 169</td>
<td>1.58 ± 0.43</td>
<td>246 ± 102</td>
<td>160</td>
<td>38 ± 5</td>
<td>1400 ± 148</td>
<td>37</td>
</tr>
<tr>
<td>PCB 126</td>
<td>0.029 ± 0.004</td>
<td>0.99 ± 0.18</td>
<td>35</td>
<td>0.24 ± 12</td>
<td>4.4 ± 2.7</td>
<td>19</td>
</tr>
<tr>
<td>PCB 77</td>
<td>0.73 ± 0.30</td>
<td>1500 ± 550</td>
<td>2000</td>
<td>13 ± 5</td>
<td>2200 ± 82</td>
<td>170</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

**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 CYPlA 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 CYPlA 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 CYPlA induction by HAH. Cells were treated as in Figures 1 and 5. CYPlA 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.
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 H4IE 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 (isoctane 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 fluoroanthene 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 \( K_s \) for TCDD binding to the AHR (\( K_s = 0.8 \) nM in \( S_0 \); 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 \( S_0 \) rather than \( S_{10} \) 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”) 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 \( S_0 \) were significantly higher than those determined in \( S_{10} \) 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; Petrulis 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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