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ETHOXYRESORUFIN-*O*-DEETHYLASE INDUCTION POTENCY OF POLYCHLORINATED DIPHENYL ETHERS IN H4IIE RAT HEPATOMA CELLS

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Abstract—Polychlorinated diphenyl ethers (PCDEs) are structurally similar to polychlorinated biphenyls (PCBs), and some PCDE congeners have been reported to cause toxic responses similar to those caused by some of the non-*ortho*-substituted PCBs, which are mediated by the aryl hydrocarbon receptor (AhR). Twenty-nine PCDEs were tested for their potency as AhR agonists relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) by measuring their ability to induce the cytochrome P-450 1A1-associated enzyme activity, ethoxyresorufin-*O*-deethylase (EROD), in the H4IIE rat hepatoma cell bioassay. All PCDE congeners tested were found to be inactive as EROD inducers except for PCDE 156, which was a weak EROD inducer with a 2,3,7,8-TCDD equivalency factor of about 1.2×10^{-5}. During this study we determined that small amounts of polychlorinated dibenzofurans (PCDFs) that occurred as impurities in the PCDE preparations were the cause of the apparent EROD induction initially measured in our experiments. Once the PCDF impurities were removed by purification on florisil, little or no activity could be attributed to the PCDEs.

Keywords—Polychlorinated diphenyl ethers H4IIE bioassay EROD Ah receptor

INTRODUCTION

Polychlorinated diphenyl ethers (PCDEs) are structurally related to polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs), which are considered environmentally harmful compounds due to their persistence, potential for biomagnification, and toxicity. In the early 1970s, chlorophenol formulations were observed to contain relatively high concentrations of PCDEs [1]. Polychlorinated diphenyl ethers have been reported to occur as impurities in some chlorinated phenol formulations at concentrations between 100 and 1,000 mg/kg of formulation [2]. The production and/or use of chlorophenol-based wood preservatives appears to be a major source of environmental PCDE contamination. Relatively high concentrations of PCDEs were measured in fish and sediment below a factory on the Kymijoki River, Finland, that formerly produced a wood preservative. These abnormally high concentrations were established to have originated from leakages of this formulation by demonstrating that the pattern of relative concentrations of PCDE in the environmental samples were similar to those in the formulation [3]. Combustion is another source of PCDEs released into the environment [4]. Polychlorinated diphenyl ether congeners have also been detected in fly ash [4,5]. The occurrence of PCDEs in environmental samples supports the conclusion that they are persistent and can bioaccumulate. Polychlorinated diphenyl ethers have been detected in mussels and clams [6], fish [3–5,7–11], seals [3; J. Koistinen et al., unpublished data], birds [12–14], sediment [3], and humans [15–18] in North America and Scandinavia.

Findings of structural similarities between PCDEs and PCBs have led to the suggestion that current concentrations of PCDEs in the environment may be contributing to adverse effects similar to those caused by PCDDs, PCDFs, and certain non-*ortho*-PCBs [19]. If the published toxic potencies of certain PCDEs relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) are correct, current concentrations of PCDEs in some environmental matrices could contribute significantly to the total 2,3,7,8-TCDD equivalents measured.

Neither the environmental fate nor the toxicological properties of PCDEs has been studied in as much detail as those of structurally similar PCDDs, PCDFs, or PCBs. Polychlorinated diphenyl ethers accumulate in fish in a manner similar to that of PCBs [20]. The uptake and excretion of PCDEs by juvenile Atlantic salmon were similar to those of PCBs, but PCDEs were more persistent [20]. Also of environmental significance is the fact that PCDEs are possible precursors of the highly toxic PCDDs and PCDFs, e.g., photochemical reactions might convert PCDEs to PCDFs [21].

The toxicity of a small number of PCDEs has been studied in trout and rat [22,23]. The acute toxicity of these PCDEs to trout occurred at about 1 mg/L [22]. In rats, 2,2′,4,4′-tetrachlorinated diphenyl ether (PCDE 47), 2,2′,4,4′,5,5′-hexachlorinated diphenyl ether (PCDE 153), and 2,2′,3,4,4′,6,6′-heptachlorinated diphenyl ether (PCDE 184) caused increased liver weights at 500 mg/kg [23]. Exposure of rats to heptachlorinated diphenyl ether caused decreased food consumption at 500 mg/kg, and all three congeners produced histological changes in liver and thyroid. Suppression of growth or food consumption was not observed with oral doses from 0.04 to 40 mg/kg per d. Polychlorinated diphenyl ethers have been reported to be immunotoxic to mice [24], but in another study a PCDE fraction isolated from an analytical-grade pentachlorophenol was not immunosupressive, whereas the PCDD/PCDF fraction was [25].

Polychlorinated diphenyl ethers have been reported to induce hepatic monooxygenases in fish and rodents [23,24,26–28] and

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Table 1. Polychlorinated diphenylether (PCDE) preparations examined for ethoxyresorufin-*O*-deethylase (EROD) induction potential in H4IIE cells

Structure no.	Substitution	Ability to induce EROD activity: Before purification	Ability to induce EROD activity: After purification
PCDE 47	2,2',4,4'–	–	–
PCDE 66	2,3',4,4'–	–	–
PCDE 77	3,3',4,4'–	+	–
PCDE 85	2,2',3,4,4'–	–	–
PCDE 99	2,2',4,4',5–	–	–
PCDE 105	2,3,3',4,4'–	+	–
PCDE 118	2,3',4,4',5–	+	–
PCDE 126	3,3',4,4',5–	+	–
PCDE 128	2,2',3,3',4,4'–	–	–
PCDE 137	2,2',3,4,4',5–	+	–
PCDE 138	2,2',3,4,4',5'–	+	–
PCDE 140	2,2',3,4,4',6'–	–	–
PCDE 147	2,2',3,4',5,6–	–	–
PCDE 153	2,2',4,4',5,5'–	+	–
PCDE 154	2,2',4,4',5,6'–	+	–
PCDE 156	2,3,3',4,4',5–	+	+
PCDE 157	2,3,3',4,4',5'–	+	–
PCDE 167	2,3',4,4',5,5'–	+	–
PCDE 170	2,2',3,3',4,4',5–	+	–
PCDE 180	2,2',3,4,4',5,5'–	+	–
PCDE 181	2,2',3,4,4',5,6–	–	–
PCDE 182	2,2',3,4,4',5,6'–	–	–
PCDE 190	2,3,3',4,4',5,6–	+	–
PCDE 194	2,2',3,3',4,4',5,5'–	+	–
PCDE 195	2,2',3,3',4,4',5,6–	+	–
PCDE 196	2,2',3,3',4,4',5',6–	–	–
PCDE 197	2,2',3,3',4,4',6,6'–	–	–
PCDE 203	2,2',3,4,4',5,5',6–	–	–
PCDE 206	2,2',3,3',4,4',5,5',6–	+	–

have been classified as either phenobarbital-, 3-methylcholanthrene-, or mixed-type inducers [26]. A number of penta- and hexachlorinated or more highly chlorinated PCDE congeners have been found to induce hepatic microsomal ethoxyresorufin-*O*-deethylase (EROD) activity in C57BL/6 mice and to a lesser extent in DBA/2 mice [24,29]. These findings indicate that an interaction of the PCDEs with the aryl hydrocarbon receptor (AhR) is responsible for the observed biological activity. The greater sensitivity of C57BL/6 mice to PCDEs is consistent with the greater sensitivity of this strain to known AhR agonists, such as 2,3,7,8-TCDD [30]. If PCDEs are indeed agonists for the AhR, they would be expected to have the ability to elicit toxic responses similar to those of PCDDs, PCDFs, and certain PCBs. To examine the hypothesis that some PCDEs are AhR agonists, 29 PCDE congeners were tested for their ability to induce EROD activity in H4IIE rat hepatoma cells. This bioassay system is a sensitive and reliable tool for assessing AhR-mediated biological activity of chlorinated compounds [31–32]. Non-, mono-, and di-*ortho*-substituted PCDE congeners were examined in order to test the structure–activity relationships proposed by other authors.

MATERIALS AND METHODS

PCDE congeners

Twenty-nine PCDE congeners were selected for study (Table 1). Selection of congeners for inclusion in the study was based on the fact that they were found in the environment or that they had previously been reported to be AhR-active. Polychlorinated diphenyl ethers were synthesized at the Department of Chemistry in the University of Jyväskylä, Finland. The method of synthesis and purification of the products was presented earlier [33].

Chemical analyses of the purity of PCDEs

The purity of PCDE congeners was determined by high-resolution gas chromatography (HRGC) with electron capture detection and HRGC–low-resolution mass spectrometry (HRGC–LRMS). The HRGC was performed with a Perkin-Elmer gas chromatograph with a DB-5 column (30 m × 0.250 mm inner diameter), and HRGC–LRMS was performed on a Hewlett-Packard 5972 Series Mass Selective Detector combined with a Hewlett-Packard 5890 Series II Plus Gas Chromatograph (with HP-5 column, 30 m × 0.250 mm). The PCDDs and PCDFs were analyzed from 10 ng/μl stock solutions of the PCDEs by injecting 2 μl splitlessly. U.S. Environmental Agency (EPA) 1613 standard was used as an external standard to quantify PCDDs and PCDFs in PCDEs. After florisil purification, purified PCDE standards were reanalyzed for PCDDs and PCDFs.

Florisil purification of PCDEs

The PCDDs and PCDFs were separated from PCDEs by a florisil column chromatographic method that had been previously developed for analyses of PCDE in environmental samples [11,14]. The PCDE standard (150–400 μl of 1 mg/ml solution) in hexane was introduced onto a 1-g column of florisil PR (60–100 mesh, 1.25% H_2O, Sigma, St. Louis, MO, USA), and PCDEs were eluted from the column by hexane. Fifteen milliliters of the eluent was collected, and then the PCDDs and PCDFs were eluted from the column with dichloromethane (12 ml).

H4IIE bioassays

The H4IIE bioassay procedure is presented in detail elsewhere [32]. Briefly, the H4IIE rat hepatoma cells were grown in 75-cm² flasks until cells were almost confluent. The cells were trypsinized and seeded in 96-well culture plates (250 μl cell suspension at 2.0 × 10^4 cells/ml). After 24 h, PCDEs at various dilutions in isooctane (5 μl) were added to each well. After dosing cells were incubated for 72 h and then analyzed for induction. EROD activity and protein content of each well was measured fluorometrically [32].

The potencies of the PCDEs relative to 2,3,7,8-TCDD were determined by measuring a standard 2,3,7,8-TCDD dose–response curve with each set of PCDE congeners. Resorufin and protein standards (bovine serum albumin) were analyzed simultaneously with each set of samples. Five concentrations and a blank (vehicle alone) were tested for each PCDE congener. Each concentration was repeated five times. Ethoxyresorufin-*O*-deethylase activities were expressed as picomoles resorufin per minute per milligram protein. Median effective doses (ED50s) were calculated by Woolf plot analysis. In addition to determining 2,3,7,8-TCDD standard dose-response curves with each experiment, the reliability and reproducibility of the assay was tested by measuring dose-response curves for PCBs 77 and 126.

RESULTS AND DISCUSSION

Prior to purification to remove PCDD and PCDF contaminants, a number PCDE congener preparations were inducers of EROD activity in H4IIE cells, while others were inactive (Table 1). According to preliminary experiments, PCDEs 77, 118, 138,

Table 2. Polychlorinated dibenzofuran (PCDF) impurities (ng/ml) in polychlorinated diphenylethers (PCDEs) (10 μg/ml)[a]

PCDE	77	105	118	126	137	138	153	154	156
2,3,7,8-Tetra-CDF	7	1	6	NA	1	–	0.2	–	0.4
1,2,3,7,8-Penta-CDF	–	–	–	–	0.7	0.5	–	–	40
2,3,4,7,8-Penta-CDF	–	–	2	–	–	3	–	–	–
1,2,3,4,7,8-Hexa-CDF	–	–	–	–	–	–	–	–	11
1,2,3,6,7,8-Hexa-CDF	–	–	–	2	–	–	–	–	–
1,2,3,7,8,9-Hexa-CDF	–	–	–	–	–	–	–	–	–
2,3,4,6,7,8-Hexa-CDF	–	–	–	–	–	2	–	2	–
2,3,7,8-Hepta-CDFs	–	–	–	–	–	–	–	–	–
Octa-CDF	–	–	–	–	–	–	–	–	–

PCDE	157	167	170	180	190	194	195	206
2,3,7,8-Tetra-CDF	–	0.6	–	–	–	–	3	–
1,2,3,7,8-Penta-CDF	NA	15	–	2	–	–	–	–
2,3,4,7,8-Penta-CDF	–	–	–	2	–	–	–	–
1,2,3,4,7,8-Hexa-CDF	–	–	–	2	20	–	–	1
1,2,3,6,7,8-Hexa-CDF	–	–	71	19	–	21	–	1
1,2,3,7,8,9-Hexa-CDF	–	–	–	–	9	–	–	–
2,3,4,6,7,8-Hexa-CDF	–	4	–	–	–	–	–	–
1,2,3,4,6,7,8-Hepta-CDF	–	–	–	–	–	4	6	–
1,2,3,4,7,8,9-Hepta-CDF	–	–	–	–	–	72	–	–
Octa-CDF	–	–	–	–	–	31	–	37

[a] NA = not analyzed; – = not detected (<0.2 for tetra-, <0.5 for penta-, <0.7 for hexa-, <1.0 for hepta-, and <2.0 ng/ml for octa-CDFs).

156, 167, 170, 180, and 194 were the most potent inducers of EROD activity (Table 1) and were comparable to PCB 77 in their potency. Since the relative potencies of the various PCDE preparations did not conform to structure–activity relationships analogous to those of PCBs, the possibility that impurities in the PCDE preparations were responsible for some or all of the EROD induction observed was investigated. Preparations of PCDEs that were initially active were analyzed for PCDD and PCDF impurities before repeating the induction experiments. Polychlorinated dibenzofuran impurities were detected in the original preparations of most of the PCDEs (Table 2). Polychlorinated dibenzo-*p*-dioxins were not detected above the detection limit of 3 pg/μl in a 10-ng/μl solution of any of the PCDE preparations. All PCDF impurities in the original PCDE preparations occurred at less than 1% by weight. However, based on the relative potencies of these PCDFs, it was determined that they could cause significant EROD induction at these levels because concentrated solutions (mg/ml) of PCDEs were tested in the H4IIE bioassay. After repurification, the concentrations of PCDF impurities in the PCDE preparations were less than the detection limit of the HRGC–LRMS method.

Florisil purification was effective in the separation of trace contaminants of PCDDs and PCDFs in the PCDE preparations, even though milligram amounts of PCDE were loaded onto the 1-g florisil columns. High-resolution gas chromatography–low-resolution mass spectrometry analyses verified that the PCDFs detected in the PCDE preparations before florisil purification eluted only in the second fraction (PCDDs, although not detected, would also elute in this fraction). The contents of PCDFs in the second fraction of florisil were similar to those of PCDFs in the unpurified PCDE preparations. Traces of PCDEs in the second fraction from the florisil column were less than 1%. As an example of the efficiency of the separation of PCDDs and PCDFs from PCDEs, the mass fragmentograms of hexachlorinated dibenzofuran impurities isolated from PCDE 156 are compared with those of hexachlorinated dibenzofuran traces in a purified PCDE 156 preparation (Fig. 1).

In the final set of induction experiments conducted with repurified preparations, most PCDEs were found to be inactive as EROD inducers in the H4IIE bioassay. When the separated PCDD and PCDF fractions were tested in the assay, they were shown to be responsible for the induction of EROD activity. An example of this is the case of PCDE 77 (Fig. 2). When the PCDF impurities were removed, the repurified PCDE 77 did not cause any EROD induction, but the fraction containing the PCDF impurities had the same potency as the unpurified preparation. After purification with florisil, only PCDE 156 remained weakly active (Fig. 3), with a TCDD equivalency factor (TEF) of 1.2×10^{-5}. Considering the loss of activity with all other congeners, the weak activity of PCDE 156 was most likely due to the presence of trace impurities.

The fact that relatively high concentrations of PCDE congeners were present in the active preparations before purification and that the activities of the trace contaminants in the presence and absence of PCDE were the same indicates that there was little interaction between the nonactive PCDEs and the active PCDF impurities. To further confirm this observation, the effect of PCDEs on the induction potency of TCDD was examined by coadministration of TCDD with either PCDE 77 or 153 (10 ng/μl). The presence of the PCDEs had no effect on the induction response caused by TCDD (Fig. 3). This is contrary to the effects of certain di-*ortho*-substituted PCB congeners and polychlorinated naphthalenes, which have been shown to antagonize the induction response by several AhR-active PCDD and PCB congeners [32,34]. Polychlorinated biphenyls 77 and 126, which were tested as additional reference substances in the H4IIE bioassay, had TEFs of 4.0×10^{-4} and 2.5×10^{-2}, respectively (Fig. 4), which are comparable to values reported previously for these congeners [32,35]. These substances were tested for their purity and did not contain PCDD and PCDF impurities at concentrations that could cause induction of EROD activity.

The results of the present study are different from those reported by others and suggest that PCDEs do not possess AhR-

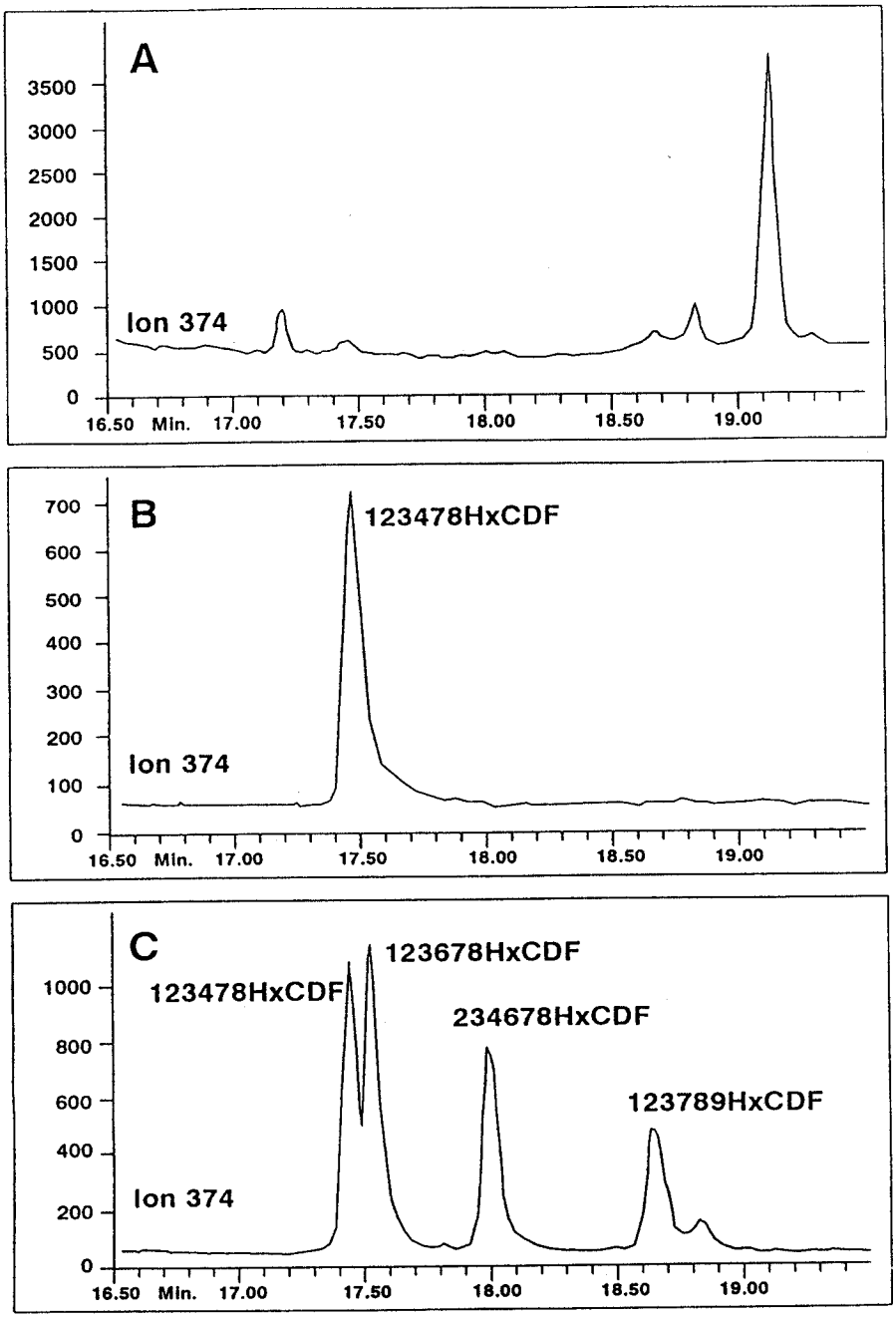

Fig. 1. Mass fragmentograms (hexachlorinated dibenzofurans) of fractions from the florisil purification of PCDE 156: (A) PCDE fraction, (B) PCDD/PCDF fraction, dilution 250-fold compared to A, and (C) PCDF standard. The observed peak (4 pg) of hexachlorinated dibenzofuran in B corresponds to an impurity of 0.1% by weight in the original PCDE fraction.

mediated biological activity. Results of previous studies on hepatic microsomal EROD induction in mice suggested certain AhR-related structure–activity relationships for the PCDEs, since AhR-mediated EROD induction potential was reduced when the number of *ortho* substituents was increased in a manner analogous to that of PCBs [24]. Polychlorinated diphenyl ether congeners with only one ortho-chlorine substituent were the most potent: PCDEs 118, 126, and 156 had the greatest EROD induction potencies and were the most immunotoxic. It is possible that the observed biological responses attributed to PCDEs in these previous studies are artifacts due to the presence of unknown quantities of PCDF impurities in the preparations used. In the present study, PCDEs 118, 126, and 156 were among the most potent inducers of EROD activity before purification on the florisil column but were found to be inactive once the PCDF impurities were removed. This result indicates that it is likely that the activities reported for these congeners in previous studies were due to the presence of PCDF impurities. Kerkvliet et al. [25] found that a PCDE fraction isolated from technical-grade pentachlorophenol (PCP) was not immunosuppressive but that the PCDD/PCDF fraction was active. Although a study recently reported that the immunotoxicity of several PCDE congeners in mice correlated with certain electronic properties of PCDEs [36], this structure–activity relationship is not reliable because of the limited availability of toxicity data on PCDEs and the confounding effect of possible contamination with PCDDs and PCDFs. Similarly to the PCDEs, the sulphur analogues of PCDEs, polychlorinated diphenyl sulphides, are

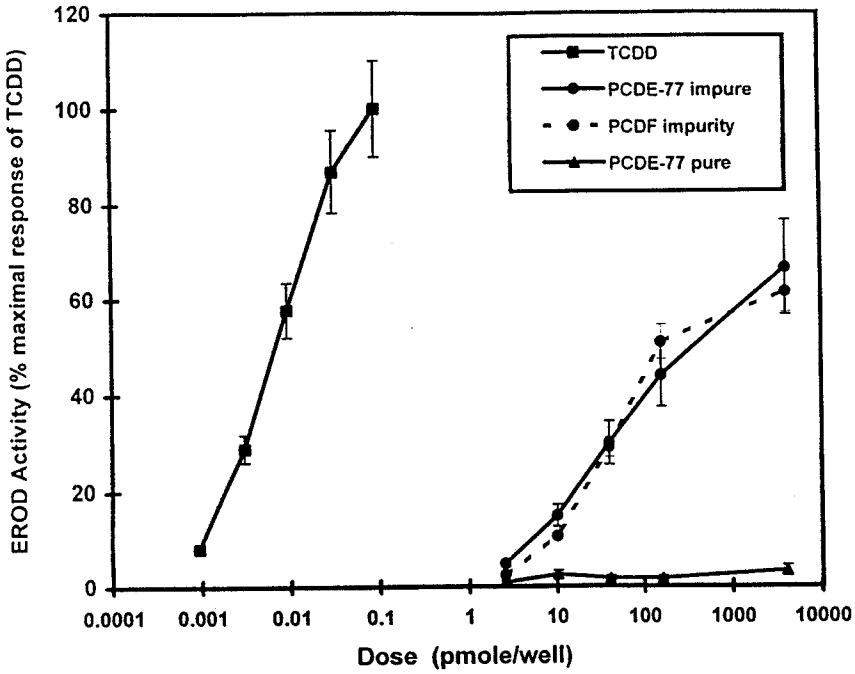

Fig. 2. Induction of EROD activity in H4IIE rat hepatoma cells by 2,3,7,8-TCDD, unpurified and florisil-purified PCDE 77, and the PCDD/PCDF impurity fraction isolated from PCDE 77. The impurity fraction was dosed to the cells at equivalent concentrations based on the amount of PCDE 77 originally present (before purification).

probably not very active as inducers of EROD activity because 3,3′,4,4′-tetrachlorodiphenyl sulfide (TCDS 77) did not cause any EROD or aryl hydrocarbon hydroxylase induction in Hepa-1 mouse hepatoma cells [37].

CONCLUSIONS

The EROD induction potencies of the 29 PCDEs in H4IIE rat hepatoma cells were found to be much less than those of analogous PCB congeners with the same substitution patterns. Any EROD induction caused by PCDE preparations initially was found to be due to the presence of trace amounts of PCDFs at concentrations high enough to account for all the observed EROD induction. After florisil purification of the preparations only PCDE 156 remained weakly active. It is felt that EROD induction and other biological responses, such as immunosuppression, that have been attributed to PCDEs by other authors

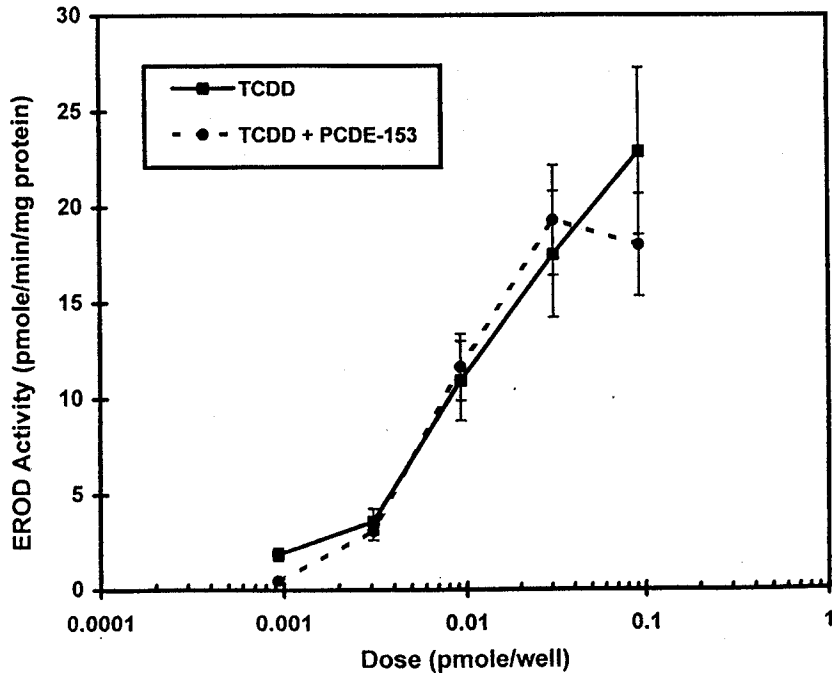

Fig. 3. Induction of EROD activity in H4IIE rat hepatoma cells by 2,3,7,8-TCDD alone and 2,3,7,8-TCDD in the presence of 65 ng/well PCDE 153.

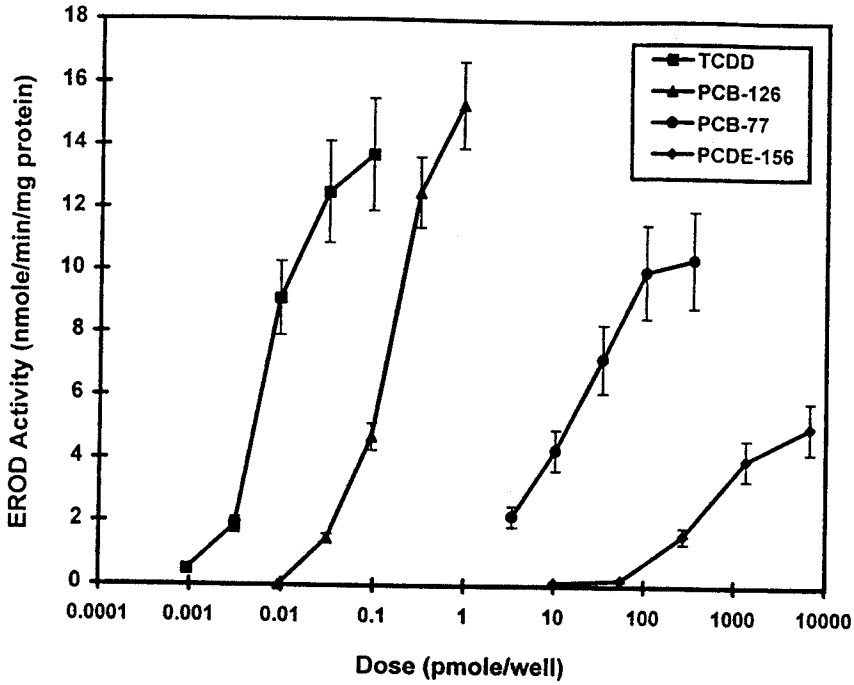

Fig. 4. Induction of EROD activity in H4IIE rat hepatoma cells by PCDE 156 (after florisil purification) compared to the induction by 2,3,7,8-TCDD, PCB 126, and PCB 77.

were due to the presence of trace quantities of PCDFs in their preparations. Based on their ability to cause EROD induction in the H4IIE assay, PCDEs are very weak AhR agonists. However, the relatively high concentrations of PCDEs that have been found in some environmental matrices remain of toxicological concern. They are persistent, bioaccumulate, may have toxicities via other mechanisms, and can be precursors for PCDDs and PCDFs under certain conditions.

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