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Maternal and developmental toxicity of polychlorinated diphenyl ethers (PCDEs) in Swiss-Webster mice and Sprague-Dawley rats

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Abstract

Polychlorinated diphenyl ethers (PCDEs) are industrial byproducts found in many ecosystems at low levels. PCDEs are not markedly toxic to adult rodents, but their developmental toxicity has not previously been examined. We evaluated the maternal and perinatal toxicity of nine PCDE congeners to outbred mice when compounds were administered from gestation day (GD) 6 through GD 15. 2,2′,4,4′,5,6′-hexaCDE and 2,3′,4′,6-tetraCDE decreased the number of pups born per female mated and the number of pups surviving per litter born. 2,2′,4,4′,5,5′-hexaCDE and 2,2′,4,5,6′-pentaCDE decreased the number of litters born per female mated, without decreasing postnatal survival. The other PCDEs did not decrease survival either pre- or postnatally. None of the PCDEs caused absence of Harderian glands in surviving offspring at the doses administered. Neither induction of cytochromes P450 nor tissue residues of individual congeners correlated well with developmental toxicity. Three PCDEs were also evaluated in outbred (Sprague-Dawley) rats: 2,2′,4,5,6′-pentaCDE and 2,3′,4′,6-tetraCDE, because of their toxicity to mice; 2,2′,4,4′,5,5′-hexaCDE, because it should exhibit PCB-like toxicity. Each congener was administered at three dose levels from GD 6 through GD 15. 2,2′,4,5,6′-pentaCDE decreased the number of litters born at 100 mg/kg/day, and the survival of pups in litters carried to term, at both 50 and 100 mg/kg per day. Postnatal weight gain was also reduced. In contrast to its action in mice, 2,3′,4′,6-tetraCDE decreased neither the numbers of litters born nor postnatal survival of rat offspring, but did suppress postnatal weight gain at least through PD 5. As in mice, induction of cytochromes P450 was not well correlated with the developmental toxicity of individual congeners. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

Chlorodiphenyl ethers (PCDEs, Fig. 1a) are unavoidable contaminants of the many chemicals synthesized from chlorinated phenols, and may constitute between 1 and 5% of technical trichlorophenol mixtures (Stanley et al., 1990). As a result, PCDEs are found in many rivers and streams in the US and Canada (Stafford, 1983). PCDEs are also produced by environmental perchlorination of industrial discharges that contain diphenyl ether (Becker et al., 1991), and have been identified as contaminants of incinerator waste (Paasivirta et al., 1986). PCDEs are environmentally persistent and bioaccumulate (Stafford, 1983). In the Great Lakes, PCDEs are found in fish at levels up to 8 ppm (Komsta et al., 1988), and induce liver enzymes (Chui et al., 1985). They have been detected in human fat at levels up to 2 ng/g lipid, while over 90% of the 48 pooled samples generated from 865 individuals contained measurable levels of PCDEs (Stanley et al., 1990). Thus there is ample evidence that PCDEs are present in the environment and reach humans.

Nonetheless, relatively few studies describing the toxicity of PCDEs to mammals exist, and those few deal with acute or subchronic effects in adults. When two hexachloro-CDEs and a pentachloro-CDE were administered to rats at 0.04, 0.4 and 4.0 mg/kg per day for 28 days, no overt toxicity was seen (Chu et al., 1989). Liver and thyroid were the target tissues, but even 40 mg/kg per day, the highest dose administered, caused only mild morphological changes in the liver and thyroid after 28 days (Chu et al., 1989). Similar results were obtained when a pentachloro-, hexachloro- and heptachloro-diphenyl ether were administered to rats at up to 500 ppm in the diet for 28 days (Chu et al., 1990). PCDEs induce cytochromes P450; the isozymes induced depend on the congener (Iverson et al., 1987). Some degree of immunotoxicity, correlated with binding to the Ah receptor, has also been identified (Howie et al., 1990; Harper et al., 1993), as has increased phorbol ester binding (Kodavanti et al., 1996). The numerous congeners included among the PCDEs will inevitably differ in their acute toxicity, and even in the range of their toxic effects; however, the available data strongly suggest that PCDEs are only moderately toxic to adult mammals. No evaluation of their carcinogenicity, mutagenicity, or developmental toxicity has been published to date. The absence of such studies is problematic, because developmentally toxic congeners are found among the closely related nitrodiphenyl ethers (NDEs).

While PCDEs are accidental contaminants and have not been studied extensively, the structurally similar NDEs comprise a large and growing class of herbicides. Nitrofen (2,4-dichlorophenyl 4′-nitrophenyl ether, Fig. 1b) was withdrawn from

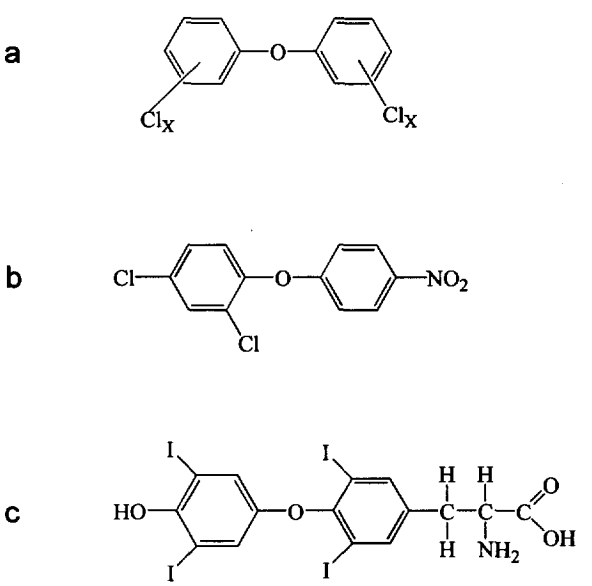

Fig. 1. Structures of diphenyl ethers. (a) 2,4-dichlorophenyl-4′-nitrophenyl ether (nitrofen), a potent developmental toxicant in rodents. (b) General structure of the polychlorinated diphenyl ethers (PCDEs) evaluated in this study. (c) Thyroxine or T_4, precursor to the active thyroid hormone tri-iodothyronine or T_3.

commercial production in the US because of its developmental toxicity (Hurt et al., 1983). Nitrofen, with a rat oral LD_{50} of 3580 mg/kg (Ambrose et al., 1971), causes malformations in both rats and mice at doses only a fraction of the levels that cause any symptoms in the dams (Costlow and Manson, 1981; Gray et al., 1983). The developmental no-effect level in rats is estimated to be 0.17 mg/kg per day (Hurt et al., 1983). Moreover, at least one experimental nitrofen analog, 2,4,5-trichlorophenyl 4′-nitrophenyl ether (245-NDE) is 10 times as fetotoxic as nitrofen (Francis, 1986, 1990), and increasing the degree of chlorination tends to increase the fetotoxicity of NDEs (Francis, 1989, 1990 and unpublished). Therefore one must consider the possibility that PCDEs—with a higher average number of chlorines per congener—will also exhibit developmental toxicity. On the assumption that PCDEs would cause the same kinds of developmental toxicity as the NDEs, we undertook the present study to determine the potential for developmental toxicity of selected PCDEs in rodents.

2. Materials and methods

2.1. Animals and treatment

Outbred Swiss-Webster-derived CD-1 mice, 50–60 days of age, were purchased from Charles River (Wilmington MA). Mice came either from the Michigan or Canadian facilities of the company. On arrival, females were caged 10 per polypropylene cage (19″ × 10.5″ × 6″) with corncob bedding. Males were individually caged in smaller polypropylene cages (11″ × 8.5″ × 6″). All animals were supplied *ad libitum* with water and rodent chow (Harlan Teklad, Indianapolis IN). The room was kept at 22 ± 2°C, with a 12-h light/dark cycle (lights on at 05:00 h).

After acclimatization, 2 females were placed overnight with each male. The morning that a semen plug was seen was designated day 0 of gestation. Mated females were weighed, caged individually, and assigned sequentially to treatment groups. Females were treated on gestation days (GD) 6 through GD 15, and were weighed on each day of treatment as well as one day after the last treatment (GD 16). After the last weighing on GD 16, a subset of pregnant females was killed by cervical dislocation for cytochrome P450 assays and for determination of residues in fetuses and in maternal fat. The remaining females were observed daily until a litter was born. If no litter was born by GD 20, the female was killed and her uterus examined for implantation sites. Females without implantation sites were removed from the study. Developmental toxicity was evaluated using the Chernoff-Kavlock assay (Chernoff and Kavlock, 1982) as described below (cf. Section 2.3.2).

Timed mated female Sprague-Dawley rats weighing approximately 250 g and at least 90 days of age were purchased from Harlan Sprague Dawley, Indianapolis IN. Additional adult male and virgin female rats were obtained from the same source and mated in our laboratory. Pregnancy was determined by the identification of sperm in the vaginal wash, and the day semen was present was considered GD 0. Rats were housed singly, either from the first day of pregnancy or on arrival, in polypropylene cages (19″ × 10.5″ × 8″) with corn cob bedding, and given access to water and 10% breeder chow (Harlan Teklad, Indianapolis IN) ad libitum. The room was kept at 22 ± 2°C, with a 12-h light/dark cycle (lights on at 05:00 h).

On GD 6, females were weighed and assigned to treatment groups. Treatment groups consisted of 10–15 animals. Females were dosed by gavage on GD 6–15 at levels of 25, 50, 75, or 100 mg/kg per day with one of three chlorinated diphenyl ether congeners or corn oil vehicle. There was an increase in the number of 'non-pregnant' females (females that were sperm positive, but did not produce litters) in the 100 mg/kg per day dose group. Previous studies in mice have shown increases in the incidence of 'pseudo-pregnancy' when the related nitrodiphenyl ethers were administered in the same dosing regimen (Francis, 1990). Because of this, we discontinued the 100 mg/kg per day dose, resulting in small sample sizes for this dose. A subset of pregnant females was killed by decapitation on GD 16, 24 h after the last dose of PCDE, for cytochrome P450

Table 1
Names and designations of the nine diphenyl ethers synthesized for this project, and of the nitrodiphenyl ether used as control for some aspects of the study

Compound	Designation
2,2′,4,4′,5,6′-hexachlorodiphenyl ether	224456-hexaCDE
2,2′,4,4′,5,5′-hexachlorodiphenyl ether	224455-hexaCDE
2,2′,4,4′,5-pentachlorodiphenyl ether	22445-pentaCDE
2,2′,4,4′,6-pentachlorodiphenyl ether	22446-pentaCDE
2,2′,4,5,6′-pentachlorodiphenyl ether	22456-pentaCDE
2,3′,4,4′,5-pentachlorodiphenyl ether	23445-pentaCDE
3,3′,4,4′,5-pentachlorodiphenyl ether	33445-pentaCDE
3,3′,4,4′-tetrachlorodiphenyl ether	3344-tetraCDE
2,3′,4′,6-tetrachlorodiphenyl ether	2346-tetraCDE
2,4,5-trichlorophenyl 4′-nitrophenyl ether	245-NDE

assays. Concurrent negative controls were dosed with vehicle, and weighed and killed on the same days as treated females. The remaining females were observed daily until a litter was born. If no litter was born by GD 23, the female was killed and her uterus examined for implantation sites. Females without implantation sites were removed from the study. Developmental toxicity was evaluated using the Chernoff-Kavlock assay (Chernoff and Kavlock, 1982) as described below (cf. Section 2.3.2).

2.2. Chemicals

Nine polychlorinated diphenyl ethers (PCDEs) were synthesized according to the method previously described (Nilsson and Renberg, 1974). The PCDEs and their designations are listed in Table 1, and the structures of the 9 PCDEs are shown in Fig. 2. Purity of each compound was greater than 98%, except for 224455-hexaCDE, which had a purity of > 96% due to contamination with 3–4% of 22445-pentaCDE. All congeners were first evaluated in mice, since their small size minimizes the amounts that must be synthesized. Three congeners were then selected for testing in rats. Two were selected for their developmental toxicity to mice: 22456-pentaCDE and 2346-tetraCDE. The third congener, 224455-hexaCDE, was selected because it should exhibit PCB-like toxicity.

Stock solutions of each PCDE were made by dissolving them in corn oil at 40 or 80 mg/ml, depending on their solubility. Dosing solutions were made up from these stocks so that not less than 50 μl and not more than 300 μl of dosing solution was administered daily to each female mouse, and not more than 2.5 ml to each female rat. PCDEs were administered by gavage, as was the corn oil vehicle. All dosage levels were determined by the weight of the female on GD 6, and were not increased as weight rose during gestation. Concurrent controls were gavaged with 300 μl corn oil (mice) or no more than 2.5 ml (rats) and weighed on the same days as treated females. Dosing solutions as well as the PCDEs were protected from light at all times.

2.3. Endpoints

2.3.1. Maternal toxicity

Females of both species were monitored daily for signs of overt illness. When symptoms occurred (ruffled fur, hunched posture and/or vaginal bleeding), dosing was stopped. Females were also weighed on each day of dosing to identify the abrupt weight loss characteristic of pregnancy loss.

2.3.2. Evaluation of fetotoxicity

Developmental toxicity was evaluated using a modification of the Chernoff-Kavlock assay (Chernoff and Kavlock, 1982). In the Chernoff-Kavlock assay, females are allowed to give birth, and pups remain with the dam until day 3. The number of pups per litter and the mean weight of pups on postnatal day (PD) 1 serve as measures of prenatal toxicity, while the number and weight of pups surviving to PD 3 measure perinatal effects of the compound (Chernoff and Kavlock, 1982). We modified the assay by keeping all pups until PD 5. In addition, a subset of mouse pups at the highest dose of each compound was reared to PD 30 so that Harderian glands could be examined (Francis, 1989, 1990).

2.3.3. Cytochrome P450 Assays

Pregnant female were killed by cervical dislocation (mice) or decapitation (rats) on GD 16, 24 h after the last dose of PCDE. Livers were perfused in situ with ice cold 0.05 M Tris–HCl/0.15 M

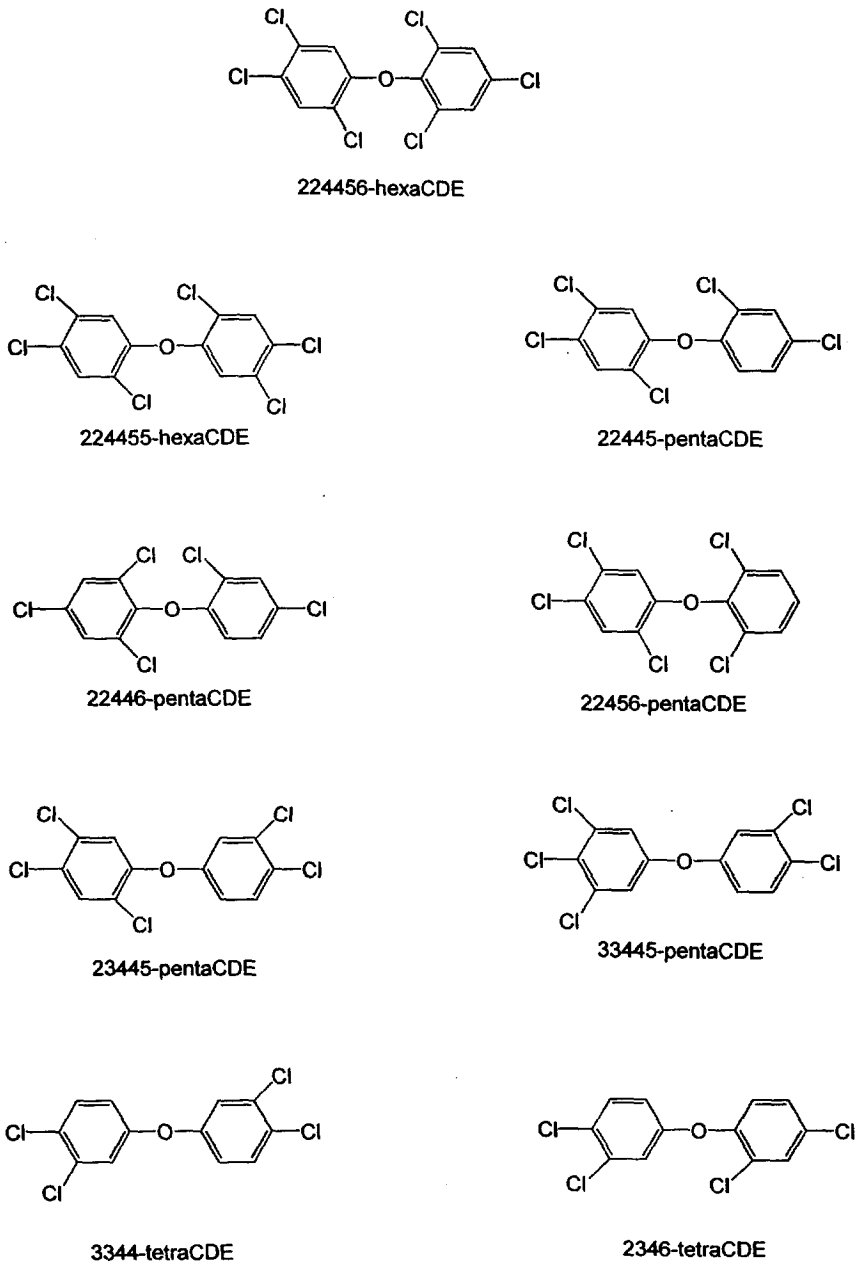

Fig. 2. Structures of the 9 polychlorinated diphenyl ethers examined in this study. The code by which each is designated is also shown.

KCl buffer (pH 7.5), excised, weighed, and homogenized in the same buffer. The homogenate was centrifuged (10 000 × *g* for 15 min, Tomy centrifuge) at 4°C; the supernatant was decanted and centrifuged at 105 000 × *g* for 60 min at 4°C (Beckman ultracentrifuge). The microsomal pellet was resuspended in 0.05M Tris–HCl buffer (pH 7.5) and used immediately (mice) or resuspended

and frozen (rats) in 1 ml aliquots in freezing buffer (0.1 M Na_2HPO_4/0.1 M NaH_2PO_4, 0.25 M sucrose, 1.0 nmol dithiothreitol, at pH 7.4) for cytochrome P450 assays. Concurrent vehicle-treated control females were included in each assay.

2.3.3.1. Total cytochrome P450. In mice, protein levels were determined using the BCA Protein Assay (Pierce, Rockford IL); in rats, protein levels were determined using a modification (Guengerich, 1982) of the Lowry method (Lowry et al., 1951). For both species, total cytochrome P450 was measured by the method of Omura and Sato (1964), in which the difference spectrum of the carbon monoxide adduct of reduced cytochrome P450 is measured by the change in absorbance at 490 nm. Comparisons between groups were analyzed using SPSS/PC +, version 5.0 (SPSS, 1992).

2.3.3.2. O-Dealkylation of alkoxyresorufins. Protein levels were determined using the Lowry method (Lowry et al., 1951) and the *O*-dealkylation of alkoxyresorufins was measured by a modification of the method of Pohl and Fouts (1980), Li and Hansen, 1996). The 1 ml reaction mixture contained 5 mM $MgCl_2$, 1 mg bovine serum albumin, 0.4–1.0 mg microsomal suspension, 2.5 μM ethoxyresorufin (EROD), 10 μM benzoxyresorufin (BROD) or 10 μM pentoxyresorufin (PROD), and an NADPH-generating system (0.8 mg $NADP^+$, 1.5 mg glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase) in 0.05 M Tris-HCl buffer at pH 7.5. The reaction was initiated by adding the NADPH-generating system and stopped by addition of 2 ml methanol. The reaction was carried out at 37°C: 7 min for EROD, 10 min for PROD and BROD. Each sample was run in duplicate. Formation of resorufin was determined by measuring fluorescence relative to a known amount of resorufin with excitation at 550 nm and emission at 585 nm in a Perkin-Elmer 203 fluorescence spectrometer.

2.4. Statistical analyses

2.4.1. Chernoff-Kavlock assay and Harderian gland weights

The litter or the adult female was the unit of analysis for all endpoints. Data were analyzed using Analysis of Variance and Regression Analysis in SPSS (SPSS, 1992). No statistical comparisons were made between congeners. Regression analysis was used to determine, for each endpoint, the probability that the dose-response slope for a given endpoint differed from zero. Covariables were used, as follows. In unmated females, weight at first dose was used as a covariable for analysis of liver-to-body-weight ratios. In pregnant females, the female's weight at the first dose, and litter size at birth, were used as covariables for maternal weight gain, since larger litters would clearly increase weight of the pregnant female. Only females that bore litters to term were included in maternal weight gain analyses. Litter size at birth was also the covariable in analyzing the effects of dose on the mean weight of pups from birth to weaning. Finally, the age and mean weight of pups at dissection were used as covariables for Harderian gland analyses. The number of comparisons made argues against giving undue consideration to a statistical significance level of $0.01 < P < 0.05$.

2.4.2. Enzyme assays

The litter or the adult female was the unit of analysis for all endpoints. Data were analyzed using Analysis of Variance and Regression Analysis in SPSS (SPSS, 1992). No statistical comparisons were made between congeners. The F test was performed to determine homogeneity of variance. Student's *t*-test was then used to examine differences between control and treated groups; when variances were not homogeneous, the modified Student's *t*-test was used (SPSS, 1992).

3. Results

3.1. Mice

The effects of each PCDE on maternal weight gain and on the number of pups per litter are

Table 2
Response of pregnant female mice to treatment with chlorinated diphenyl ethers

PCDE CODE	Dose (mg/kg per day)	*N*	No. litters born	Maternal wt gain (gm) ± S.E.	No. pups born per ♀ mated
Control	—	85	85	21.2 ± 0.5	13.6 ± 0.2
224456-hexa-CDE	10	21	20	18.8 ± 0.7*	11.9 ± 0.6**
	50	19	19	17.8 ± 0.7	12.2 ± 0.7
	100	22	21	16.4 ± 0.9	10.9 ± 0.6
224455-hexa-CDE	10	14	14	20.3 ± 1.1	13.6 ± 0.6*
	50	12	11	19.3 ± 1.8	11.6 ± 1.6
	100	13	13	20.8 ± 1.2	12.5 ± 0.5
22445-penta-CDE	10	14	14	19.8 ± 1.1	13.6 ± 0.8
	50	16	16	21.0 ± 1.5	13.6 ± 1.1
	100	16	16	18.5 ± 1.6	11.5 ± 1.2
22446-penta-CDE	10	17	17	20.1 ± 1.4	13.3 ± 1.1
	50	18	18	21.9 ± 1.2	14.1 ± 0.6
	100	18	18	21.3 ± 0.8	13.9 ± 0.5
22456-penta-CDE	10	19	19	24.0 ± 0.7***	14.7 ± 0.4***
	50	17	17	23.0 ± 0.8	13.8 ± 0.5
	100	4	1	18.5 ± 0.3	3.8 ± 3.8
33445-penta-CDE	100	19	19	21.7 ± 0.7	14.3 ± 0.7
23445-penta-CDE	100	21	21	20.5 ± 0.8	13.6 ± 0.7
2346-tetraCDE	10	19	18	22.0 ± 0.7	14.4 ± 0.5***
	50	15	15	20.7 ± 0.8	13.8 ± 1.3
	100	6	1	16.2 ± 2.7	2.8 ± 2.8
3344-tetraCDE	100	18	18	21.0 ± 0.4	14.2 ± 0.3

The regression of maternal weight gain on dose, and of the (number of pups born/♀) on dose, were determined for each CDE. *P*-levels identify the probability that the slope of the regression line does not differ from zero.
* $P<0.05$ that slope of regression = 0. ** $P<0.01$ that slope of regression = 0. *** $P<0.001$ that slope of regression = 0. *N* = Number of females.

shown in Table 2. The number of pregnancies is shown both in terms of the number of females with implantation sites (pregnant females) and of the number of litters born. Differences between these two numbers identify the prenatal whole litter loss. The number of pups born per female mated shows the extent to which overall prenatal loss (of whole litters or of pups within litters carried to term) decreased the number of pups born. Maternal weight gain was analyzed only for females giving birth to litters, and using the number of pups born per litter as covariable. Four of the congeners significantly reduced the number of pups born per female: 224456-hexaCDE, 22456-pentaCDE, 2346-tetraCDE and 224455-hexaCDE. Two of these, 224456-hexaCDE and 22456-pentaCDE, also depressed maternal weight gain. No other reductions in maternal weight gain were noted in any of the treatment groups.

Table 3 shows the number of pups born per litter born and their survival and growth to postnatal day (PD) 5. These data exclude females which did not deliver a litter between GD 17 and GD 20, even though implantation sites were

Table 3
Effects of CDEs, administered from day 6 through day 15 of gestation, on perinatal survival and growth of mice exposed in utero

Chemical and code	Dose (mg/kg per day)	*N*	Pups per litter born	Live/litter: PD 1	Mean pupwt: PD 1	Live/litter: PD 5	Mean pupwt: PD 5
Control	—	85	13.6 ± 0.2	13.4 ± 0.2	1.89 ± 0.02	13.4 ± 0.2	3.55 ± 0.05
224456-hexaCDE	10	20	12.5 ± 0.6***	12.3 ± 0.6***	1.87 ± 0.04	12.1 ± 0.5***	3.66 ± 0.10*
	50	19	12.2 ± 0.7	12.2 ± 0.7	1.88 ± 0.04	12.0 ± 0.7	3.59 ± 0.12
	100	21	11.4 ± 0.6	11.2 ± 0.7	1.98 ± 0.07	11.2 ± 0.7	3.62 ± 0.12
224455-hexaCDE	10	14	13.6 ± 0.6	13.4 ± 1.1	1.89 ± 0.10	13.2 ± 0.7	3.52 ± 0.19
	50	11	12.6 ± 1.3	12.5 ± 1.3	2.01 ± 0.09	12.3 ± 1.1	3.48 ± 0.16
	100	13	12.5 ± 0.5	12.4 ± 0.4	1.88 ± 0.06	12.0 ± 0.4	3.55 ± 0.11
22445-pentaCDE	10	14	13.6 ± 0.8*	13.9 ± 1.2	1.85 ± 0.06	12.8 ± 1.2*	3.40 ± 0.13
	50	16	13.6 ± 1.1	13.4 ± 1.1	1.80 ± 0.04	13.2 ± 1.1	3.33 ± 0.13
	100	16	11.5 ± 1.2	11.4 ± 1.2	1.91 ± 0.07	11.3 ± 1.2	3.69 ± 0.14
22446-pentaCDE	10	17	13.3 ± 0.8	13.2 ± 0.8	1.88 ± 0.05	13.2 ± 0.8	3.59 ± 0.14
	50	18	14.1 ± 0.6	13.9 ± 0.6	1.82 ± 0.06	13.7 ± 0.6	3.32 ± 0.11
	100	18	13.9 ± 0.5	13.7 ± 0.5	1.81 ± 0.06	13.7 ± 0.5	3.47 ± 0.09
22456-pentaCDE	10	19	14.7 ± 0.4	14.7 ± 0.4	1.86 ± 0.05	13.7 ± 0.8	3.42 ± 0.09
	50	17	13.8 ± 0.5	13.8 ± 0.5	1.90 ± 0.05	12.6 ± 0.9	3.56 ± 0.09
	100	1	15.0	15.0	1.76	14.0	3.51
3,3′4,4′,5-pentaCDE	100	19	14.3 ± 0.7	14.0 ± 0.7	2.04 ± 0.06***	13.9 ± 0.7	3.57 ± 0.11
2,3′4,4′,5-pentaCDE	100	21	13.6 ± 0.7	13.1 ± 0.7	1.96 ± 0.08	13.2 ± 0.7	3.54 ± 0.11
2,3′4′,6-tetraCDE	10	18	15.2 ± 0.5**	15.1 ± 0.5***	1.76 ± 0.03***	15.0 ± 0.5***	3.25 ± 0.08***
	50	15	11.2 ± 1.0	7.2 ± 1.5	1.47 ± 0.09	6.2 ± 1.6	2.72 ± 0.26
	100	1	17.0 ± –	17.0 ± –	1.63	17.0	3.25
3,3′4,4′-tetraCDE	100	18	14.2 ± 0.3	14.0 ± 0.4	1.88 ± 0.04	14.0 ± 0.4	3.64 ± 0.09*

The regression of litter size on dose, and of pup weight on dose, were determined for each CDE. *P*-levels identify the probability that the slope of the regression does not differ from zero.
* $P<0.05$ that slope of regression = 0. ** $P<0.01$ that slope of regression = 0. *** $P<0.001$ that slope of regression = 0. *N* = Number of litters born.

Table 4
Mean liver weight and mean cytochrome-P450 levels in pregnant female mice treated with CDEs from day 6 through day 15 of gestation

Compound	N	Liver (g) ± S.E.	nmol P450 per mg protein ± S.E.	Total P450 (nmol per liver) ± S.E.
Vehicle Control	16	2.97 ± 0.14	0.50 ± 0.05	11.78 ± 2.99
245-NDE	15	3.40 ± 0.14*	1.23 ± 0.14**	51.49 ± 5.18***
224456-hexaCDE	5	3.20 ± 0.15	1.06 ± 0.16*	93.41 ± 9.80**
224455-hexaCDE	5	3.98 ± 0.20**	1.35 ± 0.18**	84.02 ± 21.25*
224456-pentaCDE	5	3.50 ± 0.30	0.96 ± 0.18	40.95 ± 7.39*
22445-pentaCDE	6	3.46 ± 0.10**	1.07 ± 0.35*	71.34 ± 16.89*
22456-pentaCDE	6	2.86 ± 0.20	0.99 ± 0.17*	28.41 ± 4.30**
33445-pentaCDE	5	4.06 ± 0.29*	0.56 ± 0.12	35.59 ± 7.32*
23445-pentaCDE	5	3.23 ± 0.57	0.96 ± 0.28	42.02 ± 8.30*
3344-tetraCDE	3	3.23 ± 0.23	0.78 ± 0.35	31.92 ± 13.58
2346-tetraCDE	3	2.78 ± 0.08	0.67 ± 0.16	29.12 ± 6.40

Data for vehicle-treated controls, and for females treated with 245-NDE are also shown.
Asterisks denote probability that values differ significantly from vehicle control: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

present in the uterus. Dose-dependent decreases in litter size were seen for two congeners: 224 456-hexaCDE at birth, on PD 1 and on PD 5 ($P < 0.001$ in each case), and 2346-tetraCDE ($P < 0.01$ for number of pups born; $P < 0.001$ on PD 1 and PD 5). 22 445-pentaCDE marginally decreased the number of pups per litter born and the number surviving on PD 5 ($P < 0.05$ in each case). Postnatal survival was not decreased by the remaining PCDEs, despite the sharp decrease in the numbers of litters born to dams exposed to 22 456-pentaCDE. A marginally significant increase in mean pup weight was seen with 3344-tetraCDE on PD 5 ($P < 0.05$), but this may be spurious, given the number of statistical analyses run.

The low incidence of postnatal mortality suggested that most PCDEs did not cause the postnatal syndrome caused by some NDEs. This suspicion was confirmed by examination of the Harderian glands of weanlings. All pups examined had Harderian glands, and there was no decrease in the mean size of Harderian glands relative to those in controls for any congener (data not shown).

Effects of PCDEs on liver enzymes of dams on GD 16 are shown in Tables 4 and 5. The potent developmental toxicant, 245-NDE (Francis, 1986, 1990), was evaluated with the PCDEs for comparison. 245-NDE increased liver size ($P < 0.05$) and increased the level of total cytochromes P450 per mg protein ($P < 0.01$); total cytochromes P450 for the whole liver were almost five-fold control levels ($P < 0.001$). Increased liver size was also seen with 224455-hexaCDE ($P < 0.01$), with 22445-pentaCDE ($P < 0.01$), and with 33445-pentaCDE ($P < 0.05$), whereas increased cytochrome P450 per mg protein was seen with 224456-hexa-CDE ($P < 0.05$), 224455-hexaCDE ($P < 0.01$), 22456-pentaCDE ($P < 0.05$) and 22456-pentaCDE ($P < 0.05$). Increases in total P450, based both on enzyme levels per mg protein and on total liver weight, were significant for all PCDEs except 3344-tetraCDE and 2346-tetraCDE (Table 4). Lack of significant induction of cytochromes P450 by these congeners may be due to the smaller sample size ($N = 3$).

Induction of EROD, PROD and BROD activities was determined for 3 congeners: for the most potent developmental toxicants, 22456-pentaCDE and 2346-tetraCDE; and for 33445-pentaCDE, which has no *ortho*-Cl substituents. All three congeners significantly induced EROD and BROD activity. Only 33445-pentaCDE induced PROD (Table 5). The 245-NDE induced EROD and BROD activity, but the increases in specific activity were not significant, probably due to the considerable variation among samples.

PCDE residues in the abdominal fat of pregnant female mice and in their fetuses were assayed on GD 16, 24 h after the 10th dose of 100 mg/kg of each PCDE, in the same females used for evaluation of total cytochrome P450 levels. Residues in maternal fat were 2–3 orders of magnitude higher than fetal

Table 5
Mean liver weight and microsomal enzyme activities on GD 16 in pregnant mice treated with 100 mg/kg per day of 22456-pentaCDE, 2346-tetraCDE, or 33445-pentaCDE from day 6 through day 15 of gestation

Compound	N	Liver as % of body wt.	EROD: pmol/min per mg liver protein	PROD: pmol/min per mg liver protein	BROD: pmol/min per mg liver protein
Vehicle Control	4	5.35 ± 0.38	20.4 ± 4.7	4.0 ± 1.28	8.6 ± 3.6
245-NDE	4	6.35 ± 0.46	37.4 ± 27.0	4.0 ± 1.9	14.3 ± 5.6
22456-penta-CDE	3	5.98 ± 0.35	34.6 ± 4.2*	5.0 ± 1.0	17.2 ± 2.4*
33445-penta-CDE	4	6.13 ± 0.55	110.8 ± 19.9*	8.4 ± 3.3*	21.6 ± 9.0*
2346-tetraCDE	4	6.18 ± 0.39	43.8 ± 16.1*	5.5 ± 1.2	21.3 ± 6.9*

Data for vehicle-treated controls are also shown. Each female was dosed with 100 mg/kg of PCDE from GD 6 through 15, inclusive. Asterisks denote probability that levels differ from vehicle control: * $P \leq 0.05$; ** $P \leq 0.01$. N = Number of litters.

residues (Table 6). Levels were generally higher for hexa-Cl than for penta-Cl congeners, and were lowest for the tetra-Cl congeners. In most cases, each additional Cl substituent added residues of one order of magnitude in the maternal fat. However, 22446-pentaCDE was present in maternal fat at 1506 ppm, closer to the levels of hexa-Cl congeners than to levels of the other penta-Cl congeners, which ranged from 535 ppm for 22445-pentaCDE to 841 ppm for 23445-pentaCDE. Interestingly, residues of 22446-pentaCDE in fetuses were very low: 0.061 ppm. Of the two tetra-Cl congeners, 3344-tetraCDE left more than 40 times the residues in maternal fat (449 ppm) as did 2346-tetraCDE (10 ppm); fetal residues of 3344-tetraCDE (0.59 ppm) were also greater than for 2346-tetraCDE (0.1 ppm).

Table 6
Residues of PCDEs in maternal fat and in fetuses when pregnant mice were treated with 100 mg/kg per day, GD 6-15

Compound	Maternal fat residues (ppm ± S.E.)	Fetal residues (ppm ± S.E.)
Vehicle-treated controls	0.045 ± 0.02	Not detected
224456-hexa-CDE	2094.0 ± 1.20	5.254 ± 1.23
224455-hexa-CDE	1986.0 ± 257	6.128 ± 0.68
22445-penta-CDE	535.6 ± 69.2	0.368 ± 0.03
22446-penta-CDE	1506.0 ± 112	0.061 ± 0.04
33445-penta-CDE	785.2 ± 59.5	0.608 ± 0.16
23445-penta-CDE	841.5 ± 187.0	0.375 ± 0.08
3344-tetraCDE	448.5 ± 48.0	0.586 ± 0.08
2346-tetraCDE	10.12 ± 1.55	0.122 ± 0.09

Values are means of fat samples from three females, measured 24 h after the 10 dose (GD 16) or of three fetuses from the same dams, also measured on GD 16.

3.2. Rats

Of the PCDEs evaluated in mice, three were selected for evaluation in rats: 224455-hexaCDE, which is known to induce cytochrome P450, and the two congeners that were most potent developmental toxicants in mice: 22456-pentaCDE and 2346-tetraCDE. Occurrence of 'infertile' matings, in which females with sperm in vaginal washings did not produce litters, led to abandonment of the 100 mg/kg dose in rats after 3–5 litters were born. The number of litters born per female mated decreased with increasing dose for 22456-pentaCDE (Table 7). Dose-dependent decreases in mean litter size on PD 1 and PD 5 were seen with prenatal exposure to 22456-pentaCDE and 2346-tetraCDE. The mean weight of pups on both PD 1 and PD 5 was decreased by all of the PCDEs (Table 7), but the differences were not significant for 2346-tetraCDE. Induction of specific P450 isozymes was examined in a subset of pregnant females, killed on GD 16. These data are shown in Table 8. In the pregnant females, EROD activity was induced 3.6-fold, and PROD 5-fold, by 224455-hexaCDE ($P < 0.005$ in each case), while total cytochrome P450 was increased only 1.4-fold ($P < 0.05$). Neither 22456-pentaCDE nor 2346-

Table 7
Average number and weight of pups alive on PD 1 and PD 5 when pregnant rats were treated with 224455-hexaCDE, 22456-pentaCDE, or 2346-tetraCDE from gestation day 6 through 15

Mg/kg per day	No. ♀♀	*N*	No. pups: PD 1	Pupwt (g): PD 1	No. pups: PD 5	Pupwt (g) PD 5
Control	31	30	11.1 ± 0.78	7.06 ± 0.37	10.9 ± 0.77	12.66 ± 0.78
224455-hexaCDE						
25	13	13	8.6 ± 0.99	8.96 ± 0.17***	8.6 ± 0.99	16.91 ± 0.51***
50	18	17	11.0 ± 0.94	6.43 ± 0.59	10.2 ± 1.14	10.75 ± 1.02
100	4	3	9.3 ± 4.70	3.97 ± 2.48	8.7 ± 4.33	6.93 ± 3.97
22456-pentaCDE						
25	13	13	10.9 ± 1.05	7.47 ± 0.22	10.6 ± 1.13	12.36 ± 1.23
50	16	14	8.2 ± 1.18*	5.44 ± 0.74*	7.8 ± 1.15*	10.56 ± 1.40
100	9	5	0.8 ± 0.80***	0.32 ± 1.32**	0.8 ± 0.80***	1.71 ± 2.71*
2346-tetraCDE						
50	15	13	9.5 ± 1.55	8.96 ± 0.17	9.7 ± 1.58	16.91 ± 0.51
75	15	14	10.6 ± 1.02	6.43 ± 0.59	10.6 ± 1.00	10.75 ± 1.33
100	6	5	8.4 ± 2.58	3.97 ± 2.48	7.6 ± 2.56	N.A.

Results expressed as mean ± S.E. N = number of litters. Asterisks denote the probability that values differ significantly from vehicle control: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P < 0.001$. N.A. = not available.

tetraCDE significantly induced total cytochromes P450, EROD, or PROD activities.

4. Discussion

PCDEs are structurally similar to PCBs, to nitrodiphenyl ether herbicides, and to thyroid hormones (Fig. 1). Since both PCBs and nitrodiphenyl ethers are thyrotoxic (Gray and Kavlock, 1983; Chu et al., 1989; Ness et al., 1993; McKinney and Waller, 1994; Li and Hansen, 1996), induce cytochromes P450 (Howie et al., 1990; Hurt et al., 1983; Chu et al., 1989, 1990) and are developmental toxicants (Rogan, 1982, Costlow and Manson, 1981; Manson, 1986; Francis, 1990), it is logical to examine the structure activity relationships among PCDEs with respect to the same parameters. We chose a diverse group of PCDEs, including congeners that are environmentally relevant (Chu et al., 1989, 1990) and congeners with known effects on cytochromes P450, notably 224455-hexaCDE and 3344-tetraCDE, which are likely to induce the same P450 isozymes as coplanar PCBs (McKinney and Waller, 1994). Our dosing regimen, up to 100 mg/kg per day throughout organogenesis, was not designed to reflect plausible dietary exposures, but to identify the potential for developmental toxicity among PCDEs. Determining whether PCDEs pose a risk to the human population would require far more extensive studies.

In earlier studies with NDEs related to the herbicide nitrofen, we identified two distinct developmental syndromes: a prenatal mortality consisting primarily of whole litter death, strongly associated with maternal illness but not always with postnatal mortality (Francis, 1990); and the nitrofen syndrome (Manson, 1986, Gray et al., 1983; Ostby et al., 1985), consisting of perinatal mortality, persisting decreases in weight among surviving offspring, and absent or abnormally small Harderian glands at weaning. Both the nitrofen syndrome and the prenatal mortality occurred at lower doses as the number of chlorine substituents increased, suggesting that highly chlorinated diphenyl ethers (such as the PCDEs examined here) might be extremely potent developmental toxicants.

When the number of chlorine substituents was held constant, developmental toxicity of NDEs varied significantly with the position of the chlorines on the phenyl ring. *ortho*-CL substitution favored occurrence of prenatal mortality, while *para*-Cl substitution favored the postnatal syndrome. Moreover, among 20 congeners tested, the

Table 8
Relative liver weight, EROD activity, PROD activity, and total cytochrome P450 activity in female rats killed on gestational day (GD) 16

	N	Relative liver weight (mg/g)	EROD (pmol/min per mg protein)	PROD (pmol/min per mg protein)	Total Cytochrome P450 (nmol/min per mg protein)
Control	7	36.0 ± 1.0	2052.6 ± 1150.7	270.0 ± 47.3	0.709 ± 0.07
224455-hexa-CDE	5	41.0 ± 1.0	7497.9 ± 983.9*,**	1287.5 ± 259.5*,**	1.014 ± 0.13*
22456-penta-CDE	6	39.0 ± 3.0	2343.9 ± 1191.5	285.9 ± 34.7	0.560 ± 0.04
2346-tetraCDE	6	40.0 ± 1.0	2367.4 ± 674.8	284.0 ± 29.4	0.641 ± 0.06

Each female was dosed with 100 mg/kg of PCDE from GD 6 through 15, inclusive. Results expressed as mean ± S.E. N indicates the number of females. Asterisks denote the probability that values differ significantly from vehicle control: * $P \leq 0.05$; ** $P < 0.005$.

postnatal nitrofen syndrome showed some correspondence with increased liver size (Francis, 1990). The prenatal mortality did not correlate with increased liver size, and may be more closely related to the uncoupling of oxidative phosphorylation caused by dinitrophenols (Gasiewicz, 1991), rather than to the postnatal nitrofen syndrome. This hypothesis remains speculative.

It is interesting to note that none of the PCDEs caused the nitrofen syndrome. Despite considerable variation in both the number and distribution of chlorine substituents, none of the 9 congeners tested in mice decreased the size of Harderian glands. This suggests that the nitrofen syndrome requires the *para*-NO_2 moiety of the diphenyl ether herbicides. However, two PCDE congeners (22456-pentaCDE and 2346-tetraCDE) caused prenatal mortality of entire litters in mice without causing significant postnatal morbidity, demonstrating that the prenatal mortality does not depend on the *para*-NO_2 group. Since 22456-pentaCDE has 3, and 2346-tetraCDE 2, *ortho*-Cl substituents, the importance of *ortho*-Cl substituents is confirmed. However, since 22446-pentaCDE also has 3 *ortho*-Cl substituents and did not induce significant prenatal mortality, other parameters must also pertain.

There was no correspondence between prenatal whole litter loss and the induction of either total cytochromes P450 or of specific P450 activities. This confirms results we obtained with NDEs, for which the prenatal mortality is not strongly correlated with increased liver to body weight ratios (Francis 1990). In addition, since several PCDE congeners strongly induced overall cytochrome P450 levels, it is clear that induction of these enzymes by a halogenated diphenyl ether is not sufficient to cause the nitrofen syndrome (although it may be a necessary element).

Residue data for mice were obtained from the maternal fat and from whole fetuses on GD 16. As expected, the results demonstrated strong differences in the bioaccumulation of PCDEs. Maternal fat residues were several orders of magnitude above fetal residues, an expected result given that entire fetuses were used for residue evaluation and it is well known that PCDEs, like PCBs, accumulate selectively in fat. Also as expected, compounds with higher degrees of chlorination were generally retained to a greater extent than those with lower chlorination. However, the pattern of chlorine substitution also affected retention, and the rank order of congeners with respect to accumulation differed between dams and fetuses. Most important, there was no correlation between either maternal fat residues or fetal residues and the occurrence of fetotoxicity. These results do not support the hypothesis that differences in doses delivered to maternal or fetal targets are critical in determining the fetotoxicity of specific congeners. Nor does the pattern of residues suggest any simple structural relationships underlying the accumulation of these compounds.

Results in rats were qualitatively similar to those in mice for the 3 congeners that were evalu-

ated in both species. 22456-pentaCDE was somewhat more fetotoxic in rats than was 2346-tetraCDE, in that litter size was already decreased at 50 mg/kg/day by the former, and only at 100 mg/kg/day by the latter. 224456-hexaCDE was least active, in that it decreased only postnatal weight, not survival. As in mice, 224456-hexaCDE significantly induced both EROD and PROD activities ($P < 0.005$). Observations suggested that PCDEs were more toxic to pregnant rats than to pregnant mice, and comparison with results from other studies (Ostby et al., 1985; Hurt et al., 1983) suggest that pregnant animals may be more sensitive than adult males or nonpregnant females in both species.

In summary, it is apparent that the developmental toxicity of PCDEs as a class differs from the developmental toxicity of the NDEs related to nitrofen. Depending on their structure, NDEs may cause either prenatal mortality and/or perinatal mortality accompanied by postnatal morbidity (Francis, 1990); the latter syndrome is apparently not caused by PCDEs. The primary symptoms of adult exposure to chlorinated diphenyl ethers have been attributed to induction of cytochromes P450 by NDEs (Hurt et al., 1983) and PCDEs (Chui et al., 1985; Chu et al., 1989, 1990) and to interaction of PCDEs with the Ah receptor (Safe, 1994). Our data show that prenatal mortality in both rats and mice is not well correlated to induction of cytochromes P450. Moreover, because PCDEs do not induce the postnatal nitrofen syndrome, even when they induce cytochromes P450, other mechanisms must also be found for this developmental toxicity of the nitrodiphenyl ethers.

In mice, the two congeners with the greatest fetotoxicity, 22456-pentaCDE and 2346-tetraCDE, also bore the closest resemblance to thyroxine (Fig. 1). Several studies with NDEs have suggested that the thyroid gland is a target of nitrodiphenyl ether toxicity (Gray et al., 1983; Manson, 1986). Not only the structural similarity of the NDEs to thyroxine, but also many features of the postnatal nitrofen syndrome (notably persistent growth retardation, decreased Harderian gland size, and delayed sexual maturation) suggest hypothyroidism. High doses of nitrofen do cause transient hypothyroidism in adult mice (Gray et al., 1983) and depress T_4 levels in rat fetuses (Manson, 1986). We have shown elsewhere (Rosiak et al., 1997) that several of the congeners examined in this study also cause hypothyroidism both in pregnant rats (22456-pentaCDE and 2346-tetraCDE) and in juvenile rats exposed in utero (224455-hexaCDE, 2456-pentaCDE and 2346-tetraCDE). Thus, the connections between diphenyl ethers and thyroid hormones are sufficiently striking to argue that further investigation should be made of the role of thyrotoxicity in the developmental toxicity of diphenyl ethers. The persistence of growth retardation beyond weaning, as well as the absence or decreased size of the Harderian glands in nitrofen-exposed pups, suggests that it is the fetal thyroid, or thyroid hormone receptors, that are affected in this syndrome. The prenatal mortality, on the other hand, is more closely associated with maternal toxicity. It is also strongly associated with whole litter loss: those litters that survive to term often do well postnatally. These results suggest that the prenatal mortality may affect fetuses indirectly, and that thyroid effects consequent to PCDE exposure affect the dam, rather than being directly fetotoxic.

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