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Effects of Subchronic Exposure to Complex Mixtures of Dioxin-like and Non-Dioxin-like Polyhalogenated Aromatic Compounds on Thyroid Hormone and Vitamin A Levels in Female Sprague-Dawley Rats

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The aim of this study was to determine the effects of subchronic exposure to complex mixtures of polyhalogenated aromatic hydrocarbons (PHAHs) on the thyroid hormone and retinoid status in female Sprague-Dawley rats and to investigate the predictability of these effects by the toxic equivalency factor (TEF) concept. In the first experiment, the focus was on a complex dioxin-like PHAH mixture, which covered > 90% of the total toxic equivalents (TEQ) present in Baltic herring. In the second experiment, the contribution of non-dioxin-like polychlorinated biphenyls (PCBs) was investigated by testing the commercial PCB mixture Aroclor 1260, its 0-1 *ortho* and 2-4 ortho fractions and the reconstituted 0-4 *ortho* fraction. Hepatic retinoid levels were severely decreased (~70%) after treatment with the dioxin-like PHAH mixture, similar to the effect of a TEQ equivalent dose of 1 μg 2,3,7,8-TCDD/kg bw/week. However, the TEF concept failed to predict the effect on plasma retinol; a decrease (21%) was observed after treatment with the PHAH mixture, whereas an increase (21%) was found after treatment with TCDD. A more severe decrease of total thyroid hormone in plasma was observed after exposure to the PHAH mixture compared to treatment with TCDD (~60% vs. 38%). The discrepancy found between the predicted and observed effects for plasma retinol and thyroid hormone is possibly due to an additional effect of hydroxylated PCBs, formed from metabolizable PCBs present in the PHAH mixture. Aroclor 1260 and its fractions did not significantly alter the retinoid and thyroid hormone status at the dose levels tested, indicating that in case of exposure to complex PCB mixtures at environmental levels, no effects, or at best, only marginal effects can be expected on the retinoid and thyroid hormone status.

Key Words: **PCBs; dioxins; TCDD; mixtures; vitamin A; thyroid hormone; subchronic; rat.**

Polychlorinated biphenyls (PCBs), polychlorodibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and other polyhalogenated aromatic hydrocarbons (PHAHs) elicit a broad spectrum of toxic effects and biochemical changes, e.g., body weight loss, thymic atrophy, hepatotoxicity, carcinogenicity, induction of hepatic cytochrome P450 isoenzymes, and alterations of the retinoid status and thyroid hormone metabolism (Safe, 1990, 1994).

Most, if not all, of the toxic responses of PHAHs are suggested to be mediated by the aryl hydrocarbon (Ah) receptor (Ahlborg *et al.*, 1994; Safe, 1994). The most toxic PHAH compounds exhibit a planar molecular conformation with lateral chlorine substitution, thereby expressing a so-called dioxin-like toxicity. Di-*ortho* substituted PCBs are in general less toxic; due to their nonplanar conformation, they do not exhibit Ah-receptor agonistic activity, but have been shown to possess a phenobarbital-like toxicity (Safe, 1994).

In environmental matrices and biota PCBs, PCDDs, and PCDFs are always present as complex mixtures. The toxic equivalency factor (TEF) concept has been developed to aid the risk assessment of complex mixtures of PHAHs. Based on *in vivo* and *in vitro* studies, the relative toxic potencies of individual PHAHs have been determined relative to 2,3,7,8-TCDD as the most toxic congener. The TEF concept is based on the assumptions that all non-*ortho* and mono-*ortho* chlorine substituted PCB congeners and related dibenzo-*p*-dioxins and dibenzofurans act through the Ah receptor-based mechanism of action and that the effects of the individual compounds in a mixture, expressed as toxic equivalencies (TEQs), are additive (Ahlborg *et al.*, 1994; Safe, 1990, 1994). However, interactive effects have been observed between PHAHs and some nonplanar di-*ortho* PCB congeners or PCB mixtures (Aarts *et al*, 1995; Bager *et al.*, 1995; Biegel *et al*, 1989; Davis and Safe, 1989; Haag-Grönlund *et al.*, 1998; Sargent *et al.*, 1991; Yao and Safe, 1989; Zhao *et al.*, 1994). In addition, several di-*ortho* PCBs have been shown to possess toxic properties both *in vivo* and *in vitro*, e.g., disturbance of the vitamin A and thyroid hormone status, development of altered hepatic foci, and inhibition of intercellular communication (Bager *et al.*, 1997; van Birgelen *et al.*, 1992; de Haan *et al.*, 1995).

Several toxicity studies have been performed with com-

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plex PCB mixtures (Abraham *et al.*, 1989; Ahlborg *et al.*, 1987; Kihlström *et al.*, 1992; Kimbrough *et al.*, 1975; Morse *et al.*, 1996; Ward, 1985). However, in most studies the focus was on individual PHAH congeners or combinations of two or three congeners (Bager *et al.*, 1995; van Birgelen *et al.*, 1994a,b; Haag-Grönlund *et al.*, 1998; Sargent *et al.*, 1991). Our major aim of this study was to evaluate the applicability of the TEF concept for the tumor promotion potential of complex environmentally relevant PHAH mixtures (van der Plas *et al.*, 1999, 2000). In addition, the effects of subchronic exposure to these PHAH mixtures was determined on endocrine parameters, in particular on the vitamin A and thyroid hormone system. Vitamin A and thyroid hormone are both essential for normal tissue growth, differentiation, and fetal development, and are possibly involved in carcinogenesis (Blomhoff, 1994; Dunn, 1989; Guernsey, 1993).

Alterations in both vitamin A and thyroid hormone levels are a well-known effect of PHAHs in rodents following single-dose or subchronic treatment (van Birgelen *et al.*, 1992, 1994a,b, 1995; Brouwer *et al.*, 1988b; Chen *et al.*, 1992). In short, retinol and thyroxine (T_4) were both reduced in rat plasma following exposure to metabolizable planar (PCB 77), mono-, and di-*ortho* PCBs (Brouwer *et al.* 1988a; Morse *et al.* 1996). However, TCDD and relatively stable PCBs (PCB 126, 169, 156) were found to increase plasma retinol, whereas T_4 was still reduced (van Birgelen 1994a,b, 1995). The reasons for the effects of PHAHs on plasma levels of retinol and T_4 are partly due to direct effects of hydroxylated PHAH metabolites on the plasma transport protein complex of T_4 and retinol (Brouwer and van den Berg, 1986; Brouwer *et al.*, 1988a,b); effects on hepatic metabolism of T_4 and retinol by parent compounds, i.e., mainly increased T_4 glucuronidation; and reduced formation of retinyl esters and retinol metabolism (Brouwer *et al.*, 1998; Zile, 1992).

In this manuscript, the effects on vitamin A and thyroid hormone status in female Sprague-Dawley rats was studied after subchronic exposure to complex PHAH mixtures. The possible interactive effects between congeners and the involvement of different mechanisms are discussed. Data were obtained from two independent experiments. In the first experiment, the focus was on a complex synthetic mixture of dioxin-like, planar compounds and possible interactive effects with the non-dioxin-like PCB 153 (2,2′,4,4′,5,5′-HxCB). The composition of this mixture was based on the presence and relative ratios of PHAH in fish, one of the main contributors of PHAHs to the human diet. In the second experiment, the main focus was on complex mixtures containing only di-*ortho* PCBs, or planar (non- and mono-*ortho*) PCBs. These mixtures were obtained by fractionation of the commercial PCB mixture Aroclor 1260. The planar and di-*ortho* PCBs were tested individually and after reconstitution.

MATERIALS AND METHODS

Chemicals. N-nitrosodiethylamine (NDEA) was obtained from Fluka (Fluka Chemie, Buchs, Switzerland); 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118), 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB 156), and 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153) were kindly provided by Prof. Å. Bergman (Department of Environmental Chemistry, Stockholm University, Sweden). 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (PeCDD) was obtained from Wellington Laboratories (Canada), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) was a gift from Prof. S. H. Safe (College of Veterinary Physiology and Pharmacology, Texas A&M University, USA), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from RADIAN CIL, Inc.(USA). All compounds had a purity > 99%. Aroclor 1260 was kindly provided by Dr. M. van den Berg (Research Institute of Toxicology, University of Utrecht, The Netherlands).

Retinoid standards (retinol, retinyl palmitate, and retinyl acetate) were all obtained from Fluka Chemie (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Amerlite chemiluminescence assay kits for thyroid hormone analysis were obtained from Ortho-Clinical Diagnostics (Amersham, UK).

All other compounds were of analytical grade.

Animal experiments. Two subchronic animal experiments were performed, both approved by the animal welfare committee before starting the experiments. The treatment protocol used for these experiments was based on the altered hepatic foci (AHF) tumor promotion protocol introduced by Pitot *et al.* (1978) and described in detail by van der Plas *et al.* (1999, 2000). In short, an initiation step, consisting of a diethylnitrosamine injection (i.p. 30 mg/kg body weight) 24 h after a partial (two-thirds) hepatectomy, is followed by a promotion treatment of 20 weeks starting 6 weeks after the hepatectomy.

For these experiments, juvenile female Sprague-Dawley rats (Møllegaard Breeding Center Ltd., Denmark) about 6 weeks of age at the start of the experiment were used. The rats were kept in wire-bottom, stainless steel cages in groups of four animals under standard conditions (12 h light/dark cycle, temperature 22°C, humidity 55%) and fed *ad libitum* (pellets, Hope Farms Woerden). Test compounds were administered once a week by subcutaneous injections for 20 weeks at concentrations indicated in Table 1. A corn oil group (1 ml/kg bw/week) as a negative vehicle control and a TCDD group (1 μg/kg body weight/week) as a positive control were incorporated in both experiments. The first dose was a loading dose, which was five times the concentration of the maintenance dose given for the following 19 weeks.

One week after the last injection, the animals were sacrificed under ether anesthesia, using orbital puncture for blood collection (heparinized), followed by decapitation. The liver was collected, of which a part was stored at −80°C for gas chromatography and mass spectrometry (GC-MS) analysis and a part was placed in formaldehyde and acetone fixative for immunohistochemistry purposes. The rest of the liver was homogenized on ice in a Potter tube with 0.01 M Tris–HCl-buffer and 0.25 M sucrose (pH 7.4). Plasma was collected by centrifugation of the blood at 500 × *g* for 10 min.

PHAH exposure mixtures. Two different environmentally relevant PHAH mixtures were tested in separate subchronic animal experiments in groups and concentrations as presented in Table 1.

The composition of the PHAH mixtures tested in experiment 1 was based on PHAH contamination found in Baltic herring and covered over 90% of the TEQs present in these fish. The PHAH mixture contained the following congeners: 2,3,7,8-TCDD; 1,2,3,7,8-PeCDD; 2,3,4,7,8-PeCDF; PCB 126; PCB 118; PCB 156; and PCB 153, in relative ratios indicated in Table 2. In order to investigate possible interactive effects between planar and nonplanar congeners, the mixture was also tested without the non-dioxin-like PCB 153. In addition to the PHAH mixtures, the commercial PCB mixture Aroclor 1254 was tested. The PHAH mixture containing solely dioxin-like PHAHs is referred to as PHAH–; the mixture including PCB 153 is referred to as PHAH+. A more detailed description of the mixture preparation and the experimental setup is given in van der Plas *et al.* (1999). Calculation of the TEQs of the PHAH mixtures was based on TEF values as proposed by the WHO (Ahlborg

TABLE 1
Dose Levels of the PHAH Mixtures and Aroclor 1260 Fractions Used in the Animal Studies

Mixture	(Experiment)	Group size n	Dose/kg bw/week	Equivalent to Aroclor 1260 Amount (mg)	Toxic equivalency (TEQ/kg bw/week)
Corn oil	(1,2)	18	1 ml	—	No activity
2,3,7,8-TCDD	(1,2)	12, 10	1 μg	—	1 μg
PHAH+ mixture	(1)	12	1.1 mg	—	0.5 μg[a]
			2.3 mg	—	1 μg[a]
			4.5 mg	—	2 μg[a]
PHAH–mixture	(1)	12	1.0 mg	—	1 μg[a]
0–1 *ortho* fraction	(2)	10	1 mg	10	1.1 ng[b]
2–4 *ortho* fraction	(2)	10	1 mg	1.1	No activity[b]
			3 mg	3.3	No activity[b]
			9 mg	10	No activity[b]
0–4 *ortho* fraction	(2)	10	10 mg	10	1.1 ng[b]
Aroclor 1254	(1)	12	7.5 mg	—	0.2 μg[c]
Aroclor 1260	(2)	10	10 mg	10	1.3 ng[b]

[a] TEQ values based on literature data (see van der Plas *et al.*, 1999).
[b] TEQ values determined using the DRE-CALUX assay (van der Plas *et al.*, submitted).
[c] TEQ value based on WHO TEF values (van den Berg *et al.*, 1998) and PCB concentrations in Aroclor 1254 (Leonards *et al.*, 1995).

et al., 1994) and relative potency (REP) values obtained from tumor promotion studies (Hemming *et al.*, 1993, 1995; Wærn *et al.*, 1991).

In experiment 2, the focus was on the non-dioxin-like di- to tetra-*ortho* substituted PCBs. For that purpose the commercial PCB mixture Aroclor 1260 was chosen as the experimental mixture, as approximately 90% of Aroclor 1260 consists of non-dioxin-like congeners. Aroclor 1260 was fractionated into a 0-1 *ortho* dioxin-like fraction (~9.7 % of the total mass) containing mainly 0- and 1-*ortho* substituted PCBs and a trace of 2-*ortho* PCBs, and a 2-4 *ortho* non-dioxin-like fraction (~90% of the total mass). This was done according to a method described by Athanasiadou *et al.* (1991), with slight modifications described in more detail by van der Plas *et al.* (2000). The 0-1 *ortho* fraction and the 2-4 *ortho* fraction were tested separately and as a reconstituted 0-4 *ortho* mixture. Aroclor 1260 and PCB 153 were incorporated as an extra positive control and a model compound for the di-*ortho* PCBs, respectively. The composition of PCB congeners in the various fractions was tested by GC-MS analysis and confirmed that no 2-4 *ortho* PCBs were present in the 0-1 *ortho* fraction (van der Plas *et al.*, 2000). The TEQs of the Aroclor 1260 fractions were determined using the DRE-CALUX bio-assay (van der Plas *et al.*, 2000).

TABLE 2
Congener Composition of the PHAH Mixtures of Experiment 1

Congener	Relative level (weight base) PHAH − mixture − PCB 153	PHAH + mixture + PCB 153
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	3.3	3.3
2,3,4,7,8-PeCDF	17	17
PCB 126	61	61
PCB 118	12800	12800
PCB 156	1888	1888
PCB 153	—	20000

Note. The composition of the mixtures is based on Swedish herring oil and covers over 90% of the TEQs in Swedish herring.

Vitamin A analysis. Retinol levels were measured in plasma and retinol and retinyl palmitate levels in liver homogenates according to Brouwer *et al.* (1989) with some modifications. Plasma, or liver homogenate (50 μl) was extracted with methanol containing 0.1% BHT as an antioxidant and an internal standard (0.5 μg retinyl acetate/ml for plasma, 1 μg retinyl acetate/ml for liver homogenate), and diisopropyl ether in a 1:1:2 concentration. Samples were vortexed for 30 sec., kept overnight at –20°C, vortexed again, and centrifuged for 10 min at 5000 rpm in an Eppendorf centrifuge. The diisopropyl-ether phase was collected and filtered over a 0.45-μm filter (Millipore, Etten Leur, The Netherlands), evaporated under N_2, and resuspended in 50 μl methanol (plasma extracts) or 200 μl 1:3 ethylacetate/methanol (liver extracts) with 0.1% BHT. Extractions were carried out in duplicate. Extraction efficiencies were routinely above 80%. Twenty-microliter aliquots of resuspended extracts were analyzed with HPLC using a C18 analytical reverse-phase column (Pecosphere, 3 μm particle size, 3.3 cm length, and 4.6 mm internal diameter, Perkin Elmer) and a wavelength of 326 nm for detection of retinoids. A Merck-Hitachi HPLC system was used consisting of an L-6200 Intelligent pump, L-4200 UV-VIS detector, AS-2000 Autosampler, and a D-2500 Chromato Integrator. Plasma extracts were analyzed isocratically with 86% methanol and 14% water with a flow rate of 1 ml/min and data collection for 10 min. Hepatic retinoids were analyzed by 86% methanol and 14% water for 1.5 min, followed by a gradient to 100% methanol for 2.5 min, and subsequent elution of the retinyl esters for 12 min. The column was then re-equilibrated at 86% methanol and 14% water for 6 min.

Plasma thyroid hormone analysis. Total thyroxine (TT_4), total triiodothyronine (T_3) and free thyroxin (FT_4) levels were determined in plasma using commercially available chemiluminescence kits, according to the protocol of the supplier with the following modifications: the T_4 assay buffer was diluted five times in demineralized water. The standard curve for TT_4 ranged from 0 to 120 nmol/liter, for T_3 from 0 to 6 nmol/liter, and for FT_4 from 0 to 106 pmol/liter. Thyroid hormone levels were calculated from the luminescence data with the Elia-Securia II software program of Canberra Packerd.

Statistical analysis. Data were analyzed with the statistical package SPSS-PC 7.5. A Tukey's Honestly Significant Difference test was used to perform a multiple comparison on statistical differences between groups.

TABLE 3
Retinoid Levels in Plasma and Liver Tissue of Female Sprague-Dawley Rats Following Subchronic Exposure to Dioxin-like PHAHs (Experiment 1)

Treatment Dose/kg bw/week		Retinol in plasma (ng/ml)	Retinol in liver tissue (μg/g liver)	Retinyl palmitate in liver tissue (μg/g liver)	Retinol/retinyl palmitate (μg/mg)
Corn oil	1 ml	172.3 ± 6.8[a]	4.75 ± 0.33	1402.8 ± 43.6[a]	3.5 ± 0.3
TCDD	1 μg	208.3 ± 12.4[b]	3.63 ± 0.44	357.4 ± 17.7[b]	10.4 ± 1.4
Aroclor 1254	7.5 mg	106.8 ± 5.5[a,b]	5.67 ± 0.55	1258.4 ± 51.7[a]	4.8 ± 0.5
PHAH–	1 μg TEQ	124.3 ± 6.5[a,b]	3.37 ± 0.32	471.6 ± 20.8[a,b]	7.3 ± 0.7
PHAH+	0.5 μg TEQ	155.9 ± 8.1[a]	4.06 ± 0.43	569.9 ± 37.4[b]	7.4 ± 0.9
PHAH+	1 μg TEQ	136.6 ± 11.2[a,b]	4.04 ± 0.87	436.9 ± 16.1[a,b]	9.1 ± 1.8
PHAH+	2 μg TEQ	117.1 ± 9.2[b]	3.50 ± 0.40	303.4 ± 16.2[a,b]	11.5 ± 1.0

Note. Data are given as arithmetic mean ± the standard error. Doses are given per kg bodyweight per week.
[a] Significantly different from the TCDD group ($p < 0.05$ Tukey HSD test).
[b] Significantly different from the corn oil group ($p < 0.05$ Tukey HSD test).

RESULTS

Vitamin A Levels

Experiment 1 (Table 3). Plasma retinol levels were significantly increased by 21% in the TCDD treatment group compared to the corn oil group. In contrast, significantly decreased plasma retinol levels were observed for all dioxin-like PHAH mixture groups as well as for Aroclor 1254. A dose-dependent decrease up to 32% reduction was observed in the groups treated with the PHAH+ mixture. The largest decrease in plasma retinol was found after treatment with Aroclor 1254 (38%), which is interesting, as in this group no significant effects were observed on the hepatic retinyl palmitate levels. In all other treatment groups, hepatic retinyl palmitate levels were strongly decreased (60–80%) as compared to the corn oil control group. The lowest dose of the PHAH+ mixture, 0.5 μg TEQ/kg/week (≈70 ng TEQ/kg bw/day), still gave rise to significant plasma retinol and, in particular, hepatic retinyl palmitate reductions, indicating that this is a very sensitive response of the PHAH mixture. Retinol levels in the liver were not affected in the PHAH treatment groups compared to the corn oil group. As a consequence, the retinol/retinyl palmitate ratios were increased in all treatment groups as compared to corn oil controls.

Experiment 2 (Table 4). Similar to results from the first experiment, the plasma retinol level was significantly increased

TABLE 4
Retinoid Levels in Plasma and Liver Tissue of Female Sprague-Dawley Rats Following Subchronic Exposure to Non-dioxin and Dioxin-like Fractions of Aroclor 1260 (Experiment 2)

Treatment Dose/kg bw/week		Retinol in plasma (ng/ml)	Retinol in liver tissue (μg/g liver)	Retinyl palmitate in liver tissue (μg/g liver)	Retinol/retinyl palmitate (μg/mg)
Untreated	—	176.6 ± 6.9	8.22 ± 0.86	2052.3 ± 111.3	4.0 ± 0.3
Corn oil	1 ml	171.7 ± 6.6	7.37 ± 0.73	1733.1 ± 70.4	4.2 ± 0.4
TCDD	1 μg	223.1 ± 16.0[a]	4.05 ± 0.39[a]	474.5 ± 32.0[a]	9.1 ± 1.3[a]
Aroclor 1260	10 mg	162.1 ± 6.6	4.65 ± 0.31	1280.8 ± 46.3[a]	3.7 ± 0.3
0–4 *ortho*	10 mg	167.0 ± 6.3	5.73 ± 0.68	1400.0 ± 62.3	4.1 ± 0.5
0–1 *ortho*	1 mg	143.4 ± 9.9	6.72 ± 0.76	1731.5 ± 175.7	4.1 ± 0.6
2–4 *ortho*	1 mg	177.3 ± 11.8	5.58 ± 0.72	1688.6 ± 71.9	3.3 ± 0.4
2–4 *ortho*	3 mg	168.3 ± 12.7	4.59 ± 0.43	1529.2 ± 64.3	3.0 ± 0.2
2–4 *ortho*	9 mg	174.0 ± 11.0	5.14 ± 0.58	1476.1 ± 66.9	3.5 ± 0.4
PCB 153	1 mg	168.4 ± 8.6	6.04 ± 0.85	1770.4 ± 89.8	3.4 ± 0.4
PCB 153	9 mg	184.6 ± 12.6	4.56 ± 0.71	1233.9 ± 77.0[a]	3.6 ± 0.5

Note. Data are given as arithmetic mean ± the standard error. Doses are given per kilogram bodyweight per week. 2–4 *ortho* = 2–4 *ortho* substituted PCB fraction of Aroclor 1260; 0–1 *ortho* = 0–1 *ortho* substituted PCB fraction of Aroclor 1260 with a trace of 2 *ortho* substituted PCBs; 0–4 *ortho* = the reconstituted mixture of the 2–4 and the 0–1 *ortho* fraction.
[a] Significantly different from the corn oil group ($p < 0.05$ Tukey HSD test).

TABLE 5
Thyroid Hormone Levels in Plasma of Female Sprague-Dawley Rats Following Subchronic Exposure to Dioxin-like PHAHs (Experiment 1)

Treatment Dose/kg bw/week		Total T_3 in plasma (nmol/l)	Total T_4 in plasma (nmol/l)	Free T_4 in plasma (pmol/l)	Total T_4/free T_4 (nmol/pmol)
Corn oil	1 ml	1.56 ± 0.07	34.39 ± 1.52[a]	13.75 ± 0.89[a]	2.6 ± 0.11
TCDD	1 μg	1.46 ± 0.09	21.46 ± 1.34[b]	8.80 ± 0.60[b]	2.5 ± 0.15
Aroclor 1254	7.5 mg	1.37 ± 0.09	19.96 ± 1.75[b]	12.33 ± 1.07	1.6 ± 0.05[a,b]
PHAH–	1 μg TEQ	1.40 ± 0.07	15.71 ± 1.22[b]	10.24 ± 0.76	1.5 ± 0.06[a,b]
PHAH+	0.5 μg TEQ	1.22 ± 0.09	15.50 ± 1.67[b]	8.31 ± 0.93[b]	1.9 ± 0.10[a,b]
PHAH+	1 μg TEQ	1.32 ± 0.11	11.73 ± 1.32[a,b]	8.14 ± 0.91[b]	1.5 ± 0.06[a,b]
PHAH+	2 μg TEQ	1.18 ± 0.11	8.28 ± 0.81[a,b]	7.34 ± 0.64[b]	1.1 ± 0.08[a,b]

Note. Data are given as arithmetic mean ± the standard error. Doses are given per kilogram bodyweight per week.
[a] Significantly different from the TCDD group ($p < 0.05$ Tukey HSD test).
[b] Significantly different from the corn oil group ($p < 0.05$ Tukey HSD test).

in the TCDD treatment group (by 30%) compared to the corn oil control group. However, no significant changes in plasma retinol levels were observed after exposure to any of the PCB fractions, Aroclor 1260, or PCB 153. Hepatic retinoid levels in the corn oil group were slightly but nonsignificantly lower compared to the untreated control group. Analysis of the hepatic ethoxyresorufin-*O*-deethylase activity (van der Plas *et al.*, in press) and luciferase induction by hepatic microsomes (unpublished results) in the AhR-dependent H4IIE-Luc reporter gene (DRE-CALUX) assay did not indicate that this may be due to a PHAH contamination of the corn oil. Therefore it is concluded that the observed difference between the untreated and the corn oi1 group in hepatic retinoid levels is more likely related to the treatment procedure. In the group treated with TCDD, the hepatic retinyl palmitate level was significantly decreased to 55% of the corn oil control group. Aroclor 1260 and PCB 153, 9 mg/kg bw/week, also significantly decreased the hepatic retinyl palmitate levels to 62% of the corn oil group. The Aroclor 1260 fractions and PCB 153, 1 mg/kg bw/week, did not affect retinyl palmitate levels in the liver significantly, although there was a tendency for a dose-dependent reduction in the 2-4 *ortho* fraction. Retinol levels in the liver after PCB exposure were all nonsignificantly decreased compared to the corn oil control. Except for TCDD, none of the PHAH compounds changed the hepatic retinol/retinyl palmitate ratio.

Thyroid Hormone Levels

Experiment 1 (Table 5). A small, statistically nonsignificant decrease was observed in the total plasma T_3 levels after PHAH treatment compared to the corn oil control. However, plasma total and free T_4 levels were decreased in most PHAH treatment groups. A dose-dependent decrease of the total T_4 levels compared to the corn oil group was observed after treatment with different doses of the PHAH+ mixture up to 76% reduction in the highest dose of 2 μg TEQ/kg bw/week. At the lowest dose of 0.5 μg TEQ/kg bw/week of the PHAH+ mixture, the plasma total T_4 level was still reduced by >50% as compared to corn oil controls. The total T_4 level was much less reduced (38%) after exposure to 1 μg/kg bw/week TCDD as compared to the TEQ equivalent dose of the PHAH+ mixture. Free T_4 levels were decreased in all treatment groups, but the ratio of total T_4 over free T_4 (illustrating the effect on the T_4 fraction bound to its transport protein TTR) differed between the treatment groups. The total T_4/free T_4 ratio was not changed in the TCDD exposure group compared to the corn oil control. However, a dose-dependent decrease of the total T_4/free T_4 ratio was observed for the PHAH mixture groups. Although the PHAH– and PHAH+ group at 1 μg TEQ/kg bw/week are equipotent in theory, the effects on T_3 and T_4 levels were stronger in the latter.

Experiment 2 (data not shown). No changes were found in thyroid hormone levels after exposure to Aroclor 1260, any of its fractions, or PCB 153. A slight, nonsignificant decrease in total T_4 was observed after treatment with TCDD. The total T_4/free T_4 ratio was significantly decreased (24%) after treatment with the 0-4 *ortho* fraction.

DISCUSSION

The effects on endocrine parameters, i.e., thyroid hormone and retinoid status, by complex mixtures of PHAHs are part of a larger study aimed at investigating the predictability of subchronic effects, as tumor promotion and biochemical effects, by the TEF concept. The focus in this paper is on the impact of dioxin-like and non-dioxin-like PHAH mixtures on thyroid hormone and retinoid levels in plasma and liver. Both parameters have been suggested to be implicated in the expression of subchronic effects such as tumor promotion and reproductive and developmental effects. Until now, much research has been performed on studying mainly short-term effects of individual congeners of PCBs and dioxins on vitamin A and thyroid

hormone metabolism. Here, the impact of complex PHAH mixtures following subchronic exposure is discussed, and attention is given to the contribution of strictly dioxin-like and non-dioxin-like PCB congeners, the involvement of different mechanisms, and the predictive value of the TEF concept.

Effects on Hepatic Retinoids

A statistically significant decrease of the retinyl palmitate but not of the hepatic retinol levels was observed after treatment with TCDD (experiment 1), the PHAH+ and the PHAH– mixture, PCB 153, and the commercial PCB mixture Aroclor 1260. In experiment 2, TCDD decreased both hepatic retinol and retinyl palmitate. The effects of TCDD on vitamin A status have been extensively studied in many species and have been shown to alter the vitamin A status in all species examined to date (Zile, 1992). A severe decrease of hepatic vitamin A levels was also observed after exposure to e.g., PCB 77 (Brouwer and van den Berg, 1986; Chen *et al.*, 1992); PCB 126 (Chen *et al.*, 1992; van Birgelen *et al.*, 1994b); PCB 156 (van Birgelen *et al.*, 1994a); and to a lesser extent after treatment with e.g., PCB 153 (van Birgelen *et al.*, 1992) and Aroclor 1254 (Morse and Brouwer, 1995). The decrease of hepatic retinyl ester levels induced by TCDD and related PHAHs may be due to a combination of increased mobilization of hepatic stores of vitamin A and inhibition of the storage of newly ingested vitamin A in the liver (Håkansson and Ahlborg, 1985; Håkansson *et al.*, 1988; Kelley *et al.*, 1998; Zile, 1992;). TCDD was found to severely decrease the lecithin:retinol acyltransferase (LRAT) activity in the hepatic stellate cells (Nilsson *et al.*, 1996), an enzyme involved in the conversion of retinol into retinyl esters. Increased mobilization of hepatic retinyl esters could be the result of a direct effect of TCDD on hepatic enzyme activities or by an up regulation of a signal controlling release of vitamin A stores into circulation (Kelley *et al.*, 1998; Zile, 1992).

The decrease of the hepatic retinyl palmitate concentration appeared to be a rather sensitive effect of PHAH exposure. Even at the lowest dose of 0.5 μg TEQ/kg bw/week of the PHAH+ mixture, equivalent to ≈70 ng TEQ/kg bw/week, a reduction of 60% of the hepatic retinyl palmitate level was found. In addition, the effect of the dioxin-like PHAH mixtures appeared to be quite well predicted by the TEF concept, i.e., an almost equal decrease of hepatic retinyl palmitate levels was observed after treatment with TEQ equivalent doses of PHAH mixture and TCDD (68% vs. 75%). The effect of the PHAH+ mixture on the hepatic retinoid level is within the same range observed for the TEQ equivalent dose of the PHAH– mixture, whereas both mixtures have a slightly, but significant, smaller effect on the hepatic retinoid level as compared to the equipotent TCDD group. The Lowest Observed Adverse Effect Level (LOAEL) for the PHAH mixtures from this study is close to the LOAEL of 14 ng TEQ/kg bw/day of TCDD reported by van Birgelen *et al.* (1995). In contrast to the PHAH mixtures, Aroclor 1254 (estimated dose 0.2 μg TEQ/kg bw/week) induced only a slight decrease of the hepatic retinyl palmitate level as compared to the corn oil group. A possible explanation might be that PCB 118 and PCB 156 are the main contributors (~60%) to the TEQ value of Aroclor 1254 (Leonards *et al.*, 1995), whereas for the TEQ of the PHAH mixtures these congeners are only of minor importance (14%). Håkansson *et al.* (1994) reported that PCB 118 had no effect on the hepatic vitamin A content at dietary levels up to 2000 μg/kg. Also for PCB 156, the loss of hepatic retinoids was shown to be a less sensitive parameter than, for instance, reduction of plasma T_4 or CYP1A2 induction (van Birgelen *et al.*, 1994a).

Aroclor 1260 reduced the hepatic retinyl palmitate concentration by 30% compared to the corn oil control, whereas its 0-4 *ortho* PCB fraction decreased the retinyl palmitate levels, nonsignificantly, by 20%. The most likely explanation for this difference in effect is the loss of impurities, i.e., PCDFs, during the fractionation of Aroclor 1260 (Athanasiadou *et al.*, 1991). In fact, a slightly lower ethoxyresorufin-*O*-deethylase (EROD) activity was observed after exposure to the 0-4 *ortho* PCB fraction as compared to Aroclor 1260 (van der Plas *et al.*, 2000), which underscores the former explanation.

The effect of the 2-4 *ortho* PCB fraction on the hepatic retinoid concentration was somewhat lower but close to the effect of the di-*ortho* PCB 153. PCB 153 is one of the dominant di-*ortho* congeners in environmental PHAH mixtures and is often used as a representative for the group of 2-4 *ortho* PCBs. In this study a statistically significant effect of PCB 153 on the retinoid levels in the liver was observed at the highest dose of 9 mg/kg bw/week (≈1.2 mg/kg bw/day). This is in agreement with the results of a 13-week feeding study in female rats, in which PCB 153 was shown to decrease the hepatic retinoid level from 10 ppm (≈0.72 mg/kg bw/day) onwards (van Birgelen *et al.*, 1992). As the toxicity of di-*ortho* PCBs is not mediated by the Ah receptor, no TEF values are available, and consequently risk assessment is not possible for this category of PCBs. However, the observed effects on the hepatic retinoid level occurred at very high doses, and it is not likely that the non-dioxin-like PCBs will effect retinoid status at environmental exposure levels.

Effects on Plasma Retinol

The plasma retinol level was increased after TCDD exposure in both experiments, compared to the corn oil control. In rat, an increase of plasma retinol after TCDD treatment has been reported before (van Birgelen *et al.*, 1992, 1994, 1995; Håkansson *et al.*, 1988; Kelley *et al.* 1998), and a similar effect was seen after exposure to 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 169) or 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB 156) (van Birgelen *et al.*, 1994a; Chen *et al.*, 1992). An increase of the plasma retinol level after PHAH exposure may be the result of an enhanced mobilization of hepatic vitamin A (Zile, 1992). However, the mechanism by which hepatic mobilization of retinol is increased is still unknown, as TCDD and related

compounds either inhibit or have no effect on the activity of hepatic retinyl ester hydrolase (REH) (Chen *et al.*, 1992).

In contrast to the TCDD treatment groups, a decrease of the plasma retinol levels was observed in the animals treated with the PHAH mixtures and Aroclor 1254. A decrease of plasma retinol levels was also reported after exposure to e.g., 3,3′,4,4′-TCB (PCB 77); 2′,3,3′,4,5-PeCB (PCB 122); 3,3′,4,4′,5-PeCB (PCB 126); 2,2′,3,3′,5,5′-HxCB (PCB 133); and the commercial PCB mixture Aroclor 1254 (Brouwer and van den Berg, 1986; Chen *et al.*, 1992; Morse *et al.*, 1996). It has been suggested that a decrease in plasma retinol levels, as seen after exposure to the PHAH mixtures, may be caused by a decrease of hepatic REH activity involved in the mobilization of hepatic vitamin A stores (Zile, 1992). However, such a decrease of REH activity by PHAHs has not been observed before.

Another mechanistic explanation for a decrease of plasma retinol concentrations by PHAHs has been reported by Brouwer *et al.* (1988a). The 4-hydroxy metabolite of PCB 77 was found to displace T_4 from its transport protein transthyretin (TTR), leading to a destabilization of the RBP-TTR complex and subsequently to a decrease in plasma retinol and thyroxin concentrations (Brouwer and van den Berg, 1986; Brouwer *et al.*, 1988a). A number of other hydroxylated PHAHs were found to possess a high binding affinity for TTR as well, including hydroxylated PCBs likely to be formed of congeners that are present in Aroclor 1254 and/or the PHAH mixtures, i.e., PCB 105 (2,3,3′,4,4-PeCB), 118, 126, and 156, (Bergman *et al.* 1994; Brouwer *et al.*, 1998; Lans *et al.*, 1993, 1995a). Disruption of plasma transport of retinol and thyroxin does not play a role in the case of TCDD exposure, because only low amounts of hydroxy metabolites are formed from TCDD *in vivo* (Lans *et al.*, 1995b). From the data presented here, it became clear that there was no correlation between the exposure in TEQs and the effect on plasma retinol. It is therefore concluded that the TEF approach is not applicable to the prediction of effects on plasma retinol levels after exposure to complex PHAH mixtures.

Effects on Plasma Thyroid Hormone

Plasma T_3 concentrations were not significantly decreased after PHAH treatment compared to the corn oil control. However, severe reductions in both plasma TT_4 and FT_4 levels were observed. This phenomenon is in accordance with other reports on effects of PHAHs on thyroid hormones (van Birgelen *et al.*, 1992, 1994a,b; Lans *et al.*, 1995a). Interestingly, the decrease of the TT_4 levels in the PHAH mixture and the Aroclor 1254 treatment groups was considerably stronger compared to the effect observed in the TEQ equivalent TCDD group, whereas lesser or equal effects were observed for free T_4. Consequently, in the Aroclor 1254 and the PHAH groups, the TT_4/FT_4 ratio was decreased as compared to both the TCDD and the corn oil group. This indicates a lower proportion of protein-bound T_4 after exposure to Aroclor 1254 or the PHAH mixtures, which is in line with a disturbance of the plasma protein transport system of T_4 due to T_4-TTR binding competition by hydroxylated PCB metabolites (Brouwer *et al.*, 1998). Lans *et al.* (1995a) demonstrated that in the blood of rats exposed to a single dose of Aroclor 1254, the hydroxylated PCB metabolite 4-OH-2,3,3′,4′5-PeCB competitively inhibited T_4 binding to TTR. It might be concluded that the TEF concept failed in its prediction for the effect of PHAH exposure on the thyroid hormone status, as the TEF concept does not account for additional toxicity of hydroxylated PCBs as possibly observed here.

In the PHAH+ group, the TT_4/FT_4 ratio was similar to the TEQ equivalent-dosed PHAH– group, but the absolute decrease of the TT_4 and FT_4 concentrations was stronger in the first. This is probably an indirect effect of the non-dioxin-like PCB 153, which was added to the PHAH+ mixture. PCB 153 was shown to increase the hepatic deposition of all dioxin-like congeners, which resulted in an increased internal exposure (van der Plas *et al.*, 1998, 1999) and possible enhancement of hepatic T_4 glucuronidation. A direct effect of PCB 153 on hepatic T_4 glucuronidation was considered less likely, since the amount of PCB 153 in the PHAH mixture was below the concentration where effects of PCB 153 on the thyroid hormone status might be expected (see experiment 2; van Birgelen *et al.*, 1992).

In the second experiment, no statistically significant effects on the thyroid hormone levels were observed except for a decrease of the total T_4/free T_4 ratio after treatment with the reconstituted 0-4 *ortho* PCB fraction and a minor, nonsignificant decrease of the total T_4/free T_4 ratio after treatment with Aroclor 1260. Van der Plas *et al.* (2000) reported a 7- to 8-fold increase of EROD induction after treatment with the 0-4 *ortho* fraction and Aroclor 1260. Although the EROD activity observed after treatment with the 0-4 *ortho* fraction and Aroclor 1260 is relatively low as compared to the more than 100 times increase that can be observed after treatment with TCDD, the CYP1A induction might be high enough to stimulate formation of hydroxylated PCBs. Based on the exposure in TEQs of the 0-4 *ortho* fraction and Aroclor 1260 (see Table 1), no effects were expected, as a NOEL of TCDD for decreasing plasma TT_4 levels was estimated on 26 ng/kg bw/day (van Birgelen *et al.*, 1995).

On the basis of these studies it was concluded that the effect of complex PHAH mixtures on hepatic retinyl palmitate was quite well predictable by the TEF concept. However, the TEF concept failed in its prediction for the effects on plasma retinol and underestimated the effect on plasma thyroid hormone concentrations, possibly because the additional toxicity by hydroxylated PCBs is not taken into account. Treatment with the 0-4 *ortho* fraction at a TEQ level more than 100 times below the NOEL for TCDD (estimated by van Birgelen *et al.*, 1995) still induced a significant decrease of the total T_4/free T_4 ratio. The non-dioxin-like PCBs did not significantly alter the retinoid and thyroid hormone status at the dose levels tested,

indicating that in case of exposure to these PCBs at environmental levels, no effects, or at best, only marginal effects can be expected on the retinoid and thyroid hormone status.

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