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

Simone A. van der Plas,* Ineke Lutkeschipholt,* Bert Spenkelink,* and Abraham Brouwer*††

*Department of Food Technology & Nutritional Sciences, Toxicology Group, Wageningen University, P.O. Box 8000, 6700 EA Wageningen, The Netherlands; and †Institute of Environmental Studies (IVM), Free University of Amsterdam, De Boelelaan 1115, 1081 HV Amsterdam, The Netherlands

Received June 13, 2000; accepted September 26, 2000

The aim of this study was to determine the effects of subchronic exposure to complex mixtures of polychlorinated 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-
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 (T₄) 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 T₄ was still reduced (van Birgelen 1994a,b, 1995). The reasons for the effects of PHAHs on plasma levels of retinol and T₄ are partly due to direct effects of hydroxylated PHAH metabolites on the plasma transport protein complex of T₄ and retinol (Brouwer and van den Berg, 1986; Brouwer et al., 1988a,b); effects on hepatic metabolism of T₄ and retinol by parent compounds, i.e., mainly increased T₄ 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. A. 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-pentachlorodibenzo-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). Amerelte cholinuminescence 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 0.025 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 et al., 1994a,b, 1995; van Birgelen 1994a,b, 1995; van der Plas et al., 1999, 2000).
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., 1999).

**TABLE 1**

<table>
<thead>
<tr>
<th>Mixture</th>
<th>(Experiment)</th>
<th>Group size n</th>
<th>Dose/kg bw/week</th>
<th>Equivalent to Aroclor 1260 Amount (mg)</th>
<th>Toxic equivalency (TEQ/kg bw/week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>(1,2)</td>
<td>18</td>
<td>1 ml</td>
<td>—</td>
<td>No activity</td>
</tr>
<tr>
<td>2,3,7,8-TCDD</td>
<td>(1,2)</td>
<td>12, 10</td>
<td>1 µg</td>
<td>—</td>
<td>1 µg</td>
</tr>
<tr>
<td>PHAH + mixture</td>
<td>(1)</td>
<td>12</td>
<td>1.1 mg</td>
<td>—</td>
<td>0.5 µg a</td>
</tr>
<tr>
<td>PHAH–mixture</td>
<td>(1)</td>
<td>12</td>
<td>2.3 mg</td>
<td>—</td>
<td>1 µg b</td>
</tr>
<tr>
<td>0–1 ortho fraction</td>
<td>(2)</td>
<td>10</td>
<td>4.5 mg</td>
<td>—</td>
<td>2 µg c</td>
</tr>
<tr>
<td>2–4 ortho fraction</td>
<td>(2)</td>
<td>10</td>
<td>1.0 mg</td>
<td>—</td>
<td>1 µg d</td>
</tr>
<tr>
<td>0–4 ortho fraction</td>
<td>(2)</td>
<td>10</td>
<td>1.0 mg</td>
<td>1 mg</td>
<td>1.1 ng e</td>
</tr>
<tr>
<td>Aroclor 1254</td>
<td>(1)</td>
<td>12</td>
<td>10 mg</td>
<td>3 mg</td>
<td>1.1 ng e</td>
</tr>
<tr>
<td>Aroclor 1260</td>
<td>(2)</td>
<td>10</td>
<td>10 mg</td>
<td>9 mg</td>
<td>10 No activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.3 mg</td>
<td>9 mg</td>
<td>10 No activity</td>
</tr>
</tbody>
</table>

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).

**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 N2, 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 retinol 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 (TT4), total triiodothyronine (T3) and free thyroxin (FT4) levels were determined in plasma using commercially available chemiluminescence kits, according to the protocol of the supplier with the following modifications: the T4 assay buffer was diluted five times in demineralized water. The standard curve for TT4 ranged from 0 to 120 nmol/liter, for T3 from 0 to 6 nmol/liter, and for FT4, from 0 to 106 pmol/liter. Thyroid hormone levels were calculated from the luminesence data with the Elia-Securia II software program of Canberra Packard.

**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)

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

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...
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 oil 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 \(\mu g\) TEQ/kg bw/week. At the lowest dose of 0.5 \(\mu g\) TEQ/kg bw/week of the PHAH+ mixture, the plasma total \(T_4\) level was still reduced by >50% compared to corn oil controls. The total \(T_4\) level was much less reduced (38%) after exposure to 1 \(\mu g\) 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 \(T_4/\text{free } T_4\) ratio was not changed in the TCDD exposure group compared to the corn oil control. However, a dose-dependent decrease of the total \(T_4/\text{free } T_4\) ratio was observed for the PHAH mixture groups. Although the PHAH– and PHAH+ group at 1 \(\mu g\) 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 \(T_4/\text{free } T_4\) 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 with 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 T4 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., PCDDs, 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 hepatic 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 T₄ 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 to 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 T₄ concentrations were not significantly decreased after PHAH treatment compared to the corn oil control. However, severe reductions in both plasma TT₄ and FT₄ 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 TT₄ 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 T₄. Consequently, in the Aroclor 1254 and the PHAH groups, the TT₄/FT₄ ratio was decreased as compared to both the TCDD and the corn oil group. This indicates a lower proportion of protein-bound T₄ after exposure to Aroclor 1254 or the PHAH mixtures, which is in line with a disturbance of the plasma protein transport system of T₄ due to T₄-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 T₄ 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 TT₄/FT₄ ratio was similar to the TEQ equivalent-dosed PHAH− group, but the absolute decrease of the TT₄ and FT₄ 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 T₄ glucuronidation. A direct effect of PCB 153 on hepatic T₄ 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 T₄/free T₄ ratio after treatment with the reconstituted 0-4 ortho PCB fraction and a minor, non-significant decrease of the total T₄/free T₄ 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 TT₄ 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 T₄/free T₄ 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.

ACKNOWLEDGMENTS

We thank Gerrit van Tintelen, Jo Haas, Bert Weijers, Annelies Landman, and Maria Faassen-Peters (Center for Laboratory Animals, Agricultural University Wageningen, The Netherlands) for their assistance during the animal experiments, and all colleagues of the Division of Toxicology (Department of Food Technology & Nutritional Sciences, Agricultural University Wageningen, The Netherlands) for their help during termination of the experiments. We thank all colleagues from the Institute of Environmental Medicine of the Karolinska Institute (Stockholm, Sweden), and especially Gunilla Scheu, for their assistance. The research on which this article is based has been funded by the Dutch Ministry of Agriculture, Nature Management and Fisheries, and partially supported by the Swedish Environmental Protection Agency.

REFERENCES


