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A 28-Day Oral Dose Toxicity Study Enhanced to Detect Endocrine Effects of Hexabromocyclododecane in Wistar Rats

Leo T. M. van der Ven,*[,1] Aart Verhoef,* Ton van de Kuil,* Wout Slob,† Pim E. G. Leonards,‡ Theo J. Visser,§ Timo Hamers,‡ Maria Herlin,¶ Helen Håkansson,¶ Hanna Olausson,¶ Aldert H. Piersma,* and Josephus G. Vos*

**Laboratory for Toxicology, Pathology and Genetics and †Centre for Substances and Integrated Risk Assessment, National Institute of Public Health and the Environment (RIVM), 3720 BA Bilthoven, The Netherlands; ‡Institute for Environmental Studies, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands; §Department of Internal Medicine, Room Ee 502, Erasmus Medical Center, 3000 Dr Rotterdam, The Netherlands; and ¶The Institute of Environmental Medicine, Karolinska Institutet, 171 77 Stockholm, Sweden*

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A 28-day repeated dose study in rats (OECD407) enhanced for endocrine and immune parameters was performed with hexabromocyclododecane (HBCD). Rats were exposed by daily gavage to HBCD dissolved in corn oil in 8 dose groups with doses ranging between 0 and 200 mg/kg bw per day (mkd). Evaluation consisted of dose-response analysis with calculation of a benchmark dose at the lower 95% one-sided confidence bound (BMDL) at predefined critical effect sizes (CESs) of 10–20%. The most remarkable findings were dose-related effects on the thyroid hormone axis, that is, decreased total thyroxin (TT4, BMDL 55.5 mkd at CES – 10%), increased pituitary weight (29 mkd at 10%) and increased immunostaining of TSH in the pituitary, increased thyroid weight (1.6 mkd at 10%), and thyroid follicle cell activation. These effects were restricted to females. Female rats also showed increased absolute liver weights (22.9 mkd at 20%) and induction of T4-glucuronyl transferase (4.1 mkd at 10%), suggesting that aberrant metabolization of T4 triggers feedback activation of the thyroid hormone system. These effects were accompanied by possibly secondary effects, including increased cholesterol (7.4 mkd at 10%), increased tibial bone mineral density (> 49 mkd at 10%), both in females, and decreased splenocyte counts (0.3–6.3 mkd at 20%; only evaluated in males). Overall, female rats appeared to be more sensitive to HBCD than male rats, and an overall BMDL is proposed at 1.6 mkd, based on a 10% increase of the thyroid weight, which was the most sensitive parameter in the sequence of events.

Key Words: **hexabromocyclododecane; brominated flame retardants; endocrine disruption; thyroid hormones; rats; hazard identification; risk assessment.**

Hexabromocyclododecane (HBCD) is a brominated flame retardant (BFR) which has its main application in textile industry and in polystyrene foam for building construction (Hutzinger and Thoma, 1987). The compound is simply blended with polymers and, therefore, more likely to leach out of products than covalently bound flame retardants. Indeed, HBCD has been identified as a contaminant of environmental significance (Letcher and Behnisch, 2003). In aquatic biota in polluted areas, levels of HBCD are comparable to other BFRs, such as tetrabromobisphenol-A and some polybrominated biphenylethers (Morris *et al.*, 2004; Sellstrom *et al.*, 2003), although the production volume of HBCD is lower than of those other BFRs. Low but significant exposure levels of this potentially persisting and bioaccumulating compound are found in humans with a fluctuating increase over time (Fängström *et al.*, 2006; Ryan *et al.*, 2006). Intake *via* food is possibly the largest source of human HBCD exposure, although inhalation and dermal routes may contribute significantly, notably, in occupational exposure (KEMI, 2003). Therefore, the use of this compound is a reason for environmental and health concern (Alaee *et al.*, 2003).

The current toxicological database on HBCD is too limited to form a solid basis for an integrated risk assessment, combining data from effect and exposure studies in multiple species and environmental levels (Letcher and Behnisch, 2003). HBCD has been tested in a 28-day toxicity study in rats showing a lowest observed effect level of 125 mg/kg on the basis of liver weight effects, but no hormonal parameters were addressed (Chengelis, 1997; cited in KEMI, 2003). In a 90-day toxicity study, also in rats, liver weight increases were found at 100 mg/kg and higher without observed pathology. In addition, there were effects on the thyroid hormone axis, that is, decreased T4 and increased TSH (Chengelis, 2001; cited in KEMI, 2003). Furthermore, in mice, HBCD induced developmental neurotoxic effects (Eriksson *et al.*, 2006). Additional studies are needed to understand effects and risks of exposure to this compound in wildlife, humans, and their ecosystems,

This paper is dedicated to the memory of Prof Joseph G. Vos, inspiration of our endocrine disruption research, who died on May 15 2006.

[1] To whom correspondence should be addressed at Laboratory for Toxicology, Pathology and Genetics, National Institute of Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands. Fax: +31-30-274-4446. E-mail: l.van.der.ven@rivm.nl.

that is, to understand mechanisms of toxicity and/or biological effects and to identify risk of exposure (Darnerud, 2003).

Therefore, the current subacute oral dose study was undertaken in rats, with a focus on endpoints commonly affected by persistent organic pollutants, that is, in the immune and endocrine domains, including effects on the thyroid hormone (TH) axis, on hematology, on bone size and mineralization, and on retinoid parameters (Andrews *et al.*, 2001). So far, in line with the observations mentioned above, the TH system has been identified as a target for potential endocrine disruptive hazard of BFRs in general (Legler and Brouwer, 2003). Interaction of BFRs with the TH system was mainly described at the level of TH metabolism; several BFRs inhibit TH sulfation, modulate deiodination, and induce glucuronidase and uridine diphosphate (UDP) glucuronyl transferase. Further BFR interactions with the TH system are known at the level of transport, for example, competition with T4 on transthyretin (TTR), thus possibly facilitating transport over the placenta and the blood-brain barrier.

The rationale to test the endocrine effects of HBCD in the *in vivo* model in this study was supported by results from *in vitro* tests with indicator cell lines sensitive to endocrine activity, which showed that HBCD can interact with multiple hormone systems, particularly with TH-mediated cell proliferation and with progesterone receptors, and to a lesser extent with estrogen, androgen, and dioxin (arylhydrocarbon) receptors (Hamers *et al.*, 2006), while significant enhancement of TH-receptor (TR)–mediated gene expression was confirmed in HeLa TR cells (Yamada-Okabe *et al.*, 2005).

ANIMALS, MATERIALS, AND METHODS

Forty Wistar rats (RIVM Cpb:WU) of both sexes, aged 7 weeks, were purchased from the RIVM breeding facilities (three to four generations after original purchase from HsdCpb:WU Harlan, Horst, NL) and housed individually to allow recording of individual feed consumption, and to avoid bias from hierarchical stress. Housing was in plastic Macrolon cages of appropriate sizes with stainless steel wire cover and chopped wood bedding. Light/dark regime was 12/12 h. Standard pelleted rat feed without soy (RMH-GS, Hope Farms/ABDiets, Woerden, NL; this standardized formulation contains ca 18,800 IU vit.A/kg) and drinking water were supplied *ad libitum*. HBCD (technical mixture containing traces of tetra- and pentabromocyclododecane) was obtained as a composite mix through Bromine Science and Environmental Forum (BSEF, with kind cooperation of Dr Klaus Rothenbacher). The technical preparation is a mixture of three enantiomers, HBCD-α, -β, and -γ, and their respective proportion in the used batch was 10.28, 8.72, and 81.01%. It was dissolved in corn oil *via* primary solution in acetone, which was evaporated afterward. Target dosing was 0, 0.3, 1, 3, 10, 30, 100, and 200 mkd; the highest dose depended on maximum HBCD solubility in corn oil. Appropriate concentrations of HBCD in corn oil were based on daily gavage of 5 ml/kg bw.

The experimental protocol followed the OECD407 28-day subacute toxicity guideline, which was enhanced for endocrine and immunological endpoints (Andrews *et al.*, 2001). However, in contrast to the published protocol, the animals were distributed among more dose groups each with fewer animals, that is, five rats per sex per dose group, for improved assessment of dose-response relationships (Kavlock *et al.*, 1996; Slob, 2002).

Exposure started at the age of 11 weeks. Animals were inspected daily for general condition and clinical abnormalities. Beddings were changed weekly, at which time rats were submitted to in-hand observations and reaction to handling, and their body weight and feed consumption was recorded.

At the end of the exposure period, animals were necropsied in the course of 5 days, resulting in a total of 28–33 days of exposure. Necropsies were performed in a targeted manner to avoid clustering of animals from single exposure groups on one day and to allow clustered immunological analysis which was only performed in males to avoid variability due to the estrous cycle. Targeted necropsy of females in the first day of diestrous was abandoned because staging of the estrous cycle on the basis of vaginal smears was not completely consistent with further histological staging of uterus and vagina epithelium. Euthanasia was achieved by exsanguination from the abdominal aorta under carbon dioxide anesthesia.

During necropsy, blood was collected and the following organs were dissected: skin/mammary gland, liver, pancreas, spleen, thymus, heart, lungs, stomach, duodenum, ileum, jejunum, colon, ceacum, ovaries, uterus, urine bladder, kidney, adrenals, trachea/thyroid gland, brain, pituitary gland, testes, epididymis, seminal vesicles with coagulation gland, prostate, popliteal lymph nodes, ischiadic nerve, fat, muscle, bone marrow (from femur), sternum, and thoracic spine. All organs were visually inspected and weighed directly after dissection or after fixation (thyroid, pituitary) to reduce mechanical damage. Sperm was collected from the cauda epididymis for direct analysis of motility and morphology. Defined samples of the liver, intestines, brain, adrenals, testes, ovaries, muscle, and fat were snap frozen in liquid nitrogen and stored at − 80°C. Plasma aliquots were stored at − 20°C. A sample of whole blood and part of the spleen were freshly analyzed. All remaining dissected organs were fixed in standard formalin for further histological processing. In these collected tissues, the following endpoints were addressed: sperm quantity and quality (cauda epididymis sperm), cellular composition (blood, bone marrow), immune cell subpopulations and natural killer (NK) activity (spleen), thyroid hormones (plasma), clinical plasma components (plasma), HBCD kinetics (liver, fat), metabolic activity (liver), and histopathology (all prepared tissues/organs). A distal body preparation, including lumbar vertebrae, pelvic bones, and one intact hind limb, was frozen at − 20°C for later dissections and analysis of bone parameters.

Experiments were approved by the institutional Committee on Animal Experimentation, according to Dutch legislation.

Compound analysis. Internal dosing was verified by analysis of HBCD enantiomers in liver of three animals per dose group and in a limited number of fat samples. Liver was chosen because sufficient material is available for analysis of measurable quantities of the compound and potentially also of metabolites. The liver sample (about 100 mg) was weighed, dried with sodium sulphate (Merck, Darmstadt, Germany), and stored for 2 h. The dried sample was extracted using a Soxhlet apparatus with hexane:acetone (3:1, vol/vol, Promochem, Wesel, Germany), with a starting volume of 100 ml. After extraction, 1 ml of ^{13}C-labeled α-, β-, γ-HBCD (100 ng/ml, CIL, Andover, MA) was added, and the extracts were evaporated to 10 ml. An aliquot of the extract (1/10) was dried with nitrogen to determine the lipid content gravimetrically. The residual extract was treated with 1 ml concentrated sulfuric acid (Merck) to remove the lipids. This step was two times repeated, and the combined hexane extract was further purified with silica gel. Silica gel was eluted with 11 ml isooctane and 29 ml isooctane:diethyl ether (85:15, vol/vol, Promochem). Finally, the silica eluate was evaporated to almost dryness with nitrogen, and 1 ml of methanol was added. The α-, β-, γ-HBCD diastereomers were determined with LC-MS/MS (LCQ Advantage, Thermo Finnigan, Austin, TX) using electrospray ionization. Identification of HBCD was based on the retention times of the ^{13}C-labeled compounds and the bromine clusters of the adduct ion m/z 677 and fragment ion m/z 644. The m/z 644 ion was used for quantification. A Zorbax Eclipse XDB-C18 HPLC column (2.1 × 150 mm, 3.5 μm, Agilent, Palo Alto, CA) was used with a gradient of ammonium chloride (0.1mM) and acetonitrile (ACN) starting from 0 to 6 min at ammonium chloride:ACN (3:1, vol/vol); followed by ammonium chloride:ACN (1:9, vol/vol) from 6 to 10 min.

Measurement of the HBCD-β enantiomer in a selected number of tissue samples showed that this isomer only comprised a minor fraction of the total HBCD (< 1.5%; data not shown); measurements in the full experimental set were therefore restricted to the HBCD-α and -γ enantiomers.

HBCD partitions to adipose tissue (Yu and Atallah, 1980; cited in KEMI, 2003), therefore, internal concentrations in the liver in this study are expressed on lipid basis.

Organ weights. The relationship between organ weight and body weight in a growing animal is allometric of nature for most organs with allometric constants that vary by organ and also by stage of organ ontogeny (Trieb *et al.*, 1976). Analysis of relative organ weights does not eliminate the indirect effect of treatment on organ weight *via* its effect on body weight and was even suggested to be unreliable (Shirley, 1977; Trieb *et al.*, 1976). Furthermore, changes in body fat, muscularity, or bone density, all potential specific effects of endocrine disruption, may confound the conclusions of relative organ weights. To avoid this as well as complex and/or uncommon approaches such as to adjust each organ weight by its specific allometric exponent of the body weight, or to differentiate between organs for the specific use of body or brain as a reference, or to use body weight as a covariate (Bailey *et al.*, 2004; Trieb *et al.*, 1976), analysis of organ weights was restricted to independent dose-response analysis of absolute organ weights. The observed organ weight changes were then interpreted in the context of concomitant other changes, particularly of body weight but also of other organs.

Histology. After fixation (and subsequent weighing, see above), organs sampled for histology were dehydrated and paraffinized and embedded according to standard sampling and trimming procedures. Sections of 4 μm were stained with hematoxylin and eosin in an automated way. Microscopic observations were done by comparison of control and exposed samples. Scoring thyroid gland histopathology was done by semiquantitative assessment of parameters of thyroid activation (follicular cell size, nuclear size, vacuolization), and of follicle size, without knowledge of treatment. For each parameter, four relative stages were discerned (control, slight, moderate, and severe). Effects on TSH in thyrotropic cells in the adenohypophysis were assessed by immunohistochemistry. For this purpose, paraffin sections of routinely processed, formalin fixed, pituitaries were deparaffinized in a graded series of xylol/ethanol. Endogenous peroxidase activity was blocked in a 1/1 methanol/distilled water solution with 1/100 0.3% H_2O_2 added. Antigen exposure was improved by 30 min trypsin incubation (0.25% wt/vol trypsin with 0.02% wt/vol $CaCl_2$ in distilled water), and background staining was reduced by 15 min incubation with blocking reagent (PerkinElmer, Wellesley, MA) and 1% wt/vol in phosphate-buffered solution. Subsequent 60 min incubation with purified rabbit polyclonal IgG against rat TSH (Biogenesis, Ede, The Netherlands), 1/1000 dilution was followed by incubation with a biotinylated anti-rabbit second antibody (1/200, 30 min; Vector, Burlingame, CA) and avidin-biotin complex (Vector) according to instructions of the manufacturer; both antisera were diluted in the 1% blocking reagent solution. Immunostaining was completed with incubation with a standard diaminobenzidine (Sigma, St Louis, MO) solution for 5 min. Counterstaining was with hematoxylin (Mayer procedure). Immunostaining intensity was evaluated by measuring pixel saturation on standardized digital images of comparable areas in each pituitary, using image analysis software (AnalySIS, Olympus Soft Imaging Solutions, Münster, Germany). Saturation profiles were classified compared with two sample cells, representing low- and high-intensity staining, and ratios of low/high-intensity pixels were calculated for each image/pituitary.

Immunology/hematology. For hematology, one femoral shaft was flushed with 4 ml ICP (Impulse Cytophotometer) solution, containing 3.22 g/l trisodium-citrate-dihydrate, 3.4 g/l sodium-dihydrogenphosphate-dihydrate, 3.87 g/l disodium-hydrogenphosphate-dihydrate, 1.17 g/l citric-acid-monohydrate, 3.65 g/l dextrose, 4.96 g/l sodiumchloride in demineralized water, pH 7.4 at 20°C. The resulting cell suspension and the collected EDTA blood were kept at 4°C until automated analysis in an ADVIA120 (Bayer, Leverkusen, Germany) within 4 h. Analysis of the spleen subpopulations (T-lymphocytes (CD3), T-helper (CD4), cytotoxic T cells (CD8), NK cells (CD161a), and B-lymphocytes (CD45RA), was done on fixed cell suspensions prepared of approximately one third of the organ, with a FACS-Calibur (B&D, Franklin Lakes, NJ) in two separate runs. Numbers of these subpopulations were calculated from the ratios determined by fluorescence-activated cell sorter (FACS) analysis, cell counting with the Coulter Counter Z2 (Beckman, Fullerton, CA), and weights of spleen parts. NK-function test was essentially performed as described previously (de Jong *et al.*, 1980), with ^{51}Cr-labeled YAC-1 cells as targets, following an overnight incubation at 37°C to remove adherent cells.

Clinical chemistry. Albumin, alkaline phosphatase, alanine aminotransferase, total cholesterol, creatinine, glucose, total protein, and urea were measured routinely as single measurement on an autoanalyzer (Hitachi 912, Roche Diagnostics, Basel, Switzerland) using standard kits from Roche Diagnostics. The measurement included hemolytic, icteric, and lipemic indices to exclude interferences.

TH analysis. Total concentrations of circulating thyroid hormones thyroxin T4 and T3 were determined in serum by in-house radioimmunoassays (RIAs) (Internal Medicine Laboratory, Erasmus Medical Center, Rotterdam) validated for rats and mice, as described previously (Friedrichsen *et al.*, 2003). These measurements were done in duplicate RIAs. Within-assay coefficients of variation amounted to 2–8% for T4 and 2–6% for T3, and between-assay coefficients of variation were 5–10% for T4 and 8% for T3. Based on structure differences, no interference of HBCD was expected in this assay.

UDP-glucuronosyltransferase assay. Hepatic microsomal fractions were prepared by homogenizing liver samples on ice in 3 volumes ice-cold 10mM Tris-HCl buffer (Sigma Chemicals Co), pH 7.4, containing 0.25M sucrose and 1mM dithiothreitol (DTT, Duchefa Biochemie, Haarlem, NL), using a Potter tube. The homogenate was centrifuged for 30 min at 12,000 × g and 0–4°C. The resulting supernatant was centrifuged for 90 min at 105,000 × g and 0–4°C, and the microsomal pellet was resuspended in 2 volumes ice-cold 10mM buffer containing 3mM EDTA and 1mM DTT, pH 7.4. Microsomal protein levels were determined using BioRad Protein reagent and BSA as a standard. Hepatic T4-UDP glucuronosyltransferase (UGT) activities were determined as described previously (Schuur *et al.*, 1997). Briefly, microsomes (1 mg protein/ml) were incubated for 30 min at 37°C with 1μM T4 plus ≈100,000 cpm 125I-labeled T4 (Amersham, Buckinghamshire, UK), 3.75mM MgCl2, and 0.125% BSA (Sigma) in the presence or absence (blank) of 5mM UDP-glucuronyl acid (Sigma) as a cofactor in 200 μl 75mM Tris-HCl, pH 7.8. Reactions were stopped by the addition of 0.2 ml ice-cold methanol. After centrifugation, 0.2 ml supernatant was mixed with 0.8 ml 0.1M HCl and transferred to Sephadex LH-20 minicolumns (Pharmacia, Woerden, NL). Columns were eluted with HCl to collect free 125I and, subsequently, with water to collect the glucuronated fraction. Hepatic microsomes from beta-naphthoflavone (bNF)– and corn oil–exposed rats (Meerts *et al.*, 2000) were used as positive and negative controls, respectively, throughout all UGT assays as internal reference. Maximal T4-UGT activities induced by HBCD in this experiment were approximately 50% of those induced by the internal reference bNF (2.0 ± 0.4 pmol/min/mg protein).

Apolar retinoid analyses. Apolar retinoids were extracted from liver homogenates (20% wt/vol in water) using diisopropyl ether and separated on a Nucleosil C_{18} 5-μm HPLC column using an ethanol:water gradient elution as described previously (Nilsson *et al.*, 1996). Retinol and retinyl esters were detected with a JASCO 821-FP fluorescence detector and quantified using internal and external standards.

Bone analyses. Dissected left femur and tibia were cleaned from soft tissue and stored in Ringer solution (1 l contains 0.3 g Tris, 0.24 g CaCl2(H20)2, 0.4 g KCl, and 2.05 ml 1M HCl, pH 7.4) stored in − 20°C until analysis. The length of each bone was measured using an electronic sliding caliper to the nearest 0.01 mm (IP65, Sylvac SA, Crissier, Switzerland). The bones were scanned using the peripheral quantitative computed tomography (pQCT) system (Stratec XCT Research SA+) with software version 5.50 (Norland Stratec Medizintechnik, GmbH, Birkenfeld, Germany) as described elsewhere (Stern *et al.*, 2005). Diaphyseal pQCT scans of femur and tibia were performed at sites distanced 50% of total bone length from the end of the bone to determine cortical bone parameters. Metaphyseal pQCT scans, at sites distanced 15% (male and female) of total bone length from the distal end of

femur and at 12% (male) or 15% (female) from the proximal end of tibia, were performed to measure total and trabecular bone parameters.

Statistical analysis. Dose-response analysis of effects, based on external dosing (mg/kg bw) was done using a nested family of purely descriptive (exponential) models with the PROAST software (Slob, 2002). This method enables integrated evaluation of the complete data set. From the best curve fit, indicated by significance at the 5% level, a critical effect dose (CED, also referred to as benchmark dose, Filipsson *et al.*, 2003) was calculated at a predefined critical effect size (CES) of 10 or 20% (see Tables 1 and 2 for an overview). CES is defined as the threshold adverse effect level, determined by expert judgment for each parameter based on knowledge of the pathophysiology of each effect, including irreversibility or adverse follow-up effects. In practice, a CES of 10% was defined as the default, considering that this effect size will cover the hazard for the most sensitive subjects in a population. For liver weight and immune parameters, a CES of 20% was defined, considering that lower effect sizes could represent a nontoxic physiologic response. The analysis was completed with the calculation of a 95% confidence interval (two-sided), thus enabling the calculation of a 5% lower confidence bound of the CED estimate. This value may be considered as a benchmark dose at the lower confidence bound (BMDL) for continuous data. The CED/BMDL ratio was used as a measure for the (statistical) uncertainty in a data set and, hence, for validity of the result of the dose-response modeling. As a consequence, in case of a more than 10-fold difference between CED and BMDL, the data were considered uninformative. The controls were used as input for the modeling, although for graphical representation on a log scale, an approximation is used.

BMDL values expressed as external doses were converted to internal doses (liver concentrations) using the regression equation between external and internal dose (Tables 1 and 2). For verification, BMDLs were also recalculated based on internal doses, of which, however, maximal three data points per dose groups were available (Tables 1 and 2).

The current experimental setup, with relatively few replicates per dose group, is not suitable for comparing individual dose groups due to limited statistical power. However, some parameters, which were targeted at discerning an overall difference rather than a dose-response, were analyzed using conventional statistical tests. These included Fisher exact test (for distribution of histological scores of liver basophilia between controls and exposed specimen), two-way ANOVA (for variance of TSH immunostaining using dosing and gender as covariates), and a Student *t*-test (for comparing glucose concentrations between all males and all females).

RESULTS

In Life Observations

Two male rats died from erroneous administration on exposure day 4. They were replaced since these animals were from a single dose group (3 mg/kg bw). Another rat (female, dose group 0.3 mg/kg bw) died from the same cause in the second week of exposure; this animal was not replaced. Two animals showed transient irritation of both eyes and three other animals had transient and limited skin irritations. These aberrations were not considered to be related to the exposure. There were no further clinical anomalies, dosing was well tolerated. All animals in all dose groups showed normal feed consumption. There were no effects on body growth during the exposure period (Supplementary Data Table 1).

Internal HBCD Concentrations

Analysis of HBCD-α and -γ in the liver showed a dose-dependent increase with a plateau at the three highest doses (Fig. 1). Remarkably, the HBCD concentrations in the liver were higher in females than in males over the entire dose range (on average 5.2 times), and all females in the control group also had low levels of HBCD, whereas HBCD was below the limit of detection in control males. The average ratio γ/α, which was approximately 8.1 in the administered technical mix, appeared to be 2.9 in females and 1.9 in males and decreased with dose in both sexes; at low doses, there was a higher contribution of the γ enantiomer, while at higher doses the ratio equalized or reversed. Similarly, the higher concentration of HBCD in female compared to male livers was mainly due to contributions of the γ enantiomer at low and the α enantiomer at high doses. The lipid fraction in livers was not different between females and males, nor were there differences between dose groups. Analysis of fat samples of two animals of each sex in the 10 mg/kg bw dose groups revealed average HBCD values of 379.5 and 84.9 mg/kg retroperitoneal fat tissue in females and males, respectively, that is, 2.4 and 1.4 times higher than in liver lipid (not shown).

Sperm Analysis

Sperm collected from the epididymis during necropsy had similar characteristics in all dose groups (data available in Supplementary Data Table 2).

Organ Weights

There was a significant dose-dependent weight increase of the pituitary, the thyroid, and liver in females only. In males, there was only a decrease in thymus weight (Tables 1 and 2; Supplementary data Table 2). Dose-responses of spleen and kidney weights of female animals were too limited for calculation of BMDLs.

The applied CES and corresponding BMDLs, both based on external doses and internal concentrations for these and other variables, are shown in Tables 1 and 2; details of weights of all organs are given in Supplementary Data Table 2. The deduction of a CED at a predefined CES is illustrated in Figure 2.

Histopathology

Histopathology of the thyroid showed that in HBCD-exposed females, thyroid follicles were smaller, depleted, and had activated, hypertrophied epithelium (Fig. 3A). Histopathological grading of the thyroid activation was based on assessment of follicle cell height, cellular vacuolization, and size of the nucleus, most of which showed a dose-related increase in both sexes, and scoring of follicle size, which showed a dose-related decrease in males (Table 3). Cell height and nuclear size were most sensitive, with initiation of the effect (a progression from slight to moderate activation, i.e., size) at intermediate doses, whereas effects in follicle size and cytoplasmic vacuolization only appeared at the high end of the dose range. In males, control thyroids were already moderately

TABLE 1
Significant HBCD-Induced Dose-Response Effects in Female Rats

	Organ weights				Plasma chemistry								Liver chemistry					Total bone[a]		Trabecular bone[a]	
																		Femur		Femur	Tibia
HBCD dose mg/kg bw	*n*	Pituitary (mg)	Thyroid (mg)	Liver (g)	*n*	ALP (U/l)	CHOL (mmol/l)	GLU (mmol/l)	Total protein (g/l)	Albumin (g/l)	*n*	TT4 (nmol/l)	TT3 (nmol/)	T4-UGT (pmol/min/mg protein)[b]	Retinyl palmitate (nmol/g liver)	Retinol (nmol/g liver)	Sum retinoids (nmol/g liver)	*n*	Area (mm^2)	Mineral content (mg/mm)	Mineral density (mg/cm^3)	
0	5	5 ± 1	17 ± 2	9.7 ± 1.0	5	4.7 ± 2.9	1.8 ± 0.2	9.8 ± 0.9	56.1 ± 2.0	42.0 ± 1.6	5	41.3 ± 2.6	0.91 ± 0.10	0.56 ± 0.23	1720 ± 297	19.1 ± 2.9	1987 ± 338	3	16.1 ± 1.5	10.5 ± 0.5	228 ± 34	199 ± 45
0.3	4	12 ± 1	18 ± 2	8.9 ± 1.1	3	3.1 ± 2.8	1.7 ± 0.1	9.6 ± 1.2	56.9 ± 2.2	43.2 ± 1.3	4–5	41.9 ± 3.1	0.84 ± 0.15	0.61 ± 0.15	1831 ± 224	28.7 ± 12.5	2138 ± 234	3	14.9 ± 0.9	10.2 ± 1.1	262 ± 34	226 ± 53
1	5	11 ± 3	22 ± 4	8.6 ± 1.3	3	4.7 ± 2.5	1.7 ± 03	9.8 ± 1.0	56.4 ± 1.8	42.6 ± 1.4	5	40.2 ± 7.3	0.88 ± 0.12	0.53 ± 0.17	1690 ± 301	26.2 ± 7.1	1964 ± 349	4	13.7 ± 0.6	9.5 ± 0.6	231 ± 40	199 ± 24
3	5	13 ± 2	15 ± 4	9.5 ± 0.4	3	3.7 ± 2.1	1.9 ± 0.3	10.3 ± 1.2	56.3 ± 0.7	42.3 ± 1.1	5	37.2 ± 4.7	0.81 ± 0.11	0.43 ± 0.09	1542 ± 236	24.9 ± 2.7	1797 ± 280	5	14.4 ± 0.7	9.2 ± 0.7	219 ± 20	186 ± 23
10	5	11 ± 3	18 ± 3	8.9 ± 0.6	3	2.3 ± 1.2	1.6 ± 0.2	9.4 ± 0.4	56.8 ± 2.4	42.1 ± 1.7	5	38.6 ± 1.7	0.80 ± 0.09	0.57 ± 0.14	1368 ± 98	22.8 ± 1.2	1601 ± 103	3	14.5 ± 0.9	9.5 ± 1.1	211 ± 45	201 ± 70
30	5	8 ± 2	35 ± 17	11.0 ± 1.0	4	2.4 ± 0.3	1.9 ± 0.3	9.8 ± 1.1	59.2 ± 3.1	42.6 ± 2.8	4–5	38.0 ± 6.1	0.74 ± 0.15	0.74 ± 0.22	1157 ± 122	17.1 ± 3.5	1354 ± 135	3	15.5 ± 0.4	9.9 ± 0.4	248 ± 10	181 ± 33
100	5	13 ± 1	27 ± 7	13.0 ± 0.5	4	2.7 ± 1.6	2.4 ± 0.2	8.7 ± 0.9	64.1 ± 1.3	45.6 ± 1.1	4–5	35.8 ± 5.2	0.92 ± 0.20	1.39 ± 0.27	1152 ± 162	17.0 ± 5.0	1361 ± 169	3	16.2 ± 1	10.4 ± 0.8	228 ± 22	195 ± 29
200	5	13 ± 2	26 ± 3	11.6 ± 0.6	3	2.4 ± 2.7	1.8 ± 0.2	8.1 ± 1.8	61.0 ± 3.6	44.2 ± 2.6	5	30.4 ± 5.9	0.82 ± 0.13	0.99 ± 0.43	1193 ± 186	15.4 ± 1.3	1386 ± 205	3	15.7 ± 0.7	11.1 ± 0.5	265 ± 20	251 ± 12
CES (%)		10	10	20		− 10	10	− 10	10	10		− 10	10	10	− 10	− 10	− 10		10	10	10	10
CED (mg/kg bw)		50.6	3.4	29.9		33.9	23.6	100.0	194.3	316.8		76.1	—	14.5	3.0	16.0	3.1		> 200	134.6	139.3	91.1
BMDL (mg/kg bw)		29.9	1.6	22.9		18.9	7.4	70.8	142.7	197.5		55.5		4.1	1.6	4.8	1.6		129.8	86.7	69.9	49.3
Ratio CED/BMDL		1.7	2.1	1.3		1.8	3.2	1.4	1.4	1.6		1.4		3.5	1.9	3.4	1.9		1.8	1.6	2.0	1.8
Maximum response (% compared to control)		More than + 41[c]	+ 61	+ 36		More than − 45.3[c]	+ 19.9	More than − 17.1[c]	+ 11.1	+ 6.1		More than − 23.5[c]		+ 121	− 33.7	− 30.8	− 33.6		+ 9	More than + 15[c]	More than + 18[c]	More than + 31[c]
BMDL (ID) (μg/g liver lipid, converted[d])		223	43	192		172	102	361	534	640		315		73	43	80	43		506	504	358	295
BMDL (ID) (μg/g liver lipid, recalculated[e])		—	39	112		—	121	253	209	267		209	—	64	—	—	—		149	148	141	130

Note. Figures are average ± SD of *n* replicates per dose group, CED values indicate critical effect dose at the given critical effect size (CES). ALP, alkaline phosphatase; CHOL, cholesterol; GLU, glucose; T4-UGT, T4-uridine diphosphate glucuronyl transferase; —, no significant dose-response or ratio CED/BMDL (ID) above 10.

[a]Measured at femur and tibia metaphysis.

[b]A similar dose-response is observed when activities are expressed per min per gram liver.

[c]No plateau reached within the applied dose range.

[d]For conversion external dose (ED)-internal dose (ID), the regression equation ID = 33377 × ED 0.5587 (with $R^2 = 0.79$) was used.

[e]For recalculations, which were applied for verification of the converted IDs, dose-responses were directly analyzed using ID as *X*-variable; differences between converted and recalculated BMDL (ID) values are probably mainly due to different size of the data sets; only three or even less animals per dose group were available for recalculation, with subsequent larger confidence interval variation (particularly with bone parameters). For this reason, the recalculated BMDL (ID) values are generally below the converted BMDL (ID) values, and the converted BMDL (ID) values should be considered more reliable.

TABLE 2
Significant HBCD-Induced Dose-Response Effects in Male Rats

	Organ weights		Spleen absolute cell numbers ($\times 10^{7}$)			Plasma chemistry							Liver
HBCD dose	*n*	Thymus (g)	*n*	Total cells per spleen	CD4 (Th)	CD161a (NK)	*n*	CHOL (mmol/l)	GLU (mmol/l)	Total protein (g/l)	Albumin (g/l)	*n*	TT4 (nmol/l)	TT3 (nmol/l)	T4-UGT (pmol/min/mg protein)[a]
0	5	0.47 ± 0.08	4	48.7 ± 10.5	14.0 ± 4.7	4.8 ± 1.2	5	2.08 ± 0.20	10.6 ± 1.1	56.4 ± 2.1	39.2 ± 1.0	5	40.2 ± 3.6	0.81 ± 0.06	0.36 ± 0.05
0.3	5	0.45 ± 0.08	1	49.6	15.2	3.8	3	2.13 ± 0.31	10.4 ± 3.2	58.1 ± 2.9	39.1 ± 0.7	4–5	40.4 ± 5.0	0.84 ± 0.14	0.44 ± 0.15
1	5	0.52 ± 0.17	2	47.1 ± 15.4	13.3 ± 4.8	3.6 ± 0.1	3	2.10 ± 0.23	10.3 ± 2.6	58.6 ± 2.2	39.7 ± 1.9	5	40.6 ± 5.3	0.85 ± 0.16	0.40 ± 0.14
3	5	0.47 ± 0.07	1	44.4	11.4	4.6	3	1.74 ± 0.30	10.9 ± 1.8	58.1 ± 1.5	40.1 ± 0.3	5	49.4 ± 7.2	0.89 ± 0.04	0.69 ± 0.37
10	4	0.50 ± 0.09	2	39.4 ± 3.8	10.5 ± 0.9	4.1 ± 1.2	3	2.01 ± 0.23	10.9 ± 2.5	57.6 ± 2.0	38.8 ± 1.2	5	43.3 ± 1.3	0.97 ± 0.16	0.60 ± 0.24
30	5	0.37 ± 0.06	1	29.7	9	2.7	4	2.31 ± 0.41	10.5 ± 1.5	60.1 ± 1.2	39.2 ± 1.6	4–5	41.9 ± 4.6	0.90 ± 0.13	0.73 ± 0.26
100	5	0.42 ± 0.09	1	37	11.2	2.9	4	1.99 ± 0.39	9.7 ± 2.6	61.4 ± 1.7	40.8 ± 1.8	4–5	35.4 ± 4.2	0.82 ± 0.06	0.99 ± 0.41
200	5	0.38 ± 0.13	2	35.8 ± 1.1	10.0 ± 2.0	2.6 ± 0.1	3	1.63 ± 0.19	8.4 ± 1.2	59.6 ± 3.6	40.6 ± 1.8	5	41.4 ± 3.5	0.89 ± 0.05	0.88 ± 0.45
CES (%)		20		20	20	20		10	10	10	10		10	10	10
CED (mg/kg bw)		176.6		9.8	1.9	12.6		102.9	98.1	> 200	> 200		—	—	1.1
BMDL (mg/kg bw)		104.2		1.7	0.3	6.3		65.9	57	> 200	> 200				0.1
Ratio CED/BMDL		1.7		5.8	6.3	3		1.6	1.7	1.7	1.8				11
Maximum response (% compared to control)		− 20		− 28	− 33	− 41		Less than − 21.2[b]	Less than − 19.7[b]	5.2	More than + 3.8[b]				106
BMDL (ID) (μg/g liver lipid, converted[c])		113		8.5	2.9	19		85	77						—
BMDL (ID) (μg/g liver lipid, recalculated[d])		52		—	—	—		—	—	83	—		—	—	21

Note. Figures are average ± SD of *n* replicates per dose group. CHOL, cholesterol; GLU, glucose; T4-UGT, T4-uridine diphosphate glucuronyl transferase; —, no significant dose-response or ratio CED/BMDL (ID) above 10.

[a]A similar dose-response is observed when activities are expressed per min per gram liver.

[b]Measured at femur and tibia metaphysis.

[c]The regression equation for conversion external dose (ED)-internal dose (ID) ID = 6101 × ED 0.6276 (with $R^2 = 0.38$) was used.

[d]For recalculations, which were applied for verification of the converted IDs, dose-responses were directly analyzed using ID as *X*-variable; differences between converted and recalculated BMDL (ID) values are probably mainly due to different size of the data sets; only three or even less animals per dose group were available for recalculation, with subsequent larger confidence interval variation (particularly with spleen parameters). For this reason, the recalculated BMDL (ID) values are generally below the converted BMDL (ID) values, and the converted BMDL (ID) values should be considered more reliable.

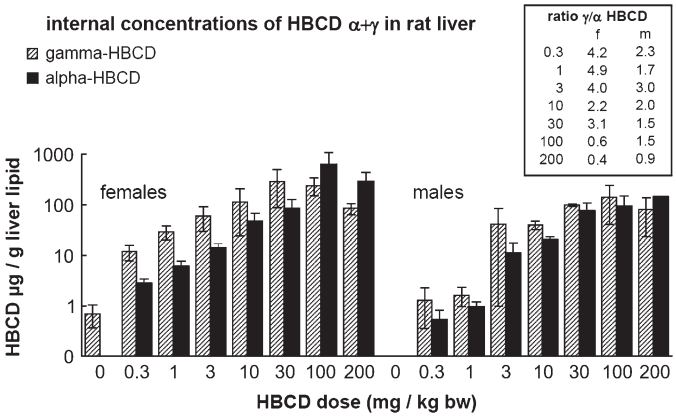

FIG. 1. Sum HBCD α and γ in liver of female and male rats, after exposure to 0.3–200 mg/kg bw of technical mix for 28 days. Concentrations are expressed per gram of lipid on a logarithmic scale. There is a dose-dependent increase in both sexes up to a plateau in the three highest doses. Females tend to have higher concentrations than males. Averages are based on measurement of three animals per group; error bars indicate SD. For correlation equations, see Tables 1 and 2.

activated, and changes to advanced activation were therefore less obvious. There were no indications of hyperplasia.

Immunohistochemical detection of TSH in the pituitary was done to elucidate whether activation of the thyroid epithelium was associated with increased expression of this stimulating hormone. Comparison of top dose animals with control animals showed a significantly increased ratio between high- and low-intensity immunostaining thyrotropic cells (Fig. 3B). When comparing dosed and control animals per gender, the effect was only significant in females, consistent with the increase of pituitary weight in females.

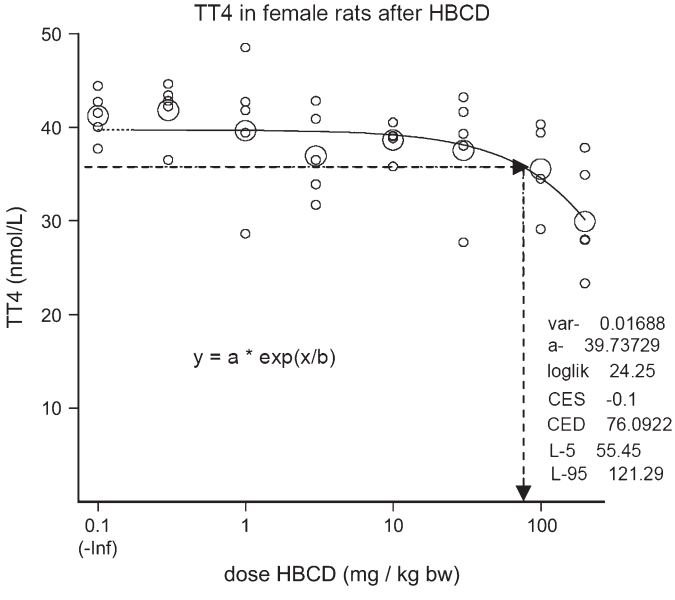

FIG. 2. Dose-response analysis illustrated with circulating TT4 in female rats. Method and parameters describing the curve were explained in Slob (2002). In this model, parameter *a* is the background level and *b* reflects the efficacy of exposure (slope). Log likelihood is used to assess to what extent the described curve deviates from no effect ($y = a$). CES, critical effect size, for TT4 set at 10% (horizontal dotted arrow); a corresponding critical effect dose (CED) is represented by the vertical dotted arrow. Exposure dose (x-axis) is on a logarithmic scale.

In the liver, HBCD-exposed specimen of both sexes could be discerned on the basis of intense basophilia, specifically in periportal zones (Fig. 3C) to an accuracy of 60–80% by repeated blind scoring (not statistically significant, Fisher exact test). This visual effect is suggestive of endoplasmatic reticulum induction, compatible with increased synthetic activity, and is in line with the increased liver weights in females. There were no further apparent HBCD exposure associated changes in the liver, nor in the other organs assessed for histopathology, after analysis of the control and (sub-) top dose ($n = 5$ per sex per group; Supplementary Data Table 3). Inflammation in Langerhans islets was observed throughout all groups, independent of dosing. This insulitis was more severe in males than in females and associated with a gender difference regarding glucose concentrations (see below) but not related to exposure (not shown).

Immunology/Hematology

FACS analysis of immune cell populations in the spleen, which was only performed in male animals, revealed reduced total spleen cell count and counts of T-helper and NK cell subpopulations (Tables 1 and 2; Supplementary Data Table 4). Fractions of these cells did not change because counts of other subpopulations also declined, although not significantly. Cytotoxic activity of NK cells was also not affected. There were no effects on blood or bone marrow white blood cell populations (Supplementary Data Table 5). In hematological parameters, which were also only measured in males, there was a marginal decrease of the mean red blood cell volume, with a concomitant increase of mean erythrocyte hemoglobin concentration (Supplementary Data Table 6). Both changes were, however, below the CES.

Clinical Chemistry

Chemical analysis of plasma showed that female rats had decreased concentrations of alkaline phosphatase and glucose and increased cholesterol and total protein/albumin. Males had decreased cholesterol, increased total protein/albumin, and decreased glucose, all in a significant dose-dependent way (Tables 1 and 2; Supplementary Data Table 7). Plasma glucose concentrations were higher in males compared to females (10.2 ± 2.1 in 40 males and 9.5 ± 1.2 mmol/l in 38 females), a nearly significant difference ($p = 0.079$).

Thyroid Hormones and T4-UGT

Measurement of thyroid hormones showed a dose-dependent decrease in total T4 (TT4) in females only (Tables 1 and 2; Supplementary Data Table 8); there were no effects on total T3

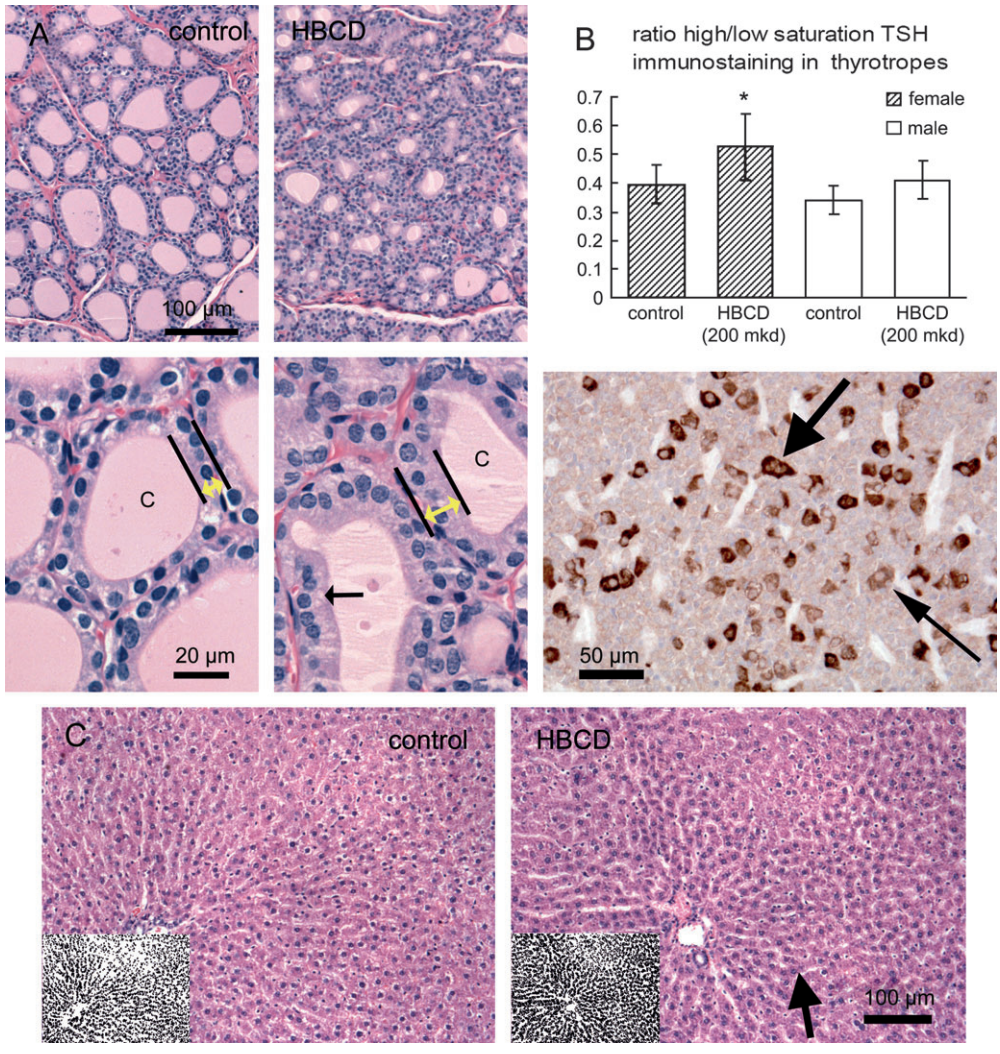

FIG. 3. (A) Histology in the central thyroid of female rats, control (left panels) and after 28 days of exposure to 100 mg/kg bw HBCD (right panels) at low (top) and high (bottom) magnification. In the exposed sample, follicles are smaller or even depleted, the intrafollicular colloid (c) stains more faintly; cell height is increased (compare yellow arrows), nuclear size increased, and cells are more vacuolated (black arrow). All effects are illustrated at a moderate degree (see Table 3). (B) Morphometrical analysis of immunostained thyrotropic cells (top). The ratio between high and low saturation of TSH immunostaining was significantly increased in females exposed to the highest dose HBCD ($p = 0.043$, t-test). In the ANOVA, HBCD exposure accounted significantly for total variance ($p = 0.038$), while there was no significant effect of gender, nor is there interaction between variables. For both groups of female pituitaries $n = 5$ and $n = 4$ for both male groups. The microphotograph (bottom) is an image sample used for the morphometry with thin and thick arrows indicating low- and high-intensity staining cells. (C) Liver microphotographs of a control (left) and a HBCD (right)-exposed female (100 mg/kg bw), illustrating increased basophilia in periportal zones after exposure (dark staining areas). A basophilic staining hepatocyte is indicated with an arrow, false color inserts show that there is more basophilia (black) in the HBCD-exposed liver.

(TT3) levels. In both male and female liver, a dose-dependent increase of T4-UGT activity was found (Tables 1 and 2; Supplementary Data Table 8). The BMDL was lower for male than for female rats (0.1 vs. 4.1), however, the high CED/BMDL ratio for T4-UGT in males implies high statistical uncertainty due to high variation of the data set, that is, general scatter (Tables 1 and 2); therefore, the BMDL for this parameter is considered not valid.

Liver Retinoids

Marked dose-related decreases in apolar liver retinoid concentrations were observed in female rats (Tables 1 and 2; Supplementary Data Table 9). The decreases were parallel to the dose-related increase in liver weight (Tables 1 and 2; Supplementary Data Table 3). No effect on the apolar liver retinoid concentrations was seen in male rats (Table 9). The total contents of apolar retinoids in the liver were not affected by HBCD at any dose neither in female nor in male rats (Table 9).

Bone Parameters

In bone, the most consistent observation was an increase in mineral density of trabecular bone at femur and tibia metaphysis in female rats (Tables 1 and 2; Supplementary Data Table 10). Further changes at the femur metaphysis of female

TABLE 3
Doses of Progression for Histological Stages in the Thyroid

	Females		Males	
	Background stage	CED for progression to next stage (mg/kg bw)	Background stage	CED for progression to next stage (mg/kg bw)
Cell height	1	47	2	—
Nuclear size	1	25	2	39
Vacuolization	1	177	2	90
Follicle size	1	—	1	199

Note. Thyroids of all available animals ($n = 38$, females; $n = 36$ males) were evaluated for the histological parameters of the first column. Stages indicated size (cell, nucleus, and follicles), or degree of vacuolization (number and size of discernible vacuoles). CEDs are the critical effect doses in mg/kg body weight at which the average thyroid progresses to a further stage in a staging range 0–3, that is, reference, slight, moderate, and severe. Reference and moderate degree of effect in all four parameters is illustrated in Figure 3. For all four endpoints, there is a maximum progression of one stage. Male samples have higher background stages than female samples. Effects in cell height and nuclear size are initiated at intermediate doses (and reach a plateau at the end of the dose range). Effects in follicle size and cytoplasmic vacuolization are initiated at high doses.

rats were increased total bone area and increased total bone mineral contents. There were no changes in any of the bone parameters in male rats.

DISCUSSION

The BFR HBCD is a potentially persisting and bioaccumulating compound, of which low but significant exposure levels are found in humans (Fängström *et al.*, 2006; Ryan *et al.*, 2006). HBCD is suspected to interfere with the endocrine system (Legler and Brouwer, 2003). For these reasons, it was tested in a subacute toxicity study, enhanced for endocrine and immune endpoints.

The daily gavage dosing resulted in dose-dependent liver concentrations of the compound, indicating bioavailability. The higher HBCD concentrations in females are consistent with results from a 90-day study in rats (Chengelis, 2001; cited in KEMI, 2003) and possibly due to a faster elimination in male compared to female rats (Yu and Atallah, 1980; cited in KEMI, 2003). The excreted HBCD in that study, both in the feces and in the urine, was probably completely metabolized, indicating complete absorption of the orally dosed compound. Since activity of all assessed metabolizing enzymes, CYP1A1, CYP2B, CYP3A (Germer *et al.*, 2006), and T4-UGT (Tables 1 and 2) was lower or comparable in males compared to females (background and exposed), other mechanisms may play a role. The same mechanisms can also explain why HBCD was recovered from control female but not male animals, assuming that these control animals received traces of the compound, presumably, through the standard feed or from ambient contamination. In the 90-day study cited above, the HBCD concentrations in adipose tissue were 4–5 times higher than the concentrations in liver lipid in top dose animals in our study. Although comparison between these two studies is hampered by differences of preparations (acetone assisted solution in our study) and top doses (five times higher in the Chengelis study), a limited analysis in our study also showed higher concentrations in adipose tissue than in liver lipid.

The γ/α HBCD isomer ratio in this study differed from the pattern in wildlife where α HBCD dominates (Zegers *et al.*, 2005). On the other hand, even the highest γ/α ratio in our rats was far lower than the γ/α ratio of the technical preparation used for exposure. This is in line with a higher susceptibility of the γ isomer to biodegradation compared to the α isomer in CYP2B-induced rat liver microsomes (Zegers *et al.*, 2005). The decreasing γ/α ratio with increasing dose can then be understood as intensified biotransformation of the γ isomer with increasing dose, whereas relatively more biotransformation-resistant α isomer will accumulate. This effect may be enhanced by the induction of CYP2B (Germer *et al.*, 2006). In this perspective, the different isomer ratios in our rats and in wildlife are most likely due to differences in exposure dose and duration.

The most marked HBCD dose-related effects were on the TH axis, including decreased TT4, increased immunostaining for TSH in the pituitary, increased weight/activation of the pituitary and the thyroid glands, and induction of T4-UGT in the liver. All these effects were restricted to females, which is in line with the higher HBCD liver concentrations in females versus males, although contrasting with observations in a 90-day study by Chengelis (Chengelis, 2001; cited in KEMI, 2003), which showed decreased TT4, increased serum TSH, and thyroid gland activation in both sexes. Decreased TT4 can be considered as a relative insufficiency of the feedback (activation of pituitary/thyroid glands), that is, the imposed stress is beyond the exposure dose that can be adequately counterbalanced by an activated feedback system (McDermott and Ridgway, 2001). On the other hand, the feedback was sufficient to maintain physiological levels of the biological active TT3 in exposed animals, but even under these circumstances, decreased plasma TT4 levels can induce hypothyroidy-like effects, because plasma T4 is a source for intracellular T3 through deiodination (Escobar del Rey *et al.*, 1989).

The increased TSH immunostaining detected in the pituitary is likely an indicative of increased levels of circulating TSH in exposed animals, as measured in the 90-days study (Chengelis, 2001; cited in KEMI, 2003). The increased weight of the pituitary must be due to indiscernible diffuse cellular hypertrophy and/or hyperplasia since no robust (clonal) changes were observed. Since, probably, only a limited number of the many cell populations of the pituitary are affected in a specific way, the detected slight weight increase of the pituitary may be symptomatic of a relatively strong effect in a single subpopulation, probably mainly the thyrotropic cells. The higher

background activity observed in male thyroid histology, compared to control females, is in line with higher levels of circulating TSH in males than in females (Capen, 1997). The associated higher sensitivity for induction of proliferative lesions is not relevant here since these were not observed.

One of several ways by which HBCD could induce hypothyroxinemia is through evoking a detoxification response in the liver, which concomitantly affects T4. Indeed, HBCD treatment led to induction of CYP2B and CYP3A (Germer *et al.*, 2006). This is in line with the induction of UGT described here since there is coexpression of and functional cooperation between P450 and UGT enzymes (Ishii *et al.*, 2005). The detoxification activity was stronger in female compared to male rats, consistent with the suggested histopathologic activation of the liver and increased liver weights in female rats, the latter also observed in a similar study (Chengelis, 1997; cited in KEMI, 2003). The higher sensitivity of females versus males for HBCD exposure could be enhanced by the lower binding capacity of TTR in female compared to male rats (Emerson *et al.*, 1990). Since TTR is the major TH carrier protein in the adult rat (Schussler, 2000), T4 might thus be more available for metabolization in females compared to males.

Other mechanisms which can induce hypothyroxinemia, such as direct interaction of HBCD with TH synthesis, or activation of other metabolizing enzymes (e.g., deiodinases, sulfotransferases) or cellular TH uptake were not explored in this study.

Multiple mechanisms can be responsible for the observed effects on plasma parameters, including interaction of HBCD with many hormone receptors (Hamers *et al.*, 2006). Furthermore, a physiologic relationship with the observed TH changes may possibly play a role, in view of the regulating potential of THs on metabolism. Specifically, associations between TH disruption and disregulation of metabolism of lipids/cholesterol (Fleischman and Shumaker, 1942), of serum proteins (Larsen and Davies, 2002), and of plasma glucose levels (Chidakel *et al.*, 2005) are known. Some of these associations, partly also observed in a previous study (Chengelis, 2001; cited in KEMI, 2003), are gender specific. We cannot exclude that the effects on plasma glucose are biased by the insulitis which was present throughout the entire population of this experiment (and in other animals from the same source).

In general, decreased alkaline phosphatase in the plasma, as observed in the female rats, can be a marker of decreased activity of the liver or of osteoblasts. Both, however, appeared to be activated (increased liver weight, liver histopathology, and increased bone density). On the other hand, osteoblast alkaline phosphatase expression, which is TH dependent (Milne *et al.*, 1998), could possibly be suppressed by HBCD through activation of TH-mediated effects (Hamers *et al.*, 2006).

The observed changes in the immune system (weight of spleen and thymus, spleen cellularity) are considered relevant because each imbalance in this system may reduce the homeostatic capacity of the individual. The effects on splenocyte counts should, however, be interpreted with caution due to small sample size and high CED/BMDL ratios, implicating statistical uncertainty. There is no unequivocal explanation for these effects, which may be related to a potential direct interaction of HBCD with steroid hormone or TH systems, the latter in view of the effects that TH can have on lymphocyte functions (Ong *et al.*, 1986).

The dose-dependent decrease of liver apolar retinoid concentrations in female rats probably reflects the dose-related increase in liver weight of these animals since there was no effect of HBCD on the total liver contents of apolar retinoids. Hepatic retinoid reduction has been proposed as an AhR-mediated response (Nilsson and Hakansson, 2002). Thus, the lack of retinoid effect, suggesting that HBCD does not activate the AhR, is consistent with the unaffected hepatic expression of CYP1A1/hepatic ethoxyresorufin-o-deethylase activity (Germer *et al.*, 2006).

Increased trabecular bone mineral density was observed at femur and tibia metaphysis in female rats, but there was no effect of HBCD on cortical bone at the diaphysis. This suggests that trabecular bone is more sensitive to the perturbations by HBCD compared to cortical bone, which may be related to the higher metabolic activity and turnover rate of trabecular compared to cortical bone (Ott, 2002). Since TH are involved in normal skeletal development, linear growth and the maintenance of adult bone mass (Bassett and Williams, 2003), the effect of HBCD in bone may be related with the observed hypothyroxinemia, supported by the occurrence of both effects in female rats only. Other mechanisms of action of HBCD can, however, not be excluded.

Evaluation

According to United States Environmental Protection Agency, adverse rodent noncancer thyroid effects due to chemically induced thyroid-pituitary disruption should be considered relevant to humans (Hill *et al.*, 1998). In the current study, HBCD evoked a sequence of events in the TH system, specifically, in female rats. Apparently, these effects did not exceed the limits of homeostasis, in view of unaffected levels of circulating T3, but decreased plasma TT4 levels may well cause hypothyroidy-like effects. The initiating event is probably activation of liver enzymes (induction of T4-UGT at a BMDL of 4.1 mkd), resulting in increased liver weight at 22.9 mkd) with increased TH turnover (decreased TT4 at 55.5 mkd) and a feedback reaction in the pituitary (increased weight at 29 mkd, increased immunostaining intensity of TSH) and the thyroid gland (increased weight at 1.6 mkd, follicle cell activation at 25 mkd) as a consequence. Additional events, which may be secondary to changes in the TH system, include effects on plasma chemistry (notably, increased cholesterol at 7.4 mkd), bone parameters (49 mkd and higher), and spleen parameters (decreased splenocyte counts 0.3–6.3 mkd, only assessed in males).

Overall, female rats appear to be more sensitive to HBCD exposure than male rats, and a BMDL for adversity of HBCD,

based on the most sensitive parameter, should be defined at 1.6 mg/kg bw (equivalent to 43 μg HBCD/g liver) for the 10% increase of the thyroid weight. This is well below the previously proposed lowest observed adverse effect level (LOAEL) of 100 mkd based on increased liver weight and disturbed TH system (Chengelis, 2001; cited in KEMI, 2003), and in the range of the LOAEL of 0.9 mkd based on behavioral effects in mice (Eriksson *et al.*, 2006). Combined with recent exposure data in human breast milk, indicating HBCD levels which are at least four orders of magnitude below this BMDL (Fängström *et al.*, 2006; Ryan *et al.*, 2006), this study does not support a major health risk of HBCD for humans.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/. Supplementary tables are provided to show average values with SD of body and organ weights and cauda epididymis sperm characteristics at necropsy, and a summary of histopathologic observations. For male rats only, average values with SD are provided of spleen subpopulations and NK activity, white cells in blood and bone marrow, and red blood cell parameters. Similar tables show average values with SD of clinical plasma chemistry, thyroid hormones and T4-UGT, retinoids in liver, and bone parameters of femur and tibia.

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