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HEXACHLOROBENZENE-INDUCED HYPOTHYROIDISM

INVOLVEMENT OF DIFFERENT MECHANISMS BY PARENT COMPOUND AND METABOLITE

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Abstract—Rats received repeated oral treatment with different doses of hexachlorobenzene (HCB) (0–3.5 mmol/kg) for 2 or 4 weeks. Measurements of thyroid hormone status after 2 weeks showed a dose-dependent decrease of total thyroxine (TT4) levels, decreased free thyroxine (FT4) levels and little change of total triiodothyronine (TT3) levels. The effects on thyroid hormone status were more pronounced after 4 weeks and also included increased thyroid stimulating hormone (TSH) levels. These conditions suggest that HCB had induced hypothyroidism in these animals. Indications for occupation of thyroid hormone binding proteins were found in serum of exposed animals. The major metabolite pentachlorophenol (PCP) also caused, by competitive interactions with thyroid hormone binding proteins in serum, a rapid and dose-dependent decrease of TT4 and FT4 levels, but not of TT3 levels in serum. The decrease of serum TT4 levels by repeated dosing with 3.5 mmol HCB/kg for 4 weeks could be attributed to competitive interactions of PCP with hormone serum binding proteins and to increased metabolism induced by HCB to an equal degree. At lower dose levels or with shorter dosing periods, increased metabolism of T4 is the main cause of decreased TT4 serum levels. This is the first indication that a similar effect is caused simultaneously by the parent compound and its metabolite through different and independent mechanisms.

Hexachlorobenzene (HCB§), a polyhalogenated aromatic compound, has been used in industry mainly as a fungicide and occurs as a by-product of industrial aromatic chlorination processes [1]. Accidental poisoning of humans with HCB by consumption of HCB-treated grain has been reported and toxic effects observed included enlargement of the thyroid gland and decreased blood thyroxine (T4) levels [2].

In experimental animal systems a number of biological and biochemical effects of HCB on thyroid homeostasis have been described. Chronic feeding of laboratory animals with HCB was found to induce thyroid adenomas [3], thyromegaly, and hypothyroidism [4–6]. Similar effects concerning the thyroid have been observed in rodents and primates chronically exposed to other halogenated aromatics such as polychlorinated biphenyls (PCBs) or polybrominated biphenyls (PBBs) [7–10].

The mechanism(s) involved in HCB-induced hypothyroidism is not clear. Microsomal enzymes are strongly induced by HCB [4, 6, 11] and this could lead to an increased T4 catabolism. Recently it was shown that HCB strongly enhanced glucuronidation of T4 through induction of several UDP-glucuronyltransferases (UDPGT) [12], resulting in an augmented disappearance of T4 from the body [12]. In animals exposed to PCBs and dioxins, such a mechanism has also been advanced [13–16].

An additional factor that might contribute to depressed thyroid hormone levels in HCB-induced thyroid changes is pentachlorophenol (PCP), the major oxidative metabolite of HCB [17–19]. PCP has been found to be more effective in reducing thyroid hormone levels than HCB itself in acute *in vivo* experiments [20]. Furthermore, *in vitro* studies have demonstrated competitive interactions of PCP and T4 for binding sites on T4 serum binding proteins with a greater specificity for binding to transthyretin (TTR) than either thyroid binding globulin (TBG) or albumin [21, 22]. Comparable results have been found in animals exposed to chlorinated benzenes or 3,4,3′,4′-tetrachlorobiphenyl (TCB), suggesting that the hydroxylated form of chlorinated aromatic compounds might be responsible for lowered T4 levels in serum through competitive interactions with T4 serum binding proteins [23–26].

Other studies showed that not only hydroxylated PCBs, but also the hydrophylic derivatives of dioxins and dibenzofurans are capable of interacting with the T4 binding sites of TTR and nuclear thyroid hormone receptors [27–30]. In addition, it was demonstrated by molecular modelling that DDT (dichlorodiphenyltrichloroethane), an insecticide that also lowers thyroid hormone levels, fitted into the T4 binding sites of TTR [31].

The aim of the present study was to determine the relative contributions of (1) increased microsomal catabolism of T4 by HCB and (2) interference of

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§ Abbreviations: HCB, hexachlorobenzene; PCBs, polychlorinated biphenyls; PBBs, polybrominated biphenyls; TCB, 3,4,3′,4′-tetrachlorobiphenyl; PCP, pentachlorophenol; UDPGT, UDP-glucuronyltransferase; TT4, total thyroxine; TT3, total triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone; TTR, transthyretin; TBG, thyroid binding globulin; RIA, radioimmunoassay; FT3, free triiodothyronine.

PCP at the thyroid hormone binding protein level in hypothyroidism induced by HCB. For this purpose, animals were repeatedly dosed with HCB to induce hypothyroidism as determined from measurements of thyroid hormone status. In addition, serum concentrations of HCB and PCP in these animals were analysed. In separate experiments the capacity of PCP to decrease thyroid hormone levels by competitive interactions was determined. The combined results were used to estimate the relative role of both types of mechanisms in hypothyroidism induced by HCB.

MATERIALS AND METHODS

Chemicals. HCB and PCP were purchased from Aldrich (Brussels, Belgium). Commercial TT4 (total thyroxine) (Amerlex-M), TT3 (total triiodothyronine) (Amerlex-M) and TSH RIA (thyroid stimulating hormone radioimmunoassay) kits (Amerlex-M, code RPA 554) were purchased from Amersham (Amersham, U.K.). The FT4 and FT3 assays (Amerlex-MAB) were obtained from Kodak Clinical Diagnostics Ltd, (Cardiff, U.K.). Tween-20 was obtained from Bio Rad Laboratories (Richmond, CA, U.S.A.).

Animals and treatments. Male rats of the inbred Wistar strain (WAG-RIJ), weighing 200–300 g and approximately 12 weeks of age, were used. They were housed in animal quarters with a 12-hr light/dark cycle and an ambient-temperature of 24° and a humidity of 50–70%. Water and food were freely accessible.

Animals were treated with oral doses of either vehicle (control animals) or different doses of HCB (3.5, 2.6, 1.7 and 0.9 mmol/kg, five animals per dose group), 3 days per week over a period of 2 weeks and with a dose of 3.5 mmol/kg for 4 weeks. These experiments (2 or 4 weeks dosing) were conducted at a different time point. HCB was administered as an emulsion of 0.14 mmol/mL in water containing 0.5% Tween-20. Approximately 2 days after cessation of dosing, blood was collected from the tail vessel, and the prepared serum stored at −20° for further analysis. During the period of HCB dosing, the general health of the animals was monitored three times a week by measuring rectal body temperature and body weight.

In experiments with PCP, the compound was dissolved in corn oil and administered i.p. on an acute basis at doses between 0 and 100 μmol/kg. Each dose of PCP was tested in groups consisting of four animals. Serum was collected 6 hr after exposure, when effects on thyroxine levels are at a maximum [20].

Biochemical analysis. TT4, TT3, free T4 (FT4), free T3 (FT3) and TSH levels were determined in sera of control and experimental rats using appropriate RIAs (Amersham). HCB and PCP concentrations in sera were analysed using HPLC as described earlier [20]. The detection limit for PCP in serum was 0.075 μmol/L and for HCB 0.351 μmol/L. The extraction efficiencies for both PCP and HCB were more than 95%.

Competitive interactions with T4 binding proteins. Occupation of thyroid hormone binding sites by HCB and/or PCP was studied by a competitive binding assay as described previously [21, 32]. Sera were obtained from control and experimental animals (day 39) and diluted with phosphate-buffered saline (PBS) (1:30, v/v) before competitive binding of radiolabeled T4 was determined using mini-Sephadex G-25 columns. Radioactivity of eluate fractions was determined in a gamma counter.

Statistics. Student's *t*-test was used for statistical evaluation of mean values of various parameters between experimental and control animals. Results are presented as means ± SD.

RESULTS

Effects of sub-chronic dosing with HCB on general health parameters

Rectal body temperature of rats treated with repeated doses of HCB (3.5 mmol/kg) for a period of 4 weeks was not altered on the last day of the dosing period (38.0 ± 0.41° vs 37.7 ± 0.49° in control and experimental animals, respectively). Body weights of the experimental and control animals were not different (285 ± 11.7 g vs 285 ± 14.7 g in control and experimental animals, respectively), and also followed a normal growth curve (results not shown).

Effect of HCB on thyroid hormone status

Treatment of rats for 2 weeks with 3.5 or 2.6 mmol/kg HCB produced a significant reduction ($P < 0.05$) of serum TT4 levels (36.9 and 24.8%, respectively, Table 1). Levels of FT4 in serum were also significantly reduced ($P < 0.05$) at a dose of 2.6 mmol/kg HCB (19.3% decrease, Table 1). After 4 weeks of dosing, TT4 and FT4 levels in animals exposed to HCB were even more depressed (41% for TT4, and 70.7% for FT4). In contrast, no significant decrease of TT3 levels in serum was found (Table 1) after a 2 or 4 week dosing period with a dose of up to 3.5 mmol HCB/kg. A trend of increased TSH levels in serum by HCB was noted after 2 weeks. TSH levels were significantly increased by 49% after a dosing period of 4 weeks with 3.5 mmol HCB/kg. An increasing effect of at least 20% was found comparing control values of TSH (Table 1).

HCB and PCP levels in serum during HCB treatment

Accumulation of HCB and its major metabolite PCP in serum after repeated exposure of animals to different doses of HCB was determined by HPLC methods. A clear dose–response relationship was found between the serum concentrations of HCB or PCP reached and the administered dose of HCB after 2 weeks (Table 2). At a given dose of HCB (3.5 mmol/kg), doubling of the exposure period from 2 to 4 weeks generally resulted in higher serum concentrations of both HCB and PCP, as also appeared from a separate time-course study (results not shown). Control animals had no detectable levels of HCB or PCP at any time point.

Occupancy of serum binding proteins

Sera of animals exposed to 3.5 mmol HCB/kg for 4 weeks did bind significantly less T4 (20% less) than sera of control animals, indicating partial

Table 1. Effect of HCB on thyroid hormone status

HCB (mmol/kg)*	N	Expt (weeks)	TT4 (nmol/L)	FT4 (pmol/L)	TT3 (nmol/L)	TSH (ng/mL)
0.0	4–5	2	21.4 ± 2.1	14.7 ± 2.2	0.44 ± 0.06	3.5 ± 0.5
0.9	3–5	2	23.7 ± 2.6	17.2 ± 2.2	0.44 ± 0.08	4.9 ± 1.1
1.7	3–5	2	19.2 ± 2.7	13.3 ± 1.9	0.42 ± 0.08	5.1 ± 1.6
2.6	5	2	16.1 ± 3.7†	11.8 ± 0.7†	0.33 ± 0.13	4.6 ± 1.6
3.5	4–5	2	13.5 ± 1.2†	12.1 ± 1.2	0.38 ± 0.07	4.8 ± 1.8
0.0	3–5	4	35.3 ± 3.8	9.2 ± 1.3	0.48 ± 0.06	7.6 ± 0.4
3.5	4–5	4	20.9 ± 3.4†	2.7 ± 0.6†	0.50 ± 0.17	11.9 ± 2.9†

Groups of rats (N = 3–5) were orally dosed three times a week with different doses of HCB for a period of 2 or 4 weeks. Within 24 hr after the last dose, blood was collected from the tail, and thyroid hormone parameters were determined.

* The doses of HCB expressed in mg/kg are 0, 250, 500, 750 and 1000, respectively. Statistical significance: †$P < 0.05$.

Table 2. HCB and PCP concentrations in serum of animals exposed to HCB

Dose of HCB (mmol/kg)	Dosing period (weeks)	HCB (μmol/L)	PCP (μmol/L)
0.0	2	DL	DL
0.9	2	9.0 ± 0.9	1.1 ± 0.51
1.7	2	14.5 ± 3.3	1.7 ± 0.58
2.6	2	23.6 ± 4.0	2.7 ± 0.45
3.5	2	23.2 ± 2.3	2.6 ± 0.52
3.5	4	49.0 ± 8.7	10.4 ± 3.25

Groups of rats (N = 4–5) were orally dosed three times a week with HCB for a period of 2 or 4 weeks. Within 24 hr after the last dose, blood was collected by tail bleeding. HCB and PCP concentrations in serum were analysed by HPLC. DL, Detection limit (see Materials and Methods).

occupancy of binding sites as a consequence of dosing with HCB (Table 3).

Effects of administered PCP on serum (free) thyroxine levels

In separate dosing experiments investigating the competitive interactions of PCP with thyroid hormone serum binding proteins, results indicated that PCP caused a rapid and substantial reduction in TT4 concentration in serum that was highly dose dependent (Fig. 1). When rats were exposed to the highest dose of PCP (0.10 mmol/kg), TT4 concentrations in serum were reduced by more than 65% (Fig. 1). FT4 levels in serum were also strongly reduced (by 60%) after dosing with PCP compared with control values (Fig. 2) PCP had neither clear effects on serum levels of (F) T3 nor on TSH levels (data not shown).

PCP levels in serum after PCP treatment

The relationship between the dose of PCP administered and the concentration of PCP in serum reached, as determined by HPLC analysis, was found to be linear (Fig. 3) up to an administered dose of 100 μmol/kg.

DISCUSSION

The results of the present experiments demonstrate that both HCB and its major metabolite PCP are able to produce a decrease in serum thyroid hormone levels. HCB produced significant reductions of TT4 in a dose-dependent manner (2 or 4 weeks exposure), while levels of TT3 were not found to be substantially altered. However, in short term experiments (2 weeks) there appeared to be no large dose-dependent effect on FT4, and hence on TSH levels (because the TSH response is to FT4 rather than to TT4). During longer exposure to HCB (4 weeks), a greater reduction of TT4 levels was observed, including decreased FT4 and hence increased TSH levels. An

Table 3. Occupancy of binding sites after HCB-dosing

Incubation conditions	N	cpm	% Inhibition
HCB sera	5	37463 ± 1321*	20
Control sera	5	46662 ± 866	0
Control sera + T4 (100 μM)	2	3083 ± 194	93

Sera were obtained from animals dosed with 3.5 mmol HCB/kg for 4 weeks or without HCB. Binding of a standard amount of [^{125}I]T4 to serum binding proteins was determined as described in the Materials and Methods. Significance: *$P < 0.05$.

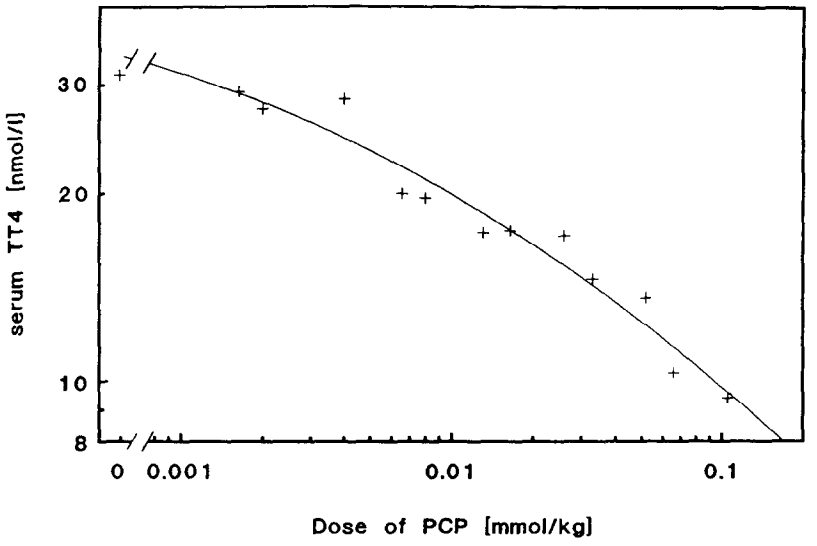

Fig. 1. Effect of the dose of PCP on serum TT4 levels. Groups of rats (N = 4) received a single i.p. injection of different doses of PCP (0–0.105 mmol/kg in corn oil). Control rats received corn oil only. Blood samples were taken 6 hr after exposure. TT4 levels in serum were determined with a RIA. The results represent combined data from two independent animal experiments.

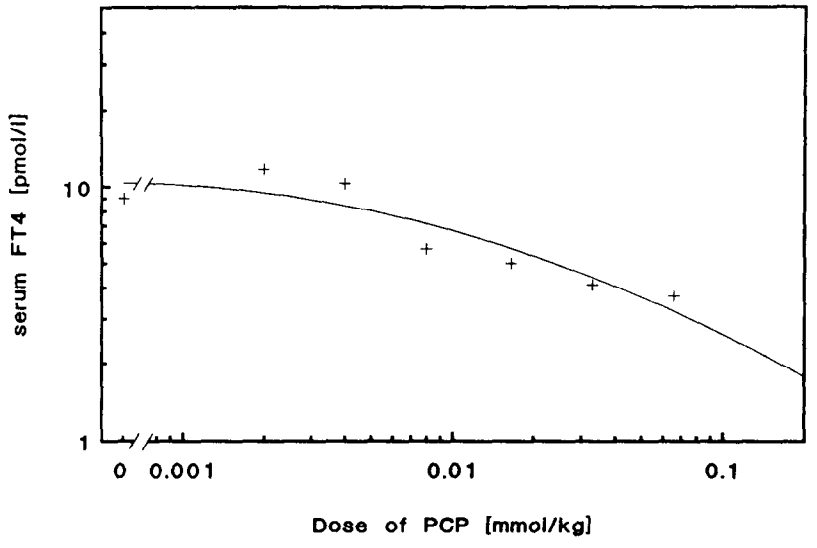

Fig. 2. Effect of the dose of PCP on serum FT4 levels. Single i.p. injections of different doses of PCP were given to groups of rats (N = 4). Blood was collected 6 hr after administration of PCP. FT4 levels in serum were determined with a RIA.

explanation may be that during the shorter period (2 weeks) the buffering capacity of serum binding proteins on T4 is still large enough to compensate the FT4 pool. With prolonged exposure this capacity may become exhausted through competitive effects of PCP, the FT4 levels can no longer be maintained and therefore TSH levels go up. TT3 levels have never been observed to be altered with PCBs, dioxins, chlorinated benzenes etc. These observations confirm and extend results of other investigators [4–6] who also found decreased TT4 levels but no significant alterations in TT3 serum levels by HCB. Recently however, evidence has been obtained for HCB-induced significant reductions of plasma TT3 levels [26]. The hypothalamus–pituitary axis is probably not impaired as indicated by an increased TSH response, while preliminary results have shown evidence for an increased activity of the thyroid gland as appeared histologically by hyperplasia in combination with increased iodine uptake in the

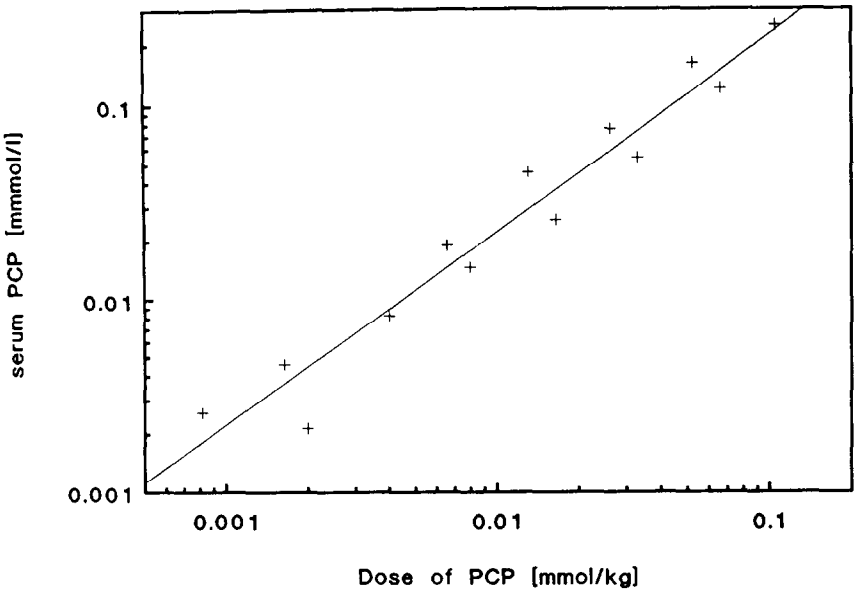

Fig. 3. Relationship between serum PCP levels and administered dose. Different doses of PCP were injected i.p. and blood was collected 6 hr later. PCP concentration in serum was analysed by HPLC. These results represent combined data from two independent animal experiments.

thyroid. The combination of decreased TT4 and FT4 levels in sera and an increased TSH response after subchronical treatment with HCB suggest the condition of hypothyroidism.

Repeated dosing with HCB leads to induction of microsomal enzymes, e.g. P450[11], ethoxyresorufin-deethylase [11] and UDPGT levels [12]. Enhanced activity of several UDPGTs have recently been shown to give rise to increased levels of T4-glucuronides and to an increased bile flow [12], the combined effect could lead to a rapid removal of T4 from the circulation [12].

A further consequence of hepatic microsomal enzymatic activity is a build-up in serum of PCP, the major metabolite of HCB [17–19]. PCP has been causally implicated in HCB-induced reductions of thyroid hormone levels because of its greater effectiveness in reducing TT4 serum levels [20]. The present experiments confirm the rapid decrease of TT4 serum levels in animals exposed to PCP. In addition, FT4 levels in serum were also equally affected. No significant alterations of TSH levels were observed, but the time period of 6 hr may not be long enough for proper operation of the hypothalamus–pituitary–thyroid feedback mechanism.

In the present study, evidence for significant competitive interactions (19.7% competition in the standard gel filtration assay) was observed in sera of animals dosed with HCB. In contrast, no alterations in serum binding of T4 in sera of animals exposed to HCB were reported using an electrophoretic technique [6]. Possible differences in sensitivity of the techniques being applied may account for the different findings. One of the conclusions from earlier studies was that there was very little binding of HCB to T4 binding sites of serum proteins. PCP however, is a very avid binder of these sites. Among the serum binding proteins PCP has relatively the highest binding to TTR, even higher than T4 itself, followed by albumin and lowest binding to TBG. By Scatchard analysis it is apparent that PCP alters the affinity of T4 binding and not the number of binding sites on serum proteins [21, 22, 25, 32].

One of the unresolved issues concerns the question to what extent the possible mechanisms (i.e. competitive interactions and increased metabolism) are involved in the decrease of thyroid hormone by HCB and related polyhalogenated aromatics. The present experiments allow an estimation of the respective contribution of either of these mechanisms. It was possible to estimate the contribution of PCP to the decrease of TT4 levels through competitive interactions, because, relationships between administered doses of PCP versus serum concentrations of PCP on the one hand (Fig. 3) and versus serum TT4 levels on the other were established (Fig. 1). After dosing for 4 weeks with 3.5 mmol HCB/kg, PCP levels in serum reached 0.01 mmol/L (Table 2). This serum level of PCP would correspond with an administered dose of 0.0044 mmol/kg PCP (by extrapolation in Fig. 3) that would be able to induce a decrease of 19.5% in serum TT4 levels (and a marginal decrease of FT4 levels) through competitive interactions (by extrapolation in Fig. 1). Since the present data show a maximum reduction of TT4 levels of 41% with a sub-chronic dosing of 3.5 mmol HCB/kg (Table 1) it can be calculated that PCP may decrease TT4 levels through competition at the hormone binding protein level by about 48% (i.e. $19.5/41 \times 100\%$). The remaining 52% decrease of TT4 levels is attributed to other causes such as increased hepatic metabolism. At the earlier time of 2 weeks, serum levels of PCP were not high enough

to cause a decrease of TT4 levels by competitive interactions with thyroid hormone binding proteins in serum and the effect on TT4 levels may be caused solely by the metabolic pathway.

The present findings suggest that the decrease of T4 levels by repeated dosing of HCB may initially be caused by induction of metabolic pathways for T4 only. With prolonged dosing, HCB accumulates, for instance, in the liver. As hepatic microsomal metabolism is also increased, PCP levels in the circulation can reach levels that are high enough to compete with thyroid hormone for binding sites of thyroxine binding proteins such as TTR and albumin [21, 22, 25, 26]. In this phase both competitive interactions and metabolism work in conjunction to lower T4 serum levels. As far as we know, this may be the first example of a common effect (decrease of serum TT4 levels) being caused by parent compound and metabolite at the same time through different and independent mechanisms.

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