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INCREASED GLUCURONIDATION OF THYROID HORMONE IN HEXACHLOROBENZENE-TREATED RATS

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Abstract—Metabolism of thyroid hormones was investigated in WAG/MBL rats that had been exposed to hexachlorobenzene (HCB). Serum thyroxine (T4) levels were lowered by 35.5%, whereas triiodothyronine (T3) levels were not changed. Bile flow, as well as T4 excretion in bile were increased by HCB treatment. Analysis of bile by HPLC revealed a more than 3-fold increase of T4 glucuronide (T4G) and a concomitant reduction of non-conjugated T4. T4 UDP-glucuronyltransferase activity (T4 UDPGT) activity in hepatic microsomes was increased more than 4.5-fold in animals exposed to HCB. *p*-Nitrophenol (PNP) UDPGT showed a comparable increase by HCB. Both T3 and androsterone UDPGT activities were low in WAG/MBL rats compared with normal Wistar rats. T3 UDPGT activity was increased 2.5-fold by HCB, but androsterone UDPGT activity was unchanged. These results suggest that T4 is a substrate for HCB-inducible PNP UDPGT and T3 for androsterone UDPGT. In the absence of the latter, T3 is also glucuronidated to some extent by PNP UDPGT. Type 1 iodothyronine deiodinase activity was decreased by HCB treatment. It is concluded that decreased T4 levels in serum of animals after exposure to HCB may be due to a combined effect of displacement of T4 from carriers, an increased glucuronidation of T4 and enhanced bile flow.

It is well known that the fungicide hexachlorobenzene (HCB‖) affects the thyroid system. Chronic exposure of different species of animals but also of humans leads to a decrease of thyroid hormone levels in the circulation [1–7]. Several chlorinated aromatic compounds, such as polychlorinated biphenyls (PCBs) and dioxins have also been found to cause hypothyroidism [8–11]. The mechanisms responsible for reduced thyroid hormone levels after chronic exposure of rats to HCB are not completely understood. There are indications that the major metabolite of HCB, i.e. pentachlorophenol (PCP), may play a role because PCP is more potent in decreasing serum thyroid hormone levels in rats than an equimolar dose of HCB [3]. In addition, *in vitro* and *ex vivo* observations suggest that PCP interacts strongly with serum thyroxine (T4) carrier proteins as compared to HCB [4, 12, ¶]. PCP was found to interact competitively with the T4 binding site of transthyretin, while the affinity of PCP is about 2-fold higher than that of T4 [12]. This competition with thyroid hormone carriers may contribute to the lowered blood T4 levels [4, 12, ¶].

Enhanced hepatic metabolism of T4 in HCB-treated rats might be an additional mechanism for reducing serum T4 levels [5, 6, 13, 14]. The most prominent metabolic routes for T4 are the deiodination pathways and hepatic conjugation of the phenolic hydroxyl group with glucuronic acid or sulfate. Recent studies reported that multiple UDP-glucuronyltransferase (UDPGT) isozymes are involved in the glucuronidation of thyroid hormones, and suggested that T4 is glucuronidated by *p*-nitrophenol (PNP) and bilirubin UDPGTs, and triiodothyronine (T3) specifically by androsterone UDPGT [15, 16].

In the present study, the role and identity of enzymes involved in the metabolic clearance of thyroid hormones in HCB- or vehicle-exposed rats were investigated by determining (i) bile flow and biliary excretion of thyroid hormone glucuronides (ii) T4 and T3 UDPGT and type I deiodinase activities in liver microsomes in parallel with PNP and androsterone UDPGT activities.

MATERIALS AND METHODS

Chemicals. HCB was obtained from Aldrich (Brussels, Belgium); [^{125}I]T4 (sp. act. > 1200 μCi/μg), [^{125}I]T3 (sp. act. 2800 μCi/μg), [^{125}I]rT3 (sp. act > 1200 μCi/μg), T4 and T3 radioimmunoassay kits (Amerlex-M) from Amersham (Amersham, U.K.); androsterone from Steraloids (Wilton, NH, U.S.A.); [^{3}H]androsterone (sp. act. 116 μCi/μg) from New England Nuclear (Boston, MA, U.S.A.); UDP glucuronic acid (UDPGA) from Boehringer (Mannheim, F.R.G.); bovine serum albumin (BSA), 3,3-cholamidopropyl-dimethylammonio-1-propane-sulfonate (CHAPS), dithiothreitol (DTT), PNP, T4

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‖ Abbreviations: HCB, hexachlorobenzene; PCBs, polychlorinated biphenyls; PCP, pentachlorophenol; UDPGT, UDP-glucuronyltransferase; PNP, *p*-nitrophenol. T4, thyroxine; T3, triiodothyronine; UDPGA, UDP glucuronic acid; BSA, bovine serum albumin; CHAPS, 3,3-cholamidopropyl-dimethylammonio-1-propane-sulfonate; DTT, dithiothreitol; T4G, T4 glucuronide.

¶ Van Raaij JAGM, Frijters CMG and van den Berg KJ, Hypothyroidism: Involvement of different mechanisms by parent compound and metabolite, submitted.

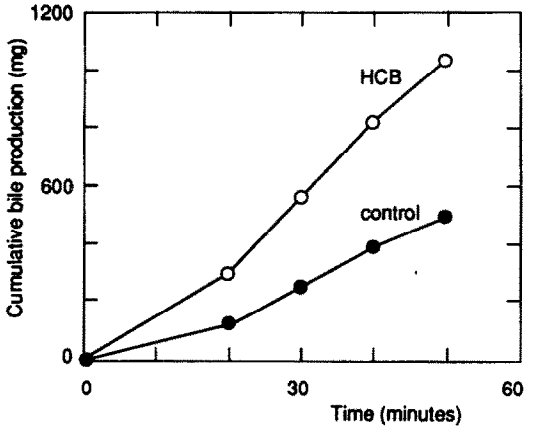

Fig. 1. Bile flow in control and HCB-treated rats. Bile ducts were cannulated from rats exposed to HCB (N = 4) or to vehicle (N = 3). Bile was collected in 10 min fractions and weighed. Results are expressed as the cumulative weight of bile collected.

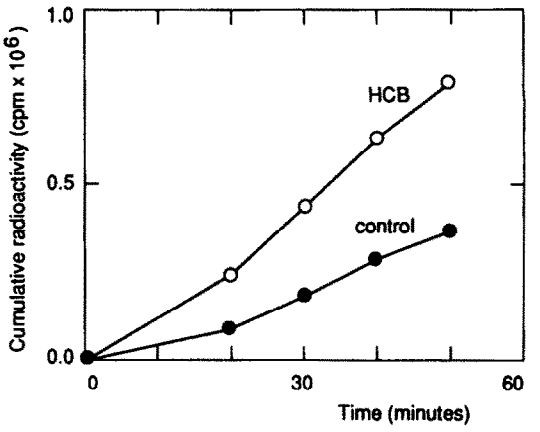

Fig. 2. Cumulative biliary T4 clearance in control and HCB-treated rats. Rats exposed to HCB (N = 4) or to vehicle (N = 3) received [^{125}I]T4 i.p. About 5 hr later bile collection was started. Bile samples were weighed and counted.

and T3 from the Sigma Chemical Co. (St Louis, MO, U.S.A.); 3,3',5'-triiodothyronine (rT3) from Henning (Berlin, F.R.G.); and Sephadex LH-20 from Pharmacia (Uppsala, Sweden). Radiolabeled T4 and T3 were purified on Sephadex LH-20 before incubation.

Animals and treatments. Male WAG/MBL (WAG) rats, weighing 200–300 g were maintained on regular diet and tap water *ad lib.* They were housed in a constant environment with a 12 hr light : 12 hr dark cycle, a temperature of 24° and a humidity of 50–70%. Animals were treated orally three times a week for 4 weeks with 3 mL of either 1 g HCB/kg (40 mg/mL, 0.5% Tween-20 in water) or water plus 0.5% Tween-20 only. After the last dose, liver and sera were collected from animals after killing by decapitation. Livers were frozen at −70° until preparation of microsomes. Sera were also stored at −70° until analysis of T4 and T3 by commercial radioimmunoassay kits.

Bile flow and biliary clearance. Animals were injected i.p. with 25 μCi [^{125}I]T4 in 1 mL saline. After about 5 hr, bile ducts were cannulated under halothane anesthesia. Bile was collected in fractions of 10 min for a 1 hr period. After termination of the experiment, serum samples were collected.

Table 1. Serum T4 and T3 levels of rats treated with HCB

	HCB	N	Control	N
T4 (nmol/L)	12.9 ± 0.58*	5	20.0 ± 2.00	5
T3 (nmol/L)	0.63 ± 0.16	5	0.65 ± 0.15	5

Rats were exposed to HCB for 4 weeks. After the last dose, serum levels of T4 and T3 were determined by radioimmunoassay.

Results are given as mean ± SEM.

* $P < 0.001$.

Radioactivity in serum and bile samples was determined in a gamma counter.

HPLC analysis of bile samples. Representative chromatograms of T4 glucuronide (T4G) excretion were obtained by injecting 20 μL of pooled bile from HCB- or vehicle-treated rats into a reverse phase HPLC C18 system, eluting with linear gradients of acetonitrile in ammonium acetate (pH 4), as described earlier [17]. This procedure results in the separation of iodide, sulfated, glucuronidated and non-conjugated iodothyronines [17]. Fractions were collected and counted for radioactivity. Recovery of applied radioactivity amounted to 80 and 100%.

Microsomal preparations. Livers from WAG rats were homogenized in 5 vol. of 0.25 M sucrose, 10 mM Hepes, 1 mM DTT (pH 7.0) at 4°. Microsomes were obtained by centrifugation for 10 min at 25,000 *g* and subsequent centrifugation of supernatants for 60 min at 100,000 *g*. The microsomal pellets were suspended at a protein concentration of 10–20 mg/mL in 0.1 M phosphate (pH 7.2), 2 mM EDTA and 1 mM DTT, and frozen in 0.5 mL aliquots at −80°. Protein contents in microsomes were measured with the bicinchoninic acid procedure [18], or with the Bio-Rad assay (Richmond, CA, U.S.A.) using BSA as the standard.

Assays. T4 and T3 UDPGT activities were assayed essentially as previously described [15] in incubations of 1 μM labeled T4 or T3 with 1 mg/mL protein and 5 mM UDPGA in 75 mM Tris (pH 7.8), 3.75 mM $MgCl_2$, containing 0.125% BSA and 0.025% CHAPS. PNP UDPGT [19], androsterone UDPGT [20] and type I deiodinase activity [21] were also measured according to published methods.

Statistics. Bile flow and biliary T4 clearance in control and HCB-treated rats were statistically evaluated by analysis of variance and covariance with repeated measures. Student's *t*-tests were used for comparing other effects in control and HCB-treated animals.

Table 2. Biliary T4 clearance in control and HCB-treated rats

	HCB (% of dose)	N	Control (% of dose)	N
Bile radioactivity	2.9 ± 0.12*	4	1.3 ± 0.22	3
T4G	1.5 ± 0.06†	4	0.4 ± 0.08	3
T4	0.07 ± 0.01	4	0.18 ± 0.05	3

Rats were exposed for 4 weeks to HCB or vehicle. At the end of the experiment, the animals were injected i.p. with 25 μCi [^{125}I]T4. About 5 hr later, bile ducts were cannulated for collection of bile. Total bile was analysed by HPLC as described in Materials and Methods.

Results are given as means ± SEM.

* $P < 0.01$; † $P < 0.001$.

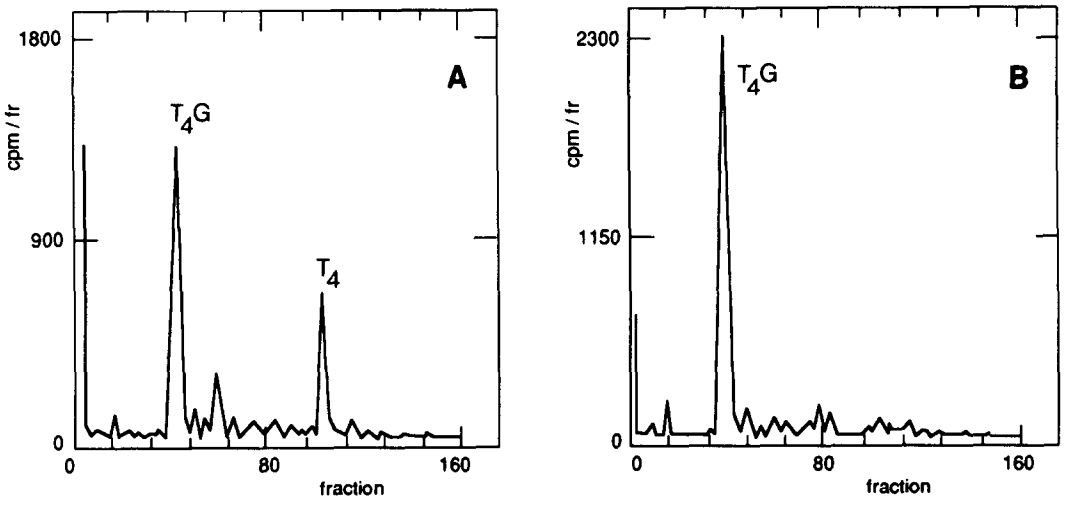

Fig. 3. HPLC analysis of bile from control (A) and HCB-treated (B) rats. Bile samples were analysed by HPLC as described in Materials and Methods. Results are expressed as radioactivity per fraction. Note differences in scale of the vertical axis.

RESULTS AND DISCUSSION

When animals had been exposed to HCB for 4 weeks, serum T4 levels were decreased by 35% compared with control animals (Table 1). Serum levels of T3 were not different between both groups (Table 1). In earlier studies [5], a transient decrease in serum levels of T3 during the second and third week of dosing was observed.

To investigate whether lowered T4 levels after dosing with HCB were the result of an increased metabolism, biliary T4 clearance was analysed. It appeared that bile flow was significantly increased ($P < 0.05$) by more than 100% after HCB treatment compared with control values (Fig. 1). This may be explained by enlargement of the liver, which was significantly increased in weight by HCB: livers of rats exposed to HCB or vehicle weighed 16.7 ± 0.5 and 10.0 ± 0.4 g, respectively ($P < 0.001$). Recently, Cuomo *et al.* [22] reported an increased bile acid independent flow (BAIF) by HCB.

In order to examine excretion of T4 in bile, animals received [^{125}I]T4. Radioactivity excreted into bile fluid was also significantly increased (more than 100%, $P < 0.01$) by HCB (Fig. 2, Table 2). Therefore, it may be concluded that HCB increases the elimination of T4 by an increased bile flow.

The composition of biliary radioactivity was analysed by HPLC. A chromatogram of bile from control animals showed the label predominantly in association with T4G and to a minor extent with non-conjugated T4 (Fig. 3A). Some iodide was present in the void volume fractions. A similar chromatogram of bile from animals exposed to HCB revealed a greater extent of T4 glucuronidation (Fig. 3B). Excretion of T4G was increased 3.7-fold by HCB exposure, whereas non-conjugated T4 was decreased 2.6-fold (Table 2).

HCB stimulation of biliary T4G excretion may be due to an enhanced T4 UDPGT activity. Indeed, a 4.7-fold increase of T4 UDPGT activity was observed (Table 3), which appeared somewhat more substantial than the excretion of T4G into bile (Table 2). Increased levels of T4G in bile were also demonstrated after exposure of rats to PCBs,

Table 3. Effects of HCB on UDPGT and type I deiodinase activities

Enzyme activity	HCB	N	Control	N
T4 UDPGT (pmol/min/mg)	1.26 ± 0.07†	5	0.27 ± 0.02	5
PNP UDPGT (nmol/min/mg)	278 ± 8.94†	5	62 ± 2.68	5
T3 UDPGT (pmol/min/mg)	0.88 ± 0.04†	5	0.35 ± 0.01	5
Androsterone UDPGT (nmol/min/mg)	0.52 ± 0.01*	5	0.47 ± 0.02	5
Deiodinase (pmol/min/mg)	442 ± 90*	5	641 ± 131	5

Dosing of animals, isolation of livers, preparation of hepatic microsomes and enzyme assays were carried out as described in Materials and Methods.
* $P < 0.05$; † $P < 0.001$.

associated with a strong induction of T4 UDPGT [15]. Thus, HCB induces processes involved in the metabolism of T4.

There are indications that in rats T4 and PNP are substrates for a common UDPGT isoenzyme and that xenobiotics, such as PCBs, methylcholantrene, dioxines and other various hepatic microsomal enzyme inducers, are potent inducers of T4 and PNP glucuronidation [13–15, 23–25]. PNP UDPGT activity in the WAG rats used in the present study was increased 4.5-fold by HCB (Table 3). In view of the broad substrate specificity, this enzyme could also be involved in glucuronidation of PCP, the major metabolite of HCB. Our findings suggest that also in the WAG rat the enzyme responsible for enhanced T4 glucuronidation by HCB may be identical to the HCB-induced PNP UDPGT.

Androsterone UDPGT activity in untreated WAG rats was ≈10% of that measured in livers of normal Wistar (HA) rats and similar to the androsterone UDPGT activity in Wistar LA and Fisher rats, which have a genetic defect in the gene coding for this isoenzyme [15, 16]. Like Wistar LA and Fisher rats, T3 UDPGT activity in WAG rats is only about one-third of that in Wistar HA rats, suggesting that T3 is a substrate for androsterone UDPGT. While HCB has little influence on the (low) androsterone UDPGT activity in WAG rats, it increases liver microsomal T3 glucuronidation 2.5-fold (Table 3). This suggests that in the absence of androsterone UDPGT, T3 is glucuronidated to some extent by the HCB-inducible PNP UDPGT.

In conclusion, decreased serum T4 levels in animals exposed to HCB may be explained by a combination of factors: displacement of T4 from serum proteins and induction of T4 UDPGT activity. The resultant increase in thyroid activity apparently does not fully compensate for the increased T4 clearance.

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