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FORMATION OF PENTACHLOROPHENOL AS THE MAJOR PRODUCT OF MICROSOMAL OXIDATION OF HEXACHLOROBENZENE

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SUMMARY: On incubation of [^{14}C]-hexachlorobenzene with microsomes from livers of rats induced with hexachlorobenzene, the major product (80-90%) was pentachlorophenol. The only other detectable metabolite, tetrachlorohydroquinone (4-15%), was presumably formed from pentachlorophenol. A considerable amount of radioactivity (5-10% of the amount of extracted metabolites) was covalently bound to protein. Microsomes derived from male hexachlorobenzene-induced rats gave by far the highest conversion (approx. 1% of substrate). Microsomes from female hexachlorobenzene-induced rats were 3 times less efficient. Microsomes from untreated and 3-methyl-cholanthrene-treated animals gave less than 5% of the amount of pentachlorophenol formed by microsomes from hexachlorobenzene-induced male rats, while phenobarbital and aroclor 1254-induction resulted in formation of 51% and 34% respectively.

The fungicide hexachlorobenzene (HCB) has been shown to be hepatocarcinogenic (1) and is known for its porphyrinogenic action in man and animal (2). The accumulation of porphyrins is a result of inhibition of the enzyme uroporphyrinogen decarboxylase (3). Although it has been reported that HCB itself has an inactivating effect upon this enzyme in its purified form (4), there is also substantial evidence that metabolism of HCB is a prerequisite for this inactivation. Selective inhibition of cytochrome P-450 in a primary liver cell culture prevents the accumulation of porphyrins after HCB-exposure (5), while *in vivo* induction of P-450 by phenobarbital enhances the porphyrinogenic action of HCB (6-10). These experiments indicate the important role of cytochrome P-450 in the metabolism of HCB. Numerous studies have appeared on the *in vivo* metabolism of HCB, resulting in the identification of a large number of metabolites, e.g. chlorophenols, chlorobenzenes and sulphur-conjugated chlorophenols and -

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benzenes (11-13). The only investigation on the microsomal metabolism of HCB reported thus far (11) was performed with microsomes of non-induced rats, and the amounts of metabolites found were too small for quantitation. In the present study the metabolism of [^{14}C]-labeled HCB by liver microsomes of rats pretreated with HCB and a number of other inducing agents was investigated and the amounts of metabolites were determined quantitatively.

METHODS

Chemicals: U-[^{14}C]-hexachlorobenzene (Amersham UK, sa. 106 mCi/mmol) was purified by HPLC, using the procedure described below for analysis of metabolites, to a radiochemical purity of 99.96% (0.03% contamination with pentachlorobenzene). Hexachlorobenzene (analytical grade, BDH chemicals, Poole UK) was purified by preparative HPLC (Chrompack Lichrosorb RP18 1.2x25 cm column, eluted isocratically with methanol) for use in microsomal incubations. NADPH was from Boehringer Manheim FRG. The HPLC marker metabolites were: tetrachlorohydroquinone (ICN Pharm., N.Y.); pentachlorophenol (Aldrich); chloranil (Merck); 2,3,4,5-tetrachlorophenol (Fluka); 1,2,3,4- (Merck), 1,2,4,5-(Aldrich) and 1,2,3,5- (Merck) tetrachlorobenzene and pentachlorobenzene (Merck).

Preparation of microsomes: 14 week old male or female Wistar rats were pretreated (2 rats per treatment) with HCB (14 days 0.1% in chow, prepared from a 1% solution in olive oil (6)), phenobarbital (7 days 0.1% in drinking water), 3-methylcholanthrene (3 daily i.p. injections of 30 mg/kg body weight) and aroclor 1254 (one i.p. injection of 600 mg/kg body weight, sacrifice after 6 days). Livers were perfused with a 20 mM Tris-HCl 250 mM sucrose buffer, pH 7.4, homogenised with a teflon potter, and centrifuged for 20 minutes at 10.000 g. The supernatant was centrifuged for 90 minutes at 105.000 g, and the pellet was washed in 150 mM KCl. The resulting pellet was resuspended in 100 mM potassium phosphate buffer, pH 7.4. Microsomes were stored at -25°C until use. Cytochrome P-450 was determined according to Omura and Sato (14). Protein concentration was determined by the method of Lowry (15).

Microsomal incubations: The standard incubation mixture contained 25µM [^{14}C]-HCB diluted to a s.a. of 20 mCi/mmol, 3mM $MgCl_2$, 0.1 M potassium phosphate buffer pH 7.4 and 0.16 - 5 µM microsomal cytochrome P-450 in a final volume of 2 ml. Incubations were started by addition of 1mM NADPH. After 30 minutes incubation at 37°C the reaction was terminated by addition of trichloroacetic acid (3% final concentration). Metabolites were extracted with 2x3 ml of acetone/ethyl acetate 1:2. This resulted in complete (>99.9%) removal of non-covalently bound radioactivity from the aqueous phase. The extracts were dried with Na_2SO_4 and after removal of the organic solvent in a stream of dry air, the residues were dissolved in 50 µl of methanol.Controls consisted of incubations with boiled microsomes, without NADPH or with carbon monoxide. To find optimal conditions the concentrations of HCB, NADPH and the incubation time were varied.

HPLC analysis of metabolites: Separation of the metabolites of HCB was achieved on a Dupont Zorbax ODS column (0.46x25 cm). After injection of 50 µl incubation sample together with 5µl of a solution containing the marker metabolites, all radioactivity was eluted with a linear gradient of 65% 20 mM tris-acetate buffer, pH 8.0/35% methanol to 100% methanol in 10 min, followed by 20 min of 100% methanol, at a flow rate of 1 ml/min. The eluent was monitored at 254 nm and 0.3 - 1 ml fractions were collected in order to determine the amount of radioactivity, in 5 ml of Packard instagel. In some runs the tris-acetate buffer was replaced by 1% acetic acid in water.

Covalent binding to proteins: After the acetone/ethyl acetate extraction the aqueous (protein) samples were extracted with 10 ml of methanol (3 times), ethanol (2 times) and ethanol: chloroform: ether 2:2:1 (2 times). The resulting pellet was dissolved in 1 ml of soluene 350 (Packard) after which 10 ml of scintillation-cocktail was added and the amount of radioactivity was determined. This method was adapted from that described by Koss (13).

RESULTS

metabolites of HCB: In order to determine and quantitate the possible metabolites of hexachlorobenzene we have developed an HPLC-procedure which separates HCB and 8 potential metabolites (fig. 1a). Using this procedure it was found that the major product of the HCB-induced microsomal metabolism of HCB co-eluted with the pentachlorophenol marker, and consisted of approximately 90% of the radioactivity that eluted before the substrate. The only other detectable radioactive peak eluted together with tetrachlorohydroquinone, and accounted for 4-15% of total metabolites. In order to prove the identity of the pentachlorophenol- and tetrachlorohydro-

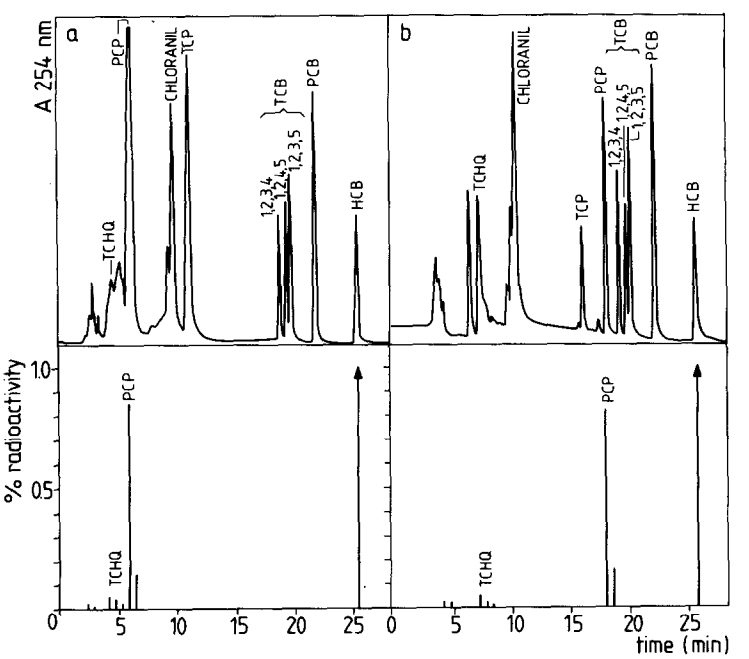

Figure 1.: HPLC-analysis of metabolites of hexachlorobenzene converted by microsomes of male hexachlorobenzene induced rats.
a) Base run (tris-acetate pH8-buffer as the aqueous component), b) acid run (1% acetic acid as the aqueous component). For exact conditions and explanation of marker metabolites see methods section.

quinone-peaks, an HPLC run was performed in which the tris-acetate buffer, pH 8, was exchanged for a 1% acetic acid solution in water (fig. 1b). As a result the tetrachlorohydroquinone eluted just prior to the chloranil, and the pentachlorophenol marker appeared close to the tetrachlorobenzenes. As expected, figure 1a and b show that only pentachlorophenol (pKa= 4,6), tetrachlorophenol (pKa= 7.0) and tetrachlorohydroquinone were sensitive to changes in pH. The shifts in the radioactivity elution profile corresponded exactly with the shifts in the UV-elution profile. Pentachlorobenzene was not generated, nor was it metabolized: the trace amount of radioactive contamination with pentachlorobenzene was found unaltered after every incubation. The conversion appeared to be an enzymatic (boiled microsomes gave no conversion) and cytochrome P-450 dependent reaction: incubations without NADPH resulted in no detectable metabolites, while only 10% of the maximal conversion was reached after saturating the microsomes with carbon monoxide (Table 1). Variation of the HCB- and NADPH-concentration showed

Table 1. Metabolism of hexachlorobenzene by microsomes of male HCB-induced rats

		conversion[1]	% of metabolism[2] %PCP	%TCHQ	covalent binding[3]
a) boiled microsomes		0.07	13	0	195
(microsomes + CO)		0.14	28	15	307
b) variation of NADPH (mM)	0	0.04	0	0	137
	0.5	0.94	90	6	
	1.0	1.08	91	6	
c) variation of HCB (μM)	5	1.65	88	8	
	10	2.19	86	8	
	25	0.66	90	7	
d) variation of P-450 (μM)	0.16	0.31	84	9	264
	0.32	0.71	81	13	752
	0.64	1.63	71	15	1581
e) variation of time (min)	5	0.77	84	6	478
	10	1.58	84	8	1000
	20	1.33	84	7	1478

Unless otherwise stated, incubation conditions were: 25μM HCB, 1mM NADPH, 2.5 μM P-450, 30 min.

1) Conversion is expressed as percentage of total radioactivity eluted from the column.
2) Metabolites are expressed as percentage of total metabolites (i.e. total radioactivity eluted before the substrate).
3) Covalent binding is expressed as p.p.m. of total added radioactivity.

that both substrates were present in saturating amounts. At low levels (0.16-0.63μM) the conversion was linear with the concentration of P-450. Variation of the incubation time showed that maximal conversion was reached after 10 minutes (Table 1). The results indicate that within one series of incubations all values were comparable but that different series of incubations could result in slight variations in the rates of conversion, although the same ratios of conversion were measured. This was presumably due to a slight evaporation of HCB during solvent removal.

Covalent binding to protein: The protein fraction of microsomes of male HCB-induced rats contained 6.5 times more radioactivity than the same incubation with boiled microsomes (Table 1). Radioactivity in the protein fraction showed a linearity with the incubation time, while incubations without NADPH or in the presence of carbon monoxide gave values similar to the incubation of boiled microsomes. This implies that, besides the extracted metabolites pentachlorophenol and tetrachlorohydroquinone, a substantial part of metabolized HCB (between 5,5 and 10,8% of total extracted metabolites) was covalently bound to microsomal protein.

Differential metabolism in microsomes of HCB-induced male and female rats Microsomes of HCB-induced female rats converted only 30% of the amount of HCB metabolized by microsomes of HCB-induced male rats (0.23% and 0.77% resp. of total added HCB, Table 2). However, in both cases the same metabolites were produced. Non-induced male nor female rat liver microsomes were able to metabolize HCB to pentachlorophenol (Table 2). The difference between the amount of pentachlorophenol and the total amount of metabolites was due to a few very small unidentified peaks in the apolar region of the HPLC-eluate which were not detectable as metabolites from microsomes of any of the tested inducers.

The effect of different inducing agents on the microsomal metabolism of HCB Besides HCB-microsomes, the microsomes of male rats induced with phenobarbital (PB), 3-methylcholanthrene (3MC) and aroclor 1254 were assayed for their ability to metabolize HCB. The results are shown in table 2; experi-

Table 2. The effect of inducing agents on the microsomal metabolism of hexachlorobenzene

microsomes	protein (mg/ml)	conversion[1]	% of metabolites[2]	
			PCP	TCHQ
HCB male	1.60	0.77	90 (100)	4 (100)
HCB female	1.79	0.23	93 (31)	2 (15)
PB male	1.81	0.38	94 (51)	1 (17)
3 MC male	1.69	0.06	32 (3)	0 (0)
Aroclor male	1.20	0.28	84 (34)	1 (12)
non induced male	3.33	0.16	19 (4)	25 (123)
non induced female	3.57	0.07	9 (1)	0 (0)

25μM HCB was incubated at 37°C for 30 minutes. P-450 concentration was 2.5μM
1) Conversion is expressed as percentage of total radioactivity, eluted from the column.
2) Individual metabolites are expressed as percentages of total metabolites, the number in brackets denotes the amount of metabolite as percentage of the amount of metabolite formed by HCB microsomes.

ments with a doubled P-450 concentration gave comparable ratios for the conversions. After PB-induction the microsomal HCB-metabolism was 50% of that after HCB-induction per nmol P-450. 3MC was not able to induce the HCB-converting P-450 ('s). Aroclor 1254, a mixed inducer, raised product formation to 1/3 of the HCB-induced microsomes. Both PB- and aroclor microsomes produced pentachlorophenol as the predominant metabolite (84-94%), together with 1.5% tetrachlorohydroquinone.

DISCUSSION

In this report we demonstrate that hexachlorobenzene is metabolized by cytochrome P-450 and that pentachlorophenol is the only resulting metabolite. The formation of tetrachlorohydroquinone can be attributed solely to the conversion of pentachlorophenol (16). Since pentachlorophenol itself is not porphyrinogenic (17), it seems likely that a reactive intermediate with a strong affinity for protein is generated by P-450 during the turnover of HCB and that this intermediate is responsible for the porphyrinogenicity of HCB. The enhancement of covalent binding of radioactivity to protein during microsomal HCB-metabolism also points in this direction.

Metabolism of labeled HCB both in primary liver cell culture (18) and in vivo (13) also results in covalently attached label to protein.

Our results however, do not explain the greater susceptibility of female rats to the porphyrinogenic action of HCB. Female rats produce up to 300 times more porphyrins than males (19). The finding that HCB-induced female rat liver microsomes metabolize HCB to a lesser extent than male rat liver microsomes is in agreement with the report of Franklin (19), who found the same difference for a number of other substrates. Rizzardini however, reports no difference in urinary excretion of pentachlorophenol between male and female rats after exposure to HCB (20).

In recent years, considerable attention has been paid to the nature of the cytochrome P-450 isozymes induced by HCB (21-24). Our findings indicate that HCB is probably metabolized by (one of the) PB-induced forms, and not by a 3-MC induced isozyme, in accord with the results of Debets (6).

To solve these questions, the mechanism of HCB-conversion and the possible generation of reactive intermediates is being investigated in a system reconstituted with purified HCB-induced cytochromes P-450.

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