Biochemical Pharmacology, Vol 35, No 13, pp 2163–2170, 1986
Printed in Great Britain

0006-2952/86 $3 00 + 0 00
Pergamon Journals Ltd

METABOLISM OF THE "MIXED" CYTOCHROME P-450 INDUCER HEXACHLOROBENZENE BY RAT LIVER MICROSOMES*

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(*Received* 5 *November* 1985, *accepted* 10 *January* 1986)

Abstract—1 Hexachlorobenzene (HCB) was metabolised by phenobarbital-induced liver microsomes from male rats to pentachlorobenzene, pentachlorophenol, tetrachloro-1,2-benzenediol and tetrachloro-1,4-benzenediol (1.88 2 9) Metabolites were identified and quantified by electron capture g l c Structures were confirmed by selective ion monitoring g.l c.-m s. The formation of pentachlorophenol was dependent on the presence of NADPH and O_2 and inhibited by CO, SKF 525A and metyrapone 2. Conversion of HCB to pentachlorophenol was stimulated by pretreatment of rats with phenobarbital (PB) but not by 3-methylcholanthrene (3-MC), or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) In contrast, the conversion of pentachlorophenol to tetrachloro-1,4-benzenediol was markedly induced by 3-MC but poorly by PB HCB, Aroclor 1254 and isosafrole stimulated both hydroxylations 3 The cytochrome P-450*c* inhibitor 9-hydroxyellipticine inhibited conversion of pentachlorophenol to tetrachlorobenzenediols by HCB and β-naphthoflavone induced micromes. 4 In addition to hydroxylation reactions, evidence was obtained for the conjugation of HCB with glutathione catalysed by a microsomal glutathione transferase 5 Radioactivity from [^{14}C]HCB was bound to microsomal protein during aerobic incubations. Binding was inhibited by GSH and *N*-acetyl-cysteine Preliminary studies suggested that the reactive species was derived from tetrachloro-1,4-benzoquinone 6 No correlation was found between levels of metabolites or covalent binding produced by the two sexes and the marked sex dependent hepatic porphyrogenic and carcinogenic effects of HCB.

Hexachlorobenzene (HCB) is a pollutant of the general environment with a number of human poisoning episodes reported, one being of major significance [1]. Amongst the toxic syndromes produced in both animals and humans is a persistent porphyria due to depression of hepatic uroporphyrinogen decarboxylase activity [EC 5.1.1.37] [2–5]. Besides causing porphyria, HCB is a hepatocarcinogen in hamsters, mice and rats [6–8]. Female rats are much more sensitive than males both to the onset of porphyria [9, 10] and to the carcinogenic potential of HCB [11, 12].

In addition to the toxic actions of HCB, this chemical is of biochemical interest as an inducer of the cytochrome P-450 group of drug metabolizing enzymes. Most inducers of the hepatic cytochrome P-450 system induce a spectrum of monooxygenase activities similar either to that produced on the one hand by phenobarbital (PB) or on the other by 3-methylcholanthrene (3-MC), β-naphthoflavone and most potently of all 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The major haemoproteins induced are cytochromes P-450$_b$ and P-450$_c$ respectively [13]. In contrast, HCB was the first to be described of only a few chemicals which induce haemoproteins and monooxygenase activities characteristic of induction by both PB and 3-MC [14–18]

It is thus important to determine how this fully chlorinated, aromatic compound is metabolised by the hepatic microsomal system and the extent to which metabolism can be modified by inducers of cytochrome P-450. *In vivo* HCB is metabolized extremely slowly [19–21] and those microsomal studies reported have been restricted to the use of [^{14}C]HCB [22, 23]. Here we demonstrate with the aid of electron capture detection g.l.c. and mass spectrometry the hydroxylation of HCB to pentachlorophenol and its subsequent conversion to tetrachlorobenzenediol by rat hepatic microsomes. The effects of inducers and inhibitors of cytochrome P-450 on these two hydroxylation steps have been studied, with emphasis on the "mixed" inducing properties of HCB. An explanation was sought for the distinct sex difference in the susceptibility of rats to the hepatotoxic actions of this chemical

MATERIALS AND METHODS

Chemicals. HCB (Organic Analytical Standard grade) and PB were purchased from BDH Chemicals Co. (Poole, Dorset, U.K.) 2,3,5,6-Tetrachloro-1,4-benzenediol, 3,4,5,6-tetrachloro-1,2-benzoquinone, diethylstilboestrol dipropionate, GSH, metyrapone, 3-MC, NADPH, NADP$^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were supplied by Sigma Chem Co. (Poole, U.K) Other chemicals were obtained from the following commercial sources, pentachlorophenol, pentachlorobenzene,

* Part of this work was reported at the International Symposium on Hexachlorobenzene, Lyon, France, June 1985

2,3,5,6-tetrachloro-1,4-benzoquinone, 2,3,5,6-tetrachlorophenol, β-naphthoflavone, 3-chloro-5-methoxyphenol, 1-butaneboronic acid and *N*-acetylcysteine from Aldrich Chem. Co. (Gillingham, Dorset, U.K.), SKF 525A (β-diethylaminoethyl-diphenylpropyl acetate), Smith, Kline & French Ltd. (Welwyn Garden City, Herts., U K); Aroclor 1254, Monsanto Co (St Louis, MI); isosafrole (*cis* + *trans*), Fluorochem. Ltd. (Glossop, Derbyshire, U.K.). All solvents were Distol pesticide grade, Fisons Co. Ld. (Loughborough, Leics., U.K.). Multi-purpose cocktail and BTS-450 solubilizer were from Beckman-R11C Ltd (High Wycombe, Bucks , U.K) [U-^{14}C]HCB (106 mCi/mmole) was purchased from Amersham International PLC (Amersham, U K.). The radiochemical purity by t.l.c. was 98% as assessed by the manufacturer. No impurities were detected by electron capture g.l.c. TCDD was a gift from Dr. J. B. Greig and 9-hydroxellipticine from Professor C Paoletti

Pentachlorophenol and tetrachloro-1,4-benzenediol were repeatedly crystallised from methanol before use. 3,4,5,6-Tetrachloro-1,2-benzenediol was prepared from tetrachloro-1,2-benzoquinone by reduction with sodium dithionite in methanol It was recrystallised until pure by g.l.c and mass spectrometry (M^+ *m/z* 246). 2,4,5,6-Tetrachloro-1,3-benzenediol was prepared from 3-chloro-5-methoxyphenol [24] by Mr I. Bird (M^+ *m/z* 246) Pentachlorothiophenol was prepared as described previously [21] Electron impact mass spectra of all compounds and appropriate methyl and ethyl ethers were obtained using a VG 70/70 mass spectrometer at 70 eV Of the three tetrachlorobenzenediols only tetrachloro-1,2-benzenediol formed a cyclic 1-butaneboronate [25] as expected for a *vic*-diol [26], i.e $C_{10}BCl_4O_2$ M^+ *m/z* 312.

Animals and pretreatments Male and female Fischer 344/N rats (about 160 and 250 *g* respectively) were fed MRC 41B diet and received one of the following treatments before being killed: no treatment i.e. control; PB in the drinking water (0 1% w/v) for 7 days; 3-MC (5 mg/ml in arachis oil) as three daily i p. injections of 20 mg/kg; Aroclor 1254 (50 mg/ml in arachis oil) 200 mg/kg once 5 days previously, TCDD dissolved in corn oil (6 μg/ml) 25 μg/kg 3 days earlier, diethylstilboestrol dipropionate (5.5 mg/ml in arachis oil) three daily i.p. injections of 20 mg/kg from 5 days previously, isosafrole dissolved in arachis oil (37 5 mg/ml) as four daily i.p injections of 150 mg/kg; β-naphthoflavone (10 5 mg/ml in arachis oil) three daily i.p. injections of 100 mg/kg from 5 days previously HCB in arachis oil (25 mg/ml) as a single i.p injection 100 mg/kg 5 days prior to death Animals were starved overnight before being killed by decapitation

Preparation of microsomes. Microsomes were prepared essentially as described by Ernster *et al.* [27]. Microsomal fractions were stored under a glycerol–0 1 M-Na_2HPO_4/NaH_2PO_4 buffer (1 · 4 v/v), pH 7 4, at −70° Protein was determined with bovine serum albumin as a standard [28]. Cytochrome(s) P-450 contents were determined by the method of Omura and Sato [29]

Incubations. Incubations consisted of 3 4 ml of 0.1M-Na_2HPO_4/NaH_2PO_4 buffer (pH 7 4), 2–4 mg of microsomal protein and 210 nmol of HCB, pentachlorophenol or pentachlorbenzene added in 30 μl of acetone Where appropriate incubations contained 1 ml of cytosol (7–10 mg protein) Metyrapone, SKF 525A or 9-hydroxyellipticine, in acetonitrile (34 μl), water (34 μl) and dimethyl formamide (1–10 μl) respectively, were added immediately before the addition of the substrate Incubations under N_2 or CO were performed in sealed flasks equilibrated with the appropriate gas mixture. After a 4-min preincubation period at 37° incubations were started by the addition of the NADPH re-generating system (1 5 μmol of NADPH, 10 μmol of glucose-6-phosphate, 0 25 units of glucose 6-phosphate dehydrogenase and 30 μmol of $MgCl_2$ in a volume of 0 2 ml) Reactions were terminated by vortexing 1 ml of incubation mixture with ethyl acetate (2 × 2 ml). Incubations containing cytosol were hydrolysed under N_2 at 70° in 0 5 M NaOH for 1 hr to cleave glutathione-derived conjugates, before extraction with ethyl acetate [30]

Extracts were dried over anhydr Na_2SO_4 and derivatised with diazomethane or diazoethane in diethyl ether Analyses were performed by g l c using a Varian Vista 6000 instrument fitted with a ^{63}Ni electron capture detector at 250°. HCB, pentachlorobenzene and the methyl or ethyl ethers of phenols and thiophenols were separated on a column (2 m × 4 mm i.d.) of 5% OV-210 coated on Gas Chrom Q (100–120 mesh) with a linear temperature program of 130–190° (6°/min) and a gas flow of 30 ml/min of N_2. Recoveries of HCB, pentachlorophenol, tetrachloro-1,4-benzenediol and pentachlorothiophenol from "spiked" samples were approximately 95%. Retention times of reference compounds relative to HCB were pentachlorbenzene, 0 50; the methyl ether of 2,3,5,6-tetrachlorophenol, 0.63; methyl ethers of pentachlorophenol 1.23, tetrachloro-1,2-benzenediol 1 25, tetrachloro-1,3-benzenediol 1 40; tetrachloro-1,4-benzenediol 1.45 and pentachlorothiophenol 2 09, and the ethyl ethers 1 45, 1 68, 1.86, 1 91 and 2 34 respectively Identifications of metabolites were confirmed by full mass spectra or by selective ion monitoring of molecule ions during g l c.-m s (50 m × 0.2 mm fused-silica open-tubular capillary columns of SE-52) by Mr J Lamb and Dr P B Farmer

Incubations with [U-^{14}C]*HCB* Radioactivity studies were carried out as described under microsomal incubations with 3 μCi (60 nmol) of [^{14}C]HCB and where indicated in the presence of 1 mM GSH, *N*-acetylcysteine or metyrapone Blanks contained no NADPH-regenerating system. Incubations were stopped by mixing with 3 ml ethyl acetate and centrifuged at 1800 *g* for 10 min. The extraction was repeated three times. Ethanol (3 ml) was then added to the aqueous phase and the precipitating protein pelleted by centrifugation at 1800 *g* for 15 min A portion of the supernatant was taken for determination of radioactivity content The resultant pellet was washed twice with 3 ml ethanol/hexane (25 10 v/v), and with methanol (3 ml) No radioactivity was detected in the final ethanol/hexane or methanol washes The protein was dissolved in 1 ml of BTS-450 solubilizer overnight Multi-purpose

cocktail acidified with 1% acetic acid was added and radioactivity estimated with a Searle Analytic Mark III liquid-scintillation system.

RESULTS

Conditions of metabolism

The metabolism of HCB by liver microsomes from PB-induced rats in the presence of an NADPH-regenerating system produced pentachlorophenol, pentachlorobenzene, tetrachloro-1,4-benzenediol and tetrachloro-1,2-benzenediol (88 1:9·2 respectively). Products were identified by comparison with standards. G.l.c. chromatographs of an incubation mixture with PB-induced microsomes are shown in Fig. 1 The identity of the pentachlorophenol formed was confirmed by a full mass spectrum during g.l.c.-m s. Levels of the other metabolites were too low for full spectra to be obtained but identifications were confirmed by electron impact selective ion monitoring for molecular ions of the ethyl ethers, i e. pentachlorophenol *m/z* 292, tetrachloro-1,2-benzenediol *m/z* 302, tetrachloro-1,4-benzenediol *m/z* 302 No tetrachloro-1,3-benzenediol was detected. Even under optimum conditions the metabolites represented conversion of only 1.4% of the substrate after 30 min. Because of this small conversion we did not attempt to equate substrate loss with product formed. The accumulation of pentachlorophenol was linear up to 30 min, to a substrate concentration of 60 μM and optimum at a protein concentration of 1.2 mg/ml. Table 1 shows that the metabolism of HCB to pentachlorophenol was dependent on the presence of NADPH and O_2 and inhibited by CO, SKF 525A and metyrapone. NADH was less effective than NADPH and produced no synergistic effect. Interestingly, CO did not inhibit phenol formation if added after HCB. Under similar conditions (no inhibitors present) pentachlorobenzene was converted to pentachlorophenol at a much greater rate (528 ± 56 pmol/min per mg protein) than HCB with 2,3,5,6-tetrachlorophenol and tetrachloro-1,4-benzenediol as minor products

Influence of inducers of cytochrome P-450

Various inducers of cytochrome P-450, including HCB, were compared for their effects on the metabolism of HCB. PB was by far the most effective inducer of the conversion of HCB to pentachlorophenol with rates 8.7- and 13 7-fold greater than controls in males and females respectively (Fig. 2) Aroclor 1254 and HCB (both "mixed" inducers) and isosafrole (inducer of cytochrome P-450$_d$) were less effective, inducing metabolism by 4.2- and 3.2- and 3.0-fold respectively. In contrast, 3-MC had no effect whilst TCDD, the most potent inducer of cytochrome P-450$_c$ [13], produced a 30% reduction in the yield of pentachlorophenol with male micro-

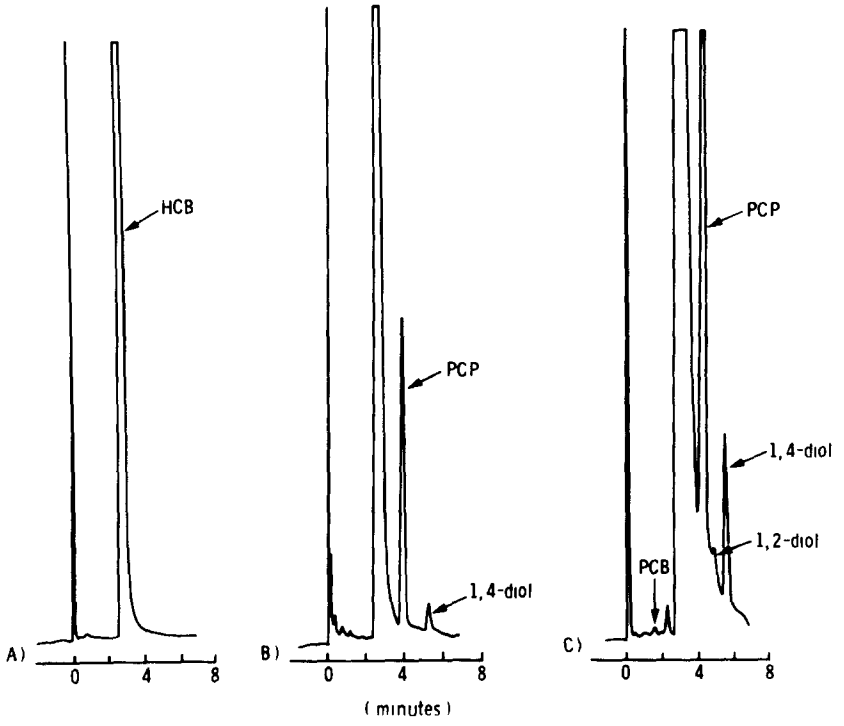


Fig 1 G l c traces of the metabolism of HCB (210 nmol/incubation) by hepatic microsomes from male rats as described in Table 1 Phenols were extracted and derivatised to the methyl or ethyl ethers for analysis and detected by electron capture as described in the Materials and Methods (A) Incubation in the absence of NADPH. (B) Methyl ethers in the presence of NADPH (C) Ethyl ethers (×8 amplification) in the presence of NADPH HCB, hexachlorobenzene, PCP, pentachlorophenol, PCB, pentachlorobenzene, 1,4-diol, tetrachloro-1,4-benzenediol, 1,2-diol, tetrachloro-1,2-benzenediol

Table 1 Dependency of the conversion of HCB to pentachlorophenol on cytochrome P-450

Conditions	Pentachlorophenol (pmol/min per mg protein)	% of control
Basic system	21 3	100
Under N_2	0 9	4
Under CO O_2 (8 2)	1 6	7 5
NADPH omitted	ND	ND
Plus NADH (1 5 μmol)	22 0	103
NADH alone	2 0	9
1 mM-SKF 525A	5 0	23
1 mM-Metyrapone	4 1	19

HCB was incubated with hepatic microsomes from male rats induced with PB as described in the Materials and Methods Incubations for 30 min were modified as described below from a basic system consisting of 3 4 ml of 0 1 M-Na_2HPO_4/NaH_2PO_4 buffer (pH 7 4), 1 5 μmol NADPH, 10 μmol glucose-6-phosphate, glucose 6-phosphate dehydrogenase, 30 μmol $MgCl_2$, 3–4 mg microsomal protein and 210 nmol of HCB (added in 30 μl acetone) Pentachlorophenol formed was estimated by g l c of the methyl ether Limit of detection 0 5 pmol/min per mg protein ND = not detected Values are means of triplicates

somes. Diethylstilboestrol, which *in vivo* stimulates HCB metabolism [21], did not influence microsomal metabolism HCB was less efficiently metabolised by microsomes from female rats pretreated with either PB or HCB than by those from equivalent males

When the influence of cytochrome P-450 inducers on the hydroxylation of pentachlorophenol to tetrachloro-1,4-benzenediol was explored with male microsomes, a different pattern of induction was observed to that seen for HCB hydroxylation (Fig 3). 3-MC was by far the most potent inducer of pentachlorophenol metabolism (9 5-fold), whereas PB was relatively ineffective (2-fold) Isosafrole and HCB were intermediate inducers of this hydroxylation With PB-induced microsomes the rate of conversion of pentachlorophenol to tetrachloro-1,4-benzenediol was twenty seven times faster than that of HCB to the phenol and similar to that for the hydroxylation of pentachlorobenzene Tetrachloro-1,2-benzenediol was also formed from penta-

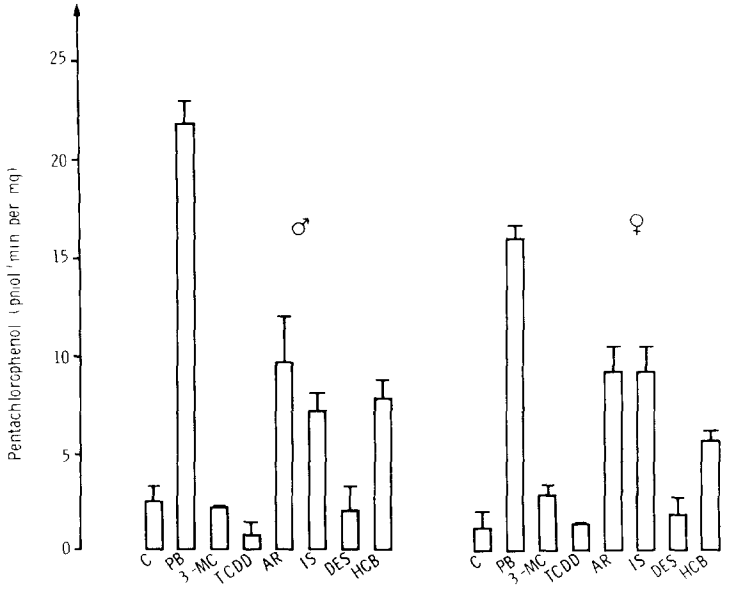


Fig 2 Influence of inducers of cytochrome P-450 on the metabolism of HCB to pentachlorophenol by male and female rat hepatic microsomes HCB (210 nmol) was incubated with microsomes and a NADPH-regenerating system as described in Table 1 Incubations were extracted, and pentachlorophenol as the methyl ether estimated by g l c as described in the Materials and Methods C, control, PB, phenobarbital, 3-MC, 3-methylcholanthrene, TCDD, 2,3,7,8-tetrachlordibenzo-*p*-dioxin, AR, Aroclor 1254, IS, isosafrole, DES, diethystilboestrol, HCB, hexachlorobenzene Results are means ±S E from 4 animals Endogenous HCB in HCB-induced microsomes was <5% of that added as substrate

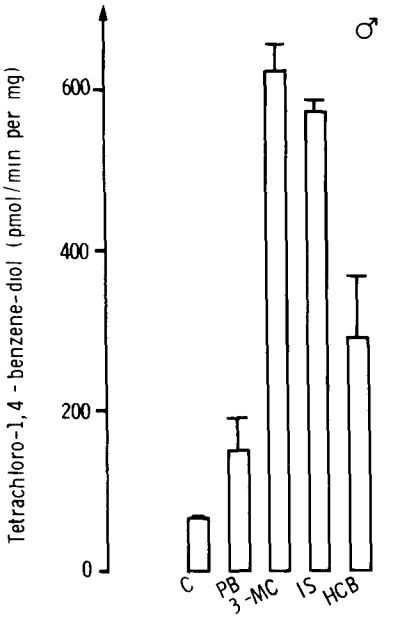
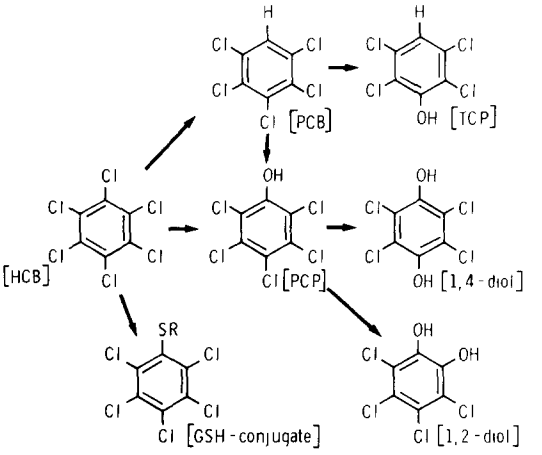


Fig 3. Influence of inducers of cytochrome P-450 on the conversion of pentachlorophenol to tetrachloro-1,4-benzenediol by male rat liver microsomes Incubations were as in Table 1, with 210 nmol of substrate The diol was estimated as the methyl ether by g.l c as described in the Materials and Methods Results are means ±S E of four animals Abbreviations as in Fig 2



Scheme 1 Scheme for the metabolism of hexachlorobenzene in PB-induced rat liver microsomes TCP, 2,3,5,6-tetrachlorophenol, other abbreviations are as in Fig 1.

chlorophenol but pentachlorobenzene, 2,3,5,6-tetrachlorophenol and tetrachloro-1,3-benzenediol were not detected.

Inhibition by 9-hydroxyellipticine

The influence of 9-hydroxyellipticine, a relatively specific inhibitor of cytochrome P-450$_c$ [31, 32], on the hydroxylations of HCB and pentachlorophenol were compared (Fig. 4). 9-Hydroxyellipticine markedly inhibited the hydroxylation of pentachlorophenol to tetrachlorobenzene-1,4-diol by β-naphthoflavone-induced microsomes (15% of control at 1 μM inhibitor concentration). In contrast, the hydroxylations of both HCB and pentachlorophenol by PB-induced microsomes were much less susceptible to inhibition (70 and 90% of control respectively at 1 μM). With HCB-induced microsomes the hydroxylation of pentachlorophenol was inhibited to a similar extent as with β-naphthoflavone-induced microsomes (20% of control at 1 μM).

Incubations in the presence of glutathione

The addition of cytosol or 1 mM-GSH to incubations of HCB with male PB-induced microsomes resulted in the formation of a metabolite which after

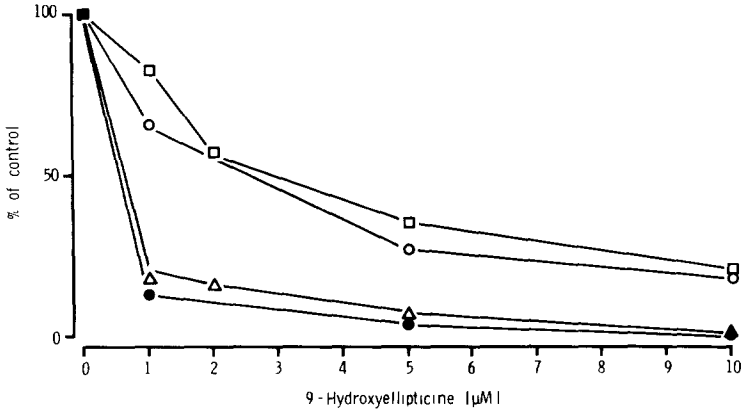


Fig 4 Inhibition of HCB and pentachlorophenol metabolism by 9-hydroxyellipticine Substrates (210 nmol) were incubated with male phenobarbital, β-naphthoflavone or HCB induced microsomes as appropriate (about 1 nmol of cytochrome P-450/ml of incubation) as described in Table 1 after a 2 min preincubation with 9-hydroxyellipticine Control values for the formation of pentachlorophenol from HCB by PB-induced microsomes (○) was 10 5 pmol/min per mg protein; tetrachloro-1,4-benzenediol from pentachlorophenol with PB-induced microsomes (□) 263 pmol/min per mg protein, tetrachloro-1,4-benzenediol from pentachlorophenol with β-naphthoflavone-induced microsomes (●) 800 pmol/min per mg protein, tetrachloro-1,4-benzenediol from pentachlorophenol with HCB-induced microsomes (△) 500 pmol/min per mg protein Values are means of 4 separate incubations

Table 2 Effects of cytosol and GSH on the metabolism of hexachlorobenzene by male phenobarbital-induced microsomes

Conditions	Pentachlorophenol (pmol/min per mg)	Tetrachlorobenzenediol (pmol/min per mg)	Pentachlorothiophenol (pmol/min per mg)
Microsomes + GSH	ND*	ND	2 5 ± 0 2
Microsomes + NADPH	18 8 ± 0 6	2 4 ± 0 1	ND
Microsomes + GSH + NADPH	27 4 ± 4 0	ND	1 9 ± 0 6
Microsomes + cytosol + NADPH	21 9 ± 4 0	ND	3 1 ± 1 5
Microsomes + cytosol + GSH	ND	ND	4 8 ± 0 3
Microsomes + cytosol + GSH + NADPH	26 0 ± 5 8	ND	3 8 ± 0 8
Cytosol + GSH + NADPH	ND	ND	ND

Incubations, hydrolyses and analyses of the methyl ethers by g l c were as described in the Materials and Methods Microsomal protein 1 2 mg/ml, cytosolic protein 3 mg/ml, GSH 1 mM Values are means ± S E from 4 rats
* ND – not detected

alkaline hydrolysis co-chromatographed during g.l c with pentachlorothiophenol (Table 2) This thiol is formed during hydrolysis of pentachlorophenyl cysteine (A G Smith, unpublished data) and might be expected to be formed from the glutathione conjugate. NADPH and cytosolic GSH-transferases were not required The yield of pentachlorophenol from HCB was not inhibited by the formation of the thiol However, under these conditions no tetrachlorobenzenediols could be detected. With pentachlorophenol as substrate GSH addition produced a reduction in the yield of tetrachloro-1,4-benzenediol from 137 ± 6 pmol/min per mg protein to 3 ± 1 pmol/min per mg protein Pentachlorophenol was not converted to the thiol In other experiments (data not shown) tetrachloro-1,4-benzenediol was stable in the presence of phosphate buffer and GSH, whereas an oxidation product, tetrachloro-1,4-benzoquinone, reacted chemically to an unidentified product. If the diol was formed by microsomes from pentachlorophenol it also rapidly disappeared to undetected products when GSH was added There was no large sex difference in the production of pentachlorothiophenol from HCB- or PB-induced microsomes but where HCB-induced microsomes were used the yield of pentachlorothiophenol was greater than that after induction by PB i e pentachlorophenol, males 7 8 ± 0.9, females 5.6 ± 0 5 pmol/min per mg, pentachlorothiophenol males 6 8 ± 0.5; females 7 8 ± 0.9 pmol/min per mg of protein (cf Table 2).

Covalent binding of [^{14}C]hexachlorobenzene to microsomal protein

When [^{14}C]HCB was incubated with microsomes from PB-induced male rats in the presence of NADPH and O_2, radioactivity was bound to microsomal protein (Table 3) representing 0 64% of the substrate added Binding was reduced by 90% under anaerobic conditions Incorporation of radioactivity into microsomes obtained from female rats was 87% (aerobic) and 47% (anaerobic) of that obtained with microsomes from male rats Binding was inhibited by GSH and *N*-acetylcysteine with no impairment of pentachlorophenol accumulation as judged by g.l.c Metyrapone inhibited both binding to microsomal protein and the formation of pentachlorophenol. Radioactivity in the aqueous phase was increased 3- to 4-fold in the presence of GSH and *N*-acetylcysteine but diminished by metyrapone Covalent binding of radioactivity to a number of different

Table 3 Covalent binding of [^{14}C]hexachlorobenzene to microsomal protein

Conditions	Sex	Bound radioactivity (pmol/incubation)	Aqueous radioactivity (pmol/incubation)
Aerobic	male	384 ± 60	126 ± 18
Anaerobic	male	12 ± 6	ND*
Aerobic	female	320 ± 59	88 ± 22
Anaerobic	female	6 ± 2	ND
Aerobic 1 mM-GSH	male	107 ± 21	533 ± 34
Aerobic 1 mM-*N*-acetyl-cysteine	male	102 ± 20	505 ± 20
Aerobic 1 mM-metyrapone	male	18 ± 2	16 ± 6

[^{14}C]HCB was incubated with hepatic microsomes from male and female rats induced with PB as described in the Materials and Methods Incubations for 30 min were modified as described above from a basic system consisting of 3 4 ml 0 1 M-Na_2HPO_4/NaH_2PO_4 buffer (pH 7 4) 1 5 μmol NADPH, 10 μmol glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 30 μmol $MgCl_2$, 3–4 mg microsomal protein and 60 nmol (3 μCi) of [^{14}C]HCB (added in 40 μl of acetone) Bound radioactivity was determined as described in the Materials and Methods and expressed as pmol of HCB equivalents bound or in the aqueous phase Blanks (<20 pmol/incubation), which consisted of the basic system without the NADPH regenerating system, were subtracted from the values obtained Values are means ± S E from 3 rats per group
* ND – not detected

microsomal proteins was demonstrated by SDS-PAGE-autoradiography, according to the method of Shimada *et al.* [33]. The major radio-labelled protein bands were at M_r 47, 51, 55 and 68.

DISCUSSION

The results demonstrate that HCB is metabolised to pentachlorophenol by the hepatic cytochrome P-450 mediated system with minor reductive dechlorination to pentachlorobenzene (Scheme 1). Pentachlorophenol is subsequently converted to tetrachloro-1,2- or 1,4-benzenediols but not to the 1,3-diol. Whether the initial or second hydroxylations occur by direct insertion or via an arene oxide remains to be investigated. However, it may be pertinent that tetrachloro-1,4-benzenediol can be formed from pentachlorophenol in the free radical generating xanthine–xanthine oxidase system (F. P. Stewart and A. G. Smith, unpublished data).

Studies on the influence of cytochrome P-450 inducers illustrate that the hydroxylation of HCB is preferentially induced by PB whereas the subsequent conversion of pentachlorophenol to tetrachloro-1,4-benzenediol is favoured by cytochrome P-450 inducers of the 3-MC type. The findings of van Ommen [23] and Alhborg and Thunburg [34] on the separate hydroxylation steps are consistent with this view. (In assessing the formation of pentachlorophenol from HCB we have ignored any contribution due to further metabolism since in the presence of the large excess of HCB secondary hydroxylation is probably greatly inhibited.) These two similar dechlorination-hydroxylation steps would therefore appear to be catalysed most efficiently by different cytochrome P-450 isozymes. This conclusion is confirmed by the preferential inhibition by 9-hydroxyellipticine. The induction of both HCB and pentachlorophenol hydroxylations by HCB itself is compatible with the "mixed" monooxygenase-inducing properties of this chemical [14–18], and comparable to that of Aroclor 1254 (a mixture of polychlorinated biphenyls). Although the hydroxylation of pentachlorophenol is inducible by PB, the inhibition shown by 9-hydroxyellipticine implicates the involvement of cytochrome $P\text{-}450_c$ in this transformation by HCB-induced microsomes. It is difficult to reconcile the 3-MC type of induction produced by HCB with the apparent strict structural requirement for induction by other polyhalogenated aromatics [35] but this cannot be attributed to contamination by TCDD or related compounds [17]. Isosafrole, an inducer of cytochrome $P\text{-}450_d$ [36], produced a pattern of induction for both hydroxylations similar to that produced by HCB. The role of cytochrome $P\text{-}450_d$ in HCB metabolism cannot therefore be excluded However, the ability of isosafrole to induce more than one cytochrome P-450 isozyme may also be of significance [36]

In addition to being hydroxylated, HCB is metabolised *in vivo* to a conjugated metabolite [37]. The excretion product, pentachlorophenyl *N*-acetylcysteine, can be hydrolysed to pentachlorothiophenol [20, 38]. During the analysis of the GSH-fortified microsomal incubations in the present work, pentachlorothiophenol was probably generated by hydrolysis of the GSH conjugate. The lack of any pentachlorothiophenol detected after incubations with cytosol and GSH alone may be the result of poor presentation of HCB to cytosolic GSH transferase but probably indicates that HCB is predominantly metabolised by GSH transferases present in the microsomes. It is unlikely that GSH reacts with a reactive metabolite prior to pentachlorophenol formation since phenol levels remained unchanged. In contrast, GSH prevented detection of tetrachloro-1,4-benzenediol. This would appear to be due to the formation in the presence of microsomes of tetrachloro-1,4-benzoquinone or perhaps the semiquinone radical [39] which reacts non-enzymically with GSH to form a water soluble product. The identity of this product is currently being investigated. The binding of [^{14}C]HCB to microsomal protein and the protection afforded by GSH and *N*-acetylcysteine may be related phenomena and similar to that described for benzene and phenol metabolism [40, 41].

An unequivocable sex difference in the induction of porphyria and hepatic tumours by HCB in rats is well established [9–12] In the present work no significant sex difference in favour of females for hydroxylation, conjugation or covalent binding to microsomal protein was observed. Hence we are unable to ascribe the much greater susceptibility of female rats to major differences in metabolism Any highly toxic metabolite still to be detected must of course be compatible with similar hepatic toxicities produced by other polyhalogenated aromatics [35]. Alternatively, the toxic properties of HCB may be due to the induction of other processes perhaps associated with the *Ah* locus [35] and of a free radical nature. Further studies are in progress to test some of these possibilities.

Acknowledgements—We thank Dr P B Farmer, Mr J Lamb, Mr I. Bird and Dr J B Greig for assistance, Dr C Paoletti for the gift of 9-hydroxyellipticine, and Drs T A Connors, F De Matteis and J Roberts for discussions Finally we thank the MRC for a research studentship (FPS)

REFERENCES

1 K D Courtney, *Environ Res* **20**, 225 (1979)
2 G H Elder, in *Heme and Hemoproteins, Handbook of Exp Pharmacol*, Vol 44, (Eds F De Matteis and W N. Aldridge) p 157 Springer, Berlin (1978)
3. A G Smith and F De Matteis, *Clin Haematol* **9**, No 2, 399 (1980)
4 G Koss, S Seubert, A Seubert, J Seidel, W Koransky and H Ippen, *Archs Toxic* **52**, 13 (1983)
5 D J Cripps, H A Peters, A Gocmen and I Dogramaci, *Br J Dermatol*, **111**, 413 (1984)
6. J R P Cabral, P. Shubik, T Mollner and F Raitano, *Nature, Lond* **269**, 510 (1977)
7 J R P Cabral, T Mollner, F Raitano and P Shubik, *Int J Cancer* **23**, 47 (1979)
8 A G Smith and J R. P Cabral, *Cancer Letts* **11**, 169 (1980)
9 L C San Martin de Viale, A A Viale, S Nacht and M Grinstein, *Clin chim Acta* **28**, 13 (1970)
10 D L Grant, F Iverson, G V Hatina and D C Villeneuve, *Environ Physiol Biochem* **4**, 159 (1974)
11 R W. Lambrecht, E Erturk, E. E Grunden, H A Peters, C R Morris and G T Bryan, *Fedn Proc* **42**, 786 (1983)

12 A G Smith, J E Francis, D Dinsdale, M M Manson and J R P Cabral, *Carcinogenesis* **6**, 631 (1985)
13 J A Goldstein, *Trends Pharmac Sci* **5**, 290 (1984)
14 M D Stonard, *Biochem Pharmac* **24**, 1959 (1975)
15 M D Stonard and J B Greig, *Chem -Biol Interact* **15**, 365 (1976)
16 W Vizethum and G Goerz, *Chem -Biol Interact* **28**, 291 (1979)
17 J A Goldstein, P Linko, J N Huckins and D L Stalling, *Chem -Biol Interact* **41**, 131 (1982)
18 R B Franklin, R K Breger and J J Lech, *J Toxicol Environ Health* **12**, 223 (1983)
19 D V Parke and R T Williams, *Biochem J* **74**, 5 (1960)
20 G Koss, W Koransky and K Steinbach, *Archs Toxic* **35**, 107 (1976)
21 M Rizzardini and A G Smith, *Biochem Pharmac* **31**, 3543 (1982)
22 H M Mehendale, M Fields and H B Matthews, *J Agr Food Chem* **23**, 261 (1975)
23 B van Ommen, P J van Bladeren, J H M Temmink and F Muller, *Biochem biophys Res Comm* **126**, 25 (1985)
24 A S Wong and D G Crosby, *J Agr Food Chem* **29**, 125 (1981)
25 A G Smith and C J W Brooks, *Biomed Mass Spectrom* **4**, 258 (1977)
26 C J W Brooks, W J Cole, J H Borthwick and G M Brown, *J Chromatogr* **239**, 191 (1982)
27 L Ernster, P Siekevitz and G E Palade, *J Cell Biol* **15**, 541 (1962)
28 G L Peterson, *Analyt Biochem* **83**, 346 (1977)
29 T Omura and R Sato, *J biol Chem* **239**, 2379 (1964)
30 A G Smith and J E Francis, *Biochem Pharmac* **32**, 3797 (1983)
31 P Lesca, E Rafidinarivo, P Lecointe and D Mansuy, *Chem -Biol Interact* **24**, 189 (1979)
32 C Ioannides, P Y Lum and D V Parke, *Xenobiotica* **14**, 119 (1984)
33 T Shimada, Y Imai and R Sato, *Chem -Biol Interact* **38**, 29 (1981)
34 U G Ahlborg and T Thunberg, *Archs Toxic* **40**, 55 (1978)
35 A Poland and J C Knutson *Ann Rev Pharmac Toxic* **22**, 517 (1982)
36 D E Ryan, P E Thomas and W Levin, *J biol Chem* **255**, 7941 (1980)
37 G Renner, *Xenobiotica* **11**, 435 (1980)
38 G Renner, E Richter and K P Schuster, *Chemosphere* **7**, 663 (1978)
39 I Witte, U Juhl and W Butte, *Mutat Res* **145**, 71 (1985)
40 A Tunek, K L Platt, P Bentley and F Oesch, *Molec Pharmac* **14**, 920 (1979)
41 T Sawahata and R A Neal, *Molec Pharmac* **23**, 453 (1983)