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Metabolism and covalent binding of hexachlorobenzene by isolated male and female rat hepatocytes

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Hexachlorobenzene (HCB) is highly persistent in humans, animals and the environment [1, 2] and a substantial episode of human poisoning has occurred in Turkey [3] with toxic effects still being apparent many years later [4]. In rats HCB causes uroporphyria [5, 6], and is also a hepatocarcinogen [7]. Both of these syndromes occur far more readily in female rats than in males perhaps suggesting that they are linked in some way [7]. Metabolites of HCB *in vivo* are mainly either hydroxylated derivatives or arise by conjugation with glutathione [8–10]. Although *in vivo* studies have shown distinct differences between males and females in levels of metabolites in urine, these result from variations in routes of excretion and have obscured conclusions as to the role of metabolism in the hepatotoxicity of HCB [10, 11]. Incubations of HCB with microsomal fractions have produced pentachlorobenzene, pentachlorophenol and tetrachlorobenzenediols [12, 13] and provided evidence for conjugation with glutathione (GSH) [13]. In addition, radioactivity from [^{14}C]HCB was covalently bound to microsomal protein [12–14]. Neither levels of metabolities nor covalent binding to microsomal protein correlated with the marked sex-dependent hepatotoxic actions of HCB [12, 13].

Isolated hepatocyte suspensions can be used to study the metabolism of a chemical as a system which resembles more closely hepatic metabolism *in vivo* than incubations with microsomes, but without the added complications of excretion. In the present work we compared the metabolism of HCB by hepatocytes from male and female rats and investigated in more detail the degree and nature of covalent binding with respect to the marked sex-dependent porphyrogenic and carcinogenic actions of this chemical.

Materials and methods

Chemicals. HCB (Organic Analytical Standard grade with no detectable pentachlorobenzene) and phenobarbitone sodium were purchased from BDH Chemicals (Poole, U.K.). 2,3,5,6-Tetrachloro-1,4-benzenediol, 3,4,5,6-tetrachloro-1,2-benzoquinone, GSH, NADPH, glucose-6-phosphate, β-glucuronidase (*E. coli* type VII) and glucose-6-phosphate dehydrogenase, were purchased from Sigma Chemical Co (Poole, Dorset, U.K.). Pentachlorobenzene, pentachlorophenol, 2,3,5,6-tetrachloro-1,4-benzoquinone and *N*-acetyl-L-cysteine were from Aldrich Chemical Co (Gillingham, Dorset, U.K.). [^{14}C]HCB (106 mCi/mmole) was purchased from Amersham International PLC (Amersham, U.K.).

Animals, pretreatments and isolation of primary hepatocytes. Male and female F344/N rats (about 160 and 250 g respectively) were fed MRC 41B diet and given phenobarbitone sodium in the drinking water (0.1%) for 7 days. Animals were starved overnight before being anaesthetised with an i.p. injection of pentobarbitone sodium (May & Baker Ltd, Dagenham, U.K.), ie 120 mg/kg body wt. Isolated hepatocytes were prepared by collagenase perfusion [15].

Incubations. Hepatocytes were incubated in 100 ml round-bottom flasks by rotation under O_2/CO_2 (95 : 5 v/v) in a modified Williams medium E, pH 7.4 (15–25 ml; 3–15 $\times$ 10^6 cells/flask) in the presence of glutamine (200 mM), gentamycin (50 μg/ml), 0.19% $NaHCO_3$ (w/v), heparin (12.5 U/ml) and 10% heat-inactivated foetal calf serum (v/v) at 37° [16]. Incubations contained 10 nmol/ml of HCB, pentachlorobenzene, pentachlorophenol or 0.25 μCi/ml of [^{14}C]HCB. Controls consisted of either cell suspensions previously heated to 100° for 2 min or suspensions maintained at 0–4°.

Analyses. Incubations were extracted and metabolites analysed after methylation by electron capture g.l.c. [13]. The mono- and dithiols were determined after hydrolysis of incubations in 0.5 M NaOH (5 vol. excess) under N_2 for 10 min at 70° [13]. Experiments to determine whether glucuronides had been formed were conducted by extracting cell suspensions with ethyl acetate until no metabolites were detected and then re-extracting after treatment with β-glucuronidase (55 Fishman units overnight at 37° [17]). Mass spectra were obtained in the chemical ionization mode (isobutane) using a VG Micromass 70/70 double focussing mass spectrometer by Mr. J. H. Lamb.

Estimation of covalent binding from [^{14}C]HCB. Cell suspensions were extacted with ethyl acetate followed by precipitation of the protein from the aqueous phase with ethanol. The protein, after centrifugation, was washed twice with ethanol/hexane and with methanol [13]. High molecular weight DNA was isolated [18] from cell suspensions which had been first frozen at −40°C. Radioactivity contents were estimated as described previously [13]. For all covalent-binding experiments the formation of pentachlorophenol was demonstrated whereas none was detected in the appropriate controls.

Results and discussion

The metabolism of HCB was examined with hepatocytes from rats treated with phenobarbitone since both the formation of pentachlorophenol and subsequent conversion to diols are induced by this drug albeit to differing extents [13]. There is no evidence that the sex difference between rats in the development of porphyria is eliminated by pretreatment with phenobarbitone; indeed, coadministration of this drug with HCB increases the sensitivity of females [19] (HCB itself is a substantial phenobarbitone-like inducer of cytochrome P-450 in rats [13]). A 2.5 hr incubation of hepatocytes from male rats with HCB gave only a 0.8% conversion to detectable products. Subsequent studies were conducted for 20 hr with approximately 4% of HCB being metabolised. Cytochrome P-450 levels in the hepatocytes were 1.34 ± 0.03 nmol/10^6 cells in males and 1.04 ± 0.06 nmol/10^6 cells in females (N = 3). Cell viability in the absence or presence of HCB fell from about 85% to 50% after 20 hr. Extraction of cells with ethyl acetate gave only pentachlorophenol.

Tetrachloro-1,4-benzenediol and pentachlorobenzene were not detected. Alkaline hydrolysis of incubations gave the additional products pentachlorothiophenol and tetrachloro-1,4-benzenedithiol probably formed from conjugates derived from glutathione [9] as observed *in vivo* for HCB [20] and pentachloronitrobenzene [11]. There was no significant sex difference in the amounts of total products formed although hepatocytes from males produced significantly greater quantities of thiols than hepatocytes from females (Fig. 1). The accumulation of pentachlorophenol, and interestingly the sulphur-containing metabolites, were inhibited by the presence in the incubation system of the cytochrome P-450 inhibitor SKF 525A (250 μM). Sulphates and glucuronides did not seem to be formed since incubation with β-glucuronidase did not release chlorophenols [17].

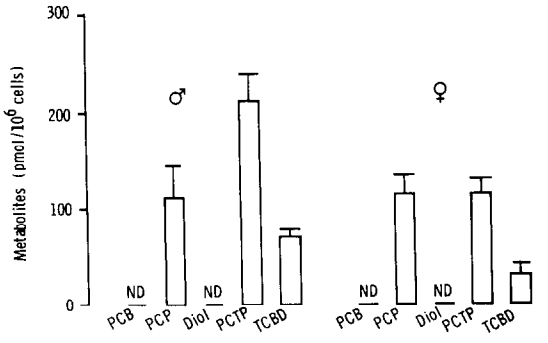


Fig. 1. Profile of metabolites formed by isolated hepatocytes from phenobarbitone-treated male and female rats. Incubations were performed in the presence of HCB (10 nmol/ml) with 15–18 × 10^6 cells in 15 ml of modified Williams medium E for 20 hr at 37°. HCB was added in dimethylsulphoxide (2 μl/ml incubation). Metabolites were extracted (sulphur conjugates being hydrolysed prior to extraction) and analysed by g.l.c. as described in the Materials and Methods. PCB, pentachlorobenzene; PCP pentachlorophenol; Diol, tetrachloro-1,4- and 1,2-benzenediols; PCTP pentachlorothiophenol; TCBD tetrachloro-1,4-benzenedithiol. Results are means ± SEM from 4 animals. N.D. not detected.

Pentachlorobenzene was metabolised much more rapidly than HCB so that with male hepatocytes 45% had been converted in 2.5 hr with 15 × 10^6 cells. Pentachlorophenol and pentachlorothiophenol were the major products representing >80% of the total metabolites detected. Tetrachlorobenzenedithiol was not found. Similarly pentachlorophenol was converted to tetrachloro-1,4-benzenediol at a much faster rate than HCB with 6.2% metabolised in 2.5 hr. After 20 hr, however, the diol was not detected suggesting that further metabolism, oxidation or binding had occurred. No sulphur-containing products were found after incubation with pentachlorophenol. Pentachlorobenzene has recently been proposed as the initial and major isolated product of NADPH-fortified rat liver microsomal metabolism of [^{14}C]HCB especially under anaerobic conditions [21]. Pentachlorophenol was apparently only a minor product. In contrast, pentachlorophenol was the major product observed in other studies [12, 13]. Only trace amounts of pentachlorobenzene were found [12–14] even after anaerobic incubations when the accumulation of pentachlorophenol was inhibited by >90% (F. P. Stewart, unpublished data). It is difficult to reconcile these two sets of findings. In the present work pentachlorobenzene was not detected even at trace levels or after short incubation times. The much faster metabolism of pentachlorobenzene to pentachlorophenol than that of HCB might, however, preclude its detection.

Hepatocytes from males showed greater binding of radioactivity to protein from [^{14}C]HCB than hepatocytes from females and this was reflected in radioactivity in the aqueous phase remaining after precipitation of protein (Table 1). In similar experiments, maximum covalent binding to DNA at 37° was <9.9 × 10^{-5}% of the substrate added and only marginally above hepatocytes held at 4°. There was no significant sex difference and the maximum binding over controls in males was estimated as approximately 11 nmol/mol of DNA nucleotide or equivalent to 1 molecule of HCB per 91 × 10^6 nucleotides.

Previously we reported that tetrachlorobenzenediols, formed from HCB or pentachlorophenol in microsomal incubations, were not detected if GSH or cytosol were present [13]. Covalent binding of radioactivity from [^{14}C]HCB to microsomal protein was also inhibited by GSH. Tetrachloro-1,4-benzenediol did not conjugate with GSH in buffer, unless microsomes were present, whereas tetrachloro-1,4-benzoquinone reacted rapidly to an unidentified product. This suggested that in the presence of microsomes tetrachloro-1,4-benzenediol was converted to the quinone (perhaps via the semiquinone radical) and that reaction of one of these species with GSH or protein led to covalent binding. As a chemical model of this reaction tetrachloro-1,4-benzoquinone was mixed in acetonitrile with *N*-acetylcysteine (which like GSH inhibits covalent binding to microsomal protein); a shift in maximum u.v. absorbance from 288 nm to 251 nm was observed. The product was methylated with diazomethane and analysed by chemical ionization m.s. The structure was identified as *N*-acetyl-*S*-(trichloro-3,6-benzenediol)-L-cysteine (*m/z* 416 $[M + H]^+$, *m/z* 358 $[M-NHCOCH_2]^+$, *m/z* 144 $[CH_2CHCOOCH_3]^+$). Similar data were obtained after the reaction of *N*-acetylcysteine with tetrachloro-1,2-benzoquinone.

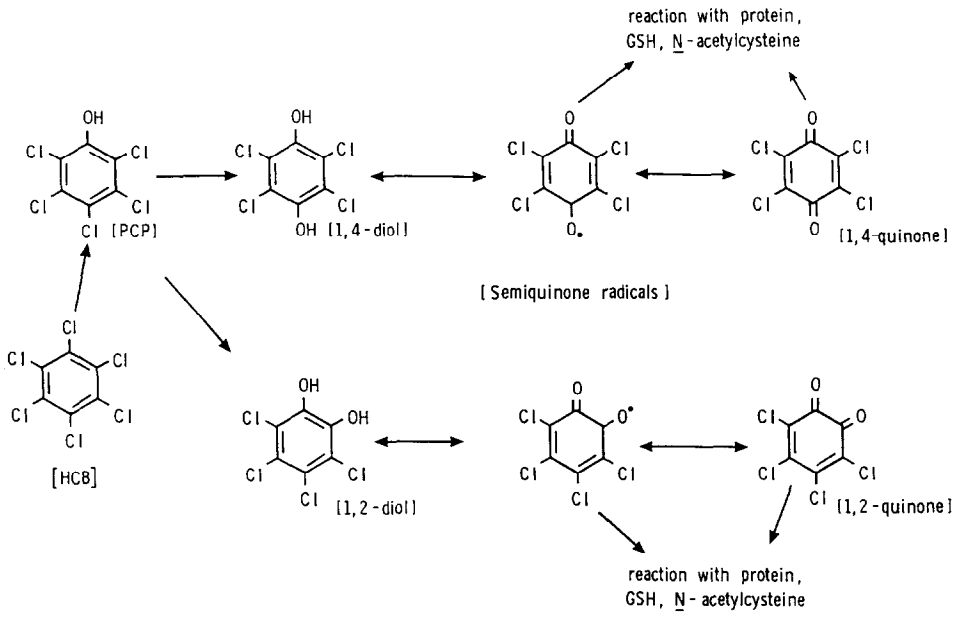
There is considerable recent evidence to suggest that most of the covalent binding of radioactivity from [^{14}C]HCB to microsomal protein occurs subsequent to the formation of pentachlorophenol and not following an initial activation of HCB [13, 14]. The production of tetrachloro-1,2- and 1,4-benzenediols appeared to be necessary for this binding. Since tetrachlorobenzenediols only slowly react with thiols in buffer (F. P. Stewart, unpublished data) but tetrachloro-1,2- and 1,4-benzoquinones rapidly react with GSH and *N*-acetylcysteine, it seems likely that it is the quinones or semiquinone radicals formed in microsomes which are the actual reactive species (Scheme 1). This would explain the loss of tetrachlorobenzenediols during the metabolism of HCB and pentachlorophenol observed in the present and previous work [13]. A free radical intermediate has been detected by e.s.r. during the conjugation of tetrachloro-1,4-benzoquinone with cysteine [22] and in the reaction of the diol with DNA [23]. Semiquinone anion radicals have been detected in the reaction of 1,4-benzoquinone and 1,4-naphthoquinone with GSH and other thiols [24]. On the other hand Wallin *et al.* [25] were unable to detect the presence of semiquinone radicals during covalent binding of [^{14}C]phenol although they did conclude that oxidation of catechol and hydroquinone was a prerequisite.

Whatever the precise mechanism of the covalent binding from HCB to protein and DNA there appears to be a poor correlation with the porphyrogenic and carcinogenic actions of this chemical in the liver of rats *in vivo*. Firstly, these phenomena are highly sex-dependent in the rat and this was not reflected in levels of metabolites produced or covalent binding with the isolated male and female hepatocytes. Secondly, the degree of covalent binding to DNA was significantly lower than is usually expected for hepatocarcinogens although not as low as that recorded for

Table 1. Covalent binding of radioactivity to protein after incubation of hepatocytes with [^{14}C]HCB.

Sex	Bound radioactivity (pmol/10^6 cells)	Aqueous radioactivity (pmol/10^6 cells)
Male	19 ± 2	155 ± 18
Female	12 ± 3	111 ± 34

[^{14}C]HCB was incubated with suspensions of hepatocytes from rats previously treated with phenobarbitone as described in the Materials and Methods. Incubations were for 20hr and contained 0.25 μCi/ml (5 nmol/ml) of [^{14}C]HCB. Bound and aqueous radioactivity is expressed as pmol of HCB equivalents. Blanks contained heat-denatured cells (<2 pmol/10^6 cells) and have been subtracted. Values are means ± SEM of hepatocytes from 4 animals.



Scheme 1. Postulated mechanism of covalent binding to protein and other thiols by the microsomal metabolism of HCB and pentachlorophenol.

TCDD [26]. This suggests that some other toxic process should be sought to explain the porphyrogenic and hepatocarcinogenic actions of HCB.

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