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EXCRETION PATTERN AND METABOLISM OF HEXACHLOROBUTADIENE IN RATS

EVIDENCE FOR METABOLIC ACTIVATION BY CONJUGATION REACTIONS*

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Abstract—Excretion, covalent binding and metabolism of hexachloro-1,3-butadiene (HCBD), a nephrotoxic and nephrocarcinogenic compound, have been studied in female rats. Seventy-two hours after administration of a single oral dose of 1 mg/kg [^{14}C]HCBD†, 5.3% of the dose were exhaled as unchanged HCBD and 76.3% were metabolized and excreted in urine and feces or exhaled as $^{14}CO_2$. After a 50 mg/kg dose of [^{14}C]HCBD, the amount of exhaled parent compound was nearly unchanged at 5.4%. At the higher dose the gastro-intestinal absorption of HCBD appeared to be saturated with the result that unchanged HCBD constituted the major portion of the 69% radioactivity eliminated. Covalent binding to proteins in kidney and liver agreed well with the organ-specific toxicity of HCBD: binding was higher in the kidney, independent of the dose. It increased significantly when the rats were pretreated with phenobarbital, an inducer of monooxygenases; it decreased when the inhibitor piperonyl butoxide was given.

Urinary radioactivity in 24 hr urine was separated by column chromatography into four fractions. High performance liquid chromatography, radio gas chromatography and gas chromatography/mass spectrometry were used for further separation and identification. Two major metabolites were identified as pentachlorobutadiene methylthio ether and pentachlorobutadiene carboxymethylthio ether. Their formation is plausibly explained via glutathione conjugation, which appears to be the first step in HCBD metabolism. The mechanism of the conjugation at the olefinic double bond of HCBD is explained by an addition–elimination reaction. This pathway, which appears to lead to a destabilization of the HCBD molecule, could explain the distinct nephrotoxic effects of HCBD.

Hexachloro-1,3-butadiene (HCBD) is a by-product in the synthesis of tri- and tetrachloroethylene [1, 2] and a decomposition product of dichloroacetylene [3]. Excessive levels of HCBD have been found in the surroundings of plants producing chlorinated aliphatic hydrocarbons [1, 4]. Portions of our food supply are contaminated with HCBD [1, 5], e.g. HCBD levels of up to 4.6 ppm have been measured in fish samples [1, 2, 4]. HCBD is relatively stable in comparison to other chlorinated aliphatic hydrocarbons. The technical uses of HCBD are based on its chemical stability, especially its non-inflammability. It has been proposed for use in hydraulic and in electrical insulation fluids, and as a lubricant and solvent [6, 7]. HCBD is known as a highly effective insecticide, herbicide (for narrow-leafed weeds) and strongly depresses the growth of algae; moreover, it combats the most serious grape disease, phylloxera [8].

HCBD is one of the few compounds in the series of chlorinated aliphatic hydrocarbons which exerts selective nephrotoxic and nephrocarcinogenic effects [9–12]. The carcinogenic activity of HCBD agrees well with a mutagenic potential in *Salmonella typhimurium* strain TA 100, induction of morphological transformation and unscheduled DNA synthesis in cultured Syrian hamster embryo fibroblasts [13, 14]. The results of the genotoxicity studies support the assumption that oxidation of HCBD serves as a possible activation mechanism for its toxicity. However, an oxidative mechanism as the only pathway in the toxication of HCBD cannot sufficiently explain the distinct organotropic properties of this compound. Oxidative activation, by e.g. microsomal monooxygenases with epoxide formation as shown for chlorinated ethylenes, is consistent with a cytotoxic and/or carcinogenic effect at the site of formation of the chemically reactive epoxide. This is the case with chlorinated ethylenes, which are hepatotoxic or hepatocarcinogenic, but is not the case with HCBD, which selectively elicits nephrotoxic and nephrocarcinogenic responses. Other

* Preliminary accounts of this study have been presented at the Third International Congress on Toxicology, San Diego, 1983, and at the Spring Meeting of the German Pharmacological Society, Mainz, 1984.

† Abbreviations: GC, gas chromatography; HCBD, hexachloro-1,3-butadiene; HPLC, high performance liquid chromatography; MS, mass spectrometry; 3-PCBAC, pentachloro-3-butenoic acid chloride; 2-PCBAM, pentachloro-2-butenoic acid methyl ester; 3-PCBAM, pentachloro-3-butenoic acid methyl ester; PCCMTB, pentachloro-carboxymethylthio-1,3-butadiene; PCMTB, pentachloro-1-methylthio-1,3-butadiene; TCBAM, trichloro-2-butynic acid methyl ester; and TPCM, triphenylphosphine carbomethoxymethylene.

mechanisms of activation are obviously involved in the metabolism of halogenated olefins.

In order to gain insight into the possible activation reactions in vivo, the chemical reactivity of HCBD was recently studied under the conditions of thermal and photochemical oxidation: pentachlorobutenoic acid chloride and a series of degradation products were found, e.g. tetrachlorosuccinic acid chloride and pentachloroacetoacetyl chloride [15]. In addition, substitution reactions leading to pentachlorobutadiene derivatives were reported by Roedig and Bernemann [16]. Since data on the biotransformation of HCBD were still lacking, we decided to investigate the absorption, metabolism and excretion of ^{14}C-labeled HCBD in rats. The results of this study should also aid in understanding the biological fate of structurally related compounds with a similar organ-specific toxicity.

MATERIALS AND METHODS

Chemicals

[^{14}C]HCBD. This was purchased from Hoechst (Frankfurt, FRG) and had a specific radioactivity of 38.85 MBq/mmole (1.05 mCi/mmole). Its radiochemical purity exceeded 99%, as shown by radio–GC.

Pentachloro-3-butenoic acid methyl ester (3-PCBAM). This was obtained from pentachloro-3-butenoic acid chloride (3-PCBAC), which was prepared as described previously [16] by oxidation of pentachloro-1-ethoxybutadiene with chlorine gas. 3-PCBAC was distilled twice on a Vigreux column until its boiling point and refraction index were in agreement with the data from the literature [16]. 3-PCBAM was spontaneously formed upon addition of methanol to 3-PCBAC. The identity of 3-PCBAM was confirmed by GC/MS (see Fig. 4).

Pentachloro-2-butenoic acid methyl ester (2-PCBAM). This was prepared as shown in Fig. 1 by using a general method for the preparation of α,β-acetylenic carboxylic acids [17]. 12.0 g (36 mmole) triphenylphosphine carbomethoxymethylene (TPCM), which was formed from triphenylphosphine and α-bromoacetic acid methyl ester [18], were dissolved in 90 ml of dry, warm benzene and 3.2 g (18 mmole) freshly distilled trichloroacetyl chloride in 18 ml benzene were added dropwise under vigorous stirring at 20°. After standing for 3 hr, the reaction mixture was filtered and the solvent evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate/methanol 1:1 v/v and

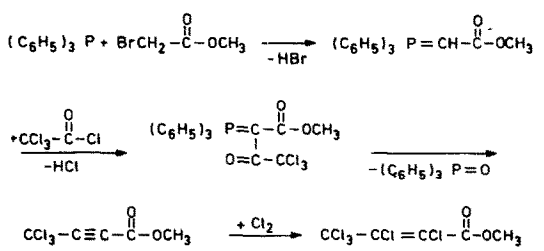

Fig. 1. Synthetic route for the preparation of 2-PCBAM.

purified by precipitation with petroleum ether to yield 5.3 g of triphenylphosphine-trichloromethyl-acetyl-carbomethoxymethylene as colorless crystals. 1.0 g of this intermediate was heated to 265° for 30 min and the product trichloro-2-butynic acid methyl ester (TCBAM) simultaneously removed by distillation under reduced pressure. 80 mg of an oily fluid were obtained with a content of 60% TCBAM, according to GC/MS. MS (only ions containing ^{35}Cl are given): *m/e* 185 ($M^+ - CH_3$, 10%), 169 ($M^+ - OCH_3$, 50%), 165 ($M^+ - Cl$, 60%), 157 (10%), 142 (40%), 141 ($C_3Cl_3^+$, 45%), 137 (60%), 106 (50%), 71 (80%), 59 ($COOCH_3^+$, 100%).

50 mg of the oily TCBAM were dissolved in 0.2 ml CCl_4, and a solution of 60 mg chlorine (dried with conc. H_2SO_4) in 0.8 ml CCl_4 was added. The chlorination was performed by irradiating the reaction mixture with a tungsten filament bulb (200 Watt) for 2 hr from a distance of 40 cm. Excess chlorine and CCl_4 were removed under a stream of N_2. GC showed a yield of 44% 2-PCBAM. MS see Fig. 4.

Pentachloro - 1 - methylthio - 1,3 - butadiene (PCMTB). 0.2 g (2.5 mmole) methane sulfenyl chloride (CH_3SCl) [19] in 10 ml anhydrous acetic acid was added to a solution, in 3 ml acetic acid, of 0.9 g (1.7 mmol) perchlorobutenyne [20]. After 3 days at 20°, the reaction mixture was poured into icewater and extracted with diethyl ether. The ethereal phase was washed with an aqueous $NaHCO_3$ solution and dried with Na_2SO_4. The solvent was evaporated *in vacuo* and the oily residue purified by column chromatography on silica gel with petroleum ether to yield a yellow oil. Purity was 99%, as determined by GC. UV spectrum (methanol): λ_{max} 224 nm, $\varepsilon = 13400$; λ_{max} 252 nm, $\varepsilon = 4900$. MS see Fig. 4. The structure of PCMTB, i.e. with the methylthio group in the 1-position, can be assigned because small amounts of two isomeric compounds were detected by GC in the crude reaction product in addition to PCMTB. One of these had a mass spectrum identical with that of PCMTB (see Fig. 4) and is assumed to be the corresponding *cis/trans* isomer. The other compound had a different mass spectrum (spectrum not shown) and presumably is the isomer with the methylthio group in the 2-position.

Pentachloro-carboxymethylthio - 1,3 - butadiene (PCCMTB). 2.0 g (25 mmole) mercaptoacetic acid were added to a solution of 1.15 g (50 mmole) sodium in 150 ml dry ethanol at 20°. After 10 min of vigorous stirring, 6.53 g (25 mmole) HCBD in 125 ml ethanol were added. The yellow-colored reaction mixture was stirred for another 90 min, filtered, and the solvent evaporated *in vacuo*. The solid residue was dissolved in water and the aqueous phase extracted twice with petroleum ether to remove unchanged HCBD. Glacial acetic acid was added to the aqueous solution and the resulting precipitate collected by centrifugation. Purification of the intensely colored oily product by column chromatography on silica gel with methanol/methylene chloride 1:9 v/v, followed by crystallization from diethyl ether/petroleum ether 1:1 v/v at 4° yielded colorless crystals of a purity greater than 99%, as determined by GC. U.V. spectrum (methanol): λ_{max} 217 nm, $\varepsilon = 17800$; λ_{max} 276 nm, $\varepsilon = 9800$. For GC/MS, the carboxylic group

of PCCMTB was esterified by 3 N methanolic HCl. MS see Fig. 5.

Animals and treatments

Female Wistar rats (180–220 g, Institut für Versuchtstierzucht, Hannover, FRG) were used in all studies. Female rats are about four times more susceptible to the nephrotoxicity of HCBD than male rats [11]. Single doses of [^{14}C]HCBD, dissolved in tricaprylin at 1.2%, were administered by gastric intubation between 09.00 and 10.00 hr. Pairs of animals were placed in all-glass metabolism cages immediately after dosing. A constant flow of air (500 ml/min) was drawn through the cages. Standard diet (Altromin) and tap water were supplied *ad libitum*.

For some experiments, rats were pretreated with either phenobarbital or piperonyl butoxide. Phenobarbital (100 mg/kg) was given as a single i.p. injection (100 mg/kg) and in the drinking water (0.1%) three days before the administration of [^{14}C]HCBD. A single i.p. injection of piperonyl butoxide (1 ml/kg) was given 1 hr before [^{14}C]HCBD gavage.

Sampling and measurement of radioactivity

Exhaled air, urine and feces were collected for 72 hr following HCBD administration. Urine and feces were collected at 6 hr intervals. At the end of the collection period, the cage was rinsed with 100 ml water. [^{14}C]HCBD and $^{14}CO_2$ were collected in half-hour and 3 hr intervals, respectively, by drawing the air from the metabolism cage through a series of four traps. Before entering the first trap, the air was dried with Drierite (Hammond Drierite Co., Xenia, Ohio, U.S.A.). The first two traps each contained 50 ml toluene, the remaining two traps each contained 100 ml of diethanolamine/2-methoxyethanol 1:1 v/v. All traps were maintained at ambient temperature.

Samples from the traps and urine samples were transferred directly into Rotiszint 22 liquid scintillation medium (Roth, Karlsruhe, F.R.G.) for determination of radioactivity. The rats were sacrificed by cervical dislocation 72 hr after HCBD gavage; organs were weighed and samples of tissues (approx. 100 mg) were removed and combusted in a Packard 306 Sample Oxidizer. The carcass was weighed and solubilized in 20% aqueous potassium hydroxide solution. Radioactivity was determined in aliquots of the digests by liquid scintillation counting (Packard TriCarb 2650).

Separation of urinary metabolites

The collected 24 hr urine of two rats was freeze-dried and the residue extracted with methanol at ambient temperature until it contained less than 1% of the initial radioactivity. The extract was concentrated to about 2 ml *in vacuo* and processed by column chromatography according to a previously described method [21]. Briefly, the column was packed with alumina (neutral, act. I) and eluted consecutively with an ethanol/water gradient, a phosphate/citrate buffer pH 6, and 20% aqueous formic acid. The fractions containing radioactivity were further analyzed by reversed-phase HPLC on a 8.0 × 250 mm LiChrosorb RP-18 column (particle size 10 μm). A linear solvent gradient shifting from 5% methanol in water to 100% methanol over a time period of 45 min was used.

HPLC fractions were methylated with diazomethane and analyzed by radio-GC and GC/MS. For radio-GC, a Packard 427 gas chromatograph equipped with a flame ionization detector was used. The separation was carried out on a 6ft × 2 mm i.d. glass column packed with 3% OV-225 on GasChrom Q 100/120 mesh. Argon was used as carrier gas at a flow rate of 30 ml/min. The temperatures were 250° for the injector, 270° for the detector and 80–270° with a heating rate of 4°/min for the column oven. GC/MS was performed in part on a Varian CH-7 mass spectrometer coupled to a Varian 2700 gas chromatograph and equipped with a Varian SS100 data system, and in part on a Finnigan 4510 GC/HSIDs. The mass spectra were recorded at an electron energy of 70 eV.

Measurement of covalent binding

Aliquots of tissue from liver and kidney were homogenized in a fourfold volume of water. One ml of this homogenate was added to 2 ml 0.9 M aqueous trichloroacetic acid and the mixture centrifuged at 1000 *g* for 15 min. The sediment was resuspended and centrifuged twice with 2 ml 0.6 M trichloroacetic acid and 5–8 times with 3 ml 80% aqueous methanol and chloroform/methanol 2:1 v/v each, until no further radioactivity could be removed. The precipitate was then dissolved in 1 N aqueous NaOH and aliquots were taken for the determination of protein and for liquid scintillation counting [22].

RESULTS

Excretion of HCBD

In Table 1, the amounts of ^{14}C-activity found in the excreta of rats 72 hr after a single oral dose of 1.0 and 50.0 mg/kg body weight are compared. The majority of the radioactivity (42–69%) was recovered from the feces, while 11–30% were excreted in the urine. The higher amount of radioactivity in the feces after the higher dosage was exclusively due to unchanged HCBD, which was extracted with toluene and identified by GC/MS.

In exhaled air, both [^{14}C]HCBD and $^{14}CO_2$ were identified. The amount of exhaled HCBD was not

Table 1. Recovery of radioactivity 72 hr after oral administration of different doses of [^{14}C]HCBD to female rats. Data represent mean ± S.E. of three experiments with two rats each

	Recovery (% of administered activity)	
Dose (mg/kg)	1.0	50.0
Exhaled air:		
HCBD	5.31 ± 0.35	5.45 ± 0.26
CO_2	3.60 ± 0.20	1.17 ± 0.03
Urine	30.61 ± 4.39	11.01 ± 0.91
Feces	42.13 ± 4.67	69.03 ± 1.63
Carcass and tissues	7.42 ± 0.47	6.33 ± 0.59
Cage rinse	6.24 ± 0.71	2.28 ± 0.47
Total recovery	95.31 ± 1.48	95.25 ± 1.33

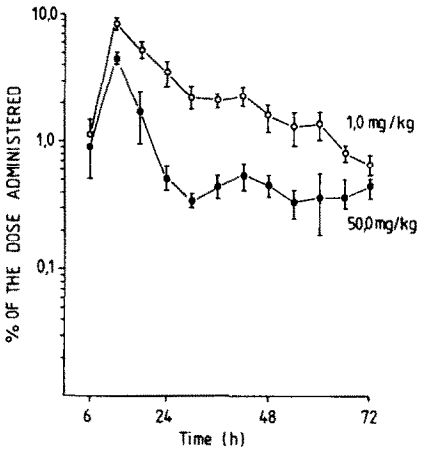

Fig. 2. Urinary excretion of radioactivity as a function of time after 1 and 50 mg/kg [^{14}C]HCBD. Each point represents the mean ± S.E. for three experiments with two rats each.

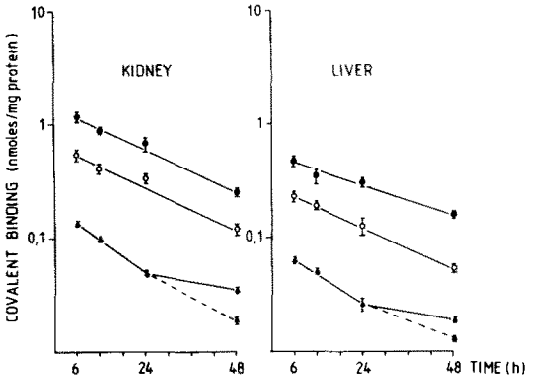

Fig. 3. Covalent binding to proteins in kidney and liver tissue as a function of time after dosage of 1 mg/kg [^{14}C]-HCBD. Each point represents the mean ± S.E. of three experiments with untreated animals (○) and after pretreatment with phenobarbital (●) or piperonyl butoxide (▲).

influenced by the different dosages. In contrast, the formation of $^{14}CO_2$ was reduced by a factor of three after administration of the higher dose of 50 mg/kg, indicating a saturation in HCBD metabolism. This assumption was supported by a corresponding decrease in renal excretion of metabolites after the high dose.

The time course of the urinary excretion of radioactivity is shown in Fig. 2. After an elimination phase of approximately 24 hr, a significant increase in the amount of urinary radioactivity occurred at about 30 hr after dosing. A possible explanation would be an initial saturation of the intestinal absorption of HCBD, followed by reabsorption of HCBD at a later time. A redistribution of HCBD from fat and brain tissue cannot contribute to a large extent to the increase in urinary radioactivity, as the amount of HCBD in these tissues is only 0.001% and 0.078%, respectively, of the administered dose of 50 mg/kg, whereas in the feces at the same time it exceeds 20%.

Distribution and covalent binding of HCBD

The distribution of HCBD in rats is mainly determined by the lipophilicity of this compound. The largest amounts of radioactivity were detected in liver, brain and kidney (data not shown). The extent of covalent binding in the kidney exceeded that in the liver more than twofold (Fig. 3), despite the fact that the amount of total radioactivity was higher in the liver. The covalently bound radioactivity increased significantly after pretreatment of the rats with phenobarbital, a known inducer of monooxygenases, and decreased very markedly when the inhibitor piperonyl butoxide was administered prior to HCBD (Fig. 3).

The covalent binding of HCBD-related radioactivity to tissue proteins was time-dependent (Fig. 3): binding was highest during the first 6 hr after dosing and decreased in a linear fashion for at least 72 hr with a half-life of 22 hr in both liver and kidney. The decrease became slower after 24 hr in the rats pretreated with piperonyl butoxide (Fig. 3); however, when the treatment with piperonyl butoxide was repeated at this time point, the bound radioactivity decreased again with a half-life corresponding to the value of the first 24 hr (Fig. 3, dotted line), suggesting that piperonyl butoxide itself undergoes metabolic transformation.

Isolation and identification of urinary metabolites

Metabolites were isolated from the pooled 24 hr urine of two rats after p.o. administration of 100 mg/kg [^{14}C]HCBD. The methanol extract of the freeze-dried urine was separated by column chromatography on alumina into four radioactive fractions (Table 2). Most of the radioactivity was found in fraction 2, which, according to the separation characteristics of the column [21], should consist of mercapturic acids and other sulphur-containing metabolites, e.g. sulfates. Fractions 1 and 2 were further separated by HPLC; three peaks were observed in

Table 2. Distribution of urinary radioactivity after column chromatography

Fraction	Metabolites	Radioactivity (%)
1	Unconjugated, nonpolar	1.2
2	Mercapturic acids and other sulphur-containing metabolites	81.6
3	Glucuronides	15.7
4	Unconjugated, polar	1.5

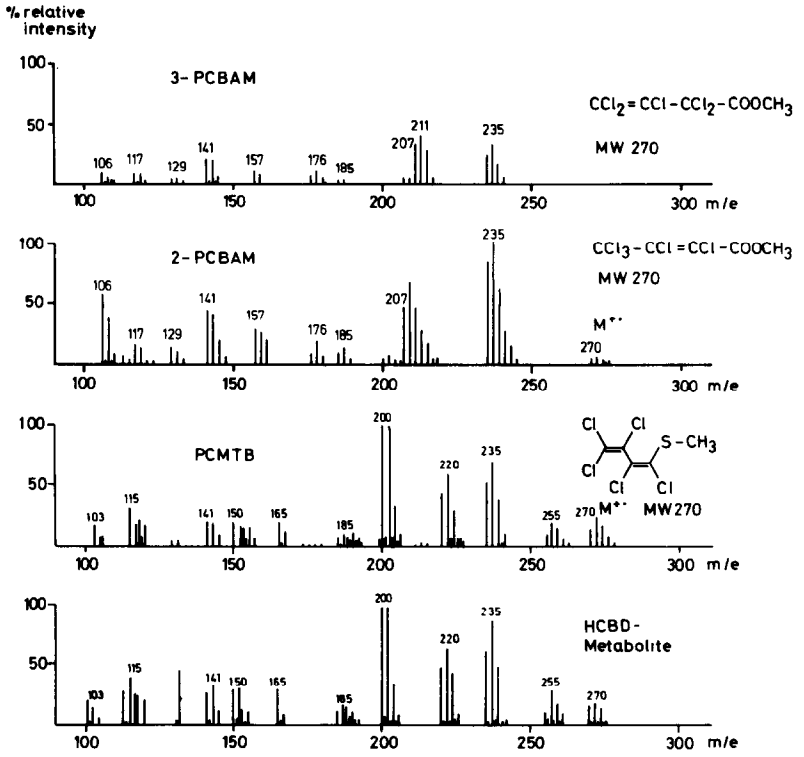

Fig. 4. Mass spectra of a HCBD metabolite and three synthetic reference compounds of identical molecular weight and chlorine content.

fraction 1 and at least 5 peaks in fraction 2. The HPLC fractions were then treated with diazomethane and analyzed by radio-GC and GC/MS. In addition to their GC retention times as revealed by radio-GC, the metabolites were easily recognized in GC/MS by the characteristic isotope patterns of chlorine in their mass spectra.

The mass spectrum of one major metabolite, which accounted for *ca.* 10% of the urinary radioactivity is shown in Fig. 4. The ion with the highest mass was 270 and the isotopic and fragmentation patterns indicated the presence of five chlorine atoms in the molecule. At least three compounds could theoretically account for this spectrum: pentachloro-2-butenoic acid methyl ester (2-PCBAM), pentachloro-3-butenoic acid methyl ester (3-PCBAM) and pentachloro-1-methylthio-1,3-butadiene (PCMTB). Comparison of the mass spectra of the synthetic reference compounds with the mass spectrum of the metabolite clearly identified this urinary HCBD metabolite as PCMTB (Fig. 4). In support of this structure assignment was the high intensity of the fragment at *m/e* 200, which could only be explained plausibly by this structure; the isomeric substance with the methylthio group in the 2-position had only a weak intensity of this fragment in its mass spectrum (see Materials and Methods).

The mass spectrum of another major metabolite

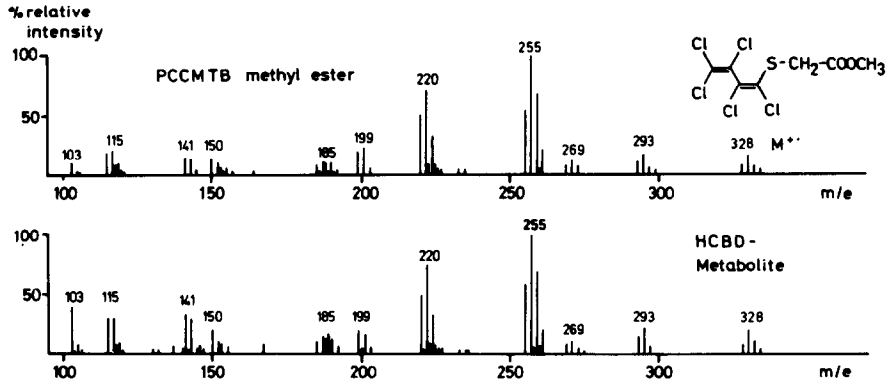

Fig. 5. Mass spectra of a HCBD metabolite and the corresponding authentic reference compound.

also showed the presence of five chlorine atoms (Fig. 5); the apparent molecular ion was 328. The structure of pentachloro-carboxymethylthio-1,3-butadiene (PCCMTB) was assigned to this metabolite. The methyl ester of an authentic sample of this substance yielded an identical mass spectrum and had the same GC retention time.

The mass spectra of the other HCBD metabolites did not lead to plausible structural suggestions as yet.

DISCUSSION

This study reports on the excretion and metabolism in rats of HCBD, a highly toxic and carcinogenic compound. HCBD is extremely lipophilic, and nearly insoluble in water (max. 2 mg/l, ref. 8). Both the extremely low water solubility and high lipophilicity account for the low gastro-intestinal absorption of [^{14}C]HCBD and its early saturation, resulting in the fecal excretion of significant amounts of unchanged HCBD at the 50 mg/kg dose as compared to the 1 mg/kg dose. Accordingly, the proportion of the dose excreted as urinary metabolites and exhaled as $^{14}CO_2$ was lower after administration of 50 mg/kg than after 1 mg/kg (Table 1).

In urine, a variety of metabolites of [^{14}C]HCBD was observed after chromatographic separation by HPLC and GC. Thus far, two of the predominant metabolites could be unequivocally identified by their mass spectra and co-chromatography with authentic reference compounds as pentachloro-1-methylthio-1,3-butadiene (PCMTB) and pentachloro-carboxymethylthio-1,3-butadiene (PCCMTB). The syntheses of these new compounds is described in detail.

The most plausible metabolic pathways leading to PCMTB and PCCMTB are shown in Fig. 6. It is proposed that conjugation of HCBD with glutathione, followed by the release of glycine and glutamic acid, are the primary metabolic steps. Deamination and subsequent oxidative decarboxylation of the cysteine adduct can then lead to PCCMTB, the carboxymethylthio derivative of HCBD. The main pathway for the formation of methylthiolated metabolites like PCMTB is suggested to involve the cysteine adduct of HCBD. This adduct undergoes a C–S bond cleavage, catalyzed by the enzyme β-lyase, to form the thiol compound. The final step in this pathway is mediated by S-methyltransferase, which transfers the methyl group of S-adenosyl-L-methionine to the thiol-containing intermediate [23–25]. It cannot be excluded that a minor pathway which forms PCMTB is the decarboxylation reaction of PCCMTB.

Thus far, the intermediate adducts with glutathione and cysteine, as well as the corresponding mercapturic acid resulting from acetylation of the cysteine adduct (Fig. 6) have not been identified among the numerous water-soluble *in vivo* metabolites of HCBD. However, the deamination of cysteine adducts to carboxymethyl derivatives is an established metabolic pathway for several halogenated ethylenes, e.g. vinylidene chloride [26] and vinyl chloride [27]. The formation of a glutathione adduct of HCBD *in vitro* has recently been demonstrated by Oesch and Wolf, who showed that HCBD is a substrate of microsomal rather than cytosolic glutathione transferases [28].

An interesting question raised by the identified metabolites concerns the mechanism of conjugation of HCBD with the sulfur-containing moiety. A direct nucleophilic substitution of one chlorine atom by the sulfhydryl group at the olefinic double bond is unlikely to occur in a mammalian organism. It is more likely that the conjugation will proceed in two steps as an addition-elimination mechanism (Fig. 7): the nucleophilic addition of the thiol to HCBD leads to a perchloroallylic anion, which eliminates a chloride ion with consequent reconstitution of the double bond. This mechanism parallels the previously described condensation of enolates with HCBD [29].

The present study also provides some evidence for the oxidative metabolism of HCBD. Covalent

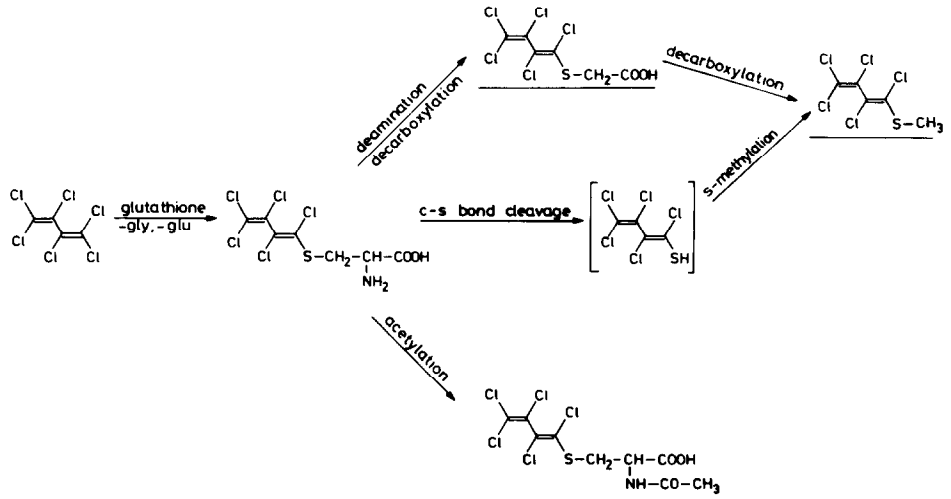

Fig. 6. Tentative pathway for the conjugation of HCBD with glutathione in rats. Identified metabolites are underlined.

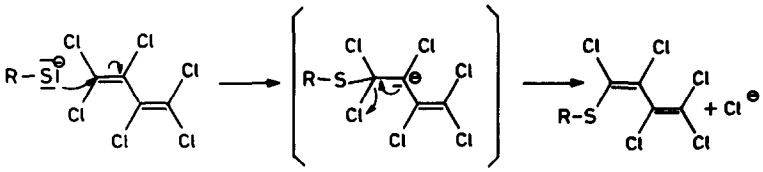

Fig. 7. Proposed mechanism for the conjugation of HCBD with thiols.

binding in kidney and liver increased very significantly when the animals were pretreated with phenobarbital, a known inducer of monooxygenases (Fig. 3); conversely, binding was decreased when piperonyl butoxide was given before HCBD gavage; piperonyl butoxide is known to inhibit oxidative metabolism by binding to cytochrome P-450 [30, 31]. These results agree well with the observation that the severity of renal necrosis is increased in rats pretreated with phenobarbital [11] and decreased after pretreatment with piperonyl butoxide [32]. These observations, along with the finding by Oesch and Wolf [28] that phenobarbital does not affect the rate of microsomal conjugation of HCBD with glutathione *in vitro*, suggest that monooxygenases are involved in the metabolic activation of HCBD. We think that an oxidation reaction most probably could occur at the sulfur containing substituent rather than at the HCBD molecule. Quantitatively the amount of unconjugated metabolites found in urine is about 2–3% (Table 2).

The conjugation reaction of HCBD with glutathione can sufficiently explain the organotropic effects of this compound and of comparable olefinic chlorinated hydrocarbons. The glutathione and the cysteine conjugate as well as the mercapturic acid of HCBD have shown to be nephrotoxic in rats [25, 33]. The conjugation reaction of HCBD is a typical example of an activation by conjugation, as described previously for some other compounds [34].

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