POSSIBLE REACTIVE INTERMEDIATES IN THE OXIDATIVE BIOTRANSFORMATION OF HEXACHLOROBENZENE

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SUMMARY

In this review the biotransformation of hexachlorobenzene is discussed, with special reference to the possible generation of reactive metabolites or intermediates during this process. Evidence is presented for the direct involvement of certain cytochrome P-450 isoenzymes in the major toxic effect of hexachlorobenzene, hepatic porphyria. The *in vivo* biotransformation is discussed and compared with *in vitro* experiments (microsomal and cell culture studies). The possible reactive metabolites and intermediates and their mechanisms of formation are presented. Special attention is directed to a very reactive metabolite, tetrachloro-1,4-benzoquinone, which has a high capacity to efficiently react with protein, thus possibly linking the oxidative biotransformation of hexachlorobenzene and the molecular mechanism of enzyme inactivation leading to hepatic porphyria.

ABBREVIATIONS

HCB	=	hexachlorobenzene;
P-450	=	cytochrome P-450;
PCB	=	polychlorinated biphenyl;
PCDD	=	polychlorinated dibenzodioxin;
PCDF	=	polychlorinated dibenzofuran;
PCP	=	pentachlorophenol;
TAO	=	triacetyloleandomycin;
TCBQ	=	tetrachlobenzoquinone;
TCHQ	=	tetrachlorohydroquinone;
URO-D	=	uroporphyrinogen decarboxylase

I. INTRODUCTION

1.1 General

The toxicity of hexachlorobenzene (HCB) has been under intensive research during the past thirty years, ever since this compound was the cause of a disastrous poisoning in the eastern part of Turkey /1, 2, 3/. In spite of all the efforts, the mechanism of toxicity has not yet been elucidated. The way in which HCB interacts with the human physiology appears to be very complicated and manifests itself in a large number of different toxic effects, the major one being hepatic porphyria. Furthermore, HCB proved to be carcinogenic in laboratory animals.

There is, however, a growing line of evidence that the interaction of HCB with the cytochrome P-450 system plays a key-role in the main toxic effect of HCB, the disturbance of the hepatic heme synthesis, resulting in a massive excretion of porphyrins. In this review the various aspects of the possible bioactivation of HCB are discussed.

1.2 Use and Occurence

Hexachlorobenzene has been used as a fungicide, especially for the protection of seed grains against bunt fungi. The production of HCB for this purpose was estimated to be about 7 tons annually /4/. Of greater importance is the production of HCB as an unwanted byproduct in the synthesis of a number of halogenated compounds such as perchloroethylene, carbon tetrachloride, chlorine, chlorinated solvents and pesticides. In the production of the pesticide pentachloronitrobenzene for example, 2000 tons of HCB were produced annually in the early seventies /4/. In many countries, the use of HCB as a fungicide has been prohibited and its production as a byproduct strictly regulated.

However, due to the large amounts of HCB introduced into the environment in the past and its poor biodegradability, HCB has become a major pollutant /5/. For instance, HCB has been shown to be present in human adipose tissue, wild mammals, birds, fish and soil /for an overview: 6/. The high degree of persistence has been the main reason for its restriction.

II. TOXICITY AND BIOTRANSFORMATION OF HEXACHLOROBENZENE

2.1 Toxicity of Hexachlorobenzene

The (sub)acute toxicity of HCB is relatively low. LD_{50} values range from 2600-4000mg/kg bodyweight /7/. Chronic exposure, however reveals a wide range of toxic effects. These effects include immunotoxicity /8/, teratogenic effects /9/, neurotoxicity /10/, porphyria and tumor formation. The last two effects mentioned will be discussed in some detail. For a more detailed review of the toxicological data on HCB see reference 11.

HCB has been shown to be carcinogenic in rats /12, 13, 14/, mice /15/, and hamsters /16/. In all species the incidence of liver tumors was significantly increased after chronic exposure to at least 75ppm HCB, while in hamsters and rats /14/, thyroid adenomas were reported as well. In the rat, renal cell adenomas were detected /17/.

The sex differences in tumor development are noteworthy. Whereas in hamsters and mice hepatomas occurred predominantly in males, in the rat HCB induced more liver tumors in females than in males /12/. This phenomenon is strikingly similar to the development of HCB-induced porphyria. Of the three species mentioned, only in the rat is there a sex difference in the development of hepatic porphyria occuring in females to a larger extent than in males /18/. However, in the rat both renal and thyroid tumors were induced to a larger extent in males than in females.

2.2 The Prophyrinogenic Action of Hexachlorobenzene

The best known toxic effect of HCB is its porphyrinogenic action in man and animals. Hepatic porphyria, the accumulation and excretion of porphyrins as a result of a disturbance of the heme synthesis, can either be the result of exposure to certain chemicals or be of congenital origin. A variety of porphyrinogenic compounds is known. Apart from HCB, well-known representatives are the polychlorinated biphenyls (PCB's), polychlorinated dibenzodioxins (PCDD's) and the polychlorinated dibenzofurans (PCDF's) /19/.

The porphyrinogenic action of HCB in humans was discovered after a case of massive poisoning in the south-eastern part of Turkey in 1955-1959 /1, 2, 3/. Consumption of grain dressed with HCB resulted in at least 3000 cases of porphyria, with a 10 percent mortality rate. Among infants the mortality amounted to 95 percent.

Chronic exposure of rats to 500 ppm HCB in the diet results in the appearance of large amounts of porphyrins after 6-10 weeks /20, 21/. The mechanism of HCB-induced porphyria has only been partially resolved. The accumulation of porphyrins in the liver is due to a selective, irreversible inhibition of the enzyme uroporphyrinogen decarboxylase (URO-D, EC 4.1.1.37), which catalyses the four successive decarboxylations of uroporphyrin (with 8 carboxyl groups) to coproporphyrin (with 4 carboxyl groups) /22/. The excreted porphyrins predominantly consist of 8, 7, 6 and 5 carboxyl group containing porphyrins. The mechanism of inhibition is not clear. Kawanishi /23/ reported the direct inhibition on purified URO-D of HCB, while Billi /24/ found no effect of HCB on cytosolic (unpurified) UROD. Smith /25/ reported that mouse liver URO-D *in vivo* is not radiolabeled by [^{14}C]-HCB. However, Rios de Molina /26/ showed that partially purified URO-D from HCB-porphyric rats is structurally different from the enzyme of untreated rats. Elder /27/, finally, showed that the amount of immunodetectable URO-D is unchanged after HCB-exposure, although its activity is greatly diminished.

2.3 The in vivo Biotransformation of hexachlorobenzene

The metabolites of HCB can be divided into three groups: phenolic metabolites, sulfur-containing metabolites and lower chlorinated benzenes.

A large number of metabolites has been detected. They are presented in Table 1. Recent reviews on the *in vivo* biotransformation of hexachlorobenzene have been published by Renner /28-30/.

The major metabolite of HCB is pentachlorophenol (PCP). During a long term exposure, the amount of excreted PCP was 25% of the dose, and appr. 70% of the metabolites /21/. PCP has been detected in the liver, faeces and urine of rats exposed to HCB /21/. The second most important oxidative metabolite is tetrachloro-1,4-hydroquinone. The other predominant metabolites are pentachlorothiophenol and penta-

TABLE 1

The In V vo Metabolites of Hexachlorobenzene

Benzenes	Sulfur Metabolites	Phenoles
pentachlorobenzene	pentachlorothiophenol	pentachlorophenol
1,2,3,4-tetrachlorobenzene	tetrachlorothiophenol	2,3,4,6-tetrachlorophenol
1,2,4,5-tetrachlorobenzene	pentachlorothioanisol	2,3,5,6-tetrachlorophenol
1,3,5-trichlorobenzene	tetrachlorothioanisol	2,3,5-trichlorophenol
	hexa(methylthio)benzene	2,4,6-trichlorophenol
	dichlorotetra(methylthio)benzene	
	tetrachloro-1,4-di(methylthio)benzene	
	4-methylthiotetrachlorothiophenol	
	1,4-dimercapto tetrachlorothiophenol	
	N-acetyl-S-(pentachlorophenyl)cysteine	

Adapted from Renner (28-30)

chlorothioanisol. Both sulphur containing metabolites are interconvertable by the rat /31/. The amount of sulphur containing metabolites as compared to the amount of pentachlorophenol excreted is 1:2.8 after long-term exposure and 1:1.3 after short-term exposure /21, 32/. The formation of lower chlorinated benzenes from HCB has been the subject of discussion for some time. Renner /33/ suggested the formation of pentachlorobenzene takes place by a pathway he described as reductive desulfuration (Figure 1). This implies the initial

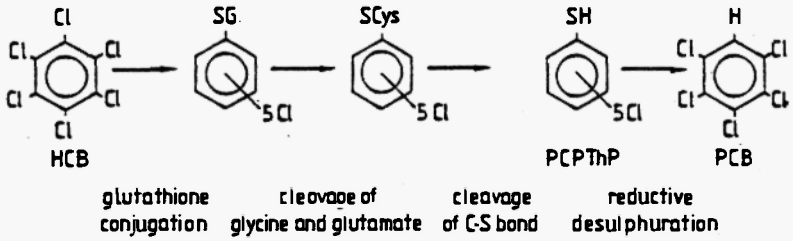

Fig. 1: Formation of pentachlorobenzene from hexachlorobenzene by the mechanism of reductive desulfuration, as adapted from Renner /33/. After formation of the glutathione conjugate of HCB, pentachlorothiophenol is formed by hydrolysis of the peptide bonds and subsequent cleavage if the carbonsulfur bond by cysteine conjugate β-lyase. Reductive desulfuration results in formation of pentachlorobenzene

formation of a glutathione conjugate of HCB, which is subsequently converted stepwise into pentachlorothiophenol and pentachlorobenzene, respectively. Stewart /34/, however, reported the direct reduction of HCB to pentachlorobenzene by rat liver microsomes. The rate of this reduction, though, is extremely low, and the role of the intestinal microflora should not be disregarded.

2.4 The Relation Between Porphyria and Biotransformation of Hexachlorobenzene

In the rat, the porphyrinogenic action of HCB can be enhanced by the cytochrome P-450 inducer phenobarbital /35, 36/. The Japanese quail, a species which develops porphyria in response to HCB very

rapidly, excretes porphyrins much earlier and to a greater extent after treatment with β-naphthoflavone. Phenobarbital pretreatment shows an inhibitory effect on the development of porphyria in this species /37/. In a primary culture of chick hepatocytes, SKF-525A and piperonyl butoxide, both selective inhibitors of cytochrome P-450, inhibit the porphyrinogenic action of chlorinated hydrocarbons /38/ and HCB /39/, while addition of 3-methylcholanthrene /40/ and β-naphthoflavone /38/, inducers of cytochrome P-450c, together with HCB, also induced porphyria. Piperonyl butoxide has also been found to inhibit the development of porphyria *in vivo* /25/. These findings have led to the hypothesis that oxidative biotransformation of HCB is a prerequisite for its porphyrinogenic action. A number of metabolites have therefore been tested for their ability to induce porphyria or to inhibit URO-D *in vivo*. The primary oxidative metabolite of HCB is pentachlorophenol (PCP) /21, 41/. Although in early studies pentachlorophenol was reported to be porphyrinogenic, later reports showed that this was due to chemical impurities, especially PCDD's and PCDF's. Pure pentachlorophenol does not cause porphyria in rats /42-44/. Pentachlorothiophenol, pentachlorothioanisol and its sulfoxide and sulfone were also found to be non-porphyrinogenic /31/. Pentachlorophenol and tetrachlorohydroquinone, although not porphyrinogenic by themselves, are able to increase the porphyrinogenic action of HCB /45, 46/, the latter being the strongest synergist. In contrast to the findings in rats, *in ovo* exposure of chick embryo's to tetrachloro-1,4-hydroquinone, and to a lesser extent exposure to other phenolic metabolites resulted in accumulation of porphyrins. HCB itself showed no porphyrinogenic effect in this study /47/. As far as the direct effect on URO-D is concerned, only tetrachlorohydroquinone and pentachlorophenol had an inhibitory effect /24/. However, the concentrations used by far exceed the concentrations these compounds may reach in the liver as metabolites of HCB.

In a recent experiment, the *in vivo* metabolism of HCB was selectively inhibited by an inhibitor of cytochrome P-450p, the isoenzyme which has been identified to be the major cytochrome P-450 form involved in the hydroxylation of HCB (see below). This inhibition was achieved by continuous treatment of rats with triacetyloleandomycin (TAO), a compound which, after being N-demethylated by cytochrome P-450p, selectively binds to, and inhibits this isoenzyme /48/. Simul-

taneous exposure to HCB and TAO resulted both in a decrease of the urinary excretion of the oxidative metabolites PCP and TCHQ, as well as in a drop in the urinary excretion of porphyrins (Figure 2). Thus, a correlation between the oxidative biotransformation and porphyria has been established. It should be stressed, however, that until this moment, no direct evidence has been presented which proves that the origin of porphyria lays in the biotransformation of HCB.

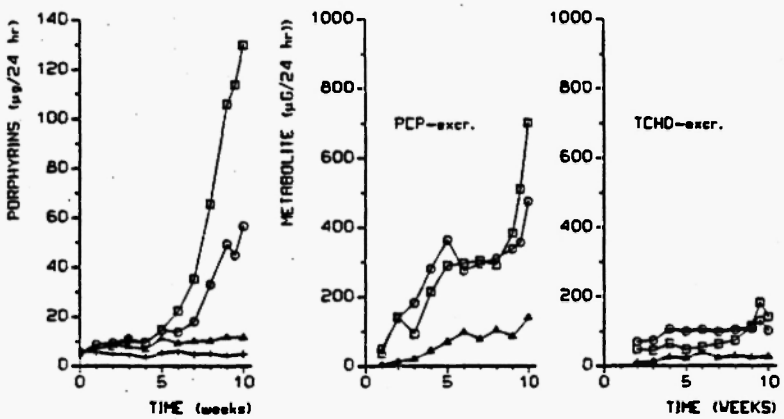

Fig. 2: The relation between urinary excretion of oxidative metabolites and porphyrins. Groups of 9 rats were continuously exposed to hexachlorobenzene (□), hexachlorobenzene together with phenobarbital (○) and HCB together with TAO (△). The urinary excretion of PCP, TCHQ and porphyrins was measured weekly. The porphyrin excretion of the control group is also presented (+).

It is possible that cytochrome P-450 is not involved as a monooxygenase, hydroxylating HCB, but as an oxidase, generating reactive oxygen species, as postulated by Ferioli /49/, Sinclair (50) and others. TCHQ has been shown to stimulate the oxidase activity of microsomal cytochorme P-540 very strongly /51/. However, besides HCB there are a large number of other compounds which stimulate the oxidase function of cytochrome P-450, and most of these are not porphyrinogenic. Other mechanisms must thus be involved which determine the relative specificity of the group of chlorinated aromatic compounds in inducing porphyria.

2.5 Induction of Cytochrome P-450 by Hexachlorobenzene

Hexachlorobenzene is an inducer of hepatic microsomal cytochromes P-450. The changes in drug metabolism which occur after exposure of rats to HCB indicate that HCB is a "mixed inducer", which implies the induction of both phenobarbital and 3-methylcholanthrene inducible cytochrome P-450 isoenzymes /52-58/. Carpenter /59, 60/ describes the same type of induction by HCB for the Japanse quail. Most authors describe an increase in transformation of typical P-450c substrates like benzo(a)pyrene, ethoxyresorufin and ethoxycoumarin to about half the level that can be reached with induction by β-naphthoflavone or 3-methylcholanthrene, while conversion of typical P-450b substrates like aminopyrine, pentoxyresorufin /65/, and biphenyl (4-hydroxylation) is increased about as much as by phenobarbital induction. HCB shares this type of induction with polychlorinated biphenyls /61/. The induction is accompanied by a significant increase in liver weight. Recently, two studies have been performed in which the total amount of cytochrome P-450 isoenzymes has been estimated by means of mRNA induction /62/, or immunochemical quantitation /63, Table 2/. Both studies confirmed the mixed nature of induction.

Recent experiments have indicated the involvement of cytochrome P-450p in the biotransformation of HCB /48 unpublished results/. Inhibition of this isoenzyme in microsomes from HCB treated male rats resulted in an 80% decrease in the rate of hydroxylation of both HCB and PCP, while *in vivo* inhibition if cytochrome P-450p resulted in a diminished excretion of oxidative metabolites /62/. Thus, evidence is presented for the presence of cytochrome P-450p after induction by HCB. Apparently, a discrepancy exists between the quantitation of individual isoenzymes and the total amount of cytochrome P-450, as published for HCB /63, Table 2/, since these values only allow for the presence of 0.02 nmol of unknown cytochromes P-450 (e g. cytochrome P-450p). Microsomes derived from untreated rats contain appr. 17% cytochrome P-450p /88/.

Other hepatic drug metabolizing enzymes which are induced by HCB are the cytochrome P-450 reductase /57/, glucuronyl transferase /64, 65/, epoxide hydrolase /37/ and cytosolic glutathione S-transferase (with CDNB as substrate) /59, 66/. The isoenzyme pattern of glutathione S-transferases after induction with HCB resembles the pattern obtained after induction with phenobarbital /66/.

TABLE 2

Induction of Cytochrome P-450 Isoenzymes by Hexachlorobenzene

Inducer	Cytochrome P-450 isoenzyme					
	P-450a	P-450b+e	P-450c	P-450d	unknown	total
	Absolute values (nmol/mg of protein)					
Control (corn oil)	0.05	0.025	0.015	0.055	0.54	0.69
	Induction Factor (induced/control)					
Hexachlorobenzene	2.3	48	13	8	0.04	2.8
Pentachlorobenzene	1.0	13	1.0	1.6	1.0	1.6
Pentachlorophenol	1.0	2.0	1.0	1.2	1.2	1.2

The concentration of the cytochrome P-450 isoenzymes in rat liver microsomes were determined by immunoquantitation. The concentration of the cytochrome P-450 isoenzymes after induction by the chlorinated benzenes is expressed relative to the concentration of control animals. The total amount of cytochrome P-450 was determined spectrally, while the amount of "unknown" isoenzymes is calculated from the difference between the total amount of cytochrome P-450 and the sum of the isoenzymes P-450a – P-450e. Data taken from Li et al (63).

It has been established that the induction of cytochrome P-450 by HCB is not caused by impurities /64/. As for the metabolites of HCB, the major metabolite pentachlorophenol does not induce P-450 /42, 62/. The confusion on this matter is due to the fact that early studies with this compound were performed with pentachlorophenol, containing PCDD's and PCDF's as impurities /64/. Lower chlorinated benzenes (penta, tetra and trichlorobenzenes) induce cytochrome P-450 to some extent /58/, but the amount of these compounds produced as metabolites of HCB is insufficient for them to act as an inducer *in situ*. This inevitably leads to the conclusion that HCB itself is responsible for the induction described above.

2.6 The Nature of the Cytochrome P-450 Isoenzymes Involved in the Hydroxylation of Hexachlorobenzene

Studies using microsomes from rats treated with various inducers of cytochrome P-450 showed that hexachlorobenzene itself induced its microsomal biotransformation to the largest extent, while phenobarbital-induced microsomes were also relatively effective. Microsomes from non-induced rats and rat treated with 3-methylchlolanthrene were not able to convert HCB to any significant extent /34, 41/. However, when using purified isoenzymes in studying the biotransformation of HCB, the rate of conversion as measured for the major isoenzymes induced by phenobarbital, P-450b and P-450e, which are also the major isoenzymes induced by HCB /62, 63/ could not account for the microsomal rates. Furthermore, using specific monoclonal antibodies against cytochrome P-450 isoenzymes, it also appeared that P-450b and P-450e were not the major isoenzymes involved in the hydroxylation of HCB and PCP /67/. Recently, microsomes from rats treated with inducers of P-450p (dexamethasone, pregnenolone α-carbonitrile) proved to be very efficient in the hydroxylation of HCB and PCP, while specific inhibition of P-450p by antibodies decreased this conversion to approximately 20% of the values without antibody (Van Ommen, unpublished results). Furthermore, selective inhibition of P-450p in microsomes by formation of a specific enzyme-metabolite complex with triacetyloleandomycin (TAO) did also reduce the biotransformation of HCB and PCP /48/.

III. POSSIBLE REACTIVE INTERMEDIATES IN THE BIOTRANSFORMATION OF HEXACHLOROBENZENE

3.1 General

As indicated, a relation is suspected between the biotransformation and porphyrinogenic action of hexachlorobenzene. A number of stable metabolites have been screened for their porphyrinogenic capacity and, at least *in vivo,* no metabolite has been found to be capable of inducing porphyria as strongly as HCB itself. A number of authors have suggested the involvement of a reactive intermediate in the inhibitory action towards uroporphyrinogen decarboxylase. Sinclair and Granick /38/ showed that a cell homogenate derived from cultures of embryonal chick hepatocytes in which porphyria had been induced, can induce porphyria in another culture, but only for a very limited period of time. However, Cantoni /68/ and Smith /87/ reported on an extractable and stable URO-D inhibitor from TCDD- and HCB-porphyric mice, while others /69/ report the same for rats. This stable inhibitor was thought to be different from the parent compounds, while indications were obtained for a covalent interaction between the TCDD derived inhibitory extract and the decarboxylase /68/. Since the extracts were free of porphyrins, inhibition of URO-D by modified substrates of the enzyme (porphyrin adducts or oxidized uroporphyrinogen) can also be ruled out. Furthermore, these experiments exclude the involvement of reactive oxygen species generated by cytochrome P-450 isoenzymes, since previous to the inhibition experiments, the extracts were treated in a manner that excludes the survival of *in vivo* generated reactive oxygen species. An analogy thus exists between the porphyrinogenic action of HCB and TCDD (both produce a stable and extractable inhibitory species which is neither the parent compound, neither a porphyrin, and neither a reactive oxygen species), but no direct evidence for the involvement of metabolites in the porphyrinogenic action of TCDD has been described. Metabolites of TCDD have not been tested for their porphyrinogenic potentials /68/. TCDD is porphyrinogenic in much lower concentrations as compared to HCB and, like HCB, is very poorly metabolized.

Furthermore, it is possible that covalent binding of metabolites is involved in the HCB-induced tumor formation. Although the data on the carcinogenicity of PCP are conflicting, this compound has been

shown to produce chromosome changes during occupational exposure /70/. The ability of TCBQ to bind to DNA has been established /71/. TCHQ was reported to cause DNA strand breaks /72/. Therefore, the mechanisms of action leading to formation of reactive intermediates and covalent binding which may occur during HCB biotransformation will be discussed.

Since cytochrome P-450 has been suggested to play an important role in the porphyrinogenic action of HCB, and the first hydroxylation product of HCB *in vivo,* pentachlorophenol, is not porphyrinogenic, it stands to reason to assume a reactive intermediate produced during the first hydroxylation. Two types of intermediates during this reaction are conceivable, epoxides and radicals. In addition, phenols can be transformed into quinones, which also have alkylating properties. These three types of reactive intermediates are discussed separately.

3.2 Radicals as a Result of Dechlorination

The mechanism of reductive dehalogenation by cytochrome P-450, as described previously for carbon tetrachloride and halothane /73, 74/, may play a role in the formation of pentachlorobenzene. In this case, a pentachlorophenyl radical would be formed after a one electron reduction by cytochrome P-450.

This radical could either be reduced to pentachlorobenzene, or bind covalently to cellular macromolecules (Figure 3). Stewart and Smith /34/ report the microsomal formation of pentachlorobenzene from HCB by rat liver microsomes. This route would be expected to be enhanced under anaerobic conditions. However, such conditions did not give rise to an increase in formation of PCB or covalent binding to proteins /75/. In contrast, both the formation of PCP and covalent binding were reduced to zero-levels. Apparently this mechanism is not involved at least in the *in vitro* biotransformation of HCB. As mentioned previously, an alternative pathway for the formation of pentachlorobenzene has been proposed involving glutathione conjugation (Figure 1).

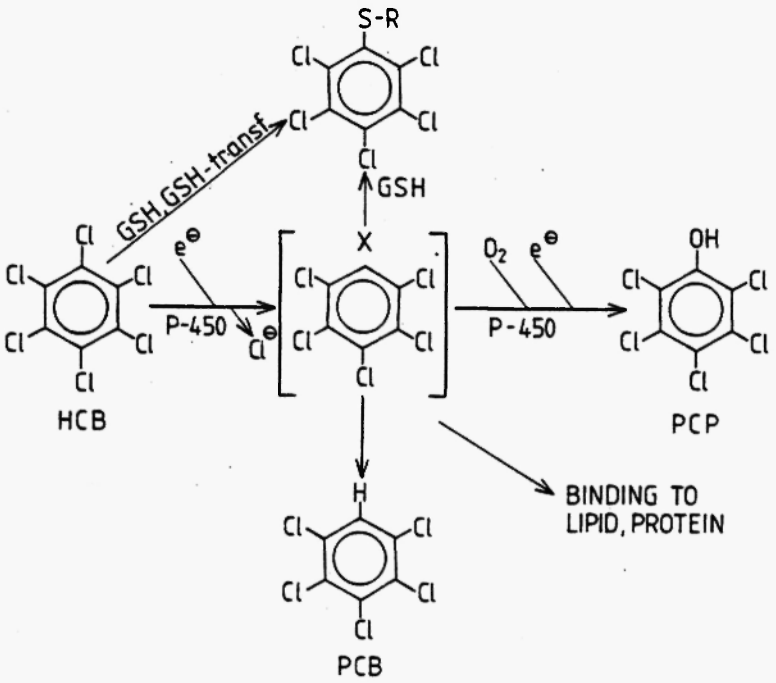

Fig. 3: Possible route of reductive dechlorination of hexachlorobenzene, and generation of an intermediate pentachlorophenyl radical. In the absence of oxygen, cytochrome P-450 bound HCB might take up one electron, resulting in loss of chlorine and the formation of a pentachlorophenyl radical. This radical may decompose to pentachlorobenzene, conjugate with glutathione or react with cellular macromolecules.

3.3 Epoxides Generated During the Oxidation of HCB and PCP

The microsomal hydroxylation of HCB occurs at a remarkably low rate. This is not due to a low affinity of cytochrome P-450 towards HCB. The spectral binding constant (apparent Ks) for binding of HCB to phenobarbital induced microsomal cytochrome P-450 was determined to be about 10μM (Van Ommen, unpublished results), while the overall apparent Km of the hydroxylation of HCB is 34μM /75/. Apparently, the chemical structure of HCB is highly resistant to hydroxylation.

There are two possible routes for the hydroxylation of HCB (Figure 4) namely: i) Attack of the reactive oxygen intermediate generated by cytochrome P-450 on an aromatic bond, resulting in epoxidation /76/ or ii) attack on an electron rich chlorine atom, resulting in the chloroso structure *4* (Fig. 4), as has been suggested to occur for other halogenated derivatives by MacDonald /77/. Substitution of the chloroso group with water would result in the formation of pentachlorophenol. However, except for the isolated example of iodosobenzene, nothing is known about the chemistry of such intermediates, making their formation and behaviour hard to predict.

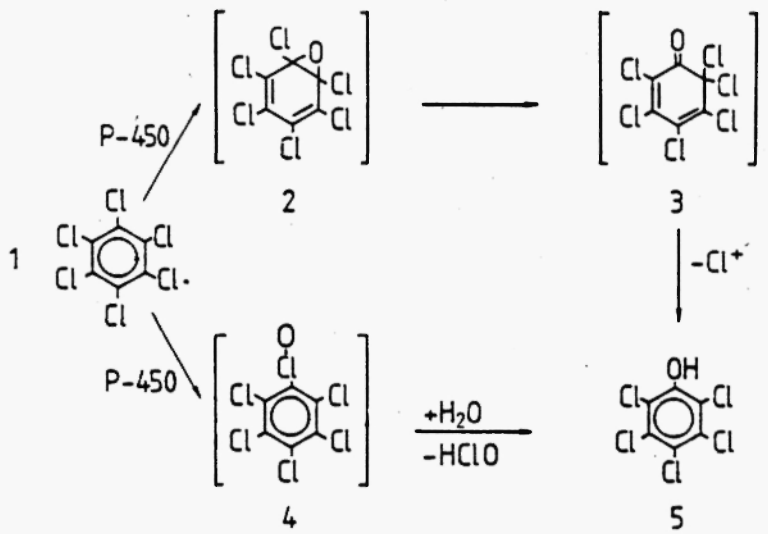

Fig. 4: Possible mechanisms of hydroxylation of hexachlorobenzene. Theoretically, pentachlorophenol /5/ may either be formed by epoxidation of HCB, followed by an NIH-like shift of chlorine and loss of a positively charged chlorine ion, or alternatively by formation of the chloroso structure 4, followed by substitution by water. Experiments with 180-1abeled H20 have ruled out the latter possibility.

Epoxidations are the usual primary reactions of cytochrome P-450 with aromatic compounds. Epoxidation of HCB, followed by a shift of a chlorine atom, analogous to the well-known NIH shift /76/, would result in compound *3*, 2,2,3,4,5,6-hexachloro-3.5-cyclodiene (Fig. 4),

which has been described as a donor of positively charged chlorine ions in organic synthesis /78/. Loss of Cl^+ results in pentachlorophenol formation. Addition of water to the 4-position of compound *3* (Fig. 4) might result in direct formation of the tetrachloro-1,4-benzoquinone. However, experiments with [^{18}O]-labeled H_2O have shown that no oxygen from H_2O is incorporated into either PCP or 1,4-TCHQ during the microsomal formation /75/. This provides indirect evidence for the involvement of epoxidation in the hydroxylation of both HCB and PCP. A scheme for the possible routes of oxidation of PCP to the 1,4- and 1,2-diols or quinones is presented in Figure 5.

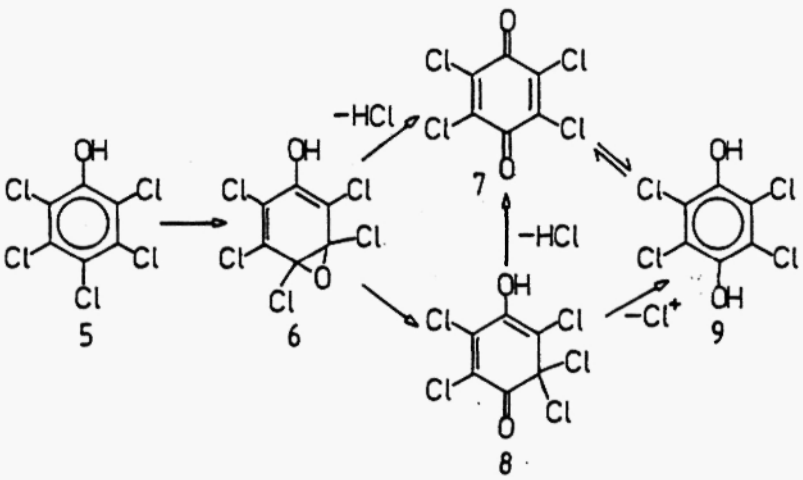

Fig. 5: Possible mechanisms of hydroxylation of pentachlorophenol. Epoxidation of PCP may either be followed by loss of chlorine and a proton, resulting in formation of tetrachloro-1,4-benzoquinone, or by loss of a positively charged chlorine, in analogy to the hydroxylation of HCB, resulting in formation of the hydroquinone.

Intermediate *3* (Fig.4) could not be detected by means of trapping with 2,4-dimethylphenol or 2,6-dimethylphenol during microsomal hydroxylation of either HCB or PCP, although this method has been successfully applied in studies on the dechlorination of CCl_4 /79/. The ketone intermediate, 2,2,3,4,5,6,-hexachloro-3,5-cyclodiene, is stable in crystalline form, but very rapidly eliminates Cl^+ in aqueous solutions (unpublished results). Epoxidation of PCP would lead to formation of

structure *6* (Fig. 5). Theoretically, *6* might lose HCl, giving rise to the benzoquinone *7* (Fig. 5), either directly or after the ("NIH") shift of the para positioned chlorine to the meta position. On the other hand, in analogy to structure *2*, compound *8* (Fig. 5) could release Cl^+, resulting in the formation of the 1,4-diol *9* (Fig. 5). The pKa value of PCP is 4.6 /34/. If the proton of compounds *6* or *8* is dissociated during the reaction catalyzed by cytochrome P-450, formation of tetrachloro-1,4-benzoquinone directly from PCP might be more likely. This would imply that oxidation of the hydroquinone /51/, is not relevant. However, in microsomal incubations tetrachloro-1,4-benzoquinone is not readily reduced to its hydroquinone form /51/, while in addition to covalent binding, a relatively large amount of the diol is detected in microsomal incubations of HCB /75/. Furthermore, the apolar nature of the cytochrome P-450 active site will not be conducive to the deprotonation of PCP. The Km for the oxidation of PCP by cytochrome P-450 is lower than the Km for the hydroxylation of HCB (13 and 34uM, respectively, Van Ommen, 71, 75), suggesting that PCP binds to the P-450 active site with a higher affinity than HCB. Since apolar interactions are very important in this binding /80/, it is likely that PCP is not deprotonated in the active site. Proof for the occurrence of these pathways will have to await the synthesis and characterization of the oxides *2* and *6* (Figures 4 and 5).

For the lower chlorinated benzenes, in general two routes of hydroxylation are possible: i) formal substitution of a chlorine atom with a hydroxyl group, analogous to HCB, and ii) formal oxidation of the carbon-hydrogen bond. In the case of pentachlorobenzene for instance, the first route results in the formation of 2,3,5,6-tetrachlorophenol, while the second route leads to PCP. Both compounds have been described as products of pentachlorobenzene formed *in vivo* /21, 81/. For tri-, di- and monochlorobenzene, chloro-substitution has not been detected.

3.4 Quinones as Reactive Metabolites

Microsomal experiments with HCB revealed the formation of tetrachloro-1,4-benzoquinone, a very reactive metabolite. Efficient reactions with proteins and DNA were observed. The formation of this

benzoquinone and covalent binding of this compound to protein was observed in microsomal incubations of HCB, PCP as well as tetrachloro-1,4-hydroquinone /51, 71, 75/. The proposed route of formation is shown in Figure 6. Besides the 1,4-quinone, the 1,2-quinone is also formed. The ratio between the 1,4- and the 1,2-isomer ranges from 1.2 to 4.9, depending on the type of microsomes used /75/.

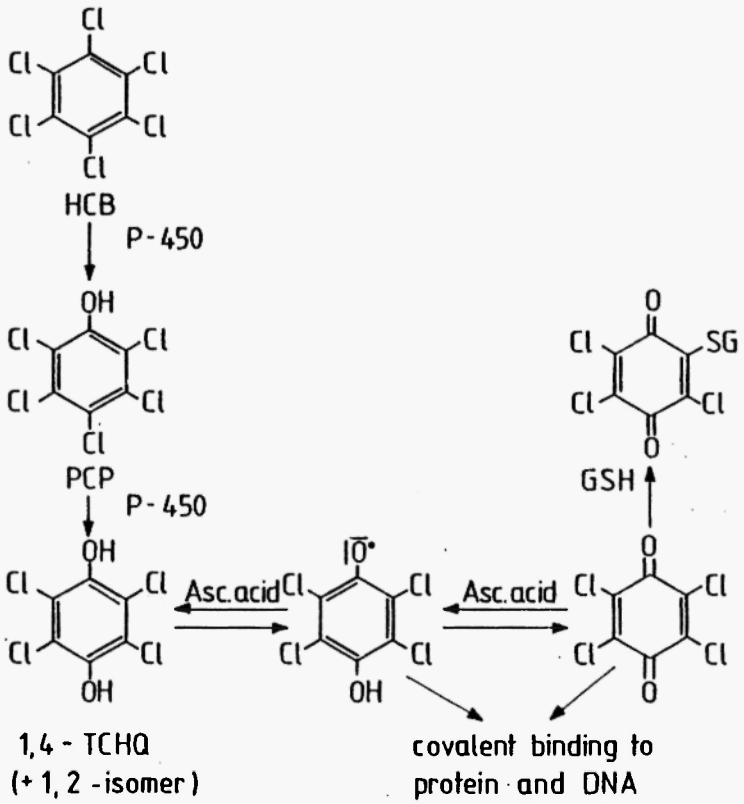

Fig. 6: Route of oxidative biotransformation of hexachlorobenzene

The quinones derived from HCB are unique in their high reactivity towards nucleophiles. This is due to the perhalogenated character of the tetrachlorobenzoquinones. The reaction with a sulfhydryl group results in loss of HCl, without reduction of the quinone. Other quinones which

have been described to be reactive towards protein, undergo a Michael type addition, resulting in a stable hydroquinone adduct (e.g. 82), which has to be reoxidized before being able to react again (Figure 7)

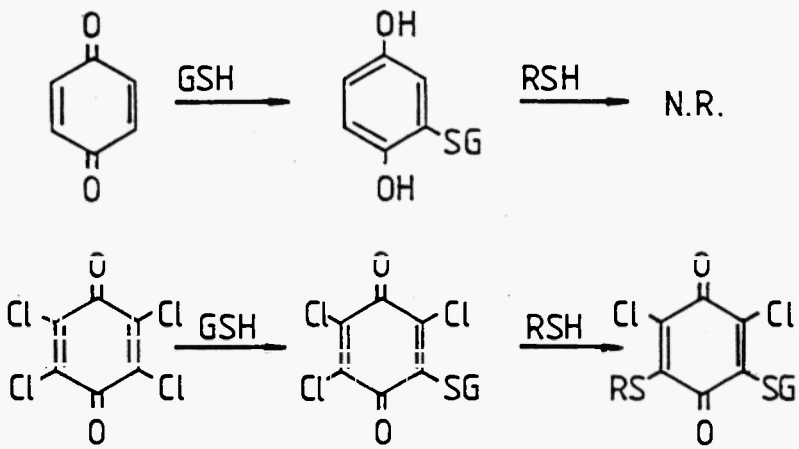

Fig. 7: The reaction of glutathione with 1,4-benzoquinone and tetrachloro-1 4-benzoquinone. In the latter case chlorine is substituted for the glutathionyl moiety, leaving the product in the oxidized form, and thus able to further react with other nucleophiles.

As a consequence, glutathione conjugates of tetrachlorobenzoquinones are still reactive towards protein. This is illustrated by the fact that in the reaction of tetrachloro-1,4-benzoquinone with glutathione not only monosubstituted conjugates are formed, but also di, tri and possibly fully substituted conjugates. /75/. A special example of the reactivity of the glutathione conjugate of tetrachloro-1,4-benzoquinone is the interaction of this compound with glutathione S-transferases. It appeared that the conjugate inhibited this enzyme in a irreversible way, at almost equimolar ratios. Evidence has been presented for the fact that the glutathione moiety of the compound "targets" the molecule into the active site of the transferase, while the quinone structure reacts with an essential sulfhydryl residue in or close to the active site, resulting in loss of activity /83/.

The involvement of the hydroquinones in the toxic action of halogenated benzenes has only been investigated for hexachlorobenzene and monobromobenzene /82/. A number of the tetra-, tri- and dichloro compounds are porphryrinogenic. It is tempting to assume a common mechanism of porphyrinogenic action for HCB and these lower chlorinated benzenes. The involvement of quinone metabolites in this mechanism is still a matter of debate.

Apart from covalent binding, a number of quinones show redox cycling capacities: a redox shuttle between the quinone and the semiquinone form, resulting in generation of reactive oxygen species during reoxidation, imposing a condition of cellular oxidative stress /84/. The factors determining the redox cycling capacities of quinones are not fully understood. Tetrachloro-1,4-benzoquinone does not possess this capacity /51/.

IV. IN VIVO COVALENT BINDING AS A RESULT OF BIOTRANSFORMATION OF HEXACHLOROBENZENE

The involvement of cytochrome P-450 in the development of HCB-induced porphyria seems to be firmly established. However, no metabolite or reactive intermediate of HCB has been positively identified as the ultimate porphyrinogenic compound. Are the benzoquinones, which are very reactive towards protein and DNA, responsible for the porphyrinogenic and carcinogenic action of HCB *in vivo*? A number of aspects of this question will be discussed.

Experiments with primary cultures of embryonal chick hepatocytes /67, 85/ have shown that, in contrast to microsomal incubations, the major route of biotransformation of HCB in intact cells does not lead to tetrachlorobenzoquinones, as was found for microsomal incubations, because there are effective protective mechanisms (reduction and conjugation) present in these cells to protect against covalent binding to protein of TCBQ. As a result, the amount of covalent binding as a percentage of total metabolites, formed from HCB is much smaller in these cultures than in microsomal incubations (appr. 1% and 7%, respectively). Incubation of 1 μM [^{14}C]-1,4-TCHQ with microsomes and NADPH results in 45% binding of radioactivity in 5 minutes, while in a hepatocyte culture only 3% binding is detected after 24 hours of incubation.

Koss /86/ detected a small amount of radioactivity attached to rat liver cytosolic proteins after a single oral dose of [^{14}C]-HCB. However, since no control experiments were performed, it is not possible to conclude that this binding stems solely from covalent binding of metabolites of HCB to protein. Smith et al /25/ partially purified the uroporphyrinogen decarboxylase from mouse livers after exposure to HCB and were not able to demonstrate covalently bound metabolites to this enzyme. However, since the ^{14}C-HCB was only administered once (5 days before section, 15 days after a single oral dose of unlabeled HCB, which was shown to be able to inhibit the uroporphyrin decarboxylase) it is possible that the major part of the enzyme had already been modified. Furthermore, the purification of the (inactive) enzyme from the HCB treated mice was performed after mixing with (active) uroporphyrinogen decarboxylase from untreated mice and screening for enzyme activity. However, Rios de Molina et al /26/ described that, with respect to the chromatographic procedures used, the modified enzyme behaved differently from the active enzyme. During the chromatographic procedures distinct radioactive peaks were observed.

The above mentioned facts suggest that the amount of covalent binding of TCBQ will be much smaller *in vivo* than in microsomes. It cannot be excluded that even a small amount of binding may cause toxicity, provided that a high degree of selectivity of the alkylating agent towards the site of modification exists. However, studies with benzene, phenol and chlorobenzene indicated that the covalent binding of these compounds, which afterwards was proven to be due to quinone metabolites, was relatively nonspecific. On the other hand the nephrotoxicity of bromobenzene appeared to be caused by glutathione conjugates of bromobenzoquinone, generated in the liver, which selectively exhibited their toxic action (which may be related to covalent binding) in the kidney /82/. As mentioned previously, the interaction of the glutathione conjugate of TCBQ with glutathione S-transferases is another example of a very specific kind of covalent binding resulting in damage to enzymes /83/. Perhaps the next search should be for the targetting effect towards the URO-D.

V. CONCLUSIONS

It is evident that a benzoquinone which is very reactive and is rather unique in its mechanism of reaction with nucleophiles, is generated during the microsomal metabolism of HCB and PCP. There is, however, no direct evidence available for the involvement of tetrachloro-1 4-benzoquinone in the toxicity of HCB. *In vivo*, powerful defence mechanisms against the effects of TCBQ exist (glucuronidation, reduction, conjugation with glutathione). On the other hand, a number of experiments suggest the involvement of TCBQ in HCB-induced porphyria. Inhibition of the major cytochrome P-450 isoenzyme involved in the hydroxylation of HCB and PCP resulted in both a strong decrease in excretion of PCP and TCHQ, as well as a diminished excretion of porphyrins. The search is still on.

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