THE IN VITRO BIOTRANSFORMATION OF HEXACHLOROBENZENE IN RELATION TO ITS TOXICITY

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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STELLINGEN

1. De tetrachloorbenzochinonen zijn de enige metabolieten van hexachloorbenzeen welke in staat zijn tot covalente binding aan eiwit.

   dit proefschrift

2. Covalente eiwitbinding van metabolieten van hexachloorbenzeen speelt geen directe rol bij de ontwikkeling van de door deze stof geïnduceerde leverporfyrie.

   dit proefschrift

3. De door Takazawa et al. vermelde spectrale bindingsconstante voor hexachloorbenzeen aan microsomaal cytochroom P-450 is te hoog omdat de oplosbaarheid van hexachloorbenzeen overschat is.

   R.S. Takazawa en H.W. Strobel, Biochemistry 25, 4804-4809 (1986)

4. De conclusie van Lunte et al. dat superoxide dismutase geen effect heeft op de oxidatie van het p-hydrochinon berust op een verkeerde interpretatie van hun resultaten.

   S.M. Lunte en D.T. Kissinger, Chemico-Biological Interactions 47, 195-212 (1983)

5. Het feit dat haem-achtige verbindingen sterke remmers zijn van glutathion S-transferases geeft aanleiding tot een herbezinning op de rol van deze enzymen in de biotransformatie van porfyrinogene stoffen.

6. Het ontbreken van een spectrale lijnverbreding van het FMN-semichinon ten opzichte van de geoxideerde vorm in het ^{31}P-NMR-spectrum van het cytochroom P-450 reductase wordt door Otvos et al. niet voldoende verklaard, met name omdat de rol van fosfolipiden buiten beschouwing wordt gelaten.

   J.D. Otvos, D.P. Krum en B.S.S. Masters, Biochemistry 25, 7220-7228 (1986)

7. De kloof tussen wetenschap en praktijk blijkt bijvoorbeeld uit het feit dat voor het commercieel belangrijkste enzym, het protease subtilisine, honderden modelmatige assays zijn ontwikkeld, terwijl de proteolytische activiteit ten opzichte van slechts twee intacte eiwitten is beschreven.

M. Philipp en M.L. Bender, Molecular and Cellular Biochemistry 51, 5-32 (1983)

8. Het gebruik van de "oxidant waarde" als maat voor de hoeveelheid oxiderende luchtverontreiniging is vanuit toxicologisch oogpunt gezien weinig relevant.

9. Biochemische oxidatie van veel hydrochinonen blijkt alleen bewerkstelligd te worden door een reductor.

10. De publieke afkeer van synthetisch bereide doch natuuridentieke geur- en smaakstoffen en de affectie voor vitaminepreparaten is blijkbaar niet alleen gebaseerd op het feit dat beide groepen verbindingen afkomstig zijn van de chemische industrie.

11. Aan in vitro onderzoek naar reactieve intermediairen dient aandacht te worden besteed in een vroege fase van het toxicologisch onderzoek.

12. Versnippering van het toxicologisch onderzoek dient vermeden te worden. Diepgaand toxicologisch onderzoek behoeft echter niet per definitie interdisciplinair te zijn.

RAWB advies inzake prioriteiten in het gezondheidsonderzoek

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6 maart 1987

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Aan Tjits,
en mijn ouders

VOORWOORD

Het is een goede zaak om in dit voorwoord duidelijk te maken dat meerdere mensen hun stempel op dit werk gedrukt hebben. In de eerste plaats geldt dit voor mijn wetenschappelijke steunpilaar en klankbord, Peter van Bladeren. Beste Peter, zonder jou had dit boekje er waarschijnlijk heel anders uitgezien. Ook mijn beide promotoren, de hoogleraren Koeman en Müller, hebben mij, door hun scherpe kijk op het geheel en hun bemoedigende woorden, op het goede pad gehouden. Van Hans Temmink durf ik te beweren dat ik zonder hem zelfs nooit aan dit werk zou zijn begonnen.
Lex Ronteltap, Roeland Hanemaaijer, Ria Vos, Eric Biessen, Luuk Brader, Lia van de Brink, Connie Oers, Ton Adang, Jos Bessems, Jan Willem Voncken en Roelofine van de Ruit hebben zich tijdens hun studie voor mij ingespannen en verdienstelijk gemaakt. Vooral Ton Adang moet ik bedanken (voor het introduceren van het koffiezetapparaat). Maarten Posthumus, bedankt voor de vele weken meettijd op jouw mooie machine. Jo Haas en de zijnen wil ik bedanken voor hun verleende expertise bij de proefdierexperimenten.
I would like to express my thanks to Paul Thomas and Wayne Levin of the Roche Institute of Molecular Biology, for the opportunity to perform some experiments at their lab, and their continuing willingness to co-operate. Their solid approach to science has impressed me.
Slechts een gering aantal mensen die een bijdrage geleverd hebben aan de tot stand komen van dit proefschrift zullen hun naam tegenkomen boven één van de hoofdstukken. Dit komt omdat niet alle bijdragen van wetenschappelijke aard waren. Zo is daar bijvoorbeeld de bijdrage van Tjits. Beste Tjits, hoewel je geen medeauteur werd, is jouw aandeel van zeer veel waarde geweest. Mijn collega's op toxicologie en biochemie hebben door raad en daad en door het uitlenen van menig apparaat, maar vooral door een prettige omgang, het samenwerken tot een plezierige bezigheid gemaakt. Ria en Roeland dank ik hartelijk voor de bewezen diensten als paranimf.
De vakmensen van de tekenkamer en fotografie van het Biotechnion verzorgden de plaatjes in en op dit proefschrift, en tot mijn genoegen is gebleken dat Manon, Janneke, Loes, Marleen, Ietje en Gré veel sneller kunnen typen dan ik.

SUMMARY

Hexachlorobenzene (HCB) has become a major environmental pollutant due to its formation as an unwanted byproduct in the industrial production of a number of chlorinated compounds, and because of its former use as fungicide. In laboratory animals, HCB induces tumor formation. In man and animals, HCB disturbes the hepatic heme biosynthesis, resulting in massive excretion of porphyrins. This porphyrinogenic action of HCB was shown to be due to a selective inhibition of the enzyme uroporphyrinogen decarboxylase. Evidence has been presented for the involvement of cytochrome P-450 in the porphyrinogenic action of HCB: in vivo induction of this enzyme by phenobarbital increased, and inhibition with piperonyl butoxide decreased the amount of excreted porphyrins. This led to the assumption that the inactivation of uroporhyrinogen decarboxylase could be attributed to a metabolite or a reactive intermediate formed during the oxidative biotransformation of HCB. The aim of the present study was to investigate the in vitro oxidative biotransformation of HCB, with special attention to the formation of reactive intermediates. In doing so, a more balanced evaluation could be made regarding the involvement of biotransformation in the toxic action of HCB.

As tools in the present study of the biotransformation of HCB, use has been made of, on the one hand, rat liver microsomes (particles of the endoplasmatic reticulum, which contain the cytochrome P-450 complex) and purified cytochrome P-450 isoenzymes, and on the other hand, a primary culture of chick embryo hepatocytes. Throughout the investigations, radiolabeled HCB and metabolites have been applied. This proved to be of great advantage in the tracing and quantification of very small amounts of products.

Evidence was obtained that rat liver microsomes were able to hydroxylate HCB. Pentachlorophenol (PCP) was detected in very small amounts (10 - 350 pmoles, depending on the type of microsomes used, in a 30 minute incubation of 50 nmoles of HCB). The hydroxylation of HCB appeared to be cytochrome P-450 dependent, as saturation of the microsomes with carbon monoxide, an inactivator of cytochrome P-450, almost completely inhibited the reaction. The formation of PCP amounted to 80-90% of the total metabolite formation. As a minor metabolite,

tetrachloro-1,4-hydroquinone (1,4-TCHQ) was identified. The apparent Km-values for the formation of PCP and 1,4-TCHQ were both determined to be 34 μM, with Vmax-values of 24 pmoles PCP/min/mg protein and 1.9 pmole 1,4-TCHQ/min/mg protein. Using ^{18}O-labeled H_2O, the origin of the oxygen atoms incorporated in PCP and 1,4-THCQ was determined to be molecular oxygen, indicating a sequential hydroxylation by cytochrome P-450. Furthermore, a small amount (5-10% of all metabolites) was detected to be covalently bound to microsomal protein. Since this binding was metabolism-dependent, attention became focussed on the identity of the metabolite or reactive intermediate involved.

Reductive dechlorination of HCB, resulting from a one electron reduction of HCB by cytochrome P-450, and giving rise to a pentachlorophenyl radical, was proven not to be involved in the process of covalent binding. Under anaerobic conditions, which in general stimulate reductive dehalogenation, the enzymatic hydroxylation was greatly reduced, while covalent binding also disappeared. Pentachlorobenzene, as a product of reductive dehalogenation, could not be detected.

Covalent binding to microsomal protein was also detected as a result of microsomal PCP-hydroxylation. If PCP was incubated at concentrations comparable to those formed in HCB-incubations, the amount of covalent binding was similar to the amount detected after HCB-incubations. This indicated that the covalent binding resulting from the microsomal conversion of HCB was due to a reaction product of PCP, formed in these incubations. Because no covalent binding could be attributed to the hydroxylation of HCB to PCP, the formation of reactive species during this reaction is improbable.

The covalent binding as a result of microsomal conversion of HCB could be prevented by addition of ascorbic acid to the incubation mixtures. The disappearance of covalent binding was accompanied by a proportional increase in the amount of TCHQ formed. Glutathione also inhibited covalent binding, but no increase in formation of 1,4-TCHQ was observed. These results led to the conclusion that the benzoquinone or semiquinone forms of the tetrachlorohydroquinones are the species involved in covalent binding to protein. Ascorbic acid prevents the oxidation of hydroquinones, while glutathione reacts with the benzoquinone, forming conjugates. The reaction of glutathione with tetrachloro-1,4-benzoquinone was studied

chemically, and evidence was obtained for the formation of mono-, di- and tri-substituted conjugates, still in the oxidized form.

Microsomal hydroxylation of PCP resulted, in addition to covalent binding, in the formation of tetrachloro-1,4-hydroquinone and tetrachloro-1,2-hydroquinone. The apparent Km-values for the formation of these hydroquinones was 13 μM, a value also measured for the formation of covalently bound products in PCP-incubations. Ascorbic acid influenced the covalent binding in the same way as observed in HCB-incubations. A conversion-dependent covalent binding to DNA was observed in incubations with DNA, which was 0.2 times the amount of binding to protein.

Using liver microsomes from rats treated with different inducers of cytochrome P-450, not only different rates of conversion of HCB and PCP were measured, but indications were also obtained for a preferential formation of either of the two isomers of the hydroquinone by different isoenzymes of cytochrome P-450. This matter has been studied in more detail, using purified cytochrome P-450 isoenzymes and monoclonal antibodies against cytochrome P-450b+e and cytochrome P-450c. It appeared that at least three different isoenzymes are involved in the hydroxylation of HCB. Purified cytochromes P-450b and P-450e exhibited a (very low) catalytic activity towards HCB, while selective inhibition of these isoenzymes in microsomes also resulted in a moderate decrease in formation of PCP. Moreover, microsomes from rats treated with dexamethasone, which induces cytochrome P-450p, displayed a 4.4 times higher rate of hydroxylation than microsomes treated with phenobarbital, which induces cytochromes P-450b and P-450e. It seems likely that P-450p is the major cytochrome P-450 isoenzyme involved in the hydroxylation of HCB. In a reconstituted system, PCP was converted to the hydroquinones by purified cytochromes P-450c and P-450d, and to a much lesser extent by cytochromes P-450b and P-450e. The first two isoenzymes mentioned preferentialy produced the 1,4-diol, while the latter two form more 1,2-TCHQ than 1,4-THCQ. Selective inhibition of the catalytic activity of P-450c in microsomes from rats treated with 3-methylcholanthrene (a potent inducer of cytochrome P-450c), and inhibition of P-450b+e in microsomes from phenobarbital treated rats did not result in a decrease in formation of diols, indicating that these isoenzymes were not involved in the microsomal hydroxylation. Microsomes from dexamethasone induced rats again

were the most potent in converting PCP, indicating the importance of cytochrome P-450p. These microsomes almost exclusively produce the 1,4-diol.

A high reactivity of tetrachloro-1,4-benzoquinone towards protein was detected. The oxidation of tetrachloro-1,4-hydroquinone to its benzoquinone was studied. Purified cytochrome P-450b appeared to be able to catalyze this reaction. However, in microsomes, cytochrome P-450 is not the only enzyme involved: carbon monoxide only partially inhibited the oxidation, as measured by covalent binding to microsomal protein, while even under anearobic conditions binding was still 39% of the amount found under aerobic conditions. Superoxide dismutase inhibited covalent binding in microsomes to almost the same extent, while oxidation by purified cytochrome P-450 was completely inhibited. This indicated that all oxygen-mediated oxidation of 1,4-TCHQ can be ascribed to the superoxide anion radical. However, covalent binding as a result of microsomal hydroxylation of PCP was not influenced by superoxide dismutase. This might indicate that TCHQ, as formed from PCP, is oxidized in the active site of cytochrome P-450, by superoxide anion radicals generated by this enzyme. The finding that 1,4-TCHQ stimulated the oxidase activity of cytochrome P-450 supports this hypothesis.

In order to obtain more insight in the biotransformation of HCB and the cellular protective mechanisms against alkylation damage, the metabolic route of HCB leading to the formation of the metabolites reacting with protein has been studied in a primary culture of chick embryo hepatocytes, using radiolabeled HCB, PCP and 1,4-TCHQ. Although covalent binding as a result of biotransformation of these compounds was detected, the relative amount was lower than found during microsomal incubations. It is clear that a number of mechanisms are available to protect against this binding. The hydroxylation of HCB and PCP in these cultures resembled the microsomal hydroxylation: the same inducers were effective, and PCP was the only extractable product detected. However, biotransformation of PCP did not result in accumulation of diols. Instead, a number of conjugation reactions prevented covalent binding by either a reaction with PCP or with the benzoquinones. Incubations with 1,4-TCHQ supported these findings: although a high degree of biotransformation was measured, only a relatively small amount of covalent binding was detected.

In view of these results, it is unlikely that covalent binding to protein, as caused by the oxidative biotransformation of HCB, is of major importance in vivo. A direct alkylation of uroporphyrinogen decarboxylase by TCBQ is most probably not involved in the process of HCB-induced porphyria. However, a relation between covalent binding of the tetrachlorobenzoquinones and the carcinogenicity of HCB and the mutagenicity of PCP may exist

CHAPTER 1

GENERAL INTRODUCTION

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 (Quinlivan, 1977). 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 (Quinlivan, 1977). In many countries, including the Netherlands (since 1973), 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 (NAS, 1975). For instance, HCB has been shown to be present in human adipose tissue, wild mammals, birds, fish and soil (for an overview: Courtney, 1979). The residual character has been the main reason for its restriction. The concern over HCB has even given rise to an international conference on this compound only (Morris and Cabral, 1987).

GENERAL TOXICITY

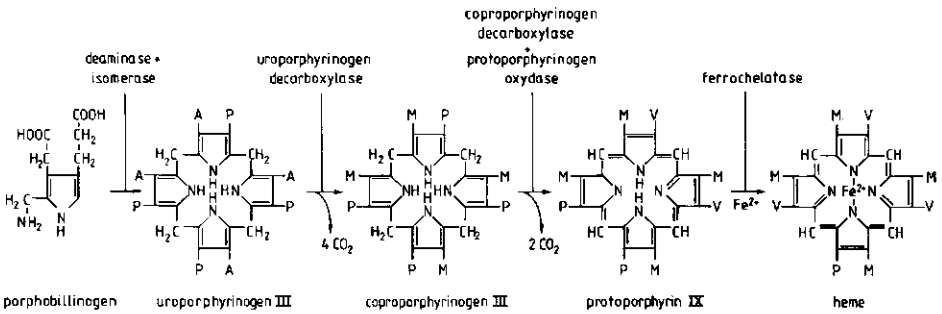
The (sub)acute toxicity of HCB is relatively low. LD50 values range from 2600-4000 mg/kg bodyweight (Strik, 1987). Chronic exposure, however, reveals a wide range of toxic effects. These effects include immunotoxicity (Vos, 1983), teratogenic effects (Khera, 1975), neurotoxicity (Peters, 1987), porphyria and tumor formation. The last two effects mentioned will be discussed in some detail. For a review of all toxicological data on HCB see Courtney (1979).

THE PORPHYRINOGENIC 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 heme synthesis (figure 1), 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) (Strik, 1980).

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 (Peters, 1976, Peters, 1982, Crips, 1984). 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. Eversince, the porphyrinogenic action of HCB has been the subject of a large number of studies.

Chronic exposure of rats to 500 mg/kg food of HCB results in the appearance of large amounts of porphyrins after 6-10 weeks (Goerz, 1977, Koss, 1978). 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



A = CH_2COOH, P = COOH, V = $CH{=}CH_2$, M = CH_3

figure 1. Part of the porphyrin biosynthetic pathway

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) (Smith and Francis, 1981). The excreted porphyrins predominantly consist of 8, 7, 6 and 5 carboxyl group containing porphyrins. The mechanism of inhibition is not clear. Kawanishi (1984) reported the direct inhibition on purified URO-D of HCB, Billy (1986) found no effect of HCB on cytosolic URO-D. Smith (1986) reported that mouse liver URO-D in vivo is not radiolabeled by ^{14}C-HCB. However, Rios de Molina (1987) showed that partially purified URO-D from HCB-porphyric rats is structurally different from the enzyme from untreated rats. Elder (1982), finally, showed that the amount of immunodetectable URO-D is unchanged after HCB-exposure, although its activity is greatly diminished.

THE EFFECT OF IRON ON THE PORPHYRINOGENIC ACTION OF HCB

Iron stimulates the development of HCB-induced porphyria (Taljaard, 1972, Smith, 1983). Khanna (1985) reported that iron stimulates the HCB biotransformation, although the time course of metabolite excretion did not coincide with the development of porphyria. Muckerji (1984) showed that 0.1 mM Fe^{2+} had a 50% inhibitory effect on URO-D. This inhibition could be a direct effect (binding to sulfhydryl groups of URO-D), or could be an indirect effect. Iron is known to stimulate the production of reactive oxygen species in the liver. A number of authors mentioned the hypothesis of HCB-porphyria mediated through lipid peroxidation (Smith 1986) or oxidation of porphyrinogens to porphyrins, which are competitive inhibitors of URO-D (Ferioli, 1984, Muckerji, 1984, Jones, 1981). However, Cantoni (1987) showed that URO-D is also inactivated in the absence of porphyrins.

There is a striking sex difference in the HCB-induced development of porphyria in the rat. After 103 days of exposure to HCB, female rats excrete 40 times more porphyrins in the urine than male rats, while the amount of porphyrins in the liver is more than 300-fold higher in females than in males (Rizzardini, 1982). Untreated female rats contain 3-5 times more hepatic non-heme iron than male rats (Smith, 1985),while the amount

of heme bound iron does not change after treatment with HCB (Wainstock de Calmanovici, 1985). Preloading of males with iron, however, did not stimulate the porphyric action of HCB, while long-term exposure to HCB resulted in a decrease in hepatic iron content in both males and females (Smith, 1985).

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 (Kerklaan, 1979, Debets, 1981, Wainstock de Calmanovici, 1984). 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 beta-naphthoflavone. Phenobarbital pretreatment shows an inhibitory effect on the development of porphyria in this species (Carpenter, 1984). In a culture of primary chicken hepatocytes, SKF-525A and piperonyl butoxide, both selective inhibitors of cytochrome P-450, inhibit the porphyrinogenic action of chlorinated hydrocarbons (Sinclair, 1974) and HCB (Debets, 1981a), while addition of 3-methylcholanthrene (Sinclair, 1984) and beta-naphthoflavone (Debets, 1981a), inducers of a cytochrome P-450 isoenzyme, together with HCB, also induced porphyria. Piperonyl butoxide also inhibits the development of porphyria in vivo (Smith, 1986). These findings have led to the hypothesis that 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. 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 (Goldstein, 1977, Goertz, 1978, Wainstock de Calmanovici, 1980). Pentachlorothiophenol, pentachlorothioanisol and its sulfoxide and sulfone were also found to be non-porphyrinogenic (Koss, 1979). Pentachlorophenol and tetrachlorohydroquinone, although not porphyrinogenic by themselves, are able to increase the porphyrinogenic action of HCB (Debets, 1980, Carpenter, 1985), the latter being the strongest synergist. In ovo exposure of chick embryo's to tetrachloro-1,4-hydroquinone, and to a lesser extent for other phenolic

metabolites, resulted in accumulation of porphyrins. HCB itself showed no porphyrinogenic effect (Billy, 1985). As far as the direct effect on URO-D is concerned, only tetrachlorohydroquinone and pentachlorophenol had an inhibitory effect (Billy, 1986). However, the concentrations used by far exceed the concentrations these compounds may reach in the liver as metabolites of HCB. HCB itself, although reported to inhibit purified URO-D (Kawanishi, 1984), caused no decrease in activity of cytosolic URO-D (Billy, 1986).

Summarizing the data presented so far, it is clear that HCB does not by itself inactivate URO-D, but whether a metabolite or reactive intermediate is involved, or by which mechanism iron is involved in this inactivation remains to be resolved.

THE CARCINOGENIC ACTION OF HEXACHLOROBENZENE

HCB has been shown to be carcinogenic in rats (Smith, 1980, 1985, Lambrecht, 1983, Arnold, 1985), mice (Cabral, 1979), and hamsters (Cabral, 1977). In all species the incidence of liver tumors was significantly increased after chronic exposure to at least 75 ppm HCB, while in hamsters and rats (Arnold, 1985), thyroid adenomas were reported as well. In the rat, renal cell adenomas were detected (Lambrecht, 1982).

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 (Smith, 1985). This phenomenon is strikingly similar to the development of HCB-induced porphyria. Of the three species mentioned, only in the rat females developed hepatic porphyria to a larger extent than males (Rizzardini, 1982). However, in the rat both renal and thyroid tumors were induced to a larger extent in males than in females.

THE BIOTRANSFORMATION OF HEXACHLOROBENZENE

GENERAL

In general, the nature of the process of biotransformation of foreign compounds is to increase the polarity of the xenobiotic, resulting in a faster elimination from the body of the product than its parent compound. Two kinds of reactions have been identified for this purpose:

1) transformation reactions (phase I reactions). The introduction of a polar functional group into the molecule. The reactions catalysed by cytochrome P-450 form the majority of this class.

2) conjugation reactions (phase II reactions). The xenobiotic, or the product of a phase I reaction, is linked to a polar endogenic molecule, mostly in an enzyme catalysed reaction.

An overview of the phase I and phase II reactions is given in table 1.

A fascinating phenomenon is that the biotransformation described above should not only be considered as a detoxification process, since there are

TABLE 1. PHASE I AND PHASE II REACTIONS INVOLVED IN BIOTRANSFORMATION OF XENOBIOTICS

reaction	enzyme	substrates
PHASE I REACTIONS (TRANSFORMATIONS)		
oxidation	mixed function oxidases	alkanes, alkenes, arenes, amines, thiones, thioethers
	FAD-containing monooxygenases	amines
	alcohol dehydrogenases	alcohols
	aldehyde dehydrogenases	aldehydes
reduction	mixed function oxidases	azo and nitro groups, N-oxides arene-oxides, alkyl halogenides aldehydes, ketones
	alcohol dehydrohgenases	aldehydes, ketones
hydrolysis	esterases	esters
PHASE II REACTIONS (CONJUGATIONS)		
with H_2O	epoxide hydrolase	epoxides
with glutathione	glutathione-S-transferases	electrofiles
with glucuronic acid	glucuronyl transferases	OH, COOH, NH
with sulfuric acid	sulfotransferases	NH, OH
with acetic acid	N- and O-methyl transferases	NH, OH
with acetic acid	N-acetyl transferases	NH

Adapted from Van Bladeren (1981)

a large number of examples available in which the foreign compound becomes toxic only after biotransformation (metabolic activation). Many of the examples known refer to substances which have mutagenic or carcinogenic properties. For example, from benzo(a)pyrene carcinogenic diolepoxides are formed by 1) oxidation catalyzed by cytochrome P-450, 2) hydrolysis of the epoxide by epoxide hydrolase and 3) oxidation by cytochrome P-450 of the remaining double bond (see figure 2)(Jerina, 1984).

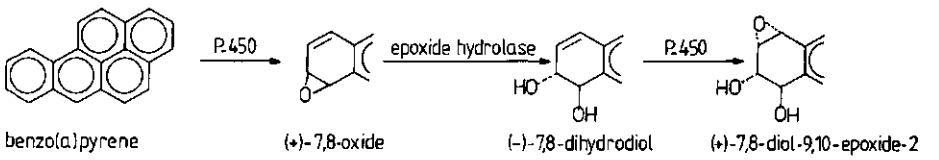


figure 2. The metabolic activation of benzo(a)pyrene. Adapted from Jerina, 1984.

Another example of a bioactivation reaction, in which glutathione is involved is represented by 1,2-dibromoethane. This compound has been shown to be carcinogenic in rats and mice (Olson, 1973). In vitro studies suggested glutathione conjugation, resulting in a reactive thiiranium ion, to be responsible for this action (Van Bladeren, 1980, figure 3). The DNA adduct of this intermediate has been isolated and characterized (Koga, 1986). Metabolic activation is dependent on the chemical structure of the compound. Examples are known for every enzyme involved in biotransformation.

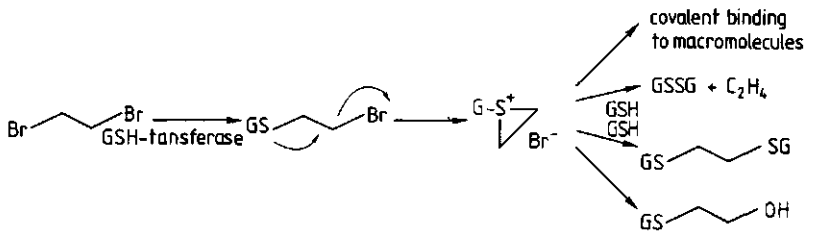


figure 3. The metabolic activation of dibromoethane. A reactive thiiranium ion is formed after conjugation with glutathione. Adapted from Van Bladeren (1980).

A noteworthy kind of activation occurs with a number of olefins, acetylenes and hydrazines. These compounds act as suicide inhibitors toward cytochrome P-450 because during the catalytic turnover of the substrate a reactive product is formed which covalently binds to the prosthetic heme group of the cytochrome P-450, thus inactivating the enzyme (Ortiz de Montellano, 1983).

CYTOCHROME P-450

The term cytochrome P-450, or P-450, is commonly used to denote a group of isoenzymes sharing a number of characteristics:

-all are iron containing hemoproteins of about 50,000 dalton molecular weight, showing an absorption maximum of approximately 450 nm, when carbon monoxide is bound to the reduced iron;

-all are membrane proteins, localized in the endoplasmatic reticulum, together with an electron donating system consisting of the NADPH-cytochrome P-450 reductase, cytochrome b5 reductase and cytochrome b5;

-besides a role in the metabolism of endogenous compounds, an important function is the oxygenase activity towards xenobiotics. They are monooxygenases, which implies the incorporation of a single oxygen atom,

TABLE 2. SUBSTRATE SPECIFICITY OF THE CYTOCHROME P-450 ISOENZYMES

SUBSTRATE	REACTION	ISOENZYME (rate in nmole/min/nmole P-450)									
		a	b	c	d	e	f	g	h	i	j
benzo(a)pyrene	3/9-hydrox.	0.2	0.4	23.4	0.3	0.1	0.1	0.1	1.8	<0.1	<0.1
hexobarbital	3-hydrox.	<0.5	42.0	0.0	<0.6	8.2	<0.5	1.1	22.6	1.0	<0.5
benzphetamine	N-demethyl.	2.3	132.0	0.7	3.9	19.8	1.3	4.9	52.1	2.8	5.5
7-ethoxucoumarin	O-deethyl.	0.6	9.6	97.0	0.6	2.0	<0.5	1.1	0.9	0.7	1.2
p-nitroanisol	O-demethyl	<0.5	1.8	21.6	0.7	<0.5	<0.5	<0.5	1.5	<0.5	1.6
aniline	p-hydrox.	<0.5	1.8	1.0	9.6	<0.5	<0.5	<0.5	1.5	<0.5	12.7
zoxazolamine	6-hydrox.	0.9	3.3	60.8	21.1	1.1	0.9	<0.5	7.4	0.7	3.4
estradiol 17B	2-hydrox.	<0.5	<0.5	1.7	13.8	0.9	0.5	0.6	8.0	1.3	0.8

Data taken from Ryan et al (Ryan, 1984, + refs)

derived from molecular oxygen, into the substrate, and catalyse the following reaction:

$$RH + NADPH + O_2 + H^+ \longrightarrow ROH + NADP^+ + H_2O$$

The highest concentration of cytochrome P-450 is found in the liver, although in many other organs its presence has also been established.
In the rat, in which the cytochrome P-450 system is most thoroughly studied, at least 11 hepatic P-450 isoenzymes have been purified, all with different activities toward a number of specific substrates (table 2). Although not uniformly adopted, the most widely used nomenclature is the one from Levin and co-workers, naming the isoenzymes alphabetically.
Most cytochrome P-450 isoenzymes are inducible. Form b, c, d and e, which are present only in trace amounts in non-induced rats, are the isoenzymes which are most frequently induced by xenobiotics (table 3). P-450b, the phenobarbital inducible isoenzyme, is the most widely studied form. Cytochrome P-448 is the obsolete name for P-450c, the isoenzyme inducible by polycyclic hydrocarbons. Isoenzymes f, g, h and i are so-called "constitutive" P-450's, and are isolated from non induced rats. At least one isoenzyme is known which is not named and charactarized by Levin et

TABLE 3. INDUCTION OF CYTOCHROME P-450 ISOENZYMES

INDUCER	TOTAL CYTOCHROME P-450 (nmole/mg)	ISOENZYME a	b+e	c	d	unknown (% of total amount of cytochrome P-450)
none	0.86	6	4	3	5	82
isosafrole	2.33	5	17	16	38	24
3-methylcholanthrene	1.83	14	<1	78	24	-16
beta-naphthoflavone	1.96	9	<1	71	24	-4
TCDD	2.51	13	<1	60	20	7
aroclor 1254	3.67	7	37	27	22	7
gamma-chlordane	1.88	4	46	3	1	46
phenobarbital	2.18	6	55	1	2	36
SKF 525-A	2.26	4	35	4	1	56
PCN	1.34	5	3	2	1	89

Data taken from Thomas et al, 1983

al. This isoenzyme, P-450-PCN or P-450p is induced by glucocorticoids and macrolide antibiotics (Wrighton, 1985, 1985a). The mechanism of induction has only been estabished for P-450c and d, for which it is receptor-mediated. For a state of the art of mechanisms of induction, see Goldstein (1984).

Against most of the purified cytochrome P-450 isoenzymes, specific monoclonal antibodies have been produced (Thomas, 1981, 1983). These antibodies are well suited as probes for the structure and function of different forms of cytochrome P-450. They can be used for immunoquantitation purposes (Parkinson, 1983), as well as for specific inhibition of isoenzymes (Thomas, 1984, Reik, 1985, chapter 7 of this thesis).

The P-450 active site is characterized by its hydrophobicity. In general, apolar molecules have a high binding affinity toward P-450. The very broad substrate specificity (Nebert 1979) can only be partly explained by the existence of multiple isoenzymes. Most individual isoenzymes are able to react with a large number of substrates. Jerina and co-workers (Van Bladeren, 1984) have established a model for the dimensions of the active site and the stereoselectivity toward polycyclic hydrocarbons of cytochrome P-450c. It appears that the active site is a relatively open structure, with physical limitations at only a few sites. The stereoselectivity of the isoenzyme is due to the limited possibilities of orientation of the substrates towards the heme-iron (figure 4).

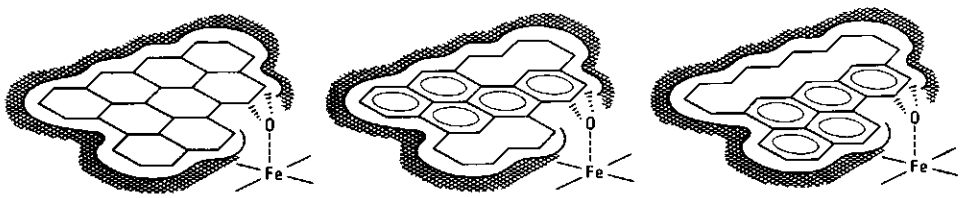


figure 4. Model of the active site of cytochrome P-450. a) The model as derived from stereochemical fittings of epoxidation sites of polycyclic hydrocarbons. b). Benzo(a)pyrene positioned for the 9,10-epoxidation. c) Benzo(a)pyrene positioned for the (favoured) (+)-7,9-epoxidation. Adapted Van Bladeren (1984).

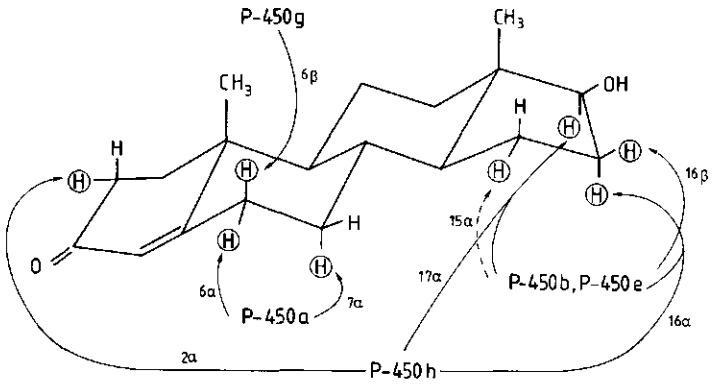


figure 5. The different stereoselective hydroxylation sites of testosterone by a number of cytochrome P-450 isoenzymes. Adapted from Waxman (1983) and the work of Levin and co-workers.

Another striking example of substrate specificity and stereoselectivity of the P-450 isoenzymes is the hydroxylation of testosterone. This steroid is a substrate for a number of isoenzymes. Each isoenzyme, however, has its own favoured stereoselective site of hydroxylation. For example, P-450a has a high affinity toward the 7-alpha position, while P-450b mostly hydroxylates the 16-alpha site (figure 5).

Three oxidative functions of cytochrome P-450 can be distinguished:

-the monooxygenase function;

-the oxidase function;

-the peroxidase function.

A scheme of these functions is presented in figure 6.

Acting as an oxygenase, the essential action of P-450 is presenting a highly reactive oxygen species to the substrate (White and Coon, 1980). The heme-iron is used to bind, reduce and stabilize oxygen, while the structure of the heme pocket is organized to present the substrate to the reactive oxygen. Starting in the oxidized (Fe^{3+}) form, the substrate binds close to the heme, thus causing the iron to shift from a low spin to a high spin state. In this state, the iron can be one-electron reduced, accepting an electron from the NADPH-cytochrome P-450 reductase. This reductase is a unique enzyme in that it possesses two flavin prosthetic groups, FAD and FMN, both of which play a role in transporting the

electrons from NADPH to P-450. Oxygen binds to the reduced iron and a second electron is presented, after which an iron stabilized oxene complex is formed, which reacts with the substrate. The product dissociates together with a molecule of H_2O, leaving the iron again in the oxidized form.

As an oxidase, the same reaction cycle is followed, but the oxygen, instead of reacting with the substrate, dissociates from the active site as a superoxide anion, leaving the iron in the oxidized form, with the substrate still bound to the active site. It appears that for many hydroxylation reactions of cytochrome P-450, the stoichiometry of the reaction (the amount of oxygen and NADPH used versus the amount of product) is not 1:1:1, as it should be, but a surplus of oxygen and reduction equivalents is used. This indicates the partial uncoupling of the oxygenase reaction (Gorsky, 1984), resulting in the formation of activated oxygen species. For example, the ethanol inducible P-450h (Ryan, 1984) exhibits a hydroxyl radical mediated oxidation of ethanol (Morgan, 1982).

Besides using molecular oxygen and reductase derived electrons, P-450 is also able to substitute O_2 and NADPH for organic peroxides, like cumene hydroperoxide (O'Brien, 1978).

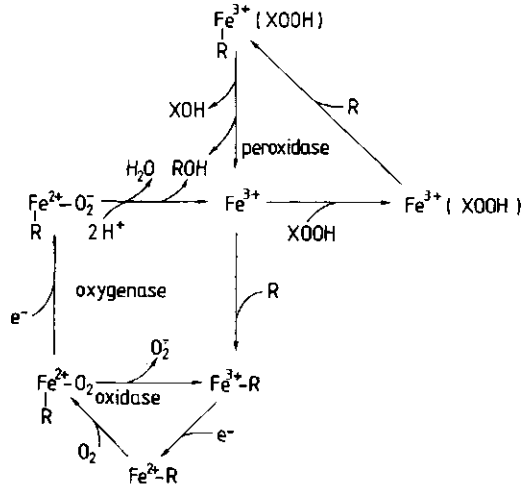


figure 6. Oxygenase, oxidase and peroxidase activity of cytochrome P-450. Adapted from Estabrook (1982).

Apart from the oxidative reactions, cytochrome P-450 sometimes acts as a reductive enzyme. If the substrate has a high affinity for the first electron donated to the complex, the iron returns to the oxidized form, preventing the binding of oxygen, and the substrate is reduced. Carbon tetrachloride, for example, dissociates into a $CCl_3^{\cdot}$ radical and a chloride ion (Uehleke, 1973). Some substrates, for example halothane and DDT, need anaerobic conditions to let the reductive route prevail (Baker and Van Dyke, 1984, 1984a). The reductive pathway is illustrated in figure 7 for DDT.

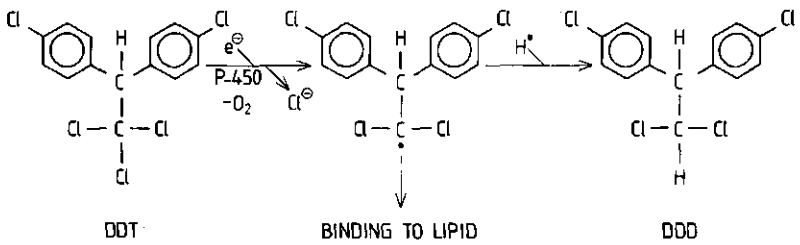


figure 7. Reductive dechlorination of DDT. Adapted from Baker (1984).

INDUCTION OF CYTOCHROME P-450 BY HEXACHLOROBENZENE

Hexachlorobenzene is an inducer of hepatic microsomal cytochrome 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 (Stonard, 1974, 1976, Blekkenhorst, 1978, Goldstein, 1978, Vizethum, 1980, Holme, 1982, Franklin, 1983). Carpenter (1985a, 1985b) 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 beta-naphthoflavone or 3-methylcholanthrene, while conversion of typical P-450b substrates like aminopyrine, pentoxyresorufin (Rozman, 1986), and

biphenyl (4-hydroxylation) is increased about as much as by phenobarbital induction. HCB shares this type of induction with polychlorinated biphenyls (Parkinson, 1983). The induction is accompanied by a significant increase in liver weight. Recently, two studies have been performed in which the total amount of P-450 isoenzymes has been estimated by means of mRNA induction (Goldstein, 1987), or immunochemical quantitation (Li, 1987). Both studies confirmed the mixed nature of induction. The results are presented in table 4. Other hepatic drug metabolizing enzymes which are induced by HCB are the cytochrome P-450 reductase (Holme, 1982), glucuronyl transferase (Goldstein, 1978, Rozman, 1986), epoxide hydrolase (Carpenter, 1985) and cytosolic glutathione S-transferase (with CDNB as substrate) (Carpenter, 1985, Debets, 1981b).

It has been established that enzyme induction by HCB is not caused by impurities (Goldstein, 1978). As for the metabolites of HCB, the major metabolite pentachlorophenol does not induce P-450 (Goldstein, 1977, San Martin de Viale, 1980, Li, 1987). 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 (Goldstein, 1977). Lower chlorinated benzenes (penta, tetra and trichlorobenzenes) induce cytochrome P-450 to some extent (Goldstein, 1982), but the amount of these compounds produced as metabolites of HCB is insufficient to act as an inducer in situ. This inevitably leads to the conclusion that HCB itself is responsible for the induction described above.

TABLE 4. INDUCTION OF CYTOCHROME P-450 ISOENZYMES BY HEXACHLOROBENZENE

INDUCER	INDUCTION FACTOR (induced/control)					
	P-450a	P-450b+e	P-450c	P-450d	unknown	total
CORN OIL	1	1	1	1	1	1
HEXACHLOROBENZENE	2.3	48	13	8	0.04	2.8
PENTACHLOROBENZENE	1.0	13	1.0	1.6	1.02	1.6
PENTACHLOROPHENOL	1.0	2.0	1.0	1.2	1.17	1.2
	ABSOLUTE VALUES (nmole/mg protein)					
	0.05	0.025	0.015	0.055	0.54	0.69

Data taken from Li, Safe, Levin et al (1987)

THE BIOTRANSFORMATION OF HEXACHLOROBENZENE

Hexachlorobenzene is very slowly metabolized. This is partly due to the fact that HCB is absorbed by the lymphatic system of the gastro-intestinal tract, thus avoiding the first pass liver circulation, and accumulates in adipose tissue (Iatropoulos, 1975, Mehendale, 1975). Twenty years after the dramatic case of HCB poisoning in Turkey, traces of HCB were still detected in maternal milk (Peters, 1976). The first study on the biotransformation of HCB revealed no metabolites (Parke and Williams, 1960). Since then, partly because of the HCB disaster in Turkey and partly because HCB became a worldwide pollution problem, the in vivo biotransformation of HCB has been widely studied. Koss (1978) showed that in a long-term exposure study in rats, 40 percent of the administered HCB is metabolized.

The metabolites of HCB can be devided into three groups:

-phenolic metabolites

-sulfur-containing metabolites

-lower chlorinated benzenes

A large number of metabolites have been detected. They are presented in table 5. Recent reviews on the biotransformation of hexachlorobenzene have been published by Renner (1981, 1984a, 1985).

The major metabolite of HCB is pentachlorophenol (PCP). PCP has been detected in the liver, faeces and urine of rats exposed to HCB (Koss,

TABLE 5. THE METABOLITES OF HEXACHLOROBENZENE

BENZENES	PHENOLES	SULFUR CONTAINING METABOLITES
pentachlorobenzene	pentachlorophenol	pentachlorothiophenol
1,2,3,4-tetrachlorobenzene	2,3,4,6-tetrachlorophenol	tetrachlorothiophenol
1,2,4,5-tetrachlorobenzene	2,3,5,6-tetrachlorophenol	pentachlorothioanisol
1,3,5-trichlorobenzene	2,4,5-trichlorophenol	tetrachlorothioanisol
	2,4,6-trichlorophenol	dichlorotetra(methylthio)benzene
		hexa(methylthio)benzene
		tetrachloro-1,4-di(methylthio)benzene
		1,4-dimercapto-tetrachlorobenzene
		4-methylthio-tetrachlorothiophenol
		N-acetyl-S-(pentachlorophenyl)cysteine

Adapted from Renner (1985)

1978). The second most important metabolite is tetrachlorohydroquinone. The other predominant metabolites are pentachlorothiophenol (PCThP) and pentachlorothioanisol. Both sulphur containing metabolites are interconvertable by the rat (Koss, 1979). 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 (Koss, 1976, 1978). The formation of lower chlorinated benzenes from HCB has been subject to discussion for some time. Renner (1984) suggested the formation of pentachlorobenzene to take place by a pathway he described as reductive desulphuration (figure 8). This implies the initial formation of a glutathione conjugate of HCB, which is subsequently converted stepwise into pentachlorothiophenol and pentachlorobenzene, respectively. Stewart (1986), however, reports the direct reduction of HCB to pentachlorobenzene by rat liver microsomes. The rate of this reduction, though, is extremely low.

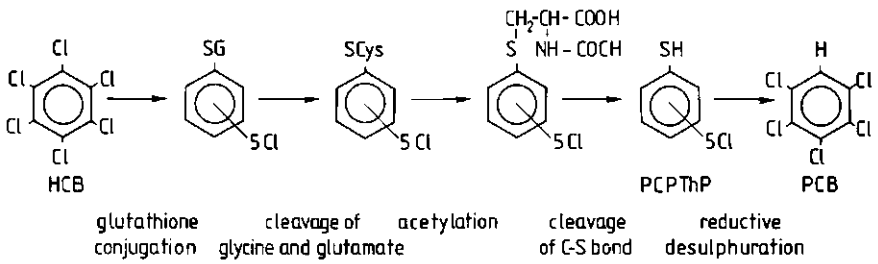


figure 8. Formation of pentachlorobenzene from hexachlorobenzene by the mechanism of reductive desulfuration, as proposed by Renner (1984).

POSSIBLE REACTIVE INTERMEDIATES IN THE BIOTRANSFORMATION OF HEXACHLOROBENZENE

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 toward

uroporphyrinogen decarboxylase. Sinclair and Granick (1974) 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 (1987) reports on an extractable and stable URO-D inhibitor from TCDD- and HCB-porphyric mice, and Billy (1987) reports the same for rats. The inhibition of URO-D may be caused by covalent binding. Therefore, the mechanisms of action leading to formation of reactive intermediates and covalent binding which may occur during HCB biotransformation will be discussed.

Since P-450 plays an important role in the porphyric 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 this reaction. Two types of intermediates 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.

1) EPOXIDES Reaction of the cytochrome P-450 generated oxene with a carbon-carbon double bond leads to the formation of an epoxide. The cytochrome P-450 mediated hydroxylation of benzene to phenol has been shown to involve an epoxide, as deduced by the nuclear induced hydrogen (NIH) shift (Jerina and Daly, 1974). The benzo(a)pyrene 7,8-diol-9,10-epoxide (figure 2) covalently binds to DNA, causing mutations and carcinogenity (Bentley (1977). Monohalobenzenes undergo epoxidation leading to specific hydroxylation products (MacDonald, 1984). Epoxides can react with water to form diols, either chemically or enzyme catalyzed. The enzyme involved is epoxide hydrolase, a microsomal enzyme which stereoselectively forms trans-diols (Oesch, 1979).

The formation of a HCB-epoxide would be greatly hindered by the presence of the bulky chlorine atoms. In the case of HCB, the NIH-shift would involve a positively charged chlorine ion as a leaving group. Other theoretical pathways of phenol formation out of a HCB-epoxide involve the formation of chloroso compounds, which can substitute with water to form pentachlorophenol. These mechanisms are discussed in more detail in chapter 8.

2) RADICALS AS A RESULT OF REDUCTIVE DECHLORINATION The mechanism of reductive dehalogenation by cytochrome P-450, as mentioned previously for carbon tetrachloride and halothane (figure 7), may play a role in the formation of pentachlorobenzene.

In this case, a pentachlorophenyl radical will 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 9). Steward (1986) reports the microsomal formation of pentachlorobenzene from HCB by rat liver microsomes. This route would be enhanced under anaerobic conditions.

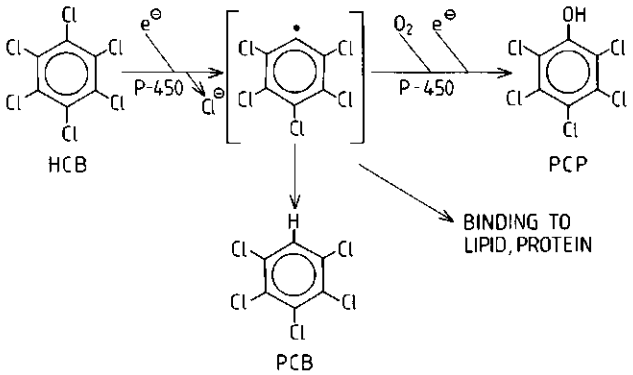


figure 9. Possible route of reductive dechlorination of hexachlorobenzene, and generation of an intermediate pentachlorophenyl radical.

3) QUINONES In the case of benzene, the ultimate reactive species appeared to be the 1,4-benzoquinone, formed after oxidation of the benzene diol, the second hydroxylation product of benzene by cytochrome P-450 (Tunek, 1980). Since tetrachlorohydroquinone is detected as an in vivo metabolite of HCB, this type of reactive compounds is of interest. Quinones are toxic by two different modes of action: covalent binding and redox cycling.

Covalent binding of quinones has for example been described for 2-acetamido-p-benzoquinone, a metabolite of 3'-hydroxyacetanilide

(Streeter and Baillie, 1985) and p-benzoquinone (Wallin 85). The proposed mechanism is a Michael addition to macromolecular nucleophilic groups, especially sulfhydryl groups. Glutathione inhibits covalent binding by forming a conjugate, most likely by the same type of reaction. An example is given in figure 10 for bromobenzoquinone (Monks, 1985).

Redox cycling as a mechanism of toxicity of quinones has been recognized for some time to be of major importance. A number of anticancer drugs, e.g. adriamycin (Smith, 1985) and toxic compounds exert their action in this way. Some enzymes are able to reduce quinones to their semiquinone radicals. For example, DT-diaphorase has a high affinity for quinones (Ernster, 1962), and the NADPH-cytochrome P-450 reductase has been shown to reduce the benzo(a)pyrene-3,6-quinone (Smith, 1985). The semiquinone radicals are able to be reoxidized, reducing molecular oxygen to superoxide anion radicals. This redox shutle results in a large comsumption of reduction equivalents, cellular oxidative stress, depletion of glutathione and cell death. (Kappus, 1985).

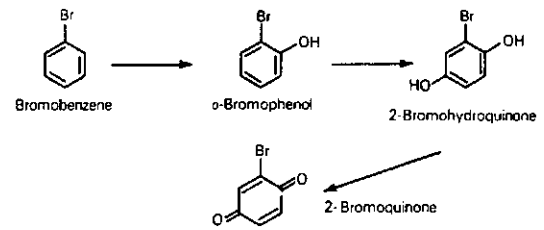


figure 10. The metabolic route of bromobenzene leading to the reactive bromobenzoquinone, and the stable glutathione-bromohydroquinone as product. Adapted from Monks (1985)

OBJECTIVES OF THE INVESTIGATION

Since the involvement of cytochrome P-450 in the development of HCB induced porphyria was established, a number of investigations have been carried out in order to determine which of the HCB metabolites was the actual porphyrinogenic agent. At the onset of the investigations presented in this thesis, no convincing evidence had appeared indicating the porphyrinogenic capacity of a particular metabolite. On the contrary, the major oxidative metabolite, PCP, had been shown to be non porphyrinogenic. This led to the hypothesis that a reactive intermediate was involved , generated during the hydroxylation of HCB to PCP (Debets, 1981). The general aim of the present study was to investigate the biotransformation of HCB, with special attention to the mechanism of generation of reactive intermediates. The approach chosen to elucidate this mechanism has been to study the reactions involved in vitro, since in vivo the reactivity of such compounds would greatly hinder their detection and isolation.

The first objective of this study was to prove the involvement of cytochrome P-450 in the hydroxylation of HCB. This is described in chapter 2. During the microsomal metabolism of HCB a small amount of metabolites was found to be covalently bound to protein. In chapter 3 the origin of this binding was studied. It appeared that PCP was further hydroxylated to tetrachlorohydroquinone, which in turn was oxidized to its benzoquinone. This tetrachlorobenzoquinone was postulated to be the actual covalently binding agent. The evidence for this mechanism of covalent binding to protein is described in chapters 4 and 5. Chapter 4 deals with the microsomal metabolism of pentachlorophenol, resulting in covalent binding to protein and DNA. In chapter 5 the driving force behind the oxidation of the tetrachlorohydroquinone to tetrachlorobenzoquinone is studied. In chapter 6, the determination of the cytochrome P-450 isoenzymes involved in the hydroxylation reactions of HCB and PCP is described. The last experimental chapter deals with the metabolism of HCB and PCP in a primary culture of chick embryo hepatocytes, in order to compare the microsomal data with a situation which more closely resembles the in vivo situation.

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CHAPTER 2

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

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ABSTRACT

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

INTRODUCTION

The fungicide hexachlorobenzene (HCB) has been shown to be hepatocarcinogenic (1) and is known for its porphyrinogenic action in man and animal (2). The accumulation of porphyrins is a result of inhibition of the enzyme uroporphyrinogen

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decarboxylase (3). Although it has been reported that HCB itself has an inactivating effect upon this enzyme in its purified form (4), there is also substantial evidence that metabolism of HCB is a prerequisite for this inactivation. Selective inhibition of cytochrome P-450 in a primary liver cell culture prevents the accumulation of porphyrins after HCB-exposure (5), while in vivo induction of P-450 by phenobarbital enhances the porphyrinogenic action of HCB (6-10). These experiments indicate the important role of cytochrome P-450 in the metabolism of HCB. Numerous studies have appeared on the in vivo metabolism of HCB, resulting in the identification of a large number of metabolites, e.g. chlorophenols, chlorobenzenes and sulphur-conjugated chlorophenols and -benzenes (11-13). The only investigation on the microsomal metabolism of HCB reported thus far (11) was performed with microsomes of non-induced rats, and the amounts of metabolites found were too small for quantitation. In the present study the metabolism of ^{14}C -labeled HCB by liver microsomes of rats pretreated with HCB and a number of other inducing agents was investigated and the amounts of metabolites were determined quantitatively.

METHODS

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

Preparation of microsomes: 14 week old male or female Wistar rats were pretreated (2 rats per treatment) with HCB (14 days 0.1% in chow, prepared from a 1% solution in olive oil (6)), phenobarbital (7 days 0.1% in drinking water), 3-methylcholanthrene (3 daily i.p. injections of 30 mg/kg body weight) and aroclor 1254 (one i.p. injection of 600 mg/kg body weight, sacrifice after 6 days). Livers were perfused with a 20 mM Tris-HCl 250 mM sucrose buffer, pH 7.4, homogenized with a teflon potter, and centrifuged for 20 minutes at 10.000 g. The supernatant was

centrifuged for 90 minutes at 105.000 g, and the pellet was washed in 150 mM KCl. The resulting pellet was resuspended in 100 mM potassium phosphate buffer, pH 7.4. Microsomes were stored at -25°C until use. Cytochrome P-450 was determined according to Omura and Sato (14). Protein concentration was determined by the method of Lowry (15).

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

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

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

RESULTS

Metabolites of HCB: In order to determine and quantitate the possible metabolites of hexachlorobenzene we have developed an HPLC-procedure which separates HCB and 8 potential metabolites (fig. 1a). Using this procedure it was found that the major product of the HCB-induced microsomal metabolism of HCB co-eluted with the pentachlorophenol marker, and consisted of approximately 90% of the radioactivity that eluted before the substrate. The only other detectable radioactive peak eluted together with tetra-chlorohydroquinone, and accounted for 4-15% of total metabolites. In order to prove the identity of the pentachlorophenol- and tetrachlorohydroquinone-peaks, an HPLC run was performed in which the tris-acetate buffer, pH 8, was exchanged for a 1% acetic acid solution in water (fig. 1b). As a result the tetrachlorohydroquinone eluted just prior to the chloranil, and the pentachlorophenol marker appeared close to the tetrachlorobenzenes. As expected, figure 1a and b show that only pentachlorophenol (pKa= 4.6), tetrachlorophenol (pKa= 7.0)

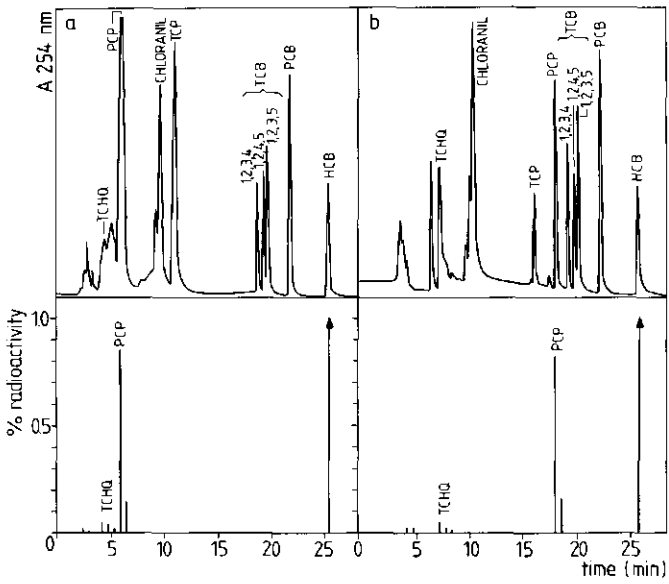

Figure 1. HPLC-analysis of metabolites of hexachlorobenzene converted by microsomes of male hexachlorobenzene induced rats.
a) Base run (tris-acetate pH8-buffer as the aqueous component), b) acid run (1% acetic acid as the aqueous component). For exact conditions and explanation of marker metabolites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

and tetrachlorohydroquinone were sensitive to changes in pH. The shifts in the radioactivity elution profile corresponded exactly with the shifts in the UV-elution profile. Pentachlorobenzene was not generated, nor was it metabolized: the trace amount of radioactive contamination with pentachlorobenzene was found unaltered after every incubation. The conversion appeared to be an enzymatic (boiled microsomes gave no conversion) and cytochrome P-450 dependent reaction: incubations without NADPH resulted in no detectable metabolites, while only 10% of the maximal conversion was reached after saturating the microsomes with carbon monoxide (Table 1). Variation of the HCB- and NADPH-concentration showed that both substrates were present in saturating amounts. At low levels (0.16-0.63μM) the

TABLE 1.
METABOLISM OF HEXACHLOROBENZENE BY MICROSOMES OF MALE HCB-INDUCED RATS

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

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

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

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

Covalent binding to protein: The protein fraction of microsomes of male HCB-induced rats contained 6.5 times more radioactivity than the same incubation with boiled microsomes (Table 1). Radioactivity in the protein fraction showed a linearity with the incubation time, while incubations without NADPH or in the presence of carbon monoxide gave values similar to the incubation of boiled microsomes. This implies that, besides the extracted metabolites pentachlorophenol and tetrachlorohydroquinone, a substantial part of metabolized HCB (between 5.5 and 10.8% of total extracted metabolites) was covalently bound to microsomal protein.

Differential metabolism in microsomes of HCB-induced male and female rats Microsomes of HCB-induced female rats converted only 30% of the amount of HCB metabolized by microsomes of HCB-induced male rats (0.23% and 0.77% resp. of total

TABLE 2.
THE EFFECT OF INDUCING AGENTS ON THE MICROSOMAL METABOLISM OF HEXACHLORO-BENZENE

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

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

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

The effect of different inducing agents on the microsomal metabolism of HCB

Besides HCB-microsomes, the microsomes of male rats induced with phenobarbital (PB), 3-methylcholanthrene (3MC) and aroclor 1254 were assayed for their ability to metabolize HCB. The results are shown in table 2; experiments with a doubled P-450 concentration gave comparable ratios for the conversions. After PB-induction the microsomal HCB-metabolism was 50% of that after HCB-induction per nmol P-450. 3MC was not able to induce the HCB-converting P-450 ('s). Aroclor 1254, a mixed inducer, raised product formation to 1/3 of the HCB-induced microsomes. Both PB- and aroclor microsomes produced pentachlorophenol as the predominant metabolite (84-94%), together with 1.5% tetrachlorohydroquinone.

DISCUSSION

In this report we demonstrate that hexachlorobenzene is metabolized by cytochrome P-450 and that pentachlorophenol is the only resulting metabolite. The formation of tetrachlorohydroquinone can be attributed solely to the conversion of pentachlorophenol (16). Since pentachlorophenol itself is not porphyrinogenic (17), it seems likely that a reactive intermediate with a strong affinity for protein is generated by P-450 during the turnover of HCB and that this intermediate is responsible for the porphyrinogenicity of HCB. The enhancement of covalent binding of radioactivity to protein during microsomal HCB-metabolism also points in this direction. Metabolism of labeled HCB both in primary liver cell culture (18) and in vivo (13) also results in covalently attached label to protein.

Our results however, do not explain the greater susceptibility of female rats to the porphyrinogenic action of HCB. Female rats produce up to 300 times more porphyrins than males (19). The finding that HCB-induced female rat liver microsomes metabolize HCB to a lesser extent than male rat liver microsomes is in agreement with the report of Franklin (19), who found the

same difference for a number of other substrates. Rizzardini however, reports no difference in urinary excretion of pentachlorophenol between male and female rats after exposure to HCB (20).

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

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

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CHAPTER 3

THE MICROSOMAL METABOLISM OF HEXACHLOROBENZENE - ORIGIN OF THE COVALENT BINDING TO PROTEIN

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ABSTRACT

The microsomal metabolism of hexachlorobenzene is studied, with special attention to the covalent binding to protein. The metabolites formed are pentachlorophenol and tetrachlorohydroquinone. In addition, a considerable amount of covalent binding to protein is detected (250 pmoles pentachlorophenol, 17 pmoles tetrachlorohydroquinone and 11 pmoles covalent binding in an incubation containing 50 µmoles of hexachlorobenzene). In order to establish the potential role of reductive dechlorination in the covalent binding, the anaerobic metabolism of hexachlorobenzene was investigated. At low oxygen concentrations no pentachlorobenzene was detected, and only very small amounts of pentachlorophenol as well as covalent binding, indicating a relationship between covalent binding and the microsomal oxidation of hexachlorobenzene. Incubations with ^{14}C-pentachlorophenol at low concentrations showed that a conversion dependent covalent binding occurs to the extent of 75 pmol binding per nmol pentachlorophenol. This is almost enough to account for the amount of label bound to protein observed in hexachlorobenzene incubations. This indicates that less than 10% of the covalent binding occurs during conversion of hexachlorobenzene to pentachlorophenol, and the remainder is

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produced during conversion of pentachlorophenol. The major product of microsomal oxidation of pentachlorophenol is tetrachlorohydroquinone, which is in redox-equilibrium with the corresponding semiquinone and quinone (chloranil). The covalent binding is inhibited by addition of ascorbic acid or glutathione to the hexachlorobenzene incubations. Ascorbic acid decreases the covalent binding with a simultaneous increase in formation of tetrachlorohydroquinone, probably due to a shift in the redox equilibrium to the reduced side. Glutathione does not act as a reducing agent, since the inhibition of covalent binding is not accompanied by an increase in tetrachlorohydroquinone formation. Instead glutathione reacts with chloranil, producing at least 3 stable products, probably in a Michael type reaction. These results strongly indicate the involvement of chloranil or the semiquinone radical in the covalent binding during microsomal hexachlorobenzene metabolism.

INTRODUCTION

Hexachlorobenzene (HCB) has been used worldwide as a fungicide and is formed as waste during the production of a number of chemicals. Although it is a hepatocarcinogen (1) it is perhaps best known for its disturbance of the hepatic porphyrin synthesis (2). Evidence has accumulated that HCB has to be metabolized in order to become porphyrinogenic: induction of cytochrome P-450 by phenobarbital enhances the porphyrinogenic action (3-6), while selective inhibition of cytochrome P-450 in a primary chick embryo liver cell culture decreases its action (7). We have previously investigated the microsomal metabolism of HCB, and shown that HCB is mainly converted to pentachlorophenol (PCP), while a small amount of tetrachlorohydroquinone (TCHQ) is also detected (8). TCHQ has been reported to be a metabolite of PCP (9). During the microsomal metabolism of HCB a substantial amount ($\pm$ 10%) of total metabolites becomes covalently bound to microsomal proteins. Since the porphyrinogenic action of HCB is due to an inhibition of the liver enzyme uroporphyrinogen decarboxylase, it is tempting to assume that this inactivation is caused by a covalent modification as a result of HCB metabolism. These considerations led us to investigate in detail the origin of the covalent binding.

MATERIALS AND METHODS

Chemicals: U-[^{14}C]-Hexachlorobenzene (Amersham UK, 70 mCi/mmol) contained 0.12% pentachlorobenzene (PCB) and 0.04% tetrachlorobenzene as radiochemical impurities. U- ^{14}C -pentachlorophenol (CEA, Gif-sur-Yvette, France, 35 mCi/mmol) contained 2.2% tetrachlorophenol as radiochemical impurity, determined using the HPLC assays described below. ^{18}O - H_2O (99%) was from Amersham. The origin of all other chemicals has been described elsewhere (8).

Microsomal incubations: Microsomes of male 12 week old Wistar rats induced with 0.1% HCB for 14 days were prepared as described previously (8). The standard incubation mixture contained 25 µM ^{14}C-HCB(3.5 µCi, in 50 µl acetone), 3 mM $MgCl_2$, 0.1 M potassium phosphate-buffer pH 7.4 and 4 mg microsomal protein in a final volume of 2 ml. The reaction was started by addition of 1 mM NADPH. PCP-incubations contained 0.05-1 µM ^{14}C-PCP (3.75-15 nCi in 50 µl acetone). After 10 minutes incubation at 37°C, the reaction was terminated with trichloroacetic acid (final conc. 3%). Total (>99.9%) soluble radioactivity was extracted with ether (2 x 3 ml). The extracts were dried with Na_2SO_4 and the ether was evaporated in a stream of nitrogen. Residues were dissolved in 50 µl of methanol for HPLC analysis. For anaerobic incubations, the incubation mixture was flushed with argon and degassed four times. HCB and NADPH were added by syringe through the seal, to start the reaction. Some anaerobic incubations also contained glucose oxidase (50 units) and glucose (10 mM). The oxygen concentration was measured in the gas-phase of tubes, treated similarly, but with non-radioactive HCB. 100 µl of the gas-phase was injected onto a GLC equipped with a catharometer (Pye Unicam CDC chromatograph, molecular sieve 5A 80-100 mesh column 5ft x 4 mm ID; carrier gas argon.

To be certain of equilibrium between gas- and liquid-phase the tubes were vigourously shaken before measurement. This method showed a range in oxygen concentration between 280-1000 ppm, while addition of glucose/glucose oxidase resulted in an oxygen concentration of 100-500 ppm.

HPLC-analysis of metabolites: Separation of HCB, TCHQ, chloranil (CAN, tetrachloro-p-benzoquinone), PCP and PCB was achieved by injection of the sample in 50 µl of methanol (see above) plus 10 µl of methanol containing markers, on a Perkin Elmer series 4 HPLC equipped with a Chrompack lichrosorb 150 x 4.6 mm 5RP18 column, eluted with a gradient of 60% methanol in a 0.1% acetic acid solution in water to 100% methanol (1.67% per minute for 15 min. followed by 1.5% per min for 10 min. and kept at 100% for 5 minutes). Flow rate was 1.0 ml/min. and detection

was at 254 nm. 1 ml fractions were collected and screened for radioactivity (5 ml atomlight scintillation fluid (NEN) in a Packard tricarb counter).

Covalent binding to protein: After ether extraction the aqueous (protein) samples were extracted with 3 x 10 ml methanol, 3 x 10 ml ethanol and 3 x 10 ml ether. After drying the protein pellets were dissolved in 1 ml Packard soluene 350 and 15 ml scintillation fluid and the amount of radioactivity was determined.

GC-MS-determination of ^{18}O-incorporation in PCP and TCHQ: Unlabeled HCB was incubated as described above for the radioactive compound, but in water containing 25% ^{18}O -H_2O. The ether extract was methylated with diazomethane and PCP-methylether and TCHQ-dimethylether were isolated by HPLC (isocratic elution in 100% acetonitril, 0.5 ml per minute on a Chrompack hypersil 290 x 4.6 mm 5 RP18 column, k' 2.0 and 1.7, respectively). After evaporation of the acetonitril the samples were dissolved in 5 ul of ether and injected into a GC-MS (VG MM7070). Samples were injected on a 3% OV-17/Chromosorb WHP column (2mm ID x 1.5m) using a temperature gradient of 160 °C to 200 °C at 6 °C per minute. Selected ion recording was performed for peaks at m/z 277.86 and 279.86 (molecular ion peaks of PCP-methylether, further termed M^+ and M^++2) and at m/z 258.89 and 260.89 (M^+ minus CH_3 peaks of TCHQ-dimethylether). The ratios between the integrated M^+ and M^++2 signals were calculated and compared with the corresponding values found for the reference compounds. Both PCP- and TCHQ-methylether derived from incubations in unlabeled water and the synthetic compounds were measured as reference.

RESULTS

Metabolism of HCB: As found previously (8), the only products of the microsomal oxidation of HCB are PCP (90% of total metabolites) and TCHQ (5-10% of metabolites), together with a small amount of material covalently bound to proteins. Of TCHQ, both the 1,2- and 1,4-isomers were detected, of which the 1,4-diol was the major fraction. The apparent K_m of the formation of PCP and TCHQ were both determined to be 34 μM with a V_{max} of 24 pmol PCP. mg protein^{-1}. min^{-1} and 1.9 pmol TCHQ/mg protein^{-1}. min^{-1} (fig. 1). The formation of TCHQ is linear with time during the 10 minutes incubation time, while the PCP-formation shows a slight deviation from linearity after 5 minutes.

Substitution of NADPH with NADH results in zero conversion (table 1), while simultaneous addition of both cofactors results in the same rate of metabolism as

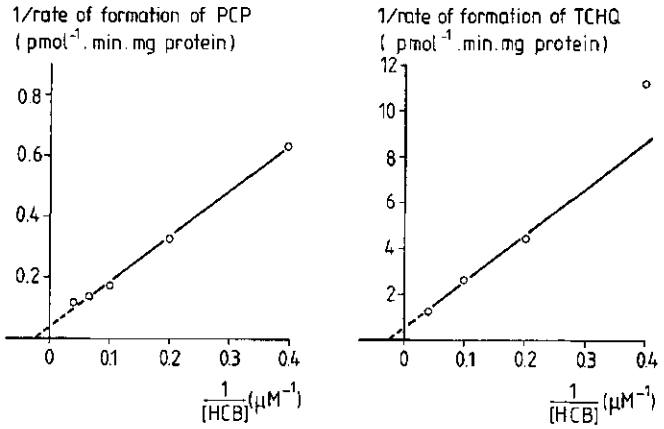

Figure 1. Lineweaver-Burke plots of the formation of PCP and TCHQ in the microsomal oxidation of HCB. 25 µm ^{14}C -HCB was incubated for 10 minutes in a suspension of microsomes derived from male HCB-induced rats (2 mg protein per ml), in the presence of 1 mM NADPH. Products were separated from HCB by HPLC and quantitated by measurement of radioactivity.

TABLE 1.
THE MICROSOMAL METABOLISM OF HCB

incubations	total conversion[a)] (pmol)	PCP (pmol)	PCB (pmol)	TCHQ (pmol)	covalent binding (pmol)
1 mM NADPH	260	220	0	14	15.2
1 mM NADH	35	0	0	4	0
1 mM NADPH + 1 mM NADH	265	225	0	20	7.3
low oxygen concentration	15-65	13-58	0	0	0-1.3

25 µM ^{14}C-HCB was incubated at a protein concentration of 2 mg/ml. Microsomes were derived from male HCB induced rats. Incubations were performed in a volume of 2 ml at 37°C. In the low oxygen concentration incubations oxygen was removed by repeated degassing and purging with argon in a sealed incubation tube. The upper and lower limit values of conversion at low oxygen pressure conditions are shown. Values in between show no PCB or covalent binding.
The variation in metabolism under low oxygen conditions is presumably due to the variation in oxygen concentration (see method section).

a) Total conversion denotes the total amount of extractable metabolites produced and is calculated from the total amount of radioactivity eluting from the HPLC before the substrate.

compared to the incubations with NADPH alone.

Under anaerobic or very low oxygen conditions, resulting in a PCP-formation of 4-26% as compared to aerobic conditions, the formation of PCP, PCB and covalent binding was measured. No PCB-formation was detected. Only at 26% PCP-formation a small amount of covalent binding was detected. (12% as compared to aerobic incubations, table 1). The variation in PCP-formation is probably due to the variation in oxygen concentration (100-1000 ppm in the gas phase, see method section).

As shown in table 2, there is no evidence for incorporation of oxygen from ^{18}O-H_2O into PCP or TCHQ. ^{18}O-incorporation would result in a 2 Dalton increase of the molecular weight, resulting in a shift in the ratios of the peaks. Both for PCP and TCHQ these ratios are unchanged after microsomal incubation of HCB in ^{18}O-H_2O, as compared to an incubation in ^{16}O-H_2O or the synthetic reference. The theoretical values for the peak ratios if oxygen is derived completely from H_2O are also stated in table 2.

TABLE 2.
ORIGIN OF THE OXYGEN IN PCP AND TCHQ

sample	PCP (M^++2/M^+ x 100)	TCHQ (M^+-13/M^+-15 x 100)
a) ^{16}O-H_2O	156.3 ± 2.7 (n=9)	127.6 ± 4.7 (n=10)
b) 25% ^{18}O-H_2O	158.3 ± 1.6 (n=7)	126.9 ± 3.6 (n=3)
synthetic reference	157.0 ± 1.0 (n=4)	126.5 ± 0.9 (n=10)
expected value, if oxygen is derived from H_2O[1)]	189.6	160.4 (1 x ^{18}O) 193.4 (2 x ^{18}O)

Unlabeled HCB incubations were performed under normal conditions (a) and in a system containing 25% ^{18}O-labeled H_2O. PCP and TCHQ were purified by extraction and HPLC of their methyl ethers and analysed by GC-MS (selective ion recording). The extent of incorporation of ^{18}O was determined by comparing the ratio's for peaks at m/z=277.9 and 279.9 for PCP-methylether (M^+ and M^++2) and at m/z=258.9 and 260.9 (M^+-15 and M^+-13) for TCHQ-dimethyl ether.

1) The expected value is calculated as follows: in the case of 25% ^{18}O-labeling, 25% of each mass peak will show a 2 mass-unit shift, resulting in a 25% decrease of each individual original mass peak and addition of this value to the peaks two mass units higher.

Covalent binding by Pentachlorophenol: At normal incubation conditions the amount of covalent binding of radioactivity to protein resulting from metabolism of HCB is 10.6 ± 2.3 pmol/4 mg protein (n=8). In order to establish whether this covalent binding originates from an intermediate generated during the formation of PCP from HCB, or from binding of PCP or a conversion product of PCP, we have incubated ^{14}C-PCP under the same conditions as ^{14}C-HCB but at PCP-concentrations which are comparable to the amount of PCP formed during HCB-metabolism (0.250 ± 0.035 nmol/4 mg protein/10 min. in a 2 ml incubation, n=8). The results are shown in figure 2. It appears that there is an almost linear relationship between the amount of covalent binding to protein and the PCP-concentration, with an initial slope of 75 pmol binding per nmol PCP. Figure 2 represents the covalent binding due to conversion of PCP, since the values are corrected for the binding of PCP itself, which amounted to 18 pmol binding per nmol PCP (derived from incubations with boiled microsomes and incubations without NADPH).

Inhibition of covalent binding: In the ^{14}C-HCB-incubations, ascorbic acid appeared to be able to completely inhibit the covalent binding at a concentration of 1 mM (fig. 3). At concentrations which show no decrease in total conversion, there is a

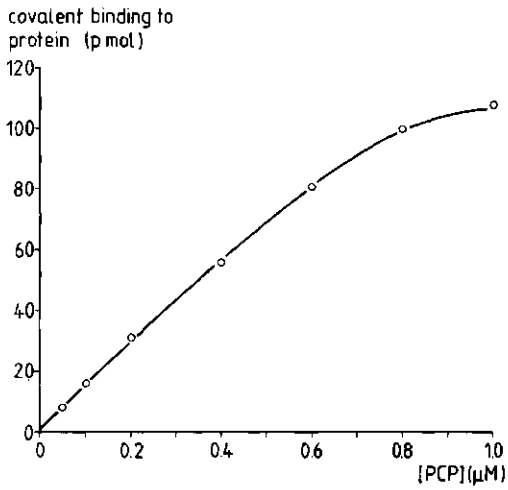

Figure 2. Covalent binding to protein as a result of metabolism of PCP in microsomal incubations at very low PCP-concentrations. The values are corrected for binding of PCP itself. Covalent binding was measured by determining the amount of radioactivity in protein fractions of microsomal incubations after extensive washing with organic solvents.

concentration-dependent decrease of covalent labeling. The loss of covalent binding is accompanied by an increase in TCHQ-formation: The concentration of TCHQ more than doubles after addition of 1 mM ascorbic acid to the incubation mixture. The amount of TCHQ which is produced due to addition of ascorbic acid is 60% of the amount of covalent binding wich disappears. In some experiments, part of the TCHQ is measured as chloranil (CAN, tetrachloroquinone), due to partial oxidation of TCHQ during solvent removal. Glutathione also inhibits covalent binding (fig. 3). As compared to ascorbic acid, glutathione shows a stronger inhibition: even at 0.01 mM an effect is observed. Up to 10 mM, the total conversion is not affected. In the case of glutathione however, no increase in TCHQ is observed. Similar results are also obtained with cysteine: inhibition of covalent binding , but no increase in TCHQ.

To find an explanation for these phenomena, the reaction of glutathione with CAN was studied. Addition of glutathione to a solution of CAN in methanol at equimolar concentrations does not give rise to the formation of TCHQ, but results in the formation of at least three stable compounds, termed 1, 2 and 3, of which 3 is the

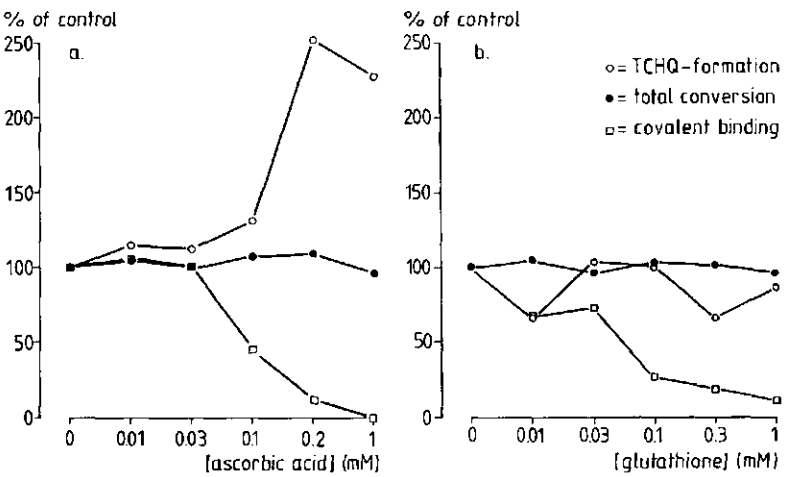

Figure 3. The effect of ascorbic acid (a) and glutathione (b) on the covalent binding, total conversion and TCHQ-formation in the microsomal metabolism of HCB. Total conversion (the total amount of soluble metabolites produced) and TCHQ-formation were determined by HPLC of extracted substrate and products. Covalent binding was measured by counting the radioactivity of protein samples after extensive washing with organic solvents. All results are expressed as the percentage of activity with respect to a control incubation without ascorbic acid or glutathione.

major compound (figure 4). These compounds are different from TCHQ, CAN and glutathione in their absorption spectra and HPLC behaviour. All products are water soluble. Upon reduction with ascorbic acid, fractions 1, 2 and 3 show a single absorption peak at 320, 330 and 335 nm, respectively. (TCHQ and CAN show single peaks at 310 and 286 nm, respectively.) Glutathione apparently does not act as a reducing agent towards the benzoquinone, but forms adducts.

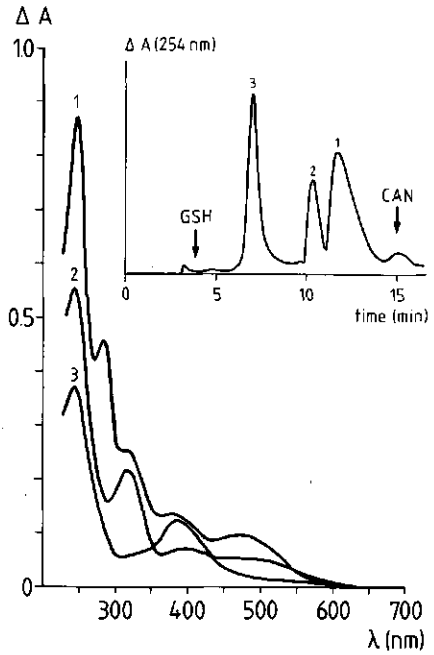

Figure 4. HPLC-analysis and absorption spectra of the 3 products of the reaction of glutathione with chloranil. HPLC separation was achieved on a reversed phase C-18 column, eluted isocratically with 50% methanol and 50% H_2O containing 0.4% acetic acid. Spectra were recorded in the same solution.

DISCUSSION

HCB is a very poor substrate for the cytochrome P-450 reaction. The low V_{max}-value corresponds with the in vivo data on the slow biotransformation of HCB (10). The HCB-concentration in the standard incubations (25 μM) is lower than the apparent K_m (34 μM). This is due to the very poor solubility of HCB. The near absence of

metabolites and covalent binding in the anaerobic incubations indicate that a reductive dechlorination, as suggested by Renner (11) is unlikely. This implies that the covalent binding probably cannot be a result of the formation of a pentachlorophenyl radical, analogous to the radicals detected under anaerobic conditions in the microsomal metabolism of carbon tetrachloride and halothane (12, 13).

Our results indicate that the covalent binding which occurs during the microsomal metabolism of HCB does not originate from an intermediate which is generated during the turnover of HCB to PCP by cytochrome P-450, but mainly results from the conversion of PCP. The amount of PCP which is produced during HCB-conversion in our incubation system after 10 minutes is 250 pmol, while the amount of covalent binding to microsomal protein is 10.6 pmol. The ^{14}C-PCP incubations show that 114

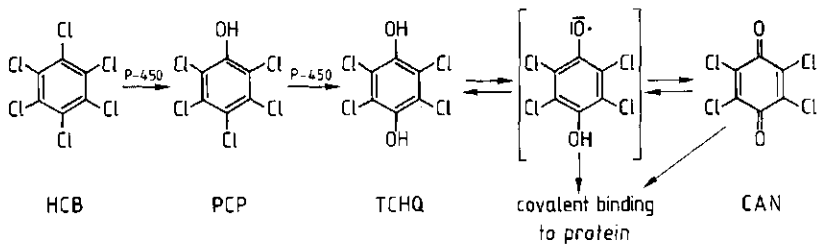

Figure 5. Proposed route of microsomal metabolism of HCB and mechanism of covalent binding as a result of metabolism.

pmol PCP is necessary for 10.6 pmol covalent binding. Although the conversion of HCB to PCP is not completely linear during the 10 minutes incubation time, the average amount of PCP available for conversion and binding during the incubation time will not largely exceed half of the amount which is formed after 10 minutes, e.g. 125 pmol PCP. This is 1.1 times the amount of PCP which produces the amount of covalent binding generated during HCB-turnover.

The major metabolite of PCP during metabolism by microsomes of HCB induced male rats is TCHQ. The results of the microsomal incubation of HCB in ^{18}O-labeled H_2O show that both oxygen atoms of TCHQ are derived from molecular oxygen. This rules out the possibility that TCHQ is (partly) formed by H_2O-substitution of PCP or an intermediate during the PCP-formation, and indicates a sequential hydroxylation of HCB to PCP and of PCP to TCHQ by cytochrome P-450. TCHQ can undergo a two step - two electron oxidation to the benzoquinone-form (CAN), with a semiquinone radical as the one electron oxidation intermediate (fig. 5). The existence of this semi-

quinone has been demonstrated by ESR and its lifetime was estimated to be 5 seconds (14). Either the tetrachloro-benzo-semiquinone radical might be responsible for the majority of the covalent binding which is measured after HCB metabolism (fig. 5) or chloranil itself, which might undergo a Michael type addition. The finding that, together with the loss of covalent binding as a result of the presence of ascorbic acid, the TCHQ-concentration is raised with a comparable amount, seems to indicate the involvement of CAN or the benzoquinone radical in the covalent binding.

The protective action of ascorbic acid is probably due to a shift in the redox equilibrium to the reduced form (TCHQ), thus lowering the concentration of the semiquinone and CAN and causing a loss of covalent binding. The redoxpotentials (E_o) of the chloranil-TCHQ and the dehydroascorbic acid-ascorbic acid equilibria are 0.71 V and 0.06 V, respectively (15), showing that 1 mM ascorbic acid is well capable of lowering the CAN concentration to zero levels. This also explains the rise of TCHQ concentration when the covalent binding is inhibited by ascorbic acid. The protective role of ascorbic acid against HCB-induced porphyria in chick embryo liver cell culture has been reported earlier (3).

Glutathione and cysteine are known to react easily with benzoquinones. The reaction with 2,6-dichloro-p-benzoquinone for instance is used in the determination of cysteine (16). In general, SH-groups react with the quinone in a Michael type reaction followed by loss of HCl. From a reaction at equimolar amounts of glutathione and CAN, 3 products are formed, but no TCHQ was observed. In this way CAN might also react with protein-SH-groups. Glutathione and cysteine will act as inhibitors in this process. This explains the fact that, in contrast to ascorbic acid, no increase in TCHQ is measured at concentrations of glutathione and cysteine which completely inhibit covalent binding.

Diethylmaleate, a GSH-depleting agent, has been reported to enhance the porphyrinogenic action of HCB in vivo (4,5). The antiporphyrinogenic activity of ascorbic acid in an in vitro culture system and the observation that ascorbic acid and glutathione inhibit the covalent binding without inhibiting the cytochrome P-450 activity suggest the involvement of covalent binding in the porphyrinogenic action of HCB. This covalent binding is mainly due to pentachlorophenol metabolism.

On the other hand, pentachlorophenol has been shown to be unable to cause porphyria or induce cytochrome P-450 isoenzymes in rats (17, 18, 19). We are presently investigating whether the lack of inducing capacity of PCP is responsible for its slow metabolism in vivo and lack of porphyrinogenic activity.

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CHAPTER 4

THE MICROSOMAL METABOLISM OF PENTACHLOROPHENOL AND ITS COVALENT BINDING TO PROTEIN AND DNA

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SUMMARY

The microsomal metabolism of pentachlorophenol (PCP) was investigated, with special attention to the conversion dependent covalent binding to protein and DNA. The two metabolites detected were tetrachloro-1,2- and -1,4-hydroquinone. Microsomes from isosafrole (ISF) induced rats were by far the most effective in catalyzing the reaction: the rate of conversion was increased 7-fold over control microsomes. All other inducers tested (hexachlorobenzene (HCB), phenobarbital (PB) and 3-methylcholanthrene) (3MC) gave 2-3 fold increases over control. There are indications that the 1,2- and 1,4-isomers are produced in different ratio's by various cytochrome P-450 isoenzymes: (a) Microsomes from PB and HCB treated rats produced the tetrachloro-1,4- and tetrachloro-1,2-hydroquinone in a ratio of about 2, while microsomes from rats induced with 3MC and ISF showed a ratio of about 1.3. (b) When pentachlorophenol was incubated with microsomes from rats treated with HCB a mixed type inducer of P-450, the ratio between formation of the 1,4- and 1,2-isomers decreased with increasing concentration of PCP suggesting the involvement of at least two P-450 isoenzymes with different Km-values. The overall apparent Km-value for HCB microsomes was 13 μM both for the formation of the

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soluble metabolites and the covalent binding to microsomal protein, suggesting both stem from the same reaction. The covalent binding could be inhibited by ascorbic acid, and this inhibition was accompanied by an increase in formation of tetrachlorohydroquinones (TCHQ). Although a large variation was observed in rates of conversion between microsomes treated with different (or no) inducers, the rate of covalent binding to microsomal protein was remarkably constant. A conversion-dependent covalent binding to DNA was observed in incubations with added DNA which was 0.2 times the amount of binding to protein (37 pmol/mg DNA).

INTRODUCTION

Pentachlorophenol (PCP) is used worldwide as a wood preservative and, due to its relatively slow biodegradation, forms a serious environmental problem. The best known toxic actions of PCP are its ability to uncouple the oxidative phosphorylation (1) and its inhibitory effect on cytochrome P-450 (2) and sulphotransferases (3). A carcinogenic effect cannot be excluded: the interpretations of long term carcinogenicity studies are controversial on this matter (for an overview: ref.4), while both in vivo (5) and in vitro (6) studies have demonstrated the DNA-damaging properties of PCP or PCP metabolites.

The metabolism of PCP has been studied in vivo and in vitro (7). In both cases, the primary oxidative metabolites were the tetrachloro-1,2- and -1,4-hydroquinones. In vivo, PCP and its metabolites are partly excreted as their glucuronides (7). The formation of diols was enhanced after treatment of rats with inducers of cytochrome P-450 both in vivo and in vitro (7,8).

Our interest in PCP originates from the fact that PCP is the primary oxidative metabolite of hexachlorobenzene. During the microsomal metabolism of hexachlorobenzene a substantial amount of covalent binding to protein was detected (9). Further investigation showed that this covalent binding did not occur during the first hydroxylation step, but rather after PCP was metabolized to the 1,2- and 1,4-diols. The diols can be oxidized to the quinone form, which binds covalently to protein (10). These results led us to investigate in detail the microsomal metabolism of PCP, with special attention to the covalent binding to protein and DNA.

METHODS

CHEMICALS. U-(^{14}C)-pentachlorophenol was purchased from CEA (Gif-sur-Yvette, France) and had a specific activity of 37.5 mCi/mmol. Depending on the batch, 0.5-1.8% tetrachlorophenol was present as a radiochemical impurity, as determined by the HPLC method described below. PCP was from Aldrich, tetrachloro-1,4-hydroquinone (1,4-TCHQ) from ICN pharm. N.Y., tetrachloro-1,4-benzoquinone (1,4-TCBQ) from Merck, 1,2-TCBQ and alpha-naphthoflavone from Janssen, Belgium, tetrachlorophenol and isosafrole from Fluka, NADPH from Boehringer Mannheim and 3-methylcholanthrene and Proteinase K (17 u/mg) from Sigma. 1,2-TCHQ was prepared by reduction of 1,2-TCBQ with ascorbic acid in methanol. The methylethers of PCP, Tetrachlorophenol, 1,2- and 1,4-TCHQ were prepared by reaction with diazomethane in diethylether (prepared from Diazald, Janssen).

ANIMALS AND TREATMENTS. 11-12 Week old male or female Wistar rats were treated with hexachlorobenzene (14 days 0.1% in chow), phenobarbital (7 days 0.1% in drinking water), 3-methylcholanthrene (3 daily i.p. injections of 30 mg/kg body weight) or isosafrole (4 daily i.p. injections of 150 mg/kg bodyweight). The preparation of microsomes has been described elsewhere (9).

MICROSOMAL INCUBATIONS. Standard microsomal incubation conditions were: ^{14}C-PCP (spec. act. 0.57 mCi/mmol, 100 µM, added in 50 µl of acetone), microsomes of HCB-induced rats (2 mg protein), 0.1 M potassium phosphate buffer pH 7.4 and 3mM $MgCl_2$, in a final volume of 2 ml. The reaction was started by addition of NADPH to a final concentration of 1 mM. After 5 or 10 minutes incubation at 37°C the reaction was stopped with HCl (final concentration 0.6N). Ascorbic acid, to a final concentration of 10 mM, was added to prevent oxidation of hydroquinones to quinones after the enzymatic reaction was stopped. Totale soluble radioactivity (99.6%) was extracted with 3x3ml acetone/ethyl acetate (1:2). The extracts were dried with Na_2SO_4 and methylethers were prepared by addition of a solution of diazomethane in ether. After removal of the solvent in a stream of nitrogen, the samples were dissolved in 50 µl of methanol for HPLC-separation.

HPLC-ANALYSIS OF METABOLITES. Separation of the methylated PCP and metabolites was achieved by injection of 50 µl of methanol containing the extracted metabolites plus 10 µl marker solution, on a Perkin Elmer series 4 HPLC, equipped with a Chrompack Lichrosorb 150x4.6 mm 5RP18 column, eluted isocratically with 82% methanol and 18% H_2O (see fig. 1). In order to check whether methylation was complete using the above conditions, the phenolic metabolites were also separated. The same

system was used, but the metabolites were eluted using a gradient of 50 to 85% methanol in a 1% aqueous acetic acid solution in 15 minutes, followed by a gradient of 85 to 100% methanol in 10 minutes. The k'-values were: 1,2-TCBQ 2.4, 1,4-TCHQ 3.2, 1,4-TCBQ 5.0, 1,2-TCHQ 7.6, PCP 8.2, 1,4-TCHQ-dme 13.7, tetrachlorophenol-me 14.2, 1,2-TCHQ-dme 15.0 and PCP-me 17.4. This method revealed a maximum of 1.2% of radioactivity to be non-methylated, of which at least 95% was PCP. The eluent was monitored at 254 nm and 0.6 ml fractions were collected for measurement of radioactivity in 3.5 ml Atomlight (NEN) in a Packard Tricarb scintillation counter.

COVALENT BINDING TO PROTEIN. After extraction of the soluble metabolites the precipitated proteins in the aqueous phase were washed with 3x10 ml of methanol, 3x10 ml of ethanol, 2x10 ml of acetone and 10 ml of diethylether. After drying the protein, pellets were dissolved in 1 ml soluene 350 (Packard). 15 ml scintillation liquid was added and the amount of radioactivity was determined. As a control on this method a number of incubations were treated as follows: the reaction was stopped by addition of sodium dodecyl sulphate (SDS) to a final concentration of 5% in order to prevent precipitation of the proteins in the presence of organic solvents. The aqueous phase was then extracted with 5x5 ml ethyl acetate, after which the aqueous (protein) phase was screened for radioactivity, while the ethyl acetate extract was prepared for HPLC-analysis as described above. Both methods showed the same amounts of covalent binding and soluble metabolites for similar incubations.

COVALENT BINDING TO DNA. To microsomal incubations of 2 mg of microsomal protein from HCB induced rats in 2 ml and containing 100 μM ^{14}C PCP with a Spec. Act. of 5 mCi/mmol, 1 mg of calf thymus DNA was added. After 5 min. incubation, the reaction was stopped by addition of 2 ml of phenol (water saturated). The water phase was extracted with equal volumes of phenol, phenol:chloroform 1:1 and chloroform, respectively. To the aqueous phase was added 0.25 mg Proteinase K in 0.5 ml of water, and this mixture was incubated for 15 hr at 37°C, after which the same extraction procedure was followed once again to remove residual protein and amino acids. Addition of 5 volumes of ethanol precipitated the DNA overnight at -20°C. After centrifugation the DNA was resuspended in Tris-acetate pH 7.4 (20 mM, 1 ml). The purity and recovery of the DNA were determined spectrophotometrically (11) and the amount of covalently bound radioactivity was determined after addition of 10 ml scintillation liquid (atomlight). DNA recovery was 38-58%, while the ratio in absorbance between 260 nm (DNA-absorption) and 280 nm (protein absorption) was the

same as measured for pure DNA (2.09 and 1.95 respectively). Formation of soluble metabolites in DNA incubations was determined in duplicate incubations which were treated as described under microsomal incubations.

RESULTS

IDENTIFICATION OF METABOLITES. Rat liver microsomes converted pentachlorophenol (PCP) into tetrachloro-1,4- and -1,2-hydroquinone. HPLC-separation of the methylated conversion products showed only radioactivity coeluting with added 1,4- and 1,2-THCQ-dimethylethers (figure 1). No other conversion products could be detected. No conversion occurred in incubations without NADPH. Using microsomes of hexachlorobenzene-induced male rats, the characteristics of the reaction were studied. With all protein concentrations used the rate of conversion was linear with time for 10 minutes. Doubling the protein concentration from 0.5 to 1 mg/ml

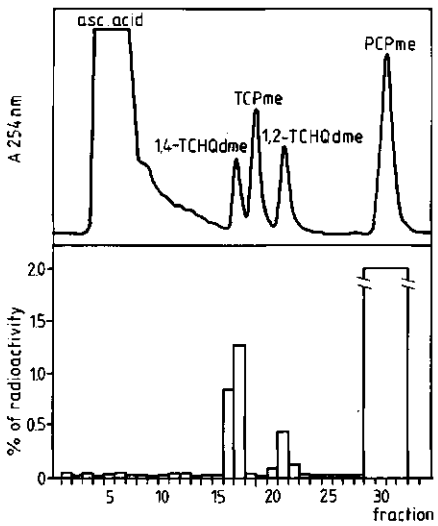

Figure 1. HPLC-analysis of methylated PCP metabolites. 2 mg of microsomal protein from HCB induced rats was incubated with 100 µM ^{14}C-PCP for 5 minutes as described in Methods. Metabolites were extracted, methylated and submitted to HPLC separation. Non radioactive methylated metabolites were added for identification. The upper trace shows the UV absorption of the eluate, while the lower trace shows the amount of radioactivity, expressed as percentage of total eluted radioactivity, of the corresponding fractions. (d)me denotes (di)methylether.

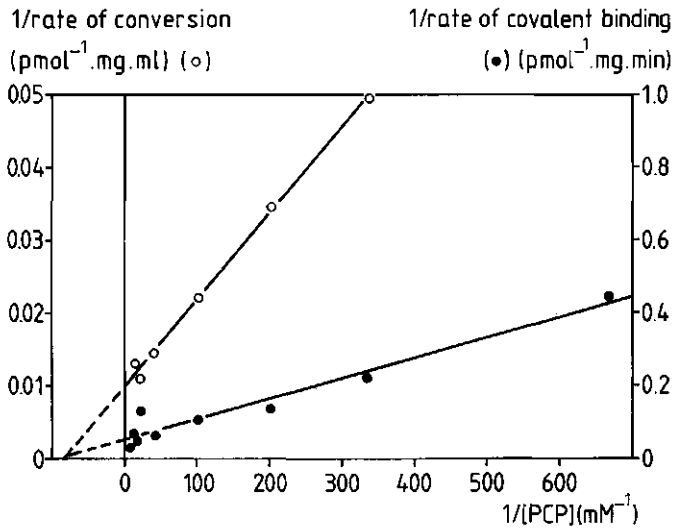

Figure 2. Lineweaver-Burk plot of the formation of soluble metabolites and covalent binding in the microsomal conversion of PCP. 2 mg/ml microsomal protein from HCB induced rats was incubated for 10 minutes. For exact conditions, see Methods.

had no effect on the rate, while 2 mg/ml showed a 30% decrease in rate as compared to 1 mg/ml. As shown in figure 2, the relation between conversion and PCP-concentration can be described by Michaelis-Menten kinetics, resulting in an apparent Km of 13 μM. It appeared that the ratio between the 1,4-TCHQ and the 1,2-TCHQ decreased with increasing PCP-concentration (figure 3): PCP-concentrations between 0.4 and 10 μM resulted in a 1,4/1,2-ratio of approximately 6, while further raising the concentration up to 200 uM showed a decrease in this ratio to 1.5.

EFFECT OF INDUCING AGENTS. In addition to microsomes from rats treated with HCB, microsomes from male and female rats induced with phenobarbital, 3-methylcholanthrene and isosafrole were also tested for their ability to convert PCP and compared to microsomes from untreated rats. The results are presented in table 1. It appeared that for both sexes isosafrole induction resulted in the highest rate of conversion (6-7 fold higher rates as compared to non-induced microsomes). HCB-induction showed a 3-4 fold increase. Both phenobarbital and 3-methylcholanthrene resulted in a 2.3 fold increase for male rats, while microsomes derived from female rats treated with 3-MC showed a 3-fold induction. PB-treatment of female

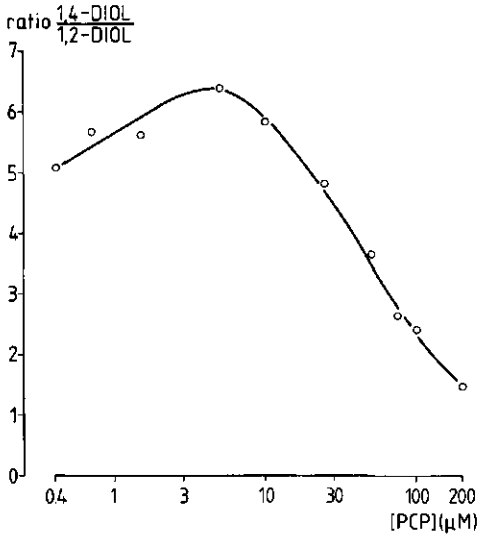

Figure 3. Ratio's of formation of the 1,4- and 1,2-TCHQ as a function of the concentration of PCP in a microsomal incubation system. 1 mg/ml microsomal protein from HCB induced rats was incubated for 5 minutes.

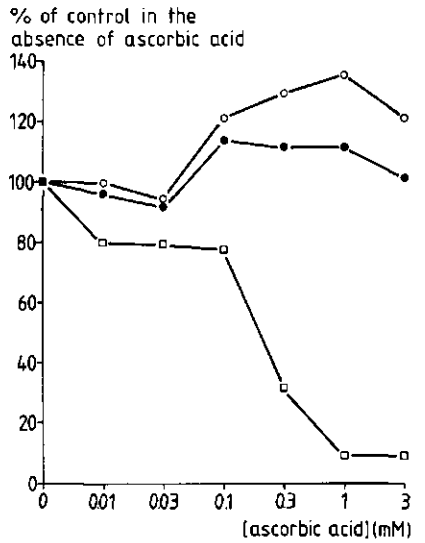

Figure 4. Effect of ascorbic acid on the covalent binding (-□-), total conversion (-○-) and TCHQ formation (-●-) in the microsomal metabolism of PCP at standard conditions. Total conversion denotes the total amount of soluble metabolites produced. All results are expressed as the percentage of activity with respect to a control incubation without ascorbic acid.

TABLE 1

THE MICROSOMAL METABOLISM OF PENTACHLOROPHENOL EFFECT OF INDUCING AGENTS AND THE EFFECT OF INHIBITION OF THE COVALENT BINDING

Inducer	Sex	Rate[a]	% 1,4-TCHQ[b]	% 1,2-TCHQ[b]	Ratio 1,4/1,2-TCHQ	Rate of covalent binding[c]
None	m	113 (212)	52 (52)	11 (11)	4.9 (4.9)	72
	f	120 (232)	24 (35)	17 (30)	1.6 (1.1)	74
HCB	m	378 (540)	78 (77)	31 (23)	2.5 (4.3)	63
	f	469 (555)	68 (59)	26 (31)	2.6 (1.9)	75
PB	m	265 (340)	52 (63)	27 (21)	2.0 (3.1)	76
	f	146 (265)	69 (58)	46 (29)	1.6 (2.0)	104
3MC	m	263 (361)	53 (50)	45 (35)	1.2 (1.4)	75
	f	365 (550)	51 (51)	44 (44)	1.2 (1.2)	16
ISF	m	798 (808)	55 (55)	42 (44)	1.3 (1.2)	66
	f	720 (648)	53 (53)	38 (38)	1.4 (1.4)	71

The incubations were performed at 37° for 5 min, with the following concentrations: 100 µM PCP, 1 mM NADPH, 3 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 7.4) and 2 mg microsomal protein in a 2 ml volume. All values are the average of duplicate incubations of a representative experiment. The figures in parenthesis represent the values derived from experiments in which teh covalent bindng was completely inhibited by the addition of 10 mM ascorbic acid.

a. The rate is expressed as pmol total soluble metabolite formed per mg protein/min. Total metabolite formation is expressed as the total amount of radioactivity which eluted from the HPLC-column before the substrate, divided by the total eluted radioacitivity.
b. 1,4-TCHQ and 1,2-TCHQ are expressed as percentage of total metabolites.
c. The rate of covalent binding (in $pmol.min^{-1}.mg^{-1}$) is calculated from the difference in covalent binding in incubations without and with ascorbic acid. Binding in the presence of ascorbic acid was the same as in incubations without NADPH.

rats had almost no effect on the rate of conversion. The ratio between formation of the 1,4-TCHQ and 1,2-TCHQ was dependent on the inducer used (table 1). Induction with isosafrole or 3-MC resulted in a 1,4/1,2-ratio of 1.2-1.4, PB-treatment gave a ratio of 1.6-2.0 and HCB-induction showed a 2.5 ratio. These results were similar for both sexes.

COVALENT BINDING TO PROTEIN. A substantial amount of covalent binding to protein was detected. This binding was dependent on metabolism of PCP. Incubations without NADPH resulted in a very small amount of binding, (1-2.5% of the amount of ^{14}C-PCP added, dependent on the batch of isotope used), which was independent of the incubation time or the amount of protein. The covalent nature of the binding was confirmed by the finding that two essentially different methods of analysis resulted in the same values for incubations both with and without NADPH. As determined for HCB-microsomes, the binding was dependent on the concentration of PCP. The apparent Km-value for covalent binding of 13 μM was very similar to the apparent Km-value found for the formation of the soluble metabolites (fig. 2).

Ascorbic acid was able to completely inhibit the metabolism-dependent covalent binding to microsomal protein. At 5 μM PCP the inhibition of covalent binding was complete at 1 mM ascorbic acid (fig. 4), while at 100 μM PCP complete inhibition was achieved at 10 mM ascorbic acid (table 1). At both concentrations the inhibition of covalent binding was accompanied by an increase in formation of 1,4- and 1,2-TCHQ. The sum of covalent binding and formation of soluble metabolites remained the same on increasing the concentration of ascorbic acid. Although the covalent binding was clearly related to conversion and PCP-concentration, the amount of binding did not differ greatly between the incubations with microsomes obtained from rats treated with different inducers (table 1). The rate of binding amounted to approximately 70 pmol/mg/min for all types of microsomes used, including microsomes from non-induced rats. In most cases, the increase in rate of formation of soluble metabolites as a result of adding 10 mM ascorbic acid to the incubation matched the decrease in covalent binding.

The ratio between the 1,4-TCHQ and 1,2-TCHQ did not change when the covalent binding was inhibited in incubations with isosafrole and 3-MC-microsomes (table 1). However, in the case of HCB- and PB-microsomes, this ratio (with the exception of female HCB-microsomes) was raised 30-60% after inhibition of covalent binding.

COVALENT BINDING TO DNA. The amount of binding to DNA was much smaller than the amount of binding to protein. Incubations (2 mg of protein from HCB induced rats, 5 min. incubation to which 1 mg DNA was added) showed a covalent binding of 12 ± 3 pmol/mg DNA/min (n=3). The average amount of binding to microsomal protein under these conditions was 63 pmol/mg protein/min. The addition of DNA had no effect on the formation of soluble metabolites. The DNA-binding was corrected for the amount of label recovered in incubations with DNA but without NADPH, which showed an average binding of 73 pmol/mg DNA in a 5 minutes incubation.

DISCUSSION

The aim of this study was to characterize and quantitate the microsomal metabolism of pentachlorophenol, with special attention to its ability to produce a metabolism-dependent covalent binding to protein and DNA. Protein binding was detected previously in a study of the nature of the covalent binding of hexachlorobenzene (10), of which PCP is the primary product (9).

The tetrachloro-1,4- and -1,2-hydroquinones, which we detected as the only microsomal oxidation products, were described previously to be the in vivo and in vitro conversion products of PCP (7). In contrast to other findings (12), no inhibitory effect of PCP on its own metabolism was found up to a concentration of 200 μM. An apparent Km-value of 13 uM was found both for the formation of the hydroquinones and the covalent binding, suggesting that both stem from the same reaction. There are strong indications for the fact that the benzoquinone or the semiquinone form of 1,4- and 1,2-TCHQ is responsible for the covalent binding properties. Glutathione has been shown to inhibit binding to protein by forming conjugates with the benzoquinones (10), while ascorbic acid reduces the benzo- and semiquinone to the hydroquinone form, thus preventing covalent binding.

The results of the experiments with microsomes derived from rats treated with different inducers indicate that PCP is best converted by cytochrome P-450d, the isosafrole induced isoenzyme (13). HCB-microsomes contain (in decreasing quantity) cytochrome P-450b, d, c and a (14). The difference in 1,4/1,2-TCHQ-ratio between on the one hand ISF and 3MC and on the other hand PB and HCB-microsomes suggests the involvement of two separate isoenzymes. The shift in this ratio with increasing PCP-concentrations in incubations with HCB microsomes can also be explained by the involvement of two isoenzymes with different Km-values and giving different 1,4/1,2-ratios. This may be the result of differences in regioselective binding of PCP to the different isoenzymes. Since microsomal incubations will not give definite answers to these questions, we intend to investigate this matter with purified P-450 isoenzymes.

The finding that in the case of experiments with PB or HCB microsomes, the ratio between 1,4- and 1,2-TCHQ raises after inhibition of the covalent binding, indicates a disproportional binding of the 1,2-TCHQ to protein under these conditions. With 3-MC or ISF microsomes, the ratio does not change, implying that both isomers bind with the same affinity.

Regardless of the origin of the microsomes used and the rates of conversion, the

amount of covalent binding found is almost constant. From the experiments in the presence of ascorbic acid it is clear that in all cases covalent binding is the result of tetrachlorobenzoquinone or semiquinone formation. Perhaps the oxidation of the hydroquinones to the benzoquinone form is the rate limiting step in the process of covalent binding. The amount of covalent binding in HCB microsomes from HCB induced rats with respect to the amount of TCHQ is about 13%. Studies in chick embryo hepatocyte cultures showed this amount to be only about 2.5% (15). This might be explained by the fact that intracellular conditions prevent oxidation of TCHQ to TCBQ.

The conversion dependent covalent binding to DNA is in agreement with the reported finding that TCHQ is able to bind to DNA (6). The amount of binding reported in the study mentioned is much smaller (53 pmol/mg DNA after 1 hour incubation with 50 mM TCHQ). This can also be explained by the necessity of oxidation to TCBQ, which in microsomes can occur enzymatically, for instance by activated oxygen species generated by cytochrome P-450 (16). Care should be taken in extrapolation of the in vitro covalent binding to the in vivo situation, since both reduction and conjugation mechanisms, which are present in living cells, are able to prevent this process.

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CHAPTER 5

THE OXIDATION OF TETRACHLORO-1,4-HYDROQUINONE BY MICROSOMES AND PURIFIED CYTOCHROME P-450. IMPLICATIONS FOR COVALENT BINDING TO PROTEIN

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ABSTRACT

The enzymatic oxidation of tetrachloro-1,4-hydroquinone (TCHQ), resulting in covalent binding to protein of tetrachloro-1,4-benzoquinone (TCBQ), was investigated, with special attention to the involvement of cytochrome P-450 and reactive oxygen species. TCBQ itself reacted very rapidly and extensively with protein (58% of the 10 nmoles added to 2 mg of protein, in a 5 minutes incubation). In microsomal incubations TCHQ oxidation was shown to be dependent on NADPH and partially dependent on cytochrome P-450 (30% inhibition by carbon monoxide) and oxygen (61% inhibition in anaerobic incubations). Ascorbic acid and glutathione prevented covalent binding of TCBQ to protein, both when added directly and when formed from TCHQ by microsomes. In a reconstituted system, purified cytochrome P-450b was able to oxidize TCHQ. Addition of superoxide dismutase to this system completely inhibited the oxidation. In microsomal incubations, superoxide dismutase prevented 61% of the covalent binding, the same amount as found in anaerobic incubations. However, covalent binding as a result of microsomal hydroxylation of pentachlorophenol to TCHQ and subsequent oxidation of TCHQ to TCBQ was not influenced by the addition of superoxide dismutase. This might indicate that the oxidation of TCHQ now takes place at the active site of cytochrome P-450, presumably still as a result of

superoxide anion production by cytochrome P-450. As a consequence, metabolically formed hydroquinones might more easily be converted to quinones, with possible toxic implications, than hydroquinones added from the outside.

INTRODUCTION

Toxic effects of quinones usually are the result of one of the two following mechanisms: i) covalent binding to cellular macromolecules (1), and ii) a redox shuttle between the quinone and semiquinone form, resulting in the generation of reactive oxygen species during reoxidation, imposing a condition of cellular oxidative stress (redox cycling) (2). Exploiting their ability to redox cycle, several quinones are used therapeutically, e.g. mitomycins in cancer chemotherapy (3).

A number of quinones have been described to be metabolically formed. Oxidation of aromatic diols, e.g. catechols like dopamine and adrenaline (4), and phenols such as 1-naphtol results in quinone formation (5). In some cases, metabolically formed quinones have been implicated in the toxic effects of the parent compound: benzene undergoes two hydroxylations by cytochrome P-450, and the resulting 1,4-hydroquinone and catechol are oxidized to their quinone forms (6). These quinones may play a role in benzene induced leukemia (7). A similar route has been described for the formation of 2-bromobenzoquinone from bromobenzene (8). The glutathione conjugate of this quinone is thought to be responsible for the nephrotoxic effects of bromobenzene, bromophenol and 2-bromohydroquinone (9). Recently, quinone metabolites have been implicated in the toxic effects of hexachlorobenzene.

Hexachlorobenzene (HCB) is known for its porphyrinogenic action (10). The HCB induced porphyria is thought to be associated with metabolic activation (11, 12). HCB has also been shown to be carcinogenic in a number of species (13, 14, 15) and for both of its primary oxidative metabolites, pentachlorophenol (PCP) and tetrachloro-1,4-hydroquinone (1,4-TCHQ), DNA damaging properties have been recorded (16, 19).

Studies on the microsomal metabolism of HCB revealed covalent binding to protein and DNA to occur after hydroxylation of both HCB and PCP (17, 18,

19). Both compounds yield tetrachloro-1,4- and -1,2-hydroquinone as products. These oxidations may be catalyzed by several forms of cytochrome P-450, and of these cytochrome P-450p, a rat liver microsomal isoenzyme induced by macrolide antibiotics like triacetyloleandomycin and glucocorticoids like dexamethasone (20), has been shown to exhibit by far the highest rate for both hydroxylations.

It is assumed that tetrachloro-1,4-hydroquinone is oxidized to the ultimate covalent binding compound tetrachloro-1,4-benzoquinone (chloranil) in microsomal incubations. Ascorbic acid inhibits the binding by preventing the oxidation of the hydroquinones, and glutathione inhibits the binding by formation of conjugates with the benzoquinone (18). Similar mechanisms for protection against binding have been described for phenol (6, 21) and bromobenzene (8).

The nature of the hydroquinone oxidizing agent and the possible enzymes involved is not always clear. E.g. for methyldopa (4), cytochrome P-450 has been shown to be involved, and in some cases the role of reactive oxygen species like the superoxide anion radical plays a role (4, 8). For 2-bromohydroquinone, NADPH promoted binding (8), while for p-hydroquinone NADPH decreased the amount of binding (21).

The aim of the present study was to investigate the role of cytochrome P-450 and possible other enzymes in the oxidation of tetrachloro-1,4-hydroquinone, the involvement of oxygen in this process and the possible protective mechanisms against this oxidation.

MATERIALS AND METHODS

chemicals. Pentachlorophenol was from Aldrich, tetrachloro-1,4-hydroquinone from ICN pharm., NY, tetrachloro-1,4-benzoquinone was from Merck, NADPH from Boehringer, superoxide dismutase from Sigma (type I, 2800 units per mg), catalase (10,000 units per mg) from Merck, dilauroylphosphatidylcholine from Sigma. All other chemicals were at least of analytical grade. The synthesis of the TCBQ-glutathione conjugates has been described previously (18).

radiochemicals. U-(^{14}C)-PCP (37.5 mCi/mmol) was from CEA, Gif sur Yvette, France. U-(^{14}C)-1,4-TCHQ was prepared by large scale incubations of

^{14}C-PCP with microsomes from isosafrole induced male rats, for 20 minutes at 37°C. The conditions were: 2mg/ml microsomal protein, 1 mM NADPH, with an extra 1 mM addition after 10 minutes, 150 nmole ^{14}C-PCP, s.a. 37.5 mCi/mmole, in 0.75 ml of acetone, 0.1 M potassium phosphate buffer, pH7.4, 3 mM $MgCl_2$ and 2 mM ascorbic acid. The total volume was 24 ml. The reaction was stopped with 0.2 ml of HCl (6N) and ascorbic acid was added to a final concentration of 10 mM. 1,4-TCHQ was purified from the extract by preparative HPLC using a gradient of 57% to 100% methanol against water containing 1% acetic acid in 8 minutes, followed by 5 minutes at 100% methanol. (k'(1,4-TCHQ)=4.5, k'(1,2-TCHQ)=7.2, k'(PCP)=9). The conversion of PCP to 1,4-TCHQ was approximately 15%, and 1,2-TCHQ formation was about 2%. During storage 1,4-TCBQ could be formed up to 15%. When necessary, TCBQ was completely removed directly before the incubations by the addition of 1 mM ascorbic acid to the TCHQ solution, extraction of TCHQ with diethylether, evaporation of the ether and dissolving the TCHQ in acetone. ^{14}C-1,4-TCBQ was prepared from ^{14}C-1,4-TCHQ by quantitative oxidation with an excess of dichlorodicyanoquinone (DDQ) in water/methanol 50/50. 1,4-TCBQ was then purified by HPLC as described below for 1,4-TCHQ and 1,4-TCBQ incubations.

purification of enzymes. Cytochrome P-450b was purified from liver microsomes of phenobarbital treated male Wistar rats according to (22), resulting in an electrophoretically pure isoenzyme with a specific concentration of 20.2 nmoles per mg protein. The NADPH-dependent cytochrome P-450 reductase was purified from liver microsomes of phenobarbital treated New Zealand white rabbits, using DEAE-cellulose and 2'5'ADP-Sepharose affinity chromatography (23). Detergent was removed from both enzymes by using a hydroxylapatite column (Biogel HTP, Biorad Laboratories).

microsomal incubations The microsomal incubations were performed with 2 mg of microsomal protein derived from 12 week old dexamethasone treated male Wistar rats (4 daily i.p. injections of 300 mg dexamethasone per kg body weight). Microsomes were prepared as described previously (17). Unless otherwise stated, the standard conditions for microsomal incubations were: a 2 ml volume, 5 minutes incubation at 37°C, 0.1 M potassium phosphate buffer, pH 7.4, 3 mM $MgCl_2$ and 1 mM NADPH. The reaction was terminated by the addition of 0.2 ml 6N HCl, after which 3 extractions with 3 ml ethyl

acetate / acetone 2:1 were performed. PCP-incubations: PCP was added in 50 µl of acetone, to a final concentration of 100 µM (specific activity:1.14 mCi/mmole). TCHQ-incubations: TCHQ was added in 50 µl of acetone, to a final concentration of 2.5 µM. The specific activity was 2 mCi/mmole. TCBQ-incubations: the same as for TCHQ, specific activity was 0.9 mCi/mmole. After drying the extracts with Na_2SO_4, the solvents were evaporated in a stream of nitrogen, and the residues were dissolved in 50 µl of methanol for HPLC analysis. Anaerobic incubations were performed as described previously (18).

HPLC analysis. The extracts of the incubations were analysed by HPLC using a Perkin Elmer series 4 HPLC equipped with a Chrompack lichrosorb 150x4.6 mm 5RP18 column. 1,4-TCHQ and 1,4-TCBQ were analysed isocratically at 55% methanol and 45% water containing 1% acetic acid. (k'(DDQ)=2.0, k'(1,4-TCHQ)=5.0, k'(1,4-TCBQ)=7.0) Detection was by UV (254nm), fractions of 0.6 ml were collected of which, after addition of 4 ml of scintillation liquid (Atomlight, NEN-Dupont), the amount of radioactivity was determined in a Packard tricarb 300 scintillation spectrometer.

measurement of covalent binding. After removal of the organic phase after the last extraction, the precipitated protein was separated from the aqueous phase by centrifugation, and washed extensively with organic solvents. For PCP incubations, the protein pellet was washed with 3x10 ml of methanol, 3x10 ml of ethanol, 2x10 ml of acetone and 10 ml of diethylether. For incubations with 1,4-TCHQ and 1,4-TCBQ, it appeared to be sufficient to wash with 10 ml of methanol, 10 ml of ethanol, 10 ml of acetone and 10 ml of diethylether. After evaporation of the remaining diethylether, 1 ml of Soluene 350 (Packard) was added in order to dissolve the protein, and the sample was screened for radioactivity in 15 ml of scintillation liquid (Atomlight).

incubations with purified enzymes. Reconstitution experiments with purified cytochrome P-450b and NADPH dependent cytochrome P-450 reductase were performed using the same conditions as described for the microsomal experiments, except for the replacement of the microsomes with a mixture of the enzymes, (1 nmole of P-450 and 3 nmoles of reductase), which were preincubated in a small (0.25 ml) volume, together with 40 µg of dilauroylphosphatidylcholine, for 3 minutes at 37°C. BSA (2 mg) was added as a target for covalent binding to protein.

oxygen consumption measurements. The microsomal oxygen consumption measurements were performed with a Clark type oxygen electrode equipped with a LKB spectroplus spectrometer, using the same conditions as in the microsomal incubations, except for the amount of acetone added (5 µl instead of 50 µl), and the amount of microsomal protein (0.5 mg of microsomal protein from dexamethasone induced male rats, instead of 2 mg), in order to decrease the rate of oxygen consumption to a measurable level.

RESULTS

1) The covalent binding to protein of tetrachloro-1,4-benzoquinone

TCBQ reacted very rapidly with microsomal protein. After a 5 minute incubation 58% of the added TCBQ was bound covalently (290 pmole/minute/mg protein)(figure 1). 13% of the label could not be extracted, while of the extracted label, 75% was in the hydroquinone form. The reaction appeared to be non enzymatic, because incubations of 5 nmoles of TCBQ with 2 mg of BSA resulted in approximately the same amount of binding (52%). NADPH and

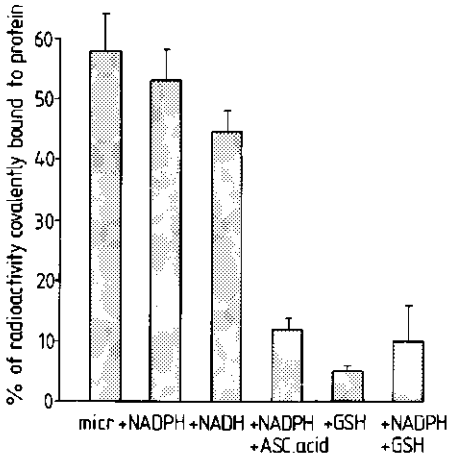

figure 1
Covalent binding of tetrachloro-1,4-benzoquinone to microsomal protein. 5 nmoles of 1,4-TCBQ were incubated for 5 minutes at 37°C with 2 mg of microsomal protein in a 2 ml volume, as described in the method section. The concentration of NADPH, NADH was 1 mM, ascorbic acid 3 mM and glutathione 1.5 mM. n=2.

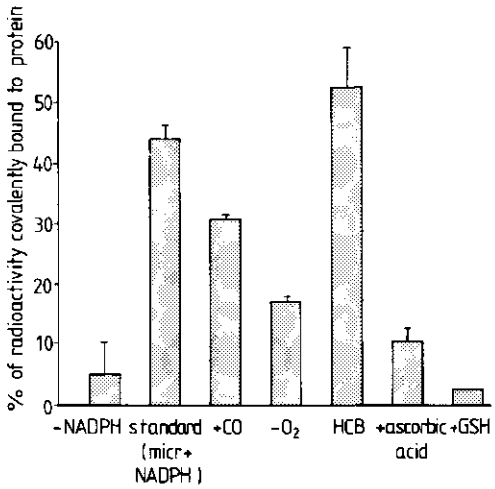

figure 2
Covalent binding of tetrachloro-1,4-hydroquinone to microsomal protein. Standard 5 minute incubations consisted of 2 mg of microsomal protein from dexamethasone induced male rats, 5 nmoles 1,4-TCHQ and 1 mM NADPH, in a 2 ml volume of a buffered solution as described in the method section. Additions were: 25 µM hexachlorobenzene, 3 mM ascorbic acid and 1.5 mM glutathione. n=2.

NADH acted as reducing agents towards TCBQ. After incubation of TCBQ with 1 mM NADPH or NADH, HPLC analysis of the extracted label revealed 78% to be in the hydroquinone form for NADPH and 75% for NADH. However, in microsomal incubations the covalent binding to protein was hardly influenced by these additions (53% and 44% of total added radioactivity, for NADPH and NADH respectively (figure 1).) Ascorbic acid, on the other hand, was a more effective protecting agent against covalent binding of TCBQ. Addition of 3 mM to the incubation mixture resulted in a decrease to 12% binding. 98% of the extracted label was TCHQ. Glutathione (1.5 mM) also inhibited covalent binding. In this case however, nearly all radioactivity (89%) remained in the aqueous phase after extraction, indicating the formation of a water soluble conjugate. HPLC analysis of the aqueous phase suggested formation of a highly conjugated molecule (at least 3 molecules of glutathione per TCBQ), since the elution time of the major radioactive fraction was shorter than that of the mono- and disubstituted TCBQ, of which the identity was established previously (18).

2) The oxidation of tetrachloro-1,4-hydroquinone, and the involvement of reactive oxygen species in this reaction

The covalent binding to protein as a result of metabolic activation of TCHQ was studied with ^{14}C-TCHQ in microsomes and in a reconstituted system consisting of purified rat liver cytochrome P-450b and NADPH-cytochrome P-450 reductase, as well as with ^{14}C-PCP in microsomes, in order to investigate the binding of TCHQ generated at the active site of cytochrome P-450.

The microsomal studies with ^{14}C-1,4-TCHQ indicated that TCHQ itself did not bind to protein. Incubations without NADPH showed a binding of 5% (figure 2), while addition of NADPH to the incubation increased the binding to 44% of the added 5 nmoles of TCHQ. The concentration dependency of the covalent binding is shown in figure 3. Up to 10 nmoles (5 uM), a linear relationship existed between binding and concentration. All incubations therefore were performed with 5 nmoles of TCHQ.

Glutathione and ascorbic acid inhibited the covalent binding in the same manner as with the incubations with TCBQ: for glutathione, total inhibition of binding with the major part of the label (96%) remaining in the aqueous phase after extraction, and for ascorbic acid total inhibition of the binding with all of the extracted label in the reduced (hydroquinone) form (figure 2).

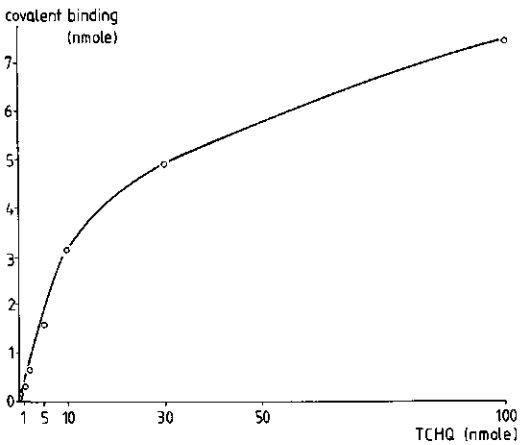

figure 3
Dependency of the covalent binding to microsomal protein on the concentration of tetrachloro-1,4-hydroquinone, in a microsomal incubation. 2 mg of protein was incubated in the presence of 1 mM NADPH. The binding is corrected for the binding in the absence of NADPH, which amounted to approximately 9% of total binding. n=2.

TABLE 1. COVALENT BINDING TO PROTEIN OF TETRACHLORO-1,4-HYDROQUINONE CATALYZED BY PURIFIED CYTOCHROME P-450b

experiment 1	COVALENT BINDING - pmole
BSA	201 ± 25 (4.0)
BSA + NADPH	168 ± 12 (3.4)
BSA + NADPH + REDUCTASE	160 ± 5 (3.2)
BSA + NADPH + REDUCTASE + CYTOCHROME P-450b	525 ± 15 (10.6)
BSA + NADPH + REDUCTASE + CYTOCHROME P-450b + CO	129 ± 3 (2.6)

experiment 2	COVALENT BINDING - pmole
BSA + NADPH + REDUCTASE + SOD	401 ± 6 (8.0)
BSA + NADPH + REDUCTASE + CYTOCHROME P-450 + SOD	423 ± 23 (8.6)

Numbers in brackets: % of total added radioactivity bound to protein
exp.1: 2 mg BSA, 1 mM NADPH, 1 nmole P-450b, 3 nmole reductase
exp.2: The same as in exp. 1, except for BSA: 1mg, SOD 1mg

In order to estimate the role of cytochrome P-450 in the oxidation of TCHQ to TCBQ, the microsomes were saturated with carbon monoxide. This resulted in a 30% decrease of covalent binding as compared to the standard (+NADPH) incubations. Anaerobic incubations showed a 61% decrease of binding (figure 2). Addition of 25 μM hexachlorobenzene resulted in a 19% increase of the covalent binding.

TABLE 2. THE EFFECT OF HCB AND METABOLITES ON THE MICROSOMAL OXYGEN CONSUMPTION.

COMPOUND	OXYGEN CONSUMPTION (% decrease of $[O_2]$ per minute)
none (acetone)	2.34 ± 0.10
HCB (25 μM)	2.80 ± 0.28
PCP (100 μM	0.98 ± 0.09
1,4-TCHQ (10 μM)	4.63 ± 0.84

0.5 mg of microsomal protein of dexamethasone induced rats was incubated at 37°C, in the presence of 1 mM NADPH. For exact conditions, see method section.

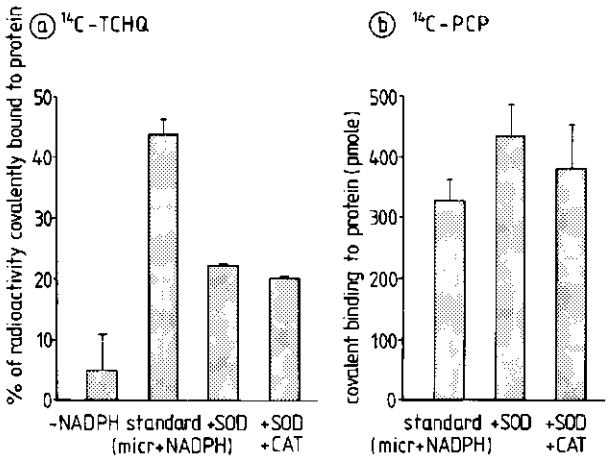

figure 4
Effect of addition of superoxide dismutase (SOD, 1 mg, 2800 units) and catalase (CAT, 1 mg, 10,000 units) on the covalent binding to microsomal protein of tetrachloro-1,4-hydroquinone (a) and pentachlorophenol (b). The 1,4-TCHQ incubations were performed as stated in figure 3, the PCP incubations were performed similarly, with a PCP concentration of 100 μM. For PCP, the covalent binding is corrected for binding in the absence of NADPH. (Without NADPH no formation of 1,4-TCHQ was observed.) n=2.

Incubations with purified cytochrome P-450b (the major isoenzyme after induction of rats with phenobarbital) and NADPH-cytochrome P-450 reductase in the presence of dilauroylphosphatidylcholine (1 nmole, 3 nmoles and 40 μg, respectively) showed the ability of cytochrome P-450 to oxidize TCHQ (table 1, exp.1). Furthermore, carbon monoxide was able to inhibit this P-450 activity completely.

In order to determine the role of activated oxygen species in the oxidation of TCHQ, a number of incubations were performed to which 2800 units of superoxide dismutase (SOD) and 10,000 units of catalase (CAT) were added. In microsomal experiments with ^{14}C-TCHQ, 1 mg of SOD decreased the covalent binding by 50%, while a further addition of 1 mg of catalase had only a minor extra effect (from 50% to 52%, see figure 4a). In the reconstituted system the binding was almost completely inhibited by the addition of 1 mg of SOD. (table 1, exp. 2). The increase in blanc binding as compared to experiment 1 is probably due to a greater susceptability of SOD than BSA to covalent binding. Incubations with ^{14}C-PCP showed no

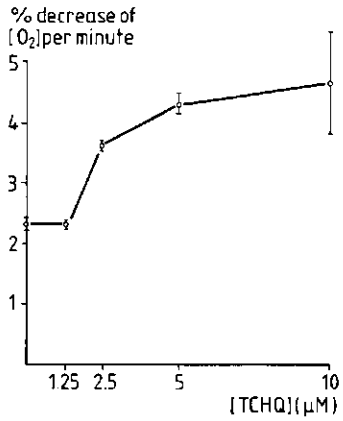

figure 5
Dependency of the microsomal oxygen consumption on the concentration of tetrachloro-1,4-hydroquinone. Microsomal incubations were performed as described for the covalent binding studies (figure 3), except for the amount of protein (0.5 mg) and the amount of acetone (5μl), in a Clark type oxygen electrode cell. n=2.

decrease in covalent binding due to the addition of SOD or SOD together with catalase (figure 4b). Instead, a small increase was measured, again most probably due to a high affinity of these proteins for covalent binding.

3) **Microsomal oxygen consumption.** In order to estimate the amount of reactive oxygen species generated during the microsomal incubations, a number of (non- radioactive) incubations were performed in a closed cell, fitted with a Clark type oxygen electrode. The blanc level of oxygen consumption, due to the addition of acetone, was a decrease of 2.34% per minute. Addition of PCP and 1,4-TCBQ decreased this amount (table 2), while HCB showed a small increase. The largest increase was due to addition of 1,4-TCHQ to the microsomal incubations (4.63% per minute). The oxygen consumption as a result of 1,4-TCHQ is of an enzymatic nature and NADPH dependent (table 3). Substitution of NADPH for NADH resulted in loss of activity. The dependency of oxygen consumption on the concentration of 1,4-TCHQ is shown in figure 5.

TABLE 3. THE EFFECT OF TETRACHLORO-1,4-HYDROQUINONE ON THE MICROSOMAL OXYGEN CONSUMPTION

SAMPLE	OXYGEN CONSUMPTION (% decrease of $[O_2]$ per minute)
TCHQ	0
TCHQ + NADPH	0
TCHQ + micr	0
TCHQ + micr + NADPH	5.15
TCHQ + micr + NADH	0
TCHQ + micr + NADPH + NADH	4.60

The concentrations used were:10 μM 1,4-TCHQ, 1 mM NADPH, 0.5 mg of microsomal protein from dexamethasone treated male rats. The incubations were performed at 37°C.

DISCUSSION

The role of cytochrome P-450 in oxidation of tetrachlorohydroquinone

Cytochrome P-450 is capable of oxidizing TCHQ. With purified rat liver cytochrome P-450b, NADPH-dependent covalent binding occurred to the extent of 365 pmole/min/nmole P-450, and this binding was completely inhibited by carbon monoxide. On the other hand, inhibition of microsomal cytochrome P-450 by carbon monoxide resulted in only a partial loss of covalent binding. The microsomal incubations thus clearly suggest that P-450 is not the only enzyme involved in this reaction. Moreover, in the total absence of molecular oxygen, covalent binding did still take place (23% as compared to $[+NADPH/+O_2]$ incubations). This indicates that in addition to cytochrome P-450, at least two other types of enzymes are involved: i) non-P450 enzymes which use oxygen and NADPH for the oxidation of TCHQ (responsible for the difference between the remaining activity after CO-inhibition and the remaining activity in incubations without oxygen) and ii) enzymes which need NADPH but do not need oxygen (responsible for the activity which remains in incubations without oxygen).

The role of activated oxygen species in the oxidation of TCHQ

A number of hydroquinones are dependent on activated oxygen species in their oxidation to quinones. On the other hand, certain quinones are formed by oxidation of hydroquinones by molecular oxygen. Lau et al(8)

described the partial inhibition of covalent binding of 4-bromocatechol by superoxide dismutase. Activated oxygen species are involved in the oxidation of dopa and dopamine (4, 24). However, in the case of 2-acetamido-p-hydroquinone, neither SOD nor catalase caused a significant decrease in 2-acetamido-p-benzoquinone formation (25). Although both molecular oxygen and superoxide anion have been shown to be involved in the p-hydroquinone oxidation (26), p-hydroquinone oxidized spontaneously in aqueous solution, and addition of superoxide dismutase increased this oxidation fivefold (27) . The binding in microsomal incubations of hydroquinone and catechol is not stimulated by the presence of NADPH (21). The one electron reduction of molecular oxygen has a much lower redox potential then the one electron reduction of superoxide anion (28). Apparently, the redox potentials of the hydroquinone/semiquinone and semiquinone/benzoquinone equilibria are of major importance in the oxidation. These values strongly depend on the environment. Under aprotic conditions, the E1/2 value of TCBQ is 0.6 V higher than that of benzoquinone (29), while in alcoholic solution the redox potentials are similar (30).

The oxidation of TCHQ by purified cytochrome P-450 appeared to be completely mediated by superoxide anion radicals, since no difference was observed between blanc incubations and incubations which, ecxept for the addition of superoxide dismutase, have shown to be able to stimulate covalent binding. Cytochrome P-450 is known for its ability to generate reactive oxygen species like superoxide anion radicals, hydroxyl radicals and hydrogen peroxide (31). The involvement of these reactive oxygen species is also apparent from the finding that addition of hexachlorobenzene to a microsomal incubation of TCHQ increased the covalent binding with 19%, a value remarkably similar to the 20% increase in microsomal oxygen consumption after addition of the same concentration of HCB. Covalent binding as a result of the incubation of ^{14}C-PCP also was stimulated by 23% by the addition of HCB.

In the microsomal incubations addition of superoxide dismutase and catalase inhibited the covalent binding almost to the extent of the incubations without oxygen (52% and 61% respectively), which implies that all oxygen consuming reactions leading to covalent binding are mediated through reactive oxygen species. Besides cytochrome P-450, a number of

other enzymes produce these compounds. It is known, for example that the flavin containing NADPH-cytochrome P-450 reductase can produce superoxide anion radicals (32). However, incubations of TCHQ with this enzyme in its purified form did not give rise to covalent binding.

The large amount of oxygen consumption in the presence of 1,4-TCHQ and NADPH is most likely due to uncoupling of cytochrome P-450. The reaction is dependent on both microsomes and NADPH and independent of NADH. This indicates that chemical oxidation of TCHQ by molecular oxygen, resulting in formation of superoxide anion, is not involved. In that case addition of superoxide dismutase would increase the amount of benzoquinone formation, as was shown for 1,2,4-benzenetriol and hydroquinone (27, 33). No oxygen was consumed on addition of 1,4-TCBQ, showing that TCBQ was unable to undergo redox cycling in a microsomal system.

We have previously shown that covalent binding in a microsomal incubation of PCP was the result of the formation of tetrachlorobenzoquinone (19). The observation that superoxide dismutase and catalase did not inhibit covalent binding as a result of PCP-metabolism might be explained by assuming that TCHQ, formed from PCP in the active site of cytochrome P-450, is oxidized by P-450 generated superoxide anion radicals before it leaves the active site. The finding that THCQ promotes the uncoupling of cytochrome P-450 supports this hypothesis. The active site would then have to be inaccessable to superoxide dismutase. This mechanism is invalid if superoxide anion is not involved in the oxidation of 1,4-TCHQ, formed by hydroxylation of PCP. However, if this assumption is correct, the consequence for the in vivo situation is that hydroquinones generated by cytochrome P-450 may more easily be oxidized to quinones and thus exert possible toxic actions linked to covalent binding than hydroquinones which are not metabolically produced.

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CHAPTER 6

THE INVOLVEMENT OF DIFFERENT CYTOCHROME P-450 ISOENZYMES IN THE HYDROXYLATION OF HEXACHLOROBENZENE AND PENTACHLOROPHENOL. THE USE OF SPECIFIC MONOCLONAL ANTIBODIES AND PURIFIED CYTOCHROME P-450 ISOENZYMES

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ABSTRACT

The toxicity of hexachlorobenzene has been shown to be cytochrome P-450 mediated. In order to determine the identity of the cytochrome P-450 isoenzyme(s) involved in the hydroxylation of hexachlorobenzene and its primary hydroxylation product pentachlorophenol, both compounds were incubated with the four purified cytochromes P-450b - P-450e. Also, in incubations of hexachlorobenzene and pentachlorophenol with microsomes from rats treated with different cytochrome P-450 inducers, monoclonal antibodies against cytochromes P-450b and P-450e, and cytochrome P-450c were used to selectively inhibit these isoenzymes.

At least 3 different isoenzymes were involved in the hydroxylation of hexachlorobenzene. This compound was very slowly hydroxylated by purified cytochromes P-450b and P-450e (0.8 and 0.7 pmole PCP formed per minute per nmole P-450, respectively). Partial suppression of the hydroxylation of hexachlorobenzene in microsomes from rats treated with phenobarbital or hexachlorobenzene by a monoclonal antibody against P-450b+e agreed with

this observation. Microsomes from rats treated with dexamethasone showed by far the highest catalytic activity toward hexachlorobenzene hydroxylation, suggesting the involvement of cytochrome P-450p. The remaining activity after inactivation of P-450b and P-450e in microsomes from rats induced by phenobarbital may be due to P-450p, since this isoenzyme is also present in these microsomes.

In a reconstituted system, pentachlorophenol was converted to tetrachloro-1,4-hydroquinone and tetrachloro-1,2-hydroquinone by purified cytochromes P-450c and P-450d, and to a lesser extent by cytochromes P-450b and P-450e (450, 440, 180 and 120 pmole diols/min/nmole P-450, respectively). However, inhibition of the catalytic activity of cytochrome P-450b in microsomes from phenobarbital treated rats, and inhibition of cytochrome P-450c in microsomes from rats treated with 3-methylcholanthrene did not affect the rate of conversion of pentachlorophenol. This illustrates that although purified cytochrome P-450 isoenzymes may possess catalytic activity towards a compound, this not necessarilly implies that they contribute to their microsomal conversion. Microsomes from dexamethasone and hexachlorobenzene induced rats were the most active in catalyzing the hydroxylation of pentachlorophenol. Probably, cytochrome P-450p is the major enzyme involved in the microsomal hydroxylation of PCP.

INTRODUCTION

The cytochrome P-450 isoenzymes are involved in the metabolism of drugs and endogenous compounds. At least 10 isoenzymes have been purified from rat liver and characterized (Ryan, 1979, 1980, 1982, 1984, 1985), all showing different substrate specificities.

Although numerous reports have appeared on the induction of cytochrome P-450 isoenzymes by HCB (Stonard, 1976, Goldstein, 1978, Vizethum, 1980, Holme and Dybing, 1982, Li, 1987), relatively little attention has been paid to the identity of the cytochrome P-450 isoenzymes involved in its metabolism. Studies with microsomes of rats treated with different inducers suggest the involvement of cytochrome P-450b in the hydroxylation of HCB to its primary metabolite pentachlorophenol (PCP) (Van Ommen, 1985,

Stewart, 1986). PCP in turn is hydroxylated to tetrachloro-1,4- and -1,2-hydroquinone, and microsomes of rats treated with inducers of cytochromes P-450c and P-450d (3-methylcholanthrene and isosafrole) have been shown to catalyze this reaction (Ahlborg, 1978, Van Ommen, 1986, Stewart, 1986). The ratio between 1,4-diol- and 1,2-diol-formation appeared to be dependent on the type of inducer used, suggesting the involvement of more than one isoenzyme in its metabolism (Van Ommen, 1986). The hydroquinones formed can be oxidized to their quinone forms, which bind covalently to protein and DNA (Van Ommen, 1986), and may be responsible for some of the adverse effects of HCB, such as its carcinogenicity and porphyrinogenic action.

Cytochrome P-450 has been shown to be involved in the development of porphyria as a result of exposure to hexachlorobenzene (HCB). Several studies have shown that treatment of rats with phenobarbital enhanced the porphyrinogenic action of HCB (Goldstein, 1978, Kerklaan, 1979, Wainstock de Calmanovici, 1984). In the japanese quail the same result was obtained with beta-naphthoflavone, but not with phenobarbital (Carpenter, 1984). In a primary culture of chick embryo hepatocytes, simultaneous treatment with HCB and 3-methylcholanthrene (Sinclair, 1974, 1984) or beta-napthoflavone (Debets, 1981) stimulated the accumulation of porphyrins, while piperonyl butoxide (an inhibitor of P-450 activity) decreased this amount. It has been suggested that a reactive intermediate or metabolite of HCB, produced by cytochrome P-450, is responsible for the porphyrinogenic action of HCB. This study is aimed at characterizing the cytochrome P-450 isoenzymes involved in the hydroxylation of HCB and PCP. The two methods used for this purpose are i) the use of purified cytochrome P-450 isoenzymes, in order to directly determine their capacity to hydroxylate HCB and PCP, and ii) the use of specific monoclonal antibodies against P-450 isoenzymes, thus selectively inhibiting isoenzymes in order to estimate their contribution to the hydroxylation of HCB and PCP in microsomes from rats treated with different inducers.

METHODS

Chemicals. U-[^{14}C]-Hexachlorobenzene (S.A. 70 mCi/mmole) was purchased

from Amersham (UK). The radiochemical contamination with pentachlorobenzene was reduced from 0.12% to 0.06% by preparative HPLC using the method described below for analysis of the metabolites. U-[^{14}C]-pentachlorophenol (S.A. 37.5 mCi/mmole) was from CEA, Gif-sur-Yvette, France, and contained 0.8% tetrachlorophenol as a radiochemical impurity. Dexamethasone was from Sigma. The origin of all other chemicals has been described elsewhere (Van Ommen, 1986).

Microsomes, purified enzymes and antibodies. Microsomes were prepared as described previously (Van Ommen, 1985, 1986), except for dexamethasone: 4 daily i.p. injections of 300 mg/kg bodyweight. The purification of cytochrome P-450b, c, d and e has been published previously (Ryan, 1979, 1980, 1982). The monoclonal antibodies (MAb) C7, C8 and C9 have been characterized and described by Thomas et al (1984). C7 and C9 bind to cytochrome P-450c without inhibiting its catalytic activity, while C8 binds and completely inhibits this isoenzyme. BE 52 has been described by Reik, (1985). This monoclonal binds and inhibits cytochromes P-450b and P-450e.

Incubations with microsomes and monoclonal antibodies. Liver microsomes from male rats treated with different inducing agents were incubated in the presence of antibodies with ^{14}C-HCB or ^{14}C-PCP, using the following method: in a volume of 0.27 ml the microsomes (2 mg) were preincubated for 10 minutes at 21°C with 0.20 mg/nmole P-450 of the appropriate antibody, after which, at 4°C, the remaining constituents were added, resulting in final concentrations of 0.1 M potassium phosphate buffer, pH 7.4, 3 mM $MgCl_2$ and 1 mM ascorbic acid, in a 1.75 ml volume. After temperature equilibration (2 minutes at 37°C), this volume was added to 50 ul of acetone, containing the substrate (50 nmoles of HCB, 1 µCi, or 200 nmoles of PCP, 0.25 µCi), and the reaction was started by the addition of NADPH to a final concentration of 1 mM in a 2 ml total incubation volume. After 5 minutes incubation at 37°C, the reaction was terminated by addition of 0.2 ml 6N HCl, and 1 ml 30 mM ascorbic acid (for the PCP incubations). After extraction (with 3x3ml diethylether for HCB and 3x3 ml ethylacetate/acetone 2:1 for PCP), the extracts were dried with Na_2SO_4, the solvents evaporated in a stream of nitrogen and the residues, dissolved in 50 µl of methanol, subjected to HPLC-analysis. All incubations were performed in duplicate. Blancs consisted of zerotime

incubations and incubations without NADPH.

The dealkylation of pentoxy- and ethoxyresorufin was performed according to Burke (1986).

Incubations with purified cytochrome P-450 isoenzymes. Incubations with purified cytochrome P-450 were performed similarly to microsomal incubations, with the replacement of the microsomes/antibody mixture by a reconstituted system consisting of 1 nmole cytochrome P-450b, c, d or e, 3 nmoles of NADPH dependent cytochrome P-450 reductase and 40 µg of dilauroylphosphatidylcholine, preincubated for 3 minutes at 37°C, in a volume of 0.2-0.25 ml. No ascorbic acid was added to these incubations. As blanc, an incubation without cytochrome P-450 was performed.

HPLC-analysis of metabolites. HPLC-analysis of the extracts of the incubations was performed on a Perkin Elmer series 4 HPLC, equipped with a Chrompack Lichrosorb 150x4.6mm 5RP18 column and UV (254nm) detection. 50 µl of the extract together with 10 ul of methanol containing marker metabolites was injected. Radiochemical detection was carried out by collecting 0.6 ml fractions, of which the radioactivity was measured in 4 ml of Atomlight (NEN) in a Tricarb III liquid scintillation spectrometer (Packard). Separation of HCB and its metabolites (PCP, pentachlorobenzene as radiochemical impurity and 1,4-TCHQ) was achieved with a gradient of 90% methanol against water containing 1% acetic acid, to 100% methanol in 5 minutes, followed by 7 minutes of 100% methanol. k'-values were 8, 3.8, 6 and 2.3, respectively. Separation of PCP, 1,4-TCHQ, 1,2-TCHQ and tetrachlorophenol was achieved by isocratic elution for 3 minutes at 65% methanol against water containing 1% acetic acid, followed by a gradient to 100% methanol in 8 minutes, followed by 7 minutes of 100% methanol. k'-values were 10.7, 3.3, 7.8 and 9.7, respectively.

Apart from the diols, an unknown metabolite was formed in the microsomal incubations of PCP, with a rate of 30-160 pmole/min/mg protein, depending on the type of microsomes used. This formation was independent of cytochrome P-450, since incubations without NADPH or without oxygen gave the same amount of this metabolite. The MAb used did not affect its formation. Hydrolysis of this unknown compound resulted in reappearance of PCP. Since it has no connection with the cytochrome P-450 supported biotransformation, this unknown metabolite is not included in the discussion.

RESULTS

1) Conversion of hexachlorobenzene and pentachlorophenol by purified cytochrome P-450 isoenzymes.

The rate of product formation for HCB and PCP was measured in a system containing purified rat liver microsomal cytochromes P-450b, P-450c, P-450d or P-450e, NADPH dependent cytochrome P-450 reductase and dilauroylphosphatidylcholine (1 nmole, 3 nmoles and 40 µg, respectively). The results are presented in table 1. Formation of PCP as a result of

TABLE 1a

CONVERSION OF HEXACHLOROBENZENE BY PURIFIED CYTOCHROME P-450 ISOENZYMES

	ISOENZYME			
	b	c	d	e
rate of PCP formation	0.8	0	0	0.7

TABLE 1b

CONVERSION OF PENTACHLOROPHENOL BY PURIFIED CYTOCHROME P-450 ISOENZYMES

	ISOENZYME			
	b	c	d	e
rate of 1,4-diol formation	80	310	290	50
rate of 1,2-diol formation	100	140	150	70
ratio between 1,4 and 1,2	0.7	2.1	1.9	0.7

1 nmole of cytochrome P-450, 3 nmoles of reductase and 40 µg of lipid were mixed, incubated for 3 minutes at 37°C and returned to ice. The other components were added (0.1 M potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$) to a volume of 1.75 ml. After temperature equilibration the sample was added to 50 µl of acetone containing 50 nmoles of HCB or 200 nmoles of PCP, and the reaction was started by the addition of 1 mM NADPH. After 5 minutes incubation at 37° the reaction was stopped and the product formation was measured by HPLC/scintillation spectrometry. The rate is expressed as pmole product per minute per nmole cytochrome P-450.

hydroxylation of HCB was found in incubations with cytochromes P-450b and P-450e. The amount of PCP produced was very low (0.8 and 0.7 pmole/min/nmole P-450, respectively) No formation of diols as secondary hydroxylation products of HCB was detected. P-450c and P-450d displayed no catalytic activity towards HCB, at a detection limit of 0.2 pmole/min/nmole P-450.

Incubation of ^{14}C-PCP with cytochromes P-450b, P-450c, P-450d and P-450e resulted in formation of tetrachloro-1,4- and tetrachloro-1,2-hydroquinone for all isoenzymes. However, cytochromes P-450c and P-450d showed a higher catalytic activity towards hydroxylation of PCP than P-450b and P-450e (table 1b). The relative amounts of the 1,4-diol and 1,2-diol also differed for the isoenzymes used. Cytochrome P-450c and d produced more 1,4-diol than 1,2-diol, while cytochromes P-450b and P-450e produced more of the tetrachloro-1,2-hydroquinone. No other conversion products were detected.

2) inhibition with monoclonal antibodies of the catalytic activity of cytochrome P-450 isoenzymes towards HCB and PCP in microsomes.

In order to estimate the contribution of the different cytochrome P-450 isoenzymes in the hydroxylation of HCB and PCP in microsomes, the isoenzymes P-450b and P-450c were selectively inhibited by monoclonal antibodies (MAb). In the presence of these antibodies, the conversion of HCB to PCP and diols, and the conversion of PCP to the tetrachloro-1,4- and -1,2-hydroquinone were measured using microsomes from male rats treated with different inducers of cytochrome P-450 isoenzymes. The antibodies used were described previously (Thomas et al, 1984, Reik at al, 1985). The BE52 antibody completely inhibits cytochrome P-450b, C8 inhibits P-450c, and the C7 and C9 MAb bind to P-450c without affecting its catalytic activity. The latter two were used as a control for the addition of the extra amount of protein to the incubations. To illustrate their functioning, and to test their ability to inhibit cytochrome P-450 isoenzymes from the Wistar strain of rats (the antibodies were raised against cytochrome P-450 isoenzymes from Long Evans rats), the dealkylation of ethoxyresorufin and pentoxyresorufin, specific substrates for P-450c and P-450b, respectively (Burke, 1986) was determined in the presence and absence of the MAb (table 2). C7 has no effect on the catalytic activity, BE52 completely inhibits the pentoxyresorufin

TABLE 2
EFFECT OF SPECIFIC MONOCLONAL ANTIBODIES ON THE DEALKYLATION OF PENTOXY- AND ETHOXYRESORUFIN.

INDUCER/ANTIBODY	O-dealkylase activity (pmole/min/mg) pentoxyresorufin		ethoxyresorufin	
PB	560	(100)		
C7	662	(118)		
BE52	0	(0)		
3MC			1040	(100)
C7			1066	(103)
C8			195	(18)
HCB	57	(100)	273	(100)
C7	54	(95)	273	(100)
BE52	0	(0)	-	
C8	-		91	(33)

The incubation of the antibodies with microsomes was performed similarly to the HCB and PCP microsomal incubations. With these preincubated microsomes the formation of resorufin was measured fluorimetrically, in a 2 ml assay with 0.15 mg of microsomal protein. The numbers in brackets represent the percentage of activity as compared to the activity of the microsomes without antibody.

dealkylation in both phenobarbital and HCB induced microsomes, and C8 inhibits most of the ethoxyresorufin deethylation activity in 3-methylcholanthrene and HCB induced microsomes.

The effect of the antibodies on the hydroxylation of HCB by microsomes of rats treated with different inducers is presented in table 3. The microsomes from dexamethasone induced rats appeared to be by far the most active in the conversion of HCB. Furthermore, microsomes from rats treated with HCB, phenobarbital and isosafrole showed catalytic activity (in decreasing order) towards HCB. The amount of product formation by microsomes from 3-methylcholanthrene induced rats and from untreated rats was negligible. Inhibition of P-450c had no effect on the activity. The BE52 MAb inhibited the formation of PCP in microsomes from HCB and phenobarbital induced rats with 58% and 44%, respectively. In these

TABLE 3

MICROSOMAL CONVERSION OF HEXACHLOROBENZENE. EFFECT OF DIFFERENT INDUCERS AND SPECIFIC MONOCLONAL ANTIBODIES

INDUCERS/ ANTIBODY	RATE PCP	DIOLS
NONE (male)	0.8 (100)	0.1 (100)
NONE (female)	1.8	0
HCB	42.0 (100)	4.2 (100)
C9	40.3 (96)	2.8 (68)
BE52	18.5 (44)	1.4 (33)
C8	41.1 (98)	4.4 (106)
PB	24.9 (100)	1.2 (100)
C9	24.4 (98)	1.3 (105)
BE52	14.3 (57)	0.2 (13)
C8	23.9 (96)	1.2 (100)
3-MC	0.8	0
ISF	8.5 (100)	0.1 (100)
C9	8.9 (105)	0.1 (127)
BE52	10.2 (121)	0 (20)
C8	10.6 (125)	0.1 (149)
DEX	109.8	14.9

The incubation conditions were: 5 minutes incubation with 2 mg of microsomal protein, 0.2 mg per nmole P-450 of the appropriate antibody, 50 nmoles of ^{14}C-HCB, and 1 mM NADPH in a 2 ml volume. For details, see method section. The rate is expressed as pmole product per minute per mg microsomal protein. Between brackets the rate as a percentage of the rate of the incubations without antibody is presented. The C9 MAb binds to cytochrome P-450c without affecting its catalytic acitivity, C8 binds and inhibits P-450c, and BE52 inactivates cytochromes P-450b and P-450e. The rate of formation of PCP and diols together always was within 90% of the total rate of conversion, as derived from the total amount of radioactivity eluting before the substrate. The values represent the average of duplicate incubation.

TABLE 4

MICROSOMAL CONVERSION OF PENTACHLOROPHENOL. EFFECT OF DIFFERENT INDUCERS AND SPECIFIC ANTIBODIES

INDUCERS/ANTIBODY	RATE (pmole/min/mg protein)		RATIO BETWEEN 1,4 AND 1,2
	1,4-DIOL	1,2-DIOL	
NONE (male)	120 (100)	29 (100)	4.1
C7	107 (89)	33 (114)	3.2
BE52	94 (78)	29 (100)	3.2
C8	103 (86)	24 (83)	4.3
NONE (female)	77	33	2.3
HCB	469 (100)	162 (199)	2.9
C7	425 (91)	144 (89)	3.0
BE52	337 (72)	61 (38)	5.5
C8	519 (111)	141 (87)	3.7
PB	219 (100)	101 (100)	2.2
C7	190 (87)	82 (81)	2.3
BE52	221 (101)	79 (78)	2.8
C8	188 (86)	80 (79)	2.4
3-MC	187 (100)	114 (100)	1.6
C7	177 (95)	88 (77)	2.0
BE52	175 (94)	112 (98)	1.6
C8	225 (120)	135 (118)	1.7
ISF	344 (100)	72 (100)	4.8
C7	326 (95)	62 (86)	5.3
BE52	276 (80)	49 (68)	5.6
C8	319 (93)	58 (81)	5.5
DEX	618 (100)	54 (100)	11.4
C7	626 (101)	45 (83)	13.9
BE52	634 (103)	43 (80)	14.7
C8	594 (96)	54 (100)	11.0

The rate is expressed as pmole product per minute per mg of microsomal protein. Between brackets the rate as percentage of the rate of the incubations without antibody is presented. Incubation conditions as stated in Table 2, with 200 nmoles of ^{14}C-PCP. Excluding the unknown metabolite produced independently from cytochrome P-450, the total diol formation always was within 85% of total product formation, as derived from the total amount of radioactivity eluting before the substrates.

incubations, the diol formation was also greatly reduced. Using microsomes from isosafrole treated rats, no decrease in activity was affected by BE52.

The microsomal conversion of PCP, and the influence of the MAb on the formation of tetrachloro-1,4- and tetrachloro-1,2-hydroquinone was measured (table 4). Again, microsomes from dexamethasone treated rats showed the highest rate of conversion (4.5 times the amount of product as compared to microsomes from untreated rats), while microsomes from HCB treated rats were 4.2 times as active as microsomes from untreated rats. Microsomes from isosafrole induced rats gave a 2.8 fold induction, and both 3-methylcholanthrene and phenobarbital treatment resulted in a twofold induction as compared to untreated microsomes. The only clear inhibitory effect of the added antibodies is a 62% decrease in formation of 1,2-diol after addition of BE52 to microsomes from HCB treated rats. In these incubations, the 1,4-formation is also slightly lowered (38%). No inhibition was measured when cytochrome P-450b was inhibited in microsomes from phenobarbital treated rats, nor was any decrease in catalytic activity detected when cytochrome P-450c was inhibited in microsomes from 3-methylcholanthrene treated rats.

DISCUSSION

1) the hydroxylation of hexachlorobenzene

The data obtained from the incubations with purified cytochrome P-450 isoenzymes and from the microsomal incubations, using microsomes from rats treated with different inducing agents, and selective inhibition of cytochrome P-450b and P-450c, suggest the involvement of at least 3 different P-450 isoenzymes in the hydroxylation of HCB, namely P-450p, P-450b and P-450e. The catalytic activity of purified cytochromes P-450b and P-450e is in agreement with the decrease of activity caused by the BE52 MAb in microsomes from rats treated with HCB and phenobarbital. However, the rate of conversion of HCB by purified P-450b and P-450e is lower than would be expected from this inhibition. This is most likely due to the very poor solubility of HCB in aqueous solutions. In microsomal

suspensions the solubility is related to the microsomal concentration, and is about 25 uM at 1 mg protein per ml.

The involvement of P-450p in the hydroxylation of HCB is indicated by the high rate of conversion displayed by microsomes from dexamethasone induced rats. Dexamethasone induces cytochrome P-450p more than 100-fold as compared to untreated female rats (Wrighton, 1984). Phenobarbital treatment results in a 20-fold induction of P-450p (Wrighton et al, 1985). The presence of P-450p in microsomes from phenobarbital treated rats probably accounts for the remaining activity after inhibition of P-450b and P-450e. Microsomes from isosafrole induced rats have also been shown to contain a small amount of cytochrome P-450p (Guengerich, 1982). HCB treatment causes induction of cytochromes P-450b, P-450c and P-450d (in decreasing order), as established by immunoquantitation (Li, 1987). The amount of P-450p has not been determined after treatment of rats with HCB.

2) the hydroxylation of pentachlorophenol

Although purified cytochromes P-450b and P-450e, and to an even larger extent, P-450c were able to hydroxylate PCP, the use of MAb indicated that these isoenzymes are not, or only to a small extent, involved in the microsomal conversion. Treatment of rats with 3-methylcholanthrene resulted in a twofold increase in the rate of microsomal hydroxylation of PCP, but inhibition of cytochrome P-450c in these microsomes had no inhibitory effect on product formation. Only in HCB-microsomes inhibition of P-450b and P-450e clearly decreased the formation of tetrachloro-1,2-hydroquinone, while tetrachloro-1,4-hydroquinone formation was hardly affected. The fact that the formation of the 1,2-isomer is more suppressed than the formation of the 1,4-diol is in agreement with the finding that P-450b and P-450e produce more 1,2-diol than 1,4-diol. Apparently, the rate of hydroxylation shown by purified isoenzymes is higher than their activity in microsomes. This is also illustrated by the rate of N-demethylation of benzphetamine by purified cytochrome P-450b, which is at least 3 times higher than the activity towards benzphetamine by P-450b in microsomes from phenobarbital induced rats (Reik, 1985).

Cytochrome P-450p probably has a high catalytic activity towards PCP, as can be concluded from the incubations with liver microsomes from

dexamethasone treated rats. Cytochrome P-450p may be partly responsible for the hydroxylation of PCP by microsomes from rats treated with phenobarbital, HCB or isosafrole, but it is unlikely that cytochrome P-450p is present in microsomes from 3-methylcholanthrene treated rats, since these microsomes have no catalytic activity towards HCB hydroxylation. The isoenzyme responsible for the turnover of PCP in microsomes from 3-methylcholanthrene and isosafrole treated rats may be cytochrome P-450d, which is also active in a reconstituted system, and is more strongly induced by isosafrole than by 3-methylcholanthrene (Thomas, 1983). This is in agreement with the higher rate of product formation by microsomes from rats treated with isosafrole than with 3-methylcholanthrene. However, the possibility remains that another, as yet unidentified cytochrome P-450 isoenzyme is involved.

As noted previously for microsomal incubations (Van Ommen, 1986), the ratio between formation of tetrachloro-1,4-hydroquinone and tetrachloro-1,2-hydroquinone differed for the various cytochrome P-450 isoenzymes. Purified cytochromes P-450b and P-450e predominantly produce the 1,2-diol, while the cytochromes P-450c and P-450d have a preference for the 1,4-diol. Microsomes from dexamethasone treated rats almost exclusively form the 1,4-diol. The finding that in microsomes of rats treated with HCB or phenobarbital an excess of the 1,4-diol is detected may be attributed to the activity of P-450p in these microsomes.

Concluding, the hydroxylation of HCB in microsomes is most likely catalyzed by cytochrome P-450p, and to a lesser extent by cytochromes P-450b and P-450e. The hydroxylation of PCP is also predominantly supported by cytochrome P-450p. It is clear that studies using specific inducers of cytochrome P-450 can be misleading in that small amounts of other P-450 isoenzymes can be of relatively major importance, and that the ability of purified cytochrome P-450 isoenzymes to convert compounds not necessarilly implicates that they contribute to the microsomal conversion.

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CHAPTER 7

BIOTRANSFORMATION OF ^{14}C-HEXACHLOROBENZENE AND ITS MAJOR OXIDATIVE METABOLITES BY PRIMARY CULTURES OF CHICK EMBRYO HEPATOCYTES

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ABSTRACT

Using radiolabeled compounds, the biotransformation of hexachlorobenzene and its major oxidative metabolites, pentachlorophenol and tetrachloro-1,4-hydroquinone, has been investigated quantitatively in primary cultures of chick embryo hepatocytes.

Hexachlorobenzene biotransformation resulted in formation of pentachlorophenol as the major product. The formation of PCP could be influenced by treatment of the cultures with different types of inducers of cytochrome P-450. The most potent inducer, dexamethasone, which induces cytochrome P-450p in the rat, increased the PCP-formation threefold (238 pmole/culture/24 hrs) as compared to none-induced cultures.

Pentachlorophenol metabolism mainly resulted in formation of conjugates. More than 85% of total product formation was attributed to an unidentified, extractable conjugate. No tetrachloro-1,4-hydroquinone could be detected. Addition of the latter compound to cultures resulted in a partial oxidation to the benzoquinone, together with the formation of a more polar extractable metabolite. For all three compounds evidence was obtained for the formation of glutathione conjugates, which, at least in the case of PCP and TCHQ metabolism, most likely are conjugates of tetrachloro-1,4-benzoquinone. Incubations with HCB, PCP and TCHQ all resulted

in the appearance of radioactivity covalently bound to cellular protein, with TCHQ giving the most and HCB giving the least binding. In no case, however, did covalent binding reach the level found in microsomal incubations.
In conclusion, the biotransformation of HCB in primary cultures of chick embryo hepatocytes is only partly oxidative, leading to covalent binding to protein. Other pathways, presumably conjugation reactions, to a large extent prevent the hydroxylation products of HCB from forming benzoquinones, thus protecting against toxic effects due to covalent binding.

INTRODUCTION

The fungicide hexachlorobenzene (HCB) is known for its porphyrinogenic and carcinogenic action (Koss, 1978; Smith and Cabral, 1980). A state of HCB-induced chemical porphyria is characterized by an accumulation of uroporphyrin in liver and urine, caused by a selective inhibition of the enzyme uroporphyrinogen decarboxylase (Elder, 1978). Biotransformation of HCB seems to be a prerequisite for this inactivation: induction and selective inhibition of the cytochrome P-450 system leads to an enhancement and a decrease of the porphyrinogenic action, respectively (Wainstock de Calmanovici, 1984; Smith, 1986). Microsomal studies have shown that HCB is hydroxylated by cytochrome P-450 to pentachlorophenol (PCP), which in turn is hydroxylated to tetrachloro-1,4- and -1,2-hydroquinone. These hydroquinones can be oxidized to their quinone forms, which display a high reactivity towards protein and DNA (Van Ommen, 1985, 1986, 1986a). This covalent binding may be involved in the inactivation of uroporphyrinogen decarboxylase.
The chick embryo hepatocyte culture system has proven to be a sensitive system for mechanistic studies of HCB-induced porphyria (Debets, 1981; Ferioli, 1984; Sinclair, 1987; Wainstock de Calmanovici, 1980). These cells uniquely combine the presence of an intact porphyrin biosynthesis pathway (Sinclair and Granick, 1974) with an inducable cytochrome P-450 system (Althaus, 1979, Topp and Van Bladeren, 1986), thus offering the possibility of linking the in vivo studies on chemical porphyria (e.g. Koss, 1978) with mechanistic microsomal studies on HCB metabolism (Van Ommen, 1985, 1986, 1986a). The induction of the P-450 system as well as the porphyrinogenic action of HCB in chick embryo hepatocytes have been studied in detail.

In the present paper the biotransformation of HCB and its primary oxidative metabolites PCP and 1,4-TCHQ is described, with special attention to the cytochrome P-450 dependent biotransformation leading to covalent binding to protein.

METHODS

CHEMICALS U-(^{14}C)-hexachlorobenzene (Amersham UK), 70 mCi/mmol, contained 0.12% pentachlorobenzene as a radiochemical impurity as determined by the HPLC method described below. U-(^{14}C)-pentachlorophenol, with a specific activity of 37.5 mCi/mmole, was purchased from CEA, Gif-sur-Yvette, France. The 2.2% radiochemical impurity of tetrachlorophenol was removed by preparative HPLC with a method described in chapter 5. (^{14}C)-tetrachloro-1,4-hydroquinone was synthesized enzymatically from (^{14}C)-PCP as described in chapter 5. Williams E culture medium containing 2 mM glutamine and 10% newborn calf serum was from Gibco biocult, Scotland, dispase grade 2 from Boehringer Mannheim FRG, phenobarbital from OPG, the Netherlands, aroclor 1254 from Monsanto, St. Louis USA, isosafrole from Fluka and beta-naphthoflavone from Aldrich, dexamethasone was from Sigma. All other chemicals used were of analytical grade.

HEPATOCYTE ISOLATION AND CULTIVATION Hepatocytes were obtained from 14 day old chick embryos. The livers were perfused in situ with Hank's solution without calcium and magnesium, containing 20 mM HEPES and 0.04% EDTA to wash out red blood cells. After cutting the collected livers into small pieces, the cells were dissociated enzymatically in Hank's HEPES pH 7.4 containing 1 U/ml dispase. The dissociated cells were sedimented by centrifugation at 1200 g for 4 minutes. Red blood cells were lysed by washing the cells with buffered ammonium chloride pH 7.65 during 4 minutes. Dissociation, centrifugation and lysis were repeated two more times. The isolated hepatocytes were resuspended in Williams E medium containing 10% serum (10 livers/100 ml). 5 cm plastic culture dishes (Costar) were filled with 5 ml of this suspension, containing 4-6x10^6 cells. The dishes were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. A monolayer was established in 4 hr. Non-attached cells were removed after 22 hr by renewing the medium.

STANDARD INDUCTION AND EXPOSURE PROCEDURE 24 hr after isolation of the hepatocytes, treatment with drug inducing agents was started. Phenobarbital (dissolved in water), beta-naphthoflavone (dissolved in DMSO), dexamethasone, isosafrole or

aroclor 1254 (all dissolved in acetone) were added. The concentrations used are given in the legends to figures and tables. The maximum concentration of the solvents in medium was 0.2%. After 24 hr the medium was replaced by medium without serum containing the same inducers, together with 7.1 μM ^{14}C-HCB (2.5 uCi), 1 μM ^{14}C-PCP (0.16 uCi) or 1 μM ^{14}C-TCHQ (12.5 nCi) dissolved in 25 μl acetone. The solution of HCB PCP or TCHQ in medium was prepared by injecting 25 μl of a solution in acetone into a tube containing 5 ml of medium, after which the tube was vigourously vortexed. The HCB appeared to be dissolved since microscopic examination revealed no crystals in the culture dishes (as can be observed when HCB is added in DMSO or ether). After 24 hr the cells were harvested. Blancs consisted of zero time incubations for metabolism and covalent binding experiments and of 24 hr incubations without cells to determine the radiochemical impurity. Incubations were stopped by separating the medium from the cells, harvesting the cells with a rubber policeman in 2x0.5 ml Tris/HCl buffer, pH 7.8 (100 mM) and finally rinsing the dish with a small volume of methanol. Medium, cells and methanol wash were combined and, after addition of HCl to a final concentration of 0.6 N, extracted with 3x3 ml of diethylether in the case of HCB, or ethylacetate/acetone (2:1) in the cae of PCP and 1,4-TCHQ. The extracts were dried with anhydrous Na_2SO_4, solvent was removed in a stream of nitrogen and the residue dissolved in 50 μl of methanol and analysed by HPLC.

ANALYSIS OF METABOLITES AND COVALENT BINDING The HPLC system used and HPLC analysis of HCB metabolites have been described previously (Van Ommen, 1986).

PCP metabolism was quantitated by separating PCP and metabolites on HPLC with a gradient of 50 to 85% methanol against water containing 1% acetic acid in 30 minutes, followed by 5 minutes isocratic elution of 85% methanol. TCHQ metabolism was analysed as described elsewhere (chapter 5).

After the ether extraction the precipitated protein was separated from the water phase and covalent binding to protein was determined as described previously (Van Ommen, 1985). The aqueous phase was treated with HCl (pH 1) for one hour at 95°C (Ahlborg, 1978), after which ether extraction and HPLC separation was performed again to estimate the amount of glucuronidated and sulfated metabolites formed during the incubation (Lilienblum, 1985). The amount of radioactivity after this second extraction procedure was determined in order to estimate the amount of glutathione conjugates produced.

RESULTS

1) Biotransformation of ^{14}C-hexachlorobenzene

HCB was mainly converted into pentachlorophenol (PCP) by chick embryo hepatocytes in culture (table 1). A decrease in the amount of pentachlorobenzene, present as a radiochemical impurity (0.12%) was also observed. Concomitant appearance of tetrachlorophenol explained this fact. The sum of tetrachlorophenol and pentachlorobenzene remained constant in all experiments, indicating that no pentachlorobenzene was formed from HCB under these conditions.

In order to estimate the amount of glucuronidated or sulfated metabolites, acid hydrolysis was performed on the aqueous phase after ether extraction (Lilienblum, 1985). Repeated ether extraction after hydrolysis resulted in partial removal of radioactivity from the water phase (table 1). HPLC analysis of ether extracts after acid hydrolysis showed that the activity consisted almost exclusively of HCB, whereas about 8.9% of total recovered PCP coeluted with the PCP marker. The method of detection and quantitation of the metabolites we used was not applicable for the detection of the previously described metabolites pentachlorothiophenol

TABLE 1. BIOTRANSFORMATION OF HEXACHLOROBENZENE IN A PRIMARY CULTURE OF CHICK EMBRYO HEPATOCYTES

METABOLITE	AMOUNT (pmol)
pentachlorophenol	69
PCP-glucuronide or sulfate ester	1.7
glutathione conjugate	15.3
metabolites covalently bound to protein	0.7

35 nmoles of ^{14}C-HCB were incubated for 24 hours with a monolayer of embryonal chick hepatocytes, as described in the method section. The amount of PCP formed was determined by HPLC of the ether extract of the medium and lysed cells, glucuronides and sulfate esters were estimated from the amount of PCP extracted from the medium after acid hydrolysis (as determined by HPLC), glutathione conjugates were estimated from the amount of radioactivity remaining in the aqueous phase after acid hydrolysis / extraction, and the amount of covalent binding was determined after extensive washing with organic solvents of the precipitated cellular proteins. Values are the average of duplicate incubations of a representative experiment, corrected for zerotime incubations.

and pentachlorothioanisol (Debets, 1981). Pentachlorothiophenol does not elute as a distinct peak from reversed phase columns in our hands and pentachlorothioanisol almost coelutes with HCB. Therefore, since the thioether bond is relatively stable under acid hydrolysis, glutathione conjugates were estimated as the residual radioactivity left in the aqueous phase after acid hydrolysis and extraction (table 1).

The biotransformation of HCB appeared to be linear with time for at least 24 hours (fig. 1). The relationship between the concentration and the amount of PCP formed is presented in figure 2. Biotransformation of HCB was linear up to 7 µM. This concentration of HCB was therefore chosen for all subsequent incubations.

The effect of exposure of the culture system to different cytochrome P-450 inducing agents on the biotransformation of HCB is presented in table 2. Dexamethasone appeared to be by far the most potent inducer of HCB-hydroxylation. Isosafrole, aroclor 1254 and phenobarbital induced PCP-formation in decreasing order, while beta-naphthoflavone decreased the hydroxylation as compared to non-induced cultures. Figure 3 shows the dependency of the hydroxylation on the dexamethasone concentration. At high concentrations of dexamethasone the amount of PCP-formation was the same in cultures with and without serum. The covalent binding as a result of biotransformation of HCB also varied with the different

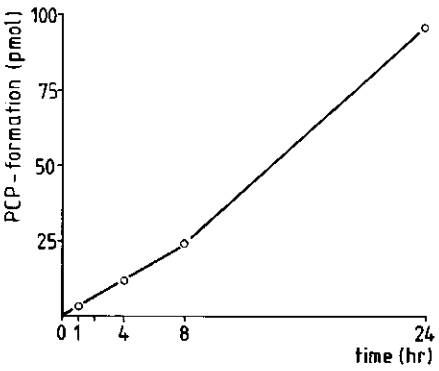

figure 1. Dependency of the formation of pentachlorophenol as a result of HCB-biotransformation on the incubation time, in a primary culture of chick embryo hepatocytes, treated with phenobarbital (400 µg/ml). For details, see method section.

TABLE 2. BIOTRANSFORMATION OF HEXACHLOROBENZENE IN A PRIMARY CULTURE OF CHICK EMBRYO HEPATOCYTES.
EFFECT OF TREATMENT WITH DIFFERENT INDUCERS OF CYTOCHROME P-450.

INDUCER	FORMATION OF PENTACHLOROPHENOL (pmol)	COVALENT BINDING (pmol)
none	77	0.7
phenobarbital (400)a	81	0.4
aroclor 1254 (2)	98	0.4
B-naphthoflavone (6)	35	1.1
isosafrole (18)	105	0.9
dexamethasone (39)	238	0.6

35 nmoles of ^{14}C-HCB were incubated with a monolayer of embryonal chick hepatocytes, as described in the method section. The amount of PCP formed after 24 hours of incubation was determined by HPLC of the ether extracts of medium and lysed cells. Covalent binding was quantified after extensive washing of the cellular protein pellet after ether extraction. The values (average of duplicate incubations) of a representative experiment are presented, and are corrected for zerotime incubations.
a) In brackets the concentration of the inducer is stated, in µg/ml.

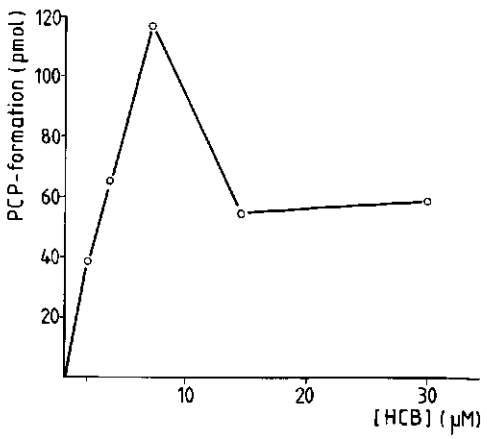

figure 2. Dependency of the formation of pentachlorophenol on the concentration of hexachlorobenzene in a primary culture of chick embryo hepatocytes, treated with phenobarbital (400 µg/ml). For details, see method section.

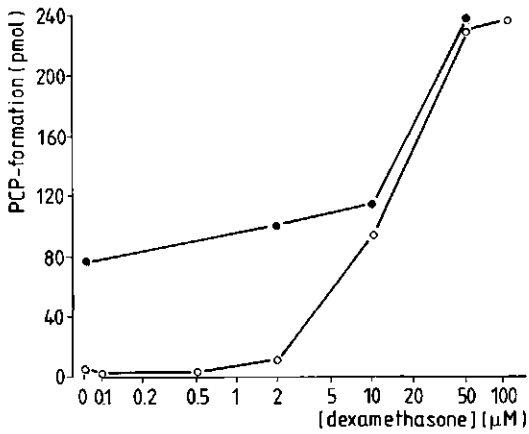

figure 3. Effect of the concentration of dexamethasone on the formation of PCP from HCB, in a primary culture of chick embryo hepatocytes, in the presence and absence of serum. For details, see method section.

inducers used (table 2). Beta-naphthoflavone induction resulted in the largest amount of covalent binding to protein (1.1 pmole).

2) Biotransformation of (^{14}C)-pentachlorophenol

The dependency of formation of extractable products in primary cultures of chick embryo hepatocytes on the concentration of PCP is presented in figure 4 for beta-naphthoflavone induced cultures. Conversion was linear with the concentration up to 10 uM, while a maximal product formation of 15 nmoles was reached at 50 uM PCP. Most experiments were performed using a PCP concentration of 1 μM. Contrary to the microsomal metabolism of PCP, in cultures no formation of hydroxylated products (tetrachloro-1,2-hydroquinone and tetrachloro-1,4-hydroquinone) was detected (table 3). The major product (85% of total product formation) was an ethyl acetate/acetone extractable conjugate of PCP, of which the structure could not be identified. Acid hydrolysis of this compound resulted in reappearance of PCP, while its instability prevented identification by MS or GC-MS: this technique also only indicated the presence of PCP. The amount of this conjugate formed in cultures was dependent on the inducer used (table 3). Beta-naphthoflavone was the best inducer of the biotransformation of PCP. The amount of non-extractable glucuronides and sulfate esters, as estimated by the amount of extractable label after

TABLE 3. BIOTRANSFORMATION OF PENTACHLOROPHENOL IN A PRIMARY CULTURE OF CHICK EMBRYO HEPATOCYTES.

METABOLITE	AMOUNT (pmol)		
	INDUCER		
	none	B-NF	DEX
1,4-TCHQ and 1,2-TCHQ	n.d.	n.d.	n.d.
extractable conjugate	360	700	480
glucuronide or sulfate ester	1	4	1
glutathione conjugate	46	84	40
metabolites covalently bound to protein	2.1	16.8	6.3

5 nmoles of ^{14}C-PCP were incubated for 24 hours with a monolayer of embryonal chick hepatocytes, treated with different inducers. Dexamethasone was added to 20 µg/ml, beta-naphthoflavone to 6 µg/ml. Metabolites were quantitated as described under table 1. The values are the average of duplicate incubations, corrected for zerotime incubations. (n.d.: non-detectable)

acid hydrolysis, was very low (4 pmole). In beta-naphthoflavone treated cell cultures 84 pmole of the added PCP was not extracted from the medium even after acid hydrolysis, and denoted as "glutathione conjugates". Covalent binding to protein was considerably higher than in HCB-exposed cells. Beta-naphthoflavone treatment resulted in 16.8 pmoles of binding, 8 times higher than measured in non-induced cells.

3) Biotransformation of (^{14}C)-tetrachloro-1,4-hydroquinone

The tetrachlorobenzoquinones are the ultimate covalent binding agents in microsomal HCB metabolism. In order to determine whether in primary cultures of chick embryo hepatocytes exposure to the hydroquinones would also result in covalent binding, small amounts (1 µM) of (^{14}C)-tetrachloro-1,4-hydroquinone were incubated with these cultures. After 24 hours of incubation 75% of the added 1,4-TCHQ was metabolized, while only 2.6% (130 pmole) was found to be covalently attached to protein (table 4). 3.8% was detected in the benzoquinone form, while the major part of the extractable radioactivity (35% of added label) eluted from the HPLC as a discrete peak before 1,4-TCHQ and 1,4-TCBQ, indicating a more polar structure. Conjugates with glucuronic acid or sulfate, and conjugates with glutathione were estimated to be formed to a maximal amount of 16% and 18%, respectively.

TABLE 4. BIOTRANSFORMATION OF TETRACHLORO-1,4-HYDROQUINONE IN A PRIMARY CULTURE OF CHICK EMBRYO HEPATOCYTES.

METABOLITE	AMOUNT (pmol)
extractable metabolites: 1,4-TCBQ	190
unknown	1740
glucuronide or sulfate ester	800
glutathione conjugate	900
metabolites covalently bound to protein	130

5 nmoles of 1,4-TCHQ were incubated for 24 hours with a monolayer of embryonal chick hepatocytes, induced with isosafrole. The metabolites were determined as described under table 1. The values are the average of duplicate incubations, corrected for zerotime incubations.

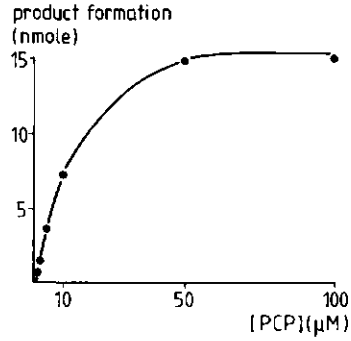

figure 4. Dependency of the total (extractable) product formation on the concentration of PCP, in a primary culture of chick embryonal hepatocytes, treated with beta-naphthoflavone (6 μg/ml). For details, see method section.

DISCUSSION

The chick embryo hepatocyte culture system provides a tool for quantitative investigation of the in vitro biotransformation of HCB. Although the rate of conversion is extremely low, the amounts of metabolites are reproducable and well above the detection limit of 1 pmole. The first part of the biotransformation of HCB is similar for microsomal incubations and hepatocyte cultures. Both systems yield PCP as the major product, together with a small amount of covalent binding.

In studies with rat liver microsomes and purified cytochrome P-450 isoenzymes (chapter 6) it appeared that P-450p is the major cytochrome P-450 isoenzyme involved in the hydroxylation of HCB. Cytochrome P-450 isoenzymes can be induced in the chick embryo culture system (Althaus, 1979; Topp and Van Bladeren, 1986) and the results obtained are in good agreement with the microsomal data: Dexamethasone pretreatment resulted in the highest conversion of HCB. The negative effect of serum on the metabolic capacity of this culture system has been described earlier for benzo(a)pyrene metabolism (Topp and Van Bladeren, 1986).

The biotransformation of HCB and PCP in chick embryo hepatocytes differs from that by rat liver microsomes in that no diols could be detected from the hepatocytes. In microsomal incubations the cytochrome P-450 dependent diol formation was the major route of metabolism of HCB, ultimately leading to covalent binding of quinones to protein (Van Ommen, 1986a, chapter 5). It is possible that all diols formed in these cultures have been oxidized to their quinone forms and were recovered as covalently bound label or as the glutathione conjugates of their benzoquinones. The high reactivity of glutathione towards tetrachloro-1,4-benzoquinone has been shown previously (Van Ommen, 1986).

Covalent binding as a result of biotransformation of HCB and PCP was best induced by beta-naphthoflavone. However, levels were considerably lower than in microsomal incubations (Van Ommen, 1986, 1986a). In agreement with this finding, the relative amount of 1,4-TCHQ which became covalently bound to protein when this compound was incubated with chick hepatocytes is very low (2.6%), as compared to the 45% binding in a 5 minute microsomal incubation in the presence of NADPH (chapter 5). Conjugation mechanisms apparently are effective in removing the benzoquinone, since also upon incubation of ^{14}C-TCHQ, almost three times more non-extractable radioactivity than benzoquinone or covalently bound metabolites was detected. Similarly, the major metabolite of PCP produced by the hepatocytes was an as yet unidentified conjugate.

In conclusion, the biotransformation of HCB and its major oxidative metabolites PCP and 1,4-TCHQ in primary cultures of chick embryo hepatocytes shows similarities with the biotransformation by rat liver microsomes, especially for the cytochrome P-450 dependent hydroxylation of HCB. However, the presence of other biotransformation routes and of efficient protective mechanisms results in much less covalent binding to protein.

Chick embryo hepatocytes in primary culture have proven to be a sensitive tool for observing the development of porphyria after exposure to HCB (Debets et al.,

1981). An earlier onset and increase of porphyrin accumulation was observed after pretreatment of the cultures with beta-naphthoflavone, 3-methylcholanthrene and phenobarbital, as compared with non-induced cultures. Now that it is possible to quantitatively investigate HCB biotransformation and porphyrinogenic action in the same culture system, it will be possible to combine results of mechanistic studies on the biotransformation with data on development of porphyria.

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CHAPTER 8

DISCUSSION

THE RELATION BETWEEN BIOTRANSFORMATION AND PORPHYRINOGENIC ACTION OF HEXACHLOROBENZENE

At the onset of the investigations presented in this thesis, the involvement of cytochrome P-450 in the development of HCB-induced porphyria had been firmly established (chapter 1). However, no metabolite or reactive intermediate of HCB had been identified as the ultimate porphyrinogenic compound. The aim of this study was to investigate the mechanisms of the oxidative in vitro biotransformation of HCB, in order to identify possible reactive intermediates.

The route of microsomal biotransformation of HCB as established in the present study is shown in figure 1. Hydroxylation of HCB and PCP results in formation of two tetrachlorohydroquinone isomers. These hydroquinones can be oxidized to their semiquinone and quinone forms, which are highly reactive towards protein and DNA. A mechanism is presented in which biotransformation of HCB leads to the generation of compounds which are potentially (geno)toxic. In this chapter the following questions will be discussed:

1 Are these quinones the only reactive species involved in the oxidative biotransformation of HCB?
2 What is the relevance of this covalent binding in vivo?
3 Is there a relation between this covalent binding and the toxic action of HCB.?

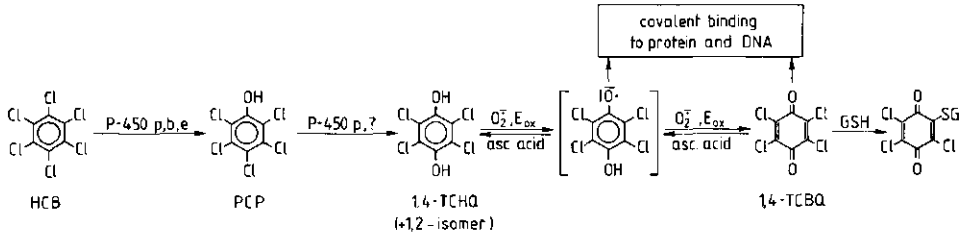

figure 1. Route of oxidative biotransformation of hexachlorobenzene.

1) The experiments described in this thesis clearly demonstrate that quinone-mediated covalent binding to protein is the only alkylating process originating from microsomal biotransformation of HCB. The amount of covalent binding produced during the microsomal metabolism of HCB can almost exclusively be attributed to reactions occurring after the formation of PCP (chapter 3). This rules out the possible involvement of radicals generated during reductive dechlorination of HCB, as mentioned in chapter 1. Anaerobic conditions, which would stimulate this route, did not result in an increase of covalent binding or formation of pentachlorobenzene. These results have been confirmed by Stewart and Smith (1986). Epoxidation, resulting in a hexachlorocyclodienone structure, analogous to the NIH-shift described for benzene (Jerina and Daly, 1974), followed by loss of a positively charged chlorine ion, probably is the mechanism of hydroxylation of HCB. Although the epoxide has alkylating properties, the absence of covalent binding during the formation of PCP suggests that no such process occurs.

The quinones derived from HCB are unique in their reactivity in that their quinone structure remains intact after reaction with a nucleofile. 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 (Lau, 1984), which has to be reoxidized before being able to react again (figure 2). As a consequence, e.g. glutathione conjugates of tetrachlorobenzoquinones are still reactive towards protein. This is illustrated by the fact that in a reaction of tetrachloro-1,4-benzoquinone with glutatione not only monosubstituted conjugates are formed, but also di- and tri-, and possibly fully substituted conjugates.

2) The experiments with primary cultures of chick embryo hepatocytes, as described in chapter 7, have shown that i) the major route of biotransformation of HCB in intact cells does not lead to tetrachlorobenzoquinones, as was found for microsomal incubations. This is mainly due to a different route of PCP-biotransformation, competing with the oxidative route, and resulting in conjugation. ii) There are efficient protective mechanisms (reduction and conjugation) present in these cells

3) Are the tetrachlorobenzoquinones responsible for the porphyrinogenic and carcinogenic action of HCB in vivo? The amount of covalent binding as a result of biotransformation of HCB in primary cultures of embryonal chick hepatocytes is extremely low (about 1 pmole, 35 ppm of the added HCB). Moreover, Smith (1986) partly purified URO-D after exposure of female mice to ^{14}C-HCB, and was not able to detect radiolabel on the enzyme.

The fact that PCP is not porphyrinogenic would suggest that tetrachlorobenzoquinones, as metabolites of PCP, have no relation with porphyria. There may be some differences between PCP given orally and PCP as a hydroxylation product of HCB. Firstly, PCP does not induce cytochrome P-450. Therefore, the possibilities for hydroxylation to hydroquinones are limited, and the route of glucuronidation might be favoured. Secondly, the site of hydroxylation of PCP, the liver endoplasmatic reticulum, is also the site of formation of PCP as a product of HCB. It is even possible that PCP, formed from HCB by cytochrome P-450p, does not leave the active site, but instead is hydroxylated by the same cytochrome P-450-molecule to TCHQ, which is, again in the active site, oxidized to the benzoquinone (chapter 5). Tetrachloro-1,4-hydroquinone has not been tested for its porphyrinogenic capacities in the rat. In the Japanese quail, a species very sensitive towards chemical porphyria, 1,4-TCHQ also proved to be non-porphyrinogenic (Carpenter, 1985), although this compound was able to increase the HCB-induced porphyrin excretion when administered simultaneously.

Summarizing and concluding, it is probable that covalent binding to protein, as caused by oxidative biotransformation of HCB, is of minor importance in vivo. Direct alkylation of uroporphyrinogen decarboxylase by quinone metabolites is most likely not a key mechanism in the porphyrinogenic action of HCB. Since no evidence for the occurrence of other reactive intermediates was obtained, it is unlikely that the inactivation of URO-D is caused by covalent modification by a metabolite of HCB. The role of cytochrome P-450 in the process of HCB-induced porphyria will then have to be reevaluated. Phenobarbital treatment of female rats enhances the porphyrinogenic action of HCB in vivo. However, no direct evidence for a higher degree of oxidative biotransformation was found: no increase in hepatic levels of oxidative metabolites has been

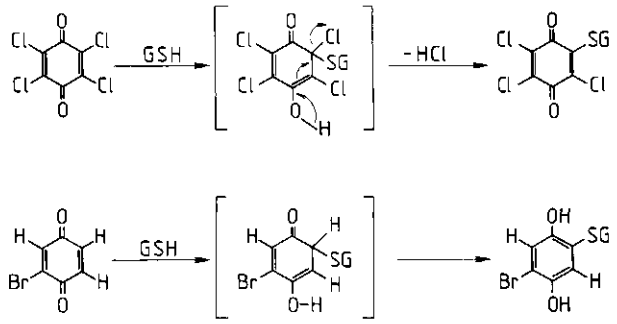

figure 2. The reaction of glutathione with tetrachloro-1,4-benzoquinone and 2-bromobenzoquinone.

to protect against covalent binding to protein of TCBQ. Probably the cellular antioxidants play an important role. As a result of these two phenomena, 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). In agreement with this finding, 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 (1980) 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.

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 non-selective (Tunek, 1979).

detected (Debets, thesis). 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 (1984), Sinclair (1987) and others. However, besides HCB there are a large number of other compounds which stimulate the oxidase function of cytochrome P-450, and many of these are non-porphyrinogenic. Other mechanisms must be involved which determinine the relative specificity of the group of chlorinated aromatic compounds in inducing porphyria.

It may be possible, however, that covalent binding is involved in the HCB-induced tumor formation. Although the data on the carcinogenicity of PCP are conflicting, this compound has been shown to provoke chromosome changes after occupational exposure (Bauchinger, 1982), In chapter 4 the ability of the TCBQ to bind to DNA was established, while TCHQ causes DNA-breaks (Witte, 1985).

In the present study (partly) purified enzyme systems were shown to be a powerful tool in studies on the relation between biotransformation and toxicity, and provide information on the mechanism of single reactions involved in metabolic routes and their consequences, and are thus of major importance in unravelling the metabolism of HCB. However, one should keep in mind that the in vivo situation is more complex than a number of single reactions placed one after the other, and that in vitro findings should be validated by in vivo studies.

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DE IN VITRO BIOTRANSFORMATIE VAN HEXACHLOORBENZEEN IN RELATIE TOT ZIJN TOXICITEIT

SAMENVATTING

Hexachloorbenzeen (HCB) wordt in grote hoeveelheden in het millieu aangetroffen. Dit is een gevolg van het feit dat HCB gevormd wordt als bijproduct in de industriele productie van verschillende gechloreerde verbindingen, en dat het tot voor kort gebruikt werd als fungicide.
In proefdieren induceert HCB tumorvorming. Zowel in de mens als in proefdieren verstoort HCB de hepatische biosynthese van haem, met als gevolg een sterk verhoogde excretie van porfyrines. Deze porfyrinogeniteit van HCB bleek te worden veroorzaakt door selectieve remming van het enzym uroporfyrinogeen decarboxylase. Aangetoond is dat cytochroom P-450 betrokken is bij de porfyrinogeniteit van HCB: In vivo inductie van dit enzym met fenobarbital verhoogt, en inhibitie met met piperonyl butoxide verlaagt de excretie van porfyrines. Dit leidde tot de veronderstelling dat de inactivatie van uroporfyrinogeen decarboxylase zou kunnen worden toegeschreven aan een metaboliet of reactief intermediair, gevormd tijdens de oxidatieve biotransformatie van HCB.
Het doel van dit onderzoek was de bestudering van de oxidatieve biotransformatie van HCB in vitro, waarbij de aandacht vooral gericht was op de vorming van reactieve intermediairen. Bij de bestudering van de biotransformatie van HCB werd gebruik gemaakt van rattelever microsomen (delen van het endoplasmatisch reticulum welke het cytochroom P-450 bevatten) en gezuiverde cytochroom P-450 enzymen, evenals een primaire cultuur van kippe-embryo hepatocyten. Bij het gehele onderzoek werd gebruik gemaakt van radioactief gelabeld HCB en metabolieten. Hiermee bleek het goed mogelijk zeer kleine hoeveelheden producten op te sporen en te kwantificeren.
Aangetoond werd dat rattelevermicrosomen in staat zijn HCB te hydroxyleren. Pentachloorphenol (PCP) werd in zeer geringe hoeveelheden gedetecteerd (10-350 pmol, afhankelijk van de herkomst van de gebruikte microsomen, in een 30 minuten durende incubatie van 50 nmol HCB). De hydroxylatie van HCB bleek afhankelijk van cytochroom P-450, aangezien verzadiging van de microsomen met koolmonoxide, een remmer van het

cytochroom P-450, de reactie bijna geheel blokkeerde. De hoeveelheid PCP bedroeg 80-90% van de totale gevormde hoeveelheid metabolieten. Als tweede metaboliet werd tetrachloor-1,4-hydrochinon (1,4-TCHQ) geidentificeerd. De Km-waarden voor de vorming van PCP en 1,4-TCHQ waren voor beide 34 µM, met Vmax-waarden van 24 pmol PCP/min/mg eiwit en 1,9 pmol 1,4-TCHQ/min/mg eiwit. Experimenten met ^{18}O-gelabeld water maakten duidelijk dat de zuurstofatomen die in PCP en 1,4-TCHQ worden geincorporeerd afkomstig waren van moleculair zuurstof. Dit duidt op twee opeenvolgende hydroxylaties door cytochroom P-450. Bovendien werd gevonden dat een geringe hoeveelheid radioactiviteit (5-10% van alle metabolieten) covalent bond aan microsomaal eiwit. Aangezien deze binding duidelijk een gevolg was van de biotransformatie van HCB, werd de aandacht gericht op de identiteit van de betrokken metaboliet of reactieve intermediair.

Reductieve dechlorering van HCB als gevolg van een 1-electron-reductie van HCB door cytochroom P-450 en resulterend in een pentachloorphenyl radicaal, bleek niet betrokken bij het proces van covalente binding. Onder anaerobe condities, die in het algemeen reductieve dehalogenering stimuleren, werd de enzymatische hydroxylatie sterk geremd, terwijl covalente binding niet meer optrad. Pentachloorbenzeen, als product van reductieve dechlorering, kon niet worden gedetecteerd.

Covalente binding aan microsomaal eiwit werd ook gevonden als gevolg van microsomale PCP-hydroxylatie. Wanneer PCP werd geincubeerd bij concentraties zoals gevormd tijdens HCB-incubaties, was de mate van covalente binding ongeveer gelijk aan de hoeveelheid gedetecteerd na HCB-incubaties. Dit duidt erop dat de covalente binding ten gevolge van de microsomale conversie van HCB wordt veroorzaakt door een in deze incubaties gevormd reactieproduct van PCP. Omdat geen covalente binding toegeschreven kon worden aan de hydroxylatie van HCB tot PCP, was vorming van reactieve verbindingen tijdens deze reactie onwaarschijnlijk.

De covalente binding ten gevolge van microsomale omzetting van HCB kon worden voorkomen door toevoeging van ascorbinezuur aan de incubaties. Het verdwijnen van covalente binding ging vergezeld van een evenredige toename in de gevormde hoeveelheid TCHQ. Glutathion remt de covalente binding ook. Een stijging in de vorming van 1,4-TCHQ werd echter niet waargenomen. Deze resultaten leidden tot de conclusie dat de benzochinon- of semichinonvormen van de tetrachloorhydrochinonen verantwoordelijk zijn

voor de covalente eiwitbinding. De reactie van glutathion met het benzochinon werd chemisch bestudeerd, waarbij duidelijk werd dat zowel mono- ,di-, als trigesubstitueerde conjugaten gevormd werden. Deze conjugaten bevonden zich in de geoxideerde (chinon)vorm.

Naast covalente binding aan eiwit werden tijdens de microsomale hydroxylatie van PCP ook tetrachloor-1,4-hydrochinon en tetrachloor-1,2-hydrochinon gedetecteerd. De Km-waarden voor de vorming van deze hydrochinonen was 13 µM. Voor de covalente binding aan eiwit werd dezelfde Km-waarde gemeten. Ascorbinezuur beinvloedde de covalente binding op dezelfde wijze als bij HCB-incubaties. In PCP-incubaties werd een metabolisme-afhankelijke covalente binding aan DNA gemeten, welke een vijfde van de binding aan eiwit bedroeg.

Bij het gebruik van microsomen van ratten die behandeld waren met verschillende inductoren bleek niet alleen dat verschillen optraden in de omzettingssnelheden voor HCB en PCP, maar werden ook aanwijzingen verkregen voor het feit dat verschillende P-450 isoenzymen de beide isomeren van TCHQ in verschillende verhoudingen vormden. Op dit verschijnsel is verder ingegaan door gebruik te maken van gezuiverde cytochroom P-450 isoenzymen en selectieve monoclonale antilichamen tegen cytochroom P-450b+e en P-450c. Het bleek dat minimaal 3 verschillende isoenzymen betrokken waren bij de hydroxylatie van HCB. Gezuiverd cytochroom P-450b en P-450e waren in staat om HCB, zij het zeer langzaam, om te zetten. Hiermee in overeenstemming was het feit dat selectieve remming van deze isoenzymen in verschillende microsomen een gedeeltelijke remming van de hydroxylatie van HCB tot gevolg had. Microsomen van ratten behandeld met dexamethason, een inductor van cytochroom P-450p, vertoonden een 4,4 maal hogere omzettingssnelheid dan microsomen van phenobarbital behandelde ratten. Het lijkt daarom waarschijnlijk dat cytochroom P-450p het belangrijkste isoenzym is in de hydroxylatie van HCB. In een gereconstitueerd systeem werd PCP het meest intensief omgezet door P-450c en P-450d, en in mindere mate door P-450b en P-450e. De eerste twee isoenzymen vormden voornamelijk het 1,4-diol, en de laatste het 1,2-diol. Selectieve remming van het P-450c in microsomen van 3-methylcholantreen behandelde ratten, en remming van P-450b en P-450e in microsomen van fenobarbital behandelde ratten had echter geen invloed op de omzettingssnelheid van PCP. Dit betekent dat deze enzymen, hoewel in staat

tot het metaboliseren van PCP, in microsomen geen rol van betekenis spelen bij deze omzetting. Microsomen van ratten behandeld met dexamethason bleken ook voor PCP de hoogste katalytische activiteit te vertonen. Deze microsomen vormden bijna uitsluitend het 1,4-diol.

Het tetrachloor-1,4-benzochinon bleek zeer snel met eiwit te reageren. Gezuiverd cytochroom P-450b bleek in staat te zijn de oxidatie van het tetrachloro-1,4-hydrochinon te katalyseren. In microsomen bleek dit enzym echter niet het enige enzym te zijn dat betrokken is bij de oxidatie van TCHQ. Koolmonoxide remde de oxidatie door microsomen slechts gedeeltelijk. In afwezigheid van zuurstof trad nog 39% covalente binding op in vergelijking met aerobe condities. Superoxide dismutase remde de binding in microsomen in vergelijkbare mate als onder anaerobe omstandigheden, terwijl in incubaties met gezuiverd P-450 de binding volledig geremd werd. Dit betekent dat alle zuurstof-afhankelijke binding via het superoxide anion verloopt. De covalente binding als gevolg van microsomaal metabolisme van PCP werd echter niet beinvloed door superoxide dismutase. Dit zou kunnen betekenen dat TCHQ, wanneer het gevormd wordt uit PCP door P-450, op de actieve plaats van dit enzym geoxideerd wordt door superoxide anion radicalen gevormd door P-450. De gemeten ontkoppeling van cytochroom P-450 door TCHQ ondersteunt deze hypothese.

Om meer inzicht te verkrijgen in de biotransformatie van HCB en de cellulaire beschermingsmechanismen tegen deze alkylerende verbindingen is, met behulp van radioactief gelabeld HCB, PCP en 1,4-TCHQ, het metabolisme van HCB onderzocht in een primaire cultuur van kippe-embryo hepatocyten. Hoewel covalente binding in deze hepatocyten kon worden aangetoond, was deze lager dan in microsomale incubaties. Een aantal beschermingsmechanismen bleek aanwezig te zijn. Hoewel de hydroxylatie van HCB overeenkomsten vertoonde met de reactie gekatalyseerd door rattelever microsomen, bleek het metabolisme van PCP niet te leiden tot ophoping van diolen. Een aantal conjugatie routes verhinderde covalente binding door ofwel een reactie met PCP, ofwel met TCBQ. Ook incubaties met het 1,4-TCHQ resulteerden in relatief weinig eiwitbinding.

Deze resultaten in ogenschouw nemend lijkt het onwaarschijnlijk dat covalente eiwitbinding als gevolg van oxidatieve biotransformatie van HCB in vivo van groot belang is. Een directe alkylering van het uroporfyrinogeen decarboxylase door TCBQ is waarschijnlijk niet de oorzaak

van de door HCB geinduceerde porfyrie. Het is echter wel mogelijk dat een relatie bestaat tussen de eiwitbinding van TCBQ en de carcinogene activiteit van HCB, en de mutageniteit van PCP.

CURRICULUM VITAE

Ben van Ommen werd geboren in Amersfoort, op 26 juni 1958. Na het voltooien van de Atheneum opleiding aan het Farel college te Amersfoort in 1977, werd begonnen met de studie Moleculaire wetenschappen aan de Landbouwhogeschool (thans Landbouwuniversiteit) te Wageningen. In september 1983 werd deze studie, met als hoofdvakken toxicologie (Prof.dr. J.H. Koeman) en biochemie (Prof.dr. C. Veeger), met lof afgesloten. Aan het in dit proefschrift beschreven onderzoek werd in de periode januari 1984 tot en met december 1986 gewerkt op de vakgroep toxicologie van de Landbouwuniversiteit.