THE RELATIONSHIP BETWEEN BIOTRANSFORMATION AND TOXICITY OF HALOGENATED BENZENES:

nature of the reactive metabolites and implications for toxicity

Promotoren: dr. J.H. Koeman,
hoogleraar in de Toxicologie

dr. P.J. van Bladeren,
bijzonder hoogleraar in de Biotransformatie en de Toxicokinetiek

THE RELATIONSHIP BETWEEN BIOTRANSFORMATION AND TOXICITY OF HALOGENATED BENZENES:
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Cathaline den Besten

Proefschrift

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STELLINGEN

1. De covalente eiwitbinding die optreedt tijdens oxidatie van chloorbenzenen kan volledig verklaard worden uit de vorming van chinonen.
   Dit proefschrift.

2. Bij de cytochroom P450 afhankelijke oxidatie van halogeenfenolen bepaalt de substituent op de positie *para* ten opzichte van de hydroxyl groep het karakter van het gevormde product (hydrochinon of benzochinon).
   Dit proefschrift.

3. De vorming van tetrachloorbenzochinon uit pentachloorfenol speelt geen rol in de door hexachloorbenzeen geïnduceerde porfyrie.
   Dit proefschrift.

4. De door de auteurs met enige verbazing geconstateerde bevinding dat na blootstelling van ratten aan broombenzeen het belangrijkste protein-*S* adduct een gedebromineerd adduct is, kan eenvoudig verklaard worden uit een directe oxidatie van *p*-broomfenol tot benzochinon onder afsplitsing van het bromide anion, gevolgd door alkylering van eiwit.
   D.E. Slaughter and R.P. Hanzlik (1991), Chem. Res. Toxicol. 4, 349-359.
   Dit proefschrift.

5. Het feit dat het broombenzeen-3,4-epoxide niet spontaan reageert met glutathion roept twijfels op bij de gangbare hypothese dat het 3,4-epoxide door alkylering van celbestanddelen verantwoordelijk is voor de hepatotoxiciteit na blootstelling aan broombenzeen.
   T.J. Monks et al. (1982), Chem.-Biol. Interact. 41, 203-216.

6. Inhibitie van glutathion transferases biedt meer mogelijkheden in de therapie van multidrug-resistente tumoren dan inhibitie van de glutathion synthese met buthionine sulfoximine.
   P.J. van Bladeren and B. van Ommen (1991), Pharmacol. Ther. 51, 35-46.
   K.D. Tew et al. (1988), Cancer Res. 48, 3622-3625.

7. Bij de huidige risicobenadering in het stoffenbeleid ligt het accent nog te veel op een stof-per-stof aanpak. In geval van stofgroepen met vergelijkbare effecten wordt het milieu en de gezondheid mogelijk onvoldoende beschermd en moet men in principe uitgaan van een gecombineerde, groepsgewijze benadering.

8. De ontwikkeling van alternatieven voor dierproeven wordt vooral in de weg gestaan door het feit dat te veel onderzoekers die zich daarmee bezig houden te conservatief zijn. Zij concentreren zich op vervanging van bestaande *in vivo* methoden in plaats van te zoeken naar werkelijk alternatieve methoden voor extrapolatie en risicoschatting.
G.J. Mulder, NRC 11/06/1992.

9. Het feit dat het rendement van de AIO-opleidingen binnen de gestelde termijn van vier jaar in de alfa- en gammawetenschappen beduidend lager is dan in de betawetenschappen suggereert ten onrechte een kwaliteitsverschil tussen de desbetreffende AIO-ers en/of wetenschappen.
Volkskrant, 08/08/1992.

10. De beperkte mogelijkheden voor vrouwen in Nederland om de opvoeding van kinderen te combineren met een carrière leidt tot een voor de maatschappij zorgwekkende ontwikkeling, namelijk dat de voortplanting en opvoeding van de komende generaties met name in handen komt van laag-opgeleide vrouwen.
L. Thooft, Opzij, Oktober 1992.

11. Het in de marketing veel toegepaste begrip 'milieuvriendelijk produkt' bestaat slechts in relatieve zin; het gebruik in absolute zin is derhalve misleidend voor de consument.

12. Het bestaansrecht van de moderne Olympische Spelen ligt, in tegenstelling tot wat velen denken, niet in de aanéénrijging van sportieve hoogtepunten, maar in de stelselmatige opsomming van commerciële aandachtspunten.

Stellingen behorend bij het proefschrift:
'The relationship between biotransformation and toxicity of halogenated benzenes: nature of the reactive metabolites and implications for toxicity.'
Cathaline den Besten, Wageningen 18 december 1992.

Voor Nico
Voor mijn ouders

Contents

Chapter 1

GENERAL INTRODUCTION

One of the major goals in the field of toxicology is to elucidate the mechanisms by which xenobiotics produce detrimental biological responses in organisms. A large number of xenobiotics are as such biologically inert, and many of their toxicological effects are mediated through the formation of 'active' metabolites. However, in spite of the extensive research in the past decades, both the nature of the interaction between such 'active' metabolites (either chemically stable or chemically reactive) and the various cellular targets, as well as the mechanisms by which these interactions eventually lead to cell death are still relatively poorly understood.

Halogenated benzenes like bromobenzene and hexachlorobenzene have served as model compounds in numerous studies, in an attempt to elucidate the actual mechanism(s) of aromatic hydrocarbon toxicity. These compounds, in itself relatively harmless because of their chemical stability, are bioactivated to products ultimately responsible for the observed toxic effects. In the present study this model approach is extended to a range of chlorinated benzene congeners with the ultimate aim of identifying metabolic activation pathways which are relevant from a toxicological point of view. Knowledge on the different pathways by which a chemical can be metabolised into reactive products increases our understanding of the relationship between the chemical structure of xenobiotics and their effects on living systems. Moreover, such knowledge will be helpful in identifying chemical groupings in a given molecule, which predispose that molecule to a sequence of processes which might eventually lead to toxicity.

Biotransformation reactions

Organisms are constantly exposed to a large number of xenobiotics (i.e., chemicals foreign to the body), such as drugs, pesticides, natural food constituents, food additives and industrial chemicals. Unlike bacteria, many of which are capable of degrading and utilising a broad range of chemicals as carbon and energy source (Reineke and Knackmuss, 1984; Schraa *et al.*, 1986), animals have evolved ways for elimination of the chemical rather than utilisation. Most xenobiotics are lipophilic and would remain in the body indefinitely were it not for metabolism resulting in more water soluble derivatives. This process of biotransformation is regulated by several enzyme systems (Table 1.1), which can be conveniently divided into two classes, referred to as Phase I and Phase II enzymes. Phase I enzymes catalyse so-called 'transformation' reactions, which introduce or unmask

a polar functional group (e.g., -OH, -NH_2, -SH) in lipophilic molecules through oxidation, reduction or hydrolysis. These functional groups can then be used to append other small endogenous moieties such as glutathione, glucuronic acid or sulphate in Phase II metabolism ('conjugation' reactions). In this fashion, lipophilic xenobiotics can, in principle, be transformed into more water soluble products capable of being excreted from the body (Sipes and Gandolfi, 1986).

Biotransformation of xenobiotics predominantly occurs in the liver, although in the last decade considerable interest has developed into extrahepatic biotransformation. The major tissues of extrahepatic metabolism are those involved in absorption and excretion of chemicals, such as lung, kidney, skin and gastrointestinal mucosa (Gram *et al.*, 1986; Dahl and Hadley, 1991; Kaminski and Fasco, 1992).

An interesting feature of biotransformation enzymes is the fact that their activities can be enhanced or depressed following treatment of organisms with a variety of chemicals. This may consequently alter the pharmacological or toxicological response, not only of the compound itself, but also of other chemicals and drugs. Studies on induction and inhibition have mostly been concerned with the cytochromes P450, and pretreatment of rodents with known P450 inducers (or inhibitors) is often used as a tool to unravel metabolic activation pathways of chemicals. However, it should be realized that many compounds which induce P450 enzymes also induce Phase II enzymes. For example, pretreatment of rats with phenobarbital (PB) or 3-methylcholanthrene (3-MC) not only increases the activity of P450 (major isoenzymes P450IIB1 and P450IA1, respectively), but also of epoxide hydrolase (Oesch, 1972), UDP-glucuronyltransferase (Owens, 1977) and glutathione *S*-transferase (Vos *et al.*, 1988). The enzyme-inducing potential of a compound needs not necessarily to be classified as a toxic event, but can also be regarded as an adaptive response of the organism in its struggle for survival in a chemically-complex world.

Cytochrome P450: general aspects

The cytochrome P450 monooxygenase system plays a crucial role in the biotransformation of numerous substrates of both exogenous (such as drugs and pesticides) and endogenous origin (such as steroids and fatty acids) (Wislocki *et al.*, 1980). Although classified as an oxygenase, conditions of low oxygen tension may favor cytochrome P450 mediated reductive biotransformation of xenobiotics like haloalkanes, nitroaromatics or azodyes (Sipes and Gandolfi, 1986). Cytochrome P450 is incorporated in the endoplasmatic reticulum in combination with an electron donating system consisting of the haemprotein cytochrome b5, and the flavoproteins NADPH-cytochrome P450 reductase and cytochrome b5 reductase. The catalytic site contains a haem prosthetic group, whose ferrous-carbon monoxide complex exhibits an absorption maximum near 450 nm relative to the complex without carbon monoxide (Omura and Sato, 1964).

Table 1.1 Major enzymes involved in the biotransformation and bioactivation of xenobiotics.

Enzyme (Action)	Substrate	Reactive metabolite	Reference
Phase I			
Cytochrome P450			
aliphatic *C*-oxidation	hexane	diketone	Couri and Milks, 1982
aromatic *C*-oxidation	aflatoxin B_1	8,9-oxide	Swenson et al., 1977
N-oxidation	acetaminophen	benzoquinone imine	Van der Straat et al, 1988
S-oxidation	parathion	paraoxon	Neal and Halpert, 1982
reduction	carbon tetrachloride	radical	Mico et al., 1982
	halothane	radical	Pohl and Gillette, 1984
Flavin monooxygenase			
N-oxidation	MPTP	MPP^+	Markey et al., 1984
S-oxidation	thioacetamide	sulfene, sulfine	Hanzlik et al., 1980
Phase II	*Conjugation with*		
Epoxide hydrolase	H_2O	benzo[a]pyrene-7,8-oxide	diolepoxides	Oesch and Guenthner, 1983
Glutathione *S*-transferase	GSH	1,2-dibromoethane	thiiranium ion	Van Bladeren et al, 1980
		tetrafluoroethylene	fluorothiono-acylfluoride	Commandeur et al., 1989
Glucuronyl-transferase	UDPGA	2-naphtylamine	hydroxylamine[a]	Young and Kadlubar, 1982
Sulfotransferase	PAPS	*N*-hydroxy-2-acetyl aminofluorene	nitrenium ion	Van den Goorbergh et al., 1985
N-methyl transferase	SAM	4,4'-bipyridinium	paraquat	Ansher and Jakoby, 1990

Abbreviations: GSH, glutathione; H_2O, water; MPTP, 1-methyl-4-phenyltetrahydropyridine; MPP^+, 1-methyl-4-phenyl pyridinum ion; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SAM, S-adenosyl-L-methionine; UDPGA, uridine 5'-diphosphoglucuronic acid. [a] proximate reactive metabolite.

Table 1.2 Nomenclature and characteristic reactions catalysed for the major inducible forms of cytochrome P450.

	Cytochrome P450			
Inducer	Sub-family	Iso-enzyme	Species	Characteristic reaction(s) catalysed
Polycyclic aromatic hydrocarbons	IA	1	rat, human[a], rabbit, mouse	7-ethoxyresorufin *O*-deethylase, benzo[a]pyrene hydroxylase
		2	rat, human, rabbit, mouse	phenacetin *O*-deethylase, caffeïne 3-demethylase, 4-aminobiphenyl *N*-hydroxylase
Phenobarbital	IIB	1	rat	7-pentoxyresorufin *O*-deethylase, 7,12-DMBA 7-methyl hydroxylase
		2	rat	7,12-DMBA 12-methyl hydroxylase
		4	rabbit	benzphetamine *N*-demethylase
Ethanol, acetone	IIE	1	rat, human, rabbit	aniline 4-hydroxylase nitrosodimethylamine *N*-demethylatase
Glucocorticoids, Macrolide antibiotics	IIIA	1,2	rat	testosterone 6ß-hydroxylase
		3,4	human	cyclosporin *N*-demethylase, nifedipine dehydrogenase, testosteron 6ß-hydroxylase
		5	human	testosteron 6ß-hydroxylase
		6	rabbit	erythromycin *N*-demethylase
		7	human (foetal)	dehydroepiandrosterone 3-sulphate 16α-hydroxylase
Peroxisome proliferators	IVA	1	rat	lauric acid *w*-hydroxylase

References: George and Farrell, 1991; Murray and Reidy, 1990; Gonzalez, 1989; Wrighton *et al.*, 1985; Guengerich and Shimada, 1991; Okey, 1990. [a] mainly extrahepatic.

Multiplicity of cytochrome P450

While mammalian cytochrome P450 was originally believed to exhibit a remarkable lack of substrate specificity, it is now apparent that the diversity of oxidative reactions towards both endogenous and foreign compounds lies in the multiplicity of the P450 proteins (Table 1.2). With the purification, isolation, and characterization of multiple forms of cytochrome P450 in the last decades (Ryan and Levin, 1990; and references cited therein), the need for a unified classification became apparent. In 1987, Nebert and co-authors introduced a systematic nomenclature in which individual P450 proteins are assigned into families or subfamilies based on the extent of structural homology (i.e., amino acid sequences)[1]. This rationale has since then been widely accepted and updates of this classification have already been published (Nebert *et al.*, 1989; 1991). The major advantage of this unified nomenclature is that structurally similar P450's are easily recognized, regardless of the species, the inducer or the catalytic activity examined.

Regulation of the cytochrome P450 isoenzymes after exposure to chemical agents is primarily the consequence of changes in the levels of P450 apoprotein. Elevation of P450 protein levels generally involves increased transcription of the P450 gene, resulting in elevated levels of specific mRNAs as in the case of induction of P450IA1 by 2,3,7,8-tetrachlorodibenzodioxin or 3-methylcholanthrene (Poland *et al.*, 1987), P450IIB1/IIB2 by phenobarbital (Atchison and Adesnik, 1983), P450IIIA1 by dexamethasone (Simmons *et al.*, 1987), and P450IVA by clofibrate (Hardwick *et al.*, 1987). In addition, regulation of some P450s occurs by a post-transcriptional mechanism, resulting in elevated mRNA levels without increased transcription as described for P450IA2 (Pasco *et al.*, 1988). Post-transcriptional regulation at the protein level (through stabilization of the protein against degradation) is observed with the ethanol/acetone inducible P450IIE1 (Song *et al.*, 1989) and the glucocorticoid-responsive P450IIIA1/2 (Watkins *et al.*, 1986).

General features of catalysis

The catalytic mechanism of cytochrome P450 mediated oxidation is depicted in Figure 1.1. Extensive reviews have been published concerning the most recent insights into reductive activation of molecular oxygen and incorporation of the reactive oxygen species into the substrate (Mansuy *et al.*, 1989; White, 1991; Guengerich and MacDonald, 1984; 1990). Binding of the substrate to the oxidized form of P450 (*step 1*) facilitates the uptake of the first electron donated by the flavoprotein NADPH cytochrome P450 reductase (*step*

[1] Those P450 proteins derived from genes with >40 % sequence identity are included in the same family (indicated by a Roman numeral); those from genes with >55 % sequential homology are included in the same subfamily (indicated by a capital letter). The individual gene products are indicated by an Arabic numeral (Nebert *et al.*, 1987).

2) (Backes and Eyer, 1989). The substrate-cytochrome P450 complex combines with molecular oxygen (*step 3*), followed by the uptake of a second electron, preferentially via NADPH cytochrome P450 reductase, but in some instances also via cytochrome b5 (*step 4*). However, before introduction of the second electron, decomposition of the oxycomplex may occur, resulting in the formation of superoxide anion ("oxidase" activity of cytochrome P450; *step 8*) (Kuthan and Ullrich, 1982). Details regarding the next step are still not precisely understood, but involve cleavage of the O-O bond, reduction of one oxygen atom to a water molecule, and the generation of an activated iron-oxene intermediate (*step 5*). Finally, oxygen insertion into the substrate (*step 6*) is believed to result from two sequential one-electron transfers (Guengerich and MacDonald, 1984; 1990). Depending on its structure, the substrate is activated via hydrogen abstraction, electron removal from a heteroatom or radical addion of the iron-oxene species to a double bond. The oxidation process is then completed by radical recombination reactions. The

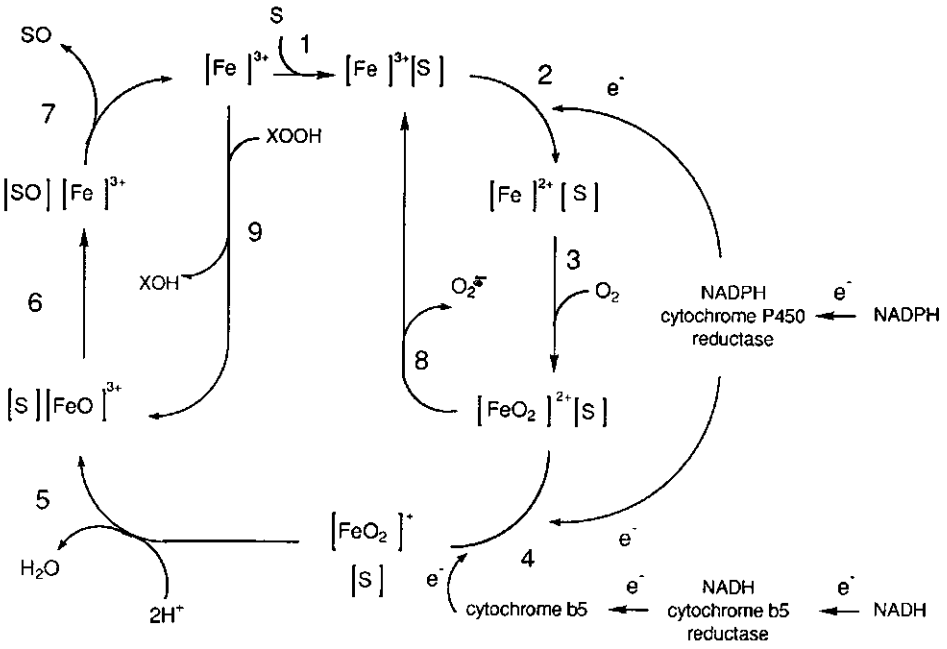


Figure 1.1 Catalytic cycle of substrate oxidation by cytochrome P450. $[Fe]^{3+}$ represents the haem group in the active site involved in the reductive activation of molecular oxygen. [S] denotes the substrate to be oxidized, [SO] represents the oxidized substrate. For further explanation, see text.

oxidized substrate diffuses out of the enzyme (*step 7*) and a new catalytic cycle can be repeated with fresh substrate. Alternatively, peroxycompounds like alkylhydroperoxides (Nordblom *et al.*, 1976), sodium periodate and -chlorite (Hrycay *et al.*, 1976), and iodosylbenzene derivatives (Gustafsson *et al.*, 1979) may serve as both oxygen and electron donor (*step 9*), and thus substitute molecular oxygen and NADPH in the cytochrome P450 mediated oxidation.

Conjugation enzymes: general aspects

Phase I biotransformation reactions are usually followed by phase II reactions to ensure rapid elimination from the organism. Conjugation enzymes attach a strongly polar group to a substrate with a suitable functional group (either a product of Phase I biotransformation, a xenobiotic which already possesses such a moiety, or an endogenous compound such as steroid hormones and bile acids). Like Phase I enzymes, Phase II enzymes are widely distributed among organisms, and can be found in many tissues including the liver, kidney, lung and intestinal mucosa. Pronounced species differences in conjugation reactions exist, which appear to be associated with evolutionary development. For example, the cat has a defective glucuronidation pathway, whereas the dog lacks the enzyme arylamine *N*-acetyl transferase and the pig is deficient in sulfation (Sipes and Gandolfi, 1986; Williams, 1975).

Conjugation of nucleophilic substrates

The most common reaction of nucleophilic substrates is the conjugation with uridine 5'-diphosphoglucuronic acid (UDPGA), catalysed by *UDP-glucuronosyltransferase* (GT). Numerous endogenous and exogenous compounds may serve as substrates, provided they have sufficient lipophilic character and possess functional group(s) of a nucleophilic nature such as alcohols, carboxylic acids, primary and secundary amines, and free sulhydryl groups. The resulting products are *O*-, *N*- or *S*-glucuronides. In addition, certain nucleophilic carbon atoms have been shown to form C-glucuronides, such as carbon atoms with 2 neighbouring carbonyl groups, or ethynilic carbons (Abolin *et al.*, 1980). An interesting aspect is the ability of man and monkey but not rodents to glucuronidate tertiary amine drugs to form quaternary ammonium glucuronides (Mulder *et al.*, 1990). Depending on the size of the substrate, glucuronides are excreted into bile (MW $>$ 350 Da) or urine (MW $<$250 Da) (Sipes and Gandolfi, 1986). In man, urinary excretion seems to be favored over biliary excretion (Klaassen and Watkins, 1984).

GT's are localized in the endoplasmatic reticulum. Multiple forms have been observed in most species, with overlapping substrate selectivity. Many GT's catalyse the conjugation of more than one xenobiotic, and vice versa, many xenobiotics are substrates for more

than one isoenzyme of GT. To date, 11 rat liver isoenzymes have been identified, of which some show activity exclusively towards endogenous substrates (3α-hydroxy steroid GT, bilirubin GT), or exclusively towards exogenous substrates (4-nitrophenol GT) and others towards both endo- and exogenous substrates (17ß-hydroxysteroid GT) (Mulder *et al.*, 1990).

A similarly important conjugation reaction for nucleophilic groups is the conjugation with inorganic sulfate (3'-phosphoadenosine 5'phosphosulfate, PAPS), catalysed by the cytosolic *sulfotransferases* (ST). Classification of ST's is based on the nature of the substrate and its functional moiety: alcohol sulfotransferases (aliphatic primary and secundary alcohols), phenol sulfotransferases (phenolic substrates) and amine *N*-sulfotransferase (aliphatic and aromatic amines). However, it should be realized that the various isoenzymes display a broad substrate selectivity, i.e., hydroxylamines may serve as substrates of phenol ST, and phenols and hydroxysteroids may act as substrates of amine ST (Mulder and Jakoby, 1990). Sulfate conjugates are mainly excreted in urine (Mulder *et al.*, 1985). After biliary excretion, both glucuronide and sulphate conjugates may be hydrolyzed by ß-glucuronidase and arylsulphatase in the gut, and the aglycone may be taken up again (enterohepatic circulation).

Since many substrates of sulfation can also be glucuronidated, there is competition between these two pathways. In addition, *O*-methylation of certain substrates such as catechols (catalysed by *catechol O-methyl transferase*) may also compete (Thakker and Creveling, 1990). In general, the sulfation pathway has a higher affinity (lower Km) for the same substrate than the glucuronidation pathway. In contrast, glucuronidation has a higher capacity. Thus, in general, at low substrate concentration sulfation is the predominant route, whereas at high substrate concentration glucuronidation is (Mulder and Jakoby, 1990).

Conjugation of electrophilic substrates

Electrophilic centres in a xenobiotic, e.g., alkylating anticancer drugs, or its metabolite, e.g., arene oxides, may be conjugated with glutathione (γ-glutamyl cysteinyl glycine, GSH). Although these reactions have spontaneous rates which vary considerably depending on the reactivity of the electrophile, conjugation with GSH is usually catalysed by the *GSH S-transferases* (GST). The glutathione conjugates are then transported to the kidney and converted into the corresponding mercapturic acids by a series of enzymatic processes: removal of the γ-glutamic acid and of glycine by γ-glutamyl transpeptidase and cysteinyl-glycine dipeptidase, respectively, followed by acetylation of the residual cysteine moiety by *N*-acetyltransferase (Sipes and Gandolfi, 1986).

The GST's are a family of isoenzymes with broad and overlapping substrate selectivity. Although membrane-bound forms of GST have been detected (Morgenstern *et al.*, 1988),

GST activity is mainly located in the cytosol. GST's are dimers of subunits and within a dimer, each subunit functions independently of the other (Mannervik and Jensson, 1982). The GST's are now known to be a multi-gene family of isoenzymes, which can be divided into three classes (*alpha, mu* and *pi*), based on similarity in structural, physical and catalytic properties of their subunits (Ketterer and Mulder, 1990; Vos and Van Bladeren, 1990).

In addition to their crucial role in catalyzing glutathione conjugation, GST's may also be important in intracellular binding and/or transport of endogenous and xenobiotic non-substrate ligands (Listowsky *et al.*, 1988).

Metabolites which contain a (usually) highly reactive, electrophilic epoxide moiety may undergo hydrolytic cleavage to less reactive vicinal dihydrodiols. This reaction is catalysed by the enzyme *epoxide hydrolase* (EH), which was first thought to be exclusively located in the endoplasmatic reticulum (microsomal epoxide hydrolase, mEH; Oesch, 1972). In later studies on the mammalian metabolism of certain alkyl epoxides, the existence of a cytosolic EH (cEH) was demonstrated (Gill *et al.*, 1974). The two forms of EH have complementary substrate specificity to some extent, in that many epoxides, e.g., arene oxides, which are good substrates for mEH are poor substrates for cEH, and vice versa, e.g., *trans*-disubstituted oxiranes are good substrates for cEH but not for mEH (Hammock and Hasagawa, 1983). More recent studies have pointed to the fact that the common nomenclature of 'microsomal' and 'cytosolic' epoxide hydrolase is not semantically precise: metabolic and immunochemical studies demonstrated the existence of membrane-bound forms of cEH (Guenthner and Oesch, 1983), whereas mEH-like activity was detected in cytosolic fractions of human tissue (Schladt *et al.*, 1988). Therefore, a revision of the nomenclature for EH is desirable, e.g., based on a system of unique identifiers for each isoenzyme such as the currently accepted P450 nomenclature.

Bioactivation

Although most xenobiotics are converted in the body to metabolites that are less toxic than the parent compound ('detoxication'), it has become increasingly clear that many toxicological effects of xenobiotics are mediated through the formation of reactive metabolites ('toxication', 'metabolic activation' or 'bioactivation'). Bioactivation of chemicals is now known to be catalysed by almost all of the enzymes involved in the process of biotransformation (*cf.* Table 1.1). For example, the conjugation of dibromoethane with glutathione catalysed by GSH *S*-transferase results in the formation of a reactive thiiraniium ion (Van Bladeren *et al.*, 1980), responsible for the carcinogenicity of the parent compound in rats and mice. Glucuronidation of certain *N*-hydroxy arylamines generates proximate carcinogens and provides a means of transportation to the bladder,

where they are converted to the ultimate carcinogenic species (Kadlubar *et al.*, 1977). The sulfate conjugate of *N*-hydroxy-2-acetylaminofluorene is converted to a reactive nitrenium ion, which binds to cellular macromolecules and induces hepatocarcinogenicity (Mulder and Jakoby, 1990). However, the majority of bioactivation reactions involve oxidation, which largely can be attributed to the action of cytochrome P450. Oxidation of (halogenated) hydrocarbons to reactive carbonyl compounds, epoxidation of polycyclic aromatic hydrocarbons to (diol-) epoxides, and *N*-oxidation of aromatic amines to hydroxylamines (and -esters) are just several model reactions of the many mechanisms of bioactivation known to date. These reactions have been reviewed in more detail elsewhere (Guengerich and Liebler, 1985; Guengerich and Shimada, 1991; Van Bladeren, 1988).

A number of key steps can be distinguished in the general mechanism responsible for the effect caused by a given compound in a given cell. Most molecules offer several primary regions of attack to biotransformation enzymes. Depending on the concentration of the various enzymes in a particular cell, the extent of formation of the reactive intermediate may be influenced, and thus also the extent of the toxic effect. A reactive intermediate usually has electrophilic properties and will react with nucleophilic sites in cellular macromolecules. Membranes, nucleic acids and proteins are obvious targets. Depending on the properties of the electrophiles, certain sites will be favored. One of the most successful theories in this respect is the theory of 'Hard and Soft Acids and Bases' (Pearson and Songstad, 1967). This theory predicts that hard electrophiles (low polarizibility and small atomic radii) will preferentially react with hard nucleophiles, while the soft species (high polarizibility and relatively greater atomic radii) will also react best with each other.

However, detoxication reactions will compete with the reaction of the reactive intermediate with tissue macromolecules, and convert them to harmless metabolites that are eliminated from the body. For epoxides, for instance, epoxide hydrolase as well as the GSH *S*-transferases are of major importance in their detoxication. For other electrophiles, in addition to the GSH *S*-transferases, glutathione itself and sometimes amino acids such as methionine constitute a first line of defence. Again, depending on both exogenous, e.g., nutritional, as well as endogenous factors, e.g., differentiation status, organ, genetic deficiencies, the relative concentration of activating and detoxifying enzyme systems may vary, with a concomitant variation in the extent of toxicity. Ultimately, the balance between formation and detoxication of the reactive intermediate will determine whether it can elicit its toxic effect.

Halogenated benzenes: occurrence in the environment

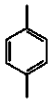
Halogenated benzenes have long been of concern regarding the possible toxic consequences of human exposure, due to their ubiquitous environmental distribution and their tendency for bioaccumulation in animal and human tissue. Virtually all chlorinated benzenes have been found in the various waterbodies (Oliver and Nicol, 1982), and reports on the presence of residues in human fat and milk have indeed been published (Mes *et al.*, 1986; Wagner *et al.*, 1991).

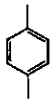
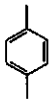
Chlorine-substituted benzenes represent by far the most important group, with widespread economical use as chemical intermediates in industry, and among others, as solvents, dye carriers, space deodorants and moth repellents. Input into the ecosystem results primarily from industrial and municipal waste water and agricultural runoff, with additional input of chlorinated phenols and benzenes by chlorination treatment processes (Deichmann, 1982). Natural sources of chlorinated benzenes in the environment have not been identified. A considerable body of data is nowadays available on their consequences of (sub-)chronic exposure or on reproductive and teratologic effects in laboratory animals, although for several isomers there are still large data gaps to fill before accurate risk estimates can be made.

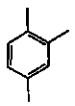
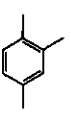
Metabolism of chlorinated benzenes

Table 1.3 gives a summary of the main studies on the biotransformation of chlorinated benzenes. For a more extensive overview, the reader is referred to documents drawn up by the United States Environmental Protection Agency (US EPA, 1985) or the World Health Organization (WHO, 1987). Some general remarks can be made. Biotransformation of the higher substituted congeners (tetra-, penta-, hexa-) is slower and a greater proportion of the compound is excreted unchanged in the faeces or expired air compared to the mono- and di-substituted congeners, although variation in kinetic behaviour is observed for the different isomers of the same congener (*cf.* metabolism of tetrachlorobenzene isomers; Chu *et al.*, 1984b). Considerable species variation exists both in qualitative and quantitative sense (*cf.* metabolism of 1,2,4-trichlorobenzene in the rat and monkey; Lingg *et al.*, 1982). This interspecies variation in metabolism could very well reflect differences in the relative concentration of the various (iso-)enzymes involved. However, with the exception of bromobenzene and hexachlorobenzene, there is a huge lack of detailed *in vitro* metabolism studies of halogenated benzenes, which would help assess the relative contribution of the various (iso-)enzymes to the overall metabolism. Ultimately, interspecies variation in metabolism could have toxicological implications. For example, extensive studies on the microsomal metabolism of bromobenzene revealed the existence of a toxic route to *p*-bromophenol (catalysed by P450IIB1) and a non-toxic route to *o*-bromophenol (catalysed by P450IA1) (Lau *et al.*, 1980).

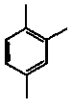
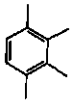
Table 1.3 Main results of studies on distribution and metabolism of chlorinated benzenes.

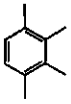
Compound (Reference)	Species; Study Protocol	Results
MCB (Lindsay Smith *et al.*, 1972) 	Dutch rabbit (♀); oral gavage of 0.5 g [^{14}C]MCB twice daily for 4 consecutive days. Collection of excreta for 7 days	After 7 days, 19.6% of the total dose was excreted into urine, 1.5% into faeces and < 1% was found in tissues. Urinary metabolites were glucuronides (33.6%), sulphates (33.9%), mercapturic acids (23.8%), MCP (2.8%, p > m > o), diphenols (4.2%), 3,4-dihydro-3,4-dihydroxychlorobenzene (0.6%).
MCB (Sullivan *et al.*, 1983) 	Sprague-Dawley rat (♂); inhalation of 100, 400 or 700 ppm for 1 or 5 days	All tissues contained radioactivity immediately following single exposure and at 48 hr post-exposure with highest levels in fat. Disproportionate increase in respiratory elimination of parent compound and dose-dependent decrease in mercapturic acid percentage of urinary metabolites from 68% at 100 ppm to 51% at 700 ppm.
1,3-DCB (Parke & Williams, 1955) 	Chinchilla rabbits; oral gavage of 0.5 g/kg in olive oil	Elimination of 1,3-DCB was virtually complete in 5 days. Metabolites consisted of 2,4-DCp-mercapturic acid (minor), and sulphate and glucuronide conjugates of 2,4-DCP (major), 3,5-DCP and 3,5-DCC.
1,4-DCB (Kimura *et al.*, 1979) 	Wistar rat (♂); oral gavage of 200 or 800 mg/kg in corn oil	At 1 hr post-dosing, levels in fat were 10x blood levels. Levels in fat, kidney, liver and lung peaked between 6-12 hr and were below detection limit at 48 hr in all tissues except fat (still detectable at 120 hr). Major metabolite was 2,5-DCP (conjugated), whereas trace amounts of methyl sulfoxide and methyl sulphone metabolites were identified.

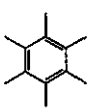
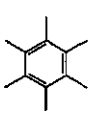
Compound (Reference)	Species; Study Protocol	Results
1,3-DCB (Kimura *et al.*, 1984) 	Wistar rat (♂); oral gavage of 200 mg/kg in corn oil	Absorption and elimination rates of 1,3-DCB were greater than of 1,4-DCB and concentration in fat was lower. Sulfur containing metabolites were detected in blood and excreta: 2,4-/ 3,5- DCp-methylsulfoxides/ -sulphones; 2,4- and 3,5-DCp-mercapturic acids were excreted into urine to a considerable extent.
1,4-DCB,  1,2-DCB  (Azouz *et al.*, 1955)	Chinchilla rabbits; oral gavage of 0.5 g/kg in olive oil (1,4-DCB) or in water (1,2-DCB)	Elimination of 1,2-DCB was complete in 5-6 days post-dosing, whereas excretion of metabolites of 1,4-DCB was still appreciable after 6 days. Major metabolites of 1,2-DCB were sulphate and glucuronide conjugates of 3,4-DCP; minor metabolites of 1,2-DCB: conjugates of 2,3-DCP, 3,4-DCC and 4,5-DCC, and 3,4-DCp-mercapturic acid. Metabolites of 1,4-DCB were 2,5-DCP (major) and DCHQ (minor).
1,4-DCB (Hawkins *et al.*, 1980) 	CFY rat (♀); inhalation of 1000 ppm [^{14}C]1,4-DCB, 3 hr/day for 10 days. P.o. and s.c. administration of 250 mg/kg for up to 10 days. Single exposure (1000 ppm inh., 250 mg/kg p.o. or s.c.) to bile-duct-cannulated rats	Tissue concentrations at 24 hr were similar for all routes of exposure with highest levels in fat, and to a lesser extent in kidney, liver and lung. Excretion of 1,4-DCB was primarily into urine (91-97%), and was complete in 5 days. Bile contained 46-63% of excreted compound at 24 hr. Major metabolites were 2,5-DCPsulphate (46-54%) and -glucuronide (31-34%). Minor metabolites were DCHQ and a mercapturic acid.

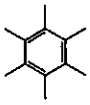
Compound (Reference)	Species; Study Protocol	Results
1,2,3-TRICB  1,2,4-TRICB  1,3,5-TRICB  (Jondorf *et al.*, 1955)	Chinchilla rabbit; oral gavage of 0.5 g/kg in arachis oil	Rate of metabolism: 1,2,3- > 1,2,4- > 1,3,5-TRICB (at 5 days post-dosing, 62%, 38%, 23% excreted as phenolic conjugates, respectively). Major metabolite of 1,2,3-TRICB was 2,3,4-TRICP with small amounts of 3,4,5-TRICP, 3,4,5-TRICC, 2,3,4-TRICp-mercapturic acid. Major metabolites of 1,2,4-TRICB were 2,4,5- and 2,3,5-TRICP with minor amounts of 3,4,6-TRICC, and 2,3,5-/2,4,5-TRICp-mercapturic acid. 2,4,6-TRICP was the only phenol detected in urine of 1,3,5-TRICB-treated animals, and unchanged compound was found in faeces.
1,2,3-TRICB 1,2,4-TRICB 1,3,5-TRICB (Chu *et al.*, 1987)	Sprague-Dawley rat (♂); oral gavage of 10 mg/kg of each [^{14}C]-labelled isomer in corn oil. Serial sacrifices at 0.5, 1 and 24 hr, and after 2, 7, 14, 28 and 56 days	For all isomers, blood levels peaked between 2-4 hr post-dosing. Fat, skin and liver had high concentrations of parent compound, whereas kidney and muscle had high levels of metabolites. 95% and 88% of the dose of 1,2,3- respectively 1,3,5-TRICB was excreted after 48 hr. Faecal excretion amounted to more than 1/3 of total excretion.
1,2,4-TRICB (Lingg *et al.*, 1982) 	Charles River albino rat (♂), Rhesus monkey (♀); oral (p.o.) or intravenous (i.v.) administration of 10 mg/kg [^{14}C]-1,2,4-TRICB	Excretion into urine and faeces at 24 hr post-dosing in rats amounted to 84% and 11% (p.o.), and 78% and 7% (i.v.), respectively. Urinary metabolites were 2,3,5-/2,4,5-TRICp-mercapturic acid (60%), 2,3,5-/2,4,5-TRICthiophenol (33%) and 2,3,5-/ 2,4,5-TRICP (1-10%).

- continued -

Compound (Reference)	Species; Study Protocol	Results
1,2,4-TRICB - continued -		Excretion into urine at 24 hr postdosing in monkeys was 40% (p.o.) and 22% (i.v.), with < 1% in faeces for both routes. Urinary metabolites consisted of glucuronides of 3,4,6-TRIC-3,5-cyclohexadiene-1,2-diol (both isomers, 48-61%) and of 2,3,5-/2,4,5-TRICP (14-37%), and free TRICP (1-37%).
1,2,4-TRICB (Tanaka *et al.*, 1986) 	Wistar rat (♂); oral gavage of 50 mg/kg [^{14}C]-1,2,4-TRICB	66% and 17% were excreted into urine and faeces, respectively, in 7 days; 2,1% was exhaled as DCB and unchanged compound. Tissue residues were evenly distributed with the exception of higher concentrations in fat. Major metabolites in urine were free 2,4,5- and 2,3,5-TRICP and their conjugates. Minor metabolites were 5- or 6-sulhydryl-, methylthio-, methylsulfoxide and methylsulphone products.
 1,2,3,4-TCB  1,2,3,5-TCB  1,2,4,5-TCB (Chu *et al.*, 1984b)	Sprague-Dawley rat (♂); oral gavage of 10 mg/kg of the [^{14}C]-labelled isomers in corn oil	For 1,2,3,4- and 1,2,3,5-TCB, 46-51% of the doses was excreted into urine and faeces within 48 hr, which increased slightly to 51-55% of the dose within 7 days. Only 8% of the dose of 1,2,4,5-TCB was excreted, increasing to 21% in 7 days. 1,2,3,4-TCB yielded 2,3,4,5- and 2,3,4,6-TCP, and traces of TCthiophenol and 2,3,4-TRICP. 1,2,3,5-TCB yielded 2,3,4,6-TCP, isomeric hydroxy-TRICthiophenols, and a TRICP. 1,2,4,5-TCB yielded 2,3,5,6-TCP, TCHQ and a TRICP.

Compound (Reference)	Species; Study Protocol	Results
1,2,3,4-TCB 1,2,3,5-TCB 1,2,4,5-TCB (Kohli *et al.*, 1976)	rabbit (♂); i.p. injection of 300 mg in vegetable oil. Collection of excreta for 10 days	1,2,3,4-TCB was the most extensively metabolised isomer, yielding 2,3,4,5-TCP (major) and 2,3,4,6-TCP (minor). 1,2,3,5-TCB was metabolised to 2,3,4,5-, 2,3,5,6- and 2,3,4,6-TCP. 1,2,4,5-TCB yielded only 2,3,5,6-TCP.
1,2,3,4-TCB  1,2,3,5-TCB  1,2,4,5-TCB  (Schwartz *et al.*, 1987)	Squirrel monkey (♂); oral gavage of 50 mg/kg or 100 mg/kg of the [^{14}C]-labelled isomers in corn oil, twice per week over 3 wk	1,2,3,4-TCB was eliminated mainly, and 1,2,3,5- and 1,2,4,5-TCB exclusively, in the faeces. Parent compound accounted for 50% (1,2,3,4- and 1,2,3,5-TCB) and > 99% (1,2,4,5-TCB) of total faecal radioactivity. Major faecal metabolites of 1,2,3,4-TCB were 2,3,4,5-TCP (22%) and 2,3,4,5-TCp-mercapturic acid (18%). Major faecal metabolites of 1,2,3,5-TCB were TCP isomers (14%, 9% and 2% for 2,3,4,6-, 2,3,5,6-, 2,3,4,5-TCP) and 2,3,4,6-TCp-sulfinic acid (15%). Urinary metabolites of 1,2,3,4-TCB were 2,3,4,5-TCP (15%) and 2,3,4,5-TCp-mercapturic acid.
PCB (Kohli *et al.*, 1976) 	rabbit (♂); i.p. injection of 300 mg in vegetable oil. Collection of excreta over 10 days	Urinary metabolites were 2,3,4,5-TCP and PCP, both detected at 1% of administered dose after 10 days post-dosing.
PCB (Koss & Koranski, 1977) 	rat (♀); i.p. injection of 403 μmol/kg in olive oil. Collection of excreta for 4 days	Only 3% of dose was eliminated in unchanged form in faeces. Metabolites excreted both in urine and faeces included PCP (9%), 2,3,4,5-TCP, hydroxy-2,3,4,5-TCthiophenol, and traces of another isomer of TCP. TCHQ was only detected in urine.

Compound (Reference)	Species; Study Protocol	Results
PCB (Rozman *et al.*, 1979) 	Rhesus monkey (♂, ♀); oral gavage of 0.5 mg/kg	Absorption was at least 95%. Faecal excretion (99% parent compound) was about twice the amount of the urinary excretion. After 40 days following a single dose, total excretion amounted to 40.2% and 33.2% for ♂ and ♀ monkeys, respectively. Highest residual concentration in fat and bone marrow, followed by lymph nodes, thymus, adrenal cortex and large intestine. Urinary metabolites were PCP > 2,3,4,5-TCP > 2,3,5,6-TCP. No sex related differences in metabolism.
HCB (Koss *et al.*, 1976) 	Wistar rat (♀); i.p. injection of of repeated single dosis of 130 mg/kg in olive oil (total dose 390 mg/kg)	After 28 days, 7% and 27% of total dose was excreted in urine and faeces, respectively. 70% and < 10% of total faecal and urinary radioactivity was excreted as parent compound. Major metabolites in urine were PCP, TCHQ, PCthiophenol, and minor metabolite was TCthiophenol. PCP and PCthiophenol were the only 2 faecal metabolites.
HCB (Koss *et al.*, 1978) 	Wistar rat (♀); oral gavage of 50 mg/kg (i.e. 178 μmol/kg) in olive oil every other day for 15 weeks. Serial sacrifices every 3 wks	An equilibrium between intake and elimination was reached after 9 weeks, with 1 g of liver containing 1 μmol HCB, 50 nmol PCP, 5 nmol TCHQ and 0.1 nmol PCthiophenol. Concentration in fat was about 30-60 times higher. After cessation of administration, the rate of elimination of HCB decreased.

Compound (Reference)	Species; Study Protocol	Results
HCB (Jansson & Bergman, 1978) 	Wistar rat (♂); two i.p. injections of 25 mg/kg in peanut oil. Collection of excreta over 12 days.	The following sulfur metabolites of HCB were detected in excreta after methylation of the extracts: HCB > PCanisole > 1,4-bis-(methylthio)-TCB > PCthioanisole.

Abbreviations: see list of abbreviations; DC, dichloro-; PC, pentachloro-; TC, tetrachloro-; TRIC, trichloro-; capital "P" as in DICP denotes 'phenol', whereas lowcast "p" as in DICp-mercapturic acid denotes 'phenyl' residu.

Toxicity of chlorinated benzenes

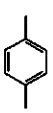
Table 1.4 presents an overview of the main studies on the toxicity of chlorinated benzenes. For an elaborate review of the toxicity of chlorinated benzenes, the reader is again referred to documents drawn up by foreign bodies (US EPA, 1985; WHO, 1987). Effects in rat are mainly confined to the liver, kidney, thyroid and lung. In mice, bone marrow also seems to be affected. Based on the sparse relevant data available it appears that, with the exception of hexachlorobenzene and 1,4-dichlorobenzene, there is little potential for progressive toxicity with chronic administration beyond that observed in the subchronic studies. The hepatocarcinogenic potential of hexachlorobenzene has been clearly demonstrated in rats, mice and hamster (Smith *et al.*, 1985; Cabral *et al.*, 1977). Interestingly, tumours occurred predominantly in females. This coincides with the greater susceptibility of this sex to the induction of porphyria by hexachlorobenzene (see below), suggesting a link between these two manifestations of toxicity (Smith *et al.*, 1985). The increased incidence of renal tumours in male rats but not in female rats after long-term oral administration of 1,4-dichlorobenzene (NTP, 1987) may very well be related to the induction of Protein Droplet Nephropathy and the resulting enhanced cell replication (Goldsworthy *et al.*, 1988).

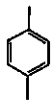
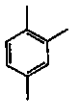
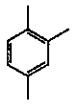
Hexachlorobenzene is known for its strong porphyrinogenic action. However, in spite of all the efforts over the past decades, the mechanism of hexachlorobenzene-induced hepatic porphyria, which becomes manifest through the accumulation and excretion of porphyrins as a result of a disturbance of the haem synthesis by irreversible inhibition of the key enzyme uroporphyrinogen decarboxylase (UROG-D), has only been partially resolved (Van Ommen and Van Bladeren, 1989). Several lower chlorinated benzenes (1,4-

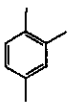
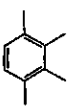
dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,3,4-tetrachlorobenzene) have also been reported to produce some increase in urinary porphyrin excretion (Rimington and Ziegler, 1963), although an extremely high dose regimen was used and doubts were raised about the purity of the compound. Chronic feeding studies with lower doses failed to show the porphyrinogenic action of lower chlorinated benzenes (Carlson, 1977).

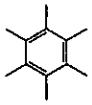
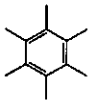
Table 1.4 Main results of studies on the subchronic toxicity of chlorinated benzenes.

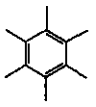
Compound (Reference)	Species; Study Protocol	Effects
MCB (Irish, 1963) 	Rat, rabbit, guinea pig; inhalation of 0 - 200 - 475 -1000 ppm, 7 h/d, 5 d/wk, 32 exposures over 44 days	No effect at 200 ppm; slight increase in Li wt and slight LI histopathological changes at 475 ppm; at 1000 ppm, histopathological changes in LU, LI and KI. Growth depression and increase in mortality (guinea pig).
MCB (Kluwe *et al.*, 1985) 	Fischer-344 rat (♂,♀) $B6C3F_1$ mouse (♂,♀); oral gavage in corn oil of 0-60 - 125 - 250 - 500 - 750 mg/kg/d, 5 d/wk, 13 wk	Reduced survival and reduction in BW gain at ≥250 mg/kg. Dose-dependent increase in LI wt, centrilobular degeneration and necrosis; slight increase in KI wt, degeneration of proximal renal tubuli; slight decrease in SP wt, with lymphoid or myeloid depletion of TH, SP and BM at ≥250 mg/kg. At 500 mg/kg, increase in serum AP, γ-GT and liver porphyrin (rat), and polyuria and porphyrinuria (rat & mouse).
1,2-DCB (Hollingsworth *et al.*, 1958) 	rat, guinea pig (♂,♀); inhalation of 0 -49 -93 ppm, 5 h/d, 5 d/wk for 6-7 months	At 93 ppm, a significant decrease in BW (♂ rat), and decrease in SP wt (♂ guinea pig) was observed. No effect at 49 ppm.
	rat (♀); oral gavage in olive oil of 0-18.8-188-376 mg/kg/d, 5 d/wk, 138 x in 192 days	No effect observed on BW gain. Significant increase in LI and KI wt (at ≥188 mg/kg) and decrease in SP wt (376 mg/kg). No effects at 18.8 mg/kg.

Compound (Reference)	Species; Study Protocol	Effects
1,2-DCB (Robinson *et al.*, 1991) 	Sprague Dawley rat (♂,♀); oral gavage in corn oil of 0 - 37.5 - 75 - 150 - 300 mg/kg/d (10 days); 0 - 25 - 100 - 400 mg/kg/d (90 days)	300 mg/kg/d, (♂): decrease in final BW and organ weights (HE, KI, SP, TE, TH), and rel. organ weight (SP, TH). Increase in abs. and rel. LI weight; 300 mg/kg/d, (♀): decrease in abs. and rel. SP weight, and increase in abs.and rel. LI weight. Both sexes showed increase in water consumption, ALT, leukocyt count (♂ only) and in incidence of LI necrosis (♂ only). In 90 d study, effects in the 400 mg/kg/d group were similar as those described above. In addition, ♀ rat showed increase in abs. and rel. KI wt. LI damage was observed in both sexes.
1,4-DCB (Hollingsworth *et al.*, 1956) 	rat, guinea pig; inhalation of 0 - 96 - 158 ppm, 7 h/day, 5 d/wk for 157-199 days	Growth depression in guinea pig. Increase in LI wt (guinea pig, rat) and KI wt (rat). Cloudy swelling or granular degeneration in LI (rat, 158 ppm). No histopathological changes observed at 96 ppm.
	rat (♀); oral gavage in olive oil of 0 - 18.8 - 188 - 376 mg/kg/d, 5 d/wk, 138x in 192 days	At 188 and 376 mg/kg, there was an increase in LI wt, KI wt, and a decrease in SP wt (376 mg/kg only). Slight cirrhosis and focal necrosis in LI at highest dose.
	rabbit; oral gavage in olive oil of 0 - 500 mg/kg/d, 5 d/wk, in 376 days; 1000 mg/kg, 5 d/wk in 219 days	Increase in mortality at 1000 mg/kg. BW loss, tremor and slight changes in liver (cloudy swelling, focal necrosis) were observed at both doses.

Compound (Reference)	Species; Study Protocol	Effects
1,4-DCB  (Loeser and Litchfield, 1983)	rat (♂,♀); inhalation of 0 - 75 - 500 ppm, 5 h/d, 5 d/wk for 76 wk followed by 36 wk unexposed	No increase in mortality. Increase observed in LI wt and KI wt at 500 ppm. Slight elevation of urinary protein and coproporphyrin output in 500 ppm group. No effects observed at 75 ppm.
1,2,3-TRICB  1,2,4-TRICB  1,3,5-TRICB  (Côte *et al.*, 1988)	Sprague Dawley rat (♂,♀); oral in diet for 90 days, 0 - 1 - 10 - 100 - 1000 ppm	1,2,3-TRICB: Growth suppression at 10 or 1000 ppm; mild to moderate morphological changes in LI and THYR at 1000 ppm. 1,2,4-TRICB: Increase in rel. LI and KI wt, and in microsomal enzyme activities at 1000 ppm; mild to moderate histological changes in LI and THYR at 1000 ppm. 1,3,5-TRICB: Moderate histological changes in KI, LI and THYR at 1000 ppm; increase both in abs. and rel. LI and KI wt at 1000 ppm. No haematological changes. Male rat is more sensitive than female. Dose-dependent accumulation observed in LI and fat: 1,3,5- > 1,2,4- > 1,2,3-TRICB.
1,2,4-TRICB (Coate *et al.*, 1977) 	Sprague Dawley rat (♂), New Zealand White rabbit (♂), cynomolgus monkey (♂); inhalation of 0 - 25 - 50 - 100 ppm, 7 h/d, 5 d/wk for 26 wk	No effect on BW gain. No haematological or serum biochemical changes. In rat, mild hepatocytomegaly and non-dose dependent hepatocyte vacuolization was observed in addition to liver granulance, biliary hyperplasia, and hyaline degeneration in KI after 4 and 13 wk of exposure. No effects were observed after 26 wk. No effects in rabbit or monkey after 26 wk.

Compound (Reference)	Species; Study Protocol	Effects
1,2,4-TRICB (Kociba *et al.*, 1981) 	Sprague Dawley rat, New Zealand white rabbit Beagle dog (all ♂); inhalation of 0 - 30 - 100 ppm, 7 hr/d, 5 d/wk, 30 exposures in 44 days	No effect on BW gain, hematologic and serum biochemical parameters. No histopathologic changes in tissues examined. Increased urinary excretion of porphyrins in rats (30 - 100 ppm). Increase in abs. LI wt (rat, 100 ppm), rel. LI wt (rat, dog, 100 ppm; rabbit, 30 - 100 ppm), rel. KI wt (rat, 100 ppm), and abs. and rel. TE wt (rabbit 100 ppm).
1,2,4,5-TCB (Braun *et al.*, 1978) 	Beagle dog (2♂, 2♀); oral in diet for 2 years at 5 mg/kg/d, followed by a recovery period of 20 months	After 18 mo of exposure, all clinical parameters were normal. After 24 mo, serum alkaline phophatase activity and bilirubin levels were slighly elevated, which returned to normal within 3-mo of recovery. No morphological changes in tissues were observed after a 20-mo recovery period.
1,2,3,4-TCB  1,2,3,5-TCB  1,2,4,5-TCB (Chu *et al.*, 1984a)	Sprague Dawley rat (♂,♀); oral in diet for 90 days, 0 - 0.5 - 5 - 50 -500 ppm	1,2,4,5-TCB: Increased LI and KI wt (♂,♀, 500 ppm). Increased serum cholesterol (♂,♀, 500 ppm) and hepatic microsomal enzyme activities (AH, APDM; 50 ppm (♂), 500 ppm (♂,♀)). Slight decrease in hemoglobin and hematocrit. Dose-dependent changes in severity and prevalence of renal lesions (extensive epithelial necrosis with intratubular casts) and of hepatic lesions (hepatocytomegaly), with male rat being more sensitive than female rat. Less severe changes observed with other two isomers. Dose-dependent accumulation in LI and fat: 1,2,4,5- > > 1,2,3,5- > 1,2,3,4-TCB.

Compound (Reference)	Species; Study Protocol	Effects
PCB (Linder *et al.*, 1980) 	Sherman rat (♂,♀); oral in diet, ♀: 0 - 125 - 250 - 500 - 1000 ppm for 280 days ♂: 0 - 125 - 1000 ppm for 100 days	No effect observed on BW gain. Dose-dependent accumulation in fat. No evidence of porphyria (♀). Increased WBC at 1000 ppm. Decrease in HB, RBC and HC (♂) at 1000 ppm. Increase in ADR wt and KI wt (♂, 1000 ppm). Increase in relative LI wt (≥250 ppm). Hepatocellular enlargement (500, 1000 ppm). Dose-related increase in hyaline droplet formation (♂) in KI; at 1000 ppm, focal renal tubular atrophy and interstitial lymphatic infiltration.
HCB (Vos *et al.*, 1971) 	Japanese quail (♂,♀); oral in diet for 90 days, 0 - 1 - 5 - 20 - 80 ppm	Tremor and mortality occurred in birds fed 80 ppm. Li damage (hepatocellular enlargement, bile duct proliferation, necrosis), SP erytrophagocytosis, pigments in KI tubules, reduced reproduction were noticed in 80 ppm group. No effect on shell-thickness index. Increased LI wt, slight LI damage and increased faecal porphyrin level in 5 ppm group. NOEL, 1 ppm.
HCB (Kuiper-Goodman *et al.*, 1977) 	Charles River rat (♂,♀); oral in diet for 3, 6, 9, 12 and 15 wk; 0 - 0.5 -2 - 8 - 32 mg/kg/d. Surviving rats fed HCB-free diet for 1 wk up to 33 wk	Hepatocytomegaly was observed at 2 mg/kg/d. Increased porphyrin levels were noticed in LI, KI, SP (♀, ≥8 mg/kg/d), increased LI wt (≥8 mg/kg/d), KI wt, SP wt (32 mg/kg/d). Centrilobular LI lesions (≥8 mg/kg/d) and splenomegaly (32 mg/kg/d). At 32 mg/kg/d, increased mortality and ataxia (♀) and intension of tremors (♂,♀). After termination of exposure, there was an increase in LI porphyrin levels (♀, 0.5 mg/kg/d (transient), 2 mg/kg/d).

Compound (Reference)	Species; Study Protocol	Effects
HCB  (Van Ommen *et al.*, 1989)	Wistar rat (♀); oral in diet for 10 wk; 0.03 % HCB with or without co-administration of TAO (0.3 %).	Increase in LI porphyrin levels (600-fold over control) and increase in urinary porphyrin levels after 3 weeks. Co-treatment of rats with HCB and TAO resulted in an near-absence of porphyria.

Abbreviations: see list of abbreviations; abs., absolute; ADR, adrenal; AH, arylhydrocarbon hydroxylase; ALT, alanine aminotransaminase; AP, alkaline phosphatase; APDM, aminopyrine demethylase; BM, bone marrow; BW, body weight; HC, hematocrit; HE, heart; HB, haemoglobin; KI, kidney; LI, liver; LU, lung; NOEL, no effect level; RBC, red blood cel; rel., relative; SP, spleen; TE, testis; TH, thymus; THYR, thyroid; WBC, white blood cel; wt, weight.

Multiple reactive metabolites of halogenated benzenes

As with many other lipophilic compounds, the toxicity observed after exposure to halogenated benzenes is believed to be due to metabolites rather than to the parent compound. Studies attempting to explain the mechanism of toxicity of (halogenated) aromatic hydrocarbons have mainly focussed on the model compounds bromobenzene and hexachlorobenzene. In recent years, the routes for metabolic activation of these compounds have been found to be more complicated than was previously assumed.

The various pathways for the metabolic activation of halogenobenzenes are summarized in Figure 1.2. Oxidative attack by cytochrome P450 results in the formation of an intermediate, most commonly depicted as an epoxide, an electrophilic intermediate which may covalently interact with tissue macromolecules. The spontaneous isomerization to phenols, the conversion of epoxides to dihydrodiols as catalysed by epoxide hydrolase, and the conjugation of epoxides with glutathione, either spontaneously or enzymatically by GSH *S*-transferases, are alternative pathways that compete with the reaction of epoxides with tissue macromolecules. For fully halogenated benzenes, an alternative mechanism of oxidation has recently been proposed, which proceeds without the obligatory formation of epoxides as intermediates in the oxidative dehalogenation to pentahalogenated phenols (Rietjens and Vervoort, 1992). This mechanism includes direct formation of a benzohaloquinone cation with a positive charge on a halogen substituent *para* with respect to the position of oxidative attack, and subsequent elimination of the halogen from the molecule as a halogen anion.

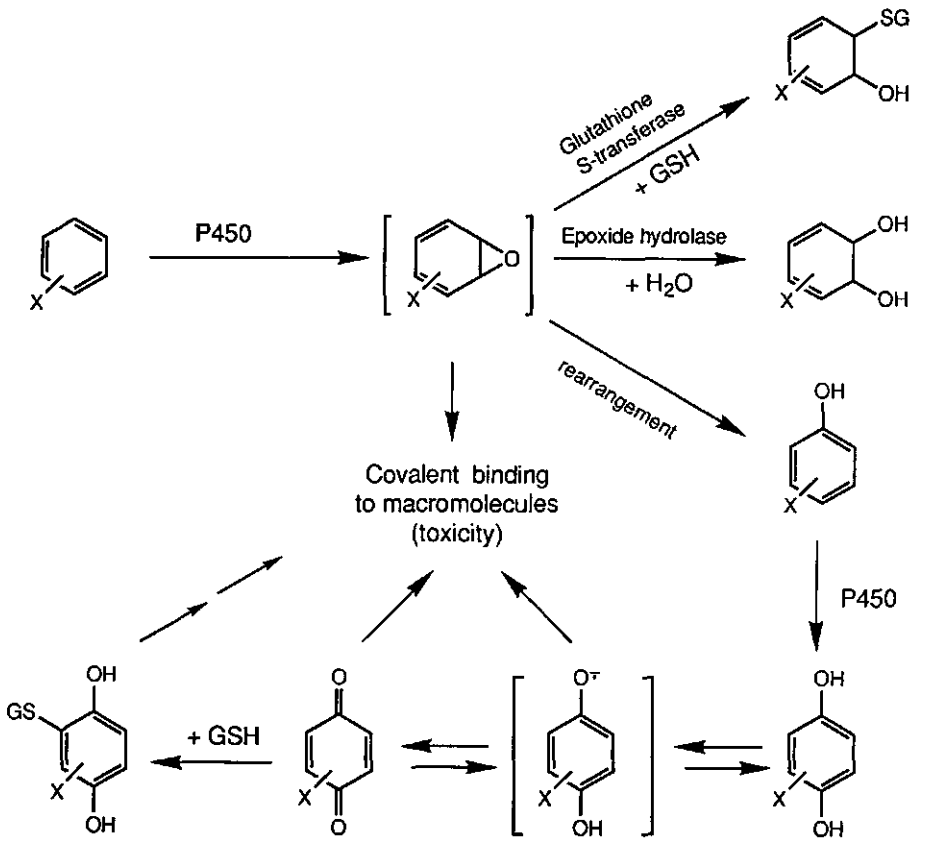


Figure 1.2 Different routes for metabolic activation of halogenated benzenes.

Secondary metabolism of phenols to hydroquinones and benzoquinones may pose additional stress to a cell in the form of sulfhydryl alkylation and/or oxidation, or in the form of oxidative stress due to redox cycling. Enzyme mediated conjugation of phenols and hydroquinones with glucuronic acid or sulphate may prevent the formation of reactive benzoquinones, whereas, once formed, benzoquinones may be quickly reduced to their corresponding hydroquinones, e.g., by the action of DT-diaphorase. Finally, conjugation of quinones with glutathione can lead to the formation of products which leave the liver and are targetted to the kidney, where they may elicit a toxic response through a mechanism that is still unknown (Monks and Lau, 1990).

Thus, multiple pathways are involved in the generation of (proximate) toxic metabolites of halogenobenzenes: epoxides as primary metabolites, benzoquinones as secondary metabolites, and benzoquinone-derived glutathione conjugates. In the next section, the role of these different reactive metabolites in the toxicity of halogenobenzenes is briefly discussed.

Epoxides and their role in hepatotoxicity

The primary target of toxicity for halogenated benzenes is the liver parenchymal cell. Although different degrees of hepatotoxic effects have been described for a broad range of halogenated benzenes, mechanistic investigations have virtually only been applied to the case of bromobenzene.

Early studies by Brodie *et al.*, (1971) and Reid and Krishna (1973) demonstrated that the development of centrilobular liver necrosis in rats treated with bromobenzene and other halogenated benzenes was associated with the covalent binding of reactive material to cellular macromolecules in the necrotic zone. Pretreatment of rats with phenobarbital potentiated both covalent binding and liver necrosis, while inhibition of oxidative metabolism by SKF-525 had the opposite effect, indicating an important role for the P450 monooxygenase system in the metabolic activation of these compounds.

The protective effect of glutathione, and hence the electrophilic nature of the reactive metabolite involved, was demonstrated by increased liver necrosis in bromobenzene-treated rats, which were pretreated with diethylmaleate, a compound known to drastically reduce glutathione levels in the liver (Reid and Krishna, 1973). This was confirmed in *in vitro* studies, which demonstrated that the toxicity of bromobenzene was much more pronounced in hepatocytes isolated from rats pretreated with phenobarbital and diethylmaleate than in those isolated from rats which were only pretreated with phenobarbital. Inclusion of precursors of glutathione in the incubation medium, i.e., cysteine or methionine, protected against bromobenzene-induced cytotoxicity, while inhibition of glutathione synthesis by methionine sulfoximine abolished this protective effect of glutathione precursors (Thor and Orrenius, 1980).

On the basis of identification of the major urinary metabolites and the correlation of hepatotoxicity with changes in their ratio, hepatotoxicity was ascribed to bromobenzene-3,4-epoxide. The increased susceptibility of PB-pretreated rats to bromobenzene-induced hepatotoxicity was associated with an increased urinary excretion of *p*-bromophenol, which is formed via the 3,4-epoxide. In contrast, 3-MC-pretreated rats showed a diminished hepatotoxicity after administration of bromobenzene, while they selectively excreted *o*-bromophenol, which is formed via the 2,3-epoxide (Lau and Zannoni, 1979). These metabolic routes were confirmed *in vitro* with liver microsomes from Pb and 3-MC treated rats (Lau and Zannoni, 1979; Monks *et al.*, 1984b). Additional evidence for the role of bromobenzene-3,4-epoxide as the hepatotoxic metabolite of bromobenzene emerged from

studies using two different strains of mice (Lau *et al.*, 1980). It was found that Balb/cJ mice possessed a significantly higher activity of the toxic 3,4-epoxide pathway than C57BL/J mice, whereas the activity of the non-toxic 2,3 epoxide pathway was similar in either strain. The higher ratio of *p*-bromophenol versus *o*-bromophenol excreted by Balb/cJ mice was associated with extensive hepatic necrosis, in contrast to the minimal pathological changes in the livers of C57BL/J mice. These studies on the metabolism of bromobenzene have generated an interesting example of the differential role of different P450 isoenzymes in the metabolism and toxicity of a xenobiotic. However, the (bio)chemical basis of this difference in the toxicity of two potentially reactive epoxides remains to be elucidated.

In a recent study, the metabolism of chlorobenzene to *o*- and *p*-chlorophenol in human and mouse microsomes paralleled the metabolism of bromobenzene, yielding kinetic parameters similar to those measured for bromobenzene (Kerger *et al.*, 1988). Apparently, chlorobenzene is metabolised by the same P450 isoenzymes as bromobenzene, and the type of halogen substitution has little effect on enzyme affinity. In this context, it is interesting to note that the ratio of *p*- to *o*-halophenol production in human microsomes amounted to 4.8 for both halogenobenzenes, compared to 1.3 and 1.4 in mouse microsomes for bromobenzene and chlorobenzene, respectively. This suggests that human microsomes preferentially metabolise halogenobenzenes through the hepatoxic 3,4-epoxide pathway, implying that humans may be rather susceptible to the hepatotoxic effects generated by halogenobenzenes.

Quinones as mediators of toxic effects

Although the role of bromobenzene-3,4-epoxide in the hepatotoxicity of the parent compound, bromobenzene, seems to be firmly established, more recent developments have stressed the importance of secondary metabolites in the toxicity and/or carcinogenicity of a variety of aromatic hydrocarbons. Secondary metabolites have been found to interact more readily with cellular macromolecules than the primary epoxides. For example, the bulk amount of covalent binding observed in microsomal incubations with benzene appeared to arise from further oxidation of initially formed phenol (Tunek *et al.*, 1980; Smart and Zannoni, 1985). Furthermore, the majority of reactive metabolites of 2,2'-dichlorobiphenyl formed by rat liver microsomes arose from secondary metabolism of phenolic metabolites, whereas arene oxides, the primary products, only played a minor role in protein binding of dichlorobiphenyl (Hesse *et al.*, 1978).

A major fraction of the covalent binding of bromobenzene to microsomal protein could be ascribed to secondary metabolites, presumably benzoquinones, of *o*- and *p*-bromophenol (Buben *et al.*, 1988). Additional support for the importance of benzoquinone metabolites in the covalent binding of bromobenzene both *in vitro* and *in vivo* comes from studies with $^{3}H/^{14}C$ labelled bromobenzene (Narasimhan *et al.*, 1988). A major decrease was observed in the $^{3}H/^{14}C$-ratio in covalently bound material compared to the substrate and soluble

metabolites formed, indicating that benzoquinones (which have a higher oxidation state, and thus a lower $^{3}H/^{14}C$-ratio) contribute to a large extent to the covalent binding. In this respect, it is interesting to note that the $^{3}H/^{14}C$-ratios for tissue adducts in lung and kidney were considerably higher suggesting a differential role for the various reactive intermediates in different organs. The exact role of these chemically reactive quinones in the hepatotoxicity of bromobenzene is still unclear, because conditions which increased the covalent binding of *p*-bromophenol *in vitro* (phenobarbital pretreatment and glutathione depletion) did not cause hepatotoxicity *in vivo* (Monks *et al.*, 1984a).

Covalent binding observed upon microsomal metabolism of the fully substituted benzene, i.e., hexachlorobenzene, was completely inhibited in the presence of the reducing agent ascorbic acid (Van Ommen *et al.*, 1986). A concomitant increase in the formation of the secondary metabolite tetrachlorohydroquinone was detected, strongly indicating tetrachlorobenzoquinone as the ultimate binding species involved.

By virtue of their electrophilic nature, quinones may spontaneously react with nucleophilic centers in the cell, and thus covalently bind to cellular protein and DNA. Quinones derived from hexachlorobenzene are unique in their reactivity toward nucleophiles. Unlike other benzoquinones, which form stable hydroquinone adducts upon reaction with sulfhydryl groups and which have to be reoxidized before being able to react again, tetrachlorobenzoquinone remains in its oxidized state and, consequently, the conjugates formed are still reactive towards nucleophiles. This is illustrated by the fact that the reaction of tetrachlorobenzoquinone with glutathione does not only result in the formation of mono-substituted glutathionyl conjugates, but also of di-, tri-, and possibly fully substituted glutathionyl conjugates (Van Ommen *et al.*, 1986). The unique reactivity of tetrachlorobenzoquinone even after conjugation with glutathione, is demonstrated by the interaction of this conjugate with GSH *S*-transferase. This enzyme was inhibited in an irreversible way at almost equimolar ratios by this compound, and evidence was presented for the fact that the glutathione-moiety of the conjugate targets the molecule to the active site, while the quinone structure reacts with an essential sulfhydryl group in or near the active site, resulting in loss of enzymatic activity (Van Ommen *et al.*, 1988).

In addition to alkylating properties, quinones also possess the capacity for redox cycling, thereby generating reactive oxygen species (Kappus, 1986). This may impose a condition of oxidative stress on cells, which may ultimately lead to cell damage. Tetrachlorobenzoquinone has been shown not to initiate redox cycling *in vitro* (Van Ommen *et al.*, 1988), but the potency for redox cycling *in vivo* may differ from the *in vitro* situation, e.g., due to differences in oxygen pressure and redox state of the cells.

The question whether tetrachlorobenzoquinone is responsible for the porphyrinogenic action of hexachlorobenzene *in vivo* still remains a matter of debate and future research. Several metabolites of hexachlorobenzene have been tested *in vivo* for their ability to induce porphyria or to inhibit uroporphyrinogen D-carboxylase, a key enzyme in the haemsynthesis (Goerz *et al.*, 1978; Wainstock de Calmanovici and San Martin de Viale, 1980). None of the metabolites tested had a direct porphyrinogenic effect, although

pentachlorophenol and tetrachlorohydroquinone were capable of increasing the porphyrinogenic action of hexachlorobenzene (Debets *et al.*, 1980). The fact that the primary metabolite pentachlorophenol is not porphyrinogenic when administered to rats would argue against a possible relationship of the secondary metabolite tetrachlorobenzoquinone with porphyria. However, the distribution, biotransformation and excretion of an orally delivered compound, and hence its toxic effects, might differ from the situation in which the compound is metabolically formed. For example, induction of P450 enzymes by hexachlorobenzene may be a prerequisite for the possible toxic action of HCB through its metabolite, by changing the relative contribution of conjugation and oxidative reactions in favor of the latter. In addition, it is conceivable that pentachlorophenol formed upon oxidation of hexachlorobenzene by cytochrome P450 does not leave the active site, but instead is oxidised by the same P450 molecule to its (hydro-)quinone derivatives. In this respect, it is interesting to note that in a recent study selective inhibition of a specific isoenzyme, cytochrome P450-p (P450IIIA1) inhibited oxidation of hexachlorobenzene *in vivo* and resulted in a decrease of the urinary excretion of porphyrins (Van Ommen *et al.*, 1989).

Glutathione conjugates as proximate toxicants in nephrotoxicity

Exposure to halogenobenzenes has not only been described to cause hepatotoxicity, but also lead to the development of renal necrosis. A single dose of bromobenzene or chlorobenzene to mice caused extensive necrosis of the proximal convoluted tubules within 24-48 hours (Reid, 1973). The development of necrosis was associated with covalent binding of radioactive material at the site of necrosis. As with hepatotoxicity, the biotransformation of the parent compound is a prerequisite for the development of nephrotoxicity, since prior inhibition of metabolism blocked both renal toxicity and covalent binding to kidney proteins. In addition, pretreatment of mice with phenobarbital, which induces hepatic but not renal xenobiotic metabolism, resulted in a significantly higher binding of bromobenzene metabolites to kidney protein.

The conclusion of Reid (1973) that a hepatic metabolite of bromobenzene was responsible for the covalent binding and, presumably, the nephrotoxicity led to investigations into the nephrotoxic potency of several hepatic metabolites of bromobenzene. In mice, all three bromophenol isomers and 4-bromocatechol were found to be nephrotoxic when injected intravenously at only 10% of a nephrotoxic dose of bromobenzene (Rush *et al.*, 1984). In contrast, *p*-bromophenol and 4-bromocatechol were not nephrotoxic in rats after intraperitoneal administration (Monks *et al.*, 1984a). Apparently, species differences in biotransformation and/or susceptibility to bromobenzene-induced toxicity exist. In addition, differences in routes of administration may influence the fate of the chemical administered and, hence, its effect.

It was subsequently shown that *o*-bromophenol, a major metabolite of bromobenzene, caused severe renal damage in non-induced rats at only 1/5 of the dose required by bromobenzene to produce similar necrosis in phenobarbital-induced rats (Lau *et al.*, 1984b). When [^{14}C]-*o*-bromophenol was administered to rats, the amount of covalently bound radioactive material to kidney proteins was four times greater than to liver proteins. Interestingly, liver microsomes converted *o*-bromophenol to covalently bound material and 2-bromohydroquinone, whereas kidney microsomes did not (Lau *et al.*, 1984a,b). These observations were consistent with the view that a hepatic metabolite of bromobenzene (and of *o*-bromophenol) was transported to the kidney, where it elicited toxicity.

Further studies revealed the formation of several isomeric mono- and disubstituted glutathione conjugates of 2-bromohydroquinone. Conjugation of potentially toxic electrophiles (like benzoquinones) with glutathione is usually associated with detoxication and excretion into urine as their corresponding mercapturic acids. However, glutathione conjugates of 2-bromohydroquinone were shown to be potent and selective nephrotoxicants (10-50 μmol/kg, i.v.; Monks *et al.*, 1985). Perhaps suprisingly, mono-glutathione conjugates exhibited less toxicity than the di-substituted conjugates.

Metabolism of the conjugate to reactive thiols by the renal tubular cells (through the ß-lyase pathway) might be the mechanism of activation. However, structure-activity studies with bromo-2,5-dihydroxy-thiophenol and isomeric bromothiophenols indicated that the quinone function of this compound is a more important determinant of toxicity than the thiol group (Monks *et al.*, 1988).

The role of γ-glutamyl transpeptidase (γ-GT), a key enzyme in the transport of glutathione conjugates into renal tubular cells, is ambiguous. Firstly, γ-GT activity may be required for transport of the hydroquinone glutathione conjugates into renal proximal tubular cells. In fact, inhibition of γ-GT by AT-125 decreased the accumulation of 2-bromohydroquinone glutathione conjugates into kidney slices (Lau *et al.*, 1988). Secondly, γ-GT-mediated formation of a relatively labile hydroquinone-cysteine conjugate may facilitate oxidation to the reactive benzoquinone form and may thus be involved in the generation of the ultimate toxic metabolite. Indeed, the oxidation potential is decreased in the following order (and thus the ease to oxidise is increased): 2-bromohydroquinone > 2-bromohydroquinone glutathion-*S*-yl conjugate > 2-bromohydroquinone cystein-*S*-yl conjugate (Monks and Lau, 1990b).

Thirdly, γ-GT mediated hydrolysis of the bromohydroquinone glutathion-*S*-yl conjugate to its cystein-*S*-ylglycine derivative may also be regarded as a detoxication route of hydroquinone glutathione conjugates by means of oxidative cyclization resulting in the formation of a 1,4-benzothiazine, which is subsequently excreted into urine (Monks *et al.*, 1990). The striking difference between the glutathione conjugate of menadione, which was not nephrotoxic, and its *N*-acetylcysteine derivative, which was nephrotoxic, may be related to their relative sensitivity towards cyclization, since cyclization is hindered in the mercapturic acid (Lau *et al.*, 1990).

In contrast to the bioactivating role of γ-GT in the nephrotoxicity of bromohydroquinone glutathione conjugates, the nephrotoxic action of the structurally related glutathione conjugates of 2,5-dichlorohydroquinone and 2,3,5-trichlorohydroquinone was actually decreased by γ-GT activity (Mertens *et al.*, 1991). Thus, multiple renal transport mechanisms may be involved in the accumulation of glutathione-conjugated hydroquinones, and/or differences may exist in the relative rate for the pathways leading to toxicity or detoxication (oxidative cyclization, *N*-acetylation vs -deacetylation, protein arylation) for different halogenated benzoquinones.

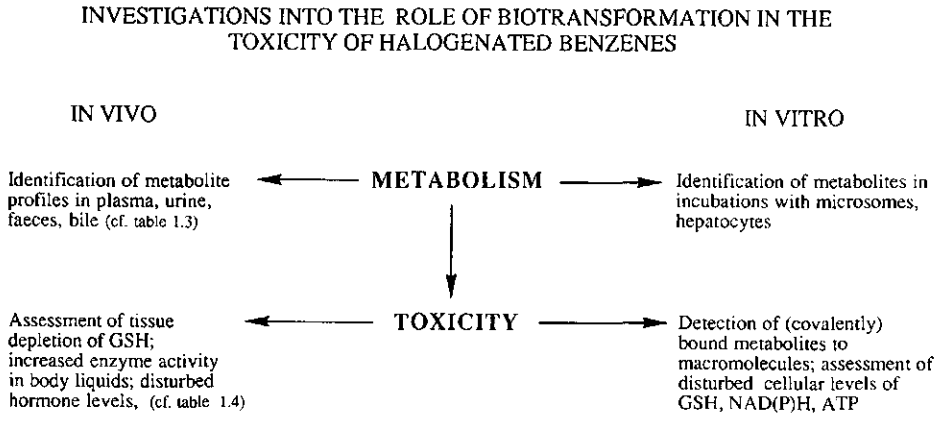


Figure 1.3 Multi-pathway approach to the study of the role of biotransformation in the toxicity of halogenated benzenes.

Scope of the present investigation

The main objective of the present investigation is to compare a range of structurally related chemicals with respect to their pathways of metabolic activation. With reference to Figure 1.2 and page 32-39, different reactive products may be formed upon (oxidative) biotransformation of halogenated benzenes: epoxides as chemical intermediates in the primary oxidative event, and benzoquinone metabolites as secondary products. Target sites of either class of metabolites in relation to toxicity still remain to be identified.

In an attempt to characterise and evaluate the contribution of the different routes to bioactivation, the whole series of halogenated benzenes should be taken into account. The ultimate aim of the present thesis includes the development of a set of general rules

concerning the role of metabolism in the toxic effects generated by these chemicals. As schematically presented in Figure 1.3, this question can be addressed in a number of ways. In order to define a meaningful correlation between the observed toxic effects and the various reactive intermediates involved in their metabolism, detailed *in vitro* studies on the microsomal oxidation of different congeners of chlorinated benzenes are described in Part I (chapter 2, 3, and 4). As a quantification of metabolic activation, covalent binding of radiolabeled compound to microsomal protein and DNA is detected. In addition, the differential role of cytochrome P450 isoenzymes in the oxidation of chlorinated benzenes is assessed. In chapter 5, a mechanism is proposed for the formation of the reactive benzoquinone metabolites.

Part II describes the toxicity and fate of chlorinated benzenes *in vivo*. In order to extrapolate data on microsomal metabolic activation of chlorinated benzenes, studies focussing on target-organ toxicity of a range of chlorinated benzenes after a single exposure have been performed (chapter 7) to determine the role of chemically reactive and/or biologically active (chemically stable) metabolites in the various toxic symptoms. Finally, a detailed semi-chronic study is performed comparing the toxicologic profile and the metabolic profile of 2 congeners, hexachlorobenzene and pentachlorobenzene, which have been shown to produce qualitatively similar products *in vitro* (chapter 8 and 9).

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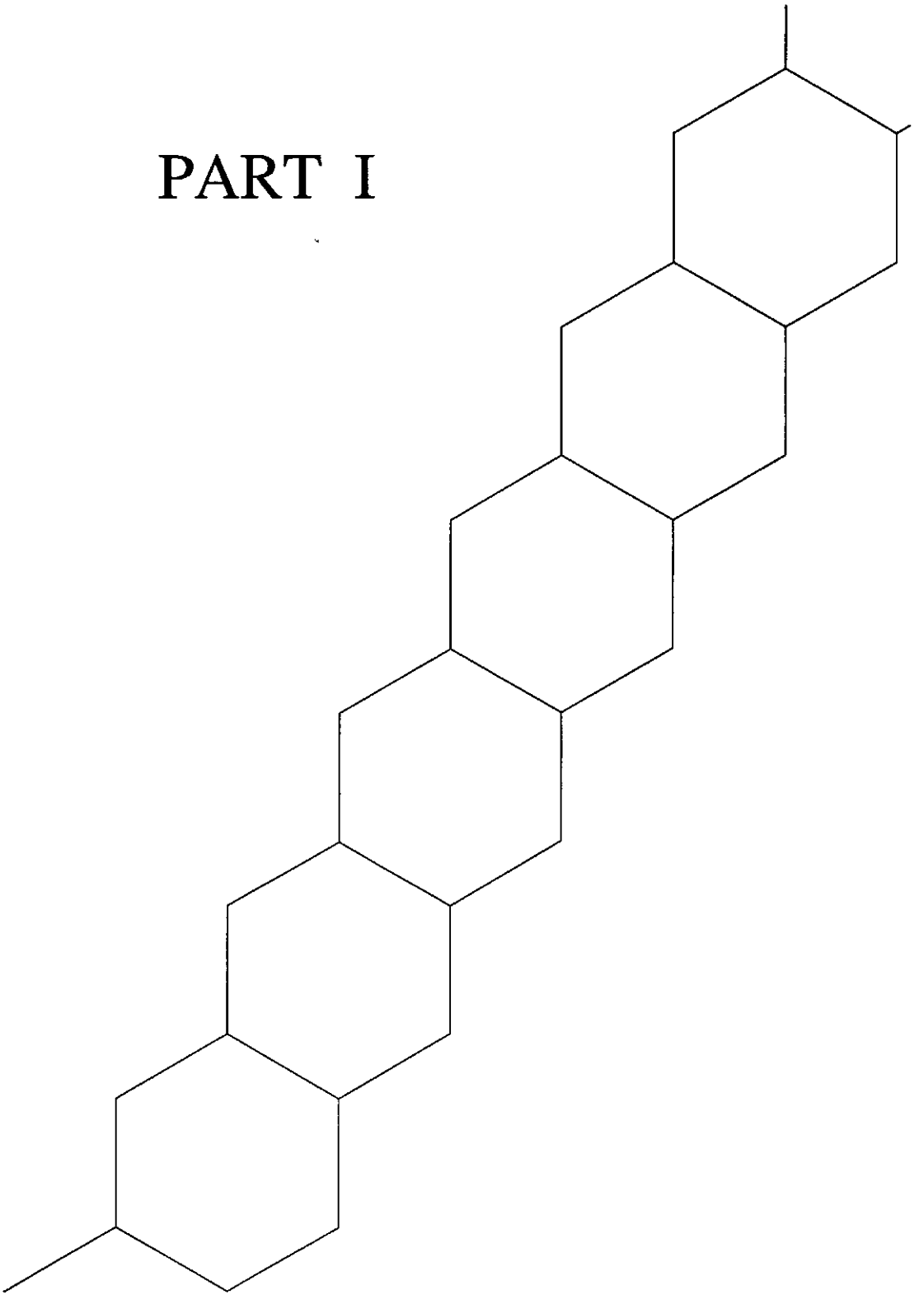
PART I



Chapter 2

THE METABOLISM OF PENTACHLOROBENZENE BY RAT LIVER MICROSOMES: The nature of the reactive intermediates formed

Abstract

Metabolism of [^{14}C]-pentachlorobenzene by liver microsomes from dexamethasone induced rats resulted in the formation of pentachlorophenol and 2,3,4,6-tetrachlorophenol as major primary metabolites in a ratio of 4:1, with 2,3,4,5- and 2,3,5,6-tetrachlorophenols as minor primary metabolites. Thus, the unsubstituted carbon atom was the favourite site of oxidative attack, but the chlorine-substituted positions also played a sizable role. As secondary metabolites both *para*- and *ortho*-tetrachlorohydroquinone are formed (1.4 and 0.9 % of total metabolites, respectively). During the cytochrome P450-dependent conversion of pentachlorobenzene, 5 - 15 % of the total amount of metabolites became covalently bound to microsomal protein. Ascorbic acid inhibited this binding to a considerable extent, indicating that benzoquinone metabolites play an important role in the binding. However, complete inhibition was never reached by ascorbic acid, nor by glutathione, suggesting that other reactive intermediates, presumably epoxides, are also involved in covalent binding.

C. den Besten, M.M.C.G. Peters, P.J. van Bladeren, *Biochem. Biophys. Res. Commun.* 163 (1989) 1275-1281.

Introduction

Chlorinated benzenes are widely used as chemical intermediates in the synthesis of organic compounds, and as solvents, pesticides, space deodorants and other industrial products (US EPA, 1985). Because of their lipophilic character and their relative resistance to chemical and biological degradation, they tend to accumulate in animal and human tissue (Veith *et al.*, 1979; Jan, 1983). Hence, there is concern over the consequences to human health of chronic exposure to these agents. Toxic symptoms after exposure to chlorinated benzenes range from adverse effects on the nervous system to liver and kidney damage, sometimes accompanied by severe porphyria (Brown *et al.*, 1969; Reid, 1973; Rimington and Ziegler, 1963).

Investigations into the metabolism of halogenated aromatics have shown that epoxide formation is involved in their hydroxylation by the cytochrome P450 containing mono-oxygenases (Jondorf *et al.*, 1958, Daly *et al.*, 1972, Kohli *et al.*, 1976). These reactive, electrophilic intermediates have been implicated in the toxicity associated with these compounds. For example, the hepatotoxic effects after bromobenzene or chlorobenzene administration to rats is thought to be mediated by their 3,4-epoxide intermediate (Lau and Zannoni, 1979).

However, in recent years a considerable role in the metabolic activation of aromatics has been ascribed to secondary metabolism, resulting in the formation of electrophilic quinones. For example, studies on the microsomal metabolism of benzene have shown that quinone metabolites (and perhaps the semiquinone intermediates) are the ultimate covalently binding species to microsomal protein (Tunek *et al.*, 1980). Furthermore, it has been demonstrated in our laboratory that hexachlorobenzene (HCB) is hydroxylated by rat liver microsomes to pentachlorophenol (PCP), which is further metabolised to tetrachlorohydroquinone (TCHQ) (Van Ommen *et al.*, 1985). Covalent binding to microsomal protein as a result of the oxidative biotransformation of hexachlorobenzene appears to be completely mediated by tetrachlorobenzoquinone (TCBQ), which is formed upon oxidation of TCHQ (Van Ommen *et al.*, 1986).

Although a number of investigations describe the urinary and faecal products of the various chlorinated benzenes in different species (Kohli *et al.*, 1976, Rozman *et al.*, 1979), there is a lack of detailed studies on the relationship between formation of reactive metabolites and the toxicity caused by these compounds. As a first step, the study presented here describes the *in vitro* metabolism of pentachlorobenzene by rat liver microsomes, with special emphasis on the formation of reactive metabolites.

Materials and Methods

Chemicals

[^{14}C]-Pentachlorobenzene (PCB, s.a. 19.2 mCi/mmole) was purchased from Sigma Chemical Co., St. Louis, USA, with a radiochemical purity of 99.0 %. Impurities consisted of 0.4 % tetrachlorobenzene and 0.5 % hexachlorobenzene that were present in identical amounts both before and after metabolism of PCB, 0.02 % 2,3,4,6-tetrachlorophenol (TCP) and 0.04 % 2,3,4,5-TCP. NADPH was from Boehringer Mannheim, Germany. PCB was from Merck, Schuchardt, Germany (> 98 %). All other chemicals have been described elsewhere (Van Ommen *et al.*, 1985).

Preparation of microsomes

Male Wistar rats (300 g) were pretreated with dexamethasone (4 daily administrations by oral gavage of 300 mg/kg body weight dissolved in 2 % Tween 80). Control rats were untreated. Microsomes were prepared as described previously (Van Ommen *et al.*, 1985) and stored at -80 °C until used. Cytochrome P-450 was determined according to Omura and Sato (1964). Protein concentrations were determined by the method of Lowry (1951).

Microsomal incubations

Unless otherwise stated, incubations were carried out with dexamethasone (DEX)-induced microsomes. A standard incubation mixture was used, containing 1 mM NADPH, 3 mM $MgCl_2$, 0.1 M potassium phosphate buffer pH 7.4, 0.25-1.0 mg microsomal protein and 50 μM PCB. PCB and NADPH were present at saturating amounts. [^{14}C]-PCB was diluted to a specific activity of 5 mCi/mmole. After a 2 min preincubation in a shaking waterbath at 37 °C of all components except NADPH, the substrate was added in 50 μl of acetone. The reaction was started by adding NADPH (final volume 2.0 ml) and, unless otherwise stated, terminated after 10 minutes by the addition of HCl (final concentration 0.6 N). Control incubations were without NADPH.

Extraction of [^{14}C]-PCB and metabolites

Extraction of the incubation mixture with 2x2 ml of diethyl ether removed > 99 % of the radioactivity from the aqueous phase. After overnight drying of the extracts on Na_2SO_4, the organic solvent was removed under a stream of nitrogen gas and the residues were dissolved in 75 μl of methanol.

HPLC analysis

Reversed phase chromatography was performed using a Perkin-Elmer Series 4 HPLC, fitted with a 150 x 4.6 mm ID Lichrosorb 5RP18 column and a Co:Pell ODS precolumn. The eluent was monitored at 254 nm. After injection of 30 μl sample together with 3 μl of a solution containing the marker metabolites (see legend to Figure

2.1), all radioactivity was eluted isocratically with 60 % methanol/ 40 % Tris-phosphate buffer (50 mM, pH 2.5 or pH 8.0, see legend to Figure 2.1) for 2 minutes, followed by a linear gradient to 100 % methanol in 10 minutes, and a stationary phase at 100 % methanol for 10 min. For determination of radioactivity in the eluent, 0.5 ml fractions were collected followed by scintillation counting (5 ml Atomlight, Du Pont) in a Tri-Carb liquid scintillation counter (Packard, Brussels, Belgium). In the assay described above, the 2,3,4,6- and the 2,3,5,6-TCP isomers co-eluted in one peak (*cf.* Figure 2.1). In order to distinguish between all 3 isomers of TCP, samples were also eluted on a Nucleosil 5RP18 column (150 x 4.6 mm) with a linear gradient from 50% methanol/ 50 % potassium dihydrogen phosphate (20 mM, pH 4.5) to 85 % methanol/ 15 % potassium dihydrogen phosphate in 30 minutes, followed by 20 minutes at 85 % methanol (k' 2,3,5,6-TCP, 12.6; k' 2,3,4,6-TCP, 13.0; k' PCP, 13.8; k' 2,3,4,5-TCP, 14.4). It became evident that 2,3,4,6-TCP was the major TCP isomer formed (ratio 2,3,5,6- : 2,3,4,6-: 2,3,4,5-TCP = 1 : 11.6 : 1.9).

Covalent binding to protein

Covalently bound material was measured after extensive washing of the protein pellet with organic solvents: 3x5 ml of methanol, 3x5 ml of ethanol, 2x5 ml of ethyl acetate, 2x5 ml of diethyl ether. No radioactivity was detected in the final ether-wash. The final pellet was solubilized in 1 M NaOH overnight at 40 °C and diluted with 1 volume of distilled water. An aliquot was taken for determination of the radioactive content. Protein content of the digests was determined according to Lowry (1951).

Results

Figure 2.1A shows the separation of PCB and 8 possible metabolites by a reversed-phase HPLC system using a Tris-phosphate buffer of pH 2.5 as the aqueous phase. Analysis of the ether extracts of microsomal incubations of [^{14}C]-PCB revealed 2 major radioactivity peaks, eluting before the substrate, which were identified as PCP and 2,3,4,6-TCP. Minor peaks of radioactivity were identified as 2,3,4,5-TCP, 2,3,5,6-TCP, *p*-TCHQ and *o*-TCHQ, respectively. The identity of the fractions was confirmed by conducting a base run, in which the acid Tris-phosphate buffer was replaced by a Tris-phosphate buffer of pH 8.0. Due to this increase in pH, the retention times of phenols and hydroquinones change, depending on their pKa value (Figure 2.1B). A small amount of radioactivity (0-2.5 % of the radioactivity present), characterized by a relatively high polarity, remained unidentified.

Oxidation of PCB by liver microsomes from DEX-treated rats was measured as a function of varying microsomal protein concentration and varying incubation times (Table 2.1). It is shown that under these circumstances, oxidation of PCB was linear up to 0.25 mg microsomal protein/ml, while the maximum conversion rate was already

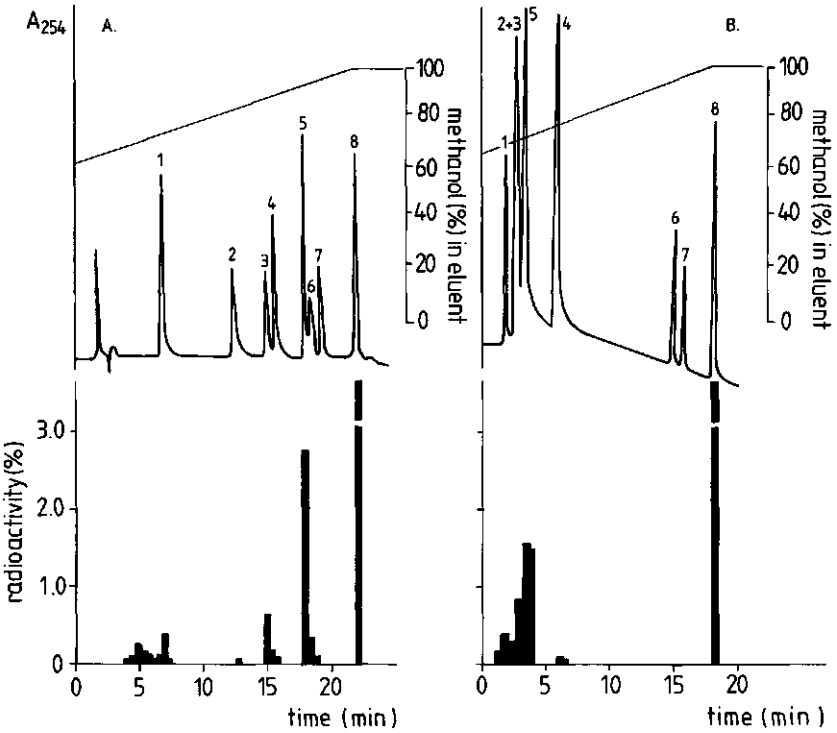


Figure 2.1 HPLC-analysis of metabolites of pentachlorobenzene converted by dexamethasone-induced rat liver microsomes. A, acid run (50 mM Tris-phosphate pH 2.5 as the aqueous component); B, base run (50 mM Tris-phosphate pH 8.0 as the aqueous component). Marker metabolites: 1, *p*-tetrachlorohydroquinone (*p*-TCHQ); 2, *o*-TCHQ; 3, 2,3,4,6-tetrachlorophenol (TCP), 2,3,5,6-TCP; 4, 2,3,4,5-TCP; 5, pentachlorophenol (PCP); 6, 1,2,3,4-tetrachlorobenzene (TCB); 7, 1,2,3,5-TCB; 8, pentachlorobenzene (PCB).

reached within 5 min.

Conversion of PCB was dependent on enzymatic activity (*cf.* no conversion with boiled microsomes) and appeared to be cytochrome P450 mediated: in incubations without NADPH, no metabolites were detected, and the use of the classical inhibitor of cytochrome P450 activity, metyrapone, decreased metabolism of PCB by 83 % (Table 2.2).

Table 2.1 Metabolism of [^{14}C]-pentachlorobenzene by liver microsomes from dexamethasone-induced rats.

Incubation	Conversion (%)	Individual metabolites as percentage of total metabolites						
		PCP	TCP (2,3,4,6+ 2,3,5,6)[a]	2,3,4,5-TCP	1,4-TCHQ	1,2-TCHQ	not identified	CVB
protein (mg/ml)								
0.125	9.9	62.2	15.9	3.2	1.4	0.9	8.5	7.9
0.25	16.0	51.4	15.7	2.8	3.3	0.9	15.7	10.2
0.5	15.9	54.9	16.3	2.8	1.3	0.7	9.8	14.2
time (min)								
5	6.6	68.0	22.9	3.3	1.7	0.3	0	3.8
10	8.6	63.5	18.4	3.3	1.5	0.6	5.1	7.6
20	11.9	60.3	19.1	2.8	1.3	0.8	7.0	8.7

Note. Incubations were performed at 37°C with a standard incubation mixture containing 3 mM $MgCl_2$, 0.1 M potassium phosphate buffer pH 7.4, 1 mM NADPH and 50 μM PCB with 0.25 mg microsomal protein (final volume 2.0 ml) for 10 minutes, unless stated otherwise. Conversion is expressed as the sum of the percentage of extracted metabolites and the percentage of radioactivity covalently bound to protein. Both the conversion, determined by HPLC analysis and the covalent binding were corrected for blank values, obtained from incubations without NADPH. Data are means of at least duplicate observations, and the standard deviation was generally less than 10%. [a] 2,3,4,6-TCP and 2,3,5,6-TCP were formed in a ratio of 12:1 (see under Materials and Methods). CVB, covalent binding.

Metabolism-dependent covalent binding of [^{14}C]-PCB to microsomal protein was detected after extensive organic extraction. In incubations with boiled microsomes or in the absence of NADPH, the protein fraction contained a very small amount of radioactivity, which was independent of incubation time or amount of protein present. Furthermore, covalent binding was dependent on the activity of the cytochrome P450 complex, since both carbon monooxide and metyrapone blocked binding to a great extent (Table 2.2).

Table 2.2 Microsomal metabolism and covalent binding of [^{14}C]-pentachlorobenzene by liver microsomes from dexamethasone-treated rats.

Incubation	Conversion (%)	Covalent binding (%)
Complete mixture (DEX)	100	100
minus NADPH	nil	3 ± 1
Boiled microsomes	nil	4 ± 0
Control microsomes	20 ± 2	4 ± 0
Complete mixture (DEX)		
+ CO	12 ± 0	5 ± 1
+ metyrapone (0.5 mM)	17 ± 4	4 ± 1
+ ascorbic acid		
1.0 mM	89 ± 12	34 ± 1
10.0 mM	85 ± 3	23 ± 1
+ glutathione (1.0 mM)	115 ± 7	22 ± 2
+ TCPO (1.0 mM)	76 ± 8	81 ± 4

Note. A complete incubation mixture (control) contained 0.25 mg DEX-induced microsomes, 3 mM $MgCl_2$, 0.1 M potassium phosphate buffer pH 7.4, 1 mM NADPH and 50 μM PCB (final volume 2.0 ml). Incubations were performed at 37°C for 10 minutes. Data are based on 2-6 observations and are expressed as a percentage of the conversion and covalent binding of control incubations (conversion 9.6 ± 1.3 nmoles/10 min/0.25 mg, covalent binding 0.74 ± 0.1 nmoles/10 min/0.25 mg, mean ± S.D., n = 6). TCPO, 1,1,1-trichloropropene oxide.

Ascorbic acid, a strong reducing agent, inhibited the covalent binding of reactive PCB metabolites by 60 - 70 %. A concomitant increase in the formation of TCHQ was detected, which was comparable to the decrease in covalent binding (data not shown). The presence of 1 mM of glutathione (GSH), a nucleophilic sulfhydryl compound, resulted in a strong decrease in the amount of protein bound metabolites, while 36 % of the total amount of metabolites formed remained in the aqueous phase after organic extraction. However, considerable binding was still observed.

In an attempt to study the involvement of epoxide intermediates in the protein binding of PCB, 1,1,1-trichloropropene oxide (TCPO), an inhibitor of epoxide hydrolase, was added to a standard incubation mixture. If epoxide metabolites were to be the ultimate

binding species, an increase in covalent binding in the presence of TCPO would be expected, due to a prolonged lifetime of the reactive epoxide metabolites. However, a slight decrease both in protein binding and metabolism was observed.

Discussion

Dexamethasone, a compound known to increase isoenzyme P450IIIA1 in rat liver (Wrighton *et al.*, 1985), is a powerful inducer of metabolism of chlorinated benzenes: previous studies in our laboratory demonstrated that conversion of HCB was by far highest in DEX-induced microsomes compared to microsomes from rats pretreated with phenobarbital, 3-methylcholanthrene, isosafrole and HCB (Van Ommen, 1987). In the present study, metabolism of PCB in DEX-induced microsomes was 5 times higher than in control microsomes.

Based on the results presented in this study, a route for microsomal metabolism of PCB is proposed (Figure 2.2). It is demonstrated that PCB can be metabolised at different positions at the aromatic ring. The primary oxidative attack is not limited to the unsubstituted carbon atom, resulting in the formation of PCP, but can also take place at the more sterically hindered chlorine-substituted position, as is demonstrated by the formation of the different TCP-isomers. Apparently, rearrangement to 2,3,4,6-TCP is the most favourable situation, since this isomer predominated over the other two isomers. However, it should be noted that secondary oxidations to hydroquinones might influence the ratio of the different primary metabolites. The formation of various TCP-isomers from PCB in different species has been described in previous studies (Kohli *et al.*, 1976, Rozman *et al.*, 1979, Engst *et al.*, 1976).

In addition to the formation of ether-soluble products, PCB is substantially metabolised to reactive metabolites that covalently bind to microsomal protein. The fact that, with increasing incubation times and increasing amounts of protein, the protein binding increases at the cost of primary metabolites, seems to support a role for secondary metabolism, i.e., benzoquinone formation, in the covalent binding of PCB.

The protective action of both glutathione and ascorbic acid on the protein binding of reactive metabolites has been described in previous reports for hexachlorobenzene (Van Ommen *et al.*, 1986) and bromobenzene (Buben *et al.*, 1988). Selective inhibition of protein binding by glutathione emphasizes the electrophilic nature of the reactive metabolite(s). The detection of radioactivity (36 % of total metabolites formed) in the aqueous phase after addition of glutathione agrees with the findings of Van Ommen *et al.*, (1986) that glutathione readily forms water-soluble adducts with TCBQ. However, both arene oxides and quinones are electrophilic species, and thus capable of reacting with sulfhydryl compounds. The finding that ascorbic acid caused a loss of protein

binding together with a comparable increase in the formation of TCHQ, seems to indicate TCBQ (and perhaps the semiquinone radical) as the most important binding species.

As expected, these data are in agreement with studies on the microsomal metabolism of HCB, in which TCBQ was suggested as the only binding species involved, since both HCB and PCB produce PCP as their primary metabolite. However, in contrast to protein binding resulting from HCB metabolism, binding by PCB metabolites could not completely be inhibited by ascorbic acid. Increasing the ascorbic acid concentration to 10 mM decreased the covalent binding only slightly more (Table 2.2), suggesting that, although to a lesser extent than TCBQ, other reactive species are involved in the protein binding of PCB. The question whether epoxides are responsible for this remaining protein binding, and whether binding selectivity is different for the different reactive species, warrants further investigations.

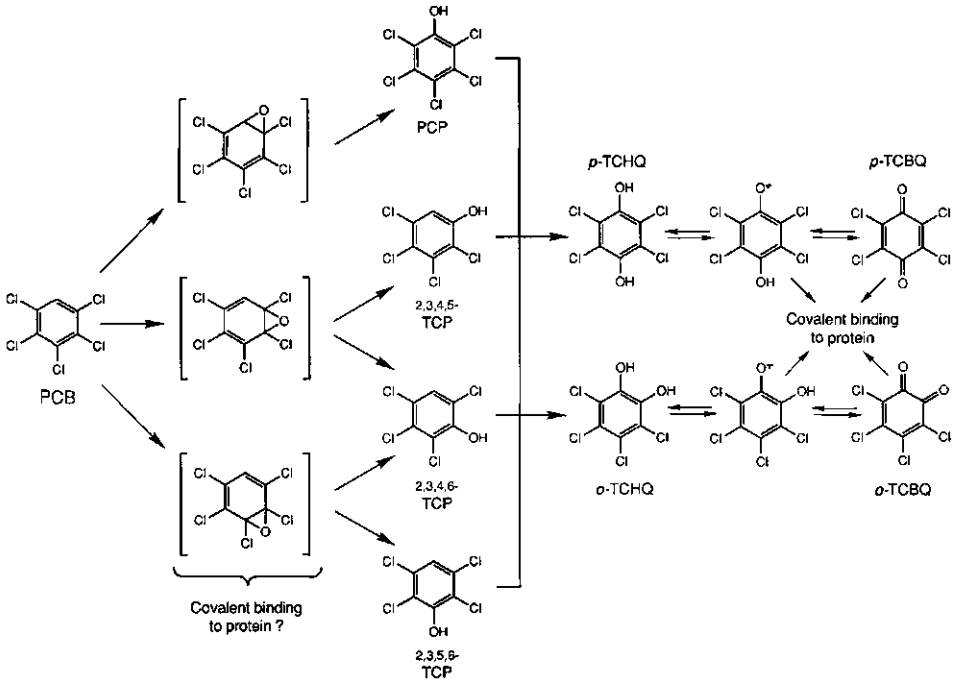


Figure 2.2 Proposed metabolic route for pentachlorobenzene in rat liver microsomes.

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Chapter 3

METABOLIC ACTIVATION OF 1,2,4-TRICHLOROBENZENE AND PENTACHLOROBENZENE BY RAT LIVER MICROSOMES: a major role for quinone metabolites

Abstract

Microsomal metabolism of [^{14}C]-1,2,4-trichlorobenzene (1,2,4-TRICB) and [^{14}C]-pentachlorobenzene (PCB) was studied with special emphasis on the conversion dependent covalent binding to protein and DNA. 1,2,4-TRICB was metabolised by microsomes from dexamethasone-pretreated rats to 2,3,6- and 2,4,5-trichlorophenol, and to a lesser extent to 2,4,6- and 2,3,5-trichlorophenol, and trichlorohydroquinone. About 10 % of all metabolites became covalently bound to protein in a rather nonselective way. Both for 1,2,4-TRICB and PCB, a strong correlation between secondary metabolism to hydroquinones and covalent binding was established. Protein binding of 1,2,4-TRICB was completely inhibited by the addition of ascorbic acid, indicating quinone metabolites as the sole reactive species formed. Both 1,2,4-TRICB and PCB alkylated DNA, although to a much lesser extent than protein (0.5 % and 0.3 % of all metabolites, respectively). Nonquinone intermediates, presumably epoxides, were responsible for a minor portion of the observed DNA binding, since complete inhibition by ascorbic acid was not reached. The differential role of cytochrome P450 both in primary and secondary metabolism was demonstrated by the use of microsomes from rats pretreated with different inducers. Dexamethasone (DEX) microsomes (cytochrome P450IIIA1) showed the highest activity towards these chlorinated benzenes (14 nmol/mg/5 min for 1,2,4-TRICB and 36 nmol/mg/10 min for PCB), both with regard to the formation of phenols and to the formation of protein-bound metabolites. In addition, DEX microsomes preferentially formed 2,3,5-trichlorophenol, whereas other microsomal suspensions formed 2,4,5-trichlorophenol as the major isomer. The present study cleary demonstrates the high alkylating potency of secondary quinone metabolites derived from chlorinated benzenes, and poses a need for reevaluation of the role of epoxides in the observed toxicity of these compounds.

C. den Besten, M.C.C. Smink, E.J. de Vries, P.J. van Bladeren, *Toxicol. Appl. Pharmacol.* 108 (1991) 223-233.

Introduction

Halogenated benzenes are produced in large quantities for use as solvents and in the synthesis of many organic compounds. As a consequence of their intense industrial use, they have become ubiquitous environmental pollutants and have been identified by the US Environmental Protection Agency as priority pollutants for hazard evaluation. Many investigations concerning their distribution in the environment (Oliver *et al.*, 1982), their bioaccumulation in animal and human tissue (Jan and Malnersic, 1980; Mes *et al.*, 1986), and their microbial (De Bont *et al.*, 1986) and mammalian degradation (Jondorf *et al.*, 1958) have been described. In addition, a substantial number of studies describes the adverse effects of halogenobenzenes, ranging from neurological disorders to effects on liver, kidneys and lungs, sometimes accompanied by porphyria (Brodie *et al.*, 1971; Rimington and Ziegler, 1963). It is generally accepted that halogenated benzenes, like many other xenobiotics, require metabolic activation before they exert their toxic effects. For example, the toxicity of bromobenzene and hexachlorobenzene, two halogenated benzenes which have been the subject of many investigations, is decreased by inhibition of mono-oxygenase activity and is increased by selective induction of specific isoenzymes of cytochrome P450 (Brodie *et al.*, 1971; Van Ommen *et al.*, 1989).

Previous studies on the metabolism of bromo- and chlorobenzene have demonstrated the involvement of arene oxides in the hydroxylation of the aromatic ring (Jerina and Daly, 1974). These electrophilic intermediates are thought to be the ultimate reactive species involved in the toxicity of many aromatics. In fact, the hepatotoxic effects of bromo- and chlorobenzene in rodents are thought to be mediated by the 3,4-epoxide derivatives (Lau *et al.*, 1980).

However, more recent developments have indicated the importance of secondary metabolites in the toxicity and/or carcinogenicity of a variety of aromatic compounds. *In vitro* studies have clearly demonstrated that some secondary aromatic metabolites like quinones or their semiquinone-anions may react more readily with cellular macromolecules than the primary arene oxides. For example, the major amount of covalent binding observed in microsomal incubations with benzene (Tunek *et al.*, 1980) or 2,2'-dichlorobiphenyl (Hesse *et al.*, 1978) appears to arise from further oxidation of initially formed phenol metabolites. In our laboratory, covalent binding to microsomal protein as a result of the oxidative biotransformation of hexachlorobenzene (Van Ommen *et al.*, 1986) was found to be mediated by tetrachlorobenzoquinone (or -semiquinone), which is formed via oxidation of the intermediate metabolite pentachlorophenol.

The lack of detailed investigations into the metabolic activation of halogenated benzenes other than the monosubstituted compounds has prompted us to investigate the *in vitro* metabolism of a range of chlorinated benzenes. In a recent study, the microsomal metabolism of pentachlorobenzene was described (Den Besten *et al.*, 1989). The present investigation describes the microsomal conversion of 1,2,4-trichlorobenzene. Special

emphasis is put on the nature of the reactive metabolites derived from 1,2,4-trichlorobenzene and pentachlorobenzene. Furthermore, the differential role of cytochrome P450 isoenzymes in the metabolism of these compounds is demonstrated.

Materials and Methods

Chemicals

[^{14}C]-Pentachlorobenzene (PCB) and 1,2,4-[^{14}C]-trichlorobenzene (1,2,4-TRICB) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Radiochemical impurities in PCB consisted of 0.4 % tetrachlorobenzene and 0.5 % hexachlorobenzene that were present in identical amounts both before and after metabolism of PCB, and 0.02 % 2,3,4,6-tetrachlorophenol and 0.04 % 2,3,4,5-tetrachlorophenol. Radiochemical impurities of 1,2,4-TRICB consisted of 0.24 % 1,2-dichlorobenzene that was present in identical amounts both before and after metabolism, 0.05 % 2,3,6-trichlorophenol, 0.05 % 2,3,5-trichlorophenol and 0.5 % polar metabolites which were not further identified. Reagent grade chemicals (Merck, Darmstadt, BRD) were used to adjust the specific activity of PCB and 1,2,4-TRICB to 10 and 5 mCi/mmole, respectively unless stated otherwise. NADPH was from Boehringer Mannheim GmbH, BRD. All other chemicals were at least of reagent grade.

Preparation of microsomes

Microsomes were isolated from male Wistar rats (300 g) which were pretreated with phenobarbital (PB, 0.1 % in drinking water for 7 days), 3-methylcholanthrene (3-MC, 3 daily i.p. administrations of 30 mg/kg), isosafrole (ISF, 4 daily i.p. administrations of 150 mg/kg) or with dexamethasone (DEX) as previously described (Den Besten *et al.*, 1989). Control rats were untreated. The livers were perfused with ice cold 0.9 % NaCl and homogenized in 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose using a teflon pestel. The 11,000 g supernatant was centrifuged at 105,000 g for 95 min, after which the pellet was washed. Pellets were finally resuspended in 0.1 M potassium phosphate buffer pH 7.4 + 20 % glycerol. Microsomes were stored at -80°C until use. Cytochrome P450 was determined according to Omura and Sato (1964) and the protein concentration was determined by the Lowry assay (Lowry *et al.*, 1951).

Microsomal incubations

Incubations were performed using glass tubes with a teflon-lined screw top in a shaking waterbath at 37°C as previously described (Den Besten *et al.*, 1989). Radiolabeled substrates (50 μM PCB or 100 μM 1,2,4-TRICB) in acetone (2.5 % final concentration) were added to the incubations containing 0.1 M potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$, and microsomal protein (0.25 to 2 mg DEX microsomes) after 2 minutes of preincubation. The reaction was started by the addition of 1 mM

NADPH (final volume of 2.0 ml) and stopped by the addition of HCl (final concentration of 0.6 N) after 10 or 5 min in the case of PCB or 1,2,4-TRICB, respectively, unless otherwise stated.

Extraction of metabolites

PCB and its metabolites were extracted and prepared for HPLC analysis as previously described (Den Besten *et al.*, 1989). Due to the volatile character of 1,2,4-TRICB, diethylether extracts of incubations with this compound were not dried under nitrogen, but extraction was performed with small volumes (0.5 ml) of ethyl acetate, which were collected separately. Four separate extractions resulted in the removal of > 99.5 % of the radioactivity from the aqueous phase. Direct HPLC analysis of the two first ethylacetate extracts (> 90 % of all radioactivity) revealed no significant differences in the relative composition of metabolites and substrate. Therefore, routine HPLC analysis was only performed on 25 μl of the first collected ethylacetate phase.

HPLC analysis

Reversed-phase High Performance Liquid Chromatography was performed using a Perkin-Elmer Series 4 HPLC, equipped with a 250 x 4.6 mm I.D. Nucleosil 5C18 column fitted with a Co:Pell ODS precolumn, and a gradient elution using a mobile phase containing methanol and 20 mM KH_2PO_4 (see legend to Figure 3.1). The flow rate was 1 ml/min and the eluent was monitored at 254 nm. The following k' values were obtained: 2,5-dichlorohydroquinone (DCHQ), 2.7; trichlorohydroquinone (TRICHQ), 4.1; 2,3-dichlorophenol (DCP), 6.5; 2,5-DCP, 6.9; 2,4-DCP, 7.3; 2,3,6-trichlorophenol (TRICP), 8.6; 2,3,4-TRICP, 9.5; 2,4,6-TRICP, 9.8; 2,4,5-TRICP, 10.2; 2,3,5-TRICP = 1,2-dichlorobenzene (DCB), 10.5; 1,4-DCB, 11.4; 1,3-DCB, 11.8; 1,2,4-TRICB, 12.8. For determination of radioactivity in the eluent, fractions of 0.5 ml (or 0.2 ml in the area where the trichlorophenol isomers eluted) were collected followed by scintillation counting (5 ml of Ultima Gold, Packard) in a Tri-Carb liquid scintillation counter (Packard). When routine HPLC analysis was performed to determine total conversion rather than exact quantification of individual trichlorophenol isomers, a simplified assay was used employing a 150 x 4.6 mm I.D. Lichrosorb 5RP18 column and a mobile phase consisting of acetonitrile, methanol and water, all phases containing 0.5 % acetic acid. The concentration of methanol in the mobile phase was kept at 30 % throughout the assay. All radioactivity was eluted isocratically for 2 minutes with 10 % acetonitrile and 60 % water, followed by a linear increase to 50 % acetonitril (2 % per minute for 10 minutes, followed by 4 % per minute for 10 minutes) and concluded isocratically with 70 % acetonitrile for 10 minutes. The k' values were: 2,5-DCHQ, 2.6; TRICHQ, 4.7; 2,3-DCP, 7.8; 2,4-DCP, 8.8; 2,3,6-TRICP, 10.0; 2,3,4-TRICP, 2,4,6-TRICP, 10.7; 2,4,5-TRICP=2,3,5-TRICP, 11.1; 1,2-DCB = 1,4-DCB, 11.8; 1,3-DCB, 12.2; 1,2,4-TRICB, 13.3. Microsomal metabolites were identified by coelution with marker metabolites which were injected together with the sample. The identification of

metabolites was confirmed by conducting a alkaline run with a mobile phase consisting of methanol and 20 mM Tris-phosphate buffer (pH 8.0) using a 250x4.6 I.D. Nucleosil 5C18 column. All radioactivity was eluted by a linear gradient from 50 % methanol to 85 % methanol in 30 minutes, and followed by 10 minutes of isocratic elution with 85 % methanol. Phenols and hydroquinones, which are sensitive to changes in pH, will move to the more polar region of the eluent, depending on their pKa value. The k' values were: TRICHQ, 1.3; 2,3,6-TRICP=2,4,6-TRICP, 1.9; 2,3,5-TRICP, 3.3; 2,4,5-TRICP, 3.9; 2,3,4-TRICP, 4.2; 1,2-DCB=1,4-DCB, 9.2; 1,3-DCB, 9.7; 1,2,4-TRICB, 10.9.

Covalent binding to protein

Covalently bound material to microsomal protein was measured after extensive washing of the protein pellet with organic solvents as previously described (Den Besten *et al.*, 1989). No radioactivity was detected in the final ether-wash. The final pellet was digested in 1 M NaOH overnight at 40°C and diluted with 1 volume of distilled water. An aliquot was taken for determination of the radioactive content. Protein content of the digests was determined according to Lowry (1951).

Covalent binding to DNA

Calf thymus DNA (1 mg) was added to a standard microsomal incubation mixture (2 ml) containing 1 mg of microsomal protein and 50 μM PCB and 100 μM 1,2,4-TRICB. After 10 minutes, the reaction was stopped by the addition of 5 ml of water-saturated phenol and the water phase was extracted with equal volumes of phenol, phenol: chloroform :isoamylalcohol (25:24:1), chloroform:isoamylalcohol (24:1) and water-saturated diethylether. After complete removal of the diethylether under nitrogen, the aqueous phase was incubated with Proteinase K (0.25 mg) for 15 hours at 37°C followed by the extraction procedure as described above, to remove residual protein and amino acids. The DNA was precipitated overnight by the addition of sodium chloride to the water phase (final concentration 0.3 M) and 2 volumes of ice-cold ethanol. After centrifugation the DNA was resuspended in 1 ml of 20 mM Tris-acetate pH 7.4. The purity of the DNA recovered was determined by measuring the ratio of the absorbance at 260 nm and at 280 nm (Markov and Ivanov,1974), and was in all experiments similar to that of pure DNA (1.92 ± 0.05). The recovery was determined by measuring the absorbance at 260 nm and varied from 30% to 51%. Aliquots (0.2 ml) were taken for determination of radioactivity.

Determination of lipid peroxidation

Peroxidation of microsomal lipids, due to possible redox cycling of quinones formed during the metabolism of chlorinated benzenes, was determined by the measurement of thiobarbituric acid reactive substances (TBRS). For these determinations, incubations were carried out with an NADPH generating system (8 mM glucose 6-phosphate, 0.1 unit/ml glucose 6-phosphate dehydrogenase, 0.3 mM NADP) for 30 minutes in the

presence of 50 μM PCB or 100 μM 1,2,4-TRICB. Control incubations lacked substrates. The formation of TBRS was measured spectrophotometrically at 532 nm as previously described (Buege and Aust, 1978).

Results

Figure 3.1 shows a typical HPLC reversed phase elution profile of the ethylacetate extracts of microsomal incubations with 1,2,4-TRICB. Two major peaks eluted before the substrate, which were identified as 2,3,6-TRICP (peak 6) and 2,4,5-TRICP (peak 9), respectively, as well as minor peaks that were identified as TRICHQ (peak 2), 2,4,6-TRICP (peak 8) and 2,3,5-TRICP (peak 10). Dichlorophenols or -benzenes were not detected. About 9 % of the radioactivity that eluted in the first 10 ml as a rather broad peak was not further identified.

In addition to extractable metabolites, products were formed that covalently interacted with microsomal protein. Table 3.1 shows that, with increasing protein concentration, the relative role of secondary metabolism, i.e., formation of hydroquinones, increased at the cost of primary metabolism, i.e., formation of phenols. Covalent binding to protein also showed an increase when the protein concentration was increased, indicating the involvement of secondary metabolism in the formation of reactive metabolites.

Both conversion and covalent binding of 1,2,4-TRICB were mediated by cytochrome P450, as is shown by the dependence on the addition of NADPH and by the inhibitory action of metyrapone (Table 3.2). The covalent binding observed in incubations without NADPH amounted to 9 % of the total binding and was independent of either amount of protein present or incubation time. Glutathione reduced the covalent binding almost completely through the formation of water soluble conjugates, as 35 % of all metabolites remained in the aqueous phase (data not shown). 1,1,1-Trichloropropene oxide (TCPO), an inhibitor of microsomal epoxide hydrolase (Oesch *et al.*, 1971), had no effect on the covalent binding to protein. Addition of 1 mM ascorbic acid, a reducing agent, resulted in a considerable reduction of the covalently bound protein metabolites with a concomitant increase in hydroquinone formation (8 % of the total conversion). When 10 mM ascorbic acid was included in the microsomal incubation of 1,2,4-TRICB, covalent binding was at the level of the minus NADPH-control. However, considerable inhibition of total conversion, i.e., the sum of ethylacetate soluble metabolites and covalently bound radioactivity, was observed with increased concentrations of ascorbic acid. This could be a result of feedback inhibition due to the accumulation of hydroquinones and/or phenol metabolites.

When the conversion of 1,2,4-TRICB was monitored with time, it was observed that the net rate of phenol production was highest in the first 30 seconds (6.7 nmol/mg/min) and gradually decreased thereafter (2.8 nmol/mg/min at 5 minutes, Figure 3.2A). In contrast, the formation of both hydroquinones and covalently bound metabolites increased

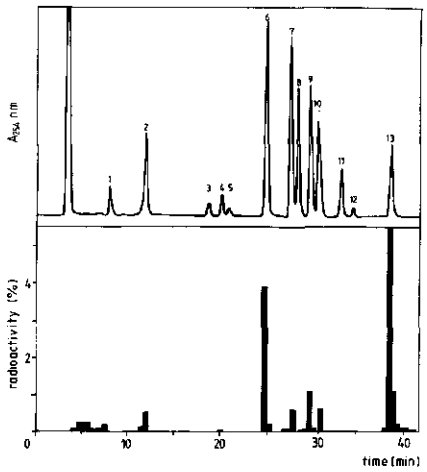

Figure 3.1 HPLC analysis of ethylacetate extracts from incubations performed with 1,2,4-TRICB and DEX-induced microsomes. Analysis was performed using a Nucleosil 5C18 (250 x 4.6 mm I.D.) column. All radioactivity was eluted employing a mobile phase containing 50% methanol and 50% KH_2PO_4 (20 mM in water) for 2 minutes, followed by a linear increase to 85 % methanol in 30 minutes, and isocratic elution at 85 % methanol for 10 minutes. (A) UV absorption spectrum (254 nm) of the marker metabolites coinjected with the sample. (B) Amount of radioactivity eluted from the column, expressed as % of total radioactivity eluted from the column. Marker metabolites: 1, DCHQ; 2, TRICHQ; 3, 2,3-DCP; 4, 2,5-DCP; 5, 2,4-DCP; 6, 2,3,6-TRICP; 7, 2,3,4-TRICP; 8, 2,4,6-TRICP; 9, 2,4,5-TRICP; 10, 2,3,5-TRICP, 1,2-DCB; 11, 1,4-DCB; 12, 1,3-DCB; 13, 1,2,4-TRICB.

sharply after a lagphase of 30 seconds (insert C), again demonstrating a correlation between secondary metabolism and covalent binding.

A similar curve of product formation was observed when PCB was used as a substrate. PCB was metabolised to pentachlorophenol (PCP), tetrachlorophenols (TCP) and tetrachlorohydroquinone (TCHQ) as previously reported (Den Besten *et al.*, 1989). Figure 3.2B demonstrates that the net formation of pentachlorophenol and tetrachlorophenols gradually decreased with time (6.3 nmol/mg/min at 30 seconds to 3.0 nmol/mg/min at 10 minutes), whereas both the formation of TCHQ and covalent binding increased with time after an initial lagphase of 1.5 min (insert D).

Quinones might be subjected to redox cycling with the concomitant generation of reactive oxygen species. Because reactive oxygen species might induce peroxidative

Table 3.1 Metabolism of 1,2,4-[^{14}C]-trichlorobenzene by liver microsomes from rats pre-treated with dexamethasone.

Incubation protein (mg/ml)	Conversion[a] (nmol/5min)	percentage of total conversion			
		TRICP[b]	TRICHQ	Covalently bound metabolites	not identified
0.5	15.0	77.1	4.5	9.4	9.0
1.0	23.0	62.8	10.0	17.0	10.2

Note. Incubations were performed with 100 μM 1,2,4-TRICB for 5 minutes at 37°C in the presence of 0.1 M potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$, 1 mM NADPH and microsomes from DEX-treated rats (final volume of 2 ml). Data are corrected for control incubations without NADPH. Variation was less than 10%. [a] Conversion is calculated as the sum of the formation of extractable metabolites, as analysed by HPLC, and the covalent binding to protein. [b] 'TRICP' denotes sum of all trichlorophenol isomers.

Table 3.2 Microsomal conversion and covalent binding of 1,2,4-trichlorobenzene by liver microsomes from dexamethasone-treated rats

Incubation	Conversion (%)	Covalent binding (%)
Complete mixture	100[a]	100[b]
- NAPDH	nil	9 ± 3
+ metyrapone (0.5 mM)	19 ± 4	15 ± 6
+ TCPO (0.5 mM)	90 ± 1	100 ± 7
+ glutathione (1.0 mM)	130 ± 7	17 ± 1
+ ascorbic acid		
1 mM	90 ± 5	26 ± 4
5 mM	74 ± 1	19 ± 1
10 mM	65 ± 5	10 ± 1

Note. A control incubation contained 100 μM 1,2,4-TRICB, 1 mM NADPH, 0.1M potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$, and 1 mg microsomal protein (final volume of 2 ml). Incubations were performed at 37°C for 5 min. Data are based on 2-6 observations and are expressed as a percentage of the conversion and covalent binding of control incubations, in which the following rates were observed (mean±S.D., n=10): [a] 14.7 ± 1.4 nmol/mg/5 min, [b] 1.77 ± 0.20 nmol/mg/5 min. TCPO, 1,1,1-trichloropropene oxide

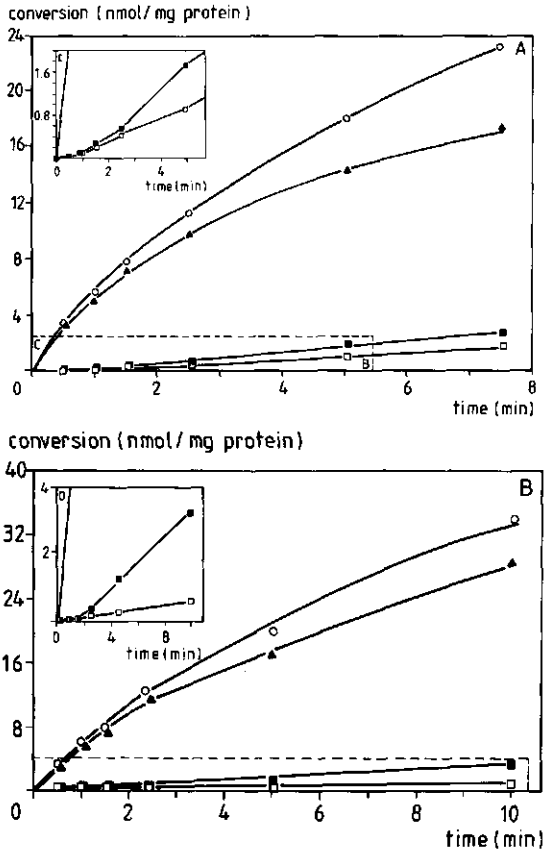

Figure 3.2 Conversion of 1,2,4-trichlorobenzene (A) and pentachlorobenzene (B) by rat liver microsomes. Standard incubations were performed with 100 μM 1,2,4-TRICB and 1 mg microsomal protein, or 50 μM PCB and 0.25 mg microsomal protein, in a final volume of 2 ml, as described under Materials and Methods. All data are corrected for the binding in the absence of NADPH. (○) Total conversion; (▲) phenols; (□) hydroquinones; (■) covalent binding. Lines without a symbol in inserts C and D represent the formation of phenols.

damage to microsomal lipids, the extent of lipid peroxidation in microsomal incubations was determined. It was observed that the formation of TBRS was not stimulated in microsomal incubations containing 1,2,4-TRICB or PCB (89% and 87% of values in control incubations, respectively; data not shown). The results suggest that the hydroquinones and benzoquinones formed during the metabolism of chlorinated benzenes do not undergo extensive redox cycling. In a previous study, tetrachlorobenzoquinone was shown not to undergo redox cycling when added exogenously to a microsomal suspension (Van Ommen *et al.*, 1988).

The role of different isoenzymes of cytochrome P450 was investigated by performing incubations with microsomes isolated from male control rats and rats which were pretreated with inducers of the cytochrome P450 mono-oxygenases other than

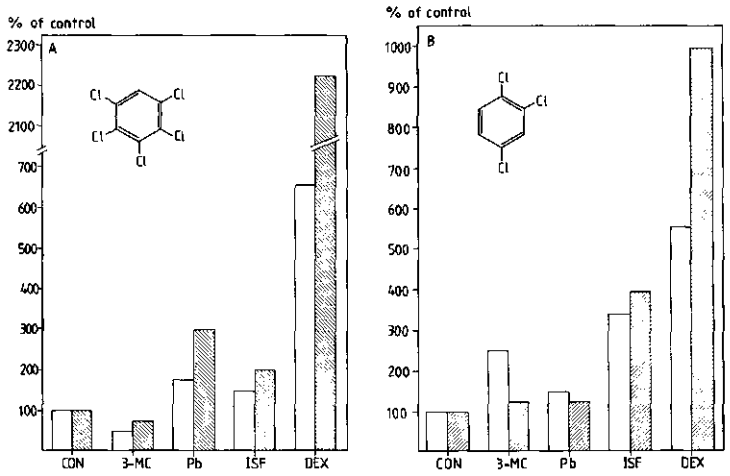

Figure 3.3 Conversion to extractable metabolites (□) and protein bound metabolites (▨) of pentachlorobenzene (A) and 1,2,4-trichlorobenzene (B) by rat liver microsomes from control rats and rats treated with different inducers of the cytochrome P450 monooxygenases. Conversion is expressed as percentage increase over the values observed with control microsomes, which amounted to 1.98 nmol of extractable metabolites/ nmol P450/ 10 min, and 40 pmol of protein bound metabolites/ nmol P450/ 10 min for PCB, and to 2.26 nmol of extractable metabolites/ nmol P450/ 5 min and 120 pmol of protein bound metabolites/ nmol P450/ 5 min, for 1,2,4-TRICB, respectively. Standard incubations were performed with 0.5 μM cytochrome P450, and 50 μM PCB or 100 μM 1,2,4-TRICB in a final volume of 2 ml, as described under Materials and Methods.

dexamethasone. Figures 3.3A and B clearly demonstrate that dexamethasone is the most powerful inducer of the microsomal metabolism of chlorinated benzenes (680% and 570% of the activity in control microsomes for PCB and 1,2,4-TRICB, respectively). In addition, the amount of covalent binding expressed as percentage of total metabolism was significantly higher in DEX microsomes compared to control microsomes or microsomes from rats treated with other inducers of cytochrome P450. Figure 3.3 also indicates that different microsomal suspensions varied considerably in their affinity and/or catalytic activity towards the two chlorinated benzenes tested.

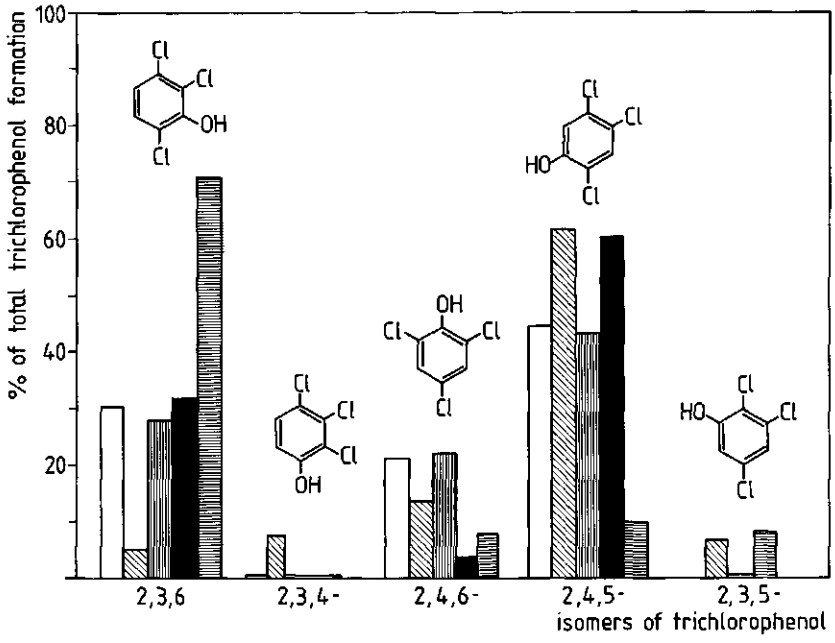

Figure 3.4 Formation of isomers of trichlorophenol in the metabolism of 1,2,4-trichlorobenzene by microsomes from control rats and rats pretreated with various inducers of the cytochrome P450 monooxygenases. (□) Control; (▨) 3-MC; (▥) ISF; (■) PB; (▤) DEX.

Microsomes from 3-methylcholanthrene-induced rats showed little activity towards PCB (53% of the activity in control microsomes), whereas they showed considerable activity towards 1,2,4-TRICB (240% of the activity in control microsomes).

Figure 3.4 displays the metabolic profile of 1,2,4-TRICB by microsomes from rats treated with different inducers. It is noteworthy that DEX-microsomes demonstrated a preference for oxidative attack at the more crowded 2,3-position, resulting in the formation of 2,3,6-TRICP as the major isomer, whereas other microsomes oxidized the substrate preferentially at the unsubstituted side of the ring, resulting in 2,4,5-TRICP as the major trichlorophenol formed. 2,4,6-TRICP was a minor metabolite in all microsomal suspensions, and 2,3,4-TRICP and 2,3,5-TRICP were only formed in trace amounts.

As shown in Table 3.3, chlorinated benzenes have the potency to covalently interact *in vitro* with exogenously added DNA, although to a much lesser extent than the interaction with proteins (2-5% of protein binding). This DNA-binding was dependent on the metabolism of the chlorinated benzenes, since binding in the absence of NADPH was negligible. The involvement of quinones in the DNA-binding is illustrated by the reduction in binding in the presence of 10 mM ascorbic acid. The addition of DNA to the incubation mixture had no effect on the formation of soluble metabolites.

Table 3.3 Covalent binding of chlorinated benzenes to DNA *in vitro*.

Incubation	Substrate	
	1,2,4-TRICB[a]	PCB[b]
	(pmol bound/ mg DNA/ 10 min)	
- NADH	3 ± 2[c]	4 ± 0
+ NADPH (1 mM)	131 ± 26	50 ± 14
+ NADPH (1 mM) + ascorbic acid (10 mM)	23 ± 5	6 ± 1

Note. Incubations were performed for 10 min in a shaking water bath at 37°C, with 1 mg of microsomal protein, 3 mM $MgCl_2$, 0.1 M potassium phosphate buffer pH 7.4, 1 mg calf thymus DNA, and 50 μM PCB or 100 μM 1,2,4-TRICB, in a final volume of 2 ml. [a] Total conversion of 1,2,4-TrCB amounted to 28.1 nmol/mg protein/10 min. [b] Total conversion of PCB amounted to 16 nmol/mg protein/10 min. [c] mean ± SD of 2-4 observations.

Discussion

Liver necrosis observed after exposure to bromobenzene is believed to be due to the formation of its 3,4-epoxide derivative which covalently interacts with cellular macromolecules and causes cell death (Lau *et al.*, 1980). However, the exact nature of the binding species to liver proteins has not yet been identified. In more recent studies the importance of secondary metabolites in the toxicity of xenobiotics has been recognized. Covalent binding of benzoquinones to proteins was observed during the microsomal conversion of phenol metabolites of bromobenzene (Lau *et al.*, 1984a,b). In

fact, during metabolism of bromobenzene, a major part of the observed covalent binding could be ascribed to these quinone metabolites (Buben *et al.*, 1988). Furthermore, during the metabolism of the fully substituted hexachlorobenzene, tetrachlorobenzoquinone (TCBQ) is the sole reactive metabolite formed that covalently interacts with protein and DNA (Van Ommen *et al.*, 1986). A recent study into the microsomal conversion of PCB also indicated an important role of TCBQ in the metabolic activation of this compound (Den Besten *et al.*, 1989).

Similarly, the present study demonstrates that in the metabolism of a lower chlorinated benzene, 1,2,4-TRICB, quinone metabolites are also the major reactive species formed. The involvement of secondary metabolism in the metabolic activation is demonstrated by (i) the correlation in the rate of secondary metabolism, i.e., formation of hydroquinones, and the rate of covalent binding, both demonstrating an initial lagphase in contrast to the initial burst of primary oxidation, and (ii) by the complete inhibition of the covalent interaction with protein in the presence of the reducing agent ascorbic acid. In contrast, the formation of reactive metabolites from PCB could never completely be inhibited (Den Besten *et al.*, 1989), which may indicate that to a small extent, reactive nonquinone species, presumably epoxides, interact with protein. However, the lack of increase in covalent binding in the presence of TCPO (Den Besten *et al.*, 1989), an inhibitor of epoxide hydrolase, and the near absence of covalent binding in the first minute of metabolism when primary metabolism is high (Figure 3.2B), are contradictory to the involvement of epoxides in the covalent binding to protein. Since the ratio of covalent binding, i.e., formation of quinones, to TCHQ is high (> 4 after 10 minutes), it might be possible that the addition of 10 mM ascorbic acid is insufficient to completely prevent the reaction of TCBQ with microsomal protein.

Binding of quinone metabolites to microsomal protein was of a rather non-selective nature and was not limited to the microsomal membrane, since soluble proteins were also arylated (data not shown).

The mutagenicity and carcinogenicity of chlorinated benzenes other than hexachlorobenzene is still a matter of debate. The present study demonstrates the low DNA alkylating properties of the quinone metabolites of both PCB and 1,2,4-TRICB, and, hence, the low genotoxic potential of these compounds. Under conditions when protein binding of 1,2,4-TRICB was completely absent, and thus the formation of quinones completely inhibited, still about 17 % of the DNA binding was observed, which illustrates the relatively high affinity of epoxide metabolites to alkylate DNA.

Cytochrome P450IIIA1, which is induced by dexamethasone over 100-fold (Wrighton *et al.*, 1985), shows a unique high affinity and/or catalytic activity towards chlorinated benzenes. This has also been demonstrated for the fully substituted hexachlorobenzene, the metabolism of which was induced more than 100 times in DEX-microsomes as compared to control microsomes (Van Ommen *et al.*, 1989). However, this substrate specificity appears to become less unique with decreasing degree of chlorination, since microsomes from ISF- and 3-MC treated rats converted 1,2,4-TRICB but not PCB, or

HCB (Van Ommen *et al.*, 1989) to a considerable extent. The ratio of the conversion of PCB and 1,2,4-TRICB by DEX-microsomes to the conversion by PB-microsomes was remarkably constant (3.8 and 3.9, respectively). This might indicate that isoenzyme P450IIIA1, which is increased 20-fold in PB-microsomes (Wrighton *et al.*, 1985), is responsible for the catalytic activity towards chlorinated benzenes observed in PB-microsomes. Future experiments with purified isoenzymes of cytochrome P450 are needed to establish the contribution of the different isoenzymes in the metabolism of a range of chlorinated benzenes.

The present study demonstrates the variation in the metabolic profile of 1,2,4-TRICB when different isoenzymes of cytochrome P450 are induced. This is illustrated by the observed variation in trichlorophenol isomers, e.g., the preferential formation of 2,3,6-TRICP by DEX-microsomes, but not by 3-MC microsomes, whereas 2,3,4-TRICP was only detected in incubations with 3-MC microsomes. In addition, secondary metabolism to quinones, measured as covalent binding to protein, also varied depending on the microsomal source used: in DEX-microsomes 8.2% of all metabolites covalently interacted with microsomal protein, whereas in 3-MC microsomes and PB-microsomes the percentage of covalently bound metabolites (2.9% and 3.9%, respectively) was lower than in control microsomes (4.9%). A similar selective stimulation of covalent binding of quinone metabolites from PCB was observed.

In the primary oxidative step of 1,2,4-TRICB the formation of 2,4,6-TRICP and 2,3,4-TRICP can be explained via a NIH-like chlorine shift of the 2,3,5-trichloro-1,2-benzene oxide and the 1,2,4-trichloro-3,4-benzene oxide, respectively. Arene oxides have been shown to be intermediates in the cytochrome P450 mediated oxidation of many aromatic compounds, but the mechanism of their formation remains controversial.

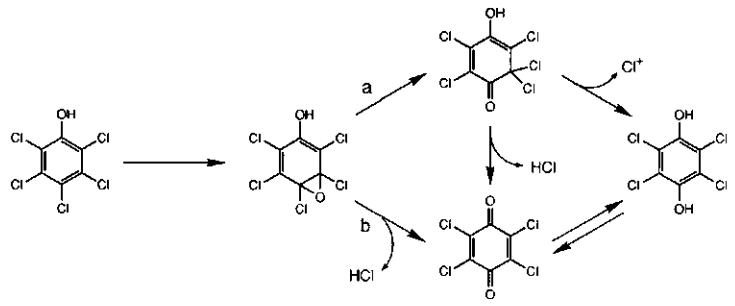

Figure 3.5 Possible pathways in the formation of tetrachlorobenzoquinone (TCBQ) via pentachlorophenol epoxide. TCBQ may be formed by (formal) loss of a positively charged chlorine and subsequent oxidation of the resulting hydroquinone (route a), or directly via loss of a negatively charged chlorine and a proton (route b).

In fact, evidence is accumulating that the cytochrome P450-mediated oxidation of unsaturated compounds does not proceed via a direct epoxidation of the aromatic ring (Burka *et al.*, 1983). A recent study on the mechanism of oxidation of aromatics using isotopically labeled (mono-) chlorobenzenes as probes suggested the formation of a tetrahedral intermediate that can rearrange to the phenol directly, or via epoxide or ketone intermediates (Korzekwa *et al.*, 1989).

The mechanism of formation of the quinones is also a matter of debate (Van Ommen and Van Bladeren, 1989). They might be formed by subsequent oxidation of hydroquinones, which would be formed via epoxidation of the primary phenol metabolites and subsequent (formal) loss of a positively charged chlorine (Figure 3.5, route a). However, electrophilic chlorine could not be trapped with 2,4- or 2,6-dimethylphenol during microsomal oxidation of pentachlorophenol or hexachlorobenzene, (Van Ommen, unpublished results), although this method has been successfully applied in studies on the dechlorination of carbon tetrachloride (Mico and Pohl, 1982). Van Ommen and co-workers demonstrated that TCHQ was oxidized to TCBQ via a cytochrome P450-dependent mechanism, most likely via the generation of reactive oxygen species (Van Ommen *et al.*, 1988). However, quinones might also be formed directly upon oxidation of the primary phenol metabolites, via loss of a negatively charged chlorine-atom and a proton from the phenol-epoxide intermediate (route b). This latter pathway does not necessarily need an epoxide as a distinct intermediate (Guengerich and MacDonald, 1990). Oxidation of the primary phenol metabolites via an initial hydrogen abstraction would yield a phenoxy radical, which could rearrange to the radical intermediates. This mechanism, which has been proposed for the oxidation of paracetamol (Koymans *et al.*, 1989), will result in the direct formation of quinones. The observation that covalent protein binding is detected in a fourfold excess compared to hydroquinone formation is supportive for such a direct formation of quinone metabolites. Additional studies need to be conducted in order to gain insight into the pathway that leads to the formation of these secondary quinone metabolites.

In summary, the present investigation demonstrates that intermediates (presumably epoxides) generated during primary metabolism of 1,2,4-TRICB do not possess the capacity to alkylate proteins *in vitro*. Instead, as previously demonstrated with higher substituted chlorinated benzenes, quinone metabolites are the species responsible for covalent binding to protein. However, the extent of quinone formation *in vivo* might differ from the one obtained under the present *in vitro* conditions. First, the relative contribution of primary and secondary metabolism to the overall disposition of these compounds will depend on the concentration of the parent compound. For example, the cytochrome P450-mediated secondary oxidation of phenol metabolites could be diminished due to (competitive) inhibition at higher concentration of the parent compound. Second, the presence of conjugative (glucuronidation and sulfation) and reductive pathways *in vivo* makes predictions difficult about the relative importance of these two potentially reactive metabolites in the toxicity of polyhalogenated benzenes.

Therefore, additional studies are necessary to assess the toxic potential and the metabolic profile of these compounds. However, in view of the considerably higher alkylating potency of quinone metabolites, the importance of arene oxides in the toxicity of halogenated aromatics might have been overestimated so far.

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Chapter 4

THE INVOLVEMENT OF PRIMARY AND SECONDARY METABOLISM IN THE COVALENT BINDING OF 1,2- AND 1,4-DICHLOROBENZENES

Abstract

The microsomal oxidation of 1,2-[^{14}C]- and 1,4-[^{14}C]-dichlorobenzene (DCB) was investigated with special attention for possible differences in biotransformation that might contribute to the isomer-specific hepatotoxicity. Major metabolites of both isomers were dichlorophenols (2,5-DCP for 1,4-DCB, and 2,3- and 3,4-DCP for 1,2-DCB, respectively), and dichlorohydroquinones. The formation of polar dihydrodiols appeared to be a major route for 1,2-DCB but not for 1,4-DCB. Both the hepatotoxic 1,2-DCB and the non-hepatotoxic 1,4-DCB were oxidized to metabolites that covalently interacted with protein, and only to a small extent with DNA. Protein binding could be inhibited by the addition of the reducing agent ascorbic acid with a concomitant increase in the formation of hydroquinones and catechols, indicating the involvement of reactive benzoquinone metabolites in protein binding. However, in the presence of ascorbic acid, a substantial amount of protein-bound metabolites of 1,2-DCB was still observed, in contrast to 1,4-DCB where binding was nearly completely inhibited.

This latter effect was ascribed to the direct formation of reactive benzoquinone metabolites in a single P450-mediated oxidation of *para*-substituted dichlorophenols (such as 3,4-DCP) in the case of 1,2-DCB. In contrast, the major phenol isomer derived from 1,4-DCB, i.e., 2,5-DCP, is oxidized to its hydroquinone derivative, which needs prior oxidation in order to generate the reactive benzoquinone species. Residual protein binding in the presence of ascorbic acid could also indicate the involvement of reactive arene oxides in the protein binding of 1,2-DCB, but not of 1,4-DCB. However, MO computer calculations did not provide indications for differences in chemical reactivity and/or stability of the various arene oxide/oxepin tautomers that can be formed from either 1,2-DCB or 1,4-DCB. In conclusion, reactive intermediates in the secondary metabolism of 1,2-DCB lead to more covalent binding than those derived from 1,4-DCB, which correlates very well with their reported hepatotoxic potency.

C. den Besten, M. Ellenbroek, M.A.E. van der Ree, I.M.C.M. Rietjens, P.J. van Bladeren, *Chem.-Biol. Interact.*, in press.

Introduction

The hepatotoxicity of halogenated benzenes is thought to be mediated by the generation of (a) reactive metabolite(s) as a result of their oxidation by cytochrome P450. Depletion of glutathione and the covalent binding of reactive metabolites to hepatic proteins are common features of halogenobenzene toxicity (Brodie *et al.*, 1971; Reid and Krishna, 1973). To derive a general mechanism of action for the bioactivation of these aromatics, bromobenzene has frequently served as a model compound (Lau and Monks, 1988). Based on the profile of urinary metabolites of bromobenzene and on the correlation between changes in this pattern and in bromobenzene hepatotoxicity effected by pretreatment with inducing agents, toxicity and covalent binding to cellular macromolecules was ascribed mainly to its 3,4-oxide metabolite (Jollow *et al.*, 1974). However, more recent studies stress the importance of secondary quinone metabolites in the covalent binding to cellular protein, both *in vitro* (Hesse *et al.*, 1980; Buben *et al.*, 1988) and *in vivo* (Narasimhan *et al.*, 1988; Slaughter and Hanzlik, 1991).

Few studies have been conducted on halobenzenes other than bromobenzene. The type of halogen substituent appeared to influence the extent of hepatotoxicity observed. For example, fluorobenzene was much less toxic than either chloro- or bromobenzene (Brodie *et al.*, 1971). In the same study, the isomer-specific differential effect on the acute hepatotoxicity of dichlorobenzenes is reported: 1,2-dichlorobenzene appeared to be highly hepatotoxic after a single dose of 1.45 mmol/kg, whereas 1,4-dichlorobenzene produced little or no effect at a dose as high as 3.8 mmol/kg. This striking difference in hepatotoxicity between the two isomers has recently been confirmed (Den Besten *et al.*, 1991b; Stine *et al.*, 1991). Furthermore, toxicity of 1,2-DCB was correlated with a higher rate of conversion and a higher extent of covalent binding to cellular protein compared to observations after exposure to 1,4-DCB (Reid and Krishna, 1973).

The metabolic profile of dichlorobenzenes in the rabbit was reported by Williams and coworkers (Azouz *et al.*, 1955), as part of an extensive series of biotransformation studies on halogenobenzenes. Both 1,2- and 1,4-DCB were excreted mainly as conjugated dichlorophenols (DCP). Minor metabolites were 3,4- and 4,5-dichlorocatechol (DCC) in the case of 1,2-DCB, and dichlorohydroquinone (DCHQ) in the case of 1,4-DCB. Formation of a mercapturic acid metabolite was reported for 1,2-DCB only. It was suggested that both the lack of hepatotoxic potency and the absence of mercapturic acid derivatives originated from the fact that *p*-disubstituted benzenes cannot form sufficiently stable epoxides. However, in later studies a dichlorophenyl mercapturic acid was identified as a minor urinary metabolite in the urine of rats treated with 1,4-DCB (Hawkins *et al.*, 1980). Furthermore, trace amounts of methyl thiolated metabolites of 1,4-DCB, very probably derived from the corresponding mercapturic acids, were identified in blood, urine and faeces (Kimura *et al.*, 1979).

Thus, the above data point at pronounced differences in, and a possible relationship between, both toxicity and biotransformation pathways of the two isomeric DCB's. In theory, differences in their biotransformation, and perhaps toxicity, may be related to differences in (i) the type of intermediate arene oxide formed upon the primary oxidation step by cytochrome P450; (ii) the reactivity and fate of these intermediates; and/or (iii) the formation of secondary quinone metabolites.

The objective of the present study was to investigate these characteristics of 1,2-DCB and 1,4-DCB metabolism in more detail. This was done by *in vitro* studies on the microsomal metabolism of both isomers, and by AM1 semi-empirical computer calculations on their primary (reactive) monooxygenated intermediates. Results obtained provided a new hypothesis for differences observed in metabolic profiles of 1,2- and 1,4-DCB.

Materials and Methods

Chemicals

1,4-[^{14}C]-Dichlorobenzene (1,4-DCB, radiochemical purity 98.8 %) and 1,2-[^{14}C]-dichlorobenzene (1,2-DCB, radiochemical purity 98.8 %) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Impurities of 1,2-DCB coeluted with 2,6-DCP and 2,3-DCP (0.01 % each), and 3,4-DCP (0.04 %). Impurities of 1,4-DCB coeluted with DCHQ (0.02 %), 4-chlorophenol (0.04 %), and 2,5-DCP (0.02 %). Both substrates contained radioactivity that coeluted with monochlorobenzene (0.14 % and 0.16 % for 1,2- and 1,4-DCB, respectively). For 1,2-DCB and 1,4-DCB, respectively, 1.04 % and 1.0 % eluted as an apolar fraction (i.e., after the parent peak on reversed phase HPLC). Thus, with both substrates, the major portion of the impurity was present in regions that did not coincide with metabolite formation. Chemicals used as marker metabolites were obtained from commercially available sources. NADPH was from Boehringer Mannheim GmbH, Germany. Calf thymus DNA was from Sigma Chemical Co. All other chemicals were of reagent grade.

Preparation of microsomes

Microsomes were prepared using standard procedures as previously described (Den Besten *et al.*, 1991a). For preparation of microsomes, livers were obtained from male Wistar rats (300 g), which were untreated (control, CON) or pretreated with phenobarbital (PB), 3-methylcholanthrene (3-MC), isosafrole (ISF) or dexamethasone (DEX) according to regimens as previously described (Den Besten *et al.*, 1991a). Microsomes were stored at -80°C until use. Cytochrome P450 was determined according to Omura and Sato (1964) and the protein concentration was determined by the Lowry assay (Lowry *et al.*, 1951).

Microsomal incubations

Incubations containing 0.1 M potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$, and 1 mg microsomal protein from DEX-pretreated rats (unless stated otherwise) in a final volume of 2.0 ml were performed using glass tubes with a teflon lined screw top in a shaking waterbath at 37°C as previously described (Den Besten *et al.*, 1991a). In short, after 2 minutes of preincubation of buffer, cofactor and protein, radiolabeled substrates (1.2 μCi) in acetone (2.5 % final concentration) were added at their maximal soluble concentration (0.8 mM). The reaction was started by the addition of NADPH (1 mM final concentration) and stopped by the addition of HCl (0.6 N final concentration). Incubation time was kept short in first instance (2.5 min), because of the high volatility of the chemicals. After 2.5 min at 37°C, 90 % of the added radioactivity was still present in the microsomal mixture, whereas the amount of radioactivity in the aqueous phase decreased to approximately 50 % after 15 min of incubation. However, incubations were also performed for 15 min to obtain higher conversions. In order to nearly completely recover all radioactivity added to the incubation (recovery >90 %), the reaction mixtures were frozen in liquid nitrogen before opening the tubes to perform the acid precipitation of the protein. The tubes were kept cool (4°C) thereafter. Incubations were also performed in the presence of ascorbic acid (for concentrations, see Table 4.2).

Extraction of metabolites

Extraction was performed with small volumes (0.5 ml) of ethyl acetate, which were collected separately. Four consecutive extractions resulted in the removal of 85 to 95 % of the total amount of radioactivity added to the incubation, whereas the sum of the radioactivity present in the aqueous phase and bound to protein amounted to less than 1%. Thus, due to the volatility of the substrate, recovery was less than 100 %. Direct HPLC analysis was performed on 50 μl of the first ethyl acetate extract, which contained about 87 % of all radioactivity extracted.

HPLC analysis

Reversed Phase High Performance Liquid Chromatography was performed using a Perking-Elmer Series 4 chromatograph, equipped with a 150x4.6 mm i.d. Nucleosil 5C18 column and a Co:Pell ODS precolumn with a flow rate of 1 ml/min and a mobile phase consisting of methanol (kept at 30 % throughout the assay), acetonitrile and water, using percentages as defined below. All phases contained 0.5 % acetic acid. Radioactivity was eluted using a gradient program, which started isocratically for 5 min with 10 % acetonitrile and 60 % water followed by a linear increase of 1.67 % acetonitrile per min for 12 min, followed by an increase of 4 % acetonitrile per min for 10 min, and concluded by 5 min at 70 % acetonitrile. Microsomal metabolites were identified by co-elution with marker metabolites, which were injected together with the sample. Fractions of 0.5 ml were collected followed by scintillation counting (5 ml of Ultima Gold, Packard, Brussels, Belgium) in a Tri-Carb liquid scintillation counter

(Packard). Using the above described HPLC analysis, 2,5-DCP and 2,4-DCP, potential metabolites of 1,4-DCB, were not sufficiently separated to identify them as separate metabolites. Similarly, complete separation of 4,5-DCC and 2,6-DCP, potential metabolites of 1,2-DCB, was not reached by the above described method. Therefore, alkaline runs were conducted using a 250x4.6 mm i.d. Nucleosil 5C18 column, a mobile phase consisting of 20 mM Tris-phosphate buffer (pH 8.0) and a gradient as described previously (Den Besten *et al.*, 1991a). The k' values observed in alkaline runs were as follows: 2,5-DCP, 9.3; 2,4-DCP, 11.5; 4,5-DCC, 5.8; 2,6-DCP, 4.7. Alkaline runs performed on extracts from incubations with 1,2-DCB failed to detect the formation of 2,6-DCP. When alkaline runs were performed on ethyl acetate extracts of 1,4-DCB incubations, the radioactivity that coeluted with the DCP markers in the routine HPLC analysis, was now split in 3 fractions: one small fraction with k' 11.5 (= k' 2,4-DCP), and 2 larger fractions of about equal amounts of radioactivity, with k' 9.3 (= k' 2,5-DCP) and k' 12.6, which could not be ascribed to any of the potential metabolites. In fact, since none of the chlorinated phenols, -catechols or -benzenes showed k' in the base and acid HPLC analysis comparable to the unknown fraction, this fraction (which amounted to 50 % of the radioactivity detected as DCP and which did not change in the presence of ascorbic acid) was not further identified. All data on formation of DCP (and total conversion) presented in this paper are corrected for this fraction.

Covalent binding to protein and DNA

Covalent binding to microsomal protein was measured after extensive washing of the protein pellet with 5 ml of the following organic solvents in series: 3x methanol, 3x ethanol, 2x ethyl acetate, 2x diethyl ether. Back ground radioactivity was detected in aliquots of the final ether wash. Data are corrected for binding observed in incubations without NADPH, which amounted to 1.3 nmol and 1.8 nmol for 1,2-DCB and 1,4-DCB respectively. This amount was independent of the amount of protein present, and slightly increased with longer incubation times. Covalent binding to exogenously-added calf thymus DNA (1 mg/ 2 ml of incubation) was measured in incubations using standard conditions as described above and extra added DNA. Incubation time was 15 min. DNA was purified as previously described (Den Besten *et al.*, 1991a). Corrections were made for binding observed in incubations without NADPH, which amounted to 0.1 nmol/mg DNA/ 15 min for both 1,2-DCB and 1,4-DCB.

Molecular orbital computer calculations

Computer calculations were carried out on a Silicon graphics Iris 4D/85 using Quanta/Charmm (Polygen Inc., UK). The semi-empirical molecular orbital method was used, applying the AM1 Hamiltonian from the AMPAC program (Quantum Chemistry Program Exchange No. 506; Indiana University, USA). All calculations were carried out with PRECISE criteria. For all calculations the self consistent field was achieved. Geometries were optimized for all bond lenghts, bond angles and torsion angles using the

Fletcher-Powell criteria. Frontier electron densities were calculated from HOMO and HOMO-1 characteristics or LUMO and LUMO+1 characteristics as described (Fukui *et al.*, 1954). Results from these computer calculations represent data from compounds *in vacuo*, a situation different from the one in which the compounds are present in microsomal incubations, or bound to the active site of an enzyme. Solvation and a different dielectric constant might influence parameters calculated for the compounds in the *in vacuo* situation. However, in the present study, related compounds are compared and it can be assumed that deviations from the *in vacuo* situation due to solvation or binding to the active site of a protein will be similar for all compounds studied, and thus will not influence relative differences between these compounds. Results from computer calculations for molecules *in vacuo* can thus be used as an approach to study relative differences between related compounds also when dissolved in buffer or when bound to the active site of an enzyme.

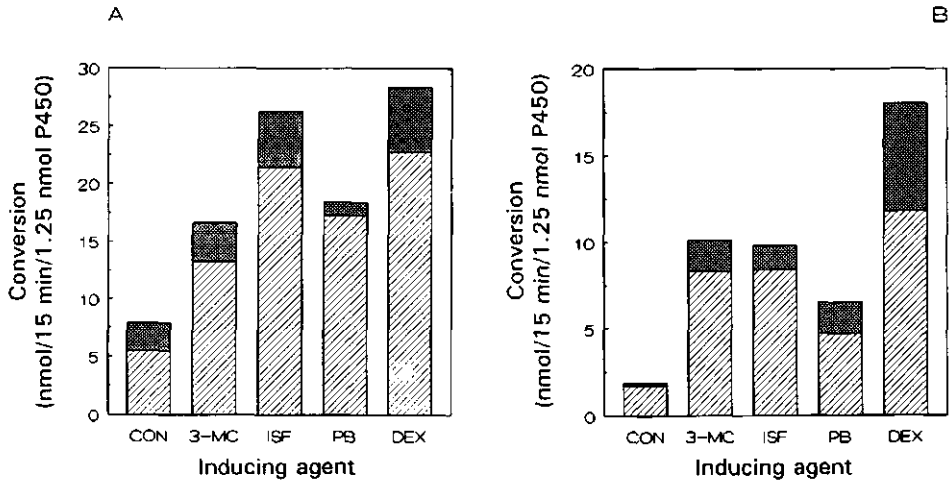

Figure 4.1 Microsomal oxidation of 1,2-DCB (A) and 1,4-DCB (B) to ethyl acetate extractable metabolites (▨) and protein-bound species (▩). Liver microsomes were isolated from control rats or rats pretreated with 3-methylcholanthrene (3-MC), isosafrole (ISF), phenobarbital (PB) or dexamethasone (DEX). Incubations were performed with 1.25 nmol P450 for 15 min (final volume 2 ml) as described under Materials and Methods. Conversion is expressed as nmol/ 15 min/ 1.25 nmol P450 and is corrected for values observed in incubations without NADPH. Variation in conversion and covalent binding was less than 10%.

Results

Microsomal oxidation of 1,2-DCB and 1,4-DCB

Using a battery of microsomes prepared from rats pretreated with selective inducers of the cytochrome P450 enzymes, the contribution of the various P450 isoenzymes to the oxidation of 1,2- and 1,4-DCB was investigated (Figure 4.1A, B). From the results obtained it follows that conversion of both dichlorobenzenes was significantly increased when using microsomes from rats pretreated with DEX. However, inducers of other P450 isoenzymes also enhanced oxidation of the dichlorobenzenes to a considerable extent.

Figure 4.2 shows the HPLC separation profiles of 1,2-DCB (A) and 1,4-DCB (B) and their potential metabolites, using the gradient elution profile as described under Materials and Methods. The metabolic profiles of the two isomers of DCB in microsomal

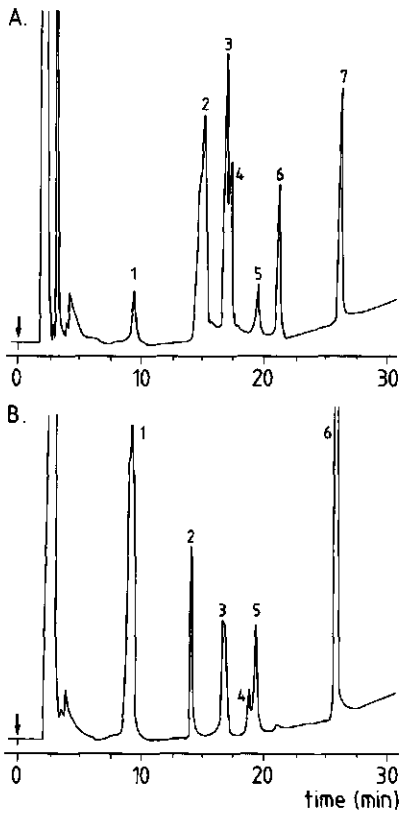

Figure 4.2 Separation of 1,2-DCB (A) and 1,4-DCB (B) and their potential metabolites by HPLC (detected at UV 254 nm), carried out as described in the Method Section. Aliquots (50 μl) of ethyl acetate extracts of microsomal incubations with the respective DCB (final concentration 0.8 mM) were co-injected with marker metabolites. Marker chemicals for 1,2-DCB (A) were: (1) 2,3-DCHQ; (2) 3,4-DCC; (3) 4,5-DCC; (4) 2,6-DCP; (5) 2,3-DCP; (6) 3,4-DCP; (7) 1,2-DCB. Marker chemicals for 1,4-DCB (B) were: (1) 2,5-DCHQ; (2) 3,6-DCC; (3) 3,5-DCC; (4) 2,5-DCP; (5) 2,4-DCP; (6) 1,4-DCB.

Table 4.1 Metabolism of 1,2- and 1,4-DCB by rat liver microsomes.

Substrate Protein (mg)	Conversion (nmol)[a]	Percentage of Total Metabolites						
		Polar[b]	DCHQ 2,3-	DCC 3,4-	DCC 4,5-	DCP 2,3-	DCP 3,4-	CVB[c]
1,2-DCB								
1.2	16	19.8	11.6	8.1	4.7	19.0	9.3	9.3
2.5	24	20.8	16.9	10.0	2.5	26.9	6.2	16.2
5.0	28	25.0	27.0	2.0	1.4	16.9	4.1	23.6

		Polar	DCHQ 2,5-	DCC 3,5-	DCP 2,4- / 2,5[d]	CVB
1,4-DCB						
1.1	7.3	4.6	24.0	4.4	38.3	28.6
2.3	12.8	4.5	23.8	1.3	26.6	43.9
4.5	21.0	4.4	26.0	2.2	18.0	49.6

Note. Incubations were performed with DEX-microsomes for 2.5 min as described under Materials and Methods. Data are corrected for blank incubations without NADPH. Data are based on 2-4 incubations. Variation was generally less than 10%, but never more than 20%.
[a] Conversion is calculated as the sum of the formation of extractable metabolites as analysed by HPLC, and the amount of covalent binding to protein. [b] Polar metabolites denotes all radioactivity that eluted in the first 8 ml. [c] CVB, covalent binding to protein. [d] Amount of 2,4-DCP amounted to approximately 5-10 % of total DCPs, as determined in later experiments by performing alkaline runs (see under Materials and Methods).

incubations are presented in Table 4.1. Both isomers are oxidized to dichlorophenols and dichlorohydroquinones as major metabolites. 3,4- and 4,5-DCC were detected as minor metabolites of 1,2-DCB. 3,5-DCC was detected in minute amounts as metabolite of 1,4-DCB, and may be formed upon secondary oxidation of the 'shifted' 2,4-DCP, which is actually detected in small amounts. In addition to ethyl acetate extractable metabolites, radioactivity remained associated with microsomal protein even after extensive washings

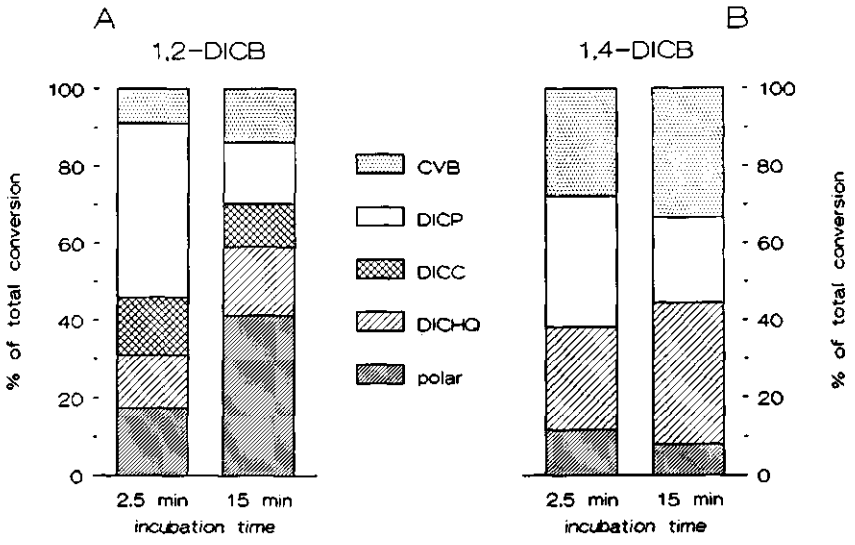

Figure 4.3 Time-dependent change in metabolic profile of 1,2-DCB (A) and 1,4-DCB (B) in microsomal incubations. Incubations were performed as described in the Method Section. Total conversion, which is calculated as the sum of ethyl acetate extractable metabolites as determined by HPLC, and radioactivity bound to protein, amounted to 12.0 nmol/ mg/ 2.5 min and 25.6 nmol/ mg/ 15 min for 1,2-DCB, respectively; total conversion amounted to 10.4 nmol/ mg/ 2.5 min and 16 nmol/ mg/ 15 min for 1,4-DCB, respectively.

with a range of solvents, indicating that both 1,2- and 1,4-DCB are metabolized to reactive species that covalently interact with protein. With increasing protein concentration the contribution of covalent binding and of DCHQ to total metabolism strongly increased, while the relative contribution of primary phenol metabolites decreased (Table 4.1).

Interestingly, in incubations with 1,2-DCB a relatively high amount of polar metabolites (presumably dihydrodiols) is detected, whereas only trace amounts of polar metabolites were formed upon microsomal metabolism of 1,4-DCB. The difference in the amount of polar metabolites derived from 1,2- and 1,4-DCB is even more noticeable when the incubation time is increased from 2.5 min to 15 min (Figure 4.3). The contribution of polar metabolites to total conversion slightly decreased upon a prolonged incubation of 1,4-DCB, whereas polar metabolites formed during prolonged incubation of 1,2-DCB amounted to more than 40 % of total conversion. The apparent low conversion

Table 4.2 Effect of ascorbic acid on the microsomal conversion of 1,2- and 1,4-DCB to ethylacetate extractable and protein bound metabolites.

Substrate	Covalent binding	Conversion	Percentage of total metabolites			
Incubation	(%)	(%)	Polar	DCHQ	DCC	DCP
1,2-DCB						
Control	100	100	42.4	26.1[a]	12.1[b] (25)	19.4[c] (12)
+ 1 mM AA	31.9± 7.8	98.5 ± 1.3	37.5	28.1	18.1 (50)	16.3 (8)
+ 10 mM AA	27.5± 4.7	72.2 ± 0.8	35.0	29.9	16.2 (63)	18.8 (9)
1,4-DCB						
Control	100	100	7.8	35.3[d]	1.9[e]	22.5[f] (8)
+ 1 mM AA	6.6± 6.3	126.5 ± 33.2	5.5	61.7	12.5	20.3 (10)

Note. Incubations were performed with DEX-microsomes for 15 min as described under Materials and Methods, in the absence (control incubation) or presence of ascorbic acid (AA). Data are based on 2-4 incubations and are expressed as percentage of the conversion and covalent binding in control incubations (25.9 ± 0.7 and 7.3 ± 0.8 nmol/mg/15 min for 1,2-DCB, respectively; 11.0 ± 0.9 and 5.3 ± 0.7 nmol/mg/15 min for 1,4-DCB, respectively; all data are corrected for blank incubations without NADPH). [a] 2,3-DCHQ, [b] sum of 3,4- and 4,5-DCC; the number in brackets denotes the amount of 4,5-DCC expressed as percentage of total DCC. [c] sum of 2,3- and 3,4-DCP; number in brackets denotes the amount of 3,4-DCP expressed as percentage of total DCP. [d] 2,5-DCHQ; [e] 3,5-DCC; [f] sum of 2,4- and 2,5-DCP; the number in brackets denotes the amount of 2,4-DCB expressed as percentage of total DCP.

rates between 2.5 min and 15 min of incubation might be due to (i) a relatively high degree of secondary metabolism (*cf.*, Table 4.1); (ii) the high volatility of the parent compound, causing lower actual substrate concentrations, thereby further increasing the contribution of secondary metabolism to overall conversion; and possibly (iii) protein inactivation due to covalent binding (see below).

Covalent binding to protein and DNA

The nature of the metabolites that bound to protein was further investigated by performing incubations in the presence of ascorbic acid, a reducing agent. As shown in Table 4.2, covalent binding upon metabolism of 1,4-DCB was nearly completely inhibited by the addition of 1 mM of ascorbic acid to the incubation mixture. A concomitant increase in the formation of 2,5-DCHQ, and to a lesser extent 3,5-DCC, was observed. Binding due to metabolism of 1,2-DCB was decreased by 68 % in the presence of 1 mM of ascorbic acid, with a less pronounced increase in the formation of DCHQ and DCC. A ten-fold increase in the concentration of ascorbic acid diminished the covalent binding only by an additional 5 %, whereas it inhibited conversion of 1,2-DCB to ethyl acetate extractable metabolites by nearly 30 %.

Covalent binding to calf-thymus DNA (1 mg) added to a standard microsomal incubation amounted to levels just slightly above background, i.e., 0.1 nmol/mg/ 15 min, for both 1,2-DCB and 1,4-DCB, indicating that these compound are not metabolized to products with a high genotoxic potential (data not shown). This is in agreement with recent observations on the low extent of DNA-binding of metabolites of 1,4-dibromobenzene (Colacci *et al.*, 1990), and is consistent with the low reactivity of chlorinated benzoquinones towards nucleic acids (Den Besten *et al.*, 1991a).

Molecular orbital computer calculations

In order to see whether theoretical data would provide support for the observed metabolite profiles of the two isomers of DCB, molecular orbital (MO) computer calculations were carried out. An important factor which has to be taken into account is that arene oxides are in dynamic equilibrium with their oxepin form (Boyd and Jerina, 1985). Experimental evidence for the existence of differences in reactivity between arene oxides and their oxepin tautomers has never been provided, hampered by difficulties associated with studying equilibria. However, theoretical MO studies can overcome this problem. Therefore, Table 4.3 presents results from AM1 semi-empirical MO calculations for the various arene oxide-oxepin tautomers from 1,2- and 1,4-DCB. These data indicate that the intrinsic chemical characteristics of the various arene oxides and their corresponding oxepins derived from 1,2-DCB are comparable to those derived from 1,4-DCB. Thus, differences in product formation and/or covalent binding of 1,2- and 1,4-DCB cannot simply be explained by differences in calculated chemical reactivity of their respective arene oxide intermediates.

Furthermore, as demonstrated by an MO study the arene oxide-oxepin equilibrium is influenced by the substituent pattern of the ring (Hayes *et al.*, 1980). Table 4.3 shows the ratio's for the number of resonance structures of the respective arene oxides and oxepins, determined as previously described (Hayes *et al.*, 1980). From the δHF,

Table 4.3 Molecular orbital characteristics for DCB arene oxides and oxepins as calculated by semi-empirical AM1 calculations.

Compound	Frontier orbital density on							
	C1	C2	C3	C4	C5	C6	O7	Rest of molecule
12-DCB-23-oxide	0.60	0.04	0.02	0.54	0.28	0.37	0.10	0.05
12-DCB-34-oxide	0.42	0.65	0.02	0.02	0.49	0.23	0.09	0.06
12-DCB-45-oxide	0.32	0.32	0.59	0.02	0.02	0.59	0.10	0.04
14-DCB-12-oxide	0.04	0.02	0.61	0.38	0.27	0.54	0.11	0.03
14-DCB-23-oxide	0.58	0.02	0.02	0.58	0.32	0.32	0.09	0.07
12-DCB-23-oxepin	0.25	0.05	0.36	0.13	0.36	0.30	0.01	0.09
12-DCB-34-oxepin	0.38	0.19	0.42	0.39	0.15	0.41	0.00	0.06
12-DCB-45-oxepin	0.44	0.44	0.14	0.38	0.38	0.14	0.00	0.08
14-DCB-12-oxepin	0.46	0.37	0.13	0.40	0.37	0.19	0.00	0.08
14-DCB-23-oxepin	0.20	0.43	0.43	0.20	0.34	0.34	0.01	0.05

Charge on	C1	C2	C3	C4	C5	C6	O7
	-0.08	+0.05	-0.01	-0.15	-0.11	-0.11	-0.21
	-0.05	-0.09	-0.01	-0.02	-0.14	-0.11	-0.22
	-0.05	-0.05	-0.14	-0.02	-0.02	-0.14	-0.22
	+0.05	-0.01	-0.15	-0.04	-0.11	-0.13	-0.21
	-0.09	-0.01	-0.01	-0.09	-0.11	-0.11	-0.22
	-0.09	+0.03	-0.02	-0.17	-0.11	-0.12	-0.16
	-0.05	-0.12	+0.01	-0.01	-0.19	-0.10	-0.18
	-0.05	-0.05	-0.19	+0.01	+0.01	-0.19	-0.19
	+0.04	-0.01	-0.17	-0.05	-0.11	-0.16	-0.17
	-0.12	-0.01	-0.01	-0.12	-0.11	-0.11	-0.17

Table 4.3 -continued-

HF (kcal/mol)	E(LUMO) (eV)	E(LUMO+1) (eV)	ΔHF oxepin-oxide (kcal/mol)	Ratio resonance structures oxide:oxepin[a]
20.36	-0.62	0.62		
15.01	-0.64	0.76		
16.12	-0.61	0.77		
19.07	-0.61	0.57		
13.76	-0.69	0.68		
8.32	-0.72	0.28	-12.04	3:5
5.45	-0.73	0.42	-9.56	4:4
4.09	-0.75	0.56	-12.03	3:5
6.48	-0.79	0.28	-12.59	2:5
4.54	-0.77	0.43	9.22	5:2

Note. For easier comparison, numbering of the atoms was chosen in such a way that in the oxide and the oxepin the same atom numbers are connected to the oxygen atom. The oxygen atom was always numbered 7. [a] Calculated as described by Hayes *et al.*, 1980.

calculated as the HF of the oxepin minus the HF obtained for the corresponding arene oxide, it follows that a higher number of resonance structures for the oxepin form is reflected by a relatively more thermodynamically stable oxepin with respect to the arene oxide, i.e., a more negative δHF. This is in accordance with observations based on MINDO3 calculations for other substituted arene oxides (Hayes *et al.*, 1980). From the δHF data presented in Table 4.3 it follows that the oxide-oxepin equilibrium will be shifted more in favor of the oxepin, when going from 1,4-DCB-1,2-oxide/oxepin > 1,2-DCB-2,3-oxide/oxepin = 1,2-DCB-4,5-oxide/oxepin > 1,2-DCB-3,4-oxide/oxepin > 1,4-DCB-2,3-oxide/ oxepin. Such a shift in the arene oxide/ oxepin equilibrium may be of importance, especially when these two tautomers would show a differential reactivity for specific reactions.

In fact, results from the AM1 calculations indicate that the frontier orbital characteristics for nucleophilic attack (Fleming, 1976) on the respective arene oxides and corresponding oxepins vary considerably (Table 4.3). In the arene oxides, the carbon

atoms connected to the oxygen atom contain almost no frontier orbital density for nucleophilic attack, making their electrophilic reactivity low. In contrast, the same carbon atoms in the oxepin contain significant frontier density for nucleophilic attack. This implies that the reactivity of these carbon atoms for nucleophilic attack is significantly increased going from the oxide to the oxepin. Since nucleophilic attack on the arene oxide-oxepin by for instance a water molecule in the active site of the enzyme epoxide hydrolase will occur at these carbon atoms, it can be derived that, if no other structural characteristics are taken into account, oxepins might be better substrates for epoxide hydrolase than the corresponding arene oxides.

Discussion

Hepatotoxicity of dichlorobenzenes has been shown to correlate with the extent of covalent binding to liver protein, which has been reported to be 6-fold respectively 10-fold higher following a single dose of 1,2-DCB compared to an equivalent dose of the 1,4-isomer (Reid and Krishna, 1973; Stine *et al.*, 1991). The involvement of oxidative metabolism in the covalent binding and hepatotoxicity of 1,2-DCB was demonstrated in rats, which were pretreated with either an inducer (phenobarbital, PB) or an inhibitor (SKF-525A) of cytochrome P450 (Reid and Krishna, 1973). Induction of the cytochrome P450 activity had no effect on the (lack of) hepatotoxic potential of 1,4-DCB (Stine *et al.*, 1991), whereas covalent binding to liver protein was actually decreased in rats pretreated with PB (Reid and Krishna, 1973). These results strongly suggest a crucial role for cytochrome P450 in the oxidation of the 1,2-substituted isomer to reactive (and toxic) species, and of the 1,4-substituted isomer to harmless metabolites. Therefore, the present study was directed at a comparison of the microsomal oxidation of the hepatotoxic 1,2-DCB and the non-hepatotoxic 1,4-DCB.

The present study indicates that the selective high contribution of cytochrome P450IIIA (induced by DEX) towards the oxidation of chlorinated benzenes as previously demonstrated for hexachlorobenzene (Van Ommen and Van Bladeren, 1989), pentachlorobenzene and 1,2,4-trichlorobenzene (Den Besten *et al.*, 1991a), appears to be less prominent for lower chlorinated benzenes like DCB. Apparently, with decreasing degree of chlorination, the contribution of other P450 isoenzymes (especially P450IA isoenzymes) becomes more important.

In Figure 4.4, a schematic overview is presented of the different pathways in the oxidation of 1,2-DCB (A) and 1,4-DCB (B) by rat liver microsomes. 1,2-DCB is mainly oxidized to 2,3-DCP, and to a lesser extent to 3,4-DCP. The absence of 2,6-DCP as a metabolite of 1,2-DCB corresponds with the absence of 2,6-dimethylphenol as a microsomal metabolite of *o*-xylene (Kaubisch *et al.*, 1972), suggesting that in a microsomal incubation the 2,3-disubstituted-1,2-epoxide is not formed. 2,5-DCP was the major DCP-isomer formed upon microsomal oxidation of 1,4-DCB, with only a trace

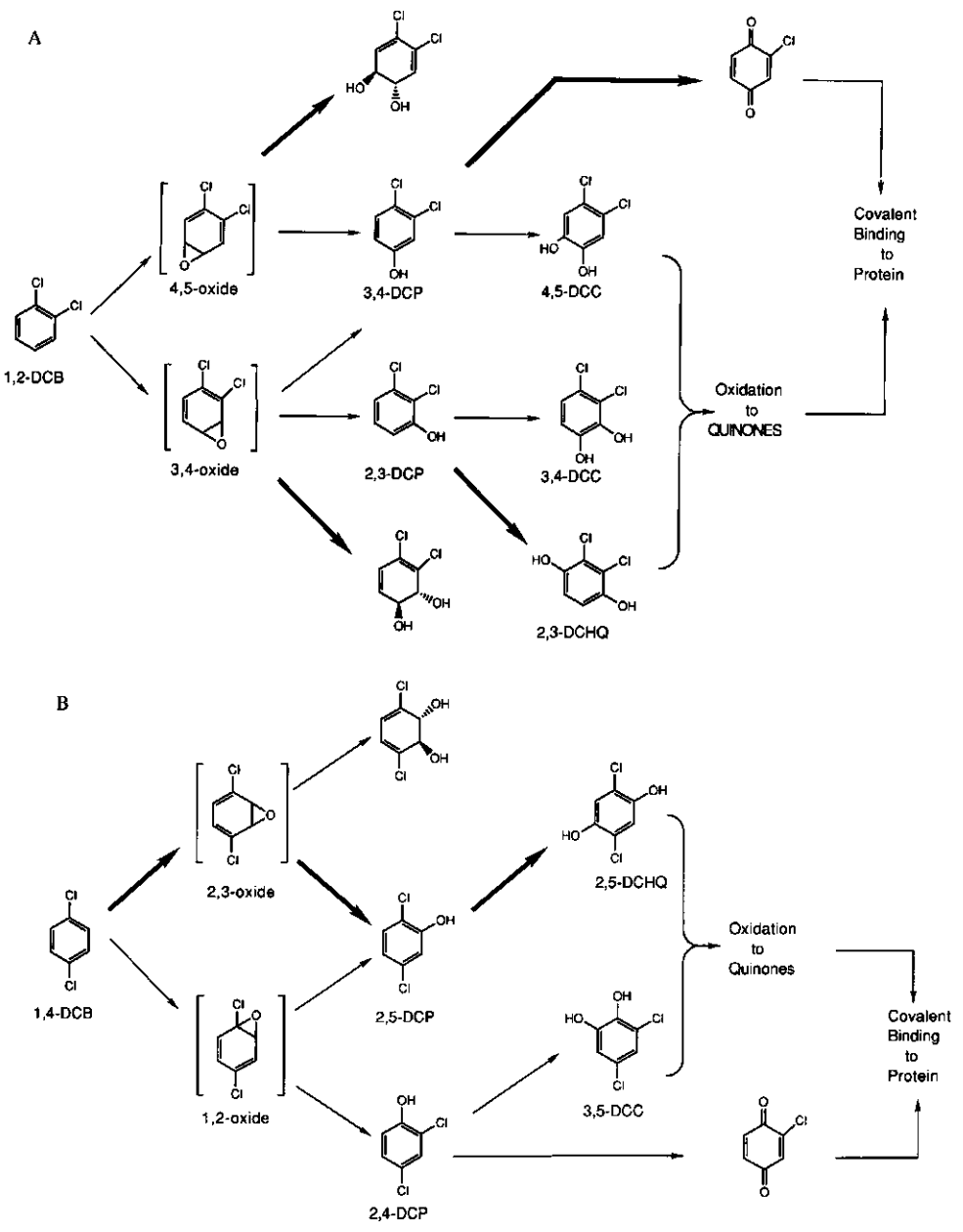

Figure 4.4 Different pathways of oxidation for 1,2-DCB (A) and 1,4-DCB (4) by rat liver microsomes.

amounts of 2,4-DCP detected. This is in agreement with the *in vivo* metabolic profile in rat urine, in which only a trace of 2,4-DCP was present (Hawkins *et al.*, 1980).

In addition to phenol formation, microsomal oxidation of dichlorobenzenes results in the formation of major amounts of hydroquinones and minor amounts of catechols, which increase in the presence of ascorbic acid, indicating that their oxidized analogs (semiquinone anions or benzoquinones) contribute to the covalent binding. *In vivo* and in isolated hepatocytes, catechols are primarily formed by oxidation of dihydrodiols. However, due to the absence of the cytosolic enzyme dihydrodiol dehydrogenase in microsomal preparations, catechol formation in microsomal incubations must arise from a subsequent oxidation of the primary phenols (Miller *et al.*, 1990).

An interesting feature in the microsomal metabolism of 1,2-DCB as compared to 1,4-DCB is the formation of a relatively high amount of dihydrodiols. These polar metabolites are formed by the epoxide hydrolase catalysed conjugation of epoxides with water. Results from AM1 semi-empirical computer calculations suggested that the oxepin forms of the various DCB arene oxides have better substrate characteristics for the nucleophilic attack by a water molecule than the corresponding arene oxide tautomers. These theoretical data can be helpful in finding a rationale for the observed differences in microsomal formation of polar dihydrodiols of 1,2- and 1,4-DCB. From arene oxides/ oxepins which are relatively more in the oxepin form, formation of polar dihydrodiol metabolites might be favored, whereas isomerization to phenols -which is well known to arise from rearrangement of the oxide tautomer (Jerina *et al.*, 1971)- and subsequent oxidation of the phenol to secondary metabolites becomes relatively more important when the arene oxides/ oxepin equilibrium is shifted in favor of the oxide tautomer. For those intermediates that actually may serve as substrate for the epoxide hydrolase-catalysed conjugation with water, the relative importance of the oxepin declines in the order: 1,2-DCB-4,5-oxide/ oxepin > 1,2-DCB-3,4-oxide/ oxepin > 1,4-DCB-2,3-oxide/ oxepin. Thus, relatively more oxepin intermediates can be expected in the oxidation of 1,2-DCB compared to 1,4-DCB, and this would explain the observation that from 1,2-DCB relatively more polar metabolites are formed than from 1,4-DCB. The near-absence of covalent binding of 1,4-DCB to liver protein *in vivo* is not simply due to the inability of cytochrome P450 to produce reactive metabolites of this substrate *per se*. The present study clearly demonstrates that in a subcellular system, 1,4-DCB is readily oxidized to reactive species which covalently interact with protein. Apparently, in the *in vivo* situation, other factors are involved which modulate the generation of reactive species - and ultimately hepatotoxicity. For example, the P450 isoenzyme status may influence the extent to which a compound is bioactivated, as illustrated by the differential effect of P450-inducers on the covalent binding observed (Figure 4.1). However, this can not be the only explanation for the isomer-specific hepatotoxicity of dichlorobenzenes, since in the absence of ascorbic acid PB-microsomes clearly increase covalent binding of 1,4-DCB (Figure 4.1), whereas exposure of PB-pretreated rats to 1,4-DCB does not results in covalent binding to liver protein (Reid and Krishna, 1973) and does not lead to

hepatotoxicity (Stine *et al.*, 1991). A main additional factor *in vivo* may be the abundance of reducing equivalents (see below).

An interesting observation of the present study is the fact that ascorbic acid only partially inhibited protein binding of 1,2-DCB, whereas protein binding of 1,4-DCB metabolites was nearly completely inhibited. These data seem to indicate that (i) covalent binding of 1,4-DCB is completely due to quinone metabolites; and (ii) upon microsomal metabolism of 1,2-DCB, reactive species other than quinone or catechol metabolites are formed that interact with macromolecules. Most likely candidates are intermediates (presumably epoxides) formed upon the primary oxidative event. However, based on AM1 computer calculations (Table 4.1) no differences are observed in intrinsic chemical parameters of the various arene oxides (and their oxepins) derived from either 1,2- or 1,4-DCB. In addition, based on studies with bromobenzene, serious questions are raised with respect to the alkylating potency of halobenzene-epoxides towards protein nucleophiles, since (i) conjugation of bromobenzene-3,4-epoxide with glutathione requires enzymatic catalysis (Monks *et al.*, 1982); and (ii) bromobenzene derived epoxide adducts account for only $< 0.5\%$ of total protein binding *in vivo* (Slaughter and Hanzlik, 1991). Moreover, the reactivity towards protein of epoxides formed in the oxidation of higher chlorinated benzenes was negligible (Den Besten *et al.*, 1989; Den Besten *et al.*, 1991a; Van Ommen and Van Bladeren, 1989).

A more likely explanation for the observed difference in the residual protein binding in the presence of ascorbic acid can be found in the secondary oxidation of the phenolic metabolites. Recent studies have pointed at the existence of different mechanisms for the cytochrome P450-mediated oxidation of *para*- and non-*para*-halogenated phenols (Rietjens and Vervoort, 1991; Den Besten *et al.*, in preparation). The cytochrome P450 dependent oxidation of halophenols with a halogen substituent at the *para*-position with respect to the hydroxyl moiety (such as 3,4-DCP) will result in the direct formation of a reactive benzoquinone and elimination of the halogen as a halogen anion. Oxidation of a non-*para*-substituted halophenol (such as 2,5-DCP) results in the formation of hydroquinone, which needs further oxidation to its benzoquinone derivative before it is sensitive to nucleophilic attack. Chemical reduction by ascorbic acid of reactive benzoquinone metabolites formed directly from the phenol precursor may be less efficient, and consequently incomplete, compared to the ascorbic acid-mediated prevention of the oxidation of the hydroquinone metabolite. This would explain the ascorbic acid mediated inhibition of covalent binding during 1,2- and 1,4-DCB metabolism in an another way than by assuming an additional and different type of binding species for the two compounds.

In conclusion, the difference in hepatotoxicity of 1,2-DCB *vs* 1,4-DCB appears to correlate with the degree of microsomal protein binding in the presence of reducing equivalents. Although the experimental data in the present study do not strictly rule out a role for epoxides in the covalent binding (and hepatotoxicity) of 1,2-DCB, theoretical

data did not provide support for significant differences in the stability and reactivity of the various epoxides (and oxepins) derived from either 1,2- or 1,4-DCB. Direct formation of quinone metabolites in a single oxidation step is suggested as an explanation for the observed differences in protein binding. This might contribute to differences in *in vivo* covalent binding (and/or toxicity) between the two isomers, because reducing equivalents present in a cell may (completely) prevent formation of quinones in the metabolism of 1,4-DCB but not in the metabolism of 1,2-DCB.

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Chapter 5

CYTOCHROME P450 MEDIATED OXIDATION OF *para*-HALOGENATED PHENOLS TO BENZOQUINONES AND NON-*para*-HALOGENATED PHENOLS TO HYDROQUINONES AS PRIMARY REACTION PRODUCTS

Abstract

The mechanism of oxidative dehalogenation of halogen-substituted arenes is still a matter of debate. In the present study, pentafluoro- and pentachlorophenol and their non-*para*-halogenated analogs were used as model compounds and metabolite patterns were determined using ^{19}F-NMR and HPLC. In order to study the primary oxidation products, microsomal incubations were performed using different electron and oxygen donors. Both in an aerobic, NADPH-driven system and in an anaerobic, cumene hydroperoxide-driven system, tetrahalogenated phenols were oxidized to their corresponding hydroquinones. However, the formation of tetrahalohydroquinone as a product of the cytochrome P450 oxidation of pentahalogenated phenols was only observed in the NADPH-driven system. Cumene hydroperoxide-dependent cytochrome P450 mediated oxidation of pentafluorophenol and [^{14}C]-pentachlorophenol resulted in the formation of fluoride anion and covalently bound material, respectively. Addition of reducing equivalents (like NADH) to these incubations resulted in the formation of the halohydroquinones with a concomitant decline in the formation of fluoride anion (in the case of pentafluorophenol) or covalently bound material (in the case of [^{14}C]-pentachlorophenol). Based on these results, it was concluded that cytochrome P450-mediated oxidation of the non-*para*-halogenated phenols results in the formation of their *para*-hydroxylated derivatives, i.e., hydroquinones, as the primary reaction products. However, oxidation at a halogenated *para*-position with respect to the hydroxyl moiety is proposed to result in the formation of the reactive benzoquinone derivative as the primary reaction product with the concomitant loss of the halogen as a halogen anion. This direct formation of reactive benzoquinones upon cytochrome P450 catalyzed oxidation of halogenated aromatic compounds may very well have toxicological implications.

C. den Besten, E. Duizer, J. Vervoort, I.M.C.M. Rietjens, P.J. van Bladeren, manuscript in preparation

Introduction

In the toxicity of halogenated benzenes, bioactivation to chemically reactive species is thought to be involved (Brodie *et al.*, 1971). Indeed, during microsomal metabolism of chlorinated benzenes a considerable amount of covalent binding to protein is observed. Secondary benzoquinone metabolites formed upon further oxidation of the primary phenols have recently been implicated to be the reactive species involved in the metabolism of hexachlorobenzene (Van Ommen *et al.*, 1986a), pentachlorobenzene (Den Besten *et al.*, 1989), 1,2,4-trichlorobenzene (Den Besten *et al.*, 1991), 1,2- and 1,4-dichlorobenzene (Den Besten *et al.*, 1992), and bromobenzene (Buben *et al.*, 1988). This prompted us to investigate more closely the mechanism of formation of these benzoquinones from the primary phenol metabolites.

Traditionally, the enzymatic oxidation of aromatic compounds has been considered in terms of epoxidation (Jerina and Daly, 1974). In the last decade, evidence has been gathered that cytochrome P450-mediated oxidation of aromatic rings does not proceed by a concerted process, i.e., direct oxygen insertion across a carbon-hydrogen bond or a carbon-carbon double bond (Burka *et al.*, 1983, Korzekwa *et al.*, 1989, Guengerich and MacDonald, 1984). Rather, a mechanism has been proposed involving the initial formation of an intermediate iron-oxo-π-complex, which proceeds via a tetrahedral intermediate (either a (bi-)radical or a nonradical cationic intermediate) and subsequently rearranges to the observed products (Guengerich and MacDonald, 1990). In addition, for compounds containing hydroxyl- or amino substituents, oxidation proceeding via an initial hydrogen radical or electron abstraction provides an additional possible mechanism (Koymans *et al.*, 1989).

In the past it has been suggested that oxidation of arenes at a halogenated position, as in the case of pentachlorophenol, proceeds by a mechanism analogous to arene oxidation at a non-halogenated position resulting in a hydroxylated product (Ahlborg *et al.*, 1978, Van Ommen *et al.*, 1986b). Tetrachlorohydroquinone has indeed been detected as a metabolite of pentachlorophenol both *in vitro* (Van Ommen *et al.*, 1986b) and *in vivo* (Renner and Hopfer, 1990). Taking the P450 reaction as a net two-electron transfer, the halogen is formally lost as a halogen cation (depicted in route a, Figure 5.1). However, the actual mechanism of (oxidative) dehalogenation resulting in a hydroxylated product is still a matter of debate; particularly, the nature of the halogen ion lost has never been unambiguously demonstrated. Direct formation of tetrachlorobenzoquinone upon oxidation of pentachlorophenol accompanied by loss of a halogen anion (route c, Figure 5.1) has been suggested as one of the alternative pathways (Van Ommen and Van Bladeren, 1989). Theoretically, formation of the semiquinone anion radical accompanied by the formal loss of a chlorine radical is also feasible upon oxidation of pentachlorophenol (route b, Figure 5.1).

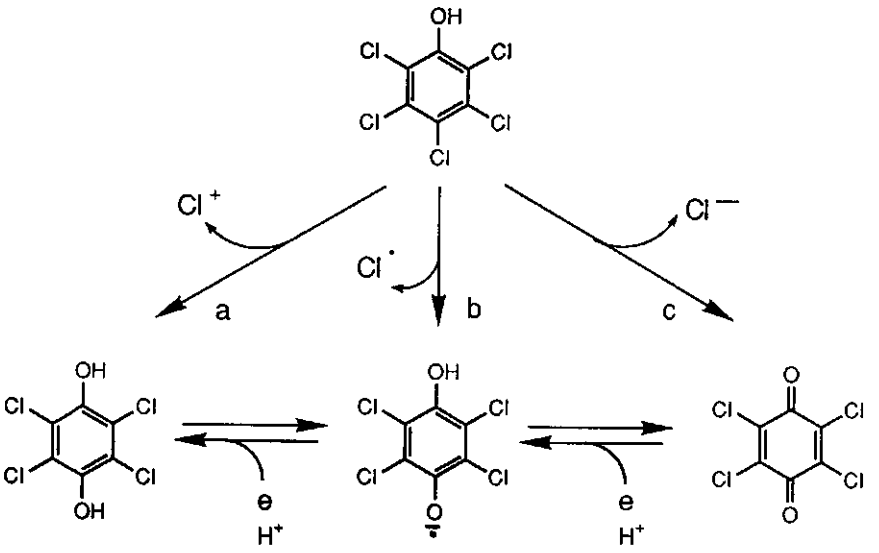

Figure 5.1 Possible pathways of cytochrome P450-mediated oxidation of PCP, taking into account the electron balance for the P450 reaction. PCP might be oxidized to tetrachlorohydroquinone via elimination of a chloride cation (*route a*), to tetrachlorosemiquinone anion radical via elimination of a chloride radical (*route b*), or to tetrachlorobenzoquinone via elimination of a chloride anion (*route c*).

The current lack of experimental evidence favouring any of these mechanisms is due to several complicating factors that are present when performing mechanistic studies with these substrates. On the one hand, direct formation of benzoquinones may be masked by their swift covalent binding to protein due to their high reactivity (Van Ommen *et al.*, 1988a). On the other hand, the presence of reducing equivalents like NADPH makes it impossible to probe the cytochrome P450-dependent oxidation, since any benzoquinone formed may subsequently be reduced to its hydroquinone.

In recent studies using fluoroanilines and ^{19}F-NMR (Rietjens *et al.*, 1990, Rietjens and Vervoort, 1991) evidence was obtained for the formation of benzoquinoneimines and a halogen anion as the primary reaction products of the cytochrome P450-dependent oxidation of *para*-fluorinated anilines. The present study was undertaken to test whether the cytochrome P450-dependent oxidation of *para*-halogenated phenols proceeds by a similar reaction pathway. Pentafluorophenol and 2,3,5,6-tetrafluorophenol were used as model substrates to investigate the oxidation at a halogenated and a non-substituted position *para* with respect to the hydroxyl moiety. In addition, the conversion of their chlorinated analogs was studied to investigate whether the type of halogen substituent

influences the mechanism of oxidation. Based on the results obtained, a plausible reaction pathway for the cytochrome P450-dependent dehalogenation and oxidation of halophenols is presented.

Materials and Methods

Chemicals

Pentafluorophenol (PFP), 2,3,5,6-tetrafluorophenol (TFP), 2,3,5,6-tetrafluorohydroquinone (TFHQ), pentachlorophenol (PCP) and 2,3,5,6-tetrachlorophenol (TCP) were purchased from Aldrich (Steinheim, Germany). 2,3,5,6-Tetrafluorobenzoquinone (TFBQ) was from Fluorochem (Derbyshire, UK). 2,3,5,6-Tetrachlorohydroquinone (TCHQ) was obtained by the ascorbic acid-mediated reduction of its tetrachlorobenzoquinone analog (Merck, Schuchard, Germany). [^{14}C]-Pentachlorophenol was from Sigma, Chemical Co. (St. Louis, MO, USA), and had a radiochemical purity of 99.9 %. NAD(P)H was from Boehringer-Mannheim (Mannheim, Germany). Cumene hydroperoxide (CumOOH) was from Aldrich (Steinheim, Germany).

Preparation of microsomes

Microsomes from untreated rats were prepared as previously described (Den Besten *et al.*, 1989) and stored at -80° C until used. Protein was determined according to Lowry using bovine serum albumin as a standard (Lowry *et al.*, 1951). Cytochrome P450 was determined as described by Omura and Sato (1964).

Microsomal incubations

Microsomal incubations (final volume of 2 ml) were carried out as previously described. In short, fluorinated phenols (3 mM final concentration) in acetone (2.5 % final concentration) were added to an incubation mixture containing 0.1 mM potassium phosphate pH 7.4 and microsomal protein (2 nmol P450/ml) in glass culture tubes with a teflonized rubber cap. After 2 min of preincubation at 37° C, the reaction was started by the addition of NADPH (1 mM final concentration). Reactions were terminated after 5 min by the addition of HCl (0.6 N final concentration). Ascorbic acid (1 mM final concentration) was also added to prevent oxidation of the hydroquinone formed. The reaction mixtures were frozen in liquid nitrogen and stored at -20°C. After centrifugation upon thawing, the supernatant was made anaerobic by four cycles of evacuation and filling with argon, and directly analysed by ^{19}F-NMR. In some incubations, cumene hydroperoxide (CumOOH, 0.25 mM final concentration, suspension in water) was used as an oxygen donor, and NADH (1 mM final concentration) as a reducing agent. For these incubations, the preincubation mixture as well as the CumOOH solution and the NADH solution were made anaerobic by four cycles of evacuation and filling with argon. The reactions were started by the addition of CumOOH and/ or NADH.

Similar incubations were performed with 2,3,5,6-tetrachlorophenol (TCP). Incubations were also performed with [^{14}C]-labeled pentachlorophenol (PCP, 1.4 μCi in a final volume of incubation of 1.0 ml) to detect covalent binding to protein in addition to metabolite formation. Extraction of acid-precipitated incubation mixtures with diethyl ether and subsequent HPLC analysis of the concentrated sample as previously described (Den Besten *et al.*, 1989) could not be used in incubations containing CumOOH due to a number of UV-detectable disturbing peaks which were also present in blank incubations (i.e., without substrate). Therefore, all incubations with chlorophenols were stopped by freezing the reaction mixture in liquid nitrogen and after centrifugation upon thawing, the supernatant was directly analysed by HPLC. Product ratio's thus analysed were comparable to those obtained after the procedure of concentrating the sample by extraction with organic solvents.

HPLC analysis

Supernatants from the chlorophenol incubations were analysed by HPLC, using a Perkin Elmer Series 4 equipment with a 150 x 4.6 mm Nucleosil 5C18 column. The eluent was monitored at 300 nm. The mobile phase consisted of an aqueous solution of 0.5 % acetic acid and methanol (50 %/ 50%) for 5 min, followed by a linear gradient of 2 % methanol per min to 90 % methanol and a second isocratic elution with 90 % methanol for 10 min (k' PCP 12.2, k' TCP 10.7, k' TCHQ 5.7, k' TCBQ 7.1). The amount of TCHQ was quantitated by comparing peak areas to the peak areas obtained from a series of standards (lower detection limit of 0.25 μM). The eluent of incubations with [^{14}C]-PCP was also assayed for radioactivity. To this end, fractions of 0.5 ml were collected followed by scintillation counting (in 5 ml of Ultima Gold, Packard, Brussels, Belgium) in a TriCarb liquid scintillation counter (Packard).

Covalent binding to protein

After sampling the supernatant for HPLC analysis, protein was precipitated in the remaining supernatant by the addition of 5 ml of methanol. Upon centrifugation, the resulting protein pellet was extensively washed with organic solvents of decreasing polarity as previously described (Den Besten *et al.*, 1991). Covalent binding to protein was measured as reported previously (Den Besten *et al.*, 1991). Protein binding was corrected for the binding observed in incubations without an electron donor, which amounted to 2.5 $\pm$ 0.05 nmol/ 5 min/ nmol P450.

^{19}F-NMR measurements

For ^{19}F-NMR measurements samples were routinely made oxygen-free by four cycles of evacuation and filling with argon gas. ^{19}F-NMR measurements were performed as previously described (Rietjens *et al.*, 1990). Briefly, a Bruker CXP 300 spectrometer operating at 282.3 MHz with a 10 mm ^{19}F Bruker probe head and Norell (Landsville, NJ, USA) 10 mm NMR tubes were used. The sample volume was 1.71 ml, containing

100 μl ^2H_2O, and the temperature was kept at 7° C. Proton decoupling was achieved with the Waltz-16 pulse sequence at -16 dB from 20W. Nuclear Overhauser effects were eliminated using the inverse gated decoupling technique. Spectra were obtained with 30° pulses (6 us), a 50 kHz spectral width, repetition time of 1 s, quadrature phase detection and quadrature phase cycling (CYCLOPS). Between 20,000 and 50,000 scans were recorded. To improve signal to noise ratio, the free induction decays were multiplied by an exponential decay resulting in an increase of the linewidth by 5 Hz. The concentrations of the substrates and products were determined from the integrals of their ^{19}F-NMR resonances. Concentrations were calculated by comparison of these integrals to the integral from the ^{19}F-NMR resonance of *para*-fluorobenzoic acid added to each sample as an internal standard. Chemical shifts are reported relative to $CFCl_3$.

Identification of ^{19}F-NMR resonances

The ^{19}F-NMR chemical shifts for the standard compounds relative to $CFCl_3$ acquired under our standard experimental conditions (incubations performed in 0.1 M potassium phosphate pH 7.4, stopped by acidifying the reaction by the addition of 0.6 N HCl (final concentration), after which 1 mM ascorbic acid was added; measured at 7° C) were as follows: fluoride anion - 164.1 ppm; pentafluorophenol, - 167.2 ppm (F2,F6), - 169.5 ppm (F3,F5), -174.7 ppm (F4); 2,3,5,6-tetrafluorophenol, - 146.0 ppm (F3,F5), - 166.9 ppm (F2,F6); 2,3,5,6-tetrafluorohydroquinone, - 168.4 ppm (F2,F3,F5,F6). Resonances could be ascribed to specific fluorine substituents on the basis of (i) relative signal intensities, (ii) splitting patterns in proton coupled and decoupled spectra, and (iii) the knowledge that *meta*-substituents only slightly influence ^{19}F-NMR chemical shifts, whereas *ortho*- and *para*-substituents have a much larger effect.

Results and Discussion

NADPH-Dependent microsomal oxidation of halophenols

Fluorophenols. Incubation of TFP or its *para*-fluorinated analog, PFP, with rat liver microsomes in the absence of an electron donor did not result in formation of metabolites (Figure 5.2a and 5.3a). In the presence of NADPH and molecular oxygen, formation of TFHQ is observed for both TFP and PFP (singlet at - 168.4 ppm, Figure 5.2b and 5.3b). In addition to TFHQ, formation of a considerable amount of fluoride anion was observed in incubations with PFP (singlet at - 164.1 ppm, Figure 5.3b), whereas in incubations with TFP only a small amount of fluoride anion was detected (Figure 5.2b). From these data, which are quantitatively presented in Table 5.1, it can be derived that PFP and TFP were metabolized to a comparable extent in a NADPH-driven P450 dependent oxidation. However, the ratio of fluoride anion to TFHQ is much higher for

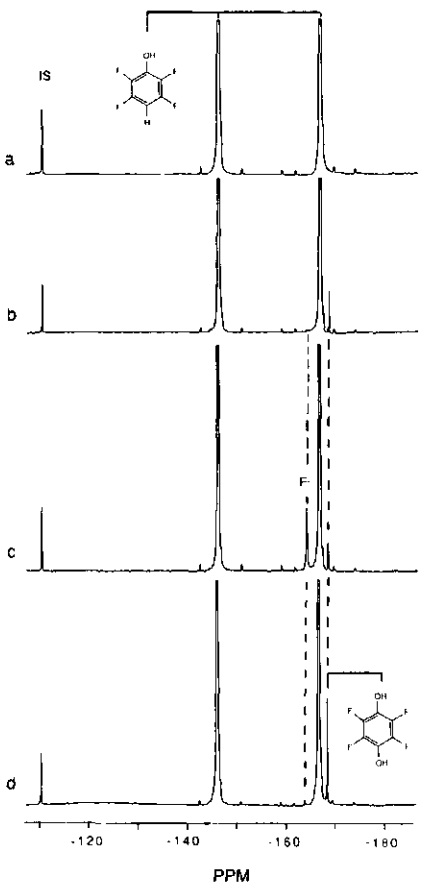

Figure 5.2 ^{19}F-NMR spectra from microsomal incubations with 2,3,5,6-tetrafluorophenol.

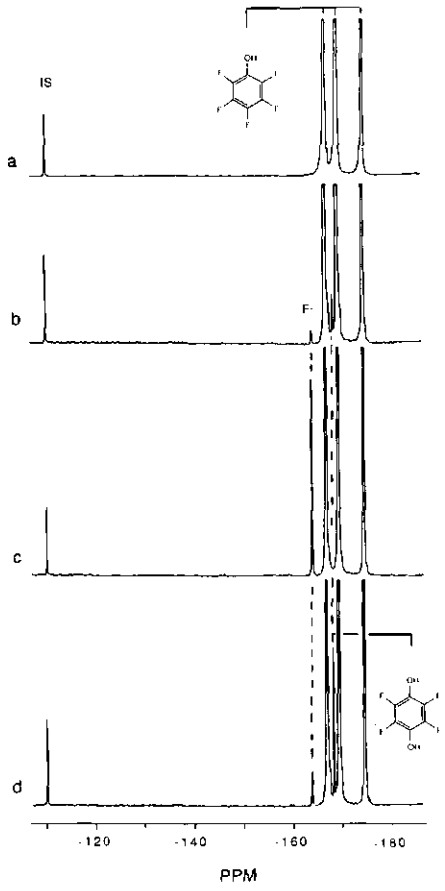

Figure 5.3 ^{19}F-NMR spectra from microsomal incubations with penta-fluorophenol.

Note to Figure 5.2 and Figure 5.3 Different electron and oxygen donors were used. (a), Blank under aerobic conditions, but without an electron donor; (b), NADPH under aerobic conditions; (c), cumene hydroperoxide under anaerobic conditions; (d), cumene hydroperoxide + NADH under anaerobic conditions. Microsomal incubations with substrate and NADH gave similar results as blank incubations (^{19}F-NMR spectra not shown). Control incubations with substrate and either cumene hydroperoxide or NADH did not result in any product formation (^{19}F-NMR spectra not shown). The resonance marked 'IS' is derived from the internal standard *para*-fluorobenzoic acid.

Table 5.1 Conversion of 2,3,5,6-TFP and PFP to TFHQ and fluoride anion by rat liver microsomes in the presence of different electron and oxygen donating systems.

Substrate Incubation conditions	F^-	TFHQ	Ratio F^-: TFHQ	Total conversion MIN	MAX
2,3,5,6-TFP					
Blank / O_2	< 0.5[a]	< 0.1[a]			
NADPH[c] / O_2	3.1	7.2	0.4	7.9[b]	10[b]
CumOOH[d]	107	3.8	29	31	111
CumOOH+NADH[d]	4.8	16	0.3	17	21
NADH	< 0.5	< 0.1			
PFP					
Blank/ O_2	< 0.5	< 0.1			
NADPH[c] / O_2	14	3.4	4.1	6.0	14
CumOOH[d]	211	< 0.1	> 2100	43	> 2100
CumOOH+NADH[d]	31	14	2.2	18	31
NADH	< 0.5	< 0.1			

Note. Incubations were carried out as described under Materials and Methods, in a final volume of 2 ml. Blank incubations contained no electron donor. Product formation was analysed by ^{19}F-NMR. [a] Data are expressed as nmol product/ 5 min/ nmol P450. [b] Data are expressed as nmol substrate converted/ 5 min/ nmol P450. A distinction is made between 'minimal' (MIN) and 'maximal' (MAX) conversion, based on the following assumptions: (i), for 2,3,5,6-TFP it is assumed that 1 molecule of TFP loses either 4 fluoride anions ('MIN') or 1 fluoride anion ('MAX'), and (ii), for PFP it is assumed that 1 fluoride anion is derived from the conversion of PFP to TFHQ, whereas 4 fluoride anions ('MIN') or 1 fluoride anion ('MAX') are derived from 1 molecule TFHQ upon binding to protein. [c] Incubations performed under aerobic conditions. [d] Incubations performed under anaerobic conditions as described under Materials and Methods.

PFP than for TFP, even when corrected for the loss of the one fluorine atom in the first oxidation step of PFP (Table 5.1).

Chlorophenols. Microsomal incubation with PCP and TCP resulted in the formation of TCHQ, although their rates of conversion were several fold lower compared to the conversion rates of their fluorinated analogs (Table 5.2). Using [^{14}C]-labeled PCP, a small amount of protein binding was detected in addition to the formation of TCHQ.

Table 5.2 Conversion of 2,3,5,6-TCP and [^{14}C]-PCP to TCHQ and covalently bound metabolites by rat liver microsomes in the presence of different electron and oxygen donating systems.

Substrate Incubation conditions	TCHQ	Protein binding	Ratio Protein binding : TCHQ	Total conversion
2,3,5,6-TCP				
Blank/ O_2	< 0.1[a]			
NADPH[b] / O_2	1.4			
CumOOH[c]	3.4			
CumOOH + NADH[c]	48			
NADH	< 0.1			
[^{14}C]-PCP				
Blank/ O_2	< 0.1	2.5		
NADPH[b]/ O_2	0.9	0.4	0.4	1.3
CumOOH[c]	< 0.1	29	> 290	29
CumOOH + NADH[c]	2.7	6.5	2.4	9.2
NADH	n.d	n.d.		

Note. Incubations were carried out as described under Materials and Methods, in a final volume of 2 ml (for 2,3,5,6-TCP) and 1 ml (for [^{14}C]-PCP). Blank incubations contained no electron donor. Product formation was analysed by HPLC as described under Materials and Methods. Protein binding data in the presence of electron donors are corrected for the amount of binding observed in blank incubations; n.d., not determined. [a] Data are expressed as nmol product/ 5 min/ nmol P450. [b] Incubations performed under aerobic conditions. [c] Incubations performed under anaerobic conditions as described under Materials and Methods.

CumOOH-dependent microsomal oxidation of halophenols

The mechanism of oxidation of halophenols to either benzoquinones or hydroquinones can not be studied under standard conditions, since any benzoquinone formed can be directly reduced by NADPH to its hydroquinone form (see below). To circumvent this problem, incubations were performed under anaerobic conditions using CumOOH as an oxygen donor. The CumOOH-dependent P450 monoxygenase activity is thought to proceed via a homolytic oxygen-oxygen bond cleavage, generating a cumyloxy radical and the equivalent of an iron-bound hydroxyl radical (Blake and Coon, 1981).

Fluorophenols. In incubations of TFP under anaerobic conditions with CumOOH as the oxygen donor, the formation of TFHQ was readily observed (Figure 5.2c). Since in

the absence of reducing equivalents TFHQ can not be formed from TFBQ, TFHQ must be the primary product resulting from the cytochrome P450 conversion of TFP. However, when PFP was used as a substrate, the formation of TFHQ was not observed in the CumOOH-driven reaction mixture (Figure 5.3c). Nevertheless, CumOOH dependent microsomal oxidation of PFP resulted in the formation of a significant amount of fluoride anion, indicating that this substrate is converted into a defluorinated product other than TFHQ. Addition of reducing equivalents in the form of NADH to incubations containing PFP as a substrate and CumOOH as the oxygen donor for the cytochrome P450 reaction, resulted again in the formation of TFHQ. In addition, a concomitant decline in the amount of fluoride anion production was observed upon NADH addition to the PFP/CumOOH incubation (Figure 5.3d). Apparently, conversion of PFP results in the formation of a metabolite that can be reduced to TFHQ by NAD(P)H, suggesting it to be TFBQ. This would imply that the cytochrome P450-dependent conversion of PFP results in formation of the reactive TFBQ as the primary reaction product. The fluorine originally present at the *para*-position is lost from the molecule as a fluoride anion. In the presence of reducing equivalents, e.g., NAD(P)H, the benzoquinone metabolite is (partly) reduced to its hydroquinone derivative. Another part might end up bound to macromolecules. The absence of a ^{19}F-NMR resonance representing TFBQ in the ^{19}F-NMR spectra can be explained by the fact that this compound avidly binds to microsomal macromolecules. The substantial increase in molecular weight of the fluorine containing metabolite due to this adduct formation gives rise to an increase in its rotational correlation time (decrease in T2) and consequently such an extensive linebroadening of its ^{19}F-resonance that the signal cannot be observed in the ^{19}F-NMR spectrum. Binding of TFBQ to protein could proceed by an addition-elimination reaction as described for binding of chlorinated benzoquinones (Van Ommen *et al.*, 1988b), resulting in loss of additional fluoride anions. To test this phenomenon, TFBQ was incubated with microsomal protein. In the ^{19}F-NMR spectrum of this incubation 99% of the fluorine intensity was present in the singlet at -164.1 representing fluoride anion, demonstrating that TFBQ indeed readily binds to protein resulting in fluoride anion production and disappearance of TFBQ (^{19}F-NMR spectra not shown).

In Table 5.1, total conversion and the ratio of fluoride anion to TFHQ are presented as well. Replacing NADPH and molecular oxygen as cofactors for the cytochrome P450 mediated oxidation of fluorophenols by CumOOH results in a more efficient oxidation of both TFP and PFP. One reason contributing to this difference in oxidation rate might be a difference in the rate limiting factor in the two respective reactions. For example, in the NADPH-mediated oxidation the rate limiting step might be the donation of the first or second electron to the iron-substrate-porphyrin or the iron-substrate-oxygen-porphyrin complex, whereas another rate limiting factor will be involved in the CumOOH-driven reaction.

Furthermore, in incubations with TFP the ratio of fluoride anion to TFHQ is increased to 29 in the CumOOH-driven reaction as compared to 0.4 in the NADPH-driven

reaction. This might be explained by 2 factors, (i) TFHQ itself is partly oxidized to TFBQ by a CumOOH dependent mechanism, thereby decreasing the amount of TFHQ detected, and (ii) TFBQ can covalently interact with microsomal protein by an addition-elimination reaction, resulting in the loss of additional fluoride anions; in the absence of reducing equivalents this might occur to a much higher extent than in the presence of reducing equivalents, where part of the TFBQ can be reduced to TFHQ.

Thus, the amount of TFHQ detected is a 'steady-state' concentration, dependent on the rate of its formation (by the P450-dependent conversion of the halophenols) and the rate of its disappearance due to its oxidation to TFBQ. Since the total conversion of both TFP and PFP at 5 min of incubation are comparable (Table 5.1), the amount of TFHQ formed from PFP and TFP should be similar. Thus, in theory, the absence of a detectable amount of TFHQ in the CumOOH/PFP incubation (Figure 5.3c) in contrast to its presence in similar TFP incubations (Figure 5.2c) could possibly be a result of inactivation of the P450, and thus inhibition of TFHQ formation occurring in these PFP incubations. However, similar experiments in which the samples were analysed at t=1, 2.5 min or 10 min (instead of at 5 min) showed no TFHQ formation in the PFP incubations, and significant amounts of TFHQ in the TFP incubations, i.e., results similar to those presented in Figure 5.2c and 5.3c. In addition, with both substrates a time-dependent increase in fluoride anion production was observed, which was significantly higher in incubations with PFP compared to incubations with TFP (data not shown). These findings support the conclusion that PFP is converted to TFBQ as the primary product, which subsequently binds to microsomal protein with the concomitant release of fluoride anion.

Chlorophenols. Incubations of chlorophenols in the presence of CumOOH produced similar results as those obtained for the fluorophenols, although both the lack of a reliable detection method for chloride anions and the lack of commercially available [^{14}C]-TCP complicated the comparison of the conversion rates between TCP and PCP. The data in Table 5.2 show that by analogy with the oxidation of fluorophenols, the CumOOH-mediated oxidation of chlorophenols by cytochrome P450 proceeds at a higher rate compared to the oxidation with molecular oxygen, and NADPH as the electron donor. The amount of TCHQ formed in incubations with TCP was increased more than 2 fold respectively 35-fold under anaerobic conditions with CumOOH and CumOOH in the presence of NADH, respectively, compared to the aerobic incubations in the presence of NADPH. In CumOOH-containing microsomal incubations with PCP, the formation of TCHQ was not observed. However, the extent of metabolism-dependent covalent binding to protein was nearly a 100-fold higher compared to the NADPH-dependent oxidation. Addition of NADH to these CumOOH incubations revealed the formation of TCHQ from PCP with a concomitant decrease in covalent binding. These results strongly suggest that, in analogy to the TFBQ formation from PFP, the reactive TCBQ is the primary P450 oxidation product of PCP, which, in the presence of NAD(P)H, can easily be reduced to TCHQ.

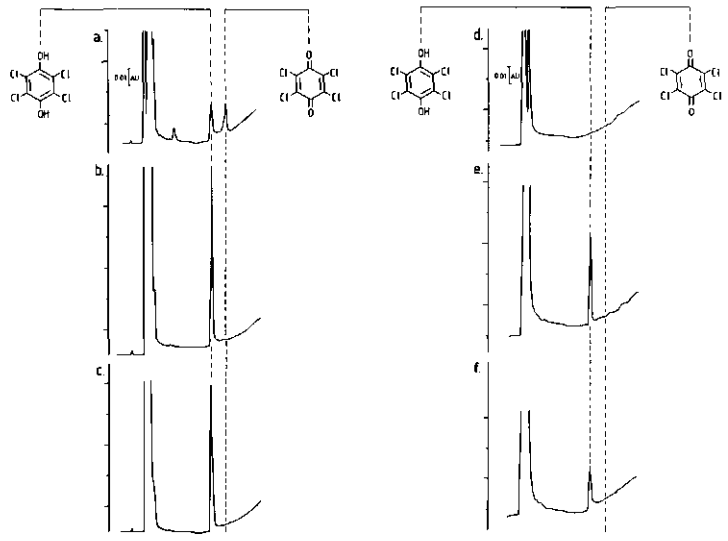

Figure 5.4 HPLC elution patterns of incubations performed with 10 μM TCBQ in the absence of electron donors, (a); in the presence of 1 mM NADH, (b) or 1 mM NADPH, (c); in the presence of microsomal protein (2 nmol/ ml) without electron donors, (d); (e), in the presence of microsomal protein and 1 mM NADH, (e) or 1 mM NADPH, (f).

Reduction of TCBQ and TFBQ by NAD(P)H

Additional experiments were performed to demonstrate that both NADH and NADPH were indeed capable of (chemically) reducing TCBQ into TCHQ thus (partly) preventing the protein binding of TCBQ (Figure 5.4). When TCBQ is added to a potassium phosphate buffer (0.1 M, pH 7.4), it is partly reduced to TCHQ (Figure 5.4a). In the presence of 1 mM NADH or NADPH, TCBQ was completely reduced to TCHQ (Figure 5.4b and 5.4c, respectively). After incubation of TCBQ in the presence of microsomal protein but in the absence of reducing equivalents, TCBQ was not observed anymore in the soluble phase nor was any other product (Figure 5.4d), indicating the strong alkylating properties of this compound. However, the addition of either NADH or NADPH to these incubations containing protein and TCBQ again resulted in the detection of TCHQ, which amounted to 56 % and 30 % of the initial concentration of TCBQ for NADH and NADPH containing incubations, respectively (Figure 5.4e and 5.4f). These

findings were irrespective of the concentration of TCBQ used (either 10 μM or 100 μM). Similar results were obtained from incubations with TFBQ in the absence or presence of microsomal protein and NAD(P)H, although the reduction of TFBQ to TFHQ was never complete (data not shown).

Concluding remarks

Based on the results presented, a scheme for the cytochrome P450-dependent oxidation of halophenols at a *para*-halogenated and a non-*para*-halogenated position is put forward in Figure 5.5. P450-dependent oxidation at a *para*-unsubstituted position proceeds via the formation of the hydroquinone as the primary product, which may subsequently be oxidized to its quinone derivative. However, the cytochrome P450-mediated oxidation at a *para*-halogenated position is proposed to proceed by direct formation of a benzoquinone and the loss of the halogen from the molecule as a halogen anion. These halobenzoquinones are highly reactive compounds and may covalently interact with cellular macromolecules. However, in the presence of NAD(P)H, or other reducing equivalents, part of the benzoquinone may be reduced to its (nonreactive) hydroxyquinone form. The proposed pathway for oxidation at a halogenated position corresponds to route c illustrated in Figure 5.1, which is favoured over the other 2 proposed pathways, i.e. route a in which the halogen is lost as a halogen cation and route b in which the halogen is lost as a halogen radical. This is further supported by the observation of a fluoride anion resonance in the ^{19}F-NMR spectra.

Recent studies on the P450-catalysed conversion of *para*-fluorinated anilines also indicated the direct formation of benzoquinoneimine with the concomitant loss of fluoride anions (Rietjens *et al.*, 1990, Rietjens and Vervoort, 1991). It becomes tempting to suggest that the proposed pathway may be a general mechanism for the cytochrome P450 dependent oxidation of substrates, which meets the following requirements: (i) the presence of an acidic proton in one of the substituents of the aromatic ring; and (ii) substituents with a 'good leaving group' character (e.g., halogens) in the *ortho*- or *para*-position. In fact, evidence has been gathered that the proposed mechanism may even be extended to compounds with electron-donating substituents in general such as fully halogenated aromatics e.g., hexahalobenzenes, resulting in the formation of a benzohaloquinone cation intermediate which may subsequently be reduced to its phenol derivative (Rietjens and Vervoort, 1992).

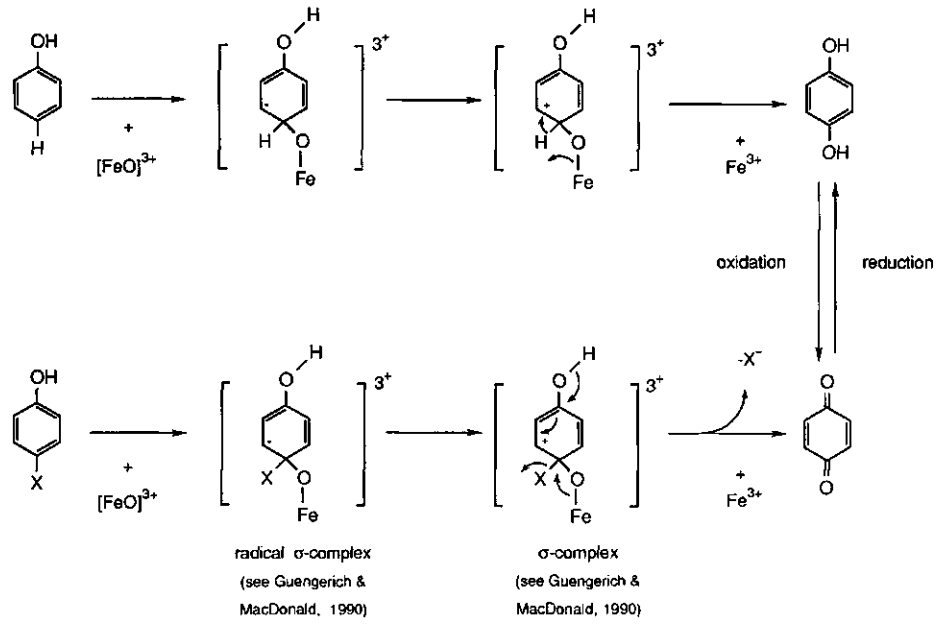

Figure 5.5 Proposed mechanism for oxidation of phenols at a halogenated and non-halogenated *para*-position (X, halogen) by cytochrome P450 in a NADPH and molecular oxygen driven system. Initial attack by the iron-oxene species ($[FeO]^{3+}$) results in the formation of a radical σ-complex and a σ-complex, as described by Guengerich and MacDonald (1990). Oxidation of substrates by cytochrome P450 in a CumOOH-driven system is believed to proceed via a mechanism involving hydrogen atom abstraction and subsequent radical combination reactions between the substrate, i.e., (phenoxy) radical, and iron-bound oxygen species (Blake and Coon, 1981). Finally, elimination of a proton in the case of a nonhalogenated position results in the formation of a hydroquinone, whereas elimination of a halogen anion results in the formation of a benzoquinone.

The proposed pathway for oxidative dehalogenation may also be a relevant pathway in the P450-dependent oxidation of bromobenzene to protein alkylating species. In a recent *in vivo* study it was shown that the majority of the protein adducts observed in rat liver after bromobenzene exposure consisted of debrominated benzoquinone adducts (Slaughter and Hanzlik, 1991). This phenomenon can readily be explained by the mechanism proposed in the present study, in which the cytochrome P-450-dependent oxidation of *para*-bromophenol, a primary metabolite of bromobenzene, results in the formation of a

debrominated benzoquinone with the loss of the bromine from the molecule as a bromide anion. The reactive benzoquinone may subsequently bind to cellular macromolecules.

Quinone metabolites may play a role in the toxicity of several xenobiotics, through arylation of cellular macromolecules and/or through their capacity to form a redox shuttle with their reduced counterparts, thereby generating reactive oxygen species and imposing a condition of cellular oxidative stress. The direct formation of benzoquinones may have toxicological implications, especially because *in vivo* it can be expected that hydroquinones will not be efficiently oxidized to their reactive quinone analogs, due to (i) competing pathways of conjugation, and (ii) the presence of reducing equivalents such as NAD(P)H, glutathione, ascorbic acid and vitamine E. However, for aromatic compounds from which benzoquinones are formed as a direct result of the cytochrome P450 mediated conversion, formation of the reactive intermediates can not be circumvented.

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Chapter 6

SUMMARY OF IN VITRO STUDIES

The first part of this thesis is directed at the question whether a range of (poly)chlorobenzene congeners are oxidized to potentially reactive products which can covalently bind to protein. To this end, incubations were performed using rat liver microsomes (i.e., particles of the endoplasmatic reticulum, which contain the cytochrome P450-complex) and radiolabeled substrates. Special attention was directed at the nature of the conversion-dependent covalent binding to protein.

In *chapter 2*, the cytochrome P450-mediated oxidation of pentachlorobenzene (PCB) to pentachlorophenol, and to a lesser extent to the various isomers of tetrachlorophenol, is described. In addition, a considerable amount of the metabolites became covalently bound to protein. Incubations performed in the presence of the reducing agent ascorbic acid indicated that benzoquinone metabolites are the most important binding species involved. However, based on the observation that the protein binding could never completely be inhibited by ascorbic acid, it was suggested that other reactive metabolites, presumably epoxide intermediates in the primary oxidation step, also take part in the protein binding.

Chapter 3 deals with the microsomal oxidation of 1,2,4-trichlorobenzene (TRICB) to the various isomers of trichlorophenol and, to a lesser extent, to trichlorohydroquinone. The intermediacy of epoxides in the oxidation of 1,2,4-TRICB was indicated by the formation of two trichlorophenol isomers (i.e., 2,3,4- and 2,4,6-trichlorophenol), which could be formed upon an 'NIH'-like shift of their corresponding epoxide intermediates. In analogy with PCB, a sizeable amount of metabolites became covalently bound to protein. However, protein binding of 1,2,4-TRICB was completely prevented by ascorbic acid. Furthermore, not only in the case of 1,2,4-TRICB but also in the case of PCB, the extent of covalent binding was strongly correlated with the extent of secondary oxidation to hydroquinones (and not with the extent of the primary oxidation to phenols). Based on these observations, it was suggested that benzoquinone metabolites were the sole reactive species formed in the oxidation of both 1,2,4-TRICB and PCB. Rather than assuming a different nature of the binding species, the residual protein binding of PCB-metabolites in the presence of ascorbic acid (cf. chapter 2) could then be explained by an incomplete reduction of the reactive tetrachlorobenzoquinone to tetrachlorohydroquinone.

Chapter 3 also describes the differential role of the cytochrome P450 isoenzymes in the oxidation of these compounds. Microsomes from rats pretreated with dexamethasone (a selective inducer of P450IIIA1) showed the highest activity towards both PCB and 1,2,4-TRICB. However, with decreasing degree of chlorination the contribution of P450IIIA1 to oxidation appears to become less prominent, and the relative contribution of other P450 isoenzymes becomes more important. This is further confirmed in *chapter 4* on the microsomal oxidation of different DCB isomers.

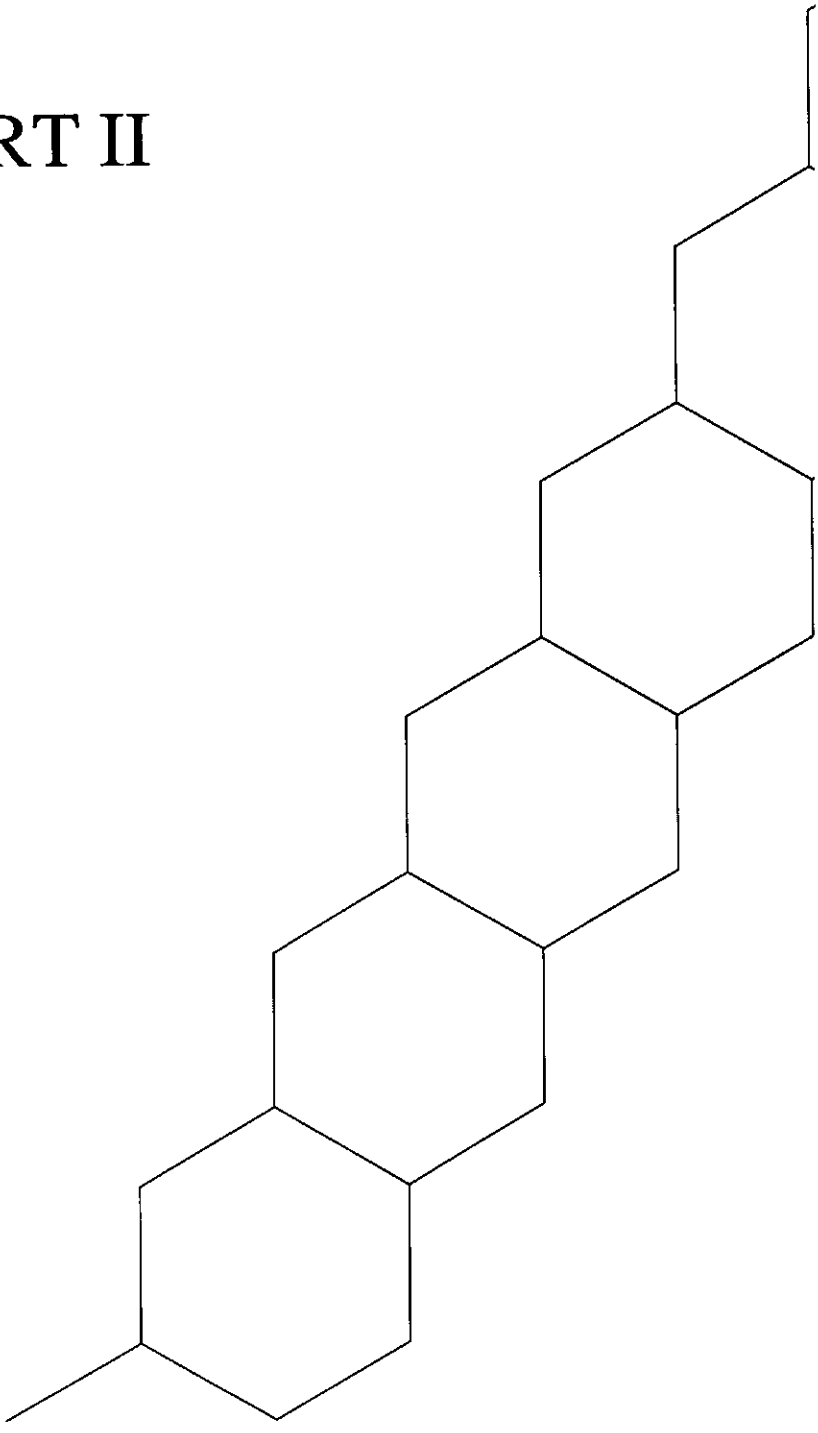
Two isomers of dichlorobenzene (DCB), i.e. 1,2- and 1,4-DCB, are known to show pronounced differences in their hepatotoxic potency. In *chapter 4,* the microsomal oxidation of the toxic 1,2-DCB and the non-toxic 1,4-DCB was studied with special attention for possible differences in metabolic profile that might contribute to the isomer-specific hepatotoxicity. Both isomers were oxidized by rat liver microsomes to reactive metabolites that became covalently bound to macromolecules. Interestingly, ascorbic acid completely inhibited the binding of 1,4-DCB metabolites, whereas ascorbic acid-mediated inhibition of protein binding by 1,2-DCB metabolites was never complete. Molecular Orbital AM1 computer calculations on the arene oxide/oxepin intermediates derived from either 1,2- or 1,4-DCB revealed no differences in chemical reactivity and/or stability of the various arene oxides. This would argue against a differential role of arene oxide intermediates in the protein binding (and toxicity?) of 1,2- and 1,4-DCB.

In anticipation of *chapter 5,* in which the mechanism of formation of the reactive benzoquinone metabolites from the primary halophenol metabolites is studied, the different binding behaviour of 1,2-DCB metabolites as compared to 1,4-DCB metabolites is explained by a direct oxidation of the primary phenols to reactive quinone metabolites in the case of 1,2-DCB, which does not take place in the case of 1,4-DCB.

The results presented thus far stress the importance of secondary benzoquinone metabolites in the metabolic activation of chlorinated benzenes. This prompted us to investigate more closely the mechanism of formation of the reactive benzoquinones from the primary phenol metabolites. To this end, pentafluoro- and pentachlorophenol, and their non-*para*-halogenated analogs were used as model compounds, and their microsomal oxidation products were determined using ^{19}F-NMR and HPLC. However, exclusive evidence for the nature of the primary oxidation products of halophenols (i.e., either benzoquinones or hydroquinones) can not be obtained under standard conditions with molecular oxygen and NADPH as the electron donor, due to the fact that any benzoquinone formed can be directly reduced by NADPH to its hydroquinone form. Therefore, anaerobic incubations were performed using cumene hydroperoxide as the oxygen donor. Based on the results described in *chapter 5*, a plausible mechanism for the P450-dependent oxidation of halophenols at a *para*-halogenated and at a non-*para*-halogenated position is put forward. Cytochrome P450-mediated oxidation at a non-*para*-halogenated position with respect to the hydroxyl moiety results in the formation of the

para-hydroxylated derivative, i.e. hydroquinone, as the primary reaction product. However, oxidation at a halogenated *para*-position is proposed to result in the direct formation of the reactive benzoquinone with the concomitant loss of the halogen from the molecule as a halogen anion. As discussed in *chapter 11*, the direct formation of reactive benzoquinone metabolites may very well have toxicological implications.

PART II



Chapter 7

THE LIVER, KIDNEY AND THYROID TOXICITY OF CHLORINATED BENZENES

Abstract

The acute toxicity of a number of chlorinated benzenes, ranging from mono-substituted to pentasubstituted benzenes, was studied in rats. Toxic effects on the liver, the kidneys and the thyroid were monitored after a single i.p. administration of 1, 2 or 4 mmol/kg monochlorobenzene (MCB), 1,2-dichlorobenzene (1,2-DCB), 1,4-dichlorobenzene (1,4-DCB), 1,2,4-trichlorobenzene (1,2,4-TRICB) and pentachlorobenzene (PCB). Due to its low solubility, 1,2,4,5-tetrachlorobenzene (TCB) was tested at a highest dose of 0.8 mmol/kg. 1,2-DCB and 1,2,4-TRICB produced the most severe hepatotoxic effects when compared with an equimolar dose of the other chlorinated benzenes, as determined by plasma ALT profile and histopathological changes after 72 hr. MCB was considerably less hepatotoxic. Severe degenerative damage to the kidney was only observed in a few rats treated with 1,2,4-TRICB. However, protein droplets in the tubular epithelial cells were observed at 72 hr after administration of 1,4-DCB, 1,2,4-TRICB, 1,2,4,5-TCB and PCB. In the latter two groups, these protein droplets were still observed nine days after administration. All chlorinated benzenes tested excluding MCB induced a reduction in plasma thyroxine levels. The extent of decrease in plasma thyroxine was more severe in rats treated with 1,2,4-TRICB or PCB and correlated well with the relative binding affinities of the phenolic metabolites to the plasma transport protein for thyroxine, i.e., transthyretin. The present study indicates that the establishment of a structure-activity relationships with regard to toxicity depends on the sensitivity of the respective target organs. In the series of (poly-)chlorinated benzenes studied, ranging from mono- to pentachlorobenzene, the most severe effects on liver, kidney and thyroid are observed for 1,2,4-substitution.

C. den Besten, J.R.M. Vet, H.T. Besselink, G.S. Kiel, B.J.M. van Berkel, R. Beems, A. Brouwer and P.J. van Bladeren. Published in: *Toxicol. Appl. Pharmacol.* 111 (1991) 69-81.

Introduction

A considerable number of studies on the toxicity of chlorinated benzenes indicates that the liver and the kidneys are the principal target sites. For example, degenerative changes in the liver and -to a lesser extent- in the kidneys were reported in rats exposed to 1,2-dichlorobenzene via inhalation (Cameron *et al.*, 1937) or after oral administration (Hollingsworth *et al.*, 1958). Short-term dermal exposure of guinea-pigs to 1,2,4-trichlorobenzene produced necrotic foci in the liver (Brown *et al.*, 1969), whereas (mild) transient effects in liver and kidneys were observed in rats after 4 weeks of inhalation exposure to 1,2,4-trichlorobenzene, but not after 26 weeks (Coate *et al.*, 1977). In addition to histological changes in the liver and the kidneys, morphological changes in the thyroids were observed in a subchronic feeding study with several trichlorobenzene isomers (Côte *et al.*, 1988) and tetrachlorobenzene isomers (Chu *et al.*, 1984). Furthermore, reduced serum thyroid hormone levels were observed during hexachlorobenzene-induced porphyria (Kleiman de Pisarev *et al.*, 1990). Adverse effects have also been reported on the immune system after exposure to monochlorobenzene (Kluwe *et al.*, 1985) and hexachlorobenzene (Vos, 1989). Furthermore, severe porphyria with accumulation of phorphyrins in liver and their excretion into urine is observed after semichronic treatment with hexachlorobenzene (Goerz *et al.*, 1977), whereas several lower chlorinated benzenes, e.g., 1,4-dichlorobenzene and 1,2,4-trichlorobenzene, have also been reported to cause a rise in urinary porphyrins after (short-term) treatment with a high dose (Rimington and Ziegler, 1963).

Investigations into the mechanism of the toxic effects elicited by halogenobenzenes have been conducted nearly exclusively for the monosubstituted compounds, bromobenzene and chlorobenzene, and the fully substituted hexachlorobenzene. Covalent binding of bromobenzene to liver proteins was detected prior to the occurrence of liver necrosis (Reid and Krishna, 1973). A correlation between the extent of formation of reactive metabolites via biotransformation and the hepatotoxic potential was established for a number of halogenated compounds among which were 1,2-dichlorobenzene and 1,4-dichlorobenzene. Subsequent studies indicated that bromobenzene was metabolized via a reactive 3,4-epoxide intermediate, which is capable of arylating nucleophilic sites on cellular macromolecules and causes a disturbance in cellular homeostasis, leading to cell death (Lau *et al.*, 1980). However, the exact nature of the covalent binding species has not yet been confirmed, and the identification of bromobenzene adducts of liver proteins has only recently been undertaken (Weller and Hanzlik, 1991).

More recent investigations have focussed attention on another class of reactive metabolites, the quinones, which arise upon further oxidation of primary phenol metabolites. In fact, the covalent interaction with proteins observed during microsomal metabolism of hexachlorobenzene could be fully ascribed to the formation of quinone metabolites (Van Ommen *et al.*, 1986). In addition, a major role for quinones is

suggested in the covalent binding to liver microsomes during the conversion of pentachlorobenzene (Den Besten *et al.*, 1989). More interestingly, glutathione conjugates of quinones appear to be involved in the tubular necrosis observed after exposure to bromobenzene (Monks *et al.*, 1985). This latter aspect has been the subject of an elegant series of investigations (Monks *et al.*, 1988), but the final details of the mechanism of action remain to be elucidated.

In an attempt to establish the generality of the metabolic activation via formation of quinone metabolites, a range of chlorinated benzenes from monochlorobenzene to pentachlorobenzene is being investigated with respect to their metabolic activation and their toxicity. Recently, we have demonstrated that the reactivity of primary (epoxide) metabolites of pentachlorobenzene and 1,2,4-trichlorobenzene towards microsomal protein is negligible, whereas their quinone metabolites exhibit a high alkylating potency towards protein (Den Besten *et al.*, 1991). However, bioactivation *in vitro* does not always imply toxicity *in vivo*. Toxicity studies in combination with studies into the urinary and biliary metabolic profile are essential in order to further assess the role of potentially reactive metabolites like quinones in the observed effects. As a start, the present report describes the adverse effects of a range of chlorinated benzenes on three target organs, the liver, the kidney, and the thyroid, after a single i.p. treatment. The role of chemically reactive intermediates and biologically active (but chemically stable) metabolites in the various toxic symptoms is discussed.

Materials and Methods

Chemicals

Monochlorobenzene (MCB), 1,2-dichlorobenzene (1,2-DCB), 1,2,4-trichlorobenzene (1,2,4-TRICB, all compounds of > 99% purity) and 1,4-dichlorobenzene (1,4-DCB, >98% purity) were obtained from Merck (Darmstadt, Germany). 1,2,4,5-Tetrachlorobenzene (1,2,4,5-TCB, > 98% purity) and pentachlorobenzene (PCB, > 99% purity) were obtained from Riedel-de Haen (Seelze, Germany). Pentachlorophenol (Aldrich, Brussels, Belgium) was purified by acid-base extraction to remove possible traces of dioxines. 2,3,4,5-Tetrachlorophenol was from Fluka Chemie AG (Buchs, Switzerland). 2,3,5,6-Tetrachlorophenol and 2,3,4-trichlorophenol were from Aldrich, and all other chlorophenols were obtained from Merck. 3,5,3',5'-tetra-L-thyroxine was from Sigma Chemical Co. (St. Louis, USA). Human transthyretin (TTR, purity 98 %) was from Calbiochem (Hoechst, Germany). [^{125}I]-L-Thyroxine was purchased from Amersham International (PLC, UK) and contained 10 % unbound iodide as determined by gelfiltration on Sephadex LH20 (Pharmacia LKB, Woerden, The Netherlands). Biogel P-6DG was obtained from Biorad laboratories, Richmond, CA, USA).

Animals

Male Wistar rats (10-14 weeks of age) were housed in air-conditioned and light controlled rooms and were allowed food and water ad libitum.

Toxicity studies

Three day observation study. Groups of three rats were treated between 9 and 11 AM with a single i.p. dose of MCB, 1,4-DCB, 1,2-DCB (groups of four rats), 1,2,4-TRICB, and PCB at 1, 2 and 4 mmol/kg in arachidis oil (3.33 ml/kg except for the high dose of PCB which was administered at 5 ml/kg due to its low solubility). 1,2,4,5-TCB was administered in doses of 0.5 and 0.8 mmol/kg/5 ml, due to its limited solubility. Control rats were treated with arachidis oil. Body weight and general appearance were checked daily. Blood was collected from the retroorbital sinus under light ether anesthesia at 24, 48, and 72 hr following exposure to the test compounds. Plasma was separated by centrifugation and stored at -20°C until analyses were performed. At 72 hr following exposure, the animals were killed by exsanguination via the abdominal aorta. The liver and the kidneys were excised and weighed.

Nine day observation study. Based on studies with bromobenzene, it is believed that secondary metabolites formed in the liver, are transported to the kidney, where they are involved in the tubular degenerative changes (Monks *et al.*, 1985). In order to take into consideration that this might be a relatively slow process, an additional study was performed in which rats (four per group) were treated with 1 and 2 mmol/kg of the test compounds (excluding 1,2-DCB) and were monitored for 9 days (216 hr). Blood samples were taken at 24, 48, 72, 144, 192, and 216 hr following exposure to the test compounds. In this study, one rat dosed with 2 mmol PCB/kg died between 48 and 72 hr after exposure. On autopsy, no gross abnormalities were observed on the liver and kidneys nor were any biochemical and histopathological indications of hepatotoxic or nephrotoxic damage observable.

Depletion of liver glutathione. Rats were treated with 1,2,4-TRICB (4 mmol/kg, i.p.). Three rats per treatment were subsequently killed at time zero (control rats) or at 2, 5, 10, 24 or 48 hr after dosing (both control rats and 1,2,4-TRICB-treated rats). The livers and kidneys were homogenized (25 % w/v) in ice-cold Tris-HCl (pH 7.4), and proteins were precipitated with a 10 % TCA-solution. Glutathione (GSH) was determined spectrophotometrically as described by Sedlack and Lindsay (1968). In a separate experiment, groups of 3 rats were treated (i.p.) with 0.8 mmol/kg 1,2,4,5-TCB, 1 mmol/kg 1,2,4-TRICB, or 4 mmol/kg of the other chlorinated benzenes, and were killed at 0 hr (control rats) and 8 hr (control and treated rats) after dosing. GSH levels in livers and kidneys were determined as described above.

Histological determinations

Sections of the liver and kidneys were preserved in 4 % buffered formalin (pH 7.4) and stained with hematoxylin and eosin for microscopic examination.

Biochemical determinations

Plasma alanine amino transaminase (ALT) activity and blood urea nitrogen level (BUN), indicative of overt hepatotoxicity and nephrotoxicity, respectively, were performed employing a Cobas-Bio centrifugal analyzer and commercially available kits. Plasma total thyroxine and total triiodothyronine levels were measured using the Amerlite TT4 and Amerlite TT3 assays (Amerham Nederland BV).

In vitro competition assay for thyroxine (T4)

A modification of the binding assay using the gel filtration procedure as described by Somack *et al.*, (1982) was employed to determine the capacity of various chlorinated benzenes and their oxidative metabolites to compete with T4 binding sites on transthyretin (TTR), a major rat plasma transport protein for thyroxine. Briefly, the assay mixture contained 60 nM [^{125}I]-L-T4 (70,000 cpm), 30 nM TTR and graded concentrations of T4 or chlorinated test compounds in methanol (final concentration 5 %) in 0.1 M Tris-HCl, pH 8.0, containing 0.1 M NaCl and 1 mM EDTA in a final volume of 200 μl. The incubation was performed overnight at 4°C in small, airtight flasks to prevent evaporation of the test compounds. The assay mixture was placed on a gel-filtration column, which was prepared in a 1 ml disposable syringe and was centrifuged (100 g, 1 min) immediately to minimize dissociation of the TTR-ligand complex. The column was washed once with 200 μl Tris buffer. Protein bound radioactivity was collected in these first two fractions, whereas free hormone binds tightly to the gel matrix and does not elute in the volumes used. The amount of radioactivity in the eluate was determined in a gamma counter (Multi Prias, Packard, Brussels, Belgium).

Statistical evaluation

Data obtained in the *in vivo* toxicity studies were tested for significant differences ($p < 0.05$) using the Kruskal-Wallis nonparametric ANOVA test.

Results

Effect on Body Weight and Organ Weight

Treatment of rats with 1,2-DCB (all doses), 1,2,4-TRICB (2 and 4 mmol/kg) and 1,2,4,5-TCB (0.8 mmol/kg) resulted in significant body weight loss after three days (ranging from -6 to -13 % of initial body weight, data not shown). At 72 hr, relative liver weight was significantly increased in rats treated with the various chlorinated

Table 7.1. Effect of a single i.p. injection of various chlorinated benzenes on relative liver weight, and treatment-related histopathological changes in the liver.

Treatment Dose (mmol/kg)	Liver wt/100 g body wt		Treatment-related histopathological findings at 72 hr			
	at 72 hr[a]	at 216 hr[b]	NRF	CLH	VAC	DEG
Control						
-	3.7 ± 0.1	3.9 ± 0.1	3	-	-	-
MCB						
1	4.4 ± 0.2*	4.0 ± 0.1	3	-	-	-
2	4.3 ± 0.1*	4.0 ± 0.1	1	2(+3)[c]	-	1(+1)
4	4.6 ± 0.1*	NT	0	-	-	3
1,4-DCB						
1	4.2 ± 0.2	4.1 ± 0.1	3	-	-	-
2	4.3 ± 0.2	4.1 ± 0.2	3	-	-	-
4	4.3 ± 0.1*	NT	2	1(+1)	-	-
1,2-DCB						
1	4.4 ± 0.1[b]*	NT	0	4(+7)	-	4
2	4.5 ± 0.1[b]*	NT	0	4(+6)	-	4(+12)
4	4.9 ± 0.2[b]*	NT	0	4(+9)	-	4(+13)
1,2,4-TRICB						
1	5.4 ± 0.2*	4.2 ± 0.1	0	3	-	1
2	5.2 ± 0.2*	4.5 ± 0.1	0	-	-	3(+8)
4	5.8 ± 0.3*	NT	0	-	2(+3)	3(+4)
1,2,4,5-TCB						
0.5	3.9 ± 0.1	NT	3	-	-	-
0.8	4.1 ± 0.1	4.7 ± 0.1*	3	-	-	-
PCB						
1	4.4 ± 0.1*	4.4 ± 0.1	0	3(+4)	-	-
2	5.1 ± 0.1*	5.0 ± 0.2*	1	2(+4)	-	-
4	5.4 ± 0.1	NT	0	1(+2)	2(+4)	1(+1)

Note. Abreviations used: NT, not tested; NRF, no treatment related findings; CLH, centrilobular hypertrophy; VAC, vacuolation; DEG, hepatocellular degeneration + fibrosis. [a] Mean±SE, n = 3; [b] Mean±SE, n = 4; [c] Figure denotes number of animals with the lesion. Within brackets, the severity of the changes is given as the sum of the gradings assigned to the lesion: very slight, +1; slight, +2; moderate, +3; severe, +4. Arterisk (*) denotes statistically significant difference from control rats, $p < 0.05$.

Table 7.2. Effect of a single i.p. injection of various chlorinated benzenes on relative kidney weight, and treatment-related histopathological changes in the kidney.

Treatment Dose (mmol/kg)	Kidney wt/100 g body wt		Treatment-related histopathological findings at 72 hr			
	at 72 hr[a]	at 216 hr[b]	NRF	TUBDIL	TUBDEG	PD
Control						
-	0.67 ± 0.02	0.66 ± 0.02	3	-	-	-
MCB						
1	0.72 ± 0.01	0.65 ± 0.02	3	-	-	-
2	0.73 ± 0.03	0.65 ± 0.02	2	1(+2)[c]	-	-
4	0.73 ± 0.01	NT	3	-	-	-
1,4-DCB						
1	0.75 ± 0.04	0.66 ± 0.05	1	1(+1)	-	1(+2)
2	0.74 ± 0.01	0.69 ± 0.02	1	-	-	2(+2)
4	0.72 ± 0.03	NT	2	-	-	1(+2)
1,2-DCB						
1	0.65 ± 0.02[b]	NT	3	-	-	-
2	0.71 ± 0.05[b]	NT	3	-	-	-
4	0.69 ± 0.01[b]	NT	3	-	-	-
1,2,4-TRICB						
1	0.67 ± 0.04	0.67 ± 0.01	0	-	-	3(+7)
2	0.71 ± 0.01	0.82 ± 0.12*	0	-	-	3(+4)
4	0.92 ± 0.19	NT	1	1(+3)	1(+2)	1(+1)
1,2,4,5-TCB						
0.5	0.71 ± 0.01	NT	0	-	-	3(+5)
0.8	0.82 ± 0.07*	0.73 ± 0.02	0	-	-	3(+6)
PCB						
1	0.73 ± 0.01	0.69 ± 0.05	0	-	-	3(+4)
2	0.78 ± 0.03*	0.80 ± 0.05*	1	1(+2)	-	1(+2)
4	0.84 ± 0.05*	NT	2	-	-	1(+2)

Note. Abbreviations used: NT, not tested; NRF, no treatment related findings; TUBDIL, tubular dilatation; TUBDEG, tubular degeneration; PD, presence of protein droplets.
[a] Mean ± SE, n = 3, [b] Mean ± SE, n = 4. [c] Figure denotes number of animals with the lesion. Within brackets, the severity of the changes is given as the sum of the gradings assigned to the lesions: very slight, +1; slight, +2, moderate, +3. Arterisk (*) denotes statistically significant difference from control rats, $p < 0.05$.

benzenes (Table 7.1). The liver-to-body weight ratio was still significantly higher 216 hr after treatment with 1,2,4-TRICB, 1,2,4,5-TCB and PCB. Relative kidney weight was increased in rats treated with the higher chlorinated benzenes (Table 7.2).

Effects on the Liver

Plasma enzyme activity. Table 7.3 shows the plasma ALT levels in rats following i.p. dosing with a range of chlorinated benzenes. A rise in plasma ALT levels was observed in rats treated with 1,2-DCB, 1,2,4-TRICB, and to a lesser degree in rats treated with MCB. Depending on the test compound and on the dose used, plasma ALT levels peaked at the 24 - 48 hr time points, and decreased again thereafter. A dose-response relationship in plasma ALT level was observed for MCB and to some extent for 1,2,4-TRICB, but not for 1,2-DCB. Furthermore, none of the rats used in the GSH-depletion study showed any signs of hepatotoxicity at 8 hr postdosing (data not shown).

Liver glutathione level. A significant decrease in GSH equivalents was already noticeable 2 hr after exposure to a high dose of 1,2,4-TRICB (4 mmol/kg, i.p.) (Figure 7.1). Although maximum GSH depletion was seen 5 hr after dosing, a significant increase in plasma ALT level was not observed until later between 10 hr and 24 hr after dosing. As the level of GSH equivalents was rebounding past control levels at 48 hr, plasma ALT levels showed a sharp decline.

As is shown in Figure 7.2, the incidence of hepatotoxicity of a series of chlorinated benzenes as measured by elevated plasma ALT levels at 24 hr coincided with a fall in GSH equivalents 8 hr after dosing. However, the extent to which GSH was depleted did not correlate with the plasma ALT levels since MCB, a moderately hepatotoxic isomer, showed a similar GSH depletion compared to both 1,2-DCB and 1,2,4-TRICB, which are severely hepatotoxic.

Histopathology of the liver. Exposure to the various chlorinated benzenes did not result in obvious microscopic liver changes after 8 hr. After 72 hr, distinct treatment related changes were observed which were characterized by hepatocellular hypertrophy, mainly in the low- and/or mid-dose groups and hepatocellular degeneration in the mid- and/or high-dose groups (Table 7.1). The degenerative changes were characterized by swelling, degeneration and necrosis of centrilobular hepatocytes, accompanied by small aggregates of RES cells and lymphocytes, and proliferation of some fibroblasts. In relatively mild cases the changes were restricted to a small rim around the central veins. In more severely affected livers the changes occupied a larger part of the centrilobular areas. In control animals hepatocellular hypertrophy and degeneration were absent at 72 hr. After 9 days, a minimal degree of centrilobular hypertrophy was observed in the liver of one or a few animals of most of the treatment groups and in a single control animal (data not shown). An increased incidence or severity of this change was observed in rats treated with 1,2,4,5-TCB and PCB. Only in the latter groups this change is considered to be due to the administration of these test compounds.

Table 7.3 Effect of a single i.p. injection of various chlorinated benzenes on plasma enzyme activity (alanine aminotransaminase, ALT).

Treatment	Dose (mmol/kg)	ALT Time after exposure (hr) 24	48	72
Control	-	33 ± 3	41 ± 3	44 ± 3
MCB	1	39 ± 6	48 ± 3	55 ± 3
	2	116 ± 20*	72 ± 13	52 ± 2
	4	154 ± 35*	831 ± 281*	149 ± 32*
1,4-DCB	1	36 ± 2	50 ± 2	52 ± 3
	2	34 ± 3	46 ± 3	49 ± 3
	4	34 ± 1	50 ± 3	61 ± 6
1,2-DCB	1	2105 ± 166[a,*]	997 ± 169[a,*]	146 ± 11[a,*]
	2	1826 ± 104[a,*]	1841 ± 487[a,*]	309 ± 46[a,*]
	4	326 ± 166[a,*]	840 ± 211[a,*]	96 ± 20[a,*]
1,2,4-TRICB	1	1160 ± 164*	373 ± 14*	99 ± 8*
	2	2969 ± 608*	631 ± 126*	127 ± 11*
	4	2294 ± 994*	570 ± 199*	159 ± 38*
1,2,4,5-TCB	0.5	33 ± 1	39 ± 6	48 ± 2
	0.8	36 ± 4	49 ± 3	50 ± 4
PCB	1	31 ± 1	39 ± 6	41 ± 5
	2	27 ± 3	39 ± 3	54 ± 4
	4	25 ± 1	42 ± 5	56 ± 9

Note. Data are taken from the 3-day observation study and represented as mean±SE, $n=3$ (Units/liter). Plasma ALT values in the 9-day observation study showed a similar profile. [a] Mean±SE, $n = 4$. *, Statistically significant differences compared to control rats, $p < 0.05$.

Effect on the Kidneys

Plasma BUN levels. The levels of plasma BUN remained within the normal range in all rats (5.4 - 8.5 mg BUN/ml), except for a single animal in the three day observation study treated with 4 mmol/kg 1,2,4-TRICB (12.6, 16.2, and 15.5 mg BUN/ml at 24, 48,

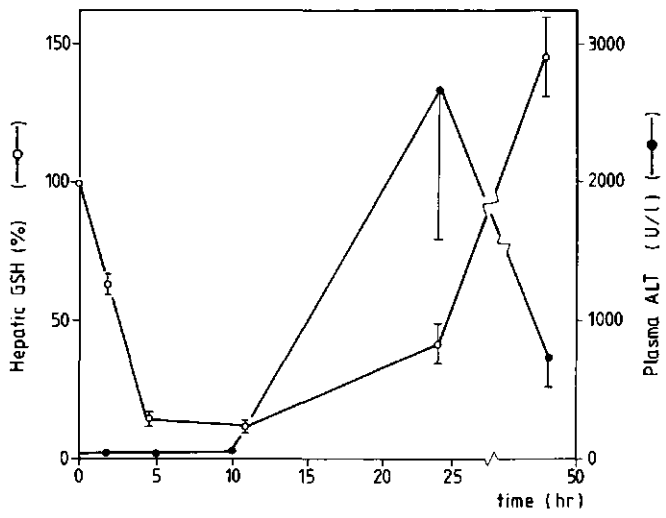


Figure 7.1 Plasma ALT levels (U/liter) (●) and hepatic glutathione content (○), expressed as percentage of time-matched oil-treated control rats, in rats treated with 4 mmol/kg 1,2,4-TRICB.

and 72 hr after dosing, respectively) and a single animal in the nine day observation study treated with 2 mmol/kg 1,2,4-TRICB (28.2, 68.4, 55.3 and 9.2 mg BUN/ml at 24, 48, 72 and 216 hr after treatment, respectively).

Kidney glutathione level. GSH levels in whole homogenates of the kidneys at 8 hr after a single i.p. dose of 4 mmol/kg MCB or PCB were slightly reduced to 76 and 71 % of the GSH content in control rats, respectively (Figure 7.2). Treatment of rats with the other chlorinated benzene isomers did not result in decreased GSH levels.

Histopathology of the kidney. In the 1,2,4-TRICB treated rats with elevated BUN levels distinct kidney damage was observed, consisting of proximal tubular degeneration and dilatation (4 mmol/kg TRICB), and severe nephrosis (2 mmol/kg). The toxicological significance of these isolated findings is questionable, but treatment relationship cannot be excluded. Slight proximal tubular degeneration was observed in rats nine days after treatment with PCB.

At 72 hr proteinaceous droplets were observed in the renal proximal tubular epithelial cells in all dose groups of rats treated with 1,4-DCB, 1,2,4-TRICB, 1,2,4,5-TCB and PCB, but not in the MCB group, the 1,2-DCB group and in the controls (Table 7.2). At 216 hr, protein droplet formation was most pronounced in 1,2,4,5-TCB and PCB treated rats.

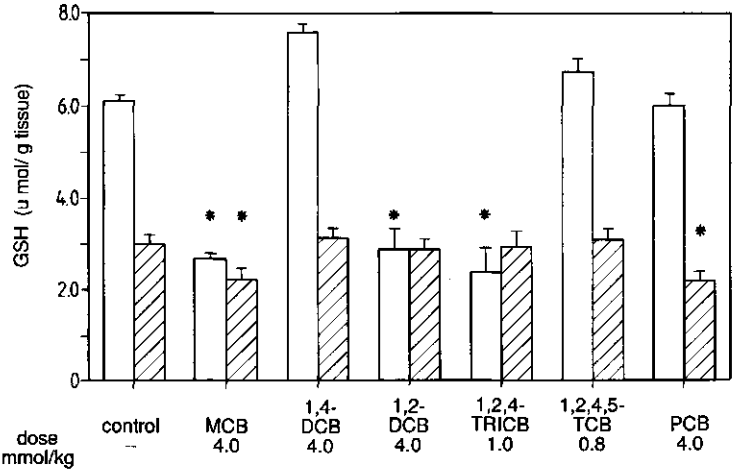


Figure 7.2 Hepatic (□) and renal (▨) glutathione content at 8 hr post-dosing (i.p.) of arachidis oil (control) or various chlorinated benzenes. *, significantly lower compared to control, $p < 0.05$.

Interaction with Thyroid Hormone Homeostasis

Plasma thyroid hormone levels. The effect of a single i.p. dose of chlorinated benzenes on plasma total thyroxine (TT4) and total triiodothyronine (TT3) levels 24 hr after exposure are shown in Table 7.4. With the exception of rats treated with MCB or the low dose of 1,4-DCB, a significant decrease in plasma TT4 was observed. No effect on plasma TT3 levels was observed, except for a slight reduction after treatment with 2 mmol/kg PCB and a dramatic reduction after treatment with 1,2-DCB.

Rats treated with 1,2,4-TRICB manifested a sharp decline in plasma TT4 levels, reaching a minimum level within 24 hr after exposure (Figure 7.3a). Plasma TT4 levels started to recover after 72 hr, and resulted in an 'overshoot' at 192 and 216 hr after exposure. The high dose PCB group demonstrated a prolonged reduction in plasma TT4 (Figure 7.3b). In rats treated with 4 mmol/kg 1,2,4-TRICB, a significantly reduced level of plasma TT4 was already observed at 5 hr after exposure (32.1 ± 2 nmol/l in 1,2,4-TRICB treated rats vs 45.2 ± 4 nmol/l in control rats), and reached minimum levels between 10 hr and 24 hr post-dosing (19 ± 0.9 and 4.0 ± 1.5 nmol/l in 1,2,4-TRICB treated rats vs 39.5 ± 1.5 and 40.5 ± 0.4 nmol/l in control rats).

In vitro binding studies with transthyretin. In order to investigate whether chlorinated benzenes and their metabolites are capable of competing with T4 for its TTR-binding site, *in vitro* binding studies were conducted with transthyretin (TTR). The binding constants derived from these competition experiments, and the binding affinities relative to T4 are shown in Table 7.5. Chlorophenols are capable of displacing T4 from its TTR-binding site, but they differ greatly in their potency to do so. The affinity of PCP for the T4-binding site was distinctly higher than that of T4 itself. With decreasing degree of chlorination, the binding affinity for the T4 binding site on TTR decreased. Moreover, monochlorophenol (4-CP) did not show any detectable interaction, neither did PCB or lower chlorinated benzenes, nor 1,4-tetrachlorobenzoquinone, a compound with strong alkylating properties. However, 1,4-tetrachlorohydroquinone demonstrated a binding constant similar to that of 2,4,6-TRICP.

Discussion

Chlorinated benzenes are compounds with a low intrinsic toxicity and require metabolism to biologically reactive products in order to induce toxic effects in target organs like the liver, the kidneys or the thyroid. Recent *in vitro* studies implicated quinones as the ultimate reactive species that bind to proteins. In an attempt to assess the meaning of the metabolic activation in a subcellular system in terms of toxicity *in vivo*, a range of chlorinated benzenes was compared with respect to their ability to induce organ toxicity after a single i.p. treatment. Interaction between reactive products and cellular macromolecules in the liver is believed to occur only after insufficiency of cellular defense mechanisms, e.g., substantial depletion of glutathione. The important role for reactive electrophilic intermediates in the hepatotoxicity of various chlorinated benzenes was indicated by the fact that liver necrosis was preceded by severe GSH depletion. However, the extent of GSH depletion did not correlate with the degree of hepatotoxicity of MCB, 1,2-DCB and 1,2,4-TRICB. The absence of such a correlation has also been observed in a recent study on the isomer-specific toxicity of dichlorobenzene (Stine *et al.*, 1991).

1,2-DCB and 1,2,4-TRICB produced the most severe signs of hepatotoxicity, whereas MCB was only moderately hepatotoxic. The apparent lack of a dose-response relationship for 1,2-DCB may be explained by a saturation of metabolism, resulting in a (competitive) inhibition of the route of metabolic activation (e.g., secondary metabolism to quinones). The striking differences in hepatotoxic properties between 1,2-DCB and 1,4-DCB has been noted in earlier reports (Brodie *et al.*, 1971) and could perhaps be ascribed to differences in the capacity to form reactive metabolites or in the nature of the reactive products. However, preliminary experiments show that during microsomal metabolism of

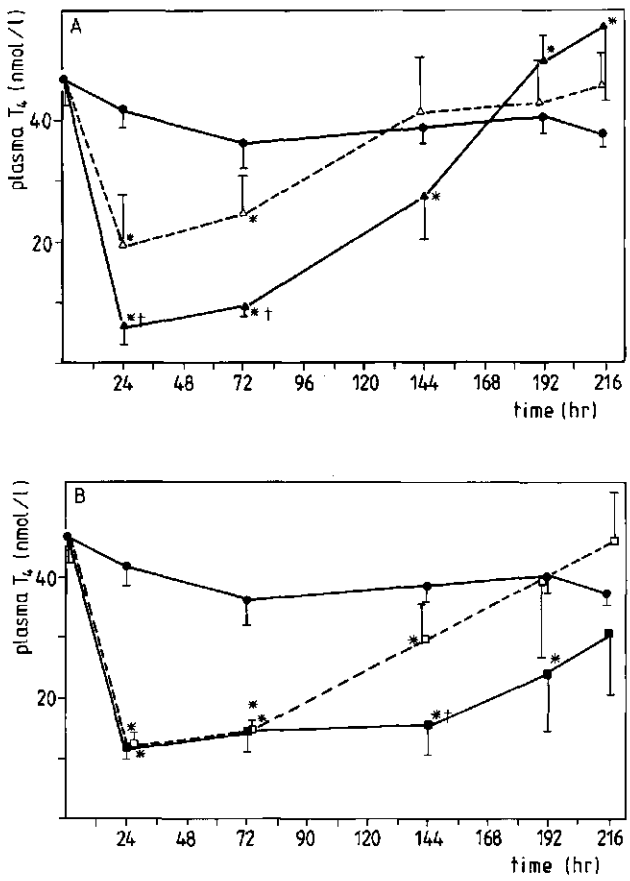


Figure 7.3 Plasma total thyroxine (T4) levels in rats after a single i.p. dose of 1,2,4-TRICB (A) and PCB (B). (●), Control rats; (▵), (□), 1 mmol/kg i.p.; (▲), (■), 2 mmol/kg. *, significantly different from control rats, $p < 0.05$.

the non-hepatotoxic 1,4-DCB *in vitro*, substantial protein binding occurs (results to be published elsewhere).

Previous studies on the microsomal metabolism of PCB (Den Besten *et al.*, 1989, 1991) have demonstrated its conversion to protein-binding quinone-metabolites. However, the lack of overt hepatotoxicity after a single i.p. treatment with PCB emphasizes that the extent to which a compound is activated to reactive products *in vitro*

Table 7.4 Plasma thyroxine and triiodothyronine concentrations at 24 hr after a single administration (i.p.) of a series of chlorinated benzenes.

Compound dose (mmol/kg):	Thyroxine[a] (TT4) (nmol/liter)		Triiodothyronine[b] (TT3) (nmol/liter)	
	1	2	1	2
MCB	42.0±1.4	39.9±5.8	0.66±0.14	0.51±0.05
1,2-DCB	32.0±2.2*	26.8±2.5*	0.15±0.04*	0.17±0.05*
1,4-DCB	40.7±4.5	34.3±1.2*	0.77±0.12	0.78±0.06
1,2,4-TRICB	19.9±4.3*	6.4±1.7*	0.69±0.10	0.65±0.10
1,2,4,5-TCB[c]	28.8±2.7*	NT	0.79±0.18	NT
PCB	13.9±0.6*	11.3±1.2*	0.51±0.09	0.48±0.06*

Note. Plasma total thyroxine (TT4) and triiodothyronine (TT3) levels were determined as described under Materials and Methods. Values represent mean±SE (n=4). [a] The level of TT4 in control rats was 42.3±1.6 nmol/liter. [b] The level of TT3 in control rats was 0.71±0.1 nmol/liter. [c] Due to its limited solubility, 1,2,4,5-TCB was administered in a dose of 0.8 mmol/kg. Values significantly different from control rats: *, $p < 0.05$. NT, not tested.

does not necessarily correlate with the observed toxic effects. On the one hand, this apparent discrepancy could be due to lack of hepatotoxicity of reactive quinone metabolites per se. On the other hand, the concentration of reactive metabolites at the target site may be influenced by factors, which play a role in the whole organism but not in a subcellular system. The lipophilicity of the compound and hence the extent to which it is bioaccumulated in the organism will determine whether the rate of formation of reactive products can reach a treshold level. Variation in the affinity of competing pathways between differently substituted benzenes may also play a critical role in the observed effects.

Metabolic activation of halogenated benzenes is also thought to be involved in the nephrotoxic symptoms. For bromobenzene, compelling evidence is obtained that glutathione conjugates of secondary hydroquinone metabolites are implicated in the proximal tubular degeneration (Monks *et al.*, 1985). In the present study, administration of up to 4 mmol/kg did not result in a distinct nephrotoxic effect. Since MCB has been described to be nephrotoxic at higher doses (9 mmol/kg, Reid, 1973), the dose-range applied in the present study might have been too low to cause tubular degeneration after treatment with the chlorinated benzenes.

Table 7.5 Relative binding affinity of chlorinated benzenes and metabolites for the T4 binding site on transthyretin (TTR).

Compound	IC50[a]	Binding affinity[b]
Thyroxine (T4)	1.0×10^{-7}	1.00
Pentachlorophenol	4.0×10^{-8}	2.50
2,3,4,5-Tetrachlorophenol	1.1×10^{-7}	0.91
2,3,5,6-Tetrachlorophenol	2.2×10^{-7}	0.45
2,4,5-Trichlorophenol	5.0×10^{-7}	0.20
2,3,6-Trichlorophenol	1.8×10^{-6}	0.06
2,3,4-Trichlorophenol	2.5×20^{-6}	0.04
2,6-Dichlorophenol	1.0×10^{-5}	0.01
2,3-Dichlorophenol	$\geqslant 10^{-5}$	$\leqslant$ 0.01
4-Chlorophenol	$\geqslant 10^{-5}$	$\leqslant$ 0.01
1,4-Tetrachlorohydroquinone	4.0×10^{-7}	0.25
1,4-Tetrachlorobenzoquinone	$\geqslant 10^{-5}$	$\leqslant$ 0.01
Pentachlorobenzene	$\geqslant 10^{-5}$	$\leqslant$ 0.01

[a] IC50 values were determined from the competition curves and represent the concentration (M) at 50 % of total [^{125}I]T4 binding to TTR.

[b] Binding affinities of the test compounds are expressed relative to T4:

$$\frac{\text{IC50 T4}}{\text{IC50 test compound}}$$

The accumulation of protein droplets, believed to be induced by a reversible interaction between certain xenobiotics or their metabolites and $\alpha 2\mu$-globulin, has been described after chronic oral exposure to PCB (Linder *et al.*, 1980) as well as after subchronic exposure to 1,4-DCB (Bomhardt *et al.*, 1988). The mechanism has been suggested to be through binding of hydroxylated metabolites to $\alpha 2\mu$-globulin (Charbonneau *et al.*, 1989). From the present results it can be concluded that this is a rather general phenomenon for chlorinated benzenes: with the exception of MCB and 1,2-DCB, all other compounds tested induced protein droplets to some extent.

In the present study an additional reversible interaction between stable metabolites and a receptor molecule is demonstrated *in vitro*, which may be responsible for the severe disturbance of thyroid hormone homeostasis observed. Chlorinated benzenes other than MCB caused a moderate to severe reduction in plasma levels of thyroid hormone (thyroxine, T4). Similar reductions in plasma T4 levels have been described in rats

exposed to structurally related compounds like the polychlorinated biphenyls. In the latter case, (part of) this decrease might be explained by an interference of hydroxylated metabolites with plasma transport (Brouwer, 1989) through a displacement of T4 from its binding site(s) on transthyretin (TTR), a major T4 transport protein in the rat. *In vitro* experiments with purified TTR demonstrated that simple chlorinated phenols also possess the capacity to displace T4 from its plasma carrier protein, confirming a recent binding study with TTR and several chlorinated aromatics (Van den Berg, 1990). Metabolism seems to be a prerequisite, since the nonhydroxylated chlorobenzenes do not show affinity for the T4-binding site on TTR. The relative binding affinity of chlorinated phenols towards TTR increased with increasing degree of chlorination, which correlated well with the observation *in vivo*, i.e., a greater reduction in plasma T4 after exposure to the higher chlorinated benzenes. Addition of an extra hydroxyl substituent as in tetrachlorohydroquinone, a secondary metabolite, did not further increase the affinity of the phenol towards the thyroxine binding site(s) on TTR. Furthermore, tetrachlorobenzoquinone, a strong alkylating compound with a high affinity towards protein thiols, did not interact with the thyroxine binding site(s), suggesting that cysteine residues do not play a role in the binding of thyroxine to TTR.

However, it should be realized that in addition to reduced plasma binding of thyroxine, alterations in hepatic thyroxine metabolism may be involved in the reduction of plasma thyroid hormone. A recent study suggested that HCB treatment increased hepatic thyroxine deiodination (Kleiman de Pisarev *et al.*, 1990). In addition, increased UDP-glucuronyltransferase (UDP-GT) activity could contribute to the reduced thyroid hormone levels, since thyroxine is glucuronidated prior to its bilary excretion. In fact, lower chlorinated benzenes are inducers of the UDP-GT activity (Carlson and Tardiff, 1976). However, a significant reduction in plasma TT4 level is already noticed at 5 hr after treatment. Liver damage could reduce plasma T4 carrier protein, but this is not a major factor in the reduction of thyroxine levels, since nonhepatotoxic compounds like PCB and 1,4-DCB also reduce plasma TT4 levels. Liver damage could also result in suppressed activity of the microsomal deiodases, thereby limiting the conversion of T4 to T3. A reduction of plasma TT3 in rats treated with 1,2-DCB was indeed observed. However, 1,2,4-TRICB showed similar degenerative damage without an effect on plasma TT3 levels.

It is evident from the results of the present study that the relationship between the extent of chlorination of the benzene ring and the type and degree of the observed adverse effects is target-organ specific. However, 1,2,4-TRICB was always among the compounds producing the most severe damage. For all of the effects observed, metabolic activation of the parent compound is believed to be involved. Several of the key-steps have been elucidated *in vitro* (Den Besten *et al.*, 1991). In order to correlate these results to the present *in vivo* data, detailed *in vivo* metabolism studies are necessary.

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Chapter 8

THE ROLE OF OXIDATIVE METABOLISM IN HEXACHLOROBENZENE-INDUCED PORPHYRIA AND THYROID HORMONE HOMEOSTASIS: a comparison with pentachlorobenzene in a 13-week feeding study.

Abstract

Hexachlorobenzene (HCB) induces a broad spectrum of effects including disturbances in the haem synthesis (porphyria) and in thyroid hormone homeostasis. For most of its effects, biotransformation of the parent compound seems to be a prerequisite. The present study was designed to assess the relevance of the oxidative metabolites in HCB-induced toxicity, with special attention for the role of the reactive tetrachlorobenzoquinone (TCBQ). To this end, toxicity and biotransformation of HCB was compared with that of pentachlorobenzene (PCB), since this chemical is oxidized to the same products as HCB, i.e., pentachlorophenol (PCP) and TCBQ. Female Wistar rats received diets containing different dose levels of HCB or PCB for 13 weeks, with or without co-treatment with triacetyloleandomycin (TAO), a selective inhibitor of cytochrome P450IIIA1/2. Both HCB and PCB were oxidized to PCP and tetrachlorohydroquinone (TCHQ), the reduced analog of TCBQ. Rats treated with HCB (high dose) had significantly elevated levels of urinary porphyrins from the 4th week on and a significant hepatic accumulation of porphyrins at the end of the study. Both urinary porphyrin excretion and hepatic porphyrin accumulation were greatly inhibited in rats receiving co-treatment with HCB and TAO. However, the inhibition of HCB-induced porphyria by TAO cannot be explained by a diminished formation of the highly reactive TCBQ, since rats treated with a high dose of PCB, which had a several fold higher urinary excretion of PCP and TCHQ compared to a high dose of HCB, did not develop porphyria. Instead, the present study points to the involvement of a putative reactive intermediate in the primary oxidative step in HCB-induced porphyria, since based on paired observations of individual rats, the degree of porphyria was correlated to a high degree with excretion of PCP, whereas correlation of porphyria with early excretion of TCHQ was much weaker. This finding fits well with the fact that the mechanisms of oxidation of HCB to PCP and PCB to PCP are different. Cytochrome P450IIIA1/2

C. den Besten, M.H.J. Bennik, I. Bruggeman, P. Schielen, F. Kuper, A. Brouwer, J.H. Koeman, J.G. Vos, P.J. van Bladeren, *submitted*

Abstract - continued-

appears to be involved in the conversion of HCB and PCB, since co-treatment of TAO resulted in a strongly diminished urinary excretion of PCP and TCHQ. Treatment with HCB as well as PCB results in disturbances of retinoid and thyroid hormone homeostasis. These effects, which have also been reported after exposure to polychlorinated biphenyls, originate from interference of hydroxylated metabolites (notably PCP) with the plasma thyroxine transport protein, transthyretin, and since this metabolite is formed from both HCB and PCB, this results in the same toxicity for both compounds.

Introduction

Hexachlorobenzene (HCB) and pentachlorobenzene (PCB) are chlorinated hydrocarbons, which have been used in the past as crop protectants. Nowadays, emission into the environment may occur due to their use as a chemical intermediate or by-product in industry, and their presence as an impurity in the formulation of several widely used pesticides (US EPA, 1985).

As a consequence of the disastrous HCB-poisoning in eastern Turkey from 1955-1959 (Peters *et al.*, 1982), this chemical has been the subject of intense research. HCB has been shown to be hepatotoxic: effects on the liver range from hepatocellular enlargement to severe disturbance of the haem synthesis, resulting in accumulation of porphyrins in the liver and (massive) excretion of porphyrins into urine (Böger *et al.*, 1979). HCB also has potent immunotoxic properties. In rats, HCB has been reported to induce immune stimulation (both humoral and cell-mediated; Vos *et al.*,1979; 1983), whereas in mice, HCB acts as an immune suppressor (Vos, 1986). More recently, effects of HCB on thyroid hormone homeostasis have been reported (Rozman *et al.*, 1986; Kleiman de Pisarev *et al.*, 1989).

The mechanisms by which HCB exerts these adverse effects have been the subject of many studies over the past decades. For example, evidence has been obtained that disturbances in thyroid hormone homeostasis by HCB is, at least in part, related to alterations in thyroid hormone metabolism (e.g., increased deiodination and/or glucuronidation), resulting in increased elimination from the body (Kleiman de Pisarev *et al.*, 1989). Impaired thyroid hormone synthesis does not seem to be involved (Kleiman de Pisarev *et al.*, 1990). Recent studies, however, pointed at an important role for hydroxylated metabolites of HCB in the observed plasma thyroid hormone decrease through a mechanism involving plasma transport (Van Raaij *et al.*, 1991).

Furthermore, HCB-induced porphyria is believed to originate from a selective and irreversible inactivation of uroporphyrinogen decarboxylase (UROG-D), a key enzyme in haem synthesis (Elder *et al.*, 1976). However, the mechanism of inactivation of

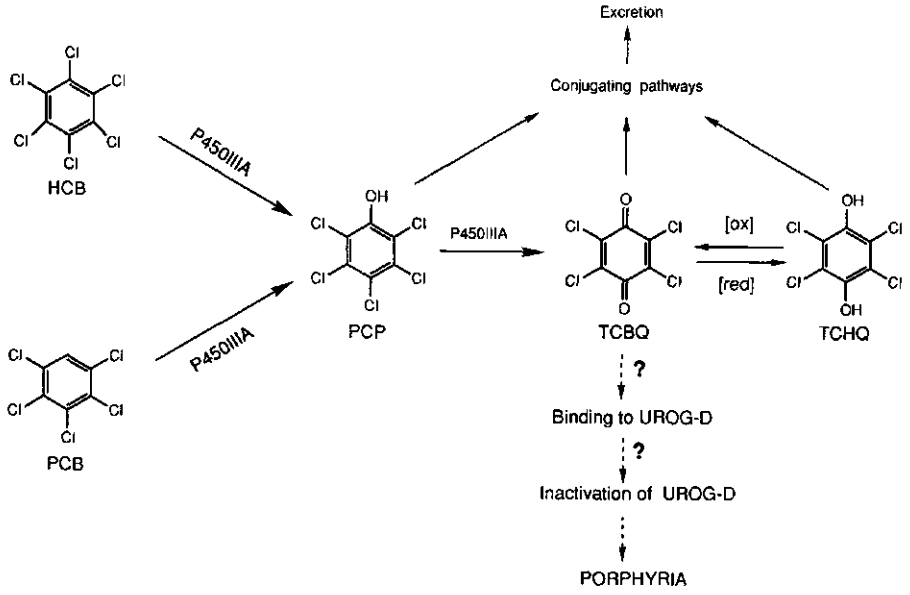


Figure 8.1 Formation of TCBQ as a common metabolite of HCB and PCB, and its proposed role in the induction of porphyria.

UROG-D is still a matter of debate. One of the main hypotheses regarding the mechanism of HCB-induced porphyria is based on the assumption that the parent compound is oxidized to reactive derivatives (Debets *et al.*, 1980b; Carpenter *et al.*, 1984), which may subsequently covalently bind in or near the active site of UROG-D, resulting in inactivation of this enzyme (Debets *et al.*, 1981; Billi de Catabbi, 1991). Although biotransformation of HCB appears to be a prerequisite for its porphyrinogenic action, none of the known metabolites is directly porphyrinogenic when administered *in vivo* (Wainstok de Calmanovici and San Martin de Viale, 1980; Koss *et al.*, 1979). Two oxidative metabolites, pentachlorophenol (PCP) and tetrachlorohydroquinone (TCHQ), displayed a direct inhibitory effect on partially purified UROG-D (Billi *et al.*, 1986); however, the concentrations used in this study by far exceeded the level these compounds may reach in the liver as metabolites of HCB. Based on these studies, it appears that if a reactive metabolite is involved in the induction of porphyria, its metabolic formation *in situ* is a prerequisite.

The formation of tetrachlorobenzoquinone (TCBQ), a secondary metabolite of HCB, can be hypothesized as a plausible pathway ultimately leading to porphyria. TCBQ has a

uniquely high reactivity towards nucleophiles, due to its perhalogenated character. After reaction of TCBQ with a nucleophile (such as e.g., glutathione), the quinone structure is retained, and thus its reactivity (Van Ommen *et al.*, 1988). The formation of TCBQ, as measured by the excretion of its reduced analog TCHQ, has been reported to be strongly correlated to the porphyrinogenic action of HCB (Van Ommen *et al.*, 1989). However, direct proof for its involvement in HCB-induced porphyria is still lacking, since studies aimed at demonstrating the direct interaction of reactive metabolites with UROG-D have not been successful thus far.

In vitro experiments with rat liver microsomes showed that the major P-450 isoenzyme involved in the oxidation of both HCB (to PCP) and PCP (to TCBQ) belongs to the P450IIIA subfamily, which is induced by glucocorticoids and macrolide antibiotics (Van Ommen *et al.*, 1989). Some macrolide antibiotics, like triacetyloleandomycin (TAO), induce P450IIIA1/2 to a high degree, but simultaneously form a catalytically inactive P450-TAO metabolite complex (Pessayre *et al.*, 1981). In a recent study, TAO was used as a tool to selectively inhibit the oxidative biotransformation of HCB. In rats co-treated with HCB and TAO, a significantly decreased excretion of both PCP and TCHQ correlated with a greatly diminished extent of porphyria compared to rats treated with HCB alone (Van Ommen *et al.*, 1989). Based on these results, it was suggested that the formation of the reactive TCBQ may be a crucial factor in the porphyrinogenic action of HCB.

Interestingly, the microsomal oxidation of pentachlorobenzene has also been shown to result in the formation of the reactive TCBQ via oxidative dehalogenation of the primary metabolite PCP (Den Besten *et al.*, 1989). Furthermore, microsomes from rats pretreated with dexamethasone, an inducer of cytochrome P450IIIA1, showed a high catalytic activity towards PCB similar to that of HCB (Den Besten *et al.*, 1991). Thus, if the *in situ* formation of TCBQ is relevant to the development of porphyria, PCB should resemble HCB in its porphyrinogenic action, as hypothetically illustrated in Figure 8.1.

Therefore, the present study was designed to seek further support for the role of the reactive TCBQ in HCB-induced porphyria by comparing both oxidation products and the development of porphyria in rats treated with HCB or PCB in their diet for 13 weeks. The contribution of P450IIIA to the observed effects is assessed by studying the effects in rats which were co-treated with TAO. Furthermore, a comparison between biotransformation of HCB and PCB, and their possible effects on other targets such as thyroid hormone homeostasis might indicate a role for common metabolites in these effects. Since structurally related compounds like the polychlorinated biphenyls have been described to disturb both thyroid hormone- and retinol homeostasis, the effects of HCB and PCB on plasma and liver retinol/ retinoid levels were also evaluated.

Materials and Methods

Chemicals

Pentachlorobenzene (>99.5 % purity) was purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Hexachlorobenzene (gold label, > 99 % purity) was purchased from BDH Chemicals Ltd., United Kingdom. Triacetyloleandomycin (TAO) was from Pfizer, Orsay, France.

Animals and treatment

One week prior to the start of the study, eighty-one female Wistar rats (160 - 200 g) were ad random divided into 9 treatment groups of 9 rats each, with each group having approximately the same mean body weight. Animals were housed in a temperature- and humidity-controlled room under a 12 hours light:dark cycle. Rats within each treatment group were randomly divided and housed in wire bottom cages (three rats per cage) in order to prevent the ingestion of urine and faeces. Food (standard laboratory chow, Hope Farms, Woerden, The Netherlands) and tap water were supplied ad libitum. After 1 week of acclimatization, the rats were fed the following diets: Control; TAO (0.3 %); HCB low (0.015 %); HCB high (0.03 %); HCB high (0.03 %) + TAO (0.3%); PCB low (0.03 %); PCB high (0.13 %); PCB low (0.03 %) + TAO (0.3 %); PCB high (0.13 %) + TAO (0.3 %). All diets contained 4 % corn oil. Diets were prepared in two batches, at the start of the experiment and in week 7, and stored in a dark, cool room of low humidity. Accurate food consumption per cage was determined weekly and corrected for the chow spilled. One day each week, rats were transferred into individual metabolism cages for 24 hr urine collection. Body weight was measured weekly at the start of the experiment, and more frequently later when animals showed signs of distress. Clinical observations were made daily, and rats were terminated when a body weight loss of more than 10 % in 24 hr over two consecutive days occurred. Plasma was collected at t=0, 1 and 5 weeks by orbita punction under marginal ether anesthesia. Rats were killed after a treatment period of 13 weeks by exsanguination via the abdominal aorta under marginal ether anesthesia. Blood (7 ml) was collected and plasma was prepared in heparin-containing tubes by centrifugation. Liver, kidneys, thyroid, adrenal glands, spleen, thymus, popliteal and mesenteric lymph nodes were excised and weighed. Thyroid, adrenal glands and a sample of liver and kidney was fixed in buffered formalin for microscopical examination (H&E staining). Lungs were intratracheally fixed with gelatin and one lung was fixed in formalin for microscopical examination. The other lung, spleen, thymus and lymph nodes were frozen in liquid nitrogen for immunohistochemical analysis (results on lung and organs related to the immune system will be published elsewhere). Histopathological examination of liver, kidney, adrenal and thyroid was only performed on tissues obtained from control rats and rats fed diets containing TAO alone, HCB high, PCB high, HCB high+TAO and PCB high+TAO.

Two samples (of approximately 1 g each) of each liver were frozen in liquid nitrogen for determination of porphyrin content, and stored at -80 °C until analysis. The remaining part of the liver was weighed, frozen on dry ice in a 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, and stored at -80 °C.

Biochemical determinations

Plasma total thyroxine (TT4), total triiodothyronine (TT3) and free thyroxine (FT4) levels were measured by standard procedures using the Amerlite TT4, TT3 and FT4 assays (Amersham, England). Plasma retinol and liver retinoids were determined in control rats and rats treated with HCB high and PCB high. Plasma retinol was extracted as previously described (Brouwer and Van den Berg, 1984). Livers were homogenized in ice-cold Tris-HCl (1/5, w/v) using a Teflon pestel. Whole homogenate samples were frozen at -80 °C for determination of retinoid content. Liver retinoids were extracted by a modification of the method of Olson (Olson *et al.*, 1979) as previously described (Brouwer *et al.*, 1988a). Extracts were analyzed for retinol and retinyl palmitate as reported (Brouwer *et al.*, 1988a). Plasma asparagine amino transaminase (AST), alanine amino transaminase (ALT) activity and blood urea nitrogen level (BUN), indicative of hepatotoxicity and nephrotoxicity respectively, were determined employing a Cobas-Bio centrifugal analyzer and commercially available kits (reagents 3146, 3147 and 3162, Baker T.J., Deventer, The Netherlands; kit 7-15379, Hoffman-Laroche, Basel, Switzerland).

Determination of porphyrins in urine and liver

The amount of total porphyrin in urine was determined fluorimetrically according to Schwartz *et al.*, (1976), modified by Debets *et al.*, (1981). Liver porphyrin content was determined according to the same method, after preparations as described by Van Ommen *et al.*, (1989).

Determination of urinary excretion of PCP and TCHQ

Urinary excretion of pentachlorophenol (PCP) and tetrachlorohydroquinone (TCHQ) was determined after 1, 4, 6, 8, 10, 12 and 13 weeks of treatment. Urinary metabolites were extracted as previously described (Van Ommen *et al.*, 1989). Separation was achieved on a Nucleosil RP18 column (150x4.6 mm I.D.) with water and methanol as mobile phases (both containing 0.5 % acetic acid). The following gradient elution profile was applied: 2 min isocratically at 50 % methanol followed by an increase to 90 % methanol in 17 min, followed by 5 min stationary at 90 % methanol and concluded with 10 min at 95 % of methanol. The flow rate was 1 ml/min, and the eluent was monitored at 300 nm. Analysis of urine extracts from HCB-treated rats revealed 2 major and 2 minor peaks, whereas 5 major and 4 minor peaks were detected in the urine extracts from PCB-treated rats. A full report on the isolation and identification of these metabolites will be given elsewhere (Chapter 9). Comparison of the HPLC elution

profiles revealed that PCP and TCHQ were the only two common urinary metabolites derived from HCB and PCB. Quantification of the amounts of PCP and TCHQ was accomplished by comparing peak areas to those obtained from a calibration curve of reference standards. Recovery was determined by the addition of [^{14}C]-PCP (10,000 dpm) to urine samples as an internal standard prior to extraction. This amount of PCP did not contribute to the UV absorption at 300 nm during HPLC analysis. Routinely, a recovery of more than 70 % was obtained.

Statistical analysis

Data were analyzed statistically using a non-parametric ANOVA according to Kruskal-Wallis using Chi-squared approximation (two-sided). Correlation between metabolite excretion and porphyria was computed using Spearman's rank correlation coefficient, which was tested for significancy (two-sided) using Student's *t*-distribution with *n-2* degrees of freedom (*n,* number of paired observations). Data on the histopathological changes were analyzed statistically with the Fischer exact probability test (two-sided).

Results

Mortality and clinical symptoms

Mortality and clinical symptoms were limited to rats in the HCB low, HCB high, and HCB high+TAO groups. In the HCB high group, 4 animals were sacrificed before the end of the study (i.e., after 57, 74, 81, and 88 days of treatment), because of severe weight loss and distress. In the HCB high+TAO group, one rat died after 85 days. These prematurely dead animals did not show signs of a higher extent of porphyria than surviving rats in the same group (data not shown). Skin lesions were observed in the HCB high group (from the 5th week on), in the HCB high+TAO group (from the 6th week on) and in the HCB low group (from the 8th week on). From week 8 on, rats in the HCB high and HCB high+TAO group developed tremors.

Food intake and body weight gain

Final body weight was significantly lower in rats treated with TAO alone, PCB high, and in the PCB +TAO treated rats (Table 8.1), which correlated with a significant decrease in food intake. As a result of decreased food intake, the amount of PCB ingested on a daily basis was slightly lower in the TAO co-treated animals (both PCB low and PCB high). Final body weight of surviving rats treated with HCB low or HCB high was not significantly different from control rats.

Table 8.1 Effect of HCB and PCB on body weight gain and food intake.

Treatment	Initial Body wt (g)	Final Body wt[a] (g)	Food Intake (g/cage/ week)	Amount of chemical ingested (mg/kg/d)[b]
Control	174 ± 3	243 ± 5	295.7 ± 0.2	0
Control+TAO	173 ± 3	216 ± 4[¶]	265.6 ± 0.6[¶]	0
HCB low	176 ± 4	254 ± 4	302.5 ± 0.6	9.5
HCB high	179 ± 3	260 ± 6	315.1 ± 3.0[¶]	19.0
HCB high+TAO	177 ± 4	240 ± 3	287.5 ± 3.4[§]	19.1
PCB low	173 ± 3	246 ± 4	297.3 ± 1.8	19.1
PCB high	173 ± 3	227 ± 3[¶]	288.1 ± 1.8[¶]	84.1
PCB low+TAO	177 ± 4	218 ± 6[¶]	263.7 ± 2.8[¶§]	17.9
PCB high+TAO	174 ± 4	222 ± 6[¶]	280.1 ± 2.7[¶]	81.4

Note. Groups of nine female rats were exposed for 13 weeks to HCB or PCB in their diet, with or without co-treatment with TAO, as described under Materials and Methods. Data are represented as Mean±SE. [a] Values represent mean±SE of 9 animals, except in the HCB high group (5 surviving rats) and in the HCB high+TAO group (8 surviving rats). [b] Approximate figure based on average food intake and body weight. [¶] Statistically significant difference compared to control group, $p < 0.05$; [§] Statistically significant difference compared to rats receiving same treatment without TAO, $p < 0.05$.

Organ weight to body weight ratio

Table 8.2 shows organ weights expressed as unit of body weight (wt). All treatments resulted in a significant increase in liver-to-body weight ratio. Rats in the HCB high, HCB high+TAO, and PCB high+TAO groups had significantly higher kidney-to-body wt ratio's, whereas rats treated with PCB high alone showed a tendency to higher kidney-to-body wt ratio's ($p = 0.057$). Significantly higher adrenal-to-body wt ratio's were observed both in rats treated with the HCB high dose and HCB high+TAO, and also in rats treated with TAO alone. No significant alterations were observed in relative thyroid weights between control and treatment groups.

Table 8.2 Effect of HCB and PCB on relative organ weights.

Treatment	Organ weight per 100 g Body wt[a] or 10,000 g Body wt[b]			
	Liver[a]	Kidney[a] (total of two)	Adrenal[b] (total of two)	Thyroid[b] (total of two)
Control	3.13 (0.17)	0.70 (0.02)	2.46 (0.11)	0.93 (0.12)
TAO	3.68[††] (0.15)	0.71 (0.02)	2.85[†] (0.10)	0.85 (0.10)
HCB low	4.10[†††] (0.10)	0.74 (0.01)	2.66 (0.07)	1.13 (0.11)
HCB high	6.34[†††] (0.25)	0.93[†††] (0.04)	4.16[††] (0.46	0.84 (0.09)
HCB high+TAO	6.40[†††] (0.37)	0.84[††] (0.03)	3.06[†] (0.20)	0.92 (0.11)
PCB low	3.56[†] (0.08)	0.70 (0.02)	2.33 (0.10)	0.71 (0.09)
PCB high	4.63[†††] (0.14)	0.78 (0.02)	2.67 (0.13)	0.82 (0.14)
PCB low+TAO	3.91[††] (0.14)	0.75 (0.02)	2.67 (0.08)	0.85 (0.09)
PCB high+TAO	4.98[†††] (0.09)	0.82[††] (0.02)	2.71 (0.09)	0.90 (0.14)

Note. Groups of nine female rats received dietary treatment with HCB or PCB for 13 weeks, with or without co-treatment with TAO, as described under Materials and Methods. Organ weights are expressed relative to [a] 100 g body wt, or relative to [b] 10,000 g body wt, and presented as mean (SE). Statistically significant differences compared to control rats are indicated as follows: [†] $p < 0.05$, [††] $p < 0.01$, [†††] $p < 0.001$.

Clinical chemistry parameters

There were no significant differences between the different diets for plasma ALT, AST and BUN after 1, 5, or 13 weeks of treatment (data not shown), although two rats in the HCB high group had relatively high ALT values after 13 weeks (122 and 367 U/l versus 63±3 U/liter for control rats, mean±SE).

Histopathology

The histopathological observations are presented in Table 8.3. Hypertrophic hepatocytes with eosinophilic cytoplasm with thread-like basophilic structures were observed in the liver of animals in the HCB high group. The hypertrophy, which was especially prominent in the centrilobular area, was less severe in PCB high-treated rats. The incidence of inflammatory cell infiltrates, especially periportal mononuclear inflammatory cells, was increased in the HCB high rats only. In addition, minimal to moderate bile duct proliferation was found in the HCB high rats only.

Effects on the kidneys and adrenals were observed only in the HCB high-treated rats. In the kidneys, an increased incidence of basophilic renal tubules and the presence of proteinaceous casts was observed in HCB-high rats, whereas in the adrenals hypertrophy and hyperplasia of the cortex cells was seen, occasionally accompanied by haemorrhage, cortical cell vacuolation and inflammatory cell infiltrates in the adrenal cortex. In several PCB-high treated rats, slight hypertrophy and hyperplasia of adrenal cortex cells was also observed. The meaning of these isolated findings remains unclear.

Slightly activated appearance of the thyroid was observed in several animals in the HCB high-group, but the incidence was just outside the statistically significance range ($p = 0.05$).

Treatment of rats with TAO alone resulted in changes in the liver, consisting of vacuolation of the hepatocytes, resembling macrovesicular fatty change. No distinct lobular pattern was observed.

Combined exposure to HCB high+TAO or PCB high+TAO did not result in a diminution of the effects of HCB and PCB, respectively (Table 8.3). The only effect was noticed in the liver, where the cytoplasm of the hypertrophic hepatocytes was altered from eosinophilic with thread-like basophilia, as seen in the HCB and PCB group to a more homogeneous lightly-eosinophilic cytoplasm with accentuation of the cell membrane by a condensed eosinophilic staining. Hepatocyte vacuolation, as observed in the TAO group was also seen in the HCB high+TAO group, but not in the PCB high+TAO group.

Table 8.3 Histopathological lesions in rats treated with HCB and PCB with or without TAO.

Tissue Observation	Control	TAO	HCB high	PCB high	HCB high +TAO	PCB high +TAO
Liver						
Hypertrophy	0	0	9^{111}(3.2)	9^{111}(2.3)	8^{111}(2.9)	9^{111}(2.4)
Periportal mc infiltrate	0	0	6^{11}(1.8)	1 (1.0)	6^{11}(1.7)	4 (1.0)
RES/necrotic hepatocytes	5 (1.2)	8 (2.3)	9 (2.2)	4 (1.3)	5 (1.8)	4 (1.3)
Bile duct proliferation	0	2 (1.0)	9^{111}(1.9)	0	8^{111}(1.9)	2 (1.0)
Kidney						
Basophilic tubules	2 (1.5)	0	9^{11}(2.6)	2 (1.0)	7^{1}(2.7)	5 (1.4)
Proteinaceous cast(s)	0	0	5^{1}(2.0)	0	6^{11} (1.8)	2 (1.0)
Adrenal						
Hypertrophy + Hyperplasia cortex cells	0	0	9^{111}(4.0)	8^{111}(3.6)	1 (2.0)	2 (2.0)
Thyroid						
Activated appearance	1 (2.0)	2 (2.0)	6 (2.0)	1 (2.0)	4 (2.0)	5 (2.2)

Note. Female rats (nine per group) received dietary treatment with HCB or PCB, with or without co-treatment with TAO for 13 weeks, as described under Materials and Methods. At the end of the exposure period, liver, kidney, adrenal and thyroid were fixed in formalin for microscopic examination. Data represent incidence of lesion (average severity score within brackets) and are based on 9 rats per group, except in the HCB high+TAO group in which n=8 due to the premature death of one animal. Tissues of prematurely killed animals in the HCB high group (see text) are included. The average severity score was calculated on a scale of 1 to 5: minimal, 1; slight, 2; moderate, 3; marked, 4; severe, 5. Statistically significant differences are indicated as follows: $^{1}p < 0.05$, $^{11}p < 0.01$, $^{111}p < 0.001$.

Urinary excretion of porphyrins

As shown in Figure 8.2, urinary excretion of porphyrins was significantly increased in rats treated with a high dose of HCB from the 4th week on. Addition of TAO to the diet greatly reduced the porphyrinogenic activity of HCB to levels observed after

exposure to a low dose of HCB (0.015 %), which were only significantly different from control values from the 10th week on. Rats treated with PCB (high dose) also had very slightly but statistically significantly elevated urinary porphyrin levels compared to control rats. However, in PCB-treated rats there was no sign of progressive porphyria with treatment time, but urinary porphyrin levels rather decreased at the end of the treatment period.

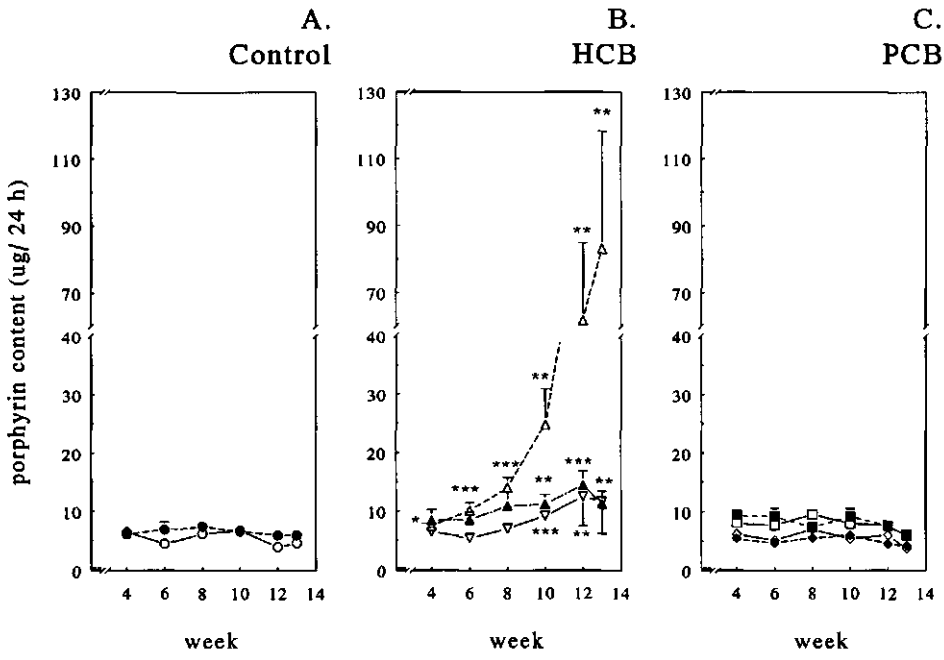


Figure 8.2 Urinary excretion of porphyrins in untreated female rats (A) and female rats, which received dietary treatment with HCB (B) or PCB (C) for 13 weeks, with or without co-treatment with TAO. Data are presented as mean±SE. Symbols: (A), ○ , control; ● , TAO-control: (B), ▽ , HCB-low; △ , HCB-high; ▲ , HCB-high+TAO; (C), ◇ , PCB-low; ◆ , PCB-low+TAO; □ , PCB-high; ■ , PCB-high+TAO. Statistically significant differences compared to control rats are indicated as follows: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. In Figure C, porphyrin content is significantly different from control rats, $p<0.05$, at all time points in PCB-high treated rats, and, with the exception of week 8 and week 10, in PCB-high+TAO treated rats.

Table 8.4 Effect of HCB and PCB on liver porphyrin levels.

Treatment	Porphyrin level[a] (μg/g liver)	(range)
Control	0.28± 0.02	(0.21 - 0.40)
TAO	0.24± 0.02	(0.16 - 0.35)
HCB low	3.23± 2.60[¶]	(0.20 - 24.0)
HCB high	42.57± 17.07[¶¶¶]	(2.72 - 147.67)
HCB high+TAO	2.34± 1.55[¶¶]	(0.24 - 14.67)
PCB low	0.25± 0.02	(0.15 - 0.29)
PCB high	0.32± 0.02	(0.25 - 0.44)
PCB low+TAO	0.22± 0.01	(0.16 - 0.27)
PCB high+TAO	0.24± 0.02	(0.17 - 0.35)

Note. Groups of nine female rats received dietary treatment with HCB or PCB, with or without co-treatment with TAO for 13 weeks, as described under Materials and Methods. Liver porphyrin levels were determined fluorimetrically as described under Materials and Methods, and are expressed as mean±SE (range) of 9 animals. Livers of prematurely dead animals are included. Statistically significant differences are indicated as follows: [¶] $p < 0.05$, [¶¶] $p < 0.01$, [¶¶¶] $p < 0.001$.

Accumulation of porphyrins in liver

As shown in Table 8.4, rats in the HCB high group had significantly elevated accumulation of porphyrins in the liver. The addition of TAO to their diet diminished this accumulation to a great extent, although not completely. A few rats in the HCB low group also had higher liver porphyrin levels. Rats treated with PCB did not show any signs of accumulation of porphyrins in liver.

Thyroid hormone homeostasis

In Figure 8.3, the effects of HCB and PCB on plasma thyroid hormone levels are presented. Rats treated with HCB (both low and high dose) had significantly decreased plasma TT4 (total thyroxine; Figure 8.3A), plasma FT4 (free thyroxine; Figure 8.3B) and plasma TT3 (total triiodothyronine; Figure 8.3C) levels after 5 and 13 weeks of treatment. In rats treated with PCB (both low and high dose), maximally decreased levels

of TT4 (Figure 8.3D), FT4 (Figure 8.3E) and TT3 (Figure 8.3F) were already observed after 1 week of treatment. After 13 weeks, however, plasma TT3 levels in PCB-treated rats were back to control levels, whereas in HCB-treated rats TT3 levels were still significantly decreased at the end of the study. Rats treated with TAO alone showed plasma thyroid hormone levels higher than untreated (control) rats ($p < 0.01$ after 5 weeks (TT4) and 13 weeks (TT3)), making the changes observed in rats receiving co-treatment with HCB or PCB rather difficult to interpret.

Retinoid homeostasis

Liver and plasma retinoid levels in rats receiving the HCB high or the PCB high treatment are presented in Table 8.5. One control rat showed an extremely high liver retinol level (248 μg/g liver) compared to the other rats in the same group (29 ± 3.7, mean$\pm$SE, n=8), resulting in a high standard error. This value was, however, not omitted, since it did not influence statistical significancy between treatment groups (control vs HCB or control vs PCB).

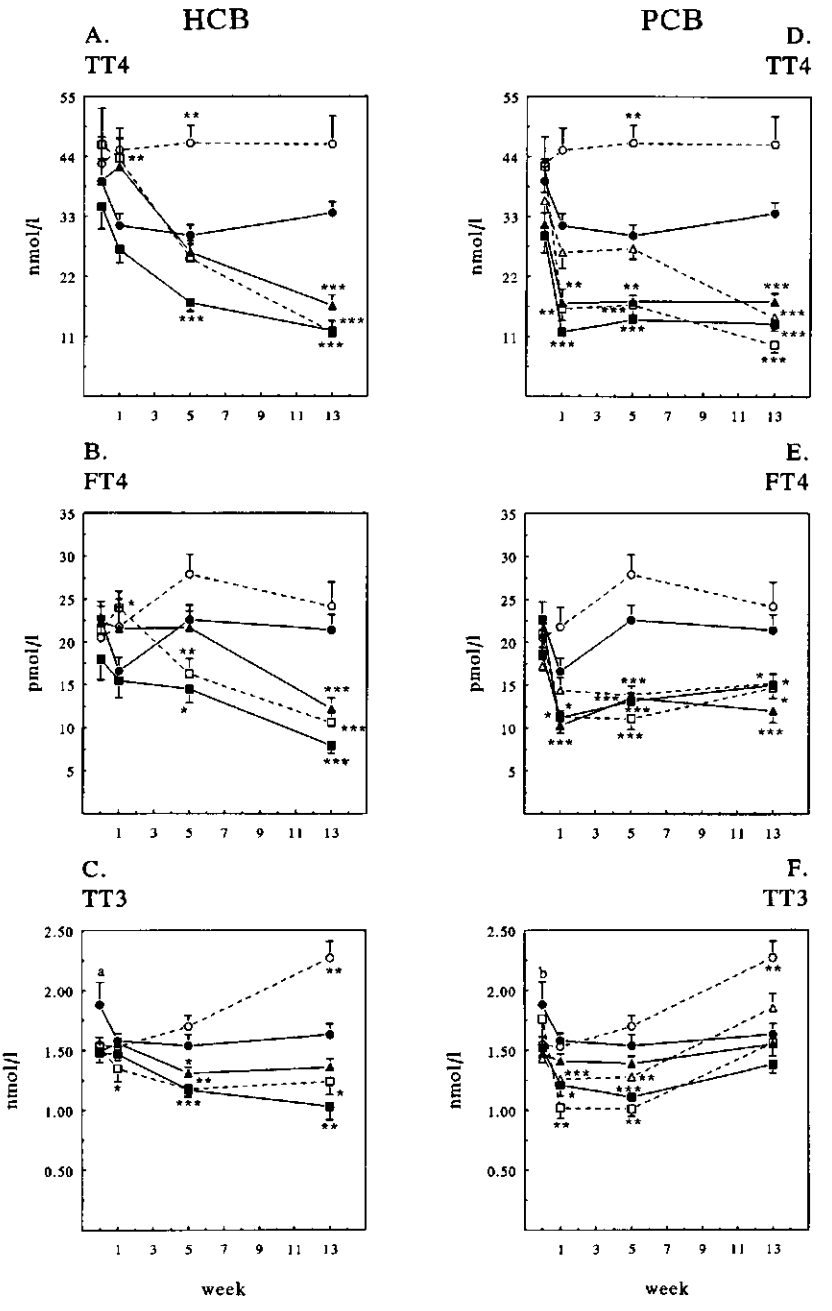
As shown in Table 8.5, liver retinol content was significantly decreased in rats treated with a high dose of HCB compared to control rats. In addition, the amount of liver retinyl palmitate was decreased both in HCB high and PCB high-treated rats.

Plasma retinol levels were decreased after 13 weeks of treatment with a high dose of HCB. In contrast, plasma retinol levels in PCB high-treated animals were significantly higher compared to levels in control rats, both after 1 week and 13 weeks of treatment.

Urinary excretion of PCP and TCHQ

Urinary excretion of PCP and TCHQ, the reduced equivalent of the reactive TCBQ, as determined by HPLC, is shown in Figure 8.4A, B, and C for HCB, and in Figure 8.4D, E and F for PCB treated rats, respectively. In fact, PCP and TCHQ were the only two common urinary metabolites after administration of HCB and PCB to rats.

Figure 8.3. Plasma total thyroxine (TT4; A, D), free thyroxine (FT4; B, E) and total triiodothyronine (TT3; C, F) levels in female rats which received dietary treatment with HCB or PCB for 13 weeks, with or without co-treatment with TAO. Data are presented as mean+SE. Symbols indicate the following: (●), control; (○) TAO-control; (▲) HCB low or PCB low; (△) PCB low+TAO; (■) HCB high or PCB high; (□) HCB high+TAO or PCB high+TAO. * significantly different from levels in control rats, $p<0.05$, ** $p<0.01$, *** $p<0.001$. ^{a} control significantly different from all other treatments, $p<0.05$; ^{b} control significantly different from all other treatments with the exception of PCB high+TAO, $p<0.05$.


HCB
PCB
A.
TT4
D.
TT4
B.
FT4
E.
FT4
C.
TT3
F.
TT3
nmol/l
pmol/l
week

A full report on the identification of urinary metabolites of HCB and PCB will be given elsewhere (Chapter 9).

When the content of HCB in the feed is doubled from 0.015 % (HCB low) to 0.03 % (HCB high), the excretion of PCP and TCHQ is increased disproportionally (cfg. Figure 8.4A,B). Co-administration of HCB high with TAO resulted in a strongly diminished excretion of both metabolites to amounts comparable to those excreted by rats treated with a low dose of HCB (Figure 8.4C). Similarly, the administration of TAO to the PCB-containing diets inhibited the oxidation of PCB to PCP and TCHQ (both PCB low dose (not shown) and high dose, Figure 8.4F).

The present study was aimed at the comparison between HCB and PCB with respect to the role of their oxidation products in toxicity. Therefore, it is important to compare the extent of formation of the two common oxidation products between treatment groups. Comparison of a similar dose (0.03 %) of HCB and PCB (i.e., HCB high vs PCB low, Figure 8.4B vs Figure 8.4D) reveals that, with the exception of the first 4 weeks, HCB was converted to PCP to a higher extent than PCB was. In contrast, urinary TCHQ excretion was significantly higher in PCB low treated rats (Figure 8.4D) compared to the HCB high treated rats (Figure 8.4B) until week 10 (with the exception of week 6), whereas after 13 weeks of treatment excretion of TCHQ was significantly higher in HCB high treated rats.

When the concentration of PCB in the feed is increased to 0.13 % (PCB high), the excretion of PCP is strongly increased in the first 2 weeks of exposure, and then reaches a plateau, indicating saturation of this pathway (Figure 8.4E). PCP excretion in rats treated with the HCB high dose (0.03%) reached the same plateau value after 12 weeks. TCHQ excretion is also increased in PCB high-treated rats compared to PCB low-treated rats, and reaches a plateau after 6 weeks. With exception of the last 2 weeks of the treatment period, excretion of these two metabolites is significantly higher in the PCB high rats (0.13 %) when compared to rats treated with the HCB high dose (0.03 %). During the whole exposure period, excretion of PCP and TCHQ is significantly higher in rats treated with the PCB high dose (0.13%) compared to rats treated with the HCB low dose (0.015 %, Figure 8.4A).

Discussion

Porphyria is one of the most striking consequences of exposure to HCB. In spite of the fact that a great number of studies appears to indicate a crucial role for oxidative biotransformation of HCB in its porphyrinogenic action (Kerklaan *et al.*, 1977; Debets, 1980a; Wainstok de Calmanovici *et al.*, 1984; 1989), direct evidence which proves that the origin of porphyria is connected to the metabolic activation of HCB is still scanty.

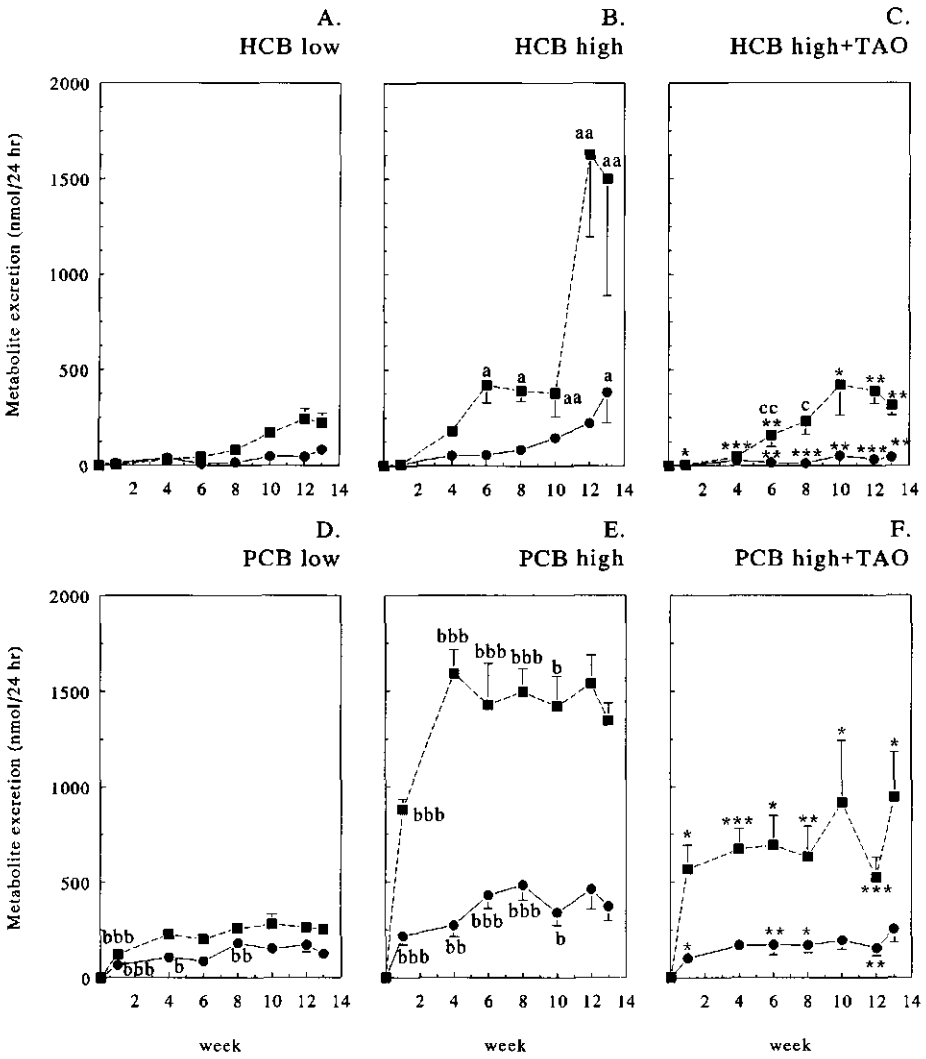


Figure 8.4. Urinary excretion of PCP (■) and TCHQ (●) in female rats which received dietary treatment with 0.015% HCB (HCB low, A), 0.03% HCB (HCB high, B) and 0.03% HCB + TAO (HCB high+TAO, C), 0.03% PCB (PCB low, D), 0.13% PCB (PCB high, E), and 0.13% PCB + TAO (PCB high+TAO, F). Data are presented as mean+SE.
* significantly different from excretion in rats receiving the same treatment without TAO, $p<0.05$, ** $p<0.01$, *** $p<0.001$; ^{a} significantly different levels compared to PCB low-treated rats (cf. Figure 8.4D), $p<0.05$, ^{aa} $P<0.01$; ^{b} significantly different levels compared to HCB high treated rats, $p<0.05$, ^{bb} $p<0.01$, ^{bbb} $p<0.001$; ^{c} significantly different levels compared to HCB low treated rats, $p<0.05$, ^{cc} $p<0.01$, ^{ccc} $p<0.001$.

Table 8.5 Effect of HCB and PCB on liver retinoid levels and plasma retinol levels.

	control	HCB high	PCB high
Liver retinoid			
retinol[a]	53.3± 24.5	15.8± 3.6[b,††]	22.7± 3.9
retinyl palmitate[c]	2.16± 0.08	1.04± 0.04[b,†††]	1.30± 0.09[†††]
Plasma retinol[d]			
at t= 0	0.34± 0.01	0.32± 0.02	0.34± 0.02
after 1 week	0.22± 0.01	0.22± 0.01	0.34± 0.02[†††]
after 13 weeks	0.21± 0.01	0.16± 0.01[e,†]	0.34± 0.03[††]

Note. Groups of nine female rats received dietary treatment with HCB or PCB with or without TAO for 13 weeks, as described under Materials and Methods. Plasma retinol and liver retinoid levels are determined as described under Materials and Methods. Data (mean±SE, n=9) are expressed per [a] μg/g liver, [c] mg/g liver, and [d] μg/ ml. [b] Liver retinoid levels in prematurely dead rats are also included. [e] Due to prematurely killed animals, data are based on n=5 rats. Statistically significant differences compared to control rats are indicated as follows: [†] $p<0.05$, [††] $p<0.01$, [†††] $p<0.001$.

The experimental design used in the present study, in which HCB and PCB were compared with respect to their main adverse effects, allowed us to assess the contribution of *in situ* formation of TCBQ, the highly reactive metabolite of HCB and thus a likely candidate for the porphyrinogenic species.

Previous reports indicate the involvement of cytochrome P450IIIA1/2 in the microsomal oxidation of HCB (Van Ommen *et al.*, 1989) and PCB (Den Besten *et al.*, 1991) to PCP and TCHQ. The present study clearly shows that extrapolation of these microsomal findings to the *in vivo* situation is valid, since selective inactivation of cytochrome P450IIIA1/2 by TAO in rats treated with HCB or PCB results in a dramatic decrease in the urinary excretion of PCP and TCHQ.

The present study confirms earlier findings by Van Ommen *et al.*, (1989) that, in addition to decreased metabolite excretion, the porphyrinogenic action of HCB was greatly reduced when rats were treated with a combination of HCB+TAO. In HCB treated groups, (mean) excretion of both PCP and TCHQ as a function of treatment time was compared with the extent of urinary porphyrin excretion as a function of treatment

time using Spearman rank correlation, and were found to be strongly correlated (r= 0.943, 0.886, 0.899 for excretion of PCP vs urinary porphyrin excretion in the HCB low, HCB high and HCB high+TAO treatments; r= 0.771, 0.999, 0.579 for excretion of TCHQ vs urinary porphyrin excretion in the HCB low, HCB high and HCB high + TAO treatments). However, a statistically significant correlation between two factors that both increase with prolonged treatment time does not necessarily imply a causal relationship, but may simply be due to a common cause, i.e., the exposure to HCB. Because variation in the extent of porphyria between rats within each HCB treatment group is considerable (cf. Figure 8.2 and Table 8.3), a more meaningful correlation can be computed based on paired observations made on individual rats within each treatment group. Thus, the question may be raised whether within each HCB-exposed group, rats which are highly porphyric also have a high metabolite excretion, and vice versa, rats which are less porphyric have a lower extent of metabolite excretion. Correlation coefficients thus calculated are presented in Table 8.6. These data indicate that the correlation between oxidative biotransformation cumulated over the entire treatment period and porphyria at the end of the study is rather weak, with a statistically significant correlation observed only between urinary excretion of PCP in rats receiving the HCB low treatment and liver porphyrin accumulation. However, when all paired observations concerning metabolite excretion and porphyria in rats receiving the HCB low, HCB high or HCB high+TAO treatments were combined, urinary excretion of both PCP and TCHQ were significantly correlated with the extent of porphyria.

Inhibition of UROG-D has been shown to be an early event in HCB-induced porphyria, preceding the manifestation of the porphyric condition (Koss *et al.*, 1983; Smith *et al.*, 1986). For example, UROG-D activity dropped soon after the start of HCB exposure in mice, but overt porphyria was not observed until weeks later (Smith and Francis, 1983). With respect to this long latency period in the induction of porphyria, it might be relevant to compare the degree of urinary metabolite formation in the first part of the exposure period with the degree of porphyria observed at the end of the study. These data are also included in Table 8.6. Both for the HCB high group and for the combined treatments, the degree of porphyria correlated well with the excretion of PCP, whereas correlation between porphyria and excretion of TCHQ was much weaker. These data are not supportive for the involvement of the reactive TCBQ, the oxidized analog of TCHQ, in HCB-induced porphyria.

In the present study, urinary metabolite excretion and the degree of porphyria was also monitored in rats treated with PCB. This chemical is also oxidized to PCP and TCHQ (Den Besten *et al.*, 1989; 1991). Rats receiving the PCB high dose had a several fold higher urinary excretion of both PCP and TCHQ compared to rats receiving the HCB high treatment. Although rats receiving the PCB high treatment had slightly elevated urinary porphyrin levels compared to control rats, PCB does not cause hepatic porphyria in a way similar to HCB, because of the fact that (i) no sign of liver porphyrin accumulation was observed, and (ii) urinary porphyrin levels actually decreased at the

end of the treatment period instead of showing a progressive increase. More likely, the slight increase in urinary porphyrins may be the result of the ability of PCB to induce hepatic haem-containing microsomal enzymes (which is indicated by the increased liver weight and centrilobular hypertrophy), rather than originating from a disturbance in the haem synthesis. A similar effect has also been described for 1,2,4-trichlorobenzene (Kociba *et al.*, 1981). Thus, although (i) TCBQ was generated in PCB-treated rats in a mechanism comparable to TCBQ formation in HCB-treated rats (i.e., via oxidative dehalogenation of the primary metabolite PCP), and (ii) urinary TCHQ levels were even significantly higher in PCB high compared to HCB-treated rats, hepatic porphyria was only observed in HCB-treated rats. This provides an additional argument against a role for the oxidized analog of TCHQ, i.e., TCBQ, in HCB-induced porphyria.

The present study does not rule out the involvement of an intermediate formed in the primary oxidation of HCB in the induction of porphyria, since the oxidation of PCB to PCP and of HCB to PCP occur via two qualitatively different pathways, resulting in a simple hydroxylation of the former and an oxidative dehalogenation of the latter. In fact, urinary excretion of PCP showed a good correlation with porphyria (Table 8.6). Recently, the cytochrome P450-mediated oxidation of hexahalobenzenes to pentahalophenols has been proposed to proceed by the formation of a reactive benzohaloquinone cation intermediate (Rietjens and Vervoort, 1992). Whether such an intermediate is actually formed upon oxidation of HCB to PCP and whether it has any relevance to HCB-induced porphyria, remains an interesting topic for future research.

Several additional hypotheses have been put forward regarding the induction of porphyria by HCB (and related chemicals such as TCDD, polychlorinated -and polybrominated biphenyls), which favor the idea of inactivation of UROG-D activity by an indirect mechanism rather than by a direct, metabolite-enzyme interaction. For example, chemicals may induce specific cytochrome P450 isoenzymes (particularly enzymes of the P450IA family, Jacobs *et al.*, 1989; Smith and De Matteis, 1990), resulting in an increased oxidation of uroporphyrinogen to uroporphyrin (De Matteis *et al.*, 1988; Jacobs *et al.*, 1989) and thereby contributing to porphyrin accumulation. In addition, the interaction of drugs with the microsomal monooxygenase system may also lead to an uncoupled electron transport, resulting in the formation of active oxygen species which can react with uroporphyrinogen or another susceptible target to produce the long-lived inhibitor of the uroporphyrinogen decarboxylase activity, which has been isolated from porphyric animals (Cantoni *et al.*, 1984). The synergistic role of iron on chemical-induced porphyria, which has been demonstrated by a number of studies (Smith and Francis, 1983; Smith *et al.*, 1986), could arise from the ferrous-iron supported radical reactions (e.g., with reduced forms of oxygen). Thus, inhibition of UROG-D may be regarded as a secondary effect, and not as the primary biochemical lesion. In spite of all the intense research in the past decades, pieces of the puzzle still need to fall in place before the mechanism of HCB-induced porphyria is fully elucidated.

Table 8.6 Relationship between excretion of urinary oxidative metabolites and porphyria.

Treatment	Correlation coefficient between urinary porphyrin excretion at 13 weeks and: cumulative excretion over week 1-13 of PCP	TCHQ	cumulative excretion over week 1-6 of PCP	TCHQ
HCB low	0.633	0.433	0.477	0.233
HCB high[a]	-0.257	0.600	0.943[¶¶¶]	0.771
	(6)	(6)	(6)	(6)
HCB high+TAO	0.479	-0.192	0.503	-0.599
	(8)	(8)	(8)	(8)
Combined treatments[b]	0.773[¶¶¶]	0.536[¶¶]	0.815[¶¶¶]	0.212
	(23)	(23)	(23)	(23)

Treatment	Correlation coefficient between liver porphyrin accumulation[c] and: cumulative excretion over week 1-13 of PCP	TCHQ	cumulative excretion over week 1-6 of PCP	TCHQ
HCB low	0.767[¶]	0.550	0.628	0.150
HCB high[a]	-0.429	0.257	0.543	0.600
	(6)	(6)	(6)	(6)
HCB high+TAO	0.250	0.200	0.350	0.033
	(8)	(8)	(8)	(8)
Combined treatments[b]	0.708[¶¶¶]	0.583[¶¶]	0.712[¶¶¶]	0.281
	(27)	(27)	(23)	(23)

Note. Urinary excretion of oxidative metabolites (PCP, TCHQ) and porphyrins were measured after 2, 4, 6, 8, 10, 12, and 13 weeks of treatment as described under Materials and Methods. Spearman's coefficient of rank correlation between these parameters were calculated for paired observations (n=9). If number of paired observations is less than 9 due to premature sacrifice of rats, the number of paired observations is expressed within brackets. [a] Data obtained from one prematurely killed rat, which nearly completed the 13-week study (+ at day 88), is included in the calculations. [b] 'Combined treatments' indicates that the correlation coefficient is calculated using paired observations of all HCB-exposed rats. [c] Liver porphyrin accumulation at the end of treatment period; prematurely sacrificied rats were not included. [¶] Statistically significant correlation between two parameters, $p < 0.05$, [¶¶] $p < 0.01$, [¶¶¶] $p < 0.001$.

Histopathological investigation revealed that HCB induced lesions in the adrenals, liver and kidneys. PCB induced identical but less severe effects in liver as HCB, whereas TAO induced liver effects not comparable to HCB and PCB. Furthermore, co-treatment of rats with TAO and either HCB or PCB did not diminish the effects of HCB or PCB alone, which would indicate that the parent compounds themselves or metabolites formed via a non-oxidative pathway (e.g., direct glutathione conjugation) were responsible for the observed histopathological changes. Alternatively, the formation of even small amounts of oxidative metabolites such as formed in rats co-treated with TAO, might be sufficient to elicit the observed effects.

A remarkable outcome of the present study is the fact that exposure to either HCB or PCB results in a marked disturbance in thyroid hormone and retinoid homeostasis. A reduction in plasma thyroid hormone levels following exposure to HCB has been previously reported, although in those studies rats were treated with a much higher dose of HCB, and the decrease in plasma TT3 was equivocal (Rozman *et al.*, 1986; Kleiman de Pisarev *et al.*, 1989, 1990). The effects on retinoid homeostasis included both liver storage and plasma levels. The decrease in the liver retinoid storage by HCB or PCB indicates an increased mobilization of retinoids into the circulation, whereas the decline in both liver and plasma retinol in HCB high-treated rats suggests an enhanced elimination of retinoids from the organism (e.g., through increased glomerular filtration). The increase in plasma retinol observed in PCB high-treated rats can be explained by a more pronounced increase in the extent of mobilization from hepatic storage sites compared to the increase in the glomerular filtration processes.

HCB and PCB share these effects on thyroid hormone and retinoid homeostasis with structurally related compounds like polychlorinated biphenyls, and a common mechanism is strongly indicated. Hydroxylated metabolites of polychlorinated biphenyls have been shown to interfere with plasma transport of thyroid hormones through displacement of thyroxine from its binding site on transthyretin (TTR), a major plasma transport protein for thyroxine in the rat (Brouwer, 1989). Recently, *in vitro* binding studies have shown that hydroxylated metabolites of HCB and PCB, i.e., PCP and TCHQ, also have a high affinity towards TTR (Van den Berg, 1990; Den Besten *et al.*, 1991). In addition, *in vivo* studies reveal an inverse relationship between serum thyroid hormone levels and serum PCP concentrations after a single i.p. dose of PCP (Van Raaij, 1991). Thus, hydroxylated metabolites of HCB and PCB may be involved in the severely reduced plasma thyroid hormone levels, possibly through interference with thyroxine plasma transport as described above. The involvement of PCP and TCHQ in plasma thyroid hormone disturbance of the parent chlorinated benzenes is further supported in the present study by the observation that PCB is more extensively oxidized to PCP and TCHQ in the first week of treatment as compared to HCB. This correlates well with the observation that plasma thyroid hormone levels in PCB-treated rats are already maximally decreased within this first week of treatment. Unfortunately, no conclusions can be drawn from data obtained from rats which were co-treated with TAO, because

TAO itself is capable of causing a disturbance in thyroid hormone homeostasis (Perret *et al.*, 1991).

The disturbance in retinoid homeostasis may, at least in part, also be ascribed to the interference of the hydroxylated metabolites with TTR (Brouwer and Van den Berg, 1986), since plasma retinol is transported bound to a complex of retinol-binding-protein (RBP) and TTR. Binding of hydroxylated metabolites of polychlorinated biphenyls to TTR has been shown to interfere with formation of the plasma transport RBP-TTR complex, probably through a conformational change in TTR (Brouwer *et al.*, 1988b). An alternative way of interference of chlorinated aromatics with retinoid homeostasis is by a direct interaction with the hepatic storage and mobilization of retinol.

Thus, polychlorinated compounds with a single aromatic ring have effects on thyroid hormone and retinoid status comparable to those observed after exposure to polychlorinated biphenyls, probably, at least in part, through a common mechanism involving their biotransformation to hydroxylated metabolites and the subsequent interaction with the plasma transport protein, TTR. An interesting topic for future studies is the relevance of the disturbance in thyroid hormone homeostasis and retinoid metabolism in the toxic effects observed for these compounds.

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Chapter 9

COMPARISON OF THE URINARY METABOLITE PROFILES OF HEXACHLOROBENZENE AND PENTACHLOROBENZENE IN THE RAT

Abstract

The urinary metabolite profile of hexachlorobenzene (HCB) and pentachlorobenzene (PCB) in the rat is compared after dietary exposure for 13 weeks. Both HCB and PCB are oxidized to pentachlorophenol (PCP) and tetrachlorohydroquinone (TCHQ), which were the only two mutual metabolites formed. Additional urinary metabolites of HCB are *N*-acetyl-*S*-(pentachlorophenyl)-cysteine (PCP-NAC), quantitatively the most important product, and mercaptotetrachlorothioanisole (MTCTA), which was excreted as a glucuronide. PCB is more extensively metabolized to the major metabolites 2,3,4,5-tetrachlorophenol (TCP), mercaptotetrachlorophenol (MTCP) and the glucuronide of pentachlorothiophenol (PCTP), and the minor metabolites methylthiotetrachlorophenol (MeTTCP), hydroxytetrachlorophenyl sulphoxide (HTCPS), and *bis*-(methylthio)-trichlorophenol (*bis*MeTTriCP).

The biotransformation of HCB and PCB was modulated by selective inhibition of cytochrome P450IIIA1/2 in rats which received combined treatment of HCB or PCB with triacetyloleandomycin (TAO). Rats receiving this diet had a strongly diminished excretion of both PCP and TCHQ, as compared to rats fed HCB or PCB alone, indicating the involvement of P450IIIA1/2 in the oxidation of both compounds. However, the excretion of 2,3,4,5-TCP was not diminished by co-treatment of rats with PCB and TAO, indicating that (i) the oxidation of PCB to PCP and to 2,3,4,5-TCP does not proceed via a common intermediate, and (ii) oxidation of PCB to 2,3,4,5-TCP is not mediated by P450IIIA1/2. Co-treatment of rats with PCB and TAO had a differential effect on the excretion of sulfur-containing metabolites, resulting in a decrease in the excretion of PCTP glucuronide, whereas no change was observed in the excretion of MTCP, as compared to rats receiving PCB alone. The data presented in this study stress the importance of mechanistic *in vitro* studies, which should be directed at the interplay of P450-mediated oxidation and conjugation of the putative electrophilic metabolites with GSH, in order to delineate the pathways leading to the various metabolites.

C. den Besten, M.M.H. Bennik, M. van Iersel, M.A.W. Peters, C. Teunis, P.J. van Bladeren, *submitted*

Introduction

The biotransformation pathways of the fungicide hexachlorobenzene (HCB)[1], which is known for its porphyrinogenic action, has been extensively studied in many species including rat (Koss *et al.*, 1976; 1978; Jansson and Bergman, 1978), rabbit and mouse (Renner and Nguyen, 1984). A range of sulfur-containing metabolites, non-sulfur phenolic metabolites and non-sulfur-non-oxygen-containing metabolites has been described (Koss *et al.*, 1986; Renner, 1988). The formation of sulfur-containing derivatives proceeds via conjugation of HCB with glutathione, which is subsequently further processed into the corresponding mercapturic acid and other derivatives from that pathway (Renner *et al.*, 1978). Conjugation with glutathione is also a major degradation route in the biotransformation of the fungicide pentachloronitrobenzene (PCNB), producing sulfur-containing metabolites similar to those described for HCB. For extensive reviews on the common biotransformation products of HCB and PCNB, the reader is referred to Renner and coworkers, (1981, 1984).

Cytochrome P450-mediated oxidation of HCB results in the formation of pentachlorophenol (PCP) and tetrachlorohydroquinone (TCHQ) (Van Ommen *et al.*, 1986). The major isoenzyme involved in the hydroxylation of HCB has been indicated to be P450IIIA1/2 (Van Ommen *et al.*, 1989). Recent studies on the microsomal oxidation of chlorinated benzenes other than HCB revealed that pentachlorobenzene (PCB) is also converted to PCP and TCHQ (Den Besten *et al.*, 1989). Again, cytochrome P450IIIA1/2 was implicated to be the major P450 isoenzyme involved (Den Besten *et al.*, 1991).

Although these microsomal studies imply that PCB is degraded through biotransformation pathways identical to HCB, the extent to which they occur *in vivo* is not known. Therefore, the present study describes and compares the urinary metabolite profiles of HCB and PCB in rat. In order to assess the contribution of cytochrome P450IIIA isoenzymes to the *in vivo* biotransformation of HCB and PCB, urinary excretion profiles of rats treated with either HCB or PCB alone were compared to those of rats which received co-treatment with TAO, a compound which selectively inactivates P450IIIA1/2 by the formation of a stable metabolite-P450 complex (Pessayre *et al.*, 1981; Wrighton *et al.*, 1985).

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[1] Abbreviations: HCB, hexachlorobenzene; HTCPS, hydroxytetrachlorophenyl sulphoxide; MeTTCP, methylthiotetrachlorophenol; *bis*MeTTriCP, *bis*(methylthio)-trichlorophenol; MTCP, mercaptotetrachlorophenol; MTCTA, mercaptotetrachloro-thioanisole; PCB, pentachlorobenzene; PCP, pentachlorophenol; PCP-NAC, *N*-acetyl-*S*-(pentachlorophenyl)cysteine; PCTP, pentachlorothiophenol; TAO, triacetyloleandomycin; TCHQ, tetrachlorohydroquinone; TCP, tetrachlorophenol.

Materials and Methods

Chemicals

Pentachlorobenzene (>99% purity) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Hexachlorobenzene (gold label, > 99% purity) was purchased from BDH (United Kingdom). Triacetyloleandomycin was from Pfizer (Orsay, France). Tetrachlorohydroquinone was from ICN Biomedicals (Zoetermeer, The Netherlands), 2,3,4,5-tetrachlorophenol was from Fluka AG (Buchs, Schwitzerland), and pentachlorothiophenol was from ICN (Costa Mesa, CA, USA). For use as reference standards pentachlorothioanisole, 2,3,5,6-tetrachlorothioanisole, pentachlorophenylsulfoxide and -sulphone were synthesized and purified by recrystallization as described by Koss et al. (1979) (cf. Debets *et al.*, 1981).

Animals and treatments

As part of a 13-week feeding study, 24 hr urine was collected weekly from female Wistar rats (10 weeks old at the start of the experiment) fed diets containing 0.015 % HCB (HCB low), 0.03 % HCB (HCB high), 0.03 % PCB (PCB low) or 0.13% PCB (PCB high), respectively (nine rats per diet). Additional groups received co-treatment with TAO (0.3 %), i.e., 0.03 % HCB+TAO and 0.13 % PCB+TAO, respectively. All diets contained 4 % corn oil. One group of control animals (nine rats) received a diet which contained 4 % corn oil. The animals had free access to food and (tap) water. Rats were housed in wired-bottom cages (3 rats per cage) in a temperature- and humidity-controlled room under a 12 hr light:dark cycle, and were transferred into individual metabolism cages every 7th day for collection of urine, which was stored at -20°C until analysis. A full report on the design and conduct of the study will be given elsewhere (Den Besten *et al.*, in preparation).

Extraction of urinary metabolites

Samples (3 ml) of urine of individual rats were treated with ascorbic acid (1 mM final concentration) and 6 N HCl (0.6 N final concentration). The samples were extracted with diethyl ether (2x4 ml). After evaporation of the ether under a stream of nitrogen, the residues were dissolved in 300 μl of methanol and subjected to HPLC analysis (see below).

Separation of urinary metabolites by HPLC

Urine extracts were analyzed by HPLC (Perkin Elmer Series 4, Woerden, The Netherlands) using a Nucleosil C18 (150x4.6 mm I.D.) column. Flow rate was 1 ml/min and the eluent was monitored at 300 nm. Extracts were eluted using a mobile phase of methanol and water, both phases containing 0.5 % acetic acid. The following gradient was applied: 4 min stationary at 50 % methanol/ 50 % H_2O, followed by a linear gradient to 80 % methanol/ 20 % H_2O in 28 min and 8 min stationary at 80 % methanol,

followed by a linear increase to 100 % methanol in 8 min and concluded by 5 min stationary at 100 % methanol. Peaks were collected separately. The mobile phase was evaporated under a stream of nitrogen, or when the peak eluted in a more polar region, water (2 ml) was added prior to extraction with diethyl ether. The residues were subjected to combined gas chromatography-mass spectrometry (GC-MS). Some metabolites were subjected to high resolution and/or field desorption mass spectrometry (FD-MS).

GC-MS

GC-MS was carried out with a Hewlett Packard 5970B instrument. GC separation was achieved on a DB17 column (film thickness 25 μm; 30 m long, 0.25 mm I.D.). The oven temperature was programmed at 7°C/min from 100°C to 280°C. The injector temperature was maintained at 200°C. Helium gas was used as the carrier gas at a flow rate of 20 ml/min. For high-resolution mass spectrometry, a MS902, equipped with a VG-ZAB console was used.

Determination of UV absorption spectra

In order to determine whether metabolites contained substituents sensitive to changes in pH, UV absorption spectra of the isolated metabolites were determined under acid and alkaline conditions during HPLC analysis using a Waters 991 photodiode array detector. Thus, isolated metabolites were run using the mobile phase (containing 0.5 % acetic acid), applying a gradient program as described below for 'quantification of metabolites'. In addition, isolated metabolites were also run under alkaline conditions using a mobile phase containing methanol and a Tris-phosphate buffer, pH 8.1, and the following gradient program: 5 min stationary at 20 % methanol, followed by a linear increase to 100 % methanol in 20 min, and concluded with 10 min stationary at 100 % methanol.

Quantification of metabolite excretion

For quantification of metabolite excretion by HPLC, the following gradient elution profile was used (0.5 % acetic acid in the mobile phase): 2 min stationary at 50 % methanol/ 50 % H_2O, followed by a linear increase to 90 % methanol/ 10 % H_2O in 17 min followed by 5 min stationary at 90 % methanol and concluded by 10 min stationary at 95 % methanol. Finally, the column was flushed with 100 % methanol for 5 min.

In urine obtained after 1, 4, 6, 8, 10, 12 and 13 weeks of treatment, metabolite excretion of individual animals (nine rats per diet) was quantified by comparing peak areas to those obtained by a calibration curve of reference standards. For the metabolites TCHQ, 2,3,4,5-TCP and PCP, authentic reference standards were used. The remaining metabolites were projected on the calibration curve for 2,3,4,5-TCP in the case of structures containing 4 chlorine substituents, and on the calibration curve for PCP in the case of structures containing 5 chlorine substituents. Thus, amounts were calculated as nmol-equivalents of TCP/ 24 hr, and nmol-equivalents of PCP/ 24 hr, respectively. In

urine extracts from PCB-treated rats, the minor metabolites P6 and P7 (see under Results) were not quantified. Recovery was determined by the addition of [^{14}C]-PCP (10,000 dpm) to urine samples as an internal standard prior to extraction. This amount of [^{14}C]-PCP did not contribute to the UV absorption of PCP at 300 nm during HPLC analysis. Recovery was generally higher than 70 %.

It should be noted that faecal excretion, which largely contains the unchanged chemical, is not taken into account. Previous studies on the biotransformation of HCB (Koss *et al.*, 1976) and a preliminary report on the biotransformation of PCB (Koss and Koransky, 1978) indicate that urinary metabolite excretion is a good representative of the total biotransformation. However, total excretion of some metabolites may be underestimated to some extent.

Hydrolytic treatment of urine

In order to identify metabolites which were excreted in the form of conjugates with glucuronic acid or sulphate, both complete urine samples and individual metabolites, which were isolated, collected as described above and concentrated in 30 μl of methanol, were subjected to enzymatic hydrolysis with ß-glucuronidase (from *E. coli* K12, Boehringer, Mannheim, Germany) and arylsulphatase/ß-glucuronidase (from *Helix Promatia*, Boehringer, Mannheim, Germany) as previously described (Vervoort *et al.*, 1990). The aqueous incubation mixtures were subsequently extracted under acidic conditions with diethyl ether, and prepared for HPLC analysis as described above. Chemical structures of the aglycones were confirmed by GC-MS and/or FD-MS.

In addition, urine samples were hydrolyzed under alkaline conditions as described by Rizzardini and Smith (1982) and subsequently processed as described above for enzymatic hydrolysis.

Results

Urinary metabolite profile of HCB and PCB

Figure 9.1A represents a typical HPLC chromatogram of extracts of urine obtained from rats fed HCB in their diet, showing 2 major metabolites (H3, H4) and 2 minor metabolites (H1, H2). Figure 9.1B represents a typical HPLC chromatogram of extracts of urine obtained from rats fed PCB in their diet, showing 5 major metabolites (P3, P4, P5, P8, P9) and 4 minor metabolites (P1, P2, P6, P7). None of the peaks were observed in HPLC chromatograms of urine extracts from control rats (not shown). All 4 HCB metabolites and all 9 PCB metabolites were collected separately and prepared for analysis by combined GC-MS. In Table 9.1, chromatographic characteristics and mass spectral data of the individual peaks are presented.

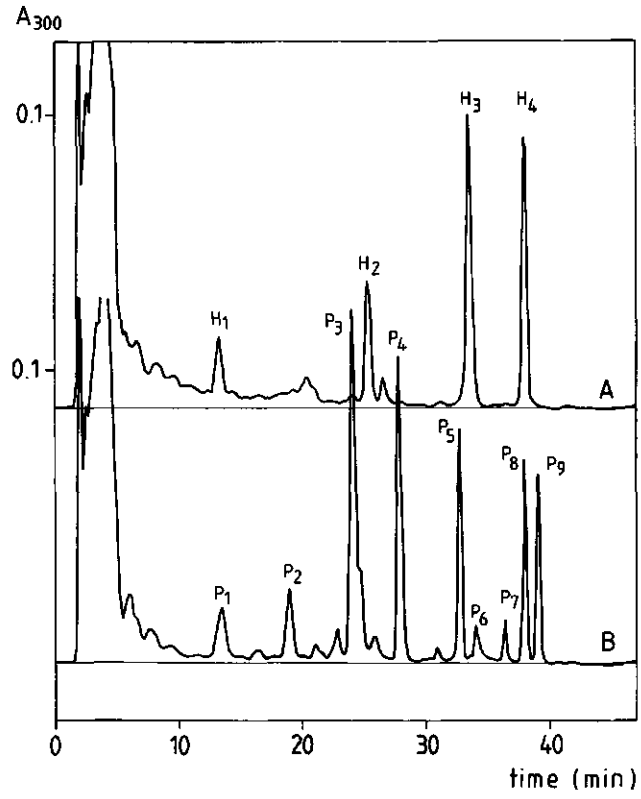

Figure 9.1 Typical HPLC chromatograms of urine extracts obtained from rats fed HCB (A) and PCB (B). Metabolites of HCB are denoted H1, H2, H3, and H4. Metabolites of PCB are denoted P1, P2, P3, P4, P5, P6, P7, P8 and P9. Full description of extraction procedure and HPLC conditions is given under Materials and Methods.

Identification of metabolites

Comparison of the two metabolite profiles revealed that urine from HCB and PCB-treated rats contained two common metabolites, one (H1 and P1) with k' 6.9 and the other one (H4 and P8) with k' 21.4, with identical chromatographic behaviour to the authentic reference compounds TCHQ and PCP, respectively. The mass spectra of these compounds, whether authentic or metabolically formed, showed molecular ions at m/z 246 (TCHQ) and m/z 264 (PCP), and chlorine clusters at the molecular ions indicating 4

and 5 chlorine atoms per molecule, respectively. The remaining 7 peaks in urine extracts from PCB-treated rats were not observed in urine extracts from HCB-treated rats.

According to the typical chlorine clusters at the molecular ion, metabolite H3 contained 5 chlorine atoms, H2 and P2, P3, P4, P5, P6 and P9 all contained 4 chlorine atoms, whereas metabolite P7 contained only 3 chlorine atoms.

The mass spectra of H2, H3 and P3, P4, P6, P7, P9 contained a signal at m/z 45, which is characteristic of organic thiocompounds (CHS-ion), and a signal at m/z 79, which could possibly indicate the presence of a SO_2CH_3 substituent on the aromatic ring (i.e., a methyl sulphone metabolite). However, the mass spectra of authentic chlorinated sulphones show a typical fragmentation pattern with a base peak at M^+-63 ($SOCH_3$) (Koss *et al.*, 1979; Mayring *et al.*, 1984; own observation from reference compounds), which was not observed in any of the metabolites (Table 9.1). Furthermore, the observation of the first mass fragment at M^+-15 in the mass fragmentation pattern of metabolite H2, P3, P6, P7 and P9 (Table 9.1) indicates the presence of a methyl group.

Metabolite P4 had a fragmentation pattern that showed characteristics of both a phenolic compound (M^+-Cl+CO, also observed with authentic chlorophenols) and a sulphydryl compound (large intensity at M^+-Cl, and fragment at m/z 79, also observed with authentic (poly)chlorothiophenols), compatible with a mercapto-tetrachlorophenol structure (MTCP). The theoretical mass of this compound is 261.8580, whereas with the aid of high resolution MS an accurate molecular weight of 261.8584 was assessed. Furthermore, in agreement with a MTCP-structure is the fluctuation in the UV absorption spectrum, which was observed when using a mobile phase in the HPLC-analysis with a low pH (λmax 224 nm, 312 nm) or a high pH (λmax 224 nm, 252 nm, 340 nm).

Chromatographic behaviour of metabolite P5 was identical to the reference compounds 2,3,4,5-/2,3,5,6-TCP. The mass spectrum of P5 was indicative for the 2,3,4,5-isomer of TCP. This substituent pattern was further confirmed by reversed-phase HPLC analysis, in which separation of all three TCP-isomers was achieved using a potassium phosphate/ methanol gradient as previously described (Den Besten *et al.*, 1989; not shown).

Metabolite P6 had a base peak at M^+-15, which is characteristic for a sulphoxide substituent at the ring (Koss, 1979; own observation with reference compounds). The fluctuation in the UV absorption spectrum under acid conditions (λmax 228 nm, 300 nm) and alkaline conditions (λmax 213 nm, 250 nm, 319 nm) is compatible with an additional hydroxyl substituent. Based on these data, it was concluded that P6 was the sulphoxide derivative of MTCP (P4).

Finally, metabolite P7 and P9 were analyzed with the aid of high-resolution MS in order to obtain further information on their molecular composition. The molecular weight of metabolite P7 amounted to 287.8997, corresponding to the molecular formula

$C_8H_7OS_2Cl_3$ (theoretical mass 287.9004). The accurate molecular weight of metabolite P9 was 275.8736, corresponding to the molecular formula $C_7H_4OSCl_4$ (theoretical mass 275.8737). Taking these data together with the fluctuation observed in the UV absorption spectra under acid and alkaline conditions, P7 was characterized as *bis*(methylthio)-trichlorophenol (*bis*MeTTriCP; λmax acid conditions 224 nm, 312 nm, λmax alkaline conditions 228 nm, 351 nm) and P9 was characterized as methylthiotetrachlorophenol (MeTTCP; λmax acid conditions 224 nm, 312 nm; λmax alkaline conditions 221 nm, 252 nm, 339 nm).

Table 9.1 Chromatographic characteristics and electron impact mass spectral data of urinary metabolites from HCB and PCB. A full description of applied HPLC and GC-MS conditions is presented under Materials and Methods. [a] Percentage between brackets indicates intensity of peaks in mass spectrum.

Parent compound	HPLC	GC-retention time	Mass spectral characteristics[a]		
peak	k'	(min)	M^+	Fragmentation pattern	Calculated chemical composition of lost fragment
HCB					
H1	6.9	16.90	246 (100 %)	M^+-36 (14 %)	HCl
				M^+-64 (19 %)	HCl+CO
				M^+-71 (20 %)	HCl+Cl
				M^+-99 (32 %)	HCl+CO+Cl
H2	14.0	22.01	292 (100 %)	M^+-15 (64 %)	CH_3
				M^+-51 (28 %)	CH_3+HCl
H3	18.8	18.00	280 (100 %)	M^+-35 (94 %)	Cl
				M^+-70 (25 %)	Cl+Cl
H4	21.4	16.16	264 (100 %)	M^+-36 (12 %)	HCl
				M^+-64 (10 %)	HCl+CO
				M^+-99 (19 %)	HCl+CO+Cl

- continued -

Table 9.1 - continued -

Parent compound peak	HPLC k'	GC-retention time (min)	Mass spectral characteristics M^+	Fragmentation pattern	Calculated chemical composition of lost fragment
PCB					
P1	6.9	16.98	246 (100 %)	M^+-36 (12 %)	HCl
				M^+-64 (18 %)	HCl+CO
				M^+-71 (16 %)	HCl+Cl
				M^+-99 (23 %)	HCl+CO+Cl
P2	10.4	12.48	230 (100 %)	M^+-36 (22 %)	HCl
				M^+-64 (18 %)	HCl+CO
				M^+-99 (37 %)	HCl+CO+Cl
P3	13.2	19.81	276 (100 %)	M^+-15 (49 %)	CH_3
				M^+-33 (12 %)	SH
				M^+-43 (39 %)	CH_3+CO
P4	15.4	19.02	262 (100 %)	M^+-35 (77 %)	Cl
				M^+-63 (24 %)	Cl+CO
				M^+-99 (26 %)	Cl+COH+Cl
P5	18.3	12.28	230 (100 %)	M^+-36 (20 %)	HCl
				M^+-64 (20 %)	HCl+CO
				M^+-99 (26 %)	HCl+CO+Cl
P6	19.1	22.19	292 (50 %)	M^+-15 (100 %)	CH_3
				M^+-49 (18 %)	HSO
				M^+-91 (32 %)	CH_3+SO+CO
P7	20.5	23.78	288 (100 %)	M^+-15 (54 %)	CH_3
				M^+-43 (30 %)	CH_3+CO
				M^+-64 (12 %)	CO+HCl
P8	21.4	16.16	264 (100 %)	M^+-36 (12 %)	HCl
				M^+-64 (10 %)	HCl+CO
				M^+-99 (19 %)	HCl+CO+Cl
P9	22.1	19.76	276 (100 %)	M^+-15 (49 %)	CH_3
				M^+-33 (14 %)	SH
				M^+-43 (28 %)	CH_3+CO

Identification of conjugated metabolites

GC-retention times and the mass spectral data of metabolite P2 and P3 were identical to those of metabolite P5 (identified as 2,3,4,5-TCP) and P9 (identified as MeTTCP). Their nature, however, was far more polar on reversed phase HPLC (see Figure 9.1B), indicating metabolite P2 and P3 to be conjugates of 2,3,4,5-TCP and MeTTCP. Similarly, the nature of the HCB-metabolites, H2 and H3 was far more polar on reversed-phase HPLC than was to be expected from their structures revealed by GC/MS (cf. Figure 9.1A and Table 9.1).

Treatment of urine samples from HCB- or PCB-treated rats with ß-glucuronidase resulted in the disappearance of metabolite H2, P2 and P3 upon HPLC-analysis, whereas all other peaks, including H3, were still observed (not shown). Treatment of urine samples with a mixture of arylsulfatase/ß-glucuronidase did not result in the disappearance of additional peaks. The individual metabolites were hydrolyzed with ß-glucuronidase, and the extracts subjected to HPLC and GC/MS. The structures of the respective aglycons of H2 and P2 were thus confirmed to be mercapto-tetrachlorothioanisole (MTCTA) and 2,3,4,5-TCP, respectively. Additional confirmation of the aglycone structure of H2 was obtained by high-resolution MS revealing an accurate mass of 291.8594 (theoretical mass of MTCTA: 291.8508). Attempts to identify the complete glucuronide structure by FD-MS were however not successful, due to the fact that the signals generated were too weak.

HPLC-analysis of the extract from the incubation of P3 with ß-glucuronidase revealed the appearance of 2 peaks, one major metabolite (P3A) with k' 25.1 (=k' pentachloro-thiophenol, PCTP), and one minor metabolite (P3B) with k' 22.1 (= k' MeTTCP, P9). The chemical structures of P3A and P3B were confirmed by GC/MS.

Metabolite H3 was not sensitive to treatment with ß-glucuronidase or arylsulfatase, but was not observed anymore upon HPLC analysis after alkaline hydrolysis of urine. Based on the facts that (i) the chemical structure of H3 derived from GC/MS analysis was identical to authentic PCTP, (ii) H3 was sensitive to alkaline hydrolysis but was shown not to be a glucuronide or a sulfate, and (iii) the UV absorption spectrum (λmax 224 nm, 294 nm) was similar to that of *N*-acetyl-*S*-(pentachlorophenyl)-cysteine (PCP-NAC) described by Renner (1978), H3 was characterized as PCP-NAC. This was confirmed by performing direct inlet MS, which showed a weak signal indicating a cluster of 5 chlorine atoms at M^+ 409, and a strong signal at M^+ 349 (M^+ - C_2H_5NO, accurate mass of 349.8291; theoretical mass of $C_9H_3O_2SCl_5$ is 349.8296). It should be noted that in HPLC runs H3 sometimes contained an additional minor peak. However, only one peak was detected when performing HPLC analysis under alkaline conditions (not shown). Since GC/MS and MS did not reveal an additional metabolite, we did not pursue any further identification of the minor peak.

Excretion of HCB-metabolites in a 13-week feeding study

Urinary excretion profiles of the 4 metabolites of HCB were quantitatively determined in the 13-week feeding study in rats treated with 0.015% HCB (low dose) and 0.03 % HCB (high dose) and are presented in Figure 9.2A, B. All 4 metabolites were detected at all time points, except for MTCTA (H2), which was not yet detected in urine after 1 week of treatment.

With the exception of week 13 in the HCB-high group, PCP-NAC (H3) was quantitatively the most important metabolite of HCB in both dose groups during the entire treatment period, ranging from approximately 87 % (HCB low) to 92 % (HCB high) of total metabolites after 1 week of treatment to 63 % (HCB low) and 42 % (HCB high) after 13 weeks of treatment. Interestingly, in both the low and the high dose group, the urinary excretion of PCP-NAC reached a plateau after 4 to 6 weeks of treatment, followed by a further increase until 12 weeks of treatment, after which excretion of PCP-NAC leveled off (low dose) or even decreased (high dose).

With respect to the oxidative biotransformation of HCB, it is noteworthy that the urinary excretion of PCP is slightly more important in rats treated with the HCB high dose compared to rats treated with the HCB low dose, ranging from approximately 2 % (HCB low) and 4 % (HCB high) of total metabolites after 1 week to 24 % (HCB low) and 50 % (HCB high) after 13 weeks of treatment.

Excretion of PCB metabolites in a 13-week feeding study

Urinary excretion profiles of metabolites by rats fed a low dose of PCB (0.03 %) or a high dose of PCB (0.13 %) for 13 weeks are quantitatively presented in Figure 9.3A,B. These data show that the relative importance of several metabolites appears to be dose-dependent. Formation of 2,3,4,5-TCP (both in free form, P5, and as a glucuronide conjugate, P2) was quantitatively the most important route for biotransformation of PCB in rats treated with a low dose (0.03%), amounting to 44 % of total metabolites after 1 week to 35 % of total metabolites after 13 weeks. In PCB-high treated rats, formation of glucuronides (P3A, P3B) and MTCP (P4) was relatively more important, and excretion of 2,3,4,5-TCP amounted to approximately 12-15 % of total metabolites. Formation of PCP (expressed as percentage of total metabolites) was comparable in both dose groups. Consequently, during the whole treatment period the ratio of formation of total 2,3,4,5-TCP-to-PCP was significantly higher in PCB-low compared to PCB-high treated rats (3.92 ± 0.34 vs 1.38 ± 0.07, PCB low vs PCB high, respectively; mean$\pm$SE over week 1, 4, 6, 8, 10, 12, 13).

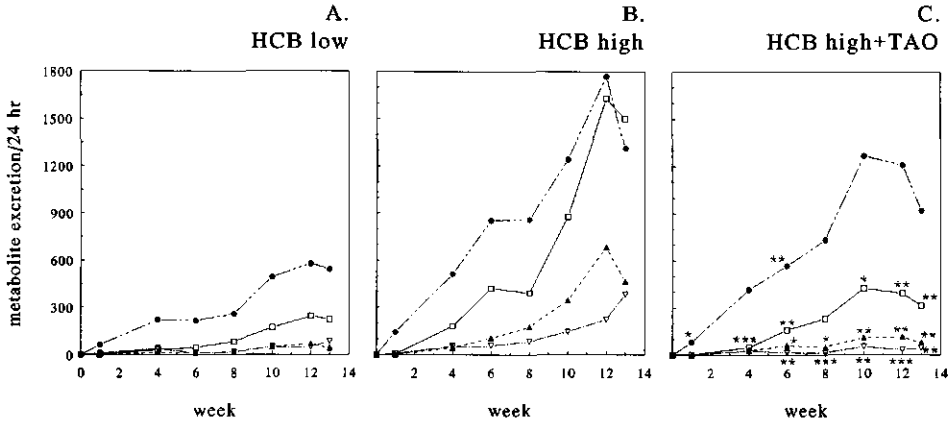

Figure 9.2. Urinary excretion of metabolites by rats fed 0.015 % HCB (A), 0.03 % HCB (B) and 0.03 % HCB + TAO (C) for 13 weeks. Metabolites were analyzed and quantified as described under Materials and Methods, and expressed as nmol equivalents PCP or TCP/ 24 hr urine. Each data point represents mean of nine rats. In figure B, data at 10, 12 and 13 weeks represent mean of respectively 8, 7, and 6 rats, due to premature kills. In Figure 9.2C, data at 13 weeks represent mean of 8 rats, due to premature death of 1 rat. Symbols indicate the following: (▽), H1; (△), H2; (●), H3; (□), H4. * denotes significant difference from levels observed in rats which received 0.03 % HCB alone, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Effect of TAO on the biotransformation of HCB and PCB

Co-treatment of rats with TAO, a selective inhibitor of P450IIIA1/2, and HCB or PCB did not result in the formation of metabolites qualitatively different from treatment with HCB or PCB alone. Urinary metabolite excretion in rats which received TAO in their diet in addition to HCB high or PCB high is presented in Figure 9.2C and 9.3C, respectively. TAO significantly inhibited the formation of TCHQ (H1), MTCTA-glucuronide (H2), and PCP (H4) in HCB-high treated rats. The urinary excretion of PCP-NAC (H3), however, was influenced to a much lower extent.

Co-treatment of rats with PCB high and TAO resulted in a significantly decreased excretion of TCHQ (P1), PCP (P8), and MeTTCP (P9). In addition, excretion of the glucuronides P3A,B was significantly lower in rats which received combined treatment with TAO. Interestingly, the urinary excretion of 2,3,4,5-TCP (P5) and MTCP (P4) was not affected by co-treatment with TAO.

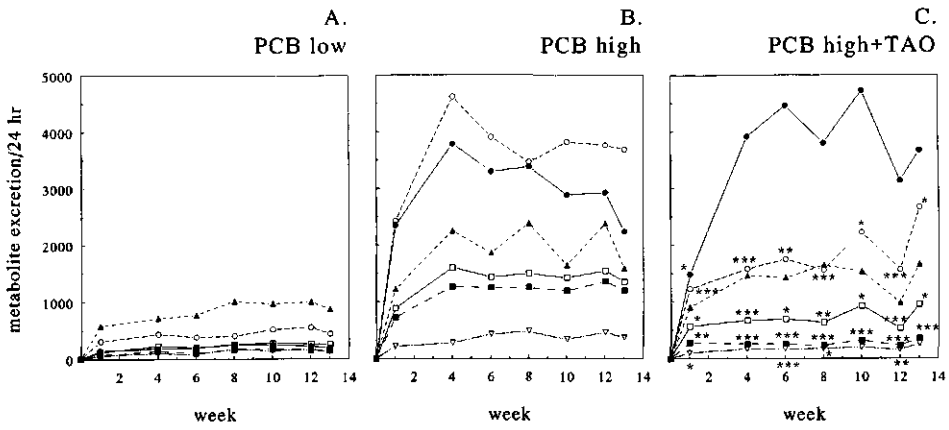

Figure 9.3. Urinary excretion of metabolites by rats fed 0.03 % PCB (A), 0.13 % PCB (B) and 0.13 % PCB + TAO (C) for 13 weeks. Metabolites were analyzed and quantified as described under Materials and Methods, and expressed as nmol equivalents PCP or TCP/ 24 hr urine. Each data point represents mean of nine rats. Symbols indicate the following: (▽), P1; (△), P2+P5; (○), P3; (●), P4; (□), P8; (■), P9. * denotes significant difference from levels observed in rats which received 0.13 % PCB alone, $p < 0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

Studies on the microsomal oxidation of HCB (Van Ommen *et al.*, 1986) and PCB (Den Besten *et al.*, 1989) revealed that both compounds are oxidized to PCP and TCHQ. The urinary metabolite profiles of HCB and PCB *in vivo* as found in the present study are summarized in Figure 9.4. It appears that the results obtained from microsomal incubations can be extrapolated to the *in vivo* situation, since both PCP and TCHQ are detected in urine derived from rats treated with HCB or PCB. However, no other mutual metabolites are formed.

In rat liver microsomal incubations with PCB, oxidation at the unsubstituted position, resulting in the formation of PCP, and oxidation at a chlorine-substituted position, resulting in the formation of TCP occurred in a ratio of roughly 3:1 in favour of PCP formation (Den Besten *et al.*, 1989). Surprisingly, the present study shows that *in vivo* formation of the oxidatively dechlorinated product (i.e., 2,3,4,5-TCP, P5) is favoured over oxidation at the unsubstituted position (i.e., PCP, P8), especially in rats treated with

the low dose. Furthermore, in a microsomal incubation all three isomers of tetrachlorophenol were detected (Den Besten *et al.*, 1989), whereas in the present study only the 2,3,4,5-TCP isomer was detected in urine.

Selective blockage of cytochrome P450IIIA1/2 by the macrolide TAO was used as a tool to delineate the contribution of P450IIIA to the biotransformation of HCB and PCB. The inhibition of oxidative metabolism of HCB by TAO is in agreement with a previous study (Van Ommen *et al.*, 1989). In the present study, the inhibitory action of TAO is extended to the oxidative biotransformation of PCB, showing a reduced formation of PCP (P8) and TCHQ (P1).

An intriguing observation in the present study is the fact that co-administration of TAO and PCB strongly affected the excretion of PCP but not of the major metabolite 2,3,4,5-TCP. This would indicate that (i) PCP and 2,3,4,5-TCP are not formed from a common intermediate (such as the putative 2,3,4,5,6-pentachloro-1,2-epoxide), and (ii) 2,3,4,5-TCP is formed by a reaction not involving cytochrome P450IIIA1/2. In a recent study, a novel pathway has been proposed in the oxidative dehalogenation of hexahalogenated benzenes involving an intermediate benzohaloquinone cation (Rietjens and Vervoort, 1992). It could be hypothesized that PCB is oxidized at the unsubstituted position via a 'classical' hydroxylation pathway into PCP by the action of P450IIIA. This reaction would involve an intermediate pentachlorobenzene-1,2-epoxide or, based on more recent insights, a tetrahedral iron-oxene radical σ-complex (Guengerich and MacDonald, 1990). On the other hand, oxidation of PCB at the chlorine substituted position resulting in the formation of 2,3,4,5-TCP, presumably catalyzed by a P450 isoenzyme different from P450IIIA1/2, might involve a 2,3,5-trichlorobenzochloroquinone intermediate. This putative intermediate can readily be reduced to 2,3,4,5-TCP. The present study clearly emphasizes the need for future studies on the mechanism of oxidative dehalogenation of (lower) halogenated benzenes, in order to characterize (i) the different isoenzymes of P450 involved in the different oxidation pathways, and (ii) the nature of the respective intermediates formed.

In addition to oxidized metabolites, both HCB and PCB are converted into sulfur-containing derivatives, but the chemical nature of these metabolites derived from either HCB or PCB is remarkably different. In urine from HCB-treated rats, PCP-NAC (H3) was the major metabolite, and this is in agreement with previous studies in which PCP-NAC was detected as PCTP by GC after alkaline hydrolysis of urine (Koss *et al.*, 1979; Rizzardini and Smith, 1982; Jansson and Bergmann, 1978; Renner and Nguyen, 1984). The mercapturate is most likely formed via a mechanism involving a nucleophilic displacement of chlorine by glutathione. Interestingly, mercapturic acids are not observed in urine from PCB-treated rats, suggesting that a direct conjugation of polychlorinated benzenes with glutathione only takes place when all ring carbons contain halogens. This view is further supported by the previous observation that in the presence of rat liver cytosol hexafluorobenzene and pentafluorochlorobenzene were readily conjugated with glutathione (Rietjens and Vervoort, 1992), whereas pentafluorobenzene was not (Rietjens,

personal communication). It should be noted that PCTP was excreted as an *S*-glucuronide by PCB-treated rats. However, its formation can not readily be explained via the formation of PCP-NAC, since the mercapturate was not observed in urine-extracts from PCB-treated rats.

In addition to PCP-NAC, HCB was converted into a disubstituted sulfur metabolite, i.e., MTCTA (H2). Formation of HCB metabolites with two sulfur-containing substituents is in agreement with previous studies (Jansson and Bergman, 1978; Renner and Nguyen, 1984). Interestingly, the present study indicates that MTCTA is excreted into urine solely as an *S*-glucuronide.

PCB is more extensively metabolized to metabolites containing both sulfur- and oxygen substituents, such as the major metabolite MTCP (P4), and the minor metabolites hydroxytetrachlorophenyl sulphoxide (HTCPS, P6), *bis*MeTTriCP (P7) and MeTTCP

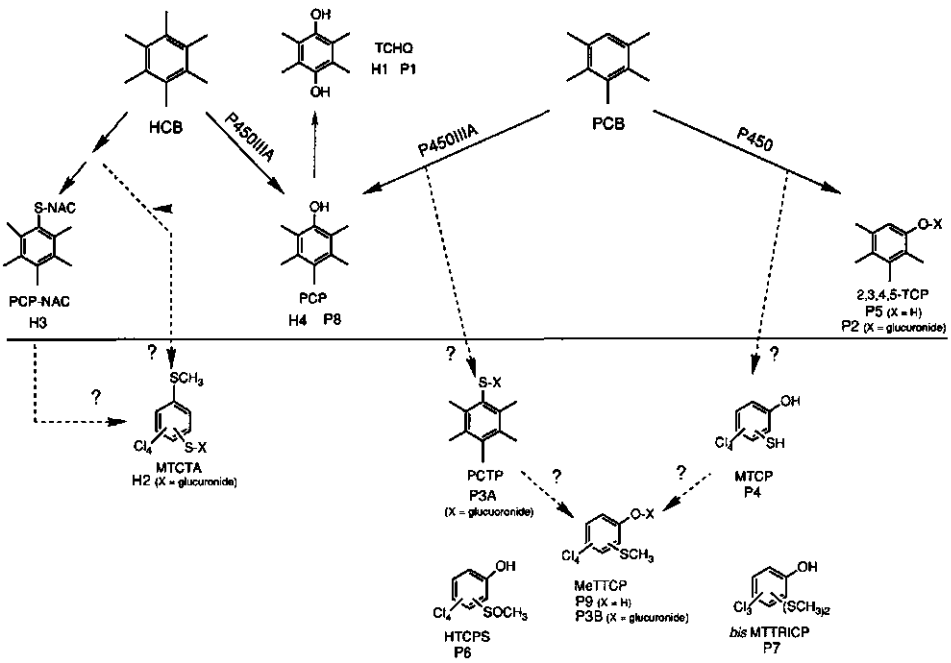

Figure 9.4. Proposed pathways for the formation of the different urinary metabolites from HCB and PCB. In the lower part below the line, the metabolic routes are more speculative.

(P9). The formation of these metabolites is generally believed to involve conjugation of glutathione with an intermediate arene oxide. In fact, the absence of mercapturic acids from the urinary metabolite profile of PCB may originate from the chemical structure of the putative primary intermediate pentachlorobenzene-1,2-oxide, since attack by glutathione, most likely to take place at the least hindered C1 carbon, will be readily followed by loss of hydrochloric acid resulting in a glutathione-*S*-yl-tetrachlorophenol structure, which may subsequently be processed in several steps to mercaptotetrachlorophenol, a major metabolite of PCB in the rat. Isomeric mercaptotrichlorophenols have been described as metabolites of 1,2,3,5-tetrachlorobenzene in the rat (Chu *et al.*, 1984). Furthermore, the biliary excretion of monochlorohydroxy-sulfur derivatives has recently been reported for 1,3-DICB (Kimura *et al.*, 1992). Thus, the formation of mercaptothiophenol derivatives from polychlorinated benzenes is indeed linked to the loss of a halogen substituent from the molecule.

However, based on comparison of urinary metabolite profiles of rats treated with PCB with or without co-treatment with TAO, questions may be raised with respect to the actual precursor leading to the formation of MTCP (P4). Excretion of MTCP, which was not affected by co-treatment with TAO, is correlated with the excretion of 2,3,4,5-TCP (P5), and not with the excretion of PCP. This would imply that MTCP and 2,3,4,5-TCP are formed via a (common) pathway not involving the action of P450IIIA1/2, whereas PCP is formed via a different pathway, catalyzed by cytochrome P450IIIA1/2. Altogether, these data stress the importance that, in order to achieve a full understanding of the metabolic fate of halogenated benzenes, mechanistic studies into the oxidative pathways should be extended to and/or combined with studies on the interplay between the P450-mediated oxidation and conjugation with glutathione.

The combined use of HPLC and GC/MS made it possible to establish the extent of excretion of conjugated metabolites. The use of GC/MS alone revealed only the aglycone structures, apparently due to the fact that the conjugates were easily hydrolyzed. The major route of conjugation appeared to be through glucuronidation of either a hydroxyl group, e.g., as in P2, or of a sulfhydryl group, e.g., as in H2 and P3. The lack of formation of sulphate conjugates from either HCB or PCB fits with the known inhibitory action of PCP on sulfotransferase activity (Meerman *et al.*, 1983).

An interesting observation is that 2,3,4,5-TCP is excreted both in its free form (P5) and as a glucuronide conjugate (P2), whereas PCP is detected in urine of HCB and PCB-treated rats exclusively in its free form. This is in agreement with a previous study on the metabolic fate of PCP, in which only 9-16% of an oral dose of PCP was excreted in the form of conjugates into urine (Ahlborg *et al.*, 1978). These data may indicate that PCP does not possess good substrate characteristics for glucuronidation. Furthermore, PCP-glucuronide has been described to be hydrolyzed under weakly acidic conditions (Lilienblum, 1985). Thus, part of the PCP detected in its free form in urine might originate from its glucuronide conjugate.

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Chapter 10

SUMMARY OF IN VIVO STUDIES

In Part II of this thesis, the results on the *in vivo* studies are described, in which the toxicity of a range of (poly) chlorinated benzenes is assessed to target organs like the liver and the kidneys. Moreover, an attempt is made to correlate adverse effects with the nature of the metabolites formed.

In *chapter 7*, a range of chlorinated benzenes was compared with respect to their ability to induce target organ toxicity after a single i.p. administration in the male rat. Toxic effects on the liver consisted of an increased liver-to-body weight ratio after 72 hr, and, in the case of the higher chlorinated benzenes, also after 216 hr. A varying degree of centrilobular hypertrophy and hepatocellular degeneration was observed in most of the treatment groups. 1,2-Dichlorobenzene and 1,2,4-trichlorobenzene produced the most severe hepatotoxic effects (as shown by a significant rise in plasma ALT levels and clear degenerative changes), whereas monochlorobenzene was also considerably hepatotoxic. In the present study, no hepatotoxic effects were observed for 1,4-dichlorobenzene and 1,2,4,5-tetrachlorobenzene, whereas pentachlorobenzene induced only slight histopathological liver changes. No obvious degenerative effects on the kidneys were observed in the dose range studied. However, formation of protein droplets in the tubular epithelial cells was shown to be a general phenomenon for polychlorinated benzenes, and was more pronounced after exposure to the higher chlorinated benzenes.

Interestingly, polychlorinated benzenes were shown to interfere with thyroid hormone homeostasis as demonstrated by a severe reduction in plasma thyroxine levels. They share this effect with the structurally related polychlorinated biphenyls (Brouwer, 1989). The most severe disturbances in thyroid hormone homeostasis were observed for the higher chlorinated congeners. Evidence is presented for a role of the phenolic metabolites in the observed decrease of plasma thyroxine, through a selective interaction with a major plasma transport protein for thyroxine, i.e., transthyretine (TTR).

In *chapter 8* and *chapter 9*, results of a 13-week feeding study with hexachlorobenzene (HCB) and pentachlorobenzene (PCB) in female rats are presented. We were interested in a comparison of these two congeners with respect to their toxicity and biotransformation, since *in vitro* studies had shown that both HCB and PCB are oxidized to pentachlorophenol and the reactive tetrachlorobenzoquinone (cf. Van Ommen *et al.*,

1986, *chapter 2, chapter 3*). Moreover, the same P450 isoenzyme (i.e., cytochrome P450IIIA1/2) appeared to be involved in the oxidation of HCB and PCB.

The study described in *chapter 8* was designed to establish the relevance of the oxidative metabolites in the various toxic effects, with special attention for the role of the reactive tetrachlorobenzoquinone in porphyria. To this end, urinary exretion of pentachlorophenol and tetrachlorohydroquinone, the reduced analog of tetrachlorobenzoquinone, was monitored throughout the study. In addition, the effect of selective modulation of cytochrome P450IIIA activity by co-treatment with the macrolide triacetyloleandomycin (TAO) on biotransformation and toxicity (especially porphyria) of HCB and PCB is established.

As demonstrated in *chapter 8*, dietary exposure to HCB resulted in highly elevated urinary porphyrin levels and a severe hepatic accumulation of porphyrins. HCB-induced porphyria was strongly inhibited by co-treatment with TAO. Co-treatment of rats with TAO also significantly inhibited oxidative metabolism of HCB (and PCB). However, inhibition of HCB-induced porphyria can not be explained by a diminished formation of the highly reactive tetrachlorobenzoquinone, since rats treated with a high dose of PCB had a several fold higher urinary excretion of tetrachlorohydroquinone compared to rats treated with HCB, but did not develop porphyria. The present data may, however, be supportive for the hypothesis suggesting the involvement of a (putative) reactive intermediate in the primary oxidation in HCB-induced porphyria, since urinary porphyrin excretion was well correlated with the excretion of the primary phenol metabolite, pentachlorophenol.

Chapter 8 also demonstrates that exposure to either HCB or PCB resulted in a disturbance of retinoid and thyroid hormone homeostasis. The involvement of a common mechanism for polychlorinated benzenes and -biphenyls is discussed, involving interference of hydroxylated metabolites with plasma transport.

Chapter 9 describes the characterization of the urinary metabolite profile of HCB and PCB. Apart from the two common oxidative metabolites, pentachlorophenol and tetrachlorohydroquinone, striking differences were observed with respect to the nature of the sulfur-containing metabolites. HCB was metabolized to *N*-acetyl-*S*-(pentachlorophenyl)cysteine, quantitatively the most important metabolite, and mercaptotetrachlorothioanisole. PCB was more extensively metabolized to sulfur- and sulfur-oxygen containing metabolites, of which the major metabolites were pentachlorothiophenol glucuronide and mercaptotetrachlorophenol .

In addition to the oxidation to pentachlorophenol, PCB is also oxidized to 2,3,4,5-tetrachlorophenol. Interestingly, excretion of 2,3,4,5-tetrachlorophenol was not inhibited by combined treatment of rats with PCB and TAO, the selective inhibitor of P450IIIA1/2. This indicates that (i) PCB is oxidized to pentachlorophenol and 2,3,4,5-tetrachlorophenol via different intermediates, and (ii) cytochrome P450IIIA is not

involved in the latter oxidation. Combined treatment of rats with PCB and TAO had a differential effect on the excretion of sulfur-containing metabolites. For example, the urinary excretion of mercaptotetrachlorophenol was not inhibited by TAO, whereas the excretion of pentachlorothiophenol glucuronide was strongly inhibited. Thus, these results stress the importance of detailed studies into the interaction between oxidizing systems (i.e., cytochrome P450 monooxygenases) and conjugating systems (i.e., glutathione S-transferases, spontaneous conjugation with glutathione) in order to delineate the different metabolic pathways.

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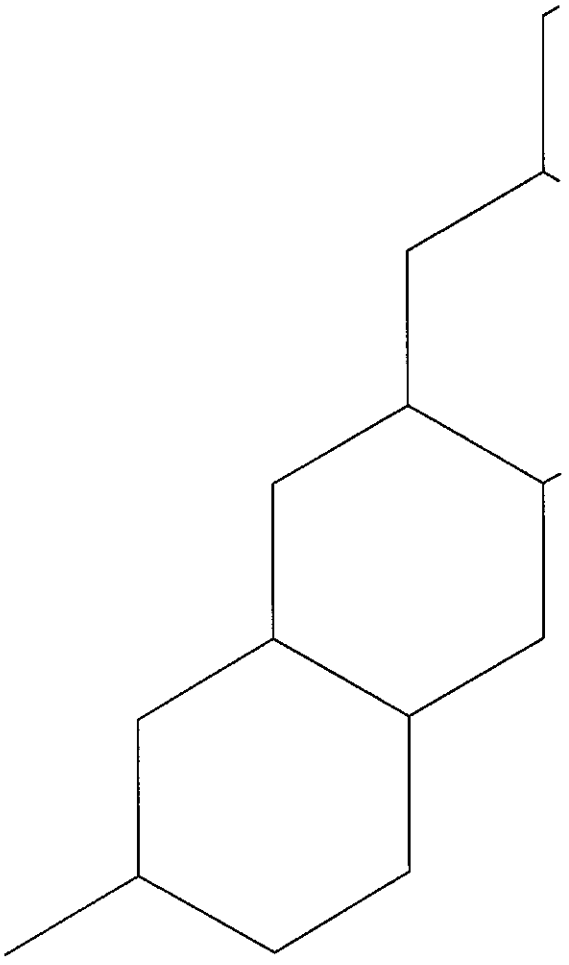
PART III



Chapter 11

CONCLUSIONS AND PERSPECTIVES

Introduction

The experiments described in this thesis were undertaken to assess the relevance of biotransformation routes to the toxic effects elicited by halogenated benzenes. The ultimate aim is to establish more precisely what chemical species is responsible for which toxic effect. As has been demonstrated for numerous unsaturated compounds (vinyl chloride, polycyclic aromatic hydrocarbons, bromobenzene), cytochrome P450 is believed to play a pivotal role in the metabolic activation of halogenated benzenes. Therefore, our first goal was to establish the route of oxidative biotransformation of a range of polychlorinated benzene congeners, with special attention for the extent of covalent binding to protein and DNA, and for the nature of the binding species. To this end, we used radiolabeled compounds and microsomal preparations (Part I, summarized in *chapter 6*). In order to assess the relevance of the formation of the various oxidized metabolites, as determined in microsomal incubations, for the toxicity *in vivo*, two studies were conducted (Part II, summarized in *chapter 10*). In the first study, a range of polychlorinated benzenes was compared with respect to their adverse effects on target organs like the liver and the kidney. Interestingly, thyroid hormone homeostasis appeared to represent an additional common target for chlorinated benzenes. In the second study, two closely related congeners, hexachlorobenzene and pentachlorobenzene, which are oxidized via a common pathway by rat liver microsomes, were compared in a 13-week feeding study with respect to their toxicity (*chapter 8*) and their urinary metabolite profiles (*chapter 9*).

Biotransformation of polychlorinated benzenes to reactive metabolites: I. nature of the reactive intermediates formed

One of the main conclusions drawn from the studies described in the present thesis is that, irrespective of the substitution degree and pattern, chlorinated benzenes are bioactivated to reactive metabolites which covalently bind to protein and DNA in a microsomal system. As discussed in *chapter 1* (cf. Figure 1.3), several reactive products can be envisioned departing from a simple halogenated benzene structure. Based on the results described in Part I, we propose that secondary benzoquinone metabolites, formed upon the oxidation of the primary phenol metabolites, are the ultimate chemically reactive species produced during oxidative biotransformation of chlorinated benzenes, although the involvement of their semiquinone anion radicals can not be excluded. New insights in the oxidative pathways and chemical nature of the (reactive) products are incorporated in Figure 11.1, and will be discussed below.

The classical view on the formation of reactive benzoquinone metabolites from the parent halogenated benzenes consists of three oxidation steps: two subsequent P450-mediated oxidations via a halophenol to a hydroquinone, which needs further oxidation, either enzymatically or chemically, to the reactive benzoquinone metabolites (Van Ommen *et al.*, 1986, Den Besten *et al.*, 1989). However, evidence presented in *chapter 5* indicates that the mechanism of formation of benzoquinone metabolites is dependent on the substitution pattern of the phenol precursor. The oxidative dehalogenation of halophenols, which results in the direct formation of benzoquinone metabolites without the obligatory intermediacy of the chemically stable hydroquinones, reflects a novel pathway of, possibly, paramount importance to the toxicity observed *in vivo* (see section II). Since the mechanism described in *chapter 5* has also been shown to be valid for *para*-fluorinated anilines, this pathway may represent a general mechanism for the P450-mediated oxidation of substrates, which possess an acidic proton in one of the substituents of the aromatic ring and an electron withdrawing substituent at the position to be oxidized.

A recent study on the conversion of fully halogenated benzenes suggests the formation of a novel intermediate in the primary oxidation step (Rietjens and Vervoort, 1992). These authors propose that the cytochrome P450-mediated formation of the primary phenol metabolite may occur without the intermediacy of an arene oxide, but rather via a halogeno-analog of a quinone (i.e., a benzohaloquinone cation). Future studies are required to validate the exact nature of the intermediate in the primary oxidation of fully halogenated benzenes, i.e., is it an epoxide (Figure 11.1 route a) or a benzohaloquinone (Figure 11.1 route b), or do they both serve as intermediates in the oxidation step (Figure 11.1 route a-c)? In addition, the generality of such a novel

mechanism needs to be established, i.e., may benzohaloquinone cations also serve as intermediates in the oxidation of lower halogenated benzenes?

An intriguing outcome of the present study is that the epoxide intermediates in the primary oxidative step to phenols apparently possess negligible alkylating properties, and this poses a need for re-evaluation of the role of this species in the toxicity of substituted benzenes. In particular, questions may be raised with respect to the presumed role of the 3,4-epoxide as the ultimate reactive species involved in bromobenzene-induced hepatotoxicity (Jollow *et al.*, 1974; Lau *et al.*, 1980). This widely accepted view on bromobenzene-induced hepatotoxicity is merely based on indirect evidence such as the observation of a good correlation between on the one hand the urinary excretion of the *para*bromophenyl mercapturic acid (which involves the intermediacy of the 3,4-epoxide),

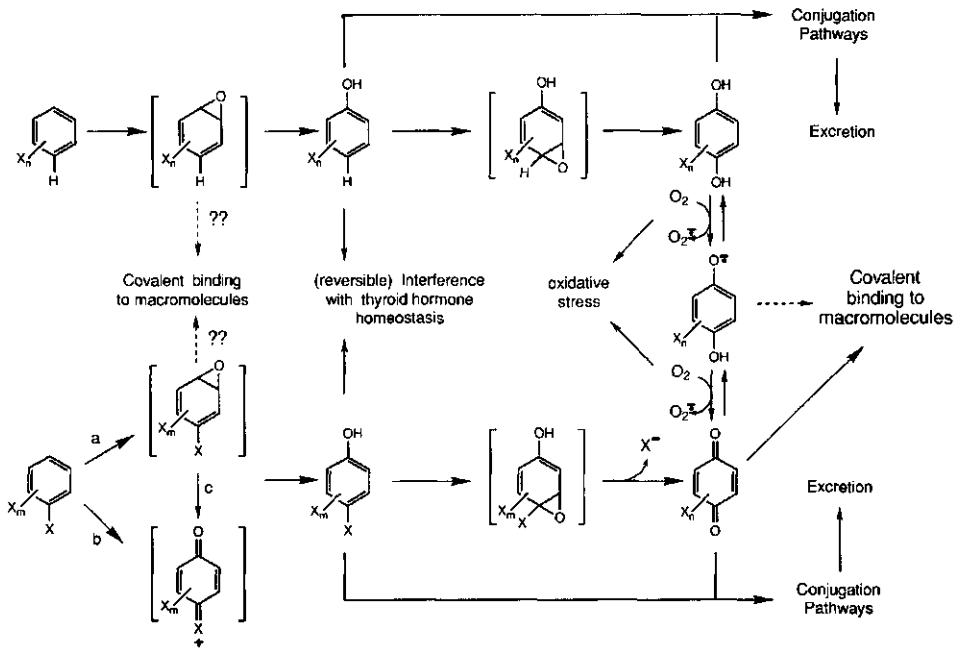


Figure 11.1 The proposed mechanisms of oxidation of halogenated benzenes and -phenols to hydroquinones and benzoquinones, depending on the substituent *para* with respect to the position to be oxidized. For explanation, see text. The formation of a distinct epoxide in the several oxidation steps has never unambigously been established, and current knowledge suggests the intermediacy of a (radical) σ-complex (Guengerich and MacDonald, 1990). For reasons of simplification, in the present figure only epoxide intermediates are presented. X, halogen; *n*, *m* denotes number of halogen substituents in which $m=n+1$.

and on the other hand depletion of cellular glutathione and subsequent covalent binding to protein. However, conjugation of bromobenzene-3,4-epoxide with glutathione has been shown to require enzymatic catalysis (Jollow *et al.*, 1974; Monks *et al.*, 1982), suggesting that this species has low spontaneous alkylating properties towards (protein-) sulfur nucleophiles. Only recently attempts were undertaken to isolate and characterize bromobenzene-derived protein adducts from rat liver. Interestingly, bromobenzene derived epoxides accounted for less than 0.5 % of total protein sulfur adducts (Weller and Hanlik, 1991, Slaughter and Hanzlik, 1991), whereas the *S*-alkylation of protein nucleophiles *in vivo* by quinone metabolites was 10-15 times more extensive (Slaughter and Hanzlik, 1991). These findings are fully in line with the observations in the present thesis that benzoquinones derived from halogenated benzenes have far greater alkylating properties than the primary epoxide intermediates (*chapter 2, 3, 4*).

The chemical structure of a compound is a main factor determining to which extent the various biotransformation pathways take place. For example, as discussed above, a main determinant for the oxidation pathway for halogenated benzenes or phenols appears to be the ring substituent at the *para*-position with respect to the position to be oxidized. However, equally important is the (iso-)enzyme status regarding the process of biotransformation in a particular cell or organ. The differential role of the various P450 isoenzymes in the conversion of chlorinated benzenes is clearly demonstrated (i) *in vitro,* by the various degrees of biotransformation and bioactivation of different chlorinated benzenes in microsomal preparations derived from rats pretreated with different P450 inducers (*chapter 3, 4*), and (ii) *in vivo,* by inhibition of the conversion of hexachlorobenzene and pentachlorobenzene through selective modulation of the cytochrome P450IIIA1/2 activity (*chapter 8, 9*). For both hexachlorobenzene and pentachlorobenzene, cytochrome P450IIIA1/2 appears to be the major isoenzyme(s) involved in their oxidation, both *in vitro* (Van Ommen *et al.*, 1989, *chapter 3*) and *in vivo* (*chapter 9*). However, with decreasing degree of chlorination, the relative contribution of other isoenzymes, especially from the P450IA and P450IIB family, becomes more important as is demonstrated by studies on the microsomal oxidation of 1,2,4-trichlorobenzene (*chapter 3*) and 1,2- and 1,4-dichlorobenzene (*chapter 4*). Interestingly, the fact that the ratio of covalent binding to total metabolism was always highest in microsomes from DEX-pretreated rats, indicates that P450IIIA1/2 is not only involved in the primary oxidation step, but may also catalyze the secondary oxidation to reactive benzoquinone metabolites.

With respect to interspecies comparison of the microsomal oxidation studies, the major role of the P450IIIA family in the oxidation and metabolic activation of chlorinated benzenes may have rather interesting consequences. This family is one of the most conserved forms across species (Smith, 1991), and in humans cytochrome P450IIIA is the most abundant P450-isoenzyme involved in the oxidation of many substrates of a chemically diverse nature (George and Farrell, 1991). We recently obtained evidence that, indeed, human P450IIIA is a major isoenzyme involved in the oxidation of

chlorinated benzenes. Using a battery of human liver microsomes, which were fully characterized with respect to their P450-isoenzyme status, it was shown that the extent of oxidation of 1,2,4-trichlorobenzene was correlated to a high degree with the amount of P450IIIA3/4 in the microsomal preparation (Table 11.1). In addition, the oxidation of 1,2,4-trichlorobenzene was weakly correlated to the P450IIA1 content. Rather surprising was the low correlation observed with isoenzymes from the P450IA family in these human liver microsomes, since both rat liver microsomes from animals pretreated with 3-MC and ISF (inducers of P450IA1 and P450IA2, respectively, *chapter 3*) and purified P450IA1 and P450IA2 in a reconstituted system (Den Besten and Henderson, in preparation) did convert 1,2,4-trichlorobenzene to a considerable extent.

Table 11.1 Spearman rank correlations of cytochrome P450 isoenzyme content with oxidation of 1,2,4-trichlorobenzene.

P450 isoenzyme	oxidation of 1,2,4-TRICB
P450IA1	0.16
P450IIA1	0.70[¶]
P450IIB1	0.57
P450IIC8	0.46
P450IIC9	0.04
P450IID6	-0.43
P450IIE1	-0.06
P450IIIA3/4	0.84[¶¶¶]
P450IVA1	-0.09

Note. The relative level of P450 isoenzymes in a panel of human liver microsomes was identified and immunoquantified as reported elsewhere (Forrester *et al.*, 1992). Conversion of 1,2,4-trichlorobenzene was determined as described in chapter 3. Values represent Spearman rank correlation coefficients. Significance of correlation: [¶] $p < 0.05$, [¶¶¶] $p < 0.001$.

Biotransformation of chlorinated benzenes to (re-)active metabolites: II. Implications for toxicity

The reactive metabolites derived from polychlorinated benzenes which attack nucleophilic sites in proteins have been identified as benzoquinones (Part I). Based on our studies and on recent evidence presented in the literature concerning protein-adducts derived from the model hepatotoxicant bromobenzene (Slaughter and Hanzlik, 1991), we propose that the hepatotoxicity of halogenated benzenes is linked to the formation of benzoquinone metabolites, and not to the formation of their primary epoxide intermediates.

The toxic potential of quinones resides in their ability to alkylate cellular nucleophiles (such as glutathione or tissue macromolecules) and/or to cause oxidative stress via redox cycling between the semiquinone anion radical and the corresponding hydroquinone or benzoquinone (Figure 11.1). Which of these two molecular mechanism (or maybe both) is involved in the toxicity induced by benzoquinones formed *in situ* from polyhalogenated benzenes remains to be established. However, covalent binding to protein has been shown to be a good correlate with hepatotoxicity induced by halogenated benzenes (Brodie *et al.*, 1971, Reid and Krishna, 1973, Stine *et al.*, 1991), and under normal circumstances hepatocytes appear to have adequate defense mechanisms to cope with oxidative stress. However, the establishment of a causal relationship between irreversible protein binding and halogenated benzene-induced hepatotoxicity remains a crucial matter for future research.

A possible approach to this question includes the characterization of the target molecules for the benzoquinone metabolites. In view of the relatively high chemical reactivity, it was not surprising that binding to microsomal protein occurred in a rather non-selective way. However, the benzoquinone metabolites were stable enough to diffuse out of the microsomal membrane and arylate soluble proteins such as exogeneously added glutathione *S*-transferases or bovine serum albumine (Den Besten, unpublished observations). In fact, chlorinated benzoquinones and their glutathione conjugates are potent irreversible inhibitors of both rat and human glutathione *S*-transferases (Vos and Van Bladeren, 1990).

As discussed in section I (and depicted in Figure 11.1), the mechanism of formation of the reactive benzoquinones appears to be dependent on the substituent of the phenol precursor at the position to be oxidized. Direct formation of benzoquinone metabolites in a single oxidation step at a halogenated position, as opposed to formation of hydroquinones which need subsequent oxidation to their reactive quinone form, may have considerable toxicological implications. For example, in the latter case multiple interactions, such as competing pathways of conjugation, or the abundance of reducing equivalents in a cell, may prevent the actual formation of the reactive species *in vivo*. In

contrast, formation of the reactive quinone species cannot be circumvented in the case of the direct oxidation pathway, and once formed, quinone metabolites may not be adequately detoxified. Thus, the hypothesis put forward in the present study is that differences observed between covalent binding to macromolecules (and hepatotoxicity) of different halogenated benzenes may (partly) be due to the differential mechanism of reactive benzoquinone formation as described in *chapter 5*.

Although a previous study indicated that the porphyrinogenic action of hexachlorobenzene was directly related to the formation of the reactive tetrachlorobenzoquinone (Van Ommen *et al.*, 1989), *chapter 8* presents evidence which argues against such a relationship. Comparison of the urinary metabolite profiles and the toxic effects elicited by hexachlorobenzene and pentachlorobenzene revealed that *in situ* oxidative dehalogenation of pentachlorophenol to tetrachlorobenzoquinone does not appear to be involved in hexachlorobenzene-induced porphyria. The involvement, however, of a putative reactive intermediate in the primary oxidation step of hexachlorobenzene in its porphyrinogenic action, such as the recently proposed benzohaloquinone cation (Rietjens and Vervoort, 1992) can not be ruled out, and future studies should be directed to tackle this enigma.

The present studies have not delivered evidence which would establish the role of glutathione conjugates of chlorinated hydroquinones formed *in situ* as proximate nephrotoxicants. However, the elegant studies conducted on bromobenzene-induced nephrotoxicity, in which (poly-)glutathione conjugates of bromohydroquinone are indicated as potent and selective nephrotoxicants, may be representive for the whole range of halogenated benzenes, since glutathione conjugates of several chlorinated hydroquinones have also been shown to be nephrotoxic (Mertens *et al.*, 1991).

In addition to the attention paid to the covalent interactions by chemically reactive metabolites, *chapter 7* and *chapter 8* emphasize the importance of selective non-covalent interactions by biologically 'active' metabolites. For example, protein droplet nephropathy observed in the male rat after exposure to polychlorinated benzenes (*chapter 7*) is believed to be induced by a reversible interaction of stable derivatives with *alpha2μ*-globulin, a male rat specific protein. This phenomenon may ultimately lead to increased incidence of renal adenomas and carcinomas, due to a sustained process of cytolysis and compensatory cell proliferation. For man, protein droplet nephropathy probably has litle relevance, because of a lack of the *alpha2μ*-globulin in the human population.

Furthermore, polychlorinated benzene congeners resemble the structurally related polychlorinated biphenyls (Brouwer, 1989) in that they markedly disturb thyroid hormone homeostasis in rat, both after a single exposure (*chapter 7*) or semi-chronic treatment (*chapter 8*). This effect may (in part) be attributed to a common mechanism involving an interaction of the primary phenol metabolites with the plasma transport protein for thyroxine, transthyretine (*chapter 7*; Van den Berg, 1990). An additional effect of polychlorinated benzenes in common with polychlorinated biphenyls includes

disturbances in retinoid homeostasis (*chapter 8*). The relevance of the disturbance in thyroid hormone and retinoid homeostasis to halogenated benzene-induced toxicity, however, remains to be established.

A few general points need to be addressed. Firstly, in interpreting the importance of covalent binding, it should be realized that microsomal systems may magnify the intensity of the oxidative pathways with respect to the more complex situation *in vivo*. In the present study this is demonstrated by the observation that the extent to which a compound is activated to reactive products *in vitro*, does not necessarily correlate with the observed toxic effects. Multiple factors may influence the ultimate outcome of a chemical insult, such as differences in absorption, distribution, biotransformation and excretion between the different polychlorinated benzenes. These factors play a role *in vivo*, but are absent in a microsomal system.

Secondly, the delicate balance between activation and detoxication pathways is important in the manifestation of chemical-induced toxicity. Competing pathways such as conjugation with glucuronic acid, sulphate or glutathione may direct the proximate reactive metabolites away from their target sites. Once the formation of reactive benzoquinone metabolites can not be circumvented, the cell possesses additional protective systems, either of an enzymatic nature (e.g., DT-diaphorase, GSH peroxidase) or a chemical nature (e.g., reducing equivalents such as pyridine nucleotides, ascorbic acid or α-tocopherol).

In order to overcome these difficulties, knowledge on biotransformation pathways should be combined with data on the actual concentrations of the relevant (iso-) enzymes in the different (target) organs, and data on the (toxico-)kinetic profiles of the different congeners. These parameters may then be used to obtain a 'Physiologically-Based Pharmaco-Kinetic' model, which should accurately describe, and ultimately predict, the concentration of a reactive intermediate or other active metabolite at the target site or in the target organ. This might provide us with explanations for the observed differences in target organ toxicity between the different (poly)chlorinated benzene congeners, and for the observed differences in the effect of different dose levels of the same congener.

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SAMENVATTING

Lipofiele xenobiotica worden in het lichaam door middel van biotransformatie omgezet in polaire metabolieten, zodat excretie via urine en/of faeces mogelijk wordt. In de meeste gevallen resulteert biotransformatie in de ontgifting van toxische stoffen. Er zijn echter vele stoffen bekend die op zichzelf weinig schadelijk zijn, maar juist als gevolg van biotransformatie worden omgezet in reactieve produkten (zgn. 'metabole activatie'), die vervolgens de normale fysiologie van een cel, weefsel of organisme kunnen verstoren. Voor een beter inzicht in de relatie tussen chemische structuur van een stof en zijn effect(en) op levende organismen is kennis van de verschillende routes, waarlangs een stof wordt omgezet, onontbeerlijk.

In *hoofstuk 1* wordt een beknopte beschrijving gegeven van de belangrijkste biotransformatie systemen, die betrokken zijn bij de omzetting van xenobiotica, en van de bijdrage die deze systemen kunnen leveren aan de metabole activering en/of toxiciteit van xenobiotica. Gehalogeneerde benzenen vormen een goed voorbeeld van een groep van stoffen, waarbij metabole activatie een rol speelt in de toxische effecten op de verschillende doelorganen (met name lever en nier). Figuur 1.2 (zie pagina 33) maakt duidelijk dat uit een betrekkelijk eenvoudige stof, zoals een halogeenbenzeen, verschillende reactieve intermediairen en metabolieten gevormd kunnen worden. Het vele onderzoek naar het mechanisme van levertoxiciteit door broombenzeen wijst op een belangrijke rol voor het intermediair 3,4-epoxide. Echter, tijdens de oxidatie van het volledig gesubstitueerde hexachloorbenzeen zijn het niet de epoxides, maar juist reactieve chinon metabolieten (met name tetrachloorbenzochinon), die covalent aan eiwit binden. Daarnaast zijn er sterke aanwijzigingen dat de niertoxiciteit door broombenzeen is toe te schrijven aan de vorming van chinon metabolieten, die na conjugatie met glutathion naar de nier getransporteerd worden en daar proximale tubulaire schade veroorzaken.

Het onderzoek dat staat beschreven in dit proefschrift is uitgevoerd om meer inzicht te verkrijgen in het belang van de verschillende intermediairen en metabolieten in de toxiciteit van een hele serie gehalogeneerde benzenen. Experimenten zijn met name uitgevoerd met (poly-)chloorbenzenen. Enerzijds zijn er *in vitro* studies gedaan met [^{14}C]-gelabelde substraten en microsomale suspensies om de verschillende biotransformatie routes te karakterizeren en het belang van deze routes in de metabole activatie te evalueren (Deel I, hoofdstuk 2, 3, 4, en 5; samengevat in hoofdstuk 6). Anderzijds zijn *in vivo* studies uitgevoerd om (i) een inschatting te krijgen van de toxiciteit van een reeks chloorbenzenen op de verschillende doel organen, en (ii) meer inzicht te krijgen in de rol van de verschillende metabolieten in de toxische effecten (Deel II, hoofdstuk 7, 8, en 9; samengevat in hoofdstuk 10).

Deel I In vitro studies

In het eerste deel van dit proefschrift komt de vraag aan de orde óf, en zo ja in welke mate, een serie polychloorbenzenen geoxideerd kan worden tot reactieve produkten die covalent aan eiwit kunnen binden. Hiertoe werden microsomale incubaties met [^{14}C]-gelabelde substraten uitgevoerd.

In *hoofdstuk 2* wordt de microsomale oxidatie van pentachloorbenzeen (PCB) naar pentachloorfenol beschreven. Daarnaast worden de verschillende isomeren van tetrachloorfenol gevormd alsmede tetrachloorhydrochinon. Tijdens de oxidatie van PCB worden metabolieten gevormd, die covalent binden aan eiwit. Incubaties uitgevoerd in de aanwezigheid van ascorbine zuur, een reductor, tonen het belang van chinon metabolieten in de eiwit binding aan. Vanwege het feit dat de eiwitbinding ondanks een grote overmaat ascorbine zuur nooit geheel voorkomen kan worden, wordt de suggestie gedaan dat andere metabolieten, waarschijnlijk de epoxides in de primaire oxidatie van PCB naar pentachloorfenol, ook een bijdrage leveren aan de eiwitbinding.

Hoofdstuk 3 beschrijft de microsomale oxidatie van 1,2,4-trichloorbenzeen (1,2,4-TRICB). 1,2,4-TRICB wordt geoxideerd tot de verschillende isomeren van trichloorfenol en, in mindere mate, tot trichloorhydrochinon. De vorming van de 2,3,4- en 2,4,6-trichloorfenol kan verlopen via een zgn. 'NIH-shift' van de overeenkomstige epoxide intermediairen in de primaire oxidatie stap. In overeenkomst met het microsomaal metabolisme van PCB blijkt een belangrijk deel van de metabolieten covalent aan eiwit te binden. Opvallend is echter dat de eiwitbinding van 1,2,4-TRICB metabolieten volledig voorkomen wordt door de aanwezigheid van ascorbine zuur in de microsomale incubatie. Daarnaast blijkt dat zowel voor 1,2,4-TRICB als voor PCB de mate van eiwit binding sterk gecorreleerd is met de mate van secundaire oxidatie van de fenol metabolieten, en *niet* met de mate van primaire oxidatie van de benzenen. Op grond van deze waarnemingen wordt geconcludeerd dat chinon metabolieten de enige reactive species[1] zijn in de oxidatie van zowel 1,2,4-TRICB als PCB. In plaats van de aanname dat de 'rest' binding van PCB metabolieten aan eiwit in de aanwezigheid van ascorbine zuur (zie hoofdstuk 2) veroorzaakt wordt door een ander type reactieve metaboliet, kan als verklaring dienen een onvolledige reductie van het reactieve tetrachloorbenzochinon tot tetrachloorhydrochinon.

Hoofdstuk 3 beschrijft ook de invloed van de cytochroom P450 samenstelling van de microsomale suspensies op de oxidatie van PCB en 1,2,4-TRICB. Microsomen van ratten

[1] hiermee wordt bedoeld 'reactieve intermediairen/ metabolieten, in het engels gewoonlijk aangeduid als 'reactive species'. Deze term is hier in het nederlands overgenomen.

voorbehandeld met dexamethason (DEX), een stof die de hoeveelheid P450IIIA1 sterk induceert, zetten de beide substraten het beste om, in analogie met wat gevonden was voor hexachloorbenzeen (HCB) (Van Ommen *et al.*, 1989). Het blijkt echter dat met afnemende substitutie graad de bijdrage van P450IIIA1 aan de oxidatie minder prominent wordt, en de relatieve bijdrage van andere isoenzymen belangrijker. De microsomale studies met twee isomeren van dichloorbenzeen bevestigen deze tendens (zie hoofdstuk 4).

Van twee dichloorbenzeen isomeren (DCB; i.e. 1,2- en 1,4-DCB) is een groot verschil in hepatotoxiciteit beschreven (zie ook hoofdstuk 7). In *hoofdstuk 4* wordt de microsomale oxidatie van de toxische 1,2-isomeer en de niet-toxische 1,4-isomeer beschreven, met speciale aandacht voor mogelijke verschillen in metabolieten profiel die een rol kunnen spelen in de isomeer-selectieve hepatotoxiciteit. Zowel 1,2- als 1,4-DCB worden geoxideerd tot metabolieten die covalent aan eiwit binden. Een interessante waarneming is echter dat de eiwitbinding van 1,4-DCB metabolieten door de reductor ascorbine zuur volledig geremd kan worden, terwijl dit voor 1,2-DCB slechts gedeeltelijk mogelijk is. 'Molecular Orbital' computer berekeningen aan de arene oxide/ oxepin intermediairen van beide substraten tonen echter geen verschillen aan in chemische reactiviteit en/of stabiliteit van de verschillende arene oxide intermediairen van 1,2-DCB versus 1,4-DCB. Dit pleit tegen een rol van arene oxide intermediairen in de ('rest') eiwit binding van 1,2-DCB.

Vooruitlopend op hoofdstuk 5, waarin het mechanisme van de vorming van reactieve chinon metabolieten wordt bestudeerd, wordt het optreden van restbinding van 1,2-DCB metabolieten aan eiwit in aanwezigheid van een reductor verklaard uit een directe oxidatie van de *para*-gesubstitueerde fenol metaboliet (i.e., 3,4,-dichloorfenol) naar het reactieve benzochinon. Een dergelijke directe oxidatie van de primaire fenol metaboliet van 1,4-DCB (i.e. 2,5-dichloorfenol; bezit geen halogeen substituent *para* ten opzichte van de hydroxyl groep) is niet mogelijk.

Bovenstaande studies benadrukken het belang van de vorming van secundaire chinon metabolieten in de metabole activatie van gechloreerde benzenen. Het is daarom van belang om het mechanisme van vorming van deze reactieve produkten te bestuderen. Hiertoe worden pentafluor- en pentachloorfenol, en hun niet *para*gesubstitueerde analogen gebruikt als model stoffen. De oxidatie produkten in een microsomale incubatie worden geïdentificeerd met ^{19}F-NMR en HPLC. Het karakter van de oxidatie produkten van de fenol substraten kan echter niet bestudeerd worden onder standaard condities met moleculaire zuurstof en NADPH als electronen donor voor cytochroom P450, vanwege het feit dat het aanwezige NADPH eventueel gevormd benzochinon (chemisch) kan reduceren tot hydrochinon. Daarom zijn anaerobe incubaties uitgevoerd, waarbij cumeen hydroperoxide werd gebruikt als zuurstof donor. Gebaseerd op de resultaten die beschreven staan in *hoofdstuk 5*, wordt een mechanisme voorgesteld voor de P450

afhankelijke oxidatie van halogeenfenolen, waarbij het al dan niet aanwezig zijn van een halogeensubstituent op de positie *para* ten opzichte van de hydroxyl groep een bepalende factor is voor het karakter van het produkt (i.e. hydrochinon vs benzochinon). Cytochroom P450 afhankelijke oxidatie op een niet-gesubstitueerde *para* positie resulteert in de vorming van het *para*-hydroxyl derivaat (i.e. hydrochinon) als primair produkt. Daarentegen resulteert cytochroom P450 afhankelijke oxidatie op een *para* positie waar een halogeen substituent zit in de directe vorming van een reactief benzochinon onder verlies van het halogeen als een halogeen anion. In *hoofdstuk 11* wordt ingegaan op mogelijke toxicologische implicaties van directe vorming van reactieve benzochinon metabolieten.

Deel II In vivo studies

In deel II van dit proefschrift staan de resultaten van twee *in vivo* studies beschreven, waarbij de toxiciteit van een hele serie chloorbenzenen op de verschillende doel organen is vastgesteld. Tevens wordt getracht de diverse effecten te correleren met de vorming van verschillende metabolieten.

In *hoofdstuk 7* wordt een hele serie chloorbenzenen vergeleken met betrekking tot hun potentie om toxiciteit te veroorzaken in verschillende doel organen na een éénmalige i.p. toediening in de rat. Effecten op de lever bestaan uit een toegenomen relatief levergewicht op 72 uur na toediening, hetgeen voor de hoger gechloreerde benzenen ook nog waarneembaar is na 216 uur. In de meeste behandelingsgroepen wordt een variabele mate van centrilobulaire hypertrofie en hepatocellulaire degeneratie waargenomen. Van alle congeneren die getest zijn, geven 1,2-dichloorbenzeen en 1,2,4-trichloorbenzeen de meest ernstige effecten, hetgeen blijkt uit een sterke stijging van de plasma ALT spiegels, en duidelijk waarneembare histopathologische degeneratieve veranderingen. Monochloorbenzeen was minder toxisch. In deze studie worden geen schadelijke effecten op de lever waargenomen na blootstelling aan 1,4-dichloorbenzeen en 1,2,4,5-tetrachloorbenzeen, terwijl blootstelling aan pentachloorbenzeen resulteert in slechts geringe histopathologische veranderingen in de lever. Hieruit kan dus geconcludeerd worden dat de mate van metabole activatie zoals waargenomen *in vitro* (deel I) geen goede weergave biedt van de potentie om leverschade te veroorzaken.

Er worden in de gebruikte dosis range geen degeneratieve effecten waargenomen op de nieren. Wel blijkt de vorming van 'Protein Droplets' in de tubulaire epitheel cellen een algemeen verschijnsel te zijn na (éénmalige) blootstelling aan gechloreerde benzenen. Dit effect was sterker en gedurende langere tijd waarneembaar na blootstelling aan de hoger gechloreerde congeneren.

Een interessante waarneming betreft het feit dat chloorbenzenen de schildklierhormoon huishouding verstoren, hetgeen tot uiting komt in een sterke daling van de plasma thyroxine spiegels. Hoger gechloreerde benzenen veroorzaken een sterker daling, en deze daling blijkt tevens op te treden na subchronische toediening van een lage dosis (hoofdstuk 8). Verstoring van de schildklierhormoon huishouding is ook beschreven voor de polychloorbiphenylen (Brouwer, 1989), verbindingen die wat betreft chemische structuur grote overeenkomsten vertonen met chloorbenzenen. Er worden in hoofdstuk 7 aanwijzingen geleverd voor een gemeenschappelijk werkingsmechanisme, waarbij de vorming van fenol metabolieten een cruciale factor is: door een selectieve interactie van fenol metabolieten met transthyretiene, een belangrijk plasma transport eiwit voor schildklierhormoon in de rat, wordt het plasma transport van thyroxine ernstig verstoord, hetgeen resulteert in verlaging van de plasma spiegels.

In hoofdstuk 8 en 9 worden de resultaten beschreven van een 13-weken dieet studie in de rat (♀) met hexachloorbenzeen (HCB) en pentachloorbenzeen (PCB). Vergelijking van de toxiciteit en van de biotransformatie van deze twee congeneren is interessant, aangezien *in vitro* studies hadden aangetoond dat zowel HCB als PCB geoxideerd worden tot pentachloorfenol (PCP) en het reactieve tetrachloorbenzochinon (TCBQ) (zie Van Ommen *et al.*, 1986; hoofdstuk 2). Daarnaast zijn er sterke aanwijzing dat hetzelfde cytochroom P450 isoenzym (P450IIIA) betrokken is bij de omzetting van deze stoffen (Van Ommen *et al.*, 1989; hoofdstuk 3).

De subchronische studie beschreven in *hoofdstuk 8* was met name gericht om meer inzicht te krijgen in de rol van de oxidatieve metabolieten in de diverse toxische effecten van HCB en PCB. Bijzondere aandacht was er voor de mogelijke rol van het reactieve TCBQ in het ontstaan van porfyrie. Hiertoe werd de excretie van PCP en tetrachloorhydrochinon, de gereduceerde vorm van TCBQ, in de urine gevolgd in de tijd. Daarnaast werd het effect bestudeerd van selectieve beinvloeding van de cytochroom P450IIIA activiteit op de toxiciteit (met name porfyrie) en de biotransformatie van HCB en PCB, door gelijktijdige behandeling met het macrolide triacetyloleandomycine (TAO).

Blootstelling van ratten (♀) aan HCB (300 ppm) via het voer resulteert in een sterke toename van de porfyrine excretie via de urine en in een sterke accumulatie van porfyrines in de lever. Deze effecten van HCB worden sterk geremd door gelijktijdige blootstelling aan TAO. Gecombineerde blootstelling van ratten aan HCB of PCB met TAO resulteert tevens in een duidelijk verlaagde uitscheiding van de oxidatieve metabolieten, PCP en TCHQ. De vermindering van de porfyrinogene effecten van HCB kunnen echter niet verklaard worden uit een verminderde vorming van het reactieve TCBQ, aangezien ratten die worden behandeld met een hoge dosis PCB (1300 ppm) een urinaire excretie van TCHQ hebben die enkele malen hoger is dan in ratten die het HCB dieet krijgen, terwijl deze ratten géén porfyrie ontwikkelen. De goede correlatie die werd waargenomen tussen porfyrie en PCP excretie kunnen echter wel de hypothese ondersteunen, waarin een reactief intermediair in de primaire oxidatie stap van HCB naar

PCP wordt voorgesteld als uiteindelijke porfyrinogene species. In dit opzicht is met name een nieuw gepostuleerd type (reactief) intermediar met een chinon-structuur (Rietjens en Vervoort, 1992) interessant voor verder onderzoek.

In *hoofdstuk 9* wordt de biotransformatie van HCB en PCB beschreven, waarin de metabolieten profielen in de urine worden vergeleken. Zoals reeds besproken in hoofdstuk 8, worden HCB en PCB *in vivo* geoxideerd tot PCP en TCHQ. Dit blijken echter de enige twee gemeenschappelijke metabolieten van HCB en PCB, die worden uitgescheiden via de urine. Overige metabolieten van HCB zijn het pentachloorfenylmercaptuurzuur, kwantitatief de belangrijkste metaboliet, en mercaptotetrachloorthioanisool. PCB wordt omgezet in een groter aantal zwavelbevattende verbindingen, waarbij mercaptotetrachloorfenol en pentachloorthiofenol de belangrijkste produkten zijn.

PCB wordt, behalve tot PCP, in belangrijke mate ook geoxideerd tot 2,3,4,5-tetrachloorfenol (TCP). Opvallend is dat de excretie van 2,3,4,5-TCP niet geremd wordt door gecombineerde blootstelling van ratten aan PCB en TAO, de remmer van P450IIIA. Dit wijst erop dat (i) de oxidatie van PCB tot PCP en tot 2,3,4,5-TCP verloopt via verschillende routes, en dat (ii) cytochroom P450IIIA niet betrokken is bij de oxidatie van PCB tot 2,3,4,5-TCP. Gecombineerde blootstelling van PCB en TAO had geen éénduidig effect op de excretie van zwavelbevattende metabolieten. Zo blijkt bijvoorbeeld dat de excretie van mercaptotetrachloorfenol niet geremd wordt door TAO, terwijl die van (het glucuronide van) pentachloorthiofenol sterk geremd wordt. De resultaten van deze studie onderstrepen het belang van meer gedetailleerde studies naar de interactie tussen fase I metabolisme (oxidatieve systemen, met name cytochroom P450) en fase II metabolisme (conjugatieve systemen, met name glutation conjugatie) om uiteindelijk een beter begrip te krijgen van de routes waarlangs de verschillende metabolieten gevormd worden.

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List of Abbreviations

AA	ascorbic acid
CumOOH	cumene hydroperoxide
CVB	covalent binding
DCB	dichlorobenzene
DCC	dichlorocatechol
DCHQ	dichlorohydroquinone
DCP	dichlorophenol
DEX	dexamethasone
EH	epoxide hydrolase
GC	gas chromatography
GSH	glutathione
GST	glutathione *S*-transferase
GT	UDP-glucuronyl transferase
γ-GT	γ-glutamyl transpeptidase
HCB	hexachlorobenzene
HF	heat of formation
HPLC	high performance liquid chromatography
i.p.	intraperitoneally
ISF	isosafrole
3-MC	3-methylcholanthrene
MCB	monochlorobenzene
MO	molecular orbital
MS	mass spectrometry
NMR	nuclear magnetic resonance
P450	cytochrome P450
PB	phenobarbital
PCB	pentachlorobenzene
PCP	pentachlorophenol
PFP	pentafluorophenol
p.o.	per os
ST	sulfotransferase
T3	triiodothyronine
T4	thyroxine
TAO	triacetyloleandomycin
TCB	tetrachlorobenzene
TCBQ	tetrachlorobenzoquinone
TCHQ	tetrachlorohydroquinone
TCP	tetrachlorophenol
TFBQ	tetrafluorobenzoquinone

TFHQ	tetrafluorohydroquinone
TFP	tetrafluorophenol
TRICB	trichlorobenzene
TRICC	trichlorocatechol
TRICHQ	trichlorohydroquinone
TRICP	trichlorophenol
TTR	transthyretine
UROG-D	uroporhyrinogen decarboxylase

Uitleg voor familie en vrienden

De toxicologie ('leer der vergiften'; toxicon= vergift) houdt zich bezig met het bestuderen van de schadelijke effecten van lichaamsvreemde stoffen, ook wel xenobiotica genoemd (xenos= vreemd, bios= leven) in organismen (mens, dier). Het fundamenteel toxicologisch onderzoek richt zich daarbij vooral op het werkingsmechanisme van giftige stoffen: bijvoorbeeld, waarom leidt overmatige alcohol consumptie tot leverschade, waarom hebben rokers een grotere kans op longkanker, hoe kan verklaard worden dat 1 tablet paracetamol de hoofdpijn doet verdwijnen, terwijl een overdosis paracetamol dodelijk kan zijn?

Omdat (ingrijpend) onderzoek met mensen ethisch niet verantwoord is, maakt men in toxicologisch onderzoek veel gebruik van proefdieren (met name de rat en de muis). De gegevens uit dergelijk onderzoek kunnen dan vervolgens 'vertaald' worden naar de mens. Echter, voor veel vraagstellingen blijkt onderzoek aan het intacte dier (*in vivo* onderzoek) veel te complex te zijn. Men kan dan zijn toevlucht nemen tot *in vitro* onderzoek. Hierbij maakt men gebruik van organen, cellen uit organen, of van celbestandelen. Behalve een simplificatie van de werkelijke situatie is een groot bijkomend voordeel van *in vitro* technieken een sterk verminderd gebruik van het aantal proefdieren.

Omzetting van stoffen in het lichaam: biotransformatie

Via o.a. voedsel, drank, geneesmiddelen en inademing van lucht krijgen we veel lichaamsvreemde stoffen binnen, zowel van natuurlijke als industriële oorsprong. Om te voorkomen dat al deze stoffen zich gedurende het leven in ons lichaam (met name vetweefsel) stapelen, met alle nadelige gevolgen vandien, moeten we ervoor zorgen dat we deze stoffen weer kwijtraken, bijvoorbeeld door uitscheiding via urine, faeces of uitademingslucht. Om de uitscheiding te vergemakkelijken, wordt het merendeel van deze xenobiotica (die vaak een vetachtig karakter hebben) tijdens de rondgang door het lichaam zodanig veranderd, dat ze beter in water oplosbaar zijn. Het lichaam bezit daartoe speciale 'fabriekjes', enzymen genoemd, die de stoffen chemisch veranderen. Het hele proces van omzetting van een oorspronkelijke stof in één of meer producten (metabolieten) wordt 'biotransformatie' genoemd. Een van de belangrijkste biotransformatie enzymen is het cytochroom P450. Organen, die een belangrijke bijdrage leveren aan het biotransformatie proces zijn de lever, en in mindere mate de nieren, longen, darmen en huid.

In het algemeen geldt dat de metabolieten van een stof minder actief (minder 'giftig') zijn dan de oorspronkelijke stof zelf. De omzetting van een stof in zijn metabolieten noemt men in dat geval ontgifting of detoxicatie. Echter, er zijn ook voorbeelden bekend, waarbij tijdens de biotransformatie metabolieten ontstaan die juist reactiever (giftiger) zijn dan de uitgangsstof. Men spreekt dan van bioactivatie. Met name de omzetting door het enzym cytochroom P450 heeft nogal eens bioactivatie tot gevolg. De reactieve

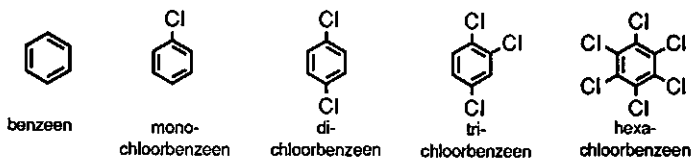
producten kunnen vervolgens reageren met bestandelen in een cel, waardoor de cel afsterft. Gelukkig kent de cel een aantal beschermingsmechanismen, zodat een éénmaal gevormd reactief product weer snel ontgiftigd kan worden (bijvoorbeeld door de werking van andere biotransformatie enzymen). Duidelijk moge zijn dat de balans tussen bioactivatie en detoxicatie heel belangrijk is voor het uiteindelijk (toxische) resultaat.

Dit proefschrift

'De relatie tussen biotransformatie en toxiciteit van halogeenbenzenen'

De chemische structuur van een stof blijkt een uitermate belangrijk gegeven te zijn voor de al dan niet schadelijke werking van die stof. De toxicoloog streeft ernaar aan de hand van de chemische structuur van de stof de toxicologische werking te kunnen voorspellen. De praktijk leert echter dat dit vreselijk moeilijk is.

In dit proefschrift staat een reeks van stoffen centraal die, wat betreft chemische structuur, veel op elkaar lijken. Deze stoffen bestaan uit een benzeen ring met één of meer 'halogeen' substituenten, zoals chloor (Cl), fluor (F) of broom (Br). Vandaar de naam: *halogeenbenzenen.* In dit onderzoek is vooral gewerkt met chloorbenzenen (zie figuur 1). Chloorbenzenen worden als product gebruikt in de chemische industrie.



Figuur 1. Enkele voorbeelden van de gebruikte chloorbenzenen. Op ieder hoekpunt van de zesring (benzeen) kunnen substituenten geplaatst worden.

Doel van het onderzoek

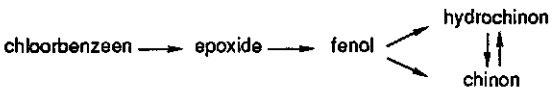
In het algemeen geldt dat halogeenbenzenen pas ná biotransformatie (dus activatie) aanleiding tot toxiciteit geven. Het is echter niet bekend welke metabolieten hiervoor verantwoordelijk zijn. Het onderzoek in dit proefschrift is dan ook gericht op de rol van biotransformatie in de toxiciteit van halogeenbenzenen.

Experimenten, beschreven in deel I, zijn uitgevoerd om aan te tonen óf er reactieve metabolieten uit halogeenbenzenen worden gevormd, en zo ja, welke chemische structuur die reactieve producten hebben. In deel II worden studies beschreven die zijn uitgevoerd om een inschatting te krijgen van de schadelijke gevolgen van de vorming van die reactieve producten.

Deel I: Wat zijn de reactieve metabolieten?

In de eerste fase is vooral 'in de reageerbuis' gewerkt (*in vitro* onderzoek). Hiertoe werden levers van ratten fijngemalen. Via een aantal voorbewerkingsstappen werden celbestanddelen geïsoleerd ('microsomen' genoemd), waarop het biotransformatie proces plaatsvindt. Aan deze microsomen zijn in de reageerbuis de verschillende chloorbenzenen toegevoegd en vervolgens is onderzocht welke metabolieten gevormd werden.

Het bleek dat de uitgangsstof werd omgezet in meerdere producten, zoals epoxides, fenolen en chinonen (zie figuur 2). Daarnaast bleek een deel van de producten zo reactief te zijn, dat zij met celbestanddelen reageerden. Deze reactieve producten bleken 'chinonen' te zijn. Verder onderzoek heeft uitgewezen dat de reactieve chinonen via verschillende 'routes' gevormd konden worden, afhankelijk van de plaats van de halogeen substituenten aan de benzeen ring.



Figuur 2. Omzetting van een chloorbenzeen in verschillende producten.

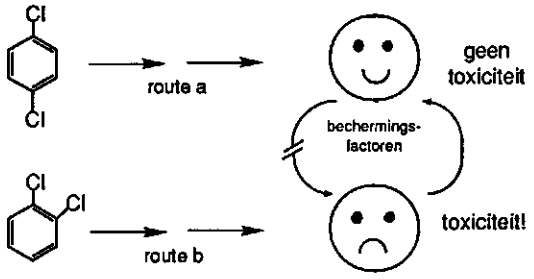
Deel II: Wat betekent dit voor de toxiciteit?

Van groot belang is om te onderzoeken wat de uiteindelijke consequenties zijn van de vorming van reactieve metabolieten, zoals beschreven in deel I, voor het levende organisme. In een levend organisme spelen dermate veel complexe processen af, waardoor de uitkomsten, die in deel I onder gecontroleerde omstandigheden gevonden zijn, niet automatisch vertaald mogen worden naar het intacte organisme. Daarom staan in deel II van dit proefschrift twee studies beschreven die zijn uitgevoerd in het intacte proefdier, de rat (*in vivo* onderzoek).

In de eerste studie is onderzocht hoe ratten reageerden op een behandeling met eenzelfde hoeveelheid van een reeks verschillende chloorbenzenen. Hierbij werd vooral gekeken naar effecten op de lever. De lever is het belangrijkste biotransformatie orgaan. Als er bioactivatie tot reactieve producten plaatsvindt, zal er in eerste instantie schade in dit orgaan optreden.

Opmerkelijk was dat sommige stoffen waarvan in deel I aangetoond was dat zij omgezet werden in reactieve produkten (dus mogelijk giftig), in het intacte dier níet giftig waren. Een mogelijke verklaring hiervoor kan gevonden worden in de chemische structuur van de stof: zoals in deel I is aangetoond, bepaald de plaatsing van halogenen (b.v. chloor) aan de benzeen ring langs welke route de reactieve producten gevormd worden. Door de aanwezigheid van bepaalde beschermingsfactoren in het intacte dier kan

één route (route a-c) geblokkeerd worden: de vorming van giftige chinonen vindt dan niet plaats, waardoor er ook geen leverschade ontstaat. Wanneer chinonen langs de tweede route (route b) gevormd worden, kunnen de beschermingsfactoren echter tekort schieten, zodat schade kan ontstaan. Voor verduidelijking, zie figuur 3.



Figuur 3. Schematische voorstelling van de route voor de vorming van reactieve producten uit chloorbenzenen.

In de tweede *in vivo* studie stonden twee stoffen centraal die zoveel op elkaar lijken, dat ze omgezet worden in hetzelfde product. Dit product bezit een chinon structuur en is derhalve zeer reactief. Volgens een bestaande hypothese zou dit chinon een rol spelen in een zeer specifiek toxisch effect dat men 'porfyrie' noemt. Indien dit het geval is, moeten beide stoffen porfyrie veroorzaken.

Om de bovenstaande hypothese te testen werden ratten gedurende 13 weken lang via het voer blootgesteld aan hexachloorbenzeen en pentachloorbenzeen. Inderdaad bleken beide stoffen omgezet te worden in het reactieve chinon. Echter, 'porfyrie' werd alleen waargenomen in ratten, die blootgesteld waren aan hexachloorbenzeen. Hieruit kan de conclusie getrokken worden dat bij het ontstaan van porfyrie andere factoren dan chinon vorming bepalend zijn. Andere effecten, zoals leverschade en effecten op schildklierhormonen, waren wel hetzelfde in beide behandelingsgroepen, en metabolieten blijken hierin een belangrijke rol te spelen.

Slotopmerking

In dit proefschrift wordt aangetoond dat chinon-metabolieten veel reactiever zijn dan epoxides. Bovendien wordt een mechanisme voorgesteld dat verband legt tussen de structuur van de stof (halogenen aan de ring) en (lever) schade in het organisme. Vooralsnog is dit een model. Toekomstig onderzoek zal zich moeten richten op de directe link tussen de vorming van chinon metabolieten en het ontstaan van schadelijke effecten.

Nawoord

Voor u ligt het tastbaar resultaat van vier jaar wetenschappelijk onderzoek. Vier jaar, waarin je als 'gedreven' onderzoeker gefixeerd bent op je eigen project: weinig andere zaken lijken belangrijker. Toch is de wereld om ons heen juist de afgelopen jaren enorm in beweging: wie had vier jaar geleden gedacht aan de val van de Muur, de hereniging van de twee Duitslanden, de uitéénvalling van de Sovjet Unie of een complete golfoorlog in het Midden-Oosten? Terugblikkend op deze gebeurtenissen, weet ik het in dit proefschrift beschreven onderzoek in het juiste perspectief geplaatst.

Wetenschap is mensenwerk. Bij het tot stand komen van dit proefschrift is de bijdrage en inzet van velen dan ook onontbeerlijk geweest. Het risico lopend sommige mensen te kort te doen, wil ik toch een aantal mensen met name noemen.

Dank, natuurlijk, aan mijn begeleider en promotor, Peter van Bladeren, die de gave bezit om met een minimale (tijds)inspanning een maximaal rendement te behalen. Peter, mocht ik in de beginfase van het onderzoek jouw standplaats bij TNO in Zeist nog als lastig beschouwd hebben, in de loop van het project heb ik er zeker mijn voordeel meegedaan. En, eenmaal in het bezit van een auto bleek Zeist plotseling heel centraal te liggen. Je wist altijd haarfijn de grote lijnen aan te geven op momenten dat ik in details dreigde te verstikken. Tevens mijn dank voor de snelle afwerking van de vele concept-manuscripten in de laatste fase.

Dank ook aan mijn tweede promotor, Jan Koeman, voor de geboden mogelijkheid om het in dit proefschrift beschreven onderzoek uit te voeren op zijn vakgroep. Jan, in het bijzonder dank voor je wezenlijke bijdrage aan hoofdstuk 8.

Ik wil Ivonne Rietjens bedanken voor de enthousiaste en motiverende samenwerking. Ivonne, met recht mag ik stellen dat zonder deze samenwerking hoofdstuk 5 er niet was geweest, en waarschijnlijk hoofdstuk 11 niet zo'n mooie figuur had gehad. Dit laatste zelfs afgezien van het feit dat ik gebruik mocht maken van jouw Apple computer! In het bijzonder dank, ook aan Jacques Vervoort, voor het uitvoeren van de NMR metingen, hetgeen vaak ten koste ging van jullie weekend rust.

Een grote bijdrage aan het in dit proefschrift beschreven onderzoek is geleverd door studenten. Zonder hun bijdrage zou dit boekje veel minder dik zijn geworden. Melanie, Anja, Brigitte, Marjan S., Marlies, Marjan van der R., Erwin en Marlou: bedankt voor jullie doorzettingsvermogen achter de HPLC. Jammer genoeg is die on-line radioactiviteitsdetector er nooit gekomen! Harry, Jurgen, Gert en Erik: 4 mannen tegelijkertijd, druk maar ó zó gezellig! Speciale dank aan Marjon, voor het produceren van een grote berg resultaten tijdens mijn afwezigheid.

De afdeling MTT van TNO Zeist, met name Ben van Ommen, wil ik bedanken voor het feit dat zij bij tijd en wijle mij, en mijn 'non-GLP compliance' op hun lab dulden. Ben, het feit dat jij onder GLP kunt werken stemt mij hoopvol: ik weet zeker dat mij dat in de toekomst ook zal lukken!

Ron Hoogenboom en Theo Polman van de afdeling Toxicologie van het Rikilt hebben gedurende enkele maanden hun, voor mij doen, geavanceerde HPLC-aparatuur beschikbaar gesteld. Zonder die auto-injector moesten er waarschijnlijk nu nog urines 'geprikt' worden! Ron en Theo, bedankt voor alle (sleutel) hulp en adviezen.

Onmisbaar in dit onderzoek zijn geweest de mensen en dieren van het CKP, met name Jo Haas en Maria Peters. Dank voor het feit dat jullie de 13-weken studie zagen 'zitten': het was een vreselijke partij werk, maar het heeft uiteindelijk geresulteerd in twee mooie hoofdstukken. Dank ook aan de mensen van het RIVM (met name Sjef Vos, Frieke Kuper en Koen Molenbeek) en RITOX (met name Peter Schielen, Nanne Bloksma) voor de prettige samenwerking, zowel achter de discussie tafel als in de sectie kamer.

Mijn collega's van de vakgroep toxicologie wil ik allen bedanken voor de gezelligheid. Bert, het leven wordt nooit meer als vroeger: ik drink tegenwoordig zelfs automaten koffie! Dank ook voor het zorgvuldige correctie werk: jouw ogen blijken zoveel beter dan de mijne. In het bijzonder dank aan Irene Bruggeman, die op het moment dat het echt nodig was de helpende hand uitstak. Irene, onze 'dames' kregen een eerste klas verzorging! En, alle resultaten overziend, heeft TAO dus wel 'gewerkt'.

In de loop van mijn onderzoek heb ik in belangrijke mate geprofiteerd van de ideeen en middelen die Bram Brouwer op onze vakgroep heeft ingebracht. Bram, bedankt voor het feit dat je altijd bereid bent mee te denken met andermans onderzoek.

Door de hulp van Gré Heitköning bij het intypen van de tabellen kon het manuscript net op tijd naar de leescommissie, zodat deze promotie nog op de valreep in 1992 kon plaatsvinden. Bedankt, Gré. De medewerkers van de tekenkamer en fotolokatie wil ik bedanken voor de bewezen diensten binnen de vaak zo krappe deadlines.

Ik ben de directie van Solvay Duphar B.V. in Weesp zeer erkentelijk voor de geboden mogelijkheid en tijd om dit proefschrift af te ronden. Natuurlijk ook een woord van dank aan mijn huidige collega's van de afdeling Toxicologie voor hun flexibele opstelling ten aanzien van mijn onregelmatige werktijden. Vanaf heden draai ik op volle kracht mee!

Marita van Amstel en Harry Besselink hebben mij als paranimfen de afgelopen maanden heel wat werk uit handen genomen. Marita, jouw steun betreft niet alleen de mooie etiketjes via WP51, maar eigenlijk veel meer de hechte vriendschap van de afgelopen jaren. Harry, de talloze loopjes van en naar de fotolokatie hebben uiteindelijk geresulteerd in mooie figuren op de juíste grootte. Bedankt ook voor het correctie werk.

Tot slot een woord voor mijn ouders, familie en vrienden: bedankt voor jullie interesse in mijn werk, alhoewel ik waarschijnlijk nooit in staat was de essentie echt over te brengen. Daarom voor jullie in dit boekje een eigen uitleg. Wie nog vragen heeft ...

De laatste woorden in dit boekje zijn bestemd voor mijn grootste steun en toeverlaat, de relativerende Noot in mijn leven, en tevens 'mede-auteur' van pagina 209-212. Lieve Nico, eindelijk is dit boekje dan af. Vanaf nu kunnen we weer samen in de tuin werken.

Cathaline

Curriculum vitae

Cathaline den Besten werd geboren op 7 mei 1963 te Rotterdam. Zij behaalde haar VWO-diploma in 1980 aan de scholengemeenschap Melanchthon te Rotterdam. Vervolgens verbleef zij een jaar in St. Louis (Missouri, USA), alwaar het high-school diploma behaald werd. In 1982 begon zij met de studie Humane Voeding aan de Landbouwuniversiteit te Wageningen. Het propadeutisch examen werd behaald in oktober 1983 (met lof). Tijdens de doctoraalfase werd onderzoek uitgevoerd op de vakgroepen Humane Voeding (prof.dr. J.G.A.J. Hautvast) en Toxicologie (prof.dr. J.H. Koeman). Aansluitend werd een stageperiode doorgebracht bij BIBRA (British Industrial Biological Research Association, Carshalton, UK). De studie werd in januari 1988 met lof afgerond.

Op 1 februari 1988 trad zij als onderzoeker in opleiding in dienst van de vakgroep Toxicologie van de landbouwuniversiteit, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd onder leiding van prof.dr. P.J. van Bladeren. Naast het verrichten van promotie onderzoek heeft zij in deze periode modules van de postdoctorale opleiding Toxicologie gevolgd. Vanaf 16 juni 1992 is zij werkzaam als toxicoloog bij Solvay Duphar B.V. te Weesp.