Contribution to the elucidation of the mechanism
of hepatic porphyria induced by hexachlorobenzene
and related polyhalogenated hydrocarbons

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Contribution to the elucidation of the mechanism of hepatic porphyria induced by hexachlorobenzene and related polyhalogenated hydrocarbons

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ter verkrijging van de graad van
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Chapter 2: Effects of pentachlorophenol on rat liver changes induced by hexachlorobenzene, with special reference to porphyria, and alterations in mixed function oxygenases. Toxicology, 15 (1980) 181-195. 57

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Chapter 4: Effects of phenobarbital and 3-methylcholanthrene on the induction of hepatic mixed function oxygenases and porphyria in hexachlorobenzene treated rats. Submitted to: Chem.-Biol. Interactions. 87

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Stellingen

1. Biotransformatie van hexachloorbenzeen door het in het gladde endoplasmatisch reticulum van de levercel gelokaliseerde "mixed function oxydase" enzymsysteem is de belangrijkste voorwaarde voor het ontstaan van leverporfyrie.

Dit proefschrift.

2. Primaire culturen van kippeëmbryo-levercellen vormen een geschikt modelsysteem voor het onderzoek naar het werkingsmechanisme van porfyrinogene en hepatotoxische stoffen.

Dit proefschrift.

3. De conclusie van Goldstein e.a., dat het door biotransformatie van hexachloorbenzeen gevormde pentachloorfenol niet verantwoordelijk is voor het ontstaan van leverporfyrie, is onvoldoende gefundeerd.

J.A. Goldstein, M. Friesen, R.E. Linder, P. Hickman, J.R. Hass & H. Bergman, Biochem. Pharmacol. 26(1977)1549-1557.

Dit proefschrift.

4. Dat celschade door ozon in zoogdiercellen hoofdzakelijk gepaard gaat met inductie van mitochondriaal en niet van cytoplasmatisch superoxide dismutase suggereert dat superoxide radicalen niet de oorzaak maar eerder het gevolg zijn van deze schade.

5. De conclusie van Knutson en Poland, dat 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) niet toxisch is voor 23 verschillende in vitro gekweekte cellijnen of primaire celculturen is onjuist, op grond van de gekozen parameters en het niet in beschouwing nemen van concentraties waarbij meer dan 90% van de cellen dood gaat.

J.C. Knutson & A. Poland, Toxicol. Appl. Pharmacol. 54(1980)377-383.

6. De vraag naar de functie van het tweede darmsegment bij postlarvale Teleosten kan vooralsnog beter vervangen worden door de vraag of het wel een functie heeft.

7. Hoewel in de literatuur gegevens te vinden zijn die wijzen op een regulerende invloed van het glycinemetabolisme en de Shemin-cyclus op de heemsynthese, is deze mogelijkheid nauwelijks onderzocht.

D. Shemin & C.S. Russell, J. Am. Chem. Soc. 75(1953)4873.

F.P. Richards & J.J. Scott, Clin. Sci. 20(1961)387-400.

J. Bruinvels, L. Pepplinkhuizen, H.R. van Tuijl, P. Moleman & W. Blom, In: Enzymes and Neurotransmitters in Mental Disease (E. Usdin, T.L. Sourkes & M.B.H. Youdim, Eds.), John Wiley & Sons Ltd., 1980, pp.139.

8. De bewering van Andrew e.a. en Cheng & Leblond, dat entero-endocriene cellen van entodermale herkomst zijn, is niet langer houdbaar gezien de bevindingen van Osaka & Kobayashi.

A. Andrew, B.B. Rawdon & B. Kramer, Gen. comp. Endocr. 40(1980)351.

H. Cheng & C.P. Leblond, Amer. J. Anat. 141(1974)537-562.

M. Osaka & S. Kobayashi, In: Endocrine gut and pancreas (T. Fujita, Ed.), Elsevier, Amsterdam, 1976, pp. 145-158.

9. De organisatie van cytoskeletaire elementen in een systeem van microtrabeculae is niet volledig te verklaren uit de brugvorming tussen eiwitten door fixatie met glutaaraldehyde.

J.J. Wolosewick & K.R. Porter, J. Cell Biol. 82(1979)114-139.

J.E. Heuser & M.W. Kirschner, J. Cell Biol. 86(1980)212-234.

10. Aangezien gechloreerde dibenzo-*p*-dioxines en dibenzofuranen in het milieu hoofdzakelijk voorkomen als mengsels van verbindingen met verschillend chloorgehalte, is het aan te bevelen om toxiciteitstesten uit te voeren met extracten van de milieumonsters waarin deze verbindingen voorkomen, in plaats van het bepalen van één van deze verbindingen namelijk het 2,3,7,8-tetrachlorodibenzo-*p*-dioxine.

11. Gezien het feit dat de jaaromzet aan voedingsreclame in Nederland thans zo'n 100 miljoen gulden bedraagt en het Voorlichtingsbureau voor de Voeding slechts één miljoen gulden voor voorlichting tot haar beschikking heeft, is optimisme over het verbeteren van de voeding van de gemiddelde Nederlander niet gerechtvaardigd.

12. Het verdient aanbeveling voor toekomstige archeologen toxicologie in hun vakkenpakket op te nemen.

F.M.H. Debets, Contribution to the elucidation of the mechanism of hepatic porphyria induced by hexachlorobenzene and related polyhalogenated hydrocarbons
Wageningen, 16 januari 1981

Chapter 5: Biotransformation and porphyrinogenic action of hexachlorobenzene and its metabolites in a primary liver cell culture. Submitted to: Toxicology. 105

Chapter 6: Effects of dietary antioxidants on the biotransformation and porphyrinogenic action of hexachlorobenzene in two strains of rats.
I. Induction of mixed function oxygenases, hepatic porphyria, glutathione-S-transferase activity, and liver glutathione levels.
II. Formation and excretion of metabolites.
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Voorwoord

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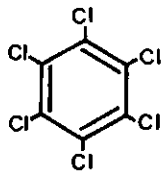
Abbreviations

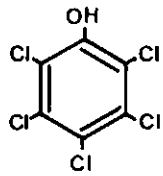
AHH	aryl hydrocarbon hydroxylase
(δ)-ALA	δ-aminolevulinic acid
(δ)-ALAS	δ-aminolevulinic acid synthase
Asc. acid	L-Ascorbic acid , vitamin C
BHT	3,5-di-tert-butyl-4-hydroxytoluene
COPRO	coproporphyrin
DPPED	N,N'-diphenyl-p-phenylendiamine
(CYT) P-450	cytochrome P-450
(CYT) P-448	cytochrome P-448
Diamide	1,1'-azobis (N,N'-dimethylformamide)
DM	diethylmaleate
ERR-O-D	ethoxyresorufin O-de-ethylase
GSH	reduced glutatione
HBB	a mixture of hexabromobiphenyl isomers (Firemaster BP-6)
HCB	hexachlorobenzene
2,4,5,2',4',5'-HCB	2,4,5,2',4',5'-hexachlorobiphenyl
HCH	hexachlorocyclohexane
H.D.	hydrolytic dechlorination
(3)-MC	3-methylcholanthrene
MFO	mixed-function oxidase
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
β-NF	β-naphthoflavone
PAPS	3'-phosphoadenylsulphate
PB	phenobarbital
PBBs	polybrominated biphenyls
PBG	porphobilinogen
PCBs	polychlorinated biphenyls
PCNs	polychlorinated naphthalenes
PCP	pentachlorophenol
PCTA	pentachlorothioanisol
PCT	porphyria cutanea tarda
PCTA-O	1-methyl-[2,3,4,5,6-pentachlorophenyl] sulfoxide
PCTA-O_2	1-methyl-[2,3,4,5,6-pentachlorophenyl] sulfone
PCThP	pentachlorothiophenol
PeCB	pentachlorobenzene
PHA	polyhalogenated aromatic compound

pip. but.	piperonyl butoxide
PROTO	protoporphyrin
R.D.	reductive dechlorination
RR	resorufin
SER	smooth endoplasmic reticulum
SKF 525A	β-diethylaminoethyldiphenylpropylacetate
2,4,5-T	trichlorophenoxyacetic acid
TCB	tetrachlorobenzene
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCH	tetrachlorohydroquinone
TCP	trichlorophenol
TCPO	1,1,1-trichloro-2-propeneoxide
TCTA	2,3,5,6-tetrachlorothioanisol
TCdi-TA	tetrachlorodithioanisol
TCThP	tetrachlorothiophenol
UDPG	uridine diphoshate-D-glucuronate
URO	uroporphyrin
UROG-D	uroporphyrinogen decarboxylase
Vit. E	DL-α-tocopherolacetate
7 COOH	heptacarboxylic porphyrin
6 COOH	hexacarboxylic porphyrin
5 COOH	pentacarboxylic porphyrin
4 COOH	coproporphyrin
3 COOH	tricarboxylic porphyrin
2 COOH	protoporphyrin

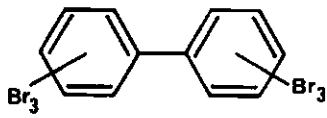
Compounds studied:



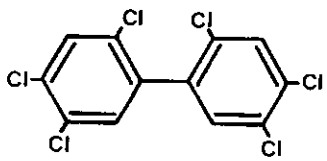
Hexachlorobenzene



Pentachlorophenol



A commercial mixture of hexabromobiphenyl isomers (Firemaster BP-6)



2,4,5,2',4',5'-Hexachlorobiphenyl

General introduction

Hexachlorobenzene in the environment

Hexachlorobenzene (HCB, C_6Cl_6) has been widely used as a fungicide, primarily to control bunt fungi (e.g. Tilletia tritici, Tilletia brevifaciens). However, its residual properties have led to a marked reduction of its application in agriculture. For instance in The Netherlands the use of all fungicides containing HCB as an active ingredient has been prohibited since january 1973. In the same year too high residues of HCB in milk and milk products were found. This problem was attributed to the foddering of cabbage leaves treated with quintozene (pentachloronitrobenzene) to prevent rotting. The quintozene used at that time contained about 3% HCB as an impurity. Since the beginning of 1975, the use of quintozene in agriculture is permitted only when it contains less than 0.1% HCB.

HCB is formed as an impurity or by-product in the manufacture of chlorine, vinyl chloride, carbon tetrachloride, trichloroethylene, perchloroethylene, other chlorinated solvents, and the following pesticides: dimethyl 2,3,5,6-tetrachloroterephthalate (Dacthal), mirex, pentachloronitrobenzene, pentachlorophenol, and hexachlorocyclohexane isomers [1-3]. The level of contamination varies from 1-10% depending on the manufacturing process. HCB is also used as a peptizing agent in the manufacture of styrene and nitrous rubber for tires. A rough estimate showed that at least 95% of the 4 million kilograms of HCB produced annually in the United States are formed as an unwanted waste by-product in the synsthesis of solvents and pesticides [3]. Approximately 20,000 kg used to enter the environment as a consequence of pecticide use. This may have decreased in recent years, thanks to improvements in the production methods. The large-scale production of HCB together with disposal of HCB-containing industrial waste has led to a ubiquitous occurrence in the environment [4]. HCB is a white crystalline solid which is volatile and enters the atmosphere by sublimation. It is an apolar compound with lipophilic properties, which is metabolized at a low rate. Therefore HCB tends to accumulate in the tissues of man and animals [5-7]. Although HCB is almost insoluble in water (6.2 µg/l at 23.5°C [3]) it has been identified as one of the major pollutants in marine waters, surface waters and industrial effluents in several countries [4,8].

Toxicity of hexachlorobenzene

The acute toxicity of HCB is low in most animal species (> 1500 mg/kg). However it produces a wide range of toxic effects after long-term exposure to low doses. Hepatic porphyria so far seems to be the predominant symptom of HCB intoxication in experimental animals and man. Other toxic effects include: Liver enlargement, neurological changes (tremors and convulsions), and cutaneous lesions [9]. The cutaneous and neurological legions develop most commonly at high level exposure to HCB and may precede the onset of hepatic porphyria. Studies of Cabral et al. [10,11] with hamsters and a certain strain of mice gave some indications that HCB might be carcinogenic. However, the incidence of tumors in these two animal species was increased only in the groups receiving HCB at rather high dose levels (> 4 mg/kg bw/ day for hamsters, and > 12 mg/kg bw/day for mice. Moreover, HCB showed mutagenic properties in S. cerevisiae test system using reversion from histidine and methionine as a measure of the induced mutation [12]. There are a number of studies which refer to immune suppressive, reproductive and teratogenic effects of HCB [9,13]; but experiments in these fields and concerning its possible carcinogenic effect need to be continued before definite conclusions can be drawn.

Hepatic porphyria

Hepatic porphyria is a disturbance in the biosynthetic pathway of heme production in the liver. It is characterized by an increased excretion of intermediates of the heme synthesis (porphyrins and earlier precursors) in mainly the urine but also in the feces. In addition, increased levels of porphyrins can be measured in the liver, skin, hemopoietic tissue, intestinal tract, and several other tissues. These free porphyrins have no biological function. Under normal conditions there is a balance between the production of heme (the end product of porphyrin metabolism) and its demand. In HCB-induced hepatic porphyria this control mechanism is disturbed and far more porphyrins are formed than are converted into heme, so that they accumulate and are excreted in excess. In man, the syndrome caused by HCB is known as Porphyria Cutanea Tarda, because the cutaneous lesions are the obvious manifestation of the disease although the underlying cause is the disturbed heme biosynthesis. The development of skin lesions in HCB-induced porphyria is supposedly due to the photosensitising action of porphyrins stored in the skin [14].

A classic case of porphyria in man due to chemical exposure is the mass poisoning with HCB-treated seed wheat in south-east Turkey from 1955 to 1959. During that period there was a shortage of wheat for bread and the seed wheat was used for food instead of its intended agricultural use. It was estimated that 3000 to 5000 people, mostly children in the age of 6 to 16, developed Porphyria Cutanea Tarda with a mortality rate between 1955 and 1959 ranging from 3-10% annually [15,16]. Several years later the porphyrinogenic effects of HCB were confirmed by the observation that HCB feeding produced porphyria in animals under experimental conditions [17,18]. Recently it has been reported that even after twenty years a large group of people in Turkey still have the toxic symptoms of Porphyria Cutanea Tarda [19]. Porphyria in man may also occur as a consequence of inborn genetic disorders [20].

The biosynthetic pathway of heme

Heme forms the end product of the porphyrin metabolism. It is combined with various apoproteins to form hemoproteins such as hemoglobin, myoglobin, and the enzymes catalase, tryptophane pyrrolase, cytochromes of the b-group and cytochrome P-450. Cytochrome P-450 is the terminal oxidase of the hepatic mixed function oxygenase system, which is involved in the biotransformation of HCB and other xenobiotics. The apoproteins are synthesized on the membranes of the endoplasmic reticulum, whereas the synthesis of heme takes place partly in the mitochondria and partly in the cytoplasm. The major steps of the heme biosynthetic pathway are shown in Fig. 1. In the mitochondria, glycine and succinyl CoA are condensed to form δ-aminolevulinic acid (ALA). This rate-limiting step is catalyzed by ALA synthase (ALAS). The ALA leaves the mitochondria and enters the cytoplasm where 2 molecules ALA are condensed to the monopyrrole porphobilinogen (PBG). Four molecules of PBG condens to give uroporphyrinogen III, a tetrapyrrole containing 8 carboxylic groups. The stepwise decarboxylation of the four acetate groups of uroporphyrinogen by uroporphyrinogen decarboxylase (UROG-D) yields coproporphyrinogen III (4-COOH groups), which re-enters the mitochondria. Coproporphyrinogen III is further decarboxylated to protoporphyrinogen (2-COOH) and finally oxidized to protoporphyrin IX. In the last step the mitochondrial enzyme ferrochelatase (protoheme ferrolyase) incorporates the ferrous iron into the protoporphyrin to form heme. The porphyrinogens do not fluoresce under ultraviolet light, but within the cells porphyrinogens are readily converted into their corresponding fluorescent porphyrins by autoxidation.

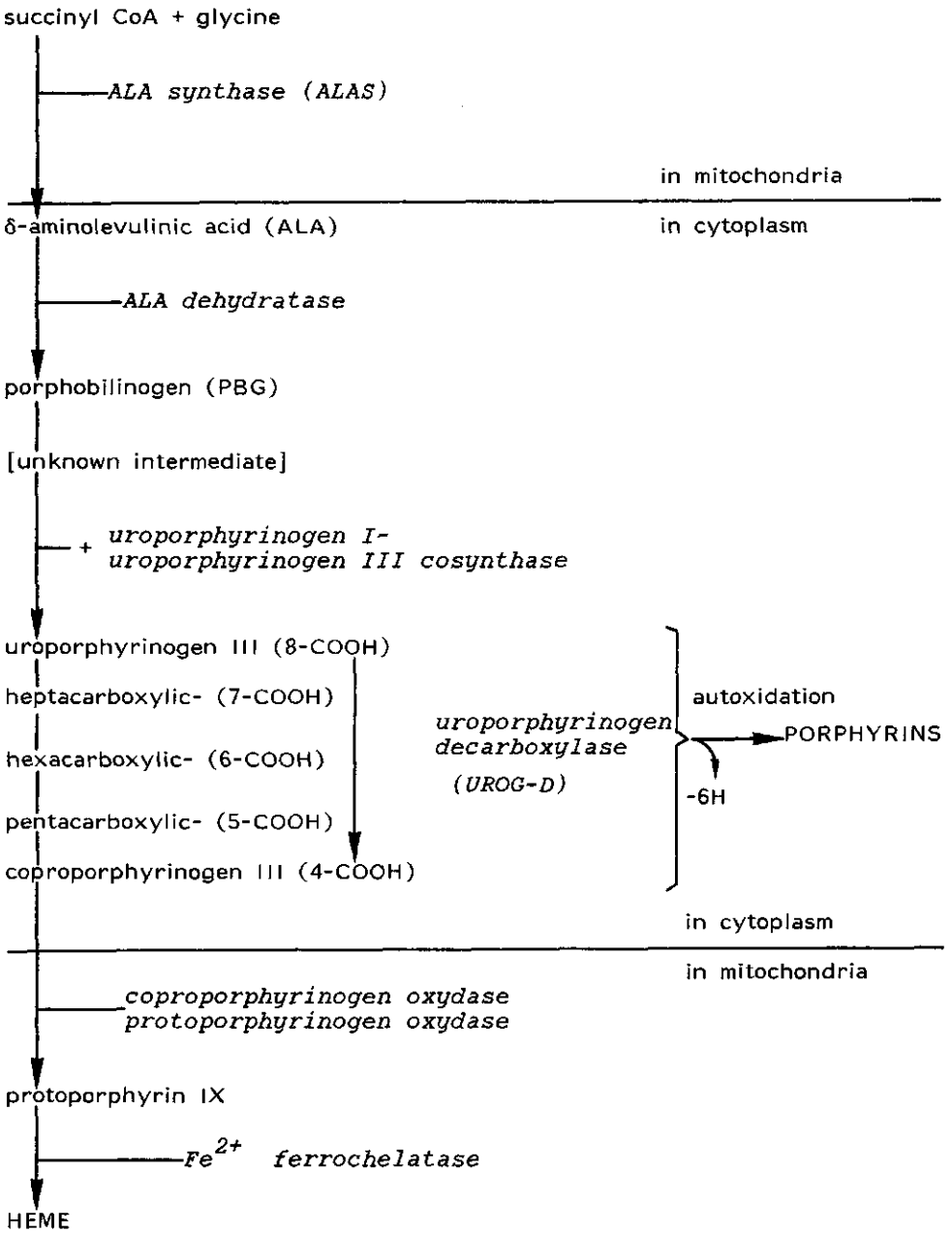


Fig. 1. The hepatic biosynthesis of heme

Hypotheses on the mechanism of HCB-induced hepatic porphyria

Under normal conditions the amounts of porphyrins that accumulate in the liver or that are excreted in the urine and feces are very small. In HCB-induced porphyria, the levels of porphyrins in liver and excreta are increased manifold. The accumulated porphyrins were found to consist mainly of porphyrins containing 8 and 7 carboxylic groups (respectively uro- and heptacarboxylic porphyrin) [21,22]. It has been shown that a decrease of the activity of the enzyme uroporphyrinogen decarboxylase (Fig. 1) forms the primary biochemical lesion underlying the disturbance of the hepatic heme synthesis in HCB-induced porphyria [23]. The inhibition of uroporphyrinogen decarboxylase leads at first to the accumulation of porphyrins at the beginning of the decarboxylation chain, thereby indicating the site of the enzyme defect. Subsequently, a shortage of heme arises as the inhibition of uroporphyrinogen decarboxylase proceeds. Since heme acts as an end-product repressor on the synthesis of ALA synthase [24], a marked induction of the activity of this enzyme can be observed in the later stage of porphyria. The increase in ALA synthase activity results in an enhanced formation of the porphyrin precursor ALA, and in consequence this exacerbates the porphyrin accumulation caused by the inhibition of uroporphyrinogen decarboxylase.

The capactity for disturbing the function of uroporphyrinogen decarboxylase is not restricted to HCB. The enzyme defect can be induced also by several other halogenated aromatic compounds including polybrominated- and polychlorinated biphenyls, chlorinated dibenzo-p-dioxins and dibenzofurans, and chlorinated naphthalenes [25]. The molecular mechanism of action underlying the inhibition of UROG-D by this group of halogenated aromatic compounds is poorly understood.

Objectives of the present study

The studies presented in this thesis were carried out to gain more insight into the mechanism of action of these compounds. Hexachlorobenzene was chosen as a model compound in the present investigations, but similar results were obtained with other representatives of this group of chemicals, as will be pointed out repeatedly.

The main discussion on the mode of action of polyhalogenated aromatic compounds as inducers of hepatic porphyria, partially based on the results of the present studies, is given in Chapter 1. Chapter 2 describes the effect of one of the main HCB metabolites - pentachlorophenol - on the induc-

tion of hepatic porphyria in HCB-treated female rats. The next two Chapters (3 and 4) show that compounds interfering with the drug-metabolizing enzyme system in the liver are able to change the porphyrinogenic effect of HCB in vitro (chick embryo liver cell cultures) as well as in vivo. Chapter 5 deals with the biotransformation and porphyrin-inducing capacity of HCB and its main metabolites in a primary chick embryo liver cell culture. The last Chapter gives the results of our studies on the influence of antioxidants and glutathione on the fate of HCB in the liver of two strains of rats, which differ in there susceptibility to the porphyrinogenic effect of HCB.

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Chapter 1

AN APPROACH TO ELUCIDATE THE MECHANISM OF HEXACHLOROBENZENE-INDUCED HEPATIC PORPHYRIA, AS A MODEL FOR THE HEPATOTOXICITY OF POLYHALOGENATED AROMATIC COMPOUNDS (PHAs)

1.1. Introduction

The mechanism of the porphyrinogenic action of hexachlorobenzene (HCB) and other polyhalogenated aromatic hydrocarbons is still unknown. Special reference will be given to hexachlorobenzene, the member of this group of chemicals that has been most extensively studied as a porphyrinogenic agent and as a model for human symptomatic porphyria (also referred to as porphyria cutanea tarda, porphyria cutanea tarda symptomatica, chronic hepatic porphyria) (Stonard, 1974; Doss et al., 1976). A better insight in the mechanism of hexachlorobenzene-induced porphyria may serve as a model for the hepatotoxic action of other polyhalogenated aromatic compounds (PHAs) (Debets and Strik, 1979). In recent years it has been shown that biotransformation of PHAs appears to be a prerequisite for the development of porphyria (Debets et al., 1979). Therefore, special emphasis will be given to the role of biotransformation of these compounds in relation to the production of porphyria.

Water-insolubility, lipophilic nature, planar or near-planar molecular structure, and different degrees of halogenation are the common physicochemical properties of this group of compounds. In addition to their porphyrinogenic action in different animal species (Table I) (De Matteis, 1967; Vos and Koeman, 1970; Goldstein et al., 1973; Strik, 1973b; Goldstein et al., 1974; Strik and Koeman, 1976), they induce microsomal drug-metabolizing enzymes (Wada et al., 1968; Sweeney et al., 1971; Rajamanickam et al., 1972; Alvares et al., 1973; Strik, 1973d; Grand et al., 1974; Stonard, 1974; Stonard and Nenov, 1974; Turner and Green, 1974; Stonard, 1975; Stonard and Greig, 1976; Dent et al., 1976), are nevertheless very slowly metabolized (Parke and Williams, 1960; Vinopal and Casida, 1973; Kimbrough, 1974; Fishbein, 1974; Villeneuve, 1975; Koss and Koransky, 1975; Koss et al., 1976; Sundström et al., 1976), and accumulate in animal (Koeman et al., 1969; Koss and Manz, 1976) and human adipose tissue (Acker and Schulte, 1970; Brady and Siyali, 1972; Hammond, 1972; Curley et al., 1973). In the past much attention has been paid to the

TABLE I

Species differences in experimental porphyria caused by polyhalogenated aromatic compounds[a]

Species	Porphyria incidence
Man	+
Rat	+
Rat (young)	-
Rabbit	+
Guinea pig	-
Mouse	-[b]
Mink	-
Minipig	-
Japanese quail	+
Chicken	+
Chicken embryo	+
Kestrel	-
Cormorant	-
Blue Heron	-
Pig	+[c]
Lamb	+[d]

a. Strik, 1973d

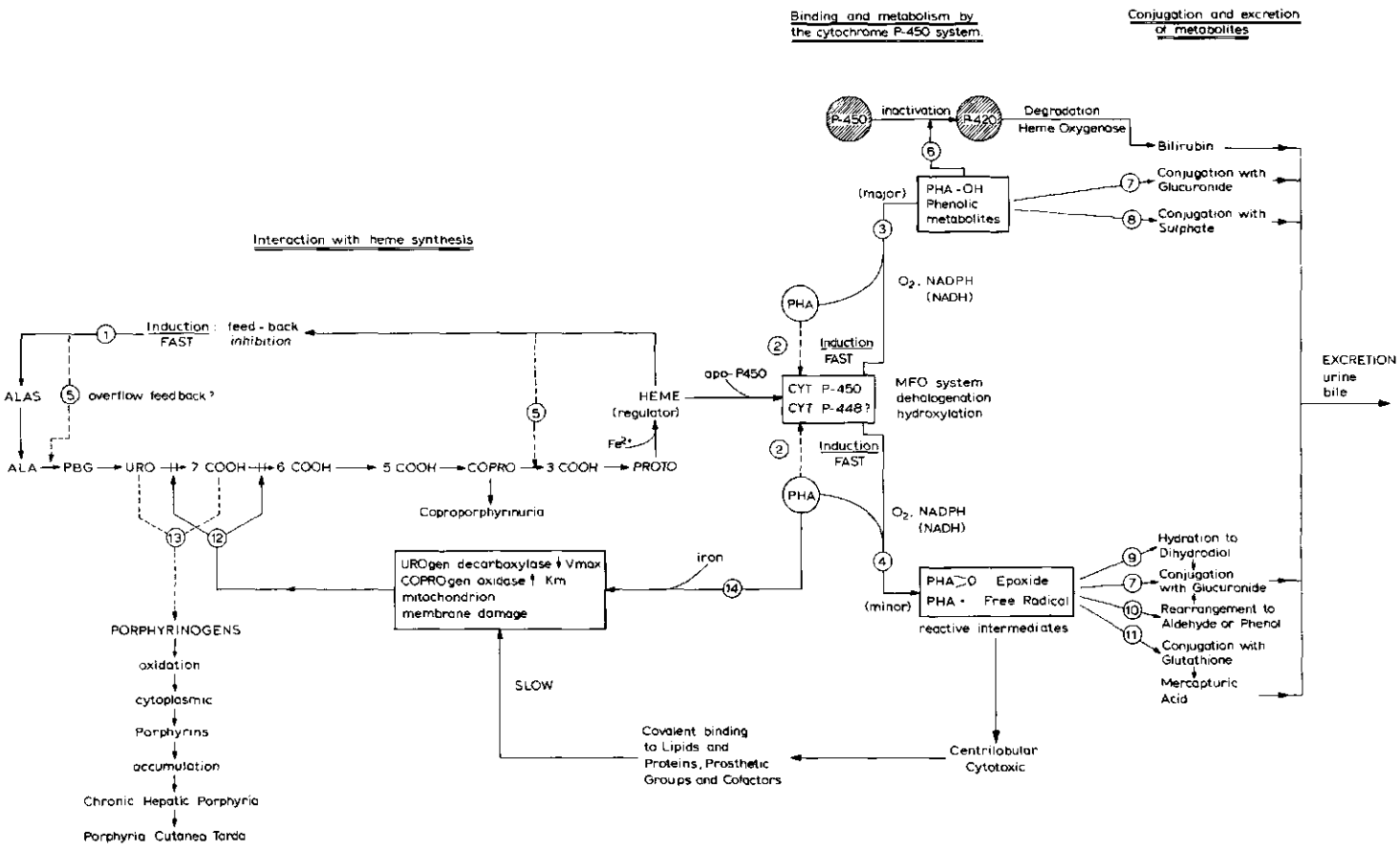
b. Mouse proved to be susceptible to the porphyrinogenic effect of TCDD (Goldstein et al., 1973)

c. Hansen et al., 1977; Den Tonkelaar et al., 1978

d. Mull et al., 1978

adaptive phase of microsomal drug-metabolizing enzyme induction (Wada et al., 1968; Sweeney et al., 1971; Rajamanickam et al., 1972; Alvares et al., 1973; Strik, 1973d; Grant et al., 1974; Stonard, 1974; Stonard and Nenov, 1974; Turner and Green, 1974; Stonard, 1975; Stonard and Greig, 1976; Dent et al., 1976) and the detection of morphological changes in the liver by the time porphyria develops (Bennet et al., 1938; Miller, 1944; Ockner and Schmid, 1961; Sweeney et al., 1971; Kimbrough et al., 1972; Medline et al., 1973; Timme et al., 1974; Mollenhauer et al., 1975; Mollenhauer et al., 1976; Kuiper-Goodman et al., 1976; Kuiper-Goodman et al., 1977; Schmodt et al., 1977). Studies in recent years on the pharmacokin-

Fig. 1. MECHANISM OF ACTION OF HEPATIC PORPHYRIA CAUSED BY POLYHALOGENATED AROMATIC COMPOUNDS. (PHA)



etics of PHAs (Piper et al., 1973; Koss and Koransky, 1975; Matthews and Anderson, 1975a, 1975b; Mehendale, 1976; Peterson et al., 1976; Koss et al., 1978a; Jansson and Bergman, 1978) on the identification of metabolites (Mio et al., 1976; Koss et al., 1976; Sunderström et al., 1976; Jansson and Bergman, 1978; Mizutani, 1978; Renner et al., 1978; Koss et al., 1979a) and their influence on porphyrin metabolism (Lui et al., 1976; Goldstein et al., 1977a; Kimbrough and Linder, 1978; Koss et al., 1979b), and on the toxicity of the parent compounds (Debets et al., 1980), are promising. They provide further insight in the mechanism of action of PHAs. The theories that have been proposed to explain the porphyrinogenic action of polyhalogenated aromatic compounds will be reviewed and discussed with the help of the scheme presented in Fig. 1.

1.2. Induction of δ-aminolevulinic acid synthase (ALAS); increase in urinary excretion of δ-aminolevulinic acid (ALA) and relative increase in urinary coproporphyrin (see Fig. 1, Nos. 1, 5)

The induction of ALAS after chronic exposure to PHAs can be divided into two phases. The first one is the adaptive phase, characterized by an immediate slight (not greater than 2-3-fold) increase in hepatic ALAS activity after commencement of the treatment. This phase probably reflects a greater demand for heme due to the induction of microsomal hemoproteins (cytochrome P-450) by PHAs (Granick, 1966; Sweeney et al., 1972; Goldstein et al., 1973; Strik, 1973c, 1973d; Goldstein et al., 1974; Stonard, 1974; Strik, 1978). The second or pathological phase, marked by a 10-20-fold increase in ALAS activity, coincides with the onset of porphyrin accumulation in the liver and increased urinary porphyrin excretion (Vos et al., 1971c; Goldstein et al., 1978). This second phase of ALAS induction is related to a decrease in the activity of uroporphyrinogen decarboxylase (UROG-D) and reflects a regulatory mechanism operating to keep the level of the free heme constant, in spite of inhibition of UROG-D (one of the enzymes in the pathway of heme synthesis, see Section 1.8). The marked increase in ALAS activity is not essential for porphyrin accumulation, but it exacerbates porphyrinuria by indirectly increasing the substrate concentration for the defective UROG-D. Goldstein et al. (1973) reported that TCDD produced a gross accumulation of hepatic porphyrins in mice, even though ALAS was induced only twofold.

Induction of ALAS and inhibition of UROG-D leads to an excessive urinary excretion of accumulating substrates of the defective enzyme, like

aminolevulinic acid (ALA), porphobilinogen (PGB) and porphyrins with 7 and 8 carboxylic groups (Koss et al., 1978a; Goldstein et al., 1978) (see Fig. 1).

1.3. Induction of cytochrome P-450 (P-448)-mediated mixed function oxidase activity in relation to porphyria (see Fig. 1, No. 2)

Polyhalogenated aromatic compounds increase the concentration of cytochromes P-450 and b-5 and the activity of other components of the mixed function oxidase system in the liver of mammals, birds and man (Wada et al., 1968; Rajamanickam et al., 1972; Sweeney et al., 1971; Alvares et al., 1973; Strik, 1973d; Grant et al., 1974; Stonard, 1974; Stonard and Nenov, 1974; Turner and Green, 1974; Farber and Baker, 1974; Stonard, 1975; Lissner et al., 1975; Stonard and Greig, 1976; Dent et al., 1976; Strik and Koeman, 1976; Alvares et al., 1977; Goldstein et al., 1978; Puzynska et al., 1979; Debets et al., 1980a).

In long-term experiments the increase in the activity of microsomal cytochrome P-450 and mixed function oxidase (aminopyrine N-demethylation; ethoxyresorufin O-de-ethylation) reaches a maximum after 2 weeks exposure time (Rajamanickam et al., 1972; Stonard, 1974; Debets et al., 1980a). However, the activity of ethylmorphine N-demethylase, aminopyrine N-demethylase and ethoxyresorufin O-de-ethylase decreases upon chronic feeding of hexachlorobenzene, by the time porphyria develops (Sweeney et al., 1971; Debets et al., 1980a). Hexachlorobenzene (Stonard, 1975; Debets et al., 1980a), polychlorinated biphenyls (Alvares et al., 1973; Stonard and Greig, 1976; Goldstein et al., 1977b) and polybrominated biphenyls (Dent et al., 1976; Babish and Stoewsand, 1977; Dent, 1978) produce a mixed pattern of microsomal enzyme induction that shows characteristics of the 3-methylcholanthrene (P-448) and characteristics of the phenobarbital (P-450) classes of inducers. Stonard (Stonard and Greig, 1976) suggests that a relationship may exist between the mixed pattern of microsomal enzyme induction and the onset of hepatic porphyria. Metabolism of PHAs and accumulation of metabolites within the liver may be a prerequisite for the observed mixed pattern of enzyme induction and/or the lesion which leads to the ultimate porphyric picture. These speculations contrast with the observation that TCDD - the most potent porphyrinogenic agent known - induces a pattern of microsomal enzymes identical to that caused by 3-methylcholanthrene (Greig, 1972; Greig and De Matteis, 1973; Lucier et al., 1973). Recently Jones and Sweeney (1977) reported that mice genetically

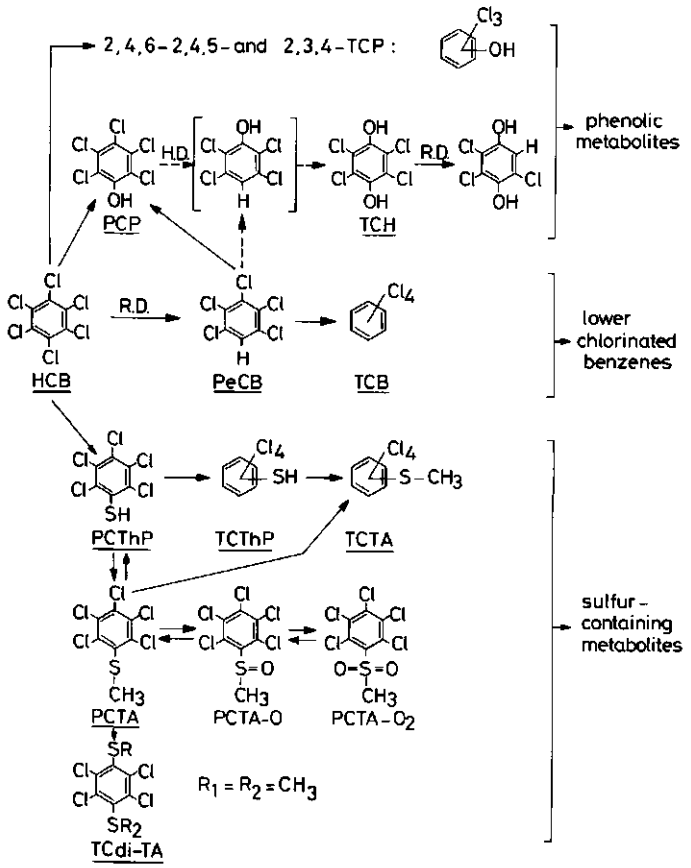
responsive to the induction of aryl hydrocarbon hydroxylase developed hepatic porphyria after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Nonresponsive mice, on the other hand, did not develop porphyria under the same conditions. Uroporphyrinogen decarboxylase activity was only depressed in the responsive mice. These observations suggest that induction of cytochrome P-448 and aryl hydrocarbon hydroxylase by PHAs is probably necessary for inhibition of uroporphyrinogen decarboxylase and hepatic porphyrin accumulation. Simultaneous administration of HCB and phenobarbital (an inducer of cytochrome P-450 and mixed-function oxygenases) promotes the metabolism of HCB and leads to an enhancement of the porphyrinogenic action of the former compound (Kerklaan et al., 1979). A metabolite or reactive intermediate of the parent compound is suspected to be responsible for the disturbance of hepatic porphyrin metabolism (see Sections 1.5 and 1.8).

1.4. Formation of phenolic metabolites (see Fig. 1, No. 3, and Fig. 2)

Parke and Williams (1960) were the first to study the fate of hexachlorobenzene in rabbits. They failed, however, to detect metabolites in the feces and urine after a single oral dose of HCB suspended in water. A poor absorption of HCB from the gastro-intestinal tract was probably the cause of the fact that no metabolites were detected. Recent studies of the fate of HCB in rat (Mehendale et al., 1975; Lui and Sweeney, 1975; Koss et al., 1976; Engst et al., 1976; Renner and Schuster, 1977; Jansson and Bergman 1978), mouse, guinea pig, Japanese quail, laying hen, rainbow trout (Koss et al., 1978b), green sunfish (Lepomis cyanellus) (Sanborn et al., 1977) and monkey (Yang et al.,1975; Rozman et al., 1975) have proved beyond any doubt that HCB in these species is metabolized into more polar compounds. In the rabbit, no phenolic metabolites of HCB have been found (Kohli et al., 1976). Iatropoulos et al. (1975) showed that the major part of a single oral dose of HCB is slowly absorbed by the lymphatic system of the gastro-intestinal tract and deposited in adipose tissue, thus bypassing the portal venous transport to the liver. This may be an explanation for the slow metabolic conversion of HCB and the failure of several investigators to quantify appreciable amounts of metabolites in urine and feces after a single dose of HCB. Pentachlorobenzene (PeCB), tetrachlorobenzene (TCB) and trichlorophenols (TCP) were only found after short-term administration of HCB to rats (Mehendale et al., 1975; Renner and Schuster, 1977; Jansson and Bergman, 1978) and rhesus monkeys (Rozman et al., 1975) (Fig. 2).

The lower chlorinated benzenes may be derived partly from HCB as contaminants (Villanueva et al., 1974; Lui et al., 1976; and Jansson and Bergman, 1978). Pentachlorobenzene could not be detected in a long-term feeding experiment (Koss et al., 1978a), which is not surprising because it has been demonstrated that pentachlorobenzene is almost completely biodegraded in the rat (Koss and Koransky, 1978).

Fig.2. The metabolic fate of hexachlorobenzene HCB in the rat.



Pharmacokinetic studies of Koss et al. (1978a) showed that during long-term administration of HCB to rats, about 60% of the xenobiotic was excreted unchanged and about 40% in the form of metabolites, once an equilibrium between intake and excretion was established. The ratio of the ex-

creted phenolic metabolites (PCP and TCH; see Fig. 2) to the sulfur-containing metabolites (PCThP and TCThP; see Fig. 2) was 2.8 after long-term administration versus 1.3 after short-term administration of HCB (Koss et al., 1976; and Koss et al., 1978a). In all animal species examined so far, with the exception of the Japanese quail (Koss et al., 1978b), pentachlorophenol was identified as the major metabolite of HCB (Koss et al., 1976; Sanborn et al., 1977; Koss et al., 1978b). Pentachlorophenol accounted for 45% of the total amount of metabolites recovered in the body, urine and feces of rats treated with a single dose of HCB. Metabolism of hexachlorobenzene to petachlorophenol is likely to proceed via hydrolytic dechlorination (Parke, 1968), as has been demonstrated in the metabolic conversion of 2,4,6-trichloroaniline to 4-amino- 3,5-dichlorophenol.

Based on the observations that prolonged administration of pentachlorophenol to female rats induces no porphyria, several authors (Lui et al., 1976; Goldstein et al., 1977a; Kimbrough and Linder, 1978; Kószó et al., 1978) conclude that pentachlorophenol as the major metabolite of HCB plays no prominent part in the disturbance of hepatic porphyrin metabolism. However, recent findings show that pure pentachlorophenol disturbs hepatic detoxification mechanisms (Arrhenius et al., 1977b) (see Section 1.5) and accelerates the onset of HCB-induced hepatic porphyria (Debets et al., 1980a). In rats treated with a combination of HCB and PCP the onset of hepatic porphyria started about 3-4 weeks earlier than in rats treated with HCB alone (Debets et al., 1980a). In this experiment PCP, when fed alone, did not cause porphyria. This finding is in agreement with the observation of others (Lui et al., 1976; Goldstein et al., 1977a; Kimbrough and Linder, 1978; Kószó et al., 1978). Hence, it appears that PCP is not the metabolite that ultimately causes porphyria. However, it should not be ruled out that PCP contributes indirectly to the disturbance of the hepatic heme synthesis and enhances porphyrin accumulation (see Section 1.5).

The metabolism of polychlorinated biphenyls (PCBs) has been thoroughly investigated by Hutzinger, Safe and co-workers and this subject has been reviewed recently (Sundström et al., 1976). As with hexachlorobenzene, the major metabolites were phenolic compounds (see Sundström et al. (1976) and papers cited therein). For example, studies on the metabolism of 4,4'-dichlorobiphenyl in the rat demonstrated the formation of four monohydroxy-, four dihydroxy- and two trihydroxy- metabolites (Tulp et al., 1976). Hydroxylated derivates are also the main metabolic products of bromobiphenyls (Safe et al., 1976) and polychloronaphthalenes (PCNs) (Ruzo et al., 1975;

Chu et al., 1976; Chu et al., 1977a, 1977b). To what extent the formation of phenolic metabolites contribute to the porphyrinogenic effects and hepatotoxicity of polyhalogenated aromatic compounds remains to be investigated further.

1.5. Disturbance of mixed function oxidase activity as a result of inactivation of microsomal cytochrome P-450 by phenolic metabolites (see Fig. 1, No. 6)

Chlorinated phenols have been known for a long time as uncouplers of mitochondrial oxidative phosphorylation both in vitro (Loomis, 1949; Weinbach, 1954) and in vivo (Jacobson and Yllner, 1971). Recently, Arrhenius et al., (1977b) and Carlson (1978) reported that phenolic compounds e.g. pentachlorophenol, di-, tri- and tetrachlorophenols exert an inhibitory effect on another electron transport chain in the smooth endoplasmic reticulum of the cell, i.e. the microsomal detoxification enzyme chain. Pentachlorophenol strongly inhibited the electron transport between a flavin and cytochrome P-450 in the microsomal mixed-function oxygenase system in vitro. This resulted in a selective blocking of the cytochrome P-450 dependent C-oxygenation of aromatic amines, favouring their flavin-mediated N-oxygenation (Arrhenius et al., 1977b). This type of change in the detoxification pattern appeared to be associated with the formation of reactive electrophilic intermediates, e.g. epoxides (Arrhenius, 1969/70; Arrhenius, 1974) which are supposed to be responsible for a disturbing effect upon several cell functions, including mutagenic and carcinogenic effects by covalent binding to cellular macromolecules. Similar effects were obtained with various phenolic metabolites of polychlorinated biphenyls (Arrhenius et al., 1977b). These results are consistent with our findings that the inhibition of the P-450 dependent O-demethylation of p-nitroanisole by pentachlorophenol in vitro is due to a selective inactivation of cytochrome P-450, which is converted to its metabolically inactive P-420 form (Förlin and Strik, 1978; Debets et al., 1980a). After the conversion of cytochrome P-450 to P-420, the heme prosthetic group of the latter can be oxidatively degraded by the microsomal heme oxygenase to form biliverdin (Maines, 1977) (Fig. 1). Biliverdin becomes subsequently reduced by biliverdin reductase to bilirubin, that can be excreted in bile and urine. Similar results were also obtained by Carlson, who found that 2,4,5-trichlorophenol at a dietary concentration of 400 mg/kg/day decreased hepatic microsomal cytochrome P-450 content. 2,3,5-, 2,3,6- and 2,4,6-trichlorophenol inhibited the

demethylation of p-nitroanisole in vitro when added to a final concentration of 0.25 mM (Carlson, 1978). The uncoupling effect of chlorophenols on the mitochondrial respiratory chain has been interpreted as a change in the properties of the lipid membrane, which carries the enzymes of the mitochondrial electron transport chain (Van Dam and Meyer, 1971).

The same might be the case with the smooth endoplasmic reticulum membrane, which carries the enzymes of the mixed-function oxidase system. Indeed, it has been reported that compounds with a amphophilic character - i.e. having a hydrophilic and hydrophobic (aromatic) part, like pentachlorophenol -readily form a complex with amphophilic phospholipids in cellular membranes (Lüllmann et al., 1973; Seydel and Wassermann, 1973), leading to an alteration in physicochemical properties of the lipid (Hruban, 1976). The observed inactivation of cytochrome P-450 after incubation of microsomes with PCP could therefore reflect a change in physicochemical properties of the lipid in which cytochrome P-450 is embedded. As a result of this, cytochrome P-450 might become detached from the microsomal membrane or its catalytic moiety might undergo a conformational change, thereby losing its metabolic functions.

Pentachlorophenol formed endogenously by metabolism of hexachlorobenzene, was demonstrated not only in the excreta, but in the liver as well (Koss et al., 1978a), Arrhenius et al. (1977a) showed that PCP administered in vivo accumulated markedly in the microsomal fraction of the liver, which encloses the drug-metabolizing system.

Pentachlorophenol generated in the smooth endoplasmic reticulum might alter the pattern of metabolism of the parent compound (HCB) or increase the turnover rate of cytochrome P-450, as it accumulates in situ.

The results of several investigators discussed above demonstrate clearly that phenolic metabolites of polyhalogenated aromatic compounds are able to disturb hepatic drug metabolism. The possibility that these phenolic metabolites play an essential part in the development of porphyria should therefore be seriously considered.

1.6. Formation of reactive intermediates and sulfur-containing metabolites (see Fig. 1, No. 4; Fig. 2)

Another pathway of metabolism of polyhalogenated aromatics probably involving glutathione, leads to formation of sulfur-containing metabolites. Pentachlorothiophenol (PCThP), pentachlorothioanisol (PCTA), tetrachlorothiophenol (TCThP), tetrachlorothioanisol (TCTA) and tetrachlorodithioanisol

(TCdi-TA) could be demonstrated in the excreta of hexachlorobenzene-treated rats (Koss et al., 1976; Jansson and Bergman, 1978; Koss et al., 1979a) (see Fig. 2). Pentachlorothiophenol and pentachlorothioanisol were also present in the livers of rats treated with hexachlorobenzene (Jansson and Bergman, 1978; Koss et al., 1979a). After short-term administration of HCB pentachlorothiophenol accounts for about a third of the total excreted amount of metabolites (Koss et al., 1976). The major part of the thiophenols could be isolated only after alkaline hydrolysis of the excreta. Free pentachlorothiophenol was, however, also extracted directly from the excreta (Jansson and Bergman, 1978; Koss et al., 1979a). A minor amount of this metabolite seems to be released from its conjugates with amino acids or proteins in vivo. Chasseaud (1976) and Koss et al. (1978a) suggested that the conjugated compounds excreted in the bile could be hydrolyzed in vivo by the intestinal enzymes and/or microflora. The metabolites that are split off may eventually be excreted in the feces, or they may be reabsorbed and undergo enterohepatic circulation. Recently Larsen and Bakke (1978) showed that an enzymic conversion of glutathione conjugates to methylthio-derivatives takes place in the intestine, probably catalyzed by a C-S lyase of the intestinal flora or tissues.

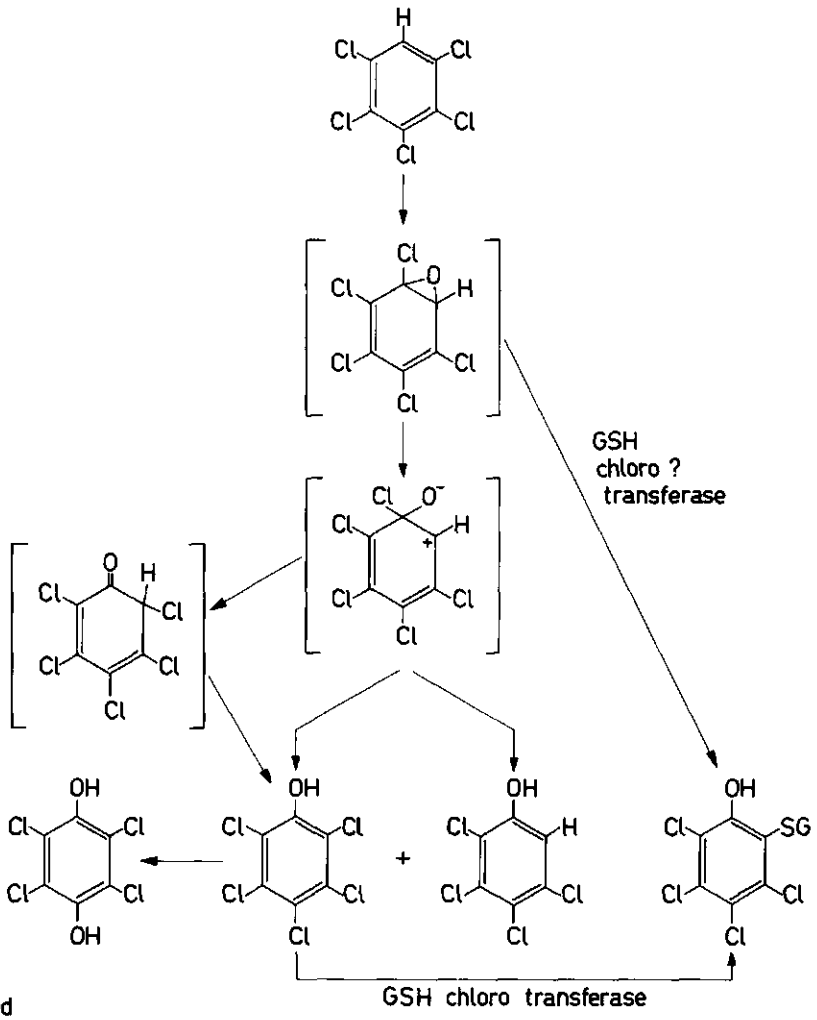
The sulfur-containing metabolites can be partly derived from conjugation with glutathione. The recent detection and isolation of pentachlorophenyl-mercapturic acid in the urine of hexachlorobenzene-treated rats adds proof to this assumption (Renner et al., 1978). Another considerable amount of sulfur-containing metabolites may stem from a reaction with methionine (Mio et al., 1976; Koss et al., 1979a) or from covalent binding of reactive intermediates to functional sulfhydryl-groups of enzymes (e.g. uroporphyrinogen decarboxylase). Since HCB is metabolized slowly, escaping reactive intermediates may react readily with glutathione without lowering its concentration in the liver. This may explain why long-term administration of HCB to rats causes no appreciable decrease in hepatic glutathione levels (Wolf et al., 1962; Rimington and Ziegler, 1963; Kerklaan et al., 1979). Combined administration of HCB and diethylmaleate (a glutathione depleting agent), however, enhanced the porphyrinogenic action of HCB (Kerklaan et al., 1979; Koss et al., 1979c). The elevated excretion of porphyrins and porphyrin precursors in rats chronically exposed to 1,2,4-trichlorobenzene could be reduced to almost normal levels by daily i.p. injections of glutathione, without stopping the treatment with 1,2,4-trichlorobenzene (Rimington and Ziegler, 1963).

These results suggest that a toxic intermediate formed during HCB metabolism and detoxified by glutathione may be responsible for disturbing the heme synthesis. Metabolic hydrolytic dechlorination of HCB via highly reactive arene oxide intermediates does not seem to be the most plausible explanation because of steric hindrance of chlorine on the aromatic ring. Enzymatic epoxidation as an intermediate step in HCB metabolism must not, however, be ruled out completely, because investigations have shown that arene oxides are plausible intermediates in the metabolism of highly substituted chlorobenzenes, like pentachlorobenzene, by the rabbit (Kohli et al., 1976) and the rat (Koss and Koransky, 1978) (Fig. 3). Formation of free radicals during reductive dechlorination of HCB - as demonstrated by the metabolism of carbon tetrachloride to chloroform (Recknagel and Glende, 1973) - seems to be more probably, since the possibility of reductive dechlorination has been reported for hexachlorobenzene (Metcalfe et al., 1973; Mehendale et al., 1975), chlorobiphenylols (Tulp et al., 1977) and chlorophenols (Ahlborg and Thunberg, 1978). The chlorine atoms of HCB are labile against nucleophilic displacement, so that the possibility of a direct attack of glutathione on a carbon-chlorine bond of HCB - catalyzed by a glutathione chlorotransferase (Fig. 3) (a hypothetical glutathione-transferring enzyme, which displaces chlorine on the aromatic ring) - should be taken into consideration.

It has been well established that PCBs, PBBs and PCNs are metabolized to hydroxylated derivatives via highly reactive arene oxide intermediates: compounds in which a formal aromatic double bond has undergone epoxidation via hepatic mono-oxygenase action (Fig. 4). (Daly et al., 1972; Sundström et al., 1976; Safe et al., 1976; Chu et al., 1977a). A low degree of chlorination of PCBs facilitates the formation of hydroxylated metabolites and, therefore, favours epoxidation. Sulfur-containing metabolites of PCBs have been detected in the feces and liver of mice injected once with 2,5,2',5'- or 2,4,2',4'-tetrachlorobiphenyl (Daly et al., 1972; Mizutani et al., 1975; Mio et al., 1976; Mizutani, 1978). Two methylthio metabolites (3- and 4-methylthio-2,5,2',5'-TCB or 5- and 6-methylthio-2,4,2',4'-TCB) and two methylsulfone metabolites (at 3- and 4- or 5- and 6-position in the phenyl ring, respectively) were excreted in the feces (Fig. 4). The total amount of the four sulfur-containing metabolites excreted during 6 days after administration of 2,4,2',4'-TCB accounted for only 0.12% of the dose (Mizutani, 1978). The substitution by a methylthio- or methylsulfone group at either of two adjacent positions in the phenyl ring points to an intermediate formation

Fig. 3. Possible metabolic pathways of pentachlorobenzene in the rat.

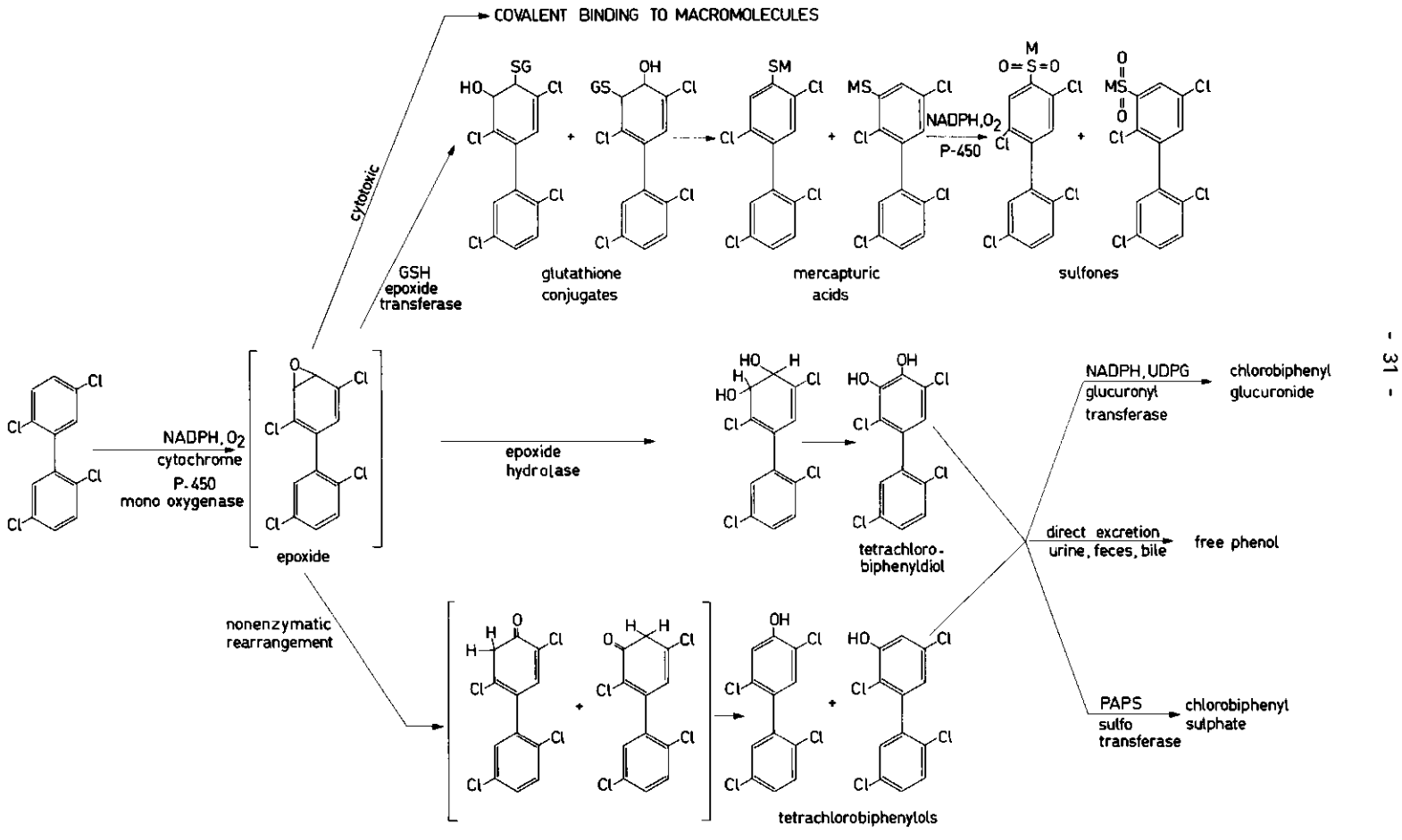
(G= glutathione)



% excreted in					
feces		4.1	18	11.3	values from Koss and Koransky (1978)
urine	1.5	6.2	35	21.6	

Fig. 4: Metabolic pathways in the mammalian metabolism of halogenated aromatic hydrocarbons

(G = glutathione, M = $CH_2CH[(NHCOCH_3)(COOH)]$)



of arene oxides in the metabolism of these PCB isomers. In the liver only the methylsulfone metabolites could be detected and Mizutani (1978) suggested that a rapid conversion of the methylthio metabolites to the corresponding methylsulfone metabolites by hepatic mono-oxygenase action could be responsible for this phenomenon. It should be noted that methylsulfone metabolites of PCBs were also found in seal blubber (Jensen and Jansson, 1976). The biological and toxicological significance of sulfur-containing metabolites of halogenated aromatic hydrocarbons in relation to hepatic porphyria is still unknown. In only one instance has the action of the HCB metabolite pentachlorothiophenol been investigated in this respect. This sulfur-containing metabolite was found to be able to disturb hepatic porphyrin metabolism in female rats in the presence of an elevated ALA level (Koss et al., 1977).

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent inducer of δ-aminolevulinic acid synthase and aryl hydrocarbon hydroxylase known (Poland and Glover, 1973, 1974). This compound causes porphyria in mammals (Goldstein et al., 1973; Cantoni et al., 1980), chick embryos (Poland and Glover, 1973) and also in man (Bleiberg et al., 1964; Jirásek et al., 1976). No metabolites of TCDD have yet been found, either following in vivo administration or after incubation with liver microsomes (Vinopal and Casida, 1973). The mechanism by which TCDD exerts its porphyrogenic and hepatotoxic action is unknown. Poland and Glover (1973) suggested that TCDD is metabolized into reactive intermediates, which might bind covalently to essential macromolecules (e.g. uroporphyrinogen decarboxylase) in liver cells. Indeed, it has been reported recently that in vitro binding of [^{14}C]TCDD to rat liver microsomes is mediated by mixed-function oxygenase activity. Binding to macromolecules could be markedly reduced by inhibition of mono-oxygenase action with SKF-525A or addition of glutathione to the incubation mixture. The observed binding was, however, not found to be covalent (Nelson et al., 1977). In the case of metabolic activation of TCDD to arene oxide intermediates, as suggested by Poland and Glover (1973), we would expect phenolic and dihydro-diol metabolites to be formed. The absence of these metabolites suggests that binding to macromolecules is mediated through other reactive metabolic products, perhaps radicals.

It is possible that different forms of cytochrome P-450 are involved in the metabolic activation of TCDD and HCB to reactive products, since TCDD belongs to the 3-methylcholanthrene (P-448) class of inducers and HCB is a mixed type inducer, sharing properties of both the 3-methylcholanthrene and phenobarbital class of inducers (Stonard, 1975; Debets et al., 1980a).

Finally, studies of Poland et al. (1979) have illustrated that for halogenated dibenzo-p-dioxin and biphenyl congeners there was a good correlation between their potency to induce AHH (P-448) and ALAS activity in chick embryo liver and their toxic potency. Moreover, all chlorinated biphenyl congeners, which showed a strong potency to induce AHH activity in chick embryo liver, were found to be porphyrinogenic in tissue culture of chick embryo liver cells (Kawanishi et al., 1978). It remains to be determined whether a common reactive metabolic intermediate is responsible for both the disturbance of hepatic heme biosynthesis and other liver lesions such as hepatocellular necrosis.

1.7. Inactivation, conjugation and excretion of reactive intermediates and metabolites (see Fig. 1, Nos. 7-11; Fig. 4)

Both hexachlorobenzene (Goldstein et al., 1978; Debets et al., 1980) and polychlorinated biphenyls (Schmoldt et al., 1977; Goldstein et al., 1977b) increase the activity of hepatic glucuronyl transferase. This enzyme catalyzes the conjugation of phenolic metabolites with glucuronic acid to form glucuronides that can be excreted easier in bile and urine. Induction of glucuronyl transferase suggests that phenolic metabolites of PHAs are excreted partly as glucuronides. Data concerning the question of to what extent phenolic metabolites are excreted free or as conjugates, are very limited. Exogenously administered PCP (the major metabolite of HCB) is excreted for the greater part in the urine. About 60% of the amount of PCP excreted in the urine was found to be present as conjugated PCP in both mice (Jacobson and Yllner, 1971) and rats (Ahlborg et al., 1974; Jansson and Bergman, 1978).

Conjugation with glutathione and subsequent mercapturic acid formation, as a mechanism to inactivate the highly reactive epoxides or free radicals arising during the metabolism of PHAs (see Section 1.6), has become more plausible since the detection of sulfur-containing metabolites of PCBs (see Fig. 4) and the isolation of pentachlorophenylmercapturic acid in the urine of hexachlorobenzene-treated rats (Renner et al., 1978). Polybrominated biphenyls (Dent et al., 1976) and probably also polychlorinated biphenyls induce hepatic epoxide hydrolase, another enzyme involved in the inactivation of electrophilically reactive intermediates of PHA metabolism. Epoxide hydrolase is probably localized in the endoplasmic reticulum, forming a complex with the mono-oxygenase system (Oesch and Daly, 1972). It catalyzes the hydration of epoxides to the electrophilically unreactive dihydrodiols

Electron microscopic examination shows that this liver cell hypertrophy is due to a considerable proliferation of the smooth endoplasmic reticulum (SER), as a result of drug-metabolizing enzyme induction. The mitochondria are often dislocated into scattered clumps, as a consequence of SER proliferation. Most mitochondria appear normal, but sometimes swollen mitochondria can be observed. It is believed that some of these mitochondria store porphyrins (Böger et al., 1979).

Centrilobular hepatocytes frequently contain lipid vacuoles. Local small spots of centrilobular necrosis (De Matteis et al., 1961; Sweeney et al., 1971; Vos et al., 1971b; Vos and Notenboom-Ram, 1972) or an increase in the plasma concentration of liver enzymes, indicating liver cell damage, are observed in several studies by the time porphyria develops (Simon et al., 1970; Strik, 1973c).

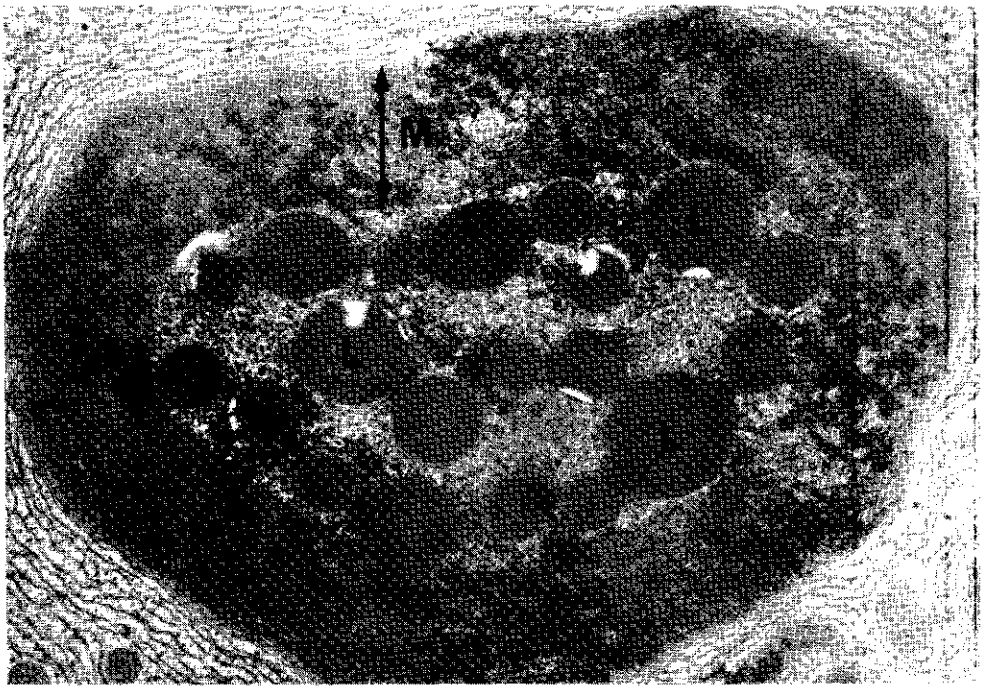


Fig. 5. Electron micrograph, showing the appearance of a large whorl in the cytoplam of a hepatocyte from a male Wistar rat after subchronic feeding of 300 ppm HCB for 12 weeks. The whorl consists of a central core of lipid droplets (L), surrounded by a thick layer of concentric arrays of tightly packed smooth membranes (M). x 10,000.

The occurrence of eosinophilic concentric membrane arrays (synonyms in current use: whorls; myelin figures; inclusion bodies; hyalin bodies) in the cytoplasm of the enlarged hepatocytes is one of the most striking features of HCB (Medline et al., 1973; Strik and Koeman, 1976; Mollenhauer et al., 1976; Kuiper-Goodman et al., 1976; Kuiper-Goodman et al., 1977) and PCB (Schmoldt et al., 1977; Hacking et al., 1978) treatment. Whorls consist of concentric layers of tightly packed membranes, probably originating from SER of which the intracisternal cavity is collapsed. They frequently enclose a central core of lipid droplets (Norback and Allen, 1969) (Fig. 5). Long-term feeding of HCB to male rats results in the formation of large whorls, varying in size from 2 to 20 µm in diameter (Medline et al., 1973) and visible in the light microscope (Fig. 5). Female rats are more susceptible to the porphyrinogenic action of HCB (Grant et al., 1974; Stonard, 1974; Strik and Koeman, 1976; Kuiper-Goodman et al., 1977) and PCBs (Kimbrough et al., 1972), but develop only small whorls ranging from about 0.2 to 3.0 µm in diameter (Fig. 6). The capacity to induce the formation of

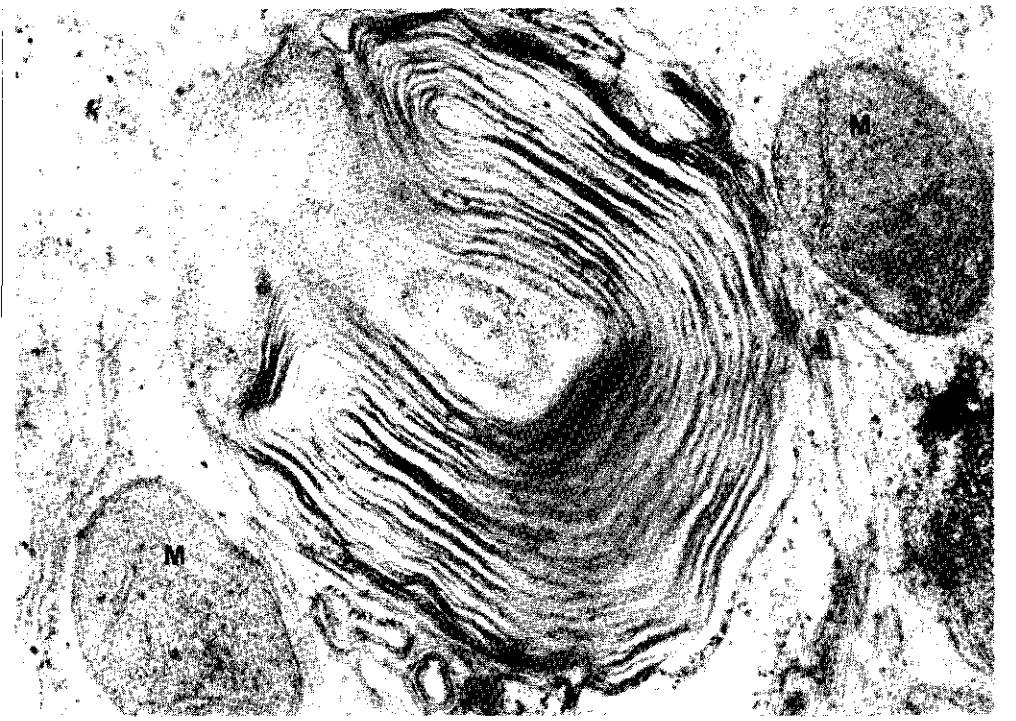


Fig. 6. Membrane whorl flanked by two mitochondria (M) in a centrilobular hepatocyte from a female rat after chronic feeding of HCB for 8 weeks. x 80,000.

whorls is not restricted to polyhalogenated aromatic compounds. Numerous drugs and other lipophilic xenobiotics, such as carbon tetrachloride (Stenger, 1966), phenobarbital (Herdson et al., 1964), DDT (Ortega, 1966), dieldrin (Hutterer et al., 1969), acetaminofluorene (Thys et al., 1973) and tetrasul (Verschuuren, 1967), are known to evoke whorls in experimental animals but are not porphyrinogenic (Kimbrough et al., 1971). A difference existing between compounds that produce both hepatic porphyria and whorls and those which induce only whorls is the presence of a great deal of brown pigment, which seems to represent lipofuscin or ceroid pigment. This pigment is thought to be the result at least in part of lipid peroxidation (Hartcroft, 1972).

The function of whorl formation and its significance in relation to the development of porphyria is still obscure. However, since many whorls appear by the time porphyria develops (Medline et al., 1973), an interrelationship seems likely. Whorl formation may be involved in the elimination of fat-stored HCB or other lipophilic xenobiotics (Mollenhauer et al., 1976). It seems reasonable to suggest that the SER starts to proliferate around lipid droplets, probably containing the lipophilic polyhalogenated aromatic compound. In an attempt to metabolize the lipophilic compound into more polar products, SER membranes can be damaged by formation of toxic metabolites and/or reactive intermediates (e.g. chlorophenols, arene oxides, free radicals; see Sections 1.4, 1.5 and 1.6). In order to continue the metabolism, new SER membranes are concentrated around the damaged membranes enclosing the central lipid core. A whorl is thus conceived of as a cluster of inactive or hypoactive SER membranes enclosing lipid and at the periphery surrounded by a few intact concentric membrane arrays. The formation of whorls is, moreover, accompanied by a decrease in drug-metabolizing enzyme activity in livers of HCB-exposed rats (Kuiper-Goodman et al., 1976). This observation suggests that the SER becomes hypoactive or even degenerative because toxic metabolites of HCB are continuously formed and subsequently accumulated up to a critical concentration in situ (see Sections 1.4 and 1.5). The mechanism of whorl formation may be comparable with the formation of myeloid bodies that can be observed in many cell types after administration of amphophilic drugs. Myeloid bodies are storage bodies containing membranes that cannot be digested further because of drug action. They should be distinguished from whorls because they are surrounded by a lysosomal membrane and have no lipid core. This mechanism presupposes that amphophilic drugs readily form a complex with membrane phospholipids.

Normally, altered membranes are removed by autophagy. In contrast, the drug-lipid complexes impair their own degradation in phagosomes and lysosomes and are transformed into myeloid bodies (see Hruban (1976) and papers cited therein). Amphophilic metabolites of PHAs, like pentachlorophenol, may react in a similar way with phospholipids of cellular membranes (see Section 1.5). Amphophilic metabolites of PHAs generated in the SER by metabolism of the parent compound may form a complex directly with phospholipids of SER membranes. This not only causes inactivation of cytochrome P-450 (see Section 1.5) and disturbance of liver mixed-function oxygenase activity, but also induces whorl formation.

1.10. Influence of iron on the porphyrinogenic effect of polyhalogenated aromatic compounds (see Fig. 1, No. 14)

There has been found no difference in the hepatic non-heme iron concentration between control and HCB-fed female rats (Elder et al., 1976a; San Martin de Viale et al., 1977; Smith et al., 1979). However, prior iron overload before HCB feeding accelerates the development of porphyria in female rats (Taljaard et al., 1971, 1972; Louw et al., 1977). The earlier onset of porphyrin accumulation in these siderotic rats is accompanied by an earlier and greater decrease in UROG-D activity (Louw et al., 1977). A direct effect of iron on UROG-D seems unlikely, because iron overload alone has no influence on the activity of UROG-D (Louw et al., 1977). Elder (1978) suggested that iron might potentiate the action of HCB or a metabolite of HCB on UROG-D.

An inbred strain of female Wistar rats (Agus) that is particularly sensitive to the porphyrinogenic action of HCB showed a higher level of total non-heme iron in their livers than female Porton-Wistar rats (Smith et al., 1979). The inhibition of uroporphyrinogen decarboxylase started earlier and proceeded at a faster rate in the Agus rats than in the Porton-Wistar strain. The difference in susceptibility to the porphyrinogenic action of HCB between the two strains could not be correlated with differences in intake or retention of HCB in their livers.

The porphyrinogenic and hepatotoxic action of TCDD to C57B1/6J mice could be markedly reduced when these animals were previously made iron-deficient (Sweeney et al., 1979). However, mice fed the iron-deficient diet and treated with TCDD showed a lower activity of cytochrome P-450 and aryl hydrocarbon hydroxylase (AHH) than the animals given a normal-iron diet and TCDD. In earlier work, Jones and Sweeney (1977) compared the

inhibition of UROG-D by TCDD in mice that were genetically responsive or nonresponsive to the induction of AHH by TCDD (see Section 1.3). The nonresponsive mice developed no porphyria. These results suggest a relationship between the induction of AHH and the development of hepatic porphyria by polyhalogenated aromatic compounds. The lower susceptibility of iron-deficient mice to the porphyrinogenic action of TCDD may, therefore, be explained in terms of the comparatively lower activity of AHH and cytochrome P-450 observed in this group.

At present, it seems likely that iron is involved in the metabolic activation of polyhalogenated aromatics to reactive intermediates that are capable of inhibiting UROG-D and causing porphyria. Moreover, iron accelerates heme catabolism by inducing heme oxygenase activity in vivo (De Matteis, 1976; Maines and Kappas, 1976). Thus, a faster turnover rate of heme in siderotic, porphyric rats causes by feed-back stimulation an increase in ALAS activity, leading to a greater accumulation of porphyrins by the UROG-D defect.

1.11. Delayed onset of hepatic porphyria: a property inherent to polyhalogenated aromatic compounds

Prolonged administration of PHAs to susceptible animal species leads to the development of hepatic porphyria. However, the time required for the appearance of symptoms may vary from a few weeks to one year, depending on the animal species and the administered dose. As yet no explanation for the delayed onset of this type of experimental porphyria has been found. Elder (1978) suggests that the delay may be due, at least in part, to the time required to reach a critical porphyrinogenic concentration of the parent compound HCB in the liver. In this connection he refers to the work of Grant et al. (1974) and Vos et al. (1972) who showed that porphyria does not develop in female rats and Japanese quail until liver HCB concentrations have reached a certain value (30 µg/g liver for female rats). However, the rate of accumulation of HCB within the liver of female rats is the same as in males (Grant et al., 1975; San Martin de Viale et al., 1976a). The role of gonadal steroid hormones in relation to the development of hepatic porphyria and the formation of large whorls in the cytoplasm of liver cells (see Section 1.9) is still unknown.

Recently, Graef et al. (1979) reported that feeding of HCB to female rats caused alterations in the metabolism of testosterone, leading to an increased production of 5β-H-steroids. The 5β-H-steroids are known to be in-

ducers of the porphyrin biosynthesis (Granick and Kappas, 1967). Similar changes in steroid metabolism have been observed following administration of TCDD to both male and female rats. The effects of TCDD on steroid metabolism in rats were more pronounced in females (Gustafsson and Ingelman-Sundberg, 1979). It is unlikely that the observed changes in the activities of steroid-metabolizing enzymes are the primary cause of accumulation of porphyrin, because it has been proved beyond any doubt that inhibition of uroporphyrinogen decarboxylase is the ultimate process responsible for the disturbance of hepatic porphyrin metabolism (see Section 1.8). The action of a reactive product of PHA biotransformation may underly the disturbances of both metabolic pathways.

One difference between female and male rats is that the livers of male rats contain a great deal more lipids after exposure to PHAs than females. This might be another cause of the insusceptibility of male rats to the porphyrinogenic effect of PHAs, because male rats can store more of the PHAs with the lipids in the liver. Consequently, PHAs stored in fat in the liver cell may be not available for biotransformation and this protects the cell from damage caused by toxic metabolites of the PHA.

The hypothesis of a critical porphyrinogenic concentration of HCB in the liver is unlikely for another reason: it has been shown that stimulation of HCB metabolism with phenobarbital accelerates the onset of hepatic porphyria (Kerklaan et al., 1979). These results are consistent with earlier suggestions of Stonard (Stonard and Greig, 1976) that the slow onset of the mixed pattern of enzyme induction, after chronic administration of PHAs, may be related to the slow onset of hepatic porphyria (see Section 1.3).

A more likely explanation for the delayed onset of hepatic porphyria is that a critical concentration of one of the metabolites is needed to cause damage to intracellular membranes and enzymes (Förlin and Strik, 1978; Debets et al., 1980a). This critical level of a given metabolite might, moreover, alter the detoxification pattern of HCB in a direction that favours the formation of reactive electrophilic intermediates. The latter phenomenon has already been observed for the influence of chlorophenols on the metabolism of aromatic amines (Arrhenius, 1974; Arrhenius et al., 1977b).

The reactive intermediate may be preferentially conjugated with glutathione. An experiment had shown that long-term administration of HCB leads to a continuous decrease of the activity of glutathione-S-epoxide transferase, which may catalyze the detoxification of a reactive electrophilic intermediate of the HCB metabolism. The decrease of this enzyme activity was

found to be accompanied by a decrease of the activity of the UROG-D in the rat liver. From this it may be concluded that an increasing amount of a reactive intermediate, which is not detoxified, attacks the uroporphyrinogen decarboxylating enzyme and thus inhibits its biological function more and more (Koss et al., in press). Moreover, liver necrosis, alkylation of cellular macromolecules and porphyria occur when glutathione is no longer available or when its transferring enzyme system is disturbed. These findings might help to understand the concomitant delayed appearance of centrilobular liver necrosis and porphyrin fluorescence in HCB porphyria.

1.12. Summary and conclusions

Polyhalogenated aromatic compounds (PHAs) seem to affect the porphyrin metabolism by a common mechanism. Increase in the urinary excretion of uroporphyrin and heptacarboxylic porphyrin in chronic hepatic porphyria induced by PHAs is accompanied by a decrease of the activity of hepatic uroporphyrinogen decarboxylase (UROG-D). These results lead to the conclusion that the decarboxylation of uroporphyrin is blocked by inhibition or inactivation of this decarboxylase. It has been shown conclusively that the porphyrinogenic effect of PHAs is not due to a direct interaction with the heme synthesis. A metabolite or reactive intermediate formed by metabolism of PHAs is suspected to be responsible for the disturbance of hepatic heme synthesis. PCBs, PBBs and possibly also HCB are metabolized via highly reactive intermediates. The detection of sulfur-containing metabolites in the excreta of animals treated with PCBs or HCB suggests that these unstable intermediates may react with catalytic SH-groups of enzymes.

The main metabolites of PHAs, however, are the hydroxylated derivatives, which have been found to disturb hepatic drug metabolism in vitro by a selective inactivation of cytochrome P-450. Damaged SER membranes, carrying cytochrome P-450, seem to be removed by formation of whorls. It remains to be investigated what metabolites are actually involved in the onset of hepatic porphyria. The hypothesis that metabolism of PHAs is a prerequisite for the development of experimental porphyria does not agree with the observations that female rats are more susceptible to the porphyrinogenic effects of PHAs than are males, although the latter show a more marked microsomal drug-metabolizing enzyme induction with these compounds. The delayed onset of hepatic porphyria might be explained in terms of time required to build up a critical porphyrinogenic concentration of a given metabolite in the liver, or as a result of this, by a change in the

pattern of metabolism of the PHA. The influence of PHAs or their metabolites on the activity of enzymes involved in the inactivation or conjugation of electrophilically reactive intermediates needs to be investigated further, because several cases in which a depressed activity was found have been reported. Additional studies on the lower chlorinated benzenes have to be carried out, because contradictory results have been published concerning their ability to induce hepatic porphyria (Rimington and Ziegler, 1963, and Carlson, 1977). Experiments with laboratory animals, carried out to gain more insight into the mechanism of chronic hepatic porphyria caused by PHAs, are time-consuming due to the long period of administration required to evoke porphyria. The use of tissue culture of primary liver cells, acting as a fast-reacting test system (Granick, 1966; Doss, 1968, 1969; Sassa and Granick, 1970; Sinclair and Granick, 1974; Granick et al., 1975; Roomi, 1975; Kawanishi et al., 1978; Grisham et al., 1978), may offer a more simple and successful alternative.

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Chapter 2

EFFECTS OF PENTACHLOROPHENOL ON RAT LIVER CHANGES INDUCED BY HEXACHLOROBENZENE, WITH SPECIAL REFERENCE TO PORPHYRIA, AND ALTERATIONS IN MIXED FUNCTION OXYGENASES

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SUMMARY

Hexachlorobenzene (HCB, 1000 ppm) and 500 ppm pentachlorophenol (PCP) were fed separately or in combination to female Wistar rats. A control group was provided with standard food without HCB or PCP. Subgroups of 4 rats were killed after 1, 2, 4, 6 and 8 weeks. No significant difference was found between the amounts of HCB accumulated in the livers of the HCB and HCB + PCP fed rats. Administering HCB together with PCP caused a noticeable accumulation of PCP in the liver, compared to the results after administering HCB and PCP separately. In the HCB and HCB + PCP fed groups liver weight increased continuously during the experiments. Microsomal cytochrome *P*-450, NADPH-cytochrome *c* reductase, ethoxyresorufin *O*-de-ethylase, aminopyrine *N*-demethylase, and glucuronyl transferase increased to a maximum in 2—4 weeks in HCB and HCB + PCP fed rats. Pentachlorophenol accelerates the onset of HCB porphyria, in other words it increases the total urinary porphyrin excretion and causes an earlier disturbance of the porphyrin pattern.

INTRODUCTION

Hexachlorobenzene (HCB) has become a world-wide major environmental

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Abbreviations: AHH, aryl hydrocarbon hydroxylase; HCB, hexachlorobenzene; PCP, pentachlorophenol; SER, smooth endoplasmic reticulum.

pollutant [1—4], since its introduction as a fungicide and its production as a by-product in manufacturing many chlorinated solvents and pesticides [5—7]. This compound is biodegraded slowly [8—10], and it is extremely persistent in body fat [11,12]. Prolonged exposure to hexachlorobenzene of susceptible mammals [13,14], birds [1,15—17] and man [18] causes massive overproduction of uroporphyrin and heptacarboxylic porphyrin and, to a lesser extent, of porphyrins with 6, 5 and 4 carboxylic groups [19—21].

The mechanism of the porphyrinogenic action of HCB and other polyhalogenated aromatic hydrocarbons is still unknown [22,23]. A better insight in the mechanism of HCB-induced porphyria may provide an explanation of the hepatotoxicity of similar compounds [24]. The recent discovery that the activity of hepatic uroporphyrinogen decarboxylase (UROG-D) is depressed in HCB porphyria, offers a possible approach to these problems [20,25].

Recent information indicates that an unstable reactive intermediate or metabolite of HCB is suspected to react with the catalytic SH-containing part of the uroporphyrinogen decarboxylating enzyme in the liver cytosol [26,27]. The possibility that HCB is metabolized via electrophilically reactive intermediates, and the toxicological significance thereof, has been discussed in a previous paper [24]. Stimulation (by phenobarbital [28]) or inhibition (by SKF 525-A [29] or piperonyl butoxide [27]) of drug metabolism, which leads respectively to an earlier development or to a prevention of porphyrin accumulation, provides indirect support to the hypothesis that a metabolic product of HCB may be the ultimate porphyrinogenic agent. However, neither PCP [30—32], the major metabolite of HCB, nor other known metabolites of HCB [33], have caused porphyria after prolonged feeding of these compounds to rats. Pharmacokinetic causes might be responsible for this apparent discrepancy. The absence of porphyria after administering PCP or other metabolites of HCB exogenously may be due to the fact that these metabolites are rapidly conjugated and excreted before they reach the site where they cause damage [34]. On the other hand, after feeding HCB, PCP is continously generated endogenously in smooth endoplasmic reticulum (SER) and may accumulate in situ. Arrhenius et al. [35] demonstrated that PCP administered in vivo is distributed unequally in the hepatic cells and accumulated conspicuously in the microsomal fraction, i.e. the endoplasmic reticulum. In addition, PCP causes a disturbance of hepatic drug metabolism [36].

Reviewing the above, it seems reasonable to assume that PCP, as the major metabolite of HCB, might contribute to the development of HCB-induced porphyria. To obtain information in support of this assumption, experiments have been carried out to study the effects of exogenous pentachlorophenol on rat liver changes induced by hexachlorobenzene. Examined were the accumulation of HCB and PCP in the liver, the onset of hepatic porphyria, and changes in mixed function oxygenases.

METHODS

Animals

Eighty adult female Wistar rats (140—160 g) were obtained from the R.I.V. (National Institute of Public Health), Bilthoven, The Netherlands. The animals were acclimatized for 2 weeks before the experiment, randomly distributed in 4 groups of 20, and had free access to standard powdered diets (Hope Farms, Woerden, The Netherlands) containing either 1000 ppm pure HCB or 500 ppm pure PCP, or both compounds in the same amounts.

A control group received food without HCB or PCP, and was kept in a separate room under identical conditions to avoid cross-contamination [37,38]. Food consumption, body weights and clinical symptoms were checked weekly. From 4 animals of each group, the urine was collected during 24 h/week in metabolic cages. After 1, 2, 4, 6 and 8 weeks, 4 animals of each group were killed by decapitation, the livers rapidly taken out, weighed and divided for preparation of microsomes, and for estimating microscopic fluorescence of porphyrins.

Chemicals

Pure HCB (organic analytical standard) was obtained from BDH Chemicals Ltd. (Poole, England), and pure PCP from Aldrich Chemical Co. (lot 060475). Both proved to be better than 99% pure. PCP was analyzed for chlorinated dibenzo-*p*-dioxins and dibenzofurans by extraction [55] and subsequent clean-up and concentration [56] of the impurities. Identification and quantitation was carried out by selected ion monitoring gas chromatography-mass spectrometry (GC-MS). 7-Ethoxyresorufin and resorufin were purchased from Pierce Eurochemie B.V. (Rotterdam, The Netherlands). All other applied compounds were of reagent grade quality.

Analytical procedures

Microsomes were prepared as previously described [39]. The level of microsomal cytochrome *P*-450 was determined in microsomal suspensions according to the method of Omura and Sato [40], and expressed per mg of microsomal protein [41].

p-Nitroanisole *O*-demethylase [42], aminopyrine *N*-demethylase [43], NADPH-cytochrome *c* reductase [44] and *p*-nitrophenol glucuronyl transferase [31] were assayed spectrophotometrically in microsomes. The specific cytochrome *P*-448-mediated ethoxyresorufin *O*-de-ethylation was measured with a fluorimetric assay [45], using an Aminco SPF-500 fluorimeter. Total urinary porphyrin and urinary porphyrin pattern analyses were made according to the method of Doss [46], with some modifications of Strik and Harmsen [47].

Residue analysis

Liver tissue samples (0.25 g) and microsomal pellets from 0.75 g wet liver tissue were homogenized in 5 ml 0.5% perchloric acid. The homogenates were refluxed for 1 h with 15 ml distilled toluene, and 2.5 μg 4-methyl-2′,5′-dichlorobiphenyl was added to these mixtures as an internal standard. The toluene phases, containing the extracted HCB and PCP residues, were reduced by evaporation to 1 ml, and then methylated overnight with 1 ml ethereal diazomethane before examination with gas chromatography-mass spectrometry (GC-MS).

GC-MS was carried out with a Hewlett-Packard 5984A system, operating in the EI mode at 70 eV. The GC-MS operating temperatures were: injector 250°C, jet separator 250°C, transfer line 280°C and ion source 180°C. The gas chromatograph was equipped with 200 × 0.2 (i.d.) cm all glass column, packed with 0.2% Carbowax 20 M on Chromosorb W 100—120 mesh [48]. Column temperature 130°C. Selected ions monitored (S.I.M.) were: 278.8 ($C_6Cl_5OCH_3$), 285.8 (C_6Cl_6) and 236.0 (internal standard).

Statistics

Two-way analysis of variance (week vs. diet) was used to analyze the results for effects of diet (treatment), time, and their interaction. In view of a positive correlation between mean values and corresponding standard deviations, logarithmic transformation was applied to equalize variances. Significant overall differences between diets and time were assessed by using the *F*-test, in which $P < 0.05$ was used as the lowest level of significance.

RESULTS

Gas chromatography-mass spectrometry

Tetrachlorodibenzo-*p*-dioxins were not detected in PCP with a detection limit of 5 ppb. However, PCP was contaminated with 170 ppm octa- and 4 ppm heptachlorodibenzo-*p*-dioxins (Table I). Sixty per cent of the latter was the 1,2,3,4,6,7,8-hepta-isomer.

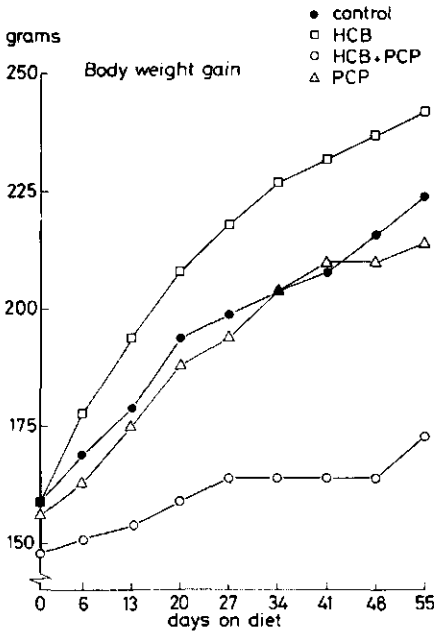
Table II shows the results of the GC-MS analyses for the HCB and PCP concentration in the livers and their microsomal fractions in individual rats after 2, 4 and 8 weeks of HCB and/or PCP treatment. Comparing the HCB contents in the livers of the HCB and the HCB + PCP treated rats produced no appreciable differences. The HCB and PCP concentrations in the livers of HCB treated rats appear to have equilibrated more or less after 4 weeks. The PCP concentration in the livers and microsomal fractions of rats fed HCB in combination with PCP was about twice as high as in PCP treated animals, whereas the liver PCP content of the latter exceeded that of the HCB rats by factors of about 40, 6, and 6, after 2, 4 and 8 weeks respectively. The 8 week values of the PCP concentration in the livers and microsomal fractions of all treated rats appear to have decreased when compared with the PCP residues after 4 weeks. About 20—25 per cent of the HCB or PCP

TABLE I

QUANTITATION OF CHLORINATED DIOXINS AND DIBENZOFURANS IN "PURE" PENTACHLOROPHENOL[a]

Dibenzo-*p*-dioxins		
Tetrachloro-	<5	ppb
Pentachloro-	<10	ppb
Hexachloro-	30	ppb
Heptachloro-	4	ppm
Octachloro-	170	ppm
Dibenzofurans		
Tetrachloro-	<5	ppb
Pentachloro-	<5	ppb
Hexachloro-	40	ppb
Heptachloro-	0.6	ppm
Octachloro-	0.8	ppm

[a] Analyzed by GC-MS; lower detection limit 5 ppb.



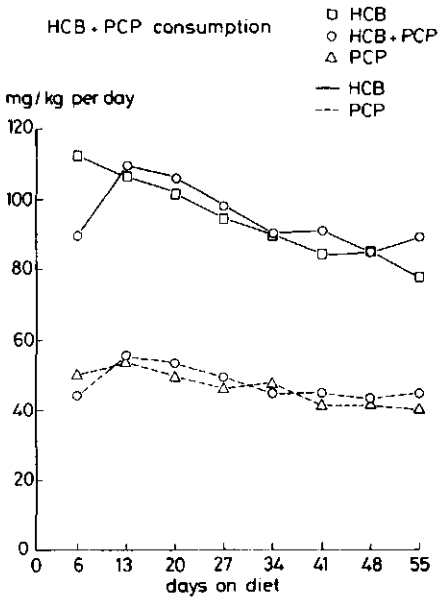


Fig. 1. Influence of hexachlorobenzene (HCB) and/or pentachlorophenol (PCP) on body weight gain of female rats. Indicated points represent means of 4 animals. S.D. within 10% of the mean values, not indicated.

Fig. 2. Consumption of hexachlorobenzene (HCB) and/or pentachlorophenol (PCP) by female rats fed different diets. Indicated points represent means of 4 animals. S.D. within 10% of the mean values.

content of the livers of the variously treated rats was always recovered in the microsomal fractions.

Body weight

The gain in body weight was significantly depressed by feeding the combined HCB and PCP diet, in contrast to the separate HCB and PCP diets. The HCB group showed an even higher gain in body weight than the control group (Fig. 1).

Dietary intake of HCB and PCP

Food consumption expressed as g/rat/day was slightly lower in the HCB + PCP treated group and higher in the HCB group. The doses of HCB and PCP on a body weight basis were practically equal in the different groups (Fig. 2). Control food contained 2 ppb HCB and 0.5 ppb PCP.

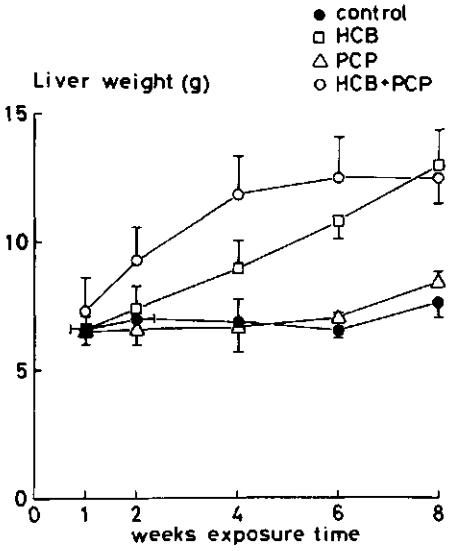
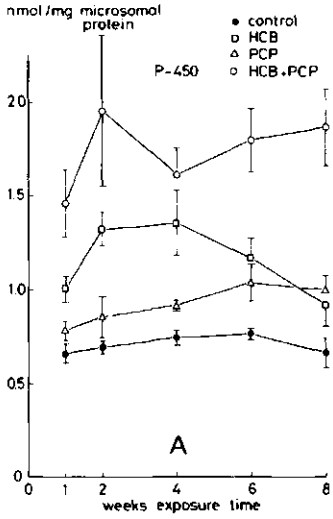
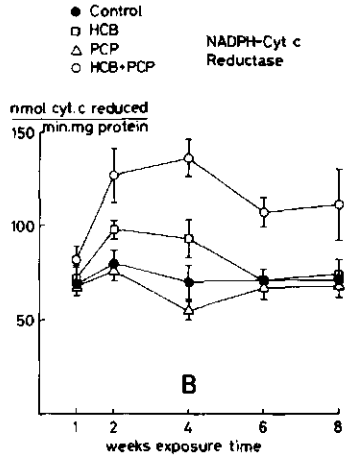
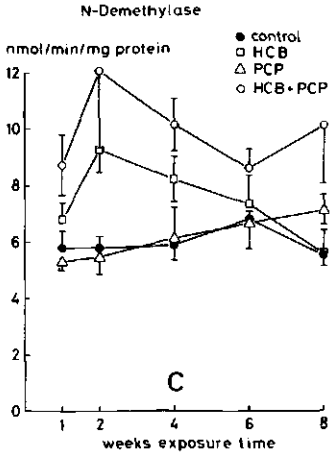


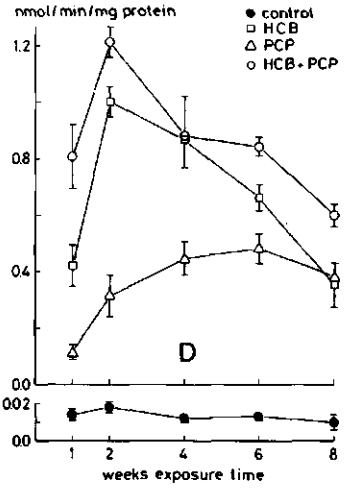
Fig. 3. Influence of hexachlorobenzene (HCB) and/or pentachlorophenol (PCP) on liver weights. Values represent means ± S.D. of 4 animals.

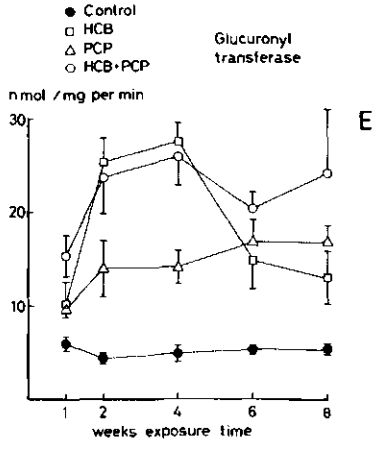
Fig. 4. Hepatic microsomal cytochrome *P*-450 content and enzyme activities at different time intervals during chronic feeding of HCB and PCP, separately or in combination, to female rats for 8 weeks. Mean values ± S.D. of 4 animals are shown. *Analysis of variance,* (A) Cytochrome *P*-450: diets produced significant overall differences (F^3_{12}-test, $P < 0.001$). (B) NADPH-cytochrome *c* reductase: a positive interaction between HCB and PCP treatment (HCB × PCP; *F*-test, $P < 0.05$). (C) Aminopyrine *N*-demethylase: a positive interaction between HCB and PCP treatment (HCB × PCP; *F*-test, $P < 0.05$). (D) Ethoxyresorufin *O*-deethylase: diets produced significant overall differences (F^3_{12}-test, $P < 0.001$). (E) Glucuronyl transferase: a negative interaction between HCB and PCP treatment (HCB × PCP; *F*-test, $P < 0.05$).


nmol/mg microsomal protein
control
HCB
PCP
HCB+PCP
P-450
A
weeks exposure time


Control
HCB
PCP
HCB+PCP
NADPH-Cyt c Reductase
nmol cyt.c reduced
min.mg protein
B
weeks exposure time


N-Demethylase
nmol/min/mg protein
control
HCB
PCP
HCB+PCP
C
weeks exposure time


Ethoxyresorufin O-deethylation
nmol/min/mg protein
control
HCB
PCP
HCB+PCP
D
weeks exposure time


Control
HCB
PCP
HCB+PCP
Glucuronyl transferase
nmol /mg per min
E
weeks exposure time

Clinical observations, mortality

In week 5 of the experiment, 6 out of 8 rats in the HCB-group and 2 out of 8 of the combined HCB and PCP treated group developed skin lesions. These were characterized by small depilated sores with hemorrhagic crusts, generally located near the shoulders or under the chin. Development of skin lesions coincided with excessive scratching of the animals. Slight chronic neurotoxic symptoms (weak tremors) were observed in the HCB treated groups during the last 2 weeks of the experiment. None of the treated animals died during the experiments.

Liver weight

A continuous increase in liver weight with the exposure time was found in the group fed HCB and in the group fed the combination of HCB and PCP (confirmed by analysis of variance (Fig. 3).

Effects on microsomal enzymes

The HCB, PCP, and combined HCB + PCP diets increased the cytochrome *P*-450 concentration to a maximum of 1.8-, 1.4-, and 2.7-fold, respectively (Fig. 4A). HCB fed alone and in combination with PCP caused a blue shift in the Soret maximum of the reduced hepatic cytochrome *P*-450 —CO complex of approx. 1 nm. In the PCP rats the blue shift in the Soret maximum

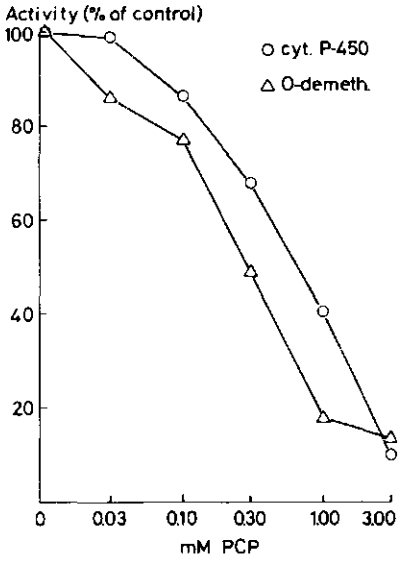


Fig. 5. Effect of PCP on cytochrome *P*-450 and *O*-demethylation of *p*-nitroanisole in vitro. Microsomes prepared as described under methods. Incubations for determination of *O*-demethylase activity, made at 37°C in 0.05 M Tris—HCl buffer (pH 7.4) for 10 min, contained (in a final vol. of 4 ml) 1.55 μmol NADPH; 17.5 μmol G-6-P; 4 I.U. G-6-PDH; 20 μmol $MgCl_2$; 4 μmol *p*-nitroanisole; 4 mg microsomal protein and PCP. Indicated points are the means of duplicate determinations.

amounted to 0.5 nm. In HCB + PCP exposed rats hepatic ethoxyresorufin *O*-de-ethylase, aminopyrine *N*-demethylase, *p*-nitrophenol glucuronyl transferase and NADPH-cytochrome *c* reductase increased to a maximum of 60-, 2-, 5-, and 2-fold respectively (Fig. 4). In the livers of HCB exposed animals the following maximal increases were found: 50-, 1.6-, 5.6-, and 1.3-fold respectively (Fig. 4). Pentachlorophenol treatment caused an increase only of ethoxyresorufin *O*-de-ethylase (20-fold) and glucuronyl transferase (3-fold).

Pentachlorophenol inhibited the cytochrome *P*-450 dependent *O*-demethylation of *p*-nitroanisole in vitro by inactivating cytochrome *P*-450 (Fig. 5). The in vitro addition of 1 mM PCP to microsomes causes a conversion of cytochrome *P*-450 into its metabolically inactive *P*-420 form, thus inhibiting *p*-nitroanisole demethylation by 80 per cent. This inactivation of cytochrome *P*-450 by PCP added in vitro could not be prevented by previous addition of 1 mM α-tocopherol or 1 mM reduced glutathione. The level of cytochrome *b*-5 was unaffected by addition in vitro of PCP to microsomes, whereas HCB had no effect in vitro on cytochrome *P*-450 or *b*-5.

Porphyrins in urine and liver

Microscopic liver porphyrin fluorescence (centrilobular foci) has been detected after 8 weeks in the HCB (2 out of 4) and HCB + PCP (3 out of 4) rats. An increase in the urinary excretion of total porphyrins was noticed as early as 3 weeks in the HCB + PCP treated rats (Fig. 6). The urinary total

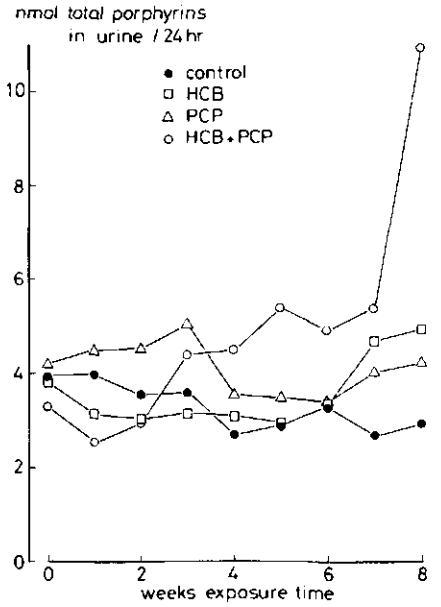


Fig. 6. Urinary total porphyrin excretion during chronic feeding of HCB and PCP, separately or in combination, to female rats for 8 weeks. Mean values of 4 animals plotted against time. Coproporphyrin used as an internal standard.

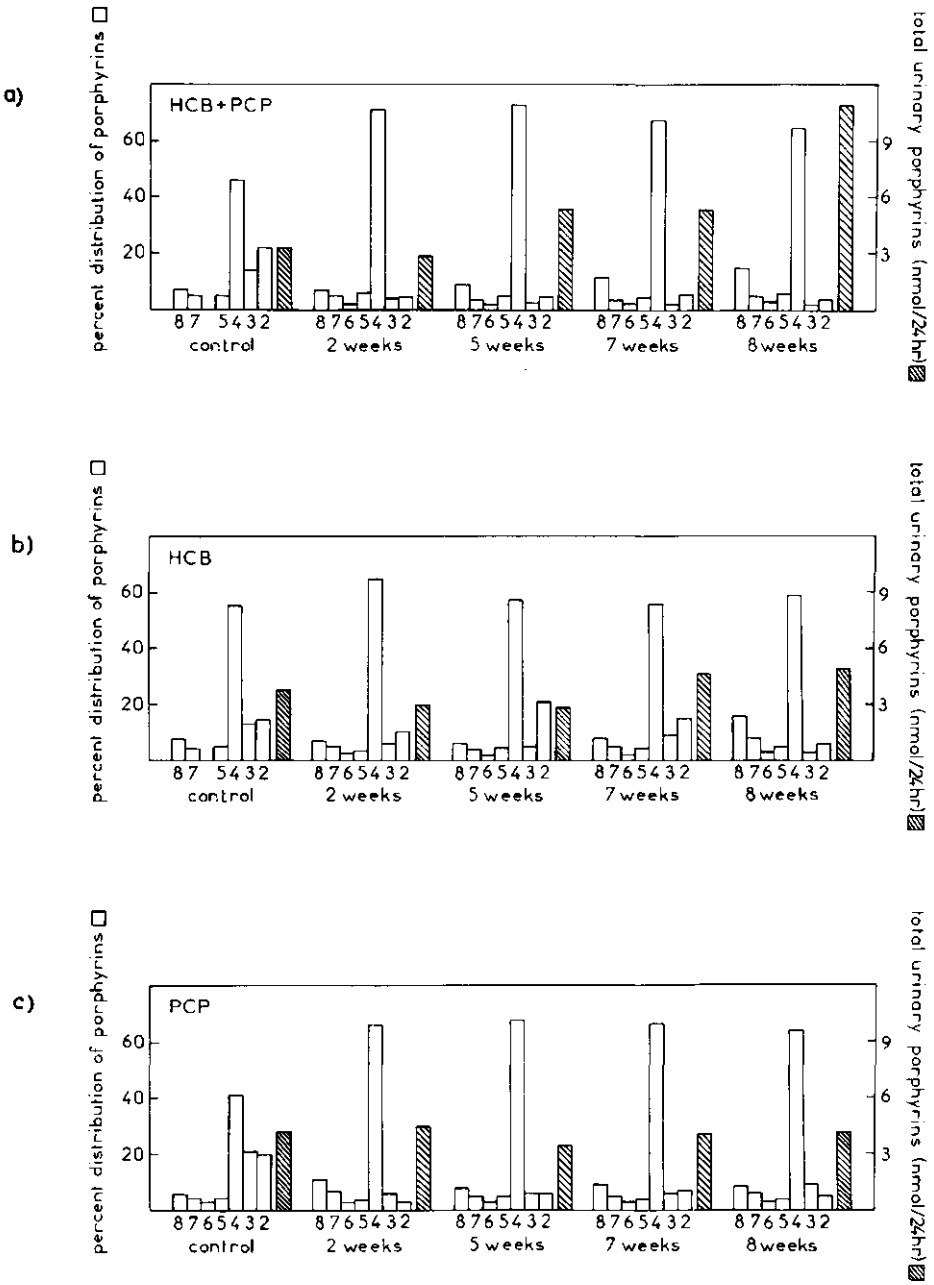


Fig. 7. (a—c) Relative distribution of porphyrins with different numbers of carboxylic groups (% of total porphyrins, open bars) at different time intervals during chronic feeding of HCB and PCP, separately or in combination, to female rats for 8 weeks. Total urinary porphyrin excretion in hatched bars and number of carboxylic groups indicated at the base of open bars. Control represents porphyrin pattern of urines collected on the first day of the experiment. Values represent analyses of pooled urine samples from 4 animals/treatment group.

TABLE II

HCB AND PCP RESIDUES IN LIVER AND MICROSOMAL FRACTION OF FEMALE RATS FED VARIOUS DIETS FOR 8 WEEKS

Time on diet (weeks)	Diet (group)	Animal No.	HCB residues (μg/g wet liver tissue)		PCP residues (μg/g wet liver tissue)	
			Whole homogenate	Microsomal fraction	Whole homogenate	Microsomal fraction
2	HCB	I[a]	132.0	31.1	0.4	0.1
		II	89.3	26.0	0.6	0.3
	HCB + PCP	I	174.3	23.0	32.7	7.2
		II	326.2	55.4	30.3	7.4
	PCP	I	ND[b]	ND	20.7	3.7
		II	ND	ND	16.3	4.0
4	HCB	I	223.4	53.8	3.4	0.8
		II	208.5	49.6	2.2	0.5
	HCB + PCP	I	181.2	44.5	29.5	6.7
		II	260.8	63.4	36.6	7.6
	PCP	I	ND	ND	20.8	4.5
		II	ND	ND	10.6	2.5
8	HCB	I	224.9	35.9	1.2	0.2
		II	261.8	39.2	2.8	0.4
	HCB + PCP	I	221.2	43.9	29.5	6.2
		II	284.8	53.0	25.8	5.3
	PCP	I	0.7	0.1	12.8	2.4
		II	0.3	0.1	12.3	2.3
	Control	I	<17.0ppb		<2.0ppb	
		II	ND[c]		ND	

[a] Values for individual animals.
[b] Not detected at 0.1 ppm detection limit.
[c] Not detected at 2 ppb detection limit.

porphyrin excretion of the HCB group, however, hardly began to rise after 6 weeks. An increase in the urinary excretion of uroporphyrin (8-carboxylic groups) and a decrease of porphyrins with 2 and 3 carboxylic groups, indicative of the onset of porphyria, began about 3 weeks earlier in the HCB + PCP treated rats than in HCB treated animals (Figs. 7a—c).

DISCUSSION

We suggest that the prophyrinogenic effects of HCB are enhanced by pentachlorophenol. This enhancement is reflected in an earlier increase in the urinary porphyrin excretion, and an earlier change in the pattern or urinary porphyrins. These results seem to contrast with the findings in this study and earlier reports [31,32] that pure PCP alone is not porphyrinogenic. However, the conclusion that PCP, as the major metabolite of HCB, plays no prominent part in the expression of HCB-induced hepatic porphyria is premature. Pentachlorophenol, when introduced exogenously, is rapidly conjugated or directly eliminated from the body [34]. Exogenous PCP loads the conjugation and excretion mechanisms in the liver cells (e.g. expressed by an increase of glucuronyl transferase, Fig. 4E). Under the latter circumstances, PCP formed by metabolism of HCB may be excessively accumulated in SER membranes. The results presented in this paper (Table II) correspond with earlier findings [35,36] that PCP has a high affinity for binding to membranes of the endoplasmic reticulum and disturbs hepatic drug metabolism by destroying cytochrome *P*-450 (Fig. 5). This increases the turnover rate of this hemoprotein, and may result in a lack of cytochrome *P*-450, thus stimulating indirectly the heme synthesis. As a result, an increased production of heme exacerbates the porphyria caused by the inhibition of uroporphyrinogen decarboxylase.

The distinct extra accumulation of PCP in the livers and microsomal fractions of the HCB + PCP treated rats may be caused by an enhanced biotransformation, as a result of a comparatively higher mixed function oxygenase activity than in rats treated only with HCB.

Results presented in this paper (Fig. 4) and those obtained by Stonard [49] prove that HCB produces a mixed pattern of microsomal enzyme induction, combining inducing properties of both the phenobarbital (*P*-450) and 3-methylcholanthrene (*P*-448) classes of inducers. These results are not in agreement with earlier observations of Goldstein et al. [50] and Carlson [51]. Their results suggest that HCB is an inducer of the phenobarbital type. The HCB-induced mixed type of enzyme induction found in the present study was not appreciably changed by an additional supply of PCP, but HCB and PCP administered together induced a higher apparent activity of aminopyrine *N*-demethylase. Our experimental results show that pure pentachlorophenol increases hepatic *P*-448-mediated ethoxyresorufin *O*-de-ethylation, which is induced preferentially by 3-methylcholanthrene [55]. This is not in accordance with Goldstein et al. [31], who reported that 500 ppm pure PCP induces no cytochrome *P*-448 and aryl hydrocarbon hydroxylase

(AHH), even after an 8-months feeding period. Comparing the purity of our PCP with that used by Goldstein et al. shows a comparable or even higher purity, regarding the presence of the potent inducers of AHH, e.g. tetra-, penta- and hexachlorodibenzo-*p*-dioxins. It is unlikely that the higher amount of octachlorodibenzo-*p*-dioxin found in our PCP is responsible for the induction of *P*-448-mediated mixed function oxygenase activity. Octachlorodibenzo-*p*-dioxin induces no AHH in the chick embryo, even at a very much higher dose level [56].

We have shown that PCP may contribute indirectly to the porphyrinogenic action of HCB. PCP is widely distributed in the environment. Moreover, PCP may be considered as a common contaminant of the human body, since it was found in 85% of urine samples from a representative group of the general American population [54]. The possibility that PCP might enhance also the toxicity of other environmental pollutants should, therefore, be seriously considered.

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Chapter 3

METABOLISM AS A PREREQUISITE FOR THE PORPHYRINOGENIC ACTION OF POLYHALOGENATED AROMATICS, WITH SPECIAL REFERENCE TO HEXACHLOROBENZENE AND POLYBROMINATED BIPHENYLS (FIREMASTER BP-6)[a]

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ABSTRACT

Pretreatment of chick embryo liver cell cultures with inducers of the drug-metabolizing enzyme system markedly stimulates the accumulation of porphyrins after exposure to polyhalogenated hydrocarbons. Inhibition of hepatic drug metabolism or addition of compounds which are known to trap electrophiles or radicals protects against the porphyrinogenic action of polyhalogenated hydrocarbons in vitro. A reactive intermediate formed by metabolism of the polyhalogenated hydrocarbon is suggested to react with the catalytic SH-containing part of the uroporphyrinogen decarboxylating enzyme in the liver cytosol. The consequent inhibition of uroporphyrinogen decarboxylase, which leads to porphyrin accumulation, may be a very first indication of cellular damage caused by toxic metabolites of these compounds. The results of this work with the chick embryo liver cell system are in good agreement with the observations in animal experiments.

INTRODUCTION

The molecular mechanism of action of porphyrinogenic polyhalogenated aromatics (PHA's) has not yet been elucidated. However, these compounds seem to affect the heme biosynthesis by a common complicated mechanism

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(Debets and Strik, 1979). The increase in the urinary excretion of uroporphyrin and heptacarboxylic porphyrin in chronic hepatic porphyria induced by PHA's is accompanied by a decrease of the activity of hepatic uroporphyrinogen decarboxylase (UROG-D) (San Martin de Viale et al., 1976; Elder et al., 1976; Blekkenhorst et al., 1976; Louw et al., 1977). These results lead to the conclusion that the decarboxylation of uroporphyrinogen is blocked by inhibition or inactivation of UROG-D.

Accumulation of uroporphyrin caused by PHA's was prevented by treatment with SKF 525-A and piperonyl butoxide, two inhibitors of hepatic drug metabolism (Sinclair and Granick, 1974). The porphyrinogenic action of hexachlorobenzene (HCB) in female rats could be enhanced by promoting its metabolism with phenobarbital (PB) (Kerklaan et al., 1979; Debets et al., in prep.). Administering HCB together with diethylmaleate (DM), a glutathione depleting agent, also led to an enhancement of the porphyrinogenic action (Kerklaan et al., 1979; Koss et al, 1979b). These findings and the isolation of sulfur-containing metabolites from the urine of rats treated with HCB and polychlorinated biphenyls (PCB's) (Jansson and Bergman, 1978; Koss et al. 1979a; Mio et al., 1976, Mizutani, 1978), strongly suggest that an electrophilic metabolic product of PHA's reacts with sulfhydryl-groups. The catalytic part of hepatic UROG-D, which contains also sulfhydryl-groups (Mauzerall and Granick, 1958; Romeo and Levin, 1971), could be inactivated through a reaction with electrophilic metabolites or intermediates of PHA's.

Experiments with laboratory animals, carried out to gain more insight into the mechanism of chronic hepatic porphyria caused by PHA's, are time consuming due to the long period of administering the compound required to induce porphyria. An in vitro model test system based on the use of primary tissue cultures of chick embryo liver cells, responding within a time span of 24 hours, offers a sensitive and much more simple experimental model for studying the mechanism of chemical porphyria.

In the present study the effects of several compounds interfering with the biotransformation reactions of PHA's on the porphyrin accumulation induced by PHA's were investigated in primary liver cell cultures of chicken embryos. These experiments were performed in order to provide indirect support for the hypothesis that a direct attack of a reactive intermediate or metabolic of the PHA on UROG-D is responsible for disturbing the heme synthesis.

MATERIALS AND METHODS

Chemicals

Williams E medium and fetal calf serum were purchased from Gibco Bio-Cult, Glasgow, Scotland. Hexachlorobenzene (organic analytical standard) was obtained from BDH Chemicals Ltd. (Poole, England); piperonyl butoxide, DL-α-tocopherolacetate, and N,N'-diphenyl-p-phenylendiamine from Riedel-De Haën AG, Seelze-Hannover, W-Germany; TCPO (1,1,1-trichloro-2-propeneoxide, 1,1'-azobis (N,N'-dimethylformamide) (diamide) and pentachlorophenol (better than 99% pure) from Aldrich. Hemin (type III, equine) and 3-methylcholanthrene were purchased from Sigma; tetrahydrofuran and ascorbic acid from Merck, Darmstadt, W-Germany. Diethylmaleate was procured from Fluka, Buchs, Switzerland; β-naphthoflavone from ICN Pharmaceuticals, Inc., Planview, N.Y. and phenobarbital (sodium salt) was obtained from O.P.G., Utrecht, The Netherlands. HBB (Firemaster BP-6, lot No. 11081), a commercial mixture of hexabromobiphenyl isomers was a gift from Michigan Chemical Co., Michigan, USA.

Preparation of a primary culture of chick embryo liver cells

Chick embryo liver cell cultures were prepared according to the procedure of Granick et al. (1975) with the following modifications. Fertilized eggs from white chickens (Hubbard strain) were obtained from a commercial supplier. After an incubation period of 15-18 days the eggs were opened under aseptic conditions in a laminar flow cabinet. The embryos were perfused through the heart with a sterile (Ca^{2+}-free). Hanks solution, containing HEPES (20 mM) and EDTA (0.04%), to wash out red cells. The livers from four embryos were cut into small pieces with a pair of scissors. The fragments were transferred into an Erlenmeyer flask and enzymatically dissociated (under slow stirring at 37°C for 20 min.) with 30 ml (Ca^{2+}-free) Hanks solution, containing 1 U/ml Dispase-II (a neutral protease from Bacillus polymyxa, [EC 3.4.24.4], Boehringer Mannheim) and 20 mM HEPES. The dissociated cells were sedimented by centrifugation at 200 g for 3 min. Remaining red blood cells were removed by washing the pellet with buffered ammonium chloride according to Sassa and Kappas (1977). The isolated hepatocytes were finally resuspended (4 livers/100 ml) in Williams E medium supplemented with 10% fetal calf serum and without the supply of any antibiotics.

Standard incubation procedure

Cell suspensions of 2 ml containing about $1.5\text{-}2\times10^6$ cells were transferred into 3 cm ϕ plastic petri dishes (Costar). The cells were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in air. The liver cells attached to the bottom of the culture dish within 2-4 hours after plating and at that time appeared as monolayer colonies. Some of the cultures were pretreated with drug enzyme-inducing compounds (β-naphthoflavone (β-NF), phenobarbital (PB) and 3-methylcholanthrene (3-MC)) between the 4th and 24th hour of culture. After 24 hours of culture the medium was replaced by 2 ml fresh medium. Thereafter the porphyrin-inducing chemicals, dissolved in ether (HCB) or ethanol (HBB), were administered at a concentration of 10 µg/ml medium. The cells were then re-incubated for another 24 hr period. The maximal concentrations of the solvents in the medium were: 0.33% (ethanol), 0.05% (dimethyl sulfoxide), 0.20% (diethyl ether).

TABLE 1

SEMIQUANTITATIVE ASSESSMENT OF THE INTENSITY OF PORPHYRIN FLUORESCENCE AFTER TREATMENT OF CHICK EMBRYO LIVER CELL CULTURES WITH PORPHYRINOGENIC COMPOUNDS

Definition	Score
no fluorescence	0
some liver cell colonies fluoresce partially	1
most liver cell colonies fluoresce partially	2
most liver cell colonies fluoresce intensely	3
all liver cell colonies fluoresce intensely	4
all liver cell colonies fluoresce very intensely	5
all liver cell colonies fluoresce very intensely and the fluorescence quenches slowly, indicating large amounts of stored porphyrins	6

Determination of porphyrin fluorescence

The morphology of cultured liver cells was examined with a phase-contrast microscope. The porphyrin fluorescence intensity of the liver cell colonies was determined semiquantitatively by fluorescence microscopy at different times after administration of the porphyrinogenic compounds (Table 1).

Analysis of the pattern of porphyrin accumulation

Porphyrins in liver cells were extracted into 100% ethanol as described by Strik (1973). The culture medium of the cells was treated as described for the urinary porphyrin pattern analysis (Strik and Harmsen, 1979). Esterification, separation of the porphyrins by thin layer chromatography and quantification of the spots was carried out according to the method of Doss (1974), with some modifications of Strik and Harmsen (1979).

RESULTS AND DISCUSSION

Phenobarbital (PB), β-naphthoflavone (β-NF) and 3-methylcholanthrene are able to induce cytochrome P-450 and mixed function oxygenase in a primary culture of chick embryo hepatocytes (Althaus et al., 1979).
Pre-exposure induction of the drug enzyme system by PB, 3-MC or β-NF, markedly stimulates the porphyrinogenic action of hexachlorobenzene (HCB) and hexabromobiphenyl (HBB) in a chick embryo liver cell culture: the onset of porphyrin accumulation within the pretreated cells occurs at an earlier time and porphyrins appear in larger amounts than in cultures treated with HCB or HBB only (Table 2). These results obtained in a cell culture system are in agreement with the observation that stimultaneous administration of HCB and phenobarbital to female rats leads to an enhancement of the porphyrinogenic action of the former compound (Kerklaan et al., 1979; Debets et al., in prep.). Pretreatment of the cell cultures with β-NF has the most stimulating effect on the porphyrinogenic activity of HCB, whereas PB can enhance the porphyrinogenic effect only when administered together with HCB (Table 2B). Pre-exposure inducation with PB or simultaneous addition of HBB and PB results in the same stimulating effect on the onset of porphyrin accumulation (Table 2A).

The monooxygenase-inhibitor piperonyl butoxide strongly inhibits the accumulation of porphyrins in liver cell cultures treated with HCB or HBB. Even when the drug enzyme system is pre-induced by β-NF, piperonyl butoxide is found to be able to inhibit the porphyrin accumulation in the presence of HCB or HBB (Table 2C). These results with inducers or inhibitors of the drug enzyme system, add indirect proof to the hypothesis that reactive intermediates or metabolites formed by metabolism of the polyhalogenated aromatic compound are the ultimate porphyrinogenic agents.

Metyrapone, a specific inhibitor of the PB-induced mixed function oxygenases, inhibits the porphyrinogenic activity of HBB in a chick embryo

TABLE 2

PORPHYRINOGENIC ACTIVITY OF HEXACHLOROBENZENE AND HEXABROMOBIPHENYL (BP-6) IN PRIMARY CULTURES OF CHICK EMBRYO LIVER CELLS AFTER INDUCTION OR INHIBITION OF LIVER DRUG-METABOLIZING ENZYME SYSTEMS[a)]

Compound(s)[b)] (24-48 hr of culture)	Pretreatment[d)] (20 hr period)	Microscopic fluorescence of cell cultures[e)] Exposure time (hr) 8	14	24
A)				
HBB		0	0	3
HBB	PB (50)	0	1	4
HBB + PB(50)[c)]		0	1	4
HBB	β-NF (3)	2	3	4[f)]
HBB	TCPO (5)	0	2	4
HBB		0	2	6
HBB	3-MC (1)	1-2	4	6
HBB + metyrapone(20)		0	1	4
HBB	PB (100)	1	3	6
HBB + metyrapone(20)	PB (100)	0	1-2	5
B)				
HCB		0	0	trace
HCB + PCP(10)		0	trace	1-2
HCB	PB (100)	0	trace	trace
HCB + PB(100)		0	2	3
HCB	3-MC (1)	1	1-2	2
HCB	β-NF (3)	2-3	2-3	3[f)]
HCB	PB (100)	0	0	trace
HCB + metyrapone(20)	PB (100)	0	trace	0-1
C)				
HBB		0	1	3
HBB + pip.but.(5)		0	0	0-1
HBB	β-NF (3)	2	2-3	4[f)]
HBB + pip.but.(5)	β-NF (3)	0	0	1-2
HCB	β-NF (3)	2	2	2
HCB + pip.but.(5)	β-NF (3)	0	0	trace

a) The results of the experiments with liver cells isolated on different days are separated by dashed lines. The results obtained with tissue cultures prepared on different days are not comparable, because of the biological variation in responsiveness of the cell cultures.
All control cultures without HCB and HBB, but containing their respective solvents and other test compounds (PB,MC,β-NF, metyrapone, hemin, diamide etc.), showed no porphyrin fluorescence within 24 hr.

b) HBB and HCB were added after 24 hr of culture at a concentration of 10 µg/ml medium and incubation was extended to 48 hr.

c) The concentration (µg/ml) of all other compounds used is indicated in parentheses.

d) Liver cells were pretreated with various inducers of microsomal drug-metabolizing enzymes between the 4th and 24th hr of culture.

e) Intensity of red fluorescence was determined semiquantitatively according to the classification given in Table 1.

f) Porphyrins in liver cells were partly leaked out into the surrounding medium.

Abbreviations: HBB, a mixture of hexabromobiphenyl isomers (Firemaster BP-6); HCB, hexachlorobenzene; PB, phenobarbital; β-NF, β-naphthoflavone; 3-MC, 3-methylcholanthrene; TCPO, trichloropropene oxide; PCP, pentachlorophenol; pip.but., piperonyl butoxide.

liver cell culture (Table 2A). In contrast, no inhibition of the HCB-induced porphyrin accumulation is found after combined treatment with HCB and metyrapone (Table 2B). These results suggest that there may exist different enzyme systems (mixed function oxygenases), which are responsible for the metabolic activation of HCB and HBB to porphyrinogenic metabolites or intermediates.

Pentachlorophenol, the main metabolite of HCB in all animal species examined so far (Koss et al., 1976; 1978), accelerates the HCB-induced porphyrin accumulation in tissue culture (Table 2B) as well as in experimental animals (Debets et al., 1980). Pentachlorophenol, when administered alone, is not porphyrinogenic in tissue culture of chicken embryo hepatocytes up to a concentration of 40 µg/ml medium. Long-term exposure to PCP induces no porphyria in female rats either, as has been reported by several investigators (Lui et al., 1976; Goldstein et al., 1977; Kimbrough and Linder, 1978). The possible role of PCP in the HCB-induced disturbance of the hepatic porphyrin metabolism has been discussed extensively in a previous paper (Debets and Strik, 1979).

Pretreatment of the liver cell culture with trichloropropene oxide (TCPO) - an uncompetitive inhibitor of expoxide hydrolase (Oesch and Daly, 1972) - enhances the HBB-mediated induction of porphyrin fluorescence (Table 2A). Diethylmaleate (DM) and 1,1'-azobis {N,N'-dimethylformamide} (diamide) - both depleting intracellular glutathione - enhance the cytotoxicity of HBB (Table 3B,C). With DM this is expressed in a higher porphyrin fluorescence. Combined diamide and HBB treatment has a much more drastic effect: within a few hours a rounding up and swelling of the hepatocytes, finally leading to detachment of the cells from the bottom of the culture dish. Porphyrin fluorescence was even weaker or completely absent in this case (Table 3C). Control cultures, treated with diamide only, showed neither morphological abnormalities nor fluorescence of porphyrins within 24 hours. The cause of this effect of HBB + diamide treatment may be that a fast depletion of glutathione leads to an increasing amount of reactive intermediates of HBB metabolism which cannot be detoxified by glutathione. Covalent interaction of reactive intermediates with cellular macromolecules may cause mitochondrial damage, resulting in a complete blocking of the heme synthesis. In consequence of this, an inhibition of the cytoplasmic uroporphyrinogen decarboxylating enzyme finds no expression in an accumulation of uro- and heptacarboxylic porphyrin. Therefore we may consider hepatic porphyrin accumulation as a very early indication of GSH shortage, result-

TABLE 3

INFLUENCE OF ANTIOXIDANTS AND GLUTATHIONE-DEPLETING AGENTS ON PORPHYRIN ACCUMULATION INDUCED BY HEXACHLOROBENZENE AND HEXABROMOBIPHENYL IN CHICK EMBRYO LIVER CELL CULTURE[a)]

Compound(s)[b)] (24-48 hr of culture)	Pretreatment[d)] (20 hr period)	Microscopic fluorescence of cell cultures[e)] Exposure time (hr)		
		8	14	24
A)				
HBB		0	2	4
HBB+ascorbic acid(0.5 mM)		0	trace	trace
HBB+Vit.E.acetate(0.5mM)		0	1-2	3
HBB+DPPED (0.1 mM)		0	0	0
HCB	β-NF (3)[c)]	2-3	2-3	2-3[f)]
HCB+ascorbic acid(0.5mM)	β-NF (3)	trace	0-1	0-1
HCB+Vit.E.acetate(0.5mM)	β-NF (3)	1-2	1-2	2
HCB+DPPED (0.1mM)	β-NF (3)	1	1	1-2
B)				
HBB		0	1	3
HBB+DM (10)		0	2	4
C)				
HBB		0	-	3
HBB+Diamide (40)		0[g)]	-	0[g)]
HBB	β-NF (3)	2	-	4
HBB+Diamide (40)	β-NF (3)	1[g)]	-	0[g)]

a,b),d-f) See footnote, Table 2.
c) In parentheses the concentration of the added test compound in µg/ml medium, unless otherwise indicated.
g) Most liver cells appeared to be rounded up or swollen. Many liver cell colonies had detached from the bottom of the culture dish.
Abbreviations: DPPED, N,N'-diphenyl-p-phenylendiamine; Vit.E.acetate, DL-α-tocopherolacetate; diamide, 1,1'-azobis (N,N'-dimethylformamide).

ng in cellular damage caused by escaping reactive intermediates generated uring metabolism of the polyhalogenated aromatic compound. The inhibition f uroporphyrinogen decarboxylase probably precedes all other known irre-ersible effects which may be induced by this group of compounds after ubacute or short term exposure.

The antioxidants ascorbic acid, vitamin E and N,N'-diphenyl-p-phenylendiamin strongly inhibit the accumulation of porphyrins in chick embryo liver cell culture, when added together with HCB or HBB (Table 3A). Granick (1966) showed that the inhibitory effect of ascorbic acid was not due to its reducing properties, which might have prevented the intracellular autoxidation of porphyrinogens (non fluorescent) to porphyrins (fluorescent). Granick attributed the effect of ascorbic acid to a more rapid oxidation, i.e. inactivation of the porphyrinogenic compound. We presume that these antioxidants trap electrophilic intermediates or radicals generated by metabolic activation of HCB and HBB. These toxic intermediates may bind preferentially to the nucleophilic catalytic sulfhydryl-groups of uroporphyrinogen decarboxylase, thereby inhibiting its biological function. The process of inhibition proceeds under normal circumstances very slowly, since

TABLE 4

EFFECT OF HEMIN-MEDIATED INHIBITION OF δ-AMINOLEVULINIC ACID SYNTHASE INDUCATION ON THE ONSET OF PORPHYRIN ACCUMULATION INDUCED BY HEXACHLOROBIPHENYL IN CHICK EMBRYO LIVER CELL CULTURE[a)]

Compound(s)[b)] (24-48 hr of culture)	Pretreatment[d)] (20 hr period)	Microscopic fluorescence of cell cultures[e)] Exposure time (hr)		
		8	14	24
HBB		0	1	3
HBB + Hemin (0.5)[h)]		0	1	3
HBB + Hemin (1.0)		0	1	3
HBB + Hemin (5.0)		0	0-1	2-3
HBB + Hemin (10)		0	trace	2
HBB	β-NF(3)[c)]	2	2-3	4
HBB + Hemin (0.5)	β-NF(3)	1-2	2-3	4
HBB + Hemin (1.0)	β-NF(3)	1-2	2	4
HBB + Hemin (5.0)	β-NF(3)	1-2	2	3
HBB + Hemin (10)	β-NF(3)	0-1	1-2	2-3

a-e) See footnote, Table 2.
h) Concentration of hemin in μM indicated in parentheses.

HCB and other polyhalogenated aromatics possess a high resistance to metabolism. Depletion of glutathione can accelerate the whole process and may cause further damage to other parts of the cell. Koss et al., (1979b) observed in experiments with radioactive labeled HCB that metabolic products of HCB were bound to liver cytosol proteins.

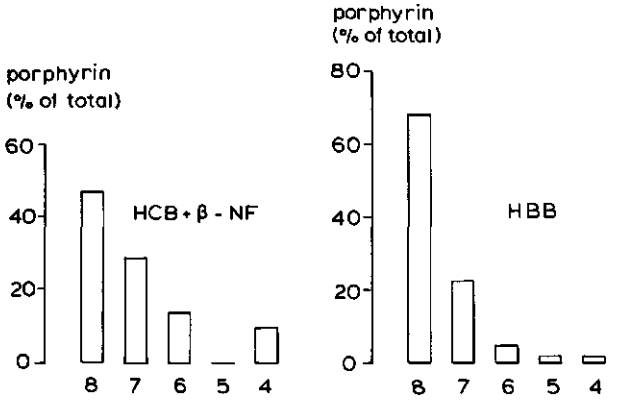

Fig. 1. The relative distribution of porphyrins with different numbers of carboxylic groups accumulated in chick embryo liver cells, maintained in Williams E medium (plus 10% fetal calf serum) and exposed to HCB (10 µg/ml) + β-NF (3 µg/ml) or HBB (Firemaster BP-6, 10 µg/ml) for 24 hr. The accumulation of the intermediates is expressed as the percentage of total porphyrins formed (mean values of 4 plates). Non-exposed cultures contained only coproporphyrin.
The intermediates are: 8, uroporphyrin; 7, heptacarboxylic porphyrin; 6, hexacarboxylic porphyrin; 5, pentacarboxylic porphyrin; 4, coproporphyrin.

Inhibition of the induction of δ-aminolevulinic acid synthase (δ-ALAS) with increasing concentrations of hemin, did not change the time of onset of porphyrin accumulation after treatment of chick embryo liver cells with HCB (Table 4). Moreover, inhibition of δ-ALAS induction has only little influence on the rate of porphyrin accumulation, as indicated by the intensity of fluorescence reached after 24 hours of incubation with HBB + hemin as compared to HBB treatment only (Table 4). Similar results were also found in Japanese quail (Coturnix c. japonica), where daily i.p. injections of hemin could not prevent HBB-induced porphyrin accumulation (Strik, 1973). These experiments show that induction of δ-ALAS is not responsible for porphyrin accumulation, but contributes to the expression of the uroporphyrinogen decarboxylase defect by an accelerated production of δ-ALA. Inhibition of the enzyme uroporphyrinogen decarboxylase also appears to be the primary

lesion in the disturbance of the heme synthesis induced by HCB and HBB in a chick embryo liver cell culture. This is indicated by the pattern of porphyrin accumulation in chick embryo liver cells, which consists mainly of uro- and heptacarboxylic porphyrin after treatment with HCB or HBB (fig. 1).

The results obtained with regard to the porphyrinogenic potential of chemicals in the chick embryo liver cell culture system are in good agreement with the findings in animal experiments. This conclusion is based on the experience with a large number of compounds, including drugs, industrial chemicals and a few pesticides (Marks, 1978; Strik et al., 1980; Koeman et al., 1980). Results similar to those described in the present paper for the porphyrinogenic activity of HCB and HBB under variable conditions in chick embryo liver cell culture, are obtained also with other polyhalogenated aromatics, like chlorinated naphthalenes and chlorinated biphenyls (Debets and Strik, in prep.). The chick embryo liver cell system may, therefore, be considered as a useful and sensitive system for the elucidation of the mechanism of chemical porphyria.

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Chapter 4

EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON THE INDUCTION OF HEPATIC MIXED FUNCTION OXYGENASES AND PORPHYRIA IN HEXACHLOROBENZENE-TREATED RATS[a]

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SUMMARY

Female Wistar rats were treated with a dietary concentration of 1000 ppm hexachlorobenzene (HCB) alone or together with either phenobarbital (PB, 0,05% in drinking water) or 3-methylcholanthrene (MC, weekly i.p. injection 80 mg/kg body weight). Control groups fed standard feed without HCB, were treated with PB or MC. Subgroups of 4 rats were killed at 1,2,4,6 and 8 weeks. The increase in body weight was depressed in the MC and HCB+MC treated rats, whereas the HCB and HCB+PB treated rats showed a higher gain in body weight than the controls. The liver weight to body weight ratios were significantly increased in all treated groups. Neither PB nor MC interfered with the liver uptake of HCB. Pentachlorophenol (PCP; one of the main metabolites of HCB) showed a high affinity for membranes of the endoplasmic reticulum, since 50 to 70% of the PCP content of the liver appeared to be bound to the microsomal fraction. Microsomal cytochrome P-450, ethoxyresorufin O-de-ethylase, ethylmorphine N-demethylase, and glucuronyl transferase increased to a maximum in 4-6 weeks in the HCB, HCB+PB, and HCB+MC treated rats. Ethylmorphine N-demethylase was not induced by MC treatment but responded to PB, whereas MC, but not PB, increased ethoxyresorufin O-de-ethylase activity. These results indi-

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cate that HCB produces a pattern of microsomal enzyme induction which shares properties of the MC and PB class of inducers. Simultaneous treatment with HCB and PB, but not with HCB and MC, enhanced the porphyrinogenic effect of HCB. This was reflected in an earlier and more marked increase in the urinary excretion of uro- and heptacarboxylic porphyrins in the HCB+PB treated group. Our results point to a specific role for the PB inducible form of cytochrome(s) P-450 in the biotransformation of HCB and the onset of hepatic porphyria.

INTRODUCTION

Hexachlorobenzene (HCB) induces, like many other xenobiotics [1], the activity of mixed function oxygenases in the liver of mammals and birds [2-4]. Inducers of hepatic mixed function oxygenases can be divided into two classes [1,5,6]: The phenobarbital-type inducers increase the concentration of cytochrome P-450 and the biotransformation of a large range of compounds, like barbiturates and aliphatic compounds. 3-Methylcholanthrene and benzo(a)pyrene belong to the second class, which induces cytochrome P-448 and increases the activity of xenobiotic-metabolizing enzymes involved in the activation of many carcinogenic polycyclic aromatic hydrocarbons. The Soret maximum of the CO-difference spectrum of the cytochrome P-450 subspecies induced by MC is shifted from 450 to 448 mm. Today, there is considerable evidence for multiple forms of cytochrome P-450 in the monooxygenase system of PB- and MC-induced microsomes [7-10].
However, the major form of cytochrome P-450 isolated from PB treated animals differs from the major form isolated from MC treated animals; although both classes have overlapping substrate specificities.

There still is much controversy about the species of cytochrome P-450 induced by HCB. Several reports refer to cytochrome P-450 as the major microsomal hemoprotein induced by HCB [11,12]. However, there is also evidence that HCB produces a mixed pattern of microsomal enzyme induction, combining inducing properties of both the PB (P-450) and MC (P-448) classes of inducers [13-15].

In addition to the ability of HCB to induce microsomal mixed function oxygenases, it produces a defect in the biosynthesis of heme which is characterized by the excretion of large amounts of mainly uro- and heptacarboxylic porphyrins in urine and feces [16-20]. It has been proved beyond any doubt that inhibition of the enzyme uroporphyrinogen decarboxylase

(UROG-D) [EC 4.1.1.37], involved in the heme biosynthetic pathway, underlies the observed accumulation of porphyrins [21-24]. Moreover, there is considerable evidence to show that biotransformation of HCB by the mixed function oxygenase system forms a prerequisite for the onset of the disease [25]. A metabolic intermediate of HCB capable of reacting with the active site of hepatic UROG-D is suspected to be responsible for enzyme inhibition [26]. In this context it is important to determine which from of cytochrome P-450 is involved in the activation of HCB, thus causing porphyria. Since simultaneous treatment of rats with PB and HCB enhanced the porphyrinogenic effect of the latter compound [27], the PB-induced form of P-450 seems predominant in the formation of reactive products of HCB. This assumption contrasts with the observation that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent porphyrinogenic polyhalogenated aromatic compound known, induces a pattern of microsomal enzymes identical to that caused by MC [28,29].

To obtain more evidence in support of the hypothesis that the porphyrinogenic effect of HCB is exerted by reactive metabolic products formed preferentially by the PB class of induced cytochrome(s) P-450, we have studied the effects of combined administration of HCB with either PB or MC on female rat liver. We examined the accumulation of HCB and pentachlorophenol (PCP) in the liver and liver microsomal fraction; the onset of hepatic porphyria; and the induction of mixed function oxygenases.

METHODS

Animals and treatments

Hundred and twenty adult female SPF Wistar (WAG/Cpb) rats (120-140 g) were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. The animals were randomly distributed over 6 groups of 20, housed in wire bottom cages, and had unlimited access to standard powdered diets (Hope Farms, Woerden, The Netherlands) supplemented with 1000 ppm HCB when required. Of the 3 groups treated with HCB, one additionally received 0.05% phenobarbital in drinking water, and a second group was weekly injected intraperitoneally with 3-methylcholanthrene (80 mg/kg of body wt.) in arachis (peanut) oil (1% solution). Three control groups - kept in a separate room to avoid cross-contamination with HCB - were fed on a diet without HCB, one group received PB and a

RESULTS

Body weight

The gain in body weight was significantly depressed in both groups treated with MC. All groups receiving HCB, with the exception of the HCB+MC group, showed a higher gain in body weight than the control group (Fig. 1); this phenomenon has also been observed in an earlier experiment [15].

Dietary intake of HCB

The dietary intake of HCB expressed in mg/kg body weight/day, in the HCB+MC treated group was about 20% lower than in the other HCB groups (Fig. 2). This was due to a decreased food consumption in both MC groups.

Clinical observations

Skin lesions were first observed in the 4th week of the experiment in the HCB group (8 out of 12). In the HCB+PB group only one animal developed skin lesions, whereas no skin lesions were found in the rats exposed to HCB+MC. Skin lesions were characterized by small depilated sores with hemorrhagic crusts located in the neck region. The development of skin lesions went together with excessive scratching by the animals at the places where skin lesion arose. There was no correlation between the light intensity in the animal cages and the number of rats in a treatment group developing skin lesions, as the light intensity in all cages was very low and nearly the same (5-15 Lux). Chronic neurotoxic symptoms (weak tremors) were observed in the HCB group during the last 4 weeks of the experiment. In the HCB+PB and HCB+MC groups tremors appeared in the 6 th week of the experiment, but to a lesser degree.

Liver weight

A significant elevation in liver weight to body weight ratio occurred in all treated groups (Fig. 3). The increase in relative liver weight of the MC, PB, and combined HCB+PB treated rats remained constant over the period of treatment. However, a continuous increase in relative liver weight with the exposure time was found after treatment with HCB alone and with the combination of HCB and MC (Fig. 3). The greatest increase in relative liver weight was induced in the HCB+MC group. This increase was approximately additive when compared with the effects of HCB and MC separately.

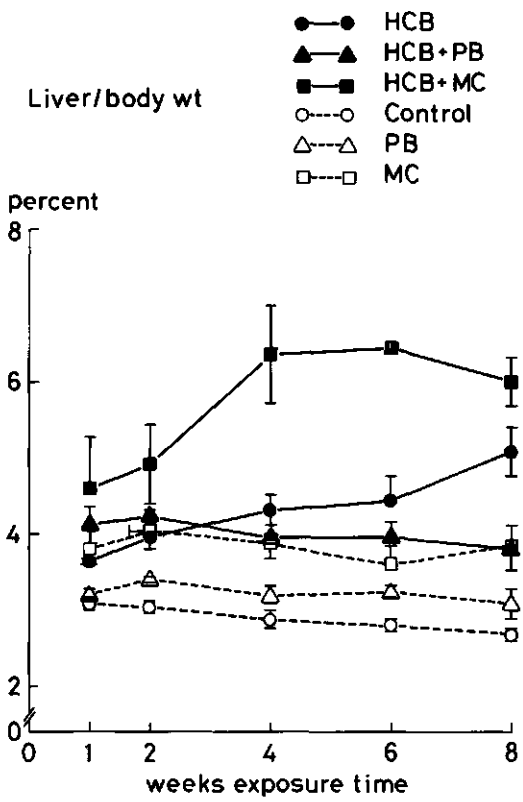


Fig. 3. Effects of phenobarbital (PB) and 3-methylcholanthrene (MC) on the liver/body weight ratios of female rats fed either a normal diet or a diet containing 0.1% hexachlorobenzene (HCB). Values represent means ± S.D. of 4 animals.

Effects on liver microsomal enzymes

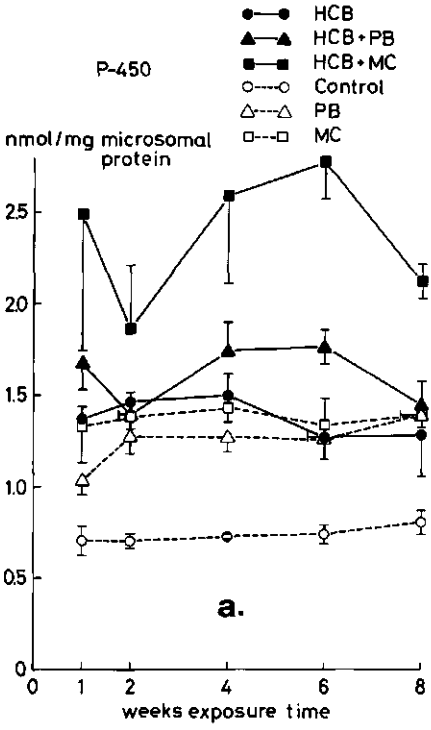
Hexachlorobenzene, HCB+PB, and HCB+MC increased the microsomal cytochrome P-450 concentration to a maximum of approximately 2.0-, 2.4- and 3.7-fold, respectively (Fig. 4a). The cytochrome P-450 concentration in animals treated with PB or MC alone was increased approximately 1.7- and 2.0-fold. Thus the effect of PB and MC on the cytochrome P-450 level in the combined treatments was at least additive. Both the combined HCB+PB treatment and MC alone caused a blue shift in the Soret maximum of the reduced cytochrome P-450-CO complex of 1 nm (maximal at 6 weeks). In microsomes from HCB and HCB+MC treated rats this blue shift amounted to 1.5 nm. at 6 weeks. PB alone shifted the λ_{max} for the reduced P-450-CO complex 0.8 nm towards the red part of the spectrum. In HCB+PB treated rats the activities of hepatic glucuronyl transferase, ethoxyresorufin O-de-

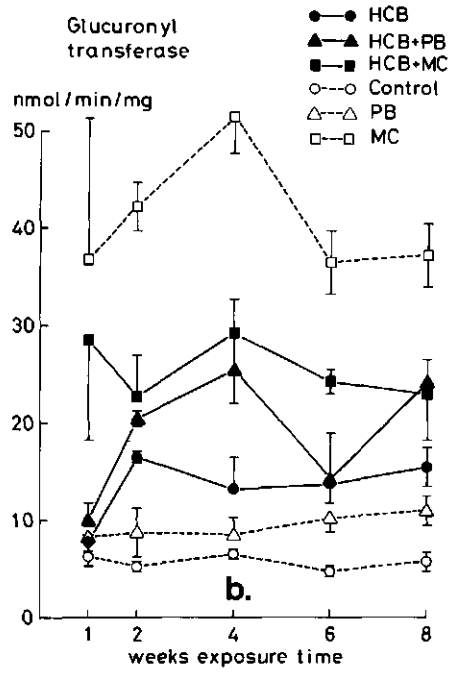
ethylase, and ethylmorphine N-demethylase were enhanced to a maximum of 3.9-, 41- and 2.3-fold, respectively (Fig. 4). For liver microsomes of HCB+MC treated rats these values were: 4.5-, 77- and 3.4-fold; and for HCB treated rats 3.1-, 62- and 1.7-fold. Phenobarbital preferentially induced ethylmorphine N-demethylase (2.7-fold) but ethoxyresurufin O-de-ethylase was not increased; on the other hand in MC treated rats, ethoxy-resorufin O-de-ethylase was induced to a maximum of 135-fold and ethyl-morphine N-demethylase activity was comparable to the control level (Fig. 4).

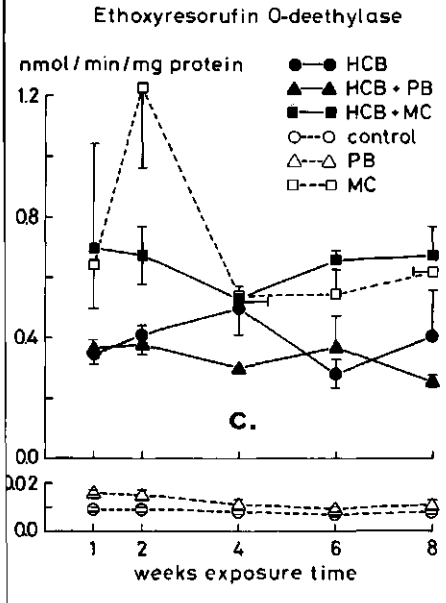
Total porphyrins and pattern of porphyrin accumulation in urine

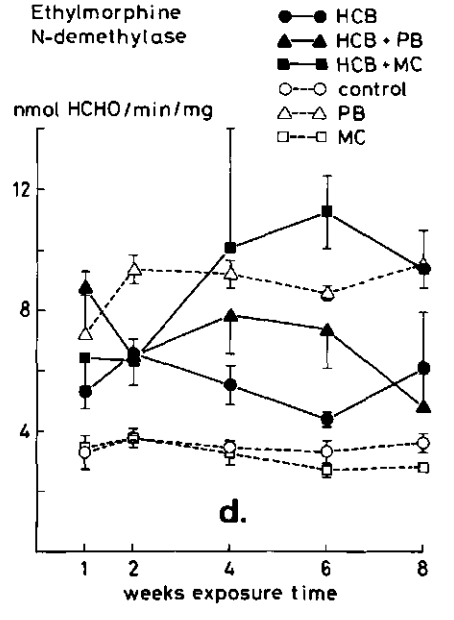
The total porphyrin excretion in the urine from HCB+PB treated rats increased abruptly after 6 weeks to a level that reached approx. 7-fold the control level after 8 weeks (Fig. 5). The increase in the total porphyrin excretion in this treatment group was accompanied by a change in the pattern of the accumulated urinary porphyrins indicative of an inhibition of uroporphyrinogen decarboxylase. The amount of uro- and heptacarboxylic porphyrin in the total porphyrin excretion markedly increased relative to the amount of copro- and protoporphyrin. The distribution of porphyrins shows that after 8 weeks uroporphyrin had already become the predominant type of porphyrin in the urine of HCB+PB treated rats (Fig. 6b). The increase in total porphyrin excretion could, therefore, be attributed primarily to an accumulation of uro- and heptacarboxylic porphyrins. In contrast, the total porphyrin excretion and porphyrin pattern of the HCB+MC and HCB treated groups began to change slowly at 8 weeks (Figs. 5; 6a,c),and at that time coproporphyrin was still the predominant porphyrin. This was also found in control, PB and MC treated rats.

Fig. 4.(a-d) Hepatic microsomal cytochrome P-450 concentration and enzyme activities in female rats in response to various treatments as in legend Fig. 1. Mean values ± S.D. of 4 animals are shown. Analysis of variance, (b) Glucuronyl transferase: a negative interaction between HCB and MC treatment (HCBxMC; F-test, $P < 0.05$). (d) Ethylmorphine N-demethylase: a positive interaction between HCB and MC treatment (HCBxMC; F-test, $P < 0.05$), and a negative interaction between HCB and PB treatment (HCBxPB); F-test, $P < 0.05$).


P-450
nmol/mg microsomal protein
HCB
HCB+PB
HCB+MC
Control
PB
MC
2.5
2.0
1.5
1.0
0.5
0
a.
0 1 2 4 6 8
weeks exposure time


Glucuronyl transferase
nmol/min/mg
HCB
HCB+PB
HCB+MC
Control
PB
MC
50
40
30
20
10
0
b.
1 2 4 6 8
weeks exposure time


Ethoxyresorufin O-deethylase
nmol/min/mg protein
HCB
HCB + PB
HCB + MC
control
PB
MC
1.2
0.8
0.4
0.0
0.02
0.0
c.
1 2 4 6 8
weeks exposure time


Ethylmorphine N-demethylase
nmol HCHO/min/mg
HCB
HCB + PB
HCB + MC
control
PB
MC
12
8
4
d.
1 2 4 6 8
weeks exposure time

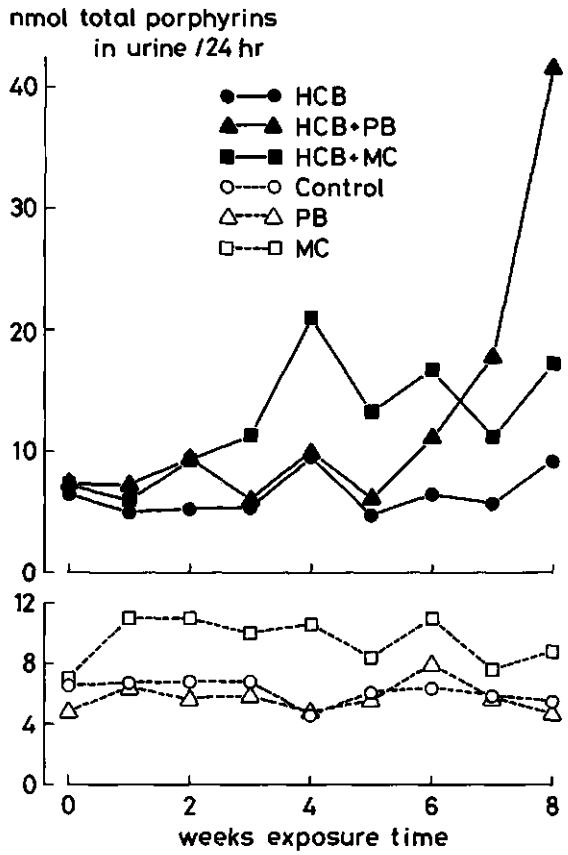
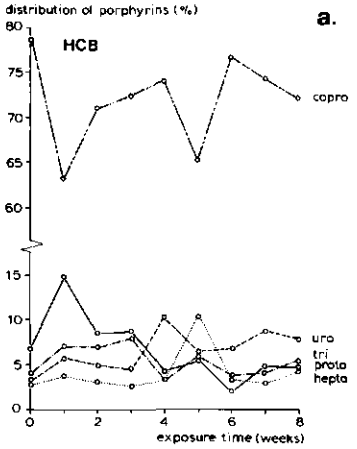
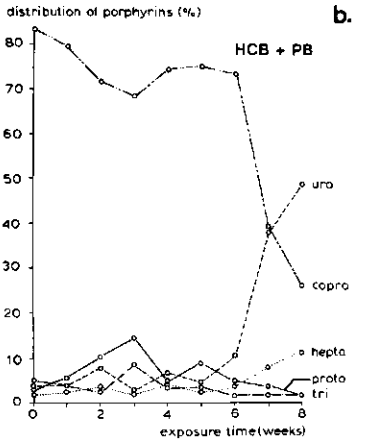
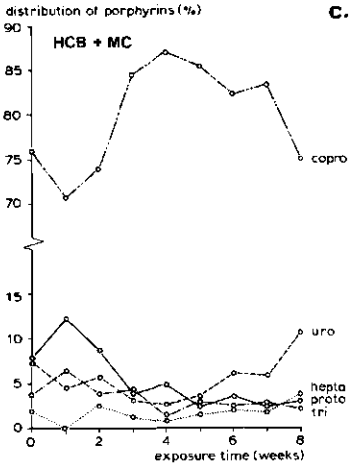


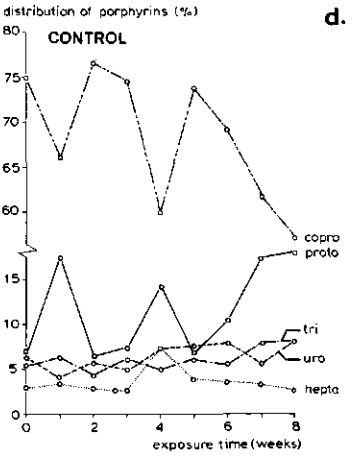
Fig. 5. Effects of phenobarbital (PB) and 3-methylcholanthrene (MC) on the total urinary porphyrin excretion of female rats fed either a normal diet or a diet containing 0.1% hexachlorobenzene (HCB). Mean values of 4 rats are plotted against time. Coproporphyrin used as an internal standard.

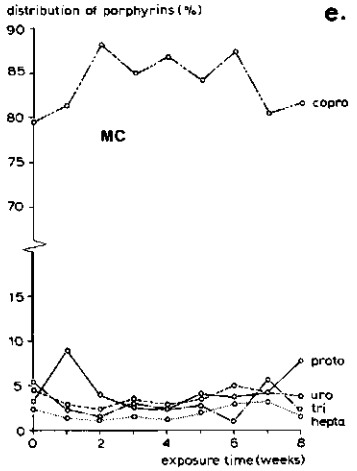
Fig. 6.(a-f) The pattern of urinary porphyrin excretion of female rats in response to various treatments as in legend Fig. 1. The excretion of porphyrins with different numbers of carboxylic groups is expressed as the percentage of total porphyrins excreted. Points represent analyses of pooled urine samples from groups of 4 rats. The porphyrins are: uro-, heptacarboxylic, copro-, tricarboxylic, and proto-porphyrin.


distribution of porphyrins (%)
a.
HCB
copro
uro
tri
proto
hepta
exposure time (weeks)


distribution of porphyrins (%)
b.
HCB + PB
uro
copro
hepta
proto
tri
exposure time (weeks)


distribution of porphyrins (%)
c.
HCB + MC
copro
uro
hepta
proto
tri
exposure time (weeks)


distribution of porphyrins (%)
d.
CONTROL
copro
proto
tri
uro
hepta
exposure time (weeks)


distribution of porphyrins (%)
e.
MC
copro
proto
uro
tri
hepta
exposure time (weeks)

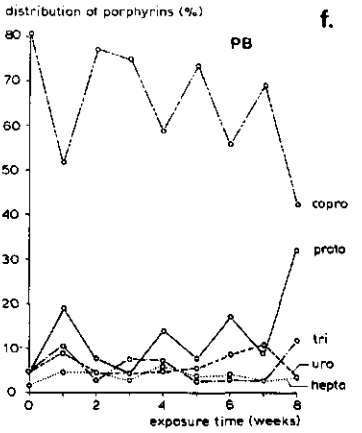

distribution of porphyrins (%)
f.
PB
copro
proto
tri
uro
hepta
exposure time (weeks)

TABLE I

EFFECTS OF PHENOBARBITAL (PB) AND 3-METHYLCHOLANTHRENE (MC) ON THE ACCUMULATION OF HEXACHLOROBENZENE (HCB) AND PENTACHLOROPHENOL (PCP) IN LIVER AND MICROSOMAL FRACTION OF FEMALE RATS FED HCB FOR 8 WEEKS

Treatment	Exposure time (weeks)	HCB content (µg/g wet liver tissue)			PCP content (µg/g wet liver tissue		
		Whole homogenate	Microsomal fraction	$\frac{\text{Micr.fr.}}{\text{whole hom.}} \times 100\%$	Whole homogenate	Microsomal fraction	$\frac{\text{Micr.fr.}}{\text{whole hom.}} \times 100\%$
HCB	4	318±147[a]	55± 8	17	4.3±2.7	1.9±0.4	44
HCB	8	446±171	64±14	14	3.9±4.6	3.3±2.5	85
HCB+PB	4	185± 63	38± 5	21	3.6±2.1	1.9±0.2	53
HCB+PB	8	305± 98	51± 5	17	2.5±1.9	1.7±0.2	68
HCB+MC	4	475±182	46±18	10	10.1±5.5	2.5±1.0	25
HCB+MC	8	297±146	65± 4	22	8.0±5.6	3.7±0.5	46

[a] Each value represents mean ± S.D. from four rats.

The maximum in total porphyrin excretion observed at 4 weeks in the HCB+MC treated rats was due to an increase in the excretion of coproporphyrin (Fig. 6c), as a result of an enormous induction of microsomal hemoproteins (Fig. 4a). This excretion pattern is not characteristic of HCB-induced experimental hepatic porphyria and is referred to as coproporphyrinuria. Coproporphyrinuria was also noticed in the group treated with MC (Fig. 6e). PB alone had no effect on either the total urinary porphyrin content or the pattern of excreted porphyrins (Figs. 5 and 6f).

Content of hexachlorobenzene and pentachlorophenol in the liver

Both MC and PB had no significant effect on the intake or retention of HCB in the liver: HCB concentrations in the livers of all HCB treated groups were about the same (Table I). Twenty per cent of the HCB found in the livers of HCB treated groups could be recovered from the microsomal fraction. The content of PCP in the livers from rats administered the combination of HCB and MC was about 2-3 times higher than in the livers from HCB and HCB+PB treated rats (Table I). The major part (70-80%, at 8 weeks) of the PCP in the livers from HCB and HCB+PB treated rats had accumulated in the microsomal fractions (Table I). In the HCB+MC group this microsome-bound PCP amounted to 25% and 45% of the total PCP content of the liver, after 4 and 8 weeks respectively.

DISCUSSION

The combined treatment of female rats with HCB and PB, markedly enhanced the prophyrinogenic effect of HCB. In contrast, MC had no significant effect on the porphyrinogenic effect of HCB. This is so, in spite of the fact that the simultaneous administration of HCB and MC induced the highest cytochrome P-450 concentration and mixed function oxygenase activity. Previous experiments had shown that the monooxygenase inhibitors SKF-525A [39] and piperonyl butoxide [25] completely prevented HCB-induced accumulation of porphyrins in a primary culture of chick embryo liver cells. Evidently, biotransformation of HCB by the mixed function oxygenase system forms a prerequisite for the porphyrinogenic effect of HCB. The present results suggest a key role for the phenobarbital inducible form of cytochrome(s) P-450 in the metabolism of HCB and the onset of porphyria. The PB-mediated alteration of HCB metabolism, resulting from a functional change in cytochrome P-450, may favour the formation of reactive metabolic

intermediates capable of reacting with the catalytic center of uroporphyrin decarboxylase, thus causing porphyria.

Effects of PB and MC, similar to those found in this study with HCB, have been reported in studies concerning the hepatotoxicity of monobromobenzene and carbon tetrachloride. Phenobarbital pretreatment enhanced the hepatotoxic effects of both compounds, whereas MC treatment even protected against CCl_4 - and monobromobenzene - induced liver necrosis [40-42]. Moreover, it has been established for both agents that their toxicity is mediated through reactive metabolic products of the parent compound [43,44].

In contrast to rats, both PB and MC were able to enhance the porphyrinogenic effect of HCB in a primary culture of chick embryo liver cells [25]. This discrepancy may be due to a difference in the pattern of microsomal enzymes induced by MC in both systems [45].

The present results prove beyond any doubt that HCB itself induces a pattern of microsomal hemoproteins, which shares characteristics of both the MC- and PB-induced pattern. HCB increased ethylmorphine N-demethylase (not induced by MC) to about half the value induced by PB. Moreover, HCB increased ethoxyresorufin O-de-ethylase (preferentially induced by MC) up to 62 times that of control values. These findings are in accordance with previously published data [14,15].

Both HCB and MC increased the microsomal cytochrome P-450 concentration in the liver and when these treatments were combined, the increase in cytochrome P-450 was additive. The same effect has been observed with combined PB and MC treatment [46]. Ethylmorphine N-demethylase was not induced by MC but the activity of this enzyme was increased by HCB. Moreover, when administered together, HCB and MC induced to a higher apparent activity than HCB alone. This contrasts with the effect on glucuronyl transferase activity, where the combination of HCB and MC induced a lower apparent activity, than MC alone. We have no explanation for this phenomenon.

The levels of PCP in the livers of HCB+MC treated rats were comparatively higher than those in the livers of animals of the HCB and HCB+PB group. This may result from an enhanced dechlorination of HCB by MC treatment, as has been reported for the stimulating effect of MC on the dechlorination of PCP [47]. Consistent with earlier observations [15,48], PCP preferentially accumulates in the endoplasmic reticulum, i.e. the site where PCP is formed by metabolism of HCB. The role of this high affinity binding

of PCP in disturbing hepatic drug metabolism and in stimulating HCB-induced accumulation of porphyrins has been discussed in a previous paper [15].

Considering the potentiating effect of PB, as found in the present study, it should be mentioned that exposure to HCB or other polyhalogenated aromatics may constitute a special hazard to those population groups that regularly use PB or other barbiturates as a medicine, e.g. as an anti-epileptic or hypnotic drug.

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Chapter 5

BIOTRANSFORMATION AND PORPHYRINOGENIC ACTION OF HEXACHLOROBENZENE AND ITS METABOLITES IN A PRIMARY LIVER CELL CULTURE[a]

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SUMMARY

Hexachlorobenzene (HCB) is metabolized in a primary culture of chick embryo liver cells and causes porphyrin accumulation within 24 hours after administration. The HCB-metabolites, pentachlorothiophenol, pentachlorobenzene and pentachlorophenol identified in liver cell culture are already known from long-term experiments with rats. The pattern of accumulated porphyrins is comparable with the pathological porphyrin pattern observed in oral feeding studies with warmblooded laboratory animals. Protein bound radioactivity was found in cell cultures treated with [^{14}C]HCB. Addition of the monooxygenase-inhibitor piperonyl butoxide or ascorbic acid decreased the irreversible binding of ^{14}C-metabolites. The results show that biotransformation of HCB fulfils an essential role in the onset of porphyria. Since none of the main HCB-metabolites could induce a pathological porphyrin pattern, a reactive intermediate capable of reacting with glutathione or thiol-groups of uroporphyrinogen decarboxylase (UROG-D) is believed to be

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[a] A part of this study has been presented at The International Conference on Xenobiochemistry, Bratislava, Czechoslovakia, June 9-13, 1980.

responsible for the inhibition of UROG-D. The chick embryo liver cell system may be considered as a useful and sensitive system for studying the metabolism of xenobiotics in relation to their toxicity.

INTRODUCTION

Hexachlorobenzene (HCB) produced an extensive outbreak of porphyria in people of south-eastern Turkey between 1955 and 1960 [1]. Since that Turkish calamity, HCB-induced porphyria in animals has been used as an experimental model for studying the stages of development of human symptomatic porphyria, and for the elucidation of the mechanism of toxic porphyria induced by the group of organohalogen compounds [2-4].
Despite extensive studies the mechanism of this type of chemical porphyria, which is characterized by an accumulation of uro- and heptacarboxylic porphyrins in the liver and urine, remains to be fully clarified. There is strong evidence that inhibition of the enzyme uroporphyrinogen decarboxylase (UROG-D) forms the key process in this disturbance of the heme synthesis [5-7]. Moreover, biotransformation of HCB appears to be a prerequisite for its porphyrinogenic action, suggesting that a metabolic product rather than the parent compound is responsible for causing porphyria [8].

Studies on the metabolism of HCB in rats showed several phenolic and sulfur-containing metabolites to be formed [9-10]. However, neither pentachlorophenol [11,12] (the main metabolite of HCB) nor several other HCB-metabolites [13] administered to rats, could induce porphyria in this animal species.

In addition to different animal species (rat, rabbit, Japanese quail), HCB produces accumulation of porphyrins in a primary culture of chick embryo liver cells [8,14]. The chick embryo liver cell culture system proved to be a sensitive experimental model to study HCB-porphyria, obviating time consuming animal experiments by responding within 24 hours after addition of HCB to the cell culture. So far nothing is known about the biotransformation of HCB in this cell culture system and the pattern of porphyrin accumulation. This, however, is particularly important for extrapolating the results to the animal model and for obtaining additional evidence that a reactive metabolic product of HCB inhibits UROG-D in the liver.

The biotransformation of HCB has been investigated in primary cultures of chick embryo liver cells, in order to find out whether the liver culture

system resembles in all respects the animal model. The main metabolites of HCB identified in rat experiments were tested for their porphyrinogenic activity in chick embryo liver cell culture. Porphyrin patterns were analysed and compared with the porphyric mammalian liver.

METHODS

Chemicals

Hexachlorobenzene was purchased from BDH Chemicals Ltd. (Poole, England); pentachlorophenol (PCP) and pentachlorothiophenol (PCThP) from Aldrich Chemical Co.; pentachlorobenzene (PeCB) and ascorbic acid from Merck (Darmstadt, W-Germany); tetrachlorohydroquinone from Ferak AG, W-Germany. The HCB-metabolites pentachlorothioanisol (PCTA), 2,3,5,6-tetrachlorothioanisol (TCTA), 1-methyl-[2,3,4,5,6-pentachlorophenyl] sulfoxide (PCTA-0) and 1-methyl-[2,3,4,5,6-pentachlorophenyl] sulfone (PCTA-O_2) were synthesized and purified by recrystallization as described by Koss et al. [10]. All compounds proved to be better than 99.5% pure after recrystallization. Piperonyl butoxide was obtained from Riedel-De Haën AG, W-Germany; β-naphthoflavone (β-NF) from ICN Pharmaceuticals, Inc., USA, and δ-aminolevulinic acid (ALA) from Sigma.
7-Ethoxyresorufin and resorufin were procured from Pierce Eurochemie BV (Rotterdam, The Netherlands). Uniformly labeled ^{14}C-HCB with a specific radioactivity of 1.1 µCi/mg was used in binding studies of HCB to cell protein. The labeled compound (cf. Koss and Koransky, [15]) and the 2,4,5, 2',4',5'-hexachlorobiphenyl were prepared by Dr. P.E. Schulze from Schering AG (W-Germany). Following recrystallization from n-hexane the melting point of the hexachlorobiphenyl proved to be 102°C. Test by means of GC-MS revealed a purity of more than 99.7%. The impurities detected were not identical with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Cell culture

Liver cells were isolated from 15-day old chick embryos as described previously [8]. The cell cultures were pretreated with β-naphthoflavone (3 µg/ml culture medium) for 18 h from the 6th h after isolation, to pre-induce the mixed function oxygenase system. Thereafter, the culture medium was replaced by fresh medium, containing HCB or one of its metabolites (dissolved in diethyl ether) at a concentration of 10 µg/ml medium. The concentration of diethyl ether in the medium did not exceed 0,2%.

After an incubation period of 24 h, the cells were harvested with a rubber policeman and used for measuring the total amount of porphyrins, for analysing the porphyrin pattern, for assaying the activity of mixed function oxygenases, and for identification of HCB metabolites by gas chromatography-mass spectrometry (GC-MS).

Identification and quantitation of HCB-metabolites

Liver cells and culture medium were sonicated and brought at pH 13 with a 10% solution of sodium hydroxide. The alkaline samples were kept at 65°C for 1 h to split the conjugates of metabolites. Thereafter, the pH of the samples was adjusted to 1 with concentrated hydrochloric acid. The digested samples were extracted twice with an equal volume of diethyl ether for 1 h. Aliquots of the combined and concentrated extracts were mixed with ethereal diazomethane to methylate the phenols and thiophenols. After 24 h the methylated extracts were submitted to column chromatography to remove the biological materials. A (13 cm x 1 cm^2) glass column, packed with silica gel 60: 70-230 mesh ASTM (Merck, W-Germany) in benzene and anhydrous sodium sulfate on the top (2 cm x 1 cm^2), was eluted with benzene. The first 20 ml of the eluate were taken and reduced in volume by evaporation. Microliter samples of the methylated ether extracts were subjected to gas chromatography (GC) or to combined gas chromatography-mass spectrometry (GC-MS).

GC was carried out with a Hewlett Packard 5750 G or 5730 A equipped with an electron capture detector and a 150 x 0.22 (i.d.) cm or 270 x 0.22 (i.d.) cm all glass column, packed with respectively 3% QF-1 or 3% OV-1 on Chromosorb Q 100-120 mesh. All other chromatographic conditions were the same as described by Koss and Koransky [15]. GC-MS was performed with an LKB 2091 instrument connected with a Digital PDP 11 computer. GC-MS analyses were carried out under the conditions described by Koss et al. [10].

Analysis of total porphyrin and porphyrin pattern

Analysis of the total porphyrin concentration in the cell culture medium was performed following as 20- to 50-fold dilution of the medium with an acid-alcohol solution of chloranil (2,3,5,6-tetrachloro-1,4-benzoquinone), as described for total porphyrin analysis in urine samples [16]. Excitation spectra (λ_{ex}: 350-500 nm, λ_{em}: 652 nm) of diluted samples of culture medium were recorded on an Aminco-Bowman Model J4-8960 spectrofluorometer

equipped with a 150-watt xenon lamp and a red sensitive photomultiplier tube type Super S-20 R4465. A solution containing equimolar amounts of 8,7,6,5,4 and 2 carboxylic porphyrins (Porphyrin Products, Logan, USA) was used as standard. Porphyrins accumulated in liver cells were determined by extraction into the acid-alcohol solution of chloranil.

The pattern of porphyrins accumulated in the culture medium was analysed as described for urine samples by Doss [17], with some modifications of Strik and Harmsen [18]. Liver cells were first digested with 100% ethanol and dried under a stream of nitrogen, prior to the esterification and separation of porphyrins by thin layer chromatography. The porphyrin methyl esters of medium and cells were pooled.

Ethoxyresorufin O-de-ethylation was measured according to Burke and Mayer [19]. Protein concentration was determined by the method of Lowry et al. [20] with bovine serum albumin as a standard.

Binding to cell protein

Liver cell cultures, pretreated with β-NF during the first 24 h of culture, were exposed to ^{14}C-HCB (1.1 µCi/mg, 10 µg/ml in medium) for 24 h. Solvent extractions were performed as described by Kappus and Remmer [21] with the following modifications: Cells and medium (7 ml/culture dish) were harvested; protein was precipitated with 2 volumes of absolute ethanol. Protein precipitate was extracted with 7 ml 70% ethanol; twice with 100% ethanol (7 ml) and twice with diethyl ether (7 ml). Extracted protein was solubilized in Soluene (Packard) and after addition of Instafluor (Packard), radioactivity was determined by liquid scintillation counting using an external standards channel ratio programme to correct for quenching. To one cell culture ^{14}C-HCB has been added just before solvent extraction, to serve as a control.

RESULTS AND DISCUSSION

Total porphyrin and the pattern of porphyrin accumulation

When added alone to the cell culture, HCB induced only little porphyrin accumulation (100-200 pmol/mg total cell protein), but pre-induction of the cell cultures with the cytochrome P-450 inducer β-NF markedly enhanced porphyrin accumulation (Table I). Addition of the monooxygenase inhibitor piperonyl butoxide (pip.but.) completely prevented the accumulation of porphyrins in cultures treated with HCB (Tabel I). These results suggest that

TABLE I

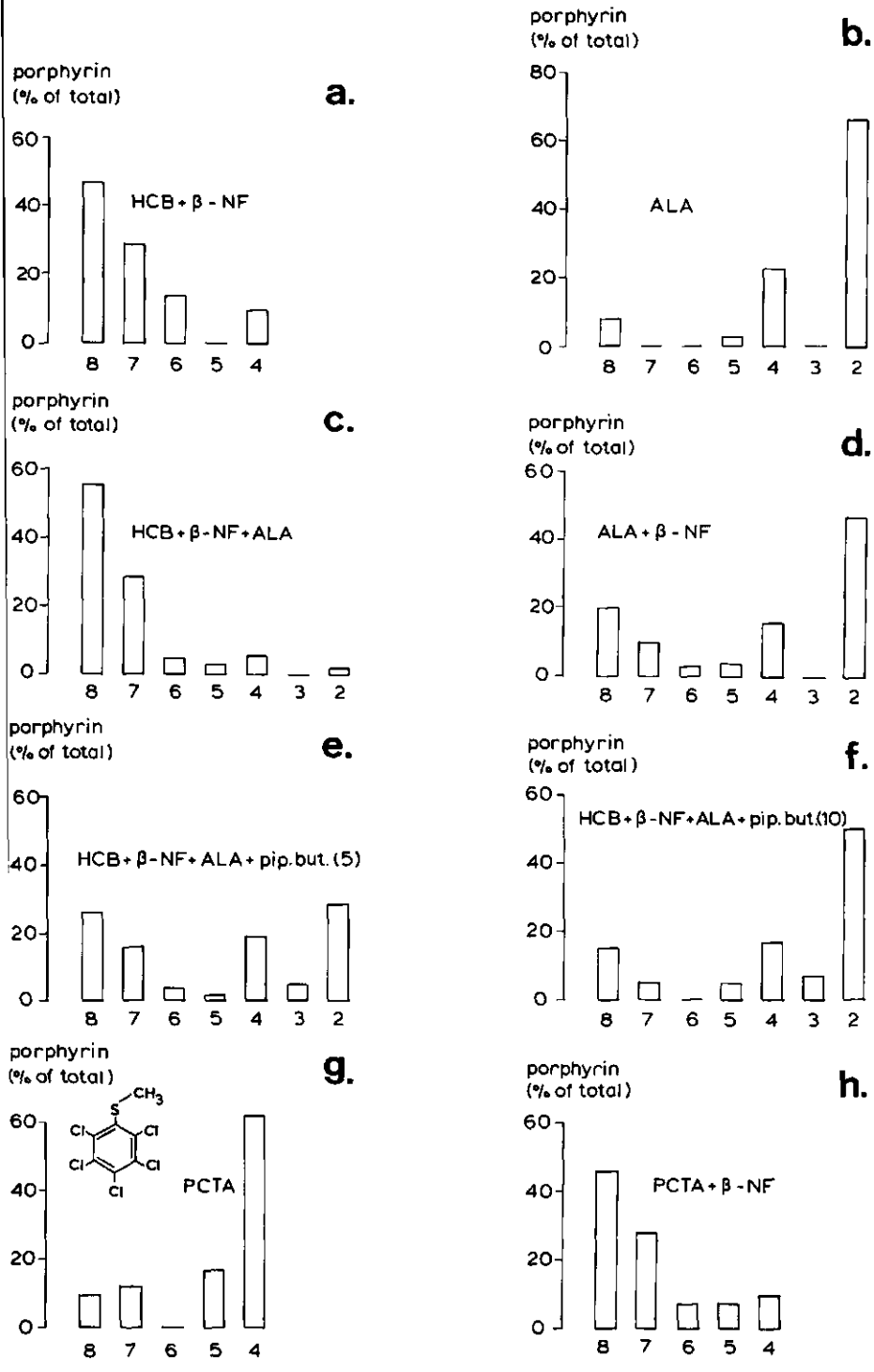
EFFECT OF β-NAPHTHOFLAVONE (β-NF) AND PIPERONYL BUTOXIDE (PIP. BUT.) ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS TREATED WITH HCB

Compound	Total porphyrins (pmol/mg cell protein)
HCB	100-200
HCB + β-NF pretreatment	600-700
HCB + pip.but. (10 µg/ml)	10-20
Control	10-20

the rate of HCB-metabolism has to be above a critical level before UROG-D, the enzyme playing the key role in HCB-induced porphyria, becomes inhibited and porphyrins start to accumulate.

The porphyrin pattern in cell culture corresponds with the porphyrin pattern found in the HCB-induced porphyric mammalian liver [2,22,23]. The pattern consists mainly of porphyrins containing 8 and 7 carboxylic groups (Fig. 1a). After addition of the porphyrin precursor ALA (25 µg/ml) to the culture medium the porphyrins accumulating in the liver cells consisted mainly of proto- and coproporphyrin (Fig. 1b). It has been assumed that porphyrins accumulate as a result of the inability of the enzymes of the heme biosynthesis pathway to cope with an increased flux of intermediates through the pathway [14]. The rate of protoporphyrin formation is probably too fast to convert all the protoporphyrin into heme by incorporation of iron in the protoporphyrin molecule. However, stimulation of the porphyrin synthesis by addition of ALA together with addition of HCB to liver cell cultures pretreated with β-NF, produced a pathological porphyrin pattern with mainly uro- and heptacarboxylic porphyrins (Fig. 1c). This indicates that

Fig. 1a-h. Patterns of porphyrin accumulation by chick embryo liver cells maintained in serum-containing Williams E medium in response to different compounds added to the medium.
Abbreviations and concentrations (µg/ml culture medium) of compounds added to the cell culture medium: β-NF, pretreatment with β-naphthoflavone (3µg/ml); HCB, hexachlorobenzene (10µg/ml); pip.but., piperonyl butoxide (5 or 10 µg/ml); δ-ALA, δ-aminolevulinic acid (25 µg/ml); PCTA, pentachlorothioanisol (10 µg/ml). The accumulation of porphyrins is expressed as a percentage of the total porphyrins formed. Values represent means of four experiments; S.D. within 10-15% of mean values, not indicated. The porphyrins are: 8, uro-; 7, heptacarboxylic; 6, hexacarboxylic; 5, pentacarboxylic; 4, copro-; 3, tricarboxylic; 2, protoporphyrin.


porphyrin
(% of total)
a.
HCB + β - NF
b.
ALA
c.
HCB + β - NF + ALA
d.
ALA + β - NF
e.
HCB + β - NF + ALA + pip. but. (5)
f.
HCB + β - NF + ALA + pip. but. (10)
g.
PCTA
h.
PCTA + β - NF

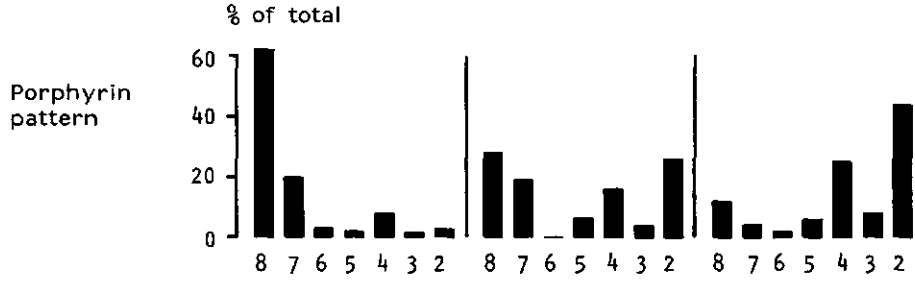
the presence of HCB disturbs the stepwise decarboxylation of uroporphyrinogen to protoporphyrinogen, or in other words inhibits UROG-D. The pathological porphyrin pattern could be altered in a pattern observed after addition of ALA, by simultaneous application of piperonyl butoxide at a concentration of 10 µg/ml medium (Fig. 1f). Piperonyl butoxide obviously prevents the inhibition of UROG-D in liver cell cultures treated with HCB. A porphyrin pattern, intermediate between a pathological and ALA-pattern, appears when a lower concentration of piperonyl butoxide (5 µg/ml medium) was used (Fig. 1e).

Similar results were obtained with 2,4,5,2',4',5'-hexachlorobiphenyl (2,4,5,2',4',5'-HCB), another representative of the group of porphyrinogenic polyhalogenated aromatic compounds (Table II): Increasing concentrations of piperonyl butoxide added together with 2,4,5,2',4',5'-HCB to liver cell cultures caused an inhibition of the porphyrinogenic activity of this polychlorinated biphenyl. The decrease of porphyrin accumulation was accompanied by a lowering of the mixed function oxygenase activity (i.e., ethoxyresorufin O-de-ethylation; ERR-O-D). The porphyrinogenic effect of 2,4,5,

TABLE II

INHIBITION OF PORPHYRINOGENIC ACTIVITY OF 2,4,5,2',4'.5'-HCB (10µg/ml) IN LIVER CELL CULTURE, BY ADDITION OF THE MONOOXYGENASE INHIBITOR PIPERONYL BUTOXIDE

	Piperonyl butoxide concentration (µg/ml medium)			
	0	5	10	25
ERR-O-D induction factor (x control)	6.0	5.7	3.7	1.4
Total porphyrins (pmol/mg protein)	1400-1600	200-500	10-20	10-20



2',4',5'-HCB could already be completely prevented at a concentration of 10 µg/ml piperonyl butoxide. The porphyrin pattern changed with increasing pip.but. concentrations from pathological (with mainly uro- and hepta-carboxylic porphyrins), via an intermediate pattern to a normal porphyrin pattern. The latter could be revealed only by adding exogenous ALA to the culture medium of the hepatocytes (Table II), in order to show to what extent UROG-D decarboxylase was inhibited. These results support the hypothesis, as suggested already by Sinclair and Granick [24], that a metabolite or reactive intermediate of the chlorinated hydrocarbon inhibits UROG-D, thus causing the accumulation of uroporphyrin.

Among several HCB-metabolites tested in the liver cell culture system (see list of chemicals in methods), only pentachlorothioanisol (PCTA) and tetrachlorothioanisol (TCTA) were able to cause an accumulation of porphyrins. Examination of the pattern of porphyrin accumulation revealed co-

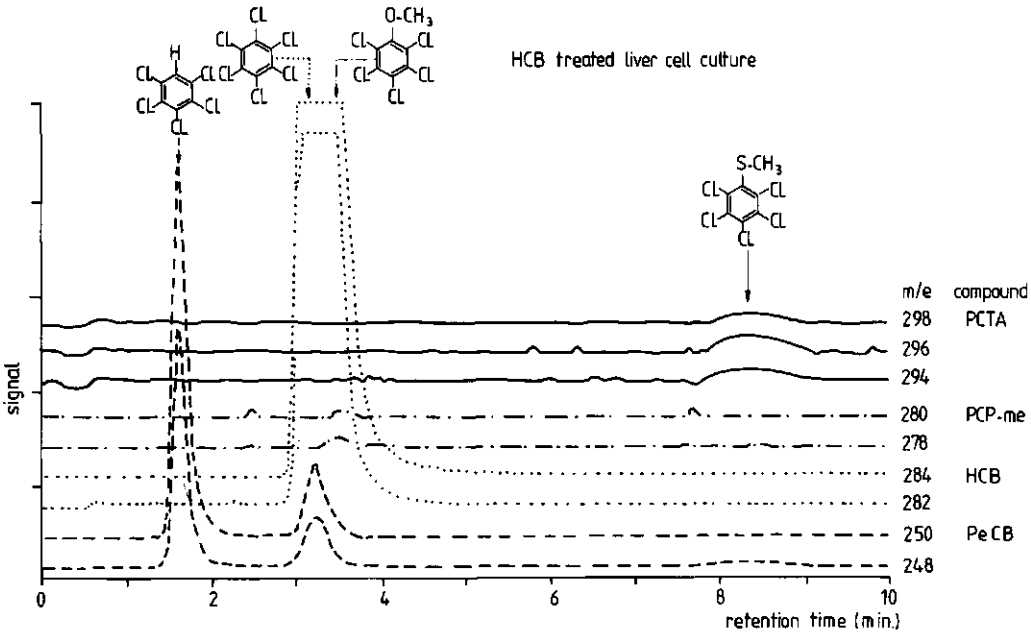


Fig. 2. Mass fragmentogram of purified and methylated ether extract of a chick embryo liver cell culture, which was exposed to HCB (10 µg/ml medium) for 24 h. Selected ions were characteristic for compounds of interest: PeCB: m/e = 248 and 250; HCB: m/e = 282 and 284; PCP-me: m/e = 278 and 280; PCTA: m/e = 294, 296 and 298. GC conditions: A 270 x 0.28 (i.d.) cm glass column packed with 3% QF-1 on Gas Chrom Q 100-120 mesh, carrier gas: helium (30 ml/min), injection port: 210°C, oven: 180 °C.

proporphyrin as the predominant type of porphyrin (Fig. 1g); but when the cultures were pre-induced with β-NF a pathological porphyrin pattern developed after administering PCTA or TCTA to the culture medium (Fig. 1h). Since PCTA does not accumulate in the liver of rats treated with PCTA and is not porphyrinogenic, in rats [13], the results of the present in vitro experiments suggests that PCTA can be porphyrinogenic, but that the in vivo liver concentrations never reach the required level to induce hepatic porphyria.

Identification and quantitation of HCB-metabolites in chick embryo liver cell culture

Fig. 2 shows the gas chromatograms for mass fragments characteristic of some known HCB-metabolites, which were derived from liver cells treated with HCB for 24 h. Besides HCB, PCTA and pentachlorobenzene (PeCB), pentachlorophenol (PCP) could be identified in the sample. The amount of PCP, however, was too small to get a clear mass spectrum. Our experience with liver material of rats is that PCP is bound very strongly to macromolecules and not easily extractable with ether, even after alkaline treatment of the sample (unpublished results). The HCB used in this study contained

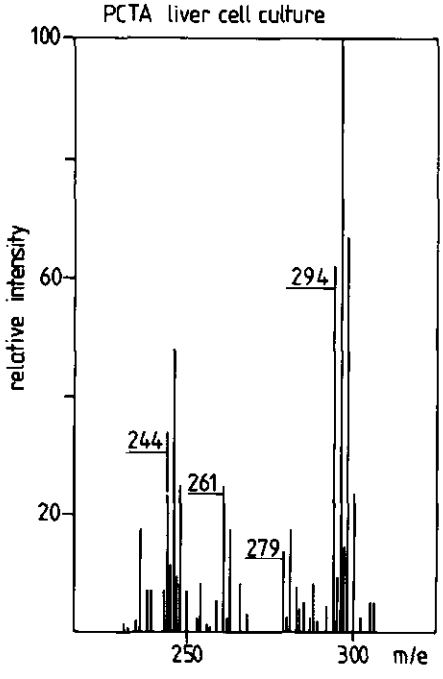


Fig. 3. Partial mass spectrum of pentachlorothioanisol (PCTA) in purified, methylated ether extract of HCB-treated chick embryo liver cells.

some PeCB, but the relative amounts of PeCB to HCB were higher in extracts of HCB treated cells, indicating that the PeCB partly originates from metabolism of HCB. A clear mass spectrum of PCTA (= methylated PCThP) was obtained (Fig. 3), which confirms that HCB is converted into PCThP as such or in the form of a conjugate. Unmethylated ether extracts contained no detectable amounts of HCB metabolites in methylated form, and neither HCB nor HCB metabolites were detected in control cultures. The HCB metabolites identified in chick embryo liver cell culture are the same as found in the excreta of different animal species after administration of HCB [9,25].

Piperonyl butoxide, which binds to the heme part of cytochrome P-450, could reduce the formation of PCThP by 50% (Table III). Although it shows that piperonyl butoxide has an effect on the biodegradation of HCB, the inhibition was not complete at the concentration of piperonyl butoxide chosen in this experiment (10 µg/ml medium). A higher concentration of piperonyl butoxide would have blocked the biotransformation of HCB to a greater extent. However, this reduction in the rate of metabolism of HCB was probably enough to prevent the binding of reactive metabolic intermediates to UROG-D, which supposedly causes the disturbance of the hepatic heme synthesis in HCB porphyria.

TABLE III

EFFECT OF PIPERONYL BUTOXIDE ON THE METABOLIC FORMATION OF "PCTA" IN HCB-TREATED CHICK EMBRYO LIVER CELL CULTURES[a]

Additions	"PCTA" on QF-1 (pmol/6.10^6 cells)	"PCTA" on OV-1 (pmol/6.10^6 cells)
None	not detected	not detected
"recovery"[b]	not detected	not detected
HCB	43	71
HCB + pip.but.	20	35

[a] Amounts of "PCTA" found in methylated ether extracts of alkaline-treated samples of chick embryo liver cell cultures, as detected by GC on a QF-1 and OV-1 column and calculated by means of an external standard.

[b] HCB was added immediately before harvesting the liver cells.

Irreversible binding of ^{14}C-HCB

When a primary culture of chick embryo liver cells was exposed for 24 h to ^{14}C-HCB, some radioactivity became tightly bound to cell protein and was not extractable by organic solvents. The binding was equivalent to 59 pmol/ 6.10^6 cells (Table IV).

TABLE IV

EFFECTS OF PIPERONYL BUTOXIDE AND ASCORBIC ACID ON THE IRREVERSIBLE BINDING OF ^{14}C-HCB TO PROTEIN

tissue culture treatment[a]	radioactivity (d.p.m.)	radioactivity -"control" (d.p.m.)	^{14}C-metabolite bound (pmol/6.10^6 cells
control[b]	39.7	-	-
^{14}C-HCB	76.5	36.8	59
^{14}C-HCB+Asc.acid (0.5 mM)	39.9	-	-
^{14}C-HCB+pip.but. (5 µg/ml)	48.3	8.6	14

a All cell cultures were pretreated with β-naphthoflavone (3 µg/ml medium) for 18 h from the 6th h after isolation.

b ^{14}C-HCB has been added to this "control" cell culture immediately before solvent extraction.

The protein-bound radioactivity could be reduced approximately four times by addition of piperonyl butoxide (5 mg/ml). Addition of the antioxidant ascorbic acid to the culture medium (0.5 mM) did prevent irreversible binding of radioactivity completely (Table IV). Moreover, earlier studies showed already that ascorbic acid could protect chick embryo liver cells against the porphyrinogenic effect of HCB [8].

After combining these results with the finding that no stable metabolite of HCB could induce a HCB-type of porphyria, it seems very plausible that a reactive metabolic intermediate is involved in the binding to UROG-D. The recent [26] discovery of a pentachlorophenyl-mercapturic acid in the urine of HCB-treated rats leaves no doubt, that the PCThP found in HCB-treated chick embryo liver cell cultures partly originates from conjugation with glutathione.

Since the amount of irreversibly bound ^{14}C-metabolites and the amount of PCThP determined in chick embryo liver cell cultures lie in the same order of magnitude (Tables III and IV), it seems that radicals formed by

metabolism of HCB and capable of reacting with catalytic SH-groups of enzymes and glutathione are responsible for the inhibition of UROG-D.

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Chapter 6

EFFECTS OF DIETARY ANTIOXIDANTS ON THE BIOTRANSFORMATION AND PORPHYRINOGENIC ACTION OF HEXACHLOROBENZENE IN TWO STRAINS OF RATS[a]

I. Induction of mixed function oxygenases, hepatic porphyria, glutathione-S-transferase activity, and liver glutathione levels

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SUMMARY

Groups of female Agus or Wistar rats were given hexachlorobenzene (HCB) (50 mg/kg bw) in olive oil by stomach tube every other day. HCB-treated animals were fed either on a basal powdered diet without extra added antioxidants or on the same basal diet supplemented with 0.15% ascorbic acid and 0.12% DL-α-tocopherolacetate. One of the HCB-treated Agus groups and one control group of Agus rats (both on a low-antioxidant diet) received by stomach tube 25 mg/kg bw of 3,5-di-tert-butyl-4-hydroxytoluene (BHT) every other day. Autopsies were made after 1,2,5 and 8 weeks. The Agus rats were highly porphyric after 8 weeks of HCB treatment, whereas the Wistar rats showed no symptoms of hepatic porphyria within that period. HCB, significantly decreased the hepatic glutathione concentration in both the Wistar strain and the Agus group fed on a low-antioxidant diet. Agus rats were found to possess lower levels of hepatic glutathione than Wistar rats.

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[a] Presented at The Seventh European Workshop on Drug Metabolism, Zurich/Switzerland, October 5-10, 1980.

Moreover, the induction of ethoxyresorufin O-de-ethylase activity was 1.5 to 1.7-fold higher in the HCB-treated Agus rats than in the corresponding Wistar rats; whereas Agus rats showed less induction of glutathione-S-transferease activity. The difference between Agus and Wistar rats in their susceptibility to the porphyrinogenic effect of HCB is discussed in the light of the above findings. In contrast to earlier observations in vitro, dietary antioxidants could not protect against the porphyrinogenic action of HCB in vivo. The dietary antioxidants even stimulated the onset of porphyria in HCB-fed Agus rats.

INTRODUCTION

An inbred strain of rats (Agus) proved to be particularly susceptible to the porphyrinogenic effect of hexachlorobenzene (HCB). These Agus rats were found to possess higher levels of total non-heme iron in their livers than randomly bred Wistar derived Porton rats [1]. Since it has been reported that iron overload potentiates the porphyrinogenic effect of HCB [2,3], Smith et al. [1] suggested that the higher liver iron content of the Agus rats might be responsible for their increased susceptibility to HCB. Although the exact mechanism is not known, it has been observed also that iron influences the induction of hepatic mixed function oxygenases [4]. It was of interest therefore to examine to what extent a difference in the pattern of induction of hepatic microsomal enzymes underlies this difference between Porton-Wistar and Agus rats for HCB porphyria; the more so as recent studies showed biotransformation of HCB to be a prerequisite for its porphyrinogenic effect [5,6].

According to one of the current hypotheses on the mechanism of HCB porphyria [7] the disturbance of hepatic porphyrin metabolism is attributed to the inhibition of uroporphyrinogen decarboxylase (UROG-D) by reactive metabolic intermediates of HCB. The isolation of more than a dozen sulfur-containing metabolites of HCB from the urine of rats treated with HCB [8,9] suggests that a reactive intermediate of HCB may react with SH-groups of proteins and peptides, like UROG-D and glutathione. However, contradictory results about hepatic glutathione levels in HCB porphyria have been reported by several workers: Goerz et al. [10] found an abrupt drop of the hepatic glutathione level in male rats by the time porphyrins started to accumulate in the urine. In contrast, several other reports make no mention of altered liver glutathione concentrations in HCB porphyria in rats

[11-13]. Furthermore, very recently Koss et al. [14] found in HCB-treated female rats a decreased activity of hepatic glutathione-S-transferase, which catalyzes the conjugation of electrophilic metabolic products with glutathione yielding less reactive glutathione conjugates.

In view of these findings and the inconsistencies therein , this study was undertaken to further evaluate the role of glutathione and glutathione-S-transferase in HCB-induced hepatic porphyria. We compared the effects of long-term HCB treatment on female rats of the Agus and Wistar strains, with special reference to induction of mixed function oxygenases, glutathione-S-transferase activity, and hepatic glutathione content. The present paper shows that in addition to iron, some other factors can be responsible for the greater susceptibility of Agus rats to the porphyrinogenic effect of HCB.

Our previous studies with primary cultures of chick embryo liver cells demonstrated that the addition of antioxidants (e.g., ascorbic acid, DL-α-tocopherol or N,N'-diphenyl-p-phenylendiamine) completely prevents the HCB-induced accumulation of porphyrins in vitro [5]. In addition, ascorbic acid could reduce the irreversible binding of radioactivity to protein of primary liver cell cultures treated with ^{14}C-HCB to control values [15]. To investigate whether the protective effect of antioxidants applies also to the situation in vivo, the present experiments on the porphyrinogenic effects of HCB in Agus and Wistar rats were performed on animals fed either on a low-antioxidant diet or on a diet rich in antioxidants.

METHODS

Animals and treatments

Thirty two female (S.P.F.) Wistar rats (12 weeks old; 150-170 g), obtained from the Central Institute for the Breeding of Laboratory Animals TNO (Zeist, The Netherlands), were randomly distributed in 2 groups of 16. Both groups received 178 $\mu mol.kg^{-1}$ (50 mg/kg bw) of HCB in olive oil DAB 8 (1% solution) by stomach tube every other day. One group was fed on a basal powdered low-antioxidant diet [-A] (without extra added antioxidants, Table I) and the second group was kept on the same basal diet supplemented with the antioxidants ascorbic acid (0.15%) and DL-α-tocopherolacetate (0.12%) [+A] (diets mixed by Hope Farms, Woerden, The Netherlands).

TABLE I

COMPOSITION OF BASAL LOW-ANTIOXIDANT (-A) DIET

Ingredient	%		%
Wheat	40.0	Mineral premix	1.0
Corn	33.5	DL-methionine	0.15
Casein	16.0	Choline	0.2
α-Cellulose	2.0	Magnesium sulphate	0.2
Sunflower oil, without ethoxyquin and BHT	2.0	Calcium phosphate	1.5
Brewer's yeast	1.0	Calcium carbonate	0.8
Vitamin premix, without ascorbic acid and DL-α-tocopherolacetate	1.0	Sodium chloride	0.4
		Potassium chloride	0.3

Three groups of 16 female Agus [16] (inbred; 12 weeks old, 150-170 g) rats, derived from a breeding nucleus from the MRC Laboratory Animals Centre (Carshalton, Surrey, U.K.) and bred at the Laboratory Animals Centre (C.K.P.) in Wageningen (The Netherlands), were treated with HCB as described above for the Wistar rats. Two of these HCB-treated Agus groups were fed on a low-antioxidant diet (Table I) and the third group received the diet rich in antioxidants. One of the HCB-treated groups and one control group of Agus rats (both on a low-antioxidant diet) received by stomach tube 25 mg/kg bw of the food antioxidant 3,5-di-tert-butyl-4-hydroxytoluene) (BHT) in olive oil DAB 8 (1% solution) every other day. Olive oil without HCB was administered to control groups. The animals were acclimatized for 2 weeks before administering HCB; during that period they were fed already on their respective low-antioxidant or antioxidant-rich diets.

Animals were housed in groups of 4 in stainless steel cages with wire-netting bottoms to avoid the intake of excrements. Consumption of feed, body weights, and clinical symptoms were recorded several times a week.

After 1,2,5 and 8 weeks, subgroups of 4 rats from each treatment group were killed by decapitation. The livers were rapidly taken out, weighed and divided for preparation of microsomes, for determination of HCB and its metabolites, for porphyrin analyses, and for measuring glutathione content.

Chemicals

HCB (organic analytical standard) was purchased from B.D.H. Chemicals Co. Ltd. (Poole, Dorset, U.K.); BHT (98%) and 1,2-dichloro-4-nitrobenzene (DCNB) from Merck (Darmstadt, F.R.G.); 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNN) from Eastman-Kodak (U.S.A.) and ethylmorphine-HCl from Brocacef B.V. (Maarssen, The Netherlands). 7-Ethoxyresorufin and resorufin were obtained from Pierce Eurochemie B.V. (Rotterdam, The Netherlands). All compounds used were of analytical grade quality.

Analytical procedures

Liver microsomes were prepared as previously described [17]. The microsomal cytochrome P-450 concentration was calculated from the sodium dithionite difference spectrum of CO-saturated samples, using an extinction coefficient of 91 $mM.^{-1}cm^{-1}$ between A_{max} and $A_{490\ nm.}$ [18]. Ethoxyresorufin O-de-ethylase activity [19] and the concentration of reduced glutathione (GSH) in the liver [20] were measured with a fluorometric assay. Ethylmorphine N-demethylase was assayed spectrophotometrically according to Van den Berg et al. [21]. The activity of GSH-S-transferases in liver cytosol was measured on the basis of the increase in conjugate concentration with GSH (2 mM) as co-substrate and 1,2-dichloro-4-nitrobenzene [DCNB] (1 mM) and 1,2-epoxy-3-(p-nitrophenoxy)propane [EPNN] (0.5 mM) as substrates [22]. Conjugate formation at 37°C in the incubation mixture containing 20 µl (DCNB) or 50 µl cytosol (EPNN) was recorded spectrophotometrically at 345 nm (DCNB) or 360 nm (EPNN). Microsomal protein concentration was determined by the method of Lowry et al. [23] with serum albumin as a standard.

Total urinary porphyrins were determined spectrofluorometrically [24], using an equimolar solution of 8,7,6,5,4 and 2 carboxylic porphyrins as a standard (Porphyrin Products, Logan, U.S.A.). Freshly passed urine (0.2 ml) was diluted with 4.8 ml of a solution of chloranil [2,3,5,6-tetrachloro-1,4-benzoquinone] (25 mg/l) in 0.6 N hydrochloric acid/acetic acid/ethanol (2 : 1 : 1, v/v/v). Excitation spectra (λ_{ex}: 350-500 nm; λ_{em}: 652 nm) of diluted urine samples were recorded on a Aminco Bowman fluorometer equipped with a 150-watt xenon lamp and a red sensitive photomultiplier tube type Super S-20 R446S; the peak maximum at 403-405 nm was compared to the standard. Total porphyrins in the liver were measured after extracting liver homogenate (0.5 ml) with 1 N perchloric acid/ethanol (1 : 1, v/v, 10 ml) and subsequently recording the fluorescence spectrum

of the supernatant from centrifuged samples, as described above for urine. The pattern of porphyrins accumulated in the urine was analysed as described by Doss [25], with some modifications made by Strik and Harmsen [26]. Pooled urine samples from 4 animals/group, containing 1 nmol total porphyrin/animal, were used for analysis. The pattern of liver porphyrins was determined according to Seubert and Seubert [27].

RESULTS

Body weight and feed consumption

The HCB-treated Agus groups showed a higher gain in body weight than

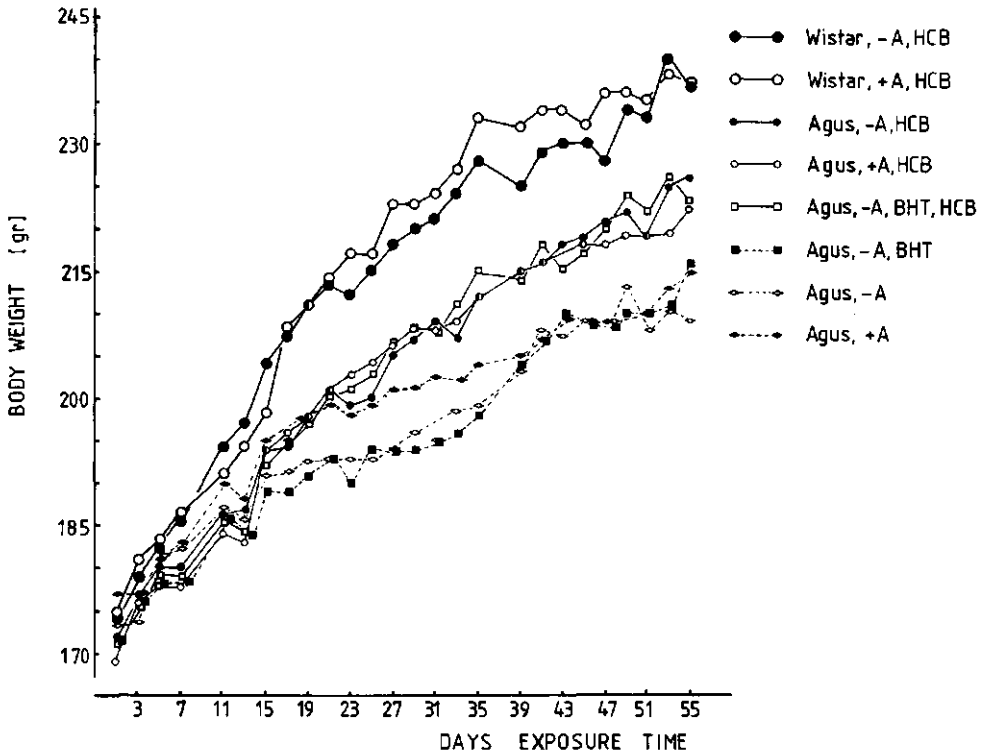

Fig. 1. Gain in body weight of female Agus and Wistar rats treated with hexachlorobenzene (HCB) and fed either a basal low-antioxidant diet (-A) or a diet (+A) supplemented with the antioxidants ascorbic acid (0.15%) and DL-α-tocopherolacetate (0.12%). Points represent means of at least 4 animals. S.D. within 10% of the mean values, not indicated. Abbreviations: HCB, hexachlorobenzene 50 mg/kg orally every other day; BHT, 3,5-di-tert-butyl-4-hydroxytoluene 25 mg/kg orally every other day; -A, low-antioxidant diet; +A, antioxidant-rich diet.

the control Agus groups during the second part of the experimental period. The gain in body weight in both Wistar groups treated with HCB was significantly higher than in the HCB-treated Agus rats throughout the experiment (Fig. 1). There were no significant differences in body weight gain between rats fed on a low-antioxidant diet (-A) and rats fed a diet rich in antioxidants (+A). Feed consumption during the experiment was not significantly different for all groups.

Clinical observations

In week 4 of the experiment, all Wistar rats treated with HCB developed skin lesions. In contrast, Agus rats developed no skin lesions even after 8 weeks treatment with HCB. Skin lesions were characterized by small depilated sores with hemorrhagic crusts located near the shoulders or under the chin. Development of skin lesions coincided with excessive scratching of the animals in the neck region. After 3 weeks the fur of all HCB-treated animals became shaggy, was neglected and showed loss of hair. The HCB-treated rats (Agus and Wistar) exhibited weak tremors after the 5th week. In the 8th week one Wistar rat of the -A, HCB group and one Agus rat treated with HCB+BHT suddenly lost 20-30 g of weight within 2 days, and came in a poor condition. Consequently, they had to be killed before the end of the experiment.

Liver weight

Liver/body weight ratios significantly increased in HCB-treated Agus rats (-A diet) after 2 weeks. In the Agus groups treated with HCB (+A diet) and HCB+BHT, and both Wistar groups a significant increase in liver weight to body weight ratio was observed after 5 weeks of treatment. At 8 weeks, the liver/body weight ratios of HCB-treated Agus and Wistar rats receiving a +A diet or BHT were significantly higher than in the corresponding treatment groups fed a -A diet ($P<0.05$; Table II).

Effects on liver mixed function oxygenases and glutathione-S-transferase

HCB treatment significantly increased liver microsomal cytochrome P-450 concentration in Agus and Wistar rats at all time points studied. The maximum concentrations of cytochrome P-450 in Agus rats receiving a -A diet, +A diet or -A+BHT in combination with HCB treatment amounted to 157%, 147% and 173% of control values, respectively (Table III). No significant differences in cytochrome P-450 concentration were found between HCB-treated Wistar and Agus rats at all time intervals studied.

TABLE II

RELATIVE LIVER WEIGHTS OF WISTAR AND AGUS RATS TREATED WITH HCB

Relative liver weights of rats exposed to HCB for 1,2,5 and 8 weeks and fed diets with different antioxidant concentrations. Relative liver weights in % of total body weight; values are means ± S.D. of 4 or 3* determinations.

Strain, diet, treatment[a]	exposure time (weeks)			
	1	2	5	8
Wistar, -A, HCB	3.84 ± 0.16	3.96 ± 0.20	4.07 ± 0.12	4.87 ± 0.54*[c]
Wistar, +A, HCB	4.07 ± 0.20	4.24 ± 0.31	4.68 ± 0.07[c,d]	5.94 ± 0.41[c,d]
Agus, -A, HCB	3.71 ± 0.16	3.74 ± 0.21[b]	4.30 ± 0.48[b]	5.00 ± 0.14[b]
Agus, +A, HCB	4.00 ± 0.10	3.85 ± 0.21	4.69 ± 0.09[b]	5.66 ± 0.36[b,d]
Agus, -A, HCB, BHT	3.83 ± 0.13	3.81 ± 0.22	4.45 ± 0.26[b]	5.49 ± 0.29*[b,d]
Agus, -A, control	3.60 ± 0.16	3.30 ± 0.12	3.54 ± 0.09	3.72 ± 0.32
Agus, +A, control	3.72 ± 0.23	3.47 ± 0.24	3.58 ± 0.19	3.88 ± 0.24
Agus, -A, BHT	3.63 ± 0.13	3.56 ± 0.17	3.53 ± 0.20	3.74 ± 0.26

[a] HCB, hexachlorobenzene; -A, low-antioxidant diet; +A, basal diet + antioxidants; BHT, 2,6-di-tert.-butyl-4-hydroxytoluene

[b] Significantly different from control group (Student's t-test, $P<0.05$)

[c] Significantly different from one week values (Student's t-test, $P<0.05$)

[d] Significantly different from corresponding HCB group on low-antioxidant diet ($P<0.05$)

TABLE III

MICROSOMAL ENZYME INDUCTION (CYTOCHROME P-450 AND ERR-O-D) IN LIVER FROM WISTAR AND AGUS RATS TREATED WITH HCB

Microsomal enzyme activities (cytochrome P-450 and ethoxyresorufin in O-de-ethylase (ERR-O-D)) in liver from rats exposed to HCB for 1,2,5 and 8 weeks and fed diets with different antioxidant concentrations. ERR-O-D is expressed in nmol resorufin (RR) formed/min/mg microsomal protein. Cytochrome P-450 is expressed in nmol/mg microsomal protein; values are means ± S.D. of 4 or 3* rats.

Strain, diet, treatment[a]		cytochrome P-450 (nmol/mg) exposure time (weeks)				ERR-O-D (nmol RR/min/mg) exposure time (weeks)			
		1	2	5	8	1	2	5	8
Wistar, -A, HCB	Mean	0.91	0.95	0.93	0.80*	0.333	0.403	0.401	0.250*
	S.D.	0.10	0.11	0.05	0.15	0.079	0.049	0.028	0.044
Wistar, +A, HCB	Mean	0.96	1.26	1.01	0.78	0.408	0.579	0.404	0.280
	S.D.	0.04	0.14	0.02	0.07	0.051	0.048	0.062	0.064
Agus, -A, HCB	Mean	0.80[c]	0.97[c]	1.02[c]	0.71[c]	0.348	0.548[b]	0.697[b]	0.370[b]
	S.D.	0.06	0.04	0.07	0.03	0.065	0.033	0.083	0.063
Agus, +A, HCB	Mean	0.97[c]	1.03[c]	0.96[c]	0.81[c]	0.435	0.550	0.656[b]	0.407[b]
	S.D.	0.06	0.16	0.03	0.04	0.120	0.143	0.063	0.021
Agus, -A, HCB, BHT	Mean	0.87[c]	0.95[c]	0.91[c]	0.69*[c]	0.336	0.488	0.672	0.381*
	S.D.	0.05	0.09	0.05	0.04	0.081	0.032	0.150	0.104
Agus, -A, control	Mean	0.64	0.70	0.65	0.58	0.008	0.007	0.009	0.005
	S.D.	0.07	0.01	0.02	0.02	0.002	0.002	0.001	0.001
Agus, +A, control	Mean	0.70	0.70	0.83	0.64	0.007	0.008	0.009	0.005
	S.D.	0.02	0.05	0.08	0.05	0.001	0.001	0.001	0.001
Agus, -A, BHT	Mean	0.66	0.55	0.66	0.51	0.006	0.008	0.009	0.006
	S.D.	0.06	0.02	0.08	0.05	0.002	0.001	0.003	0.001

[a] HCB, hexachlorobenzene; -A, low-antioxidant diet; +A, basal diet + antioxidants; BHT, 2,6-di-tert.-butyl-4-hydroxytoluene

[b] Significantly different from corresponding Wistar HCB group (Student's t-test, $P<0.05$)

[c] Significantly different from control group (Student's t-test, $P<0.05$)

At 5 weeks, ethoxyresorufin O-de-ethylase was induced maximally in HCB-treated Agus rats (Table III). The different dietary conditions dit not influence the maximum activity of ethoxyresorufin O-de-ethylase in the HCB-treated Agus rats (induced 75-fold; Table III). At 5 and 8 weeks the activities of ethoxyresorufin O-de-ethylase in HCB-treated Agus groups were about 50-70% higher than in corresponding Wistar groups (Table III). HCB dit not significantly increase ethylmorphine N-demethylase activity in either strain of rats (results not shown).

The activity of glutathione-S-transferase towards the glutathione-binding substrates DCNB and EPNN was increased in HCB-treated rats of both strains (Fig. 2). The model substrate DCNB is conjugated with glutathione by displacement of a chlorine atom, whereas EPNN is transformed to a glu-

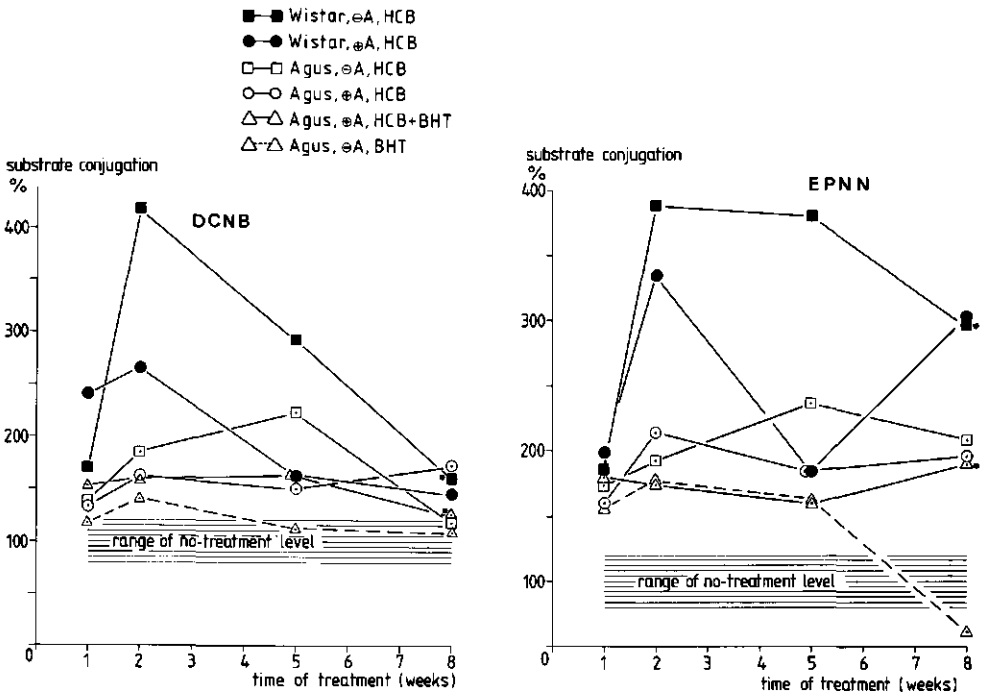

Fig. 2. Effects of dietary antioxidants on glutathione-S-transferase activity in liver cytosol of female rats of the Agus and Wistar strains, dosed orally with 50 mg/kg HCB every other day for 8 weeks. Conjugation of 1,2-dichloro-4-nitrobenzene (DCNB) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNN) with glutathione is expressed as the % of the mean activity in the corresponding Agus control group. Points represent means of 3 or 4 animals. Two-way analysis of variance (time vs. strain) showed significant overall differences between HCB-treated Agus and Wistar groups (F-test, $P<0.01$).

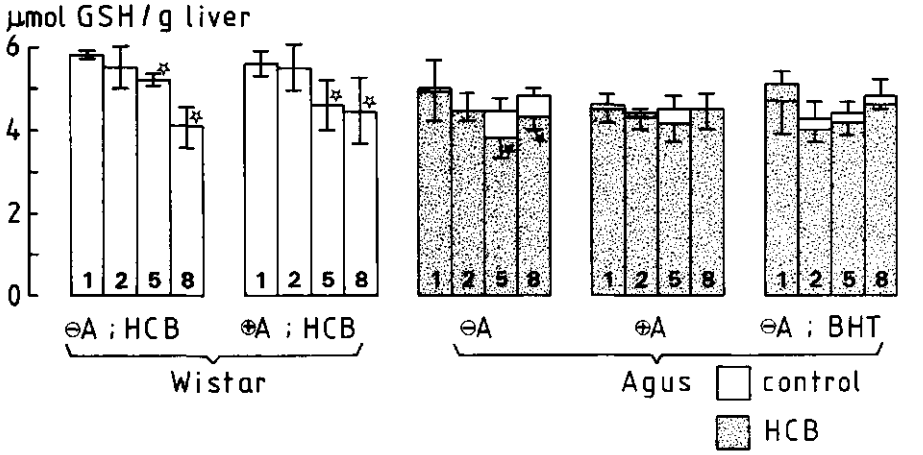

Fig. 3. Liver glutathione (GSH) content of female Agus and Wistar rats treated with hexachlorobenzene (HCB) and fed either a low-antioxidant diet (-A) or a diet rich in antioxidants (+A). Weeks exposure time indicated at the base of bars. Bars represent means ± S.D. of 4 rats. Closed asterisk denotes significant difference from control group: one-sided Student's t-test, $P<0.05$. Open asterisk denotes significant difference from 1 week values, $P<0.05$. (Consult legend Fig. 1 for more details.)

tathione conjugate by cleavage of the epoxide bond. Testing for overall significance of differences between Agus and Wistar groups by analysis of variance showed that HCB-treated Agus rats had less induced glutathione-S-transferase activities than correspondingly treated Wistar rats. Agus rats administered BHT only were found to possess a moderately increased activity of glutathione-S-transferase towards the substrates DCNB (at 2 weeks) and EPNN (at 1,2 and 5 weeks) (Fig. 2).

Liver glutathione content

The glutathione contents of the livers of HCB-treated Wistar rats killed after 5 and 8 weeks were significantly lower than the values found after 1 week of treatment. After 8 weeks, the concentration of glutathione was decreased in HCB-treated Wistar rats fed either on a -A diet or on a +A diet to 72% and 79% of the one week values, respectively (Fig. 3). In contrast, the glutathione concentration in the livers of the HCB-treated Agus rats significantly decreased only in the group administered the low-antioxidant diet. Moreover, the glutathione contents of the livers of Agus rats were generally about 20% lower than those of the Wistar rats after 1 or 2 weeks of treatment.

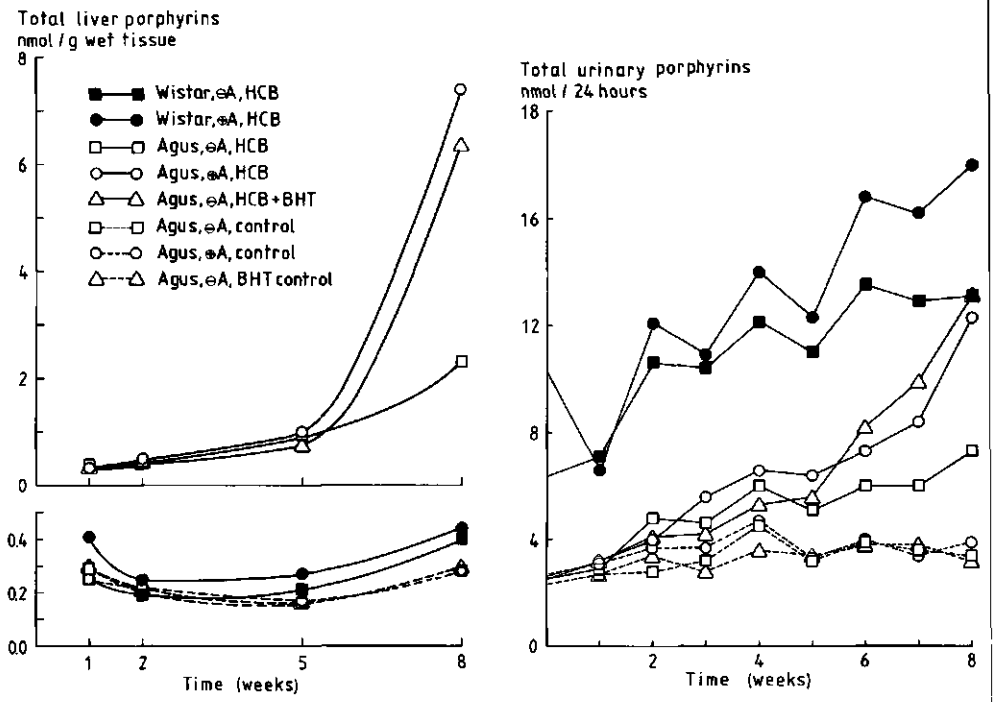

Fig. 4. Effects of dietary antioxidants on the total urinary porphyrin excretion and the total porphyrin concentration in the livers of female rats of the Agus and Wistar strains after oral dosing of hexachlorobenzene (HCB) every other day for 8 weeks. Points represent means of 4 animals. (Consult legend Fig. 1 for more details).

Porphyrins in urine and liver

Figure 3 shows that after 5 weeks the porphyrin concentration in the liver and urine of HCB-treated Agus rats began to increase rapidly, indicating the onset of porphyria (Fig. 4). At the same time the pattern of liver and urinary porphyrins changed, resulting in an increase in the amount of porphyrins with 8 and 7 carboxylic groups relative to those with 4 and 2 carboxylic groups (Table IV). After 8 weeks uroporphyrin (8 carboxylic groups) was the predominant type of porphyrin (35-45% of total) in the urine of HCB-treated Agus rats. Those changes were more pronounced in the Agus rats receiving a +A diet or BHT than in the Agus rats kept on a low-antioxidant diet (Figs. 3,4; Table IV).

Even after 8 weeks, no symptoms of hepatic porphyria could be detected in HCB-treated Wistar rats. The 2-fold increase in the urinary excretion of porphyrins in the Wistar rats shown at week 8 represented predominantly coproporphyrin (85% of total; Table IV), not uroporphyrin as is typical of the inhibition of uroporphyrinogen decarboxylase in HCB porphyria.

TABLE IV

RELATIVE DISTRIBUTION OF THE MAIN PORPHYRINS IN THE LIVER AND URINE OF WISTAR AND AGUS RATS AFTER 5 AND 8 WEEKS OF HCB TREATMENT[a]

Strain, diet, treatment	time	Relative distribution of porphyrins (% of total porphyrins)							
		URINE				LIVER			
		uro	hepta	copro	proto	uro	hepta	copro	proto
Wistar, -A, HCB	5 weeks	8.5	6.9	64.4	7.6	48.7	11.8	21.1	14.6
Wistar, +A, HCB		6.6	3.3	65.2	13.3	47.5	10.7	8.5	27.8
Agus, -A, HCB		14.6	8.6	47.6	16.5	61.5	19.6	13.2	4.3
Agus, +A, HCB		14.9	7.7	45.3	19.5	79.4	7.6	11.4	- [b]
Agus, -A, HCB, BHT		10.8	6.1	41.2	25.9	70.8	17.1	10.8	-
Agus, -A, control		10.2	7.8	45.3	19.1	40.0	14.8	21.0	9.4
Agus, +A, control		10.4	8.4	41.2	24.9	47.8	14.8	8.2	14.4
Agus, -A, BHT		16.6	7.3	47.8	16.5	51.9	10.0	11.1	13.5
Wistar, -A, HCB	8 weeks	8.7	2.8	87.6	-	44.2	10.2	18.8	19.5
Wistar, +A, HCB		10.6	-	85.7	1.1	53.0	12.4	13.2	19.4
Agus, -A, HCB		26.9	10.4	47.5	6.7	68.4	20.1	3.6	4.5
Agus, +A, HCB		34.9	10.6	31.4	6.3	77.8	17.4	2.3	1.2
Agus, -A, HCB, BHT		45.3	12.8	33.1	1.7	77.1	20.8	1.0	1.1
Agus, -A, control		9.0	13.3	41.3	15.8	40.4	8.7	15.8	20.5
Agus, +A, control		11.3	10.3	29.8	29.6	51.5	14.1	12.7	9.0
Agus, -A, BHT		11.9	14.0	47.4	13.7	28.8	20.7	23.8	22.7

[a] HCB, hexachlorobenzene; -A, low-antioxidant diet; +A, basal diet + antioxidants; BHT, 2,6-di-tert.-butyl-4-hydroxytoluene; uro, uroporphyrin; hepta, heptacarboxylic porphyrin; copro, coproporphyrin; proto, protoporphyrin

[b] Not detected

DISCUSSION

Female rats proved to be more susceptible to the porphyrinogenic effect of HCB than female Wistar rats. After 8 weeks of treatment the Agus rats showed a urinary porphyrin pattern with uroporphyrin as the predominant intermediate, whereas in the Wistar rats mainly coproporphyrin was excreted. This corresponds with earlier observations of Smith et al. [1], who reported that Agus rats developed hepatic porphyria at a much faster rate than animals of the Porton strain when treated with HCB. This difference in susceptibility is probably not only restricted to Agus and Porton strains, but exists also between Agus and Wistar rats.

We have shown that during HCB treatment the hepatic glutathione concentration significantly decreased in the Wistar strain and in Agus rats fed a low-antioxidant diet. On the other hand, no significant changes were found in the remaining two HCB-treated Agus groups, which were the most porphyric groups at the end of the 8-week period of HCB treatment. The above findings seem to contrast with the hypothesis that glutathione plays an important role in the inactivation of reactive metabolic intermediates involved in causing HCB porphyria [7]. However, recently Smith and coworkers [28] were able to show by quantitative cytochemistry that the glutathione concentration in the centrilobular hepatocytes is approximately 50% lower than in the periportal hepatocytes. Moreover, the centrilobular liver cells are more active in the biotransformation of xenobiotics [29,30] and contain more cytochrome P-450 [31] than the periportal liver cells. The higher rate of xenobiotic metabolism in the centrilobular cells leads to the formation of more reactive intermediates, which are not adequately detoxified by glutathione. Consequently, reactive metabolic intermediates of HCB will attack nucleophilic groups of enzymes including uroporphyrinogen decarboxylase, the key enzyme found to be inhibited in HCB porphyria. The above arguments would also explain why in the first stage of HCB porphyria the accumulation of porphyrins is restricted to the centrilobular regions of the liver [32,33]. Assuming further that a lowering of the glutathione content first takes place centrilobularly, a little decrease of the glutathione concentration in the affected centrilobular liver cells might have been underestimated, or even completely overlooked in studies where the decrease was so small that it lost in the standard deviation [11-13].

In our study a moderate decrease (10-15%) of the glutathione concentration in the total liver (as found in the HCB-treated Agus rats on a -A diet) means a 30-45% decrease in the centrilobular part.

In contrast to earlier reports [14], no inhibition of the activity of glutathione-S-epoxide transferase in HCB-treated rats was observed in the present study. However, the Agus rats which appeared to be more susceptible to the porphyrinogenic effect of HCB had less induced activities of glutathione-S-transferases than animals of the Wistar strain after treatment with HCB. This may be significant in terms of having less protection against electrophilic metabolites formed by biotransformation of HCB. The different dosage levels of HCB used in our experiments as compared to those of Koss et al. [14] may explain why our results did not confirm the previously reported depressed activity of glutathione-S-epoxide transferase in HCB porphyria. This clearly requires further investigation.

The induction of ethoxyresorufin O-de-ethylase was not equal in both strains of rats. The maximum activities of ethoxyresorufin O-de-ethylase were 1.5 to 1.7-fold higher in the HCB-treated Agus rats than in the HCB-treated Wistar rats. Hence, more reactive metabolic products may be formed in the Agus rats via hepatic monooxygenase activity, leading to an earlier inhibition of uroporphyrinogen decarboxylase, and therefore to porphyria.

In conclusion, based on these apparent differences between both strains of rats, it appears that in addition to the higher non-heme iron contents in the livers of the Agus rats [1] some other factors may contribute to their increased susceptibility to the porphyrinogenic effect of HCB: (1) a lower level of hepatic glutathione; (2) less induction of glutathione-S-transferase; (3) a higher mixed function oxygenase activity.

In contrast with our earlier observations in primary liver cell cultures [5], the results of the present experiments indicate that dietary antioxidants are not able to protect against the porphyrinogenic effects of HCB in vivo; it would even appear that the reverse is true. Two possible explanations can be offered. Firstly, the antioxidants (ascorbic acid and DL-α-tocopherol) in vivo never reach the concentration attainable in primary liver cell culture (0.5 mM in culture medium [5]). Secondly, liver weights of HCB-treated animals receiving a diet supplemented with antioxidants were higher than liver weights of those fed on a low-antioxidant diet; cytochrome P-450 content and ethoxyresorufin O-de-ethylase activity tended to be higher in the HCB-treated groups kept on a diet with extra antioxidants. This suggests that in vivo dietary antioxidants did enhance the biotrans-

formation capacity of the liver, resulting in a higher rate of formation of reactive metabolic products and in consequence an earlier onset of porphyrin accumulation.

BHT stimulated the porphyrinogenic action of HCB in Agus rats. Although BHT at the dosage applied here produced neither enlargement of the liver nor induction of mixed function oxygenases, it may have altered the functional state of cytochromes P-450 towards the phenobarbital-inducible forms of P-450 [34]. Since we demonstrated already that phenobarbital induction enhanced the porphyrinogenic effect of HCB in rats [35], this might explain the stimulating effect of BHT on HCB porphyria.

Finally, recent information indicates the existence of a close correlation between the responsiveness of mice to the induction of aryl hydrocarbon hydroxylase (AHH) and their susceptibility to the porphyrinogenic effects of halogenated aromatic compounds [36]. It should therefore be further investigated whether Agus rats possess a higher AHH responsiveness than Wistar rats, the more so as in the present study the highest activities of the cytochrome P-448 dependent O-de-ethylation of ethoxyresorufin were found in the HCB-treated Agus rats.

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EFFECTS OF DIETARY ANTIOXIDANTS ON THE BIOTRANSFORMATION AND PORPHYRINOGENIC ACTION OF HEXACHLOROBENZENE IN TWO STRAINS OF RATS

II. Formation and excretion of metabolites

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SUMMARY

Groups of female rats of the Agus and Wistar strains were dosed orally with hexachlorobenzene (HCB) (178 $\mu mol.kg^{-1}$) every other day for 8 weeks. Animals were fed either on a basal low-antioxidant diet or on a diet supplemented with ascorbic acid (0.15%) and DL-α-tocopherolacetate (0.12%). One group of Agus rats additionally received every other day a dosage of 25 $mg.kg^{-1}$ 3,5-di-tert.-butyl-4-hydroxytoluene (BHT). The excretion of HCB metabolites in urine and feces was determined after 1,2,5 and 8 weeks. Twenty % of the compounds excreted by the Agus rats after 8 weeks consisted of unchanged HCB, whereas 80% was excreted as metabolites. For the Wistar rats these percentages amounted to 30% and 70%, respectively. The ratio of the excreted amounts of phenolic metabolites to the sulfur-containing metabolites increased from about 0.05, after the first week, to 0.3 after the 8th week. There was no difference in the accumulation of HCB in the liver between the two strains. However, Agus rats were found to possess significantly higher concentrations of HCB metabolites in

their livers than the Wistar rats; in addition, the capacity for methylating the thiophenolic metabolites in vivo appeared to be greater in the former strain. Neither dietary antioxidants not BHT had an effect on the contents of HCB or its metabolites in the liver. On the other hand, Wistar rats fed on a low-antioxidant diet excreted 2-3 times less metabolites than Wistar rats kept on a diet rich in antioxidants. It is suggested that differences in the biotransformation of HCB between Wistar and Agus rats partly explain the increased susceptibility of the Agus strain to the porphyrinogenic action of hexachlorobenzene.

INTRODUCTION

Hexachlorobenzene (HCB) produces a disturbance of the hepatic heme biosynthesis when administered to mammals, birds and man [1]. This biochemical disorder, referred to as hepatic porphyria, proved to be attributable to a decrease in the activity of the enzyme uroporphyrinogen decarboxylase (UROG-D), which is involved in the biosynthetic pathway of heme formation [2,3]. The enzyme defect is reflected in a specific pattern of porphyrin overproduction. Porphyrins that accumulate in liver and urine mainly consist of uro- and heptacarboxylic porphyrins [4].

The porphyrinogenic action of HCB is restricted to certain species and strains of animals [5]. Recently Smith et al. [6] reported that female rats of the Agus strain are particularly sensitive to the porphyrinogenic effect of HCB. In female Agus rats the inhibition of UROG-D started earlier and proceeded at a faster rate than in female rats of the Porton-Wistar strain when treated with HCB. Since previous studies [7,8] showed biotransformation of HCB to be a prerequisite for its porphyrinogenic action, a difference in the way of biotransformation or pharmacokinetic behaviour of HCB may underly the difference in susceptibility to HCB between these two strains of rats. To investigate this, we compared the biotransformation of HCB in Agus and Wistar rats fed on diets with different antioxidant concentrations. We examined the accumulation of HCB and its main metabolites in the liver, the pattern of excretion of metabolites, and the rate of biotransformation of HCB.

METHODS

Female rats of the Agus and Wistar strains, weighing 150-170 g, were randomly divided in groups of 16. Animals were treated with oral doses of HCB (178 µmol.kg^{-1}) in olive oil and fed on either a basal low-antioxidant diet [-A] or a basal diet supplemented with the antioxidants ascorbic acid (0.15%) and DL-α-tocopherolacetate (0.12%) [+A], as detailed in the preceding paper [9]. Twenty four h collections of urine and feces were made weekly from 4 animals per treatment group in metabolism cages. Rats were put individually in metabolism cages on a day when they received no HCB, but were allowed access to feed and water. Total volume of urine and total weight of feces excreted during 24 h were measured. Samples of urine and feces were kept frozen (-18°C) until analysis.

Determination of HCB and its metabolites were performed by means of gas chromatography as described elsewhere [10-12].

Significant differences between Agus and Wistar groups were assessed by Student's t-test at $P<0.05$.

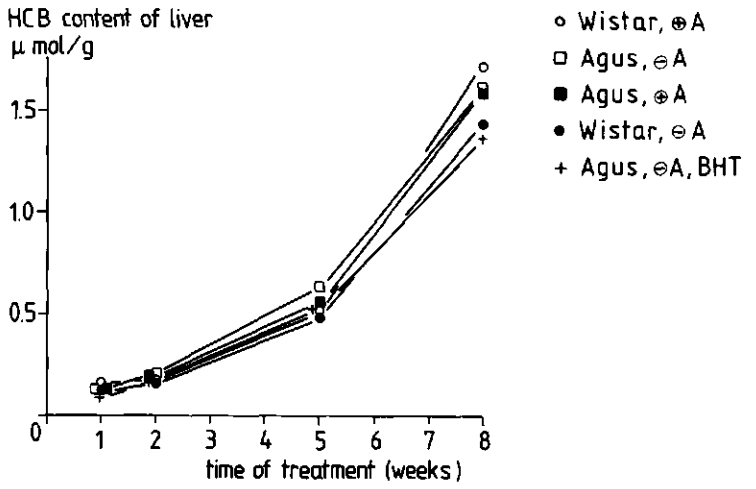

Fig. 1. Content of hexachlorobenzene (HCB) in the livers of two strains of female rats. Animals were dosed orally with HCB (50 mg/kg) every other day for 8 weeks, and fed either a basal low-antioxidant diet (-A) or a diet (+A) supplemented with the antioxidants ascorbic acid (0.15%) and DL-α-tocopherolacetate (0.12%). Points represent means of 4 rats. S.D. within 15% of the mean values, not indicated. Liver tissue of controls contained 0.2 nmol/g HCB at 5 weeks and 0.8 nmol/g HCB at 8 weeks.
Abbreviations: HCB, hexachlorobenzene 50 mg/kg orally every other day; BHT, butylated hydroxytoluene 25 mg/kg orally every other day; -A, low-antioxidant diet; +A, antioxidant-rich diet.

RESULTS

The accumulation of HCB and its main metabolites in the liver

The results presented in Fig. 1 show that no differences in the concentration of HCB in the liver were observed between Agus and Wistar rats. At 8 weeks of treatment the content of HCB in the liver amounted to about 1.5 µmol/g. In contrast to the liver HCB content, the content of metabolites in the liver was significantly higher in the Agus rats than in the Wistar rats after 5 weeks of HCB treatment (Fig. 2). Moreover, the capacity for methylating the thiophenolic metabolites of HCB in vivo appeared to be greater in the Agus rats than in the Wistar rats (Fig. 2). The dietary antioxidants and 3,5-di-tert.-butyl-4-hydroxytoluene (BHT) influenced neither the accumulation of HCB in the liver nor the content of HCB metabolites in the liver (Figs. 1, 2).

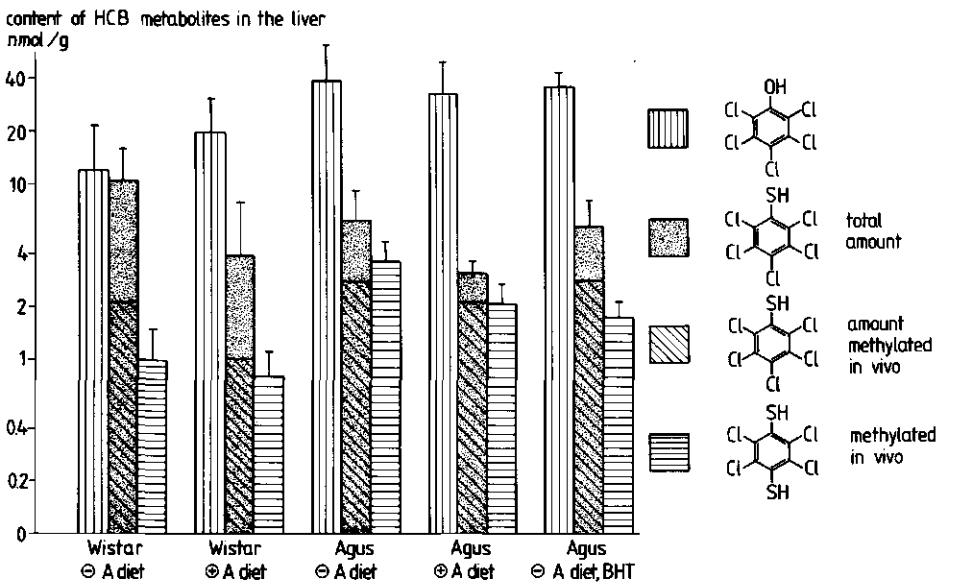

Fig. 2. Contents of the main HCB metabolites in the livers of female Agus and Wistar rats after 5 weeks of HCB administration. Bars represent mean values ± S.D. of 4 animals. Controls contained about 0.3 nmol/g PCP. Sulfur-containing metabolites were not detected in the control samples. (Consult legend Fig. 1 for more details.)

TABLE I

TOTAL EXCRETION AND RELATIVE AMOUNTS OF HCB AND ITS MAIN METABOLITES IN THE URINE AND FECES OF FEMALE AGUS AND WISTAR RATS AT THE END OF AN 8 WEEKS PERIOD OF TREATMENT WITH HCB

Strain, diet		Content of HCB and its main metabolites in the excreta (urine + feces) µmol/day					Relative amount of metabolites
		HCB[a]	PCP	TCH	PCTA	TCdi-TA	%
Wistar,	-A*	1.09 ±0.50	0.40 ±0.05	0.007 ±0.004	0.38 ±0.14	1.59 ±0.82	69.1 ± 2.5[b]
Wistar,	+A	2.63 ±0.85	1.52 ±1.41	0.037 ±0.048	0.86 ±0.41	4.33 ±2.25	69.9 ± 5.4[c]
Agus,	-A	1.63 ±0.28	1.12 ±0.15	0.024 ±0.010	0.48 ±0.13	3.51 ±0.60	76.0 ± 1.1
Agus,	+A	1.87 ±0.29	1.48 ±0.37	0.047 ±0.024	0.63 ±0.39	4.12 ±1.29	76.6 ± 3.0
Agus,	-A +BHT	1.79 ±0.48	1.68 ±0.90	0.053 ±0.015	0.48 ±0.19	4.02 ±1.16	77.4 ± 3.5

[a] Values are given in µmol/day and represent means ± S.D. of 3* or 4 animals.
Abbreviations: HCB, hexachlorobenzene; PCP, pentachlorophenol; TCH, tetrachlorohydroquinone; PCTA, pentachlorothioanisol; TCdi-TA, tetrachlorodithioanisol.

[b] Significantly different from Agus groups (Student's t-test, $P<0.05$)

[c] Significantly different from Agus groups (one-sided Student's t-test, $P<0.05$)

The biotransformation rate of HCB and the pattern of excretion of its main metabolites

After 8 weeks of HCB administration, in the Agus rats about 20% of the xenobiotic was excreted unchanged and almost 80% as metabolites. The percentage of metabolites excreted in the urine and feces of the Wistar rats was significantly lower at that time (approximately 70%, Table I). Hence it appears that after 8 weeks the rate of biotransformation of HCB in the Agus strain was higher than in the Wistar strain. This difference was not observed after 1 and 5 weeks; at those times the biotransformation rate was found to be approximately equal then for all treated groups (50-60%).

The total amount of excreted xenobiotics consisted for 50-60% of sulfur-containing metabolites (pentachlorothioanisol [PCTA] and tetrachlorodithioanisol [TCdi-TA]), whereas the relative amount of phenolic metabolites (pentachlorophenol [PCP] and tetrachlorohydroquinone [TCH]) increased from 5% after 1 week to 15-20% at the end of the experiment (8 weeks) (Fig. 3). When the relative amounts of phenolic- and sulfur-containing

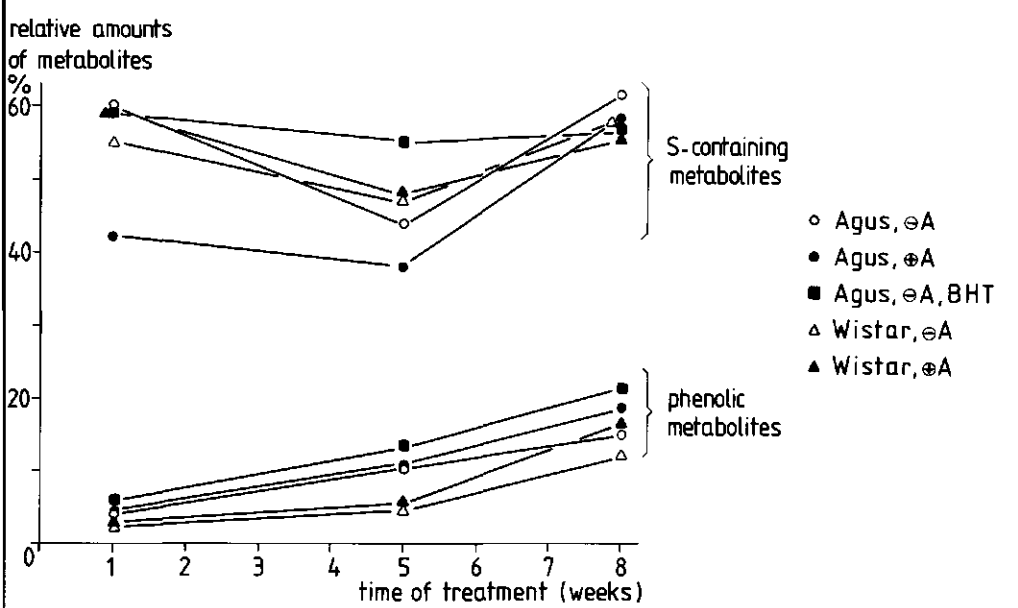

Fig. 3. Relative amounts of phenolic- and sulfur-containing metabolites of hexachlorobenzene (HCB) in the excreta (urine + feces) of female rats of the Agus and Wistar strains after oral administration of HCB every other day for 8 weeks. Values represent means of 4 rats and are expressed as the % of the sum of the amounts (µmol/day) of unchanged HCB and HCB metabolites excreted in urine and feces. (Consult legend Fig. 1 for more details.)

metabolites excreted in the urine plus feces from Agus and Wistar rats were compared, no appreciable differences could be observed (Fig. 3). However, after 8 weeks the total amounts of metabolites excreted by the Wistar rats fed a low-antioxidant diet were approximately 2-3 times lower than the amounts excreted by the Wistar rats fed a diet rich in antioxidants and the amounts excreted by the three HCB-treated Agus groups (Table I). This effect of dietary antioxidants on the total excretion of metabolites in the Wistar rats was not noticed within the Agus groups.

DISCUSSION

Based on the relative amounts of HCB and its metabolites in the excreta, the rate of biotransformation of HCB calculated in the present study was found to be almost 2-fold higher than in previous experiments [13,14]. Moreover, the ratio of the excreted phenolic metabolites to the sulfur-containing metabolites was at the end of this experiment 0.3, whereas previously a ratio of 2.8 had been determined [13]. These apparent inconsistencies may be due to the fact that the amount of the HCB metabolite tetrachlorodithiophenol had not been measured in the earlier studies. The present study showed, however, that this metabolite accounted for more than 65% of the total amount of excreted metabolites (Table I).

The higher content of metabolites in the liver of Agus rats and their higher relative amount of metabolites in the excreta (Table I) as compared to that in Wistar rats, may be attributed to the higher activity of hepatic ethoxyresorufin O-de-ethylase noted in the former strain (see preceding paper).

The higher rate of HCB biotransformation in the Agus rats may have contributed to their increased susceptibility to the porphyrinogenic effect of HCB (preceding paper), since several of our previous experiments showed metabolic conversion of HCB to be a prerequisite for inducing porphyria [7,8]. The occurrence of sulfur-containing metabolites in HCB-treated rats partly may stem from a reaction with glutathione [16]. Moreover, the large amount (60% of total amount, Fig. 3) and variety of these sulfur-containing metabolites [12] are indicative of the affinity of HCB or its intermediate metabolic products which may possess electrophilic properties, also to mercapto groups of proteins. Since the enzyme UROG-D contains functional SH-groups [17], the binding of such conversion products of HCB to this enzyme is possible. Indeed, experiments with ^{14}C-labeled HCB showed radioactivity to be irreversibly bound to cytoplasmic proteins [7,15].

Based on the above results and the observations that the livers of Agus rats contained less glutathione and possessed less induced activities of glutathione-S-transferase than those of Wistar rats (preceding paper), we suggest that in Agus rats more reactive intermediates of HCB biotransformation are binding to functional SH-groups of uroporphyrinogen decarboxylase, instead of being inactivated by glutathione and glutathione-S-transferase. We therefore propose that this, together with the comparatively high non-heme iron content of the liver of Agus rats [6], explains the peculiar susceptibility of the Agus strain to the porphyrinogenic effect of HCB.

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Conclusions

- Biotransformation of hexachlorobenzene by the hepatic microsomal mixed function oxygenase system proved to be a prerequisite for its porphyrinogenic action.

- Pentachlorophenol, as well as the other major metabolites of hexachlorobenzene, are not responsible for the porphyrinogenic action of hexachlorobenzene.

- The results show that unstable reactive intermediates formed by biotransformation of hexachlorobenzene supposedly are responsible for the disturbance of the heme biosynthesis, and not hexachlorobenzene itself.

- Antioxidants protected in vitro against the porphyrinogenic action of hexachlorobenzene, whereas this protective effect of antioxidants could not be established for the in vivo situation.

- The foregoing conclusions most probably apply to all other porphyrinogenic polyhalogenated aromatic compounds as well. For some members of this group of chemicals the present investigation has shown that this is the case.

- A primary culture of chick embryo liver cells provides a suitable model system to eludicate the mechanism of action of porphyrinogenic and hepatotoxic xenobiotics.

- It still is not definitely established that hexachlorobenzene is metabolized via reactive intermediates and that these reactive species bind to functional SH-groups of the uroporphyrinogen decarboxylating enzyme in the cytoplasm of the liver cells. This requires further studies, in which these reactive intermediates are identified by using spin trap and electron spin resonance (ESR) techniques. In addition, the complex of the reactive species with the enzyme uroporphyrinogen decarboxylase has to be isolated by immuno-adsorption with specific antibodies against the enzyme.

General summary

Polyhalogenated aromatic hydrocarbons are known to cause hepatic porphyria in man and in various species of animals. This disorder in porphyrin metabolism is attributed to a defect in the activity of the intermediary enzyme uroporphyrinogen decarboxylase in the heme biosynthetic parhway and leads to an accumulation (in the liver) and excretion (in urine and feces) of large amounts of mainly uroporphyrin. However, the mechanism underlying this block in the hepatic heme biosynthesis is not known.

The present studies have been carried out to further elucidate the mechanism of action of polyhalogenated aromatic compounds (PHAs) on the hepatic heme biosynthesis. Hexachlorobenzene (HCB), a member of this group of foreign chemicals, was used as a model compound in our investigations.

Chapter 1 gives a review of literature data on those aspects of the toxicology, pharmacokinetics and biotransformation of PHAs that are relevant to explain their effects on the hepatic heme synthesis. Special emphasis is given to HCB.

Chapter 2 describes the effects of pentachlorophenol (one of the main metabolites of HCB) on the induction of hepatic porphyria and mixed function oxygenases in female rats administered HCB. The porphyrinogenic effect of HCB was enhanced by simultaneous treatment with pentachlorophenol, whereas rats receiving pentachlorophenol alone developed no symptoms of hepatic porphyria. Pentachlorophenol showed a high affinity for membranes of the endoplasmic reticulum (see also Chapter 4) and it was found to destroy cyrochrome P-450 in vitro. From these results it is concluded that pentachlorophenol is not the metabolite that ultimately causes hepatic porphyria. However, pentachlorophenol may contribute indirectly to the porphyrinogenic action of HCB by stimulating the heme biosynthesis as a result of an accelerated breakdown of cytochrome P-450 heme. The concomitant increased production of heme exacerbates the porphyria caused by the defect of uroporphyrinogen decarboxylase.

Chapter 3 deals with the effects of several compounds interfering with the biotransformation reactions of PHAs on the accumulation of porphyrins in primary cultures of chick embryo liver cells treated with PHAs. Pre-induction of the enzyme system involved in the biotransformation of PHAs markedly enhanced the porphyrinogenic effect of PHAs in chick embryo liver cells. Inhibition of the induction of δ-aminolevulinic acid synthase with increasing concentrations of hemin could not prevent PHA-induced porphy-

rin accumulation. Inhibition of the mixed function oxygenase system or addition of elec trophile-trapping agents protected chick embryo liver cells against the porphyrinogenic effect of PHAs. A decrease of the level of intracellular glutathione with glutathione-depleting agents led to an enhancement of the cytotoxicity of PHAs in chick embryo liver cell cultures. It is suggested that a reactive intermediate, formed by biotransformation of the PHA, reacts with the catalytic SH-containing part of the uroporphyrinogen decarboxylating enzyme in the cytoplasm of the liver cells.

Chapter 4 gives a report of the effects of combined administration of HCB with either phenobarbital or 3-methylcholanthrene on female rat liver. The results presented in this Chapter and in Chapter 2 show that HCB induces a pattern of hepatic mixed function oxygenases which shares characteristics of the enzyme pattern induced by both phenobarbital and 3-methylcholanthrene. Simultaneous treatment with HCB and phenobarbital, but not with HCB and 3-methylcholanthrene, markedly enhanced the porphyrinogenic effect of HCB in female rats. These results suggest a key role for the phenobarbital-inducible form of cytochrome(s) P-450 in the biotransformation of HCB and the induction of hepatic porphyria.

Chapter 5 shows that HCB is metabolized in chick embryo liver cell cultures. The HCB-metabolites identified in chick embryo liver cell culture are the same as those found in HCB-treated rats. Since none of the major phenolic- and sulfur-containing metabolites of HCB were able to cause porphyrin accumulation in chick embryo liver cell culture, an unstable reactive intermediate capable of reacting with SH-groups of proteins is suspected to be responsible for the inhibition of uroporphyrinogen decarboxylase and the onset of hepatic porphyria. In liver cell cultures treated with [^{14}C] HCB some radioactivity became irreversibly bound to cell protein. Addition of the monooxygenase-inhibitor piperonyl butoxide or ascorbic acid reduced the protein binding of ^{14}C-metabolites. Based on the results, it appears that the rate of biotransformation of HCB has to be above a critical level before the enzyme uroporphyrinogen decarboxylase becomes inhibited and hepatic porphyria develops.

In Chapter 6 a comparison is made of the effects of dietary antioxidants on the biotransformation and porphyrinogenic action of HCB in two strains of female rats, which differ in their susceptibility to HCB. Female Agus rats were much more susceptible to the porphyrinogenic effect of HCB than female rats of the Wistar strain. The following differences between the Wistar and Agus strains of rats in their responses to HCB were noticed:

(1) HCB induced in Agus rats a higher mixed function oxygenase activity than in Wistar rats. In addition, the biotransformation rate of HCB appeared to be higher in the Agus rats. (2) Glutathione-S-transferase activity was less induced in HCB-treated Agus rats than in correspondingly treated Wistar rats. (3) Agus rats had significantly lower levels of glutathione in their livers than the Wistar rats. These differences may be responsible for the increased susceptibility of the Agus strain to the porphyrinogenic effect of HCB. Moreover, these findings support the hypothesis that binding of reactive intermediates of HCB biotransformation to functional SH-groups of uroporphyrinogen decarboxylase forms the key process in the disturbance of the hepatic heme biosynthesis. The observation that HCB was excreted in this experiment for almost 60% as sulfur-containing metabolites again confirmed the affinity of intermediate metabolic products of HCB for SH-groups.

In contrast to the results obtained with primary chick embryo liver cell cultures (Chapter 3), dietary antioxidants could not protect against the porphyrinogenic effect of HCB in vivo. Some possible explanations for this discrepancy are given in the discussion of the final Chapter.

Conclusies

- Biotransformatie van hexachloorbenzeen door het in het gladde endoplasmatisch reticulum van de levercel gelokaliseerde "mixed function oxydase" enzymsysteem blijkt een eerste voorwaarde te zijn voor het ontstaan van leverporfyrie.

- Zowel pentachloorfenol als de belangrijkste overige stabiele metabolieten van hexachloorbenzeen blijken niet verantwoordelijk te zijn voor de na chronische blootstelling aan hexachloorbenzeen optredende leverporfyrie.

- De resultaten van het onderhavige onderzoek wijzen erop dat niet, zoals men aanvankelijk dacht, hexachloorbenzeen zelf aanzet tot het ontstaan van leverporfyrie, maar instabiele reactieve intermediairen van hexachloorbenzeen.

- Antioxidantia zijn in staat om in vitro volledige bescherming te bieden tegen de porfyrinogene werking van hexachloorbenzeen. Dit beschermend effect van antioxidantia blijkt niet op te gaan voor de situatie in vivo.

- Bovenstaande conclusies gelden behalve voor hexachloorbenzeen hoogstwaarschijnlijk ook voor alle overige porfyrinogene polyhalogeen-aromaten, aangezien dit voor enkele tot deze groep behorende stoffen kon worden aangetoond in dit onderzoek.

- Primaire culturen van kippeëmbryo-levercellen vormen een geschikt modelsysteem om het porfyrinogene en hepatotoxische werkingsmechanisme van xenobiotica te bestuderen.

- Het onomstotelijke bewijs voor de vorming van reactieve intermediairen uit hexachloorbenzeen en de binding hiervan aan functionele SH-groepen van het uroporfyrinogeen-decarboxylase is vooralsnog niet geleverd. Hiertoe zal men met behulp van een 'spin trap' en elektron spin resonantiemetingen (ESR) de vorming van instabiele reactieve deeltjes daadwerkelijk moeten aantonen. Bovendien zal het reactieprodukt van het reactief intermediair met het enzym uroporfyrinogeen-decarboxylase geïsoleerd moeten worden door immuno-adsorptie met specifieke antistoffen tegen het enzym.

Samenvatting

Polyhalogeen aromatische koolwaterstoffen veroorzaken hepatische porfyrie bij de mens en verschillende diersoorten na chronische blootstelling aan deze chemische stoffen. Hepatische porfyrie is een ontregeling van de heemsynthese in de lever, die kan ontstaan door een erfelijke afwijking of door blootstelling aan lichaamsvreemde stoffen. De door polyhalogeen aromatische koolwaterstoffen geïnduceerde hepatische porfyrie wordt gekenmerkt door een ophoping van porfyrines (de voorstadia van heem) in de lever en een excessieve uitscheiding van porfyrines (voornamelijk het uroporfyrine) in de urine. Deze ophoping van porfyrines in de lever wordt veroorzaakt door een remming van de activiteit van een enzym dat betrokken is bij de biosynthese van heem: het uroporfyrinogeen-decarboxylase. Het werkingsmechanisme dat ten grondslag ligt aan de remming van dit enzym is nog onbekend.

Het in dit proefschrift beschreven onderzoek werd uitgevoerd om meer inzicht te krijgen in het werkingsmechanisme van de door polyhalogeen aromatische koolwaterstoffen veroorzaakte leverporfyrie. Hexachloorbenzeen, behorend tot deze groep van chemische stoffen, werd gekozen als een modelstof voor dit onderzoek.

In hoofdstuk 1 worden, voornamelijk op basis van literatuurgegevens, de verschillende aspecten van de toxicologie, farmacokinetiek en biotransformatie van polyhalogeen-aromaten besproken in het kader van hun porfyrinogene werking. Hierbij wordt in het bijzonder aandacht geschonken aan hexachloorbenzeen.

In hoofdstuk 2 wordt het onderzoek beschreven naar de invloed van pentachloorfenol (een van de voornaamste metabolieten van hexachloorbenzeen) op de inductie van porfyrie en het "mixed function oxydase" enzymsysteem in de lever van vrouwelijke ratten die chronisch belast werden met hexachloorbenzeen. Het genoemde "mixed function oxydase" enzymsysteem is o.a. verantwoordelijk voor de biotransformatie van lichaamsvreemde stoffen. De porfyrinogene werking van hexachloorbenzeen in vrouwelijke ratten werd versterkt wanneer de dieren gelijktijdig pentachloorfenol kregen toegediend. Daarentegen bleek chronische belasting met alleen pentachloorfenol geen leverporfyrie te kunnen opwekken. Pentachloorfenol bindt sterk aan het endoplasmatisch reticulum in de levercel (zie ook hoofdstuk 4) en kan bovendien het in deze membranen ingebouwde hemoproteïn, het cytochroom P-450 (een onderdeel van het "mixed function oxydase" systeem), in vitro omzetten in een inactieve vorm. Op basis van de resultaten kan men con-

cluderen dat pentachloorfenol als een van de belangrijkste metabolieten van hexachloorbenzeen niet verantwoordelijk kan zijn voor het ontstaan van leverporfyrie. Pentachloorfenol draagt waarschijnlijk indirect bij tot de porfyrinogene werking van hexachloorbenzeen, omdat de heemsynthese extra wordt gestimuleerd door de afbraak van cytochroom P-450 door pentachloorfenol. Een blokkade van de heemsynthese, door remming van het enzym uroporfyrinogeen-decarboxylase, zal hierdoor sneller aanleiding geven tot ophoping van porfyrines.

Hoofstuk 3 handelt over de invloed van stoffen, die aangrijpen op de biotransformatie van polyhalogeen-aromaten, op de porfyrinogene werking van laatstgenoemde verbindingen in een primaire kweek van kippeëmbryo-levercellen. Levercelculturen die vooraf behandeld werden met inductoren van het "mixed function oxydase" systeem bleken veel gevoeliger te zijn voor de porfyrinogene werking van polyhalogeen-aromaten. Remming van het "mixed function oxydase" systeem of toevoeging van stoffen die in staat zijn om electrofiele deeltjes weg te vangen, voorkwam de ophoping van porfyrines in de aan polyhalogeen-aromaten blootgestelde culturen van kippeëmbryo-levercellen. Een verlaging van het intracellulaire gehalte aan glutathion leidde tot een toename in de toxiciteit van polyhalogeen-aromaten in deze levercelculturen. De resultaten van deze in vitro experimenten met levercelculturen leverden sterke aanwijzingen op dat er tijdens de biotransformatie van polyhalogeen-aromaten reactieve intermediaren ontstaan, die door binding aan uroporfyrinogeen-decarboxylase leiden tot het ontstaan van porfyrie.

In hoofdstuk 4 is onderzocht in hoeverre stoffen zoals fenobarbital en 3-methylcholanthreen, die de lever aanzetten tot een verhoogde activiteit van het "mixed function oxydase" systeem, in staat zijn om het ontstaan van porfyrie in aan hexachloorbenzeen blootgestelde ratten te versnellen. Uit de resultaten in dit hoofdstuk en in hoofdstuk 2 blijkt dat hexachloorbenzeen een groep microsomale "mixed function oxydase" enzymen induceert, die wat betreft eigenschappen overeenkomsten vertoont met zowel de door fenobarbital als door 3-methylcholanthreen geïnduceerde groep van biotransformatie enzymen. Gelijktijdige toediening van fenobarbital en hexachloorbenzeen aan vrouwelijke ratten resulteerde in een stimulatie van de porfyrinogene werking van de laatstgenoemde stof. Methylcholanthreen had geen invloed op de porfyrinogene werking van hexachloorbenzeen. De resultaten wijzen erop dat de door fenobarbital geïnduceerde vormen van cytochroom P-450 een belangrijke rol spelen bij de metabole activatie van hexachloorbenzeen en het daaruit voortvloeiende ontstaan van leverporfyrie.

In hoofdstuk 5 wordt aangetoond dat hexachloorbenzeen gemetaboliseerd wordt in primaire culturen van kippeëmbryo-levercellen. De in deze celculturen geïdentificeerde metabolieten van hexachloorbenzeen (pentachloorbenzeen, pentachloorfenol, pentachloorthiofenol) komen overeen met de metabolieten die geïsoleerd werden uit de urine en faeces van met hexachloorbenzeen belaste ratten (hoofdstuk 6). Aangezien de diverse metabolieten van hexachloorbenzeen geen porfyrie veroorzaakten in dit in vitro celkweek systeem, ligt het voor de hand te veronderstellen dat een reactief intermediair door covalente binding aan functionele SH-groepen van uroporfyrinogeen-decarboxylase verantwoordelijk is voor het ontstaan van porfyrie. In levercelculturen die blootgesteld waren aan radioactief gemerkt hexachloorbenzeen bleek, na extractie met organische oplosmiddelen, een bepaalde hoeveelheid radioactiviteit sterk gebonden te zijn aan eiwitmateriaal. Toevoeging van een remmer van het "mixed function oxydase" systeem of ascorbinezuur (antioxidant) aan het celkweekmedium had een vermindering van de hoeveelheid eiwit gebonden radioactiviteit tot gevolg. Uit de experimenten kan de conclusie worden getrokken dat de snelheid waarmee hexachloorbenzeen in de lever gemetaboliseerd wordt eerst een kritiek niveau moet bereiken, voordat de remming van uroporfyrinogeen-decarboxylase en de hiermee samengaande ophoping van porfyrines kan optreden.

In hoofdstuk 6 wordt de invloed van antioxidantia op de biotransformatie en porfyrinogene werking van hexachloorbenzeen vergeleken in twee rattestammen (Agus en Wistar stam). Vrouwelijke Agus ratten bleken veel gevoeliger te zijn voor de porfyrinogene werking van hexachloorbenzeen dan vrouwtjes van de Wistar stam. Waarschijnlijk houdt de grotere gevoeligheid van de Agus stam voor de porfyrinogene werking van hexachloorbenzeen verband met de volgende opmerkelijke verschillen tussen de stammen, die werden waargenomen na chronische belasting met hexachloorbenzeen: (1) Hexachloorbenzeen stimuleerde het "mixed function oxydase" enzymsysteem in de lever van Agus ratten tot een hogere activiteit dan in Wistar ratten. In overeenstemming hiermee vertoonden de Agus ratten een grotere biotransformatie snelheid van hexachloorbenzeen. (2) De activiteit van glutathion-S-transferase in de lever was opvallend lager in Agus ratten. (3) Het glutathion gehalte in de lever van Agus ratten was significant lager dan in Wistar ratten. Bovenstaande bevindingen ondersteunen de hypothese dat binding van tijdens de biotransformatie van hexachloorbenzeen vrijkomende reactieve intermediairen aan functionele SH-groepen van uroporfyrinogeen-decarboxylase uiteindelijk verantwoordelijk is voor het ontstaan van

leverporfyrie. Het feit dat in dit experiment door de ratten 60% van het hexachloorbenzeen werd uitgescheiden via urine en faeces als metabolieten met één of meer thio-groepen, bevestigt nogmaals de grote affiniteit van een reactief metabool produkt van hexachloorbenzeen voor SH-groepen.

In tegenstelling tot de resultaten verkregen uit in vitro experimenten met primaire culturen van kippeëmbryo-levercellen (hoofdstuk 3), konden extra antioxidantia in het dieet geen bescherming bieden tegen de porfyrinogene werking van hexachloorbenzeen in de rat. Enkele mogelijke verklaringen voor deze tegenstrijdige resultaten worden gegeven in de discussie van het laatste hoofdstuk.

Curriculum vitae

Felix Debets werd geboren in Heerlen op 25 maart 1955. In 1972 behaalde hij zijn HBS-B diploma aan het St. Antonius Doctor College te Kerkrade, waarna hij in datzelfde jaar begon met zijn studie biologie aan de Landbouwhogeschool te Wageningen. Het ingenieursdiploma, met als hoofdvakken celbiologie (prof.dr. L.P.M. Timmermans) en toxicologie (prof.dr. J.H. Koeman) behaalde hij in januari 1978.

Op 1 februari 1978 startte hij met een promotie-onderzoek bij de vakgroep Toxicologie van de Landbouwhogeschool. De resultaten van dit onderzoek zijn vastgelegd in het proefschrift dat nu voor u ligt.