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CHLORDANE AND HEPTACHLOR ARE METABOLIZED ENANTIOSELECTIVELY BY RAT LIVER MICROSOMES

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Abstract

Chlordane, heptachlor and their metabolites are chiral persistent organic pollutants that undergo enantiomeric enrichment in the environment. This study investigated the enantioselective metabolism of both chlordane isomers and heptachlor, major components of technical chlordane, by liver microsomes prepared from male rats treated with corn oil (CO) or inducers of CYP2B (PB; Phenobarbital) and CYP3A enzymes (DX; dexamethasone), isoforms induced by chlordane treatment. The extent of the metabolism of all three parent compounds was dependent on the microsomal preparation used, and followed the rank order PB > DX > CO. The mass balances ranged from 49-130 % of the parent compound added to the microsomal incubations. Both *cis*- and *trans*-chlordane were enantioselectively metabolized to oxychlordane (EF=0.45-0.89) and 1,2-dichlorochlordene (EF=0.42-0.90). Heptachlor was metabolized enantioselectively, with heptachlor epoxide B (EF=0.44-0.54) being the only metabolite. Interestingly, the direction on the enrichment for oxychlordane, 1,2-dichlorochlordene and heptachlor epoxide differed depending on the microsomal preparation. These findings demonstrate that the direction and extent of the enantioselective metabolism of both chlordane isomers and heptachlor is P450 isoform-dependent and can be modulated by the induction of P450 enzymes.

Keywords

Chirality; environmental pollutants; insecticide; cytochrome P450 enzymes; rats; stereoisomerism

INTRODUCTION

Chlordane and heptachlor belong to the class of homologous cyclopentadiene pesticides and were widely used as effective insecticides against termites.¹ Technical chlordane is a complex mixture of structurally related chemicals, including *cis*-chlordane (C-CHLORD), *trans*-chlordane (T-CHLORD) and heptachlor (HEPTA).² Depending on the manufacturer, these three compounds constitute up to 25-50% of technical chlordane.^{3,4} The production of chlordane and heptachlor was banned because of their environmental persistence,⁵ their

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SUPPORTING INFORMATION PARAGRAPH Structures, chemical names, abbreviations and resolution of target analytes (i.e. chlordanes, heptachlor and their metabolites) on various enantioselective columns at different temperatures; description of enantioselective columns used in the project; optimization of analytical procedure used to extract target analytes from microsomal incubation samples; concentrations and enantiomeric fractions of target analytes in microsomal incubations with various microsomal preparations; range of enantiomeric fractions in various environmental matrices; resolution of OXY on various enantioselective columns; temperature effect on resolution of enantiomers of target analytes on various enantioselective columns; resolution of enantiomers of target analytes on BGB columns; representative chromatograms and NCI spectra of target analytes formed in microsomal incubations; HRMS spectrum of 1,2-dichlorochlordene. This material is available free of charge via the Internet at <http://pubs.acs.org>.

tendency to bioaccumulate and biomagnify in terrestrial and aquatic food webs,^{6,7} and environmental and human health concerns.^{8,9} In the United States, cyclopentadiene pesticides are present in appreciable amounts in the general population.¹⁰ For example, heptachlor epoxide and oxychlordane (OXY) blood levels in individuals 12 years and older ranged from 11.1-15.9 ng/g lipid and 8.7-10.1 ng/g lipid, respectively, in samples collected in 2003 and 2004. Exposure to those legacy pesticides still occurs via air,¹¹ especially indoor air in chlordane-treated buildings^{9,12} and diet.¹³ Cyclopentadiene pesticides have been implicated in a number of adverse human health outcomes, including neurotoxicity⁹ and, more recently, the epidemics of diabetes.^{14,15}

Many cyclopentadiene pesticides are chiral and exist as pairs of non-superimposable mirror images called enantiomers.¹⁶ Technical organochlorine compounds, including technical chlordane mixtures, are racemic (i.e., they contain 1:1 mixtures of both enantiomers of each chiral ingredient). There is growing evidence that chiral organochlorine compounds cause enantioselective or even enantiospecific biological effects. For example, *o,p'*-DDT and its biotransformation products enantioselectively cause apoptosis in the rat adrenal pheochromocytoma cell line PC12¹⁷ and enantiospecifically activate the human estrogen receptor.¹⁸ Chiral polychlorinated biphenyls (PCBs) enantioselectively affect cellular targets implicated in the neurodevelopmental toxicity of PCBs. For example, two important neurochemical measures, [³H]phorbol ester binding in rat cerebellar granule cells and ⁴⁵Ca²⁺-uptake in rat cerebellum, are enantioselectively altered by 2,2',3,3',6-pentachlorobiphenyl (PCB 84) enantiomers.¹⁹ (-)-2,2',3,3',6,6'-Hexachlorobiphenyl (PCB 136), but not (+)-PCB 136 enantioselectively sensitizes Ryanodine receptors, receptors involved in cellular calcium homeostasis.²⁰ Although the toxicity of pure enantiomers of cyclopentadiene pesticides has not been investigated to date, it is likely that, similar to other chiral organochlorine compounds, their toxicity is enantioselective.

Numerous studies have documented an enantiomeric enrichment of chlordane and related compounds in environmental and laboratory samples (Figure 1). Not only are the toxicological implications of this enantiomeric enrichment unknown, but the processes leading to the enantiomeric enrichment are also poorly investigated. In particular biotransformation processes are expected to make important contributions to the enantiomeric enrichment observed in many studies, especially at higher trophic levels. The major metabolic pathways of chlordane and heptachlor in mammalian systems are well established. CYP2B and CYP3A enzymes are thought to be involved in the metabolism of those chemicals²¹ because these cytochrome P450 (P450) isoforms are induced by chlordane treatment, for example in rats.²² While there is evidence that microsomal P450 enzymes enantioselectively oxidize chlordane and heptachlor,²³⁻²⁶ studies systematically investigating the role of different P450 isoforms in the enantioselective metabolism of those chemicals are currently missing.

The present study used microsomes prepared from male rats treated with phenobarbital (PB, CYP2B inducer) or dexamethasone (DX, CYP3A inducer) to study the role of CYP2A and CYP3A in the enantioselective metabolism of T-CHLORD, C-CHLORD and HEPTA, the major components of technical chlordane. Microsomes prepared from corn oil-pretreated animals (VH) were used as control microsomes representing the P450 enzyme composition in naïve animals.

Experimental procedures

Chemicals

cis-Chlordane (C-CHLORD, CAS 5103-71-9, purity 99.16%), *trans*-chlordane (T-CHLORD, CAS 5103-74-2, purity 99.9 %) and *trans*-nonachlor (CAS 39765-80-5, purity

98.9 %) were obtained from the EPA National Pesticide Standard Repository. Heptachlor (HEPTA, CAS 76-44-8, purity 99.6 %), heptachlor epoxide isomer A (HEPOXA, CAS 28044-83-9, purity 99.8 %), heptachlor epoxide isomer B (HEPOXB, CAS 1024-57-3, purity 99.8 %) and oxychlordan (OXY, CAS 27304-13-8, purity 97.9 %) were purchased from AccuStandard (New Haven, CT, USA). For the nomenclature and chemical structures see Figure 2 and Table S1.

Preparation and extraction of microsomal incubations

The preparation of rat liver microsomes and their characterization were previously described.^{27,28} In short, male rats received intraperitoneal injections of phenobarbital (PB, 3 consecutive doses of 102 mg/kg bw/d in saline), dexamethasone (DX, 4 consecutive doses of 50 mg/kg bw/d in corn oil) or corn oil alone (VH, 4 consecutive doses of 5 mL/kg bw/d). Animals were euthanized 24 h after the last treatment and livers were excised. Microsomes were prepared by differential centrifugation²⁸ and stored in sucrose (0.25 M) at -80°C. Microsomal protein was determined by the method of Lowry.²⁹ P450 enzyme activities were measured with fluorometric assays using alkoxyresorufins (ethoxyresorufin for CYP1A and benzyloxyresorufin for CYP2B)³⁰ and 7-benzyloxyquinoline (CYP3A) as substrates.³¹ The results were published elsewhere.²⁷

The microsomal incubations contained of 0.1 M phosphate buffer (pH 7.4), 3 mM magnesium chloride, 0.5 mM NADPH and 0.5 mg/mL of protein in a final volume of 2 mL. After 5 min of pre-incubation at 37 ± 1 °C, 50 µL of 0.2 mM solution of the respective organochlorine pesticide in acetonitrile was added to start the reaction. The samples were incubated for 30 min in a shaking water bath. The reactions were quenched with 2 mL of 2-propanol and kept on ice until placing them for 10 min in an oven at 110 °C to deactivate the microsomes. After addition of *trans*-nonachlor as surrogate standard (RS) (2.5 µg), the incubation samples were extracted with hexane/methyl-*tert*-butyl ether (1:1 v/v, 4 mL) followed by 4 mL of hexane. The combined extracts were washed with KCl (1 %, 3 mL) and analyzed after solvent exchange to 1 mL of hexane. Control samples with acetonitrile alone, without NADPH, without microsomes or with thermally deactivated microsomes were incubated at the same time and no metabolites were found in those samples. The mean recovery of *trans*-nonachlor was $84 \pm 21\%$ (mean $\pm$ SD). Samples with recoveries <100% were corrected for the recovery of the surrogate standard. Data are presented as mean $\pm$ standard deviation. Experiments were performed in triplicate if not stated otherwise (see Tables S4 and S5).

Gas chromatography-mass spectrometry analysis

The extracts were analyzed on an Agilent 6890 gas chromatograph with a 5975 mass selective detector with negative chemical ionization (GC-MS-NCI). Methane was used as reagent gas. The transfer line was set to 280 °C, and both MS source and quadrupole operated at 150 °C. Both total and selective ion monitoring chromatograms were recorded. The ions monitored for each organochlorine pesticide are presented in Table 1. A SLB-5ms column (30 m long $\times$ 250 µm internal diameter $\times$ 0.25 µm film thickness, Supelco, St. Louis, MO) was used for the qualitative and quantitative analysis. The following temperature program was employed: 50 °C for 5.5 min, 20 °C/min to 220 °C, 5 °C to 280 °C. The injector temperature was 250 °C. The quantifications were performed using PCB 204 (250 ng) as internal standard (IS) in the linear range of mass selective detector (5 to 500 ng).

Accurate mass determination of unknown T-CHLORD metabolite

One of T-CHLORD incubation samples was submitted for accurate mass determination to the High Resolution Mass Spectrometry facility at the University of California, Riverside

(Riverside, CA). The sample was introduced via an Agilent 7800 gas chromatograph into a Waters GCT Premier mass spectrometer. The mass spectrometer was operated in the electron ionization (EI) mode with an ionization energy of 70 eV. The accurate mass of the unknown metabolite was determined using an internal standard (perfluorotributyl amine) with a lock mass at m/z 413.9775.

Optimization of enantioselective separations

The enantiomers of all target analytes were separated on an Agilent 7890A gas chromatograph with an micro-electron capture detector (μ ECD) using the following enantioselective columns: HP-Chiral-20B (20B) and Cyclosil-B (CB) from Agilent, Chirasil-Dex (CD) from Varian, BGB-172 (BGB) from BGB Analytic, and Chiral-Dex B-DM (BDM), Chiral-Dex B-PM (B-PM) and Chiral-Dex G-TA (GTA) columns from Supelco. For details on column phases and dimensions see Table S2.

Initially, all compounds were analyzed individually using the following temperature program: 50 °C for 1 min, 10 °C/min to 140 °C, hold for 20 min, 1 °C/min to maximum column temperature (see Table S2), hold for 20 min. The injector and detector temperature was 250 °C, and helium flow was kept at 1 mL/min. Afterwards, enantioselective separations were further optimized for analytes partially separating on a particular column using isothermal conditions with decreasing temperatures. The following temperature program was used: 50°C for 1 min, 10°C/min to X, hold for Y, 10°C/min to maximum column temperature, hold for 10 min; where X varied from 140°C to 180°C and Y was between 220 min at 180°C to 500 min at 140°C. Helium flow was set to 3 mL/min.

Enantioselective analysis of metabolites from microsomal incubation

All enantioselective analyses were performed with a BGB column using GC-MS-NCI as described above. The temperature program used for these analyses employed an isothermal step at 180 °C, which allowed optimal resolution of all target analytes. The resolutions for each compound on BGB column are summarized in Table 1. The chromatograms were integrated using the Valley Drop method.³² The enantiomeric fractions (EF)³³ were defined as $E_{(+)} / (E_{(+)} + E_{(-)})$ for enantiomers with a known elution order, where $E_{(+)}$, $E_{(-)}$ are peak areas of respective enantiomers. If the elution order of enantiomers is not known, EF was defined as $E_1 / (E_1 + E_2)$, where E_1 and E_2 are peak areas of respective enantiomers. The order of elution of (+) and (-)-enantiomers of T-CHLORD, C-CHLORD, OXY and HEPOXB on the BGB column was determined based on published data.³⁴⁻³⁶

Statistical analysis

The statistical tests were conducted in R open-source software (<http://www.r-project.org/>), version 2.15.1. The differences between control and incubation samples were tested with two-sample, two tailed t-test with 95% confidence interval. Significant differences of enantiomeric fractions from racemic (EF = 0.50) were checked with one-sample two tailed t-test with 95% confidence interval. The differences were considered statistically significant for p values below 0.05.

Results

Microsomal metabolism of chlordane isomers

Rat liver microsomal preparations isolated from PB-, DX- and VH-pretreated male rats were used for microsomal metabolism studies with T-CHLORD, C-CHLORD and HEPTA. The inducer treatment resulted in different levels and activities of various P450 isoforms in the microsomal preparation, thus allowing us to assess the role of different P450 isoforms in the metabolism of chlordane and heptachlor. As published previously,²⁷ CYP1A, CYP2B and

CYP3A activities in microsomes from PB pretreated rats were 4-, 96- and 2-times higher compared to microsomes from VH-pretreated animals. Microsomes from DEX-pretreated animals had 7-times higher CYP2B and 8-times higher CYP3A activities compared to microsomes from VH-pretreated rats.

Initial microsomal metabolism experiments employed a method developed for the extraction of polychlorinated biphenyls and their metabolites from microsomal incubation samples.^{27,37} These preliminary liquid-liquid experiments yielded poor recoveries for all target analytes. Subsequent method optimization experiments demonstrated that the poor recoveries (0.4 to 77 %) of all chlordane and heptachlor derivatives are due to cleanup steps involving strong acids and bases (Table S3). Therefore, the extraction and cleanup protocol finally adopted for the analysis of all target analytes in microsomal incubations avoided the use of strong acid or base solutions, resulting in acceptable recoveries (> 70 %, with exception of HEPTA ~ 60 % and OXY ~ 60 %).

In experiments using T-CHLORD, we were able to account for 49 %, 82 % and 94 % of the total T-CHLORD added to each incubation. T-CHLORD concentrations in the microsomal incubations decreased in rank order PB >> DX > VH (Figures 3A and S4, Table S4). Specifically, only 32 % of the initial T-CHLORD amount was detected in incubations with microsomes from PB-pretreated rats, whereas 80% and 94 % were found in incubations using microsomes from DX- and VH-pretreated rats, respectively. The formation of OXY from T-CHLORD was observed in incubations with microsomes prepared from PB- and DX-pretreated rats, with 9-times higher OXY levels formed in incubations with microsomes from PB-pretreated animals (Figure 3B). An additional metabolite was observed with all three microsomal incubations (Figure 3C). Relative levels of this metabolite increased in the order PB >> DX > VH. The isotope pattern of the molecular ion suggested the presence of eight chlorines in this molecule (Figure S5). Accurate mass determination (for details, see Experimental procedures) gave m/z 403.7819, which corresponds to a chemical formula of $C_{10}H_4Cl_8$. Based on the metabolism scheme of chlordane proposed by Noimer and Hajjar,³⁸ the unknown metabolite was identified as 1,2-dichlorochlordene (DCC). Unfortunately, the absolute quantification of this metabolite was not possible because no authentic standard was available (relative comparisons are shown in Figure 3C and Table S4).

The mass balance of C-CHLORD incubations ranged from 68% to 130 % (Table S4). Overall, C-CHLORD was less readily metabolized by hepatic microsomes than T-CHLORD. For example, C-CHLORD levels clearly decreased in incubations with microsomes from PB-pretreated animals, but showed no statistically significant change in experiments using microsomes from DX- and VH-pretreated rats (Figures 3A and S6; Table S4). The putative metabolite 1,2-dichlorochlordene (DCC) was formed from C-CHLORD by all three microsomal preparations, with levels increasing in the order PB > DX ~ VH (Figure 3C, Table S4). OXY was only formed in incubation with microsomes from PB-pretreated rats, accounting for approximately 2 % of the total C-CHLORD added to each incubation (Figure 3B, Table S4). In comparison to T-CHLORD, less of DCC and OXY were formed from C-CHLORD in the microsomal incubations.

Microsomal metabolism of HEPTA

We were able to account for 88 %, 59 % and 82 % of HEPTA added to the microsomal incubations. Incubation with all three microsomal preparations resulted in a significant decrease of HEPTA levels, with a rank order of PB >> DX ~ VH (Figures 3A; Table S4). The largest decrease in HEPTA levels was observed in incubation with microsomes from PB-pretreated rats, where only 6 % of HEPTA remained at the end of the experiment. Only HEPOXB, one of the two possible isomers of HEPOX,^{39,40} was detected in the microsomal incubations. The largest amount of HEPOXB was formed in incubation with microsomes

from PB-pretreated rats (Figure S7). The levels of HEPOXB depended on the microsomal preparation and decreased in the rank order PB > DX >> VH, accounting for 82 %, 47% and 33% of the HEPTA added to the incubations (Table S4). No other metabolites were detected.

Separation of cyclopentadiene pesticide enantiomers

Since no systematic study of the resolution of cyclopentadiene pesticide enantiomers on different columns has been reported in the literature, the resolution of the enantiomers of all three parent compounds and their metabolites was assessed on a series of β - and γ -cyclodextrin-based columns (see Table S2 for column details). With exception of HEPTA, the BGB column was the only column providing baseline resolution of the enantiomers of all target analytes (Table S1, Figure S3). However, HEPTA enantiomers were partially resolved on the BGB column, whereas no other enantioselective column provided any resolution of HEPTA enantiomers. The BDM column was the second most versatile column and, with exception of HEPTA, separated the enantiomers of all target analytes. Based on our systematic exploration of different enantioselective columns, subsequent studies of the enantioselective microsomal metabolism of chlordanes and heptachlor employed the BGB column.

Enantiomeric enrichment in incubations with chlordane isomers

In the case of racemic T-CHLORD, (-)-T-CHLORD was highly enriched in incubations with microsomes from PB-pretreated rats ($EF = 0.26$) (Figures 4 and S8, Table S5). Only a slight enrichment of (-)-T-CHLORD was observed in incubations with microsomes from DX-pretreated animals; however, this enrichment was significantly different from racemic T-CHLORD. No enantiomeric enrichment was observed in the incubation with microsomes from VH-pretreated animals. Enrichment of (+)-OXY was observed in incubations with microsomes from PB-pretreated animals. In contrast, (-)-OXY was slightly enriched in incubations with microsomes from DX-pretreated animals. DCC showed an enrichment of the 2nd eluting enantiomer (E_2 -DCC) in incubations with microsomes from PB-pretreated animals, whereas the 1st eluting enantiomer (E_1 -DCC) was enriched in incubations with microsomes from both DX- and VH-pretreated rats.

(-)-C-CHLORD was only modestly enriched in incubations of racemic C-CHLORD with microsomes obtained from PB- and VH-pretreated rats (Figures 4 and S9, Table S5). However, the enrichment was still significantly different from the racemic mixture. The enrichment of (-)-C-CHLORD in incubations with microsomes from DX-pretreated rats did not reach statistical significance, but the mean EF value was comparable to incubations with microsomes from PB-pretreated animals ($EF = 0.47$). Similar to incubations with racemic T-CHLORD, (+)-OXY was enriched in incubations with microsomes from PB-pretreated animals. DCC displayed an enrichment of E_2 -DCC in incubations with microsomes obtained from PB-pretreated rats. However, clear enrichment of E_1 -DCC was observed in incubations with microsomes from DX- and VH-pretreated animals, which is in contrast to only modest enrichment of E_1 -DCC in the corresponding experiments with T-CHLORD.

Enantiomeric enrichment in incubations with racemic HEPTA

The separation of HEPTA enantiomers was possible with a low resolution ($R_s = 0.57$) for incubations with microsomes from PB-pretreated animals. A significant enrichment of the 2nd eluting enantiomer of HEPTA (E_2 -HEPTA) was observed ($EF = 0.22$) (Figures 4 and S10, Table S5). HEPOXB showed a slight but significant enrichment of (+)-HEPOXB in incubations with microsomes from PB-pretreated rats. In contrast, an enrichment of (-)-HEPOXB was noted in incubations with microsomes from DX- and VH-pretreated animals.

Discussion

The current study used microsomal preparations obtained from untreated male rats (VH), rats pretreated with PB to induce CYP2B enzymes and rats pretreated with DX to induce CYP3A enzymes to study isoform-dependent differences in the enantioselective metabolism of T-CHLORD, C-CHLORD and HEPTA. Metabolic pathways of C-CHLORD and T-CHLORD are well established (Figure 2, for a review see ³⁸). The first step is the P450 enzyme-mediated hydroxylation of C-CHLORD and T-CHLORD to 3-hydroxychlordane, followed by elimination of water.⁴¹ The resulting metabolite, DCC, has been found in low levels in kidneys and livers of male rats fed chlordane.⁴² DCC is also formed from chlordanes by rat liver microsomes.⁴³ Subsequently, a number of mostly oxygenated metabolites are formed from DCC in rats^{42,44} and rabbits.⁴⁵ OXY is one noteworthy metabolite that is formed via a minor metabolism pathway by epoxidation of DCC.^{43,44,46} OXY is a persistent metabolite that is stored preferentially in adipose^{42,46-49} and can still be detected in serum from the general United States population,¹⁰ almost 25 years after the production and use of chlordane was banned in the United States.

The metabolism of HEPTA is less complicated compared to both chlordane isomers. HEPTA can theoretically be oxidized to two isomeric heptachlor epoxides, HEPOXA and HEPOXB, as ultimate metabolites. Heptachlor epoxides have been detected in the adipose tissue from HEPTA-treated dogs and rats.^{50,51} Epoxides are also formed from HEPTA by liver microsomes from rats,²⁴ rabbits²³ and sheep.⁵² The microsomal oxidation of HEPTA requires NADPH²³⁻²⁵ and can be inhibited by classic P450 inhibitors (e.g., SKF 525-A, piperonyl butoxide, parathion, and γ -BHC),²³ which indicates that the oxidation is catalyzed by P450 enzymes. Studies using authentic standards of both heptachlor epoxides have shown that HEPOXB is selectively formed from HEPTA in biological systems.^{39,40}

The metabolite profiles and relative metabolism rates of both chlordane isomers and heptachlor in our metabolism experiments were consistent with earlier *in vitro* and *in vivo* studies.^{42-46,49} Briefly, T-CHLORD was transformed faster than C-CHLORD by all three microsomal preparations, with more OXY and DCC being formed from TCHLORD than C-CHLORD, as reported previously.^{43,44,46} DCC and OXY were not detected in control incubations, which suggests that DCC and OXY are formed by P450 enzyme-mediated metabolism. Several studies have suggested the formation of HEPTA from both chlordane isomers.^{43,44,53} Since HEPTA was not detected in microsomal incubations with both chlordane isomers in this study, its formation in earlier studies either does not involve P450 enzymes or represents an experimental artifact. Also consistent with earlier studies,^{39,54} HEPTA was selectively oxidized by all three rat liver microsomal preparations to HEPOXB, but not to HEPOXA.

Our study provides first insights into the P450 isoforms responsible for the hepatic metabolism of chlordanes and heptachlor by using rat liver microsomes from PB-, DEX- and VH-pretreated male rats. The depletion of both chlordanes and heptachlor roughly followed the order PB > DX > VH. In agreement with this rank order, more DCC was formed from both chlordane isomers with microsomes from PB-pretreated animals compared to the other two microsomal preparations. Furthermore, oxidation of DCC and HEPTA to the corresponding epoxides was more pronounced in incubations with microsomes from PB-pretreated rats compared to incubations with the other two microsomal preparations. For example, a clear rank order was observed for the formation of HEPOXB, with HEPOXB formation increasing in the order PB > DX > VH. Since CYP2B activity in the microsomal incubation used in this study increases in the order PB >> DX > VH, the trends observed in the microsomal metabolism of all three pesticides are consistent with a role of CYP2B enzymes in their metabolism. The comparable levels of DCC in incubations

of T-CHLORD and C-CHLORD with microsomes from DX- and VH-pretreated animals (Figure 3C) indicate that CYP3A enzymes play no apparent role in the metabolism of both chlordane isomers; however, this possibility cannot be completely dismissed and further studies using recombinant P450 enzymes are needed.

Enantioselective analysis demonstrated that the P450 enzyme-mediated metabolism of all three pesticides studied is enantioselective and results in an enrichment of (-)-T-CHLORD, (+)-C-CHLORD and E₂-HEPTA in incubations with microsomes from PB-pretreated animals. Near racemic signatures of the parent chlordanes and heptachlor were observed in incubations of both chlordane isomers with microsomes from DX- or VH-pretreated animals. With both microsomal preparations, the large excess of the racemic parent compound masks any enantiomeric enrichment, especially compared to incubations with microsomes from PB-pretreated animals. Consistent with our findings, earlier studies have shown that (-)-T-CHLORD is enriched in tissues from rats treated with T-CHLORD.²¹ Microsomal metabolism studies have also demonstrated that HEPTA is enantioselectively transformed to HEPOXB by rat liver microsomes;⁴⁰ however, it is unclear which HEPTA isomer was enriched in this early study.

The most intriguing findings from the present study were differences in the direction of the enantiomeric enrichment of HEPOXB, OXY and DCC in experiments using different microsomal preparations (Figure 4). The enantiomeric enrichment of metabolites isolated from incubations with microsomes from PB-pretreated animals had the opposite direction compared to metabolites obtained with the other two microsomal preparations. For example, (+)-OXY was enriched in incubations of T-CHLORD with microsomes from PB-pretreated rats, whereas a moderate enrichment of (-)-OXY was observed in incubations with microsomes from DX-pretreated animals. Treatment-dependent differences in the direction of the enantiomeric enrichment of OXY have been reported previously in rats.²¹ In this *in vivo* study, an enrichment of (+)-OXY was observed in female rats treated for 28 days with *trans*-nonachlor or T-CHLORD. In contrast, (-)-OXY was enriched in females rats dosed for 28 days, followed by a 28 or 56 day depuration period. In the same study, (-)-OXY was enriched in male rats at all time-points investigated.

The enantioselective metabolism of chlordane and related compounds by different rat P450 isoforms is the most likely explanation for the differences in the direction and extent of the enantiomeric enrichment documented in Figure 4. Currently unidentified, constitutively expressed P450 isoforms preferentially metabolize both chlordane isomers to E₂-DCC and, subsequently, DCC to (+)-OXY in incubations with microsomes from DX- and/or VH-pretreated animals. In incubations with microsomes from PB-pretreated rats, both chlordane isomers are preferentially metabolized by CYP2B enzymes to E₁-DCC, followed by preferential, also CYP2B enzyme-mediated metabolism of DCC to (-)-OXY. Similar to both chlordane isomers, the enantioselective formation of HEPOXB was P450 isoform-dependent. Our findings suggest that rat CYP2B enzymes more rapidly metabolize E₁-HEPTA, thus resulting in the enrichment of (+)-HEPOXB observed in microsomal incubations from PB-pretreated rats. As with both chlordane isomers, currently unidentified P450 isoforms are likely responsible for the enrichment of (-)-HEPOXB noted in incubations with microsomes from DX- and VH-pretreated animals. Further studies are warranted to identify the relevant isoforms using P450 isoform specific inhibitors and/or recombinant enzymes and to investigate the kinetics of the metabolism of pure enantiomers of both chlordanes and HEPTA in more detail.

Although technical chlordane and heptachlor were released into the environment as racemic mixtures, non-racemic residues with highly variable enantiomeric enrichment of chlordane-related compounds and their metabolites have been reported in abiotic environmental sample

and aquatic and terrestrial food webs (Figure 1; see also Table S6 and references therein). The extent and direction of the enantiomeric enrichment appears to be species dependent and can be highly variable, especially in fish, bird and mammals. It is noteworthy that both chlordane isomers can be highly enriched in aquatic mammals (Figures 1A and 1B, entries &, † and ‡), with the extent of the enantiomeric enrichment being comparable or higher compared to the enrichment observed in rats *in vivo* (Figure 1A, entry a2) or in rat liver microsomal incubations (Figure 1B, entry a1).

Similar observations have been reported for other chiral environmental contaminants, such as PCBs, in environmental samples.⁵⁵ As with other chiral molecules, only biological, but not physicochemical processes contribute to the enantiomeric enrichment of chlordane and related compounds in the environment as well as in terrestrial and aquatic food webs.⁵⁶ Non-racemic residues of chlordane, heptachlor and related compounds may be due to exposure to enantiomerically enriched chemicals (e.g., via the diet), species-dependent enantioselective biological processes (e.g., biotransformation by P450 enzymes) or a combination of both processes working together. Our study demonstrates that the extent and direction of enantioselective metabolism of chlordanes and heptachlor is P450 isoform-dependent in rats. This isoform-dependent metabolism partly explains the variability in the enantiomeric enrichment of chlordane-related compounds across species (Figure 1). Similar species-dependent enantiomeric enrichment has been documented for other chiral environmental pollutants. For example, the enantioselective metabolism of PCB 95, an environmentally relevant PCB congener, results in an enrichment of E₂-PCB 95 in female mice,⁵⁷ E₁-PCB 95 in rat liver microsomal incubations^{37,58} and E₂-PCB 95 in poplar plants.⁵⁹

Our study also demonstrates that changes in the composition of hepatic P450 enzymes due to the induction of certain P450 isoforms following exposure to chlordanes, heptachlor and other environmental contaminants likely explains differences in the direction and extent of their enantiomeric enrichment observed *in vivo*. For example, our study provides evidence that time-dependent differences in the enantiomeric enrichment of OXY observed in T-CHLORD-treated female rats might be due to T-CHLORD-induced changes in hepatic P450 enzyme profiles.²¹ Analogously, sex differences in the enrichment of the enantiomers of chlordane isomers in cod, with an enrichment of (-)-T-CHLORD and (-)-C-CHLORD in female and (+)-T-CHLORD and (+)-C-CHLORD in male cod,⁶⁰ are likely a reflection of sex differences in the expression of P450 isoforms involved in the metabolism of chlordane-related compounds. Consequently, future *in vivo* studies of chiral signatures of chlordane-related compounds and other chiral environmental contaminants need to consider the composition of relevant P450 enzymes to better understand factors influencing the extent and direction of the enantiomeric enrichment of chiral environmental pollutants. Ultimately, such studies will enhance our understanding of the migration of chiral environmental pollutants through aquatic and terrestrial food webs and allow us to better assess the environmental and human health risks associated with the exposure to chiral environmental pollutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Enantiomeric fractions of chlordanes, heptachlor and their metabolites in various environmental matrices. On the left there are animals and environmental samples from terrestrial food chain, on the right- from aquatic food chain. The dashed line at 0.50 denotes racemic mixture. (A) T-CHLORD; (B) C-CHLORD; (C) HEPTA; (D) HEPOXB; (E) OXY. (a1) rat liver microsomes, current study; (a2) rat *in vivo* laboratory study; (b) wolverine; (c) fox; (d) hare; (e) roe deer; (f) various species of raptor birds; (g1) cockerel *in vivo* laboratory study; (h) various parts of plants; (i) soil; (j) air; (k) water; (l) sediment; (m) ice fauna; (n) zooplankton; (o) benthos; (p) various species of fish; (q) trout; (r) cod; (s) char; (t) salmon; (u) herring; (v) alligator; (x) various species of sea birds; (y) gull; (z) penguin; (&) whale; (†) seal; (‡) polar bear. For table with source data and literature references, see Supporting Information, Table S6.

Figure 2. Simplified metabolism scheme of chlordanes and heptachlor enantiomers (adopted from reference ³⁸). Only (+)-enantiomers are shown for clarity reasons.

Figure 3.

Depletion of chlordanes and heptachlor by rat liver microsomal metabolism decreases in the order PB > DX > VH and HEPTA > T-CHLORD > C-CHLORD, whereas the formation of the respective epoxide metabolites appears to increase in the order PB > DX > VH and HEPTA > T-CHLORD > C-CHLORD. (A) Depletion of the parent chemical by different rat liver microsomes; (B) formation of oxychlordane (OXY) from *trans*-chlordanes (T-CHLORD) and *cis*-chlordanes (C-CHLORD) and heptachlor epoxide (HEPOXB) from heptachlor (HEPTA) by different microsomal preparation; (C) formation of 1,2-dichlorochlordene (DCC) from T-CHLORD and C-CHLORD by different microsomal preparations. ND - not detected. Values are presented as mean $\pm$ standard deviation of three incubations.

Figure 4.

The extent and direction of the enantiomeric enrichment of (A) T-CHLORD, (B) C-CHLORD and (C) HEPTA and the corresponding metabolites depends of the microsomal preparation. Metabolism studies were performed using rat liver microsomes from PB-, DX- or VH-pretreated male rats as described under Experimental procedures. ND - not detected; NR – not resolved. Values are presented as mean $\pm$ standard deviation of three incubations. * - significantly different from EF=0.50, one-sample two-tailed t-test, 95% confidence interval.

Table 1

Selective ion monitoring ions used for the GC-MS-NCI analysis of chlordanes, heptachlor and their metabolites, elution order of enantiomers and resolution on BGB column

Compound	<i>m/z</i> (NCI)	Elution order (BGB)	Resolution (EF)
C-CHLORD	410, 266	(+)/(–) ^{33,34,36}	0.8
T-CHLORD	410, 374	(+)/(–) ³⁶	2.2
OXY	350, 424, 316, 237	(+)/(–) ³⁶	1.4
HEPTA	266, 300, 232	unknown	0.57
HEPOXA	237, 354, 388, 282	unknown	1.4
HEPOXB	237, 318, 282	(+)/(–) ^{33,34,36}	1.5