Metabolic Hydroxylation of the Aromatic Rings of 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p'*-DDE) by the Rat

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Four phenolic metabolites of *p,p'*-DDE were identified in rat feces after dosing with *p,p'*-DDE. The structures of the metabolites, 2-hydroxy-4,4'-DDE, 3-hydroxy-4,4'-DDE, 4-hydroxy-3,4'-DDE, and 4-hydroxy-4'-DDE, were ascertained by spectral and chromatographic comparisons with synthetic material. Two of the metabolites show structures which suggest the initial formation of an arene oxide in the metabolic process.

During studies on the presence of metabolites of PCB (polychlorinated biphenyls) in biological material from the Baltic area we detected, beside a number of PCB metabolites, two compounds which showed spectral properties in agreement with hydroxylated products of *p,p'*-DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene, I, Scheme I) (Jansson et al., 1975). To confirm these findings rats were fed *p,p'*-DDE and their feces investigated for the presence of similar compounds (Sundström et al., 1975b). Three phenolic metabolites, II–IV, Scheme I, were thereby detected, the structures of which were determined by comparison with synthetic material or by mass spectrometry. On the basis of the structure of the metabolites it was also suggested that the metabolic hydroxylation proceeds via an arene oxide intermediate. Closer investigations of the fecal material have now revealed the presence of a fourth phenolic metabolite.

The present paper describes in detail the isolation and characterization of the four metabolites of *p,p'*-DDE excreted by the rat. Structure assignments were based on spectral and chromatographic comparisons with synthetic compounds the preparation of which is also reported.

Metabolic hydroxylation of the aromatic rings of the para,para' isomers of DDT-type compounds (DDT, DDD, DDE, etc.) have not been conclusively described previously, although the possible formation of phenolic metabolites of *p,p'*-DDT (1,1,1-trichloro-2,2'-bis(4-chlorophenyl)-ethane) has been indicated in several reports (e.g., Jensen et al., 1957; Dinamarca et al., 1962; Agosin et al., 1964; Morello, 1965; Sanchez, 1966; Schuntner and Schnitzerling, 1966; Oppenoorth and Houx, 1968; Welch et al., 1969; Agosin et al., 1969).

p,p'-DDE has been considered as a very stable metabolite of *p,p'*-DDT and the only breakdown product proved so far, DBP (4,4'-dichlorobenzophenone) and possibly DDA (2,2-bis(4-chlorophenyl)acetic acid), is the result of metabolic action on the aliphatic part of the molecule (for a review see Fishbein, 1974). It has been shown though that the ortho,para' isomer of DDT is extensively metabolized, enzymatic reactions occurring both at the aliphatic and the aromatic part of the molecule, by rats and chicken to yield a number of phenolic metabolites (Feil et al., 1973, 1975). Similarly, *o,p'*-DDD is metabolized by humans and rats to ring-hydroxylated compounds and products in which the structure of the aliphatic chain has been altered (Reif et al., 1974; Reif and Sinsheimer, 1975). Oxidation of the aliphatic part is also a major route in the metabolism of *p,p'*-DDT (see, e.g., Pinto et al., 1965).

It should be pointed out that in the present study no attempts were made to isolate or detect metabolites in which side-chain oxidation of the *p,p'*-DDE molecule had occurred.

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EXPERIMENTAL SECTION

Apparatus. Mass spectra (MS) were obtained on a Varian MAT CH7 instrument via the direct probe or on a Hewlett-Packard 5930A instrument with a 5933 computer on-line via a Hewlett-Packard 5830A gas chromatograph (GC–MS). Spectra were obtained at 70 eV and all calculations were based on ions containing ^{35}Cl only.

Gas chromatography (GC) was performed on a Hewlett-Packard 5830A chromatograph (flame ionization detector) with 200 × 0.2 cm glass columns packed with 0.2% Carbowax 20M on Chromosorb W, 100–120 mesh (Aue et al., 1973), which were programmed from 150 °C (3 min) to 230 °C with 5 °C/min (column A), or on a Varian 1400 instrument (electron capture detector) with 520 × 0.2 cm glass columns containing 2% Apiezon L (purified according to Jensen and Sundström, 1974a) on Chromosorb W, 100–120 mesh, at 245 °C (column B). Gas flow (He and N_2, respectively) for column A was 30 ml/min and for column B 25 ml/min.

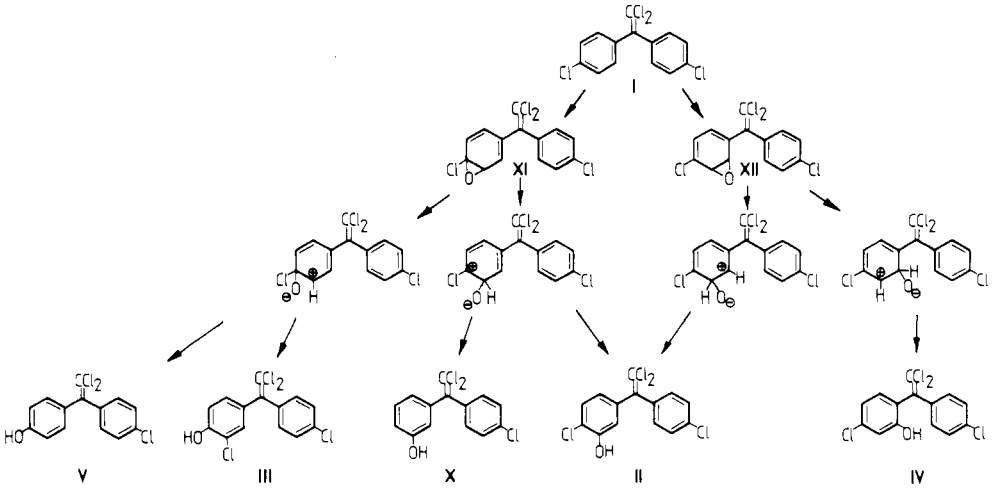
Proton nuclear magnetic resonance (^1H NMR) spectra were obtained on a Varian HR-220 or HA-100 instrument using $CDCl_3$ or acetone-d_6 solutions with tetramethylsilane as internal standard.

Analytical and preparative thin-layer chromatography (TLC) was performed on precoated silica gel plates (0.25 and 2 mm, respectively, Kieselgel 60 F_{254}, Merck) which were developed with hexane (solvent A) or hexane–ethyl acetate (4:1) (solvent B). Since the TLC plates usually were developed repeatedly to ca. 15 cm height in the same solvent system, R_f values are given relative to standard compounds (Tables I and III). Column chromatography was performed using the same adsorbent (70–230 mesh).

Animal Experiments. *p,p'*-DDE of high purity (>99% as determined by GC–MS) was dissolved in peanut oil (about 20 mg/ml) and rats were allowed to ingest the solution freely for 2–3 days (each animal consumed about 20 mg of *p,p'*-DDE during this time). The feces were collected for 1 week and investigated for metabolites as described below. The animals had free access to food and water during the experimental period.

Isolation and Purification of Metabolites. The excreta were repeatedly suspended in tetrahydrofuran and filtered. The combined extracts were reduced to ca. 50 ml and after addition of water the resulting solution was extracted with hexane–diethyl ether (1:1). Evaporation of the solvent after drying ($MgSO_4$) gave an oily residue which was treated overnight with an excess of diazomethane in diethyl ether. Solvent was evaporated again and the residue dissolved in hexane. To remove most unwanted extractives the hexane solution was treated with concentrated sulfuric acid (see Jansson et al., 1975) until the hexane solution became colorless. Finally, the hexane

Scheme I. Suggested Metabolic Route for *p,p'*-DDE in the Rat



containing methylated phenolic metabolites, *p,p'*-DDE, and some residual fat and hydrocarbons was subjected to TLC purification with hexane as solvent. A band with a relative R_f value of 10–90 (ca. 12 cm development distance of *p,p'*-DDE = 100) containing the methylated metabolites was collected and investigated by GC and GC–MS.

Synthesis of Compounds. The hydroxy-DDE compounds were synthesized by Friedel–Crafts acylations of chlorobenzene with the appropriate benzoyl chlorides. The keto group of the benzophenones so obtained was in the final step converted to 1,1-dichloroethylene groups by the Wittig-like reaction described by Seyferth et al. (1960).

4-Chloro-3-methoxybenzoic acid was synthesized from 2-chloro-5-methylphenol (Aldrich Chemical Co.) via methylation with potassium carbonate–methyl iodide in acetone solution followed by oxidation with potassium permanganate in water, mp 213–214 °C (lit. mp 215–216 °C; Hodgson and Beard, 1926).

3-Chloro-4-methoxybenzoic acid was obtained from 3-chloro-4-hydroxybenzoic acid (Riedel-de Haen Ag.) via methylation as above and subsequent hydrolysis of the methyl ester group in aqueous sodium hydroxide, mp 213–214 °C (lit. mp 214–215 °C; Schall and Dralle, 1884).

4-Chloro-2-methoxybenzoic acid was purchased from Aldrich Chemical Co., Inc., mp 146–148 °C (lit. mp 148 °C; Hodgson and Jenkinson, 1927).

4-Methoxybenzoic acid was obtained from Merck-Schuchardt, mp 182–185 °C (lit. mp 185 °C; Harris and Frankforter, 1926).

Acid chlorides of the above benzoic acids were obtained by treatment overnight with an excess of thionyl chloride at reflux temperature. After removal of excess reagent at reduced pressure the crude acid chlorides were used in the Friedel–Crafts acylations without further purifications.

Friedel–Crafts Acylations. The acid chlorides were dissolved in chlorobenzene (about 1 g in 25 ml) and aluminum chloride (2 equiv) was slowly added. After stirring overnight at room temperature another equivalent of aluminum chloride was added and the mixture was heated at 120 °C for 3 h. The reaction mixture was poured on ice and after addition of chloroform the aqueous layer was separated and discarded. The organic phase was extracted with a mixture of methanol and 2 M sodium hydroxide solution (1:1) and the combined extracts acidified and reextracted with chloroform. After drying of the organic phase ($MgSO_4$) and evaporation of solvent oily products were obtained which contained the 2-chlorophenyl and 4-chlorophenyl isomers of the benzophenones formed. Upon addition of hexane to these oils the desired benzophenones 3-hydroxy-4,4'-dichlorobenzophenone (VI), 4-hydroxy-3,4'-dichlorobenzophenone (VII), 2-hydroxy-4,4'-dichlorodibenzophenone (VIII), and 4-hydroxy-4'-chlorobenzophenone (IX) crystallized in an almost pure state in yields of 50–70%. Further purification was achieved by crystallization from hexane or by column chromatography (solvent B). Spectral, chromatographic, and physical data of the benzophenones VI–IX are given in Tables I and II.

Conversion of the Carbonyl Groups to 1,1-Dichloroethylene Groups. After methylation of the hydroxybenzophenones VI–IX with potassium carbonate–methyl iodide the methyl ethers were dissolved in dry benzene (about 1 g in 25 ml) and an equimolar amount of a 1:1 molar mixture of triphenylphosphine and phenyldichlorobromomethylmercury (Ventron Corporation) was added. The mixture was slowly heated with stirring and at 60–70 °C reaction started under discoloration and deposit of an oily precipitate. After reflux overnight hexane was added and the precipitate formed was filtered off. The solvent was evaporated and hexane–ethyl acetate (4:1) was added to the oily residue whereby unreacted benzophenones crystallized upon standing overnight and were filtered off. The mother liquor was evaporated again and the residue dissolved in hexane and shaken with concentrated sulfuric acid (see above). Evaporation of hexane gave chromatographically pure methyl ethers of compounds II, III, and V in 15–25% yield while the methyl ether of compound IV was obtained only in very low yield (<5%). The mass spectral and some chromatographic data of the methyl ethers are given in Table III.

To obtain the free phenols the above methyl ethers were demethylated with boron tribromide in dichloromethane solution (McOmie and Watts, 1963). Again the methyl ether of the 2-substituted compound IV behaved abnormally and gave only traces of demethylated product. The structure of this compound follows though by analogy on the basis of the structure of the original benzophenone VIII. Final purification of the phenols II–V was performed by preparative TLC. Some spectral, chromatographic, and physical data are given in Tables I and II.

RESULTS AND DISCUSSION

GC–MS investigations of the methylated and purified extracts of rat excreta revealed the presence of one major and three minor components not present in excreta of control animals. The mass spectra of three of these compounds indicated that they originated from a monohydroxylation of the DDE molecule (M^+ 346) while one

Table I. ^{1}H NMR Spectra, Relative R_f Values and Melting Points of Synthesized Compounds

Compound	Chemical shift, ppm H_2	H_3	H_5	H_6	$H_{2',6'}$	$H_{3',5'}$	Relative R_f-value[a]	m.p.
II[b]	6.94 (d, J~2)	—	7.29 (d, J~8)	6.79 (q, J~2,8)	7.33 (d, J~8)	7.21 (d, J~8)	—	oil
III[b]	7.25 (d, J~2)	—	6.99 (d, J~8)	7.11 (q, J~2,8)	7.34 (d, J~8)	7.21 (d, J~8)	—	101-103
IV	—	—	—	—	—	—	—	oil
V[b]	6.73 (d, J~9)	6.93 (d, J~9)	6.93 (d, J~9)	6.73 (d, J~9)	7.78 (d, J~9)	7.47 (d, J~9)	—	135-137
VI[c]	7.39 (d, J~2)	—	7.45 (d, J~8)	7.19 (q, J~2,8)	7.76 (d, J~9)	7.52 (d, J~9)	45	160-162
VII[b]	7.75 (d J~2)	—	7.09 (d, J~7)	7.57 (q, J~2,7)	7.61 (d, J~7)	7.41 (d, J~7)	24	178-179[e]
VIII[d]	—	7.09 (d, J~2)	6.85 (q, J~2,9)	7.47 (d, J~9)	7.63 (d, J~9)	7.47 (d, J~9)	107	78-79
IX[b]	7.33 (d, J~9)	6.93 (d, J~9)	6.93 (d, J~9)	7.73 (d, J~9)	7.78 (d, J~9)	7.47 (d, J~9)	20	173-175[f]

[a] solvent B, 4,4'-dichlorobenzophenone = 100. [b] 220 MHz, $CDCl_3$. [c] 100 MHz, acetone-d_6. [d] 100 MHz, $CDCl_3$. [e] lit. 168° (Buu-Hoï et al., 1954). [f] lit. 179° (Montagne, 1920).

Table II. Mass Spectral Data of Synthesized Compounds

Compound	M	M-Cl	M-2Cl	M-R_1[a]	M-R_2[a]	R_2[a]	R_1[a]
II	100	10	104	–	–	–	3
III	100	23	153	–	–	2	6
IV	–	–	–	–	–	–	–
V	100	17	100	–	–	–	3
VI	100	38	3	146	237	30	96
VII	100	36	4	279	155	32	86
VIII	100	78	2	92	65	13	45
IX	100	2	–	416	61	42	54

[a] R_1 denotes 4-chlorophenyl ring, R_2 hydroxylated ring.

Table III. Mass Spectral and Chromatographic Data of the Methyl Ethers of Synthetic and Natural Metabolites of *p,p'*-DDE Excreted by the Rat

Methyl ether of metabolite		Mass spectrum M	M-15	M-35	M-50	M-70	M-113	Retention time column A	Retention time column B	Relative R_f-value[a]
II	synthetic	100	–	18	11	97	51	11.7	13.1	24
	natural	100	–	9	8	73	43			
III	synthetic	100	11	12	8	61	58	12.8	14.8	21
	natural	100	5	12	9	73	38			
IV	synthetic	100	5	4	94	12	48	10.2	11.4	48
	natural	100	5	2	97	13	76			
V	synthetic	100	2	18	4	94	70	9.5	–	25
	natural	100	4	20	7	71	50			

[a] solvent A, *p,p'*-DDE = 100 (corresponding to a development distance of ca. 12 cm).

early eluting compound had resulted from the loss of one chlorine atom upon hydroxylation (M^+ 312). Conclusive proof for the structure of the metabolites was obtained by chromatographic and mass spectral comparisons with the synthesized compounds.

Thus, the major metabolite was shown to be 1,1-dichloro-2-(4-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)-ethylene (II) and the three minor ones were 1,1-dichloro-2-(3-chloro-4-hydroxyphenyl)-2-(4-chlorophenyl)-ethylene (III), 1,1-dichloro-2-(4-chloro-2-hydroxyphenyl)-2-(4-chlorophenyl)ethylene (IV), and 1,1-dichloro-2-(4-hydroxyphenyl)-2-(4-chlorophenyl)ethylene (V), Scheme I. The methyl ethers of compounds II–IV were isolated in the proportions 100:4:8:1.2, respectively, as estimated by GC. Their mass spectra and chromatographic behavior were virtually identical with those of the synthetic compound as indicated in Table III.

As was previously suggested the most plausible mechanism for the formation of the metabolites II–IV would involve the intermediacy of the arene oxides XI and XII as depicted in Scheme I and the discovery of compound V gives further evidence for this metabolic route. Rearrangement of the arene oxide XI with concomitant migration (NIH shift) or loss of the chlorine atom gives rise to the metabolites III and V, respectively. The complete metabolic scheme should also include the formation of compound X. However, the amount of this metabolite should presumably be very low and we were not able to detect it in the fecal material. Also, in other studies on the NIH shifts in chlorinated compounds this type of product has not been found (see, e.g., Safe et al., 1976).

NIH shifts of heavy hydrogen isotopes, halogens, or alkyl substituents have been observed during the metabolism by higher animals of a number of aromatic compounds and have been considered as a proof for the involvement of arene oxides in the enzymatic hydroxylation process (Daly et al., 1972). Such shifts of halogen or deuterium atoms were recently detected also in the enzymatic hydroxylation of PCBs (Safe et al., 1975, 1976), chloronaphthalenes (Ruzo et al., 1976a; Sundström et al., 1975a), and halogenated benzenes (Ruzo et al., 1976b; Kohli et al., 1976). Arene oxide intermediates have been suggested as being the active species involved in, e.g., the hepatotoxicity of halobenzenes, the bone-marrow toxicity of benzene, and the cytotoxicity of benzopyrenes (for references see Daly et al., 1972).

The results presented here together with the recent findings on the metabolic hydroxylation of highly chlorinated biphenyls (Jensen and Sundström, 1974b; Hutzinger et al., 1974; Goto et al., 1975) show that routes exist for the metabolic breakdown of a number of compounds previously considered being stable to metabolism. In the present case the total amounts of phenolic metabolites were estimated not to exceed 5% of the dose given. However, a higher figure for the metabolic conversion of *p,p'*-DDE may be obtained if one takes into account the

possibility for the formation of metabolites in which both the aromatic and the aliphatic parts of the molecule have been oxidized, e.g., to phenolic *p,p'*-DDA metabolites.

A closer investigation on the metabolism of other para,para'-disubstituted DDT compounds such as *p,p'*-DDT and *p,p'*-DDD will probably give conclusive evidence for the formation of phenolic metabolites also from these compounds as suggested previously (see references above).

It would be of interest to determine whether the above metabolic process or the metabolites are responsible for any biological effects caused by *p,p'*-DDE. In the case of *p,p'*-DDT and *o,p'*-DDT it has been questioned if phenolic metabolites are the ultimate cause of the estrogenic activity of these compounds (Welch et al., 1969; Feil et al., 1973). Likewise, it was proposed that a metabolite of *o,p'*-DDD containing a hydroxy group in a 4 position of the phenyl rings may be responsible for the adrenolytic activity of this compound (Reif et al., 1974). Investigations on the biological activity of the major metabolite II of *p,p'*-DDE are under way in this laboratory.

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Metabolism of Hexachlorophene in the Rabbit. Excretion, Tissue Distribution, and Characterization of the Urinary Glucuronide Conjugate

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Rabbits were given intraperitoneal doses of 10 or 15 mg/kg [^{14}C]hexachlorophene. About 48–52% of the administered radioactivity was excreted in the feces and 21–25% in the urine over a 3.5–4-day period. Unchanged hexachlorophene (HCP) was a major constituent of feces (78%) and urine (29%) of rabbits receiving a 15 mg/kg dose of [^{14}C]HCP. The major urinary metabolite (56%), HCP glucuronide, was isolated and characterized.

Hexachlorophene (HCP) was used extensively in many commercial products as a germicide until the recent finding of central nervous system disorder (Kimbrough, 1974), optic nerve atrophy (Udall and Malone, 1970), hyperthermia (Nakaue et al., 1973), and teratogenicity (Kimmel et al., 1974). Early investigations concerning the metabolism of HCP suggested that orally administered HCP was slowly and incompletely absorbed (Wit and Van Genderen, 1962). Subsequent studies showed, however, that an oral dose of HCP was rapidly and almost completely absorbed (Buhler et al., 1977), and that the bis-

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