Metabolism of *o,p'*-DDT in Rats

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The following compounds were identified in rat feces following a single oral dose of *o,p'*-DDT-^{14}C: *o,p'*-DDD, *o,p'*-DDT, 3-hydroxy-2,4'-DDD, 3-hydroxy-2,4'-DDT, 4-hydroxy-3-methoxy-2,4'-DDD, 4-hydroxy-3-methoxy-2,4'-DDT, *o,p'*-DDA, 3-hydroxy-2,4'-DDA, 4-hydroxy-3-methoxy-2,4'-DDA, glycine conjugate of *o,p'*-DDA, *o,p'*-dichlorobenzhydrol, serine conjugate of *o,p'*-DDA, 4-hydroxy-2,4'-DDA, and 5-hydroxy-2,4'-DDA. *o,p'*-Dichlorobenzhydrol was identified in rat urine. *o,p'*-DDD, *o,p'*-DDT, and *p,p'*-DDT were identified in abdominal fat. The presence of *p,p'*-DDT in the fat was due to biological concentration of a very low level in the dose rather than by biological conversion of *o,p'*-DDT.

p,p'-DDT is one of the most thoroughly studied xenobiotics known to man (396 Chemical Abstracts entries on biological studies in 1971). Reports of studies on such topics as biological effects, interactions with other compounds, and environmental distribution are abundant. Its metabolism has been investigated in many species and has been reviewed by Menzie (1969). *o,p'*-DDT has not been so extensively studied. The metabolism studies of *o,p'*-DDT have been concerned primarily with its claimed biological conversion to *p,p'*-DDT (Bitman *et al.*, 1971). Recently, considerable interest has been shown in the estrogenic activity of *o,p'*-DDT and related compounds (Cecil *et al.*, 1971).

Interest in the biological activity and metabolism of *o,p'*-DDD has increased because of its use in the treatment of adrenocortical hyperfunction (Cushing's syndrome) and adrenocortical carcinoma (Physicians' Desk Reference, 1972). Sinsheimer *et al.* (1972) reported the isolation of *o,p'*-DDA as a metabolite of *o,p'*-DDD in rabbits and in man.

The possibility that one or more metabolites of *o,p'*-DDT were responsible for effects observed on reproduction in several species prompted us to study its metabolism. Dehydrochlorination and hydroxylation at the vacant para position of the ortho-substituted ring would yield 1,1-dichloro-2-(2-chloro-4-hydroxyphenyl)-2-(4-chlorophenyl)-ethylene, a compound similar in structure to a number of highly estrogenic compounds (Bitman and Cecil, 1970; Grundy, 1957).

The isolation and characterization of 13 metabolites of *o,p'*-DDT from rat excreta and the synthesis of some of these metabolites are presented in this report.

EXPERIMENTAL SECTION

Apparatus. Column chromatographic effluents were continuously monitored with a Picker Nuclear Scinti/Flow using cerium-activated silicate glass beads with 2.5% natural lithium (Picker Nuclear) as a scintillator. Gas–liquid chromatographic (glc) separations were done with a Barber-Colman Series 5000 gas chromatograph equipped with effluent splitters so that simultaneous flame ionization and radiocarbon detection or flame ionization detection and effluent trapping could be done; columns were 4-mm i.d. and of the length specified. Infrared spectra were taken with a Perkin-Elmer 337 infrared spectrometer using the Micro KBr disk technique. Mass spectra were obtained with a Varian M-66 mass spectrometer equipped with a V-5500 control console, using the solid sample inlet system (ionizing voltage, 70 eV; source temperature, 180°; analyzer temperature, 125°). Nuclear magnetic resonance spectra were taken with a Varian A-60A spectrometer in conjunction with a Fabri-Tek 1062 computer of average transients, or a Digilab FTS/NMR-3 Fourier transform system, or a Varian Fourier transform HR-220 spectrometer (Applications Laboratory, Analytical Instrument Division, Varian Associates, Palo Alto, Calif.).

Purification of *o,p'*-DDT (ring-UL-^{14}C). Crude 1,1,1-trichloro-2-(*o*-chlorophenyl-UL-^{14}C)-2-(*p*-chlorophenyl-UL-^{14}C)ethane [byproduct from the synthesis of *p,p'*-DDT (ring-UL-^{14}C)] was obtained from Amersham/Searle Corp., Arlington Heights, Ill. This material contained a substantial amount of the p,p' isomer and small amounts of several unidentified impurities. Recrystallization of a mixture of 200 mg of unlabeled *o,p'*-DDT (purified by repeated recrystallization from ethanol) and 9.2 mg (0.5 mCi) of the crude radiolabeled material from ethanol yielded a product in which no impurities could be detected by glc (6-ft, 2% Carbowax, Chromosorb W, 210° isothermal; retention times, for *o,p'*-DDT 10 min, for *p,p'*-DDT 16 min). This material was assumed to be of high purity and was used in all of the animal experiments. Product identification in fat extracts (see Results and Discussion, Identity of Product in Fat) suggested the presence of *p,p'*-DDT in the dosing material; therefore, a chromatographic method of purification was developed to evaluate this possibility.

Chromatography on activated Florisil (1 × 25 cm) with hexane resulted in the rapid elution of at least two unidentified compounds, followed by *o,p'*-DDT and *p,p'*-DDT. Since the DDT's were only partially resolved on this column, several repetitive chromatographic separations, taking the front portion of the peak in each case, were required to remove all of the *p,p'*-DDT. Likewise, *p,p'*-DDT could be concentrated by repetitive chromatography of the latter eluents.

The chromatographic procedure also yielded good recoveries in the purification of the high specific activity material.

Animal Experiments. In one experiment, three rats (avg wt, 200 g) each received 50 mg (15 μCi) of *o,p'*-DDT (ring-UL-^{14}C) in 0.5 ml of corn oil by stomach tube, and the urine and feces were collected for 3 days. In a second experiment, six rats (avg wt, 225 g) were each given 50 mg (15 μCi) of *o,p'*-DDT (ring-UL-^{14}C) and the urine and feces were collected for 9 days. The 3-day experiment yielded 4.0% of the activity in the urine and 63% in the feces, while the 9-day experiment yielded 3.6% in the urine and 76% in the feces. Finally, one rat was fed 1.0 g of nonradioactive *o,p'*-DDT over a 50-day period, and the

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feces were collected for isolation of metabolites 3 and 5 in sufficient quantity for nmr studies.

Extraction of Metabolites. The feces were generally freeze-dried prior to extraction. Hexane extracted 15 to 20% of the original dose. An additional 15 to 20% of the original dose was extractable with ethyl acetate. Alternatively, the feces were extracted with either ethyl acetate or methanol, the solvent was removed, and the residue was partitioned between hexane and water. The aqueous layer was subsequently extracted with ethyl acetate. Comparable proportions of "hexane-soluble" and "ethyl acetate-soluble" fractions were obtained by these procedures.

The feces from a rat fed nonradioactive *o,p'*-DDT were extracted with hexane without prior freeze drying.

Isolation of Metabolites from Hexane Extracts. The hexane extracts were concentrated and chromatographed on a Sephadex LH-20 column (2.4 × 50 cm) with methanol. All of the radioactive material eluted as a single broad peak with an elution volume of 200–300 ml. The broad peak was divided into three fractions: fraction A, material having an elution volume of 200–240 ml; fraction B, material having an elution volume of 241–265 ml; and fraction C, material having an elution volume of 266–300 ml. Each of the fractions was analyzed by glc (4-ft, 2% SE-30, 0.2% Epon 1001, Chromosorb W, 150–200° at 3°/min). Fraction A contained primarily metabolites 1 and 2 (retention times, 8 and 10 min, respectively). Fraction B contained primarily metabolites 5 and 6 (retention times, 17.5 and 18.5 min). Fraction C contained primarily metabolites 3 and 4 (retention times, 13.5 and 15 min).

The isolation of metabolites 3 and 5 in sufficient quantity for nmr determinations was based on the partial separation achieved by chromatography on LH-20 with methanol. Through judicious selection of fractions (analyzed for composition by glc as described above) and repeated LH-20 chromatography of these fractions, an adequate though laborious separation was obtained; the problem was simplified because metabolites 4 and 6 were present in very small quantities in the fecal material used for isolating nmr samples.

Isolation of Metabolites from Ethyl Acetate Extracts. The isolation of metabolites or their derivatives was accomplished by combinations of extractions, derivatizations, and chromatographic separations, as shown in Figure 1. The extractions with sodium bicarbonate and sodium hydroxide were probably more effective in partitioning impurities than in separating metabolites. For example, the fraction that contained metabolites 7, 9, and 14 may also have contained metabolites 8 and 13, but these could not be obtained in sufficient purity to prove their presence. In a similar context, the nonacidic material remaining in the ether may have consisted of some of the hexane-soluble metabolites, metabolites 1 to 6; however, no compound could be isolated in sufficient purity and quantity for identification.

Chromatography of metabolites 8, 13, and 14 (hydroxy-*o,p'*-DDA's) was accomplished with several glc systems with varying degrees of success. A 6-ft, 5% FFAP, Chromosorb W column, temperature programed 225–270° at 2°/min, was most effective in separating the isomeric hydroxy-*o,p'*-DDA's and was, therefore, used to obtain cochromatographic retention times which served to supplement mass spectral data in identifications. The following glc systems were frequently more effective in purifying metabolites: 4-ft, 2% SE-30, 0.2% Epon 1001, Chromosorb W, 150–200° at 3°/min; 6-ft, 3% OV-225, Gas Chrom Q, 200–270° at 2°/min; 3-ft, 2% OV-1, Gas Chrom Q, 100–200° at 5°/min.

Amino Acid Identification. Based on ^{14}C activity, an amount of metabolic material that would correspond to 10 μg of parent *o,p'*-DDT was hydrolyzed with 6 *N* HCl by

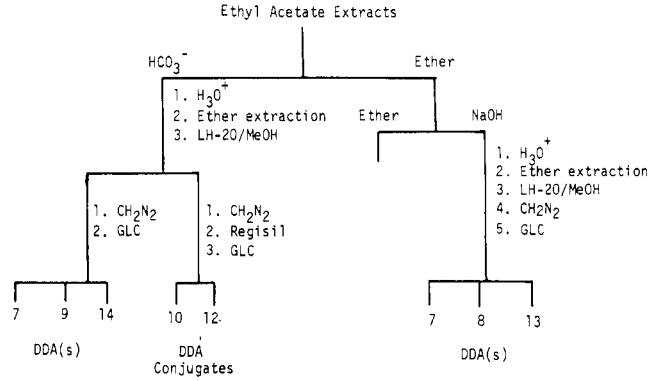

Figure 1. Isolation of ethyl acetate extractable metabolites from rat feces.

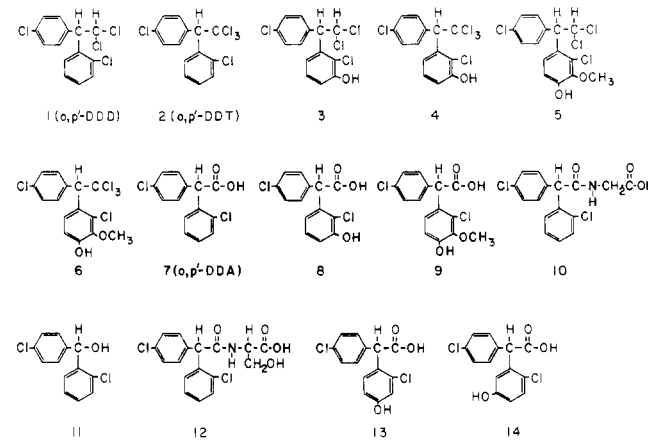

Figure 2. Metabolites identified from excreta of rats fed *o,p'*-DDT.

heating in a sealed tube for 16 hr. The hydrolyzed material was freeze-dried, dissolved in acetonitrile, and the residue reacted with Regisil. Gas chromatography was done on a 4-ft, 2% SE-30, 0.2% Epon 1001, Gas Chrom Q, 150–200° at 3°/min or on an 8-ft, 10% OV-11, Supelcoport, 120–156° at 3°/min, and then 156–275° at 5°/min. Cochromatography with standards provided evidence of the amino acids present.

Alternatively, the hydrolysates were chromatographed on Whatman No. 1 paper, using 88% phenol, water, concentrated ammonia (100 ml:20 ml:1 drop) or by two-dimensional chromatography, using butanol, glacial acetic acid, water (120:30:50) and then the previous phenol, water, ammonia system. Ninhydrin was used for detecting amino acids present.

Synthesis of Compounds Used in Identifications. Melting points were taken with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Structures of the metabolites are shown in Figure 2, and infrared spectra of some of the metabolites or their derivatives are shown in Figure 3. Some nmr, mass, and ir spectral data are listed after the procedures. Normalizations of mass spectra are based on the ^{35}Cl isotope peak.

2-Chloro-3-methoxybenzaldehyde. 2-Methoxy-6-methylaniline was prepared by catalytic reduction of the corresponding nitro compound and converted to 2-chloro-3-methylanisole through a diazonium reaction (Hartman and Brethen, 1961). A mixture of 11.8 g (0.075 mol) of 2-chloro-3-methylanisole, 29.6 g (0.166 mol) of *N*-bromosuccinimide, 0.1 g of benzoyl peroxide, and 250 ml of carbon tetrachloride was refluxed overnight. The reaction mixture was cooled and the succinimide removed by filtration. The solvent was removed, and 125 ml of morpholine

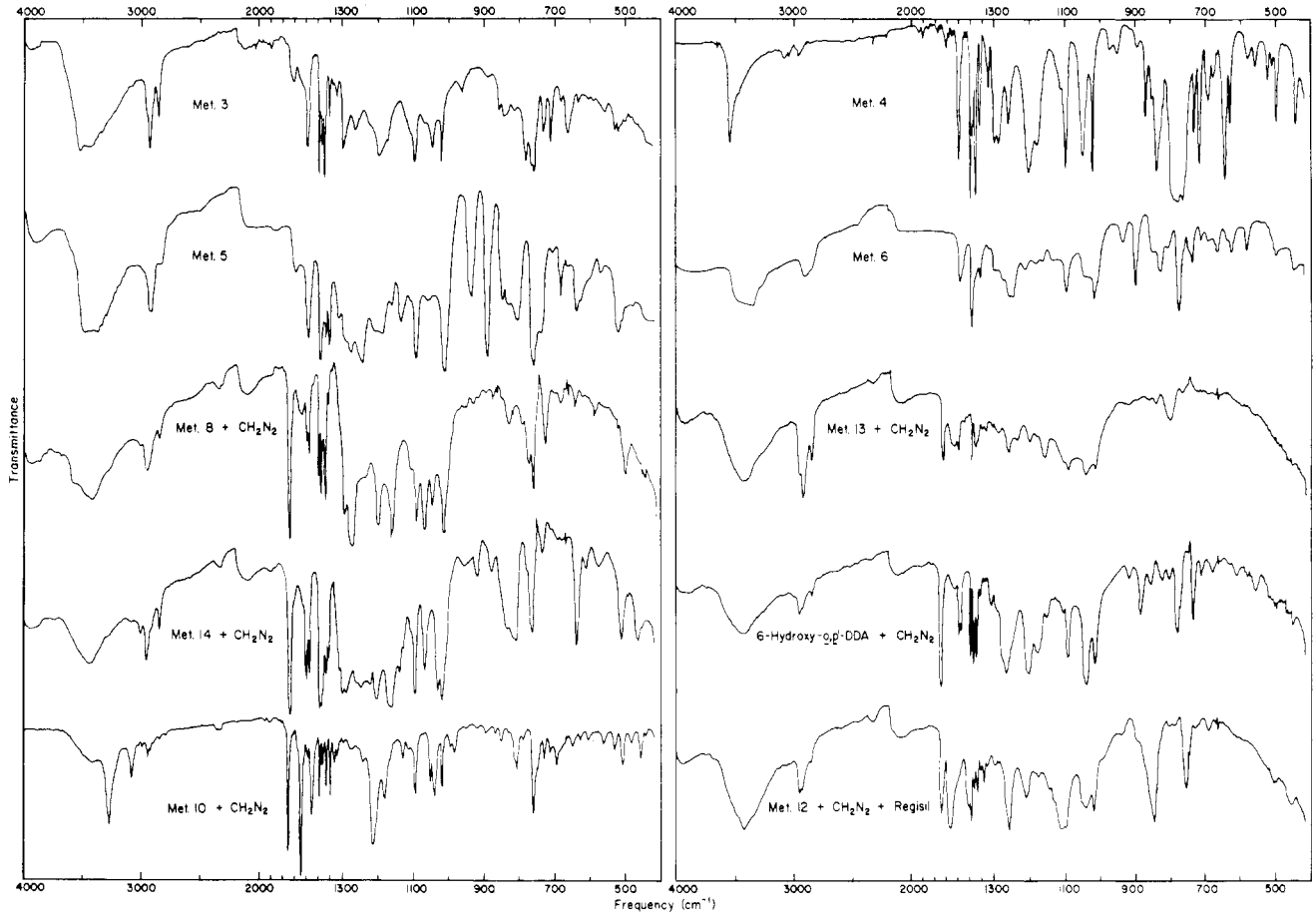

Figure 3. Infrared spectra of some metabolites and derivatives of metabolites.

was added slowly with cooling to the crude product. The morpholine solution was heated on a steam bath for 3 hr, and then poured into cold dilute hydrochloric acid and extracted with ether. The ether extracts were dried over magnesium sulfate and the solvent was removed to yield an oil which solidified (mp 47–53°) after distillation at water aspirator vacuum: ir (KBr) 1695 cm^{-1} (C=O).

2-Chloro-4-methoxybenzaldehyde. 3-Chloro-4-methylphenol was prepared from 3-chloro-4-methylaniline through a diazonium reaction (Ungnade and Orwoll, 1955) and methylated with sodium hydroxide and dimethylsulfate. 3-Chloro-4-methylanisole was converted to 2-chloro-4-methoxybenzaldehyde by the procedure used for 2-chloro-3-methoxybenzaldehyde. The product (mp 58–61°) was purified by recrystallization from ether–hexane: nmr ($CDCl_3$) δ 3.87 (s, OCH_3), 6.83 (dd, ArH, J = 10 and 3 Hz), 6.9 (d, ArH, J = 3 Hz), 7.87 (d, ArH, J = 10 Hz), 10.3 (s, O=CH).

2-Chloro-5-methoxybenzaldehyde. 4-Chloro-3-methylphenol was methylated with sodium hydroxide and dimethyl sulfate and converted to 2-chloro-5-methoxybenzaldehyde by the procedure used for 2-chloro-3-methoxybenzaldehyde. The product (mp 59–62°) was purified by crystallization from dilute acetic acid and recrystallization from hexane: nmr ($CDCl_3$) δ 3.83 (s, OCH_3), 6.95–7.4 (m, 3 H, ArH), 10.4 (s, O=CH).

2-Chloro-3,4-dimethoxybenzaldehyde. 2-Nitrovanillin was prepared from vanillin *via* *O*-acetylvanillin by the procedure of Pschorr and Sumuleanu (1899). 2-Nitrovanillin was reduced with ferrous hydroxide (ferrous sulfate and ammonia) by the procedure of Raiford and Stoesser (1928), and the resulting amino compound was converted to 2-chlorovanillin through a diazonium reaction similar to that used by Freudenberg and Jovanovic (1963) for the preparation of the 2-bromo analog. The product was purified by recrystallization from ether–hexane (mp 127–129°) and methylated by refluxing for 16 hr with sodium hydride and dimethyl sulfate in tetrahydrofuran. The product was recrystallized from hexane (mp 65–69°): ir (KBr) 1685 cm^{-1} (C=O); nmr (CCl_4) δ 3.86 (s, OCH_3), 3.97 (s, OCH_3), 6.9 (d, ArH, J = 9 Hz), 7.66 (d, ArH, J = 9 Hz), 10.32 (s, O=CH); nmr (benzene-d_6) δ 3.14 (s, OCH_3), 3.64 (s, OCH_3), 6.23 (d, ArH, J = 9 Hz), 7.7 (d, ArH, J = 9 Hz), 10.42 (s, O=CH).

1,1,1-Trichloro-2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)ethane (Metabolite 4 Methyl Ether). A solution of 22 ml of tetrahydrofuran, 5.5 ml of petroleum ether, 5.5 ml of ether, and 1.3 ml (13.5 mmol) of carbon tetrachloride was stirred under a nitrogen atmosphere and cooled to −110° using liquid nitrogen and an intermediary bath of petroleum ether and methylcyclohexane. A hexane solution of butyllithium (15 mmol) was added dropwise while keeping the temperature below −100°. The solution was stirred for 15 min after the addition was completed and a solution of 2.3 g (13.5 mmol) of 2-chloro-3-methoxybenzaldehyde in 25 ml of ether was added dropwise. The reaction mixture was allowed to warm slowly to −25° and then poured into an ammonium chloride solution and extracted with ether. The organic layer was dried over magnesium sulfate and the solvents were removed to yield an oil which distilled at 130–135° at 0.2 Torr.

A solution containing 0.5 g (1.73 mmol) of the above carbinol, 5 ml (40 mmol) of chlorobenzene, and 15 ml of concentrated sulfuric acid was stirred at room temperature overnight (Haller *et al.*, 1945). The reaction mixture was poured into ice water, extracted with ether, and the ether layer washed with a solution of sodium bicarbonate. The organic layer was dried over magnesium sulfate and

the solvent removed to yield a yellow oil that gas chromatographed as one peak (6-ft, 3% OV-1, Gas Chrom Q, isothermal at 200° for 4 min, then 200–220° at 10°/min): mass spectrum m/e 382 (5 Cl, 8%, $M^+\cdot$), 265 (2 Cl, 100%, $M - CCl_3$).

1,1,1-Trichloro-2-(2-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)ethane (Metabolite 4). Boron tribromide, 3 ml, was added to a solution of 2.0 g of 1,1,1-trichloro-2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)ethane, and the suspension was stirred at room temperature overnight under an atmosphere of nitrogen. Ether was added, and the solution was washed with sodium bicarbonate. The organic layer was dried over magnesium sulfate and the solvent removed. The residue was chromatographed on a 4.8 × 75 cm column of silica gel with carbon tetrachloride using a "dry-column" procedure (Burger, 1967; Loev and Goodman, 1967). Product of high purity was found at 12 to 16 cm; further purification was accomplished by glc (4-ft, 2% SE-30, 0.2% Epon 1001, Chromosorb W, 150–200° at 3°/min): nmr (acetone-d_6) δ 5.9 (s, CH), 7.06 (dd, p-ArH, J = 8 and 2 Hz), 7.32 (t, m-ArH, J = 8 Hz), 7.42 (d, o'- or m'-ArH, J = 8.5 Hz), 7.73 (d, o'- or m'-ArH, J = 8.5 Hz), 7,83 (dd, o-ArH, J = 8 and 2 Hz), 7.6–7.9 (br OH); mass spectrum m/e 368 (5 Cl, 3.2%, $M^+\cdot$), 333 (4 Cl, 1.8%, M − Cl), 297 (3 Cl, 1%, $M - HCl_2$), 296 (3 Cl, 0.2%, $M - H_2Cl_2$), 262 (2 Cl, 8.5%, $M - HCl_3$), 251 (2 Cl, 100%, $M - CCl_3$), 215 (1 Cl, 7.5%, $M - HCCl_4$).

1,1-Dichloro-2-(2-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)ethane (Metabolite 3). An aqueous ethanol solution of 1,1,1-trichloro-2-(2-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)ethane (metabolite 4) was refluxed with aluminum amalgam for 2 hr (Inoi *et al.*, 1962). Most of the ethanol was removed, and the remaining solution was extracted with ether. The ether layer was dried over magnesium sulfate, and the solvent was removed. The product was purified by glc (4-ft, 2% SE-30, 0.2% Epon 1001, Chromosorb W, 150–200° at 3°/min): nmr (see Figure 4); mass spectrum m/e 334 (4 Cl, 6.6%, $M^+\cdot$), 299 (3 Cl, 1%, M − Cl), 298 (3 Cl, 1.4%, M − HCl), 264 (2 Cl, 5.7%, $M - Cl_2$), 263 (2 Cl, 1%, $M - HCl_2$), 262 (2 Cl, 1.6%, $M - H_2Cl_2$), 251 (2 Cl, 100%, $M - CHCl_2$), 228 (1 Cl, 5.9%, $M - HCl_3$), 215 (1 Cl, 7.3%, $M - H_2CCl_3$).

1,1,1-Trichloro-2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)ethane (metabolite 6 methyl ether) was prepared from 2-chloro-3,4-dimethoxybenzaldehyde through the sequence of reactions used in the preparation of 1,1,1-trichloro-2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)ethane. 2-Chloro-3,4-dimethoxyphenyl trichloromethyl carbinol was purified by chromatography (alumina–methylene chloride) and by recrystallization from ether–hexane (mp 121–129°): nmr ($CDCl_3$) δ 3.87 (s, OCH_3), 3.90 (s, OCH_3), 4.71 (s, OH), 5.82 (s, CH), 6.92 (d, ArH, J = 9 Hz), 7.66 (d, ArH, J = 9 Hz). 1,1,1-Trichloro-2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)ethane could not be obtained in crystalline form; therefore, it was purified by chromatography on alumina with methylene chloride: mass spectrum m/e 412 (5 Cl, 2.6%, $M^+\cdot$), 376 (4 Cl, 3.4%, M − HCl), 342 (3 Cl, 7.5%, $M - Cl_2$), 307 (2 Cl, 1.5%, $M - Cl_3$), 306 (2 Cl, 2.5%, $M - HCl_3$), 295 (2 Cl, 100%, $M - CCl_3$).

1,1-Dichloro-2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)ethane (Metabolite 5 Methyl Ether). 1,1,1-Trichloro-2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)ethane was reacted with aluminum amalgam as in the preparation of 1,1-dichloro-2-(2-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)ethane (metabolite 3). The product was purified by chromatography on Sephadex LH-20 with methanol: nmr ($CDCl_3$) δ 3.84 (s, OCH_3), 5.08 (d, CH, J = 8 Hz), 6.27 (d, $CHCl_2$, J = 8 Hz), 6.8 (d, o- or m-ArH, J = 9 Hz), 7.1 (d, o- or m-ArH, J = 9 Hz), 7.28 (s, o'- and m'-ArH); nmr (benzene-d_6) δ 3.23 (s, p-OCH_3), 3.62 (s, m-OCH_3), 5.12 (d, CH, J = 8 Hz), 5.94 (d, $CHCl_2$, J = 8 Hz), 6.34 (d, o- or m-ArH, J = 9 Hz), 6.74 (d, o- or m-ArH, J = 9 Hz), 7.0 (s, o'- and m'-ArH); mass spectrum m/e 378 (4 Cl, 8.5%, $M^+\cdot$), 308 (2 Cl, 2.4%, $M - Cl_2$), 295 (2 Cl, 100%, $M - CHCl_2$).

Methyl 2-(2-Chloro-3-methoxyphenyl)-2-(4-chlorophenyl)acetate (Metabolite 8 Methyl Ether Methyl Ester). 2-Chloro-3-methoxybenzaldehyde was reacted with a fourfold excess of 4-chlorophenylmagnesium bromide, and the resulting product was chromatographed on alumina. Methylene chloride eluted some impurities and methanol removed the benzhydrol, which melted at 90 to 94° after recrystallization from ether–hexane. The benzhydrol was converted to the chloride with thionyl chloride (Gilman and Kirby, 1926) and purified by distillation, bp 165–170° at 0.05 Torr. The benzhydryl chloride was reacted with cuprous cyanide in the procedure of Lock and Rieger (1953) except that the reaction temperature was increased from 120–150° to 180–195°. The crude cyano compound was hydrolyzed in a solution of glacial acetic acid, concentrated sulfuric acid, and water (1:1:1) by refluxing for 4 hr. The reaction mixture was extracted with ether and nonacidic compounds were removed by a bicarbonate extraction–acidification–ether extraction sequence. 2-(2-Chloro-3-methoxyphenyl)-2-(4-chlorophenyl)acetic acid, melting at 149–152°, crystallized from ether–hexane. Reaction with diazomethane yielded the methyl ester (mp 82–84°) which was recrystallized from ether–hexane: nmr (CCl_4) δ 3.71 (s, OCH_3), 3.86 (s, OCH_3), 5.42 (s, CH), 6.7–7.2 (m, o-, m-, and p-ArH), 7.25 (s, o'- and m'-ArH); mass spectrum m/e 324 (2 Cl, 29%, $M^+\cdot$), 289 (1 Cl, 15%, M − Cl), 265 (2 Cl, 100%, $M - COOCH_3$), 230 (1 Cl, 5.5%), 229 (1 Cl, 7.6%), 215 (1 Cl, 11%, $M - COOCH_3$, CH_3, Cl), 199 (1 Cl, 4.8%), 195 (17%, $M - COOCH_3$, Cl_2), 186 (1 Cl, 6.9%), 152 (23%).

Methyl 2-(2-Chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)acetate (Metabolite 9 Methyl Ether Methyl Ester). When 2-chloro-3,4-dimethoxybenzaldehyde was subjected to the sequence of reactions used in the preparation of methyl 2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)acetate, methyl 2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)acetate was obtained. The product could not be obtained in crystalline form but could be purified by chromatography on alumina with methylene chloride: nmr (CCl_4) δ 3.72 (s, OCH_3), 3.84 (s, OCH_3), 3.86 (s, OCH_3), 5.33 (s, CH), 6.74 (d, o- or m-ArH, J = 9 Hz), 6.99 (d, o- or m-ArH, J = 9 Hz), 7.28 (s, o'- and m'-ArH); mass spectrum m/e 354 (2 Cl, 30.8%, $M^+\cdot$), 295 (2 Cl, 100%, $M - C_2H_3O_2$).

Methyl 2-(2-Chloro-4-methoxyphenyl)-2-(4-chlorophenyl)acetate (metabolite 13 methyl ether methyl ester) was prepared from 2-chloro-4-methoxybenzaldehyde through the sequence used in preparation of the 3-methoxy compound above. Neither the acid nor its methyl ester could be obtained in crystalline form. The methyl ester was purified by glc (6-ft, 2% OV-1, Gas Chrom Q, 150–200° at 5°/min): nmr (CCl_4) δ 3.70 (s, OCH_3), 3.73 (s, OCH_3), 3.51 (s, CH), 6.71 (dd, 5-ArH, J = 8.5 and 2.5 Hz), 6.90 (d, 3-ArH, J = 2.5 Hz), 7.19 (d, 6-ArH, J = 8.5 Hz), 7.23 (s, 2′-, 3′-, 5′-, and 6′-ArH); mass spectrum m/e 324 (2 Cl, 14%, $M^+\cdot$), 265 (2 Cl, 100%, $M - CO_3$), 230 (1 Cl, 1%), 229 (1 Cl, 12.5%), 215 (1 Cl, 1%), 199 (1 Cl, 1.5%), 195 (7.5%, $M - COOCH_3$, Cl_2), 186 (1 Cl, 3.8%), 152 (12%).

Methyl 2-(2-Chloro-5-methoxyphenyl)-2-(4-chlorophenyl)acetate (metabolite 14 methyl ether methyl ester) was prepared from 2-chloro-5-methoxybenzaldehyde through the sequence used previously in preparation of the 3-methoxy analog. The acid crystallized from ether–hexane, mp 123 to 126°, but the methyl ester could not be obtained in crystalline form; therefore, purification was done by glc (6-ft, 3% OV-1, Gas Chrom Q, 150–200° at

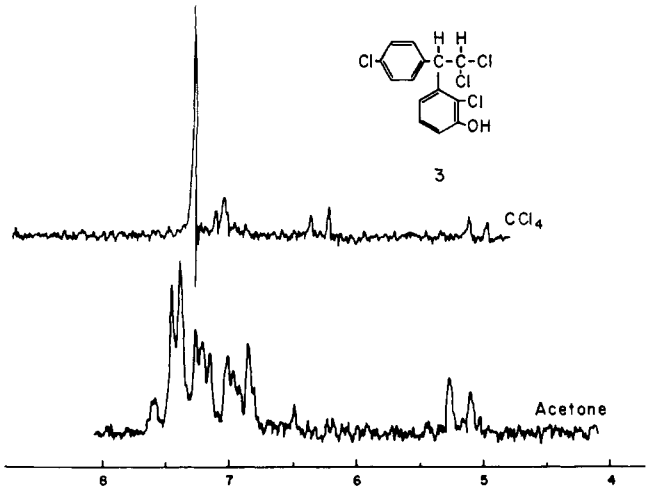

Figure 4. 60 MHz nmr spectra of metabolite 3 in carbon tetrachloride and acetone-d_6.

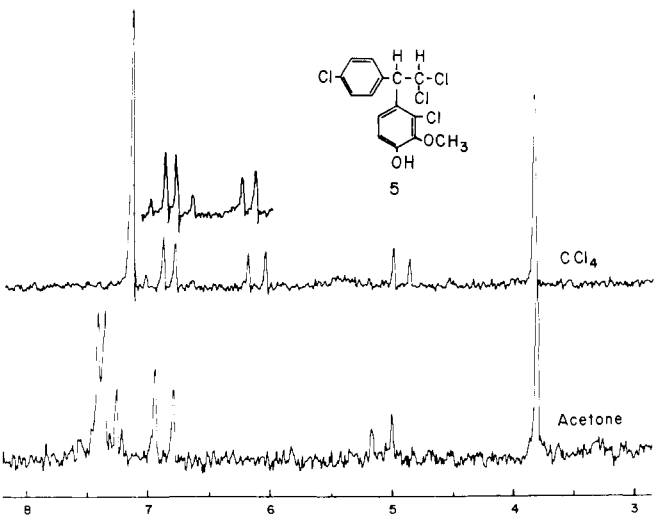

Figure 5. 60 MHz nmr spectra of metabolite 5 in carbon tetrachloride and acetone-d_6.

5°/min); nmr ($CDCl_3$) δ 3.72 (s, OCH_3), 3.75 (s, OCH_3), 5.4 (s, CH), 6.78 (dd, 4-ArH, J = 9 and 2.5 Hz), 6.82 (d, 6-ArH, J = 2.5 Hz), 7.27 (s, 2′-, 3′-, 5′- and 6′-ArH), 7.3 (d, 3-ArH, J = 9 Hz); mass spectrum m/e 324 (2 Cl, 34%, $M^{+}\cdot$), 289 (1 Cl, 15%, M − Cl), 265 (2 Cl, 100%, M − $COOCH_3$), 230 (1 Cl, 3.6%), 229 (1 Cl, 6.8%), 215 (1 Cl, 11%, M − $COOCH_3$, CH_3, Cl), 199 (1 Cl, 6%), 195 (20%, M − $COOCH_3$, Cl_2), 186 (1 Cl, 8.6%), 152 (29%).

Methyl 2-(2-Chloro-6-methoxyphenyl)-2-(4-chlorophenyl)acetate was prepared from 2-chloro-6-methoxybenzaldehyde (Postmus *et al.*, 1964) through the sequence used previously in preparation of the 3-methoxy analog. The acid crystallized from ether–hexane, mp 169 to 172°, but the methyl ester could not be obtained in crystalline form; therefore, purification was done by glc (6-ft, 2% OV-1, Gas Chrom Q, 150–200° at 5°/min): mass spectrum m/e 324 (2 Cl, 13%, $M^{+}\cdot$), 292 (2 Cl, 41%, M − CH_3OH), 265 (2 Cl, 21%, M − $COOCH_3$), 249 (2 Cl, 4.7%), 229 (2 Cl, 2.4%), 215 (1 Cl, 7.8%), 199 (1 Cl, 4.4%), 186 (1 Cl, 3.3%), 165 (12%), 152 (10.5%), 125 (100%).

Methyl *N*-(2,4′-Dichlorodiphenylacetyl)glycinate (Metabolite 10 Methyl Ester). 2,4′-Dichlorodiphenylacetyl chloride was prepared by reacting the acid with an excess of thionyl chloride and was used without purification in the procedure of Suyama *et al.* (1965). Since the product could not be crystallized, it was extracted with ether, dried over magnesium sulfate, and reacted with diazomethane. The methyl ester was chromatographed on silica gel using ethyl acetate and was subsequently recrystallized from ether–hexane to yield product melting at 129–131°: nmr ($CDCl_3$) δ 3.76 (s, OCH_3), 4.03 and 4.12 (2 s, CH_2), 5.38 (s, CH), 5.8–6.2 (br s, NH), 7.28 (s, ArH); mass spectrum m/e 316 (1 Cl, 42%, M − Cl), 235 (2 Cl, 34%, M − $CONHCH_2COOCH_3$), 201 (1 or 2 Cl, 24%), 199 (1 or 2 Cl, 22%), 165 (56%), 153 (12%), 116 (39%, $CH_3OOCCH_2NHCO^{+}$), 88 (100%, $CH_3OOCCH_2NH^{+}$).

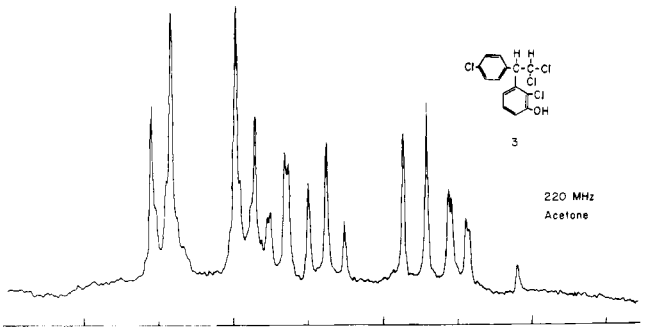

Figure 6. A portion of the 220 MHz nmr spectrum of metabolite 3 in acetone-d_6.

Methyl *N*-(2,4′-Dichlorodiphenylacetyl)serinate (Metabolite 12 Methyl Ester). The serine conjugate of *o,p′*-DDA was prepared by the method used in the preparation of the glycine conjugate above (Suyama *et al.*, 1965). Again, the acid could not be crystallized; therefore, it was extracted with ether, dried over magnesium sulfate, and reacted with diazomethane. The material obtained from the methylation reaction was washed with sodium bicarbonate and dried over magnesium sulfate. The residue that remained after removal of solvent was triturated repeatedly with hexane and crystallized from cold ether–hexane. Several recrystallizations from benzene–cyclohexane yielded product melting at 109–111°: nmr ($CDCl_3$) δ 2.32 (s, OH), 3.77 (s, OCH_3), 394 (d, CH_2, J = 4 Hz), 4.68 (m, NCH), 5.42 (s, Ar_2CH), 7.31 (s, ArH); mass spectrum m/e 346 (1 Cl, 23%, M − Cl), 328 (1 Cl, 5.7%, M − Cl, H_2O), 235 (2 Cl, 53%, M − $CONHCH(CH_2OH)COOCH_3$), 201 (0 or 1 Cl, 25.5%), 199 (1 or 2 Cl, 41%), 165 (100%), 163 (21%), 146 (68%, $CONHCH(CH_2OH)COOCH_3^{+}$), 118 (72%, $NHCH(CH_2OH)COOCH_3^{+}$).

RESULTS AND DISCUSSION

Hexane-Soluble Metabolites in Feces. Infrared and mass spectral comparisons with authentic samples of *o,p′*-DDD and *o,p′*-DDT proved the identity of metabolites 1 and 2.

Mass spectral data indicated that metabolites 3 and 4 were DDD and DDT derivatives, respectively, that had increased by 16 mass units. Mass spectral data likewise suggested that metabolites 5 and 6 were DDD and DDT derivatives that had increased by 46 mass units. Infrared spectra of these compounds indicated the presence of hydroxy groups. Limited solubility in alkali (partitioning between ether and 2.5 N sodium hydroxide yielded approximately 90% of the ^{14}C activity in the ether layer) suggested that these compounds were benzhydrols, *i.e.*, 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethanol; however, comparison with synthetic samples disproved this possibility. Thus, the compounds appeared to be atypical phenols. Because of the number of structural possibilities, the use of nmr as an aid in determining structures of at least some of these metabolites became imperative.

Sufficient quantities of metabolites 3 and 5 were isolated for nmr determinations from feces of a rat fed nonradioactive *o,p′*-DDT. The 60 MHz spectra of metabolites 3 and 5 provided clear evidence (doublets at approximately

δ 5.0 and 6.2, J = 8.5 Hz, in the carbon tetrachloride spectra) for a dichloromethane moiety (Figures 4 and 5). The spectrum of metabolite 5 in the carbon tetrachloride also provided evidence that substitution was in the *ortho*-chloro-substituted ring (doublets at δ 6.8 and 7.0, J = 8.5 Hz) and that one of the substituents was a methoxy group (singlet at 3.85). No definite assignment of substitution could be made on the basis of the aromatic splitting pattern in the 60 MHz spectrum of metabolite 3; however, a 220 MHz spectrum (Figure 6) provided clear evidence that the *ortho*-chloro ring had been hydroxylated at either the 3 or 6 position. The initial assignment of position 6 as the point of hydroxylation (Feil *et al.*, 1971) was made on the basis of the nmr work of Keith *et al.* (1969) and on the lack of base solubility. Keith *et al.* (1969) assigned the absorption that takes place at δ 8.1 (significantly downfield from the other aromatic protons) in the CCl_4 spectrum of *o,p'*-DDT to the proton at position 6 of the ortho-substituted ring because of its expected close proximity to the $-CCl_3$ group. *o,p'*-DDD did not show a significant downfield shift for this ortho proton in CCl_4, but did show a downfield shift in acetone. The downfield protons in both *o,p'*-DDT and *o,p'*-DDD did not show first-order splitting patterns, indicating restricted rotation. A temperature study in 3-pentanone showed a change in splitting pattern and an upfield shift, with increasing temperature for *o,p'*-DDD, but a first-order splitting pattern could not be obtained; however, a model compound, 1,1,1-trichloro-2-(4-chlorophenyl)-2-(2-methoxyphenyl)ethane, yielded a downfield absorption, with a first-order splitting pattern for the ortho proton, supporting the assignment. The lack of downfield absorption in the nmr spectrum of metabolite 3 suggested that substitution had taken place at the 6 position of the ortho-substituted ring; however, subsequent identification of metabolite 8 as 2-(2-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)acetic acid (3-hydroxy-2,4'-DDA) raised questions on the validity of this assignment. Several attempts at synthesis of the 6-hydroxy-2,4'-DDT failed, but synthesis of the 3-hydroxy analog was successful. Infrared and mass spectra of 3-hydroxy-2,4'-DDT were identical to those of metabolite 4. Identical spectra were also obtained for 3-hydroxy-2,4'-DDD and metabolite 3, showing the importance of hydroxylation at the 3 position as a metabolic process.

Structures of metabolite 5 and 6 were not established directly by synthesis but were established through extensive use of nmr. Fales and Warren (1967) have proposed the use of benzene-induced shifts of nmr absorbances in determining structure. Benzene-d_6 shifted the absorption due to two of the methoxy groups of 1,2,3-trimethoxybenzene upfield about 0.4 ppm from the absorption in deuteriochloroform; the absorption due to the remaining methoxy group was shifted less than 0.1 ppm. Spectra of metabolite 5 in the two solvents showed a difference of 0.65 ppm, suggesting that the methoxy group was "external" (Feil *et al.*, 1972); however, 2-chloro-3-methoxy-4-hydroxybenzaldehyde and 2-amino-3-methoxy-4-hydroxybenzaldehyde showed shifts of 0.78 and 0.75 ppm, respectively, indicating that large shifts are not necessarily restricted to external methoxy groups. The methyl ether of metabolite 5 proved to be 1,1-dichloro-2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)ethane by ir, nmr, and mass spectral comparisons; nmr spectra taken in deuteriochloroform and in benzene-d_6 showed upfield shifts of 0.32 and 0.7 ppm. This difference in shift allowed the unambiguous assignment of the position of the hydroxyl group through the use of diazomethane-d_2. Nmr spectra of metabolite 5 which had been reacted with diazomethane-d_2, and spectra of 1,1-dichloro-2-(2-chloro-3-methoxy-4-methoxy-d_2-phenyl)-2-(4-chlorophenyl)ethane (prepared from 2-chloro-3-methoxy-4-methoxy-d_2 benzaldehyde) were identical and proved that the methoxy group in the para position was the one that was shifted by 0.7 ppm. Thus, the structure of the metabolite is 1,1-dichloro-2-(2-chloro-3-methoxy-4-hydroxyphenyl)-2-(4-chlorophenyl)-ethane. The structure of metabolite 6 was not proven but is proposed on the basis of ir and mass spectral similarities to metabolite 5.

A very small amount of *o,p'*-dichlorobenzhydrol, metabolite 11, was isolated in one of the experiments. The identification was carried only to a moderate degree of certainty, being based on glc retention time and mass spectral comparisons of an impure metabolite sample and an authentic sample.

The anticipated dehydrochlorination and hydroxylation in the vacant para position of the ortho-substituted ring to form a highly estrogenic compound was not observed; however, the mass spectrum of one of the samples of metabolite 4 indicated the presence of a hydroxymethoxy-*o,p'*-DDE. Because of the limited amount of data on this possible metabolite, it can only be considered as speculative.

Acidic Metabolites in Feces. Infrared and mass spectra of methylated metabolite 7 were identical to those obtained on an authentic sample of methyl 2-(2-chlorophenyl)-2-(4-chlorophenyl)acetate (methyl ester of *o,p'*-DDA).

Mass spectral studies suggested that metabolite 8 was a hydroxy-*o,p'*-DDA. Because of the initial incorrect assignment of 6-hydroxy-2,4'-DDD for the structure of metabolite 3 (Feil *et al.*, 1971) and the assumption that the DDA had been hydroxylated at the same position as the DDD, methyl 2-(2-chloro-6-methoxyphenyl)-2-(4-chlorophenyl)-acetate was synthesized. Comparison of its mass spectrum with that of methylated metabolite 8 showed that metabolite 8 was not 6-hydroxy-2,4'-DDA. The mass spectra of the methyl derivatives of metabolite 8 and 5-hydroxy-2,4'-DDA were identical; however, their glc retention times were different. Subsequently, the 4- and 3-hydroxy isomers were also synthesized and a glc method (6-ft, 5% FFAP, Chromosorb W, 225–270° at 2°/min) was developed for the separation of the four isomeric hydroxy-DDA's. Through co-glc and comparison of mass spectra, metabolites 8, 13, and 14 were shown to be 3-hydroxy-, 4-hydroxy-, and 5-hydroxy-2,4'-DDA, respectively; comparative infrared spectra were also obtained on metabolites 8 and 13.

Metabolite 9 could not be obtained in pure form. Mass spectra of crude samples yielded a molecular ion at 326 (2 Cl) and a large peak at 281 (2 Cl). These moved to 354 and 295 when the metabolite was reacted with diazomethane. Mass spectral and glc retention time comparisons with a synthetic sample suggested that the derivatized metabolite was 3,4-dimethoxy-2,4'-DDA. The proposal that the metabolite is 3-methoxy-4-hydroxy-2,4'-DDA is speculative, based on the structure of metabolite 5.

One of the LH-20 chromatographic fractions from the bicarbonate extracts (Figure 1) appeared to contain *o,p'*-DDA conjugates since *o,p'*-DDA could be isolated only after hydrolysis. Examination of the hydrolysates by gas and paper chromatography for the presence of amino acids yielded highly variable evidence for the presence of several amino acids, with evidence for the presence of serine being most consistent. On the assumption that this material contained the serine conjugate of *o,p'*-DDA, it was derivatized first with diazomethane and then with Regisil. Gas chromatography (6-ft, 3% OV-1, Gas Chrom Q, 190–280° at 5°/min) yielded the methyl ester of the glycine conjugate of *o,p'*-DDA, metabolite 10, and the methyl ester–trimethylsilyl derivative of the serine conjugate of *o,p'*-DDA, metabolite 12. The structures of these compounds were verified by comparison of mass spectra and glc retention times with those of synthetic samples. An

apparent artifact was also isolated on several occasions from this fraction; its mass spectrum suggested a dehydrated serine .conjugate of *o,p'*-DDA. This compound could be produced from a synthetic sample of the methyl ester of the serine conjugate of *o,p'*-DDA, occasionally by the reaction of Regisil, and, more consistently, by the reaction of acetic anhydride and a trace of methane sulfonic or sulfuric acid.

Metabolites in Urine. Purification of metabolites from urine proved to be extremely difficult because of the small amount (3.5–4.0% of the dose) of metabolic material present. Although chromatography indicated the presence of several metabolites, only one compound was isolated in sufficient purity for identification purposes. Metabolite 11 was identified as *o,p'*-dichlorobenzhydrol by mass spectral comparison with that of an authentic sample.

Identity of Products in Fat. Hexane extracts of abdominal fat, purified by the procedure for nonionic organochlorine residues in the Pesticide Analytical Manual (Vol. 1, section 211.102) yielded a large peak on chromatography with Florisil. Gas chromatography of various portions of this peak revealed the presence of *o,p'*-DDD, *o,p'*-DDT, and *p,p'*-DDT. Specific activity determinations revealed that the *o,p'*-DDD and *o,p'*-DDT had essentially the specific activity of the original dose, but the *p,p'*-DDT had a specific activity at least ten times *higher* than the dose material. The presence of high specific activity *p,p'*-DDT in the fat can be accounted for by its presence as a contaminant in the original dose, but *not* by conversion of *o,p'*-DDT to *p,p'*-DDT. For conversion to prevail would require a specific activity equal to or lower than the dose material, *i.e.*, environmental contamination would lower the specific activity of the isolated *p,p'*-DDT. Since the *o,p'*-DDT used for dosing had been purified by an isotopic dilution procedure, any residual *p,p'*-DDT would have a high specific activity.

CONCLUSIONS

The isolation and identification of at least 13 *o,p'*-DDT metabolites (Figure 5) from rat excreta indicate that *o,p'*-DDT was extensively metabolized by the rat. Although difficulties with isolation procedures prevented accurate assignments of the relative percentages of the original dose that each metabolite represented, crude approximations placed most of them in the 1–3% range. Other metabolites were undoubtedly present, as indicated by poor recoveries in some of the purification steps and by the failure to obtain some of the chromatography fractions in sufficient purity to propose structures.

Most of the metabolites have been rigorously identified by comparison of infrared and mass spectra with those of synthetic samples. Metabolites 6 and 9 are, in part, speculative since spectral comparisons were made on methylated samples; the choice of 4-hydroxy-3-methoxy rather than 3-hydroxy-4-methoxy was based on metabolite 5, which was rigorously identified. Metabolites 10, 11, 12, and 14 were somewhat less rigorously identified since only comparisons of mass spectra and glc retention times were obtained with synthetic samples.

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