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BOVINE ADRENAL CORTEX TRANSFORMATIONS OF MITOTANE [1-(2-CHLOROPHENYL)-1-(4-CHLOROPHENYL)-2,2-DICHLOROETHANE; *o,p'*-DDD] AND ITS *p,p'*- AND *m,p'*-ISOMERS

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Abstract—The adrenalytic activity of mitotane (*o,p'*-DDD) has made it useful in the treatment of adrenocortical carcinoma and Cushing's syndrome. In support of a study to develop mitotane analogs as more effective therapeutic agents and as a basis for understanding the toxicity of related compounds in the adrenals, the biotransformations of *o,p'*-DDD in adrenocortical homogenate preparations have been studied and compared with those of its *m,p'*- and *p,p'*-isomers. Aliphatic oxidation to the corresponding acetic acid derivative, *o,p'*-, *m,p'*- or *p,p'*-DDA, was the major transformation for all the preparations. In the comparisons of the DDD isomers, the order of both DDA formation and apparent covalent binding was *o,p'*- > *m,p'*- > *p,p'*-DDD. There was also evidence for α-hydroxylation at the benzylic carbon with subsequent loss of water to form ethylene derivatives. This was a minor pathway for *o,p'*-DDD, but was the major pathway for the other two isomers. Thus, while the total yields of metabolites of *o,p'*- and *m,p'*-DDD were similar and at least twice that of the *p,p'*-isomer, their distribution of metabolites differed significantly. The effects of the three isomers on cell growth and cortisol production with the human adrenocortical carcinoma cell line, NCI H-295, followed the same order as their DDA formation and tissue binding. It is proposed that hydroxylation by the adrenal cortex at the β-carbon leads to an adrenalytic effect, whereas hydroxylation at the α-carbon would represent an alternate deactivation pathway.

Key words: mitotane; metabolic activation and binding; adrenal transformations; adrenal carcinoma

The localization of mitotane (*o,p'*-DDD‖) in adrenocortical tissue causes destruction of both normal and cancerous cells. Therefore, it has been useful in the treatment of patients with adrenocortical carcinoma [1] and has been employed in the therapy of pituitary ACTH-dependent Cushing's syndrome [2]. To understand the adrenalytic activity of mitotane, its metabolism has been studied both *in vivo* [3–5] and *in vitro* in adrenal preparations [6–10]. *In vitro* studies have shown that metabolic transformation of mitotane can lead to covalent binding to adrenocortical macromolecules [6, 7]. We have shown previously using adrenal perfusion that both aromatic and aliphatic oxidations of mitotane can occur in the adrenals [10]. Such transformations by the adrenal cortex may have significance in understanding the mode of action of mitotane.

Moreover, the ability of adrenals to efficiently transform endogenous hydrophobic substrates could also play a role in the activation of other xenobiotic hydrophobic compounds. This is consistent with adrenal glands having large blood supplies, rich enzyme content and high lipid composition. In this regard, it is of interest that Ribelin [11] in his survey of chemically induced lesions of endocrine glands found that the adrenals were the most frequently affected gland. Also, Colby [12] in a review of chemically induced adrenal gland toxicity lists over forty compounds that have been found to produce lesions in the adrenal cortex.

The purpose of the present study is to report the metabolic activation of *o,p'*-DDD, *m,p'*-DDD and *p,p'*-DDD in adrenal homogenates. This structure–activity information will be utilized in a study to develop mitotane analogs as more effective agents in the treatment of adrenal cancer and as a basis for understanding the toxicity of related compounds in normal adrenals.

MATERIALS AND METHODS

Chemicals. BSA (fraction V, fatty acid-free),

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‖ Abbreviations: DDD, 1-(2, 3 or 4-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichlorethane; DDE, 1-(2, 3 or 4-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethylene; DDA, 1-(2, 3 or 4-chlorophenyl)-1-(4-chlorophenyl)acetic acid; α-OH-DDD, 1-(2, 3 or 4-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethanol; α-OH-*o,p'*-DDA, 2,4'-dichlorobenzilic acid; *p*-OH-DDA, (2 or 3-chloro-4-hydroxyphenyl)-(4-chlorophenyl)acetic acid; and TCA, trichloroacetic acid.

glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type IX), HEPES, and $NADP^+$ were purchased from the Sigma Chemical Co. (St. Louis, MO). The liquid scintillant, Ecolite, was obtained from ICN (Irvine, CA) and the tissue solubilizer, Protosol, from New England Nuclear (Boston, MA).

The reference compounds, 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD, mitotane), bis(4-chlorophenyl)acetic acid (*p,p'*-DDA), 1,1-bis(4-chlorophenyl)-2,2-dichloroethane (*p,p'*-DDD) and 1,1-bis(4-chlorophenyl)-2,2-dichloroethylene (*p,p'*-DDE), were purchased from the Aldrich Chemical Co. (Milwaukee, WI). 2,4'-Dichlorodiphenylacetic acid (*o,p'*-DDA), 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethylene (*o,p'*-DDE) and 1-(3-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*m,p'*-DDD) were obtained from the Environmental Protection Agency (Research Triangle Park, NC). 1,1-Bis(4-chlorophenyl)-2,2-dichloroethanol (α-OH-*p,p'*-DDD), a gift from Rohm & Hass (Philadelphia, PA), was purified as previously noted [9], and our synthesis of 1-(4-hydroxy-2-chlorophenyl)-1-(4-chlorophenyl)acetic acid (*p*-OH-*o,p'*-DDA) has been reported [10]. 2,4-Dichlorobenzilic acid (α-OH-*o,p'*-DDA) was synthesized by the procedure of Inoi *et al.* [13].

The synthesis of the [^{14}C]DDD isomers by the condensation of ^{14}C uniformly labeled chlorobenzene with 2,2-dichloro-1-(2, 3 or 4-chlorophenyl)ethanols was as previously reported [14]. The specific activity and GC analysis of purity for these isomers were: *o,p'*-DDD, 83.2 μCi/mmol, 94.0%; *p,p'*-DDD, 99.9 μCi/mmol, 99.4%; and *m,p'*-DDD, 95.5 μCi/mmol, 97.8%.

In vitro *bovine adrenal metabolism*. Bovine adrenal glands obtained from a local slaughterhouse were trimmed of fat and connective tissue, and sliced open; cortex was separated from medulla. The cortices from four glands were weighed, minced and homogenized in 5 vol of 0.25 M sucrose–0.05 M HEPES buffer (pH 7.4) with a Teflon–glass Potter–Elvehjem homogenizer. The mixture was centrifuged at 1000 *g* at 4° for 15 min. The fat on top of the supernatant and tissue debris were discarded.

The whole homogenate was further centrifuged at 10,000 *g* for 20 min to precipitate mitochondria, which were washed in sucrose–HEPES buffer and recentrifuged. To obtain the same adrenal wet weight suspension of mitochondria, a volume of buffer was added to the washed pellet to equal the weight of the original homogenate. The 10,000 *g* supernatant was then recentrifuged at 109,000 *g* at 4° for 60 min to obtain the microsomal and cytosolic fractions. The microsomal pellet was washed, recentrifuged and adjusted with buffer to the same concentration as the original homogenate. The homogenate fractions were stored at −80° prior to use. Protein concentrations were measured by the Lowry method as modified by Miller [15] with BSA as the standard.

Incubations were carried out in 15-mL centrifuge tubes. The incubation mixture consisted of the following: (A) 1 mL of 2% BSA in 0.25 M sucrose–0.05 M HEPES buffer (pH 7.4) containing 0.17 mg of [^{14}C]*o,p'*-DDD (0.14 mM, 0.045 μCi) or 0.14 mg of [^{14}C]*p,p'*-DDD (0.11 mM, 0.045 μCi) or 0.15 mg of [^{14}C]*m,p'*-DDD (0.12 mM, 0.045 μCi) in 10 μL ethanol; (B) 0.5 mL of 44 μmol/mL $MgCl_2$; (C) 0.5 mL of NADPH generating mixture (3.77 μmol of $NADP^+$, 44 μmol of glycose-6-phosphate, 2.3 units of glucose-6-phosphate dehydrogenase in sucrose–HEPES buffer); and (D) 2 mL enzyme suspension.

The mixtures were incubated at 37° for 17 hr before freezing in dry ice acetone and drying overnight in a freeze drier (Virtis, Consol 12). Each dried incubation mixture was moistened with 0.2 mL of water, acidified with 1 mL of 10% TCA before the addition of methanol (5 mL), vortexed and centrifuged (1500 *g*). The TCA residue was remixed with methanol (5 mL), and the combined extracts were evaporated to dryness under nitrogen. The washed TCA precipitates were saved for binding studies, while their extraction residues were used for HPLC analysis. These residues were redissolved in 100–120 μL of a methanol stock solution containing reference compounds (DDA, α-OH-DDD, DDD and DDE: 4, 1.5, 1.5 and 1 mg/mL). After vortexing and centrifuging, three aliquots (4 μL) were counted for radioactivity, and the extract (70–100 μL) was analyzed by reverse-phase HPLC as described below.

Covalent binding studies. The TCA precipitates were extracted further with methanol (2 × 5 mL) and suspended in 1 mL of 50% methanol. Protosol (1 mL) was added, and the mixture was shaken overnight in a 45° water bath to dissolve the residue. The sample was acidified with 0.5 mL of concentrated HCl and decolorized with 0.5 mL of 30% hydrogen peroxide. The mixture was transferred to a 20-mL scintillation vial, methanol (2 × 1 mL) was used to rinse the tube, and Ecolite (11.6 mL) was added to the vial before counting.

High performance liquid chromatography. A reverse phase columns (5 mm i.d. × 10 cm NOVAK-PAK C-18 Radial Pak Cartridge, Waters) with a radial compressor (Waters RCM-100), protected with a guard cartridge (C-18, 10 μM spherical, 3 cm × 4.6 mm RP-18, MCP™, Brownlee Laboratories) was used for the separation of metabolites. The solvent system employed in the separation was methanol–water–acetic acid (50 : 50 : 0.2) at a flow rate of 1.2 mL/min for 40 min and changed over a 10-min period to acetonitrile–water–acetic acid (60 : 40 : 0.2). The ratio of solvents in the mobile phase was adjusted for each individual column to obtain a constant retention time for DDA.

After the peak of DDD appeared, the ratio of solvents for acetonitrile–water–acetic acid was changed to 70 : 30 : 0.2 at the same flow rate to detect metabolites, *m,p'*-DDE and *p,p'*-DDE. Fractions (1-min) were collected in 7-mL scintillation vials, Ecolite (5.8 mL) was added, and radioactivity was measured by liquid scintillation counting.

Gas chromatography and mass spectrometry. A Finnigan 4021 GC/MS equipped with a fused silica column (3 m × 0.32 mm) coated with DB-5 was used to identify the metabolites collected from ten HPLC separations of whole homogenate incubations of *m,p'*-DDD. The column temperature was at 50° for the first 2 min and increased to 275° with a 25°/min program; the injection temperature was 200°. The filament was at 70 eV with the ion source temperature

Table 1. *In vitro* biotransformation of [14C]o,p'-DDD by fractions from the cortex of bovine adrenals

Fraction	% Metabolite formation and total recovery*						% Apparent binding†
	α-OH-DDA	p-OH-DDA	o,p'-DDA	α-OH-DDD	o,p'-DDE	Total recovery	
Whole homogenate	1.76 ± 1.16	3.20 ± 1.13	7.53 ± 0.86	0.26 ± 0.06‡	0.17 ± 0.06	95.26 ± 2.45	4.57 ± 0.89
Blank§	—‖	—	0.33	0.11	0.17	98.06	0.16
Cytosolic	—	—	0.42 ± 0.07	—	—	97.65 ± 0.37	0.57 ± 0.07
Blank	—	—	0.32	—	—		0.02
Mitochondrial	—	0.78 ± 0.13	4.40 ± 0.30	0.27 ± 0.17	0.21 ± 0.10	94.52 ± 2.17	2.15 ± 0.21
Blank	—	—	0.05	0.28	0.10	98.81	0.05
Microsomal	—	—	0.47 ± 0.10	—	—	96.33 ± 1.13	0.56 ± 0.19
Blank	—	—	0.24	—	—	98.58	0.01
Mitochondrial + cytosolic	—	2.11 ± 0.98	8.34 ± 0.44	0.52 ± 0.29	0.49 ± 0.23	94.43 ± 0.64	4.61 ± 0.90
Blank	—	—	0.08	0.19		98.73	0.07

* The mean ± SD (N = 3) percent of the radioactivity applied to HPLC analysis for individual metabolite peaks with corresponding blank values subtracted. The total recovery is the percent of the applied radioactivity represented by the sum of the metabolite peaks and recovered unmetabolized substrate.

† The mean ± SD (N = 3) percent of total substrate incubated that is bound to protein.

‡ Assignment of this peak is by comparison to retention times of the α-OH-DDD metabolites of the other isomers.

§ Enzyme preparations had been placed in boiling water prior to incubation.

‖ None detected.

Table 2. *In vitro* biotransformations of [^{14}C]*o,p*′-, *m,p*′- and *p,p*′-DDD by bovine adrenal cortex

Fraction	% Metabolite formation and total recovery*						% Apparent binding†
	α-OH-DDA	*p*-OH-DDA	DDA	α-OH-DDD	DDE	Total	
o,p′-DDD	1.76 ± 1.16	3.20 ± 1.13	7.53 ± 0.86	0.26 ± 0.06‡	0.17 ± 0.06	95.26 ± 2.45	4.57 ± 0.89
Blank§	—‖	—	0.33	0.11	0.17	98.73	0.16
m,p′-DDD	—	1.02 ± 0.26	3.08 ± 0.25	6.75 ± 0.32	2.14 ± 0.13	96.58 ± 0.37	2.42 ± 0.62
Blank	—	—	0.22	1.16	0.75	97.43	0.10
p,p′-DDD	—	—	1.09 ± 0.27	3.85 ± 0.34	0.83 ± 0.34	97.67 ± 0.49	0.91 ± 0.62
Blank	—	—	0.14	0.11	0.11	99.02	0.02

* The mean ± SD (N = 3) percent of the radioactivity applied to HPLC analysis for individual metabolite peaks with corresponding blank values subtracted. The total recovery is the percent of the applied radioactivity represented by the sum of the metabolite peaks and recovered unmetabolized substrate.

† The mean ± SD (N = 3) percent of total substrate incubated that is bound to protein.

‡ Assignment of this peak is by comparison to retention times of the α-OH-DDD metabolites of the other isomers.

§ Enzyme preparations had been placed in boiling water prior to incubation.

‖ None detected.

at 150°. Helium with 10 psi pressure was used as a carrier gas. The *m,p*′-DDA collected by HPLC from the ten separations of metabolites was methylated prior to analysis by GC/MS This was carried out in a diazomethane-generation apparatus (Aldrich, Z 10, 100-1) and by the general method described by Fales *et al.* [16].

Cell culture studies. The human adrenocortical carcinoma cell line NCI H-295 [17] was used as we previously described for mitotane [18] but with the modification that 2% BSA was added to the medium to solubilize the DDD isomers. The effects of the isomers upon cell proliferation and cortisol production were compared for this steroid secreting cell line.

RESULTS

The relative abundance of the major metabolites of *o,p*′-DDD formed during incubation in whole adrenal cortex homogenates and their subfractions, together with the total recovery of metabolites and original substrate, is shown in Table 1. In Table 2, the abundance of metabolites and total recovery of *o,p*′-DDD in the whole homogenate is compared with those of the *m,p*′- and *p,p*′-DDD isomers. There was reasonable total recovery of radioactivity as shown in these tables. This recovery with the absence of other radioactive chromatographic peaks is an indication of the stability of the metabolites and substrates under our incubation and isolation conditions. Assignment of HPLC peaks in these tables was based on co-chromatography with the following reference standards: for *o,p*′-DDD: *p*-OH-DDA, DDA and DDE; for *p,p*′-DDD: DDA, α-OH-DDD and DDE. In the absence of reference standards for the metabolites of *m,p*′-DDD, assignments for their HPLC peaks are on the basis of comparisons of mass spectra and the retention times (t_R) of these metabolites to those of the *o,p*′- and *p,p*′-DDD series. The assignments deduced for peaks (A–C) and the comparisons to reference data in bold face are as follows:

(A) *m,p*′-DDA vs ***p,p*′-DDA** (t_R: 51,**52.5** min) *m/z* (relative intensity) of the methyl esters: 296 (6,**5**) [M + 2]; 294 (10,**8**) [M]; 237 (65,**64**) [M + 2-$COOCH_3$]; 235 (100,**100**) [M-$COOCH_3$]; 201 (7,**6**) [M + 2-$COOCH_3$-HCl]; 199 (17,**29**) [M-$COOCH_3$-HCl]; 165 (43,**57**) [M-$COOCH_3$-Cl_2].

(B) α-OH-*m,p*′-DDD vs **α-OH-*p,p*′-DDD** (t_R: 54,**55** min) *m/z* (relative intensity): **253 (57) [M + 2-$CHCl_2$]**; 252 (39) [M + 2-CH_2Cl_2]; **251 (89) [M-$CHCl_2$]**; 250 (58) [M-CH_2Cl_2]; 141 (39,**32**) [M + 2-CH_2Cl_2-C_6H_4Cl]; 139 (100,**100**) [M-CH_2Cl_2-C_6H_4Cl]; 113 (9,**13**) [M + 2-CH_2Cl_2-C_6H_4Cl-CO); 111 (27,**38**) [M-CH_2Cl_2-C_6H_4Cl-CO].

(C) *m,p*′-DDE vs ***p,p*′-DDE** (t_R: 90,**86** min) *m/z* (relative intensity): 320 (40,**44**) [M + 4]; 318 (76,**81**) [M + 2]; 316 (65,**68**) [M]; 281 (11,**13**) [M-Cl]; 248 (70,**71**) [M + 2-Cl_2]; 246 (100,**100**) [M-Cl_2]; 212 (8,**6**) [M + 2-Cl_2-HCl]; 210 (22,**16**) [M-Cl_2-HCl]; 176 (48,**34**) [M + 2-Cl_2-2HCl].

The results for the three DDD isomers examined for their adrenalytic effects using the human adrenocortical carcinoma cell line NCI H-295 are shown in Table 3. The rank order of the effects of the DDD isomers upon cell proliferation and cortisol production was *o,p*′- > *m,p*′- > *p,p*′-DDD.

DISCUSSION

Mitotane metabolism in bovine adrenal subcellular fractions showed the majority of metabolic transformations as well as apparent covalent binding to occur in the whole homogenate with localization in the mitochondria (Table 1). These results are in agreement with previous reports citing the mitochondria as the primary source of enzymes for *in vitro* adrenal transformation of mitotane [5–8]. In addition to *o,p*′-DDA as the major metabolite, α-OH-DDA, *p*-OH-DDA and a small amount of *o,p*′-DDE metabolites were also observed. Although adrenal microsomes contain $P450_{C21}$ and $P450_{17\alpha}$, two cytochrome P450 isozymes involved in adrenal steroidogenesis, the biotransformation of mitotane in this fraction was negligible. This is in contrast to

Table 3. Cell growth and cortisol production of human adrenal cancer cells (NCI H-295) treated for 7 days with *o,p'*-DDD, *m,p'*-DDD or *p,p'*-DDD

Treatment	Cell proliferation (A_{520})*				Cortisol level (μg/dL)†			
(50 μM)	1	2	3	4	1	2	3	4
Zero time	0.143 ± 0.002	0.197 ± 0.004	0.379 ± 0.006	0.380 ± 0.008				
o,p'-DDD	0.238 ± 0.010	0.354 ± 0.009	0.870 ± 0.010	0.960 ± 0.009	0.77 ± 0.10	1.00 ± 0.24	6.66 ± 0.05	6.30 ± 0.10
m,p'-DDD	0.265 ± 0.008	0.429 ± 0.012	1.040 ± 0.014	1.100 ± 0.009	0.87 ± 0.13	1.28 ± 0.05	8.45 ± 0.02	9.63 ± 0.03
p,p'-DDD	0.353 ± 0.010	0.505 ± 0.012	1.160 ± 0.018	1.200 ± 0.012	1.11 ± 0.20	1.81 ± 0.05	11.6 ± 0.33	11.4 ± 0.29
Control	0.409 ± 0.019	0.544 ± 0.008	1.150 ± 0.021	1.230 ± 0.017	2.21 ± 0.35	4.52 ± 0.36	15.8 ± 1.39	16.4 ± 1.85

* Seven days before compound addition, H-295 cells were dispensed into 96-well plates, at 10,000 cells/well. At the beginning of the treatment period, the sulforhodamine B dye assay was performed on one plate to obtain the zero time absorbance (A_{520}) values (mean ± SEM, N = 24 wells), and the compounds were added to the remaining plate. At the end of the 7-day treatment period, the sulforhodamine B dye assay was performed on this plate. The A_{520} value shown for each compound and untreated control in experiments 1–4 is the mean ± SEM of 16 measurements (wells). All values have been corrected for the absorbance of blank well without cells.

† Cortisol measurements of the medium as previously described [18] (mean ± SEM, N = 4 wells). No cortisol was detected in the medium of the blank wells without cells.

liver microsomal incubations where the microsomal fraction is the major source of enzymes for transformations of *o,p'*-DDD [7].

The biotransformation of mitotane in whole homogenates was equal to that in the recombined mitochondria and cytosol, while mitochondria alone exhibited only about half of this level of activity (Table 1). A possible explanation for these results is the increase in the availability of mitotane to mitochondrial enzyme systems brought about by the addition of cytosol. Vahouny *et al.* [19] have reported that sterol carrier protein$_2$ present in cytosol caused the stimulation of cholesterol transport across mitochondrial membranes. This protein is reported to be identical to non-specific lipid transfer protein [20]. In our system, such carriers might serve as carriers of mitotane and enhance its mitochondrial metabolism.

Aliphatic oxidation to the acetic acid derivative, DDA, is the major route of transformation of *o,p'*-DDD in all the incubations (Table 1). Aromatic hydroxylation, which was accompanied by aliphatic oxidation to yield *p*-OH-DDA, was a lesser route of metabolism for both *o,p'*- and *m,p'*-DDD (Table 2). This is in agreement with previous *in vivo* [4, 5] and *in vitro* perfusion studies [10] for mitotane.

The major route for the formation of DDA is postulated to consist of β-hydroxylation followed by dehydrochlorination to yield an acyl chloride that in the presence of water would result in the formation of DDA (Fig. 1). This mechanism follows a common route of metabolism for the oxidative dehydrohalogenation of dihalogenated methine moieties [21] as has been studied extensively in the case of chloramphenicol [22, 23] and has been proposed as the major route of metabolism of *p,p'*-DDD to *p,p'*-DDA *in vivo* [24–26]. Also by analogy to the chloramphenicol studies, acyl chloride formation in the presence of a bionucleophile could result in covalent binding such as that noted for the DDD isomers in the present study (Tables 1 and 2).

o,p'-DDE, a known human metabolite of mitotane [27, 28], was also found in trace amounts in the incubations of *o,p'*-DDD (Table 1). An additional minor metabolite from *o,p'*-DDD was detected at a 4-min greater retention time than that of the major *o,p'*-DDA peak. On the basis of the similarity of the retention time to that of the reference α-OH-*p,p'*-DDD and the corresponding α-OH-*m,p'*-DDD isolated from our *m,p'*-DDD incubations, this metabolite is postulated to be α-OH-*o,p'*-DDD (Table 1).

The total yields of metabolites for the *o,p'*- and *m,p'*-isomers were similar, whereas that for the *p,p'*-isomer was significantly less. Also, there was a definite difference in the distribution of metabolites when the chloro substituent was moved from the ortho position. The resulting decrease in steric hindrance to oxidation at the benzylic α-carbon may account for the increased formation of α-OH-DDD. With this increase in α-OH-DDD for both the *m,p'*- and *p,p'*-isomers, there was also an increase in corresponding DDE formation (Table 2). These DDE metabolites could be formed as the dehydration products of their corresponding α-OH-DDD metabolites. This route would represent a deactivation

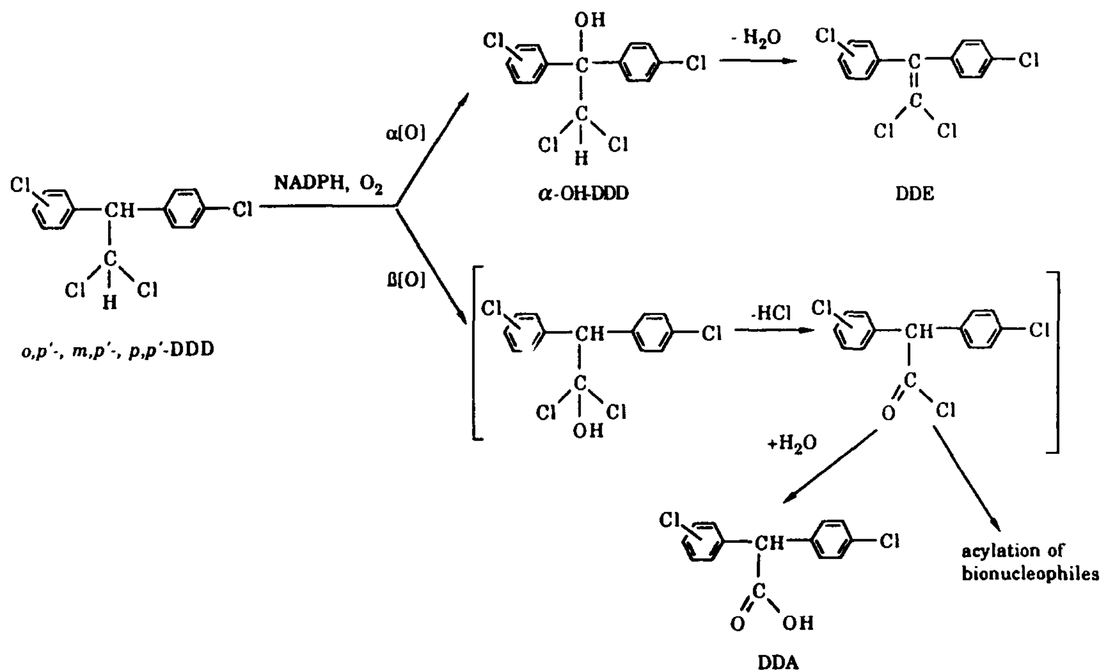

Fig. 1. Proposed pathways for the metabolism of DDD isomers through aliphatic oxidation.

pathway that would compete with metabolic activation leading to tissue binding, as proposed in Fig. 1.

An additional metabolite hydroxylated at the α-position of DDA, 2,4'-dichlorobenzylic acid (α-OH-DDA), was only detected for mitotane in the whole adrenocortical homogenates (Table 1). This metabolite has been found previously for *in vitro* adrenal perfusion studies [10]. While direct hydroxylation at the α-carbon as discussed above could contribute to the formation of α-OH-DDA, a more likely pathway would be through an epoxide intermediate as established for a secondary route of metabolism of p,p'-DDD *in vivo* [24, 25]. That is, dehydrochlorination of mitotane to 1-(4-chlorophenyl)-1-(4-chlorophenyl)-2-chloroethyene could occur followed by epoxidation of this unsaturated intermediate with subsequent hydrolysis and further oxidation at the β-carbon. If this dehydrochlorination pathway does occur with adrenal homogenates, it would be limited in m,p'- and p,p'-DDD metabolism by the increased competing direct α-hydroxylation of these isomers compared with mitotane and by the overall low level of p,p'-DDD metabolism. It is of interest in this connection that hydroylsis studies of the radioactivity bound to adrenal proteins with mitotane metabolism yields efficient recoveries of radioactivity in the form of o,p'-DDA with no α-OH-DDA detected [29]. This is in agreement with the acylation and not an epoxide pathway being the major contributor to protein binding.

In vivo studies, conducted in dogs as we previously described for mitotane [18], indicated that the m,p'-isomer retained adrenalytic activity while the p,p'-isomer did not. Nichols [30] in an earlier *in vivo* comparison of the three isomers reported that the order of the adrenalytic effect of the three isomers was $m,p' > o,p'$ with the p,p'-isomer having only marginal activity. Nichols proposed that this *in vivo* order of adrenalytic activity was related to the relative lipid solubility of the three isomers and their relative absorption after administration.

Differences in the availability to adrenal tissue among the o,p'-DDD isomers could be an important variable in their *in vivo* activity. Therefore, it was of interest to determine their relative activity at the cellular level. Towards this end, the activities of the three isomers were compared in the human adrenocortical carcinoma cell line NCI H-295. As noted in Table 3 for each of four experiments, the rank order for suppression of cell growth and cortisol production for the DDD isomers compared with their controls was $o,p' > m,p' > p,p'$. These results correlate to the extent of DDA formation and binding in incubations with adrenal cortex preparations, as summarized in Table 2. This correlation is consistent with the concept that the extent of β-hydroxylation, which leads to formation of a reactive intermediate, is an important factor in the adrenalytic activity of these isomers and should be considered in the design of an improved drug for the treatment of adrenocortical carcinoma.

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