Estrogenic Activity of o,p'-DDT Metabolites and Related Compounds

Joel Bitman,* Helene C. Cecil, Susan J. Harris, and Vernon J. Feil

Rat uterine glycogen responses were determined for 20 metabolites or compounds related to metabolites of o,p'-DDT to determine relationships of structure to estrogenic activity. Methoxy analogues of o,p'-DDA, o,p'-DDD, and o,p'-DDE and a glycine conjugate of o,p'-DDA exhibited only minimal estrogenic activity. Esterification of o,p'-DDA reduced this activity as did conjugation with serine. The presence of two methoxy groups rendered the o,p'-DDA completely inactive. Only o,p'-DDT analogues were active, demonstrating that a stable ethane chain was necessary. Activity was retained in the o,p'-DDT series if an hydroxy or methoxy group was present in the 3 or 4 position of the o-chloro ring. Estrogenic activity in decreasing order was: 3-hydroxy- > unsubstituted ≃ 4-methoxy- ≃ 5-hydroxy- > 5-methoxy-o,p'-DDT.

The estrogenic activity of commercial DDT has been demonstrated to be due to the presence of the ortho,para' isomer of DDT (Welch et al., 1969, Bitman, et al., 1968). The geometric similarity of the DDT molecule to the synthetic estrogen, stilbestrol, led us to examine DDT and 52 analogues, homologues, and structurally related compounds in an attempt to determine relationships of structure to estrogenic activity (Bitman and Cecil, 1970). We postulated that active estrogens would be derived from o,p'-DDT by metabolism of the ring bearing the ortho-chlorine atom and would be phenolic metabolites.

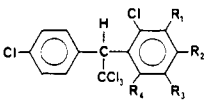
Whereas the metabolism of p,p'-DDT has been investigated in many species (Menzie, 1969; Matsumura, 1975), the metabolism of o,p'-DDT has received little attention. Recently, the metabolism of o,p'-DDT was studied in rats (Feil et al., 1973) and in chickens (Feil et al., 1975). After the administration of ^{14}C-labeled o,p'-DDT to rats, 13 compounds were identified in rat feces. After dosing with o,p'-DDT-^{14}C, 16 compounds were identified in chicken excreta. These studies demonstrated that the ortho chloro ring was extensively metabolized, whereas the para chloro ring remained intact (Feil et al., 1975). Eight of the 13 metabolites in rats and 13 of the 16 metabolites in chickens were hydroxy and methoxy compounds. Since certain of these compounds fit the theoretical requirements we had formulated for estrogenicity, we decided to test several of these metabolites to confirm relationships of structure to estrogenic activity. Several closely related analogues, also prepared synthetically, were tested because they may be metabolites at low concentrations even though they were not isolated as metabolites.

METHODS

We used the sensitive 18-h glycogen response of the rat uterus as a measure of estrogenic activty (Bitman et al., 1965; Cecil et al., 1971). The potency of active compounds is reported in terms of the minimal subcutaneous dose which will increase glycogen to a level significantly different from control. Test substances were dissolved in olive oil or an aqueous ethanol solution and injected subcutaneously into groups of six–eight rats at a screening dose rate of 8 mg/rat. Immature female Wistar rats (21–23 days old; 36–48 g) were killed 18 h after the injection; uteri were quickly excised, weighed, and analyzed for glycogen by the anthrone procedure (Seifter et al., 1950). Substances showing activity at the 8-mg screening level were successively tested at lower dosages, 4, 2, 1, etc. to 0.10 mg.

Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705 and Fargo, North Dakota 58102.

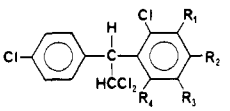
Table I. o,p'-Dichlorodiphenyl Trichloroethane Compounds with Substitutions in the o-chloro Ring



No.	Groups R1	R2	R3	R4	Name	Activity MED mg
1	H	H	H	H	o,p'-DDT	0.20
2	OH	H	H	H	3-hydroxy-o,p'-DDT	<0.10
3	H	OCH_3	H	H	4-methoxy-o,p'-DDT	0.20
4	H	H	OH	H	5-hydroxy-o,p'-DDT	0.20
5	H	H	OCH_3	H	5-methoxy-o,p'-DDT	0.80

MED = Minimum effective dose

Table II. o,p'-Dichlorodiphenyl Dichloroethane Compounds with Substitutions in the o-chloro Ring



No.	Groups R1	R2	R3	R4	Name	Activity MED mg
6	H	H	H	H	o,p'-DDD	I
7	H	H	OCH_3	H	5-methoxy-o,p'-DDD	8

MED = Minimum effective dose I = Inactive

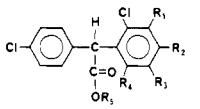
Positive responses were attributed to results showing statistical significance when analyzed by the Student's t test; data with a $P < 0.05$ were accepted as significant.

All metabolites were compared to o,p'-DDT, which was the standard for potency evaluation in the present study. A dose of 0.20 mg of o,p'-DDT was defined as the minimum effective dose (M.E.D). A dose–response curve for o,p'-DDT was published previously (Bitman and Cecil, 1970).

RESULTS AND DISCUSSION

The compounds evaluated in Table I are hydroxy- and methoxy-substituted derivatives of o,p'-DDT. The hydroxy compounds appear to be more active than the corresponding methoxy compounds (compound **4** vs. compound **5**). Hydroxylation at the 3 position (**2**) resulted in the only compound that was more active than the parent o,p'-DDT (**2**). Compound **2**, 3-hydroxy-o,p'-DDT, was a metabolite found in the feces of both rats and chickens when o,p'-DDT was administered. The other compounds tested were also prepared synthetically as potential metabolites but were not actually isolated after administration

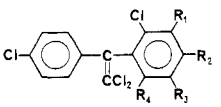
Table III. o,p'-Dichlorodiphenyl Acetic Acid Compounds with Substitutions in the o-chloro Ring



No.	Groups R1	R2	R3	R4	R5	Name	Activity MED mg
8	H	H	H	H	H	o,p'-DDA	8
9	OCH_3	H	H	H	H	3-methoxy-o,p'-DDA	8
10	H	H	OCH_3	H	H	5-methoxy-o,p'-DDA	8
11	OCH_3	OCH_3	H	H	H	3,4-dimethoxy-o,p'-DDA	I
12	OCH_3	H	H	H	CH_3	3-methoxy-methylester-o,p'-DDA	I
13	H	OCH_3	H	H	CH_3	4-methoxy-methylester-o,p'-DDA	I
14	H	H	OCH_3	H	CH_3	5-methoxy-methylester-o,p'-DDA	I
15	H	H	H	H	$NHCH_2COOH$	o,p'-DDA glycinate	8
16	H	H	H	H	$NHCH(CH_2OH)COOCH_3$	o,p'-DDA methyl serinate	I

MED = Minimum effective dose I = Inactive

Table IV. o,p'-Dichlorodiphenyl Dichloroethylene Compounds with Substitutions in the o-chloro Ring



No.	Groups R1	R2	R3	R4	Name	Activity MED mg
17	H	H	H	H	o,p'-DDE	4
18	OCH_3	H	H	H	3-methoxy-o,p'-DDE	8
19	H	OCH_3	H	H	4-methoxy-o,p'-DDE	8
20	H	H	OCH_3	H	5-methoxy-o,p'-DDE	8

MED = Minimum effective dose

of *o,p'*-DDT. These data in which the hydroxylated metabolite actually isolated was demonstrated to have high estrogenic activity support previous hypotheses for the configuration of the active estrogen structure arising from *o,p'*-DDT (Bitman and Cecil, 1970).

Two compounds with the dichloroethane chain were tested (Table II). *o,p'*-DDD, which was isolated from both rat and chicken feces, was inactive, and 5-methoxy-*o,p'*-DDD (**7**) was active only at the 8-mg dose level. Previously, *m,p'*-DDD and *p,p'*-DDD had been tested for estrogenic activity and were found to be inactive (Bitman and Cecil, 1970). We had concluded previously that rapid in vivo metabolism of compounds of this type was responsible for their lack of activity.

The compounds tested in Table III are *o,p'*-DDA derivatives. The presence of methoxy groups in the ortho chloro ring did not confer any greater estrogenic activity upon these compounds (**9** and **10**). Esterification of the acidic group of *o,p'*-DDA reduced this low level of activity (**12**, **13**, and **14**). The two conjugates tested, the glycinate of *o,p'*-DDA (**15**) and the serinate (**16**), were isolated from rat feces, but not from chicken feces, after the administration of *o,p'*-DDT. The glycinate showed only minimal activity and the serinate was inactive, again suggesting that methylation of the acidic group reduced activity.

Compounds containing the dichloro vinyl group showed only minimal activity (Table IV). *o,p'*-DDE, 3-hydroxy-, 4-hydroxy-, and 3-methoxy-4-hydroxy-*o,p'*-DDE were isolated from chicken feces but not from rat feces. The compounds evaluated in Table IV, containing methoxy substitutions at the 3, 4, or 5 positions, do not correspond exactly to the metabolites isolated by Feil et al. (1975). It seems likely, however, that the actual metabolites would have only slightly greater estrogenic activity, due to the greater activity of the hydroxy as compared to the methoxy group.

CORRELATIONS BETWEEN CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

We had previously postulated that active estrogens derived from *o,p'*-DDT would contain hydroxy or methoxy functions in the ortho chlorine ring. We have now tested for estrogenic activity several metabolites of *o,p'*-DDT isolated from rat and chicken excreta and several closely related analogues containing these functional groups. The data, in general, confirm previous observations regarding the structures necessary for estrogenic activity (Bitman and Cecil, 1970). Correlations of structure with activity were consistent with previously determined rules governing the estrogenic activity of diphenylethane compounds, i.e., compounds were active when a para or para' position was unoccupied or occupied by an hydroxy or methoxy group and a stable ethane chain was also found to be necessary for activity.

Recently, McBlain and Wolfe (1975) reported that *o,p'*-DDT was resolved into *d* and *l* enantiomeric forms. Subsequently, McBlain et al. (1976) demonstrated that the levorotatory enantiomer was the estrogenically active form. Only racemic compounds were used in the present study. Based upon this new work of McBlain and his associates (1975, 1976), the active optical isomer would exert much greater estrogenic activity than was exhibited by the racemic mixture in our study.

The biochemical mechanism by which *o,p'*-DDT exerts its estrogenic activity has not been elucidated. Several investigators have demonstrated by a variety of in vivo and in vitro tests that *o,p'*-DDT behaves in a similar manner to steroidal estrogens. Thus, Welch et al. (1969) showed that *o,p'*-DDT treatment caused a severalfold stimulation in the incorporation of glucose-*U*-^{14}C into lipid, protein, RNA, and acid-soluble constituents in the rat uterus. Singhal et al. (1970) also demonstrated an action on carbohydrate systems in vivo, showing that *o,p'*-DDT elevated uterine glycolytic and hexose monophosphate shunt enzymes in the rat. Kupfer and Bulger (1976) observed an increase in the induction of ornithine decarboxylase in the uterus after *o,p'*-DDT treatment of immature rats. In other in vivo studies it has been shown that *o,p'*-DDT competitively inhibits the uptake of labeled estradiol in the uterus (Welch et al., 1969) and that substances which block estrogen action, such as MER-25 or Actinomycin D, also inhibited *o,p'*-DDT action in the uterus (Cecil et al., 1971). Another aspect of the mechanism of estrogen action in the uterus has centered upon the initial step of binding of estradiol to the cytosolic uterine receptor. Nelson (1974) showed that *o,p'*-DDT inhibits the binding of labeled estradiol to rat uterine receptors. More recently, Kupfer and Bulger (1976) also demonstrated that *o,p'*-DDT inhibited the binding of [^3H]estradiol to rat uterine cytosol at various concentrations. It seems likely that active hydroxy or methoxy *o,p'*-DDT metabolites or the model compounds we studied in the current experiment would have exhibited inhibition equal to or greater than that reported.

It should be stressed that the estrogenicity ascribed to the *o,p'*-DDT metabolites in this report was determined by an in vivo test in which an increase in uterine glycogen was the criterion for estrogenicity. Chemicals testing negatively in this in vivo bioassay might well be estrogenic per se but might be rapidly metabolized and/or stored at sites other than the uterus. As pointed out by Kupfer and Bulger (1976), the structure–activity relationship observed in this study might represent an intrinsic property of the

parent compound, signifying the proper geometrical configuration for producing a glycogen response, or might denote the metabolism and pharmacokinetics of the compound and activity via a metabolite. Welch et al. (1969) and Bitman and Cecil (1970) suggested that active estrogens are probably derived from o,p'-DDT by metabolic conversion to a hydroxylated metabolite. The present study has demonstrated that such phenolic analogues are indeed estrogenically active.

This study has demonstrated the critical importance of the nature of the ethane chain for estrogenicity. The compounds studied have been considered in Tables I–IV according to the structure of this chain. Even though hydroxy and methoxy groups were present, estrogenic activity of any significance was only observed if the stable trichloroethane chain was also present (Table I). It is probable that rapid metabolism and excretion of nonactive compound occurs because of metabolism at the aliphatic chain.

Several structural requirements regarding estrogenic activity of the metabolites and model compounds which contained oxygen functions (hydroxy or methoxy) in the ortho chloro ring were apparent: (1) Only o,p'-DDT analogues exhibited high degrees of activity, demonstrating that a stable ethane chain was necessary. (2) Activity was present when the o,p'-DDT analogues contained an hydroxy or methoxy group in the ortho chloro ring. (3) Hydroxy groups conferred greater activity than methoxy substituents. (4) Methoxy analogues of o,p'-DDA, o,p'-DDD, and o,p'-DDE exhibited only minimal estrogenic activity at 10 to 20 times the dose levels of o,p'-DDT.

ABBREVIATIONS

Table I: o,p'-DDT, 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane; 3-hydroxy-o,p'-DDT, 1,1,1-trichloro-2-(2-chloro-3-hydroxyphenyl)-2-(4-chlorophenyl)ethane; 4-methoxy-o,p'-DDT, 1,1,1-trichloro-2-(2-chloro-4-methoxyphenyl)-2-(4-chlorophenyl)ethane; 5-hydroxy-o,p'-DDT, 1,1,1-trichloro-2-(2-chloro-5-hydroxyphenyl)-2-(4-chlorophenyl)ethane; 5-methoxy-o,p'-DDT, 1,1,1-trichloro-2-(2-chloro-5-methoxyphenyl)-2-(4-chlorophenyl)ethane.

Table II: o,p'-DDD, 1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane; 5-methoxy-o,p'-DDD, 1,1-dichloro-2-(2-chloro-5-methoxyphenyl)-2-(4-chlorophenyl)ethane.

Table III: o,p'-DDA, 2-(2-chlorophenyl)-2-(4-chlorophenyl)acetic acid; 3-methoxy-o,p'-DDA, 2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)acetic acid; 5-methoxy-o,p'-DDA, 2-(2-chloro-5-methoxyphenyl)-2-(4-chlorophenyl)acetic acid; 3,4-dimethoxy-o,p'-DDA, 2-(2-chloro-3,4-dimethoxyphenyl)-2-(4-chlorophenyl)acetic acid; 3-methoxy-methylester-o,p'-DDA, methyl 2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)acetate; 4-methoxy-methylester-o,p'-DDA, methyl 2-(2-chloro-4-methoxyphenyl)-2-(4-chlorophenyl)acetate; 5-methoxy-methylester-o,p'-DDA, methyl 2-(2-chloro-5-methoxyphenyl)-2-(4-chlorophenyl)acetate; o,p'-DDA glycinate, N-(2,4'-dichlorodiphenyl acetyl)glycine; o,p'-DDA methyl serinate, methyl N-(2,4'-dichlorodiphenyl acetyl)serinate.

Table IV: o,p'-DDE, 1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethylene; 3-methoxy-o,p'-DDE, 1,1-dichloro-2-(2-chloro-3-methoxyphenyl)-2-(4-chlorophenyl)ethylene; 4-methoxy-o,p'-DDE, 1,1-dichloro-2-(2-chloro-4-methoxyphenyl)-2-(4-chlorophenyl)ethylene; 5-methoxy-o,p'-DDE, 1,1-dichloro-2-(2-chloro-5-methoxyphenyl)-2-(4-chlorophenyl)ethylene.

Sources of the compounds used in this study were: No. **1**, **6**, **17**, Aldrich Chemical Co., Inc., Milwaukee (purity: 99+%); **2–5**, **7–16**, **18–20**, Feil et al. (1973, 1975).

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Received for review April 5, 1977. Accepted August 29, 1977.