Journal of Steroid Biochemistry, 1973. Vol. 4, pp. 585–591. Pergamon Press. Printed in Great Britain

COMPLEXING OF DDT AND O,P'DDD WITH ADRENAL CYTOCHROME P-450 HYDROXYLATING SYSTEMS

RICHARD B. YOUNG, MELVIN J. BRYSON, MAX L. SWEAT, and JOSEPH C. STREET*

Department of Obstetrics and Gynecology, Departments of Obstetrics and Gynecology and Biopharmaceutical Sciences, University of Utah, Salt Lake City, Utah 84112; and the Department of Chemistry and Biochemistry,* Utah State University, Logan, Utah, U.S.A.

(*Received* 12 *March* 1973)

SUMMARY

The chlorinated hydrocarbon pesticides, DDT and o,p'DDD were examined to determine their influence on adrenocortical steroid biosynthesis. Steroid 11β- and 21-hydroxylation, and cholesterol side chain cleavage were inhibited to significant degrees when the pesticides were present in preparations of bovine adrenocortical mitochondrial or microsomal cytochrome P-450. Spectrophotometric studies show that spectra induced in bovine mitochondrial and microsomal cytochrome P-450 preparations closely resemble those induced in the same preparations by steroid substrates. The influence of these pesticides was also studied in adrenal glands from rats on diets containing small quantities of DDT. Steroid 11β-hydroxylation was markedly reduced in these studies as with *in vitro* studies with the partially purified bovine cytochrome P-450 system.

The studies implicate an interaction between DDT or o,p'DDD and adrenocortical cytochrome P-450 which inhibits steroid hormone formation.

INTRODUCTION

SINCE Nelson and Woodward[1] observed pathological changes induced in the cortex of the dog adrenal gland by feeding o,p'DDD (1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane), a wide range of parameters relevant to inhibition of adrenal corticosteroid synthesis have been explored. Previous observations in this laboratory have shown that metyrapone, a widely employed clinical agent known to inhibit steroidogenesis, complexes with the terminal oxidase, cytochrome P-450[2]. While surveying various compounds which are known to inhibit steroidogenesis, an investigation was initiated to determine whether o,p'DDD or DDT (1,1,1-trichloro-2, 2-bis(p-chlorophenyl) ethane) also influence adrenocortical steroid hormone synthesis through complexing with cytochrome P-450.

Studies to the present show that DDT and o,p'DDD associate themselves with at least three enzymic steps involved in human and bovine adrenal steroid hormone synthesis[3]. These mechanisms afford a basic explanation for many of the physiological changes induced in the intact animal exposed to chlorinated hydrocarbons. Hart *et al.*[4] have confirmed our original findings that o,p'DDD blocks steroid C-11β-hydroxylation, although they were unable to extend their work to show the effect on the basic cytochrome P-450 system.

METHODS

1. *Collection and preparation of tissue*

Bovine adrenal glands collected at a local slaughterhouse were transported to a laboratory on ice, trimmed of fat, and demedullated. The cortices were minced in

a meat grinder, then homogenized in 0·25 M sucrose (20% suspension) for 50 sec with a Waring Blendor. All preparative procedures were performed at 0–5°.

(a) *Preparation of mitochondria*

The homogenate from the Waring Blendor was centrifuged at 600 *g* for 10 min to remove the cellular debris and nuclei. The supernatant was filtered through two layers of cheesecloth to entrap yellow fat which appeared on the homogenate surface during centrifugation, then centrifuged at 9000 *g* for 20 min to sediment the mitochondria. The mitochondrial pellet was suspended in 0·25 M sucrose (10% suspension), sedimented at 9000 *g* for 20 min, resuspended in a minimal amount of distilled water and treated with cold acetone (−5°) to form an acetone powder [5] which was stored at −80°C.

Endogenous cholesterol and other steroids exist in varying quantities in adrenal mitochondria. Consequently, when [4-^{14}C-] cholesterol is employed in mitochondrial incubation studies, side chain cleavage cannot be detected due to dilution by endogenous cholesterol. By employing acetone powder, endogenous cholesterol is eliminated and side chain cleavage of exogenous [4-^{14}C]-cholesterol can be readily demonstrated as previously described [6].

(b) *Preparation of microsomes*

The homogenate from the Waring Blendor was initially centrifuged at 700 *g* for 15 min to remove nuclei and cellular debris. The supernatant fraction was filtered through cheese cloth and centrifuged at 7000 *g* for 30 min, then at 14,500 *g* for 30 min to remove the heavy and light mitochondria, respectively. Centrifugation of the resulting supernatant at 105,000 *g* for 1 h yielded a microsomal pellet which was finally suspended in 0·25 M sucrose and employed in the incubation and spectrophotometric studies.

2. *Preparation of mitochondrial and microsomal cytochrome P-450 and P-450 reductase*

The mitochondrial acetone powder extract was prepared by suspending 10 grams of the powder in 100 ml of 0·001 M sodium-potassium phosphate buffer, pH 7·4, sonicating with a MSE ultrasonic disintegrator at 10 KC for 10 min, and centrifuging at 105,000 *g* for 1 h to remove fragments of the organelles. Centrifugation of the supernatant at 198,000 *g* for 2 h sedimented the cytochrome P-450, leaving the adrenal flavoprotein and nonheme iron protein in the supernatant fraction. The nonheme iron protein and flavoprotein moieties were resolved from the supernatant as previously described [5].

The cytochrome P-450 fraction was washed by resuspension in 100 ml of 0·001 M sodium-potassium phosphate buffer (pH 7·4), sonicated at 10 KC (maximum output) for 1 min, and recentrifugation at 198,000 *g* for 2 h. The final sediment was suspended in 50 ml of 0·001 M phosphate buffer and sonicated for clarification. The preparation was diluted to contain 2·5 mg protein/ml. Protein determinations throughout were performed with the Lowry method [7].

Purification of microsomal cytochrome P-450 beyond that reported under B-"Preparation of microsomes" has not been possible. Experiments requiring microsomal P-450 preparations were diluted to contain 8 mg protein/ml.

3. In vivo *studies with rats treated with DDT*

84 Holtzmann rats averaging 110 grams were divided into 4 groups: male-control, male-treated, female-control, female-treated. The treated rats were fed 47 days with food containing 50 ppm of purified p,p'DDT. Total DDT consumed per treated animal averaged 45 mg per male and 40 mg per female. Food consumption was uniform throughout the 4 groups, and no significant difference in weight gain between the groups was noted. No deaths occurred in the study, nor was there any manifestation of toxicity in the treated animals.

At the end of 47 days, no significant difference in adrenal weights was observed. The adrenals were excised, weighed rapidly, frozen immediately with liquid nitrogen, pooled into groups and stored at − 120°. The adrenals were minced in the frozen state, placed in 10 volumes of 0·001 M sodium–potassium phosphate buffer (pH 7·4) and homogenized in an all-glass homogenizer. The homogenate was centrifuged at 600 *g* for 10 min to sediment cellular debris. Capacity of the homogenate 11β-hydroxylation was assessed by incubation procedures described elsewhere in the text. Samples of the pooled adrenals were analyzed for chlorinated hydrocarbon content (Table 2) following published procedures [8, 9].

4. *Incubation of preparations*

Cholesterol side chain cleavage and steroid 11β-hydroxylation occurs in cytochrome P-450 preparations of adrenocortical mitochondria fortified with nonheme iron protein and adrenal flavoprotein [6]. Steroid carbon 21-hydroxylation occurs in adrenal microsomal preparations. Quantities of these preparations with the enzymic capacity sufficient to effect a 60% conversion of steroid substrate in control flasks under the conditions described elsewhere were employed in these studies.

Radiochemically pure ^{14}C-steroids obtained from New England Nuclear Co., with the following specific activities, were utilized in incubations.

Substrate (4^{14}C)	Am't Used	Specific Activity	Reaction Monitored
Cholesterol	1·0 n mol	21·5μ C/μ mol	Side chain cleavage
DOC	56·2 n mol	0·2μ C/μ mol	11β-hydroxylation
17α-OHP	3·0 n mol	21·5μ C/μ mol	21-hydroxylation

All incubation media employed in these studies consisted of 33 mM sodium-potassium phosphate buffer (pH 7·4), 1 mM NADP, 1 mM glucose-6-phosphate, 0·5 Kornberg unit glucose-6-phosphate dehydrogenase, and 0·5 mM $MgCl_2$. Total volume per incubation flask was 1·0 ml. Incubations were performed at 37° in air for 15 min. Cholesterol side chain cleavage and steroid C-11β-hydroxylation were examined by adding an equivalent of 2·4 mg protein mitochondrial cytochrome P-450, 0·5 mg nonheme iron protein, and 0·5 mg protein adrenal flavoprotein preparations. Capacity for steroid C-21-hydroxylation was evaluated by adding microsomal preparation equivalent to 1·5 mg protein. Varying concentrations of DDT and o,p'DDD ranging from 1×10^{-3}M to 1×10^{-7}M were introduced into the media to determine quantities necessary for 50% inhibition of the particular reaction being examined. It is emphasized, however, that these compounds are highly insoluble in an aqueous media. It is likely that much of the pesticides employed remains inaccessible as insoluble crystals.

5. *Extraction and analytical procedures*

Incubations were terminated by the addition of 5 ml of chloroform and extracted with 7 additional 5 ml portions. The chloroform was evaporated to dryness under a stream of nitrogen and the residue chromatographed in Zaffaroni systems for paper chromatography[10]. Products from [4-^{14}C]-cholesterol, pregnenolone and progesterone, were chromatographed in the hexane-formamide chromatographic system; the product from [4-^{14}C]-17α-hydroxy-4-pregnen-3, 20-dione, 17α, 21-dihydroxy-4-pregn-3, 20 dione, was chromatographed in the benzene-formamide system. The product of [4-^{14}C]-21-hydroxy-4-pregnen-3,20-dione incubation, corticosterone (11β, 21-dihydroxy-4-pregnene-3,20-dione), was also chromatographed in the benzene–formamide system.

The extracted steroids were located on the paper chromatograms by means of a Micromil window gas-flow scanner with rate-meter and recorder. Quantitation was accomplished by calculation of the areas underlying the recorded peaks. Confirmatory identification of the product was attained by crystallization to constant activity, as described elsewhere[6]. Spectrophotometric data were obtained with a Cary model 15 dual beam recording spectrophotometer.

RESULTS AND DISCUSSION

Data in Table I illustrate the inhibitory effects of DDT and o,p'DDD on three separate enzymatic reactions involved in the sequential pathway of adrenal steroid hormone synthesis. Associated with each of these steps (C-11β-hydroxylation, C-21-hydroxylation, and cholesterol side chain cleavage) is a cytochrome P-450 terminal oxidase found in adrenal mitochondria and microsomes. The influence of o,p'DDD or DDT on 18-hydroxylation, an intermediate step in the formation of aldosterone from DOC, was not evaluated in these studies as low temperature storage of rat adrenals has been shown in this laboratory to consistently disrupt capacity for 18-hydroxylation.

Figure 1 shows the spectral responses occurring when DDT and o,p'DDD are introduced into preparations of mitochondrial cytochrome P-450. Identical spectra are induced in microsomal cytochrome P-450 preparations, but are of a lower magnitude. As DDT and o,p'DDD induce spectra similar to those induced by steroid substrates in these cytochrome P-450 preparations, it is likely that cytochrome P-450 is the pigment responding to these compounds. Schenkman, *et al.*[11] have also reported a DDT-induced difference spectrum in cytochrome

Table 1. Inhibition of Steroid Hormone Synthesis by DDT and O, P'DDD

Steroid synthesizing enzyme system (bovine)	Concentration of pesticide required for 50% inhibition*	
	DDT	o,p'DDD
Cholesterol side chain cleavage	$1{\cdot}25 \times 10^{-5}$ M	4×10^{-5} M
Steroid C-11β-hydroxylation	$1{\cdot}25 \times 10^{-5}$ M	4×10^{-5} M
Steroid C-21-hydroxylation	$3{\cdot}7 \times 10^{-4}$ M	6×10^{-4} M

*Concentrations of bovine mitochondrial or microsomal cytochrome P-450 and reductase, and DDT or o,p'DDD are described under Methods.

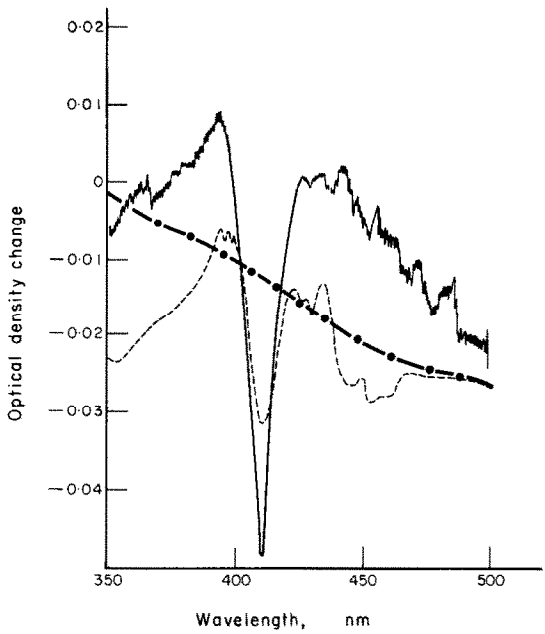

Fig. 1. Mitochondrial cytochrome P-450 preparation equivalent to 2·5 mg protein per ml was added to sample and reference cuvettes, and a baseline recorded (-.-.-.-.-.-.). Excess crystalline DDT was introduced into the sample cuvette and alternately shaken and scanned spectrophotometrically until the maximal induced difference spectrum was observed (_____). The cuvette was emptied and refilled with additional preparation. Excess crystalline o,p'DDD was introduced into the sample cuvette, and the difference spectrum (----------) was recorded.

P-450 preparations of the liver. If DDT is generally associated with this ubiquitous terminal oxidase, the physiological implications are undoubtedly extensive.

When rats were fed a diet containing purified p,p'DDT, the capacity for steroid C-11β-hydroxylation was markedly altered (Table II) with a significant diminution of 11β-hydroxylation occurring in the treated groups. Synthesis of corticosterone from 11β-deoxycorticosterone was diminished by approximately 50 percent in the animals receiving the designated quantity of chlorinated hydrocarbon in their diet. It is noteworthy that DDT and analogues were found to have accumulated in the adrenals of treated rats (Table 2). These pesticides associate themselves with the lipid component of tissues, and the adrenal cortex is distinctively involved with a number of aspects of lipid metabolism.

As other inhibitors of steroidogenic enzymes have been reported generally to cause adrenal atrophy or hypertrophy, the fact that there was no distinction in

Table 2. Steroid 11β-hydroxylation in rat adrenal homogenates from DDT-treated and control rats

Sex	Treatment	11β-hydroxylase Activity[1]	Inhibition %	Pesticide Content ppDDE	ppDDD	ppDDT
Female	control	24·6 n moles/mg protein		4ppB	10ppB	30ppB
Female	treated	12·1 n moles/mg protein	50·8	46ppB	85ppB	454ppB
Male	control	22·4 n moles/mg protein		8ppB	49ppB	100ppB
Male	treated	15·4 n moles/mg protein	31·2	37ppB	71ppB	580ppB

*Mean value of triplicate assay. Control values were typical for this tissue.

adrenal weight gain observed between treated and control rats deserves comment. It is suggested that, if the amounts of pesticides were elevated or the period of treatment was extended to toxicity levels, weight would likely have been altered. However, as stated previously, there were no deaths or manifestation of toxicity in the animals studied. Results within the magnitudes in which the experiments were performed afford additional evidence that DDT and o,p'DDD exert their influence by interfering directly within the chain of steroid hormone synthesis and not indirectly as a general or diffuse disruption of non-specific somatic cellular entities.

Since our original report[3] that DDT and o,p'DDD inhibit steroid 11β-hydroxylation, Hart *et al.*[4] have also reported a partial blockade of 11β-hydroxylation by o,p'DDD. However, their studies were limited to examination of the intact 11β-hydroxylation system in adrenal slices, making it impossible to distinguish between the basic cytochrome P-450 system and the supporting systems. Both our original and current studies have employed discrete components of the basic steroid hormone synthesizing systems. Not only o,p'DDD, but also DDT have been associated with adrenocortical cytochrome P-450. The current *in vivo* studies are consistent with the concept that accumulation of these pesticides within the adrenals would result in a diminution of steroid hormone synthesis.

It has long been recognized that DDT and o, p'DDD exert an influence on the adrenal gland. Morphological and physiological studies have been extensive. From the results of the present study, it appears that a basic enzymic interaction on the molecular level has been identified. Association of this group of chlorinated hydrocarbons with steroid hydroxylating and cholesterol side chain cleavage enzymic mechanisms offers a partial explanation concerning eggshell fragility and other reproductive problems in predacious birds. Disruption of estrogen synthesis at the cholesterol side chain cleavage step resulting in a curtailment of estrogen is likely one of the fundamental problems.

The diminution of steroid hormones observed in animals exposed to DDT or o,p'DDD has often been attributed to hepatic microsomal enzyme induction[12, 13] and adrenal atrophy[12, 14]. Accelerated hepatic biodegradation of steroid hormones, coupled with the diminution of steroid hormone synthesis is perhaps a more complete explanation concerning steroid hormone depletion in exposed animals. The current discovery that chlorinated diphenylmethane pesticides impair steroid hormone synthesis by disrupting a basic respiratory system not only serves as a point of embarkation for future investigations of the influence of these compounds in animal species, but also suggests that their well known influence on carbon fixation (photosynthesis) may be through prototype systems of plant respiration.

ACKNOWLEDGEMENTS

These studies were supported by Grant AM-01803 from the National Institute of Arthritis and Metabolic Diseases, and by Grant HD-00472 from the National Institute of Child Health and Human Development, National Institutes of Health, U.S. Public Health Service. The authors also wish to express appreciation to the Utah Community Study on Pesticides, Utah State Health Department, for the pesticide analyses.

REFERENCES

1. Nelson A. A. and Woodward G.: *A.M.A. Arch. Path.* **48** (1949) 387.
2. Sweat M. L., Young R. B. and Bryson M. J.: *Archs. Biochem. Biophys.* **130** (1969) 66.

3. Young R. B., Bryson M. J. and Sweat M. L.: *Utah Acad. Proc.* **47** (1970) 281.
4. Hart M. M., Swackhamer E. S. and Straw. J. A.: *Steroids* **17** (1971) 575.
5. Sweat M. L., Dutcher J. S., Young R. B. and Bryson M. J.: *Biochemistry* **8** (1969) 4956.
6. Bryson M. J. and Sweat M. L.: *J. Biochem.* **243** (1968) 2799.
7. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. Biochem.* **193** (1951) 265.
8. Radomski J. L. and Rey A.: *J. chrom. Sci.* **8** (1970) 108.
9. Dale W. E., Curley A. and Cueto C.: *Life Sciences* **5** (1966) 47.
10. Zaffaroni A.: *Recent Prog. Horm. Res.* **8** (1953) 51.
11. Schenkman J. B., Remmer H. and Estabrook R. W.: *Molec. Pharmacol.* **3** (1967) 113.
12. Bledsoe T., Island D. P., Ney R. L. and Liddle G. W.: *J. clin. Endocrl. Me'ab.* **24** (1964) 1303.
13. Peakall D. B.: *Nature* **216** (1967) 505.
14. Zimmerman B., Bloch H. S., Williams W. L., Hitchcock L. R. and Hoelscher B.: *Cancer* **9** (1956) 940.