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EFFECT OF PESTICIDE INTERACTIONS UPON THE REPRODUCTIVE SYSTEM

by

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report evaluates the effects of different pesticides upon the mammalian reproductive system, and to what extent these agents can induce changes in biochemical and hormonal activities.

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PREFACE

Metabolic interactions can occur between a variety of chemicals in the environment (xenobiotics) that can produce biologic effects different from those caused by the compounds individually. Many of these effects have been found to be mediated by alterations in the hepatic microsomal enzyme system(s). Changes in the activities of the microsomal enzymes can affect the biologic activity of xenobiotics, such as pesticides, herbicides, carcinogens, drugs, and hormones. Hormonal imbalances may be magnified by pesticide-induced changes in certain enzyme systems.

While the volume of literature regarding the effects of a single pesticide upon the male reproductive system of mammals continues to increase, few studies have been devoted to investigating the simultaneous effects of more than one pesticide.

The present report reveals the interaction of major classes of pesticides on hormone metabolism, and attempts to explain molecular actions of certain pesticides by demonstrating their affinity for hormone receptor molecules.

ABSTRACT

The metabolism of 1,2-^{3}H-testosterone *in vitro* was studied in prostate glands and livers of rats and mice treated with different pesticides including dieldrin and parathion. The metabolism of 1,2-^{3}H-testosterone (T-^{3}H) *in vitro* by mouse anterior prostate glands or hepatic microsomes has been studied after the oral administration of dieldrin (2.5 mg/kg daily x 5 or 10) and/or parathion (1.3,2.6,or 5.2 mg/kg daily x 5 or 10). T-^{3}H metabolism in the prostate was unaffected by the various treatment regimens. Dieldrin (10 days) caused some reduction in the microsomal production of androstenedione-^{3}H but failed to affect the biotransformation to androstanediol-^{3}H or dihydrotestosterone-^{3}H. Only treatment regimens with dieldrin stimulated hepatic testosterone hydroxylases; parathion alone had no effect. This study revealed that dieldrin and parathion can interact and produce biological effects different from those caused by either pesticide alone.

Liver microsomal steroid hydroxylating enzymes and prostatic testosterone-5α-reductase were studied in rat and mouse. Organochlorine and organophosphate pesticides tended to inhibit liver steroid hydroxylations, while carbofuran slightly stimulated them. Neither species was consistently more sensitive to pesticide effects than the other. All the pesticides (*viz*, heptachlor, carbofuran, diazinon and parathion) bound to cytochrome P-450, producing type I spectral charges. Values of Ks ranged from 1.9 to 8.7mM for organochlorine and organophosphate compounds. Affinity for carbofuran was much lower (Ks=100-200mM).

The binding of [^{3}H] dihydrotestosterone ([^{3}H]DHT) to cellular components from prostate, seminal vesicles, kidney, and liver of the male mouse was studied using a dextran-coated charcoal method to separate bound steroid from free steroid. Optimum conditions for binding include incubating tissues from animals 3 days postcastration for 12 hr at 0°C. Separation of bound steroid from free steroid was found to be optimal when the samples were incubated with the charcoal suspension for 15 min. Two components of DHT binding were found in all tissues studied, but a higher capacity was noted in androgen target tissues such as the prostate gland. The high affinity binding was also very specific for DHT as evidenced by competition studies employing various hromones, such as estriol, corticosterone, estrone, progesterone, estradiol, cyproterone acetate, testosterone, and DHT. The effects of various pesticides of ^{3}H-DHT binding in these tissue cytosols were also assessed. Parathion (10^{-8} - 10^{-5}M) was found to be an effective inhibitor of total ^{3}H-DHT binding in the prostate, seminal vesicle, kidney and liver. This organophosphate was unable to compete with ^{3}H-DHT for cytosol binding sites in the intestine. Similar *in vitro* binding studies using bieldrin, DDT, or carbaryl failed to reveal any interference with ^{3}H-DHT binding in any of the tissues studied. The mechanism of parathion's interference with ^{3}H-DHT binding in unclear.

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ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

DHT	--	dihydrotestosterone
^3H	--	tritium
S.E.M.	--	standard error of mean
$\bar{X}$	--	mean
dpm	--	disintegrations/minate
p.o.	--	per os, oral administration
K_a	--	binding affinity (liters/mol x 10^9)
$\pm$	--	plus one and minus one S.E.M.

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SECTION I

INTRODUCTION

The presence of many chemical classes of pesticides in the environment offers numerous opportunities for metabolic interactions to occur in various species of animals including man. Investigations have demonstrated that many environmental chemicals (xenobiotics) can interact to produce effects different from those effects produced by the compounds individually (Richardson et al., 1952; Conney et al., 1956; Conney, 1967; Schein and Thomas, 1976). Many of these pesticide interactions have been found to be due to altered hepatic microsomal enzyme activity (Conney and Burns, 1972; Krampl et al., 1973).

Such pesticide interactions may result in lowered chronic toxicity of certain pesticide combinations (DuBois, 1969; Murphy, 1969). Endocrine imbalances may be caused due to pesticide-induced alterations in certain enzymes (Peakal, 1967; Street et al., 1969). Methoxychlor decreases the storage of dieldrin in the body fat of rats (Cueto and Hayes, 1965). Joint lethality of DDT and dieldrin appeared to be additive in both Japanese quail and ring-necked pheasants (Kreitzer and Spann, 1973).

Although there has been an increasing interest in studying the effects of single pesticides on the mammalian reproductive system, few investigations have examined the simultaneous effects of more than one pesticide upon the male reproductive system. DDT and dieldrin have been shown to interfere with male rodent sex accessory gland metabolism of testosterone (Smith et al., 1972; Schein and Thomas, 1975). Studies by Wakeling and Visek (1973) have shown that o,p-DDT can inhibit the binding of dihydrotestosterone to specific receptor proteins in the cytoplasmic fraction of the rat prostate gland.

Various effects of pesticides have been demonstrated on hepatic metabolism of androgens in rodents. Studies by Kuntzman et al. (1966) have shown that DDT treatment caused a marked increase in testosterone 16α-hydroxylase activity in immature male rats. In the mouse DDT-inhibited hepatic microsomal testosterone 16α-hydroxylase activity while dieldrin stimulated this activity. Dieldrin stimulated the 16α- hydroxylase to a much greated extent than either the 6β- or 7α-hydroxylase (Thomas and Lloyd, 1973).

The presence of parathion has been shown to inhibit oxidation of hexobarbital and hydroxylation of aniline by mouse hepatic microsomes in vitro (Stevens et al., 1971; Welch et al., 1967). On the other hand, administration of one-half the oral LD_{50} of this pesticide to mice for 5 consecutive days reduced hexobarbital sleeping time, indicating stimulation of oxidation by hepatic microsomal enzymes (Stevens et al., 1972). Thomas and Schein (1974) using oral doses of one-sixteenth to one-fourth the LD_{50} demonstrated no alterations in mouse hepatic microsomal androgen hydroxylase activity. Swann et al. (1958) reported that the acute toxicity of parathion was related to the sex of the animal, and that sexual maturity conferred added protection against the toxic action of this organophosphate.

SECTION 2

CONCLUSIONS

1. Dieldrin and/or parathion can affect androgen metabolism in the prostate gland and the liver, but the extent of this effect is related to the dose and duration of the pesticide(s).

2. Both organochlorine-type and organophosphate-types pesticides tend to inhibit androgen hydroxylation reactions in hepatic microsomes.

3. Several pesticides such as heptachlor, carbofuran, diazinon and parathion can bind in vitro to hepatic cytochrome P-450 producing type I spectral changes.

4. Isolation of a cytosolic protein reveals that in some tissues, it not only has an affinity for hormones and certain drugs, but also for pesticides.

5. The effects of a single pesticide on hormone metabolism and the reproductive system are different than those occurring from the administration of more than one pesticide.

SECTION 3

RECOMMENDATIONS

1. Efforts should continue to disclose the toxicological effects of the different chemical classes of pesticides upon the reproductive system, particularly since so little is known about their molecular mechanism(s) of action(s).

2. Further consideration should be given to correlating pesticide-induced changes in different hormone target organs with the entire endocrine system.

3. Studies must continue to be devoted to examining the effects of new (and even "old") pesticides on the male reproductive system with some consideration devoted to spermatogenesis, fertility and possible teratogenicity.

SECTION 4

MATERIALS AND METHODS

METHODS

Animals--Male Swiss-Webster mice and sprague-Dawley rats (Hilltop Laboratory Animals; Scottsdale, PA) were acclimated for at least two weeks in the Medical Center Animal Quarters and were maintained on a standard diet of laboratory chow (Purina laboratory chow) and water ad libitum. Castrations were performed via abdominal incision under pentobarbital anesthesia.

Preparation of cytosol--All preparative procedures were performed at 0° to 4°C. Immediately upon sacrifice of the animals by cervical dislocation, whole anterior prostate glands, seminal vesicles, kidneys, and samples of liver were excised, blotted, and placed temporarily in 0.05 M Tris-HCl (pH 7.2) containing 0.60 mM Na_2EDTA and 2.6 mM mercaptoethanol (Tris/EDTA buffer). Tissues were subsequently blotted, weighed, and homogenized (Kontes Teflon-glass homogenizer, 15-20 manual strokes) in 10 vol of ice-cold Tris/EDTA buffer. The prostate and seminal vesicle were chopped (McIlwain tissue chopper) just before homogenization. Homogenates were centrifuged(2000g for 20 min), and the supernatants were decanted and recentrifuged (100,000g for 60 min) on a Beckman Model L ultracentrifuge. The resulting supernatants were designated as the cytosols or cytoplasmic fractions.

Steroid binding--Purity of the [^{3}H] DHT was assessed using thin-layer chromatography with a chloroform: ether (7:3, v/v) solvent system and was considered acceptable only when it exceeded 95%. Stock solutions of [^{3}H] DHT in ethanol/benzene were dried under N_2 and redissolved in an appropriate volume of Tris/EDTA buffer immediately before each experiment.

Quantities of [^{3}H] DHT (sp. act. 80 Ci/mmol; New England Nuclear, Boston, Mass.) were incubated with cytosol prepared in Tris/EDTA buffer in a total volume of 0.45 ml. The concentrations of cytosol protein, [^{3}H] DHT, and competing steroid were among the experimental variables studied, as were the duration of incubation and the incubation temperature. Corticosterone, cyproterone acetate, dihydrotestosterone, estradiol, estrone, progesterone, and testosterone were used as competing steroids. Samples of stock solutions of these compounds in methanol were dried under nitrogen in the incubation tubes before cytosol protein and [^{3}H] DHT were added. Final concentrations of competing steroids were varied from 10^{-8} to 10^{-6}M.

Separation of bound and free steroid--To remove free steroid from the incubation mixture and allow the counting on only the bound steroid, a dextran-coated charcoal method was employed (Binoux and Odell, 1973). A suspension of activated charcoal (0.25%, w/v) was prepared in 0.9% NaCl containing 0.025%

dextran T-70 and stirred at 0-4°C for at least 1 hr. After incubation of cytosol and [^{3}H] DHT, 1.0 ml of the charcoal suspension was added. The duration of incubation was varied from 1 to 15 min. The charcoal and the free steroid adsorbed from the solution were sedimented by centrifugation (1000g for 10 min), and the supernatants were decanted into scintillation vials.

Initial studies established the conditions for the optimum removal of free steroid by adsorption to charcoal and consistently revealed that no additional steroid was removed after 10 min of exposure to charcoal. Therefore, a 15-min charcoal incubation interval was routinely employed. No so-called "stripping" of steroid from protein binding sites occurred during this 15-min incubation period. Suitable blank tubes assessed the efficiency of removal of unbound steroids, and revealed that more than 98% of the free [^{3}H] DHT was removed under these experimental conditions.

Cytochrome P-450--Difference spectra were recorded in a Cary 15 spectrophotometer using hepatic microsomal suspensions at approximately 2 mg protein/ml at room temperature. Pesticides were added to the sample cuvette in acetone solutions; acetone alone was added to the control cuvette. Total acetone added never exceeded 1%. Spectral dissociation constants (K_S) were calculated by the method of Schenkman, et al. (1967). Cytochrome P-450 was determined from CO difference spectra (Omura and Sato, 1964).

Tissue Protein--Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Incubation techniques--Twenty-four hours after the last daily pesticide dose, animals were sacrificed by cervical dislocation and anterior prostate glands and samples of liver were rapidly excised. The liver samples were homogenized in ice-cold 0.154 M KCl-0.05 M Tris HCl (pH 7.4). Cell debris, nuclei, and mitochondria were removed by centrifugation of the homogenate at 10,000g for 25 minutes. Microsomes were sedimented from the postmitochondrial supernatant at 100,000g for 60 minutes, then resuspended in cold buffer.

Whole prostates or aliquots of hepatic microsomes (about 600 µg of protein) were incubated with 1,2-^{3}H-testosterone (New England Nuclear, sp. act. 40 Ci/mmole, 98.5%) in 0.1 M sodium phosphate (pH 7.4) containing 4 mM NADP, 5 mM glucose-6-phosphate, 5 mM MgSo4, 4 mM nicotinamide, and 5 I.U./ml of glucose-6-phosphate dehydrogenase. Incubations were carried out aerobically in a total volume of 1 ml at 37°C with shaking.

Incubations of hepatic microsomes were terminated by adding cold chloroform-ether (7:3). Prostate incubations were stopped by immersing the tissue in cold 0.4 N perchloric acid and simultaneously adding chloroform-ether to the medium. The prostate tissues were homogenized and radiometabolites of ^{3}H-T extracted from these homogenates as well as the incubation mixtures with chloroform-ether. The extracts were evaporated to dryness under nitrogen and redissolved in 500 µl of chloroform.

Twenty-five-microliter samples of the redissolved extracts were spotted on prepared thin-layer chromatography plates (Eastman Company) or on hand-made Silica Gel-G plates. Chromatograms were developed with chloroform-ether (7:3) and spots visualized by iodine vapor (nonpolar metabolites) on ultraviolet light

(polar metabolites). Spots were scrapped from the plates and radioactivity determined by a Packard Tri-Carb Scintillation Counter. These methods are described in detail elsewhere (Schein and Thomas, 1976).

Liquid scintillation counting--Quantitation of radioactivity was carried out in scintillation counting solution containing 0.05 g POPOP, 4 gm PPO, and 200 ml of Beckman Biosolv in 1 liter of toluene. Samples were counted on a Packard Tricarb scintillation spectrometer at an efficiency of 25 to 30%, using [^{3}H] toluene (New England Nuclear) as an external standard. All data were expressed in dpm or moles of [^{3}H] DHT.

Statistics--Data were analyzed by Dunnett's method for comparing multiple treatment means (Dunnett, 1955). Scatchard analyses (1949) were calculated for several tissues to determine binding affinity of both hormones and pesticides.

Chemicals and Pesticides--Solutions of dieldrin [1,2,3,4,10,10a-hexachloro-6,7-epoxy-1,4 4a, 5,6,7,8,8a-octahydroendoexo-1,1,4:5,8-dimethanonaphthalene] (K & K Laboratories, Plainfiew, NY., 95-99%) and parathion [0,0-diethyl-O-(p-nitrophenyl) ester phosphothioic acid] (City Chemical Corp., NY, 98.76%) were prepared in corn oil. Daily doses of dieldrin (2.5 mg/kg) and/or parathion (1.3,2.6 or 5.2 mg/kg) were administered by gastric intubation in a volume of 0.1 ml. Methoxychlor (DuPont), carbofuran (FMC), and diazinon (CIBA-Geigy) were received from the Environmental Protection Agency. Henry K. Suzuki, Velsicol Chemical Corporation, Chicago, kindly supplied a sample of chlordane. DDT was obtained from Nutritional Biochemical Corporation. Cyproterone Acetate (1,2α-methylene-6-chloro-Δ4,6-pregnadien-17βol 3,20-dion-17α-acetate) was obtained from Schering Corporation. DHT (5α-androst-2-ane-17β-ol) and corticosterone (4-pregnene-11β, 21-diol-3,20-dione) were obtained from Sigma Corporation. Dextran was purchased from Pharmacia Fine Chemicals, and scintillation fluors and toluene from Fisher Scientific Company. All other reagents were obtained commercially from Sigma Chemical Corporation.

SECTION 5

RESULTS

Regardless of the dose or dose sequence, neither parathion nor dieldrin caused any significant changes in prostate gland weights (Table 1). Similarly these dose regimens produced no significant alterations in hepatic microsomal proteins. Thus, these dosing protocols represent no-effect levels insofar as weights and protein levels are concerned.

Testosterone-^{3}H metabolism by the prostate gland was largely unaffected by the pesticide regimens (Table 2). There was an unexplained increase in production of androstanediol-^{3}H in a dieldrin-parathion group, but it is evident that the enzymes catalyzing formation of the major nonpolar radiometabolites of testosterone-^{3}H are not substantially altered by the organochlorine-or the organophosphate dose regimens.

Unlike the prostate gland (Table 2), hepatic microsomal testosterone-^{3}H metabolism was extensively altered by certain of the pesticide regimens (Table 3A). With the exception of dieldrin plus the highest dose of parathion, a 10-day regimen of dieldrin significantly reduced the formation of androstenedione-^{3}H ($P<0.05$). The highest dose of parathion administered concomitantly with dieldrin appeared to abolish or negate the inhibitory effect of the organochlorine on formation of androstenedione-^{3}H. Testosterone-^{3}H levels were generally lower in those groups receiving dieldrin for a period of 10 days. Again, the highest dose of parathion seemed to counteract the lowering effects of the dieldrin upon testosterone-^{3}H levels. Neither androstanediol-^{3}H nor dihydrotestosterone-^{3}H was significantly affected by the different pesticide dose regimens.

The most conspicuous alterations produced by parathion and/or dieldrin were observed in the hepatic polar metabolites (Table 3B). Of the three testosterone hydroxylase activities examined, two (the 6β- and 7α-hydroxylases) were significantly stimulated by several treatment regimens. These stimulatory effects most consistently seen on the 6β- and 7α-hydroxylases were not restricted to either dieldrin or to parathion, but were recorded after all combined treatments as well as after administration of dieldrin only. The greatest stimulatory effect appeared to occur in the 7α-hydroxytestosterone levels. No significant changes were evident in the 16α-hydroxytestosterone-^{3}H levels. It is apparent that only some of the androgen hydroxylases present in hepatic microsomes (viz., 6β- and 7α-hydroxytestosterone) were affected by these dose regimens of pesticides.

TABLE 1

DIELDRIN (D) AND/OR PARATHION (P) EFFECTS ON PROSTATE WEIGHT AND HEPATIC PROTEIN OF THE MOUSE[a]

Ten-day treatment regimen										Prostate wt (mg)	Hepatic microsomal protein (mg/g)
1	2	3	4	5	6	7	8	9	10		
C	C	C	C	C	C	C	C	C	C[b]	20.10 ± 1.53[c]	4.05 ± 0.36
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	22.82 ± 1.21	4.10 ± 0.31
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	17.70 ± 0.62	4.20 ± 0.24
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	18.92 ± 0.94	4.37 ± 0.28
D	D	D	D	D	D	D	D	D	D	18.62 ± 0.51	4.66 ± 0.31
P_1	P_1	P_1	P_1	P_1	D	D	D	D	D	16.76 ± 1.42	4.26 ± 0.22
P_2	P_2	P_2	P_2	P_2	D	D	D	D	D	17.02 ± 0.76	4.29 ± 0.41
P_3	P_3	P_3	P_3	P_3	D	D	D	D	D	16.02 ± 1.42	3.85 ± 0.22
D	D	D	D	D	P_1	P_1	P_1	P_1	P_1	17.82 ± 0.69	3.78 ± 0.44
D	D	D	D	D	P_2	P_2	P_2	P_2	P_2	18.26 ± 0.46	3.09 ± 0.17
D	D	D	D	D	P_3	P_3	P_3	P_3	P_3	18.40 ± 1.13	3.68 ± 0.17
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	18.40 ± 0.13	4.20 ± 0.26
D	D	D	D	D	D	D	D	D	D		
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	19.04 ± 0.40	3.82 ± 0.17
D	D	D	D	D	D	D	D	D	D		
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	12.15 ± 1.91	4.62 ± 0.34
D	D	D	D	D	D	D	D	D	D		

[a] Dieldrin (D) (2.50 mg/kg daily × 5 or 10) and/or parathion (P) (P_1, 1.3; P_2, 2.6; P_3, 5.2 mg/kg daily × 5 or 10).

[b] Corn oil vehicle.

[c] $\bar{x}$ ± SEM of at least six samples.

TABLE 2

EFFECTS OF DIELDRIN (D) AND/OR PARATHION (P) ON THE 10-MINUTE *In Vitro* METABOLISM OF ^{3}H–TESTERONE BY THE MOUSE PROSTATE GLAND[a]

Ten-day treatment regimen										Radiometabolites (dpm/100 μg protein)			
										^{3}H–Testosterone	^{3}H–Androstanediol	^{3}H–Dihydrotestosterone	^{3}H–Androstenedione
1	2	3	4	5	6	7	8	9	10				
C	C	C	C	C	C	C	C	C	C[b]	544 ± 28[c]	215 ± 62	428 ± 45	43 ± 13
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	552 ± 116	220 ± 70	460 ± 82	30 ± 8
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	729 ± 127	198 ± 56	568 ± 94	40 ± 20
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	564 ± 96	179 ± 20	382 ± 58	38 ± 13
D	D	D	D	D	D	D	D	D	D	534 ± 52	212 ± 23	436 ± 72	38 ± 8
P_1	P_1	P_1	P_1	P_1	D	D	D	D	D	610 ± 62	174 ± 47	394 ± 45	44 ± 10
P_2	P_2	P_2	P_2	P_2	D	D	D	D	D	513 ± 69	216 ± 39	383 ± 14	30 ± 6
P_3	P_3	P_3	P_3	P_3	D	D	D	D	D	495 ± 59	295 ± 49	401 ± 48	49 ± 11
D	D	D	D	D	P_1	P_1	P_1	P_1	P_1	401 ± 23	369 ± 51[d]	389 ± 27	45 ± 10
D	D	D	D	D	P_2	P_2	P_2	P_2	P_2	575 ± 72	316 ± 52	391 ± 33	43 ± 17
D	D	D	D	D	P_3	P_3	P_3	P_3	P_3	556 ± 36	199 ± 22	398 ± 48	29 ± 8
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	465 ± 57	219 ± 46	370 ± 31	43 ± 8
D	D	D	D	D	D	D	D	D	D				
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	611 ± 40	200 ± 19	343 ± 26	40 ± 7
D	D	D	D	D	D	D	D	D	D				
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	614 ± 105	228 ± 50	395 ± 40	40 ± 10
D	D	D	D	D	D	D	D	D	D				

[a] Dieldrin (D) (2.50 mg/kg daily × 5 or 10) and/or parathion (P) (P_1, 1.3; P_2, 2.6; P_3, 5.2 mg/kg daily × 5 or 10).

[b] Corn oil vehicle.

[c] $\bar{x}$ ± SEM of at least six samples.

[d] Significantly different from control ($P < 0.05$).

TABLE 3

EFFECTS OF DIELDRIN (D) AND/OR PARATHION (P) ON THE 5-MINUTE *in Vitro* METABOLISM OF ^{3}H-TESTOSTERONE BY MOUSE HEPATIC MICROSOMES[a]

A. Nonpolar radiometabolites (dpm/μg microsomal protein)

Ten-day treatment regimen										^{3}H–Testosterone	^{3}H–Androstanediol	^{3}H–Dihydrotestosterone	^{3}H–Androstenedione
1	2	3	4	5	6	7	8	9	10				
C	C	C	C	C	C	C	C	C	C[b]	1550 ± 262[c]	178 ± 30	98 ± 19	1296 ± 299
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	1241 ± 148	152 ± 17	71 ± 9	782 ± 176
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	1384 ± 111	201 ± 36	84 ± 13	922 ± 190
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	1368 ± 54	180 ± 12	86 ± 7	937 ± 153
D	D	D	D	D	D	D	D	D	D	588 ± 111[d]	236 ± 30	89 ± 13	546 ± 46[d]
P_1	P_1	P_1	P_1	P_1	D	D	D	D	D	1134 ± 163	203 ± 20	109 ± 4	1128 ± 218
P_2	P_2	P_2	P_2	P_2	D	D	D	D	D	1180 ± 64	209 ± 13	116 ± 17	1210 ± 132
P_3	P_3	P_3	P_3	P_3	D	D	D	D	D	1235 ± 225	220 ± 67	112 ± 18	1169 ± 184
D	D	D	D	D	P_1	P_1	P_1	P_1	P_1	1756 ± 617	237 ± 9	134 ± 20	1504 ± 369
D	D	D	D	D	P_2	P_2	P_2	P_2	P_2	810 ± 81[d]	263 ± 12	94 ± 3	746 ± 42
D	D	D	D	D	P_3	P_3	P_3	P_3	P_3	909 ± 157[d]	231 ± 24	99 ± 16	1001 ± 141
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	592 ± 106[d]	286 ± 20	113 ± 8	603 ± 161[d]
D	D	D	D	D	D	D	D	D	D				
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	591 ± 60[d]	186 ± 10	82 ± 3	636 ± 61[d]
D	D	D	D	D	D	D	D	D	D				
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	1958 ± 647	242 ± 17	132 ± 10	1821 ± 466
D	D	D	D	D	D	D	D	D	D				

TABLE 3—*Continued*

B. Polar metabolites (dpm/μg microsomal protein)

										^{3}H-16α-Hydroxytestosterone	^{3}H-7α-Hydroxytestosterone	^{3}H-6β-Hydroxytestosterone
C	C	C	C	C	C	C	C	C	C[b]	462 ± 85	356 ± 59	255 ± 31
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	350 ± 5	371 ± 11	197 ± 28
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	413 ± 46	347 ± 74	394 ± 50
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	423 ± 38	362 ± 85	357 ± 43
D	D	D	D	D	D	D	D	D	D	305 ± 11	854 ± 90[d]	450 ± 27[d]
P_1	P_1	P_1	P_1	P_1	D	D	D	D	D	498 ± 36	618 ± 113[d]	391 ± 80
P_2	P_2	P_2	P_2	P_2	D	D	D	D	D	552 ± 45	544 ± 68[d]	456 ± 26[d]
P_3	P_3	P_3	P_3	P_3	D	D	D	D	D	475 ± 49	789 ± 73[d]	452 ± 89[d]
D	D	D	D	D	P_1	P_1	P_1	P_1	P_1	597 ± 43	906 ± 40[d]	542 ± 88[d]
D	D	D	D	D	P_2	P_2	P_2	P_2	P_2	544 ± 24	765 ± 138[d]	424 ± 61[d]
D	D	D	D	D	P_3	P_3	P_3	P_3	P_3	440 ± 68	794 ± 87[d]	459 ± 61[d]
P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	P_1	428 ± 35	706 ± 26[d]	520 ± 20[d]
D	D	D	D	D	D	D	D	D	D			
P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	P_2	406 ± 24	684 ± 20[d]	356 ± 45
D	D	D	D	D	D	D	D	D	D			
P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	P_3	300 ± 45	928 ± 125[d]	719 ± 90[d]
D	D	D	D	D	D	D	D	D	D			

[a] Dieldrin (D) (2.50 mg/kg daily × 5 or 10) and/or parathion (P) (P_1, 1.3; P_2, 2.6; P_3, 5.2 mg/kg daily × 5 or 10).
[b] Corn oil vehicle.
[c] $\bar{x}$ ± SEM of at least six samples.
[d] Significantly different from control ($P < 0.05$).

Pesticides were tested at three concentrations for their ability to inhibit ^{3}H-testosterone metabolism. With rat liver microsomes, none were particularly potent (Table 4). Heptachlor and methoxychlor significantly inhibited hydroxylation reactions at 10^{-4}M but not at 10^{-6}M or less. Carbofuran did not inhibit hydroxylations, but actually seemed to stimulate formation of 16α-hydroxytestosterone and 6β-hydroxytestosterone when present at low concentration. Organophosphates had mixed effects (Table 4).

Diazinon inhibited 16α hydroxylation at 10^{-4}M to a greater extent than it reduced formation of the other hydroxysteroids. Parathion, on the other hand, had no effect on 16α-hydroxylation but at 10^{-4}M severely reduced 7α and 6β hydroxylations.

A similar array of effects was seen when hepatic microsomes from mice were studied (Table 4). In general, the steroid hydroxylations were somewhat less sensitive to organochlorines than were these reactions in the rat. The exception was 16α and 6β hydroxylations which were inhibited by low concentrations of heptachlor. Carbofuran had the same tendency to slightly stimulate metabolism that it showed in the rat. Mouse microsomes were very sensitive to diazinon at all concentrations, but were nearly refractory to parathion even at 10^{-4}M.

Spectral changes produced by direct interaction of pesticides with hepatic microsomal cytochrome P-450 were measured because this hemoprotein is a part of the enzymic complex responsible for steroid hydroxylation. All the pesticides in this study produced typical Type I spectral changes with an absorbance peak at 385nm and a trough at 420nm.

Double reciprocal plots of the pesticide concentration dependence of the peak-trough difference showed complex interactions with rat (Figs. 1A and 2A) and mouse (Figs. 1B and 2B) microsomes. In both species, heptachlor appeared to bind at two sites, one having a high affinity and the other having low affinity (Fig. 1). The total capacity of the low affinity binding sites was about twice that of the high affinity type (Table 5). Methoxychlor also bound to sites in both species with affinities comparable to the high affinity heptachlor binding (Fig. 1). However, at concentrations greater than 2×10^{-5}M, methoxychlor apparently disrupts microsomal membranes with consequent loss of the Type I cytochrome P-450 spectrum. This change was accompanied by an increase in turbidity which was visible to the unaided eye and accounts for the increase in 1ΔA at low values of 1/S.

Carbofuran produced Type I spectral changes in both rat and mouse (Fig. 2), but only at concentrations greater than 10^{-4}M. The affinity for this compound was very low and the concentration of binding sites for it was also low (Table 5).

Organophosphates had complex binding properties. In both species, diazinon bound to one type of high affinity site and caused disruption of membranes and reversal of spectral changes when its concentration exceeded 10^{-4}M (Fig. 2). The only major differences between the species was in the binding of parathion (Fig. 2). Both mouse and rat liver microsomes showed high affinity binding with K_s approximately 7mM (Table 6). Only mice, however, had low affinity binding sites for parathion.

The pesticides studied had very little apparent effect on reduction of ^{3}H-testosterone to ^{3}H-dihydrotestosterone by prostate glands from these animals. Only mouse tissue showed reduced formation of ^{3}H-dihydrotestosterone and that only when $10^{-4}M$ heptachlor or parathion was present.

Chlordane showed similar inhibitory properties to the other organochlorines, that is, it inhibited microsomal hydroxylations in both species at high concentrations and showed weak inhibition of mouse prostate testosterone reduction. Since chlordane is a mixture of many components, its cytochrome P-450 binding spectra were not recorded.

TABLE 4

EFFECTS OF PESTICIDES ON FORMATION OF ^{3}H-HYDROXYTESTOSTERONE ISOMERS FROM ^{3}H-TESTOSTERONE BY RODENT LIVER MICROSOMES

	RAT			MOUSE		
	16α-OHT	7α-OHT	6βOHT	16α-OHT	7α-OHT	6β-OHT
Heptachlor						
$10^{-8}M$	82[A]	111	96	40	90	52
$10^{-6}M$	77	121	104	56	108	63
$10^{-4}M$	25	38	54	36	93	42
Methoxychlor						
$10^{-8}M$	85	113	93	104	93	84
$10^{-6}M$	79	120	106	100	111	100
$10^{-4}M$	25	37	53	63	85	84
Carbofuran						
$10^{-8}M$	294	93	150	139	86	104
$10^{-6}M$	224	83	149	141	84	117
$10^{-4}M$	137	83	114	173	84	130
Diazinon						
$10^{-8}M$	90	77	104	31	50	67
$10^{-6}M$	76	78	142	39	32	70
$10^{-4}M$	33	67	60	20	32	32
Parathion						
$10^{-8}M$	105	108	92	96	86	100
$10^{-6}M$	133	144	96	112	81	105
$10^{-4}M$	96	22	22	80	78	76

A. All values are expressed as % of control dpm/mg Protein/5min.

TABLE 5

BINDING PARAMETERS OF PESTICIDES IN RODENT LIVER MICROSOMES

A.	Ks (μM)	ΔAmax/nmP-450
Rat		
Heptachlor	2.8	14.3
	28	30.1
Methoxychlor	1.9	15.0
Carbofuran	100	10.5
Diazinon	3.6	28.9
Parathion	7.1	41.4
B.		
Mouse		
Heptachlor	8.7	14.3
	35	26.7
Methoxychlor	3.8	9.5
Carbofuran	200	14.1
Diazinon	2.0	26.7
Parathion	6.7	22.7
	42	38.6

FIGURE 1a

(Rat Liver Microsomes)

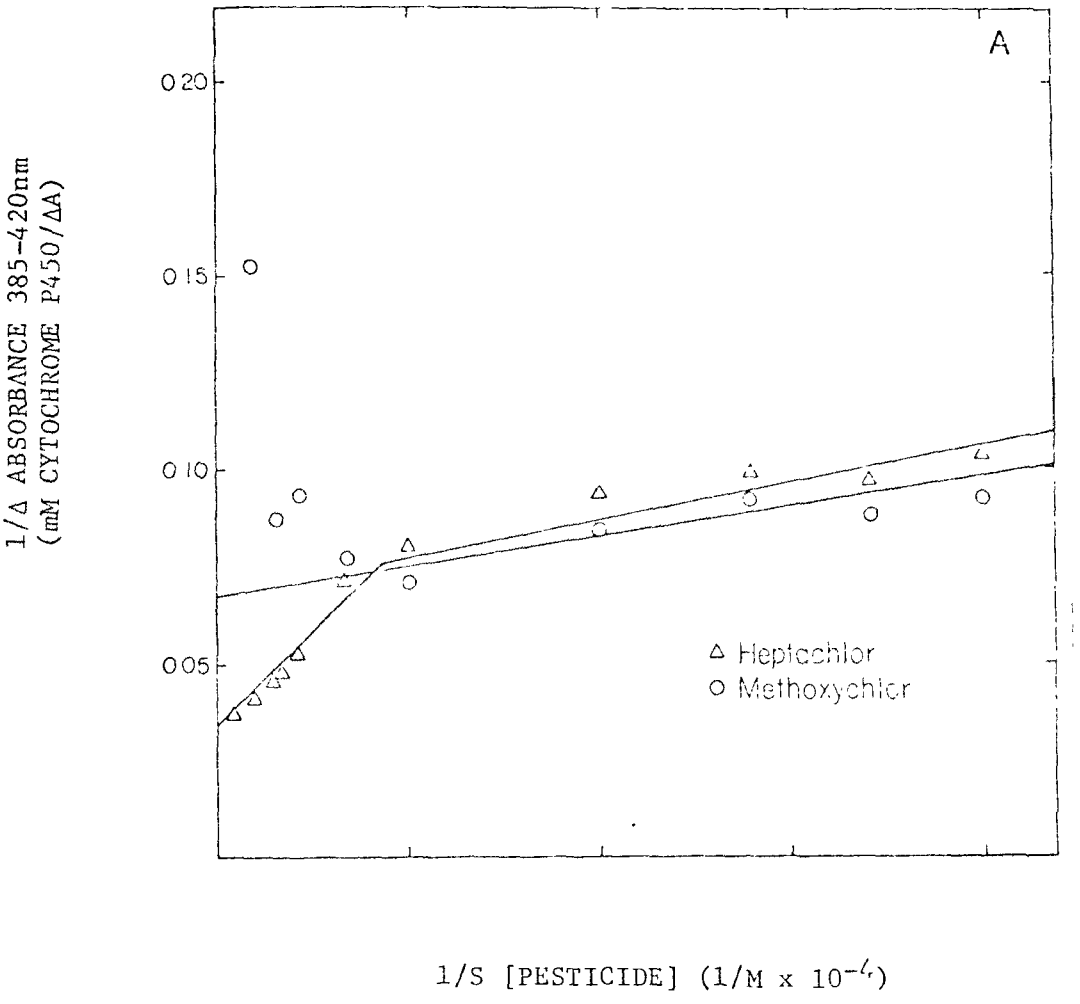


Figure 1. Double reciprocal plot of binding of organochlorine pesticides to rodent liver microsomes. Absorbance spectra were recorded using a sample containing 2 mg/ml microsomal protein plus pesticide and a reference containing only microsomes. The peak-trough difference (385-420 nm) was divided by the cytochrome P-450 concentration as determined from CO difference spectra and the reciprocal of this quantity was plotted as a function of the reciprocal of the pesticide concentration.

FIGURE 1b

(Mouse Liver Microsomes)

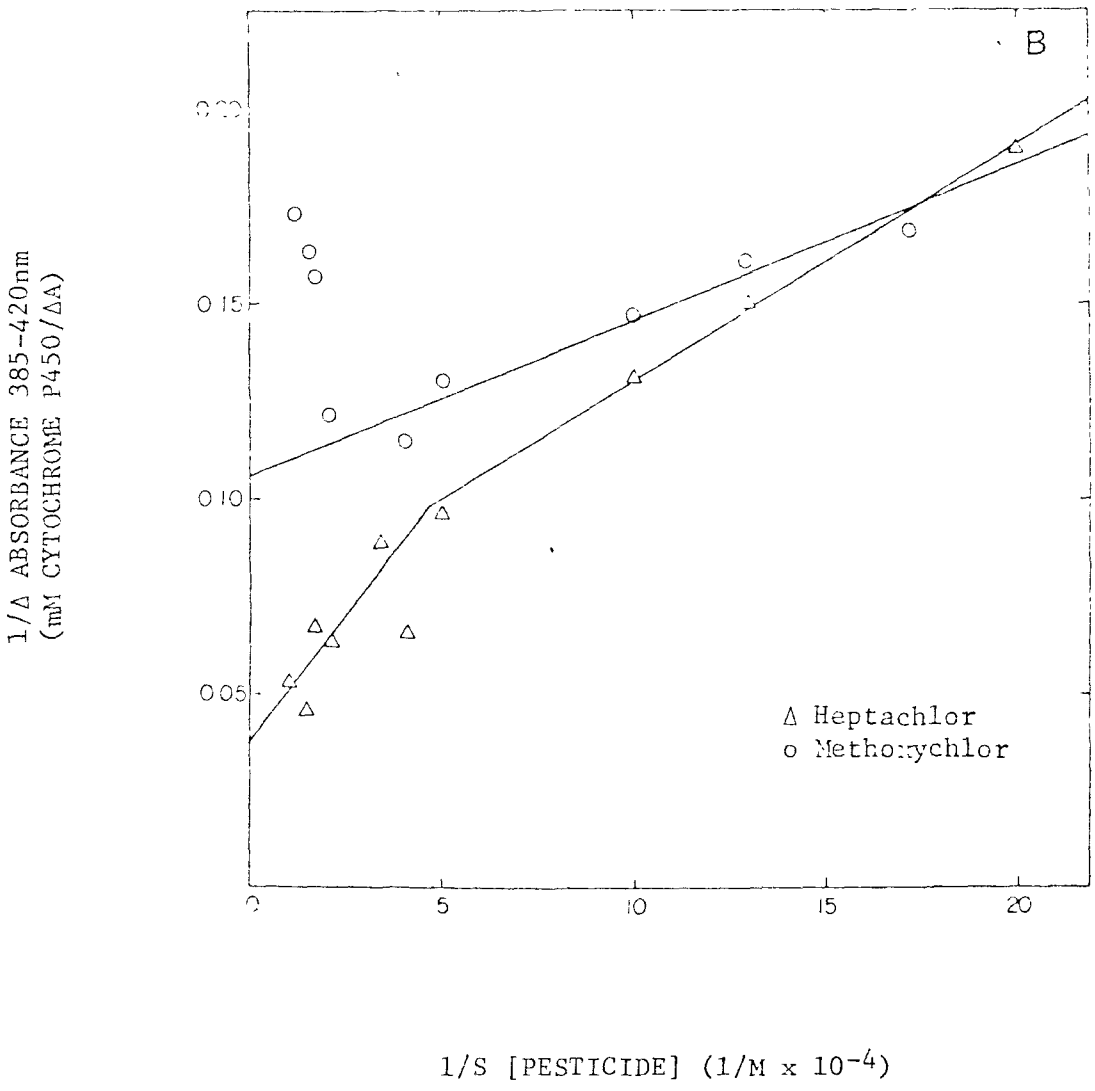


Figure 1. Double reciprocal plot of binding of organochlorine pesticides to rodent liver microsomes. Absorbance spectra were recorded using a sample containing 2 mg/ml microsomal protein plus pesticide and a reference containing only microsomes. The peak-trough difference (385-420 nm) was divided by the cytochrome P-450 concentration as determined from CO difference spectra and the reciprocal of this quantity was plotted as a function of the reciprocal of the pesticide concentration.

FIGURE 2a

(RAT LIVER MICROSOMES)

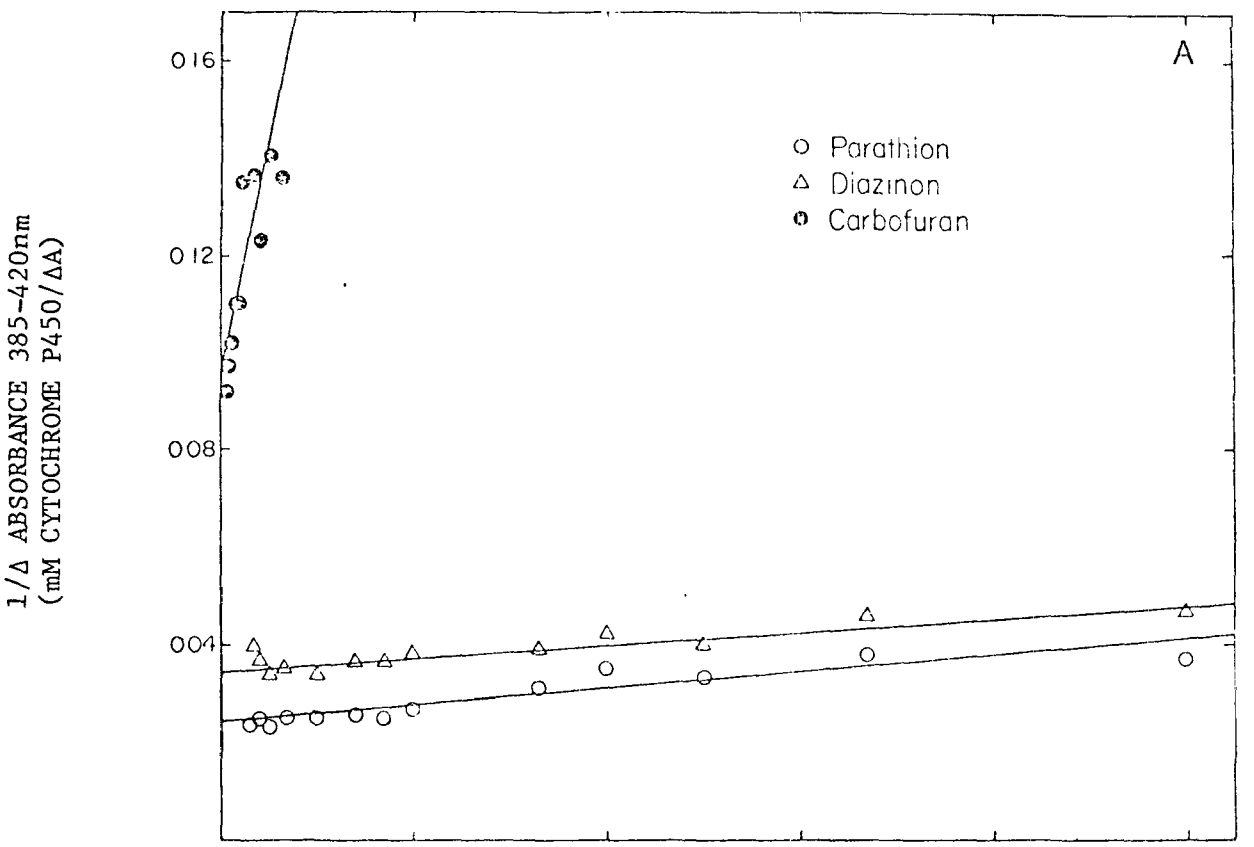


Figure 2. Double reciprocal plot of binding of organophosphate and carbamate pesticides to rodent liver microsomes. Conditions and calculations were as described in Figure 1.

FIGURE 2b

(MOUSE LIVER MICROSOMES)

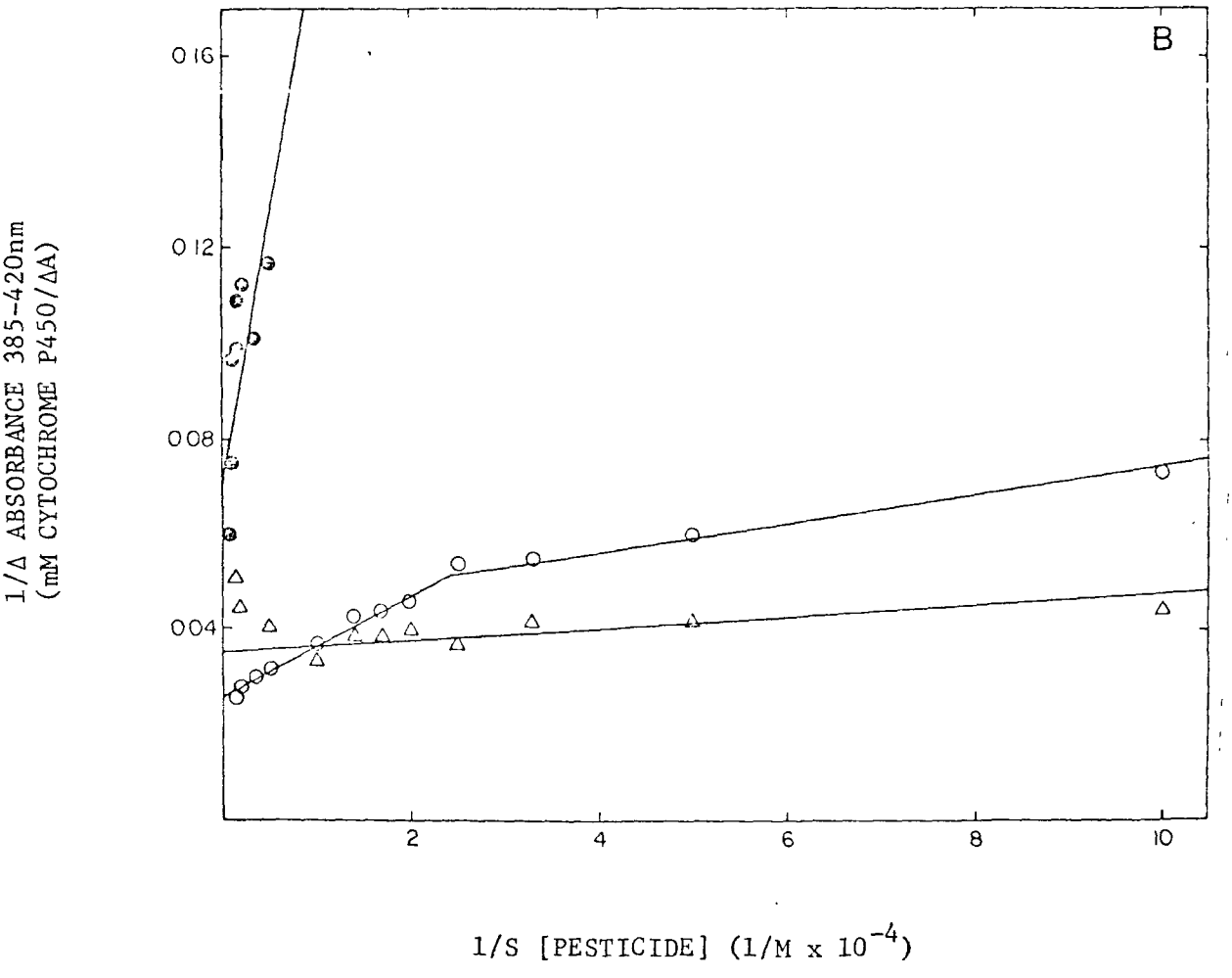


Figure 2. Double reciprocal plot of binding of organophosphate and carbamate pesticides to rodent liver microsomes. Conditions and calculations were as described in Figure 1.

Figure 3 reveals that several parameters can influence the amount of bound [^{3}H] DHT. Increasing the duration of postcastration time expectedly led to a loss of endogenous androgen resulting in more [^{3}H] DHT being bound to cytoplasmic receptors in several tissues (Fig. 3A). The increased availability of binding sites following castration was particularly evident in the sex accessory organs (viz., prostate and seminal vesicles). A 3-day postcastration time seemed to reveal considerable binding of [^{3}H] DHT, and it was arbitrarily chosen as the interval affording substantial binding (Fig. 3A). Using several in vitro incubation temperatures indicated that the total binding of [^{3}H] DHT to prostate cytosol receptor increased exponentially (Fig. 3B). However, no specific binding, as determined according to method of Chamnes and McGuire (1975), was detected when the incubation temperature was elevated beyond 10°C, and all further experiments were carried out at 0°C to maximize the degree of specific [^{3}H] DHT binding by prostate cytosol proteins. Although not shown, similar temperature curves were also obtained for other organ cytosols (viz., seminal vesicles, liver, and kidney).

If the duration of incubation was monitored over a 24-hr period, it was observed that the total binding of [^{3}H] DHT increased until about 10 hr and thereafter remained on a plateau until 24 hr (Fig. 3C). Subsequent experiments therefore utilized a 12-hr incubation interval in order to ensure the establishment of an equilibrium between bount and free [^{3}H] DHT in the tissue cytosols.

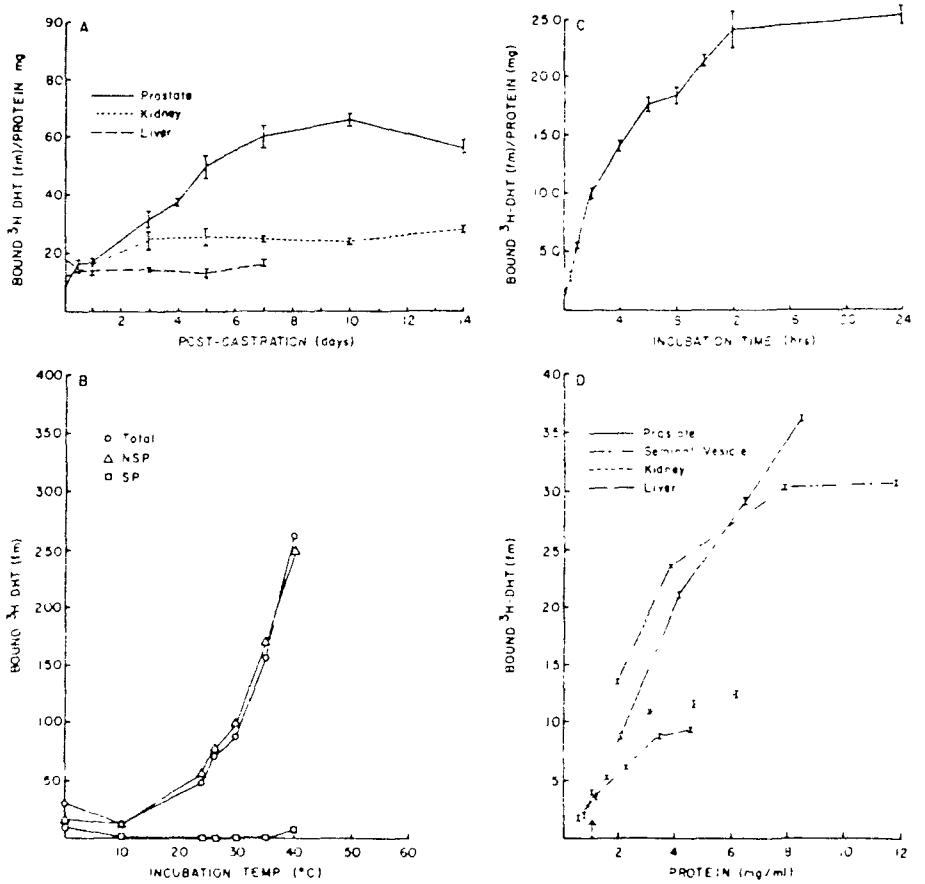
Just as an increase in incubation time led to an increase in binding of [^{3}H] DHT, as increase in the concentration of prostate cytosolic protein in the incubation mixture lead to increased [^{3}H] DHT binding (Fig. 3D). Based on this dependence pattern, a protein concentration of 2 mg/ml was selected for subsequent experiments. The dependence of binding on protein concentration was similar in all tissues studied, and the same standard concentration was chosen.

Using the optimum experimental conditions of postcastration interval, temperature, incubation time, and protein concentration, Scatchard analysis (1949) was performed on [^{3}H] DHT binding data from prostate gland and other organs (Fig. 4). The affinity of the cytosolic protein for [^{3}H] DHT was similar in all the tissues, but the total binding capacity varied among them (Table 6). Capacity was highest in the prostate and lowest in the liver.

The specificity of the prostate cytosolic steroidophil was demonstrated by competition studies using various agents (Fig. 5). As expected, nonradioactive DHT and testosterone were very effective competitors of [^{3}H] DHT binding by prostate cytosol. Of intermediate inhibitory activity were cyproterone acetate and estradiol followed by progesterone. Over a wide range of in vitro concentrations, estrone, estriol, and corticosterone failed to compete effectively with [^{3}H] DHT for its binding sites (Fig. 5). The specificity of the seminal vesicles, liver, and kidney binding profiles for the various steroids was analogous to the prostate response (not shown).

Figure 3. Effects of incubation conditions on [^{3}H] DHT binding by cytosol. All cytosol incubations were performed in the presence of 5 x 10^{-9} M [^{3}H] DHT in Tris/EDTA buffer (pH 7.2). Vertical bars show SE of the mean, and each point represents the mean of at least six values. (A) Relationship of [^{3}H] DHT bound/mg of protein to cytosols prepared from tissues taken from animals at varying times postcastration and incubated for 12 hr at 0°C. (B) Relationship of total nonspecific (NSP) and specific (SP) [^{3}H] DHT bound to cytosols prepared from tissues taken from animals 3 days postcastration and incubated for 12 hr at varying temperatures. The SE of each point is approximately 10%. (C) Relationship of [^{3}H] DHT bound/mg of protein to cytosols prepared from tissues taken from animals 3 days postcastration and incubated for varying times at 0°C. (D) Relationship of [^{3}H] DHT bound to protein concentration of cytosols prepared from tissues taken from animals 3 days postcastration and incubated for 12 hr at 0°C.

FIGURE 3



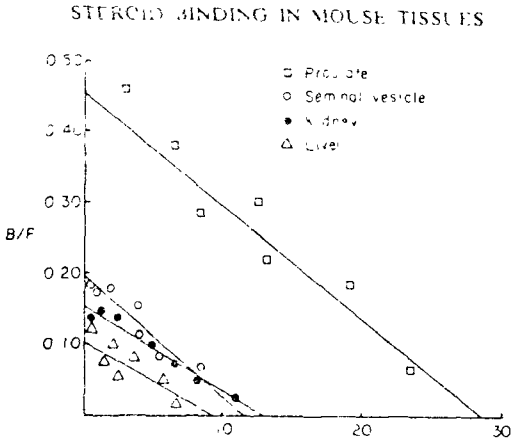


Figure 4. Scatchard analysis of [^{3}H] DHT (radioactive) binding to specific cytosol-binding proteins in various tissues from mice 3 days postcastration. Incubations of the cytosols were performed in the presence of various concentrations of [^{3}H] DHT for 12 hr at 0°C.

TABLE 6

SCATCHARD ANALYSIS OF ^{3}H-DHT BINDING TO SPECIFIC CYTOSOL RECEPTORS FROM SWISS-WEBSTER MICE 3 DAYS AFTER CASTRATION

	K_a (liters/mol × 10^9)	Binding sites (mol/g of protein × 10^{-11})
Prostate	1.7 ± 0.1[a]	5.1 ± 0.1
Seminal vesicle	1.7 ± 0.2	3.7 ± 0.1
Kidney	1.3 ± 0.1	2.9 ± 1.6
Liver	1.3 ± 0.2	1.2 ± 0.5

[a] Values are expressed as means ± SE of results of three experiments, each containing triplicate values

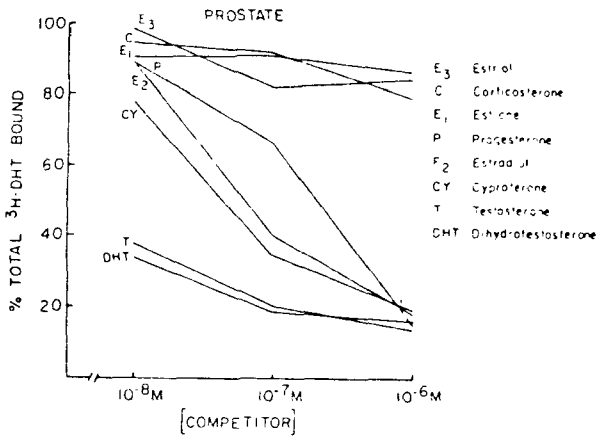


Figure 5. Ability of various hormones to compete with [^{3}H] DHT for its binding sites in prostate cytosol from castrate mice. Incubations of prostate cytosol, prepared from animals 3 days postcastration with 5 x 10^{-9}M [^{3}H] DHT either alone or with competitor were carried out for 12 hr at 0°C. Percentage total [^{3}H] DHT bound was determined by comparing the amount of [^{3}H] DHT bound in the presence of the competitor to the amount bound in the absence of the competitor.

Having established the specificity of the prostate cytosol binding components for ^{3}H-DHT, another series of experiments was conducted in order to examine the ability of certain pesticides to perturb the interaction of DHT with its cytosolic binding components (Fig. 6). Parathion (10^{-8} - 10^{-5}M) was observed to be an effective inhibitor of ^{3}H-DHT binding in the prostate cytosol (Table 7). Comparable concentrations of carbaryl and the organochlorines, DDT and dieldrin, had no effect of ^{3}H-DHT binding in the cytosol fraction of the mouse prostate. These findings of the lack of effect of the organochlorines on mouse prostate cytosol binding of ^{3}H-DHT are in contrast to those in the rat wherein either DDT or dieldrin inhibited ^{3}H-DHT binding (Wakeling and Visek, 1973). Although not shown, ^{3}H-DHT binding in the seminal vesicle, kidney and liver was similarly affected by the various pesticides investigated. Again, parathion was a potent inhibitor of ^{3}H-DHT binding in the cytosols of these tissues, whereas carbaryl, DDT, and dieldrin had no effect.

In order to determine whether parathion's inhibitory effects on ^{3}H-DHT binding were specific for organs known to be influenced by androgens (e.g. sex accessory organs, liver and kidney), a series of studies were undertaken using cytosol prepared from the small intestine of the mouse (Fig. 7). Although ^{3}H-DHT can be bound to cytosol components of the intestine, such binding is totally non-specific (Schein and Donovan, unpublished). None of the pesticides studied caused inhibition of ^{3}H-DHT binding in the cytosol of the small intestine (Fig. 7). It is evident, therefore, that among the tissues utilized in these investigations parathion's inhibitory effect on ^{3}H-DHT binding resides principally in those that are influenced by male sex steroids, and not in a non-target tissue such as the small intestine.

TABLE 7: INHIBITORY ACTIONS OF PARATHION IN VITRO ON CYTOSOL BINDING OF ^{3}H-DHT (% TOTAL ^{3}H-DHT BOUND) IN MICE

PARATHION CONC.(M)	PROSTATE	SEM. VES.	KIDNEY	LIVER	INTESTINE
0	100	100	100	100	100
10^{-8}	95.9 ± 2.9[a]	92.5 ± 4.1	87.1 ± 2.6	95.7 ± 2.2	88.8 ± 8.1
10^{-7}	84.1 ± 6.4	92.1 ± 8.5	86.1 ± 6.6	86.8 ± 6.6	100.0 ± 3.3
10^{-6}	65.6 ± 3.9	73.0 ± 9.7	59.6 ± 2.1	82.1 ± 6.1	100.0 ± 8.7
10^{-5}	51.1 ± 3.4	55.0 ± 5.7	39.6 ± 1.4	75.8 ± 3.1	99.0 ± 12.4

a $\bar{X}$ ± SE of at least 6 observations

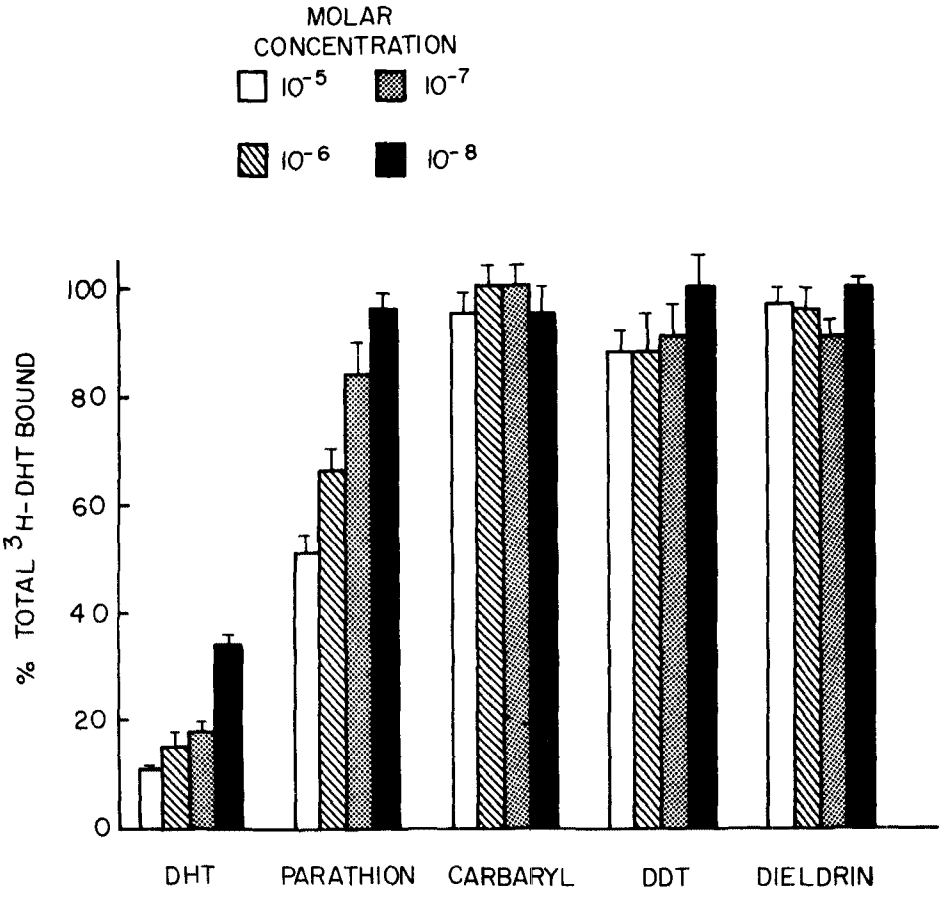


Figure 6. Percent of total ^{3}H-DHT bound in vitro to cytosol of the mouse anterior prostate gland in the presence of various concentrations of different pesticides. Each value represents the mean of at least 10 observations. Cytosols prepared from tissues taken from animals 3 days post-castration were incubated with 10^{-9}M ^{3}H-DHT and various concentrations of pesticides for 12 hours at 0°C.

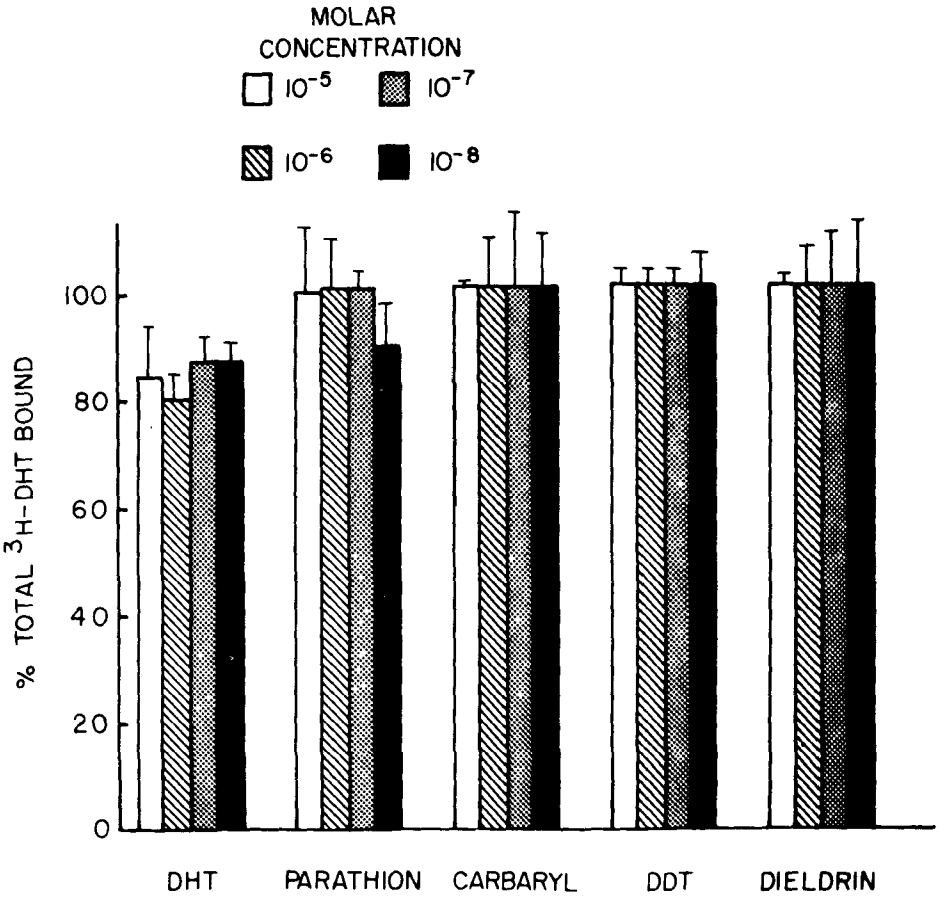


Figure 7. Percent total ^{3}H-DHT bound *in vitro* to cytosol of the mouse intestine in the presence of various concentrations of different pesticides. Each value represents the mean of at least 10 observations. Cytosols prepared from tissues taken from animals 3 days post-castration were incubated with 10^{-9}M ^{3}H-DHT and various concentrations of pesticides for 12 hours at 0°C.

SECTION 6

DISCUSSION

The effects of various pesticides on hepatic steroid metabolism have been previously reported (Conney et al., 1967; Welch et al., 1967; DuBois, 1969). In the liver, the effects of organochlorine pesticides seem to be mediated through stimulation of the microsomal hydroxylating enzyme systems (Hart and Fouts, 1965; Kuntzmann et al., 1966; Murphy, 1969). In the prostate gland, pesticides have been shown to alter the uptake and metabolism of radiolabeled testosterone (Schein and Thomas, 1975), to interfere with the binding of androgen metabolites to proteins (Wakeling and Visek, 1973) and to change the composition of prostatic secretions (Blend and Visek, 1972).

Early studies showed that the toxicity of various pesticides could be either increased or decreased by the previous or simultaneous exposure to other agents (Murphy 1969; DuBois 1969). These interactions among pesticides seemed to occur as a result of differential effects on oxidative enzyme activities (Conney and Burns, 1972). Resultant changes occurred in residue accumulation (Street and Blau, 1966), detoxication (Murphy, 1969) and hormonal balance (Street et al., 1969). Such changes, resulting from pesticide interactions might be responsible for altered sexual function (impotence) in farm workers exposed to pesticides (Espir et al., 1970).

Recently, the administration of dieldrin and parathion was shown to produce effects on testosterone metabolism different from those produced by either agent alone. Such effects occurred in liver and in androgen-dependent organs (Schein and Thomas, 1976). Likewise, the present studies demonstrate some differential effects of these two pesticides on androgen metabolism, but using dieldrin doses twice as large as previously administered.

In the mouse prostate gland, parathion treatment followed by administration of dieldrin stimulated the production of nonpolar metabolites of ^{3}H-testosterone in vitro (Schein and Thomas, 1976). In the present study, using twice the dose of dieldrin previously reported (Schein and Thomas, 1976), the effect upon nonpolar metabolites was absent and had presumably been abolished (Table 2). Unlike the previous study using lower doses of dieldrin (Schein and Thomas, 1976), the formation of metabolites of ^{3}H-T was not significantly different from that of the controls.

Similar to the response in the prostate gland (Table 2), there were changes in androgen metabolism in hepatic microsomes (Table 3). Previous studies showed that parathion alone stimulated the production of ^{3}H-androstanediol (Thomas and Schein, 1974) while dieldrin alone reduced the formation of ^{3}H-androstenedione (Schein and Thomas, 1975). When both pesticides were administered simultaneously, both effects were observed; formation of ^{3}H-androstanediol was enhanced

and of ^{3}H-androstenedione was reduced (Schein and Thomas, 1976). Table 3 shows, however, that when the dose of dieldrin was doubled, both the stimulation of ^{3}H-androstanediol and the inhibition of ^{3}H-androstenedione were abolished.

Parathion is an inhibitor of hepatic microsomal steroid hydroxylases (Kuntzman et al., 1966) while dieldrin induces these activities (Welch et al., 1971). When these two pesticides were administered simultaneously for 10 days or when dieldrin was administered for 5 days followed by 5 days of treatment with parathion, no significant changes were recorded in androgen hydroxylations (Schein and Thomas, 1976). The present findings extend these earlier studies since higher doses of dieldrin resulted in further stimulation of androgen hydroxylases (Table 3).

The present studies reveal that different pesticides can interact in mammalian systems to produce effects different from those produced by a single pesticide. The mechanism of interaction may be similar with different doses or dose regimens. This seems apparent in the response of hepatic androgen hydroxylases to various doses of dieldrin and parathion. Increasing doses of pesticides result in increasing stimulation of enzyme activities. It is possible, however, that pesticide interactions change eqalitatively with different dose regimens. This was made evident by the effects of these same pesticides on nonpolar metabolites of testosterone in liver and prostate. The effects of lower doses of pesticides on these activities disappear when higher doses are administered. Such pesticide interactions should be considered when evaluating environmental and toxicological impacts. The effects shown here on hepatic androgen metabolism have the potential to alter the hormonal balance. In addition, the effects on the prostate gland androgen metabolism could represent a direct action upon hormone-dependent organs. The importance of pesticide interactions is emphasized by the widespread agricultural use of many different types of these agents.

Organochlorines have high affinity for the cytochrome P-450 protein (Table 5) as well as high lipid solubility. This could easily lead to their accumulation in cellular membranes and produce concentrations sufficient to inhibit steroid hydroxylations (Table 4). Likewise organophosphates bind with high affinity to liver microsomes, and at high concentrations inhibit testosterone hydroxylases. Mice seem especially sensitive to diazinon with a low Ks value and significant enzyme inhibition at 10^{-8}M pesticide. In addition, methoxychlor and diazinon both appear to physically disrupt membranes at higher concentrations.

Carbofuran appears to have a different sort of toxic liability. It interacts only minimally with hepatic cytochrome P-450, but by some mechanism it appears to stimulate microsomal hydroxylations reactions.

Direct effects on prostate metabolism did not appear to be very important in this study. It is always possible that these pesticides did not adequately permeate the tissue and were not present in sufficient amounts at potential sites of action.

Another objective in this study was to determine the optimum conditions for [^{3}H] DHT cytoplasmic binding in various tissues of the mouse. It was decided that the best postcastration interval was 3 days. At this time the total binding capacity of the prostate gland is at a maximum, but tissue regression is minimal. It is essential that both preparation and incubation with labeled steroid be carried out at 0 to 4°C. The high-affinity, hormone-specific component of binding is extremely heat-labile and is destroyed at 10°C; but the amount of the low-affinity, nonspecific component was increased by higher temperatures. The heat lability of the specific binding component in the present studies was typical of androgen binding in various species (Mainwaring and Morgan, 1973). The optimum total concentration of protein in the incubation mixture is about 2 mg/ml. At protein concentrations greater than 3 mg/ml [^{3}H] DHT binding was no longer a linear function of protein concentration. A 12-hr period of incubation is necessary in order to reach equilibrium between free and bound hormone.

The slow kinetics of clearance of endogenous androgen after castration and of approach to [^{3}H] DHT binding equilibrium in vitro in the mouse are unusual. In the rat (Robinette and Mawhinney, 1978) and guinea pig (Belis et al., 1978), both of these processes are much more rapid. For example, rat prostates obtained 24 hr after castration and incubated for just 4 hr with steroid yielded maximum binding of androgen to cytosol fractions (Robinette and Mawhinney, 1978). In the present studies, it is not clear whether both high and low affinity components of [^{3}H] DHT binding exhibit this slow reaction rate nor is it clear just what structural of functional differences in the mouse prostate account for this slow clearance rate of endogenous androgens following castration.

Still another purpose was to examine the manner in which [^{3}H] DHT was bound in the cytosols prepared from the various tissues of the mouse. Two types of binding were found: (1) a heat-stable, high capacity type with low affinity, and (2) a heat-labile type with high affinity (K_a 1.5 x 10^9 liter/mol) and low capacity. This two-component androgen binding is commonly found in cytosols of mammalian cells (Wilson and French, 1976; Aakvaag et al., 1972).

Table 6 shows that the affinity constants of the high affinity [^{3}H] DHT binding component are similar in all tissues studied. On the other hand, the capacity of this binding component varies considerably among the tissues studied, being greatest in androgen-dependent organs. These findings are consistent with the distribution of binding activities among tissues of other animals (Mainwaring and Morgan, 1973).

The high-affinity type of binding present in most tissues was very specific with respect to competitors. At 10^{-8}M (i.e., at ten times the concentration [^{3}H] DHT), either DHT or testosterone significantly reduced binding of [^{3}H] DHT to this binding component. Cyproterone acetate, estradiol, or progesterone competed with [^{3}H] DHT for the high affinity binding sites at concentrations 100- to 1000-fold greater than the [^{3}H] DHT concentration. Estriol, esterone, or corticosterone did not reduce [^{3}H] DHT binding at any concentration studied.

The high affinity component of [^{3}H] DHT binding in mouse tissues behaves in the manner expected of a cytoplasmic androgen receptor. Its affinity for androgens is high. It is present in cells of tissues dependent on androgens for normal function and is also found in tissues responsive to but not dependent on androgens. Binding to this high affinity component is specific for androgens and is inhibited by antiandrogens such as cyproterone acetate, progesterone, and estradiol.

These investigations have demonstrated a relatively simple method for studying the *in vitro* effects of various compounds on the interaction of [^{3}H] DHT with its specific binding proteins in cytosol preparations of various tissues of the mouse. These methods can be useful to determine whether or not exogenous compounds (e.g., steroids, pesticides, *etc.*) have the ability to interfere with androgen-receptor dynamics at the cellular level.

Previous studies in our laboratory have shown that parathion has little or no direct effect upon ^{3}H-testosterone assimilation and/or metabolism by the mouse prostate gland if administered orally for a period of 5 days (Thomas and Schein, 1974). In the present studies, using an *in vitro* cytosol system, this pesticide was particularly effective in interfering with androgen binding. Parathion was a potent inhibitor of ^{3}H-DHT binding in the cytosol of several tissues including the anterior prostate gland, seminal vesicle, kidney and liver (Table 7). All of these tissues are subject to androgenic stimulation, but vary in their magnitude of response. On the other hand, the intestine does not appear to be stimulated by male sex hormones. Correspondingly, parathion had little effect on ^{3}H-DHT binding to cytosol components from this organ.

Although the organochlorine pesticides DDT and dieldrin have been shown to interfere with the assimilation and metabolism of ^{3}H-testosterone in mouse sex accessory organs (Thomas and Lloyd, 1973; Thomas *et al.*, 1973), neither of these compounds altered the binding of ^{3}H-DHT to cytosol binding components from any of the tissues studied in this series of experiments. Cytosol binding studies with the rat prostate (Wakeling and Visek, 1973), but not with the mouse prostate (Fig. 6), reveal that either dieldrin or DDT can inhibit binding of ^{3}H-DHT to cytosol binding components. The differences seen in these two studies might be due to species differences or technique differences. Wakeling and Visek (1973) used sedimentation gradient techniques to study the binding of ^{3}H-DHT. These studies used Dextran-coated charcoal method to isolate the bound steroid. Also, the constituents of their incubation buffer system (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [pH 7.4], containing 1.5 mM EDTA, 2.0 mM mercaptoethanol and 0.4 M KCl) differed from that used in these investigations. Furthermore, their *in vitro* incubation procedure included freezing the incubate overnight. These studies did not allow samples to freeze. Finally, the DDT used in these investigations was not analysed to determine the amount of the o,p'-DDT isomer present. This isomer has been found to be much more potent as an estrogen that the p,p'-DDT isomer (Welch *et al.*, 1969). Therefore, o,p'-DDT should be a more potent antiandrogen than the p,p'-DDT isomer. The lack of effect in this series of experiments may be due to a relatively large percent of p,p'-DDT present.

In the present studies, carbaryl was found to have little effect on ^{3}H-DHT binding to cytosol binding components of the various tissues studied. This finding was not unexpected, since previous studies have shown that this pesticide had little effect on ^{3}H-testosterone uptake and metabolism in the various androgen dependent tissues (Dieringer and Thomas, 1974; Thomas et al., 1974). As a chemical class, the carbamates seem to be unable to produce changes in the reproductive system (Guthrie et al., 1971).

Conney et al. (1974) and Kuntzman et al. (1966) have suggested that organochlorine and organophosphate pesticides, respectively, can perturb endocrine responses in mammals by induction of liver microsomal steroid hydroxylase enzymes. On the basis of the results presented in this report, an alternative mechanism for modifying hormonal action in male reproductive organs may be through the more direct inhibition of DHT binding to target tissue cytosol binding proteins.

In summary, the results of this investigation reveal that parathion is a potent inhibitor of ^{3}H-DHT binding to cytosol binding components present in androgen sensitive tissues. It would be of interest to examine the effects of paraoxon (the active metabolite of parathion) on ^{3}H-DHT binding in these tissues.

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GLOSSARY

Carbaryl: (1-Naphthyl-N-methyl carbamate)

Carbofuran: (2,3-dihydro-2,2-dimethyl-benzofuran-7-yl N-methylcarbamate)

DDT: (1,1-bis[p-chlorophenyl]-2,2,2-trichloroethane)

DHT: (dihydrotestosterone)

Diazinon: (diethyl 2-isoprophyl-6-methyl-4-pyrimidinyl phosphorothionate)

Dieldrin: (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,6,7,8,8a-octahydro-endo-exo-1,4:5,8-dimethanonaphthalene)

Heptachlor: (1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene)

Methoxychlor: (1,1,1-trichloro-2,2,-bis-4-(methoxyphenyl) ethane)

Parathion: (0,0-diethyl-0-[p-nitrophenyl]ester phosphorothioic acid

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

The metabolism of 1,2-^{3}H-testosterone *in vitro* was studied in prostate glands and livers of rats and mice treated with different pesticides including dieldrin and parathion. The metabolism of 1,2-^{3}H-testosterone (T-^{3}H) *in vitro* by mouse anterior prostate glands or hepatic microsomes has been studied after the oral administration of dieldrin (2.5 mg/kg daily x 5 or 10) and/or parathion (1.3,2.6, or 5.2 mg/kg daily x 5 or 10). T-^{3}H metabolism in the prostate was unaffected by the various treatment regimens. Dieldrin (10 days) caused some reduction in the microsomal production of androstenedione-^{3}H or dihydrotestosterone-^{3}H. Only treatment regimes with dieldrin stimulated hepatic testosterone hydroxylases; parathion alone had no effect. This study revealed that dieldrin and parathion can interact and produce biological effects different from those caused by either pesticide alone.

Liver microsomal steroid hydroxylating enzymes and prostatic testosterone-5 - reductase were studied in rat and mouse. Organochlorine and organophosphate slightly stimulated them. Neither species was consistently more sensitive to pesticide effects than the other. All the pesticides (*viz*, heptachlor, carbofuran, diazinon and parathion) bound to cytochrome P-450, producing type I spectral charges. Values of Ks ranged from 1.9 to 8.7mM for organochlorine and organophosphate compounds. Affinity for carbofuran was much lower (Ks=100-200mM).

17. KEY WORDS AND DOCUMENT ANALYSIS

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