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STRUCTURE-ACTIVITY RELATIONSHIPS ON THE INDUCTION OF HEPATIC MICROSOMAL ENZYMES IN THE MOUSE BY 1,1,1-TRICHLORO-2,2-BIS(*p*-CHLOROPHENYL) ETHANE(DDT) ANALOGS*

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Abstract—The structure–activity relationships among 28 compounds structurally related to DDT with respect to induction of the microsomal mixed function oxidases in mice have been examined utilizing NADPH oxidation, cytochrome P_{450} level and aniline hydroxylation as indices of mixed function oxidase activity. Eleven of these compounds stimulated the mixed function oxidase system at the dose applied (100 mg/kg, i.p.).

The structural requirements for maximal induction were a halogen on each phenyl ring and three halogens on the α-position of the ethane bridge. Substitutions on the β-carbon of the ethane moiety did not prevent induction, but changing substituents on the α-position greatly affected the inductive ability of the analog. The molecular dimensions, between phenyl substituents, varied from 10·1 to 11·7 Å for maximal induction. The molecular dimensions between the phenyl substituent and the ethane substituents for maximal induction varied between 7·4 and 9·0 Å. No correlations were found between toxicity of compounds and their inductive effects.

DDE, a major metabolite of DDT and an important contaminant of animal fat, increased microsomal enzyme activity at a treatment level of 200 mg/kg, but DDD had no effect even at this level. DDA, the major water soluble metabolite of DDT, also did not induce the mixed function oxidases.

OVER 200 different compounds have been reported to increase the activity of the liver microsomal xenobiotic oxidizing system. Induction has been caused by such diverse compounds as phenobarbital, polycyclic hydrocarbons and chlorinated hydrocarbon insecticides.[1] In the first report of induction by an insecticide, chlordane was shown to increase the level of xenobiotic metabolizing enzymes in the rat.[2] In a later paper, DDT was shown to elevate the level of the microsomal oxidase system in the rat, whereas DDT did not induce these microsomal enzymes in mice.[3] Subsequently, Cram and Fouts[4] reported that although DDT could induce drug metabolism in mice, it was a more selective and less potent inducer than in rats. DDT caused decreased zoxazolamine paralysis time but not hexobarbital sleeping time while chlordane stimulated the metabolism of both compounds in mice.

Structure–activity studies relative to induction have primarily been limited to the polycyclic hydrocarbons. The optimal size for induction by polycyclic hydrocarbons was found to be 85 to 150 Å^2 and a coplanar structure was necessary for activity.[5]

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No direct correlation could be drawn between the carcinogenicity of the compounds studied and ability to induce microsomal oxidations. However, subsequent work has indicated that there are parallels between these two phenomena. Among the 33 carcinogens studies by Buu-Hoi and Hien,[6] 28 were inducers. The addition of an electron-withdrawing group, i.e. formyl, lessened the carcinogenicity of the compound and also its ability to induce. Some structural requirements appear to be species dependent. The 6-carboxaldehyde derivative of benzpyrene shortened zoxazolamine paralysis time in rats[6] while it increased paralysis time in mice.[7]

The structure–activity relationship among chlorinated hydrocarbon insecticides has not been extensively studied. Hart and Fouts[3] studied six analogs of DDT and reported that only Perthane decreased hexobarbital sleeping time in mice. DDD, DDE, DDT, methoxychlor, Perthane and *o,p*-DDD have also been reported to stimulate drug metabolism in mammalian species.[8]

Since there are a number of compounds structurally related to DDT which are used as commercial pesticides or appear in technical formulations that may contribute to environmental contamination, it seemed desirable to initiate a study to examine the structure–activity relationships among DDT and a number of its analogs with respect to induction.

MATERIALS AND METHODS

Animals. Five-week-old male mice were used in this study. They were obtained from an inbred colony maintained by the North Carolina Department of Health since 1910. The animals were housed in plastic cages, and San-I-Cell Animal Bedding was used for litter. The mice were fed Purina Lab Chow and water *ad lib.* No insecticides were permitted in the rearing room.

Chemicals. DDT, *o,p*-DDT, DDE, Technical DDT and 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane were provided by Geigy Chemical Co. Rohm and Haas Chemical Co. donated Perthane, DDT and dicofol, while DuPont Chemical Co. sent samples of methoxychlor and 2,2-bis-(*p*-ethoxyphenyl)-1,1,1-trichloroethane. Prolan and Bulan were obtained from Commercial Solvents Co. Dr. R. L. Metcalf provided the 2,2-bis-(*p*-dibromophenyl)-, 2,2-bis-(*p*-fluorophenyl)-, 2,2-bis-(*p*-diiodophenyl)-, and 2,2-bis-(*p*-methylphenyl)-1,1,1-trichloroethane analogs plus 2,2-bis-(*p*-methylphenyl)-1,1,1-trimethylethane. Dr. D. Hennessey donated analogs in which fluorine atoms replace the chlorine atoms in the ethane moiety, i.e. the 1,1 difluoro- and 1,1,1-trifluoroethane analogs, along with a sample of *m,p*-DDT. The other analogs were purchased from Aldrich Chemical Co. All compounds were recrystallized and the purity was confirmed by thin-layer chromatography on Silica gel (ether–hexane, 4:1). Chemicals for enzyme assays were reagent grade.

Pretreatment. Compounds were given by interperitoneal injection using methoxytriglycol as the carrier. The dosage was adjusted to deliver 0·05 ml of solvent per 20 g of animal weight. All animals were injected with 100 mg/kg daily for 3 days and sacrificed on the fourth day unless otherwise stated. Control mice received solvent only.

Preparation for assays in vitro. Mice were sacrificed by decapitation, the liver was excised, rinsed with KCl-tris buffer and placed in ice-cold 0·15 M KCl-50 mM tris HCl buffer (pH 7·4). The livers were then blotted, weighed and homogenized in 4 vol.

(4 ml:g wet weight) of the KCl-tris buffer. The livers were homogenized by using 15 complete strokes of the pestle in a 15-ml all-glass homogenizer.

The homogenate was centrifuged at 10,000 *g* for 15 min and the resulting supernatant was sedimented at 100,000 *g* for 1 hr. The supernatant was discarded and the pellet suspended in 50 mM tris HCl buffer (pH 7·5) (4:1; vol. g wet weight). This preparation was subsequently used in the assays *in vitro*. The temperature was maintained at 0–4° during preparation.

Enzyme assays. Oxidation of NADPH was determined using a Beckman DU spectrophotometer by following the decrease in absorption at 340 nm. The reaction was followed for 3 min after allowing 30 sec for stabilization of the mixture. The assay media contained 100 μM nicotinamide, 0·5 ml of the microsomal suspension and 0·25 μM NADPH in a total volume of 2·75 ml.[9] The change in optical density was converted to micromoles using an extinction coefficient of $6{\cdot}22 \times 10^6$ cm^2/mole.[10] NADPH oxidase activity is known to be induced, and induction parallels other components of the oxidative system.[11]

The cytochrome P_{450} content was measured by diluting 1 ml of microsomal suspension with 5 ml of the 50 mM tris HCl buffer. This preparation was reduced with sodium dithionite, and the sample cuvette was gassed with CO for 1 min. The cytochrome P_{450} level was determined using a Beckman DK-2 ratio-recording spectrophotometer, and the difference in absorption at 450 nm minus 500 nm was recorded.[12,13]

The hydroxylation of aniline was determined by incubating 1 ml of the microsomal suspension, 7·4 mM aniline, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 μM NADP, and one unit of glucose 6-phosphate dehydrogenase in a total of 3 ml. The mixture was preincubated for 15 min to reduce the NADP, and the reaction initiated by the addition of the substrate. The incubation time was 15 min, and the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The denatured protein was removed by centrifuging at 18,000 *g* for 5 min. One ml of the supernatant was added to 1 ml of 0·5 N NaOH containing 1% phenol. After mixing, 1 ml of 1 M Na_2CO_3 was added. The mixture was incubated for 20 min at room temperature and the amount of *p*-aminophenol determined by measuring the absorbance at 630 nm.[14] Aniline is a type II substrate for cytochrome P_{450} and is not necessarily typical of all substrates for mixed function oxidases.

Protein was measured by the method of Lowry *et al.*[15] using bovine serum albumin as a standard.

Separate controls were run with each treatment, and the control mice were from the same litter as the treated mice. Six replications of each paired experiment were used. A paired *t*-test was used to determine the significant difference among treatments and controls, i.e. the presence or absence of induction. To compare compounds with respect to degree of induction, an average standard error was computed for the inducing compounds only, using the fact that each individual standard error was obtained from an independent experiment and using the theorem on the standard errors of linear functions of independent variables.[16] The 0·05 level was arbitrarily selected to establish significance.

RESULTS

Different levels of *p,p'*-DDT were given to determine the level required for stimulation of microsomal reactions. Treatment at the 50 and 75 mg/kg levels did not result

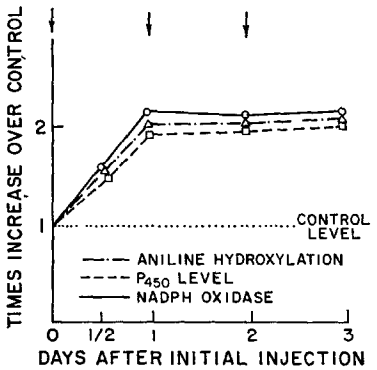


FIG. 1. Time course of induction of mouse hepatic microsomal enzymes by *p,p'*-DDT (100 mg/kg). Arrows indicate time injections were given.

in appreciable induction of microsomal enzymes, but 100 mg/kg did raise the levels of all three indices used. Treatment above the 100 mg/kg level resulted in greater than 50 per cent mortality and indicated the desirability of utilizing 100 mg/kg as the maximum dose.

Figure 1 shows the time course of the induction process. There was a significant increase in the mixed function oxidase system 12 hr after the first injection. Twenty-four hr after treatment, the level increased to approximately twice that of the controls and remained at this level for the 3-day period studied.

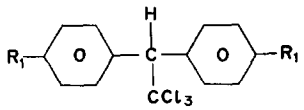
Phenyl substitutions. The effect of changing substituents on the phenyl rings was examined (Tables 1 and 2). The dichloro-analog (DDT) had the greatest effect on the three parameters while the difluoro-analog was the next. The dibromo- and diiodo-compounds were less effective inducers. Rearranging the position of one of the chlorine atoms to the *o*-position lessens the inductive effect but the *m,p*-analog is more potent than the parent DDT. If no substituents are present on the phenyl rings (diphenyl), induction does not occur. Similarly, replacing the chlorine atoms on the phenyl rings with methyl, hydroxyl, methoxy or ethoxy groups causes the resultant compound to lose its ability to induce.

Ethane substitutions. Modification of the ethane moiety of DDT also has a considerable effect upon the compound's ability to induce microsomal enzymes in the mouse.

A chloro group substituted for the H in the β-position gives an analog which shows appreciable induction but less than the parent DDT (Tables 2 and 3), whereas an OH substituent, dicofol, shows a slightly higher level of induction than the β-chloro derivatives. If there are two chlorine atoms present in the α-position (DDD and DDE), no induction of cytochrome P_{450} or aniline hydroxylase occurs. DDE, a dichloroethylene analog, does raise the activity of NADPH oxidase while DDD, the dichloroethane analog, does not affect NADPH oxidase. DDA, a water-soluble metabolite of DDT, does not induce any of the enzyme activities studied.

When all three chlorine atoms at the α-position are replaced by fluorine atoms (DDTF), the analog acts in a similar way to the parent compound, DDT. If only two fluorine atoms are present, it behaves like the dichloro analog, DDD. Two nitro derivatives, Prolan and Bulan, were examined and found not to induce microsomal enzymes.

TABLE 1. EFFECTS OF DDT ANALOGS ON THE MICROSOMAL OXIDASE SYSTEM OF MICE—MODIFICATIONS OF PHENYL GROUPS*



R_1	Abbreviated or common name	Microsomal activity and (times control) NADPH oxidase (mμ/moles NADPH oxidized/min/ mg protein)	Cytochrome P_{450} level (ΔO.D. 450–500 nm $\times 10^3$/ mg protein)	Aniline hydroxylation (mμmoles *p*-amino-phenol/15 min/ mg protein)
Cl	DDT	2·89 ± 0·2† (1·6)	180 ± 27† (2·0)	65 ± 7·3† (3·4)
Control		1·76	89	19
F	DFDT	2·25 ± 0·3† (2·0)	100 ± 9† (1·7)	19 ± 1·8† (1·6)
Control		1·12	60	12
Br	DBrDT	1·93 ± 0·1 (1·7)	85 ± 9† (1·4)	18 ± 1·0† (1·4)
Control		1·12	60	13
I	DIDT	1·93 ± 0·1 (2·0)	90 ± 9† (1·5)	18 ± 9·9† (1·4)
Control		0·96	60	13
Cl(*o,p'*)	*o,p*-DDT	1·93 ± 0·2† (1·5)	90 ± 9† (1·3)	18 ± 1·3† (1·5)
Control		1·29	69	12
Cl(*m,p'*)	*m,p*-DDT	5·6 ± 0·4† (2·4)	229 ± 18† (2·1)	72 ± 4·7† (4·2)
Control		2·3	110	17
Cl	Tech. DDT	1·93 ± 0·2 (2·0)	90 ± 9† (1·5)	18 ± 1·5† (1·4)
Control		0·96	60	13
H		1·29 ± 0·2 (1·1)	70 ± 9 (1·0)	14 ± 1·7 (1·0)
Control		1·13	70	14
OH		0·96 ± 0·1 (1·0)	60 ± 18 (1·0)	14 ± 1·2 (0·9)
Control		0·96	60	16
CH_3		1·28 ± 0·2 (1·1)	70 ± 18 (1·0)	15 ± 1·0 (0·9)
Control		1·13	70	14
CH_3O	Methoychlor	1·28 ± 0·1 (1·0)	60 ± 18 (0·9)	12 ± 0·9 (0·9)
Control		1·28	70	13
C_2H_5O	Ethoxychlor	1·45 ± 0·2 (1·1)	70 ± 27 (0·9)	14 ± 0·8 (0·9)
Control		1·29	80	15

* Mice received 100 mg/kg daily for 3 days.

† When standard errors of differences between treatments and controls in paired *t*-tests were determined, the differences were significant at the 0·05 level.

Substitutions on both phenyl and ethane groups. Where fluorine atoms replace all five chlorine atoms, induction occurs (Tables 2 and 4). However, if the five chlorine atoms are replaced by methyl groups, no induction occurs. Two DDD derivatives, *o,p*-DDD, and Perthane, have no effect upon the activity of the microsomal indices studied.

Three other organochlorine compounds were studied for their effects on drug metabolism. Whereas 1,1,1-trichloroethane did not affect the activity of the three enzymes studied, 2-(*p*-chlorophenyl)-2-chloro-1,1,1-trichloroethane and chlorobenzene significantly lowered the cytochrome P_{450} levels and aniline hydroxylase activities. Neither compound affected NADPH oxidase activity.

Since DDE induced NADPH oxidase, higher levels of DDE and DDD, both with two chlorine atoms at the α-position, were tested to ascertain their effects. At 200 mg/kg for 3 days, DDE increased all three parameters studied while DDD had no effect.

TABLE 2. RELATIVE EFFECTS OF INDUCING COMPOUNDS ON THE MICROSOMAL MIXED FUNCTION OXIDASE SYSTEM OF MICE*

Compound	Activity above control d		
	NADPH oxidase (mμmoles NADPH oxidized/min/ mg protein)	Cytochrome P_{450} level (ΔO.D. 450–500 nm × 10^3/mg protein)	Aniline hydroxylation (mμmoles *p*-amino phenol/15 min/ mg protein)
m,p-DDT	3·30	13	55
p,p'-DDT	1·13	10	46
DDE	1·28		
DFDTF	1·28	8	28
DDTF	1·28	8	24
Dicofol	0·64	7	29
DFDT	1·13	4	7
DDTET	0·67	4	10
o,p-DDT	0·64	2	6
Tech. DDT	0·97	3	5
DBrDT	0·81	3	5
DIDT	0·97	3	5
Av. S.D.	0·355	2·1	5·9
Av. LSD_{05}	0·913	5·3	15·2

* This table compares relative differences among inducing compounds shown in Tables 1, 3, 4.

DISCUSSION

At the level selected for comparison of inductive effects on the three parameters of the microsomal enzyme system studied, the following conclusions appear to be justified:

(1) Halogen substituents on the phenyl rings are a prerequisite to activity. Chlorine and fluorine atoms show maximum effect while the bromine and iodine analogs, both larger atoms, are less effective. Changing the positions of the chlorine atoms to *o,p*-positions causes a reduction in the effect, but the *m,p*-analog is as effective an inducer as DDT.

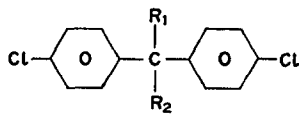
(2) Substitutions in the *p,p'*-positions of atoms other than halogens proved ineffective in stimulating microsomal activity even though the methyl and hydroxyl groups are isosteres of chlorine (C, 1·8 Å; OH, 1·7 Å and CH_3 2·0 Å). Their sizes are within the limits required for serological reaction.[17] An electron-withdrawing substituent, the hydroxyl group, does not cause induction so it appears that the effects of the halogens are not completely due to their electronegativity.

(3) Substitution of the hydrogen in the β-position by a hydroxyl group (dicofol) or a chlorine atom decreases the level of induction but does not prevent it.

(4) The trichloroethane (DDT) and trifluoroethane analogs are inducers, but if there is one less halogen atom, the inductive effect is lost except that the unsaturated analog (i.e. DDE) induces one component, NADPH oxidase, without affecting the other parameters studied. The DDT metabolite, DDA, does not affect this system.

(5) When the level of pretreatment by DDE is raised to 200 mg/kg it induces all three indices studied, but another dichloro-analog, DDD, at the same concentration does not induce.

TABLE 3. EFFECTS OF DDT ANALOGS ON MICROSOMAL OXIDASE SYSTEM OF MICE—MODIFICATIONS OF ETHANE MOIETY*



R_1	R_2	Abbreviated or common name	Microsomal activity and (times control) NADPH oxidase (mμmoles NADPH oxidized/min/mg protein)	Cytochrome P_{450} level (ΔO.D. 450–500 nm × 10^3/mg protein)	Aniline hydroxylation (mμmoles *p*-aminophenol/15 min/mg protein)
OH	CCl_3	Dicofol	1·92 ± 0·2† (1·5)	121 ± 18† (2·0)	46 ± 5·8† (2·7)
Control			1·28	60	17
Cl	CCl_3	DDTET	1·92 ± 0·2† (1·5)	100 ± 9† (1·7)	22 ± 2·5† (1·8)
Control			1·25	60	12
H	$CHCl_2$	DDD	1·61 ± 0·1 (1·1)	70 ± 36 (1·2)	20 ± 1·4 (1·1)
Control			1·45	60	18
	$=CCl_2$	DDE	3·37 ± 0·4† (1·6)	80 ± 45 (0·9)	18 ± 1·8 (1·2)
Control			2·09	90	15
H	COOH	DDA	1·45 ± 0·2 (0·9)	80 ± 36 (1·1)	15 ± 0·9 (1·0)
Control			1·60	70	15
H	CF_3	DDTF	2·89 ± 0·3† (1·8)	140 ± 27† (2·0)	42 ± 4·6† (2·3)
Control			1·61	70	18
H	CHF_2		1·25 ± 0·2 (1·3)	70 ± 9 (1·0)	16 ± 1·6 (1·1)
Control			0·97	70	14
H	$CH(NO_2)—CH_3$	Prolan	1·28 ± 0·2 (1·1)	80 ± 18 (1·1)	25 ± 1·3 (1·1)
Control			1·13	70	23
H	$CH(NO_2)—C_2H_5$	Bulan	1·28 ± 0·1 (0·9)	80 ± 18 (1·3)	23 ± 1·1 (1·0)
Control			1·45	60	23
H	CCl_3	DDT	2·89 ± 0·2† (1·6)	180 ± 27† (2·0)	65 ± 7·3† (3·4)
Control			1·76	89	19

* Mice received 100 mg/kg of experimental compound daily for 3 days.

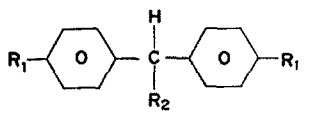
† When standard errors of differences between treatments and control in paired *t*-tests were determined, the differences were significant at the 0·05 level.

(6) When fluorine atoms replace the five chlorine atoms the analog behaves quite similarly to DDT, but if methyl groups replace the chlorine atoms, the inductive effect is lost.

(7) Induction by DDT increases linearly for 24 hr but then appears to reach a plateau over the 3-day period studied.

In this study, no conclusive and consistent correlations were found between the ability of a compound to induce and the acute toxicity as available in the published literature on mammalian toxicities.[18] DDT and DFDT are equitoxic and approximately equivalent in their inductive effects. Compounds such as DDC, methoxychlor and Perthane, which are relatively nontoxic to small mammals, are not effective inducers. Dicofol and DDE, which are also of very low toxicity to rats and mice,

TABLE 4. EFFECTS OF DDT ANALOGS ON THE MICROSOMAL OXIDASE SYSTEM OF MICE—MODIFICATIONS OF PHENYL GROUPS AND ETHANE MOIETY*



R_1	R_2	Abbreviated or common name	Microsomal activity and (times control) NADPH oxidase (mμmoles NADPH oxidized/min/mg protein)	Cytochrome P_{450} (ΔO.D. 450–500 nm × 10^3/mg protein)	Aniline hydroxylation (mμmoles *p*-aminophenol/15 min/mg protein)
F	CF_3	DFDTF	2·73 ± 0·2† (1·9)	140 ± 18† (2·0)	40 ± 6·8† (3·3)
Control			1·45	70	12
C_2H_5	$CHCl_2$	Perthane	1·45 ± 0·1 (1·1)	60 ± 9 (1·0)	15 ± 0·8 (1·1)
Control			1·29	60	14
CH_3	$C(CH_3)_3$		1·15 ± 0·2 (0·9)	60 ± 18 (1·0)	20 ± 1·1 (1·0)
Control			1·29	60	20
Cl(*o*,*p*′)	$CHCl_2$	*o*,*p*-DDD	1·29 ± 0·1 (1·1)	70 ± 9 (0·9)	13 ± 1·2 (1·0)
Control			1·15	80	13
Cl	CCl_3	DDT	2·89 ± 0·2† (1·6)	180 ± 27† 2·0)	65 ± 7·3† (3·4)
Control			1·76	89	19

* Mice received 100 mg/kg of experimental compound daily for 3 days.
† When standard errors of differences between treatments and controls in paired *t*-tests were determined, the differences were significant at the 0·05 level.

induced microsomal enzymes at levels of 100 mg/kg and 200 mg/kg respectively. These results did not allow generalizations on the relationship between the inductive effect and acute toxicity of the compounds studied. Factors such as absorption, distribution and metabolism of the compounds studied may be involved in the explanation of some of the results reported.

Measurements were made with Stuart–Briegleb models to investigate possible correlations between dimensions of the chemicals studied and their ability to induce. Free rotation of the phenyl rings was consistent among the analogs except DDE, *o*,*p*-DDT and *o*,*p*-DDD. Measurements were taken from the substituents on the other phenyl groups. The molecular size for optimal induction was found to be between 10·1 and 11·7 Å. Measurements from the phenyl substituent to the substituents on the ethane moiety indicated a size range for maximal effect to be between 7·4 and 9·0 Å.

In some species of animals, it has been postulated that intact DDT is not the most potent inducer among this family of compounds. With rats and pigeons, DDE, a major metabolite of DDT and a major storage component of DDT equivalents in fat, appears to be the most potent inducer.[19] Bunyan *et al.*[19] found that DDE produced greater increases in the cytochrome P_{450} level and aniline oxidation than did DDT. Also, when rats were fed DDT or DDE at the same rate, DDE was the more potent inducer at low concentrations. In dogs, it has been found that DDE is a stronger inducer of microsomal oxidations than is DDT.* However, in the strain of mice used in these experiments, this was not found to be the case. DDE, injected at a level of 100 mg/kg, did not increase the level of cytochrome P_{450} or aniline hydroxylation, the two indices used in the experiments of Bunyan *et al.*[19] When the level of DDE

* H. Remmer, personal communication.

pretreatment was raised to 200 mg/kg, increases in the cytochrome P_{450} and aniline hydroxylation levels were noted. On the other hand, DDT was shown to be a potent stimulator of microsomal oxidations at a level of 100 mg/kg.

Differences between strains of mice in response to pretreatment by chlorinated hydrocarbon insecticides also exist. Perthane was reported to stimulate the metabolism of hexobarbital in the mouse strain used by Hart and Fouts.[3] However, in the strain of mice studied in these experiments, Perthane had no effect on the microsomal oxidase system.

Since there are intra- and interspecific differences in induction of microsomal enzymes by DDT analogs, comparative studies need to be made to ascertain similarities and differences between the structure–activity requirements of induction by DDT analogs. The work with primates initiated by Juchau *et al.*[20] particularly needs to be extended to include other analogs. These studies would aid in understanding the nature of the mixed function oxidase system and possible ramifications of the role of DDT and its analogs in the over-all metabolic pathway and as a component of environmental contamination, particularly relating to interactions in the microsomal enzyme system.

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