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Environmental Health Effects Research Series

ROLE OF MIXED FUNCTION OXIDASES IN INSECTICIDE ACTION

Health Effects Research Laboratory
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ROLE OF MIXED FUNCTION OXIDASES IN INSECTICIDE ACTION

by

Robert L. Metcalf

Department of Entomology
University of Illinois
Urbana-Champaign, Illinois

R802022

Project Officer

Ronald L. Baron

Environmental Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, North Carolina 27711

U. S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
HEALTH EFFECTS RESEARCH LABORATORY
Research Triangle Park, North Carolina 27711

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ABSTRACT

The role of the microsomal oxidase enzymes (MFO) in the biochemistry and toxicology of insecticides has been studied. Insects contain greatly varying titres of these enzymes. A survey of 74 species from 40 families in 8 orders, using the topical LD_{50} of carbaryl alone and together with the inhibitor piperonyl butoxide showed a 55,000-fold variability in LD_{50} largely due to MFO detoxication. In individual species of Diptera, MFO activity is highly variable with age, sex, and stage of development.

The DDT-type molecule has been as a model for the study of degradophores, i.e. molecular groupings that can serve as MFO substrates. Their oxidation thus converts lipophilic compounds into more water-partitioning moieties and thus promotes excretion rather than lipid storage. Suitable degradophores for the DDT-type molecule are alkyl and alkoxy groups on the aryl rings. Compounds with judicious combinations of these provide relatively long persistence on inert surfaces and ready biodegradability in vivo. Such compounds are much less toxic to mice and to fish than DDT but because of the generally lower MFO of insects, can be effective insecticides. The role of degradophores incorporated into the aliphatic moiety of DDT has also been explored, where the $-CH(CH_3)_2$, $-CHCH_3Cl$ and $-CHCH_3NO_2$ groups are useful. Induction experiments with the biodegradable DDT analogues in mice has demonstrated that unlike DDT, these compounds do not elevate liver MFO.

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ACKNOWLEDGEMENTS

The principal investigator gratefully acknowledges the participation and invaluable assstance of students and coworkers in this investigation. Mr. Al Tegen faithfully reared large numbers of insects used for study. Mrs. Penny Tegen and Mrs. Ruth Millholin worked tirelessly at essential bioassays. Compounds involved in the study were prepared by Dr. Inder P. Kapoor, Dr. Asha Hirwe, and Dr. Joel Coats. Dr. W. F. Childers supplied fish and helped conduct bioassays. Dr. Larry Hansen, Dr. Lena Brattsten, and Dr. R. A. Metcalf carried out biochemical studies. Portions of the thesis research of Dr. Inder P. Kapoor "Comparative metabolism of DDT, methoxychlor and methiochlor in mammals, insects and in a model ecosystem"; Dr. Lena Brattsten "Role of mixed function oxidases of insects in their response to xenobiotics"; Dr. Joel R. Coats "Toxicity and metabolism of three methoxychlor analogues with altered aliphatic moieties, and the development of some novel DDT analogues"; and Dr. Keturah A. Reinbold "Effects of the synergist piperonyl butoxide on toxicity and metabolism of pesticies in the green sunfish (Lepomis cyanellus Rafinesque)", were involved in the studies reported.

1. CONCLUSIONS

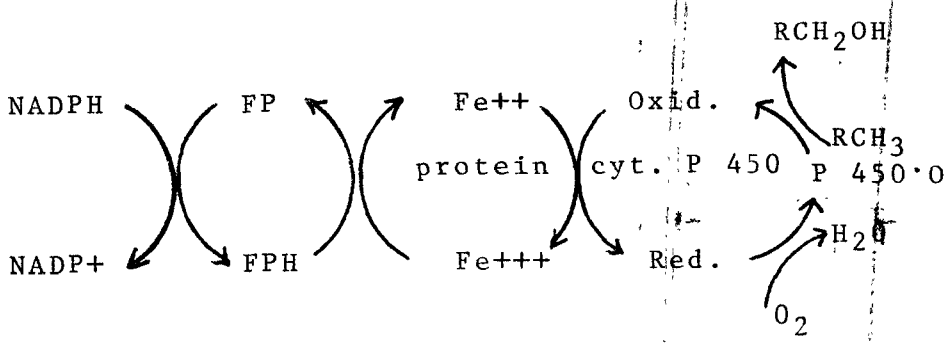
The mixed function oxidase enzymes play a critical role in insecticide action both on target and non-target species. The susceptibility of target insects appears to be inversely related to the titre of these enzymes which is highly variable over a wide range of insect species. The use of inhibitors of mixed function oxidase action such as piperonyl butoxide restores susceptibility of insects with high mixed function oxidase levels to carbaryl. Non-target organisms such as fish accumulate xenobiotic compounds such as pesticides in direct proportion to the levels of mixed function oxidases and bioaccumulation of pesticides in fish is greatly increased by exposire to mixed function oxidase inhibitors such as piperonyl butoxide.

Molecular groupings or degradophores which can serve as substrates for mixed function oxidase enzymes can improve the biodegradability of pesticides by promoting in vivo conversion to groups which are predominately water partitioning. Effective examples include CH_3-converted to -COOH, CH_3O- converted to -OH, and CH_3S converted to CH_3SO. Incorporation of these moieties into the DDT-type compound greatly increased biodegradability and decreased ecological magnification. These biodegradable DDT analogues were also largely inactive in the induction of microsomal oxidase activity when given to mice.

II. INTRODUCTION

The biological activity of nearly all o· pesticides, and toxic-substances is determine<u>vivo</u> metabolism in living organisms by a group introduce molecular oxygen combined with a uniq into readily modifiable sites of the organic mole enzymes were termed by Mason et al (1965) as "mixed oxidases" and have been variously referred to as mul oxidases after their ubiquity of reaction, microsomal from their typical location in the endoplasmic retic· sedimentation by ultracentrifugation, oxygenases from biochemical mode of action, and drug metabolizing enzymes from their typical action on xenobiotical compounds foreign to the organism. In this report we shall refer to them as MFO.

The MFO enzymes require reduced diphosphopyridine nucleotide NADPH and cytochrome P.450 to form an election transport chain which can be represented in general as:



The MFO enzymes are generally located in the endoplasmic reticulum of the vertebrate liver, in the gut wall, fat body, and malpighian tubules of invertebrates, and in uncharacterized locations in plant tissues. Typically the MFO enzymes can be sedimented as the "microsomal fraction" by ultracentrifugation at 105,000 G. Brodie and Maickel (1961) believe that the MFO enzymes arose phyllogenetically to enable terrestrial organisms to degrade and eliminate lipid soluble foreign molecules from the body. Thus fish and other aquatic organisms where xenobiotic exposure was mininal in an evolutionary sense seem to have relatively low levels of MFO compared to typical terrestrial vertebrates (Terriere 1969). Adamson et al (1965) view the MFO enzymes as normally present for metabolism of endogenous products such as steroids and acting on xenobiotics only where structural similarities permit. In any event the MFO enzymes are under genetic control and the evolutionary processes can be discerned by the selection of living organisms for tolerance to various xenobiotics e.g. insecticide resistance. In many instances survival occurs through enhanced MFO activity.

The types of biochemical reactions mediated <u>in vivo</u> by the mixed function oxidases include: (a) epoxidation of C=C, (b) hydroxylation of aromatic rings, (c) hydroxylation of alkyl groups, (d) O-dealkylation, (e) N-dealkylation, (f) S-dealkylation, (g) thioether oxidation, (h) desulfuration of C=S, (i) desulfuration of P=S, and (j) deamination. This great variety of reactions is thought to occur by attack of the .OH, or .OOH radical formed by activation of molecular oxygen in combination with cytochrome P.450. There appear to be few qualitative differences in the nature of the various MFO induced reactions in the wide variety of living organisms but a wide variety of quantitative differences in the rates with which various xenobiotics are degraded.

III. DISTRIBUTION OF MFO IN INSECTA

A rather complete survey of representative insect species and their susceptibility to carbaryl has been made by Brattsten and Metcalf (1970, 1973). Carbaryl is rapidly detoxified by the MFO enzymes by attack on the aryl rings to produce the degradation products 4-OH, 5-OH, and 6, 7-di-OH-dihydrocarbaryls. These are readily conjugated *in vivo* and are subject to further N-dealkylation and subsequent hydrolysis at the carbaryl group to give a series of hydroxylated naphthols and conjugates. Piperonyl butoxide is very effective in retarding these reactions and thus in synergizing the insecticidal activity of carbaryl. Accurate quantitative LD_{50} values were obtained for carbaryl alone and with the synergist piperonyl butoxide for 74 species of insects from 40 families. As shown in Table 1, the variation in susceptibility to carbaryl as determined by the typical LD_{50} in micrograms per body weight is ca. 55,000X, ranging from the chrysomelid beetle *Trirhabda adela* to the ant *Pogonomyronex barbatus*. Much of this variation in susceptibility is determined by MFO detoxication as the synergized LD_{50} values (with piperonyl butoxide) range over only about 200X (Table 1).

It is difficult to discern any obvious correlations between the carbaryl tolerance and synergistic ratio and the phyllogenetic position, food habits, and biological speciation of the 74 species representing 40 families and 8 orders. In general the Coleoptera showed high susceptibility and low synergistic ratio, indicating very poor MFO capacity. The Diptera showed an amazing range from an LD_{50} of 0.66 in *Stomoxys calcitrans* to 4000 for *Sarcophaga bullata*. These characteristics must relate to basic biochemical processes in the insects. It is clear, however, that the performance of carbaryl as an insecticide relates to the intrinsic MFO activity of the insect species, and this is a basic consideration in the use of degradophores, i.e. chemical groups which can serve as substrates for MFO enzymes, in the design of new biodegradable pesticides.

Table 1. SUSCEPTIBILITY OF INSECTS TO CARBARYL AND PIPERONYL BUTOXIDE

Species	Topical LD_{50} µg/g carbaryl	µg/g carbaryl + piperonyl butoxide	Synergistic Ratio
Trirhabda adela	0.11	0.14	0.8
Tetraopes tetrophthalmus	0.3	0.11	2.7
Stomoxys calcitrans	0.66	0.46	1.4
Spodoptera frugiperda	1.3	0.3	4.3
Apis mellifera	2.3	0.8	2.9
Epilachna varivestis	2.7	1.6	1.7
Chrysopa carnea	8.5	1.6	5.3
Blattella germanica	22	5.3	4.2
Musca antumnalis	8.8	1.2	7.3
Ostrinia nubilalis	12.3	7.2	1.7
Phormia regina	29	4.3	6.7
Periplaneta americana	190	10.5	18.1
Musca domestica	>900	12.5	>72
Dermestes ater	3500	110	31.8
Sarcophaga bullata	4000	10	400
Pogonomyrmex barbatus	>5800	26	>223

data from Brattsten and Metcalf (1970, 1973).

IV. AGE DEPENDENT VARIATIONS IN INSECT MFO ACTIVITY

The MFO enzymes have been shown to undergo dramatic variation in levels in insects depending upon sex, stage of development, and upon age (Brattsten and Metcalf 1973). These factors have been investigated in detail using 6 species of Diptera: Sarcophaga bullata, S. crassipalpis, S. argyrostoma, Phormia regina, Musca autumnalis, and Stomoxys calcitrans. The LD_{50} of carbaryl alone and when synergized with piperonyl butoxide has been used as an indicator of MFO activity as shown in Table 2.

In vitro assays for MFO activity in N-dealkylation, O-demethylation, and epoxidation were also performed with adults and pupae of S. bullata, S. crassipalpis, and P. regina (Brattsten and Metcalf 1973). Maximum activity was generally associated with the microsomal pellet following 100,000X g centrifugation. N-demethylase activity showed only slight variation during pupa and adult flies of 1 to 8 days of age. However, O-demethylation was lowest in nearly emerged flies and rapidly increased from 4 to 10-fold at 8 days of age. Activity was generally lower in males than in females. Epoxidation was low and virtually constant with age. O-demethylation was shown to be associated both with microsomal and soluble fractions and several enzymes may be involved. From the data in Table 2 and supplementary evidence it can be concluded that the major factors responsible for variations in the susceptibility to carbaryl associated with sex and age of typical dipterous insects are levels of MFO enzymes and related enzymes as associated with the complex patterns of metabolism involved in development and reproduction, rather than by other factors such as penetration or excretion.

Table 2. EFFECTS OF SEX, AGE, AND DEVELOPMENTAL STAGE ON SUSCEPTIBILITY OF FLIES TO CARBARYL

			Topical LD_{50} µg/g		
			carbaryl	carbaryl + piperonyl butoxide	Synergistic Ratio
Sarcophaga bullata	♀	1d	6000	7.5	800
		8d	--	14.5	> 69
	♂	1d	1100	6.1	180
		8d	>500	13	> 38
Sarcophaga crossipalpis	♀	1d	27	5.9	4.6
		8d	72	10.5	6.9
	♂	1d	52	6.6	7.9
		8d	60	6.6	9.1
Sarcophaga argyrostoma	♀	1d	110	4.5	24.4
		8d	52	10	5.2
	♂	1d	56	5.8	9.7
		8d	37	10	3.7
Phormia regina	♀	1d	13	4.5	2.9
		8d	90	11	8.2
	♂	1d	17	7	2.4
		8d	160	8.9	18
Musca antumnalis	♀	1d	8.8	1.2	7.3
		8d	3.0	1.3	2.3
	♂	1d	6.9	0.8	9.2
		8d	3.7	0.9	4
Stomoxys calcitrans	♀	1d	0.7	0.5	1.4
		8d	0.5	0.1	5
	♂	1d	0.8	0.6	1.3
		8d	0.6	0.2	3.0

V. MFO OXIDATION OF PESTICIDES IN GREEN SUNFISH AND EFFECTS OF PIPERONYL BUTOXIDE

As indicated above fish have generally low levels of MFO enzymes and this poses severe disadvantages to them in regard to low levels of micropollutant exposure and consequent biomagnification. We have evaluated the role of MFO enzymes in the green sunfish, Lepomis cyanellus by exposing the fish for up to 16 days to 0.01 ppm concentrations of ^{14}C radiolabeled methoxychlor, aldrin, and trifluralin followed by quantitative and qualitative radiochemical assay using thin-layer chromatography, radioautography, and liquid scintillation counting to determine rates of storage and degradation of the xenobiotics. To determine the total role of the MFO enzymes, a companion set of fish were exposed to each of the three pesticides together with 0.1 ppm piperonyl butoxide synergist (P.B.) which inhibits the MFO enzymes (Casida 1971). The data obtained with the three pesticides is shown in Tables 3-5 (Reinbold and Metcalf 1975).

Methoxychlor or 2,2-bis-(p-methoxy phenyl)-1,1,1-trichloroethane is degraded largely by O-demethylation and this reaction is greatly retarded by P.B. Thus with methoxychlor alone, the dihydroxy degradation product was present after 16 days at 0.216 ppm but with the addition of 0.1 ppm P.B., the concentration of this degradation product was only 0.055 ppm. Similarly, with the methoxychlor treatment alone, the fish contained 0.04 ppm parent compound after 16 days as compared to 0.605 ppm when P.B. was present (Table 3).

Aldrin or 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-dimethanonaphthalene is readily oxidized to the 3,4-epoxide dieldrin by MFO. In the green sunfish, with aldrin alone at 0.01 ppm the tissues contained 0.051 ppm aldrin after 16 days, but with the addition of 0.1 ppm P.B. the tissues contained 1.076 ppm aldrin or a 21 fold increase (Table 4). The effect of PB was also evident in the production of further

Table 3. CONCENTRATIONS OF ^{14}C-METHOXYCHLOR AND DEGRADATION PRODUCTS IN GREEN SUNFISH FROM TREATMENT AT 0.01 PPM.

	Methoxychlor									
	ppm-methoxychlor equivalents									
	Fish									
	Day 1 alone	Day 1 +PB	Day 2 alone	Day 2 +PB	Day 4 alone	Day 4 +PB	Day 8 alone	Day 8 +PB	Day 16 alone	Day 16 +PB
Total ^{14}C	5.496	4.593	3.729	7.272	4.036	7.512	1.781	4.696	2.055	2.753
Methoxychlor ethylene (R_f 0.80[a]; 0.29[b])	0.098	0.237	0.049	0.347	0.027	0.472	0.008	0.259	0.008	0.133
Methoxychlor (R_f 0.80: 0.22)	0.805	2.986	0.867	4.276	0.331	4.652	0.073	2.474	0.040	0.605
Unknown I (R_f 0.65; 0.17)	0.015	0.006	0.021	0.011	0.020	0.020	0.008	0.034	0.019	0.010
Mono-OH (R_f 0.53; 0.13)	0.357	0.102	0.255	0.219	0.791	0.320	0.310	0.251	0.417	0.522
DiOh + (R_f 0.40: 0.00) DiOH ethylene	0.041	0.009	0.042	0.013	0.122	0.041	0.126	0.023	0.216	0.055
Unknown II (R_f 0.25: 0.00)										
DiOH COOH (R_f 0.07; 0.00	0.040	0.005	0.049	0.012	0.051	0.022	0.039	0.024	0.047	0.023
Polar (R_f 0.0)	0.063	0.028	0.079	0.026	0.067	0.071	0.041	0.028	0.039	0.063
Unextractable	4.077	1.220	2.367	2.367	2.628	1.913	1.176	1.603	1.269	1.341

[a] TLC with petroleum ether (b.p. 60-68°C) - chloroform - methanol, 3:2:1/

[b] TLC with diethyl ether = petroleum ether, 1:9.

Table 4. CONCENTRATIONS OF ^{14}C-ALDRIN AND DEGRADATION PRODUCTS IN GREEN SUNFISH FROM TREATMENT AT 0.01 PPM.

	Aldrin									
	ppm-aldrin equivalents									
	Fish									
	Day 1 alone	Day 1 +PB	Day 2 alone	Day 2 +PB	Day 4 alone	Day 4 +PB	Day 8 alone	Day 8 +PB	Day 16 alone	Day 16 +PB
Total ^{14}C	5.632[a]	5.532	5.694[a]	7.611	4.934	7.688	5.537	6.700	3.800	4.738
Aldrin (R_f 0.68[b])	2.015	3.9599	1.293	5.179	0.403	4.572	0.179	3.455	0.051	1.076
Dieldrin (R_f 0.56)	3.180	1.1881	3.971	1.572	3.907	2.422	4.938	2.693	3.289	3.021
Unknown I (R_f 0.44)	0.009	0.00085	0.004	0.045	0.072	0.018	0.044	0.011	0.010	0.002
9-OH dieldrin (R_f 0.31)	0.017	0.0112	0.025	0.017	0.110	0.0225	0.063	0.017	0.062	0.014
9-C+O dieldrin (R_f 0.23)	0.023	0.0015	0.018	0.020	0.009	0.015	0.021	0.008	0.016	0.006
Unknown II (R_f 0.15)	0.007	0.0060	0.003	0.018	--	0.017	0.0008	0.007	--	0.001
DiOH trans aldrin (R_f 0.05)	0.0045	0.0056	0.003	0.005	0.005	0.004	0.007	0.003	0.007	0.009
Polar (R_f 0.0)	0.006	0.0078	0.007	0.012	0.0165	0.0185	0.009	0.013	0.006	0.019
Unextractable	na[c]	0.351	na[c]	0.743	0.412	0.599	0.275	0.493	0.357	0.590

[a] Including estimated ppm unextractable.

[b] TLC with hexane - diethyl ether, 1:1.

[c] Not analyzed.

Table 5. CONCENTRATIONS OF ^{14}C-TRIFLURALIN AND DEGRADATION PRODUCTS IN GREEN SUNFISH FROM TREATMENT AT 0.01 PPM

	Trifluralin									
	ppm-trifluralin equivalents									
	Fish									
	Day 1 alone	Day 1 +PB	Day 2 alone	Day 2 +PB	Day 4 alone	Day 4 +PB	Day 8 alone	Day 8 +PB	Day 16 alone	Day 16 +PB
Total ^{14}C	3.031	4.014	2.709	4.215	2.761	5.233	0.650	3.461	0.234	0.580
Trifluralin ($2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2N(C_3H_7)_2$) ($R_f$ 0.93[a])	1.979	3.634	1.611	3.557	1.058	4.683	0.137	3.101	0.005	0.225
$2,6\text{-}NO_2\text{-}4CF_3C_6H_2NHC_3H_7$ (R_f 0.84)	0.111	0.023	0.084	0.030	0.091	0.048	0.022	0.027	0.002	0.006
$2\text{-}NO_2\text{-}4\text{-}CF_3\text{-}6NH_2\text{-}C_6H_2N(C_3H_7)_2$ (R_f 0.63)	0.018	0.033	0.009	0.018	0.010	0.025	0.004	0.006	0.001	0.0015
$1,2\text{-}(N\text{-}CH(C_2H_5)N\text{-}C_3H_7)\text{-}3NO_2\text{-}5\text{-}CF_3$ (R_f 0.53)	0.002	0.001	0.001	--	0.001	0.0025	0.002	--	0.003	0.001
$2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2NH_2$ (R_f 0.47)	0.017	0.015	0.005	0.016	0.011	0.011	0.002	0.008	--	0.001
Unknown I (R_f 0.39)	0.075	0.015	0.095	0.018	0.082	0.008	0.050	0.010	0.009	0.013
Unknown II (R_f 0.31)	0.027	0.004	0.012	0.009	0.020	0.007	0.005	0.004	0.005	0.004
$1,2\text{-}(N\text{-}CH9C_2H_5)\text{-}NH)\text{-}3NO_2\text{-}5\text{-}CF_3\text{-}C_6H_2$ (R_f 0.19)	0.020	0.0115	0.028	0.007	0.048	0.011	0.017	0.006	0.021	0.0016
Unknown III (R_f 0.17)	0.044	0.008	0.028	0.002	0.055	0.007	0.015	0.007	0.017	0.008
Unknown IV (R_f 0.13)										
Unknown V (R_f 0.10)	0.068	0.007	0.081	0.025	0.116	0.011	0.066	0.013	0.042	0.022
Polar (R_f 0.0)	0.374	0.074	0.492	0.292	1.010	0.105	0.263	0.099	0.086	0.2125
Unextractable	0.295	0.188	0.263	0.241	0.259	0.313	0.067	0.180	0.044	0.070

[a] TLC with hexane - acetone - methanol, 90:10:2 in unsaturated tank (9).

metabolic products such as 9-OH aldrin and 9-C=O dieldrin both of which formed more slowly.

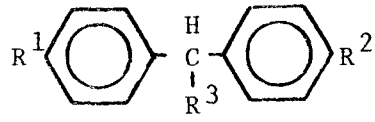
Trifluralin or α,α,α-trifluro-2,6-dinitro-N,N-dipropyl-p-toluidine is degraded largely by N-dealkylation by MFO. With trifluralin alone, at 0.01 ppm, the green sunfish contained 0.005 ppm parent compound after 16 days as compared to 0.225 ppm in the presence of P.B., for a 45-fold difference (Table 5).

In summary the MFO enzymes of the green sunfish provide substantial protection against storage and accumulation of these xenobiotics. The inhibition of these MFO enzymes with piperonyl butoxide resulted in the accumulation of 15X as much methoxychlor, 21X as much aldrin, and 45X as much trifluralin. These data stress the importance of MFO enzymes in the survival of fish in polluted aquatic environments and demonstrate the danger of traces of MFO inhibitors, such as P.B., which may also be present as aquatic pollutants in blocking normal biochemical survival mechanisms. The value of degradophore groups which can be oxidized by MFO reactions such as O-dealkylation or N-dealkylation in the design of biodegradable pesticides is readily apparent.

VI. ROLE OF DEGRADOPHORES IN DESIGN OF DEGRADABLE INSECTICIDES

The experience of the past 25 years with DDT, dieldrin and other persistent non-degradable organochlorine insecticides has shown the gross incompatibility of these materials with desirable environmental quality. Their use has resulted in definition of and focus on the phenomenon of biomagnification whereby trace amounts of these micropollutants below the limits of water solubility have been absorbed and concentrated by living organisms and deposited in their body lipids. The importance of this phenomenon is shown by the experience in Lake Michigan where DDT at an average concentration in the open lake of 6 ppt is found in mature lake trout Salvelinus namaycush at an average of 18.8 ppm, bioconcentration 3.13×10^6. The corresponding values for dieldrin are 2 ppt in water and 0.26 ppm in trout, bioconcentration 1.30×10^5 (EPA 1972). Bioconcentration in Lake Michigan has nearly destroyed the commercial fishing industry because the contaminated fish are unsafe for consumption. Moreover because of the carcinogenic properties of DDT and dieldrin, the ubiquitous presence of tissue residues in humans, averaging about 11 ppm DDT plus DDE, and about 0.29 ppm dieldrin (Durham 1969) is most disquieting from a public health viewpoint. Nevertheless, there is clearly a need for insecticides which are of some degree of environmental persistence on inert surfaces yet which are readily degradable in vivo. We refer to these compounds as persistent biodegradable insecticides.

In this research grant we have concentrated study on the DDT-type molecule:



This has been shown to be generally of low cost, relatively stable on inert surfaces and modifiable in a large number of ways while preserving substantial insecticidal properties. In DDT itself

R^1 and R^2 are Cl and R^3 is $-CCl_3$. The presence of the very stable C-Cl bonds in these three areas of the DDT molecule is largely responsible for the high *in vivo* stability of DDT and its storage in tissue lipids of animals. The DDT-type molecule in which other less environmentally stable molecular groupings are substituted for Cl atoms, is an ideal tool for studying the principles of biodegradability. The problems of developing biodegradable analogues of DDT are two-fold: (1) incorporating molecular groups or degradophores which can serve as suitable substrates for MFO enzymes, and (2) preserving an overall configuration to the molecule so that it will have DDT-like biological activity. The second phase falls outside the scope of this grant research but has been discussed by Metcalf et al (1971) and by Coats and Metcalf (1975).

Development of Degradophores

From the state of the art in 1969 when this research began we considered potential degradophores for DDT-like action to be: (a) for R^1 and R^2 CH_3O, C_2H_5O, C_3H_7O, OCH_2O, CH_3,C_2H_5, C_3H_7, and CH_3S, C_2H_5S, C_3H_7S; e.g. groups that would be readily oxidized by MFO enzymes in the vertebrate liver, thus converting the compounds into water partitioning moieties which would be excreted rather than stored in tissue lipids. We also considered (b) for R^3 degradophores with stereochemistry resembling that of the CCl_3 groups i.e. CMe_3, CMe_2Cl, CMe_2NO_2, CHMeCl, $CHMe_2$.

Methoxychlor and Methylchlor. The simplest degradophores for incorporation into the DDT molecule are p,p'-CH_3O, and p,p'CH_3 groups. Methoxychlor or 2,2-bis-(p-methoxyphenyl)-1, 1,1-trichloroethane was developed at the same time as DDT (Muller 1944) and was known as a very safe DDT analogue which did not accumulate in milk or store in animal fat as did DDT. However, these were no satisfactory studies of its degradative pathways in animals. Using ^{3}H-radiolabeled methoxychlor, Kapoor et al (1970) showed that methoxychlor was readily dealkylated

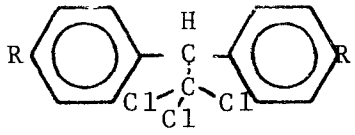
by MFO enzymes in mouse liver, to give mono- and di-hydroxy derivatives (Table 6). The result of this biochemical conversion was to change methoxychlor, H_2O solubility 0.62 ppm, to 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, H_2O solubility 76 ppm.

Similar study with methylchlor or 2,2-bis-(p-tolyl)-1,1,1-trichloroethane showed the conversion of methylchlor, H_2O solubility 2.21 ppm, to 2,2-bis-(p-carboxyphenyl)-1,1,1-trichloroethane, H_2O solubility 50 ppm. (Table 6) Methiochlor or 2,2-bis-(p-methylthiophenyl)-1,1,1-trichloroethane, H_2O solubility 0.57 ppm was converted by MFO enzymes to 2,2-bis-(p-methylthiophenyl)-1,1,1-trichloroethane, H_2O solubility 29 ppm (Table 6).

Studies of these radiolabeled DDT analogues in the laboratory model ecosystem (Metcalf et al 1971, Kapoor et al 1972) showed that the analogues incorporating degradophores were truly biodegradable and accumulated in the tissues of fish and other organisms to substantially lower levels than did DDT (Table 7). The degradative pathways found in the model ecosystem investigations were predominately those predicted from the microsomal enzyme incubation studies (Table 6).

Further investigations of this nature (Kapoor et al 1973) showed that the presence of a single degradophore e.g. CH_3, in the DDT-type molecule to form 2-(p-chlorophenyl)- 2-(p-tolyl) -1,1,1-trichloroethane was sufficient to produce a desirable degree of biodegradability and to prevent the compound from bioaccumulating to high levels in aquatic organisms (Table 7). Evaluation of the insecticidal properties (Metcalf et al 1971) and biodegradability of additional DDT analogues with asymmetrical arrangement of degradophores (Kapoor et al 1973) indicated that the most favorable combination of insecticidal properties and biodegradability was obtained with asymmetrical DDT analogues containing two different types of degradophores, e.g. 2-(p-methoxyphenyl)-2-(p-tolyl)-1,1,1-trichloroethane, and 2-(p-ethoxyphenyl)-2-(p-tolyl)-1,1,1-trichloroethane, as shown in Table 7.

Table 6. DEGRADATION OF DDT AND ANALOGUES BY MOUSE LIVER MICROSOMES [1/]

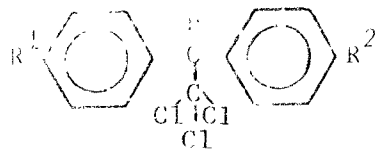


R	products recovered [2/]
Cl (DDT)	DDT with traces of α-OH compound
CH_3O (methoxychlor)	$HOC_6H_4CHCCl_3C_6H_4OCH_3$
	$HOC_6H_4CHCCl_3C_6H_4OH$
C_2H_5O (ethoxychlor)	$HOC_6H_4CHCCl_3C_6H_4OC_2H_5$
CH_3 (methylchlor)	$HOCH_2C_6H_4CHCCl_3C_6H_4CH_3$
	$HOOCC_6H_4CHCCl_3C_6H_4CH_3$
	$HOCH_2C_6H_4CHCCl_3C_6H_4CH_2OH$
	$HOOCC_6H_4CHCCl_3C_6H_4COOH$
CH_3S (methiochlor)	$CH_3SOC_6H_4CHCCl_3C_6H_4SCH_3$
	$CH_3SOC_6H_4CHCCl_3C_6H_4SOCH_3$

1/ Kapoor et al (1970, 1972)

2/ by TLC and radioautography after 2 hours incubation with microsomal suspension

Table 7. ECOLOGICAL MAGNIFICATION AND BIODEGRADABILITY OF DDT ANALOGUES [1/]



R^1	R^2	ecological magnification [2/] fish	ecological magnification [2/] snail	biodegradability index [3/] fish	biodegradability index [3/] snail
Cl	Cl	84,500	34,500	0.015	0.045
CH_3O	CH_3O	1,545	120,000	0.94	0.13
C_2H_5O	C_2H_5O	1,536	97,645	2.69	0.39
CH_3	CH_3	140	120,270	7.14	0.08
CH_3S	CH_3S	5.5	300	47	0.77
CH_3O	CH_3S	310	3,400	2.75	105
CH_3	C_2H_5O	400	42,000	1.20	0.25
CH_3	Cl	1,400	21,000	3.43	2.0

1/ Kapoor et al (1973)

2/ ratio of concentration in organism/concentration in water

3/ ratio of polar/nonpolar metabolites

Development of Synergaphores

Molecular moieties which inhibit the MFO enzymes are termed synergaphores. The best known is the $-OCH_2O-$ or methylenedioxy group. Methylenedioxyphenyl derivatives are widely used as insecticide synergists, e.g. piperonyl butoxide, sesamex, propyl isome; and it appears that these compounds combine or complex with cytochrome P.450 to prevent its function as a carrier of the .OH radical (Casida 1971). Another molecular moiety which has this property is the propynyloxy group $-OCH_2C{\equiv}CH$ (Sacher et al 1968). Synergaphores attached to various active insecticidal groupings have been shown to substantially increase the toxicity of phenyl N-methylcarbamates by autosynergism (Metcalf 1968) and this technique has also been used with the DDT-type molecule (Metcalf et al 1971). Attachment of the 3,4-methylenedioxy group to the methoxychlor type molecule as in 2-(p-methoxyphenyl) -2-(3,4-methylenedioxyphenyl)-1,1,1-trichloroethane reduced the LD_{50} values to the house fly from 45 to 22 µg per g. and reduced the synergistic ratio from 12.8 to 4.9. The addition of the propynyl group as in 2-(p-methoxyphenyl)-2-(p-propynyloxy phenyl) 1,1,1-trichloroethane reduced the LD_{50} value to 34 and the synergistic ratio to 10.3, indicating a lesser degree of autosynergism.

When the 2-(p-methoxyphenyl)-2-(3,4-methylenedioxyphenyl)-1,1,1-trichloroethane was compared with methoxychlor in toxicity studies to the green sunfish at 0.1 ppm the former compound was found to be concentrated and persistent in fish tissues with an average residue of 18.0 ppm after 4 weeks compared to 1.75 ppm for methoxychlor. Thus although the $-OCH_2O-$ group may increase the insecticidal potency of specific compounds it may also have adverse environmental effects in increasing persistence and bioaccumulation.

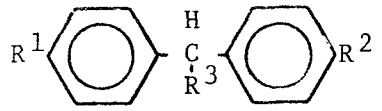
VII. DEGRADATION PATHWAYS FOR DDT ANALOGUES WITH ALTERED ALIPHATIC MOIETIES

The most important deficiency of DDT as an insecticide is the environmental dehydrochlorination of the $-CCl_3$ group to form the very stable ethylene DDE (CCl_2). DDT analogues incorporating isosteric groups such as $-CMe_3$ and $CH(Me)NO_2$ in place of $-CCl_3$ have typical DDT-like biological activity (Table 8). Although Holan (1971) has proposed the use of such compounds as biodegradable substitutes for DDT, little or no information is available about their degradative pathways and the effects of MFO enzymes on these moieties.

Dianisylneopentane - We have investigated the metabolism and degradation of ^{3}H-labeled dianisylneopentane (1,1-bis-p-methoxyphenyl)-2, 2-dimethylpropane in the house fly, the salt marsh caterpillar, by mouse liver microsomes and in a laboratory model ecosystem (Coats et al 1974). All of the results were in agreement in showing the remarkable biological stability of the neopentyl moiety. In the mouse, the major metabolites were the mono- and bis-phenols produced by microsomal O-dealkylation (see Table 9). The neopentyl group was only slightly attacked. In the model ecosystem, substantial amounts of dianisylneopentane persisted in snail and fish and this compound behaved almost identically to methoxychlor. Thus the neopentyl group is not a satisfactory substrate for the MFO enzymes.

Prolan or 1,1-bis-(p-chlorophenyl)-2-nitropropane is another DDT isostere with good insecticidal activity. Investigation of its metabolic and environmental fate using ^{14}C radiolabeled compound has been undertaken (Hirwe et al 1975). Prolan is substantially more degradable than DDT and although it accumulated to high levels in the snail Physa, was readily degraded in the fish Gambusia, of the model ecoystem. Degradation of Prolan occurred by two general mechanisms (a) dehydrogenation of the α-H and release of the NO_2^-

Table 8. EFFECTS OF STRUCTURAL CHANGES ON TOXICITY OF DDT ANALOGUES [1/]



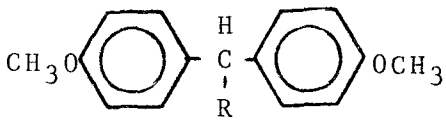
			topical LD_{50} µg per g.			
			Musca (S)		Phormia	
R^1	R^2	R^3	alone	p.b.	alone	p.b.
Cl	Cl	CCl_3	14.0	5.5	11.5	8.25
CH_3	CH_3	CCl_3	100	17.5	61.2	21.5
CH_3O	CH_3O	CCl_3	45	3.5	10.0	4.6
C_2H_5O	C_2H_5O	CCl_3	7.0	1.75	6.9	7.4
Cl	Cl	CMe_3	85	15.5	70	55
CH_3	CH_3	CMe_3	1250	35		
CH_3O	CH_3O	CMe_3	95	19	17	5.75
C_2H_5O	C_2H_5O	CMe_3	37.5	9.0	100	92.5
Cl	Cl	$HC(Me)NO_2$	20.5	7.0	11.0	11.0
CH_3	CH_3	$HC(Me)NO_2$	145	1.9	13.0	6.0
CH_3O	CH_3O	$HC(Me)NO_2$	>500	2.75	12.5	7.75
C_2H_5O	C_2H_5O	$HC(Me)NO_2$	5.0	1.25	32.5	1.35

Table 8 (continued)

Cl	Cl	HCMeCl	180	37.0	>250	107.5
CH_3	CH_3	HCMeCl	500	24.5	>250	125
CH_3O	CH_3O	HCMeCl	>500	3.5	>250	40
C_2H_5O	C_2H_5O	HCMeCl	9.5	2.0	4.6	4.6
Cl	Cl	$HCMe_2$	>500	500	>250	>250
CH_3	CH_3	$HCMe_2$	>500	300	>250	>250
CH_3O	CH_3O	$HCMe_2$	>500	36	122.5	95
C_2H_5O	C_2H_5O	$HCMe_2$	21.5	3.5	14.5	6.7
CL	Cl	$HCCl_2$	72.5	35	15.2	9.0
CH_3	CH_3	$HCCl_2$	120	11.5	10.7	8.5
CH_3O	CH_3O	$HCCl_2$	>500	10.0	250	7.5
C_2H_5O	C_2H_5O	$HCCl_2$	9.0	2.15	3.75	2.75

1/ Coats (1974) and Coats and Metcalf (1975)

Table 9. DEGRADATION OF METHOXYCHLOR AND ANALOGUES BY SHEEP LIVER MICROSOMES [1/]



R	products	recovered [2/]
CCl_3 (methoxychlor)	parent	86.6%
	monophenol	8.1%
	diphenol	3.4%
	polar	1.9%
CMe_3 (dianisylneopentane)	parent	93.5%
	monophenol	3.8%
	diphenol	0.8%
	polar	--
$HC(Me)NO_2$	parent	91.5%
	monophenol	5.7%
	diphenol	0.3%
	polar	trace
HC(Me)Cl	parent	90.0%
	monophenol	5.8%
	diphenol	1.0%
	polar	1.2%

1/ Coats (1974)

2/ by TLC and radioautography after 30 minutes incubation with microsomal suspension

moiety to produce the ethylene 1,1-bis-(p-chlorophenyl)-propene and (b) by oxidation of the propyl CH_3 group to produce 2,2-bis(p-chlorophenyl)-pyruvic acid and eventually bis-(p-chlorophenyl)-acetic acid. These pathways were demonstrated in fly, mouse, and in the model ecosystem. In the mouse the principal excretory products were bis-(p-chlorophenyl)acetone, bis-(p-chlorophenyl)-pyruvic acid, and bis-(p-chlorophenyl)-acetic acid. (DDA) The ultimate environmental degradation products from Prolan are thus DDA and 4,4'-dichlorobenzophenone both of which are also formed from DDT. The ultimate environmental distinction is that Prolan forms a biodegradable propene while DDT forms the non-biodegradable dichloroethylene DDE. Thus the nitropropane moiety $CHMeNO_2$ is a suitable degradophore.

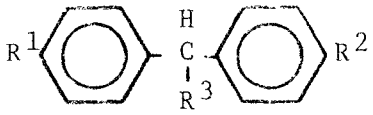
VIII. TOXICITY TO MOUSE AND GREEN SUNFISH

An important feature of use of new DDT-type analogues is their relative toxicity to mammals and to fish. The high toxicity of DDT to a variety of fish has been a major drawback of its use and this toxicity extends to methoxychlor and many other analogues. Evidently, as discussed in the Introduction, O-dealkylation and certain other MFO catalyzed processes are not readily carried out in the fish.

We have explored, in a preliminary way, the toxicity of a number of the DDT-type analogues incorporating degradophores to the Swiss mouse, as measured by the oral LD_{50} of solutions in olive oil. In addition we have studied the toxicity of the analogues at 0.1 ppm in water from acetone, using the duration of toxicity to the green sunfish Lepomis cyanellus. These latter studies were carried out in 1700 l. tanks at both summer (average 70-90°F) and winter temperatures (average 40-50°F) and are so reported in Table 10. It is apparent from this data that the nature of the aryl and alkyl substituents R^1, R^2, and R^3 has a pronounced effect on the toxicity of the compound to both mouse and fish. Surprisingly, the most toxic DDT analogue evaluated to the mouse was the C_2H_5O-C_3H_7O analogue which was more than twice as toxic as DDT. The least toxic was the CH_3-CH_3 analogue followed by CH_3O-CH_3, and methoxychlor CH_3-CH_3O. All of the combinations of C_2H_5O-C_2H_5O were safer than DDT and enhanced safety over CCl_3 (ethoxychlor) was gained by other alkyl groupings, showing that these do function as degradophores.

The toxicity picture to the green sunfish is more complex and reflects the generally low level of MFO enzymes. Thus groups such as aryl C_2H_5O which function as degradophores in the mouse are not as effective in fish and all combinations of C_2H_5O-C_2H_5O except with CMe_2Cl showed prolonged toxicity to the green sunfish especially in winter. The marked enhancement of safety to fish provided by a single aryl CH_3 is demonstrated in Table 10, by comparing the C_2H_5O-C_2H_5O compounds with their CH_3-C_2H_5O analogues for CCl_3, HCMeCl, and $HCMe_2$.

Table 10. TOXICITY OF DDT ANALOGUES TO MOUSE AND GREEN SUNFISH



R^1	R^2	R^3	oral LD_{50} mg per kg Swiss mouse [1]	duration of toxicity at 0.1 ppm - days green sunfish [2]
Cl	Cl	CCl_3	200	18 (S)
CH_3	CH_3	CCl_3	3350	--
CH_3O	CH_3O	CCl_3	1850	2 (S) 41 (W)
C_2H_5O	C_2H_5O	CCl_3	300-325	22 (S)
C_2H_5O	C_3H_7O	CCl_3	75-100	41 (W)
CH_3	CH_3O	CCl_3	>1000	0(S) 15 (W)
CH_3	C_2H_5O	CCl_3	1000	0(S) 15 (W)
C_2H_5O	C_2H_5O	CMe_3	>1000	76 (W)
C_2H_5O	C_2H_5O	$HCMe_2$	>2100	52 (W)
CH_3	C_2H_5O	$HCMe_2$	>1000	0(S)
C_2H_5O	C_2H_5O	HCMeCl	1000	4(S) 60(W)
CH_3	C_2H_5O	HCMeCl	>1000	0(W)
C_2H_5O	C_2H_5O	$HCMeNO_2$	>1000	64 (W)
C_2H_5O	C_2H_5O	CCl_2Me	800	80 (W)
C_2H_5O	C_2H_5O	CMe_2Cl	>1000	0(W)

[1] Metcalf et al (1974), Coats (1974)

[2] 5 fish used per tank, when killed restocked at weekly intervals. (Metcalf et al 1974), (s) summer (w) winter

Altered Aliphatic Moieties. An important objective was the evaluation of the effects of alterations in the CCl_3 group of the DDT-type compound. As shown in Table 8, a considerable variety of other groupings can be substituted with preservation of the insecticidal activity. However, the biological effects of the DDT-type molecule are a function of the size and shape of the entire molecule and optimum activity for a given aliphatic moiety will require one set of p,p'-substituents while another set will be required for a different aliphatic moiety (Table 8). The effects of molecular groupings readily attacked by MFO enzymes are shown by the comparisons between LD_{50} to Musca alone and LD_{50} after pretreatment with piperonyl butoxide (p.b.) which inhibits most of the MFO activity. Aliphatic moieties for which MFO detoxication was maximal were CHMeCl and $CHMe_2$. This is well demonstrated also by the fish toxicity data in Table 10. However, when exposed to MFO enzymes directly in microsomes, the part of the DDT-type molecule subjected to initial attack is the degradaphores on the aromatic rings rather than the aliphatic moiety. This is clearly shown by the experiments with sheep liver microsomes as shown in Table 9. Regardless of the nature of the aliphatic moiety, O-demethylation was the dominant reaction.

IX. DDT ANALOGUES AS INDUCERS OF MFO ENZYMES

A major objection to persistent residues of DDT concentrating in living organisms is the role of DDT and similar lipophilic substances as inducers of MFO enzyme activity. This effect has been deemed responsible for the syndrome of egg-shell thinning and lowered reproductive efficiency in birds and may give rise to abnormal metabolism of both endogenous steroids and hormones as well as exogenuous drugs (Corney and Baines (1973). DDT and some of its analogues are potent inducers of microsomal enzymes in mouse liver (Hart and Fouts 1965, Abernathy et al 1971). We have compared the inductive effects of a variety of DDT analogues some incorporating degradophores upon the induction of mouse liver microsomal enzymes producing O-dealkylation, N-dealkylation, sulfoxidation, epoxidation and ring hydroxylation as shown in Table II. (Nigg et al 1975). The analogues were injected intraperitoneally into Swiss mice at 100 μg per kg. per day for 5 days before assay of liver homogenates.

The data shows the importance of degradophores in reducing accumulation of the compound in the endoplasmic reticulum and consequent induction. Both the COOH and OH derivatives formed by degradation of alkyl or alkoxy groups were non-inducers. However CH_3S which is a degradophore was highly inductive because of metabolism to the inducer CH_3SO_2.

X. BIOCHEMISTRY OF SELECTIVE TOXICITY

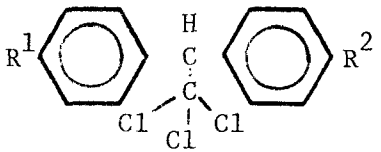
The concept of degradophores and the possibilities of building them into toxic moieties to enhance biodegradability has enhanced interest in the comparative biochemistry of selective toxicity. Clearly selective and biodegradable insecticides incorporating this principle will be effective only if detoxication in vertebrates is substantially more rapid than in invertebrates. We have investigated this area by studying O-dealkylation, a typical microsomal process on a comparative basis. In a comparison of rates of O-demethylation and O-deethylation of p-nitrophenyl ethers and DDT analogues it was found that Culex mosquito larva, Physa and Lymnaea snails, and the fish Gambusia affinis and Pimephales promelas were all more active in deethylation of p-nitrophenetol than in demethylation of p-nitroanisole. Longer chain propyl and butyl ethers were less readily O-dealkylated. Within the organisms, Gambusia was most active in O-dealkylation determined on a body weight basis, followed by Physa, Lymnaea, Culex, and Pimephales (Hansen et al 1972). The toxicity of methoxychlor and ethoxychlor was correlated with metabolism data except for snails which were naturally tolerant to these organochlorine insecticides.

The comparative in vivo and microsomal O-dealkylation of both p-nitrophenyl ethers and methoxychlor and ethoxychlor was studied by Hansen et al (1974) using the house fly Musca domestica, the flesh fly Sarcophaga bullata, and the white mouse. The rates of p- nitrophenyl alkyl ether O-dealkylation were found to vary with chain length: house fly-methyl>ethyl>n-propyl>n-butyl, flesh fly methyl=ethyl>n-propyl>n-butyl; mouse ethyl>methyl>n-propyl >n-butyl. When a comparison was made with DDT analogues, methoxy= chlor was O-dealkylated by both house fly and mouse at substantially

greater rates than ethoxychlor. Detailed kinetic studies showed the presence of both high and low affinity sites in microsomes of mouse liver and house fly abdomen. The mouse liver microsomes had a greater capacity for O-dealkylation than the fly abdomen microsomes and also converted more of the primary product to dihydroxy and polar metabolites (Hansen et al 1974).

These studies illustrate the importance of comparative biochemistry in understanding the relative effectiveness of various xenobiotics to specific organisms. There are astonishing qualitative and quantitative differences in the manner in which organic compounds are degraded by various species and the subject is of considerable complexity.

Table 11. INDUCTION OF MOUSE LIVER MFO BY DDT ANALOGUES [1/]



		MFO activity at per cent of controls					
R^1	R^2	P-450	N-demethylation	O-demethylation	S-oxidation	epoxidation	hydroxylation
Cl	Cl (DDT)	191*	97	161*	185*	278*	168*
CH_3O	CH_3O (methoxychlor)	79*	81*	79	167*	69	104
CH_3	CH_3	121	100	106	145*	160	86
NO_2	NO_2	103	100	144	83	93	--
C_2H_5O	C_2H_5O	149*	112	109	157	195*	107
CH_3S	CH_3S	209*	87	191*	175	386*	--
CH_3SO_2	CH_3SO_2	225*	110	149*	362*	273*	175*
OH	OH	104	113	104	115	170	
COOH	COOH	104	110	92	108	76	
CH_3S	CH_3O	--	111	114	--	91	--
CH_3	C_2H_5O	--	118	75	--	79	--
Cl	CH_3	--	76*	87	--	67	--
DDE		197*	99	140*	189*	246*	

* statistically significant at 5% level

1/ Nigg et al (1975)

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16. ABSTRACT

The role of the microsomal oxidase enzymes (MFO) in the biochemistry and toxicology of insecticides has been studied. Insects contain greatly varying titres of these enzymes. A survey of 74 species from 40 families in 8 orders, using the topical LD_{50} of carbaryl alone and together with the inhibitor piperonyl butoxide showed a 55,000-fold variability in LD_{50} largely due to MFO detoxication. In individual species of Diptera, MFO activity is highly variable with age, sex, and stage of development

The DDT-type molecule has been as a model for the study of degradophores, i.e. molecule groupings that can serve as MFO substrates. Their oxidation thus converts lipophilic compounds into more water-partitioning moieties and thus promotes excretion rather than lipid storage. Suitable degradophores for the DDT-type molecule are alkyl and alkoxy groups on the aryl rings. Compounds with judicious combinations of these provide relatively long persistence on inert surfaces and ready biodegradability in vivo. Such compounds are much less toxic to mice and to fish than DDT but because of the generally lower MFO of insects, can be effective insecticides. The role of degradophores incorporated into the aliphatic moiety of DDT has also been explored, where the $-CH(CH_3)_2$, $-CHCH_3Cl$ and $-CHCH_3NO_2$ groups are useful. Induction experiments with the biodegradable DDT analogues in mice has demonstrated that unlike DDT, these compounds do not elevate liver MFO.

17. KEY WORDS AND DOCUMENT ANALYSIS

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