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Cytochrome P-450 Optical Difference Spectra of Insecticides. A Comparative Study

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The cytochrome P-450 optical difference spectra of insecticides were examined using hepatic microsomes from sheep, rabbit, rat, and mouse as well as abdominal microsomes from insecticide-resistant (Fc) and insecticide-susceptible (CSMA) house flies. The results generally conform to the hypothesis that type II binding spectra are elicited by compounds containing a nitrogen atom with a sterically accessible pair of nonbonded electrons and, in addition, certain compounds with nucleophilic oxygen atoms. Other compounds generally exhibit type I spectral binding or do not form detectable spectra. Exceptions include certain pyrethroids which, while giving typical type I difference spectra with mammalian microsomes, gave rise to an unusual spectrum in insects, one with a peak at 415–418 nm and trough at 445–447 nm. Rotenone also gave rise to unusual difference spectra with hepatic microsomes of sheep and rabbit. Differences in the ratios of spectral size between species indicate that qualitative differences in the cytochrome P-450 of different species are common.

The microsomal hemoprotein, cytochrome P-450, has been implicated as the terminal oxidase in the microsomal mixed-function oxidase system responsible for the metabolism of xenobiotics (including insecticides) in mammals (Hodgson, 1968; Gillette *et al.*, 1972; Parke, 1968) and insects (Hodgson, 1968; Hodgson and Plapp, 1970; Wilkinson and Brattsten, 1972). The first step involved in the oxidation of a xenobiotic by the mixed-function oxidase system is its binding to oxidized cytochrome P-450 (Gillette *et al.*, 1972), a step which can be studied by optical difference spectroscopy. Two principal types of difference spectra are recognized with oxidized cytochrome P-450: type I, with a peak at 385–390 nm and a trough at 416–420 nm (Cooper *et al.*, 1965; Remmer *et al.*, 1966), and type II, with a peak at 424–430 nm and a trough(s) between 390 and 410 nm (Cooper *et al.*, 1965; Remmer *et al.*, 1966; Schenkman *et al.*, 1967; Sasame and Gillette, 1969). Type I spectra are known to be caused by many compounds including drugs and pesticides (Schenkman *et al.*, 1967; Sasame and Gillette, 1969; Kuwatsuka, 1970; Mailman and Hodgson, 1972; Baker *et al.*, 1972) while type II is characteristic of nitrogen-containing compounds with a sterically accessible pair of nonbonded electrons as well as certain alcohols (Cooper *et al.*, 1965; Remmer *et al.*, 1966; Schenkman *et al.*, 1967; Jefcoate *et al.*, 1969; Jefcoate and Gaylor, 1969; Diehl *et al.*, 1970; Mailman *et al.*, 1974; Kulkarni *et al.*, 1974).

Compounds which give rise to type I spectra are usually substrates for the microsomal mixed-function oxidase system. Previous investigators, with few exceptions (Schenkman *et al.*, 1967; Imai and Sato, 1967; Remmer *et al.*, 1969), have failed to demonstrate a correlation between spectrum formation by different compounds and the rates of their oxidative metabolism (Temple, 1971). This is to say K_s, the spectral binding constant, is not correlated in any systematic way with K_m, the Michaelis constant for the enzymatic reaction. Insecticides are not only metabolized by the mixed-function oxidase system but may also function as inhibitors or inducers (Hodgson, 1968; Hodgson and Plapp, 1970; Wilkinson and Brattsten, 1972; Baker *et al.*, 1972; Stevens *et al.*, 1973; Stevens and Greene, 1973; Greene, 1972; Greene and Stevens, 1973). Little is known concerning either the binding of insecticides to cytochrome P-450 or the variation between

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species in this regard. The present investigations were undertaken to provide comparative data in both of these areas.

MATERIALS AND METHODS

Insecticides. Analytical grade insecticides were either purchased from City Chemical Corporation or were generous gifts from the following sources: Perrine Primate Lab, Perrine, Fla.; Union Carbide Chemical Company; Ortho Division, Chevron; American Cyanamid; Imperial Chemical Industries; Dow Chemical Co.; Pyrethrum Board, Kenya; FMC Corporation, Niagara Chemical Division; E. I. duPont de Nemours and Co.; Mobil Chemical Co.; and Dr. Elliott, Department of Insecticides and Fungicides, Rothamsted Experimental Station, Harpenden, Hertfordshire, England. The chemical names of all insecticides are as follows: aldrin, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene; allethrin, 2-allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one ester of 2,2-dimethyl-3-(2-methylpropenyl)cyclopropanecarboxylic acid; anabasine, L-2-(3-pyridyl)piperidine; Azodrin, 3-hydroxyl-*N*-methyl-*cis*-crotonamide dimethyl phosphate; barthrin, 6-chloropiperonyl 2,2-dimethyl-3-(2-methylpropenyl)cyclopropanecarboxylate; Baygon, 2-isopropoxyphenyl *N*-methylcarbamate; bioallethrin, (±)-allethronyl (+)-*trans*-chrysanthemate; bioresemethrin, (5-benzyl-3-furyl)methyl (+)-*trans*-chrysanthemate; Bux, mixture of *m*-(1-methylbutyl)phenyl methylcarbamate (52.7%) and *m*-(1-ethylpropyl)phenyl methylcarbamate (19.1%); carbaryl, 1-naphthyl *N*-methylcarbamate; carbofuran, 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate; chlordane, 1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan; coumaphos, *O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-(2*H*)-1-benzopyran-7-yl) phosphorothioate; cyclethrin, *dl*-2-(2-furfuryl)-3-methyl-2-cyclopenten-4-ol-1-onyl *dl-cis-trans*-chrysanthemate; *p,p'*-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; diazinon, *O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate; dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8a-octahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene; dimethoate, *O,O*-dimethyl *S*-(*N*-methylcarbamoyl)methyl phosphorodithioate; dimethrin, 2,4-dimethylbenzyl-2,2-dimethyl-3-(2-methylpropenyl) cyclopropanecarboxylate; dimetilan, 1-(dimethylcarbamoyl)-5-methyl-3-pyrazolyl dimethylcarbamate; endosulfan, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide; endrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo-endo*-5,8-dimethanonaphthalene; Guthion, *O,O*-dimethyl *S*-[4-oxo-1,2,3-benzotriazin-3(4*H*)-yl]methyl phosphorodithioate; heptachlor, 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene; Kelthane, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol; Kepone, decachlorooctahydro-1,3,4-methano-2*H*-cyclobuta[*cd*]pentalen-2-one; lindane, 1,2,3,4,5,6-hexachlorocyclohexane; malathion, diethyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate; Menazon, *O,O*-dimethyl *S*-(4,6-diamino-*s*-triazin-2-yl)methyl phosphorodithioate; methomyl, methyl *N*-[(methylcarbamoyl)oxy]-thioacetimidate; Methoxychlor, 2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane; Mirex, dodecachlorooctahydro-1,3,4-metheno-1*H*-cyclobuta[*cd*]pentalene; Mobam, benzo[*b*]thien-4-yl methylcarbamate; Neopynamin, 2,2-dimethyl-3-(2-methylpropenyl)cyclopropane carboxylic acid esters with *N*-(hydroxymethyl)-1-cyclohexene-1,2-dicarboximide; nicotine, *l*-3-(1-methyl-2-pyrrolidyl)pyridine; NRDC-108, (5-benzyl-3-furyl)methyl 2,2,3,3-tetramethylcyclopropanecarboxylate; NRDC-119, (5-benzyl-3-furyl)methyl (+)-*cis*-chrysanthemate; pyrethrins, mixture of pyrethrins I and II; pyrethrum extract, mixture of pyrethroids; rotenone, 1,2,12,12a-tetrahydro-2-isopropenyl-8,9-dimethoxy-(1)-benzopyrano[3,4-*b*]furo[2,3-*b*](1)-benzopyran-6(6a*H*)-one; Ruelene, 4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate; SBP-1382, (5-benzyl-3-furyl)methyl 2,2-dimethyl-3-(2-methylpropenyl)cyclopropanecarboxylate; TDE, mixture of 2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethane (25%) and 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethane (70%); Temik, 2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime; toxaphene, polychlorinated camphene; trichlorfon, *O,O*-dimethyl (1-hydroxy-2,2,2-trichloroethyl)phosphonate; Zectran, 4-dimethylamino-3,5-xylyl methylcarbamate; Zinophos, *O,O*-diethyl *O*-(2-pyrazinyl) phosphorothioate.

Assays. The livers of 6–8-week-old male mice (North Carolina Department of Health Strain), rats (Wistar Strain), rabbits (weighing 5–6 lb), and female sheep were used as well as dissected abdomens of 6-day-old female house flies (*Musca domestica* L.) of the CSMA insecticide-susceptible strain and the Fc insecticide-resistant strain (Tate *et al.*, 1973).

Animals were killed by decapitation and the liver removed, rinsed, diced, washed, and homogenized in 0.05 *M* Tris-HCl buffer containing 0.1 *M* KCl (pH 7.5). The brei was then passed through two layers of cheesecloth and centrifuged at 10,000*g* for 15 min. The resulting supernatant was filtered through glasswool, and the microsomes were sedimented at 105,000*g* for 1 hr. The microsomal pellet was gently washed and resuspended in 0.5 *M* K_2HPO_4–KH_2PO_4 buffer (pH 7.5). Except for the initial washing, an identical procedure was used for obtaining microsomes from house fly abdomens. The final suspension was of a concentration equal to 25 abdomens/ml. The protein content (Lowry *et al.*, 1951) of the microsomal suspensions used varied between 1 and 2 mg/ml in the case of mouse, rat, rabbit, and sheep microsomes and between 2 and 4 mg/ml in the case of house fly microsomes of either strain.

The substrate difference spectra of insecticides were recorded using a Beckman Acta V spectrophotometer equipped with a turbid sample accessory. Since most of the insecticides are water insoluble, they were deposited on the bottom of 25-ml erlenmeyer flasks by evaporation of acetone solutions prior to the addition of the microsomal suspension. Three milliliters of microsomal suspension was added to each flask and, following aerobic incubation, with constant shaking, at ambient temperature for 15 min, was transferred to the sample cuvette. Corresponding incubations of microsomes without added insecticide were transferred to the reference cuvette. Nicotine was added directly, by microsyringe, to the microsomes in the sample cuvette while an equal volume of buffer was added to the reference cuvette. Several concentrations of each insecticide were incubated with freshly prepared microsomes to determine the concentration required for maximal spectral change. Spectral size of a given candidate insecticide as used in Tables I–IV is defined as the maximum peak to trough ΔOD relative to the reduced cytochrome P-450–carbon monoxide difference spectrum (ΔOD 450–490 nm) for the same preparation. Formal guidelines for the interpretation of optical difference spectra (Kulkarni and Hodgson, 1975; Mailman *et al.*, 1974) were used in order to eliminate discrepancies due to shifting base lines, denaturation of cytochrome P-450, etc. A variation of 10–20% was observed from one batch of microsomes to another. Cytochrome P-450 was estimated as the reduced CO complex in each microsomal preparation by the method of Omura and Sato (1964). The cytochrome P-420 level was negligible while no hemoglobin was detected in the preparations used. The average level of cytochrome P-450, measured as the reduced CO-difference spectrum after appropriate dilution with phosphate buffer, was 0.100 ± 0.010 ΔOD unit for mouse, rat, rabbit, and sheep microsomes and 0.060 ± 0.010 and 0.070 ± 0.015 ΔOD units for CSMA and Fc house fly microsomes, respectively. If the extinction coefficient of 91 cm^{-1}

mM^{-1} for the reduced CO complex of cytochrome P-450, as shown by Omura and Sato (1964) for the rabbit, is assumed to be correct for all animal species, then the observed levels of cytochrome P-450 would be 1.10 ± 0.11 nmol/ml for mouse, rat, rabbit, and sheep microsomes and 0.66 ± 0.07 and 0.77 ± 0.12 nmol/ml for CSMA and Fc house fly microsomes, respectively.

RESULTS AND DISCUSSION

The data obtained with various botanical insecticides and synthetic pyrethroids are given in Table I. This group of insecticides exhibits different types of spectral binding with oxidized cytochrome P-450 obtained from different species. Only nicotine and anabasine cause a type II spectral shift (Figure 1A) in all species studied. Rotenone does not produce any detectable spectrum with rat, mouse, and both strains of house fly while an unusual difference spectrum was observed with sheep and rabbit microsomes. As shown in Figure 1B this spectrum has a characteristic trough at 395 nm while the absorption maxima are at 415 and 417 nm, respectively, in microsomes from sheep and rabbit. These results confirm those reported by Kuwatsuka (1970) for rabbit and by Mailman and Hodgson (1972) for mouse microsomes and disagree with those of Schenkman *et al.* (1967) who reported a modified type II interaction of rotenone with rat microsomes.

The remaining insecticides (Table I) elicit a type I (Figure 1C) spectral interaction with the mammalian hepatic microsomes except for bioresemethrin which showed no detectable spectrum with rabbit microsomes. Further, in this species an additional peak at 417 nm is apparent when higher concentrations of pyrethrum extract, pyrethrins, allethrin, or bioallethrin were used.

In contrast to mammalian preparations, house fly microsomes from either strain exhibited unusual difference spectra with pyrethrins, allethrin, and bioallethrin. As illustrated in Figure 1D this spectrum has a characteristic peak at 415–418 nm and a trough at 445–447 nm. This spectral response differs from that caused by rotenone (Figure 1B) in that it has a distinct trough at 445–447 nm rather than at 395 nm. In addition, these spectral characteristics are observed at low as well as at higher concentrations and, therefore, cannot be considered those of an inverse type I spectrum (Schenkman *et al.*, 1972). The CSMA house fly microsomes do not yield any spectrum with the remaining compounds from this group while pyrethrum extract, dimethrin, Neopynamin, NRDC-119, and cyclethrin exhibit type I spectral interactions with Fc house fly microsomes. This is in agreement with earlier observations on the absence of type I spectral interactions in susceptible house flies and their presence in resistant strains (Philpot and Hodgson, 1972; Tate *et al.*, 1973; Kulkarni *et al.*, 1974).

In general, the order of spectral magnitude was observed to be rat > sheep > mouse > rabbit > Fc > CSMA house flies for type I spectral interactions, and rabbit > rat > mouse > sheep > Fc > CSMA house flies for type II spectral shifts indicating variation between species.

The data obtained with chlorinated hydrocarbon insecticides are given in Table II. Except for CSMA microsomes, which show no type I spectra, and Fc microsomes, which show a type II spectral shift with Kepone, all of these compounds give rise to type I spectral interaction with cytochrome from all sources.

The spectral amplitude of TDE and Kelthane was found to be much larger (Table II) than the parent compound DDT or its closely related analog, methoxychlor. The spectral size of aldrin is smaller than that of its epoxide, dieldrin, in the case of rat microsomes while the reverse is true with rabbit and Fc microsomes. Both sheep and mouse microsomes exhibit an almost equal response to aldrin and dieldrin. Similar results were observed with

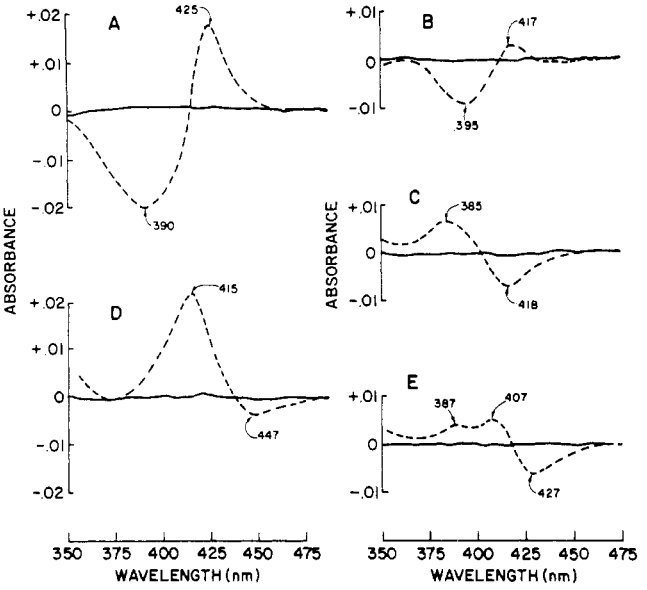

Figure 1. Oxidized cytochrome P-450 substrate optical difference spectrum of: (A) nicotine with hepatic microsomes of mouse; (B) rotenone (2.5×10^{-3} *M*) with hepatic microsomes of rabbit; (C) pyrethrins (7.5×10^{-4} *M*) with hepatic microsomes of mouse; (D) pyrethrins (5.0×10^{-6} *M*) with abdominal microsomes of Fc house flies; (E) carbaryl (2.0×10^{-3} *M*) with hepatic microsomes of rabbit. See Materials and Methods for additional details.

these insecticides by Greene (1972) and Greene and Stevens (1973) using either rat or mouse microsomes. However, a quantitative comparison is not possible since these authors added ethanol solutions of the insecticides directly to the sample cuvette and reported spectral size as the ΔOD between 470 nm and the trough. With the exception of rabbit microsomes, the spectral interactions of endrin, an endo-endo isomer of dieldrin, were equal or of greater magnitude than dieldrin although the endrin concentration required for maximal spectral change was found to be equal to or less than dieldrin (Table II). In general, the spectral magnitudes observed for these insecticides are rat > sheep > mouse > rabbit > Fc > CSMA house flies.

The CSMA microsomes did not yield a detectable spectrum with any of the carbamate insecticides tested (Table III) while, with minor exceptions, type I binding was observed with the microsomes from other sources. Carbaryl induced a spectrum with rabbit microsomes which shows a major peak at 407 nm and a minor peak at 385 nm while the trough is located at 427 nm (Figure 1E). These results agree in part with those of Kuwatsuka (1970) although he did not report the minor peak. Stevens *et al.* (1973) reported that carbaryl exhibited unstable binding with rat and mouse microsomes. However, we observe quite stable binding of this insecticide with all the cytochrome P-450's studied except that of CSMA house fly microsomes with which no detectable spectrum is produced. With sheep microsomes, methomyl was found to cause a mixed spectrum at all concentrations tested. A similar spectral response was observed with rat and rabbit but not with the mouse microsomes when higher concentrations were employed ($>1 \times 10^{-3}$ *M*). In addition, higher concentrations of Baygon and Temik with rat microsomes gave rise to a spectrum which contained a type II component in addition to type I. Bux gave the largest spectral magnitude with all cytochrome P-450's studied while the smallest spectrum was produced by Mobam with sheep and rabbit, by carbofuran with rat, by Temik and methomyl with mouse, and by dimetilan with Fc house fly microsomes.

The spectral data obtained with various organophosphate insecticides are given in Table IV. Cytochrome P-450 from all sources exhibited a type II spectral shift

Table I. Spectral Interactions of Botanical Insecticides and Synthetic Pyrethroids with Cytochrome P-450 of Mammalian and Insect Microsomes

	Sheep			Rabbit			Rat			Mouse			House fly: Fc			House fly: CSMA		
Insecticide	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size
Nicotine	II		0.434	II		0.540	II		0.505	II		0.522	II		0.412	II		0.255
Anabasine	II	3.0×10^{-3}	0.333	II	5.0×10^{-4}	0.680	II	2.0×10^{-3}	0.383	II	1.0×10^{-3}	0.461	II	1.0×10^{-2}	0.240	II	2.5×10^{-2}	0.183
Rotenone	*a*	2.5×10^{-3}	0.094	*a*	2.5×10^{-3}	0.130	ND			ND			ND			ND		
Pyrethrum extract	I	2.00 mg	0.115	I	3.00 mg	0.099	I	0.75 mg	0.202	I	1.00 mg	0.096	I	0.50 mg	0.156	ND		
Pyrethrins	I	1.0×10^{-3}	0.113	I	1.0×10^{-3}	0.080	I	1.5×10^{-3}	0.223	I	7.4×10^{-4}	0.104	*b*	5.0×10^{-6}	0.374	*b*	5.0×10^{-6}	0.140
Allethrin	I	7.4×10^{-4}	0.270	I	1.0×10^{-4}	0.160	I	5.0×10^{-4}	0.362	I	1.0×10^{-3}	0.278	*b*	3.0×10^{-5}	0.300	*b*	5.0×10^{-5}	0.286
Bioallethrin	I	7.5×10^{-4}	0.280	I	1.5×10^{-3}	0.120	I	5.0×10^{-4}	0.383	I	2.5×10^{-4}	0.287	*b*	1.0×10^{-5}	0.214	*b*	3.0×10^{-5}	0.160
Dimethrin	I	1.5×10^{-3}	0.260	I	1.0×10^{-3}	0.070	I	2.0×10^{-3}	0.298	I	5.0×10^{-4}	0.174	I	2.0×10^{-5}	0.241	ND		
Neopynamin	I	1.0×10^{-3}	0.300	I	1.0×10^{-3}	0.140	I	1.5×10^{-3}	0.390	I	2.5×10^{-4}	0.242	I	1.0×10^{-5}	0.259	ND		
Bioresemethrin	I	1.0×10^{-3}	0.122	ND			I	1.5×10^{-3}	0.192	I	2.5×10^{-4}	0.142	ND			ND		
NRDC-119	I	$>2.0 \times 10^{-3}$	0.266	I	1.0×10^{-3}	0.090	I	2.0×10^{-3}	0.223	I	2.0×10^{-3}	0.191	I	1.0×10^{-5}	0.151	ND		
NRDC-108	I	$>7.5 \times 10^{-3}$	0.110	I	1.5×10^{-3}	0.140	I	7.5×10^{-4}	0.253	I	2.5×10^{-4}	0.158	ND			ND		
SBP-1382	I	$>1.0 \times 10^{-3}$	0.170	I	1.0×10^{-3}	0.060	I	5.0×10^{-4}	0.200	I	5.0×10^{-4}	0.122	ND			ND		
Cyclethrin	I	3.8×10^{-4}	0.333	I	5.0×10^{-4}	0.200	I	2.5×10^{-4}	0.324	I	5.0×10^{-4}	0.278	I	1.0×10^{-5}	0.100	ND		
Barthrin	I	1.5×10^{-3}	0.208	I	1.0×10^{-3}	0.088	I	1.0×10^{-3}	0.242	I	7.5×10^{-4}	0.191	I	3.0×10^{-3}	0.150	ND		

[a] Absorption minimum at 395 nm and absorption maximum at 415 nm in sheep or at 417 nm in rabbit. [b] Absorption minimum at 445–447 nm and absorption maximum at 415–418 nm; ND, no detectable spectrum.

Table II. Spectral Interactions of Chlorinated Hydrocarbon Insecticides with Cytochrome P-450 of Mammalian and Insect Microsomes

	Sheep			Rabbit			Rat			Mouse			House fly: Fc			House fly: CSMA		
Insecticide	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size	Type	Final concn, M	Size
p,p'-DDT	I	4.0×10^{-3}	0.166	I	3.0×10^{-3}	0.076	I	0.5×10^{-3}	0.103	I	7.5×10^{-5}	0.112	I	5.0×10^{-3}	0.120	ND[b]		
TDE[a]	I	3.0×10^{-3}	0.283	I	2.0×10^{-3}	0.219	I	2.5×10^{-3}	0.310	I	5.0×10^{-3}	0.330	I	2.0×10^{-3}	0.100	ND		
Kelthane	I	1.0×10^{-3}	0.314	I	0.5×10^{-3}	0.057	I	0.5×10^{-3}	0.345	I	7.5×10^{-4}	0.295	I	1.0×10^{-4}	0.154	ND		
Methoxychlor	I	0.5×10^{-3}	0.214	I	7.5×10^{-4}	0.085	I	7.5×10^{-5}	0.138	I	2.0×10^{-5}	0.170	I	1.0×10^{-3}	0.143	ND		
Lindane	I	2.0×10^{-3}	0.183	I	1.0×10^{-3}	0.157	I	7.5×10^{-4}	0.397	I	4.0×10^{-4}	0.364	I	6.0×10^{-3}	0.150	ND		
Endrin	I	2.0×10^{-3}	0.300	I	1.0×10^{-3}	0.181	I	2.5×10^{-3}	0.353	I	4.0×10^{-3}	0.205	I	3.5×10^{-3}	0.128	ND		
Aldrin	I	2.0×10^{-3}	0.271	I	3.0×10^{-3}	0.219	I	1.0×10^{-3}	0.247	I	5.0×10^{-4}	0.169	I	3.0×10^{-5}	0.133	ND		
Dieldrin	I	4.0×10^{-3}	0.266	I	3.0×10^{-3}	0.167	I	2.0×10^{-3}	0.328	I	4.0×10^{-3}	0.170	I	6.6×10^{-3}	0.114	ND		
Chlordane	I	2.5×10^{-3}	0.357	I	2.0×10^{-3}	0.259	I	2.0×10^{-3}	0.379	I	4.0×10^{-3}	0.307	I	3.3×10^{-3}	0.147	ND		
Heptachlor	I	2.0×10^{-3}	0.333	I	1.0×10^{-3}	0.240	I	1.0×10^{-3}	0.302	I	3.0×10^{-3}	0.250	I	3.0×10^{-3}	0.114	ND		

Toxaphene	I	1.0 mg	0.250	I	0.8 mg	0.257	I	0.5 mg	0.354	I	0.3 mg	0.239	I	2.0 mg	0.133	ND
Kepone	I	1.0×10^{-3}	0.185	I	1.5×10^{-3}	0.162	I	1.0×10^{-3}	0.366	I	1.5×10^{-3}	0.193	II	1.6×10^{-3}	0.120	ND
Mirex	I	2.0×10^{-3}	0.083	I	3.0×10^{-3}	0.057	I	1.0×10^{-3}	0.097	I	4.0×10^{-4}	0.080	I	3.0×10^{-3}	0.063	ND
Endosulfan	I	1.0×10^{-3}	0.348	I	1.0×10^{-3}	0.143	I	1.3×10^{-4}	0.336	I	2.0×10^{-4}	0.205	I	1.5×10^{-3}	0.211	ND

[a] Mixture of *p,p'*- (70%) and *o,p'*-TDE (25%) isomers containing <5% *p,p'*-DDT. [b] ND, no detectable spectrum.

Table III. Spectral Interactions of Carbamate Insecticides with Cytochrome P-450 of Mammalian and Insect Microsomes

	Sheep			Rabbit			Rat			Mouse			House fly: Fc			House fly: CSMA		
Insecticide	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size
Carbaryl	I	1.5×10^{-3}	0.174	*b*	3.0×10^{-3}	0.088	I	1.5×10^{-3}	0.270	I	2.0×10^{-3}	0.200	I	1.5×10^{-3}	0.133	ND[d]		
Baygon	I	1.5×10^{-3}	0.362	I	1.0×10^{-3}	0.104	I	6.0×10^{-6}	0.105	I	2.0×10^{-3}	0.215	I	1.5×10^{-3}	0.136	ND		
Zectran	I	1.5×10^{-3}	0.261	I	1.0×10^{-3}	0.171	I	1.0×10^{-4}	0.200	I		0.103[c]	I	2.0×10^{-3}	0.115	ND		
Carbofuran	I	5.0×10^{-5}	0.188	I	2.0×10^{-4}	0.103	I	1.0×10^{-5}	0.090	I	1.0×10^{-3}	0.135	I	3.0×10^{-3}	0.124	ND		
Methomyl	*a*			I	2.0×10^{-4}	0.082	I	1.0×10^{-5}	0.140	I	6.0×10^{-6}	0.089	I	3.0×10^{-3}	0.058	ND		
Mobam	I	1.0×10^{-4}	0.077	I	5.0×10^{-4}	0.072	I	1.0×10^{-5}	0.140	I	1.0×10^{-4}	0.144	I	2.0×10^{-3}	0.115	ND		
Dimetilan	I	7.5×10^{-4}	0.101	I	5.0×10^{-4}	0.093	I	1.0×10^{-3}	0.180	I	2.0×10^{-3}	0.122	I	1.5×10^{-3}	0.062	ND		
Temik	I	1.0×10^{-3}	0.087	I	1.0×10^{-3}	0.082	I	1.0×10^{-5}	0.100	I	1.0×10^{-4}	0.089	I	2.0×10^{-3}	0.126	ND		
Bux	I	1.5×10^{-3}	0.449	I	2.0×10^{-4}	0.196	I	1.5×10^{-4}	0.326	I	2.0×10^{-5}	0.244	I	1.0×10^{-4}	0.211	ND		

[a] Mixture of type I and type II. [b] ΔOD calculated as difference in OD at 407 and 427 nm; see also Figure 1E. [c] Data from Mailman and Hodgson (1972). [d] ND, no detectable spectrum.

Table IV. Spectral Interactions of Organophosphate Insecticides with Cytochrome P-450 of Mammalian and Insect Microsomes

	Sheep			Rabbit			Rat			Mouse			House fly: Fc			House fly: CSMA		
Insecticides	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size	Type	Final concn, *M*	Size
Diazinon	I	6.0×10^{-5}	0.290	I	5.0×10^{-4}	0.376	I	1.0×10^{-4}	0.370	I	5.0×10^{-4}	0.337	I	1.5×10^{-3}	0.148	ND[d]		
Menazon[c]	*a*			*a*			*a*			II		0.101[b]	I		0.086	ND		
Zinophos	II	4.0×10^{-4}	0.377	II	1.0×10^{-4}	0.321	II	1.5×10^{-4}	0.242	II		0.312[b]	II	1.0×10^{-3}	0.321	II	1.0×10^{-3}	0.166
Guthion	I	1.0×10^{-4}	0.134	I	5.0×10^{-5}	0.155	I	1.0×10^{-5}	0.170	*			I	1.0×10^{-3}	0.222	ND		
Coumaphos	I	4.0×10^{-5}	0.210	I	1.0×10^{-5}	0.223	I	1.5×10^{-4}	0.400	I	1.0×10^{-4}	0.289	I	2.0×10^{-5}	0.164	ND		
Dimethoate	*a*			*a*			*a*			I		0.052[b]	I	7.5×10^{-4}	0.148	ND		
Trichlorfon	*a*			*a*			I	1.0×10^{-5}	0.095	I		0.021[b]	I	1.0×10^{-3}	0.193	ND		
Azodrin	*a*			I	2.0×10^{-4}	0.082	I	1.0×10^{-5}	0.120	I	1.5×10^{-5}	0.090	I	1.0×10^{-4}	0.123	ND		
Malathion	I	2.0×10^{-4}	0.269	I	5.0×10^{-4}	0.093	I	1.0×10^{-3}	0.263	I	1.0×10^{-3}	0.150	I	1.0×10^{-4}	0.228	ND		
Ruelene	I	6.0×10^{-5}	0.179	I	1.0×10^{-5}	0.134	I	1.0×10^{-5}	0.210	I	1.0×10^{-4}	0.144	I	2.0×10^{-5}	0.164	ND		

[a] Mixture of type I and type II or concentration-dependent changes in spectral response noticed. [b] Data from Mailman and Hodgson (1972). [c] Accurate value of the concentrations could not be obtained because of its low solubility in acetone. [d] ND, no detectable spectrum.

(peak 420–422 nm, trough 390–395 nm) with Zinophos although the spectral magnitude differed from species to species. Menazon, which produced a type II spectral response with mouse microsomes, caused a type I interaction with Fc microsomes. All other compounds except Guthion (Table IV) showed type I spectra with either mouse or Fc microsomes. Guthion caused a mixed spectrum at higher concentrations with mouse microsomes.

Certain concentration-dependent shifts in the spectra were also apparent. Rat microsomes with Menazon and dimethoate show a type I spectrum at low concentrations, a mixed spectrum at intermediate concentrations, and a type II spectrum (peak 420 nm, trough 390 nm) at higher concentrations. An additional peak at 415 nm appeared when the Guthion concentration was increased above 5×10^{-5} M and a shift in the locus of the trough from 420 to 425 nm was noted when malathion concentration was gradually changed from 1×10^{-5} to 1×10^{-3} M. This response to malathion was also seen with mouse microsomes. All other phosphate insecticides tested showed a type I spectral response with rat microsomes.

Rabbit microsomes exhibited mixed spectra (peaks at 385 and 422–425 nm) with Menazon and trichlorfon at low as well as higher concentrations. Dimethoate showed similar concentration-dependent changes in the difference spectrum to those seen in the case of rat microsomes.

Sheep microsomes also exhibited mixed spectra with Menazon at all concentrations tested with peaks located at 385 and 415 nm. Both trichlorfon and Azodrin caused a type I spectral interaction at lower concentrations and a mixed spectrum with peaks at 385 and 420 nm at higher concentration. Dimethoate either did not yield a detectable spectrum or the spectrum was a mixture of types I and II at all the concentrations tested.

In the case of CSMA microsomes, the results obtained with organophosphate insecticides were extremely variable and inconsistent from batch to batch of microsomal preparations. Mixed spectra or type II interactions, all of very small magnitude, were noted in some experiments. Only Zinophos consistently exhibited type II spectra with these microsomes.

These results are consistent with previous reports (Mailman and Hodgson, 1972; Roth and Neal, 1972; Rao and Anders, 1973; Stevens *et al.*, 1973; Stevens and Greene, 1973). Using rat and mouse microsomes, Stevens *et al.* (1973) and Stevens and Greene (1973) reported malathion to cause a type I spectrum with an abnormally broad trough and the spectrum disappeared in about 5 min. In the present investigations, the difference spectrum was found to be quite stable and the location of the absorption minimum was observed to be a function of ligand concentration. A gradual increase in the malathion concentration tended to shift the trough from 420 to 425 nm. A similar concentration-dependent change, from 414 to 419 nm, was reported by Roth and Neal (1972) for the parathion difference spectrum of rabbit liver microsomes.

Compounds containing aromatic nitrogen in the molecules have been reported to exhibit type II difference spectra with cytochrome P-450 of several species (Schenkman *et al.*, 1967; Jefcoate *et al.*, 1969; Temple, 1971; Mailman *et al.*, 1974; Kulkarni *et al.*, 1974). Thus, both nicotine and anabasine as well as Zinophos, with a 2-pyrazinyl leaving group, exhibited type II difference spectra. At the same time carbamate and some organophosphate insecticides, although containing nitrogen atoms, did not produce such a response but rather exhibited type I spectral interactions. It has been postulated (Jefcoate *et al.*, 1969; Temple, 1971; Mailman and Hodgson, 1972) that type II spectra are due to the presence of a nitrogen atom in the ligand which has a pair of unshared electrons and is sterically accessible. Further experiments with several compounds of different chemical grouping have confirmed this hypothesis using mouse (Mailman *et al.*, 1974) as well as Fc and CSMA microsomes (Kulkarni *et al.*, 1974). Data reported here agree with these observations.

Some of the organophosphate insecticides exhibit difference spectra of a mixed nature at low as well as high concentrations possibly indicating their ability to bind at both type I and type II sites. Since the peaks in such spectra were located at 385–390 and 415–425 nm (depending upon the source of microsomes and insecticide in question), the presence of a type II component in the spectrum was inferred.

In some cases, concentration-dependent changes from type I to type II were noted. Such responses have been interpreted as being modified type II spectra due to the displacement of an endogenous substrate by the added ligand. This results in abolition of a native type I spectrum in the sample cuvette and the appearance of a reverse type I difference spectrum (Schenkman *et al.*, 1972). In the present investigation this becomes questionable, primarily because (a) the spectra observed are very small in magnitude indicating a simple additive effect between two types of spectra, (b) parathion, another organophosphate insecticide and a type I substrate (Rao and Anders, 1973), has also been reported to have a type II binding component (Roth and Neal, 1972), and (c) aniline, a type II compound, also has a type I binding component in its difference spectrum (Gorrod and Temple, 1973). In addition, it has been suggested, on the basis of structure–function studies, that a nucleophilic oxygen atom in an appropriate configuration can mimic a nitrogen atom in terms of spectral response. Such spectra are typified by having the peak and trough at a somewhat lower wavelength (415–420 nm) (Mailman *et al.*, 1974; Kulkarni *et al.*, 1974). The presence of oxygen atoms in the leaving groups of these insecticides may also be partly responsible for the observed spectra. However, more critical experimentation is needed to clarify this possibility.

A substantial type I binding of metabolites, as compared to their respective parent compounds, has been observed (Table II) in the present study and has been previously reported (Greene and Stevens, 1973; Stevens and Greene, 1973; Stevens *et al.*, 1973). The requirement for NADPH in the *in vitro* oxidative metabolism of insecticides by the mixed-function oxidase system is well established and little or no oxidation occurs during incubation without added NADPH. The spectral data obtained in this study should, therefore, represent the binding of only the parent insecticide.

Many of those compounds causing type I spectral interactions are substrates of the mixed-function oxidase system, and, therefore, it has been stated that type I binding is, in general, related to the metabolism of those xenobiotics which cause it. Type II spectra, with the exception of that caused by aniline, are not related to metabolism. This view finds support in the observations that type II compounds such as aniline, nicotinamide, and 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) inhibit or slow down the rate of cytochrome P-450 reduction by NADPH, a step essential for the oxidative metabolism of xenobiotics by the microsomal mixed-function oxidase system (Schenkman *et al.*, 1967; Gigon *et al.*, 1969; Sasame and Gillette, 1969). However, Zinophos, Menazon (in mouse), nicotine, anabasine, and Kepone (in Fc house fly), which are known to be metabolized by the mixed-function oxidase system, exhibit type II spectral interactions with cytochrome P-450. It is, therefore, questionable to presume that all type II interactions are not related to metabolism. The results, however, indicate that most insecticides cause type I spectral interactions with cytochrome P-450 and are in agreement with the hypothesis.

The observation that CSMA microsomes do not show any detectable binding with the insecticides tested is in conformity with other reports (Philpot and Hodgson, 1972; Tate *et al.*, 1973; Kulkarni *et al.*, 1974). However, insecti-

Table V. Comparison of Type II to Type I Spectral Binding of Insecticides with Microsomal Cytochrome P-450 from Sheep, Rabbit, Rat, Mouse, and Fc and CSMA House Fly[a]

Type II/ type I	Sheep	Rabbit	Rat	Mouse	House fly Fc	House fly CSMA
Nicotine/ dimethrin	1.67	7.71	1.70	3.00	1.71	0.00
Nicotine/ DDT	2.61	7.11	4.90	4.66	3.43	0.00
Nicotine/ Baygon	1.20	5.19	4.81	2.43	3.03	0.00
Nicotine/ malathion	1.61	5.80	1.92	3.48	1.81	0.00
Zinophos/ dimethrin	1.45	4.59	0.81	1.79	1.33	0.00
Zinophos/ DDT	2.27	4.22	2.35	2.79	2.68	0.00
Zinophos/ Baygon	1.04	3.09	2.31	1.45	2.36	0.00
Zinophos/ malathion	1.40	3.45	0.92	2.08	1.41	0.00

[a] Ratios calculated from data presented in Tables I–IV.

cides which show type I binding with the microsomes of other species are known to be metabolized by the mixed-function oxidase system of CSMA house fly microsomes (Hodgson, 1968; Hodgson and Plapp, 1970). Similarly, microsomes of Baygon-resistant house flies which catalyze the oxidation of Baygon do not yield any detectable difference spectrum with this substrate (unpublished observations). These results, therefore, illustrate that detectable type I binding is not obligatory for metabolism. Similar conclusions have been drawn by Chaplin and Mannering (1970) for different substrates. However, these examples may be taken as exceptions.

The absence of observable type I binding and relatively smaller magnitudes of type II spectra with CSMA house fly microsomes also indicate that cytochrome P-450 present in the CSMA house fly is different from that of the Fc house fly. Even in the mammalian microsomes studied, cytochrome P-450 seems to differ from one species to another. If the cytochrome P-450 of all the mammals tested were alike, similarity in the spectral size as related to the CO spectrum, the concentration of a test insecticide required to produce a given spectrum, and the ratio of type II to type I binding would be expected. However, wide species variation is evident (Table V) and this is further illustrated by Menazon which produces no spectrum in CSMA house flies, type I in Fc house flies, type II in mouse, and a mixture of types I and II in rat, rabbit and sheep microsomes (Table IV). This view finds support from the observation that phenacetin, which causes a type I spectral interaction with rabbit microsomes (Imai and Sato, 1966), elicits a modified type II response in the rat (Schenkman *et al.*, 1972), type II in the mouse (Mailman *et al.*, 1974), type I in Fc house fly microsomes (Kulkarni *et al.*, 1974), and no detectable spectrum with CSMA house fly microsomes (Kulkarni *et al.*, 1974).

Qualitative differences between the microsomes from different species may represent differences in the cytochromes themselves or in the environment within the membrane in which the cytochrome is located. Changes in membrane structure could create differences in accessibility to the binding sites as well as the orientation of the ligand relative to the cytochrome P-450 molecule. The data on the binding of insecticides presented here cannot resolve this problem.

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