The Cytochrome P-450 Substrate Optical Difference Spectra of Pesticides with Mouse Hepatic Microsomes

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The interaction of pesticides with microsomal oxidative enzyme systems in both target (HODGSON and PLAPP 1970; HODGSON 1968) and non-target organisms (FOURNIER 1970) has been under increased scrutiny. An important consideration in the use of pesticides is the effect they may have on non-target species. In mammals, the hepatic microsomal enzymes have been shown to be of primary importance in the oxidation of xenobiotics as well as being induced by many of these same compounds (HODGSON 1968; GILLETTE et al. 1969; CONNEY 1967).

The oxidation of foreign compounds by microsomal enzymes is now thought to involve an initial binding to the oxidized form of cytochrome P-450 (REMMER et al. 1966). This complex can then accept electrons from NADPH by way of NADPH-cytochrome P-450 reductase and react with molecular oxygen to give a variety of oxidized products (HODGSON 1968). It has also been shown that compounds of a variety of different chemical structures bind to the oxidized cytochrome causing perturbations of the Soret optical spectrum of several characteristic types. The first type, known as type I, results in an optical difference spectrum with an absorption minimum at 416-420 nm and an absorption maximum at 385-390 nm (COOPER et al. 1965). The second type of perturbation, type II, has a maximum in the range of 424-435 nm and a minimum at 390-410 nm (SCHENKMAN et al. 1967; IMAI and SATO 1966). A "type III" spectrum featuring double Soret peaks between 420 and 460 nm has been reported (PHILPOT and HODGSON 1971; OMURA and SATO 1964) but since it involves binding to the reduced form of cytochrome P-450, it will not be considered here. Compounds of a variety of structures have been shown to give type I binding (SASAME and GILLETTE 1969; IMAI and SATO 1969), whereas it has been stated that aromatic nitrogen compounds (T'SAI et al. 1970) aliphatic amines (JEFCOATE et al. 1969), alcohols (DIEHL et al. 1970) and certain other compounds (SATO et al. 1970; HEWICK and FOUTS 1970; ICHIKAWA and YAMAMO 1965) produce type II binding.

METHODS AND MATERIALS

Microsomes were prepared from the livers of six week old male mice from the North Carolina Department of Health strain, inbred since 1910. The mice were fed Purina Lab Chow and water ad libitum. The mice were killed by decapitation, the livers

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TABLE I

Previous reports of cytochrome P-450 optical difference spectra of pesticides.

Compound	Hepatic microsome source	Type spectrum	Reference
Rotenone	rabbit	II[a]	KUWATSUKA 1970
Allethrin	rabbit	II[a]	KUWATSUKA 1970
Carbaryl	rabbit	I[b]	KUWATSUKA 1970
Safrole	rabbit	I	KUWATSUKA 1970
Dihydrosafrole	rabbit	I	KUWATSUKA 1970
DDT	rat	I	SCHENKMAN et al. 1967
Nicotine	rat	II	SCHENKMAN et al. 1967
Rotenone	rat	II[a]	SCHENKMAN et al. 1967
Mirex	mouse	I	BAKER et al. 1972
Kepone	mouse	I	BAKER et al. 1972
Piperonyl butoxide	mouse	I	PHILPOT and HODGSON 1971
Propyl isome	mouse	I	PHILPOT and HODGSON 1971
Parathion	rabbit	I	ROTH and NEAL 1972

[a]Type II: peak 420 nm; trough 385 nm
[b]Type I: peak 400 nm; trough 430 nm

removed, rinsed first in distilled water, and then in 0.05 M Tris-1.15% KCl buffer (pH 7.4). They were then diced, washed in Tris-KCl, and homogenized in an all-glass homogenizer with 4.5 ml Tris-KCl per liver. The homogenate was centrifuged at 10,000 g for 15 minutes, the resulting supernatant filtered through glass wool, and then recentrifuged at 105,000 g for one hour. The pellet was resuspended in KCl-Tris to give a protein concentration of approximately 20 mg/ml. All operations were carried out at 4 C. The resulting suspension was diluted as needed.

Difference spectra were recorded on a Beckman Acta V spectrophotometer with a turbid sample accessory. The majority of the pesticides used were insoluble; therefore, they were deposited on the bottom of 25 ml Erlenmeyer flasks by evaporation of acetone solution. Care was taken to insure that saturating levels of all pesticides were deposited by increasing the amount until there was no further increase in spectral size. Microsomes were then added to the flask and incubated for fifteen minutes at ambient temperature. Parallel incubations of a microsomal suspension without added pesticides were performed and the control was used in each case to establish a zero difference line. Cytochrome P-450 levels were determined in each microsomal suspension by the method of OMURA and SATO (1964). P-420 levels were found to be negligible (< 10%).

Liquid pesticides were added directly, by microsyringe, to the microsomes in the sample spectrophotometer cuvette with an equal volume of buffer added to the reference cuvette. Each

sample was shaken vigorously and allowed to stablize for 5 minutes before reading. Care was again taken to insure saturation. Water soluble pesticides were added in the same manner as the liquid pesticides. In all cases the spectrum reported was one in which additional substrate or incubation time gave no further increase in spectral size. Spectral size was calculated as the peak to trough distance (based on the zero difference line).

Analytical grade pesticides[1] were obtained from City Chemical Corporation with the following exceptions: pyrethrins from Perrine Primate Lab, Perrine, Florida; carbaryl from Union Carbide; Monitor^{R} from Ortho Division, Chevron; Zinophos^{R} from American Cyanamid; herbicides from Dr. D. E. Moreland, North Carolina State University; menazon from Imperial Chemical Industries; benomyl and 3 substituted imidazoles from E. I. DuPont de Nemours Inc.; Zectran^{R} from Dow Chemical Company; 3 benzothiadiazoles from Dr. C. F. Wilkinson, Cornell University; and apholate from Olin Mathieson Chemical Corporation. All were used without further purification.

RESULTS AND DISCUSSION

The results obtained are shown in Tables 2 and 3. It may be observed that the results clearly indicate that classical chemical grouping of pesticides is not correlated with the type of binding spectrum. It will be observed that in every group of compounds with the exception of the chlorinated aryl hydrocarbons (including the DDT family) both type I and type II binding occur. Since classical chemical grouping did not reveal any trend, all type II compounds were compared to determine if a common chemical characteristic might explain why different pesticides give common difference spectra with cytochrome P-450. It may be observed that the compounds causing type II perturbations have the common structural units of a nitrogen atom which appears relatively unhindered spatially with respect to other substituted groups. Such a nitrogen might be expected to be more accessible to a binding site such as the heme iron. Many of the other pesticides contained nitrogen but did not produce type II binding. It does appear clear, however, that in these pesticides, spatial access to the nitrogen was hindered by the presence of substituent groups which can be observed in both Fisher projections and in "space filling" models to block access to the nitrogen. Thus we observed that the synergist 1-(1-naphthyl)-imidazole gives a strong type II spectrum whereas carbaryl (1-naphthylmethylcarbamate), containing a nitrogen atom hindered by two substituent groups, one large and one small, does not give type II binding. Further, picloram, a pentasubstituted pyridine, does not give type II binding whereas

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[1] refer to Bull. Ent. Soc. Amer. 12, 161 (1966) for structures of insecticides; Herbicide Handbook of Weed Society of America (1967) for herbicides.

TABLE 2

Cytochrome P-450 optical difference spectra of botanicals, chlorinated hydrocarbons, synergists, and a thiocyanate.

Compound	Type	Δ O.D. (pk-tr)/O.D.(P-450)
Botanicals		
Rotenone	ND*	-
Pyrethrum	ND	-
Pyrethrins (21% solution)	ND	-
Allethrin	I	.294
Nicotine	II	.162
Anabasine	II	.264
Chlorinated aryl hydrocarbons		
Lindane	I	.232
Heptachlor	I	.141
Aldrin	I	.054
Deldrin	I	.114
Endrin	I	.178
Kepone^{R}	I	.240
Mirex	I	.052
p,p'-DDT	I	.032
TDE	I	.011
Chloropropylate^{R}	I	.09
Synergists		
Piperonyl butoxide	I	.210
1-(2-isopropylphenyl)-imidazole	II	.625
1-(1-naphthyl)imidazole	II	.595
1-(2-cyanophenyl)-imidazole	II	.555
5-nitrobenzothiadiazole	ND	-
4-chlorobenzothiadiazole	ND	-
6-methyl benzothiadiazole	II	.007
Thiocyanates		
Thanite^{R}	I	.270

*ND = no detectable spectrum

TABLE 3

Cytochrome P-450 optical difference spectra of organophosphate and carbamate insecticides, herbicides, and fungicides.

Compound	Type	Δ O.D. (pk-tr)/O.D.(P-450)
Organophosphates		
Diazinon	I	.443
Trichlorfon	I	.021
Dimethoate	I	.052
Zinophos^{R}	II	.312
Apholate	ND	-
Monitor^{R}	I	.054
Menazon	II	.101
Carbamates		
Baygon^{R}	I	.062
Zectran^{R}	I	.103
Carbaryl	I	.175
Herbicides		
Diuron	I	.126
TH 052-H	ND	-
Oryzalin	I	.034
Dichlorobenil	I	.05
Propanil	I	.117
Bromacil	I	.004
2,3,6-TBA	I	.045
Atrazine	I	.075
Amitrole	II	.170
Picloram	I	.016
Ioxynil	II	.225
CIPC	I	.105
2,4-D	I	.024
Fungicides		
Benomyl	I	.110

Compounds whose native absorbance or breakdown product absorbance obscured binding

Parathion
Diquat
Maleic hydrazide

nicotine and anabasine, both pyridine derivatives containing a large substituent group opposed to the ring nitrogen, will give this interaction. This data may be taken to indicate that aromatic nitrogen is a necessity for type II binding in this species since Monitor^{R} and picloram, containing free amino groups do not show type II binding. However, the steric factor previously mentioned, is probably the reason these two compounds do not give type II spectra since neither have amino groups which may be considered freely accessible.

Amitrole (3-aminotriazole) gave a strong type II spectrum whereas the substituted benzothiadiazoles gave small or undetectable type II spectra. This further reinforces the idea of an "accessible nitrogen" being of some importance in type II binding. In fact, two organophosphorous insecticides were deliberately chosen, after the random choices were examined, in an attempt to verify or disprove this idea. Menazon (4,6-diamino-s-triazin-2-ylmethyl leaving group) and Zinophos^{R} (2-pyrazinyl leaving group) both gave type II spectra, as was expected. However, Thanite^{R}, expected to form a type II spectrum, gave a type I spectrum indicating that factors other than the accessibility of the nitrogen may affect binding.

In this strain of mice, pesticides in general seem to show patterns similar to the majority of observations made with drugs (SCHENKMAN et al. 1967; SASAME and GILLETTE 1969) and other compounds (PHILPOT and HODGSON 1971; BAKER et al. 1972) using a variety of mammals. Aromatic compounds generally cause type I binding whereas compounds containing an accessible nitrogen atom give type II interactions. This data, in agreement with that previously cited, supports the view of the cytochrome P-450 type I site being a hydrophobic region removed some short distance from the heme whereas type II interactions may occur more directly with the heme (NETTER et al. 1969).

The data quoted in Table I is in partial agreement with our results. Specifically, disagreement occurs with respect to rotenone (SCHENKMAN et al. 1967; KUWATSUKA 1970) and allethrin (KUWATSUKA 1970). We could not demonstrate detectable binding of rotenone with cytochrome P-450 despite several modifications of experimental procedure and the use of several different microsomal preparations. Furthermore, allethrin always yielded a strong type I spectrum. The compound had a maxima at 385 nm and a minimum at 419 nm, which are the classical type I measurements. Previous reports of the type II interactions of both rotenone and allethrin gave maxima at 420 nm and minima at 390. This is not a classical type II spectra but corresponds to an inverted type I spectrum and may also be interpreted as either a type I spectrum in the reference cuvette or the abolishment of a "native" type I spectrum in the sample cuvette.

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