Methylenedioxyphenyl Compounds as Inhibitors of the Hydroxylation of Naphthalene in Houseflies

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Hydroxylation by housefly microsomes, using naphthalene-1-C^{14} as substrate, has been tested as a possible site of action of the pyrethrin synergists. Five commercial and nine noncommercial compounds containing the methylenedioxyphenyl structure were inhibitory of this process. Inhibitory concentrations range from $10^{-2}M$ for piperonylic acid to $10^{-5}M$ for safrole and isosafrole. Of the commercial synergists, piperonyl cyclonene is the most potent inhibitor of microsomal hydroxylation. The commercial synergists, sesamex, sulfoxide, piperonyl butoxide, and *n*-propyl isome, and the noncommercial compounds, safrole, isosafrole, and piperonal, were found to be synergistic with naphthalene in in vivo tests with female houseflies.

Sun and Johnson (*13*) have suggested that the pyrethrin synergists based on the methylenedioxyphenyl structure exert their action by interfering with biological oxidation of the toxicant. Other workers also have drawn attention to the possible antidetoxication role of such synergists (*11*, *12*).

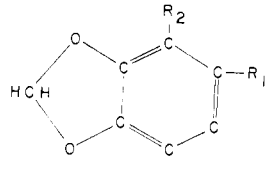
A biological oxidation of importance in the metabolism and excretion of naphthalene in houseflies is hydroxylation (*14*). The in vitro demonstration of this activity, using housefly microsomes (*2*), has provided an opportunity to test the action of methylenedioxyphenyl synergists on this system. The results of such experiments are presented here.

Materials

The following commercial synergists were used: sesamex (Sesoxane) [2-(2-ethoxyethoxy)ethyl 3,4-methylenedioxyphenyl acetal of acetaldehyde]; sulfoxide {1 - methyl - 2 - [3,4 - (methylenedioxy)-phenyl] ethyl octyl sulfoxide}; piperonyl butoxide {α-[2-(2-butoxyethoxy)ethoxy]-4,5 - (methylenedioxy) - 2 - propyltoluene}; *n*-propyl isome [dipropyl 5,6,7,8 - tetrahydro - 7 - methylnaphtho-(2,3 - *d*) - 1,3 - dioxole - 5,6 - dicarboxylate]; piperonyl cyclonene {3-[hexyl or isopentyl]-5-[3,4-(methylenedioxy)-phenyl]-2-cyclohexen-1-one, mixture with 6-carbethoxy-3-(hexyl or isopentyl) - 5 - [3,4 - (methylenedioxy)-phenyl]-2-cyclohexen-1-one}.

SKF-525A (β-diethylaminoethyl diphenylpropyl acetate HCl) was tested because of its known activity as an inhibitor of various biological oxidations (*9*).

Several noncommercial methylenedioxyphenyl compounds were obtained from Morton Beroza, U. S. Department of Agriculture, Beltsville, Md. These compounds with substituent groups identified, are referred to by common name, where available.



Compound	Common Name	Substituent
3,4-Methylenedioxyallylbenzene	Safrole	R_1, $-CH_2-CH=CH_2$
2,3-Methylenedioxyallylbenzene	*o*-Safrole	R_2, $-CH_2-CH=CH_2$
3,4-Methylenedioxypropenylbenzene	Isosafrole	R_1, $-CH=CH-CH_3$
3,4-Methylenedioxybenzaldehyde	Piperonal	R_1, $-CHO$
3,4-Methylenedioxybenzyl acetate		R_1, $-CH_2-O-C(=O)-CH_3$
3,4-Methylenedioxybenzoic acid	Piperonylic acid	R_1, $-COOH$
3,4-Methylenedioxyphenyl acetate		R_1, $-O-C(=O)-CH_3$
Methyl-(3,4-methylenedioxy)benzoate		R_1, $-C(=O)-O-CH_3$
Methyl-(3,4-methylenedioxy)phenylacetate		R_1, $-CH_2-C(=O)-O-CH_3$

All compounds were used as received without further purification.

Methods

Two strains of houseflies, *Musca domestica* L., one highly resistant to DDT and dieldrin (Orlando R), and the other moderately resistant to these insecticides (Corvallis R), were used in these experiments. The level of resistance of these two strains to DDT and dieldrin was checked at approximately 6-month intervals during the study, using the topical application method with the insecticides dissolved in redistilled acetone. The LD_{50}'s obtained were, respectively, for DDT and dieldrin: Orlando R; 50 to 100 μg. and over 100 μg. per fly; and Corvallis R, 1 to 10 and 2 μg. per fly.

For the in vitro tests, insects of mixed sexes (10 to 14 days of age) were homogenized 30 seconds in 0.154*M* KCl in a Waring Blendor. The homogenate was filtered through cheesecloth, centrifuged at 6780 × G for 10 minutes, and the supernatant recentrifuged at 21,500 × G for an additional 10 minutes. The remaining supernatant was centrifuged at 40,000 × G for 75 minutes to obtain the microsomal pellet. All operations were conducted at 0° to 3° C.

The microsomes were resuspended in 0.154*M* KCl. A quantity of microsomes equivalent to 15 mg. of protein was incubated with the standard incubation mixture which consisted of 2 ml. of tris-HCl buffer (0.11*M*, pH 8.2), glucose-6-phosphate (17.1 μmoles), NADP (1.73 μmoles), glucose-6-phosphate dehydrogenase (0.45 Kornberg unit), 0.05 ml. of naphthalene-1-C^{14} solution (0.22 μmole or 0.45 μcurie), and distilled water to make a total volume of 6 ml. The sub-

Table I. Concentrations of Several Synergists Inhibiting Hydroxylation of Naphthalene by Housefly Microsomes[a]

Synergist	Average % Inhibition of Hydroxylation at Various Inhibitory Concentrations					IN_{50} Values of Separate Assays ($\times 10^{-5}$M)	
	10^{-2}M	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M	a	b
Isosafrole			83	54	26	0.4	1.0
Safrole			86	50		0.9	1.0
Methyl-(3,4-methylenedioxy)-phenylacetate			82	49		0.8	1.2
Piperonyl cyclonene			86	46		1.0	1.4
Methyl-(3,4-methylenedioxy)-benzoate		86	67	47	28	0.9	2.3
Piperonyl butoxide			71	31		2.5	3.5
3,4-Methylenedioxyphenyl acetate			63	30		3.5	5.0
Sulfoxide			58	23		4.5	6.5
Sesamex			57	24		5.0	6.0
3,4-Methylenedioxybenzyl acetate		94	56	18		7.0	12
o-Safrole		84[b]	54	15[b]		6.5	10
Piperonal		94	49	6		9.0	11
n-Propyl isome		77	45	14		10	15
SKF-525A	70	29				300	400
Piperonylic acid	55	41	27	13		200	600

[a] Orlando R strain adults, mixed sex, 10 to 14 days of age.
[b] Actual concentrations for indicated inhibitions were $3.0 \times 10^{-4}M$ and $2.0 \times 10^{-5}M$, respectively.

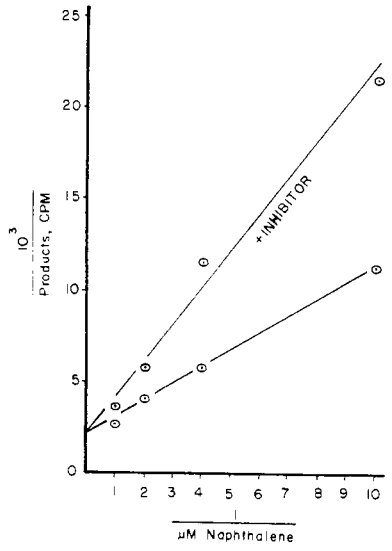


Figure 1. Microsomal hydroxylation of naphthalene in presence of inhibitor

Sesamex at 1×10^{-5}M. Lineweaver-Burke plot showing competitive inhibition

strate and the inhibitors were added in double-distilled methyl Cellosolve (ethylene glycol monomethyl ether). All control incubations contained a quantity of methyl Cellosolve equal to that used in the inhibition experiments.

The mixtures were incubated in 50-ml. glass-stoppered Erlenmeyer flasks, which were mechanically shaken in an incubator. The reaction was allowed to proceed for 30 minutes at 34° C. before being stopped by the addition of 15 ml. of ethyl ether.

The hydroxylation of naphthalene was measured radiometrically by counting aliquots of ether extracts of the incubates. Unreacted naphthalene readily sublimed as the ether was evaporated from the counting planchet, and thus was not included in the radioactivity measurements.

The inhibiting concentrations of the test compounds were determined by incubating microsome suspensions with at least four different levels of inhibitor. Each series of incubations was repeated at least once, using a different population of test insects. The results of the separate tests were averaged and log molar concentration of inhibitors was plotted against per cent inhibition of hydroxylation.

The resulting straight-line plots provided the per cent inhibition values shown in Table I. Plots of data from the individual experiments were extrapolated to provide the IN_{50} concentrations (molarity at which hydroxylation was 50% of controls) shown in the last two columns in Table I. These data indicate the agreement between individual assays. Another measurement of the precision of the incubation tests is indicated in several experiments with sesamex. In 16 incubations using this synergist at a molar concentration of 5×10^{-5}, the mean per cent inhibition of hydroxylation of naphthalene was 42.3 ± 5.54.

The effect of the synergists on the generation of NADPH, which is known to be required for the microsomal hydroxylation of naphthalene (*2*), was tested with several of the compounds using a spectrophotometric measurement. The components of this system (glucose-6-phosphate, NADP, G-6-P-dehydrogenase, and pH 7.8 tris buffer) were measured into spectrophotometer cuvettes and mixed with the test compound. The production of NADPH was followed by readings taken at 340 mμ (*4*). In the case of SKF-525A it was necessary to make the mixture neutral before taking the spectrophotometric reading. None of the compounds tested —*n*-propyl isome, sesamex, sulfoxide, piperonyl butoxide, or SKF-525A— were found to prevent NADPH generation.

Several of the methylenedioxyphenyl compounds and SKF-525A were tested for synergistic activity with naphthalene against 9- to 12-day-old adult female flies of the Orlando R strain, which was used because of its greater in vitro hydroxylating activity. Preliminary tests showed that female flies were affected only slightly (mortalities less than 10%) by a 2-hour exposure to naphthalene vapors.

The tests were conducted on groups of 50 insects held in 4-inch screened cages. The insects were exposed to the synergists in a spray tunnel similar to that described by Dorman and Hall (*6*) using methyl Cellosolve as solvent. After periods up to 60 minutes the flies were placed in glass jars where crystalline naphthalene had been allowed to equilibrate for at least 24 hours. After 2 hours in the naphthalene vapors, the insects were released in recovery cages containing sugar and water. Mortality counts were made 21 hours later. Groups of flies exposed to naphthalene only, or to test compounds only, were used as controls in all experiments. Each treatment was replicated three times.

The synergists were tested at several exposure times to provide a wide basis of comparison. The synergist concentration used was the amount which showed measurable activity without producing significant mortality when used alone. Concentrations ranged from 0.5% in the case of sesamex and piperonyl butoxide to 8% for SKF-525A.

Results and Discussion

As others have suggested (*13*), the pyrethrin synergists may modify the action of insecticides by interfering with biological oxidations. The results presented in Table I show that hydroxylation may also be inhibited by these compounds.

Other possible modes of action by the inhibitory compounds include interference with one or more of the conjugating reactions by which the naphthalene hydroxylation products are further metabolized. A second possibility would be a selective inhibition of hydroxylation, so that the production of one of the primary hydroxylation products (1-naphthol or 1,2 - dihydro - 1,2 - dihydroxynaphthalene) is prevented. Either of these actions would be revealed in a comparison of paper chromatograms of inhibited and normal incubates. Such tests, using solvent systems previously described (*2*), showed that the reduction in yields of hydroxylated products was general rather than selective, the chromatograms being qualitatively identical. Inhibition of an initial step in detoxication was thus indicated.

An attempt was made to determine the nature of the inhibitory action using the graphical methods suggested by Dixon and Webb (*5*). Sesamex was the inhibi-

Table II. Effect of Pretreatment with Synergist on Mortality of Female Houseflies Exposed to Naphthalene Vapors for 2 Hours[a]

Synergist	Concn., %	21-Hour Mortality, % Time between Treatment and Exposure, Minutes 0	15	30	60	Exposed to Synergist Only
Methyl Cellosolve		1	3	2	1	1
Isosafrole	1	23	5	9	5	2
	2	46	20	22	7	3
Safrole	1	87	1	7	2	2
Sesamex	0.5			35		4
	0.5	12	4	18	4	1
	1	54	9	34	14	0
	1	100		78	28	1
Piperonal	5	85	11	10	9	9
n-Propyl isome	5	82	14	20	6	2
	6	26	10	22	6	2
	6	97		87	27	1
Piperonyl butoxide	0.5	15	13	25	10	0
	1	100		22	5	0
Sulfoxide	2			43		4
	2	34	4	18	2	2
SKF-525A	8			33		2
	8	24	10	16	6	0

[a] Orlando R strain, 9 to 12 days of age.

tor used in these experiments. Its concentration and that of the microsomes were held constant while the level of substrate was increased in a series of incubations. The resulting plot (Figure 1) indicates that the inhibition is competitive, although the lack of information regarding the real nature of the system makes this a tentative conclusion.

All of the compounds tested in vivo (Table II) gave evidence of synergism, although the data are somewhat erratic. There was no evidence that the narcotizing action of naphthalene was affected by the synergist treatment. The commercial synergists ranked in activities in approximately the same order in both methods of testing: piperonyl butoxide = sesamex > sulfoxide > *n*-propyl isome. These rankings agree with those obtained by Sun and Johnson (*13*) in their tests with various insecticides. The noncommercial inhibitors (safrole, isosafrole, and piperonal) which were tested with both methods ranked similarly in each. Quantities of the remaining compounds were not sufficient to permit the in vivo tests.

Of the six most active inhibitors of hydroxylation (Table I) only piperonyl cyclonene and piperonyl butoxide are used commercially as synergists. The activity of two of these compounds, safrole and isosafrole, is confirmed in the in vivo tests.

In their study of the structural requirements of the methylenedioxyphenyl compounds, Beroza and Barthel (*3*) noted that polar compounds were poor synergists. These ideas are supported by the in vitro results with piperonal and piperonylic acid. The reduced activity of these two compounds might be due to their inability to penetrate the microsomes, which are generally considered to be lipoidal in nature.

As can be seen in Table II, the activity of most of the compounds was lost within 1 hour after treatment. These results may be due to mechanical loss from the insect body surface during the time prior to naphthalene exposure, to metabolism of the inhibitors by the treated insects, or to a combination of these two effects. The reduced activity of several of the compounds at the 15-minute pre-exposure period cannot be explained.

The effect of sesamex and other commercial synergists on the microsomal hydroxylation of naphthalene is consistent with the demonstrated synergism of these compounds (sesamex) with Sevin (*8*). The importance of hydroxylation in the metabolism of Sevin has been shown by Dorough and Casida (*7*). A compound interfering with this basic step in detoxication would be expected to synergize such a toxicant.

Sun and Johnson noted a slight increase in the toxicity of dieldrin and DDT when applied with sesamex (*13*) and attributed the increase to a stabilizing action. The action may be that of interference with hydroxylation: converting DDT to kelthane or other hydroxylated compounds (*7*), or rupturing the epoxide ring of dieldrin to produce a diol.

Several types of biological oxidations are now thought to be inhibited or blocked by the methylenedioxyphenyl compounds—the conversion of P = S to P = O (*13*), formation of amine oxides (*13*), prevention of epoxidation (*10*, *13*), and hydroxylation as indicated here. This diversity of action would seem to indicate that these inhibitors interfere with some process, such as the utilization of oxygen, which is common to a variety of oxidations.

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