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SYNERGISM

Mode of Action of Carbamate Synergists

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The insecticidal carbamates are synergized by a wide variety of methylenedioxyphenyl compounds. These act as inhibitors of phenolase enzymes which detoxify the carbamates largely by ring hydroxylation. The active inhibitors appear to require a three-point attachment to the phenolase enzyme to orient the methylene carbon so that interaction with a nucleophilic group at the enzyme active site takes place. Tyrosinase, which is abundant in the housefly, has served as a model enzyme for study of the kinetics of this interaction. Soluble housefly tyrosinase has been highly purified and accepts insecticidal carbamates as substrates for hydroxylation. The susceptibility of individual carbamates to enzymatic detoxication is greatly influenced by the nature of the aryl ring. The methylenedioxynaphthalenes and piperonyl carbamates are exceptionally active carbamate synergists.

ALTHOUGH THE ACTION of methylenedioxyphenyl compounds, such as piperonyl butoxide, sulfoxide, sesamex, and propyl isome, as synergists for the pyrethrins has been studied for many years, Moorefield's (*38*) demonstration of the remarkable activating properties of these synergists when used with the *N*-methyl- and *N,N*-dimethylcarbamates, such as carbaryl and isolan, provided the impetus for detailed studies of the mode of action of synergists in relation to the metabolism of the relatively simple structures of the carbamate insecticides. Moorefield (*39*) found that a combination of five parts of piperonyl butoxide (3,4 - methylenedioxy - 6 - propylbenzyl butyldiethyleneglycol ether), and either carbaryl or 3-*tert*-butylphenyl *N*-methylcarbamate, straightened and steepened the dosage mortality curve to the housefly and displaced it some 50-fold to the left. Sesoxane [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane] was found by Eldefrawi, Miskus, and Sutcher (*9*) to enhance the activity of carbaryl and other carbamates not only against susceptible houseflies but also to strains resistant to DDT and parathion and at ratios of synergist to insecticide as low as 1:10. Georghiou, Metcalf, and March (*18*) selected susceptible houseflies with 3-isopropylphenyl *N*-methylcarbamate and found that, after seven generations, the female flies had developed complete tolerance to this carbamate and to a variety of related substituted phenyl *N*-methylcarbamates. However, synergism with piperonyl butoxide almost completely restored the effectiveness of the carbamates to the resistant strain. Georghiou (*14, 16*) showed that the carbamate resistant R_{MIP} flies lost their resistance after 54 generations without selection pressure, and that the carbamate resistance results from a partially dominant single factor inheritance.

Georghiou and Metcalf (*17*) demonstrated that the R_{MIP} flies metabolized 3-isopropylphenyl *N*-methylcarbamate about 10 times as fast as the S_{NAIDM} flies, and that pretreatment with piperonyl butoxide substantially reduced the rate of metabolism of the carbamate. Similar studies with C^{14}-labeled Zectran (4-dimethylamino-3,5-xylenyl *N*-methylcarbamate) (*33*) also demonstrated the rapid metabolism of the carbamate in the R_{MIP} flies and the inhibition of this detoxication by pretreatment with piperonyl butoxide.

The mode of carbamate detoxication is complex as the studies of Dorough and

Casida (*8*) have detected more than eight metabolites produced by exposing C^{14}-carbaryl to rat liver microsomes. These studies have shown that hydroxylation of both the *N*-methyl group and of the 4 and 5 positions of the naphthyl rings is an important step in the metabolism of carbaryl in mammals and insects together with hydrolysis of the carbamate ester linkage. Similar hydroxylations appear to occur with the substituted phenyl *N*-methylcarbamates.

Materials and Methods

Compounds. The piperonyl *N,N*-disubstituted carbamates (Table VI) were prepared from piperonyl alcohol and the corresponding carbamyl chloride in pyridine (*46*). The *N*-monosubstituted carbamates were prepared from piperonyl alcohol and the appropriate isocyanate in dry ether with triethylamine catalyst. The 6-bromopiperonyl alcohol was obtained through the lithium aluminum hydride reduction of 6-bromopiperonal (*3*).

4-Fluoronaphthol was prepared from 1 - bromo - 4 - fluoronaphthalene through isopropylmagnesium bromide and subsequent oxidation to the naphthol (*27*).

5-Fluoronaphthol was prepared from 5-bromo-1-naphthylamine as the starting material. The bromonaphthylamine was diazotized and converted to the fluoborate which when heated gave a 52% yield of 1-bromo-5-fluoronaphthalene, b.p. 90–96° C. (4.0 mm., n_D^{25} 1.6312). Oxidation of the Grignard reagent from this material with dry oxygen gave 2.5 grams of 5-fluoro-1-naphthol. The fluoronaphthol was sensitive to air and was converted directly to the carbamate m.p. 127–8.5° C. by treatment with methylisocyanate.

5,8-Dihydro-1-naphthol was prepared according to Cason (*6*). The *N*-methylcarbamates of the naphthols were prepared by the reaction with a slight excess of methyl isocyanate and a few drops of triethylamine catalyst in a pressure bottle.

Enzymes. Purified mushroom tyrosinase was obtained from Nutritional Biochemicals, Inc. Two fractions of housefly, *Musca domestica*, phenolase were studied. Microsomal phenolase was obtained from chilled homogenates selectively centrifuged to 59,400 G and resuspended in 1.15% KCl as described by Schonbrod, Philleo, and Terriere (*43*). Soluble phenolase was prepared as the supernatant from homogenates of 2- to 4-day old houseflies in distilled water, centrifuged at 3200 G at 0° C. This enzyme was purified approximately 100-fold by adding ammonium sulfate to 50% concentration and centrifuging to 12,800 G for 15 minutes. The pellet was resuspended in phosphate buffer pH 8.0 and passed through a 35 × 2.5 cm. column containing Sephadex G-5. The main fraction was obtained after one holdup volume and was diluted to have one unit of activity per milliliter with *p*-methoxyphenol as the substrate (*2*). The catecholase activity of the enzyme (quinone formation) was measured by the chronometric method described by Dawson and Magee (*7*). In this method, the activity of the enzyme is determined from the length of time required to form sufficient *o*-benzoquinone from catechol to oxidize a known amount of ascorbic acid.

Using the optimum concentration of catechol, the ascorbic acid concentration was varied, and the reciprocal plot of $1/Q$ (per mole of *o*-benzoquinone produced) against $1/t$ (per second) was linear. From these reciprocal plots, the initial reaction velocity was calculated. End point determinations were usually made with four or five concentrations of ascorbic acid at each of three or four levels of inhibitor concentration.

Per cent inhibition values were calculated by comparing the initial reaction velocities in the presence of the several inhibitors with that of the uninhibited enzyme, and I_{50} values were obtained from plots of per cent inhibition *vs.* inhibitor concentration on semilogarithmic paper.

In the Lineweaver-Burk treatment of tyrosinase inhibition, initial reaction velocities were determined in the presence of varying amounts of the substrate (below the optimum) both with and without the addition of the inhibitor 1,3-benzodioxole. Daily variations in enzyme concentration were compensated for by application of a correction factor, thus allowing direct comparisons to be made from day to day.

The cresolase activity of the enzymes (hydroxylation) was determined by rate of O_2 uptake in the Warburg respirometer at pH 7.0 and 25° C., using a variety of substituted phenols and carbamates as substrates (*2*). Inhibitors were added to either enzyme assay as alcoholic solutions or as aqueous suspensions with Triton X-100 emulsifier.

The insecticidal activity, degree of synergism, relative affinity, and anticholinesterase activities of the compounds were evaluated as described previously (*36*).

Species Specificity, Synergism, and Detoxication

Although the insecticidal carbamates are effective inhibitors of the cholinesterases of various species of insects and mammals, the in vivo toxicity is often greatly different giving rise to the well known selectivity of the carbamates (*33*). This species selectivity appears to be due largely to differences in the relative rates of detoxication. As an example, carbaryl has the following LD_{50} values in mg. per kg.: housefly 900, German cockroach >133, honey bee 2.3, and rat 540. In contrast the corresponding figures for *m*-isopropylphenyl *N*-methylcarbamate are: fly 90, cockroach 15, honey bee 1, and rat 16. As indicated, the honey bee and other hymenoptera are extremely susceptible to a wide variety of carbamates and seem to be virtually without the protection of a detoxifying system. This is shown by the response to carbaryl which to *Apis mellifera* L. is synergized by five parts of piperonyl butoxide to only 2.9 times (*15*) as compared with 72 times for *Musca domestica*.

Carbamate Structure and Synergism

The degree to which an individual carbamate is synergized by a given methylenedioxyphenyl synergist is dependent upon both the susceptibility of the carbamate to attack by the detoxication enzyme and the effectiveness of the synergist in reaching the site of action and in inhibiting the detoxication enzyme. A wide range of compounds has been studied by Wilkinson, Metcalf, and Fukuto (*49*).

Fukuto *et al.* (*13*) have pointed out that the ratio of the topical LD_{50} of carbamate alone to LD_{50} of carbamate plus synergist—the synergistic ratio—is a measure of the relative detoxication of the compound by the housefly *Musca domestica*. Uniformly developed information of this type is available for a series of 27 complete sets of ortho-, meta-, and para-substituted phenyl *N*-methylcarbamates (*34, 36*). The synergistic ratios for these compounds vary from 70 to 1.2. The average ratio was 5.7 for ortho, 8.2 for meta, and 5.2 for para substituents. This suggests that, if ring hydroxylation is the most important avenue of detoxication, it takes place most readily in an unsubstituted ortho or para position. If one assumes, as discussed later, that the hydroxylating moiety is the copper stabilized peroxy radical $Cu^{+}O.OCu^{+}$ (*4*), these data are in accord with the net electron charges about the phenol ring as determined by MO calculations: ortho −0.049, meta +0.003, and para −0.036 (*40*).

A more specific evaluation of the role of various substituent groups is given by the data in Table I showing various substituent groups related to maximum (>10) and minimum (>2.0) synergism in the three ring positions. In general, maximum synergism was related to longer and branched chains, alkyl, alkoxy, and alkylthio groups, while minimum synergism was related to short alkylthio and to alkynylthio and alkynyloxy groups.

A selection of some of the greatest variations in synergistic ratio for closely related pairs of carbamates is shown in Table II. Dramatic reductions in detoxication are produced by a change from saturated to unsaturated ether or thioether, a change from branched to straight chain thioether, a change from ortho to meta position, a change in position of dimethoxy groups, a change in position of trimethyl groups, and the degree of aromaticity of the ring of 1-naphthol.

Side Chains and Detoxication. The interaction between the phenyl *N*-methylcarbamate and the detoxication enzyme must involve both the nature and number of the phenyl substituents, as shown by the variations encompassed in Table II. Some of the most striking effects are found with the dimethoxyphenyl *N*-methylcarbamates where despite relatively constant I_{50} values for cholinesterase, the 2,5- and 3,5-compounds were highly toxic to the housefly, and the 2,3-, 2,4-, and 3,4-compounds were of low toxicity but were synergized from 12 to 30 times (*13*). These data agree with substrate studies

Table I. Synergism with Piperonyl Butoxide as Related to Group and Position in Monosubstituted Phenyl *N*-Methylcarbamates

	Minimum		Maximum	
Ortho	$CH{\equiv}C{-}CH_2O$	1.4×	cyclo-C_5H_9O	10.0×
	$CH_2{=}CHCH_2S$	1.8×	iso-$C_5H_{11}S$	18.0×
	C_2H_5	1.3×	cyclo-C_6H_{11}	15.0×
	$(CH_3)_2CHS$	1.9×	C_4H_9O	11.3×
Meta	$CH{\equiv}CCH_2O$	1.2×	$(CH_3)_3Si$	30 ×
	CH_3	1.8×	C_4H_9O	16 ×
	CH_3S	1.3×	cyclo-C_5H_9	23.5×
			$(CH_3)_2N$	14.2×
Para	$CH_2{=}CHCH_2S$	1.2×	*sec*-C_4H_9	14.3×
	CH_3S	1.4×	$(CH_3)_2CHS$	39.0×
	$CH{\equiv}CCH_2S$	1.4×	$CH{\equiv}CCH_2O$	>15.0×
			$(CH_3)_2CH$	>10.0×

Table II. Relative Detoxication and Synergism of Pairs of Closely Related Carbamates

N-Methylcarbamate	Topical LD_{50} *Musca domestica*, μg. per Gram: Alone	1:5 P.B.[a]	Synergistic Ratio
1-Naphthyl	900	12.5	72
4-Benzothienyl	18.5	8.0	2.3
3,4-Dimethoxyphenyl	400	12	33
3,5-Dimethoxyphenyl	11.0	4.4	2.5
2-Propoxyphenyl	100	13.5	7.4
2-Propargyloxyphenyl	6.5	4.6	1.4
2,4,5-Trimethylphenyl	>500	40.0	>12.5
3,4,5-Trimethylphenyl	65.0	13.5	4.8
p-iso-C_3H_7S-phenyl	700	18.5	39
p-C_3H_7S-phenyl	32	8.0	4.0
m-$(CH_3)_3Si$-phenyl	>500	17	>30
o-$(CH_3)_3Si$-phenyl	100	29	3.4

[a] Piperonyl butoxide synergist.

of tyrosinase (see later section) which have shown that 3,4-dihydroxybenzoic acid is a substrate while the 3,5-isomer is not (*50*) and that 2,3- and 3,4-dihydroxyphenyl alanines are substrates, while the 3,5-isomer is not (*32*).

The relative configuration between the phenyl ring and side chain of the carbamate molecule has a marked effect on detoxication as the synergistic ratio with piperonyl butoxide was 7.3× for *l*-2-*sec*-butylphenyl *N*-methylcarbamate and 12.6× for the *d*-isomer. In contrast, there was no significant difference between the synergistic ratio for *l*-2-(*sec*-butylthio) - phenyl *N* - methylcarbamate 3.5× and the *d*-isomer 3.2× (*12*). These data can be interpreted only in terms of a three-point attachment between the carbamate substrate and the phenolase enzyme as will be discussed later.

Naphthyl Ring and Detoxication. Carbaryl or Sevin is an example of a carbamate detoxified very rapidly by the housefly and consequently synergized to a high degree, synergistic ratio 72 (Table III). Dorough and Casida (*8*) have demonstrated that detoxication in mammals and insects consists predominantly in ring hydroxylation with the 4 and 5 positions being attacked most readily. An analysis of the effects of variations in the naphthyl ring system on toxicity and detoxication, as shown in Table III, provides interesting data regarding the nature of the detoxication process. The behavior of the suspected initial detoxication products 4-OH (XII) and 5-OH (XIII) naphthyl *N*-methyl-

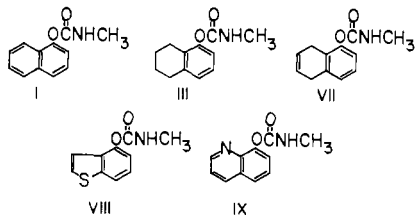
Table III. Toxicity and Synergism of Some Carbamates

N-Methylcarbamate	M.P., °C	Analysis, % Theory	Found	I_{50} M Fly ChE	Relative Affinity	*Musca domestica* LD_{50}, μg./G. A (alone)	B (1:5 P.B.)[a]	Synergistic Ratio, A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
I 1-Naphthyl (α)	139–40	Carbaryl		9.0×10^{-7}	222	900	12.5	72	1.0
II 2-Naphthyl (β)	116–17	C = 71.6 H = 5.51	C = 72.23 H = 5.85	1.4×10^{-5}	14	>500	125	>4	>10
III 5-Tetralyl (α)	110–111.5	C = 70.24 H = 7.31	C = 69.45 H = 7.26	1.4×10^{-6}	140	>500	19.0	26.3	0.12
IV 6-Tetralyl (β)	84–7	C = 70.24 H = 7.31	C = 69.76 H = 7.17	8.4×10^{-6}	24	>500	27.0	>18	>10
V 4-Indanyl (α)	87–9	(*48*)		2.4×10^{-6}	83	140	...		0.4
VI 5-Indanyl (β)	90–2	C = 69.06 H = 6.85	C = 68.90 H = 6.90	1.8×10^{-5}	11	85	34	2.5	2.0
VII 5,8-Dihydro-1-naphthyl	99–103	C = 70.98 H = 6.89	C = 71.05 H = 6.42	2.1×10^{-6}	95	167.5	33	5.1	0.53
VIII 4-Benzothienyl	119–124	[b]		2.5×10^{-7}	800	18.5	8.0	2.3	0.58
IX 8-Quinolinyl	168–69	(*45*)		4.3×10^{-5}	45	>500	21.5	>23	10
X 2,3-Dihydro-2,2-dimethyl-7-benzofuranyl	147–8 107–9	[c] C = 58.52 H = 5.40	C = 58.14 H = 5.13	2.5×10^{-7} 1.4×10^{-4}	800 14	6.7 >500	2.5 500	2.7 >1	0.054 >10
XI 4-Quinazolinyl									
XII 4-Hydroxy-1-naphthyl	162–3.5	(*28*)		5.2×10^{-6}	37.5	>500	>500	1.0	>10
XIII 5-Hydroxy-1-naphthyl	165–7	(*28*)		5.0×10^{-7}	400	>500	>500	1.0	>10
XIV 4-Fluoro-1-naphthyl	130–2	C = 65.75 H = 4.56	C = 66.17 H = 4.70	1.2×10^{-6}	166	>500	34	>15	1.5
XV 5-Fluoro-1-naphthyl	128–9	C = 65.75 H = 4.60	C = 66.09 H = 4.72	1.5×10^{-6}	133	>500	16.5	>30	1.0
XVI Phenyl	85–6	(*36*)		2.0×10^{-4}	1.0	500	38	13.2	>10
XVII 2-Fluorophenyl	75–7	(*36*)		1.6×10^{-5}	12.5	250	40	6.2	>10
XVIII 3-Fluorophenyl	52–4	(*36*)		8.4×10^{-5}	24	390	60	6.5	>10
XIX 4-Fluorophenyl	101–3	(*36*)		2.3×10^{-4}	0.87	480	135	3.7	>10
XX Pentafluorophenyl	117–20	C = 39.84 H = 1.67	C = 39.69 H = 1.76	4.8×10^{-4}	0.36	>500	>500	1.0	>10

[a] Piperonyl butoxide synergist.
[b] Mobil MC-A 600.
[c] Niagara 10242.

carbamates is surprising. These compounds, as shown in Table III, still retain their high anticholinesterase activity especially in the heteronuclear 5-OH compound, yet are devoid of toxicity even in the presence of synergist. This suggests that the introduction of the phenolic OH as a center for detoxication drastically alters the partitioning of the compound in the lipoid structure of the nerve and prevents it from reaching the site of action.

Evidently, from the data in Table III, detoxication is related to the aromaticity of the rings. This is well demonstrated by comparing the synergistic ratios of carbaryl with its isosteres 5,8-dihydro-1-naphthyl *N* - methylcarbamate (VII) which is considerably more toxic and of lower synergistic ratio, and especially with 4-benzothienyl *N*-methylcarbamate (VIII) which is very toxic to the housefly and has an insignificant synergistic ratio.



Further hydrogenation of the heteronuclear ring of carbaryl to form 5,6,7,8-tetrahydro-1-naphthyl *N*-methylcarbamate (III) resulted in a compound of low toxicity and a high synergistic ratio indicating rapid detoxication. The 8-quinolinyl *N*-methylcarbamate (IX) also has a high synergistic ratio and is readily detoxified. These remarkable variations in rate of detoxication seem to relate to the principal attack of the hydroxylating enzyme at the 5-position of carbaryl. Diner (*10*) has suggested that these metabolic hydroxylations cannot be explained satisfactorily on the basis of the electronegativity of the ground state. Rather, the perturbed molecular properties seem to be involved in a hydroxylation mechanism involving bond characteristics whereby activated molecular oxygen first forms a peroxide which then rearranges to a phenol. The capacity of the heteronuclear ring to form peroxide may be then the limiting factor in rapid detoxication. Evidently, much remains to be learned about the site and nature of the attack of the hydroxylating enzyme.

The data of Table III indicate that these various alterations of the heteronuclear ring of carbaryl do not dramatically affect the in vitro anticholinesterase activity of the molecule except for the nitrogen compounds IX and XI. This is consistent with the view that the heteronuclear ring in carbaryl interacts with the anionic site of cholinesterase (*33*). As is shown in Table III, this interaction is markedly decreased by a shift of the *N*-methylcarbamoyl group from the 1- to the 2-position.

Effects of Fluorine in the Aryl Ring. The substitution of fluorine for hydrogen is the basis of many isosteric molecules and the fluorine substituted compounds are not sterically hindered in their interaction with enzymes or other receptors (*1*). The binding energy of the C—F bond (107 kcal. per mole) is substantially higher than the C—H bond (87.3 kcal. per mole) suggesting that specific fluorine substitutions may lead to carbamates less susceptible to detoxication by hydroxylation yet active as anticholinesterases.

The three monofluorine substituted phenyl *N*-methylcarbamates when compared with the unsubstituted compound present interesting results as shown in Table III. The *p*-fluorophenyl isomer was detoxified the least as measured by the synergistic ratio, and the *o*- and *m*-isomers were detoxified substantially less than the unsubstituted compound. The fully substituted pentafluorophenyl *N*-methylcarbamate (XX), surprisingly, was inactive and unsynergizable. These data suggest that *p*-hydroxylation is the predominant pathway of detoxication of the phenyl *N*-methylcarbamate. The aggregate electron withdrawing effects of the five F atoms upon the stability of the pentafluoro compound contribute to its inactivity.

The substitution of fluorine into the carbaryl molecule at the favored detoxication positions 4- (XIV) and 5- (XV) (*8*) provides similar information about this ring system. As shown in Table III, neither compound was particularly toxic to the housefly by topical application; although both were well synergized by piperonyl butoxide, the heteronuclear compound being more toxic as would be expected from the electron withdrawing power of the F atom and its effects in decreasing the stability of the carbamate ester. This adverse affect is obviously greater when the F is in the homonuclear ring. To obtain a more meaningful comparison of the relative activities of these weakly active compounds, they were made up as 1% dry baits with sucrose, and 100 mg. was exposed to 20 female houseflies in petri dishes. The following times in hours were required for knockdown of the flies:

	KD_{50}	KD_{100}
Carbaryl	9	>48
4-OH-carbaryl	>48	...
5-OH-carbaryl	>48	...
4-F-carbaryl	>48	...
5-F-carbaryl	4	6

The demonstrably greater activity of the 5-F carbaryl confirms the importance of detoxication by hydroxylation at the 5 position of the naphthyl ring. However, apparently the C—F bond is still susceptible to a reduced rate of hydroxylation. This is in accord with Kaufman's (*25*) study of mammalian microsomal oxidases which converted 4-fluorophenyl alanine to tyrosine by direct substitution at the C—F bond.

Relation of Benzodioxole Structure to Synergism

Methylene Hydrogens. The critical importance of the bicyclic benzodioxole (methylenedioxyphenyl) ring system or its thio analogs for effective synergism of the carbamates has been demonstrated by Wilkinson, Metcalf, and Fukuto (*49*). The authors have been able to examine the comparative synergistic activity of several pairs of methylenedioxyphenyl synergists with and without dideutero - substituted methylene groups as shown in Table IV. In every case, the dideutero analog had a significantly lower synergistic ratio with carbaryl against the housefly, the deuterium isotope effect ranging from 0.57 to 0.87. These data provide strong evidence of the importance of hydride transfer to form an electrophilic benzodioxolium ion which may function as Hennessy (*21*) has suggested in acylating the active site of the detoxifying enzyme.

Nuclear Substitution. As shown by Wilkinson, Metcalf, and Fukuto (*49*) the unsubstituted benzodioxole (1,2-methylenedioxybenzene) is completely inactive as a carbamate synergist but, when substituted in the 5 position with a variety of simple substituents, becomes highly active reaching maximum synergistic ratios with carbaryl of CH_3O-82 and NO_2-90. The authors have been able to examine the synergistic activities of a large series of naphthodioxoles and related materials, some of which are shown in Table V. In contrast to the inactivity of methylenedioxybenzene, 1,2-methylenedioxynaphthalene [naphtho-(1,2-*d*)-1,3-dioxole] (XXI) and 2,3-methylenedioxynaphthalene [naphtho-(2,3-*d*)-1,3-dioxole] (XXII) with synergistic ratios of 224 and 180 were among the most effective synergists yet evaluated. The corresponding 1,8-methylenedioxynaphthalene [naphtho-(1,8-*d*)-1,8-dioxole], in which the dioxole ring bridges the ends of the two naphthalene rings, was inactive. This information emphasizes the need for a double attachment of the synergist to the surface of the detoxifying enzyme in order to inhibit its action.

As shown in Table V, a high degree of activity was retained through nuclear substitution of the 1,2- or 2,3-methylenedioxynaphthalenes. Substitution with electron-donating groups such as methoxy-produced compounds with a molar activity equivalent to the unsubstituted compounds regardless of whether the methoxy group was homonuclear or heteronuclear. Electron withdrawing groups such as halogen or nitro produced somewhat less effective compounds, and with the latter, the activity

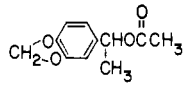
Table IV. Effects of Deuteration of Methylene Group on Synergistic Activity with Carbaryl

Synergist	Ratio to Carbaryl	Topical LD_{50} *Musca domestica* as Carbaryl, μG./G.		Deuterium Isotope Effect
		Dihydro	Dideutero	
Piperonyl butoxide	1:1	40	50	0.80
Sesamex	5:1	8.25	14.5	0.57
5-Nitro-1,3-benzodioxole	5:1	16.5	19.0	0.87
5-Bromo-1,3-benzodioxole	5:1	31.0	52.5	0.59

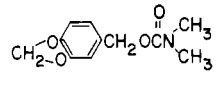
was one half for heteronuclear *vs.* homonuclear substitution. Substitution with groups such as OH or CH_2OH promoting water solubility tended to decrease synergistic activity. These results show that the synergistic effect is a property of the 1,2- or 2,3-methylenedioxynaphthalenes, and this can be subtly modified by ring substitution with a wide variety of simple or more complex groupings. This series of methylenedioxynaphthalenes appears to have considerable potentiality as practical synergists and as tools for the study of the nature of the interaction between phenolase detoxifying enzyme and substrate.

Piperonyl Carbamates. The compound 3,4-methylenedioxyphenyl *N*-methylcarbamate was shown in previous studies from this laboratory (*13*, *35*) to be substantially more toxic to the housefly than its anticholinesterase activity would suggest and unsynergizable by piperonyl butoxide. Apparently, this compound was acting as its own synergist. Thayer, Hartle, and Mallis (*48*) recently showed that related *N*-substituted carbamates such as 3,4-methylenedioxyphenyl *N*-butoxyethoxyethylcarbamate were effective pyrethrins synergists. Prill (*42*) has claimed 3,4-methylenedioxybenzyl *N*-(*n*-heptyl)carbamate as a pyrethrins synergist. This information suggested that the carbamoyl moiety might act as an appropriate anchoring group for piperonyl alcohol and improve its synergistic action for carbamates.

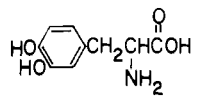
The data shown in Table VI prove that this is indeed the case as the synergistic ratios with carbaryl ranged from about 200 for the *N*,*N*-dimethylcarbamates **XXXIV** and **XXXV** to about 1.0 for the *N*,*N*-diphenyl derivative (**XXXVIII**) which was devoid of activity. These results suggest that the carbamoyl moiety unsubstituted or with small substituents is specifically complementary to a portion of the active site of the detoxication enzyme, thus explaining the extraordinary rapidity of detoxication of many of the *N*-methylcarbamate insecticides. The very effective synergism of the piperonyl carbamates, therefore, may be the result of the role of the carbamoyl group in providing the methylenedioxyphenyl group with maximum orientation to a key nucleophilic group at the active site of the detoxication enzyme. This speculation is attractive in view of the obvious structural similarities between the aryl, C=O, and NH moieties of the phenyl *N*-methylcarbamates and of tyrosine which, for the reasons presented in the next section, seems a very likely candidate as the normal substrate of the detoxication enzyme in the housefly. These data also are in accord



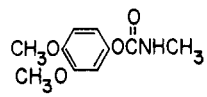
α-Methylpiperonyl acetate



Piperonyl *N*,*N*-dimethylcarbamate



3,4-Dihydroxyphenylalanine



3,4-Dimethoxyphenyl *N*-methylcarbamate

with the outstanding synergistic activity of α-methylpiperonyl acetate which had a synergistic ratio of 128 with carbaryl (*49*).

In Vivo Sensitivity of Detoxication System in the Housefly

The very high synergistic ratios for carbaryl, with a variety of the methylenedioxyphenyl synergists, reflect the extreme ease of its detoxication in the housefly, and the activity of the detoxication enzymes can be estimated further by the contrast with the high toxicity of carbaryl for the honey bee. That the detoxication enzymes are highly sensitive in vivo to the presence of minute amounts of synergist is shown by the data in Table VII. The presence of a good synergist, 2,3-methylenedioxynaphthalene at a ratio of 0.001 of that of the carbamate and at a dosage of 0.01 μg. per female fly, appreciably lowered the rate of carbamate detoxication. Maximum inhibition of the enzyme system occurs somewhere about 0.2 to 0.4 μg. per fly, or at a synergist-carbamate ratio of 2:1 to 5:1 in agreement with the data of Wilkinson, Metcalf, and Fukuto (*49*).

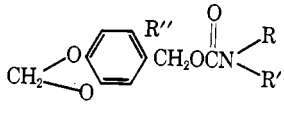
Inhibition of Phenolases by Benzodioxoles

From the results indicated above as well as from a great deal of research on factors affecting the potentiation of drug metabolism (*5*, *19*), apparently the mixed function oxidase enzymes are involved in the detoxication of the carbamate insecticides. Specifically, rat liver microsomes fortified with $NADPH_2$ have been shown to degrade oxidatively *N*,*N*-dimethyl (*22*, *23*) and *N*-methylcarbamates (*8*) presumably via the car-

Table V. Synergistic Properties of Methylenedioxy Naphthalenes

Substituted Naphthalene	Topical LD_{50} *Musca domestica* 5:1 with Carbaryl, μG. per G.	μM.	Synergistic Ratio
XXI 1,2-Methylenedioxy	4.0	23	224
XXII 2,3-Methylenedioxy	5.0	29	180
XXIII 1,8-Methylenedioxy	>100		...
XXIV 2,3-Perfluoromethylenedioxy	>100		...
XXV 3-Methoxy-1,2-methylenedioxy	6.5	32	139
XXVI 4-Methoxy-1,2-methylenedioxy	5.0	25	180
XXVII 7-Methoxy-1,2-methylenedioxy	4.6	23	196
XXVIII 3-Methyl-1,2-methylenedioxy	8.0	43	112
XXIX 4-Chloro-2,3-methylenedioxy	14.0	68	64
XXX 4-Nitro-2,3-methylenedioxy	11.5	53	78
XXXI 6-Nitro-2,3-methylenedioxy	23.0	106	39

Table VI. Synergistic Properties of Piperonyl Carbamates



Compound	R	R′	R″	M.P., ° C.	Analysis, % Theory	Analysis, % Found	*Musca domestica* topical LD_{50}, μg./G. 5:1 with Carbaryl	Synergistic Ratio
XXXII	H	H	H	103–7	C = 55.38 H = 4.62	C = 55.09 H = 4.61	9.75	92
XXXIII	H	H	CH_3	53–8	C = 57.41 H = 5.26	C = 57.40 H = 5.49	28.0	32
XXXIV	H	CH_3	CH_3	53–5 B.p. 120–6/ 0.7 mm.	C = 59.19 H = 5.83	C = 60.16 H = 5.88	4.6	196
XXXV	Br	CH_3	CH_3	78–81	C = 43.70 H = 3.97	C = 44.10 H = 4.22	4.2	214
XXXVI	H	C_2H_5	C_2H_5	B.p. 130–4/ 0.5 mm.	C = 62.55 H = 6.76	C = 62.53 H = 7.27	5.5	163
XXXVII	H	H	2-ClC_6H_5	74–6	C = 59.00 H = 3.94	C = 59.39 H = 3.53	165.0	5.5
XXXVIII	H	C_6H_5	C_6H_5	110–4	C = 72.62 H = 4.90	C = 73.03 H = 5.16	>500	ca. 1.0
	Piperonyl butoxide						12.5	72

Table VII. Effect of Varying Ratios of Carbaryl to 2,3-Methylenedioxynaphthalene upon Synergism to the Housefly

Ratio of Carbaryl to Synergist	Topical LD_{50}, μG./G. As carbaryl	As synergist	Synergistic Ratio
Carbaryl alone	900		
1000:1	500	0.5	1.8
100:1	350	3.5	2.6
10:1	110	11.0	8.2
5:1	31	6.2	29
2:1	10	5.0	90
1:1	8	8.0	112
1:2	6	12.0	150
1:5	5	25.0	180

bamyl moiety. Hydroxylation of the naphthyl ring of carbaryl has been found to occur in vivo in insects and mammals (*8*), and housefly microsomes have been shown to hydroxylate naphthalene (*47*). Methylenedioxyphenyl synergists, such as piperonyl butoxide and sesamex, are known to inhibit these oxidations (*41*). The *N*-methylcarbamates under study here are all uniform in regard to the carbamyl moiety, and evaluation of the pronounced differences in detoxication rates that occur with minor structural changes (Table III) in terms of hydroxylation of the carbamyl group is difficult. Moreover, major changes in this structure—i.e., from NH_2 to $NHCH_3$ to $N(CH_2)_3$ to $NHCF_3$—can be made without greatly altering the synergistic ratio of the carbamate with piperonyl butoxide (*11*). Therefore, seeking the major carbamate detoxifying enzymes among the phenolases, which are able to place molecular oxygen directly in aromatic rings possibly through a HOO· radical formed with Fe^{+2} or Cu^{+} (*44*), seemed most logical.

The high inhibitory activity specifically associated with the methylenedioxyphenyl (benzodioxole) group strongly suggests tyrosinase or a very similar phenolase as the principal carbamate detoxifying enzyme. However, the studies with housefly microsomes produced several puzzling anomalies which suggest that microsomal tyrosinase is not the principal site of this carbamate detoxication. For example, male houseflies are considerably more susceptible to carbamates than female flies—LD_{50} for *m*-isopropylphenyl *N*-methylcarbamate is 1.0 for males and 2.0 μg. per gram for females—and males did not develop an appreciable resistance during a selection process with this compound which produced the carbamate-resistant R_{MIP} strain—LD_{50} >5000 mg. per kg. for females (*18*). Yet the microsomal phenolase activity found in three replicate determinations, 0.65 unit for males and 0.62 unit per gram of protein for females showed no significant difference. Similar comparisons between the females of the susceptible S_{NAIDM} strain and the R_{MIP} strain gave an average of 0.55 unit of phenolase per mg. protein for the S_{NAIDM} and 0.25 unit per mg. for R_{MIP}. However, with the latter strain piperonyl butoxide at 5 to 1 with *m*-isopropylphenyl *N*-methylcarbamate produced an LD_{50} of 55 μg. per gram and a synergistic ratio of >100.

Some of these anomalies have been resolved by further investigation of the soluble tyrosinase of the housefly (*2*). This enzyme increases rapidly during larval life and reaches maximum activity at pupation. The activity declines slowly in the adult stage, and the enzyme generally is believed to be involved in the hardening and darkening of the cuticle (*20*). The soluble tyrosinase in the carbamate-resistant R_{MIP} strain has an activity of two times that of the S_{NAIDM}. The cresolase or hydroxylating activity of the purified soluble tyrosinase is inhibited readily by the methylenedioxyphenyl compounds as shown in Table VIII. The purified enzyme rapidly hydroxylates a variety of phenols and phenyl carbamates. As an example, 1.5 ml. of purified enzyme was reacted with 50 μmoles of C^{14}-ring-labeled phenyl *N*-methylcarbamate and 0.59 μmole of $NADP^+$ for two hours at 25° C. in phosphate buffer pH 7.0. The products were extracted with ether and examined by two-dimensional thin layer chromatography on silicic acid using ether–hexane (3:1) and butanone–acetone–water (1:1:1). Twenty per cent of the C^{14} was recovered as metabolites of which 10.2% was *p*-hydroxyphenyl *N*-methylcarbamate and 2.9% was *o*-hydroxyphenyl *N*-methylcarbamate as tentatively identified by comparison with known compounds and by spraying with ninhydrin—for carbamate nitrogen—and diazonium fluoborate—for phenolic

Table VIII. In Vitro Inhibition of Tyrosinase

	Compound	I_{50} M Tyrosinase: Purified Catechalase	Housefly: Catechalase	Housefly: Cresolase	Synergistic Ratio, 5:1 Carbaryl (*49*)
XXXIX	1,3-Benzodioxole (Methylenedioxybenzene)	3.0×10^{-4}			1
XL	1,3-Benzoxathiole	1.5×10^{-4}			1
XLI	1,3-Benzdithiole	1.8×10^{-5}			...
XLII	2,3-Dihydrobenzofuran	1.6×10^{-3}			1
XLIII	Indane	1.4×10^{-3}			1
XLIV	1,4-Benzodioxane	1.3×10^{-3}			1
XLV	1,2-Dimethoxybenzene	$>1 \times 10^{-1}$			1
XLVI	2-Methyl-1,3-benzodioxole	6.6×10^{-3}			1
XLVII	2,2-Dimethyl-1,3-benzodioxole	$>1 \times 10^{-1}$			1
XLVIII	2-Methyl-2-ethyl-1,3-benzodioxole	$>1 \times 10^{-1}$			1
XLIX	5-Formyl-1,3-benzodioxole (Piperonal)	4.1×10^{-4}		3.6×10^{-5}	9.0
L	5-Acetoxy-1,3-benzodioxole	9.6×10^{-5}	1.4×10^{-4}		...
LI	3,4-Dimethoxyphenyl acetate	$>1 \times 10^{-1}$			1.0
LII	5-Hydroxy-1,3-benzodioxole (Sesamol)	3.8×10^{-6}			4.8
LIII	5-Carboxy-1,3-benzodioxole (Piperonylic acid)	2.1×10^{-6}		4.8×10^{-4}	1.0
LIV	5-Methylol-1,3-benzodioxole (Piperonyl alcohol)	6.6×10^{-4}	5.0×10^{-4}	2.8×10^{-3}	12.4
LV	3,3-Dimethoxybenzyl alcohol (Veratryl alcohol)	9.5×10^{-2}			1
LVI	5-Chloro-1,3-benzodioxole	1.3×10^{-5}	1.2×10^{-3}	1.3×10^{-3}	30
LVII	5-Nitro-1,3-benzodioxole	2.1×10^{-4}	4.7×10^{-4}	8.6×10^{-4}	90
LVIII	5-Methyl-1,3-benzodioxole	1.6×10^{-4}	8.4×10^{-4}	3.1×10^{-3}	31.5
LIX	5-Methoxy-1,3-benzodioxole	5.4×10^{-5}	2.5×10^{-4}	1.7×10^{-3}	81.8
LX	5-*tert*-Butyl-1,3-benzodioxole	$>1.0 \times 10^{-3}$		6.0×10^{-3}	37.5
LXI	2,2-Dideutero-1,3-benzodioxole	6.0×10^{-4}			...
LXII	5-(1-Hydroxyethyl)-1,3-benzodioxole	3.3×10^{-5}			90
LXIII	5-(1-Hydroxyethyl)-2,3-dihydrobenzofuran	8.4×10^{-5}			19
LXIV	5-(1-Hydroxyethyl)-2,3-dihydrobenzothiophene	1.4×10^{-3}			8
LXV	5-(1-Hydroxyethyl)-1,4-benzoxathiole	4.9×10^{-4}			67
LXVI	5-(1-Hydroxyethyl)indane	3.6×10^{-5}			1
LXVII	5-(1-Hydroxyethyl)-2,2-dimethyl-1,3-benzodioxole	$>1 \times 10^{-2}$			1
LXVIII	5-(1-Hydroxyethyl)-1,4-benzodioxane	5.6×10^{-6}			3.6
LXIX	4-(1-Hydroxyethyl)-1,2-dimethoxybenzene	10^{1}			2.9

hydroxyl. A detailed account of these investigations has been prepared. Apparently this soluble tyrosinase is the enzyme largely responsible for carbamate detoxication in the housefly. The enzyme plays an essential role in the larval and pupal development of the housefly as piperonyl butoxide, added to the breeding medium at concentrations of 0.074 to 0.25%, markedly increased the larval development period and caused death in the third instar or early in pupation (*37*). No soluble tyrosinase activity can be detected in homogenates of the honey bee which is so extremely susceptible to the carbamates.

Inhibition of Tyrosinase

Purified tyrosinase was selected as a model phenolase enzyme for inhibition studies with methylenedioxyphenyl compounds, both because of its ubiquity in insect tissues (*20*) and its ready availability in purified form. A number of the more water soluble compounds evaluated as synergists (*49*) were studied further as in vitro inhibitors of the catecholase activity of this tyrosinase as shown in Table VIII. In some instances, the I_{50} values also are given for the inhibition of soluble tyrosinase from the housefly. The structural formulas for these compounds and their synergistic ratios have been given by Wilkinson, Metcalf, and Fukuto (*49*).

The data show that, among the unsubstituted bicyclic compounds, 1,3-benzodioxole (XXXIX) is a good inhibitor, and replacement of the oxygen atoms in the methylenedioxy ring by sulfur to give 1,3-benzoxathiole (XL) and 1,3-benzdithiole (XLI) results in increased inhibitor activity. Any other variations in the five-membered ring, however, result in general decreases in activity—e.g., enlargement to a six-membered ring (XLIV), or when the hydrogen atoms of the ring methylene group are substituted with alkyl groups (XLVI), (XLVII), and (XLVIII). Substitution of methylene hydrogens with deuterium gave 2,2-dideutero-1,3-benzodioxole (LXI) which was one half as active as 1,3-benzodioxole.

Ring opening to the dimethoxybenzene analog resulted in marked decrease in inhibitory activity. Of the several pairs of analogous methylenedioxy and dimethoxy compounds evaluated, there is between 100-fold and 10,000-fold decrease in inhibition associated with the replacement of methylenedioxy with dimethoxy (compare XXXIX and XLV, L and LI, LIV and LV). Evidently, the methylenedioxy group itself provides some rather specific inhibitory property to the molecule. The twofold lower activity of 2,2-dideutero-1,3-benzodioxole compared with benzodioxole and the much lower activity of the alkyl-substituted methylenedioxy compounds indicate that the hydrogen atoms are involved in the inhibition process (*21*).

The effect of substituents on the benzene ring in benzodioxole upon phenolase inhibition and in some cases on synergistic activity, also, is presented in Table VIII. Of the simpler compounds evaluated with substituents in the 5 position, the most active were those containing hydroxy (LII), chloro (LVI), and methoxy (LIX), groups in which the atoms bonded to the benzene ring provide a region of high electron density. Substitution in the same position with methyl (LVIII), and nitro (LVII) caused little change in activity, whereas the *tert*-butyl group (LX) resulted in considerable decrease in activity compared with the unsubstituted benzodioxole. The low inhibitory activity of LX suggests that the bulky *tert*-butyl group interferes with the inhibitory process. There was no direct correlation between inhibitory activity and Hammett's sigma constants of the substituents and, therefore, the change in activity associated with the substituents probably is not due to alterations in electronic properties of the methylenedioxy moiety exerted through the ring system by the substituent. Substituents, particularly those containing atoms of high electron density adjacent to the benzene ring, could enhance activity by secondary binding onto some other part of the detoxifying enzyme molecule.

Data on the synergistic activity of the compounds in Table VIII (*49*), where comparisons can be made, show that most of those which were active inhibitors of tyrosinase were also good synergists. Since previous work (*49*) has shown that 1,3-benzodioxole (XXXIX) itself showed poor synergistic activity and that substitution of the α-hydroxyethyl moiety in the 5 position produced a compound with high activity, comparison of synergism and tyrosinase inhibition was studied with analogs of α-methylpiperonyl alcohol or 5-(1-hydroxyethyl) - 1,3 - benzodioxole. The compounds LXII-LXIX are, therefore, similar in that they are all methylcarbinols with variation in the heterocyclic ring. With the exception of the indane (LXVI) and dihydrobenzofuran (LXIII) derivatives, which were strong tyrosinase inhibitors but poor synergists, there is, in general, direct correlation between enzyme inhibition and synergism.

The inhibition of the housefly microsomal tyrosinase was demonstrated with 1,3-benzodioxole (XXXIX), 1,3-benzoxathiole (XL), and 2,3-dihydrobenzofuran (XLII), although the I_{50} values were a factor of about 10 greater than those obtained with purified mushroom tyrosinase. This may be due to the tighter binding of the microsomal enzyme onto the particulate surface (*29*) thus making it less susceptible to the action of inhibitors.

The nature of tyrosinase inhibition was investigated in greater detail with 1,3 - benzodioxole. Lineweaver - Burk plots shown in Figure 1 indicate that inhibition is noncompetitive. The Michaelis-Menten constant was 1.4 ×

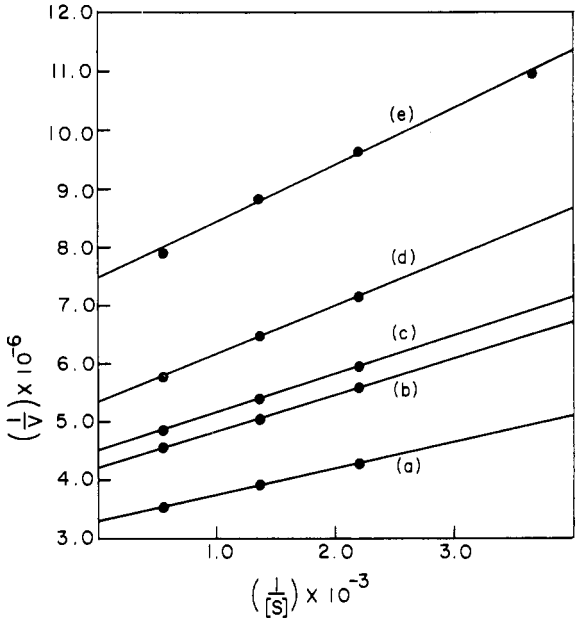


Figure 1. Lineweaver-Burk reciprocal plot of the enzyme tyrosinase in the presence of the inhibitor 1,3-benzodioxole

(a) Uninhibited
(b) 9.75×10^{-5}M
(c) 1.46×10^{-4}M
(d) 1.95×10^{-4}M
(e) 2.45×10^{-4}M

$10^{-4}M$ both with and without inhibitor. Tyrosinase is known to be a copper-containing enzyme, probably in the cuprous state (*26*) and other evidence (*24*) suggests that the enzyme is able to form a reversible complex (EO or EO_2) with molecular oxygen, probably involving the percupryl ion $[Cu^{+}O \cdot OCu^{+}]$ (*4*). With the use of O_2^{18}, tyrosinase-catalyzed hydroxylations of monophenols to diphenols have been shown to occur with the incorporation of one atom of oxygen into the substrate, the other being reduced to water (*30, 31*). Inhibition of the intermediate oxygen complex, interaction between copper and synergist, or other reaction between synergist and enzyme, therefore, could lead to noncompetitive kinetics.

Acknowledgment

The dideuterobenzodioxoles of Table IV and compound LXI of Table VIII were provided through the cooperation of D. J. Hennessy of Fordham University, Bronx, N.Y.; 4-benzothienyl *N*-methylcarbamate by John Kilsheimer of Mobil Chemical Co., Metuchen, N.J.; and the naphthodioxoles of Table V by Julius Hyman, Fundamental Research Laboratory, Berkeley, Calif.

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