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Inhibition of Acetylcholinesterase from Mammals and Insects by Carbofuran and Its Related Compounds and Their Toxicities toward These Animals

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Carbofuran or 2,3-dihydro-2,2-dimethylbenzofuranyl-7 *N*-methylcarbamate and closely related analogs were studied for selective toxicity to mammals *vs.* insects and for details of their inhibition of acetylcholinesterases from bovine erythrocyte and housefly and honeybee brains. Main kinetic analysis of the inhibitory process showed that the binding energy for all three cholinesterases was increased by mono- and dimethylation of 2,3-dihydrobenzofuranyl-7 *N*-methylcarbamate. However, effects on carbamylation were not uniform. The toxicity values for the compounds to rat or mouse and for housefly or *Culex* mosquito larva were well correlated with the bimolecular rate constants for the inhibition of erythrocyte and housefly AChE.

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuranyl-7 *N*-methylcarbamate) is one of the most active and persistent carbamate insecticides and is widely used in soil applications for control of insects attacking corn (Petty and Kuhlman, 1972). However, it is essentially nonselective to insects as compared with mammals, with a topical LD_{50} to the housefly of 4.6 mg per kg compared to an oral LD_{50} of 4.0 mg per kg to the rat. This high mammalian toxicity severely limits its insecticidal applications and there is considerable interest in developing related carbamates with much higher mammalian selectivity ratios. Experimentally, this has been defined as msr, the oral LD_{50} for rat (or mouse)/topical LD_{50} to the housefly (Metcalf, 1971).

The metabolism of carbofuran in mammals and insects has been studied extensively (Metcalf *et al.*, 1968; Dorough, 1968; Knaak *et al.*, 1970a,b). However, inhibition at the target site, another important component of selective toxicity (O'Brien, 1967), has not been explored on a comparative basis. This paper reports detailed studies on the *in vitro* inhibition of mammalian and insect acetylcholinesterases (AChE, E.C. 3.1.1.7) by carbofuran and closely related analogs and on their *in vivo* toxicity to mammal and insect. These investigations were carried out to seek new leads for the development of better selective insecticides.

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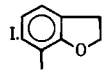
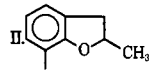
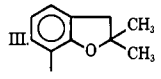
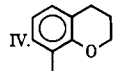
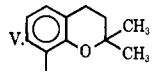
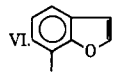
MATERIALS AND METHODS

Compounds Investigated. Carbofuran (compound III, Table I) and 2,2-dimethylchromanyl-8 *N*-methylcarbamate (V) were obtained from the FMC Corp., Niagara Chemical Division, as Niagara 10242 and 10856. The 2,3-dihydro-2-methylbenzofuranyl-7 *N*-methylcarbamate (II) was obtained from Farbenfabriken Bayer as Bayer 62863. The benzofuranyl-7 *N*-methylcarbamate (VI) and 2,3-dihydrobenzofuranyl-7 *N*-methylcarbamate (I) were prepared according to a reported procedure (FMC Corp., 1965) and chromanyl-8 *N*-methylcarbamate (IV) was prepared as described by Heiss *et al.* (1968).

Enzymes. Bovine erythrocyte AChE was purchased from Nutritional Biochemicals Co. Housefly (*Musca domestica* L.) and honeybee (*Apis mellifera* L.) head AChE's were partially purified as described by Yu *et al.* (1971).

Table I. LD_{50} Values for Carbofuran and Analogs on Mammals and Insects

R—OCNHCH$_3$ (O) R =	mg/kg ♀ rat	μg/g ♀ housefly	ppm culex larvae
I. 	30[a]	125(0.875)[b]	71.0
II. 	43	5.5(0.205)	0.19
III. 	4–11	4.6(0.10)	0.054
IV. 	20[a]	>500(0.43)	0.22
V. 	3–4	35(0.20)	0.046
VI. 	10[a]	28(1.6)	1.0

[a] Treatment on female mice. [b] Value given in parentheses represents synergized LD_{50} numbers following pretreatment for 1 hr with 50 μg/female of piperonyl butoxide.

Bioassays. The topical LD_{50} values to the 2–4-day-old female housefly were obtained by application of standard w/v acetone solutions to the prothorax with a micrometer-driven syringe. Mortalities were determined after 24 hr and LD_{50} values estimated from plots of the average of three replicates per dosage, on logarithm-probit paper. The synergized LD_{50} values to the housefly were obtained in the same manner using female houseflies pretreated for 1 hr with 50 μg of piperonyl butoxide applied to the ventral side of the abdomen. The oral LD_{50} values for 6–8-week-old male Swiss white mice were determined by oral administration of standard w/v solutions in olive oil by micrometer-driven syringe and by observations of mortality after 72 hr. The LC_{50} values for the compounds to fourth instar *Culex pipiens quinquefasciatus* larvae were determined by pipetting appropriate fractions of a milliliter of standard w/v acetone solutions into 100 ml of water containing the larvae. Mortalities were determined at 24 hr and replicates and determination of the LC_{50} value were carried out as described for the housefly.

Inhibition Studies. Inhibitor solutions were prepared in reagent grade acetone. A quantity of 0.01 ml of the inhibitor solution was delivered by means of a microsyringe to a test tube containing 0.19 ml of enzyme solution (in 0.1 *M* potassium phosphate buffer at pH 7.8 with enzyme activity of about 0.04 μmol of acetylthiocholine hydrolyzed per minute). After a specific incubation time (between 15 sec and 75 sec), a quantity of 0.05 ml of the reaction mixture was withdrawn by a microsyringe and delivered to an assay medium of 2.5 ml of 0.1 *M* potassium phosphate buffer pH 7.8 with following components: acetylthiocholine iodide, 7.5×10^{-4} *M* and 5,5′-dithiobis-2-nitrobenzoic acid, 4×10^{-4} *M*. The residual enzyme activities were measured by a Hitachi Perkin-Elmer double beam spectrophotometer (Coleman 124) at 412 nm and recorded by a Coleman recorder (Hitachi 165). The combination of inhibitor concentration and incubation time was selected so that no more than 90% of the enzyme was inhibited at that combination. The reaction temperature was at 25°C. Six inhibitor concentrations for each compound and four different incubation times for each inhibitor concentration were measured throughout the study. Main's kinetic constants (binding, carbamylation, and overall bimolecular constants) were calculated by the method similar to Main and Iverson (1966). However, the least-square method was employed to obtain the best fit of log v against time and $i\Delta t/2.303\ \Delta \log v$ against i plots (where v is enzyme activity, i is inhibitor concentration, and t is incubation time).

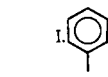
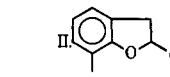
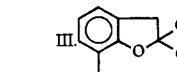
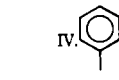
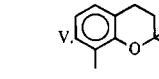
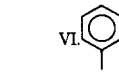
RESULTS AND DISCUSSION

It is generally proposed that the inhibition of AChE by carbamates occurs by the following reactions:

$$\underline{EH + CX} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH\cdots CX \xrightarrow{k_2} \underline{EC + HX}$$

$$\underline{EH + CX} \xrightarrow{k_i} \underline{EC + HX}$$

Table II. Kinetic Constants for Inhibition of AChE from Insect and Mammals by Carbofuran and Analogs

R—OCNHCH$_3$ (O) R =	Acetylcholinesterase								
	Bovine AChE			Housefly			Honeybee		
	K_a	k_2	k_i	K_a	k_2	k_i	K_a	k_2	k_i
I. 	4.9×10^{-3}	7.6	1.5×10^{3}	1.8×10^{-3}	25.8	1.5×10^{4}	1.4×10^{-3}	45	3.3×10^{4}
II. 	1.3×10^{-3}	14.8	1.1×10^{4}	3.4×10^{-5}	6	1.8×10^{5}	6.6×10^{-5}	29	4.4×10^{5}
III. 	2.1×10^{-4}	21.6	1×10^{5}	9.4×10^{-6}	10	1.1×10^{6}	3×10^{-5}	28	9.3×10^{5}
IV. 	1.24×10^{-3}	9.8	7.9×10^{3}	3.0×10^{-4}	13.5	4.6×10^{4}	3.9×10^{-5}	16.7	4.3×10^{5}
V. 	2.1×10^{-4}	33.5	1.6×10^{5}	6.4×10^{-6}	14.2	2.2×10^{6}	1.1×10^{-4}	51.7	4.5×10^{5}
VI. 	1.2×10^{-3}	5	3.7×10^{3}	5.4×10^{-4}	23	4.3×10^{4}	5.8×10^{-5}	50	8.7×10^{4}

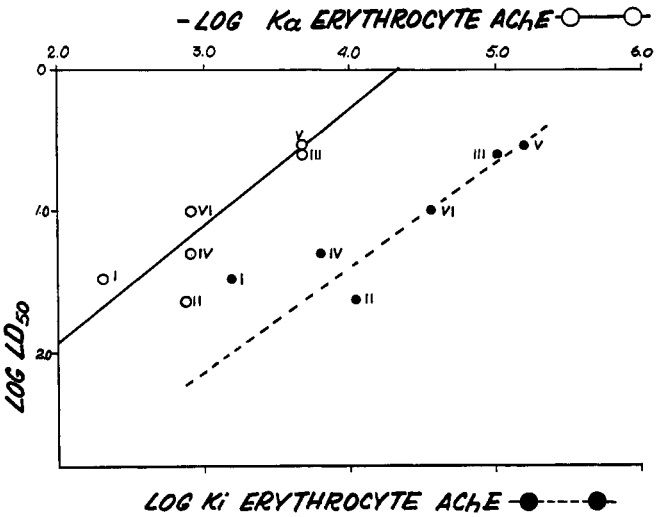


Figure 1. Graph showing the relationship between log rat (or mouse) oral LD_{50} and log k_i and $-\log K_a$ for erythrocyte AChE. Note the high degree of correlation between the bimolecular rate constant for AChE inhibition by the individual carbamate and its toxicity to the mammal. A simultaneous plot of the affinity constant ($-\log K_a$) and log LD_{50} shows that binding is a key factor in determining the toxicity of these carbamates

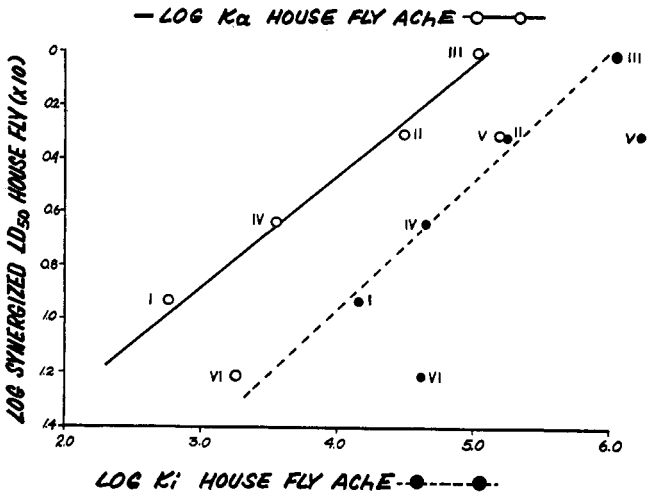


Figure 2. A graph showing the relationship between log housefly LD_{50} (synergized) and log k_i and $-\log K_a$ for housefly head AChE. There is a good correlation between the bimolecular rate constant for AChE inhibition and toxicity. A plot of the affinity constant ($-\log K_a$) and log LD_{50} shows that binding is largely reponsible for toxicity of these carbamates to houseflies

where EH and CX are the enzyme and carbamate, respectively, and EH · · · CX is enzyme–inhibitor complex and the binding or affinity constant $K_a = k_{-1}/k_1$. Main (1964) has described a procedure to measure the binding constant K_a, carbamylation constant k_2, and overall bimolecular rate constant k_i and the applications of the Main equation to inhibition of AChE by carbamates has been studied in detail by O'Brien (1968) and Hastings *et al.* (1970).

The effects of carbofuran and related compounds on the inhibition constants of AChE and toxicity toward mammals and insects are summarized in Tables I and II. It is clear that the binding energy of enzyme–inhibitor complex increases with methyl and dimethyl substitution on the 2 position of 2,3-dihydrobenzofuranyl-7 *N*-methylcarbamate molecule (compare K_a values for compounds I, II, and III). The average increase of the binding energies per methyl group, calculated from the equation

$$\Delta\Delta F = 2.303\ RT\ \Delta\log K_a$$

(where ΔF is free energy, R is gas constant, T is reaction temperature, and K_a is binding constant of enzyme–inhibitor complex), is 940 cal/mol for bovine erythrocyte, 1130 cal/mol for honeybee, and 1550 cal/mol for housefly AChE.

Wilson (1952) found an increase of 1140 cal/mol per methyl group in the methyl-substituted ammonium ions on the binding of AChE. He interpreted this increase as due to the dispersion forces between the methyl group and the protein. It is generally proposed that a hydrophobic or anionic site is located near the esteratic (catalytic) site of AChE (Wilson, 1952; Metcalf and Fukuto, 1965). Therefore, the increase of binding energy for methyl substitution in the 2 position of 2,3-dihydrobenzofuranyl-7 *N*-methylcarbamate molecule is interpreted as due to the interaction of methyl group with the anionic or hydrophobic binding site of the AChE through van der Waals' and hydrophobic forces. Such interactions can be illustrated with Fisher, Hirschfelder, Taylor molecular models of the benzofuranyl *N*-methylcarbamates and a plaster cast of the substrate acetylcholine (Metcalf and Fukuto, 1965). It appears that the binding on these substituted benzofuranyl *N*-methylcarbamates to AChE from mammal and insects is analogous to that of the normal substrate acetylcholine. For chromanyl *N*-methylcarbamates, the binding energy of the 2,-2-dimethyl-substituted compound is higher than the unsubstituted compound for bovine erythrocyte and housefly AChE, but slightly lower for honeybee AChE.

Unlike the K_a values, carbamylation rates (k_2) are not uniformly affected by the methyl substitutions in 2,3-dihydrobenzofuranyl-7 *N*-methylcarbamates (compare k_2 for compounds I, II, and III). For bovine erythrocyte AChE, methyl substitutions on the molecule accompany the increase of carbamylation rate. The opposite is true for housefly and honeybee AChE. For chromanyl *N*-methylcarbamates, k_2 for 2,2-dimethyl substitution is higher than for the unsubstituted compound for bovine erythrocyte and honeybee AChE. Therefore, there appears to be no consistency on the effect of substitution on carbamylation rate. It has been shown that the orientation of the ring substituents in phenyl *N*-methylcarbamates has important effects upon the carbamylation rate (Hastings *et al.*, 1970). It is conceivable that the relative positions of the anionic or hydrophobic binding sites in relation to the catalytic sites of AChE from different species are sufficiently different to produce substantial differences in carbamylation rates.

Figure 1 shows the relationship between log rat (or mouse) oral LD_{50} and log k_i and $-\log K_a$ for erythrocyte AChE. There is a high degree of correlation between the bimolecular rate constant for AChE inhibition by the individual carbamate and its toxicity to the mammal. As shown by the plot of $-\log K_a$ and log LD_{50}, the binding or affinity of the carbamate to AChE is the key factor in this series of carbamates in determining mammalian toxicity. The generally high toxicity of these compounds to mammals suggests that metabolic detoxication does not take place very rapidly in the mammal.

Figure 2 shows a substantially similar picture for the relationship between log housefly LD_{50} (synergized) and log k_i and $-\log K_a$ for housefly head AChE. There is good correlation, especially for the 2,3-dihydrobenzofuranyl carbamates, between the bimolecular rate constant for AChE inhibition and toxicity, and binding or affinity of the carbamate to AChE is clearly the key factor in determining insect

toxicity (see also mosquito LC_{50} values in Tables I and II). The substantial synergism shown by pretreatment of the fly with piperonyl butoxide, especially for the unsubstituted compounds I and IV, indicates that the multifunction oxidases of the housefly play an important role in detoxication of these compounds and suggests that detoxication is hindered by 2-methyl substitution.

In summary, the results shown in Tables I and II suggest that there is little significant difference in the way in which the benzofuranyl and benzopyranyl *N*-methylcarbamates react with both mammalian and insect AChE. Toxicity for this series of carbamates to both mammal and insect is predominantly a function of the affinity or binding constant K_a for the AChE of the species and selective detoxication plays a lesser role. Improvement in the mammalian selectivity ratio for this type of compound would seem to result most logically from introduction of substituent groups which are more readily metabolized by the mammalian liver than in the insect.

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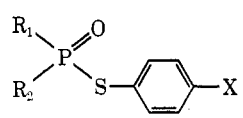
Insecticidal, Anticholinesterase, and Hydrolytic Properties of *S*-Aryl Phosphoramidothioates

James R. Sanborn and T. R. Fukuto*

The insecticidal, anticholinesterase, and hydrolytic properties for a series of *S*-phenyl phosphoramidothioates and *S*-phenyl phosphonamidothioates were examined. The compounds were moderately toxic to the housefly and were effective inhibitors of cholinesterase. Attempts to correlate cholinesterase inhibition of housefly toxicity with physical organic parameters were unsuccessful. However, an excellent linear relationship was obtained between Hammett's σ constant and alkaline hydrolysis rates of the *O*-ethyl substituted *S*-phenyl phosphoramidothioates. In addition, a kinetic study of the alkaline hydrolysis of these esters was carried out for the purpose of examining the mechanism of reaction. The results indicate that hydrolysis takes place by a direct nucleophilic attack on the phosphoryl center by hydroxide ion and the increase in hydrolytic stability with progressive nitrogen substitution can be accounted for by less favorable polar and steric effects.

Previous studies (Quistad *et al.*, 1970) in this laboratory concerning the relationship between structure, reactivity, and insecticidal activity of *O*-alkyl *S*-alkyl phosphoramidothioates revealed that several of the compounds were exceptionally toxic to the housefly, *Musca domestica*, although they were relatively weak inhibitors of fly-head acetylcholinesterase (AChE). These esters, however, produced typically cholinergic symptoms of intoxication. One of these compounds, Monitor or *O,S*-dimethyl phosphoramidothioate (Chevron Research Corp., 1967; Lorenz *et al.*, 1965), currently is undergoing evaluation as a potential insecticide.

The outstanding insecticidal properties of compounds of this type, combined with the limited amount of information available on the chemistry and mode of action of phosphoramidothioate esters, prompted us to extend our investigations to include phosphoramidothioates containing aryl moieties. This paper is concerned with the chemical, biochemical, and



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