A further effect of the metabolism of the methylenedioxybenzenes at the site of insecticide metabolism could be the result of a nonspecific competition at some limiting step by the catechol derivatives resulting from reaction of the benzodioxolium ion with water. A combination of both of these suggested modes of inhibition is not unreasonable.

The testing of methylene-deuterated methylenedioxybenzene synergists prepared in this laboratory is under way and preliminary data support the view that the synergistic action involves chemical participation of the methylenedioxy group (*33*)

Analogs of the synergistic methylenedioxybenzenes which can be expected by hydride transfer to form cationic electrophiles of reactivity similar (*2*) to the benzodioxolium ions are in the course of preparation for testing.

Acknowledgment

The author is indebted to Christopher Wilkinson, graduate student in the Department of Entomology of the University of California, Riverside, for stimulating discussions which led to the hypothesis presented in this paper. The author acknowledges the financial assistance of the New York City Health Research Council under contract HRC U1353 with Fordham University and of a Faculty Fellowship from Fordham University.

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CARBAMATE INSECTICIDES

Effects of Chemical Structure on Intoxication and Detoxication of Phenyl *N*-Methylcarbamates in Insects

ROBERT L. METCALF and T. R. FUKUTO

Department of Entomology, University of California, Riverside, Calif.

These investigations of aryl carbamates as potential insecticides began in 1949 with a study of the insecticidal activities of physostigmine sulfate, the *N,N*-dimethylcarbamic acid ester of 3-hydroxy-2-pyridylmethyl dimethylamine dihydrochloride, and other quaternary ammonium derivatives of aryl *N*-methylcarbamates (*34*). The quaternary compounds inhibited fly brain cholinesterase at concentrations of 10^{-8} *M* but were inactive as contact toxicants when applied at dosages of 500 μg. per gram. Therefore it became of interest to determine, from the analogy with the organophosphorus anticholinesterases, whether modification of the structures of these very active carbamate

This paper reviews 15 years of research by the authors on the chemistry and mode of action of substituted phenyl N-methylcarbamate insecticides. The biological activities of some 130 compounds are described. Detailed consideration is given to the interaction of these carbamates with the active site of insect cholinesterase. Detoxication mechanisms are described in relation to synergism and insect resistance.

anticholinesterases would produce contact insecticides. At the same time it was visualized that the crystalline aryl carbamates would serve as elegant and readily modifiable tools for exacting studies of the nature and dimensions of the active sites of insect cholinesterases and of the forces binding enzyme and substrate. This paper reviews briefly progress which has been made in this laboratory and summarizes a considerable amount of previously unpublished data.

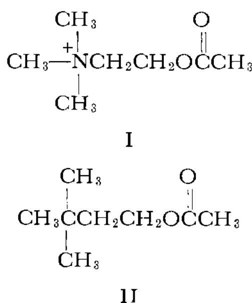
The data in the tables are taken from the original publications which summarize the techniques used for the determination of the molar concentration of carbamate for 50% cholinesterase inhibition (I_{50}), the topical LD_{50} to *Musca domestica* female houseflies, and the LC_{50} to *Culex pipiens quinquefasciatus* mosquito larvae. The precision of these determinations is indicated by a standard error of $\pm 7\%$ of the mean for five replicates of the determination of the I_{50} and a standard error of $\pm 7.5\%$ of the mean for five replicates of the determination of the LD_{50} to the housefly. The affinity for cholinesterase is the relative value I_{50} of phenyl N-methylcarbamate–substituted phenyl N-methylcarbamate.

Properties of Insect Cholinesterase

To provide background information for this research it was necessary to characterize insect cholinesterase in some detail. Thus it was demonstrated that the cholinesterase in the housefly brain hydrolyzed 11 μmoles of acetylcholine per mg. of tissue per hour at 37° as compared with 2.5 for the honeybee and 0.36 for the mouse. The activity in the fly brain was of the same order as found in the electric organ of the electric eel (*Electrophorus electricus*) (*34*). Studies of the activity *pS* curves for fly and bee cholinesterase with acetylcholine yielded *pS* optima of approximately 2 and enzyme substrate dissociation constants of 1.75×10^{-3} for the fly enzyme and 1.95×10^{-3} for the bee enzyme. Investigations with propionyl, butyryl, benzoyl, and acetyl β-methylcholines showed that the insect enzymes had properties very closely resembling the specific acetyl cholinesterase of mammalian erythrocytes and electric organ of the eel—i.e., the insect enzymes hydrolyzed acetylcholine more rapidly than any other choline ester at its substrate optimum and the activity *pS* curves with acetylcholine were bell-shaped with a sharply defined substrate optimum and pronounced inhibition by excess substrate.

The presence of a negatively charged anionic site was demonstrated by a marked competitive inhibition of fly and bee cholinesterase between choline chloride or tetraethylammonium iodide and the choline esters. The essential similarity of insect cholinesterases was demonstrated by experiments on 23 species of insects from the orders Diptera, Hymenoptera, Lepidoptera, Coleoptera, Hemiptera, Homoptera, and Orthoptera. However, through the use of various substrates and inhibitors it was demonstrated that the central nerve tissues of many species—e.g., the honeybee—contained a carbamate-sensitive specific cholinesterase and a carbamate-insensitive aliesterase; both enzymes were sensitive to organophosphorus inhibitors. The true cholinesterase was purified from 10- to 20-fold by fractional precipitation with ammonium sulfate (*36*).

Evaluation of a series of aliphatic esters of normal and branched-chain hydrocarbons ranging from ethyl acetate to 3,3-dimethylbutyl propionate, as substrates for fly and bee brain cholinesterase (*35*), showed that hydrolysis is dependent upon chain length and configuration of both alcohol and acid moieties. Hydrolysis increases with alcohol chain length up to C_5 and then decreases, increases with branching of four and five carbon alcohols (*n*-butyl < isoamyl < 3,3-dimethylbutyl), and decreases, in general, with increasing chain length from acetate through caproate. With the specific acetyl cholinesterase of fly brain, hydrolysis was maximum with 3,3-dimethylbutyl acetate (II), which was hydrolyzed about 0.83 as rapidly as acetylcholine at 0.03*M*. This compound is the carbon isostere of acetylcholine (I) and these data provide additional evidence of the stereochemical complementarity of the active site of insect cholinesterase to the acetyl choline molecular structure.



Active Site of Cholinesterase

These observations, then, are in agreement with a vast amount of study of cholinesterases from other sources (*23*), which demonstrates that acetylcholine is bound to cholinesterase by a multiple attachment. At the esteratic site, the electrophilic C=O group of the substrate is bound by a nucleophilic attack on a cyclic nitrogen, either imidazole or oxazoline of the peptide sequence glycylaspartyl-seryl-glycine. Approximately 5 A. away along the protein chain of the enzyme is the negatively charged anionic site, which is probably the COO^- group of aspartic or glutamic acid. This anionic site attracts the positively charged N^+ of acetylcholine through electrostatic forces and is also shaped to accommodate two of the approximately spherical CH_3 groups which are bound by short-range van der Waals forces (*32*).

Nerve Sheath Penetrability

A number of investigators beginning with Stedman (*40*) who first synthesized a synthetic miotic drug, *m*-dimethylaminophenyl N-methylcarbamate methiodide, and including Aeschlimann and Stemple (*1*), Haworth, Lamberton, and Woodcock (*20*), Stevens and Beutel (*41*), and Elder *et al.* (*5*), synthesized large numbers of dimethylaminophenyl methyl and dimethylcarbamates. Their quaternary ammonium salts were extensively evaluated for biological activity as miotics for pharmacological purposes and as possible antipersonnel agents for chemical warfare. However, none of the materials seems to have been considered as an insecticide nor were the unquaternized compounds evaluated.

An explanation was sought for the complete lack of insecticidal activity of substances such as *m*-dimethylaminophenyl N-methylcarbamate methiodide (VI) and the parallel case of the lack of pharmacological activity of acetylcholine, also containing a quaternary nitrogen, when injected into insects was considered (LD_{50} > 10,000 per gram for the fly as compared with 250 for the rat). Roeder (*39*) showed further that acetylcholine at $10^{-2}M$ produced neither discharge nor synaptic transmission when applied directly to the insect nerve. Therefore, it was surmised that the lipoid sheath surrounding insect nerves was impenetrable to the formally charged quaternary compounds. This was confirmed subse-

Table I. Biological Activities of Dimethylaminophenyl *N*-Methylcarbamates

N-Methylcarbamate	I_{50} M ChE	Affinity	LD_{50} µG. per g. *Musca*	LD_{50} P.p.m. *Culex*
III *o*-Dimethylaminophenyl	2.0×10^{-6}	100	45	1.6
IV *o*-Dimethylaminophenyl methiodide	1.0×10^{-5}	20	>50	>10
V *m*-Dimethylaminophenyl	8.0×10^{-6}	25	270	1.7
VI *m*-Dimethylaminophenyl methiodide	1.8×10^{-8}	11,000	>50	>10
VII *p*-Dimethylaminophenyl	2.4×10^{-4}	0.84	>500	>10
VIII *p*-Dimethylaminophenyl methiodide	3.5×10^{-6}	57	>50	>10

Table II. Inhibition Constants (K_i) of Fly Head Cholinesterase by Carbamates with Acetylcholine as Substrate (9)

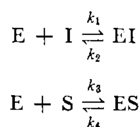
N-Methylcarbamate	K_i
3,5-Dimethoxyphenyl	1.0×10^5
3-*tert*-Butylphenyl	1.3×10^6
3-Isopropylphenyl	1.0×10^6
3-Dimethylaminophenyl methiodide	1.3×10^8
1-Naphthyl	4.6×10^6

quently by histochemical studies using acetyl thiocholine (*47*). This substance, which can be readily detected after hydrolysis by cholinesterase by the black precipitate which the thiocholine moiety forms after treatment with copper and ammonium sulfide, failed to penetrate the unbroken surface of the cockroach nerve cord, whereas the uncharged phenyl thioacetate penetrated readily.

From an appreciation of the inability of charged molecules to penetrate insect nerve sheaths the comparative behavior of the uncharged isomeric dimethylaminophenyl *N*-methylcarbamates was contrasted with the corresponding charged quaternary compounds (Table I). The results were in general agreement with those of Stedman (*40*) on the relative miotic properties of the compounds and showed the enhancement of anticholinesterase activity by quaternization in the *m*- and *p*-positions and the decreasing effect in the *o*-position. The quaternary compounds were ineffective insecticides at the highest levels which could be tested (because of their insolubility in acetone), while the *o*-dimethylaminophenyl *N*-methylcarbamate (III) and, to a lesser degree, the *m*-compound (V) were effective insecticides, in perfect agreement with theory.

Kinetics of Cholinesterase Inhibition

The inhibition of the cholinesterase enzymes by carbamates has long been assumed to be competitive in nature—i.e., they compete with the natural substrate for the active site on the enzyme surface, thus preventing the combination of the enzyme and substrate. In the simplest form the competitive nature may be represented as follows



$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

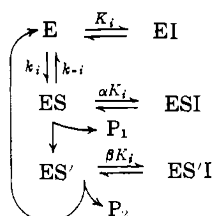
$$E + S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES$$

where E is enzyme, I is inhibitor, and S is substrate. There seems to have been some confusion in the early analysis of kinetic results of cholinesterase inhibition by prostigmine and physostigmine due to the relatively small values of k_2 for these compounds (*16–18*) which caused a time lag in the attainment of equilibrium. For example, mixing of the enzyme, inhibitor, and substrate simultaneously gave typical Lineweaver-Burk plots which could be interpreted only as resulting from competitive mechanism. However, when enzyme and inhibitor were incubated prior to substrate addition, a noncompetitive mechanism was indicated.

To add to the confusion, there have been numerous reports that certain carbamates irreversibly carbamoylate cholinesterase at the esteratic site (*2, 44, 45*). Wilson *et al.* (*44, 45*), for example, show that dimethylcarbamylcholine and dimethylcarbamyl fluoride reacted with electric eel cholinesterase to produce the dimethylcarbamoyl enzyme. It was shown that the rate of reactivation of enzyme carbamoylated by these two different carbamates was the same in water and in aqueous hydroxylamine.

It is evident that certain reactive carbamates, such as dimethylcarbamyl fluoride, have the ability to inactivate cholinesterase by reacting with the esteratic site in a manner analogous to the organophosphorus inhibitors. However, it is generally concluded (*4*) that the primary mode of inhibition is competitive in view of the relatively slow rates of carbamoylation compared to the fast rate of complex formation. The very high anticholinesterase activity of prostigmine and physostigmine relative to their very low turnover numbers when hydrolyzed by human plasma would support this view (*18*).

Recently Krupka and Laidler (*23*) have shown by kinetic means that such compounds as prostigmine, physostigmine, and carbamylcholine, do compete with the substrate for bovine cholinesterase. The reaction scheme proposed by these authors is as follows:



where E is free enzyme, ES is the enzyme-substrate complex, ES′ is the acylated enzyme, and EI, ESI, and ES′I are the forms complexed with inhibitor. K_i, αK_i, and βK_i are the respective association constants, α and β being positive numbers. In this general scheme the inhibitor may combine with the free enzyme, the Michaelis enzyme-substrate complex, and the acylated enzyme. By application of the steady-state treatment Krupka and Laidler derived equations from which they were able to determine the values for K_i, αK_i, and βK_i for physostigmine and carbamylcholine. From their results it was concluded that these compounds compete for the free enzyme, the ESI complex does not form, and the ES′I complex occurs but does not interfere in the deacylation reaction. Thus, it appears that the value of K_i alone determines the degree of inhibition.

We have carried out similar studies with fly head cholinesterase inhibition by a number of substituted phenyl *N*-methylcarbamates and Sevin and our preliminary data indicate results similar to those of Krupka and Laidler. The values for K_i obtained from the intercept of the reciprocal of the velocity *vs.* reciprocal of the substrate concentration at different inhibitor concentrations are given in Table II.

In view of the variation in the structure of these carbamates it can be concluded that the substituted phenyl *N*-methylcarbamates discussed in this paper inhibit cholinesterase primarily by a competitive mechanism.

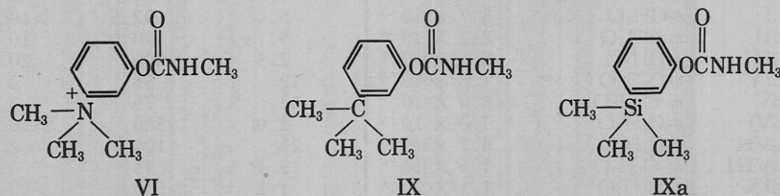
Molecular Complementarity to Active Site of Cholinesterase

The original ideas for the development of insecticidal carbamates were based on stable competitive inhibitors having an appreciable resemblance to the size and shape of acetylcholine, so as to be strongly attracted to both the esteratic and anionic sites of cholinesterase yet free from the electrically charged quaternary structure so that they would readily penetrate the insect nerve sheath (*22*). In view of the isosteric nature of trimethylammonium and *tert*-butyl groups as evidenced by the ready hydrolysis of 3,3-dimethylbutyl acetate by fly cholin-

Table III. Biological Activities of Halogen-Substituted Phenyl *N*-Methylcarbamates

Substituent		I_{50} M ChE	Affinity	LD_{50} μG. per g. Musca	LD_{50} P.p.m. Culex
X	Unsubstituted	2.0×10^{-4}	1.0	500	>10
XI	*o*-F	1.6×10^{-5}	12	250	>10
XII	*m*-F	8.5×10^{-5}	2.4	390	>10
XIII	*p*-F	2.3×10^{-4}	0.9	480	>10
XIV	*o*-Cl	5.0×10^{-6}	40	75	>10
XV	*m*-Cl	5.0×10^{-5}	4	>500	>10
XVI	*p*-Cl	2.4×10^{-4}	0.8	>500	>10
XVII	*o*-Br	2.2×10^{-6}	91	60	>10
XVIII	*m*-Br	1.3×10^{-5}	15	170	>10
XIX	*p*-Br	1.0×10^{-4}	2.0	>500	>10
XX	*o*-I	8.0×10^{-7}	250	90	>10
XXI	*m*-I	7.0×10^{-6}	29	260	>10
XXII	*p*-I	8.8×10^{-5}	2.3	>500	>10

esterase, the isomeric *tert*-butylphenyl *N*-methylcarbamates were investigated and the *m*-isomer (IX) was found to be a highly effective anticholinesterase (I_{50} $4 \times 10^{-7}M$, affinity 500) and an active insecticide.



In this molecule it seems evident that the *tert*-butyl group interacts strongly through van der Waals forces with the anionic site of cholinesterase, while the carbonyl group and ester bond of the carbamate interact with the esteratic site. The differences in activity among the *o*-, *m*-, and *p*-isomers of this and related molecules indicate that there is an optimum distance between the center of the anionic-site interactant and the carbonyl group, which for optimum affinity to cholinesterase should conform to the distance between quaternary nitrogen and carbonyl in acetylcholine. This is about 5 A. when the molecule is in its extended configuration (*8*, *46*). The importance of molecular complementarity has been further emphasized by the recent investigation of the three isomeric trimethylsilylphenyl *N*-methylcarbamates (*27*). The meta isomer (IXa), I_{50} 7.0×10^{-7} and affinity 285, displayed insecticidal activity nearly equivalent to that of its isostere *m-tert*-butylphenyl *N*-methylcarbamate (IX).

Subsequent investigations of substituted-phenyl *N*-methylcarbamates may be characterized as explorations of the dimensions and characteristics of the anionic and esteratic sites of cholinesterase and of the effects of changes in chemical structure upon cholinesterase inhibition and insecticidal properties.

Halogen-Substituted Phenyl *N*-Methylcarbamates. By oversimplification the anionic site may be pictured as a craterlike cavity and maximum affinity will depend upon the snugness of fit of the phenyl substituents. The halogens provide a series of spherical atomic substituents with a progressive increase in size as shown by their van der Waals radii of F = 1.35, Cl = 1.80, Br = 1.95, and I = 2.15 A. The inductive and electromeric properties of phenyl *N*-methylcarbamates substituted by halogens remain relatively constant, as shown by Hammett's σ values for meta substituents (F = 0.337, Cl = 0.373, Br = 0.391, and I = 0.352) and for para substituents (F = 0.062, Cl = 0.227, Br = 0.232, and I = 0.276), and Taft's σ^* values for the ortho substituents (F = +0.24, Cl = +0.20, Br = +0.21, and I = +0.21). Thus the changes in affinity for cholinesterase shown by the various halogen isomers in Table III must represent almost entirely the closeness of fit of the carbamate to the active site of cholinesterase. The para isomers show little alteration with substituent, but ortho and meta isomers progressively increase in affinity with increasing size of halogen atom.

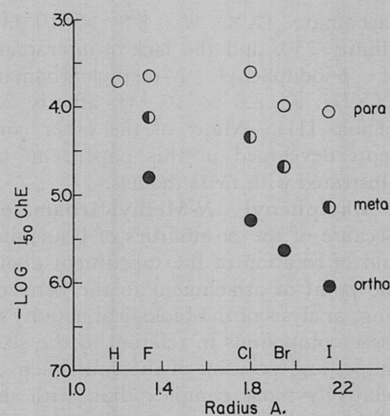


Figure 1. Relationship of −log I_{50} for fly cholinesterase of o-, m-, and p-halophenyl N-methylcarbamates to van der Waals radius of halogen atom

Activity is maximal with the ortho substituents. We have interpreted these data as indicating that the para substituents are directed away from the enzyme surface and fall outside of the confines of the anionic site, while the ortho substituents are most optimally placed to fall within the anionic site. Complementarity increases with increasing size of substituent, reaching a maximum with the iodo substituents which are the largest, and insecticidal activity parallels the affinity to a remarkable degree (*32*) (Figure 1).

Complementarity Demonstrated with Molecular Models. The degree of fit shown by Fischer-Hirschfelder models of carbamates with a plaster cast of a model of acetylcholine in its extended configuration, which simulates the active site of cholinesterase, has proved instructive. Such a model (*7*) shows, for example, the interaction of 1-naphthyl *N*-methylcarbamate (Sevin) I_{50} 9.0×10^{-7}, affinity 222, with the anionic site and the failure of 2-naphthyl *N*-methylcarbamate, I_{50} 1.4×10^{-5}, affinity 14, to interact appreciably (Figure 2). Thus in the 1-naphthyl derivative the second aromatic ring is essentially an *o-m* substituent, while in the 2-naphthyl derivative it is a *m-p* substituent. These model observations also clearly demonstrate the strong interaction of *o*-iodophenyl *N*-methyl-

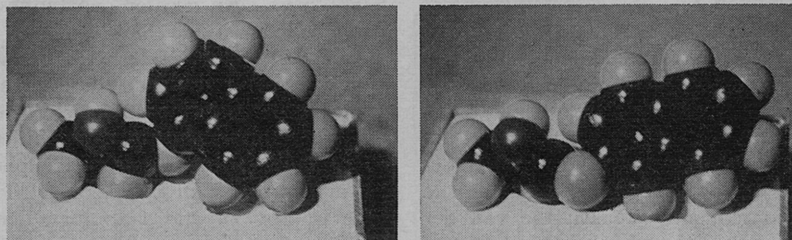


Figure 2. Complementarity of molecular models to plaster cast of molecular model of acetylcholine in extended configuration

Left. 1-Naphthyl N-methylcarbamate
Right. 2-Naphthyl N-methylcarbamate

carbamate (XX), I_{50} 8.0 × $10^{-7}M$, affinity 250, and the lack of interaction of *p*-iodophenyl *N*-methylcarbamate (XXII), I_{50} 8.8 × $10^{-5}M$, affinity 2.3 (Table III). Many of the other concepts developed in this paper can be illustrated with these models.

Alkylphenyl *N*-Methylcarbamates. Because of the possibilities of branching and of rotation of the substituent about the point of attachment to the benzene ring, analysis of the biological activity of these compounds in relation to the size, shape, and position of the substituent is relatively more complex than with the analogous halogen compounds. As summarized in Table IV, the order of activity for cholinesterase inhibition by alkyl-substituted phenyl *N*-methylcarbamates is unsubstituted <methyl < ethyl < isopropyl = *tert*-butyl < *sec*-butyl. For these compounds, the most favorable point of attachment is clearly in the meta position which studies of molecular models indicate conforms most closely to the optimum 5-A. distance between the center of the anionic interactant and the C=O group (*32*).

The differences in affinity of the carbamates with increasing methylation of the side chain are explainable in terms of the van der Waals dispersion forces between the methyl groups and the surface of the anionic site. The tetrahedral carbon atom adjacent to the benzene ring forces the third methyl group, as in *tert*-butyl, to project directly away from the enzyme surface so that it cannot interact with the anionic site. This concept is therefore in accord with the equivalence in affinities between *m*-isopropyl (I_{50} 3.4 × $10^{-7}M$) and *m*-*tert*-butyl (I_{50} 4.0 × $10^{-7}M$). These data are consistent with the effects of progressive methylation of aminoethyl acetate, where the affinity for cholinesterase increased fourfold for the first and 11-fold for the second, and remained constant with the addition of the third methyl group to give acetylcholine (*43*).

The increased affinity of *sec*-butyl over isopropyl or *tert*-butyl is presumably due to the added van der Waals attraction from the additional methylene group which lies in the direction of the anionic site and not away from it as in the *tert*-butyl compound. This agrees with data for the prostigmine derivatives, where the ethiodide of *m*-dimethylaminophenyl *N*-methylcarbamate was twice as toxic to the mouse as the methiodide (*5*).

The decreased affinity exhibited by the cyclopentyl- and cyclohexyl-substituted compounds is probably caused by their greater bulk and rigidity, which decrease the approach to the anionic site and consequently weaken the van der Waals attraction. In these two series of compounds there is much less difference between the effects of changes in ring position, which would also suggest a lack of critical interaction.

Table IV. Biological Activities of Some Alkyl Phenyl *N*-Methylcarbamates

Alkyl Substituent		I_{50} M ChE	Affinity	LD_{50} µG. per g. Musca	LD_{50} P.p.m. Culex
XXIII	*o*-CH_3	1.4 × 10^{-4}	1.4	500	>10
XXIV	*m*-CH_3	1.4 × 10^{-5}	14	50	3.3
XXV	*p*-CH_3	1.0 × 10^{-4}	2.0	500	8.2
XXVI	*o*-C_2H_5	1.3 × 10^{-5}	15	95	5.2
XXVII	*m*-C_2H_5	4.8 × 10^{-6}	42	140	0.44
XXVIII	*p*-C_2H_5	3.8 × 10^{-5}	5.3	250	>10
XXIX	*o*-IsoC_3H_7	6.0 × 10^{-6}	33	95	0.56
XXX	*m*-IsoC_3H_7	3.4 × 10^{-7}	590	90	0.03
XXXI	*p*-IsoC_3H_7	7.0 × 10^{-5}	2.9	>500	>10
XXXII	*o*-*sec*-Butyl	1.1 × 10^{-6}	180	135	0.35
XXXIII	*m*-*sec*-Butyl	1.6 × 10^{-7}	1250	100	0.03
XXXIV	*p*-*sec*-Butyl	1.8 × 10^{-6}	110	500	0.36
XXXV	*o*-CycloC_5H_9	1.1 × 10^{-6}	180	135	0.57
XXXVI	*m*-CycloC_5H_9	1.5 × 10^{-6}	130	400	0.14
XXXVII	*p*-CycloC_5H_9	2.7 × 10^{-5}	7.5	>500	>10
XXXVIII	*o*-CycloC_6H_{11}	1.4 × 10^{-6}	140	>500	2.3
XXXIX	*m*-CycloC_6H_{11}	2.0 × 10^{-6}	100	>500	1.5
XL	*p*-CycloC_6H_{11}	9.0 × 10^{-6}	22	>500	>10

Table V. Biological Activities of Alkoxyphenyl *N*-Methylcarbamates

Alkoxy	Substituent	I_{50} M ChE	Affinity	LD_{50} µG. per g. Musca	LD_{50} P.p.m. Culex
XLI	*o*-CH_3O	3.7 × 10^{-5}	5.4	92.5	>10
XLII	*m*-CH_3O	2.2 × 10^{-5}	9.1	90.0	10
XLIII	*p*-CH_3O	8.0 × 10^{-5}	2.5	>500	20
XLIV	*o*-C_2H_5O	1.6 × 10^{-5}	12	55	2
XLV	*m*-C_2H_5O	6.0 × 10^{-6}	33	75	3
XLVI	*p*-C_2H_5O	7.0 × 10^{-5}	2.9	>500	>10
XLVII	*o*-C_3H_7O	8.7 × 10^{-6}	23	105	2
XLVIII	*m*-C_3H_7O	1.6 × 10^{-5}	12.4	95	0.9
XLIX	*p*-C_3H_7O	1.1 × 10^{-4}	1.2	>500	>10
L	*o*-IsoC_3H_7O	6.9 × 10^{-7}	290	25.5	0.3
LI	*m*-IsoC_3H_7O	9.2 × 10^{-6}	22	180	3
LII	*p*-IsoC_3H_7O	8.8 × 10^{-5}	2.3	500	>10
LIII	*o*-C_4H_9O	1.2 × 10^{-5}	16.5	175	5
LIV	*m*-C_4H_9O	9.4 × 10^{-6}	21.0	280	0.3
LV	*p*-C_4H_9O	2.0 × 10^{-5}	10.0	>500	>10
LVI	*o*-*sec*-C_4H_9O	3.1 × 10^{-7}	650	50	0.28
LVII	*m*-*sec*-C_4H_9O	7.0 × 10^{-6}	29	220	1.5
LVIII	*p*-*sec*-C_4H_9O	3.2 × 10^{-5}	6.3	>500	>10
LIX	*o*-CycloC_5H_9O	4.0 × 10^{-7}	500	90	0.7
LX	*m*-CycloC_5H_9O	8.0 × 10^{-6}	25	>500	>10
LXI	*p*-CycloC_5H_9O	1.7 × 10^{-5}	12	>500	>10

Para substituents have in general the lowest activity. However, with longer chain interactants such as *sec*-butyl and isopentyl it appears that the chain is sufficiently flexible to interact with the anionic site. Thus the affinity for *p*-isopentylphenyl *N*-methylcarbamate of 22 (I_{50} 9.0 × 10^{-6}) is slightly greater than that of the *o*-isopentylphenyl *N*-methylcarbamate of 13 (I_{50} 1.5 × 10^{-5}). Other larger para substituents such as 1,1,3,3-tetramethylbutyl (affinity 66) and α,α-dimethylbenzyl (affinity 338) have even greater capabilities for anionic interaction (*27*).

Alkoxyphenyl *N*-Methylcarbamates. The elements of anionic site interaction with alkoxy-substituted phenyl *N*-methylcarbamates are not greatly different from those discussed for the alkylphenyl *N*-methylcarbamates (*30*, *32*). Thus, the isopropoxy and *sec*-butoxy derivatives had the highest affinity for cholinesterase and were the most insecticidal of a considerable series, again demonstrating the importance of interaction with the anionic site of cholinesterase.

There is, however, one key feature with the alkoxy series, as shown in Table V. In order to preserve the critical distance of 5 ± 0.3 A. between the center of the anionic interactant and the C=O group, allowance must be made for the offsetting of the phenyl substituent by the distance of the C—O bond length (1.42 A. as compared with the 1.39 A. of the C—C bond). This is clearly shown by comparing the affinity values in Tables IV and V. For the alkoxy substituents, the ortho position confers maximum activity in contrast to the meta position which is optimum for the alkyl substituents. For any given substituent the distance from center to C=O is equivalent within a few one-hundredths angstrom unit for the two series. The importance of this critical distance upon affinity for cholinesterase is shown in Figure 3.

Alkylthiophenyl *N*-Methylcarbamates. By analogy with the alkoxyphenyl *N*-methylcarbamates, the corresponding thioethers should prove to be interesting insecticides and have been carefully investigated (*29*). Table VI

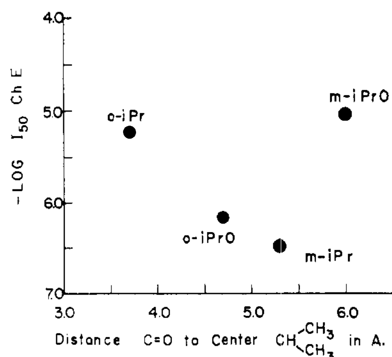


Figure 3. Relationship of $-\log I_{50}$ for fly cholinesterase of substituted phenyl *N*-methylcarbamates to approximate distance from carbonyl oxygen to center of isopropyl group

presents the biological activities of some alkylthiophenyl *N*-methylcarbamates. Many of these are highly active as anticholinesterases and toxic to flies and mosquitoes. These compounds resemble the corresponding alkoxy derivatives in demonstrating the critical nature of position isomerism and of the size and shape of the alkyl group. These features would appear to be reflections of the need for complementarity of the carbamate to the anionic site of cholinesterase. However, the alkylthiophenyl compounds are of substantially higher anticholinesterase and insecticidal activity than the alkoxyphenyl compounds and the structural requirements for this high activity appear to be substantially less critical (compare Tables V and VI). The only reasons which can be advanced for these differences in activity are the larger radius of the sulfur atom, which permits greater flexibility in the bending of the attached alkyl chain, and the expansibility of the valence shell of sulfur to accommodate a decet of electrons.

In the thioether compounds studied, the activity as anticholinesterases increased with chain branching in the order methyl < propyl < butyl < isopropyl < *sec*-butyl; and highest activity, in general, was determined by substitution in the ortho position, thus conforming to the concept of an optimum 5-A. distance between carbonyl group and the center atom interacting with the anionic site.

Table VI. Biological Activities of Some Alkylthiophenyl *N*-Methylcarbamates

Alkylthio Substituent		$I_{50}M$ ChE	Affinity	LD_{50} µG. per g. Musca	LD_{50} P.p.m. Culex
LXII	o-CH_3S	9.0×10^{-7}	222	48.5	3.9
LXIII	m-CH_3S	7.0×10^{-6}	29	8.5	1.5
LXIV	p-CH_3S	3.4×10^{-5}	6	26.5	4.3
LXV	o-C_3H_7S	1.8×10^{-7}	1100	20.0	0.18
LXVI	m-C_3H_7S	1.1×10^{-6}	180	23.5	0.10
LXVII	p-C_3H_7S	1.2×10^{-5}	17	32.0	0.41
LXVIII	o-IsoC_3H_7S	1.4×10^{-7}	1420	23.0	0.20
LXIX	m-IsoC_3H_7S	1.8×10^{-6}	110	46.5	0.13
LXX	p-IsoC_3H_7S	9.0×10^{-6}	22	700	16.5
LXXI	o-C_4H_9S	1.6×10^{-7}	1250	34.0	0.28
LXXII	m-C_4H_9S	7.8×10^{-7}	260	25.0	0.17
LXXIII	p-C_4H_9S	3.0×10^{-6}	67	27.0	1.35

Table VII. Biological Activities of Simple Disubstituted Phenyl *N*-Methylcarbamates

Substituent		$I_{50}M$ ChE	Affinity	LD_{50} µG. per g. Musca	LD_{50} P.p.m. Culex
LXXIV	2,3-DiCH_3	8.1×10^{-6}	25	190	3.3
LXXV	2,4-DiCH_3	1.3×10^{-4}	1.8	260	>10
LXXVI	2,5-DiCH_3	9.0×10^{-6}	22	320	>10
LXXVII	2,6-DiCH_3	1.0×10^{-2}	0.02	>500	>10
LXXVIII	3,4-DiCH_3	2.6×10^{-5}	7.7	120	0.9
LXXIX	3,5-DiCH_3	6.0×10^{-6}	33	60	3.0
LXXX	2,3-DiCH_3O	1.4×10^{-5}	14	>500	>10
LXXXI	2,4-DiCH_3O	2.8×10^{-5}	7.1	155	>10
LXXXII	2,5-DiCH_3O	1.3×10^{-5}	15	13	>10
LXXXIII	2,6-DiCH_3O	2.1×10^{-3}	0.09	>500	>10
LXXXIV	3,4-DiCH_3O	1.9×10^{-5}	10.5	400	>10
LXXXV	3,5-DiCH_3O	8.0×10^{-6}	25	11	>10
LXXXVI	2,3-DiCl	4.8×10^{-5}	4.2	125	20
LXXXVII	2,4-DiCl	1.4×10^{-5}	14	>500	32
LXXXVIII	2,5-DiCl	5.0×10^{-5}	4	>500	>100
LXXXIX	2,6-DiCl	1.3×10^{-3}	0.15	>500	82
XC	3,4-DiCl	1.8×10^{-5}	11	>500	24
XCI	3,5-DiCl	1.2×10^{-5}	17	>500	40

Multiple Substituents. Three complete sets of disubstituted phenyl *N*-methylcarbamates—dimethyl-, dimethoxy-, and dichloro—have been investigated as shown in Table VII (*26*, *30*). Because of the comparable size of the chlorine atom (radius 1.8 A.), methyl group (radius 2.0 A.), and methoxy group (radius 3.38 A.), the comparable isomers are isosteres and may be expected to have similar biological properties. In all cases, the 2,6-disubstituted compounds (LXXVII, LXXXIII, LXXXIX) were of very low affinity and noninsecticidal. It is concluded that the presence of two ortho substituents prevents the proper orientation of the carbamate at the anionic and esteratic sites of the enzyme. The 3,5-disubstituted compounds had the highest affinity in all cases. The marked insecticidal activity of the 3,5-dimethoxy derivative (LXXXV) as compared to the 3,4-dimethoxy (LXXXIV) has been shown to result from preferential detoxication of the latter, as discussed later. The data in Table VII also indicate that the polar properties of the substituents are important in determining activity. The methyl and methoxy substituents which are electron-donating produce compounds which have higher activity over a wider range of concentrations than the chloro substituents which are electron-withdrawing.

It is of interest to examine the effects of doubling the alkyl ring substituent in the optimumly active meta position (*27*). 3,5-Dimethylphenyl (xylenyl) *N*-methylcarbamate (LXXIX) has an affinity for cholinesterase slightly more than twice that of 3-methylphenyl *N*-methylcarbamate (XXIV). However, with the optimumly sized isopropyl and *tert*-butyl groups, doubling the substituent produces dramatic changes in affinity of about 10 and five times, respectively (Table VIII, XCII and XCIV). Incorporation of the *m*-methyl group with *m*-isopropyl (3-methyl-5-isopropyl, XCIII) increased the affinity six times and with *m*-*tert*-butyl (3-methyl-5-*tert*-butyl, XCV) increased the affinity two times. The toxicity to the housefly was also substantially increased by these changes, but the activity to the mosquito larvae decreased.

The effects of doubling with alkoxy substituents are complicated by the fact that maximal activity for isopropoxy is in the ortho position. However, as shown in Table VIII, incorporation of a *m*-methyl with *o*-isopropoxy more than doubled the affinity (2-isopropoxy-5-methyl, XCIX) and *m*-methoxy (2-isopropoxy-5-methoxy, CIII) also increased the affinity with a substantial increase in fly toxicity. The 2-isopropoxy-5-isopropylphenyl *N*-methylcarbamate (CI) was, unexpectedly, of intermediate affinity between *m*-isopropyl-(XXX) and *o*-isopropoxy (L).

These results may be explained by assuming that the aromatic ring of the phenyl *N*-methylcarbamates is capable of free rotation about the ester

oxygen, as can be observed with molecular models. Thus the presence of two ring substituents, each equally capable of interacting with the anionic site, would double the probability of the formation of the enzyme inhibitor complex, as is the case with the 3-methylphenyl (XXIV) and 3,5-dimethylphenyl (LXXIX) *N*-methylcarbamates. The marked five- to 10-fold enhancement produced by symmetrical disubstitution with isopropyl or *tert*-butyl groups can perhaps be explained by picturing an umbrella effect where a canopy of methyl groups surrounds the aromatic ring so that interaction with the anionic site may occur not only with both edges but also with both sides of the aromatic ring. The 3,5-diisopropylphenyl *N*-methylcarbamate (XCII) has the highest affinity for cholinesterase of any of the uncharged carbamates examined (I_{50} $3.3 \times 10^{-8}M$) and this is nearly equal to the quaternary *m*-trimethylammoniumphenyl *N*-methylcarbamate (VI) (I_{50} $1.8 \times 10^{-8}M$).

Unsymmetrical disubstitution has much less effect in enhancing affinity. It appears that where one of the substituents has a lower attractivity to the anionic site, the affinity is largely determined by the substituent with the favored position. Thus 2-isopropoxy-5-isopropylphenyl (CI) is intermediate between 2-isopropoxy- and 3-isopropylphenyl *N*-methylcarbamates and 2-isopropoxy-5-methyl (XCIX) is only slightly better than 2-isopropoxyphenyl *N*-methylcarbamate. When the second substituent is in a decidedly inferior position, as in 2-methyl-5-isopropylphenyl *N*-methylcarbamate (XCVI), the affinity is decreased (Table VIII).

Where substituents of markedly different polarity are mixed, as with chloro- and methyl-, the analysis of results is more complex. However, chlorine and methyl substituents can be mixed without much alteration in the affinity. Thus 3-methyl-4-chloro- (affinity 7) is little different from 3-methyl- (affinity 14) or 3,4-dimethylphenyl *N*-methylcarbamates (affinity 7.7). Addition of *o*-chlorine as in 3,4-dimethyl-6-chlorophenyl *N*-methylcarbamate resulted in an increased affinity of 13. Other more highly substituted methyl- and chloro-substituted carbamates were of generally lowered biological activity (*26*).

It is more difficult to generalize about the insecticidal effects of multisubstitutions because of species specificity. However, doubling the *m*-alkyl substituent generally increased the toxicity to the housefly, although it decreased it for the *Culex* mosquito larva. The 2-isopropoxy-5-methoxyphenyl *N*-methylcarbamate (CIII) had the highest toxicity to the housefly of any compounds of this series. In seeking to evaluate the relative insecticidal action of such compounds it must be remembered that each additional substituent provides a possible avenue for attack by a detoxication enzyme and, conversely, that increasing the molecular weight may decrease volatility and increase insecticidal persistence.

Table VIII. Biological Activities of Some Complex Disubstituted Phenyl *N*-Methylcarbamates

	Substituent	$I_{50}M$ ChE	Affinity	LD_{50} μG. per g. Musca	LD_{50} P.p.m. Culex
XCII	3,5-Di$(CH_3)_2CH$	3.3×10^{-8}	6060	17.5	0.08
XCIII	3-CH_3,5-$(CH_3)_2CH$	5.6×10^{-8}	3570	29.0	0.07
XCIV	3,5-Di$(CH_3)_3C$	7.8×10^{-8}	2560	60.0	>10
XCV	3-CH_3,5$(CH_3)_3C$	1.7×10^{-7}	1180	31.0	0.32
XCVI	2-CH_3,5-$(CH_3)_2CH$	2.0×10^{-6}	100	>500	0.46
XCVII	2-$(CH_3)_2CH$,5-CH_3	1.4×10^{-6}	140	65	3.4
XCVIII	2-CH_3O,5-CH_3	8.6×10^{-6}	23	47.5	>100
XCIX	2-$(CH_3)_2CHO$,5-CH_3	2.8×10^{-7}	715	40.0	>10
C	2-CH_3O,5-$(CH_2)_3CH$	2.8×10^{-6}	71	105	0.5
CI	2-$(CH_3)_2CHO$,5-$(CH_3)_2CH$	4.3×10^{-7}	465	48.5	8.8
CII	2-CH_3O,5-$(CH_3)_2CHO$	1.5×10^{-5}	133	150	>10
CIII	2-$(CH_3)_2CHO$,5-CH_3O	5.6×10^{-7}	364	6.5	>10

Activity of Enantiomorphic Carbamates

The critical influence of the size and shape of the substituent of the phenyl *N*-methylcarbamates upon the biological behavior of the molecule suggested that asymmetric centers in the side chain substituents might provide interesting variations in cholinesterase inhibition and insect toxicity. The *d*- and *l*-isomers of miotine or 2-(1-dimethylamino)-ethylphenyl *N*-methylcarbamate methiodide were investigated by White and Stedman (*42*) who found that the *l*-isomer was 2 to 10 times more toxic to laboratory animals than the *d*-isomer. The anticholinesterase and insecticidal activities of D- and L-2-*sec*-butylphenyl *N*-methylcarbamate were investigated by the writers (*10*), who found the L-isomer to be six times more active as an anticholinesterase, three times more toxic to the housefly, and eight times more toxic to the *Culex* mosquito larva than the D-isomer. In contrast to this highly significant difference in the activity of the two enantiomorphs, there was no very significant difference in anticholinesterase or insecticidal activity between *d*- and *l*-2-(*sec*-butylthio)-phenyl *N*-methylcarbamate, in which a sulfur atom is interposed between the asymmetric carbon and the aromatic ring:

Substituted Phenyl N-Methylcarbamate	$I_{50}M$ ChE	Affinity	LD_{50} μG. per g. Musca	LD_{50} P.p.m. Culex
D-2-*sec*-butyl-	6.0×10^{-6}	33	515	2.12
L-2-*sec*-butyl-	1.0×10^{-6}	200	170	0.26
d-2-*sec*-butylthio-	1.2×10^{-7}	1670	38	0.21
l-2-*sec*-butylthio-	8.0×10^{-8}	2500	37	0.17

These results emphasize the critical nature of the spatial interaction between the anionic site of cholinesterase (and/or the acetylcholine receptor protein) and the substituent of the phenyl *N*-methylcarbamate. The pronounced differences in the activity of the D- and L-2-*sec*-butylphenyl *N*-methylcarbamates can be explained only in terms of the classical three-point attachment theory (*24*) between enzyme and inhibitor and support the suggestion that there is a two-point interaction at the anionic site with a third point at the esteratic site about 5 A. away (*32*). The absence of pronounced stereospecificity with *d*- and *l*-2-(*sec*-butylthio)-phenyl *N*-methylcarbamates suggests that the possibility of rotation of the asymmetric center about the sulfur atom permits a fluctuation in the critical distances of the anionic interactants which nullifies the effects of asymmetry.

Inductive and Mesomeric Effects of Substituents. The phenyl *N*-methylcarbamates are competitive inhibitors of cholinesterase and steric effects, especially interaction with the anionic site of cholinesterase, are the dominant factors in determining biological activity. However, the polar effects of substituents on the aromatic ring exercise a role in determining activity, presumably through their effect on the electrophilic properties of the carbonyl carbon (*22*). Thus a number of investigators have proposed that the esteratic site makes a nucleophilic attack on this carbonyl carbon in a manner resembling the basic hydrolysis of an ester. This suggests that substituted phenyl *N*-methylcarbamates with predominantly electron-withdrawing groups behave more like substrates, while those with electron-donating groups have much lower turnover numbers with the enzyme and are far more effective competitive inhibitors. This relationship is best demonstrated by the results with small para substituents, where the picture is

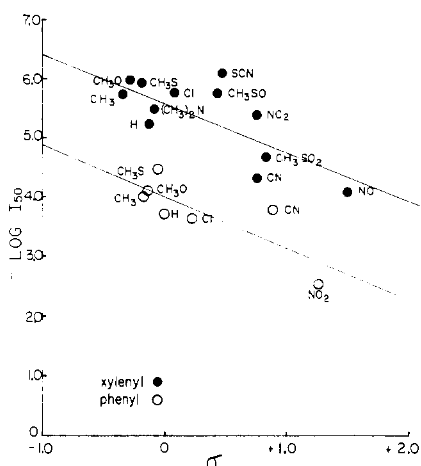


Figure 4. Relationship of $-\log I_{50}$ for fly cholinesterase inhibition to sigma values for p-substituted phenyl and xylenyl *N*-methylcarbamates

not appreciably complicated by steric interaction with the anionic site. This is illustrated by the data in Figure 4, where the polar effects of the substituent as measured by Hammett's sigma coefficient are plotted against the affinity for cholinesterase (*28*). An orderly relationship exists between electron-withdrawing power and affinity for cholinesterase which has about a 1000-fold range between the extremes of CH_3O to NO_2 substitution in phenyl *N*-methylcarbamate.

The second series of compounds illustrated in Figure 4 is the para-substituted 3,5-xylenyl *N*-methylcarbamates (see Table IX). As discussed previously, 3,5-xylenyl *N*-methylcarbamate (LXXIX) has an affinity for cholinesterase of 33 times that of the unsubstituted phenyl *N*-methylcarbamate (X), the methyl groups providing considerable interaction with the anionic site. The effect of various para substituents upon the biological activity of this xylenyl *N*-methylcarbamate must be due to inductive and mesomeric effects upon the electron density at the carbonyl carbon. It will be noted in Figure 4 that the relative electron-withdrawing power of the para substituent has exactly the same effect as with the phenyl *N*-methylcarbamates, except that the over-all affinity of the xylenyl series is 33 times greater, suggesting the dominant role of steric attraction over induction and mesomerism.

In the 3,5-xylenyl series the effect of the *p*-nitro group is relatively weaker than in the phenyl series. This is the result of steric inhibition of resonance of the nitro groups by the interferences of the methyl groups; these twist the nitro group which is out of the plane of the aromatic ring. The methylsulfonyl group, which does not require coplanarity for maximum resonance, is actually a stronger electron-withdrawing agent in the xylenyl series than the nitro group.

Table IX. Biological Activities of 4-Substituted 3,5-Xylenyl *N*-Methylcarbamates

	Substituent	$I_{50}M$ ChE	Affinity	LD_{50} µG. per g. Musca	LD_{50} P.p.m. Culex
CIV	H	6.0×10^{-6}	33	60	3.0
CV	CH_3	1.9×10^{-6}	105	65	0.28
CVI	CH_3O	1.1×10^{-6}	180	35.5	0.29
CVII	CH_3S	1.2×10^{-6}	166	24.0	0.23
CVIII	$(CH_3)_2N$	3.3×10^{-6}	60	60.0	0.49
CIX	Cl	2.1×10^{-6}	95	>500	0.89
CX	CNS	8.0×10^{-7}	250	>500	0.50
CXI	CN	4.8×10^{-5}	4.2	>500	>10
CXII	CH_3SO	1.8×10^{-6}	110	410	4.7
CXIII	CH_3SO_2	2.1×10^{-5}	9.5	>500	>10
CXIV	NO_2	4.2×10^{-6}	47	>500	>10
CXV	NO	8.3×10^{-5}	2.4	>500	>10

The effects of induction and mesomerism upon the phenyl *N*-methyl carbamates are exactly opposite to the action upon the phenyl dialkyl phosphates. In the phosphate esters strong electron-withdrawing groups such as nitro promote insecticidal activity by producing a highly electrophilic and consequently reactive phosphorus atom which attaches the esteratic site of cholinesterase and irreversibly phosphorylates it. In the carbamate esters such strong electron-withdrawing groups also promote the instability of the carbamate and make it less effective as a competitive inhibitor. This general effect is shown by the following data (*22*).

Substituted Phenyl N-Methylcarbamate	37° C., K_{hyd}, Min.$^{-1}$	$I_{50}M$ ChE	Affinity
o-NO_2	3.4×10^6	5.0×10^{-3}	0.04
p-NO_2	3.4×10^5	3.0×10^{-3}	0.066
o-Cl	2.0×10^3	5.0×10^{-6}	40
m-Cl	1.7×10^3	5.0×10^{-5}	4
p-Cl	1.0×10^3	2.4×10^{-4}	0.83
m-CH_3	3.0×10^2	1.4×10^{-5}	14
o-CH_3	2.6×10^2	1.4×10^{-4}	1.4
H	2.5×10^2	2.0×10^{-4}	1.0
o-$(CH_3)_2CH$	54.5	6.0×10^{-6}	33
m-$(CH_3)_2N$	20	8.0×10^{-6}	25
m-$(CH_3)_3C$	4	4.0×10^{-7}	500

Comparisons of the insecticidal activity of the *N*-methyl and *N*,*N*-dimethylcarbamates of several substituted phenols are shown in Table X (*31*). In all cases the *N*-methylcarbamate was superior in anticholinesterase activity and insecticidal action, generally by a factor of five- to 10-fold. This was attributed to the greater attraction of the *N*-methylcarbamate to the nucleophilic centers of the esteratic site of cholinesterase. Table X shows that the relative insecticidal specificity of the carbamate is almost entirely dependent upon the phenol moiety, indicating that specificity is a function of detoxication mechanisms which attack the aryl portion of the molecule.

Kolbezen, Metcalf, and Fukuto (*22*) have investigated the insecticidal activity of other *N*-substituted carbamates. For the *m-tert*-butylphenyl esters the relative I_{50} values for cholinesterase were: *N*-methyl, 4×10^{-7}; *N*-ethyl, 2×10^{-5}; and *N*-benzyl, $1 \times 10^{-3}M$. Comparable values for the thymyl esters were: *N*-methyl, 1.4×10^{-6}; *N*-ethyl, 2×10^{-5}; *N*-benzyl, 3×10^{-3}; and *N*-phenyl, $>10^{-3}M$. The two *N*-ethylcarbamates were slightly insecticidal but none of the other esters had any activity.

Substituted N-Alkylcarbamates

The earliest investigations of the biological action of synthetic carbamates were made with *N*-methylcarbamates patterned after physostigmine (*40*). However, because of the pharmacological employment of aqueous solution of these cholinergic substances, stability to hydrolysis became an important factor and the corresponding *N*,*N*-dimethylcarbamates, which are of the order of 10^4 to 10^5 times as stable to aqueous hydrolysis, became the important drugs—e.g., prostigmine or neostigmine, *m*-dimethylaminophenyl *N*,*N*-dimethylcarbamate methosulfate. This undoubtedly had considerable influence on subsequent investigators and the extensive work of Gysin (*19*) on the heterocyclic *N*,*N*-dimethylcarbamates such as isolan and pyrolan makes no mention of *N*-methylcarbamates.

Physiological Actions of Carbamates

Much remains to be learned regarding the physiological manifestations of carbamate intoxication in insects. The rate of beating of the isolated heart of the American cockroach (*Periplaneta*

Table X. Biological Activities of *N*-Methyl and *N,N*-Dimethylcarbamates

	Carbamate	I_{50} M ChE	Affinity	LD_{50} µG. per g. *Musca*	LD_{50} P.p.m. *Culex*	LC Mg. *Estigmene*
CXVI	1-Naphthyl *N*-methyl	9.0×10^{-7}	220	>500	0.5	1–3
CXVII	1-Naphthyl *N,N*-dimethyl	4.5×10^{-6}	44	>500	6.8	>10
CXVIII	Phenyl *N*-methyl	2.0×10^{-4}	1	500	>10	>10
CXIX	Phenyl *N,N*-dimethyl	8.0×10^{-4}	0.25	>500	>10	>10
CXX	*m*-Isopropylphenyl *N*-methyl	3.4×10^{-7}	580	90	0.03	0.1–0.3
CXXI	*m*-Isopropylphenyl *N,N*-dimethyl	5.0×10^{-5}	4	450	0.25	0.1–0.3
CXXII	*o*-Isopropoxyphenyl *N*-methyl	6.9×10^{-7}	290	25.5	0.3	0.3–1.0
CXXIII	*o*-Isopropoxyphenyl *N,N*-dimethyl	1.3×10^{-5}	15	375	>10	>10
CXXIV	2-Isopropoxy-5-methoxyphenyl *N*-methyl	5.6×10^{-7}	360	6.5	>10	0.3–1.0
CXXV	2-Isopropoxy-5-methoxyphenyl *N,N*-dimethyl	2.0×10^{-6}	100	33	>10	>10
CXXVI	3,5-Dimethoxyphenyl *N*-methyl	8.0×10^{-6}	25	11.0	>10	0.3–1.0
CXXVII	3,5-Dimethoxyphenyl *N,N*-dimethyl	5.2×10^{-5}	3.8	260	>10	3–10
CXXVIII	4-Dimethylamino-3,5-xylenyl *N*-methyl	3.3×10^{-6}	61	60	0.49	0.03–0.1
CXXIX	4-Dimethylamino-3,5-xylenyl *N,N*-dimethyl	2.2×10^{-5}	9.1	500	2.4	0.1–0.3

americana) is accelerated by acetylcholine at dilutions as low as $10^{-9}M$. Similar results are obtained by applications of organophosphorus insecticides which increase the acetylcholine concentration through inactivation of cholinesterase. When *m*-isopropylphenyl and *o*-isopropylthiophenyl *N*-methylcarbamate were applied to these heart preparations, the rate of beating was increased above a threshold concentration of $5 \times 10^{-7}M$. In contrast to para-oxon and DDVP, which at threshold values of about $1 \times 10^{-7}M$ caused only a change in rate of beating, treatment with the carbamates resulted in an erratic beat with incomplete systolic and diastolic movements leading often to complete arrest (*37*).

Investigations of the effect of carbamates, applied as perfusates or from paraffin blocks to the isolated abdominal ganglia of the nerve cord of *Periplaneta americana*, have given interesting results (*33*). The carbamates at dilutions in paraffin of 10^{-4} to $10^{-2}M$ diffused rapidly from the paraffin into the nerve cord and produced a marked increase in the level of spontaneous electrical activity in the cord. The average level of effect was well correlated with the position of the isopropyl group in the substituted phenyl *N*-methylcarbamate molecule, and with the activity of the various isomers in cholinesterase inhibition.

Isopropylphenyl N-Methylcarbamate Isomer	Time, Seconds: Threshold activity	Maximum cascade	Synaptic Block
Ortho-	77	225	240
Meta-	41	58	79
Para-	100	235	290

The data strongly suggest that the carbamates facilitate synaptic transmission.

One may speculate from the close structural resemblance of the active carbamates to acetylcholine and from the pharmacological action on the insect heartbeat and the effect on the spontaneous activity of the insect nerve, that these carbamates may have a direct action upon the acetylcholine receptors as well as their well recognized inhibitory action on cholinesterase. The latter effect has been demonstrated histochemically in the cockroach nerve cord (*47*).

Table XI. Cross Tolerances of Carbamates to Resistant Houseflies

N-Methylcarbamate	Topical LD_{50}, µg. per ♀ Fly: S_{LAB}	S_{NAIDM}	R_{MIP-1}	R_{MIP-2}
m-Isopropylphenyl	2.0	1.4	>100	
o-Isopropylphenyl	2.15	1.9	>100	
o-Isopropoxyphenyl	0.47	0.47	>100	
2-Isopropoxy-5-methoxy		0.13		>100
m-sec-Butylphenyl		2.0		>100
4-Methylthio-3,5-dimethyl		0.48		>100
4-Dimethylamino-3,5-dimethyl	1.2	1.2		>100
3,5-Dimethoxyphenyl	0.19	0.22	1.85	
1-Naphthyl	>100	>100	>100	>100
m-Isopropylphenyl +P.B.[a]	0.33	0.2	1.0	1.1
1-Naphthyl +P.B.[a]	0.24		1.9	
4-Dimethylamino-3,5-dimethyl +P.B.[a]	0.27	0.27	0.76	1.3
2-Isopropoxyphenyl +P.B.[a]	0.19	0.14	0.56	0.64

[a] 5 parts piperonyl butoxide to 1 part carbamate.

Insect Resistance to Carbamates

The development of essentially homozygous strains of insects resistant to various toxicants by continuous selection and breeding of the least susceptible individuals in the population is a well known phenomenon (*25*). Using such a selection process with the housefly (*Musca domestica*), Moorefield (*38*) produced virtual immunity to *m-tert*-butylphenyl *N*-methylcarbamate and to Sevin within 10 generations. However, with *m*-isopropylphenyl *N*-methylcarbamate, selection for 67 generations produced only eightfold tolerance. In this laboratory (*15*) selection of susceptible S_{LAB} flies with this compound produced marked resistance (R_{MIP-1}) very rapidly as shown by the following.

Generation	Topical LD_{50}, µG. per Fly: Male	Female
F_1	1.08	2.0
F_4	. . .	3.5
F_7	. . .	>100
F_{14}	2.48	>100
F_{22}	3.7	>100

The selection experiments were repeated (*12*) using the S_{NAIDM} strain, and although resistance developed somewhat more slowly, by the 40th generation topical application of 100 µg. of *m*-isopropyl phenyl *N*-methylcarbamate killed only 10% of the female flies (R_{MIP-2}).

The cross tolerance of these carbamate-resistant strains extended to a variety of substituted phenyl *N*-methylcarbamates, as shown in Table XI. The 3,5-dimethoxyphenyl *N*-methylcarbamate was the only carbamate evaluated which retained appreciable effectiveness against the resistant strain.

The effects of the methylenedioxyphenyl synergists—e.g., piperonyl butoxide—upon the effectiveness of the carbamates to the R_{MIP} flies is particularly interesting, as shown in Table XI. In all cases, the synergized carbamates were fully effective against the carbamate-resistant strains. In additional investigations of the activity of the carbamates against R-strains of houseflies selected with other types of insecticides (*12*) it was found that although resistance to DDT, cyclodienes, and various organophosphorus insecticides also resulted in pronounced cross tolerance to the substituted phenyl *N*-methylcarbamates, these carbamates synergized with piperonyl butoxide were highly effective against the resistant

Table XII. Metabolism of C^{14} Zectran[a] Injected into R_{MIP} Flies as Influenced by Piperonyl Butoxide

Time after Injection, Hr.	% Zectran Recovered	
	R_{MIP}	R_{MIP} pretreated piperonyl butoxide
4	42	74
12	24	64
24	9	67
24, flies boiled immediately after treatment	90	...

[a] Trade-mark Dow Chemical Co. for 4-dimethylamino-3,5-xylenyl N-methylcarbamate.

Table XIII. Synergism of Carbamates by Various Compounds

	Musca S_{NAIDM}, Topical LD_{50}, μG. per G.		
	Sevin	Phenyl N-methyl-carbamate	m-Isopropylphenyl N-methyl-carbamate
Carbamate alone	ca. 900	500	90
Carbamate + piperonyl butoxide	12	38	9.0
Carbamate + 1,2,3,4,7,7-hexachloro-5-isothiocyanomethyl bicyclo-(2.2.1)-5-heptene	50	45	3.8
Carbamate + tri-o-cresyl phosphate	210	112.5	..
Carbamate + 2-(3,5-dichlorobiphenyloxy)-triethylamine (Lilly 18947)	106	...	11.0
Carbamate + β-diethylaminoethyl diphenyl-propyl acetate (SKF 525A)	58.5	...	15.3

All synergists used at 5 parts to 1 part carbamate. Mortality data at 24 hours; for isothiocyanate, 48 hours.

flies. These interesting results with the methylene dioxyphenyl synergists focus attention on the role of detoxication mechanisms in determining carbamate susceptibility as discussed under Synergism and Detoxication.

Synergism and Detoxication

The marked synergistic action of the methylenedioxy compounds such as piperonyl butoxide, sesamex, sulfoxide, and N-propyl isome for Sevin, M-isopropylphenyl N-methylcarbamate, and other carbamates was first demonstrated by Moorefield (*38*), who showed that cotreatment with synergist steepened the slope of the carbamate dosage mortality curve to the housefly and displaced it toward 10-fold or greater activity. Moorefield (*38*) also showed that incorporation of piperonyl butoxide with Sevin in selection experiments for resistance suppressed the further development of resistance, a conclusion which was confirmed by Georghiou *et al.* (*15*) using m-isopropylphenyl N-methylcarbamate. As is shown in Table XI, various carbamate-resistant strains remain almost fully susceptible to carbamate–piperonyl butoxide combinations.

From a vast amount of study it has become apparent that nearly all insecticides are subject to rapid detoxication in the insect body and that, in general, insecticide-resistant strains arise from the selection of individual variants containing preadaptive genes controlling the level of detoxication enzymes. Georghiou and Metcalf (*13*) investigated the detoxication of m-isopropylphenyl N-methylcarbamate in susceptible (S_{NAIDM}) and resistant (R_{MIP-1}) flies and the effect of piperonyl butoxide synergist (P.B.). Using the anticholinesterase activity of the carbamate as an analytical tool it was shown that the S-flies accumulated more than four times as much intact carbamate within their bodies as the R-flies and the R-flies pretreated with piperonyl butoxide had 17 times more intact carbamate after 48 hours than those not pretreated. Thus, 2 hours following application of the carbamate, S-flies had metabolized about 23% of the absorbed toxicant as compared to 85% metabolized by R-flies and 65% by R-flies pretreated with piperonyl butoxide.

The evident conclusion that detoxication by R_{MIP} flies is the primary factor conferring resistance has been confirmed with experiments involving C^{14}-labeled carbamates (*27*). C^{14} (ring and 3,5-methyl–labeled) 4-dimethylamino-3,5-xylenyl N-methylcarbamate was injected into the abdomens of flies and after appropriate intervals the labeled metabolites were extracted and separated by paper chromatography. The results, summarized in Table XII, show the pronounced effect of pretreatment of 1.5 hours with piperonyl butoxide in reducing the detoxication of the carbamate within the fly.

In addition to the methylenedioxyphenyl synergists, other types of compounds known to have unique biochemical activities have been shown to have considerable effect as carbamate synergists, thus demonstrating the complex biochemical pathways involved in carbamate detoxication. The effects of some of these synergists are shown in Table XIII.

El-Sebae, Metcalf, and Fukuto (*6*) have described the synergistic activity of organic compounds containing the thiocyanate (SCN) moiety. Isobornyl thiocyanoacetate (Thanite), dodecylthiocyanate, and 1,2,3,4,7,7-hexachloro-5-isothiocyanomethyl-bicyclo-(2.2.1)-5-heptene were all active synergists for a number of carbamates against both S_{NAIDM} and R_{MIP-2} houseflies. Studies with C^{14}-labeled Zectran and phenyl N-methylcarbamate showed that in S_{NAIDM} flies the pretreatment of the flies with Thanite at a level which produced no mortality reduced the metabolic detoxication at 4 hours after treatment from 90% to 58% with Zectran and from 68% to 25% with phenyl N-methylcarbamate. Further studies are underway to elucidate the unknown biochemical mechanism of action of the thiocyanoacetates as synergists.

Tri-o-cresyl phosphate (TOCP) is nontoxic to houseflies at high dosages, but these almost completely inhibit aliesterase activity in the fly. The concomitant carbamate-synergizing effect of tri-o-cresyl phosphate as shown in Table XIII suggests that aliesterase may play a role in the detoxication pathways of Sevin and other carbamates.

Brodie, Gillette, and La Due (*3*) have studied at length the curious properties of compounds such as 2-(3,5-dichloro-2-biphenylyloxy) triethylamine (Lilly 18947) and β-diethylaminoethyl diphenylpropyl acetate (SKF 525A). These compounds inhibit the metabolic detoxication of a variety of pharmacological agents, such as procaine and barbiturates, through a general suppression of microsomal oxidations. As shown by Moorefield (*38*), Lilly 18947 is a synergist for Sevin, and SKF 525A has been demonstrated by Hodgson and Casida (*21*) to inhibit the metabolism of N,N-dialkylcarbamates by rat liver microsomes. Both of these compounds showed interesting synergistic activity with the insecticidal carbamates, as shown in Table XIII, and in studies currently in progress with radiotracer carbamates it has been shown that pretreatment with these microsomal oxidant inhibitors appreciably retard the in vivo detoxication of the carbamates.

The various substituted phenyl N-methylcarbamates are subject to widely differing rates of in vivo detoxication and various insect and mammalian species may detoxify the carbamate by a number of different pathways. These differences in detoxication mechanisms account for most of the almost inexplicable variations in carbamate toxicity and in the many anomalies of species specificity or selectivity.

One of the interesting examples of the former relates to the activity of the isomeric dimethoxyphenyl N-methylcarbamates shown in Table XIV. With the exception of the 2,6-isomer, all of the other isomers have comparable anticholinesterase activities which cover a threefold range. However, their toxicity values to the housefly cover a

Table XIV. Synergism of Isomeric Dimethoxyphenyl N-Methylcarbamates with Piperonyl Butoxide

	Musca		
	Topical LD_{50}, µg. per G.		
Isomer	*A alone*	*B 1:5 p.b.*	*Degree of synergism, A/B*
2,3	>500	16.5	>30
2,4	155	13.0	11.9
2,5	13	4.9	2.7
2,6	>500	>500	1.0
3,4	400	12.0	33
3,5	11	4.4	2.5

40-fold range. The explanation of this anomaly became apparent only when the LD_{50} values were measured as synergized with piperonyl butoxide (*11*), as shown in Table XIV. These values for the synergized LD_{50} fall within approximately a threefold range. Thus it is clear that a specific detoxication mechanism attacks the 3,4-dimethoxyphenyl N-methylcarbamate much more rapidly than the 3,5- isomer. Very similar results were obtained with the isomeric dimethylphenyl N-methylcarbamates and with a variety of other substituted-phenyl N-methylcarbamates, so that plots of log synergized LD_{50} *vs.* I_{50} for cholinesterase inhibition yielded very satisfactory agreements (see Figure 5). Fukuto *et al.* (*11*) have called attention to the ready detoxication of 2,3- and 3,4-dimethoxyphenyl N-methylcarbamate and of Sevin, all of which have structural similarities in that they have regions of high electron density ortho to one another—i.e., from the oxygen atoms of the methoxy groups or the resonating aromatic bonds of the naphthalene moiety.

Selective Toxicity

The selective toxicity of the substituted-phenyl N-methylcarbamates is particularly interesting and reflects the presence of specific detoxication systems. Georghiou and Metcalf (*14*) have compared the toxicity of a number of carbamate insecticides of commercial importance to several species of insects with the results summarized in Table XV. Especially noteworthy are (1) the extraordinary activity of 4-dimethylamino-3,5-xylenyl N-methylcarbamate (Zectran) to lepidopterous larvae such as *Bucculatrix thuberiella*, the cotton leaf perforator, and *Trichoplusia ni*, the cabbage looper (This compound was also the most active against *Estigmene acrea*, the salt marsh caterpillar, and *Panonychus citri*, the citrus red mite.); (2) the high toxicity of *o*-isopropoxyphenyl N-methylcarbamate to the housefly and *Blattella germanica*, the German cockroach. This compound is also very active against *Aphis spiraecola*, the spirea aphid. The *m*-isopropylphenyl N-methylcarbamate was very effective against *Culex pipiens quinquefasciatus* and *Anopheles*

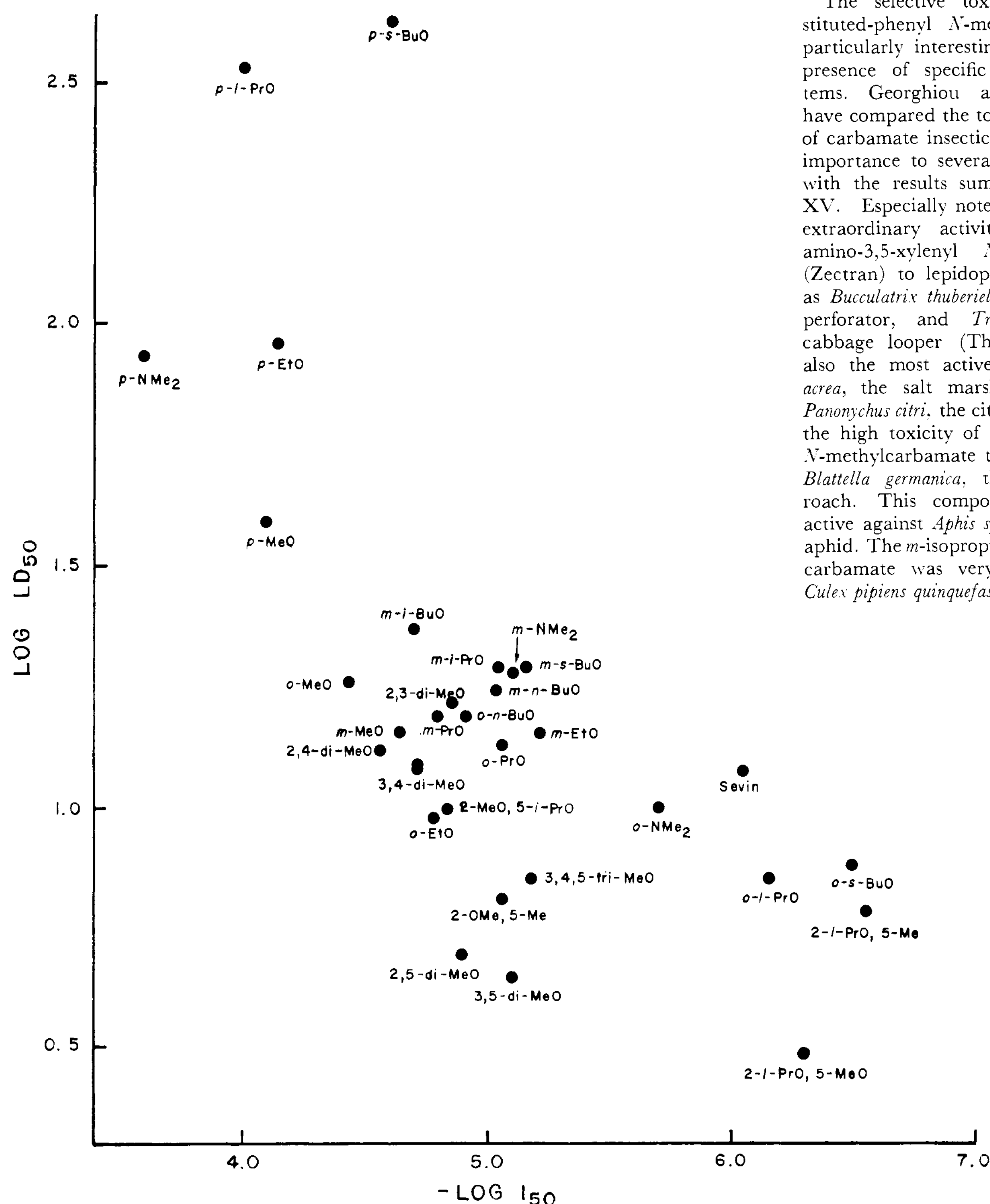


Figure 5. Plot of $-\log I_{50}$ for fly cholinesterase inhibition vs. log LD_{50} for housefly for carbamates synergized with piperonyl butoxide

Table XV. Selective Toxicity of Aryl N-Methylcarbamates

N-Methylcarbamate	Topical LD_{50}, µG. per G.			LD_{50}					
				Culex		Bucculatrix	Trichoplusia	Oral LD_{50},	
	Musca	Apis	Blattella	Larva, p.p.m.	Adult, µg./sq. cm.	larva, µg./sq. cm.	larva, µg./sq. cm.	Mg. per Kg. Rat
m-Isopropylphenyl	90	1.0	14.7	0.03	0.2	0.031	1.3	16
o-Isopropylphenyl	95	2.8	>130	0.56	1.0	...	...	500
o-Isopropoxyphenyl	25.5	0.8	11.3	0.35	1.9	0.023	1.5	250
m-sec-Butylphenyl	100	0.64	52	0.03	0.43	0.072	1.2	30
o-sec-Butylphenyl	135	...	...	0.35	...	...	...	400
4-Dimethylamino-3,5-xylenyl	60	0.6	>133	0.49	10.7	0.028	0.07	60
4-Methylthio-3,5-xylenyl	24	1.1	>133	0.23	8.8	0.082	10.2	100
1-Naphthyl	>500	2.3	>133	1.0	>16	0.61	23	540

albimanus adults and larvae and was the most active compound against *Sitophilus granarius*, the granary weevil. All of the carbamates were very toxic to *Apis mellifera*, the honeybee. However, it is surprising that 2-isopropoxy-5-methoxyphenyl N-methylcarbamate, which was the most toxic carbamate evaluated against the housefly (LD_{50}, 6.5 µg. per gram), was only about 0.1 as toxic as Sevin (which is virtually nontoxic to the housefly) to the honeybee (LD_{50}, 25 µg. per gram).

The entire group of lipoid-soluble substituted phenyl N-methylcarbamates represents a decidedly selective approach to insect toxicity. Some of the quaternary compounds such as 2-methyl-5-dimethylaminophenyl N-methylcarbamate methochloride are among the most toxic compounds known. This compound has a subcutaneous LD_{50} value to the mouse of 0.07 and an oral LD_{50} of 2.5 mg. per kg. (5). Yet these quaternary analogs are virtually nontoxic to insects. None of the compounds in Table XV approach this mammalian toxicity and the over-all safety of Sevin is well recognized. However, among these compounds other possibilities of selectivity exist. Thus in contrast to the relatively high mammalian toxicity of the *m*-isopropyl and *m-sec*-butylphenyl N-methylcarbamates, the corresponding *o*-isopropyl and *o-sec*-butylphenyl N-methylcarbamates seem to combine good insecticidal activity with low mammalian toxicity. These compounds demonstrate the specificity of mammalian detoxication pathways. It seems clear that additional information on the detoxication systems in the two classes Insecta and Mammalia should provide clues to the synthesis of carbamates with structural features blocking insect detoxication and favoring mammalian detoxication, thus leading to important advances in selective toxicity.

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