Some Effects of Molecular Structure upon Anticholinesterase and Insecticidal Activity of Substituted Phenyl *N*-Methylcarbamates

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N-methylcarbamates of various phenols, many of them new, were investigated as anticholinesterases and as insecticides. These were chosen to represent minor changes in the basic phenyl *N*-methylcarbamate structure with regard to ring position, unsaturation of side chain, multiple and branched substituents, formal charge, and dicarbamoyl groups. The results are discussed in terms of the competition of these carbamates with acetylcholine for interaction with the esteratic and anionic sites of cholinesterase and their detoxication by phenolase enzymes which can be inhibited by the synergist piperonyl butoxide.

A previous paper (Metcalf and Fukuto, 1965) has summarized the relationships between the structures of simple phenyl *N*-methylcarbamate insecticides and their biological action as inhibitors of insect cholinesterase (ChE) and as insecticides. The key feature in highly active carbamates was the presence of a relatively bulky group having a large van der Waals' energy of interaction with the anionic site of ChE, located about 5 A. from the carbonyl group. The pronounced difference in the activities of the D- and L-isomers of *sec*-butylphenyl *N*-methylcarbamate showed that the stereochemistry of the substituent of the aromatic ring plays a dominant role in determining their toxicity, while inductive and mesomeric effects are of lesser importance.

The most acceptable hypothesis for the mechanism of inhibition of ChE by these carbamates (Wilson *et al.*, 1961; Main and Hastings, 1966; O'Brien *et al.*, 1966; Fukuto *et al.*, 1967) assumes that these compounds behave as competitive substrates for ChE with high affinities for the enzyme and very low turnover numbers as originally shown by Goldstein and Hamlisch (1952). This situation is depicted as:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI' \xrightarrow{k_3} E + P$$

O'Brien *et al.* (1966) have determined separate values for the level of complex formation k_1/k_{-1}, the affinity constant K_I, and the rate of carbamylation or the carbamylation constant k_2, in a series of substituted phenyl *N*-methylcarbamates. The k_2 values for the relatively inactive phenyl *N*-methylcarbamate and the highly active 2-isopropoxyphenyl and 3,5-diisopropylphenyl *N*-methylcarbamates varied over a less than twofold range. However, the ratios of the K_I values for these three compounds were 1, 853, and 1292, respectively. These data therefore show the level of reversible complex formation as established by k_1/k_{-1} is the determining factor in ChE inhibition. They also emphasize the utility of the "relative affinity" or the ratio of I_{50} phenyl *N*-methylcarbamate/I_{50} substituted phenyl *N*-methylcarbamate (Metcalf and Fukuto, 1965) as providing a quantitative expression of the comparative effects of various ring substituents.

The present study represents an extension of the spatial exploration of carbamate-substrate interaction to a variety of other structural modifications of the phenyl *N*-methylcarbamate framework and an attempt to interpret the results in terms of interaction between the carbamate molecule and the anionic and esteratic sites of ChE.

The insecticidal activities of these aryl *N*-methylcarbamates is substantially affected by detoxication in the insect. This is accomplished both by hydroxylation by phenolase enzymes and by hydrolysis (Dorough and Casida, 1964). The hydroxylation reaction can be inhibited by methylenedioxyphenyl synergists such as piperonyl butoxide and the "synergistic ratio" provides a quantitative measure of the detoxication process. Comparative data on the synergism of related compounds discussed in this paper, provide additional information on the nature of the detoxication process (Metcalf *et al.*, 1966).

EXPERIMENTAL

Materials. The carbamates were prepared by reacting the appropriate phenol with methyl isocyanate using a trace of triethylamine as a catalyst. The phenols were prepared by conventional methods or obtained from commercial sources. The 3-isopropyl-6-bromophenol (b.p. 52–6° C./0.1 mm., $n_D^{25.5}$ 1.5504) was prepared by reduction, diazotization, and hydrolysis of 3-nitro-4-bromocumene. The 3-isopropyl-6-chlorophenol (b.p. 54° C./0.1 mm.) was prepared in the same way. The 3-nitro-4-halocumenes were prepared from 4-nitrocumene by reduction, nitration to 3-nitro-4-aminocumene, diazotization, and reaction with cuprous bromide or chloride. The 3-isopropyl-4-fluorophenol was prepared from 3-isopropyl-4-nitrophenol by formation of the methyl ether, reduction, diazotization, and treatment with HBF_4 followed by cleavage of the methyl ether. The 3-isopropyl-6-fluorophenol was prepared from 3-isopropyl-2-nitrophenol in the same way. The 3,5-bisdimethylaminophenol was prepared from 1,3,5-triaminobenzene by conversion to 3,5-diaminophenol dihydrochloride (Kruger and Hayes, 1948). This was refluxed overnight with methyl iodide and sodium carbonate in methyl alcohol and the methiodide precipitated with ether. The crude

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3,5-bisdimethylaminophenyl dimethiodide was heated at 180° to 200° C. under water-aspirator vacuum to give the phenol. These phenols were characterized by elemental analyses of their *N*-methylcarbamates as were the other previously unreported compounds (see Tables I through V). Compounds XXVII, XXIX, XLIV, LXIX, LI (Kolbezen *et al.*, 1954); XLI, XLII, XLIII (Fukuto *et al.*, 1962); XXXIX, LIV (Metcalf *et al.*, 1962); LV, LVI (Gilman *et al.*, 1954); LII, LIII (Smith *et al.*, 1945); LXI, LXIII (Stevens and Beutel, 1941); and LXXII (Metcalf *et al.*, 1964) have been reported previously. Compounds XXII (Hercules Powder Co.), XXX (Farbenfabriken Bayer), XXXI (The Upjohn Co.), XXVIII (Schering Corp.), and XXXVII (Hooker Electrochemical Corp.) were obtained through the cooperation of commercial sources.

The molar concentration of carbamate reducing the housefly head cholinesterase activity to half its normal velocity (I_{50}) was determined by the manometric method in which the inhibitor in acetone solution was reacted with enzyme for 15 minutes at 37.5° C. prior to addition of acetylcholine at a final concentration of 0.02*M*, and the CO_2 liberated from 0.025*M* sodium bicarbonate was measured over an additional 30-minute period. The rate of production of CO_2 by the inhibited enzyme remained relatively constant over this period, and no appreciable change in rate of inhibition could be measured following incubation of the enzyme and the carbamate for periods as short as 2 minutes or as long as 120 minutes.

The standard error of the mean for five complete replications of the I_{50} determination of *m*-isopropylphenyl *N*-methylcarbamate was ±7%.

The topical LD_{50} values to the female housefly *Musca domestica* were determined by application of 1-μl. drops of acetone solutions to the pronota of 20 2- to 4-day-old flies using four to six concentrations and replicating each dosage at least three times. The LD_{50} values were determined from the log dosage *vs.* probit mortality lines. The synergistic ratio (SR) in the tables is the ratio of topical LD_{50} for carbamate alone/topical LD_{50} for carbamate plus 5 parts of piperonyl butoxide. The standard error of the mean for these toxicity values was approximately ±7%. The LC_{50} values for *Culex pipiens quinquefasciatus* mosquito larvae were determined similarly by adding w./v. acetone solutions to 100 ml. of water containing 20 fourth instar larvae.

DISCUSSION OF RESULTS

Effects of Aromaticity. The results of replacement of the planar aromatic nucleus by the chair-shaped cyclohexane moiety are shown in Table I. This alteration destroys the activity of the phenyl *N*-methylcarbamates as shown by comparing I with III and IV with V. Similarly, displacement of the ester configuration by insertion of a methylene group between ring and ester linkage to give the analogous benzyl esters greatly reduced activity (compare I with II and VIII with IX). The inactivity of the cyclohexyl and benzyl *N*-methylcarbamates lends support to the carbamylation mechanism for inhibition since the carbamate moiety in these compounds, particularly the cyclohexyl derivative, is less reactive than in those directly connected to an aromatic nucleus. Aliphatic carbamates are inherently less reactive molecules than aromatic carbamates as established by hydrolysis studies and consistent with the low acidity of aliphatic alcohols compared with phenols. Aliphatic alcohols thus are poor leaving groups and the low anticholinesterase activity of aliphatic carbamates may be attributed to a slower carbamylation step.

Table I. Comparison of Aromatic and Nonaromatic Carbamates

	N-Methylcarbamate of	M.P., ° C.	Analysis, % Theory	Analysis, % Found	I_{50} *M* Fly ChE	Relative Affinity	*Musca domestica* LD_{50}, μg./G. A (alone)	*Musca domestica* LD_{50}, μg./G. B (1:5 P.B.)	Synergistic Ratio A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
I	Phenol	85–6			2×10^{-4}	1.0	500	38	13.1	>10
II	Benzyl alcohol	b. 102–4/ 3 mm.	C = 65.44 H = 6.71	C = 65.88 H = 6.44	$>2 \times 10^{-3}$	<0.1	>500	>500	1.0	>10
III	Cyclohexyl carbinol	b. 107–9/ 1.4 mm.	C = 63.15 H = 9.94	C = 62.61 H = 10.90	$>2 \times 10^{-3}$	<0.1	>500	>500	1.0	>10
IV	2-Isopropylphenol	96–7			6.0×10^{-6}	33	95	24	4	0.56
V	2-Isopropylcyclohexanol	78–81	C = 66.63 H = 10.17	C = 66.24 H = 10.73	$>1.0 \times 10^{-3}$	<0.2	>500	>500	1.0	>10
VI	2-Isopropyl-5-methylphenol	89.5–91			1.4×10^{-6}	143	65	10.5	6.2	4.0
VII	2-Isopropyl-5-methylcyclohexanol	107–8	C = 67.97 H = 10.45	C = 68.24 H = 11.10	$>1.0 \times 10^{-3}$	<0.2	>500	>500	1.0	>10
VIII	2-Isopropoxyphenol				6.9×10^{-7}	290	25.5	7	3.6	0.30
IX	2-Isopropoxybenzyl alcohol	b. 124/ 0.5 mm.	C = 64.55 H = 7.67	C = 63.91 H = 7.99	6.3×10^{-4}	<0.3	>500	>500	1.0	>10
X	Benzaldoxime	94–6	C = 60.66 H = 6.04	C = 60.60 H = 6.02	9.2×10^{-5}	2.2	500	55	9.0	>10
XI	Cyclohexanone oxime	b. 138–40/ 1 mm.	C = 58.99 H = 8.35	C = 58.19 H = 8.45	9.2×10^{-5}	2.2	500	85	5.9	>10
XII	*anti*-Isophorone oxime	97.5–9	C = 62.83 H = 8.63	C = 63.24 H = 8.74	1.0×10^{-6}	200	>500	14.5	34	1.85
XIII	*syn*-Isophorone oxime	116–18	C = 62.83 H = 8.63	C = 63.75 H = 8.96	9.0×10^{-7}	222	>500	12.5	40	1.15

Recently, oxime *N*-methylcarbamates have been shown to have insecticidal properties (Addor, 1965; Weiden *et al.*, 1965). It is of interest to compare the *N*-methylcarbamates of benzaldoxime (X), cyclohexanone oxime (XI) and the anti- and syn-isomers of isophorone oxime (XII and XIII), all of which have the —C=NO— configuration between ring and ester group, with the related *N*-methylcarbamates of benzyl alcohol (II) and cyclohexyl carbinol (III). The data of Table I show that the oxime carbamates of both benzaldehyde and cyclohexanone are effective anticholinesterases and have appreciable insecticidal activity when used with piperonyl butoxide synergist. The approximately 100 times greater affinity of the isophorone (3,3,5-trimethyl-5-cyclohexenone) oxime *N*-methylcarbamate over cyclohexanone oxime *N*-methylcarbamate is another indication of the requirement for complementarity with the anionic site of ChE comparable with the 590 times greater affinity of 3-isopropylphenyl over phenyl *N*-methylcarbamate (Metcalf and Fukuto, 1965). The *syn*-isophorone oxime *N*-methylcarbamate (high melting) is only slightly more active than the anti-isomer (low melting).

The stability of the oxime carbamate esters in Table I does not appear to be a major factor in determining activity as the acidity of all the parent alcohols and oximes is low (pK_a values—phenol 9.8, benzaldoxime 10.9, cyclohexanone oxime >12; Addor, 1965) and they may be expected to form stable esters of approximately equal carbamylating activity. Two other factors may enhance interaction of the oxime carbamates with ChE—the rigidity of the —C=N— double bond which maintains the molecule in an extended configuration, and a possible secondary interaction between the oxime *N* with its unshared electron pair and an electrophilic center at the active site of ChE. Further research on this interesting problem is in progress.

Effects of Side Chains. The critical nature of side chain geometry has been pointed out in studies with branched (Metcalf *et al.*, 1962) and asymmetrical (Fukuto *et al.*, 1964) alkylphenyl *N*-methylcarbamates. However, little attention has been given to the effects of unsaturation. In earlier work comparison of the isomeric propylthio-, allylthio-, and propargylthiophenyl *N*-methylcarbamates (Metcalf *et al.*, 1965) showed that the C=C bonds progressively increased the level of ChE inhibition but had less effect on the toxicity to flies and mosquitoes. The unsaturated compounds had lower synergistic ratios with piperonyl butoxide indicating decreased susceptibility to detoxication. However, the sulfur atom in these compounds seems to have a dominant effect on activity, probably because of the acquisition of a positive center on sulfur from either protonation at physiological pH or by sulfoxide formation. A better comparison of the effects of unsaturation is given by the isomeric propargyloxy and propoxyphenyl *N*-methylcarbamates. Here the C=C bond substantially improved the affinity for ChE and insect toxicity (Metcalf *et al.*, 1965).

The data in Table II indicate the effects of C=C bonds at three positions in alkenylphenyl *N*-methylcarbamates. The presence of C=C in the *o*-(2-propenyl)-(XV) or *o*-(1-isobutenyl)-(XVII) phenyl *N*-methylcarbamates approximately doubled the affinity for ChE and the housefly toxicity over the corresponding *o*-propyl- and *o*-isobutylphenyl *N*-methylcarbamates. The effects of the C=C bond in *o*-(2-isobutenyl)phenyl *N*-methylcarbamate (XVIII) were not as great, but the activity of the compound was improved.

Effects of Para-Substituents. Substituted phenyl *N*-methylcarbamates with small para-substituents have affinities for ChE which are generally little different from the unsubstituted phenyl *N*-methylcarbamate (Metcalf *et al.*, 1962). The *p*-CH_3S compound has an affinity of 6.0 because of the dominant attraction of the S atom to the anionic site of ChE (Metcalf *et al.*, 1965). However, for all of these substituents the *o*- and *m*-isomers have much greater affinities than the *p*-isomers. With larger para-substituents as shown in Table II, the affinity increases with chain length—compare *p*-isoamyl (XIX, A = 22) and *p*-1-methyl-3,3-dimethylbutyl (XX, A = 66) with *p*-methylphenyl *N*-methylcarbamate (A = 2.0). This increased affinity may result from the ability of the side chain to fold under the ring so as to interact with the anionic site of ChE. This idea is supported by a comparison of *p*-biphenylyl *N*-methylcarbamate (XXI, A = 8.6) with *p*-benzylphenyl- (XXII, A = 148), *p*-α,α-dimethylbenzylphenyl (XXIII, A = 338), and *p*-benzyloxyphenyl (XXIV, A = 64) *N*-methylcarbamates. A similar effect was observed with a series of *p*-alkylthiophenyl *N*-methylcarbamates where affinities increased with increasing chain length (Metcalf *et al.*, 1965). The LD_{50} values in Table II suggest, however, that the longer chains and aromatic groups provide additional opportunities for detoxication in the insect or alter the physical properties to prevent penetration. Further work is in progress to define these interesting phenomena.

Multiple Substituents. The effects of two or even three ring substituents upon the biological activities of phenyl *N*-methylcarbamates are particularly interesting. Investigations from this laboratory have explored the anticholinesterase and insecticidal activities of all of the isomeric dimethoxy-, dimethyl-, dichloro-, and a number of molecules with mixed simple substituents as well as the thymyl- and carvacryl-, and several isopropyl methoxy-, and isopropoxymethoxy-*N*-methylcarbamates (Metcalf and Fukuto, 1965).

Stevens and Beutel (1941) and Haworth *et al.* (1947) carried out extensive studies of the effects of ring alkylation upon the subcutaneous toxicity to mice of various quaternary ammonium phenyl methylcarbamates. As examples of the remarkable enhancement of activity obtained, the introduction of a *m*-isopropyl group into *p*-trimethylammoniumphenyl *N*,*N*-dimethylcarbamate increased the toxicity about 1600 times, and of *p*-methyl into *o*-trimethylammoniumphenyl *N*-methylcarbamate, about 2700 times. Some of the more active of these compounds have been prepared and evaluated along with related new compounds (Table III).

In these phenyl *N*-methylcarbamates the aryl ring is freely rotatable about the ester oxygen. Therefore, the disubstituted compounds (Table III) may be classified into four categories with regard to their capabilities for interaction with the anionic site of ChE. The symmetrical-identical compounds such as 3,5-diisopropylphenyl *N*-methylcarbamate (XXXVII) should interact with equal probability on either edge of the aromatic ring. The

Table II. Effects of Side Chains

	Phenyl *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.)	Synergistic Ratio A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
XIV	*o*-C_3H_7	64–6	C = 68.36 H = 7.82	C = 68.37 H = 7.42	6.0×10^{-6}	33	290	34.0	8.5	10
XV	*o*-$CH_2CH{=}CH_2$	70–2	C = 69.08 H = 6.85	C = 69.40 H = 6.72	3.4×10^{-6}	59	145	24.3	6.0	10
XVI	*o*-$CH_2CH(CH_3)_2$	67–72	C = 69.50 H = 8.26	C = 70.03 H = 8.61	2.3×10^{-6}	87	350	24.5	13.7	1.4
XVII	*o*-$CH{=}C(CH_3)_2$	66–8	C = 70.22 H = 7.37	C = 70.04 H = 7.20	1.2×10^{-6}	165	150	16.4	9.1	0.78
XVIII	*o*-$CH_2C(CH_3){=}CH_2$	67–72	C = 70.22 H = 7.37	C = 70.39 H = 7.44	1.8×10^{-6}	110	280	24.9	11.2	1.1
XIX	*p*-$CH_2CH_2CH(CH_3)_2$	90–3	C = 70.55 H = 8.65	C = 71.23 H = 8.87	9.0×10^{-6}	22	>500	>500	1.0	10
XX	*p*-$C(CH_3)_2CH_2C(CH_3)_2$-CH_3	101–3	C = 72.96 H = 9.57	C = 72.85 H = 9.77	3.0×10^{-6}	66	>500	>500	1.0	>10
XXI	*p*-C_6H_5	132–33.5	C = 73.98 H = 5.76	C = 74.29 H = 5.99	2.6×10^{-5}	8.6	>500	>500	1.0	>10
XXII	*p*-$CH_2C_6H_5$	95–7	C = 74.66 H = 6.26	C = 75.25 H = 6.30	1.5×10^{-6}	148	>500	440	1.3	>10
XXIII	*p*-$C(CH_3)_2C_6H_5$	117–19	C = 75.81 H = 7.11	C = 76.01 H = 6.98	7.6×10^{-7}	338	>500	200	2.5	>10
XXIV	*p*-$OCH_2C_6H_5$	121.5–5	C = 70.02 H = 5.87	C = 70.31 H = 5.98	3.5×10^{-6}	64	>500	20.6	24.2	>10
XXV	*o*-$OCH_2C_6H_5$	72–3	C = 70.02 H = 5.88	C = 69.40 H = 5.99	3.0×10^{-4}	0.66	>500	ca. 500	1	>10

symmetrical-unidentical compounds have symmetrically placed 3,5-substituents of different steric and polarizability characteristics, which may have equal opportunity to interact but which may have greatly differing attractions to the anionic site. An example is 3-isopropyl-5-trimethylammoniumphenyl *N*-methylcarbamate (LIII) where the affinity of the quaternary nitrogen group (A = 28,500) is almost 20 times that of the isopropyl group (A = 590). In the unsymmetrical-identical compounds, the affinities of the same substituent in different positions on the ring may differ manyfold; as in 2,4-diisopropylphenyl *N*-methylcarbamate (XXXVIII) (*o*-iPr A = 33, *p*-iPr A = 3). In the unsymmetrical-unidentical compounds substituents of different steric and polarizability characteristics may be placed in more or less advantageous positions for interaction, as for example, with 3-isopropyl-6-chlorophenyl *N*-methylcarbamate (XXXIII) (*m*-iPr A = 590, *o*-Cl A = 40).

There is a linear relationship between the logarithm of affinity (log A) and the van der Waals' attractivity of the anionic interactant (Metcalf *et al.*, 1962). Therefore, if probability alone favors interaction of these disubstituted carbamates with the anionic and esteratic sites of ChE, then:

$$\log A_t = k \log (A_x + A_y)$$

where A_t = affinity of the disubstituted compound, A_x and A_y = affinities of individual substituents. A plot of this situation, where k = 1, is shown in Figure 1. Clearly, the multisubstituted compounds of Table III, both symmetrical and unsymmetrical, are substantially better inhibitors of ChE than can be accounted for if probability alone determines that a single ring substituent interacts with the anionic site. Therefore, the individual substituents must reinforce one another in their interaction with the enzyme. This enhancement of activity is very small with substituents of low affinity such as CH_3 and CH_3O but increases materially with substituents of high affinity such as isopropyl and *tert*-butyl suggesting an "umbrella effect" so that the aryl ring becomes complementarily

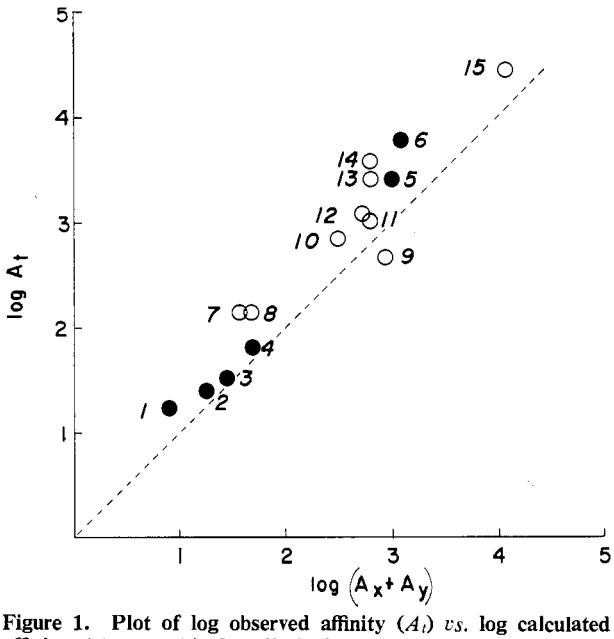

Figure 1. Plot of log observed affinity (A_t) *vs.* log calculated affinity ($A_x + A_y$) for disubstituted phenyl *N*-methylcarbamates

● 3,5-Symmetrically substituted compounds: Cl (1), CH_3O (2), Me (3), Me_2N (4), *tert*-Bu (5), i-Pr (6)

○ 2,4-Di-i-Pr (7), 2-iPr,5-Me (8), 3-iPr, 6-iPrO (9), 2-iPrO,5-Me (10), 3-iPr, 5-Me_2N (11), 3-*tert*-Bu,5-Me (12), 3-iPr,6-Cl (13), 3-iPr,5-Me (14), 3-i-Pr,5N^+Me_3 (15)

Dotted line indicates theoretical probability relationship

Table III. Effects of Multiple Substituents

Phenyl *N*-Methylcarbamate		M.P., ° C.	Analysis, % Theory	Analysis, % Found	I_{50} *M* Fly ChE	Relative Affinity	*Musca domestica* LD_{50}, μg./G. A (alone)	*Musca domestica* LD_{50}, μg./G. B (1:5 P.B.)[a]	Synergistic Ratio A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
XXVI	3-$CH(CH_3)_2$	72–4			3.4×10^{-7}	590	90	9.0	10	0.03
XXVII	3-$CH(CH_3)_2$, 6-CH_3	89.5–91			2.0×10^{-6}	100	>500	18.5	>27	0.46
XXVIII	3-$CH(CH_3)_2$, 5-CH_3	87–9			5.6×10^{-8}	3850	29	5.5	5.3	0.07
XXIX	2-$CH(CH_3)_2$, 5-CH_3	96–8			1.4×10^{-6}	140	65	9.0	7.2	3.4
XXX	2-$CH(CH_3)_2$, 4-CH_3	97–9			4.4×10^{-6}	45	500	12.0	41	2.5
XXXI	3-CH_3, 4-$CH(CH_3)_2$	b. 120/0.25			1.1×10^{-6}	182	46	8.5	5.4	0.39
XXXII	3-$CH(CH_3)_2$, 6-CH_3O	118–20	C = 64.53 H = 7.68	C = 65.29 H = 8.01	2.8×10^{-6}	72	105	90	1.2	0.50
XXXIII	3-$CH(CH_3)_2$, 6-Cl	90–1	C = 58.03 H = 6.20	C = 58.63 H = 5.74	7.8×10^{-8}	2560	39	7.5	5.0	0.071
XXXIV	3-$CH(CH_3)_2$, 6-Br	77.5–9	C = 48.54 H = 5.19	C = 48.82 H = 5.00	7.2×10^{-8}	2777	49	7.2	6.8	0.15
XXXV	3-$CH(CH_3)_2$, 6-F	100–2	C = 62.54 H = 6.60	C = 62.79 H = 6.72	5.5×10^{-6}	36	115	17	6.7	0.14
XXXVI	3-$CH(CH_3)_2$, 4-F	107.5–108.5	C = 62.54 H = 6.60	C = 63.05 H = 6.64	5.8×10^{-6}	35	400	25.5	15.7	0.062
XXXVII	3,5-di-$CH(CH_3)_2$	80			3.3×10^{-8}	6060	17.5	5.2	3.4	0.082
XXXVIII	2,4-di-$CH(CH_3)_2$	66–7	C = 71.48 H = 8.93	C = 71.84 H = 8.96	1.4×10^{-6}	141	>500	23.5	21.2	>10
XXXIX	2-$OCH(CH_3)_2$	77–9			6.9×10^{-7}	290	25.5	7.0	3.6	0.3
XL	3-$CH(CH_3)_2$, 6-$OCH(CH_3)_2$	b. 124/0.6	C = 66.90 H = 8.42	C = 65.21 H = 8.61	4.3×10^{-7}	466	48.5	9.5	5.1	8.8
XLI	2-$OCH(CH_3)_2$, 5-CH_3	63–6			2.8×10^{-7}	715	40.0	6.0	6.7	>10
XLII	2-$OCH(CH_3)_2$, 5-OCH_3	96.5–8			5.6×10^{-7}	358	6.5	3.0	2.1	>10
XLIII	3-$OCH(CH_3)_2$, 6-OCH_3	116–17.5			1.5×10^{-5}	13	150	10	15.0	>10
XLIV	3-$C(CH_3)_3$	144–5			4.0×10^{-7}	500	500	8.0	63	0.15
XLV	3-$C(CH_3)_3$, 5-CH_3	110–12	C = 70.52 H = 8.65	C = 70.56 H = 7.91	1.7×10^{-7}	1180	31	4.9	6.3	0.32
XLVI	2-$C(CH_3)_3$, 5-CH_3	110–13	C = 70.52 H = 8.65	C = 70.95 H = 9.24	8.0×10^{-4}	0.25	>500	300	>1.6	>10
XLVII	2-$C(CH_3)_3$, 5-OCH_3	84–9	C = 65.78 H = 8.08	C = 66.22 H = 8.37	1.9×10^{-4}	1.05	>500	>500	1.0	>100
XLVIII	3-$C(CH_3)_3$, 6-OCH_3	121–3	C = 65.78 H = 8.08	C = 66.01 H = 8.34	4.2×10^{-6}	48	>500	14.5	>34	3.3

[a] Piperonyl butoxide synergist.

substituted on all sides because of the overlap of methyl groups. This effect as studied with a molecular model of 3,5-diisopropylphenyl *N*-methylcarbamate (XXXVII) and a plaster cast of acetylcholine (Metcalf and Fukuto, 1965) suggests that the carbamate can interact strongly with the anionic site on both sides as well as edges of the aryl ring. This effect should increase the probability of anionic interaction severalfold over that of a single substituent.

Substitution of the 3-isopropylphenyl *N*-methylcarbamate with Cl or Br, in the 6-position (XXXIII, XXXIV) increased the affinity by more than four times, similar to 5-CH_3 substitution. However, 6-F substitution (XXXV) reduced activity to 0.06 that of the parent compound and 4-F substitution had an almost identical effect. With the 2-isopropyl-5-methyl (thymyl) *N*-methylcarbamate (XXIX), 4-Cl substitution (LXVI) decreased affinity to about 0.05 because of double ortho-substitution. These effects of halogen substitution show that the principal interaction with the anionic site is through the isopropyl group. The ortho- or para-fluorine substituent seems to behave in a way sterically similar to a hydrogen atom but the greater electron-withdrawing capacity of F in either position lowers the stability of the compound and reduces its activity. These results suggest that carbamylation, which should be more rapid with the F derivatives (O'Brien *et al.*, 1966), is not the rate determining factor in ChE inhibition. The enhancement of activity with 6-Cl and -Br analogs of 3-isopropylphenyl *N*-methylcarbamate may be the result of secondary interaction with the enzyme similar to that resulting from addition of 5-CH_3 to 3-isopropyl (XXVIII). In the monosubstituted phenyl *N*-methylcarbamates, the halogens are most active in the ortho-position and alkyl-substituents in the meta-position (Metcalf *et al.*, 1962).

Possibly, however, the increased activity of the 6-chloro- and 6-bromo-3-isopropylphenyl *N*-methylcarbamates may be due to the steric effect of the bulky ortho-substituent in preventing free rotation of the carbamoyl moiety about the benzene-oxygen bond. This should force the carbonyl carbon atom into a position which would result in greater complementarity of the molecule with the anionic and esteratic sites of the enzyme as shown below:

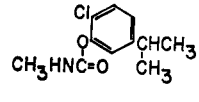

Table III. Continued

Phenyl *N*-Methylcarbamate	M.P., ° C.	Analysis, % Theory	Analysis, % Found	I_{50} *M* Fly ChE	Relative Affinity	*Musca domestica* LD_{50}, μg./G. A (alone)	*Musca domestica* LD_{50}, μg./G. B (1:5 P.B.)[a]	Synergistic Ratio A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
XLIX 3,5-di-$C(CH_3)_3$	97–9	C = 72.97 H = 9.56	C = 72.82 H = 9.87	7.8×10^{-8}	2551	39.0	6.0	6.5	>10
L 2,5-di-$C(CH_3)_3$	132–5	C = 72.97 H = 9.56	C = 72.79 H = 9.82	$>1 \times 10^{-3}$	<0.2	>500	>500	1.0	>10
LI 3-$N(CH_3)_2$	86–7			8.0×10^{-6}	25	270	19	14.2	1.7
LII 3-$CH(CH_3)_2$, 5-$N(CH_3)_2$	77–81			1.9×10^{-7}	1050	13.5	4.7	2.9	2.3
LIII 3-$CH(CH_3)_2$, 5-$N^+(CH_3)_3$	143–50			7.0×10^{-9}	28500	>500			>10
LIV 4-$N(CH_3)_2$	131–2			2.4×10^{-4}	0.84	>500	85	>5.9	>10
LV 3-$CH(CH_3)_2$, 4-$N(CH_3)_2$	85–6			1.5×10^{-7}	1330	29.5	11.5	2.6	0.32
LVI 3-$CH(CH_3)_2$, 4-$N^+(CH_3)_3$	165–6			5.0×10^{-9}	40000	>500			>10
LVII 3,5-di-$N(CH_3)_2$	103–6	C = 60.74 H = 8.07	C = 60.29 H = 8.09	2.6×10^{-6}	78	9.0	8.5	1.1	6.8
LVIII 3-$N(CH_3)_2$, 5-$N^+(CH_3)_3$	170			3.7×10^{-8}	5400	>500			>10
LIX 3,5-di-$N^+(CH_3)_3$	90 dec.			1.2×10^{-7}	1670	>500			>10
LX 3-$CH(CH_3)_2$, 4-$N(CH_3)_2$, 6-CH_3	73–5	C = 67.16 H = 8.85	C = 67.06 H = 9.06	3.9×10^{-7}	514	325	16.5	19.7	4.3
LXI 3-$CH(CH_3)_2$, 4-$N^+(CH_3)_3$, 6-CH_3	166–8 dec.			1.1×10^{-8}	18200	>500			>10
LXII 2-$CH(CH_3)_2$, 4-$N(CH_3)_2$, 5-CH_3	68–70	C = 67.16 H = 8.85	C = 66.82 H = 8.96	1.3×10^{-6}	154	115	12.5	9.2	>10
LXIII 2-$CH(CH_3)_2$, 4-$N^+(CH_3)_3$, 5-CH_3	188–9 dec.			1.0×10^{-8}	20000	>500			>10
LXIV 2-C_6H_{11}, 5-CH_3O	105–7	C = 68.41 H = 8.04	C = 67.09 H = 7.99	2.7×10^{-7}	810	>500	16	>31	>10
LXV 2-$C(CH_3)_3$, 5-C_6H_5	143–7	C = 76.29 H = 7.47	C = 76.41 H = 8.02	5.4×10^{-6}	27	>500	500	>1.0	>100
LXVI 2-$CH(CH_3)_2$, 4-Cl, 5-CH_3	91–4	C = 59.13 H = 7.45	C = 59.48 H = 6.89	5.3×10^{-7}	377	>500	11.7	43	>10
LXVII 2-$CH(CH_3)_2$, 4-Cl, 5-CH_3, 6-Cl	154–8	C = 51.81 H = 6.15	C = 52.38 H = 5.74	2.8×10^{-5}	7.1	>500	>500	1.0	>10

Dreiding models of the carbamate moiety in this conformation show that the distance between the carbonyl carbon and the central carbon of the isopropyl group is about 4.8 A. as compared with 6.0 A. in the extended conformation. As the distance between anionic and esteratic sites in fly head ChE is approximately 5.0 to 5.5 A. (Hollingworth *et al.*, 1967) greater interaction should be expected when the carbamate moiety is in a conformation closer to the carbonyl group. This hypothesis receives some support from the higher relative affinity of the larger Br derivative as compared to the Cl derivative.

The insect toxicity of these multisubstituted *N*-methylcarbamates was also generally improved by the addition of a second substituent CH_3, $(CH_3)_2CH$, $(CH_3)_3C$, $(CH_3)_2N$, or Cl or Br to the appropriate position of the ring. Thus, the 3,5-diisopropyl compound (XXXVII) was about 5 times, the 3,5-di-*tert*-butyl (XLIX) 10 times, and the 3,5-dimethylamino (LVII) 30 times as toxic as the corresponding monosubstituted analog. Some of this increase in housefly toxicity was evident after synergism (Table III) and was due to increased interaction at target site.

Effects of Quaternization. The addition of a formal charge to the dimethylamino group of the prostigmine-type compound has been shown to increase the mammalian toxicity by as much as 100-fold (Stevens and Beutel, 1941). The quaternary nitrogen atoms of such compounds have increased affinities for the ChE of both mammals and insects because of the coulombic attraction to the negatively charged anionic site of the enzyme. Thus, *m*-trimethylammoniumphenyl *N*-methylcarbamate had an affinity for fly ChE of 450 times the unquaternized compound, and of 25 times that of its uncharged isostere *m-tert*-butylphenyl *N*-methylcarbamate, but, because of the nerve sheath barrier about the insect synapses, the quaternary compound was insecticidally inactive as were all the quaternary compounds of Table III. However, investigation of the quaternary salts of the substituted dimethylaminophenyl *N*-methylcarbamates of Table III should yield additional information regarding the interaction of the inhibitors with the active site of the enzyme. Monoquaternization (LVIII) of the 3,5-bis(dimethylamino)phenyl *N*-methylcarbamate (LVII) increased the affinity for ChE by 70 times but diquaternization (LIX) was actually less effective, increasing the affinity only 8.8 times over that of the unquaternized compound. The decrease in activity of the diquaternary compound compared with its monoquater-

nary analog may be due to the high hydrolytic instability of the former. Summation of the σ constants for the 3,5-diquaternary phenyl *N*-methylcarbamate gives a value of 1.718, suggesting that the compound is highly unstable to hydrolysis. For example, *p*-nitrophenyl *N*-methylcarbamate (σ 1.267) is rapidly hydrolyzed under the pH of 7.4 used in the anticholinesterase determinations and hence is a poor inhibitor.

Quaternization of 3-isopropyl-4-dimethylaminophenyl (LV) and 3-isopropyl-5-dimethylaminophenyl (LII) *N*-methylcarbamates had approximately equal effects, increasing the affinities 25 and 21 times, respectively, and rather surprisingly (as *p*-dimethylaminophenyl *N*-methylcarbamate methiodide is only about 0.05 as active as the meta-isomer), the 3-isopropyl-4-dimethylaminophenyl methiodide (LVI) was slightly more active than the 3-isopropyl-5-dimethylaminophenyl methiodide (LIII). These two compounds were the most effective inhibitors investigated in the entire study. Quaternization of 4-dimethylaminothymyl *N*-methylcarbamate (LXII) increased affinity 130 times and quaternization of its isomer 4-dimethylaminocarvacryl *N*-methylcarbamate (LX) increased affinity only 35 times, although both the quaternary compounds were of equivalent activity.

Apparently, therefore, the better the complementarity of the uncharged carbamate to the anionic site of ChE, the less the enhancement of affinity by quaternization. Thus, the substituted 3-isopropylphenyl *N*-methylcarbamates (LII, LV, LX) which have optimum complementarity were increased in affinity by quaternization by only 21 to 35 times in contrast to the substituted 2-isopropylphenyl *N*-methylcarbamate (LXII-thymyl) where the increase was 130 times. The 3-dimethylaminophenyl *N*-methylcarbamate (LI), unlike the 3-isopropyl analog, does not have the optimum complementarity which is found in the 2-dimethylamino analog (Metcalf and Fukuto, 1965). Quaternization of the 3-dimethylamino derivatives, however, produces optimum complementarity. Hence, the increase in affinity is greater with the 3-dimethylaminophenyl carbamates than with the substituted 3-isopropylphenyl carbamates. The maximum activity in these quaternary carbamates occurs at I_{50} values of 6×10^{-9} to $2 \times 10^{-8} M$, or in the same range as monosubstituted *m*-trimethylammoniumphenyl *N*-methylcarbamate (I_{50} $1.8 \times 10^{-8} M$). This suggests that in all these compounds the positively charged trimethylammonium group, which acts through coulombic attraction over a longer range than do the van der Waals' forces, is preferentially attracted to the negatively charged anionic site of ChE thus orienting the molecule so that the other groups are of lesser importance in interaction with the enzyme surface. Thus, the addition of *p*-quaternary ammonium to *m*-isopropyl (LVI) increased affinity 68 times and the addition of *m*-isopropyl to *m*-quaternary ammonium (LIII) increased affinity only 2.5 times.

The isopropyl group and the dimethylamino group are almost equivalent in van der Waals' radii and in bond angles. Yet in monosubstituted phenyl *N*-methylcarbamates the optimum position is ortho for dimethylamino, and meta for isopropyl (Metcalf and Fukuto, 1965). The disubstituted compounds of Table III provide an even more striking example of the differences in enzyme inhibition. The 3,5-diisopropyl (XXXVII A = 6060) is some 80 times as active as 3,5-bisdimethylamino (LVII A = 78), with 3-isopropyl-5-dimethylamino (LII A = 1050) intermediate.

Trimethylphenyl *N*-Methylcarbamates. The biological activities of these six isomeric trisubstituted carbamates as shown in Table IV, provide useful data both on the complementarity of the carbamates for fly ChE (affinity), and on the susceptibility of these to attack by the phenolase detoxifying enzyme, as shown by the synergistic ratio. As has been observed with disubstituted compounds (Metcalf *et al.*, 1963) two ortho-substituents greatly reduce the affinity and toxicity. Thus the symmetrical 2,4,6-compound (LXXIII) is of very low activity but the 2,3,6-compound (LXX), which has adjacent and optimumly located methyl groups is 16 times greater in affinity and has some toxicity when synergized. The presence of two *o*-methyl groups appears to block the proper approach of the carbamate to the esteratic and anionic sites of ChE since the closely related 2,3,4- (LXVIII) and 2,3,5-(LXIX) carbamates each with a single *o*-methyl and with two contiguous methyl groups have 3 and 8 times greater affinities and are substantially more toxic. The most effective compound of this series is the 3,4,5-isomer (LXXII) which has an affinity of 8 times that of the 2,3,4-isomer and is appreciably more toxic. This 3,4,5-compound has an affinity of about 3 times that of the 3,5-dimethylphenyl *N*-methylcarbamate (I_{50} $6.0 \times 10^{-6} M$) and is of about the same toxicity indicating again the importance of the double anionic interactant.

With regard to detoxication, the 2,3,5- and 2,4,5-compounds showed much higher synergistic ratios than the

Table IV. Isomeric Trimethylphenyl *N*-Methylcarbamates

Isomer		M.P., °C.	Analysis, % Theory	Analysis, % Found	I_{50} *M* Fly ChE	Relative Affinity	*Musca domestica* LD_{50}, μg./G. A (alone)	*Musca domestica* LD_{50}, μg./G. B (1:5 P.B.)	Synergistic Ratio A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
LXVIII	2,3,4-	91–4	C = 68.37 H = 7.82	C = 68.34 H = 8.07	1.5×10^{-5}	13.3	69	17.5	3.8	0.62
LXIX	2,3,5-	123–4			6.0×10^{-6}	33.3	>500	24.0	20.8	>10
LXX	2,3,6-	117–20	C = 68.37 H = 7.82	C = 69.13 H = 7.97	5.0×10^{-5}	4.0	>500	140	3.6	>10
LXXI	2,4,5-	116–19	C = 68.37 H = 7.82	C = 67.96 H = 7.67	8.5×10^{-6}	18.2	>500	40	12.5	3.8
LXXII	3,4,5-	117–19			1.9×10^{-6}	105	65	13.5	4.8	0.28
LXXIII	2,4,6-	136–7	C = 68.37 H = 7.82	C = 68.77 H = 8.11	8.0×10^{-4}	0.25	>500	>500	1.0	>10

Table V. Carbamates with Altered Esteratic Interactants

		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.)	Synergistic Ratio A/B	*Culex pipiens 5-fasciatus* LC_{50}, P.P.M.
LXXIV	Phenyl bis(1,2-*N*-methylcarbamate)	147–50	C = 53.55 H = 5.40	C = 53.82 H = 5.28	2.6×10^{-5}	8	>500	110	>5.0	>10
LXXV	Phenyl bis(1,3-*N*-methylcarbamate)	147–52	C = 53.55 H = 5.40	C = 54.61 H = 5.84	3.4×10^{-5}	6	>500	31	>16	>10
LXXVI	Phenyl bis(1.4-*N*-methylcarbamate)	209–12	C = 53.55 H = 5.40	C = 54.01 H = 5.61	2.6×10^{-5}	8	>500	>500	1.0	>10
LXXVII	4,6-Diisopropyl phenyl bis(1,3-*N*-methylcarbamate)	259–62	C = 62.31 H = 7.84	C = 62.09 H = 7.96	$>1 \times 10^{-3}$	<0.2	>500	>500	1.0	>10
LXXVIII	$C_6H_4OC(O)$-$NHCH_3$	180–5	C = 64.43 H = 4.73	C = 65.39 H = 4.45	$>1 \times 10^{-4}$ (0%)		>500	>500	1.0	>10

others. This can be related to a previous study of the isomeric dimethylphenyl *N*-methylcarbamates in which the highest synergistic ratios were found with the 2,5-compound (10.6) and the 2,3-compound (5.0) (Metcalf *et al.*, 1963). The 2,3,5-trimethyl derivative obviously contains the elements of both 2,3- and 2,5-disubstitution, and the 2,4,5-trimethyl compound closely resembles the 2,5-dimethyl carbamate. The data suggest the need for a structural complementarity to the detoxication (phenolase) enzyme which is enhanced by two adjacent methyl groups (Metcalf *et al.*, 1966).

Effects of Doubling the Methylcarbamoyl Group. The profound effects on the affinity of the substituted *N*-methylcarbamates produced by doubling the anionic interactants as shown in Table III suggested investigation of doubling the methylcarbamoyl moiety which is the esteratic interactant. The biological evaluation of the three isomeric phenylene bis(*N*-methylcarbamates) and alkylated derivatives is shown in Table V. The three bis compounds have nearly identical affinities of 6 to 8 times that of the singly substituted phenyl *N*-methylcarbamate. This suggests that although only a single methylcarbamoyl group can interact with the esteratic site, the presence of two identical groups greatly increases the probability of attraction to the enzyme surface. The magnitude of this effect is almost exactly equal to that resulting from the doubling of anionic interactants such as isopropyl or *tert*-butyl (Table III). Compound LXXVII with two esteratic and two anionic interactants, not, however, optimally spaced, has greatly reduced affinity for ChE.

The lack of insecticidal activities of these compounds suggests that they are rapidly detoxified and they clearly have a twofold opportunity for destructive hydrolysis. However, the differences in degree of synergism are extraordinary. These suggest that the para-compound (LXXVI) has the best fit to the detoxication enzyme and the meta-compound (LXXV), the least.

The complete inactivity of LXXVIII in which the *N*-methylcarbamoyl group is bound to the aryl ring as a 1,3-benzoxazolone suggests the exacting steric requirements necessary both for complex formation and for carbamylation. A free methylcarbamoyl group is apparently required for esteratic interaction.

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