INSECTICIDE STRUCTURE AND ACTIVITY

Insecticidal Activity of Carbamate Cholinesterase Inhibitors

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The hypothesis that a number of derivatives of carbamic acids, containing the quaternary nitrogen structure and possessing high biological activity, are not active as contact insecticides because of their inability to penetrate insect cuticle and fatty nerve sheath, has been investigated. Forty-nine lipoid-soluble carbamates have been synthesized and evaluated as contact insecticides and as in vitro inhibitors of fly brain cholinesterase. The N-methylcarbamates of m-tert-butylphenol, thymol, and carvacrol were highly toxic to the housefly and to greenhouse thrips. The more toxic compounds were also tested on the green citrus aphid and the citrus red mite. Certain physicochemical relationships were shown to exist; cholinergic activity and toxicity are inversely dependent on the rates of hydrolysis of the carbamates, and in vitro cholinesterase inhibition is related to contact toxicity. The second-order hydrolysis constants for 15 carbamates have been determined, and a relationship between stability to hydrolysis and cholinesterase inhibition can be shown. As a corollary, Hammett's "sigma" coefficient can be related to cholinesterase inhibition for certain compounds. A mechanism for the mode of action of the carbamate inhibitors has been proposed. Data on 40 new carbamic acid derivatives are presented.

MOST OF THE ORGANIC INSECTICIDES in common use today were discovered during the routine screening of many miscellaneous chemicals or by the planned synthesis of derivatives of compounds already known to be effective. Few if any have been developed from the biochemical viewpoint of devising an antimetabolite or enzyme antagonist that would possess the proper prerequisites for contact toxicity, although the present status of knowledge in this field seems adequate for such an undertaking (3).

With this general concept in mind, the authors selected the cholinesterase (ChE) enzyme system of insects as a starting point because of its vital function in the neural behavior of the organism and the considerable information available concerning its properties and functions (5, 37). The organic phosphorus compounds such as TEPP and parathion have been shown to function largely if not entirely because of their inhibition of this enzyme system (3, 11, 12, 30) by acting as preferential substrates, which when adsorbed at the active center of the enzyme are not hydrolyzed and discarded as are the choline esters, but remain fixed and effectively block the site of action of the enzyme by phosphorylation (6, 7, 10, 24, 27).

The carbamic acid esters have long been known to be highly effective inhibitors of cholinesterases, and as such they have pharmacological applications. These compounds have structural con-figurations that closely resemble the choline esters, and their inhibitory activity occurs from the resulting strong attraction to the active center of the enzyme and their much greater stability to hydrolysis (1, 19). Certain of these compounds, such as physostigmine and prostigmine, are very effective inhibitors of insect cholinesterases (37). Therefore, this group of compounds was chosen as the starting point of an investigation of the requirements for converting potent enzyme inhibitors into contact insecticides.

The first demonstration of the effectiveness of synthetic phenyl carbamates came from the work of Stedman (38), who showed that m-dimethylamino-phenyl N-methylcarbamate methiodide (miotin) was more effective than the o- and p-isomers in promoting miotic activity in cats, and recognized the possible utility of this substance in medicine. In order to promote greater stability in solution, the corresponding N,N-dimethylcarbamate (prostigmine) was developed and has become an important clinical entity, largely replacing the alkaloid physostigmine as a cholinergic drug. Elaborate studies of the relation of chemical structure of related carbamates to toxicity and to cholinergic action have been carried out (2, 25, 39) with carbamic acid esters containing quaternary nitrogen or amine hydrochloride structures that were toxic to mammals intravenously or subcutaneously at 0.1 to 1 mg. per kg. and inhibited mammalian cholinesterase at concentrations of 10^{-8} to $10^{-6} M$.

Some of the more toxic compounds of this type, prostigmine, N,N-dimethyl carbamic acid ester of (2-hydroxy-5-phenylbenzyl) dimethylammonium chloride (Nu-683), and N,N-dimethylcar-bamate of (3-hydroxy-2-pyridylmethyl)-dimethylamine dihydrochloride, were evaluated as contact toxicants to the housefly, and as inhibitors of fly-brain cholinesterase. All three compounds were nontoxic at 500 mg. per kg. but showed 50% cholinesterase inhibition at concentrations of $4 \times 10^{-8} M$, $3 \times 10^{-8} M$, and $8 \times 10^{-8} M$, respectively. It seemed from this that the highly ionized quaternary ammonium structures might be unable to penetrate the lipoid barriers of the insect cuticle and nerve sheaths. This is confirmed by investigations of contact toxicity of the N-methylcarbamates of m-diethylaminophenol methochloride, methiodide, and methosulfate, and of 2 - methyl - 5 - dimethylaminophenol methochloride, and methiodide, which were virtually nontoxic to seven species of insects (8, 9).

Additionally it has been found that the LD_{50} of physostigmine injected into Locusta migratoria migratoriodes R. & F. was 20 to 25 γ per gram, while prostigmine killed only 10% at 1200 γ per gram (26). These results are perhaps to be expected, as Nachmansohn (33) demonstrated that although prostigmine and physostigmine were equally effective

as in vitro anticholinesterases, prostigmine when applied to the nerve of the squid did not affect conduction and did not penetrate into the axon, while physostigmine under equivalent conditions interrupted conduction and penetrated into the axon. Trimethylamine also penetrated readily, but the tetramethylammonium ion did not. However, at neuromuscular junctions and synapses the quaternary salt was able to penetrate, and produced profound physiological effects.

Wilson and Cohen (44) also found that prostigmine is ineffective in penetrating the axon of a crab nerve and does not affect conduction, but the uncharged tertiary amine analog, m-dimethylaminophenyl N,N-dimethylcarbamate, although only $1/100$ as effective in inhibiting cholinesterase in vitro, readily penetrated the axon and blocked conduction.

Insect nerves are known to be surrounded by lipoid sheaths (36), and it is probable that ready penetration of cholinesterase inhibitors into the nervous system is facilitated by nonpolar lipoid-soluble molecules rather than by the highly polar quaternary ammonium compounds. Therefore, attention was turned toward the synthesis of a representative series of N-substituted carbamates of substituted phenols. This series of compounds was obtained as highly purified crystalline products with a very wide range of enzyme-inhibitory and insecticidal action. An excellent opportunity was thus afforded to evaluate carefully the relations of chemical structure to activity, especially from the physicochemical viewpoint.

Since this investigation was begun, the validity of the assumption that nonpolar lipoid-soluble carbamate derivatives should be contact toxicants has been amply confirmed by extensive investigations of a large series of N-alkyl carbamates of psuedoaromatic and cyclic alcohols (16, 21). Certain of these

Figure 1. Relationship between log K_{hyd} and logarithm of median lethal concentration for substituted phenyl N-methylcarbamates

materials, particularly 1-phenyl-3-methylpyrazolyl-(5)-dimethylcarbamate (pyrolan) and 1-isopropyl-3-methyl-pyrazolyl-(5)-dimethylcarbamate (isolan), showed outstanding toxicity to aphids, thrips, flies, and other insects, both by contact and per os. Wiesmann and Kocher (42) investigated the mode of action of pyrolan extensively and concluded that the typical tremors produced are dependent upon an intact reflex area, and that the compound acts centrally on the motor region of the ganglia. The action was antagonized by barbituric acid and nicotine, and it was concluded that death resulted from exhaustion and autointoxication.

Experimental

The anticholinesterase activity of the carbamates was measured by the standard Warburg manometric method (37), using a brei of three homogenized fly brains per ml. of 0.025M sodium bicarbonate, 0.15M sodium chloride, 0.04-M magnesium chloride, and 0.01M acetylcholine bromide. The inhibitors were added as standard molar concentrations in 0.1-ml. aliquots in acetone solution, and after flushing with 95% nitrogen–5% carbon dioxide and 15 minutes' equilibration the volume of carbon dioxide produced at 37.5° in 30 minutes was measured and compared to a standard without inhibitor. The values obtained were plotted as log molar concentration against percentage inhibition, and the 50% inhibition values were determined by inspection of the resulting straight-line plots.

The toxicity tests to the housefly were made by the application of 1-microliter drops to 2- to 4-day-old females. Residual toxicities of the compounds to the greenhouse thrips were measured on mature Valencia oranges dipped in standard weight/volume solutions in acetone (29).

Methyl Isocyanate. The methyl isocyanate was prepared by heating sodium azide and acetic anhydride in n-hexyl ether (13). Better yields were obtained if water, warmed to 40° C., was passed through the reflux condenser that was used to prevent distillation of the acetic anhydride. The product was sealed under nitrogen in weighed glass ampoules and stored in the refrigerator.

Benzyl Isocyanate (25). Ten milliliters (9.8 grams) of benzylamine was dissolved in 300 ml. of dry toluene contained in a 500-ml. 3-necked flask fitted with a mechanical stirrer, a reflux condenser, and a glass delivery tube 5 mm. in inside diameter. If a coarse, fritted-glass gas bubbler was used as the delivery tube, it quickly became blocked, creating dangerous pressures in the system. The toluene solution was heated nearly to the boiling point and tank phosgene (hood), passed first through cottonseed

oil to remove excess chlorine and then through concentrated sulfuric acid, was bubbled into the stirred toluene solution of benzylamine. A white solid quickly formed, which later dissolved; the gas was passed for 2 hours. The toluene was evaporated under line vacuum (ca. 50 mm. of mercury), and the residue was fractionally distilled. The benzyl isocyanate, a colorless oil boiling at 85° to 87° C. at 10 mm., weighed 8.0 grams; yield was 66%.

Phenols. All phenols were purified by distillation, crystallization, or the preparation of crystalline esters, if these were known. In every case, the phenolic specimen was made to conform to the physical properties recorded for these compounds.

**N-Methyl- and N-Ethylcarbamates.** One gram of phenol or substituted phenol was dissolved in 1 ml. of dry isopropyl ether—more was used only if necessary for complete solution—in a glass-stoppered test tube or small flask. An ampoule containing a known weight of methyl isocyanate was cooled in a dry ice chest, and opened, and the contents were quickly added to a measured quantity of cold isopropyl ether to prepare a standard solution. Aliquots of this solution, containing only a slight molar excess of methyl isocyanate, were added to the phenolic solution; the tube was sealed with the glass stopper, and the reaction mixture was allowed to stand for several days at room temperature. In many cases a crystalline precipitate formed, which was filtered and recrystallized. In other cases the addition of light petroleum ether to the reaction mixture and cooling yielded a crystalline product. (It has been the authors' experience that if large excesses of methyl isocyanate are used, a product that fails to crystallize or a crystalline product that is definitely a mixture is often obtained.) The desired products were finally recrystallized from the following solvents: compounds II and XII (Table I), from methanol-water (2 to 1, volume); XIII and XXVIII from methanol; XXVI from 95% ethanol; XXV from n-octane; the remainder of the compounds from n-hexane. The N-ethylcarbamates (Table II) were prepared in a similar manner and were recrystallized from n-hexane.

N-Phenyl- and N-Benzylcarbamates. One gram of the phenolic compound in 5 ml. of dry toluene was refluxed for 3 hours with a 1 to 1 molar ratio of benzyl isocyanate or phenyl isocyanate. On cooling, a crystalline product separated; this was filtered and recrystallized from n-hexane for the N-phenyl- and from n-octane for the N-benzylcarbamates. The physical and toxicity data for these compounds are presented in Table II.

Hydrolysis Of Carbamates The rate of hydrolysis of a number of the carbamates was determined at 37.5° C., using the colorimetric
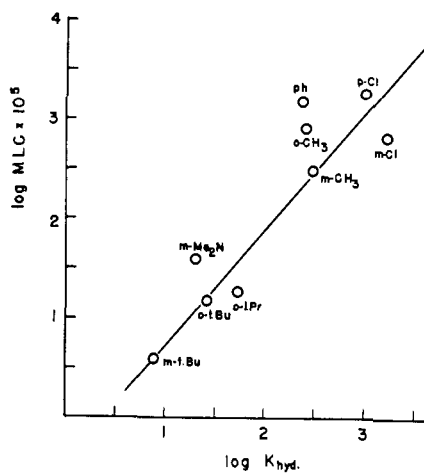
method of Gottlieb and Marsh (20) to determine the amount of free phenol by reaction with 4-aminoantipyrine and alkaline potassium ferricyanide. The carbamic acid esters were dissolved in acetone and 1 ml. of 0.10% solution added to 99 ml. of buffer, at the desired pH, containing the color-developing reagents. The solution was quickly shaken and maintained at 37.5° C. Aliquots were removed at intervals, and the absorbance at 514 mμ was

Table I. Properties and Biological Activity of N-Methylcarbamates of Various Phenols

	N-Methylcarbamate	M.P.[a], ° C.	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N	I_{50}[b]	Housefly LD$_{50}$, γ/g.	MLC[c] thrips
I	Phenyl	85–6	63.56	6.00	...	63.66	5.83	...	2×10^{-4}	70	0.015
II	o-Tolyl	101.5–102	65.43	6.71	...	65.46	6.87	...	1×10^{-4}	500	0.008
III	m-Tolyl	75.5–76.5	65.43	6.71	8.48	65.47	6.90	8.48	8×10^{-6}	50	0.003
IV	p-Tolyl	95–6	65.43	6.71	...	65.71	6.51	...	1×10^{-4}	500	>0.1
V	m-Ethylphenyl	73.5–74.5	67.00	7.30	...	67.27	7.45	...	1×10^{-5}	250	0.02
VI	o-Isopropylphenyl	96–7	68.36	7.82	...	68.63	7.90	...	6×10^{-6}	100	0.00018
VII	p-Isopropylphenyl	96.5–97.0	68.36	7.82	...	68.63	8.03	...	7×10^{-5}	>500	0.023
VIII	o-tert-Butylphenyl	95.5–96.5	69.53	8.26	...	69.29	8.29	...	6×10^{-6}	75	0.00015
IX	m-tert-Butylphenyl	144–5	69.53	8.26	...	69.62	8.36	...	4×10^{-7}	50	0.00008
X	p-tert-Butylphenyl	111–12	69.53	8.26	...	69.00	8.12	...	1.5×10^{-4}	>500	>0.1
XI	o-Nitrophenyl	87–8	48.98	4.11	...	48.68	4.12	...	5×10^{-3}	250	...
XII	m-Nitrophenyl	130–1	48.98	4.11	...	49.23	4.27	...	2×10^{-4}	>500	0.02
XIII	p-Nitrophenyl	160.5–162	48.98	4.11	...	49.23	4.37	...	3×10^{-3}	>500	>0.01
XIV	o-Chlorophenyl	90–1	...	...	7.55	...	...	7.49	5×10^{-6}	75	>0.01
XV	m-Chlorophenyl	80.5–81	51.76	4.34	...	52.12	4.31	...	5×10^{-5}	100	0.007
XVI	p-Chlorophenyl	115–16	51.76	4.34	...	52.27	4.66	...	2.4×10^{-4}	>500	0.018
XVII	o-Cyclohexylphenyl	100.5–101.5	72.07	8.20	...	72.38	8.33	...	1.4×10^{-6}	>500	0.0007
XVIII	3,5-Dimethylphenyl	100.5–102	67.00	7.30	...	66.67	6.99	...	6×10^{-6}	60	0.0015
XIX	m-Dimethylaminophenyl[d]	86–7	...	...	...	...	...	...	8×10^{-6}	500	0.0005
XX	m-Dimethylaminophenyl[e] methiodide	178–9 (efferv.)	...	...	...	...	...	...	1.6×10^{-8}	>500	0.02
XXI	2-Methyl-5-isopropylphenyl	89.5–91	69.53	8.26	...	69.76	8.39	...	2×10^{-6}	>500	0.00025
XXII	5-Methyl-2-isopropylphenyl	96–8	...	...	6.76	...	...	6.83	1.4×10^{-6}	30	0.0003
XXIII	2,3,5-Trimethylphenyl	123–4	...	...	7.25	...	...	7.19	6×10^{-6}	500	0.00023
XXIV	2,4-Di-tert-butylphenyl	128–128.5	...	...	5.32	...	...	5.20	$>1 \times 10^{-3}$	>500	>0.1
XXV	2,4-Dichlorophenyl	131–131.5	43.66	3.21	...	44.05	3.37	...	5×10^{-4}	>500	0.009
XXVI	2,4-Dinitrophenyl	130–1	39.84	2.93	...	39.71	3.11	...	$>1 \times 10^{-3}$	>500	0.03
XXVII	2-Methyl-4,6-dinitrophenyl	133.5–4.5	42.35	3.56	...	42.44	3.60	...	4×10^{-3}	>500	>0.01
XXVIII	2,6-Dimethoxy-3,5-dinitrophenyl	149–150.5	39.80	3.68	...	39.92	3.87	...	7×10^{-5}	>500	0.004
XXIX	2,4,5-Trichlorophenyl	164–6	...	...	5.50	...	...	5.59	1.4×10^{-5}	>500	0.02
XXX	2,4,6-Trichlorophenyl	178–80	...	...	5.50	...	...	5.56	1.7×10^{-5}	>500	0.04

[a] Total immersion thermometer.
[b] Molar concentration for 50% inhibition of fly-brain cholinesterase.
[c] Median lethal concentration, %.
[d] Stedman (38), gives m.p. 85–6°.
[e] Calculated for $C_{11}H_{17}IN_2O_2$: I, 37.75; found: I, 37.75. Stedman (38) gives m.p. 165° (effervescence).

Table II. Properties and Biological Activities of N-Ethyl-, N-Benzyl-, and N-Phenylcarbamates of Various Phenols

	N-Ethylcarbamate	M.P., ° C.	Analysis N calcd.	Analysis N found	I_{50}[a]	Housefly, LD$_{50}$, γ/G.	MLC[b], Thrips
XXXI	o-Tolyl	39.5–40.5	7.82	7.87	$>1.8 \times 10^{-4}$	>500	>0.1
XXXII	m-Tolyl	41–2	7.82	7.80	4.6×10^{-4}	>500	>0.1
XXXIII	p-Tolyl	110–11	7.82	7.95	$>2 \times 10^{-4}$	>500	>0.1
XXXIV	m-tert-Butylphenyl	83–4	6.33	6.40	2×10^{-5}	>500	0.0017
XXXV	2-Isopropyl-5-methylphenyl	84–5	6.33	6.39	2×10^{-5}	>500	0.003
XXXVI	Phenyl	48–9	8.48	8.45	7×10^{-4}[c]	>500	>0.1

	N-Benzylcarbamate	M.P., ° C.	Calculated C	Calculated H	Found C	Found H	I_{50}[a]	LD$_{50}$ γ/G.	MLC[b] Thrips
XXXVII	m-tert-Butylphenyl	64.5	76.29	7.47	76.51	7.42	1×10^{-3}	500	>0.01
XXXVIII	o-tert-Butylphenyl	123.5–124.5	76.29	7.47	76.39	7.71	$>1 \times 10^{-3}$	>500	>0.01
XXXIX	p-tert-Butylphenyl	89–90	76.29	7.47	76.36	7.66	$>1 \times 10^{-3}$	>500	>0.1
XL	m-Tolyl[d]	77.0–77.5	74.66	6.27	73.82	6.07	$>1 \times 10^{-3}$	>500	>0.1
XLI	2-Isopropyl-5-methylphenyl	84–85	76.29	7.47	76.70	7.68	3×10^{-3}[c]	500	>0.01
XLII	Phenyl	83.5–84.5	73.99	5.76	74.30	6.05	8×10^{-3}[c]	>500	>0.1

	N-Phenylcarbamate	M.P., ° C.	C	H	C	H	I_{50}[a]	LD$_{50}$ γ/G.	MLC[b] Thrips
XLIII	Phenyl[e]	126.5–127.5	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.1
XLIV	m-Tolyl[f]	126–127	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.1
XLV	p-Tolyl[f]	115–16	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.01
XLVI	2-Methyl-5-isopropylphenyl[g]	139–40	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.1
XLVII	2-Isopropyl-5-methylphenyl[h]	106–7	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.1
XLVIII	p-Chlorophenyl[i]	137–8	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.1
XLIX	p-Bromophenyl[i]	144–5	...	...	...	...	$>1 \times 10^{-3}$	>500	>0.1

[a] Molar concentration for 50% fly-brain cholinesterase inhibition.
[b] Median lethal concentration, %.
[c] Extrapolated.
[d] Calcd. for $C_{15}H_{15}NO_2$: N, 5.81; found: N, 5.46.

[e] Eckenroth (14) reported m.p. 126° C.
[f] Fromm and Eckard (15) reported m.p., XLIV, 125° C.; XLV, 115° C.
[g] Weehuizen (40) reported m.p., 134–5° C.
[h] Weehuizen (41) reported m.p., 107° C.
[i] Hantzsch and Mai (23) reported m.p., XLVIII, 138° C.; XLIX, 144° C.

Table III. Cholinesterase Inhibition and Toxicity of Carbanilates and Phenyl Esters

	Compound	Fly Cholinesterase % Inhibition, $10^{-3}M$	Thrips % concn.	% mortality
L	Isopropyl m-chlorocarbanilate	0	0.1	3
LI	Isopropyl m-methylcarbanilate	0	0.1	43
LII	Isopropyl m-ethoxycarbanilate	0	0.1	65
LIII	m-tert-Butylphenyl propionate	13	...	..
LIV	o-tert-Butylphenyl isobutyrate	36	0.01	1
LV	Thymyl isobutyrate	5	0.01	0

determined on a Beckman DU spectrophotometer, with a constant temperature cell maintained at 37.5°. By comparison with standard curves relating concentration of phenol to absorbance, the concentration of phenol was determined.

The buffer used was 0.1M diethyl barbituric acid, which has a pH of 9.5, the approximate basicity required for development of color. This resulted in a convenient rate of hydrolysis with most of the compounds studied. However, the nitrophenyl carbamates hydrolyzed so rapidly at this pH that accurate measurements could not be made. Therefore, the hydrolysis of these compounds was studied in phosphate buffer (pH 7.3) and followed directly by measurement of the absorbance of the nitrophenate ion at 400 mμ. In some cases where the colored compound from the phenol and 4-aminoantipyrine was unstable, aliquots of the solution of carbamate in buffer were removed at intervals, and the color was developed and measured immediately.

The data for each compound were plotted as log percentage hydrolyzed against time, giving straight-line relationships. The true second-order K_{hyd} was then obtained from the expression:

$$K_{hyd} = \frac{2.303}{t} \log \frac{Co}{C} \times \frac{1}{[OH^-]} \ min.^{-1},$$

where C = concentration in time t and Co = original concentration of carbamic ester.

Discussion of Results

Relation of Structure To Toxicity The results presented in Tables I and II indicate the identity of the carbamates synthesized, their toxicity as contact insecticides to the female housefly, Musca domestica L., and to the greenhouse thrips, Heliothrips haemorrhoidalis (Bouché), and the molar concentration required in vitro for 50% inhibition of fly-brain cholinesterase. The more toxic compounds were also evaluated as contact toxicants to the green citrus aphid, Aphis spiraecola Patch, and to the citrus red mite, Paratetranychus citri (McG.).

They exhibited approximately the same degree of toxicity to the aphid as to the thrips, but the most effective compound (IX) produced only 73% mortality of the citrus red mite adult at 0.1% concentration. It is apparent that the phenyl esters of N-methylcarbamic acid are considerably more toxic than the corresponding N-ethylcarbamates. For example, to the thrips, m-tert-butylphenyl N-methylcarbamate (IX) was 21 times as toxic as m-tert-butylphenyl N-ethylcarbamate (XXXIV), while thymyl N-methylcarbamate (XXII) was 10 times as toxic as thymyl N-ethylcarbamate (XXXV). Similar differentials of 50 times and 14 times, respectively, occurred in the anticholinesterase activities of these pairs of compounds. The other N-ethyl compounds were nearly inactive, as were the N-benzyl and N-phenylcarbamates. These results are in good agreement with those of Haworth et al. (25), Gysin (27), and Stevens and Beutel (39), who found maximum toxicity to be associated with N-methyl substitution.

The nature and extent of substitution of the phenyl rings of the N-methylcarbamates exert a remarkable influence on toxicity. The order of effectiveness of single ring substituents upon cholinesterase inhibition was NO_2 < Cl < CH_3 < C_2H_5 < iso-C_3H_7 < $(CH_3)_2$-N < tert-C_4H_9 < $(CH_3)_3N^+I$, and the contact toxicity was roughly in the same order, with the exception of the $(CH_3)_3N^+I$ derivative (XX), which is apparently too polar in character to penetrate to the site of action. Thus, quaternization of m-dimethylaminophenyl N-methylcarbamate (XIX) increased the cholinesterase inhibition 50 times but decreased the contact toxicity to $^1/_{50}$. The order of effectiveness of ring position was $p < o < m$, both for cholinesterase inhibition and for toxicity. This, too, is in agreement with previous work involving $(CH_3)_3N^+$-substituted phenylcarbamates (38), and seems to be a definite indication of maximum fit or orientation at the enzyme surface, as reflected in similarity of configuration to the normal substrate, probably acetylcholine (7). Certain of the ortho-substituted derivatives such as chlorophenyl (XIV) and cyclohexylphenyl (XVII) are of considerable activity, and it is conceivable that the degree of fit with o-substituents, especially with such large groups as cyclohexyl, may be more nearly optimum than with m-derivatives.

Where multiple substituents in the ring are concerned, reinforcement of the toxicity of meta- alkylates results from additional alkylation in the ortho- or meta- positions, as in the 3,5-dimethylphenyl (XVIII), 2,3,5-trimethylphenyl (XXIII), 2-methyl-5-isopropylphenyl
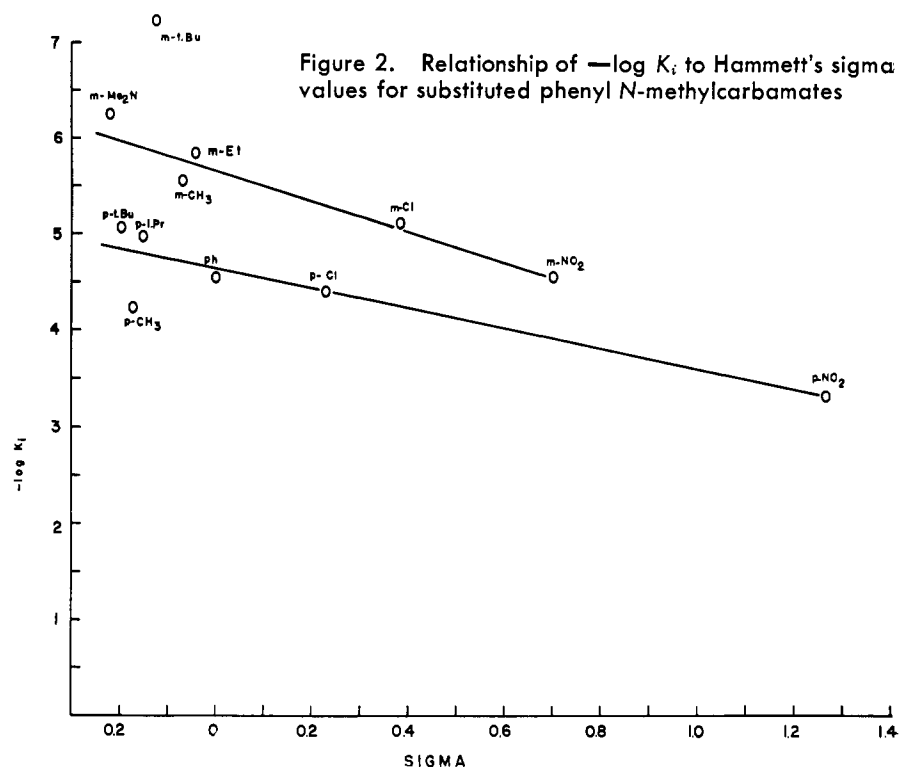

Figure 2. Relationship of —log K_i to Hammett's sigma values for substituted phenyl N-methylcarbamates

Table IV. **Hydrolysis Constants of Substituted Phenyl Esters of Some Carbamic Acids**

	Carbamate	True Second-Order K_{hyd} at 37.5° C., Min.$^{-1}$ in 1 M OH$^-$
XI	o-Nitrophenyl N-methyl	3.40×10^6
XIII	p-Nitrophenyl N-methyl	3.45×10^5
XLIII	Phenyl N-phenyl	5.76×10^3
XIV	o-Chlorophenyl N-methyl	2.0×10^3
XV	m-Chlorophenyl N-methyl	1.72×10^3
XVI	p-Chlorophenyl N-methyl	1.04×10^3
XLII	Phenyl N-benzyl	8.2×10^2
XXXVI	Phenyl N-ethyl	5.0×10^2
III	m-Tolyl N-methyl	3.01×10^2
II	o-Tolyl N-methyl	2.58×10^2
I	Phenyl N-methyl	2.46×10^2
VI	o-Isopropylphenyl N-methyl	54.5
VIII	o-tert-Butylphenyl N-methyl	28
XIX	m-Dimethylaminophenyl N-methyl	20
IX	m-tert-Butylphenyl N-methyl	4

(XXI), and 5-methyl-2-isopropylphenyl (XXII) N-methylcarbamates. There is an exception, however, in that an additional tert-butyl substituent in other than the meta position does not produce this effect, probably because of steric factors which prevent closeness of fit to the enzyme (XXIV). Additional ring substituents such as chloro- or nitro- do not enhance the toxicity (XXV, XXVI, XXVII, XXVIII, XXIX, XXX).

It was of interest to consider the behavior of two additional types of acetylcholine isoesters as inhibitors of fly cholinesterase and as toxicants. Alkylated phenyl propionates and iso-butyrates and isopropyl carbanilates have pronounced structural resemblances to the phenyl N-methylcarbamates. Several representative compounds of each type were evaluated (Table III).

Both classes of compounds were of very low activity in both tests. The inactivity of the phenyl esters is explained by the ease with which they are hydrolyzed by the insect esterase, appreciable competitive inhibition occurring only at high substrate concentration. (This will be discussed in a subsequent paper.) The inactivity of isopropyl carbanilate cannot be explained with certainty, but is probably due to steric factors which prevent attraction to the active site of the cholinesterase enzyme.

The view already expressed, that the toxic carbamates act as substrates for cholinesterase enzymes, which have a much lower turnover rate than acetylcholine, indicates that the most toxic compounds should be the most stable. This factor has been investigated for a representative series of compounds, and the true second-order hydrolysis constants are given in Table IV. These are plotted in Figure 1 as the logarithm of the hydrolysis constant, K_{hyd}, against the logarithm of the median lethal concentration to thrips.

The results indicate a good approximation to a straight-line relationship, and it seems well demonstrated that

in this series of compounds hydrolytic stability is essential for maximum enzyme inhibition and toxicity. The N-ethyl, N-benzyl, and N-phenylcarbamic acid esters are much more readily hydrolyzed than the corresponding N-methyl derivatives; this may account for part of the lack of toxicity, although steric and electronic factors, discussed later, may be involved.

Aldridge and Davison (4) have shown with a series of substituted diethyl phenyl phosphate compounds that exactly the converse is true—i.e., the most effective inhibitors are the ones most readily hydrolyzed. These apparently anomalous results can be reconciled by recalling that the phosphate inhibitors must be hydrolyzed by the enzyme before irreversible phosphorylation can occur, while the carbamates must be resistant to hydrolysis to effect inhibition.

An analysis of the biochemical activity of the substituted-phenyl N-methyl-

carbamates was made using the "sigma" coefficients of Hammett (22), who has summarized the changes in reactivity to be expected from meta- and para-substitution of the benzene ring. When log K_i (inhibition constant) of the carbamate (28):

$$K_i = \frac{I_{50} \times K_s}{K_s + S}$$

where

I_{50} = molar concentration of inhibitor for 50% inhibition

K_s = Michaelis constant for fly cholinesterase and acetyl-choline = 1.75×10^{-3} (37)

S = acetylcholine concentration = $10^{-2}M$

was plotted against "sigma," as in Figure 2, there was found to be good agreement to straight-line relationships for the meta- series and the para- series of substituents. The difference in the position of these two lines apparently results from the fact that optimum enzyme fit occurs with the meta- series as discussed above. The most pronounced deviation was that of m-tert-butyl, which can be accounted for by the relatively large bulk of this grouping, which permits maximum "fit" to the enzyme surface. A similar degree of correlation between the "sigma" coefficients and hydrolysis of ring-substituted benzylcholine derivatives by horse-serum cholinesterase has been demonstrated by Ormerod (34).

Toxicity vs. Cholinesterase Inhibition. From the data in Tables II and III it is clear that there is a direct relationship between the toxicity of the carbamate compounds and their activity as cholinesterase inhibitors. This is best illustrated by Figure 3, where the
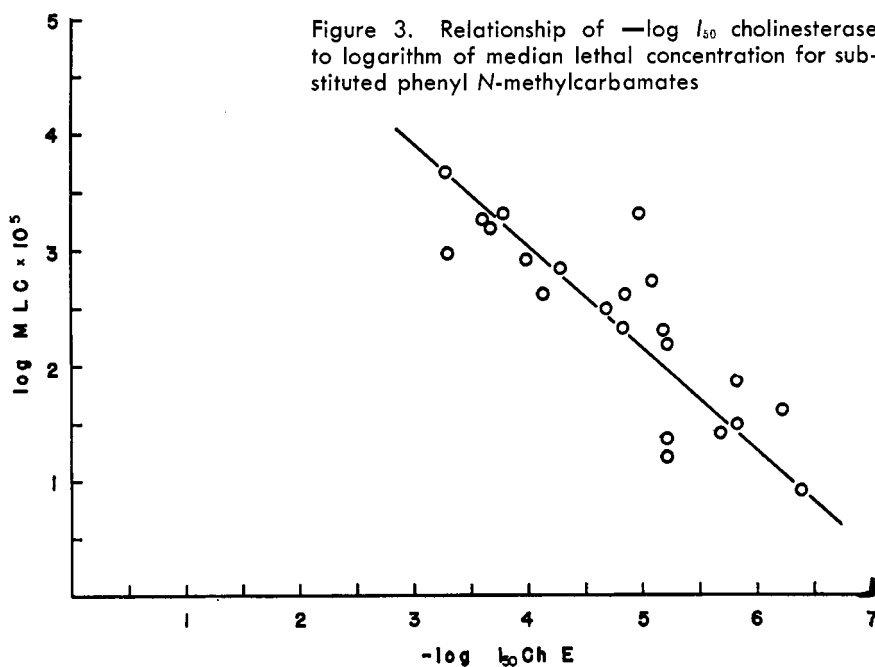

Figure 3. Relationship of —log I_{50} cholinesterase to logarithm of median lethal concentration for substituted phenyl N-methylcarbamates

logarithm of the median lethal concentration to the greenhouse thrips is plotted against the negative logarithm of the 50% inhibition for cholinesterase inhibition. The factors are clearly interdependent, and, considering the possible errors in the determination of the two sets of biological values, the agreement appears to be excellent. The data for thrips toxicity are more suitable for this purpose than the fly toxicity data because of the much greater range of toxicity exhibited by the carbamates to the thrips.

As a further demonstration of the mode of action of these compounds, 30 female houseflies were topically treated with each of two of the most toxic of the carbamates studied, and when in the prostrate stage of poisoning the heads were removed and homogenized, and the cholinesterase activity was measured and compared with an untreated lot. The following results show clearly the considerable inhibition of brain cholinesterase.

m-tert-Butylphenyl *N*-methylcarbamate, 5 γ per fly, 71% cholinesterase inhibition
m-Dimethylaminophenyl *N*-methylcarbamate, 2 γ per fly, 52% cholinesterase inhibition

The degrees of fly-brain cholinesterase inhibition found were not so complete as with flies poisoned with the organic phosphorus insecticides (*30, 32*). This is probably due to the well-known rapidly reversible nature of the carbamate cholinesterase inhibitors, which often partially dissociate from the enzyme during preparation of the samples for assay, and was reflected by the tendency of some of the flies paralyzed by these compounds to recover after several hours, as is characteristic with the pyrethrins.

Possible Mechanism Of Cholinesterase Inhibition Enzymatic reactions involving the hydrolysis and esterification of substrates appear to fall in the general class of acid-base catalyzed reactions. Schematically, the mechanism of the acid-catalyzed hydrolysis of acetylcholine may be formulated as follows (*18*):

$$(CH_3)_3\overset{+}{N}CH_2CH_2O-\underset{\underset{O}{\|}}{C}-CH_3 + HA \rightleftharpoons (CH_3)_3\overset{-}{N}CH_2CH_2-\underset{\overset{+}{X^-}}{\overset{H}{\underset{|}{O}}}-\underset{\underset{O}{\|}}{C}-CH_3 + A^-$$

$$\Big\updownarrow H_2O$$

$$BH + CH_3-\underset{\underset{O}{\|}}{C}-OH \underset{\overset{B}{\rightleftharpoons}}{} CH_3\underset{\underset{O}{\|}}{C}-\overset{+}{O}H_2 + (CH_3)_3\overset{+}{N}CH_2CH_2OH$$
$$X^-$$

HA and B are generalized acids and bases, respectively. An alternative mechanism which results in the same products attaches the proton to the carbonyl oxygen instead of the choline oxygen:

$$-CH_2-O-\underset{\underset{O}{\|}}{C}-CH_3 + HA \rightleftharpoons -CH_2-O-\underset{\underset{\overset{+}{O}}{\|}\atop H}{C}-CH_3 \underset{H_2O}{\rightleftharpoons} -CH_2-O-\underset{\underset{OH}{|}}{\overset{\overset{+}{HOH}\atop |}{C}}-CH_3$$

$$\downarrow$$

$$BH + CH_3-\underset{\overset{\diagup}{OH}}{\overset{O}{\diagdown}}C \rightleftharpoons CH_3-\underset{\underset{+}{OH}}{\overset{\diagup}{C}}\diagdown +-CH_2OH \rightleftharpoons -CH_2-\underset{\overset{+}{O}}{\overset{H}{\underset{|}{O}}}-\underset{\underset{OH}{|}}{\overset{|}{C}}-CH_3$$

Whether the proton is placed on the ether oxygen or on the carbonyl oxygen is immaterial in our argument, as is pointed out below, although the first scheme represented above is more generally accepted in view of the greater nucleophilic nature of the ether oxygen. The general belief is that enzymes consist almost entirely of proteins, in which are found sulfhydryl, carbonyl, amino, and other acidic or basic groups. The hydrolysis of an ester may, therefore, take place near or on the surface of an enzyme which furnishes both the acid and base necessary for a rapid reaction.

The concept of an acid-base enzyme-substrate intermediate has been advanced by a number of workers. Wilson, Bergmann, and Nachmansohn (*43*) have postulated for the cholinesterase-catalyzed hydrolysis of esters an intermediate in which a nucleophilic attack is made by the esteratic site on the carbonyl carbon atom, which closely resembles the basic hydrolysis of an ester mechanistically. Gawron, Grelecki, and Duggan (*17*) have suggested a similar mechanism for the wheat germ lipase hydrolysis of substituted phenyl acetates, but this differs in that both the carbonyl carbon and the ether oxygen of the ester group are considered to take part in the enzyme-substrate complex. Ormerod (*34*), from studies on the hydrolysis of substituted benzoylcholines by serum cholinesterase, suggests that the enzyme is first coordinated to the carbonyl carbon atom, displacing the alcohol moiety. Scott (*37*) postulates a similar mechanism for lipase-catalyzed hydrolysis in which the electrophilic carboxyl oxygen atom coordinates with a nucleophilic center on the enzyme.

The inhibition of cholinesterase hydrolysis of acetylcholine by the substituted phenylcarbamates appears to be one of competition and should satisfy the following criteria: The carbamate must possess the structural requirements to "fit well" on the enzyme; the carbamate must be reasonably stable to hydrolysis at the site; and the carbamate must remain firmly attached to the enzyme by a combination of its conformal fit and other intermolecular forces involved. It may be assumed, also, that the inhibiting carbamate is attached to the active site of the enzyme by the same forces and in the same manner as the substrate. From examination of Fisher-Taylor-Hirschfelder atomic models it can be seen that the interspatial distance between the carboxyl oxygens and the quaternary ammonium moiety of acetylcholine is remarkably similar to the distance between the carbamate oxygens and the *m*- positions of the benzene ring.

In line with recent investigations it appears that in addition to fit, the effect of structural changes on the reactivity of the substrate must also be considered. It is necessary, therefore, to examine the effect of R and the

substituent X on the reactivity of the carbamate linkage. An examination of the variation in R, the rest of the molecule remaining unchanged, shows that the order of effectiveness for cholinesterase inhibition is $R = CH_3 > CH_3CH_2 >> C_6H_5CH_2 > C_6H_5$. This variation in activity may be due to fit; however, it is possible that a part of the difference in activity may be attributed to the variation in electron-donating properties of the amido nitrogen atom. Examination of Hammett's sigma values shows that the ease with which the alkyl groups in question can release electrons is in the same order: $R = CH_3 > CH_3CH_2 > C_6H_5CH_2 > C_6H_5$.

Variations in the benzene ring substituent X, R constant, show that the order of increasing inhibitory activity corresponds roughly to the order of increasing electron-donating abilities. This is evident in the plot of Hammett's sigma constants against $-\log K_i$ shown in Figure 2. Also supporting this hypothesis is the fact that the addition of one or more electron-releasing substituents, such as an alkyl group besides the one already present, further increases the degree of inhibition. In view of these results a mechanism of inhibitory action may be suggested which clearly falls in line with the acid-base concept of substrate hydrolysis. The enzyme-carbamate complex may be depicted as follows:

OR
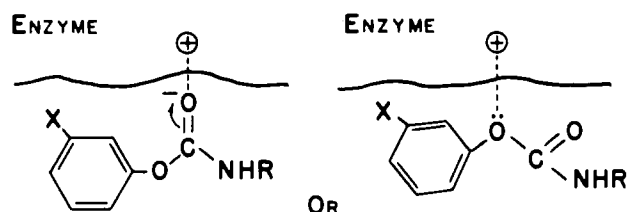
It is clear from the figures shown that any substituent X which releases electrons into the ring and toward the carbamate linkage will facilitate the formation of the complex by the increased electron densities in the neighborhood of the oxygen atoms. It is not clear, however, whether the complex formation takes place at the carbonyl or ether oxygen. Pfeiffer (35) suggests for acetylcholine and its analogs a three-point attachment to the enzyme at both the ether and the carbonyl oxygens and at the quaternary nitrogen. Whether the attachment is at either oxygen atom or at both, electron-donating substituents on the ring will still enhance complex formation. Inactivation of the carbamate may take place by hydrolysis, which is analogous
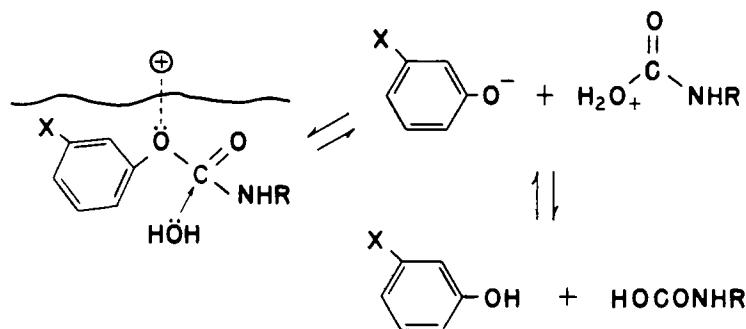
to the acid-base catalyzed mechanism. Although most of the data fit nicely into the mechanism suggested, the unusually high activity of m-dimethylaminophenyl N-methylcarbamate methiodide ($I_{50} = 1.2 \times 10^{-8}M$) and of o-chlorophenyl N-methylcarbamate ($I_{50} = 5 \times 10^{-6}M$) is unusual in that both

substituents are electron-attracting groups. The activity of m-dimethylamino methiodide substituted carbamate can be attributed to the positive charge of the quaternary nitrogen which plays a more active role in the complex formation. The activity of the o-chlorophenylcarbamate is readily explainable by the fact that the chlorine, which is naturally electronegative, can also release electrons into the benzene ring by an electromeric effect, depending upon the requirements of the reaction involved.

Acknowledgment

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