Alkaline Hydrolysis, Anticholinesterase, and Insecticidal Properties of Some Nitro-Substituted Phenyl Carbamates

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A number of alkyl-substituted nitrophenyl *N*-methyl- and *N,N*-dimethylcarbamates have been prepared and examined for alkaline hydrolysis, anticholinesterase activity, and toxicity to insects. The nitrophenyl *N*-methylcarbamates are generally exceedingly unstable under alkaline conditions and their poor anticholinesterase activity may in part be attributed to this high hydrolytic instability. The alkyl-substituted nitrophenyl *N*-methylcarbamates were slightly more stable and generally possessed higher anticholinesterase properties. Nitrophenyl *N,N*-dimethylcarbamates are stable under the same alkaline conditions and alkyl substituents in the three-position caused higher anticholinesterase activity. Practically all of the compounds used alone were poor in housefly and mosquito larvae toxicity, but several were strongly synergized by cotreatment with piperonyl butoxide. A detailed kinetic study of the alkaline hydrolysis of 4-nitrophenyl *N*-methylcarbamate was undertaken and mechanisms for the hydrolytic process and for the carbamylation of cholinesterase consistent with the hydrolytic mechanism are proposed.

Earlier investigations in this laboratory regarding the relationship among chemical structure, anticholinesterase activity, and insecticidal properties of substituted phenyl *N*-methylcarbamates have been based on the premise that these compounds inhibit the cholinesterase enzymes in a reversible manner by noncovalent bonding between the ring substituent and carbamyl moiety with the anionic and esteratic sites of the enzyme to form a tight enzyme-carbamate complex (*21*). This assumption was supported by previous work of others (*1*, *10*, *12*) who showed by kinetic means that the inhibition of acetylcholinesterase by prostigmine and physostigmine was reversible and competitive. Although there is other evidence supporting this mechanism (*27*, *33*), recent work by Wilson *et al.* (*34*, *35*) and others (*29*) indicates that these esters may inactivate cholinesterase by a reaction in which the esteratic site on the enzyme molecule is carbamylated in a manner analogous to inhibition by organophosphorus esters. This view has recently gained wide support.

One of the chief arguments against the carbamylation mechanism is the fact that compounds with a carbamate moiety of high intrinsic reactivity—e.g., *p*-nitrophenyl *N*-methylcarbamate—are generally poor inhibitors. On the other hand, carbamates of reasonable stability and whose structures are ideally complementary to the active site of the cholinesterase enzyme are the most potent inhibitors. More recently, Hansch and Deutsch (*14*) have examined data from this laboratory by π-σ analysis and have shown that the reactivity of the molecule as expressed by the σ constant does not improve correlation between structure and anticholinesterase activity, and that anticholinesterase activity can be excellently correlated with the π constants, a measure of the hydrophobic bonding between the substituent and active site of the enzyme. Since their analysis and other data from this laboratory (*21*) suggest that initial binding of the carbamate to the enzyme is an important factor in inhibition, the authors decided to examine a series of nitro-substituted phenyl *N*-methyl- and *N,N*-dimethylcarbamates in which an alkyl group was substituted also on the phenyl ring. The authors anticipated that the additional alkyl group would alter the properties of the carbamate molecule in two ways: The alkyl group would lower the reactivity of the carbamyl group by electronic and steric effects and would serve as a center of attraction to the anionic site on the enzyme molecule. In addition to the assessment of the effect of this added alkyl group, a detailed kinetic study of the alkaline hydrolysis of *p*-nitrophenyl *N*-methylcarbamate was undertaken to establish the mechanism of hydrolysis and for possible aid in elucidating the mechanism of the inhibition process. A recent report on certain aspects of the alkaline hydrolysis of *p*-nitrophenyl *N*-methylcarbamate (*3*) also prompts the authors to report their kinetic results at this time.

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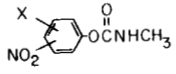
Experimental

Materials. All carbamates were prepared by reaction of the appropriate phenol with methyl isocyanate in anhydrous ether, using a trace of triethylamine as catalyst. The crystalline products were purified by repeated crystallization from benzene. The phenols were synthesized by known methods or obtained from commercial sources. New compounds were characterized by carbon and hydrogen analysis as shown in Table I. 2-,3-,4-Nitrophenyl, 2,4-dinitrophenyl,

Table I. Properties of Alkylnitrophenyl *N*-Methyl- and *N,N*-Dimethylcarbamates



	Substituent	M.P., ° C.	Analysis Calcd.	Analysis Found	I_{50}	k_o (pH 8.0), 27° C.	*Musca domestica*, μg. per Gram of Fly Alone	*Musca domestica*, μg. per Gram of Fly P.b.	*Culex pipiens quinque fasciatus*, P.P.M.
I	2-NO_2	87–8			5.0×10^{-3}	1.19	250	47.5	>10
II	3-NO_2	130–1			2.0×10^{-3}	0.039	>500	>500	>10
III	4-NO_2	156–61			3.0×10^{-3}	0.38	>500	>500	>10
IV	4-NO_2, 3-CH_3	98–103	C 53.14 H 4.76	52.34 5.14	3.2×10^{-4}	0.18	>500	>500	>10
V	4-NO_2, 3-C_2H_5	82–4	C 53.57 H 5.39	53.82 5.37	2.0×10^{-4}	0.14	>500	86.5	>10
VI	4-NO_2, 3-*i*-C_3H_7	87–90	C 55.45 H 5.92	55.58 6.02	2.8×10^{-6}	0.073	>500	14.5	5.5
VII	4-NO_2, 3,5-di-CH_3	107–8			4.2×10^{-6}	1.49×10^{-5}	>500	23.0	>10
VIII	2-NO_2, 3-CH_3	91–3			2.0×10^{-4}	0.44	67.5	17.5	>10
IX	2-NO_2, 4-CH_3	115–19			1.3×10^{-4}	0.31	>500	215	>10
X	2-NO_2, 5-CH_3	100–1	C 51.43 H 4.76	51.72 4.97	2.5×10^{-4}	0.59	>500	39.0	>10
XI	2-NO_2, 5-*i*-C_3H_7	105–10	C 55.45 H 5.92	55.40 6.01	2.3×10^{-4}	0.50	500	19.0	>10
XII	2-NO_2, 3,5-di-CH_3	129–33	C 53.57 H 5.96	53.20 5.48	$>1.0 \times 10^{-3}$	8.0×10^{-4}	>500	>500	>10
XIII	3-NO_2, 6-CH_3	99–102			2.3×10^{-5}	5.54×10^{-3}	>500	50	>10
XIV	3-NO_2, 4-CH_3	110–13			1.6×10^{-5}	0.01	>500	190	>10
XV	2,4-di-NO_2	98–101			$>1.0 \times 10^{-3}$	...	>500	>500	>10
XVI	3,5-di-NO_2	106.5–8.5	C 39.84 H 2.93	39.90 3.23	$>1.0 \times 10^{-3}$	...	>500	>500	>10
XVII	4,6-di-NO_2, 2-CH_3	133.5–4.5			4.0×10^{-3}	...	>500	...	...

N,N-DIMETHYLCARBAMATES

XVIII	4-NO_2	105–6	C 51.42 H 4.86	51.74 4.69	6.3×10^{-4}	3.8×10^{-4} [a]	>500	200	>10
XIX	4-NO_2, 3-CH_3	56–7	C 53.57 H 5.40	53.98 5.08	9.5×10^{-5}	2.8×10^{-4}	>500	105	>10
XX	4-NO_2, 3-C_2H_5	126–7	C 55.45 H 5.92	55.77 6.06	2.5×10^{-5}	3.0×10^{-4}	>500	185	>10
XXI	4-NO_2, 3-*i*-C_3H_7	120–2 (0.2 mm.)[b]	C 57.11 H 6.39	57.01 6.10	3.0×10^{-6}	...	>500	28	4.9
XXII	2,4-di-NO_2	100–2	C 42.36 H 3.55	42.73 3.76	3.0×10^{-4}	...	>500	>500	>10
XXIII	3,5-di-NO_2	77–9	C 42.36 H 3.55	43.51 3.26	5.7×10^{-4}	...	>500	>500	>10

[a] First-order rate constants in 0.01 *M* sodium hydroxide for *N,N*-dimethylcarbamates. [b] Boiling point.

3,5-dinitrophenyl *N*-methylcarbamates (*16*), and 3,5-dimethyl-4-nitrophenyl *N*-methylcarbamate (*22*) have been reported. 3-Methyl-2-nitrophenyl, 4-methyl-2-nitrophenyl, 6-methyl-3-nitrophenyl, and 4-methyl-3-nitrophenyl *N*-methylcarbamates were obtained from the Upjohn Co., Kalamazoo, Mich. The techniques for the determination of the inhibition of fly head cholinesterase (*25*) and the insecticidal activities of the female housefly (*Musca domestica*) and the mosquito larva (*Culex pipiens quinquefasciatus*) have been described (*20*).

The *p*-nitrophenyl *N*-methylcarbamate used for kinetic studies was recrystallized several times from benzene (m.p. 159–61° C.). The product reported by Dittert and Higuchi (*9*) in their kinetic work was prepared by nitration of phenyl *N*-methylcarbamate (m.p. given as 105–109° C.). *tert*-Butyl alcohol was refluxed and distilled over lithium aluminum hydride under prepurified nitrogen [b.p. 81–81.2° C. (atm.)] and stored under nitrogen. Potassium *tert*-butoxide was prepared by dissolving potassium in *tert*-butyl alcohol and its molarity was estimated with standard hydrochloric acid. Acetone was dried over calcium chloride and distilled, (b.p. 56–7° C.). Benzenethiol, Eastman White Label grade, and phenol, Matheson Coleman & Bell reagent grade, were purified according to Quale and Royals (*28*). Imidazole [m.p. 88–90° C.] was Eastman White Label grade, used without further purification. Pyridine, dried over sodium hydroxide pellets, was Baker and Adamson reagent grade material.

Buffer Solutions. Sorensen's phosphate buffer solutions were prepared according to the usual method (*13*) by mixing appropriate amounts of 0.1*M* monobasic potassium phosphate and 0.1*M* dibasic sodium phosphate to obtain the desired pH. The ionic strengths listed in Table I were calculated from the concentrations of the phosphate salts before and after appropriate dilution. Phenoxide ion–catalyzed hydrolyses were carried out in 0.025*M* phosphate buffer with sodium chloride added to give an ionic strength of 0.075. Imidazole and pyridine buffers were prepared by adding appropriate amounts of hydrochloric acid to the base to obtain the desired pH and sodium chloride was added to maintain constant ionic strength. All pH values were checked before and after each kinetic run. The concentration of the base in each buffer solution was calculated from the pK_a values of the conjugate acids.

Kinetic Methods. Decomposition rates of the various nitrophenyl *N*-methylcarbamates were determined colorimetrically by estimating the amount of the corresponding nitrophenoxide ion formed after measured time intervals in a Beckman DU spectrophotometer at 394 to 404 mμ. All nitrophenols and the corresponding carbamates were examined in a Unicam SP 800 recording spectrophotometer to show that overlaps in pertinent absorption peaks were not present. All solutions were brought up to the desired temperature by incubation in a water bath (±0.05° C.) prior to mixing. The cell compartment of the spectrophotometer was maintained at the same temperature by means of a circulating water bath. The following general procedure was used in the kinetic runs.

Five milliliters of buffer solution or *tert*-butyl alcohol solution were brought to thermal equilibrium in a standard tapered test tube. To this was added 0.2 ml. of 0.01% solution of *p*-nitrophenyl *N*-methylcarbamate (w./v.) in acetone. The mixture was shaken rapidly, an aliquot was transferred to a thermostated cell, and the color was estimated at different time intervals at 400 mμ. Time zero was taken as the moment of addition of carbamate and the initial reading followed after 30 to 60 seconds. Pseudo-first-order rate constants, k_o, in buffer were determined by plotting $\log e_\infty - e_t$ against time, where e_∞ is the absorbance at completion and e_t is the absorbance at time t. Good first-order plots were obtained over the period of reaction. Second-order rate constants in *tert*-butyl alcohol were calculated from the usual equation,

$$\frac{2.3}{a-b}\log\frac{b(a-x)}{a(b-x)} = kt$$

where a is concentration of carbamate, b is concentration of base, and x is concentration of *p*-nitrophenoxide determined from a standard curve. The liberation of *p*-nitrophenoxide followed good second-order kinetics over 90% reaction.

Results and Discussion

Cholinesterase Inhibition. The fly head anticholinesterase data in Table I show that nitro-substituted phenyl *N*-methylcarbamates are generally poor inhibitors of this enzyme. Much of this poor activity may be attributed to their very high susceptibility to hydrolysis. The values for the first-order rate constants for hydrolysis, k_o, in 0.1*M* Sorensen's phosphate buffer at pH 8.0 and 27° C. for representative nitrophenyl *N*-methylcarbamates are given in Table I. Obviously, from the hydrolysis constants, the various nitrophenyl *N*-methylcarbamates are extraordinarily unstable in alkaline media, even at pH 8.0. From first-order hydrolysis constant of 4-nitrophenyl *N*-methylcarbamate at pH 7.5, as given in Table II, the authors estimate that about 97% of this compound is hydrolyzed during the period when determinations are being made for cholinesterase inhibition. Thus, during the inhibition process the actual concentration of carbamate at any given time during inhibition evidently is considerably lower than the initial concentration. The fact that inhibition does occur to some extent would indicate that the compounds given in Table I are intrinsically better inhibitors than estimated from the I_{50} values.

Substitution of alkyl groups in the three-position of the phenyl ring gave compounds (IV to VI) which increase in anticholinesterase activity as the alkyl group is changed from methyl to ethyl to isopropyl. This increase may be attributed in part to increased stability to hydrolysis. For example, the authors estimate that approximately 50% of 3-isopropyl-4-nitrophenyl *N*-methylcarbamate (VI) remains unhydrolyzed at the end of the determination for anticholinesterase activity. This value was obtained by assuming that the change in k_o for this compound in going from pH 8.0 (value given in Table I) to pH 7.5 is in the same relative proportion as the change in k_o for *p*-nitrophenyl *N*-methylcarbamate under the same conditions. The value of 50% unhydrolyzed must be taken as approximate but is probably close to the actual value, since these two compounds should have similar activation energies for hydrolysis. Thus, under equal conditions approximately 17 times more 3-isopropyl-4-nitrophenyl carbamate (VI) would remain unhydrolyzed than the 4-nitrophenyl analog during the period needed for analysis. However, since VI is over 1000 times better as an inhibitor than III, apparently the 3-alkyl group, in addition to increasing stability, plays an even more important role in the inhibition process by increasing

Table II. Hydrolysis Rate Constants of *p*-Nitrophenyl *N*-Methylcarbamate in Phosphate Buffer Solutions Containing 3.8% Acetone at 27° C.

Total Phosphate Ions, *M*	Temp., °C.	pH	μ	k_{obsd} × 10^2	pH	μ	k_{obsd} × 10^2	pH	μ	k_{obsd} × 10^2
0.10		7.0	0.222	3.50	7.5	0.268	11.8	8.0	0.290	38.0
0.05			0.111	4.19		0.134	11.4		0.145	40.6
0.01			0.022	5.36		0.027	17.0		0.029	50.0
0.00			0.00	6.0		0.00	20.0		0.00	55.4
0.10		7.3	0.254	7.55	7.8	0.283	25.3			
0.05			0.127	8.55		0.141	26.1			
0.01			0.025	10.6		0.028	33.5			
0.00			0.00	11.6		0.00	38.8			
0.10	18.8	8.0	0.290	13.7						
0.05			0.145	14.4						
0.01			0.029	20.0						
0.00			0.00	22.0						
0.01	20.8	7.0	0.022	1.7						
	27.0		0.022	3.92						
	38.8	7.0	0.022	9.42						

noncovalent binding between the enzyme and carbamate by interaction between the alkyl substituent and anionic site.

Of some interest is the high anticholinesterase activity of 3,5-dimethyl-4-nitrophenyl *N*-methylcarbamate (VII) relative to the 3,5-dimethyl-2-nitrophenyl isomer (XII). Compared to the other monomethylcarbamates for which hydrolysis data are given, VII is exceedingly stable with a k_o value of 1.49×10^{-5} per minute. Thus this compound is about 4800 times more stable to hydrolysis than 3-isopropyl-4-nitrophenyl *N*-methylcarbamate (VI), but both compounds are about equal in anticholinesterase activity. The very low inhibitory activity of XII is difficult to explain, since the other monomethyl-substituted 2-nitrophenyl *N*-methylcarbamates (VIII to XI) showed slight but definite activity. Like VII, XII shows high stability to alkaline hydrolysis and degradation to inactive phenol is not a factor.

The two methyl-3-nitrophenyl carbamates (XIII and XIV) were somewhat more active as cholinesterase inhibitors than any of the methyl-2-nitro or methyl-4-nitro isomers examined. This probably is due largely to the greater stability of 3-nitro-substituted carbamates compared to 2- or 4-nitro compounds (compare k_o for I, II, and III). The dinitro-substituted compounds (XV, XVI, and XVII) showed little or no activity and this may be explained on the basis of their intrinsically greater instability to hydrolysis.

In comparison to the monomethylcarbamates the nitrophenyl *N,N*-dimethylcarbamates are exceedingly stable to hydrolysis. The first-order rate constants given in Table I were determined in 0.01*M* sodium hydroxide instead of phosphate buffer, and the hydrolysis needed to proceed for several weeks before a rate constant could be calculated. The data also show that except for the 3-isopropyl-4-nitro isomers (VI and XXI) the *N,N*-dimethylcarbamates are more potent anticholinesterases than the corresponding monomethylcarbamates (compare II with XVIII, IV with XIX, V with XX, XV with XXII, and XVI with XXIII). VI and XXI are about equal as inhibitors. As with the monomethylcarbamates, there was a gradual increase in inhibitory activity with increasing size in the alkyl group in the three-position.

Several points of interest emerge from the data in Table I and the preceding discussion. Obviously, compounds of extremely high intrinsic reactivity are generally poor inhibitors of fly head cholinesterase. Low anticholinesterase activity in these cases may be attributed to their high instability in aqueous media, leading to rapid breakdown of the carbamate during the determination of cholinesterase inhibition by the Warburg method. High instability, however, does not account for all examples of low inhibitory activity—e.g., 3-nitrophenyl *N*-methylcarbamate (II) is ten times more stable than the 4-nitro isomer, yet these compounds are about equal in activity, although both are poor.

Evidently, from the data, alkyl groups in the ring greatly enhance activity and enhancement probably is due to greater initial binding between the alkyl-substituted nitrophenyl carbamate and the enzyme. Regardless of the mechanism accepted for inhibition of cholinesterase by carbamates (carbamylation or reversible), there appears to be general agreement that the enzyme and carbamate interact to form a complex. The carbamylation mechanism as postulated by Wilson (*35*) is shown below, where $k_{-1}/k_1 = K_i$ determines the

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

degree of EI complex formation between enzyme and carbamate, k_2 is the carbamylation constant, and k_3 the decarbamylation constant. For reversible inhibition, the reaction sequence will end with EI, and inhibition is presumed to be due to a tightly bound complex. By either scheme, alkyl substitutes in key positions in the ring will increase the degree of inhibition by increasing the level of complex formation. Thus, by the carbamylation mechanism the over-all rate of carbamylation to EI′ will increase because of more favorable K_i values.

The carbamylation constant, k_2, should decrease with alkyl substitution, since this process should be a function of the reactivity of the molecule. The high anticholinesterase activity of compounds with low inherent reactivity as estimated by hydrolysis data—e.g., 3,5-dimethyl-4-nitrophenyl *N*-methylcarbamate (VII) and 3-isopropyl-4-nitrophenyl *N,N*-dimethylcarbamate (XXI)—therefore must be attributed to low K_i values which favor EI complex formation. 3,5-Dimethylphenyl *N*-methylcarbamate, $I_{50} = 6.0 \times 10^{-6}M$ (*11*), is slightly less active as an inhibitor than VII and this difference may be due to a higher k_2 value for VII, assuming, of course, that carbamylation is the principal means of inhibition, as has been suggested by others (*29*). On the other hand, the substantially lower anticholinesterase activity of 3-isopropyl-4-nitrophenyl *N*-methylcarbamate (VI) compared to 3-isopropylphenyl *N*-methylcarbamate, $I_{50} = 3.4 \times 10^{-7}$ (*24*), is difficult to rationalize by a carbamylation mechanism since VII is probably considerably more reactive than the nitro-deficient compound. However, the nitro moiety adjacent to the 3-isopropyl group may alter the K_i value for VII by steric or polar effects.

Insect Toxicity. Insect toxicity data in Table I show that practically all of the carbamates without synergist are nontoxic to the housefly and mosquito larvae, results which are generally consistent with the low anticholinesterase activity. A few of the compounds, however, including those with both low and high inhibitory activity, were synergized to different degrees by cotreatment with piperonyl butoxide. For example, the toxicities of 3-isopropyl-4-nitro- (VI) and 3-isopropyl-2-nitrophenyl *N*-methylcarbamate (XI) were increased significantly to almost equal toxic levels in spite of their approximately 80-fold difference in anticholinesterase activity. Since enhancement of toxicity by such compounds as piperonyl butoxide has been attributed to the inhibition of detoxication of the toxicant (*23*), the high degree of synergism obtained for VI is in line with its relatively high anticholinesterase activity. On the other hand, the equally high synergism shown by XI, a compound of low anticholinesterase activity, is without explanation.

The relatively high toxicity of 3-methyl-2-nitrophenyl *N*-methylcarbamate (VIII) against houseflies defies explanation because of its poor anticholinesterase activity and high hydrolytic instability. The phenol, 3-methyl-2-nitrophenol, also was examined against houseflies and found to be nontoxic at 500 μg. per gram of fly.

The hydrolytically more stable dimethylcarbamates also were synergized to different degrees. Highest synergism was found with 3-isopropyl-4-nitrophenyl *N,N*-dimethylcarbamate (XXI), the most effective anticholinesterase among the dimethylcarbamates examined.

The fact that compounds of both low and high hydrolytic stability (compare VI and VII) are each strongly synergized suggests that a hydrolytic detoxication mechanism is not involved in piperonyl butoxide synergism. As has been demonstrated with other carbamates, piperonyl butoxide probably interferes with phenolase-type enzymes and these enzymes appear to be degrading nitro-substituted carbamates at a rate even greater than that occurring by nonenzymatic hydrolysis.

p-Nitrophenyl N-Methylcarbamate Hydrolysis. Previous reports on carbamate hydrolysis have dwelled largely on alkaline hydrolysis of a number of different carbamate esters, primarily for comparative purposes to establish a relationship between anticholinesterase activity and hydrolytic stability (*6*, *16*). However, aside from the work of Bender and Homer (*3*) in which the catalytic effect of imidazole and phosphate dianion is reported, the effects of bases other than hydroxide ion have not been studied nor whether the rate of hydrolysis is a function of the nucleophilicity or of the basicity of the attacking basic molecule. For this study, *p*-nitrophenyl *N*-methylcarbamate was selected because of its unusual sensitivity to basic hydrolysis and for the ease with which the *p*-nitrophenoxide ion can be measured spectrophotometrically. Dittert and Higuchi (*9*) have reported on a detailed kinetic study of the hydroxide ion–catalyzed hydrolysis of this compound, but their data indicate that the compound examined as *p*-nitrophenyl *N*-methylcarbamate is not correct. Bender and Homer (*3*) have more recently reported on the kinetic analysis of the hydrolysis of this carbamate and their results generally are consistent with those reported here.

The observed first-order rate constants, k_o, for the liberation of *p*-nitrophenoxide from *p*-nitrophenyl *N*-methylcarbamate in phosphate buffers of different ionic strength and pH values between 7.0 and 8.0 at different temperatures are given in Table II. This compound was found to be extremely sensitive to hydroxide ion and at high concentrations of base the liberation of *p*-nitrophenoxide was too fast to be estimated by conventional methods. In fact, even in Sorensen's phosphate buffer of pH 8.0 (0.1*M* mixture of disodium hydrogen and monopotassium dihydrogen phosphate) at 29.0° C. the hydrolysis of the carbamate was essentially complete in less than 10 minutes.

The data in Table II show that k_o increases with decreasing values of ionic strength μ, μ being established by varying the concentration of the phosphate buffer. This indicates that the different phosphate anions do not participate in the hydrolytic reaction and the lowering of the rate constant with increasing μ is due to the decrease in the activity of hydroxide ion or of the carbamate. This is in disagreement with the results of Bender and Homer (*3*), who found a slight acceleration in rate with increasing phosphate concentration, particularly at higher pH (7.712).

The second-order constant, k_b, for hydroxide ion and carbamate and the solvolysis constant, k_w, for water were calculated by plotting the extrapolated values of k_o at $\mu = 0$ against hydroxide ion concentration, assuming that the following equation is valid:

$$k_o = k_b[OH^-] + k_w[H_2O]$$

From the intercept and slope of the straight line obtained in Figure 1, the values of k_b and k_w at 29.0° C. were calculated to be 5.44×10^3 and 1.22×10^{-4} liter per mole per minute, respectively. These rate constants indicate that solvolysis by water contributes negligibly to the observed hydrolytic rate despite the preponderance of water molecules in the reaction mixture. At pH 8.0 approximately 98.5% of the rate is calculated to be due to hydroxide ions.

From the observed first-order rate constants at different temperatures, $\Delta H\pm$ was calculated to be 14.4 kcal. and $\Delta S\pm$ was 4.4 entropy units.

Imidazole and pyridine, both weak bases, showed little or no catalytic effect in aqueous media (Table III). Increasing the concentration of base did not significantly

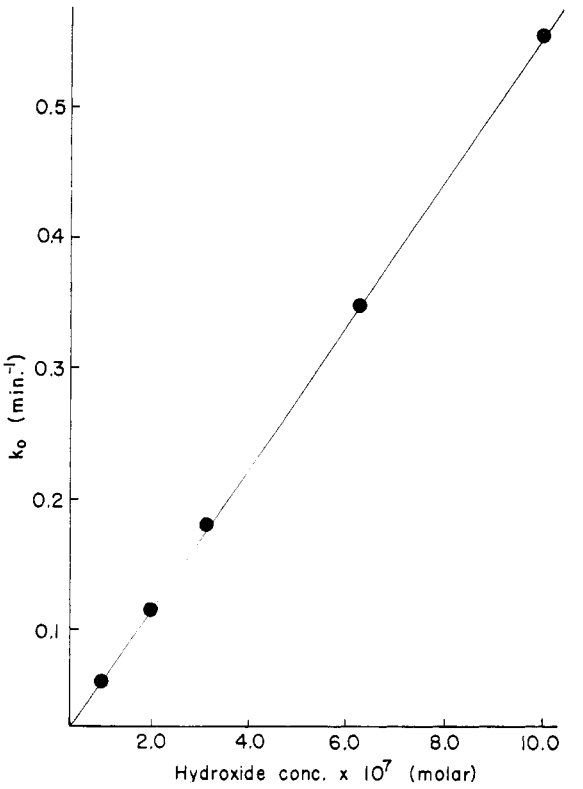


Figure 1. Linear relationship between observed first-order rate constant, k_o, and hydroxide ion concentration for *p*-nitrophenyl *N*-methylcarbamate hydrolysis in phosphate buffer

Table III. Effect of Phenolate Ion, Imidazole, and Pyridine on Hydrolysis of *p*-Nitrophenyl *N*-Methylcarbamate in Aqueous Solutions at 27° C.

Base	pH	μ	$k_{obsd} \times 10^2$ (Control)[a]	Concn. of Base, *M*	$k_{obsd} \times 10^2$
Phenolate[b]	7.0	0.075	2.88	6.44×10^{-6}	3.84
ion	7.3	0.075	7.0	12.8	8.25
	7.5	0.075	11.8	20.3	13.7
	7.8	0.075	21.0	40.6	26.2
	8.0	0.075	32.6	64.4	39.3
Imidazole[b]	7.0	0.236		2.61×10^{-1}	3.16
		0.236		1.32×10^{-1}	3.20
		0.236		7.92×10^{-2}	3.13
		0.236		2.64×10^{-2}	3.02
		0.236		2.64×10^{-3}	3.01
Pyridine[b]	6.6	0.017		9.83×10^{-1}	1.61
		0.017		4.93×10^{-1}	1.67

[a] Control presents hydrolysis rates in absence of phenolate ions. [b] Ionic strength kept constant by addition of sodium chloride.

increase the observed rate constant and in these cases k_o is due essentially to hydroxide ion in the system. In contrast to these, phenoxide ion strongly catalyzed the hydrolysis of *p*-nitrophenyl *N*-methylcarbamate, the second-order rate constant, k_p, being calculated as 1.13×10^3 liters per mole per minute ($\mu = 0.075$). The value of k_p for phenoxide was determined from the following relationship, where k_o' is the observed rate constant in phenoxide solution:

$$k_o' = k_w[H_2O] + k_b[OH^-] + k_p[C_6H_5O^-] = k_o + k_p[C_6H_5O^-]$$

Thus, by plotting the values of $k_o' - k_o$—i.e., the observed rate constant with phenol added, minus the observed rate constant for buffer solution alone—against the phenoxide ion concentration calculated from the pH of the buffer and ionization constant of phenol (*26*) ($K_a = 1.15 \times 10^{-10}$), a straight line is obtained (Figure 2) with zero intercept and slope equal to k_p.

p-Nitrophenyl *N*-methylcarbamate is extremely sensitive to hydroxide ion in aqueous solutions as shown by the second-order rate constant for the liberation of *p*-nitrophenol. The value for k_b for this compound is approximately 10^7 times greater than that found for the corresponding *p*-nitrophenyl *N,N*-dimethylcarbamate ($k_b = 3.4 \times 10^{-2}$ liter per mole per minute at 27° C.), a value which is in close agreement with that reported by other investigators (*6*, *9*). This large difference in k_b cannot be accounted for by electronic or steric effects imposed upon the carbamyl moiety by an additional methyl group on nitrogen. The difference in rates more likely is due to a different mechanism of reaction. The reaction between hydroxide ion and *p*-nitrophenyl *N,N*-dimethylcarbamate probably occurs by attack of the base on the carbonyl carbon atom in which there is a direct displacement of *p*-nitrophenoxide in a manner similar to base-catalyzed cleavage of *p*-nitrophenyl acetate (*4*, *5*). On the other hand, *N*-methylcarbamate hydrolysis can proceed by attack of the base either at the carbonyl carbon or at the *N*-proton. This alternative route has recently been invoked to explain the facile hydrolysis of *N,N'*-dialkylphosphorodiamidic chloride compared to the *N,N'*-tetraalkyl derivative (*8*, *32*). Because of this large difference in k_b between the two carbamates and the authors' findings that *p*-nitrophenoxide liberation is a function of the basicity and not the nucleophilicity of the base, *N*-methylcarbamate hydrolysis probably occurs by attack on the nitrogen hydrogen atom.

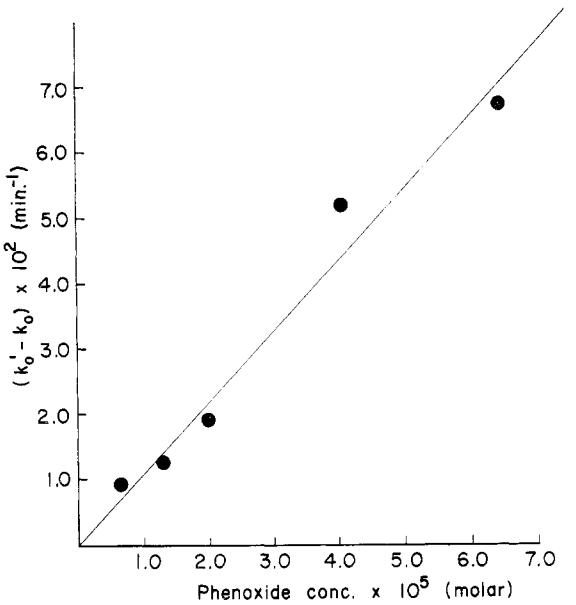


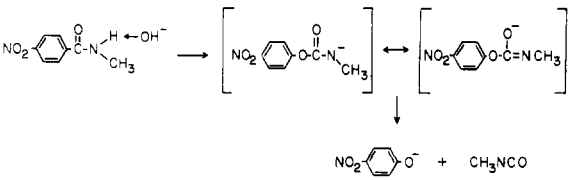
Figure 2. Relationship between $k_o' - k_o$ and phenoxide ion concentration for phenoxide-catalyzed hydrolysis of *p*-nitrophenyl *N*-methylcarbamate

Catalytic effects found with bases of different basicities and nucleophilicities provide evidence that *p*-nitrophenyl *N*-methylcarbamate hydrolysis occurs by attack of the base on the *N*-proton. Since basicity is determined by the free energy difference between species when free and when bonded to a hydrogen 1*s* orbital,

while nucleophilicity is a function of the free energy difference between species free and bonded to a carbon 2*p* orbital (*30*), *N*-proton removal from the carbamyl moiety should be a function of basicity and not nucleophilicity of the attacking base. With the bases examined in water and in *tert*-butyl alcohol there was, in general, good correlation between *p*-nitrophenoxide liberation and basicity. The calculated second-order rate constant with hydroxide ion was approximately 7.4×10^6 times greater than with imidazole ($k = 7.35 \times 10^{-2}$ liter per mole per minute at 27° C.), in general agreement with the 10^7-fold difference between the dissociation constants of water and the conjugate acid of imidazole. In contrast to this, the hydrolysis of *p*-nitrophenyl acetate which has been shown to occur by nucleophilic attack by imidazole at the carbonyl carbon atom in which an *N*-acetylimidazole intermediate is formed, takes place at a rate only $^1/_{23}$ of that with hydroxide ion (*4*, *5*). Pyridine in water gave little or no catalysis, as expected because the basicity of hydroxide ion is roughly 10^9-fold greater (*15*). The large difference in *p*-nitrophenyl *N*- methylcarbamate hydrolysis between hydroxide ion and pyridine is also in considerable variance to their relative nucleophilicities (*30*) (hydroxide ion 4.2, pyridine 3.6) as estimated by their nucleophilic constants for reaction at a saturated carbon atom and also to their relative reactivity with *p*-nitrophenyl acetate (k_b, liter per mole per minute, pyridine 0.1, hydroxide ion 890) (*15*).

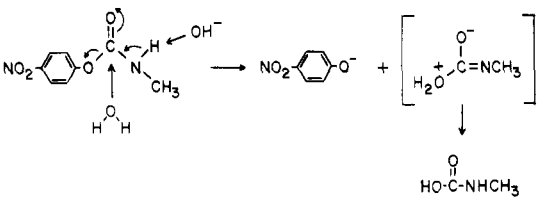
Because of the high sensitivity of *p*-nitrophenyl *N*-methylcarbamate to hydroxide ion and the limitations of an aqueous system due to the reaction of weak bases with water to give hydroxide ion, studies were carried out also in anhydrous *tert*-butyl alcohol. Miller (*26*) has used this solvent in examining rates of reaction between *O*,*O*-diphenyl phosphorochloridothioate and different bases. The data on *tert*-butyl alcohol in Table IV give the relationship between *p*-nitrophenyl *N*-methylcarbamate decomposition and basicity of *tert*-butoxide, phenoxide, *tert*-butylmercaptide, and phenylmercaptide ions. The significantly lower activity of phenylmercaptide compared to phenoxide in spite of 10^3 greater nucleophilicity of the former in displacement reactions at a carbon center adds support that the primary step in the reaction occurs at the *N*-proton. In general, mercaptide ions have been shown to be more reactive than analogous oxygen anions toward several carboxylic acid derivatives (*2*, *15*).

Two possible mechanisms in which the *N*-proton is involved can be suggested. The first postulates the formation of methylisocyanate as an intermediate in the manner shown below:



Methylisocyanate, of course, will react instantaneously with water to form *N*-methylcarbamic acid, which will eventually give methylamine and carbon dioxide. This mechanism was first suggested by Dittert and Higuchi (*9*), but is based on data obtained from a compound of dubious structure. Bender and Homer (*3*) also have suggested the same mechanism from their kinetic data and isotopic exchange studies.

The alternative mechanism which is kinetically indistinguishable from the first involves a water molecule in the transition state as follows:



The two mechanisms differ in that an ion intermediate is postulated in the first one, while the second involves a water molecule in the transition state. This assistance might be partially responsible for the large magnitude of the rate constant. This alternate mechanism has the advantage that the carbamate never becomes a charged ion at any stage. The ion intermediate in the first mechanism is probably one of high energy, even in water solution; hence a route involving it may not be preferred (*31*).

Carbamylation Mechanism. Recent evidence (*29*, *34*, *35*) has indicated that esters of carbamic acid which inhibit the cholinesterase enzymes do so by a process in which the enzyme molecule is carbamylated in a manner somewhat similar to inhibition by organophosphorus esters. Although there is no general agreement on the over-all mechanism of carbamate inhibition, the evidence is conclusive that carbamylation does occur and

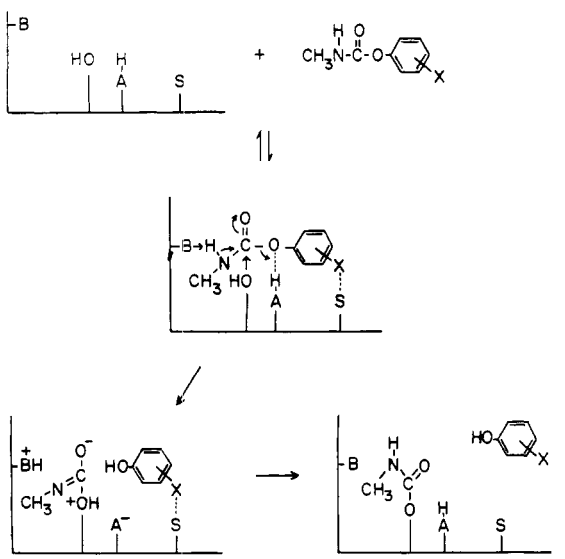
Table IV. Hydrolysis Rate Constants in *tert*-Butyl Alcohol by Different Bases at 27° C.

Conjugated Acid of Base	p*K*	Base Concn. × 10^5	Carbamate Concn. × 10^5	Second-Order Rate Constant, Liters/Mole/Minute
Phenol	9.94	6.2	2.1	1.10×10^4
Benzenethiol	6.52	6.2	1.96	2.91×10^3
tert-Butylmercaptan[a]	10.6	6.2	2.06	8.1×10^3
tert-Butyl alcohol	19.0	6.2	2.16	2.28×10^4

[a] Taken to be about equal to *n*-butylmercaptan, since *tert*-butyl alcohol has a p*K* about equal to that of *n*-butyl alcohol (*19*).

therefore is responsible for at least part of the inhibition. In the light of the results obtained in this investigation, postulation of a mechanism for the carbamylation process for monomethylcarbamates which is consistent with the authors' hydrolytic data seems appropriate.

There have been numerous investigations concerned with the nature of the vital centers in the cholinesterase molecule (*7*). Recently, Krupka and Laidler (*17, 18*) proposed a mechanism for cholinesterase hydrolysis of acetylcholine which logically accounts for the function of the various groups believed to be part of the active site. By using their simplified model of the active site, the following mechanism for carbamylation is postulated:



Here, B is the basic group, HA the acidic group, HO— the serine hydroxyl, and S the anionic site. According to this scheme, carbamylation of the serine hydroxyl group is effected by a process closely analogous to mechanism 2 postulated for the hydrolysis of *p*-nitrophenyl *N*-methylcarbamate. The serine hydroxyl, thus carbamylated, is unable to serve its normal function in acetylcholine hydrolysis. Regeneration of the decarbamylated enzyme is expected to occur somewhat slowly, since carbamates of aliphatic alcohols are considerably more stable to alkaline hydrolysis than aromatic carbamates—e.g., the second-order rate constant for hydroxide ion with ethyl *N,N*-dimethylcarbamate, ethyl *N*-methylcarbamate, and unsubstituted ethyl carbamate has been reported as 2.7×10^{-4}, 3.4×10^{-4}, and 1.2×10^{-3} liter per mole per minute at 25° C., respectively (*9*). Further, the spontaneous regeneration rates of cholinesterase from enzyme carbamylated with *N,N*-dimethyl-, *N*-methyl-, and unsubstituted carbamates are consistent with hydrolysis constants for the three ethyl carbamates.

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