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Effect of Charge on the Carbamylation and Binding Constants of Eel Acetylcholinesterase in Reaction with Neostigmine and Related Carbamates*

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ABSTRACT: The effect of charged quaternary ammonium groups on the carbamylation, k_2, and binding, K_a, constants governing inhibition of eel acetylcholinesterase by neostigmine and 13 related charged and uncharged phenyl *N*-methyl- and phenyl *N,N*-dimethylcarbamates was studied. Carbamates with substituents which bound well to the anionic site, whether charged or uncharged, were characterized by relatively high rates of carbamylation. For example, the k_2 values of *N*-methylneostigmine and of one of its carbon isosteres were 142 $\pm$ 4 and 97 $\pm$ 4 min^{-1} at 25°, respectively.

The phenyl substituents of the *N*-methylcarbamates appeared to bind more strongly to the anionic site than did those of the comparable *N,N*-dimethyl compounds. Orientation with respect to the anionic site seemed to be the predominant factor determining rates of carbamylation. The coulombic and noncoulombic energies associated with binding of substituents to the anionic site were calculated. The K_a and k_2 values of five carbamates and of diisopropyl phosphorofluoridate in reaction with eel and erythrocyte acetylcholinesterase were compared.

Esters of carbamic acid containing a charged quaternary ammonium group are more potent inhibitors of acetylcholinesterase (EC 3.1.1.7) than their uncharged analogs (Stedman, 1926; Stevens and Beutel, 1941; Kolbezen *et al.*, 1954; Kitz *et al.*, 1967b). The charge is generally considered to increase initial binding and to improve affinity through coulombic attraction to the anionic portion of the active site (Wilson and Bergmann, 1950). It is now widely accepted that inhibition by carbamates occurs by the following reaction

$$\mathrm{EH} + \mathrm{CX} \overset{K_a}{\rightleftharpoons} \mathrm{EHCX} \xrightarrow{k_2} \mathrm{EC} + \mathrm{HX} \qquad (1)$$

$$\mathrm{EH} + \mathrm{CX} \xrightarrow{\quad k_i \quad} \mathrm{EC} + \mathrm{HX}$$

where EH and CX are the enzyme and carbamate, respectively, and EHCX is an intermediate enzyme–inhibitor complex whose formation is controlled by the equilibrium affinity constant, K_a (Wilson *et al.*, 1960; Main and Hastings, 1966; O'Brien, 1968). EC is the carbamylated enzyme and the rate of its formation is controlled by k_2, the carbamylation rate constant. HX is the leaving group and k_i ($=k_2/K_a$) is the rate constant governing the over-all rate of inhibition.

Although charge has been clearly implicated in initial binding, its effect on the following carbamylation step is uncertain. Metzger and Wilson (1963) reported that certain alkylammonium ions accelerated the inhibition of acetylcholinesterase by dimethylcarbamyl fluoride. Presumably, the quaternary ammonium ions exerted their effect by occupying the anionic site which the relatively small fluoride leaving group of the carbamate would have left empty. Clearly such occupation could have affected either initial binding or the rate of carbamylation or both. However, the issue could not be decided because the criterion used to measure relative rates of inhibition did not separate initial binding from the carbamylation step. Moreover, it is not certain that the influence of the charge on a simple quaternary am-

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monium ion would be equivalent to that of a similar group attached to a carbamate.

Recent investigations that have considered the K_s and k_2 steps have either not employed charged carbamates (O'Brien *et al.*, 1966) or have failed to reveal formation of the reversible enzyme–inhibitor complex (Reiner and Aldridge, 1967).

In the present study, the inhibition procedure of Main and Iverson (1966) was used to determine K_s and k_2 values for the inhibition of eel acetylcholinesterase by neostigmine, two of its charged analogs, and their tertiary amine and carbon isosteres. The effect of the quaternary ammonium group on binding and carbamylation was then determined by comparing, in turn, the K_s and k_2 values of the appropriate charged and uncharged inhibitors.

The K_s and k_2 values for the inhibition of erythrocyte acetylcholinesterase by some of these compounds were also determined. This permitted the study of the relationship between the eel and erythrocyte enzymes to be extended within the context of separate affinity and carbamylation steps. Previous studies have been confined to comparing over-all rates of inhibition (Aldridge, 1954; Kitz *et al.*, 1967a).

Experimental Procedures

Inhibitors. 3-*t*-Butylphenyl *N*-methylcarbamate (mp 144°), 3-isopropylphenyl *N*-methylcarbamate (mp 72–73°), and 3-isopropylphenyl *N,N*-dimethylcarbamate (bp 99–102° (0.3 mm)) were supplied by Union Carbide, Clayton, N. C. Neostigmine (mp 166°) was donated by Hoffman-La Roche, Nutley, N. J. Phenyl *N*-methylcarbamate (mp 85–86°) and phenyl *N,N*-dimethylcarbamate (mp 45–46°) were gifts from Dr. R. D. O'Brien.

N-Methylneostigmine (mp 165°), 3-dimethylaminophenyl *N*-methylcarbamate (mp 86–87°), 2-isopropylphenyl *N*-methylcarbamate (mp 95–96°), 4-trimethylaminophenyl *N*-methylcarbamate (mp 178–179°), and 4-dimethylaminophenyl *N*-methylcarbamate (mp 132°) were synthesized by a method similar to that of Kolbezen *et al.* (1954) except that 5 g of phenol were treated with a 5% excess of methyl isocyanate in a round-bottomed pressure flask.

The following phenyl *N,N*-dimethylcarbamates, 3-dimethylamino- (bp 199–202° (20 mm)) and 3-*t*-butyl- (mp 52–54°), were prepared according to the method of Stevens and Beutel (1941).

The phenol intermediates, 3-dimethylamino-, 4-amino-, 2-amino-, and 3-*t*-butyl-, were obtained from the Aldrich Chemical Co., Milwaukee, Wis. 2-Isopropylphenol was purchased from K & K Laboratories, Plainsview, N. Y. 2-Dimethylaminophenol and 4-dimethylaminophenol were obtained by destructive distillation of the appropriate methiodides (Gardner and Stevens, 1947). The melting points found were in agreement with the literature. Thin-layer chromatography indicated no impurities and the infrared spectra were in accord with expected chemical structures.

The oxalate salt of *O,O*-diethyl *S*-(diethylaminoethyl) phosphorothiolate (tetram, mp 98–99°) was a gift from Dr. W. C. Dauterman.

Inhibitor Solutions. Stock solutions of the carbamates and of tetram were prepared either in absolute ethanol or in absolute methanol. Several stock solutions were prepared so that on dilution with distilled water the working solutions contained no more than 0.5% alcohol.

The *m*- and *p*-*N*-methylneostigmines were relatively unstable. Solutions of these inhibitors were therefore prepared immediately before use.

Substrate. Acetylcholine chloride was obtained from Sigma Chemical Co., St. Louis, Mo. A 30 mM solution containing 2% w/v 1-butanol was prepared in distilled water for use as the assay medium. The high concentration of substrate was necessary to prevent the inhibition in the presence of substrate that occurs with potent inhibitors, *e.g.*, neostigmine. The 1-butanol was used to activate the enzyme–substrate reaction so that excessive amounts of enzyme need not be used for each determination (Main, 1967).

Enzymes. Acetylcholine acetyl-hydrolase (EC 3.1.1.7) prepared from bovine erythrocytes was purchased from the Sigma Chemical Co., St. Louis, Mo. A 1000-unit vial contained 50 mg of erythrocytic protein, 100 mg of gelatin, 30 mg of sodium chloride, and 250 mg of phosphate salts. No other salts were added. A stock solution was prepared by diluting the contents of the vial to 25 ml with distilled water and storing at 2° under 1 drop of toluene. Working solutions of 7.5 units/ml were prepared from the stock by dilution with 10 mM sodium phosphate buffer (pH 7.0 or 7.6).

Acetylcholine acetyl-hydrolase (EC 3.1.1.7) from electric eel was purchased from the Worthington Biochemical Co., Freehold, N. J. Working solutions of 4.8 units/ml were prepared in 10 mM sodium phosphate buffer (pH 7.0) containing 0.05% gelatin. No other salts were added; 1 unit hydrolyzes 1 μmole of acetylcholine/min, pH 7.0, 25°.

Inhibition Procedure. The inhibition reactions were measured at 25° (pH 7.0 or 7.6) according to the procedure of Main and Iverson (1966) using the modified inhibition reaction vessel described by Main (1967). Typically 0.5 ml of enzyme solution and 0.5 ml of inhibitor solution were pipetted into the side arms of the reaction vessel. In some instances, where solubility of the inhibitor was limiting, 0.25 ml of enzyme and 1.0 ml of carbamate were used to attain a higher final concentration of inhibitor. Assay of the remaining enzyme activity was accomplished with a Radiometer pH-Stat (Copenhagen, Denmark) using 50 ml of substrate, at 25°, and pH 7.0 unless otherwise noted. The titrant was 20 mM sodium hydroxide prepared with CO_2-free distilled water.

Results

First-Order Kinetics and the Multiple Forms of Acetylcholinesterase. The validity of the K_s and k_2 estimations made depended upon the assumption that inhibition followed first-order kinetics when i was constant. Experimentally, first-order kinetics were assumed when the plot of log v against t was linear, where v was the velocity of a substrate reaction catalyzed by the remaining free enzyme, e, after inhibition for time t since $v \propto e$. While the initial phase of this study was in progress,

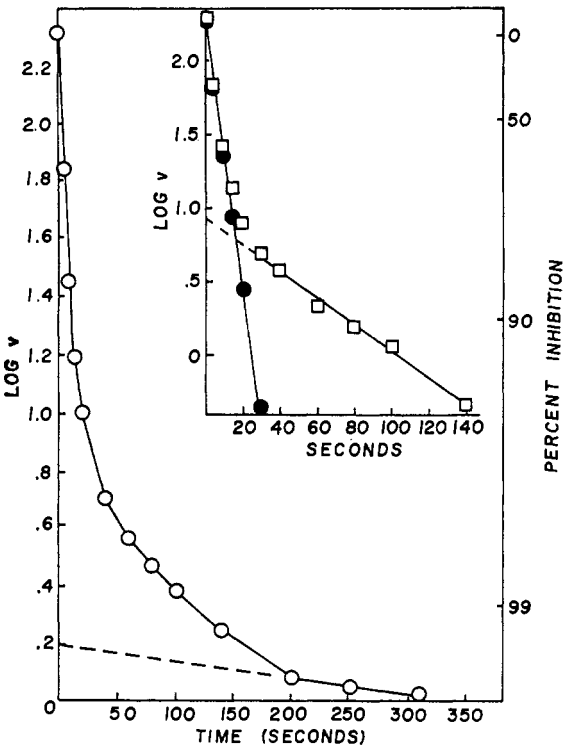

FIGURE 1: Log v against t plot for the inhibition of erythrocyte acetylcholinesterase by 5×10^{-6} M tetram at 5°, pH 7.0 (○). Inhibition was first order to about 92% inactivation. The insert compares the plot corrected for both minor components (●) with that corrected for the component represented by the linear terminal phase of inhibition between 200 and 310 sec. The linear terminal phase in the insert (□) represents the second minor component.

Main (1969a) observed that the log v against t plots of serum cholinesterase inhibited by the organophosphate tetram were not linear, but curved significantly. These studies were extended to erythrocyte acetylcholinesterase where significant curving also occurred as shown, for example, in Figure 1. Curving was demonstrated to reflect the presence of several molecular forms of acetylcholinesterase, each of which was inhibited at a different rate by tetram. A method was developed by which the curves could be resolved into their linear components. Each linear component represented the first-order rate plot for the inhibition of one form of acetylcholinesterase. Two resolved components are shown in the inset of Figure 1 while the plot of a third of low activity was estimated from the final points between 200 and 315 sec.

Similar curving was observed with neostigmine as shown in Figure 2. At the times used, regeneration would have been negligible. While it is evident that one form, the most rapidly inhibited, predominated in the preparation of erythrocyte acetylcholinesterase used, the presence of the other forms would have resulted in small but significant errors unless corrections for them were made.

A highly purified preparation of eel acetylcholinesterase was then obtained in the hope that one form would predominate to the extent that corrections would not be necessary. As shown in Figure 3 the log v against

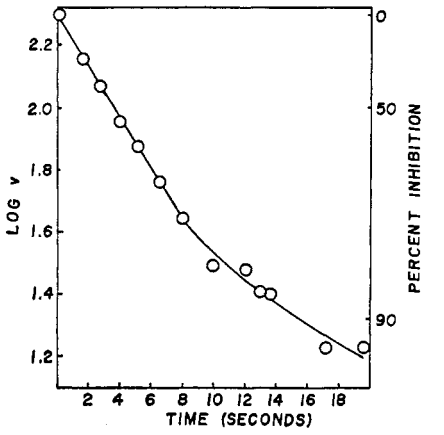

FIGURE 2: Log v *vs.* t plot for the inhibition of erythrocyte acetylcholinesterase by 4×10^{-6} M neostigmine, pH 7.0, 25°. Inhibition was first order to approximately 84% inactivation.

t plot did curve, but curving was not significant until inhibition has reached about 99% of completion. The predominant form accounted for 99.2% of the activity and the correction associated with the remaining activity were negligible and could be ignored. Eel acetylcholinesterase was therefore chosen to continue the study of the effect of charge.

The log v against t plots obtained with various concentrations of neostigmine in reaction with the eel en-

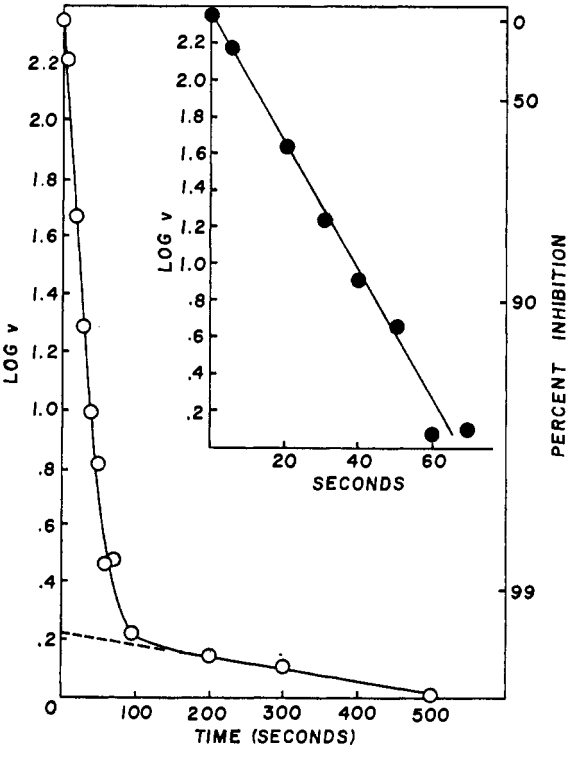

FIGURE 3: Log v *vs.* t plot for the inhibition of eel acetylcholinesterase by 5×10^{-6} M tetram at pH 7.0, 25°. Inhibition was first order to about 98% inactivation. The inset shows the plot of the major enzyme form after subtraction of the activity present in the linear, terminal inhibition phase of the primary data.

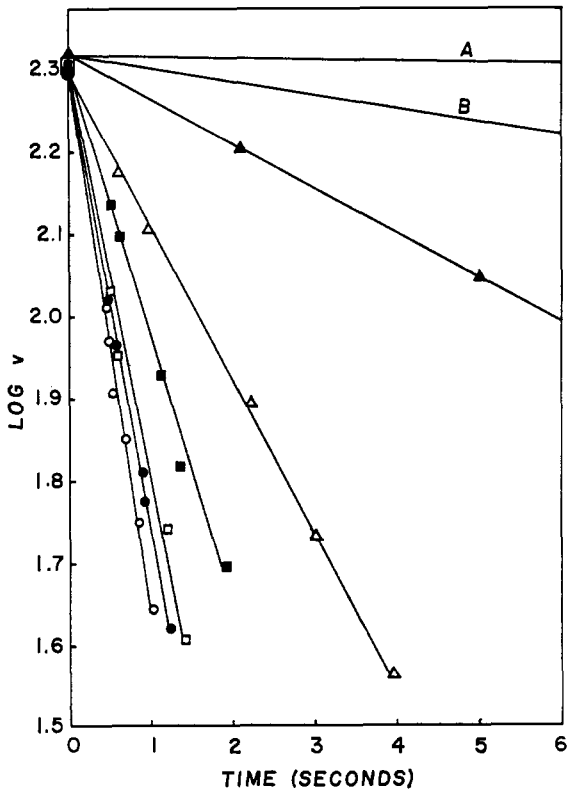

FIGURE 4: The log v *vs.* t plots for the inhibition of eel acetylcholinesterase by neostigmine (○) 1×10^{-4} M, (●) 8×10^{-5} M, (□) 6×10^{-5} M, (■) 4×10^{-5} M, (△) 2×10^{-5} M, and (▲) 5×10^{-6} M. The two solid lines are (A) 1×10^{-7} M and (B) 2×10^{-6} M. Neostigmine velocities were not determined for the latter two concentrations at times of less than 6 sec. Inhibition occurred at pH 7.0, 25°, in 10 mM sodium phosphate buffer.

zyme are shown in Figure 4. The slopes were calculated by regression analysis to give the first-order rate constant, ρ, for each concentration of i. When inhibition occurs according to reaction 1, then eq 2, where k_i =

$$i/\rho = i/k_2 + 1/k_i \qquad (2)$$

k_2/K_a, is valid. The i/ρ against i plots of neostigmine and of three other carbamates are shown in Figure 5. These were typical and suggested that eq 2 was a valid model for the inhibition reaction studied.

The Effect of Charge. The binding constants K_a, carbamylation constants k_2, and bimolecular velocity constants k_i for the inhibition of eel acetylcholinesterase by 14 carbamates are given in Table I. Three contained charged quaternary ammonium groups: neostigmine, *N*-methylneostigmine, and the *para*-substituted analog of *N*-methylneostigmine.

The *t*-butyl carbon isostere of each of the charged compounds was prepared, but the isostere of *N*-methylneostigmine was the only one for which K_a and k_2 values could be determined. The others were too insoluble. The more soluble isopropyl analogs were then made and K_a and k_2 values for both *meta*-substituted compounds were determined. Removing a methyl group from the *t*-butyl substituent to produce its isopropyl analog had no

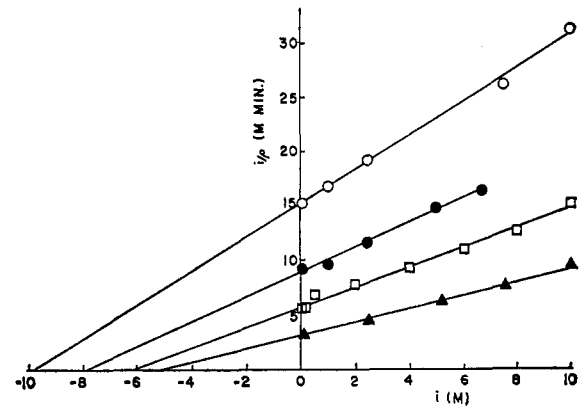

FIGURE 5: i/ρ against i plots for eel acetylcholinesterase, pH 7.0, 25°, and (○) 3-*t*-butylphenyl *N*-methylcarbamate, i/ρ is $\times 10^{-7}$, i is $\times 10^{-5}$. (●) 2-Isopropylphenyl *N*-methylcarbamate, i/ρ is $\times 10^{-6}$, i is $\times 10^{-4}$. (□) Neostigmine, i/ρ is $\times 10^{-7}$, i is $\times 10^{-5}$. (▲) 3-Dimethylaminophenyl *N*,*N*-dimethylcarbamate, i/ρ is $\times 10^{-4}$, i is $\times 10^{-4}$.

significant effect on K_a and increased k_2 by 30% in the case of the *N*-methyl analog. This suggested that valid estimates could similarly be made by comparing neostigmine with its *N*,*N*-dimethylisopropyl analog.

The tertiary amine homologs of each of the three charged compounds were also made and they were sufficiently soluble so that K_a and k_2 values with each could be determined.

Removing a methyl group from the quaternary ammonium group results in a marked increase in the electronegativity of the carbonyl group and a consequent increase in the stability of the carbamate to hydrolysis. This is reflected in the Hammett substituent constants (Hammett, 1940) of the tertiary amine compounds which were quite close to those of the carbon isosteres. It followed that in addition to the enhanced binding energy from coulombic attraction, charge might also increase inhibitory power through its effect on the bond cleaved. The first effect would be reflected by K_a while the second would influence k_2. To gain an insight into the significance of the electronic shift with respect to the effect of charge on k_2, the K_a and k_2 values of the reference unsubstituted phenyl *N*-methyl- and phenyl *N*,*N*-dimethylcarbamates were determined. These values were also useful in estimating the contribution of noncoulombic attraction to binding at the anionic site.

The Effect of Charge on Binding. It was convenient to compare the K_a values of the charged compounds, K_a^+, with those of the uncharged compounds, K_a^0, as ratios, K_a^0/K_a^+, and also in terms of the free energy of binding, ΔF and $\Delta\Delta F$, since $\Delta F = 2.3RT \log K_a$ and $\Delta\Delta F = 2.3RT \log K_a^+/K_a^0$, where R is the gas constant and T is the reaction temperature in absolute degrees (Adams and Whittaker, 1950; Wilson, 1960). The nature of the log v against t plots and the linear i/ρ against i plots suggested that K_a could be treated as a simple equilibrium constant (Main, 1969b).

The K_a^0/K_a^+ ratios of *N*-methylneostigmine compared successively with its *t*-butyl and isopropyl isosteres and tertiary amine analog were 15, 17.6, and 214, respectively. Similarly the $\Delta\Delta F$ values were 1506, 1605, and

TABLE I: Binding Constants, K_a, Carbamylation Constants, k_2, and Bimolecular Rate Constants, k_i, for the Inhibition of Eel Acetylcholinesterase by Neostigmine and Analogs.

Phenyl Substituent	Compound	K_a (M)	k_2 (min^{-1})	k_i (M^{-1} min^{-1})	i Range	Points
$N^+(CH_3)_3$	3-Trimethylaminophenyl *N,N*-dimethylcarbamate (neostigmine)	$6.20 \times 10^{-5} \pm 0.53$	110.4 ± 5.08	1.72×10^6	1×10^{-7}–1×10^{-4}	8
$N(CH_3)_2$	3-Dimethylaminophenyl *N,N*-dimethylcarbamate	$5.17 \times 10^{-4} \pm 0.25$	1.53 ± 0.12	3.16×10^3	1×10^{-5}–1×10^{-3}	5
$C(CH_3)_3$	3-*t*-Butylphenyl *N,N*-dimethylcarbamate			1.17×10^3[a]	1×10^{-5}–7.25×10^{-4}	5
$C(CH_3)_2$	3-Isopropylphenyl *N,N*-dimethylcarbamate	7.27×10^{-4}	2.53 ± 0.18	3.59×10^3	1×10^{-5}–1×10^{-3}	5
H	Phenyl *N,N*-dimethylcarbamate	$8.90 \times 10^{-3} \pm 1.36$	0.37 ± 0.04	3.94×10^1	1×10^{-3}–1.5×10^{-2}	5
$N^+(CH_3)_3$	3-Trimethylaminophenyl *N*-methylcarbamate (*N*-methylneostigmine)	$6.56 \times 10^{-6} \pm 0.13$	143.7 ± 4.9	2.11×10^7	1×10^{-7}–9.8×10^{-6}	4
$N(CH_3)_2$	3-Dimethylaminophenyl *N*-methylcarbamate	$1.41 \times 10^{-3} \pm 0.06$	48.3 ± 1.5	3.39×10^4	2×10^{-5}–1.5×10^{-3}	6
$C(CH_3)_3$	3-*t*-Butylphenyl *N*-methylcarbamate	$9.74 \times 10^{-5} \pm 0.49$	64.1 ± 2.6	6.56×10^5	1×10^{-6}–1×10^{-4}	5
$C(CH_3)_2$	3-Isopropylphenyl *N*-methylcarbamate	$1.15 \times 10^{-4} \pm 0.08$	97.1 ± 4.1	8.38×10^5	1×10^{-6}–1×10^{-4}	5
H	Phenyl *N*-methylcarbamate	$2.26 \times 10^{-2} \pm 0.26$	9.1 ± 0.9	3.7×10^2	1×10^{-4}–1.9×10^{-2}	5
		para substituted				
$N^+(CH_3)_3$	4-Trimethylaminophenyl *N*-methylcarbamate	$5.23 \times 10^{-4} \pm 0.62$	23.20 ± 1.39	4.28×10^4	6.25×10^{-7}–1×10^{-3}	6
$N(CH_3)_2$	4-Dimethylaminophenyl *N*-methylcarbamate	$1.67 \times 10^{-3} \pm 0.24$	2.97 ± 0.30	1.55×10^3	5×10^{-5}–1×10^{-3}	5
		ortho substituted				
$C(CH_3)_2$	2-Isopropylphenyl *N*-methylcarbamate	$7.87 \times 10^{-4} \pm 0.75$	87.20 ± 5.32	1.12×10^5	1×10^{-6}–6.67×10^{-4}	5
$N(CH_3)_2$	2-Dimethylaminophenyl *N*-methylcarbamate	$8.39 \times 10^{-4} \pm 0.78$	24.03 ± 1.33	2.64×10^4	1×10^{-5}–1×10^{-3}	5

[a] Determined in 2% ethanol.

TABLE II: Binding Constants, K_a, Carbamylation Constants, k_2, and Bimolecular Rate Constants, k_i, for the Inhibition of Erythrocyte Acetylcholinesterase by Five Carbamates and the Organophosphate DFP.

Inhibitor	K_a (M)	k_2 (min^{-1})	k_i (M^{-1} min^{-1})	i Range	Points	pH
3-Trimethylaminophenyl *N,N*-dimethylcarbamate (neostigmine)	$1.16 \times 10^{-5} \pm 0.19$	46.5 ± 5.4	4.0×10^6	1×10^{-7}–1.2×10^{-5}	5	7.6
3-*t*-Butylphenyl *N*-methylcarbamate	$5.48 \times 10^{-5} \pm 0.44$	21.3 ± 1.2	3.72×10^5	5×10^{-7}–5×10^{-5}	6	7.6
3-Isopropylphenyl *N*-methylcarbamate	$1.59 \times 10^{-4} \pm 0.13$	76.2 ± 7.2	4.8×10^5	1×10^{-6}–1×10^{-4}	6	7.0
Phenyl *N*-methylcarbamate	$2.41 \times 10^{-2} \pm 0.76$	8.1 ± 1.1	2.62×10^2	1×10^{-4}–2×10^{-2}	5	7.0
Phenyl *N,N*-dimethylcarbamate	$1.7 \times 10^{-2} \pm 0.29$	0.38 ± 0.1	2.37×10^1	2×10^{-4}–2×10^{-2}	4	7.0
DFP[a]	$1.58 \times 10^{-3} \pm 0.22$	11.9 ± 0.7	7.54×10^3	1×10^{-5}–5×10^{-3}	8	7.6

[a] Taken from Main and Iverson (1966). Inhibition occurred at 5°.

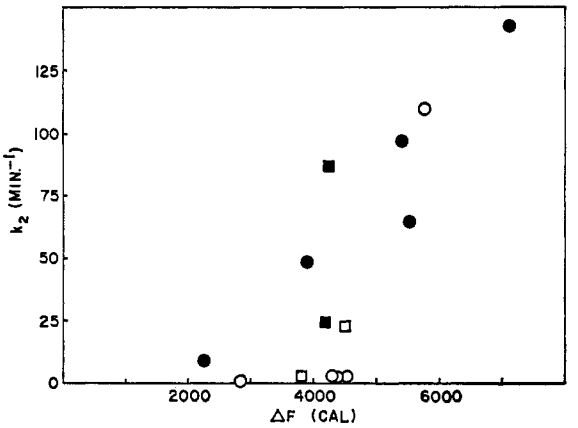

FIGURE 6: The relationship of the carbamylation rate constants, k_2, to the binding energies, ΔF, for the inhibition of eel acetylcholinesterase by various carbamates: (●) *m*-*N*-methylcarbamates, (○) *m*-*N*,*N*-dimethylcarbamates, (□) *p*-*N*-methylcarbamates, and (■) *o*-*N*-methylcarbamates. The constants were determined at 25°, pH 7.0, in 10 mM sodium phosphate buffer. The K_a values from which ΔF values were calculated are given in Table I.

3200 cal mole^{-1}. The K_a^0/K_a^+ ratios of neostigmine compared with its isopropyl and tertiary amine analogs were 11.7 and 8.3, respectively, and the $\Delta\Delta F$ values were 1467 and 1264 cal mole^{-1}. The K_a^0/K_a^+ of the *para*-substituted *N*-methylneostigmine and its tertiary amine analog was 3.2 and $\Delta\Delta F$ was 691 cal mole^{-1}.

In each case charge increased the binding energy, but the effect was not uniform, varying from 3200 to 691 cal mole^{-1}. Moreover orientation of the substituents on the phenyl ring and the number of carbamyl *N*-methyl groups binding to the acyl site appeared to be as significant as charge in determining the binding energy.

The Effect of Charge on k_2. As with the K_a values, it was convenient to compare the k_2 values of the charged and uncharged compounds as ratios k_2^+/k_2^0. The k_2^+/k_2^0 ratios of *N*-methylneostigmine compared successively with its *t*-butyl and isopropyl isosteres and tertiary amine analog were 2.2, 1.5, and 3.0, respectively. The k_2^+/k_2^0 ratios of neostigmine and its isopropyl and tertiary amine analogs were by comparison much higher, 42 and 72, respectively. The k_2^+/k_2^0 ratio of *p*-*N*-methylneostigmine and its tertiary amine analog was 7.8. In every instance charge significantly increased k_2, but as with K_a the effect was by no means uniform varying from 1.5 to 72. The k_2 values of the *N*-methyl as compared to their *N*,*N*-dimethyl analogs were about 39-fold higher; the exception was *N*-methylneostigmine compared with neostigmine where the k_2 was only 1.2-fold higher. The effect of charge also appeared to be greater with *para*- as compared with the *meta*-substituted series.

The k_2 values of the charged inhibitors were the highest obtained. This is consistent with their relatively large, positive, Hammett substituent constant, σ. Yet when the remainder of the compounds are considered, no relationship of k_2 to σ is found. This suggests that ring induction effects are not a predominant influence on k_2.

Comparison of Eel and Erythrocyte Acetylcholinesterase. The K_a and k_2 values of five carbamates and of one organophosphate, DFP, in reaction with bovine erythrocyte acetylcholinesterase are given in Table II. The ratios of the K_a, k_2, and k_i values obtained with erythrocyte as compared to eel acetylcholinesterase in reaction with these compounds is given in Table III. The binding of the erythrocyte enzyme to neostigmine was significantly better than with the eel, but the carbamylation rate was 2.4-fold lower. However, both binding and carbamylation rates were in the same order of magnitude and this was true to an even closer degree of the other compounds. Considering that the range of K_a values varied by a factor of almost 1500-fold while the k_2 values varied over a 300-fold range, the similarity of the reaction constants was close, indicating that the two enzymes possessed remarkably similar binding and carbamylation sites, particularly when their very different specie and tissue origin is remembered.

TABLE III: Comparison of Binding, K_a, Carbamylation, k_2, and Bimolecular, k_i, Rate Constants of Eel and Erythrocyte Acetylcholinesterases.

Inhibitor	K_a Eel/ K_a Erythrocyte	k_2 Eel/ k_2 Erythrocyte	k_i Eel/ k_i Erythrocyte
Neostigmine	5.35	2.37	0.43
3-*t*-Butylphenyl *N*-methylcarbamate	1.77	3.01	1.76
3-Isopropylphenyl *N*-methylcarbamate	0.73	1.27	1.73
Phenyl *N*-methylcarbamate	0.94	1.12	1.40
Phenyl *N*,*N*-dimethylcarbamate	0.53	0.98	1.66
DFP[a]	0.74	0.47	0.65

[a] Eel data for inhibition at 5°, pH 7.0: $K_a = 1.16 \times 10^{-3} \pm 0.06$ M; $k_2 = 5.5 \pm 0.2$ min^{-1}; $k_i = 4.8 \times 10^3$ M^{-1} min^{-1}.

Discussion

The results were consistent with the concept of an an ionic binding site on the active site of acetylcholinesterase. They indicated that in addition to its widely accepted role in initial binding, the anionic site also played a significant part in the following carbamylation step. As shown in Figure 6, high binding energies were usually followed by relatively high carbamylation rates, suggesting that initial binding and the carbamylation step were related, although the relationship was not precise.

Coulombic binding to the anionic site was estimated from the free-energy differences, $\Delta\Delta F$, between the charged compounds and their carbon isosteres, or in the case of *p*-*N*-methylneostigmine, the tertiary amine

analog. The noncoulombic contribution (Bernhard, 1955; Wilson, 1960) was in turn estimated from the differences between the free energies of the carbon isosteres or tertiary amine analogs and those of the parent unsubstituted phenyl carbamates. With the series based on *N*-methylneostigmine, $\Delta\Delta F$ was about 1550 cal mole^{-1}. Binding of the quaternary ammonium group to the anionic site then accounted for 68% of the total binding energy, and of this 22% was from coulombic binding and 46% from hydrophobic and other forms of binding. These calculations were based on the *t*-butyl and unsubstituted phenyl *N*-methylcarbamates.

A similar analysis of the neostigmine series suggested that the coulombic binding energy was 1400 cal mole^{-1} while the hydrophobic binding contributed about 1560 cal mole^{-1}. The quaternary ammonium group then accounted for 52% of the total binding energy of which 25% was coulombic in nature and 27% was hydrophobic binding.

The relationship between the binding energy, ΔF, and k_2 suggested that the rate of carbamylation depended upon the degree to which the anionic site was occupied. However, the nature of the binding energy, whether coulombic or hydrophobic, did not appear to be critical since the k_2 values of both charged and uncharged compounds were of the same order. The presence of the charge would increase the binding energy, but it would also serve to orient the inhibitor molecule with respect to the anionic site.

The better binding of phenyl *N,N*-dimethyl- as compared with the phenyl *N*-methylcarbamate can be attributed to binding of the extra carbamyl *N*-methyl group to the acyl binding site. But binding this additional methyl group apparently resulted in poorer orientation of the substituents on the phenyl ring with respect to the anionic site, since with the exception of the tertiary amine analogs (Table I), the binding energy of the *N*-methyl series was from 1000 to 1300 cal mole^{-1} greater than the binding energies of comparable members of the *N,N*-dimethyl series. This disorientation then led to lower carbamylation rates of the uncharged *N,N*-dimethyl analogs. The presence of the charge on neostigmine resulted in improved orientation as indicated by the increased binding and the dramatic increase in k_2 which followed.

This interpretation is reasonably consistent with the results. It lays great stress on orientation as a dominant factor determining carbamylation rates and gives relatively little weight to the effects of electronic shifts which accompany a charged group. Occupation of the anionic site, whether by charged or uncharged compounds, led not only to increased binding, but also to higher rates of carbamylation. Indeed, the carbamylation rates measured were much higher than any previously reported, either for uncharged compounds (O'Brien *et al.*, 1966) or for the charged form of eserine (Main and Hastings, 1966).

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