Carbamylation and Affinity Constants of Some Carbamate Inhibitors of Acetylcholinesterase and Their Relation to Analogous Substrate Constants

Felton L. Hastings,[1] Alexander R. Main,[2] and Frank Iverson[2]

The carbamylation (k_2) and binding (K_a) constants governing inhibition of bovine erythrocyte acetylcholinesterase at 25° C, pH 7.6, by 12 methyl and dimethylcarbamates, including Temik, neostigmine, and substituted phenyl carbamates, were determined. The k_2 values ranged from 146 min^{-1} to 0.14 min^{-1}, while K_a ranged from 2.4×10^{-2}M to 1.0×10^{-5}M, indicating that inhibitory power was as much dependent on variations in carbamylation rates as it was on initial binding. The k_2 and K_a values of the *ortho*-, *meta*-, and *para*-substituted nitrophenyl carbamates were compared with the K_m and V_{max} values of related nitrophenyl acetate substrates. In each series, *ortho*-substitution resulted in the highest k_2 or V_{max} values, whereas *meta*- and *para*-substitution gave much lower values, the *meta* being about twice that of the *para*. The effect of ring orientation on K_a and K_m was less dramatic, but similar. These findings suggested a fairly close analogy between the substrate and carbamate reaction, but the analogy did not hold for the comparable nitrophenyl phosphates.

It is now generally accepted that inhibition of acetylcholinesterase by carbamates occurs by the following reaction sequence (*e.g.*, Wilson *et al.*, 1960; O'Brien *et al.*, 1966)

$$EH + CX \underset{}{\overset{K_a}{\rightleftharpoons}} EHCX \xrightarrow{k_2} EC \xrightarrow{k_3} EH + COH \qquad (1)$$

$$EHCX \xrightarrow{k_1} HX + HOH$$

where EH is the enzyme, CX is a carbamate, $EHCX$ is a complex controlled by an equilibrium affinity constant, K_a, EC is the carbamylated active site, HX is the leaving group, k_2 is the carbamylation rate constant, and k_3 is the regeneration constant. Overall rates of inhibition are given by k_i where $k_i = k_2/K_a$ (Main, 1964). Analogous schemes apply also to inhibition by organophosphates and to the substrate reaction (Wilson, 1960).

The significance of carbamylation rates (k_2) as compared with affinity (K_a) in determining overall inhibitory power (k_i) is fundamental to understanding both the inhibitory process and the analogy which appears to exist between the substrate and inhibitor reactions (Aldridge, 1954; Main and Hastings, 1966a).

O'Brien *et al.* (1966) made the first comprehensive study of the K_a—k_2 relationship with carbamates and gave values for a series of 13 mono-substituted phenyl methyl and dimethyl analogs. The k_2 values reported were relatively low (≤ 3 min^{-1} at 38° C), and it was concluded that "virtually all the differences in anticholinesterase activity among the 13 carbamates described herein were due to differences in complexing ability" (*i.e.*, affinity).

The experimental methods of Main and Iverson (1966) have permitted the measurement of higher k_2 values (up to 146 min^{-1} at 25° C) so that a significantly wider range of phenyl substituents could be studied. The effect of ring orientation (Kolbezen *et al.*, 1954) has also been considered and attempts were made to determine the K_a and k_2 constants of the insecticide Temik, its sulfone, and of carbaryl. Low solubility or instability in aqueous solution precluded estimation of the K_a and k_2 values of some of the compounds studied.

The relative novelty of K_a estimations suggested that results reflecting on the validity of their interpretation as affinity constants were still important. Comparison of the K_m and K_a values of analogous substrates and inhibitors is one means by which this interpretation can be tested, since the reasonable expectation is that the range of such values would be of the same order. The degree to which the analogy between the substrate and inhibition reactions holds can also be judged by comparison of K_a with K_m and k_2 with V_{max}. With these considerations in mind, the K_m and V_{max} values of the substrate reactions of the acetate analogs of the phenyl and nitrophenyl carbamates and phosphates were determined.

MATERIALS AND METHODS

Enzyme. Bovine erythrocyte acetylcholinesterase (AChE), EC 3.1.1.7, was obtained as a partially purified preparation from Sigma Chemical Company, St. Louis, Mo. Stock solutions were prepared by dissolving the contents of a 1000-unit bottle (1 unit hydrolyzed 1 μmole acetylcholine per min at pH 8.0, 37° C) containing 50 mg of erythrocytic protein, 100 mg of gelatin, and 250 mg of sodium phosphate in 20 ml of water to which one drop of toluene was added. The solution was 6 mM in sodium phosphate, pH 7.6. Dilutions made from this stock were readjusted to this concentration and pH. The stock was stored at about 2° C and appeared to be stable for weeks (Main, 1969a).

Inhibitors. The phenyl-, *m*-nitrophenyl-, and *o*-nitrophenyl methylcarbamates were prepared according to the method of Kolbezen *et al.* (1954). The method of Stevens and Beutel (1941) was used in the synthesis of the *o*-nitrophenyl- and *m*-nitrophenyl dimethylcarbamates. Other carbamates used were: *m*-isopropylphenyl methylcarbamate, *m*-tertbutylphenyl methylcarbamate, carbaryl (1-naphthyl methylcarbamate), Temik [2-methyl-2-(methylthio) propionaldehyde *o*-(methylcarbamoyl) oxime], Temik Sulfone, neostigmine [dimethylcarbamate of (*m*-hydroxyphenyl) trimethylammonium bromide], *m*-isopropylphenyl dimethylcarbamate, phenyl dimethylcarbamate, and *p*-nitrophenyl dimethylcarbamate. The organophosphates studied were paraoxon and its *ortho*- and *meta*-nitrophenyl homologs. Inhibitor

[1] USDA Forest Service, Southeastern Forest Experiment Station, Forestry Sciences Laboratory, Research Triangle Park, N.C. 27709

[2] Department of Biochemistry, School of Agriculture and Life Sciences and School of Physical Sciences and Applied Mathematics, North Carolina State University, Raleigh, N.C. 27607

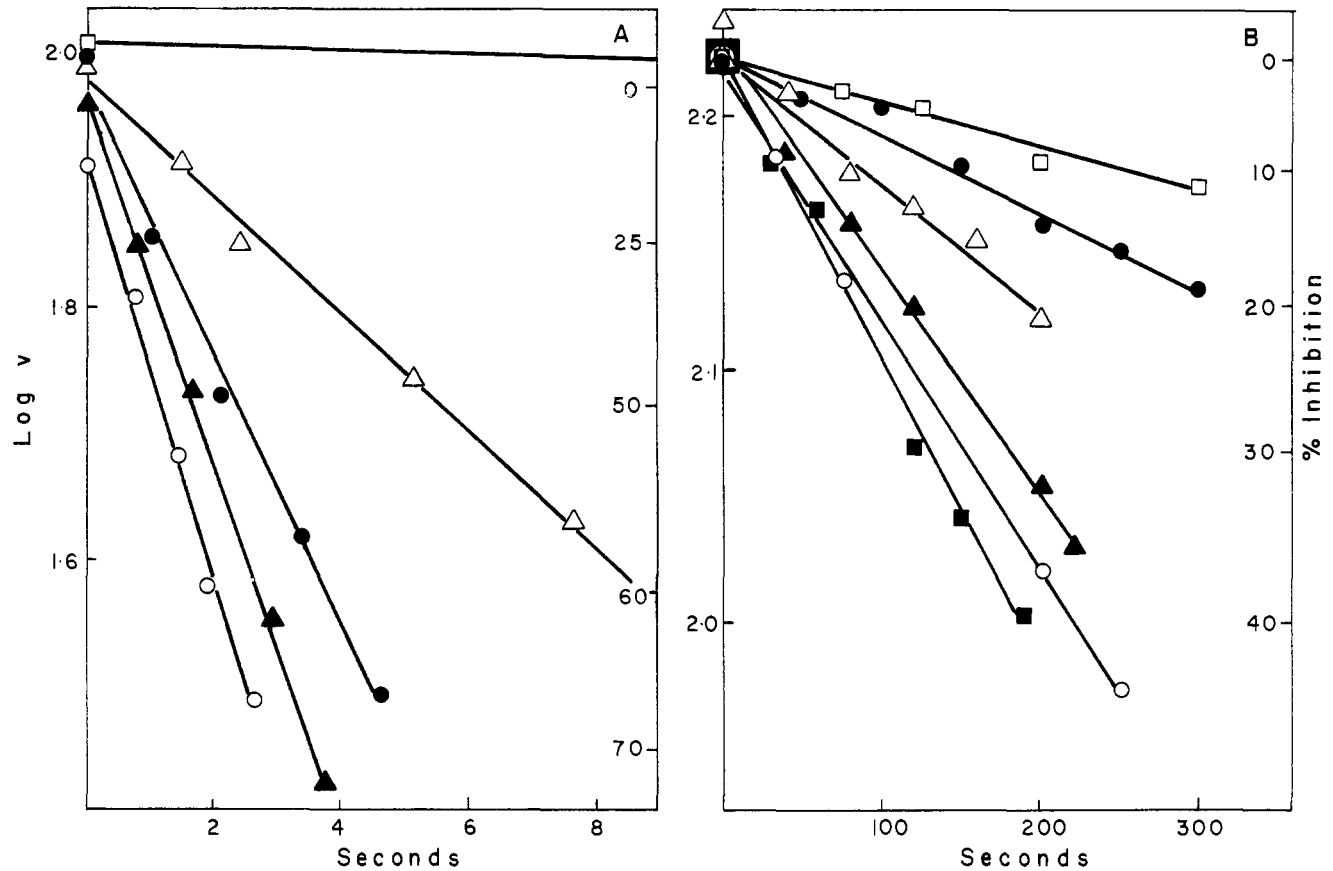

Figure 1. Plots of log v against time for the inhibition of acetylcholinesterase by (A) neostigmine and (B) phenyl dimethylcarbamate

The velocity, v, refers to the rate of acetylcholine hydrolysis at 25° C and pH 7.6 by the esterase remaining uninhibited after reacting with inhibitor for time t. The slopes give $\rho = 2.3\ \Delta \log v/\Delta t$ for various inhibitor concentrations and these are used in construction of the i/ρ against i plots. The concentrations of inhibitor were: (A) □, 0.1μM; △, 2μM; ●, 5μM; ▲, 8μM; ○, 12μM (B)□, 1mM; ●, 2mM; △, 5mM; ▲, 10mM; ○, 15mM; ■, 20mM

solutions were prepared fresh daily in water, either by stirring a weighed amount of inhibitor directly into the water, or, with the more insoluble inhibitors, by preparing stock solutions in methanol and blowing a measured amount into the water. Final concentrations of methanol in the inhibition medium were 0.5% or less; for *m*- and *p*-nitrophenyl dimethylcarbamates the methanol concentration was 1.0%.

Substrates. The *o*-, *m*-, and *p*-nitrophenyl acetates were prepared according to the method of Huggins and Lapides (1947). Phenyl acetate was obtained from Eastman Chemical Company and was distilled before use. Acetylcholine chloride was obtained from Sigma Chemical Company.

Determination of K_a and k_2 Values. The procedure of Main and Iverson (1966) was followed. Inhibition rates were measured at pH 7.6 and 25° C in 6 mM sodium phosphate buffer using inhibition reaction vessels described by Main (1969a). Usually, 0.5 ml of AChE solution containing 5.0 activity units were pipetted into one side arm and 0.5 ml of inhibitor solution were then pipetted into the other. With insoluble inhibitors, the saturation concentration was approximated by increasing the volume of inhibitor (*e.g.*, 0.9 ml) and by decreasing the volume of AChE (*e.g.*, 0.1 ml); the combined volume was 1.0 ml. Residual substrate activities were measured with 40 ml of 3 mM acetylcholine at 25° C and pH 7.6 in a Radiometer pH-Stat.

Determination of K_m and V_{max} Values. These values were determined with a Brinkmann pH-Stat using 40 ml of substrate and from 1.0 to 10 enzyme activity units (Main and Hastings, 1966a). Corrections for the partial ionization of the liberated nitrophenols were based on the following pK values: unsubstituted phenol, 9.89; *o*-nitrophenol, 7.17; *m*-nitrophenol, 8.28; and *p*-nitrophenol, 7.15.

RESULTS AND DISCUSSION

K_a and k_2 values and their standard errors (S.E.) were calculated by regression analysis from the first order rate constants (ρ) obtained at various inhibitor concentrations (i) with the following equations (Main, 1969a).

$$\frac{i}{\rho} = \frac{i}{k_2} + \frac{1}{k_i} \tag{2}$$

where $1/k_2$ is the slope and

$$\rho = -\frac{\rho}{i} K_a + k_2 \tag{3}$$

where $-K_a$ is the slope.

The validity of the assumption that inhibition was first order over the time course of the inhibition reaction depended on the linearity of the log v against t plots. When these plots are linear, the velocity of the substrate reaction is a measure of the enzyme remaining uninhibited after incubation for time t. For K_a to be a valid measure of initial binding, that is $K_a = k_{-1}/k_1$, the log v against t plots, in addition to being straight, should pass through log v_0 at $t = 0$ (Main, 1969b). The rate plots appeared to satisfy these criteria. Log v against t plots typical of those obtained are shown, for example, in

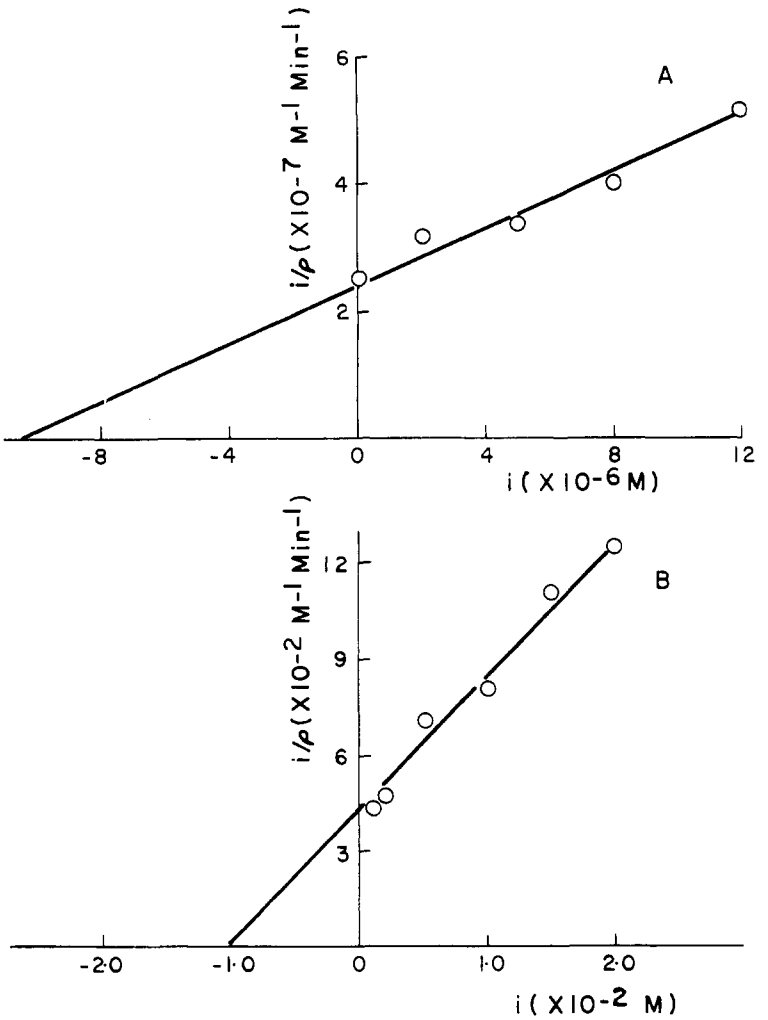

Figure 2. Plots of i/ρ *vs.* i according to Equation 2 for the inhibition of acetylcholinesterase by neostigmine (A) and phenyl dimethylcarbamate (B)

Figure 1. The average S.E. associated with the ρ values was $\pm 4.2\%$; the extremes were $\pm 8.0\%$ and $\pm 1.4\%$.

It is now recognized that bovine erythrocyte AChE exists in multiple molecular forms which may be inhibited at significantly different rates, depending on the inhibitor (Main, 1969a). Under the conditions used here, 25° C and pH 7.6, one form, the most rapidly inhibited, predominated and curving of the log v against t plots would not have been significant until at least 95% inhibition had been reached. The errors involved in neglecting inhibition of the other multiple forms, while measurable, would not have had a significant effect on the results.

Examples of i/ρ against i plots typical of those obtained are shown in Figure 2. They were reasonably linear and suggested that Equations 2 and 3 were valid for the reactions studied. The $1/v$ against $1/s$ plots for the substrate reactions are shown in Figure 3. The curving of the *o*-nitrophenyl acetate plot as its concentration approached the saturation point was significant and suggested substrate inhibition similar to that observed with acetylcholine (Wilson, 1960).

Relationship of Binding to Carbamylation Rates in Determining Inhibitory Power. The K_a, k_2, and k_i values of 12 carbamates and the k_i value of carbaryl in reaction with AChE are given in Table I. The k_2 values of the six dimethylcarbamates ranged from 0.14 to 44 min^{-1}, varying by a factor of 314, whereas the K_a values ranged from 1.03×10^{-2}M to 1.02×10^{-5}M, or by a factor of 1000. Neostigmine, the best inhibitor in the group, was characterized primarily by good binding, but its k_2 value was also the highest of the group. The k_2 values of the six methylcarbamates ranged from 0.45 to 146 min^{-1}, varying by a factor of 325, while the K_a values ranged from 2.37×10^{-2}M to 6.66×10^{-5}M, or by a factor of 360. In both groups, variation in inhibitory power depended as much on variation in carbamylation rates (k_2) as on variation in binding (K_a). Indeed, Temik and Temik Sulfone owed their considerable inhibitory power primarily to high carbamylation rates, for both compounds were characterized by relatively poor binding to AChE.

The K_a and k_2 of carbaryl could not be determined because its maximum solubility, about 5×10^{-4}M, was evidently lower than its K_a value. The highest first order rate constant (ρ) measured was 9.1 min^{-1} at 4×10^{-4}M, suggesting that k_2 was at least 20 min^{-1} and quite probably higher. Similarly, K_a was probably greater than 1×10^{-3}M. Thus, carbaryl, like Temik, appeared to owe its inhibitory power primarily to relatively high carbamylation rates rather than to good binding.

Dimethylcarbamates are poorer inhibitors of AChE than their methyl analogs (O'Brien *et al.*, 1966). When the k_2 values of the three pairs of methyl and dimethyl analogs given in Table I were compared, the k_2 of the methyl analog was, in each case, higher than that of its dimethyl counterpart. The differences were not uniform, however, varying from threefold when the phenyl substituent was *m*-nitro, to thirty-

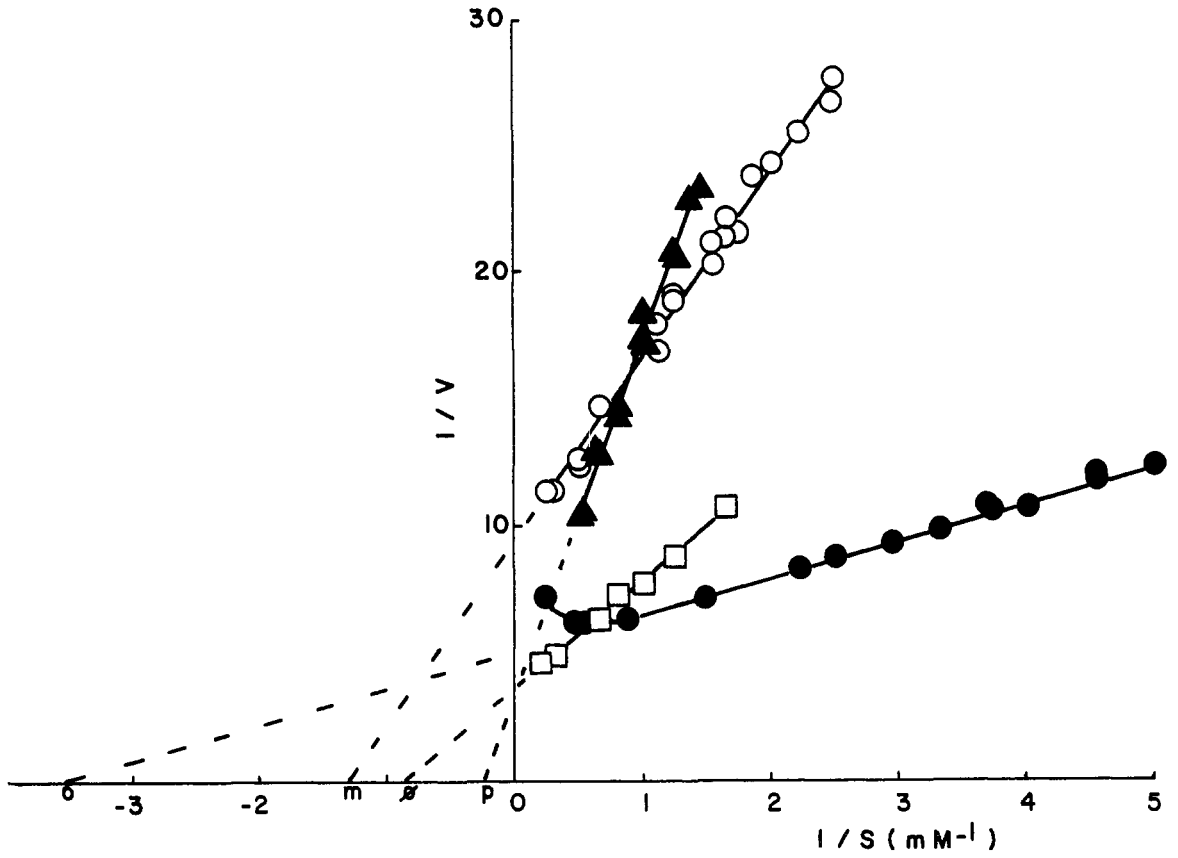

Figure 3. Plots of $1/v$ *vs.* $1/s$ for the substrate reactions of phenyl and substituted nitrophenyl acetates in reaction with bovine erythrocyte acetylcholinesterase at 25°C, pH 7.6

For convenience, velocities are expressed in arbitrary units; the amount of enzyme used and the legend is as follows: *ortho*-nitrophenyl acetate, 1 unit, ●; *meta*-nitrophenyl acetate, 2 units, ○; *para*-nitrophenyl acetate, 10 units, ▲; phenylacetate, 1.5 units, □

fold for the unsubstituted phenyl carbamate. Although the presence of the second methyl group lowered k_2 dramatically, its effect on K_a was mixed; it increased K_a in the case of the *m*-isopropyl and *m*-nitro substituents, while lowering K_a when the leaving group was unsubstituted phenyl. This decrease in k_2 and mixed effect on K_a was similar to that observed by Iverson and Main (1969) in a study with eel AChE, in which a series of neostigmine-related carbamates were used.

Thus, the poorer inhibitory power of dimethyl as compared with methyl carbamates appeared to be due to the lower k_2 values of the dimethyl compounds, an effect which was either weakened or reinforced by the variable influence of the extra methyl group on binding.

Effect of Orientation on the Phenyl Ring. Kolbezen *et al.* (1954) studied the effect of ring position with methyl, *tert*-butyl, and nitro substituents of phenyl methylcarbamates.

Table I. Affinity Constants, Carbamylation Rate Constants, and Bimolecular Rate Constants for some Carbamate Inhibitors of Acetylcholinesterase from Bovine Erythrocytes Determined at 25° C, pH 7.6[a]

Dimethylcarbamates	K_a(mM)	k_2(min^{-1})	k_i(M^{-1}min^{-1})	Range (M)	No. of Points
Phenyl	10.3 ± 1.9	0.24 ± 0.02	2.33 ± 0.24 × 10	1×10^{-3}–2×10^{-2}	6
o-nitrophenyl	1.43 ± 0.25	7.25 ± 0.9	5.06 ± 0.28 × 10^3	5×10^{-5}–1.08×10^{-3}	5
p-nitrophenyl	3.2 ± 0.63	0.14 ± 0.02	4.5 ± 0.03 × 10	5×10^{-4}–2.7×10^{-3}	5
m-nitrophenyl	0.92 ± 0.19	0.15 ± 0.02	1.65 ± 0.17 × 10^2	1×10^{-4}–1.8×10^{-3}	5
m-isopropyl	2.47 ± 0.07	6.31 ± 0.13	2.56 ± 0.02 × 10^3	1×10^{-4}–1×10^{-3}	4
m-trimethylammonium-phenyl	0.0102 ± 0.002	44.0 ± 4.7	4.30 ± 0.36 × 10^6	1×10^{-7}–1.2×10^{-5}	5
Methylcarbamates					
Phenyl	23.7 ± 1.9	6.82 ± 0.35	2.88 ± 0.09 × 10^2	1×10^{-4}–2×10^{-2}	5
m-nitrophenyl	0.68 ± 0.12	0.45 ± 0.04	6.6 ± 0.6 × 10^2	1×10^{-4}–1×10^{-3}	4
m-isopropyl	0.177 ± 0.059	80.7 ± 19.6	4.56 ± 0.47 × 10^5	2×10^{-6}–1×10^{-4}	5
m-tert-butyl	0.0666 ± 0.0085	23.4 ± 1.9	3.51 ± 0.17 × 10^5	5×10^{-7}–5×10^{-5}	6
Temik	10.3 ± 1.0	146 ± 10	1.6 ± 0.1 × 10^4	1×10^{-5}–6×10^{-3}	9
Temik Sulfone	17.02 ± 2.9	74 ± 8	4.4 ± 0.3 × 10^3	5×10^{-4}–1.44×10^{-2}	6
1-naphthyl[b]	>0.5	>20	2.18 × 10^4	1×10^{-4}–4×10^{-4}	5
Eserine[c]	0.003 ± 0.0003	10.8 ± 0.3	3.25 × 10^6	2×10^{-7}–1×10^{-5}	7

[a] Substrate velocity measurements were at pH 7.6 and 25° C in 3 mM acetylcholine chloride. [b] K_a and k_2 could not be measured because of insolubility. [c] Main and Hastings (1966b).

In each case, substitution in the *meta*-position gave the highest inhibitory power as measured by I_{50}, using housefly brain AChE. In the present work, attempts were made to determine the k_2 and K_a values of the *o*-, *m*-, and *p*-nitrophenyl methylcarbamates, but reliable values were obtained for only the relatively stable *m*-nitro compound. The others were too unstable at pH 7.6. From the alkaline hydrolysis constants (K_{hyd}) given by Kolbezen *et al.* (1954), the half-life of the *ortho*-compound was estimated to be 1–2 min and that of the *para*-compound, 10–20 min, both at 25° C and pH 7.6. Our results tentatively suggested that the *o*-nitro compound was a significantly more powerful inhibitor than either the *m*- or *p*-analogs.

The nitrophenyl dimethylcarbamates were more stable and the K_a and k_2 values for these were obtained. *Ortho*-substitution resulted in much higher k_2 values than either the *m*- or *p*-analogs, but the effect on binding was less dramatic and the *meta*-analog appeared to bind with slightly greater energy than did the *ortho*. The greater inhibitory power of the *o*-nitro substituted compound was then primarily due to its higher k_2 value. Kolbezen *et al.* (1954) reported that *o*-chloro substituents gave the highest inhibitory power, but that the *meta*-position favored the nitro substituents. This latter observation may not be valid in view of the very high alkaline hydrolysis rates which they reported for these compounds.

The k_2 values of *o*-, *m*- and *p*-dimethylamino substituted phenyl methylcarbamates in reaction with eel AChE (25° C, pH 7.0) were 24, 48.3, and 3.0 min^{-1}, respectively. With this series, the *meta*-substitution gave the highest values (Iverson and Main, 1969). As with nitro substituents, the effect on binding energy was relatively insignificant.

Evidently the ring position resulting in maximal binding and carbamylation rates will vary, depending on the nature of the substituent. As Kolbezen's results indicated, when the substituent was relatively nonpolar, *meta*-substitution appeared to give optimal inhibitory power. This was also true of quaternary ammonium substituents (Iverson and Main, 1969). The nitro groups evidently bind to a different region, since *ortho*-substitution rather than *meta* resulted in the highest k_2 values and hence inhibitory power.

The k_2 values had little or no relationship to the electron withdrawing power of the substituent, as reflected by the Hammett substituent constant, σ (Hammett, 1940). For example, σ for an *m*-isopropyl substituent is −0.190, whereas that of *m*-nitro is +0.710, but the k_2 values for the respective methyl carbamates were 80.7 and 0.45 min^{-1}, a relationship which is opposite to that expected from the electronic hypothesis (O'Brien, 1960).

The Analogy Between the Substrate and Inhibition Reactions. The K_m and V_{max} values of the *o*-, *m*-, and *p*-nitrophenyl acetates and of phenyl acetate and acetylcholine in reaction with AChE at pH 7.6, 25° C, are given in Table II. Variations of K_m with ring orientation are compared with variations in K_a of the similarly oriented phenyl dimethyl carbamates in Figure 4B. The V_{max} and k_2 values are similarly compared in Figure 4A.

Considering V_{max} and k_2 first, in each series *ortho*-substitution resulted in the highest rates, followed by the unsubstituted phenyl compounds, while *meta*- and *para*-substitution gave the lowest values. In each series the latter were close. Qualitatively, the effect of ring orientation was the same in both the substrate and inhibitor series, but the relative changes differed. For example, the V_{max} ratio of the *ortho*- compared with the *meta*-substituted compounds was 3.7, while the comparable k_2 ratio was 48.

Table II. Michaelis Constants (K_m) and Maximum Velocities (V_{max}) of Phenyl and Nitrophenyl Acetates and of Acetylcholine for Bovine Erythrocyte Acetylcholinesterase at 25° C, pH 7.6

Substrate	K_m(mM)	V_{max} (μMoles/min/ml)	Range (mM)	No. of Points
Phenyl acetate	1.14 ± 0.08	21.8 ± 0.1	0.6–5.0	7
o-nitrophenyl acetate	0.28 ± 0.02	32.0 ± 0.8	0.2–4.5	15
m-nitrophenyl acetate	0.78 ± 0.04	8.66 ± 0.08	0.4–4.0	20
p-nitrophenyl acetate	3.86 ± 0.37	4.84 ± 0.27	0.7–2.0	14
Acetylcholine	0.02 ± 0.001	19.4 ± 0.2	0.02–0.3	10

Table III. K_a and k_2 of the Diethyl, *o*-, *m*-, and *p*-Nitrophenyl Phosphates and of Tetram at 5° C, pH 7.0 for the Inhibition of Bovine Erythrocyte Acetylcholinesterase

Compound	k_2 min^{-1}	K_a mM	k_i $M^{-1}min^{-1}$
o-nitrophenyl	2.97 ± 0.5	1.44 ± 0.13	2.06×10^3
m-nitrophenyl	0.81 ± 0.13[a]	0.22 ± 0.12[a]	3.7×10^{3a}
p-nitrophenyl[b]	42.7 ± 2.8	0.36 ± 0.05	1.20×10^5
Tetram[c]	157 ± 27	0.28 ± 0.5	5.6×10^5

[a] Determined at 25° C: at 5° C the estimated $k_2 = 0.2$ min^{-1} and $k_i = 0.9 \times 10^3 M^{-1}min^{-1}$ assuming $Q_{10} = 2$ and K_a remained constant. [b] From Chiu *et al.*, 1969. [c] From Main, 1969a.

The results suggested also that the substrate reaction was k_2 rather than k_3 controlled. This was further supported by the observation that while the V_{max} values of acetylcholine and phenyl acetate were close, the V_{max} of the *o*-nitrophenyl acetate reaction was about 50% higher. On the basis of the similar V_{max} values of the phenyl acetate and acetylcholine reactions, Krupka (1966) suggested that the k_3 or deacetylation step was rate controlling. The present results are not consistent with this interpretation.

The K_m and K_a values of the two series were similar, except for the K_a of the phenyl dimethylcarbamate reaction, which was 10 times greater than the K_m of the phenyl acetate reaction. Although the order of increasing K_a values (*meta* < *ortho* < *para* < phenyl) differed from that of the K_m values (*ortho* < *meta* < phenyl < *para*), Figure 4B suggested similar trends and indicated that the differences were not great, except for the phenyl pair just mentioned. In summary, the results suggested a parallelism between the ring position and reactivity in the two series.

Consideration of the *o*-, *m*-, and *p*-nitrophenyl diethylphosphates presented a radically different picture, as shown by the results given in Table III. Here, *para*-orientation resulted in the highest k_2 values, followed by *ortho*, then *meta*, the *meta* being much lower. K_a in order of increasing values was *meta* < *para* < *ortho*.

The substrate and carbamate reactions evidently were more closely related than were those of the organophosphates. This suggested that the phosphorylation reaction may occur at a different region of the active site than did acylation.

The constants governing the reactions of Temik, tetram, and acetylcholine were also of interest, since both tetram and Temik have structures which are analogous to that of acetylcholine. Both have groups which could occupy the anionic site. The k_2 values of Temik and tetram were relatively high, suggesting that occupation of the anionic site led to high k_2 values. This was consistent with the k_2 value of neostigmine, which also has a charged group positioned to occupy the

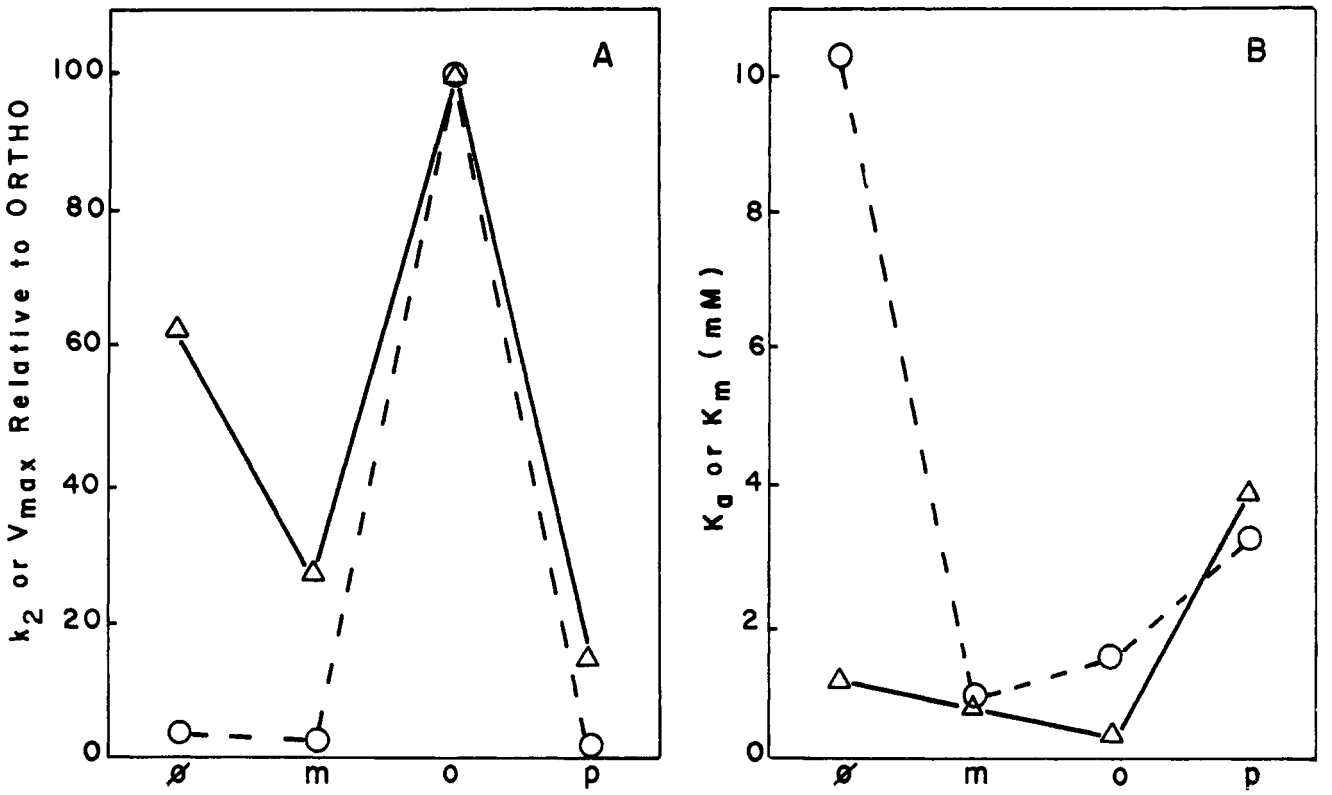

Figure 4. A comparison of phenyl and nitrophenyl dimethylcarbamate inhibition reaction constants with the substrate constants obtained with the analogous acetates in reaction with bovine erythrocyte acetylcholinesterase

(A) contrasts carbamylation rate constants (k_2), o, to maximum velocities (V_{max}), Δ; (B) contrasts affinity constants (K_a), o, to Michaelis Constants (K_m), Δ. ϕ, unsubstituted phenyl; *m*, *meta*-nitrophenyl; *o*, *ortho*-nitrophenyl; and *p*, *para*-nitrophenyl

anionic site. However, positioning with respect to the anionic site did not necessarily lead to particularly good binding. For example, the K_a of neostigmine was lower than that of acetylcholine, indicating better binding, whereas that of Temik was relatively high.

The K_a values of the organophosphorus and carbamate inhibitors were in the same order as the K_m values of comparable substrates, indicating that K_a was a valid measure of affinity.

The results do not indicate any close relation of k_2 with K_a. However, K_a is a measure of binding energy and would not necessarily reflect the molecular orientation which would lead to high k_2 values. That orientation is an important factor in determining k_2 values seems evident, first from the effects of groups which tend to bind to the anionic site, and second, from the comparison of the methyl and dimethyl carbamate pairs which have groups that bind to the acyl binding site of the enzyme. The complex effects of ring orientation and of different substituents would then, at least in part, be a reflection of the way in which the substituents were accommodated on the active site with respect to the various binding sites.

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