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Electron-Donor and Affinity Constants and Their Application to the Inhibition of Acetylcholinesterase by Carbamates

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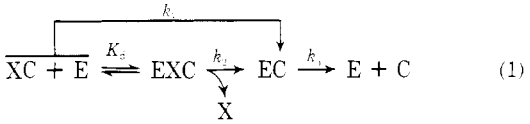
Twenty-three methylcarbamates were studied, and the effects of variations in their hydrophobicity and ability to form charge-transfer complexes upon their affinity, reactivity, and overall potency for acetylcholinesterase were explored. Variations in these factors accounted for most of the variation in enzymic effects, but the compounds fell into two distinct classes with respect to the relation between, for instance, reactivity and affinity.

Most carbamates inhibit acetylcholinesterase (AChE) by reacting with it in two steps (Wilson et al., 1961; O'Brien et al., 1966; O'Brien, 1968). The first step is characterized by the dissociation constant for the reversible complex of AChE and the carbamate (Michaelis complex); the second involves carbamylation of a serine hydroxyl in the AChE active site and is usually characterized by the reaction constant, k_2. If C is the methylcarbamyl group and X is the leaving group, the inhibition process may be represented as follows:



$$XC + E \rightleftharpoons EXC \rightarrow EC \rightarrow E + C \quad (1)$$

where k_3 is the rate constant of enzyme regeneration. The overall potency of the carbamate is measured by k_i, which equals k_2/K_d (Main, 1964).

In para-substituted phenyl methylcarbamates, the variation in potency (for instance, as measured by I_{50}, the concentration of a carbamate to inhibit AChE by 50% in a fixed time) is primarily due to variation in K_d (O'Brien et al., 1966). For 12 such compounds, we showed that their I_{50} values were closely correlated with the ability of their aromatic portions to donate electrons to a model electron acceptor (tetracyanoethylene [TCNE]) and form charge-transfer complexes (CTC) (Hetnarski and O'Brien, 1972). The CTC formation ability is measured by K_x, the association constant for CTC formation. Later we showed (Hetnarski and O'Brien, 1973) that for seven arylmethyl methylcarbamates, which are virtually noncarbamylating, and therefore simple reversible inhibitors, their K_d and K_x values were excellently correlated.

In the present study we have extended this approach to additional para-substituted aromatic methylcarbamates and also to nine meta-substituted compounds. We have also introduced new constants, the electron-donor and affinity factors, to simplify the quantitation of structure–activity relation, and to examine the processes in question from the viewpoint of the linear free-energy relationship. The current work also includes investigations on kinetic constants of the second stage (k_2), and hence covers the whole inhibition process, characterized by k_i.

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METHODS

K_d values were determined by the zero-time method (O'Brien, 1968; Hart and O'Brien, 1973). The assays were performed at 25° by the following procedure, modified from Ellman et al. (1961). A mixture was prepared consisting of 15 vol of buffer (0.2 *M* sodium phosphate, pH 7.6), 0.2 vol of acetylcholine chloride (Sigma) designed to give a final concentration of $0.73K_m$ and $0.88K_m$ ($K_m = 0.29 \times 10^{-3}$ *M*, found under conditions described here) in freshly distilled water, and 1 vol of a 0.014 *M* solution of Nbs_2 [5,5′-dithiobis(2-nitrobenzoic acid)] plus 0.02 *M* sodium carbonate in 0.2 *M* sodium phosphate buffer (pH 7). Of this mixture, 1.62 ml was placed into the cuvette and 1 ml of 1.5 units/ml of bovine erythrocyte AChE (Sterwin) solution in the aforementioned buffer solution (pH 7.6) was added; the final concentration of enzyme was 0.57 unit/ml, of Nbs_2 0.53 m*M*, and of sodium phosphate (the dominant ions) 0.2 *M*. The final pH was 7.56. The reaction was followed over 0.5 min at 412 nm using a Beckman Acta III spectrophotometer, then 0.02 ml of a freshly prepared solution of inhibitor was added, the contents of the cuvette were mixed instantly, and readings were taken at 412 nm (Figure 1). In order to find K_d values we plotted i (inhibitor final concentration) against$(v_c/v_0) - 1$, where v_c and v_0 are the reaction velocities in the absence and presence of an inhibitor, respectively. Four or five concentrations of inhibitor were used. The v_0 data were obtained by extrapolating velocities of the substrate hydrolysis to zero time, using the plot of the logarithm of hydrolysis rate as a function of time (Main, 1967) (Figure 2). The inhibitor concentrations ranged between 10^{-3} and 10^{-6} *M*, depending on solubility and potency; the actual range for each compound is in Table II.

K_x values (association constants of CTC between aryl methylcarbamates and TCNE) were determined by the Benesi–Hildebrand (1949) method in 1,2-dichloroethane at 23° (Hetnarski, 1964, 1965). The method involved the preparation of a series of concentrations of carbamates (acting as donors) in solvent, with mole fractions of from 0.003 to 0.007. Each was made 5×10^{-3} *M* with respect to TCNE. The resultant absorption was measured and obeyed

Table I. Anticholinesterase Activity and Kinetic Properties of Aryl Methylcarbamates (R—OC(O)NHCH$_3$)

No.	R	I_{50} (found)[a]	I_{50}(found)/ I_{50}(calcd)	k_2, min^{-1}	C_{k_2}	k_i, M^{-1} min^{-1}	C_{k_i}
1	4-Fluorophenyl	2.3×10^{-4}	0.5	1.11 ± 0.10	−0.43	171	−0.47
2	4-Chlorophenyl	2.4×10^{-4}	1.1	1.28 ± 0.10	−0.37	284	−0.25
3	4-Bromophenyl	8.8×10^{-5}	0.5	1.44 ± 0.01	−0.32	379	−0.12
4	4-Iodophenyl	2.0×10^{-4}	0.8	0.99 ± 0.14	−0.49	298	−0.22
5	Phenyl	2.0×10^{-4}	1.4	3.02 ± 0.11	0.00	499	0.00
6	4-Tolyl	1.0×10^{-4}	1.6	4.41 ± 0.06	0.16	1,125	0.45
7	4-Anisyl	8.0×10^{-5}	0.4	1.52 ± 0.10	−0.30	322	−0.19
8	4-Ethylphenyl	7.0×10^{-5}	0.8	2.45 ± 0.04	0.09	770	0.19
9	4-Isopropylphenyl[b]	7.0×10^{-5}					
10	4-Butoxyphenyl	2.0×10^{-5}	0.8	1.01 ± 0.05	−0.48	2,971	0.78
11	1-Naphthyl	9.0×10^{-7}	1.8	1.83 ± 0.03	−0.22	140,759	2.45
12	3-Fluorophenyl	8.5×10^{-5}	0.9	1.97 ± 0.05	−0.19	784	0.20
13	3-Chlorophenyl	5.0×10^{-5}	1.9	4.78 ± 0.42	0.20	2,555	0.17
14	3-Bromophenyl	1.3×10^{-5}	1.1	4.60 ± 0.21	0.18	5,974	1.09
15	3-Iodophenyl	7.0×10^{-6}	1.5	1.85 ± 0.10	−0.21	16,818	1.49
16	3-Tolyl	1.4×10^{-4}	9.5	7.01 ± 0.43	0.37	4,705	0.97
17	3-Ethylphenyl	3.8×10^{-5}	23.8	1.57 ± 0.10	−0.28	46,176	1.97
18	3-Isopropylphenyl	3.4×10^{-7}	3.9	1.90 ± 0.10	−0.20	678,571	3.13
19	3-Anisyl	2.2×10^{-5}	0.6	3.42 ± 0.39	0.05	1,965	0.60
20	3-Isopropoxyphenyl	9.2×10^{-6}	9.3	1.47 ± 0.12	−0.31	73,500	2.17
21	3-Dimethylaminophenyl	8.0×10^{-6}	3.0	1.10 ± 0.14	−0.44	27,500	1.74
22	3,5-Dimethylphenyl	6.0×10^{-6}	0.6	1.00 ± 0.05	−0.48	7,142	1.16
23	3,5-Dimethyl-4-methylthiophenyl			1.93 ± 0.05	−0.13	128,667	2.4

[a] I_{50} found data were reported by Metcalf and Fukuto in 1965. [b] Traces from the recording spectrophotometer, under conditions applied here, had inadequate curvatures for tangent drawing; the solubility of the compound was very poor and consequently its concentration was the lowest of all para-substituted phenyl methylcarbamates investigated. As a result, k_2 and k_i for that compound are not reported.

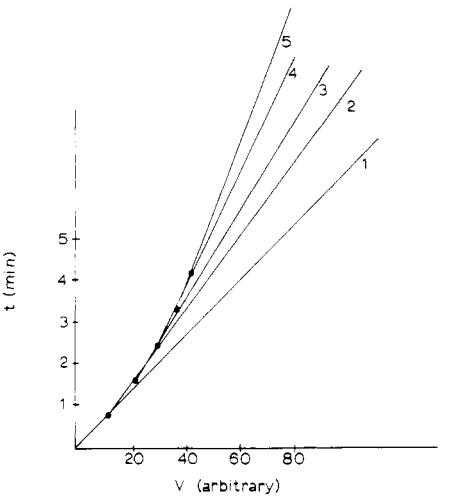

Figure 1. Progressive inhibition of acetylcholinesterase. A trace from the recording spectrophotometer is displayed. The origin corresponds to the moment of injecting 3,5-dimethyl-4-thiomethylphenyl methylcarbamate (final concentration 5×10^{-6} *M*). Reaction velocities at various times were found by means of tangents.

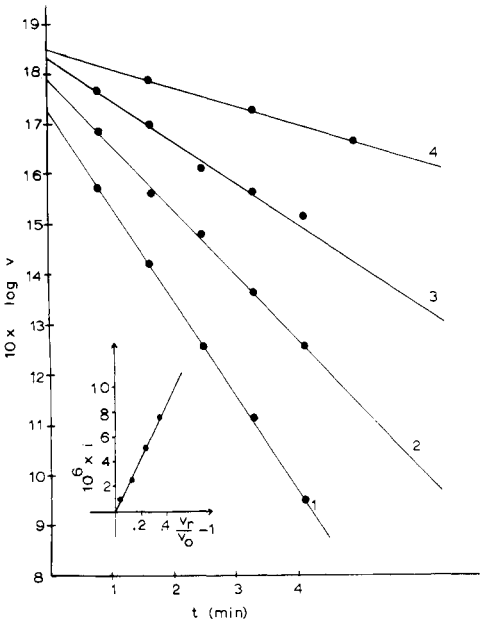

Figure 2. Analysis of tangent data from Figure 1. (Top) Logarithms of the reaction velocities were plotted as a function of time for four values of *i* (inhibitor concentration). Points of the intersection of plots with the *Y* axis are zero-time velocities (v_0); v_c denotes the velocities in the absence of inhibitor. (Inset) Plot of $i = f(v_c/v_0 - 1)$, from which the K_d value was found.

the Benesi–Hildebrand equation (for a 1-cm pathlength):

$$M_A/A = 1/\epsilon_c K_x C_D + 1/\epsilon_c \quad (2)$$

where A is the observed absorption and M_A is the molar concentration of acceptor, ϵ_c is the molar extinction coefficient of the complex, K_x is the association constant of donor and acceptor, and C_D is the concentration of donor (carbamate) in mole fractions. A plot of M_A/A as a function of $1/C_D$ permitted the calculation of K_x, for which the least-squares method was used.

K_x values for compounds **21** and **23** (Table II) were not determined because the electron-donor site is located on the highly electron-positive atoms of nitrogen and sulfur (Hetnarski and Grabowska, 1969) rather than on the aromatic ring, as in the case with the remaining compounds.

RESULTS AND DISCUSSION

Comparison with the Literature. Kinetic data have been published for many of the compounds reported here-

Table II. Affinity for AChE and Some Physicochemical Properties of Aryl Methylcarbamates (ROC(O)NHCH$_3$)

No.	Range of carbamate concns applied for K_d determinations, M	K_d(found), mM	K_d(calcd), mM[a]	C_A	K_x(mole fract)$^{-1}$	λ_{max}, nm	C_T	π
1	10^{-3}–10^{-4}	6.49 ± 1.30	6.91	−0.03	5.5 ± 2.1	357	−0.41	0.15
2	10^{-3}–10^{-4}	4.51 ± 0.96	4.97	0.13	7.6 ± 1.5	360	−0.27	0.70
3	10^{-3}–10^{-4}	3.80 ± 0.16	3.33	0.20	12.9 ± 1.0	400	−0.04	1.02
4	10^{-3}–10^{-4}	3.32 ± 0.23	3.07	0.25	10.0 ± 6.2	420	−0.15	1.26
5	10^{-3}–10^{-4}	6.05 ± 0.85	5.88	0.00	14.2 ± 3.6	365	0.00	0.00
6	10^{-3}–10^{-4}	3.92 ± 0.36	3.78	0.19	23.1 ± 3.9	405	0.21	0.52
7	10^{-3}–10^{-4}	4.71 ± 0.26	4.56	0.11	36.4 ± 3.1	510	0.41	−0.04
8	10^{-3}–10^{-4}	3.18 ± 0.07	2.80	0.28	19.9 ± 3.5	405	0.15	0.97
9	10^{-4}–10^{-5}	0.85 ± 0.22	1.00	0.85	31.1 ± 1.7	405	0.34	1.40
10	5×10^{-4}–7.5×10^{-5}	0.34 ± 0.01	0.49	1.25	39.1 ± 3.0	525	0.44	1.46
11	10^{-5}–10^{-6}	0.013 ± 0.001	0.38	2.67	75.5 ± 10.6	540	0.73	1.12
12	10^{-3}–10^{-4}	2.51 ± 0.16	2.70	0.38	5.6 ± 1.4	360	−0.42	0.13
13	10^{-3}–10^{-4}	1.87 ± 0.12	1.61	0.51	4.2 ± 1.9	365	−0.53	0.76
14	10^{-4}–10^{-5}	0.77 ± 0.08	0.60	0.90	14.2 ± 1.1	390	0.00	0.94
15	10^{-5}–10^{-6}	0.12 ± 0.01	0.36	1.74	10.3 ± 1.6	395	−0.14	1.15
16	10^{-4}–10^{-5}	1.49 ± 0.49	1.09	0.61	27.7 ± 3.7	400	0.29	0.51
17	2.5×10^{-5}–10^{-6}	0.35 ± 0.06	0.42	2.25	17.7 ± 2.6	400	0.10	1.30
18	10^{-5}–10^{-6}	0.0028 ± 0.0003	−0.25	3.33	18.5 ± 6.4	400	0.12	1.30
19	10^{-3}–10^{-4}	1.74 ± 0.03	1.78	0.54	31.9 ± 8.2	495	0.35	0.12
20	10^{-5}–10^{-6}	0.020 ± 0.0001	0.24	2.48	30.2 ± 2.3	490	0.33	0.92
21	2.5×10^{-5}–5×10^{-6}	0.04 ± 0.001		2.18				
22	10^{-4}–10^{-5}	0.14 ± 0.01		1.63	46.6 ± 3.4	430	0.51	1.02
23	10^{-5}–10^{-6}	0.015 ± 0.0002		2.61				

[a] K_d found was calculated from eq 10.

in; we now compare our findings with them. Table I lists, for all except compound **23**, the values of I_{50} reported by Metcalf and Fukuto in 1965 for a 10-min incubation time. If decarbamylation is negligible, then using the symbols of the present paper, one can rewrite eq (6) of Main (1964) which was applied to the precisely parallel case of organophosphates:

$$1/i = 1/K_d[(tk_2/2.3\ \Delta \log V) - 1] \quad (3)$$

where i is the inhibitor concentration, t is the incubation time, and $\Delta \log V$ is the change in the logarithm of the enzyme velocity at time t. For 50% inhibition, i is the I_{50} and V is halved, so $\Delta \log V = 0.3$, and:

$$1/I_{50} = 1/K_d[(tk_2/0.69) - 1] \quad (4)$$

$$I_{50}(\text{calcd}) = K_d/[(tk_2/0.69) - 1] \quad (5)$$

In view of the fact that in a 10-min period decarbamylation plays a significant role, the agreement of our calculated I_{50} with the literature values is surprisingly good: for the 21 cases which can be compared, the ratio (literature data/calculated data) averages 3.1, and is usually (i.e. in 15 cases) within the range of 0.5–2.0.

Hastings et al. (1970) report for compounds **5**, **11**, and **18** values of k_2 which are greater than ours by factors of 2, >11, and 42, and values of K_d which are greater than ours by factors of 4, >38, and 0.2. We are unable to account for these differences. Our values agree quite well with those we reported previously (O'Brien et al., 1966) for seven methylcarbamates (**1**, **2**, **5**, **6**, **7**, **11**, and **22**) using a quite different method: those K_d values averaged 1.7-fold less than reported herein, and those k_2 values averaged 1.2-fold less.

Relations between Structure and Enzyme Activity. As a measure of the electron donor capacity of aromatics we have proposed the C_T value (Hetnarski and O'Brien, 1975) which is a new constant, defined similarly to the Hammett σ constant, as follows:

$$C_T = \log K_x - \log K_H \quad (6)$$

where K_x is the association constant for CTC formation with tetracyanoethylene of the substituted compound and K_H is that for the unsubstituted compound, in this case phenyl methylcarbamate. There is a general parallelism between substituent effects upon C_T and Hammett's σ, in that electron-withdrawing substituents will tend to deplete the electrons of the whole π system, and hence reduce CTC formation and affect C_T, and also to reduce the electron density at a reaction center, and hence reduce σ. But the substituent effects upon C_T are more sensitive to steric factors, and so the parallelism is incomplete; thus, bromine in the meta position had no effect on C_T (Table II) but had a substantial effect upon σ. C_T provides a more direct comparison if one's interest is in π complexes of any kind; furthermore, it can be applied to compounds not suited for σ evaluation, such as ortho-substituted compounds and extended three-dimensional molecules. The measurement of C_T is particularly simple when a compound forms complexes which absorb in the visible region.

We found that the variation in affinity for the enzyme could be largely accounted for in terms of two factors only: variations in hydrophobicity and ability to donate π electrons to form CTC's, as measured with a model acceptor (tetracyanoethylene) (Table II). The relative contribution of these two factors varies in the series of inhibitors on which we presently report. We measured the hydrophobicity of each substituent by Hansch's constant π (Leo et al., 1971), defined in a fashion analogous to the Hammett σ constant:

$$\pi_x = \log P_x - \log P_H \quad (7)$$

where P_x and P_H are the partition coefficients (usually in the octanol–water system) of the substituted and the unsubstituted phenyl methylcarbamates, respectively.

In order to examine whether a linear free-energy relationship holds for complex formation between AChE and

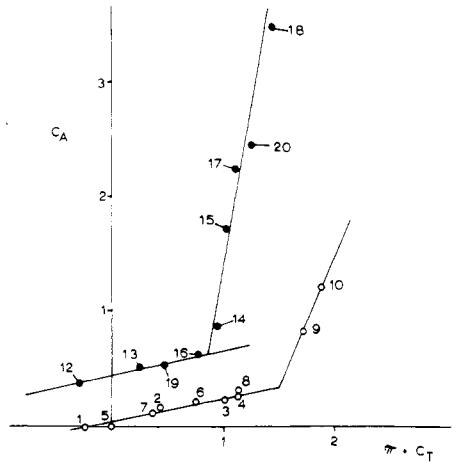

Figure 3. Dependence of affinity for acetylcholinesterase, C_A (eq 8), upon hydrophobicity, π (eq 7), and ability to form CTC, C_T (eq 6).

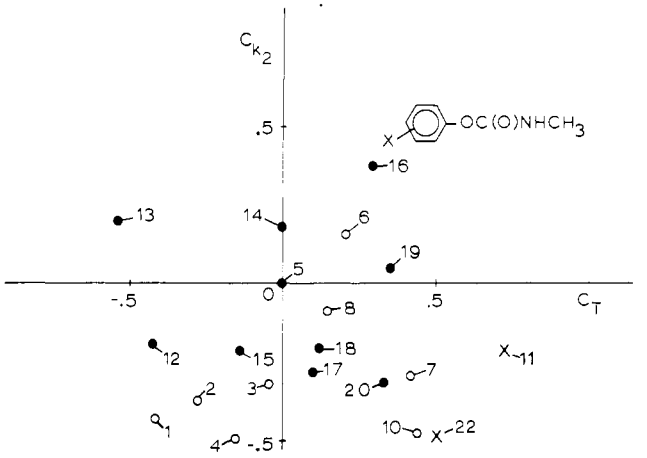

Figure 5. Lack of correlation between carbamylation constants (C_{k_2}, eq 11) and ability to form CTC (C_T, eq 6).

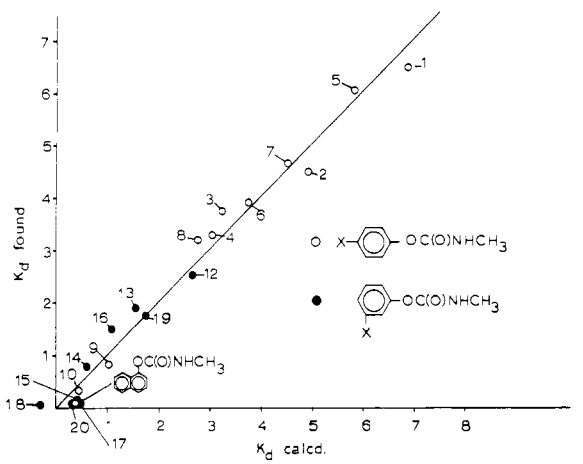

Figure 4. Relation between calculated and found dissociation constants for the enzyme–inhibitor complex. K_d calculated values were obtained by eq 9 and 10: (●) meta-substituted compounds; (○) para-substituted compounds.

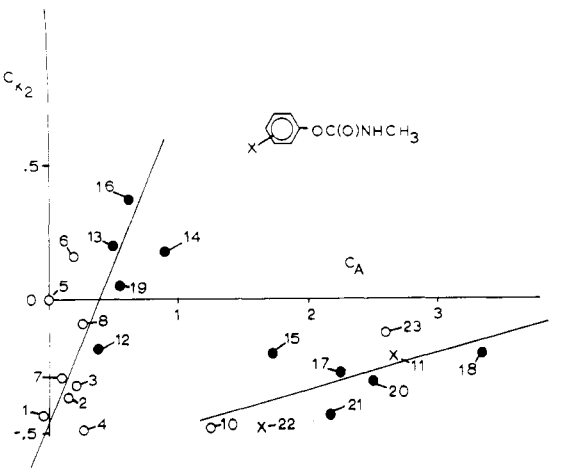

Figure 6. Relation between carbamylation constant (C_{k_2}, eq 11) and affinity of carbamates for enzyme (C_A, eq 8).

an inhibitor with respect to charge-transfer complex formation, we defined the affinity constant for complex formation, C_A, as follows:

$$C_A = \log K_a^x - \log K_a^H \quad (8)$$

where K_a^x is the affinity ($1/K_d$) of the substituted compound and K_a^H is that of the phenyl methylcarbamate.

We explored the dependencies of C_A upon C_T and π for para- and meta-substituted phenyl methylcarbamates and found that a linear free-energy relationship is satisfied in both cases over limited ranges; however, in each of them, graphs plotted on a three-dimensional device consisted of two planes. For example, in the case of meta-substituted phenyl methylcarbamates for the first four compounds (F, Cl, CH_3O, and CH_3 derivatives) the correlation coefficient was 0.999 and for the second five [Br, I, C_2H_5, $(CH_3)_2CHO$, and $(CH_3)_2CH$] it was 0.956. These good correlations contrast with $r = 0.831$ for the whole series. Because π and C_T are independent vectors which vary in the same direction, it was possible to observe the two planes of the three-dimensional model as two lines in a two-dimensional plot of $C_A = f(\pi + C_T)$, Figure 3.

The broken-plane three-dimensional plots are well linearized by a semilogarithmic plot. With a computerized least-squares method we found for para-substituted phenyl methylcarbamates the following relations:

$$K_d = -(3.476 \pm 0.359)C_T - (2.645 \pm 0.221)\pi + 5.880 \quad (9)$$
$$r = 0.989,\ n = 11,\ s = 0.367$$

For meta-substituted phenyl methylcarbamates we found:

$$K_d = -(1.228 \pm 0.357)C_T - (1.960 \pm 0.271)\pi + 2.444 \quad (10)$$
$$r = 0.958,\ n = 9,\ s = 0.322$$

where r is the correlation coefficient, n is the number of compounds, and s is the standard deviation. From these equations we were able to calculate values of K_d for all 20 compounds, and compare them with the measured values. Figure 4 shows that there was an excellent correlation (r = 0.990).

Relations between Structure and Carbamylating Activity. One might expect that electron-donating ring substituents would reduce the electrophilic character of the carbamyl carbon, and hence reduce k_2. If so, attempts to improve the overall potency $k_i = k_2/K_d$, by introducing electron-donating substituents to improve K_d, might be offset by adverse effects on k_2. Once again we defined a term, in this case C_{k_2}, to compare the relative k_2 values:

$$C_{k_2} = \log k_2^x - \log k_2^H \quad (11)$$

where k_2^x is the k_2 value for a substituted compound and k_2^H is for the parent phenyl methylcarbamate. A plot of

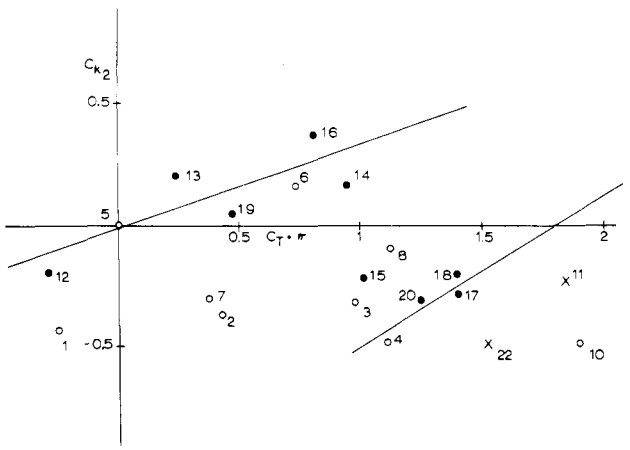

Figure 7. Approximate broken-line dependence of carbamylation constant (C_{k_2}) upon hydrophobicity and CTC-forming ability ($\pi + C_T$).

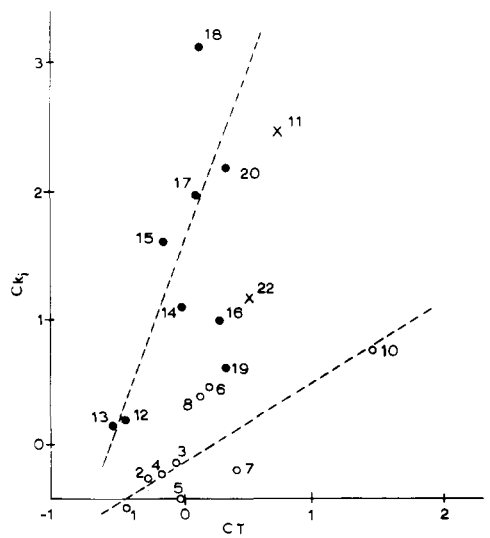

Figure 8. Relation between overall anticholinesterase activity (C_{k_i}, eq 12) and affinity of carbamate for enzyme.

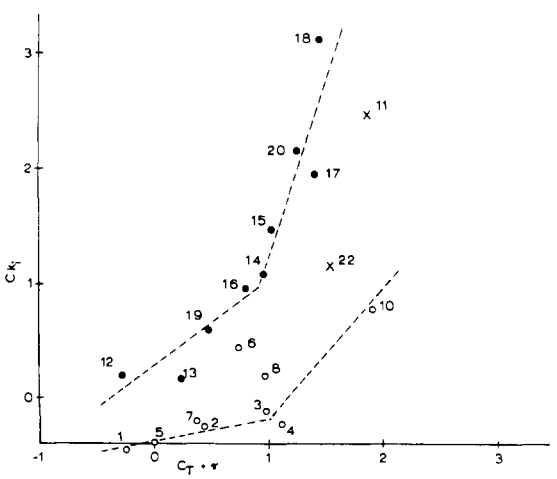

Figure 9. Dependency of C_{k_i} vs. ($C_T + \pi$). C_{k_i} is defined in eq 12.

C_{k_2} against C_T (Figure 5) showed no correlation between these parameters, yet k_2 was strongly sensitive to changes in affinity, showing a most unusual relationship (Figure 6). Values of C_{k_2} increased with increases in C_A (the reverse of expectation).

Compounds whose K_a is less than that for phenyl methylcarbamate (i.e., with C_A less than 1), a series which contains virtually all the para-substituted and half of the meta-substituted compounds, showed a sharp positive correlation between C_{k_2} and C_A. The remaining compounds all showed a less sharp positive correlation, and it is particularly noteworthy that C_{k_2} drops precipitously as C_A passes through 1. The two series of compounds appear to follow two completely different dependencies. In both cases, improved affinity gives improved k_2, suggesting that a close fit improves the effectiveness of the carbamylation step. The two kinds of dependence might imply two ways of fitting onto the enzyme surface.

In view of the fact that C_{k_2} is correlated with C_A, which in turn is correlated with ($C_T + \pi$), it was not surprising to find that C_{k_2} shows a relation to ($C_T + \pi$) (Figure 7); the same broken line dependency is seen, but the scatter is much greater, suggesting that the primary effect upon k_2 is determined by affinity, rather than that CTC-forming ability and hydrophobicity exert a direct effect upon k_2.

Relation between Structure and Overall Potency. For the designer of toxicants, the question of special importance is the role of C_T and π in determining overall potency, measured by k_i. In order to express k_i in a relative logarithmic form we use the term C_{k_i} which we define as:

$$C_{k_i} = \log k_i^x - \log k_i^H \qquad (12)$$

where k_i^x is the k_i value of a substituted compound, whereas k_i^H is that of the parent phenyl methylcarbamate. Because $k_i = k_2/K_d$, the outcome is determined by the combination of the effects of C_T and π upon k_2 and K_d. Figure 8 shows that the effect of C_T upon k_i is clearly different for para- and meta-substituted compounds. Figure 9 shows that insertion of π as well as C_T greatly improves the accounting for variation in k_i, and the effects upon para- and meta-substituted compounds again differ greatly, there being no overlap between the two series. The "broken-line" relationship, evident in the effect on C_A (Figure 3), is still visible here.

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