Charge Transfer in Cholinesterase Inhibition. Role of the Conjugation between Carbamyl and Aryl Groups of Aromatic Carbamates†

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ABSTRACT: The abilities of seven arylmethyl methylcarbamates to inhibit acetylcholinesterase were explored. Unlike the corresponding aryl methylcarbamates which form complexes with the enzyme and then carbamylate it, these compounds were found to be virtually noncarbamylating, and inhibit only by complex formation. Their inhibitory activity was well correlated with their ability to form charge-transfer complexes with a model acceptor, tetracyanoethylene. It was therefore postulated that charge-transfer complex formation was involved in the inhibition, and some puzzling structure–activity relations of aromatic carbamates were thereby accounted for.

Previous studies on carbamates which inhibit acetylcholinesterase have shown that they carbamylate the enzyme, as judged by the facts that the reaction is progressive; that the properties of the inhibited enzyme are dependent only on the nature of the N substituents (Wilson *et al.*, 1961); and the rate of release of leaving group parallels the rate of inhibition (O'Brien *et al.*, 1966). Complex formation prior to carbamylation has been proposed (Wilson *et al.*, 1961) and the values of the constants for complex formation and carbamylation were separately evaluated (O'Brien *et al.*, 1966, O'Brien, 1968).

We have suggested for aryl methylcarbamates that the complexing step involves a charge-transfer complex (CTC)[1] with the aromatic ring (Hetnarski and O'Brien 1972). However, in such compounds the situation is complicated by the fact that ring substituents which modify CTC forming ability also modify carbamylating activity, since the ring is conjugated with the carbamyl group. We therefore set out to explore benzyl carbamates, in which the ring and the carbamyl groups are not conjugated. As we shall show, these compounds proved to carbamylate the enzyme very little, and their inhibitory activity was therefore due almost exclusively to complex formation. Consequently, the relations between structure and complexing ability are particularly easy to study in these carbamates.

Materials and Methods

We studied several derivatives of arylmethyl methylcarbamates, which are listed in Table I. The carbamates selected were those with minimal possibilities of hydrophobic interaction between the ring substituent and the enzyme surface; the hydrophobic coefficients of the substituents were not higher than 0.7 (Hansch, 1970). Only para-substituted compounds were used, to minimize direct interaction between the substituent and the carbamyl group.

The compounds were prepared from commercially available appropriate aromatic carbinols and methyl isocyanate in ether solution. With the exception of benzyl methylcarbamate (Metcalf and Fukuto, 1967), none of the compounds of Table I has been previously examined for anticholinesterase activity. Compounds **3** and **7** (Table I) were prepared by Tartler *et al.* for evaluation of their bacteriostatic properties (Tartler *et al.*, 1966). Compound **6** belongs to a group of benzyl methylcarbamate derivatives reserved by a French patent (French Patent, 1965) as herbicides, but its properties have not been described.

The enzyme assays were performed at 25° by the following modification of the method of Ellman *et al.* (1961). To 3 ml of buffer (0.2 M sodium phosphate, pH 7.6) was added 0.05 ml of enzyme solution containing 30 or 15 units of bovine erythrocyte acetylcholinesterase (Winthrop) in buffer and 0.04 ml of inhibitor in methanol. After 3 min, 0.02 ml of 0.75 M acetylcholine chloride (Sigma) (in freshly distilled water) was added plus 0.1 ml of a solution of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) and 0.01 M sodium bicarbonate buffer (pH 7). Absorption was measured at 412 nm in a Beckman Acta III spectrophotometer against a blank without enzyme. The I_{50} was then calculated, *i.e.*, the concentration of inhibitor for 50% inhibition, in order to permit comparison with aryl methylcarbamates. The method of Dixon (1953) was used to calculate K_D, the dissociation constant for inhibitor and enzyme.

Results

The most striking feature of the benzyl methylcarbamates was their failure to show progressive inhibition typical of other carbamates. Figure 1 is an actual trace from the recording spectrophotometer, illustrating the way that phenyl methylcarbamate gives progressive inhibition, caused by carbamylation subsequent to complex formation (O'Brien, 1968). By contrast, the figure shows that benzyl methylcarbamate shows nonprogressive inhibition, which can only mean that the complex formation is not followed by measurable carbamylation within the time span of the reaction. No progressive inhibition was seen within about 40 min. Consequently, the benzyl methylcarbamate inhibition can be

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[1] Abbreviations used are: I_{50}, the concentration of given compound to inhibit acetylcholinesterase by 50% in 10 min; CTC, charge-transfer complex(es); K_x, association constant of CTC expressed in (mole fraction)$^{-1}$; k_i, bimolecular rate constant of the inhibitory reaction; k_2, monomolecular rate constant of the inhibitory reaction; K_D, dissociation constant of the complex of acetylcholinesterase and inhibitor; r, correlation coefficient.

TABLE I: Analytical Data of Investigated Compound ($XCH_2OC(O)NHCH_3$).

No.	X	Mp (°C)	% C Calcd	% C Found	% H Calcd	% H Found	% N Calcd	% N Found
1	*p*-Nitrophenyl	125–127	51.4	51.5	4.8	4.6	13.3	13.2
2	*p*-Fluorophenyl	75–77	59.0	59.5	5.5	5.7	7.7	7.4
3	*p*-Chlorophenyl[a]	73–75	54.1	54.0	5.0	5.0	7.0	7.1
4	Phenyl[b]	30–32					8.5	8.6
5	*p*-Toyl	58–60	67.0	67.3	7.3	7.3	7.8	7.6
6	*p*-Anisyl	83–84	61.5	61.4	6.7	6.8	7.2	7.2
7	α-Naphthyl[c]	80–82	72.5	72.1	6.0	6.1	6.5	6.8

[a] The compound was characterized by mp 67.5–68.5° (Tartler *et al.*, 1966). [b] The compound was reported by Metcalf and Fukuto (1967) as liquid. [c] The compound was reported by Tartler *et al.* (1966).

TABLE II: Summary of Anticholinesterase Potency of Carbamates and Their Complex Formation Abilities.

X	I_{50} (A) $XCH_2OC(O)NHCH_3$	I_{50} (B) $XOC(O)NHCH_3$	A/B	K_D[a] (mM)	K_X[b] [(mole fraction)$^{-1}$]
p-Nitrophenyl	3.7×10^{-3}			0.83	
p-Fluorophenyl	9.5×10^{-3}			3.40	
p-Chlorophenyl	9.0×10^{-3}	2.4×10^{-4}	37.5	3.10	12.0
Phenyl	6.5×10^{-3}	1.7×10^{-4}	38.2	2.80	31.1
p-Tolyl	5.0×10^{-3}	9.0×10^{-5}	55.6	2.35	57.6
p-Anisyl	5.0×10^{-3}	9.0×10^{-5}	55.6	2.30	68.7
α-Naphthyl	1.5×10^{-3}	2.0×10^{-6}	750.0	0.13	182.0

[a] Reciprocal of affinity of arylmethyl methylcarbamates and acetylcholinesterase measured by Dixon method (Dixon, 1953). [b] Association constants of aromatic hydrocarbons (suitable aryl portions of investigated carbamates) and tetracyanoethylene in 1,2-dichloroethane at 22°, according to Briegleb (1961).

characterized by a simple dissociation constant K_D, which was measured by the Dixon method. Table II shows the K_D values for the substituted benzyl methylcarbamates, the I_{50} values for the benzyl methylcarbamates and the corresponding phenyl methylcarbamates, and K_x, the association constants of CTC between aryl portions of the carbamates and tetracyanoethylene.

In the case of the unsubstituted benzyl methylcarbamate, we measured inhibition after long periods (1–3 hr) and estimated

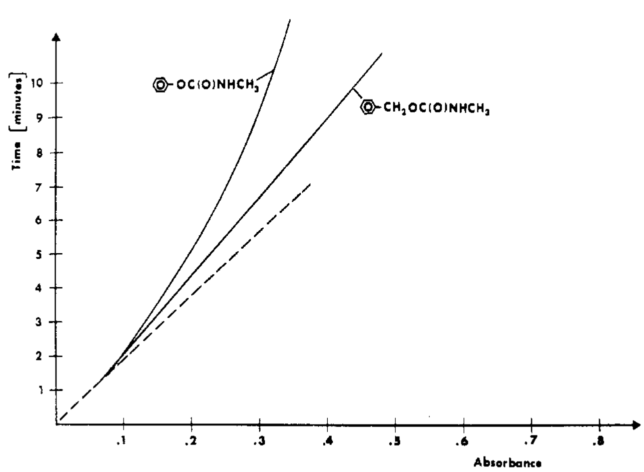

FIGURE 1: Trace from the recording spectrophotometer. Procedure as described in Methods with inhibitor at a final concentration of 10^{-3} M.

K_D and k_2 (the carbamylation constant) by the method of Main (Main, 1969) at 25° and pH 7.6. Where CX is the carbamate, with X as the methylcarbamyl group, and E is acetylcholinesterase

$$CX + E \overset{K_D}{\rightleftharpoons} CXE \xrightarrow{k_2} CE + X \qquad (CX + E \xrightarrow{k_i} CE + X)$$

The values found were $K_D = 3.0 \pm 0.9$ mM (in good agreement with $K_D = 2.8$ mM from the Dixon method, Table II) and $k_2 = 5.3 \pm 2.8 \times 10^{-3}$ min^{-1}. These values compare with those of Hastings *et al.* (1970) for phenyl methylcarbamate under the same conditions of $K_D = 23.7 \pm 1.9$ mM (*i.e.*, the affinity was 7.9 times less than that of the benzyl analog) and $k_2 = 6.82 \pm 0.35$ min^{-1} (*i.e.*, the carbamylation rate was 1287 times greater than that of the benzyl analog). The bimolecular rate constant k_i, which is equal to k_2/K_D (Main and Iverson, 1966), and is a measure of overall potency, was thus 160 times greater for the phenyl than the benzyl methylcarbamate. Main's method was not used for the other benzyl methylcarbamates because of the large errors associated with the small increases in reaction with time.

All the benzyl methylcarbamates except the *p*-nitro derivative formed CTC with tetracyanoethylene. One case of this property is illustrated by Figure 2. Job plots, of which an example for the case of the *p*-methoxy derivative is shown in Figure 3, indicated a mole-for-mole complex was formed in every case. The charge-transfer absorption maxima are shown

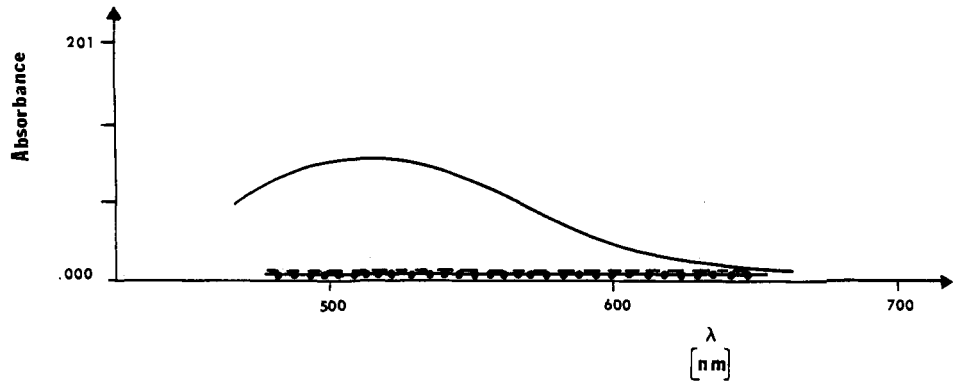

FIGURE 2: Absorption curve for tetracyanoethylene (- - -), concentration 5×10^{-2} M, and *p*-methoxybenzyl methylcarbamate (-●-●-●-●-), concentration 5×10^{-3} M, and for their mixture (—). The solvent was 1,2-dichloroethane.

TABLE III: Comparison of CTC Absorption Maxima of Tetracyanoethylene with Aryl (A) and Arylmethyl (B) Methylcarbamates.

X[a]	λ_{max}, A (nm)	λ_{max}, B (nm)
p-Chlorophenyl	355–360	340
Phenyl	365	350
p-Tolyl	405	395
p-Anisyl	510	520
α-Naphthyl	540	545

[a] $XOC(O)NHCH_3$ (A); $XCH_2OC(O)NHCH_3$.

in Table III, along with those previously reported for the corresponding phenyl methylcarbamate.

Figure 4 shows that for benzyl methylcarbamates there was an excellent correlation between K_D, the reciprocal of the affinity of carbamates for the enzyme, and K_X, the association constants of CTC of their aromatic portions which measure the ability to donate electrons to tetracyanoethylene. We have previously shown (Hetnarski and O'Brien, 1972) that the use of the K_x for the aromatic portion provides as good a correlation with I_{50} as does the use of the K_x for the corresponding aromatic carbamate. There is a pronounced exception for *p*-nitrobenzyl methylcarbamate; if it is omitted, the correlation coefficient $r = 0.99$. If one correlates $-\log I_{50}$ with K_D (Figure 5), excluding the *p*-nitro compound, a value of $r = 0.99$ is found. In view of these considerations, a good correlation between $-\log I_{50}$ and K_X was expected and found (Figure 6) with $r = 0.96$. The figure also shows the relation between $-\log I_{50}$ and K_x for the phenyl analogs (Hetnarski and O'Brien, 1972).

Discussion

It is clear that the benzyl methylcarbamates differ from their phenyl analogs in being reversible, nonprogressive inhibitors, rather than pseudoreversible,[2] progressive inhibitors. Very recently a parallel situation was described: six substituted phenyl carbamates having *N*-hydroxy-*N*-methyl or *N*-methoxy-*N*-methyl substituents were shown by Chiu *et al.* (Chiu and Fukuto, 1973) to be reversible, nonprogressive inhibitors, even though their *N*-methyl analogs were pseudoreversible, progressive inhibitors. Presumably in the former cases there is steric or other hindrance to the carbamylation reaction. In the case of the benzyl methylcarbamates, a different explanation must hold: the insertion of the methylene group blocks the inductive effect of the ring upon the carbamyl group, so that the innate carbamylating ability is profoundly reduced, to the extent of 1287-fold, as we have shown above.

The most conclusive proof that these compounds form CTC with acetylcholinesterase would of course be direct spectrophotometric evidence. Unfortunately, this kind of evidence is not available for any of the reactions of acetylcholinesterase, because of the unavailability of significant amounts of pure enzyme. And for CTC formations in general the "... complicated nature of biochemical reactions has so far prevented any demonstration of the direct involvement of CTC in the mechanisms of such reactions" (Foster, 1969). At present we cannot specify the chemical nature of the receptor site; a possibility is that an interaction between protons on the enzyme surface and the π-electron cloud of the aromatic carbamate is involved. Consequently, our evidence is of two kinds: the correlation between abilities to form CTC and to inhibit acetylcholinesterase, and the explanation of relations

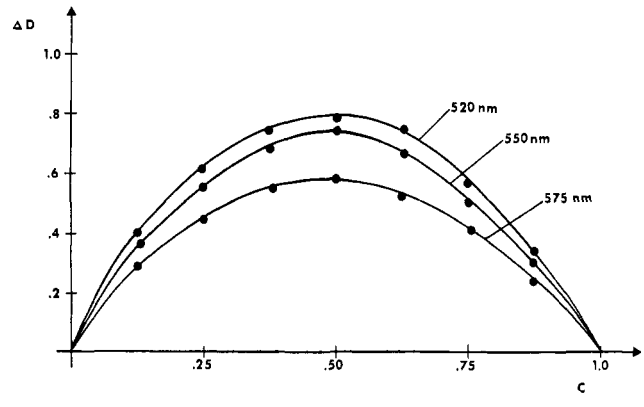

FIGURE 3: Job plot for the complex of tetracyanoethylene with *p*-methoxybenzyl methylcarbamate. ΔD is the difference between the absorption for the complex solution and that for the sum of the individual components. C is the mole fraction of the carbamate.

[2] Most carbamates give rise to a carbamylated enzyme. Because the decarbamylation reaction is fairly fast, the enzyme recovers if removed from surplus inhibitor. But this is "pesudoreversibility" in the sense that although the enzyme recovers, the carbamate is hydrolyzed in the course of the reaction. The overall system is no more reversible than in the corresponding case of organophosphates, where the dephosphorylation reaction is slow, so that one is less tempted to think of the reaction as reversible.

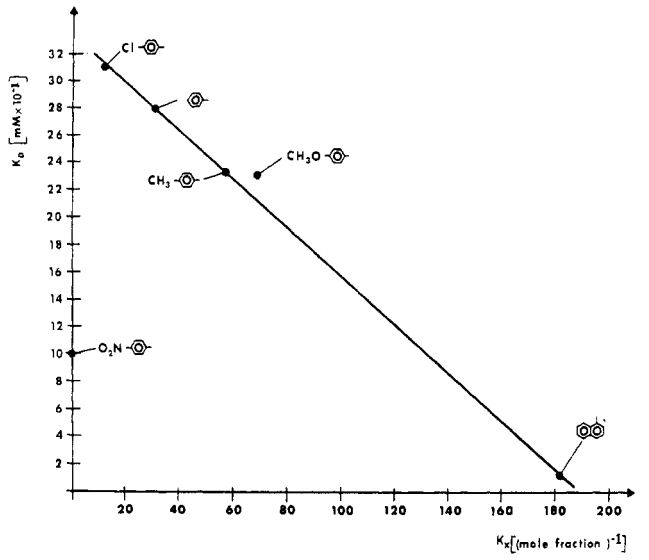

FIGURE 4: Relation between K_D, the dissociation constant of the complex of arylmethyl mehtylcarbamate and enzymes, and K_x, the association constant for the CTC with tetracyanoethylene and the aromatic moiety (X) of the carbamates ($XCH_2OC(O)NHCH_3$).

between structure and activity which have hiterto been paradoxical.

The excellence of correlations in Figures 4–6 permits the conclusion that the variations in inhibitory potency of benzyl methylcarbamates (with the exception of the *p*-nitrophenyl derivatives) are related principally to the variations in ability to form a complex with the enzyme, and this in turn is correlated with variations in the ability to form CTC. In view of the fact that these compounds are almost totally ineffectual as carbamylators, their inhibitory effect is thus almost exclusively determined by their excellence as complex formers. Because the ability to form CTC with tetracyanoethylene predicts the anticholinesterase activity so accurately, we hypothesize that

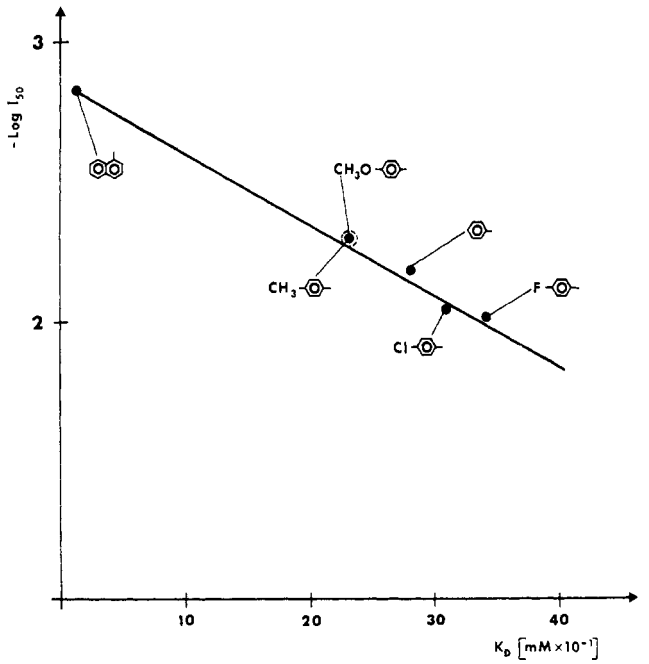

FIGURE 5: Relation between the negative logarithm of anticholinesterase potency (I_{50}) and the dissociation constant K_D of enzyme and arylmethyl methylcarbamates.

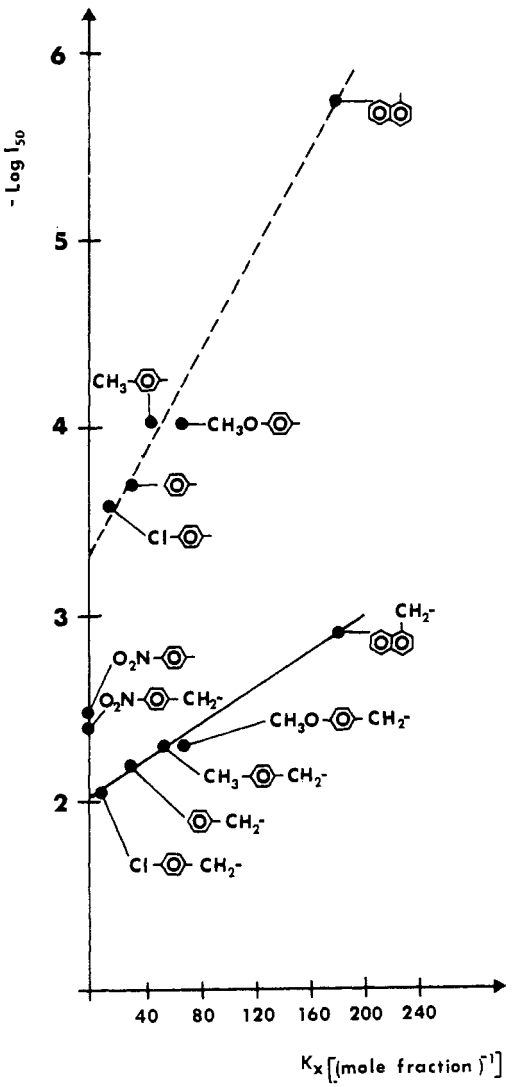

FIGURE 6: Relation between $-\log I_{50}$ and K_x (units as in Figures 4 and 5) for substituted aryl methylcarbamates (- - - -) and arylmethyl methylcarbamates (——).

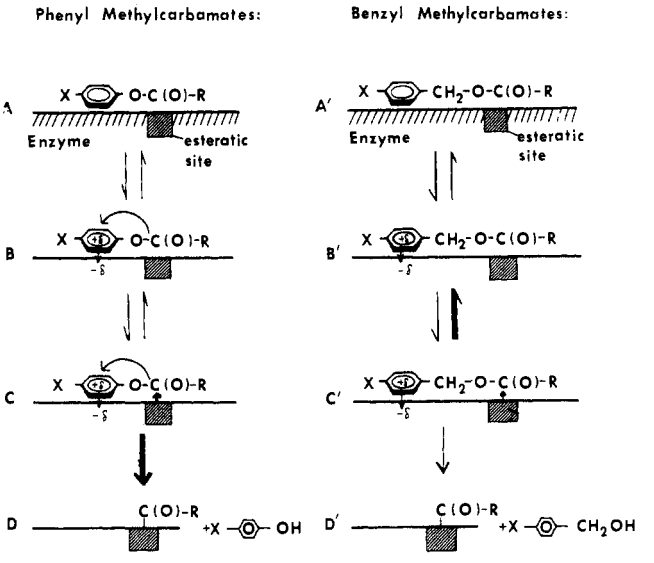

FIGURE 7: Described in text.

a CTC is formed between inhibitor and enzyme. If a CTC is not involved, then there must be some other kind of complex formation which is promoted by high electron density in the π system or aromatic inhibitors, and the CTC formation with tetracyanoethylene provides a convenient index of such density, applicable (as in this paper) to polycyclic as well as to single ring compounds.

Why should the carbamylating activity of the benzyl carbamates be so much smaller than that of phenyl carbamates, by a factor of 1287-fold in the k_2 values of the unsubstituted compounds? Two factors may be responsible: the direct electrophilic effect of the ring upon the carbamyl group is almost lost in the benzyl compounds; a parallel is the tenfold difference in the hydrolysis constants of the benzyl and phenyl esters of several acids (Baranov and Vizgert, 1957). But the very large difference in ability to carbamylate the enzyme is probably due to the fact that the ring electrons are partially donated to the enzyme in the course of complex formation, and this enhanced electrophilic effect of the ring is transmitted to the carbamyl group only in the phenyl series (Figure 7).

The *p*-nitrobenzyl methylcarbamate is an interesting exception to the above conclusion. It has no ability to act as a donor in CTC formation, at least when tetracyanoethylene is the acceptor. From Figure 4 one would estimate that it should have the poorest binding constant, with K_D greater than 3.3 mM. But it is three times better than expected, with $K_D = 1$ mM. We speculate that in fact it is acting as an electron acceptor, and complexing with a donor region of the enzyme which has a high electron density. This suggestion is supported by the reports that phenyl carbamates with strong electronegative substituents (NO_2–, CN–, F_3C–) are characterized by K_D values lower than unsubstituted compounds (Hastings *et al.*, 1970; O'Brien *et al.*, 1966).

Hydrophobic interactions doubtless contribute to the size of K_D. In this paper we have minimized this factor by selecting compounds with little variation in hydrophobic character; the hydrophobic constants of the substituents were not higher than 0.7 (Hansch, 1970). A later study will deal with situations where such is not the case.

Finally, the question must be considered: why are not the two lines in Figure 5 parallel? *I.e.*, if most of the inhibitory potency of both aryl and arylmethyl methylcarbamates is due to binding potency, why does not the extra binding bestowed by (say) a naphthyl as compared with a phenyl ring give an equal factorial increase for both kinds of methylcarbamate? In fact, the difference is roughly 100-fold for the aryl but 4.3-fold for the arylmethyl series. Presumably the reason is that the same factors which improve donor ability in aryl methylcarbamates also improve the intrinsic carbamylating activity, perhaps because of improved electronic polarizability. In the case of arylmethyl methylcarbamates this effect is blocked by the interposed methylene group.

The literature on the relation between structure and inhibitory activity of aromatic carbamates has been confused. Early work (reviewed by Metcalf and Fukuto, 1965) showed a marked negative dependence of potency upon σ in 12 xylenyl and seven phenyl methylcarbamates, which is quite the opposite of what one finds in analogous organophosphates. Later it was shown that for numerous substituted phenyl methylcarbamates, σ was unrelated to anticholinesterase potency (Metcalf and Fukuto, 1967; O'Brien *et al.*, 1966), whereas in dimethylcarbamates there was a positive dependence on σ (O'Brien *et al.*, 1966). These discrepant results can be resolved now that it is clear that "potency" is normally a function of both affinity and innate carbamylating activity. Electron withdrawal may favor innate carbamylating activity but worsen affinity, and consequently electron-withdrawing substituents may have quite different effects in systems with different relative contributions of these two factors. Although σ has been widely used to predict effects on reactions such as carbamylation, the use of K_x values is preferable in discussing affinity effects, partly because it is a direct measure of complexing ability with suitable systems, and partly because it is entirely general in its applicability (*e.g.*, to polynuclear aromatics) whereas the usual σ values refer only to meta- and para-substituted benzenes.

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