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THE ROLE OF HYDROPHOBIC AND ELECTRON DONOR PROPERTIES IN ACETYLCHOLINESTERASE INHIBITION BY CARBAMATES

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Abstract—The abilities of seven m-substituted phenyl methylcarbamates to inhibit acetylcholinesterases from honey bee and cotton leafworm (*Spodoptera littoralis*, Boisd.) were explored. The extra inhibition bestowed by m-substituted phenyl methylcarbamate as compared with the unsubstituted ($\Delta \log k_i = \log k_i(X)/k_i(H)$) was well correlated with the calculated contribution of each substituent through its hydrophobic and electron donating properties ($0.69\ \pi - 0.95\ \sigma + 1.19$). The correlation coefficient $r = 0.95$ for cotton leafworm acetylcholinesterase and 0.92 for that of honey bee. Analysis of these data, in addition to those of the housefly from the literature, resulted in a direct linear relationship in spite of enzyme source, with $r = 0.91$. It was therefore postulated that hydrophobic interaction and electron donating properties of the substituent were involved in inhibition through complex formation with the enzyme acetylcholinesterase prior to the carbamylation step.

Anticholinesterase activity of phenyl methylcarbamates appears to be much less responsive to electronic effects [1, 2] or shows a marked negative dependence of potency upon σ [3–5], a parameter that provides an estimate of the electron-withdrawing or electron-donating properties of the substituents. Therefore, substituents possessing large positive σ values should produce substituted phenyl methylcarbamates with low anticholinesterase activity, which is quite the opposite of what one finds in analogous organophosphates. Linear combination of electronic and hydrophobic effects as estimated by σ and π gave a reasonable correlation for a rather complex set of carbamates [6] on housefly head acetylcholinesterase.

In the present paper we have extended these findings [6] to include the action of these compounds on cholinesterases from honey bee (*Apis mellifera*) and cotton leafworm (*Spodoptera littoralis* Boisd.).

MATERIALS AND METHODS

Inhibitors. Seven methylcarbamates were prepared from commercially available appropriate phenols and methylisocyanate in ether solution using triethylamine as a catalyst. The compounds were isolated as crystalline solids and purified by repeated crystallization from a mixture of ether and *n*-hexane. These compounds were characterized by their melting points according to the literature. Stock solutions (0.1 M) were prepared in acetone and stored at −20°. Further dilutions from these stock solutions were made in acetone immediately before use.

Enzyme preparation. Adult bee workers were frozen at −20° and heads were ground in a cold solution of 0.1 M sodium phosphate buffer, pH 7.0 (25 heads/ml). The slurry was centrifuged at 6,400 rpm for 15 min and the supernatant was withdrawn from beneath this with a syringe. Similarly cotton leafworm cholinesterase was from heads of the 6th instar larvae by the same technique (40 heads/ml).

Enzyme activity. The enzyme assays were performed at 30° by the colorimetric method of Ellman *et al.* [7]. A mixture was prepared consisting of 4.5 ml of buffer (0.1 M sodium phosphate, pH 7.6), 0.1 ml of 0.01 M solution of DTNB [5,5′ dithiobis-(-2-nitrobenzoic acid)] plus 0.018 M sodium bicarbonate in 0.1 M sodium phosphate buffer (pH 7.0). This mixture was placed into the cuvette and 0.05 ml of the enzyme preparation was added followed by 0.1 ml acetone or acetone-inhibitor solutions. At suitable time intervals (0.25–4 min), 0.1 ml of a 0.1 M of acetylthiocholine iodide (in freshly distilled water) was added. After rapid mixing, the extinction at 412 nm was measured at 30 sec intervals for 4 min, the first reading being taken 15 sec after the substrate had been added. The final concentration of DTNB was 0.206 mM, and of acetylthiocholine 2.06 mM. The increase in extinction was linear with time for both inhibited and uninhibited enzyme samples. No correction for spontaneous hydrolysis of the substrate was necessary. Activity was estimated from the initial slope of the plot of absorbance against time. The inhibitor concentrations ranged between 4.8×10^{-4} to 2.0×10^{-8} M depending on solubility and potency.

Inhibition constants. For the study of inhibition reaction of these compounds with cholinesterases, pseudofirst order plots of log per cent residual activity (log V) against preincubation time (t) were plotted. These plots were sensibly linear of slope $= -K_i(i)/2.303$ enabling K_i for the reaction to be evaluated [8], since the slope equals the change in log V per unit time. Detailed kinetic treatments [9] was developed for bee head acetylcholinesterase to allow the separation of K_i, the overall bimolecular inhibition constant into a carbamylation rate constant (K_2) and a dissociation constant (K_d). The slope of pseudofirst order plots ($\Delta \log V/\Delta t$) were transformed into the form (i) $\Delta t/2.303\ \Delta \log V$ and the latter was plotted vs the inhibitor concentration (i). The plots fit a straight line rather well according to the equation (i) $\Delta t/2.303\ \Delta \log V = (i)/K_2 + 1/K_i$ [9].

The slope will be $1/K_2$, the intercept on the (i) axis will be $-K_d$, and the intercept on the [(i) $\Delta t/2.303\,\Delta \log V$] axis will be $(1/K_i)$.

RESULTS AND DISCUSSION

Linear combination of π and σ in eq. (1) gave a reasonable correlation for 53 mostly monofunctional phenyl methylcarbamates [6].

$$\log 1/C = 0.69\,\pi - 0.95\,\sigma + 1.19\,X + 3.5 \quad (1)$$

where C is the concentration of the inhibitor for 50 per cent inhibition of housefly head acetylcholinesterase [4].

In the above equation, X indicates whether we have meta or para derivatives. The value of 1.00 was assigned to all meta isomers, and the value of 0.0 was assigned to all para isomers [6]. This suggests that a steric factor is involved.

As pointed out [10], I_{50} values can be converted to k_i values by the relationship

$$k_i = 0.695/I_{50}\,t \quad (2)$$

where t is the incubation time of enzyme and inhibitor.

The introduction of eq. (2) into eq. (1) gives eq. (3) which is better formulated than eq. (1) since the k_i value is relatively independent of preincubation time.

$$\log k_i = \log 1/C + \log (0.695/t)$$
$$= 0.69\,\pi - 0.95\,\sigma + 1.19\,X + 3.5 + \log (0.695/t). \quad (3)$$

The enzyme source doubtless contributes in selective inhibition by these compounds. In this study this factor was minimized by calculation of the contribution of m-substituent to the inhibitory potency of the parent phenyl methylcarbamate, eqs. (4) and (5).

$$\Delta \log k_i = \log k_i(X) - \log k_i(H) \quad (4)$$

or

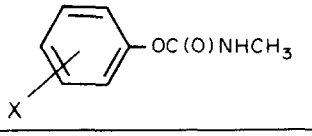
$$\Delta \log k_i = 0.69\,\pi - 0.95\,\sigma + 1.19 \quad (5)$$

where k_i (X) is the k_i value of a m-substituted compound whereas k_i (H) is that of the unsubstituted compound.

If most of the inhibitory potency of phenyl methylcarbamates is due to binding potency, an extra binding bestowed of a given substituent will give equal factorial increase in spite of the acetylcholinesterase source. Table 1 shows $\Delta \log k_i$ calculated from Hansch's π constant [11] and σ values according to eq. (5) vs the observed $\Delta \log k_i$ for housefly [4], honey bee, and cotton leafworm. Closer examination of the data reveals an approximate but direct relationship between the observed and calculated $\Delta \log k_i$ with a correlation coefficient of 0.89, 0.92, and 0.95 for the three enzymes respectively. The higher correlation associated with honey bee and cotton leafworm acetylcholinesterases as compared with that of housefly for which the basic eq. (1) has been formulated [6] means that the contribution of m-substituent on inhibitory potency was readily accounted for by its electronic and hydrophobic effects as estimated by σ and π respectively.

The predicted vs observed values, of $\Delta \log k_i$ are shown graphically in Fig. 1. The plot indicates a good approximation to a straight-line relationship in spite of the acetylcholinesterase source, with a correlation coefficient of 0.91. Therefore substituents possessing large positive π and large negative σ values should produce m-substituted phenyl methylcarbamates with high anticholinesterase activity. A noteworthy point in the above equations is the negative value for the

Table 1. Structure-activity relationship



X	$\Sigma\pi$	$\Sigma\sigma$	$\Delta \log k_i$ calcd[a]	$\Delta \log k_i$ observed: Housefly	Honey bee	Cotton leafworm
3-Cl	0.76	0.37	1.36	0.60	0.61	0.25
3-MeO	0.12	0.12	1.16	0.96	0.91	0.08
3-Me	0.51	−0.07	1.61	1.15	0.82	1.21
3,5-Me_2	1.02	−0.14	2.03	1.52	—	1.21
3-Et	0.97	−0.04	1.90	1.62	1.79	1.86
3-$C(Me)_3$	1.68	−0.12	2.46	2.70	2.81	2.95
3-$CH(Me)_2$	1.30	−0.04	2.13	2.77	2.75	2.25
n				7	6	7
			20			
r				0.888**	0.923**	0.948**
				0.909**		

[a] Calculated from eq. (5).
** r values are highly significant at the 0.01 level of probability.

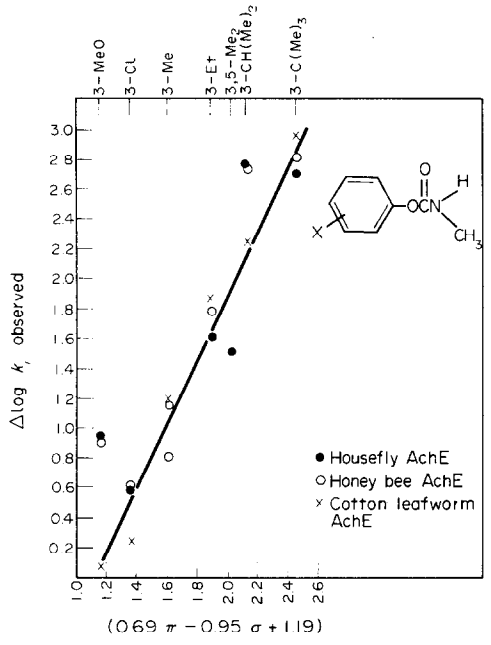


Fig. 1. Correlation between observed Δ log k_i and Δ log k_i calculated from σ and π values according to eq. (5) for m-substituted phenyl methylcarbamates.

coefficient of σ. In view of the fact that these compounds are relatively ineffectual as carbamylators as compared with analogous organophosphates, their inhibitory effect is thus almost exclusively determined by their excellence as complex formers. This is the fact, as Table 2 indicates, that the inhibitory power is not dependent on variation in carbamylation rate constants as it is on initial binding—i.e. the contribution of *m*-tert. butyl group in anticholinesterase activity, which is 641-fold, lies mainly in affinity (423-fold) rather than carbamylation (1.5-fold). These findings are in agreement with that of O'Brien *et al.* [1]. It was suggested [12, 13] that the aromatic ring in aryl carbamates is the donor in charge transfer complexes (CTCs) and that inhibitory activity of aromatic carbamates is related to their ability to donate electrons to form a complex with the enzyme, and is therefore improved by electron-donating substituents.

Presumably the contribution of m-substituent in anti-cholinesterase activity is exclusively accounted for by its ability to form enzyme-inhibitor complex.

Equation (5) shows that hydrophobic effects (π) and electron donating effects (σ) are about equally weighted, suggesting that steric interaction between the m-substituent and hydrophobic patch on the enzyme surface (positive π), and electron donating ability (negative σ) to the benzene ring and subsequent donating to the enzyme surface to form CTC play about equally important roles in the overall inhibition process. This suggestion is in agreement with the findings of Hetnarski and O'Brien [14] who observed that affinity for acetylcholinesterase correlated well with that calculated from hydrophobicity (π) and ability to donate electrons to form CTCs as measured with a model acceptor (tetracyanoethylene). In addition, the same factor which improves donor ability in aryl methylcarbamates may also improve the intrinsic carbamylating activity perhaps because of improved electronic polarizability [13]. A parallel situation is described in Table 2 as *m*-tert butyl group ($\sigma = -0.12$) increases the carbamylation constant 1.5 times that of the parent phenyl methylcarbamate ($\sigma = 0$). Electron withdrawing substituents (3-CH_3O; 3-Cl) either have no effect or even reduce k_2 value. Also it was suggested [14] that a close fit improves the effectiveness of the carbamylation step. This suggestion gives another explanation to the increase in carbamylation rate constant caused by electron donating substituents.

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Table 2. Anticholinesterase activity and kinetic properties of: X-$C_6H_4OC(0)NHCH_3$ on honey bee head acetylcholinesterase

X	$K_d(M)$	k_2 (min^{-1})	k_i (M^{-1} min^{-1})
H	2.96×10^{-4}	2.75	9.17×10^3
3-Cl	8.60×10^{-5}	2.80	3.70×10^4
3-MeO	2.50×10^{-5}	2.00	7.40×10^4
3-Me	5.60×10^{-5}	3.50	6.06×10^4
3-Et	4.30×10^{-6}	2.50	5.56×10^5
3-$C(Me)_3$	7.00×10^{-7}	4.17	5.88×10^6
3-$CH(Me)_2$	4.68×10^{-7}	2.40	5.15×10^6