4. Inhibition Constants

Detailed kinetic treatment⁷⁾ were developed for bee head acetylcholinesterase to allow the separation of k_i , the overall bimolecular inhibition reaction constant into a carbamylation rate constant (k_2) and an affinity constant, K_a ($1/K_d$). Pseudo first order plots of log per cent residual activity ($\log V$) against pre-incubation time (t) were plotted. The slope of these plots ($\Delta \log V / \Delta t$) was 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 $(i) \Delta t / 2.303 \Delta \log V = (i) / k_2 + 1 / k_1$.⁷⁾ The slope will be $1 / k_2$, the intercept on the (i) axis will be $-K_a$, and the intercept on the (i) $\Delta t / 2.303 \Delta \log V$ axis will be $1 / k_i$.

RESULTS AND DISCUSSION

The effect of meta and para alkyl substitution of phenyl methylcarbamate on honey bee acetylcholinesterase inhibition was examined at two different temperatures (Table 1) enabling some thermodynamic quantities to be evaluated (Table 2). For relating the anti-cholinesterase activity with structural change, the bimolecular inhibition reaction constant

(k_i) of the tested compounds at the same temperature (298K) was compared. As seen in Table 1, substitution with methyl, ethyl, isopropyl and *tert*-butyl in the meta position of phenyl methylcarbamate increases the anti-cholinesterase activity 7.13-, 75.4-, 772.4- and 483.7 times, respectively. This indicates that the activity increases as the substituent increases in size to three carbon atoms. The increased inhibitory activity of meta alkyl-phenyl methylcarbamates was also demonstrated against the enzyme acetylcholinesterase from other insect species.^{4,8)} Theoretically this increase in reaction constant may be due to a decrease in the activation energy (E_a), an increase in the PZ factor, or their combined effect according to the collision theory of reaction rates which denotes:

$$k_i = PZ \exp(-E_a/RT) \quad (2)$$

where Z is the number of collisions occurring between carbamate and acetylcholinesterase (AChE) in unit volume per unit time, $\exp(-E_a/RT)$ is the frequency factor which measures the probability that any molecule will have sufficient energy to react, and P is the steric factor giving the fraction of energetically

Table 1 Inhibition kinetic parameters of honey bee acetylcholinesterase by *m*- and *p*-alkylphenyl methylcarbamates.

General formula: $XC_6H_4OC(O)NHCH_3$

<i>X</i>	Temp. (K)	k_i (l/mol, sec $\times 10^3$)	K_a (M ⁻¹)	k_2 (sec ⁻¹ $\times 10^{-2}$)
H	298	1.23 $\pm$ 0.05	6.25 $\times 10^3$	1.97 $\pm$ 0.09
	308	1.41 $\pm$ 0.03	1.49 $\times 10^3$	9.46 $\pm$ 0.40
3-CH ₃	298	8.77 $\pm$ 0.16	1.25 $\times 10^4$	7.02 $\pm$ 0.47
	303	10.10 $\pm$ 0.29	1.79 $\times 10^4$	5.64 $\pm$ 0.27
3-C ₂ H ₅	298	92.70 $\pm$ 3.89	2.08 $\times 10^5$	4.46 $\pm$ 0.34
	308	150.00 $\pm$ 6.11	3.29 $\times 10^5$	4.56 $\pm$ 0.20
3-CH(CH ₃) ₂	291	575.00 $\pm$ 29.09	1.25 $\times 10^6$	4.60 $\pm$ 0.29
	298	950.00 $\pm$ 84.45	1.92 $\times 10^6$	4.95 $\pm$ 0.29
3-C(CH ₃) ₃	298	595.00 $\pm$ 14.93	1.25 $\times 10^6$	4.76 $\pm$ 0.30
	303	980.00 $\pm$ 63.00	1.43 $\times 10^6$	6.85 $\pm$ 0.28
4-CH ₃	298	0.92 $\pm$ 0.01	8.55 $\times 10^2$	11.00 $\pm$ 0.50
	303	1.19 $\pm$ 0.03	3.52 $\times 10^2$	34.00 $\pm$ 3.06
4-C ₂ H ₅	298	0.65 $\pm$ 0.02	3.58 $\times 10^2$	18.00 $\pm$ 2.65
	303	0.97 $\pm$ 0.12	8.33 $\times 10^2$	12.00 $\pm$ 1.15
4-C(CH ₃) ₃	298	0.22 $\pm$ 0.006	5.75 $\times 10^2$	3.83 $\pm$ 0.24
	311	1.13 $\pm$ 0.04	1.37 $\times 10^3$	8.25 $\pm$ 0.96

Table 2 Thermodynamic quantities of honey bee acetylcholinesterase inhibition by *m*- and *p*-alkylphenyl methylcarbamates.General formula: $\text{XC}_6\text{H}_4\text{OC(O)NHCH}_3$

<i>X</i>	E_a (kcal/mol)	<i>PZ</i> (l/mol, sec)	ΔS^* (e.u.)	$\pi^{a)}$
H	2.59	9.72×10^3	-42.28	0.0
3-CH ₃	5.06	4.51×10^6	-30.07	0.51
3-C ₂ H ₅	8.78	2.55×10^{10}	-12.92	0.97
3-CH(CH ₃) ₂	12.36	1.10×10^{14}	+ 3.72	1.30
3-C(CH ₃) ₃	17.90	8.00×10^{17}	+21.38	1.68
4-CH ₃	9.42	7.42×10^8	-19.93	0.52
4-C ₂ H ₅	14.30	2.00×10^{12}	- 4.23	0.97
4-C(CH ₃) ₃	23.14	3.06×10^{18}	+23.26	1.68

^{a)} From Hansch.¹³⁾

favourable collisions that are also sterically favourable for inhibition to take place. From k_i values for each compound in its reaction with honey bee AChE at the two temperatures, E_a was calculated according to the Arrhenius equation which denotes:

$$\ln \frac{k_{i2}}{k_{i1}} = \frac{E_a}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (3)$$

where R is the gas constant, 1.987 cal/deg mole. Listed in Table 2 are the activation energies of the tested carbamates in their reaction with honey bee AChE. Substituting for the activation energy and using k_i values at 298K, PZ factors were calculated from Eq. (2). These show, for simple alkyl substitution in the meta position, that the effect on E_a and PZ factor is startling (Table 2). The increase in anticholinesterase potency is accompanied by an increase in the activation energy and the PZ factor. The gradual increase in activation energy from 2.59 kcal/mol for unsubstituted phenyl methylcarbamate to 5.06, 8.78, 12.36 and 17.9, respectively, for *m*-methyl-, *m*-ethyl-, *m*-isopropyl- and *m*-*tert*-butylphenyl methylcarbamates indicates that the activation energy alone would have led to a reduction in the activity of the meta alkylphenyl methylcarbamates as compared with the unsubstituted parent compound. However, an increase in the PZ factor as the substituent increases in size indicates conclusively that the main factor controlling the potency of the meta alkylphenyl methylcarbamates is the favourable orientation and distribution of

potential energy among colliding molecules that is required for inhibition to take place. The above thermodynamic quantities in relation to meta alkyl substitution may explain the fact that these compounds are relatively ineffectual as carbamylators and their inhibitory effect is thus almost exclusively determined by their excellence as complex formers (Table 1).

In contrary, substitution with methyl, ethyl and *tert*-butyl in the para position slightly decrease the anticholinesterase activity, respectively, by 1.34-, 1.89- and 5.59-fold as compared with the unsubstituted phenyl methylcarbamate (Table 1). This reduction in the rate constant cannot be explained by the PZ factor since the latter increases drastically over that of the parent compound. The change is particularly striking in moving from *H* to *tert*-butyl. There is an increase of 2.12×10^{14} . However, greater energy barrier in the reaction of the para substituted compounds with honey bee AChE is absolutely responsible for their lower potency. Among all the tested analogues *p*-*tert*-butylphenyl methylcarbamate has the highest activation energy (23.14 kcal/mol). Comparing the meta and para substituted compounds of the same alkyl group (Table 1) indicates that meta substituted analogues are much more potent than their para isomers. In this reaction para alkylphenyl methylcarbamates are little more effective in raising the PZ term but their meta analogues are still more active because of lowered activation energy.

According to the theory of absolute reaction rates, a rate constant can be expressed in terms of the change in standard enthalpy ΔH^* and of the change in standard entropy ΔS^* of activation, Eq. (4).

$$k_i = \frac{kT}{h} \exp(-\Delta H^*/RT) \exp(\Delta S^*/R) \quad (4)$$

k is the Boltzman constant (3.298×10^{-24} cal·degree⁻¹) and h is the Plank constant (1.58×10^{-34} cal·sec). From E_a , the change in the standard enthalpy of activation (ΔH^*) is obtained from Eq. (5).

$$\Delta H^* = E_a - RT \quad (5)$$

By substituting for the values of ΔH^* and using k_i values at 298K, ΔS^* values can be obtained from Eq. (4). As would be expected, the system is more ordered or more restricted in its freedom to move, as reactants change to transition state and, therefore, ΔS^* will be of negative magnitude which is the case in 5 of the 8 tested compounds (Table 2). For *m*-alkylphenyl methylcarbamates, there is a decrease in the negative entropy from unsubstituted ($\Delta S^* = -42.28$ e.u.) to methyl (-30.07) and ethyl (-12.92) and it then becomes positive in isopropyl ($+3.72$) and *tert*-butyl ($+21.38$). As Laidler¹²⁾ pointed out, extensive binding to an enzyme surface displaces water molecules formerly bound to that surface and consequent entropy increase can exceed the entropy loss associated with the formation of the enzyme inhibitor complex. The above data can be explained assuming that many water molecules are displaced as the substituent size or more fairly its hydrophobicity increases. The same relation was obtained with *p*-alkylphenyl methylcarbamates with slightly higher ΔS^* as compared with their meta analogues. Higher ΔS^* values of the para alkyl compounds over that of their meta analogues may be due to less complementarity of the para substituted compounds to the enzyme surface, and then less restriction of the activated complex of these compounds with the enzyme AChE. Interestingly, the contribution of the alkyl groups to the entropy of activation depends on the number of carbon atoms of the substituent. This contribution seems to be additive since a high and signifi-

cant correlation ($r = +0.99$) was obtained between ΔS^* and the calculated molar refraction of the substituents.

In order to test whether the ejection of water molecules from the hydrophobic patch around the enzyme active site might be responsible for the contribution of substituent in the change in entropy of activation, ΔS^* values were related to the substituent hydrophobicity, π substituent constant.¹³⁾ This relation shows a better approximation to a straight-line relationship, with a correlation coefficient of $+0.99$.

CONCLUSION

In summary, the anticholinesterase activity of meta alkylphenyl methylcarbamates is greater than that of the unsubstituted parent compound and the activity increases as the substituent increases in size. In contrast, the para analogues are far less potent. Lower energy barrier, in addition to less decrease or increase in the standard entropy of activation, explains the higher potency of meta analogues. However, the higher energy barrier seems to be responsible for the reduced activity of the para analogues. Linear relations between the change in the standard entropy of activation and either the hydrophobicity or the molar refraction of the substituents were obtained with high and significant correlation coefficients.

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要 約

アルキルフェニルメチルカーバメートによるアセチルコリンエステラーゼ阻害のエネルギー論的解析

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メタアルキル置換フェニルメチルカーバメートの抗コリンエステラーゼ活性は、無置換親化合物のそれよりも大きく、置換基の大きさが増加するに従って活性も増加する。これに反して、パラ置換体はその活性が低い。活性化標準エントロピーのより少ない増減に加えるに、エネルギー障壁がより低いためにメタ置換体の活性が高くなると説明される。しかしながら、パラ置換体においてはエネルギー障壁がより高いため活性が低くなると考えられる。活性化標準エントロピーの変化と置換基の疎水性または分子屈折との間には高度の有意な相関係数をもって直線の関係が得られている。