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STUDIES ON THE INHIBITION OF HONEY BEE CHOLINESTERASE BY CARBAMATES

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The kinetic constants for the inhibition (I_{50}, Ka, k_{2c}, Ki, k_3 and Ka' values) of honey bee cholinesterase by furadan and its two analogues were determined and evaluated. Furadan proved to be a potent inhibitor of honey bee enzyme. All the three carbamates showed a high affinity for honey-bee ChE. The affinity constants ranged from 0.22 to 2.68×10^{-7} M. There was little variation among the carbamylation constants, indicating a minor role for the carbamylation step in the inhibition process. The significant variation in the bimolecular reaction constants of the three carbamates could be attributed to the variations in the affinity constants rather than to the carbamylation constants. The average apparent affinity constants were, in most cases, comparable with the Ka values and they increased progressively with the concentration of inhibitor.

INTRODUCTION

Eventhough many studies have been undertaken on the inhibition of cholinesterase (ChE) by organophosphates, studies undertaken on the enzymic inhibition by carbamates are relatively scanty.

Carbamate hydrolysis follows a scheme similar to that for the normal substrate:

$$E + I \underset{k-i}{\overset{k_i}{\rightleftarrows}} E.I \xrightarrow{k_{2c}} E' \xrightarrow{k_3} E$$

(E = free enzyme, I = free inhibitor, E.I = reversible enzyme-inhibitor complex and E' = carbamylated enzyme).

MAIN & HASTINGS (1966) derived equations for the carbamylation reaction of ChE's:

$$\frac{(I)\,\Delta t}{2.3\,\Delta \log v} = \frac{(I)}{k_{2c}} + \frac{1}{k_2'}$$

where, (I) = inhibitor concentration and $t/2.3\,\Delta \log v$ = first order rate constant at constant (I). Affinity, carbamylation and bimolecular reaction constants in the present studies were derived from the above equation. The rate constants for decarbamylation (k_3) have been calculated according to the first order equation,

$$\ln (E'/Eo) = -k_3 t$$

where E' = carbamylated enzyme, Eo = uninhibited enzyme and t = time in minutes.

A study was also undertaken to find out the influence of substrate concentration on the rate of enzymic hydrolysis under conditions of competitive inhibition according to the linear equation,

$$v = Vm - Km\,(1 + I/_{KI}).\ v/S.$$

MATERIALS AND METHODS

Cholinesterase was obtained from the heads of Indian honey bee, *Apis indica* F. and ten heads were used per ml of buffer-saline for homogenization. It was carried out in an all-glass electrically operated POTTER-ELVEHJEM type homogenizer in ice cold Tris-HCl buffer (0.05 M, pH 7.5) containing 1.5 per cent NaCl. The homogenates were centrifuged for 10 minutes at 6000 rpm in a refrigerated centrifuge at O°C. The supernatants were collected in vials and stored at -5°C until used.

Estimations of enzyme activity were done by the method of ELLMAN *et al.*, (1961) as modified by

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SMISSAERT (1964). Acetylthiocholine (ATCh) and dithionitrobenzoic acid (DTNB) used for the assay were the products of Sigma chemical company, St. Louis, Missouri, U.S.A. Furadan and its two analogues, 3-OH carbofuran and 3-keto carbofuran were supplied by the Niagara Chemical Division, FMC Corporation, Middleport, New York, U.S.A. Other chemicals used in the present study were obtained locally and were analytical reagents of the highest purity.

For the determination of I_{50} values, the enzyme (0.1 ml) and inhibitor (0.1 ml) were incubated at 30°C for a period of 30 minutes. Then a reaction mixture of 1.5 ml was constituted by adding to the above mixture 0.3 ml of buffer-saline, 0.5 ml of DTNB and 0.5 ml of ATCh (1×10^{-3} M). A reaction mixture with buffer-saline instead of inhibitor served as control, while one without enzyme and inhibitor was used as blank. The change in the optical density was recorded after an interval of 5 minutes. The I_{50} values were derived from the $e/E^1 - 1/(I)$ graphs (where E^1 = percentage of inhibited enzyme, e = percentage of enzyme remaining active and (I) = inhibitor concentration).

In the experiment to find out the carbamylation, affinity and bimolecular reaction constants, 0.1 ml enzyme was allowed to react with various concentrations of inhibitors in a total volume of 1.3 ml for 30 seconds, after which a 0.2 ml mixture of ATCh and DTNB was added. A reaction mixture with buffer-saline instead of inhibitor served as control.

For reactivation experiment, 0.2 ml of enzyme and 0.2 ml of inhibitor producing about 70 per cent inhibition were incubated at 30°C for half an hour. The enzyme-inhibitor mixture was then diluted to 5.0 ml and the increase in optical density was measured for an hour at regular intervals in the presence of 1×10^{-3} M ATCh and DTNB. A control without inhibitor showed a constant hydrolysis rate during the period of observation.

In the experiment to study the influence of substrate concentration on the rate of enzymic hydrolysis, the reaction mixture contained 0.05 M Tris-HCl buffer pH 7.5, 1.5 per cent NaCl, 3.3×10^{-4} M DTNB and the desired concentrations of inhibitor and substrate were simultaneously added followed by 0.1 ml of enzyme. The volume of total reaction mixture amounted to 1.5 ml. A reaction mixture without inhibitor served as control while one without both enzyme and inhibitor was kept as blank. The increase in inhibition was measured at one minute intervals until the steady state condition was achieved.

RESULTS AND DISCUSSION

All the parameters of inhibition obtained in the present study are given in Table 1.

The I_{50} value is the inhibitor concentration in moles per litre required to give 50% inhibition when the inhibitor is incubated with enzyme for 30 minutes. The low I_{50} values clearly indicate the high susceptibility of honeybee ChE to carbamate inhibition. This observation was in agreement with the results of EL-AZIZ *et al.* (1969), who also reported an extremely high susceptibility of honey bee to carbamates. The order of inhibition was furadan > 3-OH carbofuran > 3-keto carbofuran. The differences among the I_{50} values obtained for the three inhibitors are not significant. The results gave a clear proof for the high inhibitor potency of furadan towards insect ChE.

Since the affinity constant Ka measures the ability of the enzyme-inhibitor complex to dissociate, the lower the numerical value of this constant, the more effective the binding at the active site of the enzyme. From the data it is apparent that 3-keto carbofuran showed the highest affinity for honey bee ChE, while there was a 12-fold increase in the Ka value with furadan and a 25-fold difference with 3-OH carbofuran. So the order of carbamate inhibitors in the descending pattern of affinity for honey bee ChE was 3-keto carbofuran, furadan and 3-OH carbofuran. The inhibitor constants of 3-keto carbofuran clearly indicate that the selection of a synthetic carbamate for commercial use as an insecticide is largely dependent on its I_{50} value than on its affinity constant.

A general perusal of the carbamylation constants (k_{+2}) indicate that these indices did not differ significantly. The order of

TABLE 1. Inhibition constants for honey bee cholinesterase by various carbamates. Each datum is an average of five replicates.

Sl. No.	Inhibition constant	Inhibitor			Unit
		Furadan	3-OH carbofuran	3-keto carbofuran	
1.	I^{50} value	1.77	1.90	3.35	X 10^{-8}M
2.	Affinity constant, Ka	2.68	5.56	0.22	X 10^{-7}M
3.	Carbamylation constant, k_{2c}	2.80	4.92	6.09	min^{-1}
4.	Bimolecular reaction constant. Ki.	1.40	0.88	27.10	X 10^{7}M^{-1} min^{-1}
5.	Decarbamylation constant, k_3	0.0016	0.0030	0.0030	min^{-1}
6.	Half-life value, $t_{0.5}$	433	231	231	minutes
7.	Average apparent affinity constant, Ka'	2.01	13.30	16.70	X 10^{-7}M.

k_{2c} of honey bee ChE was 3-keto carbofuran > 3-OH carbofuran > furadan. A subsequent study with house sparrow and rat ChE's (data not presented in this paper) also showed only an insignificant difference among k_{2c} values, probably indicating a minor role of the carbamylation step in the overall specificity of each enzyme source to various carbamate inhibitors.

From the data it would be clear that the bimolecular reaction constants, Ki, varied among themselves for all the three carbamate inhibitors. The Ki of honey bee ChE for furadan was almost double than that of its hydroxy analogue while for 3-keto carbofuran, it was about 30 times higher than that for 3-OH carbofuran. The differences in the Ki values would largely be attributable to the variations in the Ka values rather than to the k_{2c} values.

The decarbamylation constants (k_3 values) obtained in the present studies are less than those reported with eel ChE by WILSON *et al.*, (1960). The k_3 value is liable to be affected by various experimental conditions like the source of enzyme, assay, inhibitor, substrate concentration, temperature, pH etc. The results reported in the present investigation may also be taken to suggest the probable difference in the chemical bondage in the honey bee carbamylated enzyme which forms a hindrance for the inhibitor moiety to separate out from the enzyme part. This difference in k_3 value of honey bee enzyme could also be interpreted to indicate the difference at the active site of the enzyme.

The half-life values of the different carbamylated enzymes were calculated using the equation, $t_{0.5} = \frac{0.693}{k_3}$. The carbamylated enzyme of honey bee with furadan took about 7 hours for recovering 50 per cent of its original enzymic activity while those with the two other analogues regained their half-activity after about 4 hours. Hence the higher $t_{0.5}$ value with insect ChE can be considered as an attribute for a good insecticide.

The average apparent affinity constants (Ka') as coined by DAVIES *et al.*, (1970) were obtained from the experiment in which the inhibitor was added simultaneously with the substrate. Ka and Ka' values of furadan for honey bee ChE were almost equal. There was a 2-fold difference in 3-OH carbofuran and a 76-fold difference in the case of 3-keto carbofuran.

In conclusion, it may be pointed out that the results obtained in the present studies

suggest that furadan and its analogues are competitive inhibitors at low concentrations (below 10^{-6} M) and the inhibitions could be predicted by invoking the mechanisms proposed by MAIN & HASTINGS (1966). At higher concentrations of the inhibitors, some other mechanism is also enforced. The deviation observed at higher inhibitor concentrations could perhaps be easily explained on the basis of allosteric inhibition.

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