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Non-Competitive Inhibition of Flyhead Acetylcholinesterase by Oxime Carbamates. Kinetic Evidence for Non-productive Binding to the Catalytic Site

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Reversible inhibition of flyhead acetylcholinesterase (E. C. 3.1.1.7) by two oxime carbamates possessing large *N*-substituents (isopropyl and allyl) was found to follow competitive kinetics of a biphasic nature with acetylcholine as the substrate. The derived values for the substrate dissociation constants of high and low affinity were in approximate agreement with the Michaelis and the non-competitive substrate inhibition constant for acetylcholine respectively. Data for the dependence of carbamoylation rates of the enzyme upon substrate concentration did not agree with a model derived from reversible inhibition kinetics. Reversible inhibition studies indicated low active site competitive inhibition constants, showing good binding to the active site. Studies upon the carbamoylation rates indicated (*i*) a non competitive interaction, (*ii*) very low concentrations of a reversibly-formed enzyme/carbamate complex prior to carbamoylation of the active site.

A possible explanation for the discrepancy is discussed whereby a reversible K_i determined from inhibition rate saturation by a »Main plot« measures the concentration of carbamate aligned in the active site following an induced shift of enzyme conformation, whereas competitive inhibition constants (K_i) determined from reversible inhibition experiments determine all binding modes at the active site which interfere with substrate attachment. Carbamates with large *N*-substituents show this effect more because overlap of the carbamoyl moiety with the catalytic site is less likely due to steric hinderance.

INTRODUCTION

Kinetic studies of the inhibition of acetylcholinesterase by carbamates which are very poor carbamoylating agents have shown that these inhibitors will compete for the active site of the enzyme with acetylcholine[1,2]. In one case[1] good agreement has been demonstrated between the derived reversible competitive inhibition constants and the equilibrium constants derived from plots of the rates of irreversible inhibition versus inhibitor concentration (»Main plots«).

We have found that the second order rates of carbamoylation of flyhead acetylcholinesterase for a homologous series of *N*-alkyl substituted oxime carbamates can be correlated with the size of the *N*-alkyl substituent[3]. With larger substituent groups (*N*-allyl and *N*-isopropyl) the rate of carbamoylation is slow and reversible inhibition of acetylcholinesterase can be demonstrated and measured.

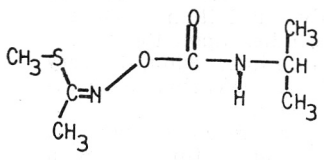
A plot of reversible inhibition data by the method of Hunter and Downs[4] shows a biphasic curve. At low concentrations of substrate (acetylcholine), inhibitor and substrate compete for the active site (true competitive inhibition). At higher concentrations substrate and inhibitor compete for a site on the enzyme which has been implicated in substrate inhibition by acetylcholine. This latter type of inhibition has been reported for the organophosphorus compound Haloxon[5], although true reversible competitive inhibition was not demonstrated for haloxon. This low affinity binding site can be shown to be kinetically distinct from the site which binds certain non-competitive phenolic inhibitors such as 2-(2-hydroxylphenyl)-1,3-dithiolane[6].

MATERIALS AND METHODS

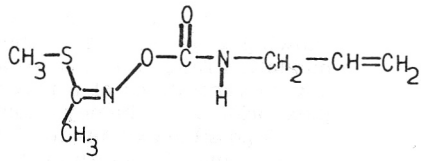
Enzyme

The flyhead acetylcholinesterase used for kinetic measurements was a butanol--solubilized preparation purified by DEAE cellulose and Sepharose 6 B chromatography. The specific activity was in the range of 800 to 2000 μmol of acetylcholine hydrolysed per min at 25 °C pH = 7.5. Disc gel electrophoresis of the enzyme followed by activity staining for arylesterase and acetylthiocholinesterase activity showed an iso-enzymic pattern somewhat similar to that reported by Tripathi, Chill and O'Brien[6]. Staining of the gels for protein using Coomassie blue showed a superimposable pattern to that developed by esteratic staining and showed no observable protein of a distinct electrophoretic mobility to the esterase bands.

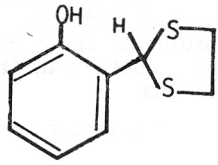
Inhibitors



I
S-methylthioacetate *N*-isopropyl carbamoyl oxime



II
S-methylthioacetate *N*-allylcarbamoyl oxime



III
2-(2-hydroxyphenyl)-1,3-dithiolane

These compounds were synthesised by conventional methods and characterised by elemental analysis, i. r. and n. m. r. spectroscopy. Purification was by recrystalling twice from ether. The stereochemistry of the oxime carbamates is such that the methyl-thio group is *syn* to the ester carbonyl. The kinetics of acetylcholinesterase inhibition by all inhibitors was not affected by further recrystallisation.

Kinetic measurements

All measurements were carried out at pH=7.5 in a solution containing 0.1 M NaCl, 0.002 M $MgCl_2$ and 10^{-4} M sodium phosphate in carbon dioxide-free distilled water. Enzyme activity was measured by a standard »Radiometer« pH-stat assay in which the acetic acid produced during the reaction was titrated continuously

to pH = 7.5 using 0.01 M NaOH in a 0.25 ml syringe. Substrate depletion at low concentrations of acetylcholine was compensated for by simultaneous addition of 0.01 M acetylcholine pH=7.5 from a matched syringe. The total volume of the assay system was 10 ml. Unless otherwise stated all measurements were carried out at 25 °C.

Reversible inhibition was measured by simultaneous addition of substrate and inhibitior.

Addition of a solution of (II) to acetylcholine and acetylcholinesterase produced reversible and progressive inhibition. In this case tangents were drawn to the curve produced at timed intervals after addition of inhibitor. A plot of log (slope) versus time (t) extrapolated to $t = 0$ gave the reversible component of inhibition. Enzyme-catalysed rates of hydrolysis of high concentrations of acetylcholine were corrected for base catalysed hydrolysis.

Rates of irreversible inhibition were measured by the addition of a known concentration of inhibitor to a sample of enzyme. At timed intervals 1.0 ml samples were withdrawn and added to 10 ml of acetylcholine (3×10^{-3} M) in the assay cell. Short times of inhibition were measured using a method similar to that used by Main[8] using separate enzyme samples.

Decarbamoylation rates were measured using a technique involving 1000 fold dilution of 90% inhibited enzyme.

RESULTS

Reversible inhibition

Fig. 1 shows the reversible inhibition of flyhead acetylcholinesterase by I and II in the presence of 3×10^{-3} M acetylcholine.

The data were treated by the method of Hunter and Downs[4] where the rate of substrate hydrolysis in the absence of inhibitor is designated V_o and in the presence of inhibitor with the same concentration of substrate V. A plot of V_o/V versus [I] for several concentrations of substrate is plotted. These plots were linear for inhibitors I, II and III and for all concentrations of acetylcholine. The function $\frac{V\,[I]}{V_o - V}$ is then calculated from the slopes and plotted versus the substrate concentration. These graphs are shown in Figs. 2 and 3 for inhibitors I and III respectively. Compound (II) exhibited similar reversible inhibition kinetics to (I).

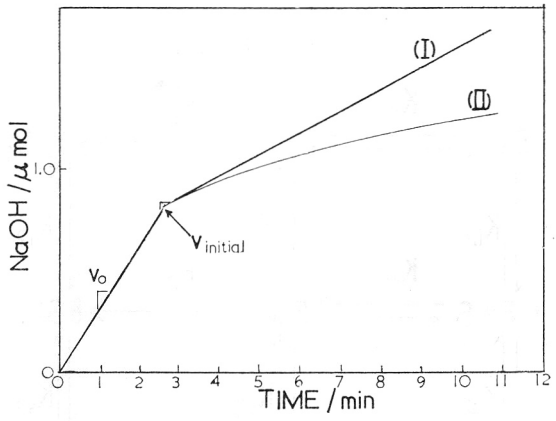


Fig. 1. Radiometer trace produced by adding a solution of oxime carbamate I or II to a mixture of acetylcholine and flyhead acetylcholinesterase. Sodium hydroxide (0.01M) was used to continuously titrate the acetic acid produced from a mixture of acetylcholine (0.03 M) and flyhead acetylcholinesterase to pH = 7.5. Compound II can be seen to inhibit progressively whereas I shows only the reversible component of inhibition. The determination of reversible inhibition data from the traces is described in the text.

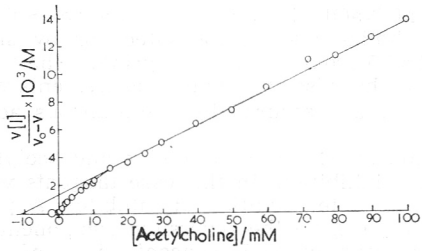


Fig. 2. Reversible inhibition of flyhead acetylcholinesterase by I at 25 °C, pH = 7.5 in the presence of acetylcholine-bromide. The data were plotted by the method of Hunter and Downs. Each point represents the average of five determinations made using five different inhibitor concentrations. The curve represents the best fit equation (2) to the points and was used to determine the constants recorded in Table I.

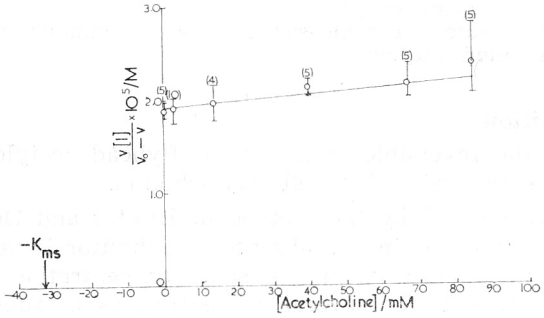
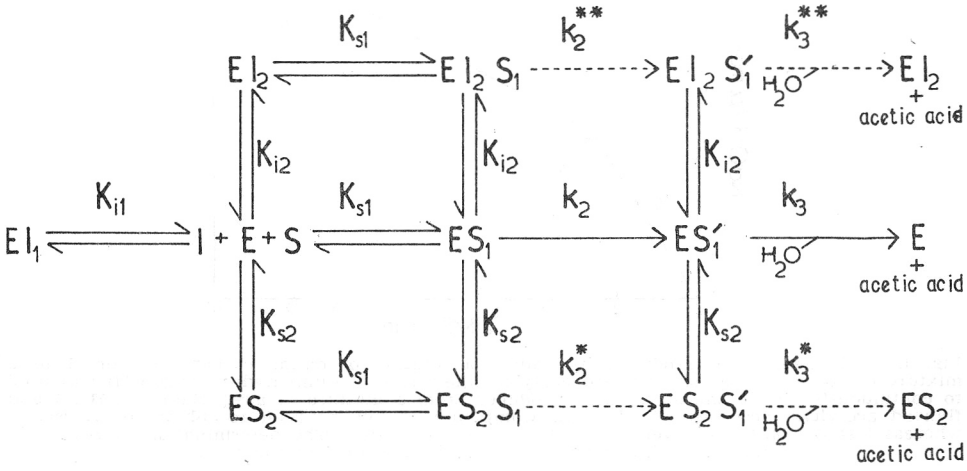


Fig. 3. Reversible inhibition of flyhead acetylcholinesterase by III at 25 °C, pH = 7.5. The data were plotted by the method of Hunter and Downs. The number of determinations are in parentheses. The error bars represent the range of values and the points the arithmetical mean.

The data for the reversible inhibition for compounds (I) and (II) can be fitted to a scheme in which the inhibitors compete with substrates at the active site (site 1) and at second site (site 2) responsible for substrate inhibition. This is shown in kinetic scheme 1.

Kinetic Scheme I



$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{1}$$

The simplifying assumptions can then be made that sites 1 and 2 are independent and occupation of a single site influences only the rates of reaction at the other site. If it is assumed that the acetyl enzyme of flyhead acetylcholinesterase does not bind ligands[9] then occupation of site 2 will influence only the acetylation rate at site 1. In the case of substrate inhibition for flyhead acetylcholinesterase we have found[5] that $k^*_{cat}/k_{cat} = 0.10$. The fact that plots of V_o/V *vs.* [S] are linear would suggest that $k^{**}_{cat} = 0$.

Simplifying the scheme still further by ignoring the acetylenzyme states yields from the Hunter-Downs approach the following expression:

$$V[I]/(V_o - V) = \frac{1 + K_{s_1}/[S] + [S]/K_{s_2} + (K_{s_1}/K_{s_2})}{K_{s_1}/K_{i_1}\ [S] + K_{s_1}/K_{i_2}\ [S] + 1/K_{i_2} + K_{s_1}/K_i \cdot K_{s_2} + (K_{s_1}\ [I]/K_{i_1}\ K_{i_2}\ [S])} \tag{2}$$

The terms in brackets are considered small compared with other terms and are thus ignored. Equation (2) thus simplifies to:

$$V\ [I]/(V_o - V) = \frac{1 + K_{s_1}/[S] + [S]/K_{s_2}}{K_{s_1}/K_{i_1}\ [S] + K_{s_1}/K_{i_2}[S] + 1/K_{i_2} + K_{s_1}/K_{i_1} \cdot K_{s_2}} \tag{3}$$

A plot of $V\ [I]/(V_o - V)$ *versus* [S] produces a curve having two asymptotes, one at high [S] and one at low [S]. Graphical analysis yields a line at high [S] of the slope K_{i_2}/K_{s_2} and intercept K_{i_2}. While at low [S] the slope is K_{i_2}/K_{s_2} and intercept K_{i_1} (Fig. 4).

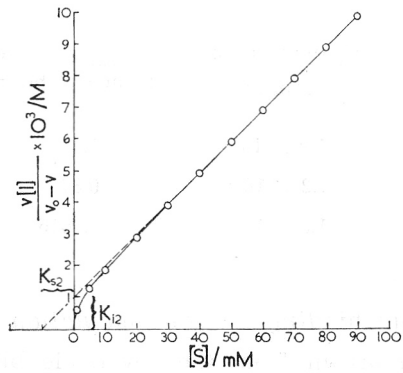


Fig. 4. Plot of equation (3) at high substrate concentration. Equation 3 was fitted to the following parameters: $K_{s_1} = 10^{-5}$ M, $K_{i_1} = 10^{-5}$ M, $K_{s_2} = 10^{-2}$ M, $K_{i_2} = 10^{-3}$ M.

The derived data in terms of the constants K_{i_1}, K_{i_2}, K_{s_1} and K_{s_2} are shown in Table I. The values of K_{s_1} and K_{s_2} derived from Figs. 1 and 2 can be compared with the Michaelis constant K_m and the binding constant for substrate

TABLE I.

Reversible inhibition of flyhead acetylcholinesterase by the carbamate inhibitors (I) and (II), 25 °C, pH = 7.5

Compound	K_{s_1}/mol l^{-1}	K_{s_2}/mol l^{-1}	K_{i_1}/mol l^{-1}	K_{i_2}/mol l^{-1}
(I)	4.8×10^{-4}	9.0×10^{-3}	4.8×10^{-5}	4.5×10^{-4}
(II)	2.7×10^{-4}	1.1×10^{-2}	8.5×10^{-3}	1.3×10^{-3}
	K_m/mol l^{-1}	K_{ms}/mol l^{-1}		
Acetylcholine	2.1×10^{-5}	2.7×10^{-2}		

inhibition by acetylcholine, K_{ms} measured independently. It is important to note that competition for site 2 can only be measured at high substrate concentrations and would be indistinguishable from the purely non-competitive inhibition by the usual reciprocal plots measured at low substrate concentration. The Hunter-Downs plot for a purely non-competitive inhibitor (III) is shown in Fig. 3 for comparison. The non-competitive constants derived are presented in Table II for three substrates together with the V_{max} for the substrates. This data suggests that (III) binds to the Michaelis complex and not the acetyl enzyme[9].

TABLE II.

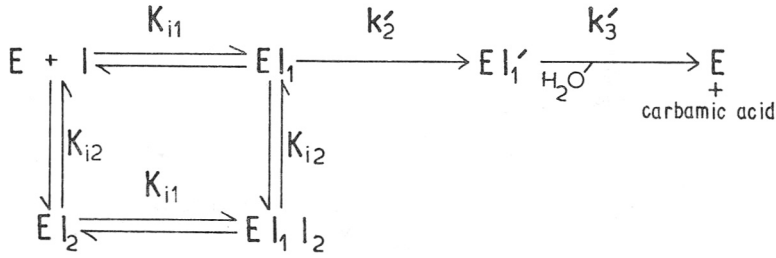
Reversible non-competitive inhibition of flyhead acetylcholinesterase by 2-(2-hydroxylphenyl)-1,3, dithiolane (III), 25 °C, pH = 7.5

Substrate	K_i (non-comp.) / mol l^{-1}	V_{max} relative to acetylcholine	V_{max} / K_i (non-comp.)
Acetylcholine	1.9×10^{-5}	1.0	5.3×10^{4}
Phenylacetate	1.2×10^{-3}	0.67	5.6×10^{2}
o-Nitroacetanilide	1.3×10^{-3}	0.0015	1.2×10^{0}

Influence of reversible binding on carbomoylation rates

If the conclusions drawn from the reversible binding experiments are correct, the rates of carbamoylation of acetylcholinesterase by I and II will be influenced by binding of I and II to site 2 and will be subject to a saturation affect due to formation of an enzyme—inhibitor Michaelis complex prior to carbamoylation. Assuming that site 1 is carbamoylated by the inhibitors a scheme for the carbamoylation of site 1 in the absence of substrate can be represented as:

Kinetic Scheme II



For compounds I and II, the decarbamoylation rate (k_3) is slow and can be measured independently. Since k'_3 is small, a plot of log V_t/V_o *versus* time is linear and the slope (ϱ) can be measured. The relationship between (ϱ) and the inhibitor concentrations [I] for the case $k'_3 = 0$ can be derived as:

$$[I]/\varrho = 1/k_2' \, [1 + K_{i1}/[I] + K_{i1}/K_{i2}] + [I]/K_{i2} \qquad (4)$$

If we make the assumption that the constants K_{i1}, and K_{i2} measured from reversible inhibition studies hold for the above scheme, a series of curves can be plotted for $1/\varrho$ versus 1/[I]. Fig. 5 shows a series of curves plotted from

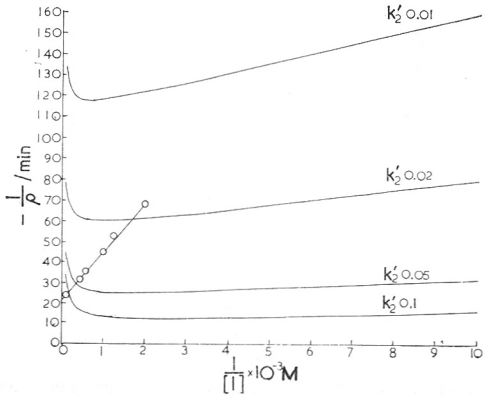


Fig. 5. Plot of equation 4 fitted to four values of carbamoylation rate constants (k_2') (»Main plot«). Equation (4) was fitted using the values of the reversible inhibition constants determined for compound (I) at 25 °C: $K_{i1} = 4.8 \times 10^{-5}$ M, $K_{i2} = 4.5 \times 10^{-4}$ M. Actual data from carbamoylation rate studies with I at 25 °C are shown inset (circles).

equation 2 fitted to the reversible binding data for (I) at 25 °C. This situation is analogous to a reciprocal Lineweaver-Burke plot demonstrating substrate inhibition. The actual data from progressive inhibition studies using I is also shown in Fig. 5 and clearly does not fit the equation derived from kinetic scheme II. Further data from progressive inhibition studies using I at four different temperatures is shown in Fig. 6. If the data from Fig. 7 is fitted to the usual interpretation of saturation of progressive inhibition, that of the formation of a Michaelis complex prior to carbamoylation, the intercept $1/\varrho$ equals $1/k'_2$ (the reciprocal of the carbamoylation rate constant) at $1/[I] = 0$. The intercepts derived from these plots cannot be described to a single kinetic rate constant since the reciprocal of the intercept does not increase with increasing temperature.

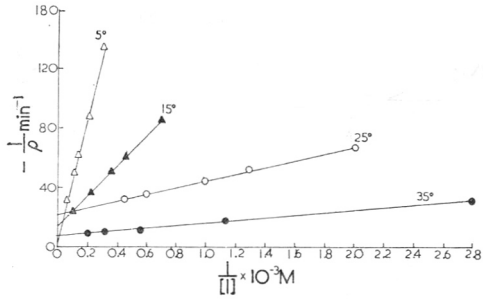


Fig. 6. Progressive inhibition of flyhead acetylcholinesterase by I at pH = 7.5. The data were used to determine the values of k_1 and K_{12} in Table III.

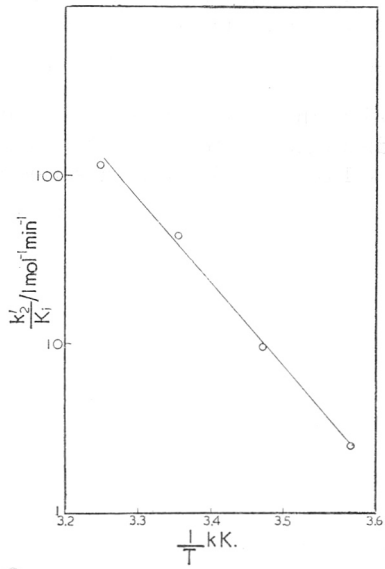
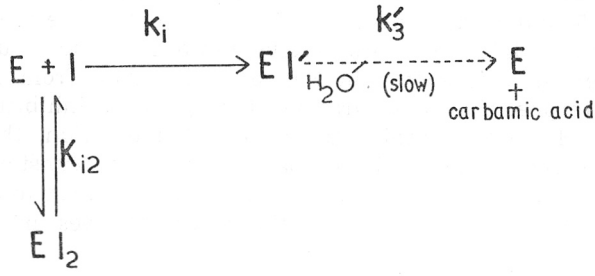


Fig. 7. Arrhenius plot for carbamoylation of flyhead acetylcholinesterase by I at pH = 7.5. The derived data are shown in Table III.

A kinetic scheme which does fit the data in Fig. 6 is the one which has been used to explain the irreversible inhibition by Haloxon[5].

Kinetic Scheme III



An expression for the first order rate for progressive inhibition ϱ, can be derived as:

$$1/\varrho = 1/k_i\,[I] + K_{i_2}\,k_i \qquad (5)$$

where k_i is the second order rate constant for progressive inhibition. The intercepts derived from Fig. 7 then yield the complex term $K_{i_2} \cdot k_i$ and the slopes $1/k_i$. A consequence of the mechanism described by kinetic scheme III is that the concentration of Michaelis complex between enzyme and carbamoylated enzyme (EI′) is very small, *i. e.* K_{i_1} is very large. The data derived from Fig. 7 in terms of equation (5) is shown in Table II for (I) and (II).

TABLE III.

Progressive inhibition of flyhead acetylcholinesterase in the absence of substrate by compounds (I) and (II). Data derived from the fit of experimental results to equation (5)

Compound	T °C	k_i / l mol^{-1} min^{-1}	K_{i_2} / mol l^{-1}	k_3' / min^{-1}
(I)	5	2.5	2.5	
	15	9.6	1.4	
	25	44.0	0.5	6.0×10^{-4}
	35	114.	0.07	1.2×10^{-3}
(II)	25	1.4×10^{3}	2.1×10^{-4}	7.5×10^{-4}
	35	4.2×10^{3}	1.2×10^{-4}	1.8×10^{-3}

Activation constants derived from the above data

		ΔG^* / kcal mol^{-1}	ΔH^* / kcal mol^{-1}	ΔS^* / cal mol^{-1} K^{-1}
(a) Carbamoylation (k_i)	(I)	17.6	20.9	11.1
	(II)	15.6	19.2	12.1
(b) Decarbamoylation (k_3')	(I)	23.9	12.0	—40
	(II)	24.1	15.3	—29.4

There is no correlation between K_{i_2} derived from the fit of irreversible binding data to equation (3) and the low affinity reversible binding to site 2 (K_{i_2}) derived from reversible inhibition data. This holds particularly for the very poorly carbamoylating inhibitor I.

DISCUSSION

The poorly carbamoylating inhibitors (I) and (II) compete with acetylcholine for the active site and for the site implicated in acetylcholine substrate inhibition. This latter type of inhibition appears to be kinetically distinct from a less specific non-competitive inhibition shown by (III). Compound (III) binds to the esterase-acetylcholine Michaelis complex and not to the acetyl enzyme. The site to which III binds is presumably part of the active site

complex which does not overlap with the acetylcholine binding site since carbamates of III are good carbamoylating inhibitors of acetylcholinesterase[6].

Compounds (I) and (II) can compete for acetylcholine for occupation of the active site in a conformation which does not lead to carbamoylation of the active site. A possible explanation of the discrepancy between the competitive inhibition and complexation leading to carbamoylation would involve good binding of (I) and (II) to the part of the acetylcholine binding site but steric interactions then lead to poor overlap with the esteratic site. This non-productive binding explanation would also explain the discrepancy between the apparent K_{i_2} measured from progressive inhibition and from the reversible experiments. Non-competitive inhibition of carbamoylation by I and II will only occur if these compounds inhibit acylation of the active site. If substrate inhibition (binding to site 2 in kinetic scheme I) is due to inhibition of deacylation only (obligate binding to acylenzyme) non-competitive inhibition of carbamoylation will not be controlled by site 2 binding determined from reversible inhibition studies.

An Arrhenius plot of the second order rate of inhibition (k_i) of compound I is shown in Fig. 7 and the derived activation constants of carbamoylation and decarbamoylation for I and II in Table II.

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DISCUSSION

I. Silman:

Is *o*-nitroacetanilide a substrate for both, flyhead and vertebrate AChE?

P. J. Jewess:

Yes. Bender and Stoops originally showed that bovine erythrocyte AChE hydrolysed this substrate.

M. E. Eldefrawi:

In characterizing the flyhead enzyme as AChE have you measured hydrolysis of butyrylthiocholine? And if you did, was there any substrate inhibition of the enzyme by this substrate?

P. J. Jewess:

I am aware of the reported catalytic activity of the flyhead AChE preparation towards butyrylthiocholine and butyrylcholine, but I have not checked the activity of our preparation using these substrates.

SAŽETAK

Nekompetitivna inhibicija acetilkolinesteraze glave muhe oksimima karbamata. Kinetički dokaz za neproduktivno vezanje u katalitičkom mjestu

P. J. Jewess i N. R. McFarlane

Nadjeno je da reversibilna inhibicija acetilkolinesteraze (E. C. 3.1.1.7.) glave muhe s dva oksima-karbamata koji na dušikovom atomu nose krupne supstituente (izopropil- i alil-), slijedi s acetilkolinom kao supstratom kompetitivnu kinetiku bifazne prirode. Dobivene vrijednosti konstanata disocijacije za visoki i niski afinitet približno se slažu s Michaelisovom konstantom i konstantom za nekompetitivnu supstratnu inhibiciju s acetilkolinom. Rezultati dobiveni za ovisnost brzina karbamilacije enzima o koncentraciji supstrata ne slažu se s modelom izvedenim za kinetiku reversibilne inhibicije. Studij reversibilne inhibicije pokazuje niske vrijednosti konstante za kompetitivnu inhibiciju u aktivnom mjestu, što ukazuje na dobro vezanje za aktivno mjesto. Istraživanja karbamilacije ukazuju na (*i*) nekompetitivnu interakciju i (*ii*) vrlo nisku koncentraciju reversibilno nastalog enzim-karbamatnog kompleksa u toku karbamilacije aktivnog mjesta.

Prodiskutirano je moguće objašnjenje za nađeno neslaganje. Pri tome vrijednost za reversibilnu K_i, određenu iz zasićenja brzine inhibicije grafičkim prikazom po Mainu, predstavlja mjeru za koncentraciju karbamata vezanog u aktivnom mjestu nakon inducirane konformacijske promjene enzima, a konstanta kompetitivne inhibicije (K_i) određena eksperimentima reversibilne inhibicije, uključuje sve načine vezanja u aktivnom mjestu koji se kose s vezanjem supstrata. Karbamati s krupnim supstituentima na dušikovom atomu pokazuju izrazit učinak jer je malo vjerojatno da je preklapanje karbamilne strane s katalitičkim mjestom uvjetovano prostornom smetnjom.

LABORATORIJ WOODSTOCK, SHELL,
SITTINGBOURNE, KENT, ENGLESKA