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Kinetic Study of the Effect of Substrates on Reversible Inhibition of Cholinesterase and Acetylcholinesterase by Two Coumarin Derivatives

Elsa Reiner and Vera Simeon

Biochemical Laboratory, Institute for Medical Research and Occupational Health, Yugoslav Academy of Sciences and Arts, Zagreb, Croatia, Yugoslavia

The reversible inhibition of cholinesterase and acetylcholinesterase by coumarin and haloxon was studied in the presence of phenylacetate and acetylthiocholine as substrates. The degree of inhibition, expressed as $v[I]/(v_o - v)$, was shown to be linear function of the substrate concentration. The linearity is maintained for the inhibition by both inhibitors, and regardless of either the kinetics of the enzyme-substrate reaction, or the enzyme inhibition by the substrate itself. At given substrate concentrations the ratio $v[I]/(v_o - v)$ is constant, regardless of the inhibitor concentration. The intercept on the ordinate of the line $v[I]/(v_o - v)$ *vs.* [S], represents the dissociation constant $K(I)$ of the enzyme-inhibitor complex. The intercept on the abscissa $K(S)$ is characteristic of the enzyme-substrate reaction; the experimentally obtained values were shown to be larger than the corresponding Michaelis constants. Theoretical possibilities are discussed of the enzyme--inhibitor and enzyme-substrate binding sites in order to interpret the $K(S)$ constants. Equations are derived for the binding of the inhibitor to the catalytic site (according to the Krupka and Laidler theory) or an allosteric site on the enzyme (according to the Aldridge and Reiner theory). Neither theoretical equation corresponds to the experimentally obtained results. The kinetics of the studied reactions is apparently more simple than theoretically predicted.

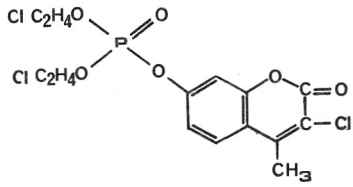
It is known that acylating inhibitors (organophosphates and carbamates) form a reversible complex (Michaelis complex) with acetylcholinesterase (E.C. 3.1.1.7) and cholinesterase (E.C. 3.1.1.8) prior to the acylation step. It is further known that some acylating inhibitors also form with acetylcholinesterase a reversible complex which is different from the Michaelis complex; this is the case with two coumarin derivatives[1] and with two bis-dimethylcarbamate derivatives[2]. The difference between this complex and the Michaelis complex is revealed in the ability of acetylcholine and acetylthiocholine to displace the inhibitor from the enzyme. The displacement of the inhibitor from the Michaelis complex is achieved with much lower substrate concentrations than the displacement from the other enzyme-inhibitor complex. Both substrates, acetylcholine and acetylthiocholine, are themselves reversible inhibitors of acetylcholinesterase.

A hypothesis has been put forward[1,3] concerning the kinetics of reversible inhibition of acetylcholinesterase by the coumarin derivatives. It has been

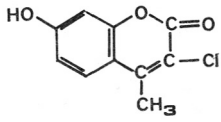
postulated that the inhibition by the coumarin compounds is an allosteric reaction, and that the inhibition by excess substrate is also due to allosteric binding. To verify if this model is more generally applicable, studies were expanded to cholinesterase as enzyme and phenylacetate as substrate.

MATERIALS AND METHODS

The enzymes were bovine erythrocyte acetylcholinesterase (Winthrop Ltd., New York, N. Y. USA) and horse serum cholinesterase (Sigma Chemical Comp., St. Louis, Mo., USA). The substrates were acetylthiocholine iodide and phenylacetate, and the inhibitors haloxon and coumarin, both from Cooper Technical Bureau, Berkhamsted, Hert., England. Coumarin is the leaving group in haloxon and was shown to follow the same kinetics of reversible inhibition like haloxon and coroxon (the diethyl analogue of haloxon)[1].



Haloxon



Coumarin

All experiments were done in 100 mM phosphate buffer pH = 7.4 at 25 °C. The activity towards acetylthiocholine was measured by the spectrophotometric method of Ellman *et al.*[4] and towards phenylacetate by the spectrophotometric method of Krupka[5]. The procedure was as follows: the enzyme solution (0.3 ml) in buffer was added to a buffer solution (2.7 ml) containing the substrate and inhibitor. The absorbance was read in 1.0 cm cuvettes against a blank containing the same final concentration of substrate and inhibitor. When acetylthiocholine was the substrate, the reaction mixture also contained the thiol reagent DTNB (final conc. 0.33 mM), which was added to the experimental and blank tube, and it also contained a final ethanol conc. of 2.0% (v/v); the absorbance was read at 412 nm. When phenylacetate was the substrate, the final ethanol concentration was 4.0% (v/v), and the absorbance due to phenol was read at 270 nm. The absorbance was read for 2—3 min at 20 s intervals on a Unicam SP 600 spectrophotometer or recorded on a Varian Techtron spectrophotometer Model 635. For both substrates, the increase in absorbance was linear with time for both, inhibited and uninhibited enzyme samples.

The reaction kinetics was studied within the following concentration range of substrates and inhibitors. The concentration of acetylthiocholine ranged from 0.1 to 10 mM, and of phenylacetate from 0.4 to 10 mM. At each substrate concentration the inhibition was determined with 2—3 different concentrations of haloxon and coumarin. The concentrations of the inhibitors were such that the degree of enzyme inhibition was more than 10% and less than 90%. The ratio $v[I]/(v_0 - v)$ was calculated, where v is the enzyme activity in the presence of the inhibitor, and v_0 the enzyme activity measured at the same substrate concentration as v, but in the absence of the inhibitor. That ratio was plotted against the substrate concentration.

RESULTS

The reactions, in the absence of inhibitor, of acetylthiocholine and phenylacetate with acetylcholinesterase and cholinesterase are presented in Fig. 1, where the enzyme activity is plotted against the substrate concentration. For cholinesterase, the activity increases with increasing substrate concentrations, but the kinetics of the reaction is different for the two substrates. For phenylacetate, the reaction follows the Michaelis equation and the Michaelis constant, calculated from the Wilkinson plot, is 3.80 mM (Table I). For acetylthiocholine,

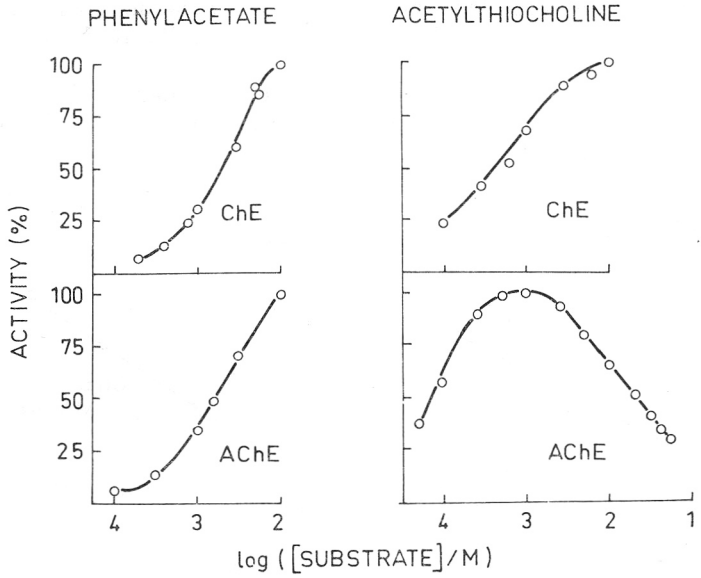


Fig. 1. Activitiy of horse serum cholinesterase (ChE) and bovine erythrocyte acetylcholinesterase (ChE) as a function of the phenylacetate and acetylthiocholine concentrations. Each point is the mean of three separate experiments, each of which was made in duplicates. The results with acetylthiocholine are from Simeon[6].

TABLE I.

Derived K (S) constants for the listed enzyme-substrate reactions determined in the presence of the indicated inhibitors

Substrate	Enzyme	K_m/mM	Inhibitor	K (S)/mM
Acetylthiocholine	ChE	0.6 [6]	Coumarin	2.40 ± 0.29 (5)
	AChE	0.11 [6]	Coumarin	2.3[1]
			Haloxon	2.34 ± 0.48 (10)
Phenylacetate	ChE	3.80 ± 0.17 (8)	Coumarin	8.03 ± 0.66 (7)
	AChE	2.64 ± 0.06 (6)	Coumarin	8.0 ± 2.5 (4)
			Haloxon	10.03 ± 0.57 (8)

The experiments were done in 100 mM buffer pH = 7.4 at 25 °C. ChE stands for horse serum cholinesterase and AChE for bovine erythrocyte acetylcholinesterase. K_m is the Michaelis constant for the enzyme-substrate reaction determined in the absence of the inhibitors. The numbers in brackets indicate the number of different substrate concentrations used in the experiment.

the reaction follows the Hill equation with a Hill coefficient of 0.7—0.8 and a K_m of 0.60 mM[6]. The reaction of acetylcholinesterase and phenylacetate follows the Michaelis equation and the Michaelis constant, calculated from the Wilkinson plot, is 2.64 mM (Table I). The reaction of acetylcholinesterase and acetylthiocholine is bell-shaped with a Michaelis constant of 0.11 mM and a substrate inhibition constant K_{ss} of 14 mM[6].

The inhibition of cholinesterase and acetylcholinesterase by haloxon and coumarin was measured with phenylacetate and acetylthiocholine as substrates, and is presented in Fig. 2. Coumarin is a reversible inhibitor of cholinesterase as well as acetylcholinesterase, while haloxon does not inhibit the cholinesterase reversibly, which is different from its reaction with acetylcholinesterase[1].

The six reactions presented in Fig. 2 have two characteristics in common. At a given substrate concentration, the ratio $v\,[I]/(v_o - v)$ is constant irrespective of the inhibitor concentration. Each point therefore is the mean

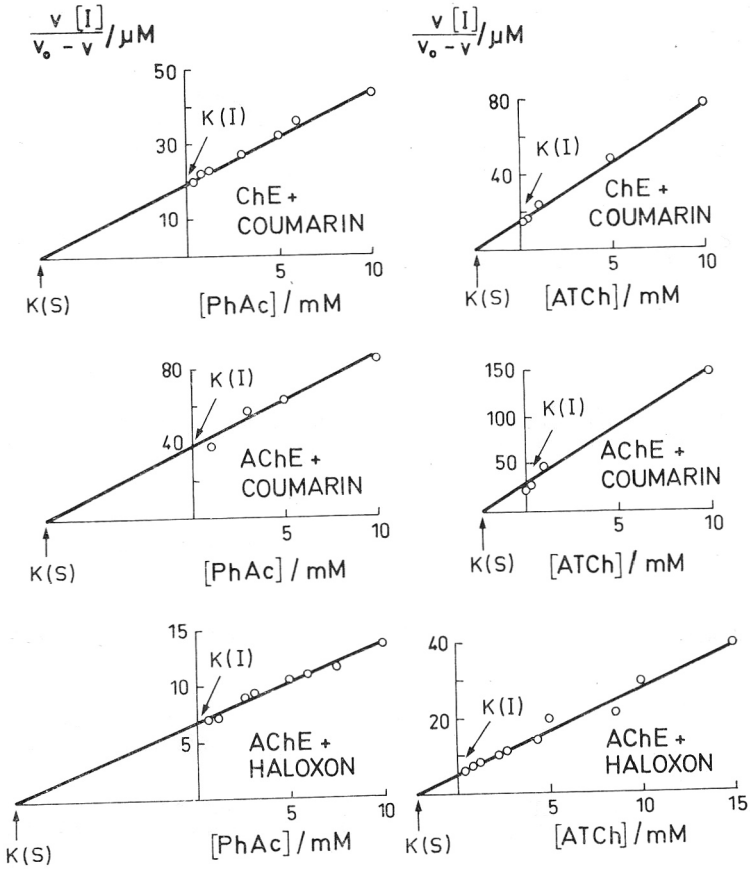


Fig. 2. Reversible inhibition of horse serum cholinesterase (ChE) and bovine erythrocyte acetylcholinesterase (AChE) with coumarin and haloxon. The substrates were phenylacetate (PhAc) and acetylthiocholine (ATCh). The symbols on the ordinate are defined in the text. $K(S)$ and $K(I)$ are the calculated intercepts of the lines on the abscissa and ordinate. The results for the reaction AChE + coumarin + ATCh are from Aldridge and Reiner[1].

value obtained with different inhibitor concentrations, but one substrate concentration. The second characteristic is the linearity between the ratio $v\,[I]/(v_o - v)$ and the substrate concentration. This is least obvious for the reaction of acetylcholinesterase + acetylthiocholine + coumarin because only three substrate concentrations were tested (*cf.* ref.[1]), but more obvious for the other five reactions presented in Fig. 2. However, for all six reactions the degree of inhibition decreases with increasing substrate concentration, and this is characteristic of a competitive reaction between substrate and inhibitor. This way of plotting the results is very convenient particularly when substrates themselves are enzyme inhibitors. The ratio $v\,[I]/(v_o - v)$ reflects only the effect of haloxon or coumarin, and not the effect of the substrate itself, because the ratio does not depend on the absolute values of the enzyme activity. The calculated intercepts of the lines in Fig. 2 with the ordinate K (I) and abscissa K (S) are given in Tables I and II, resp.

K (I) is the dissociation constant for the enzyme-inhibitor complex. This constant is essentially the same irrespective of which substrate is used to measure the inhibition (Table II). When phenylacetate is the substrate, the K (I)

TABLE II.

Derived K (I) constants for the listed enzyme-inhibitor reactions determined in the presence of the indicated substrates

Inhibitor	Enzyme	Substrate	K (I)/mM
Coumarin	ChE	Acetylthiocholine	15.3 ± 1.3 (5)
		Phenylacetate	19.54 ± 0.65 (7)
	AChE	Acetylthiocholine	28[1]
		Phenylacetate	36.6 ± 5.2 (4)
Haloxon	AChE	Acetylthiocholine	5.28 ± 0.85 (10)
		Phenylacetate	6.76 ± 0.14 (8)

The experiments were done in 100 mM buffer pH = 7.4 at 25 °C. ChE stands for horse serum cholinesterase and AChE for bovine erythrocyte acetylcholinesterase. The numbers in brackets indicate the number of different substrate concentrations used in the experiment.

value is 20% larger than that determined with acetylthiocholine as substrate. We assume that this might be due to the ethanol concentration which is 4.0% in the experiments with phenylacetate and 2.0% in the experiments with acetylthiocholine. This assumption was not proved experimentally. The K (I) value for coumarin and acetylcholinesterase, in Table II, was determined at the same pH and temperature like the other constants, but in different buffers (phosphate, phosphate-pyrophosphate, bicarbonate) and different buffer concentrations; when the value of K (I) for haloxon and acetylcholinesterase was measured in these different buffers, its value was 4.95 μM[1] which agrees well with the value in Table II. Comparing the K (I) constant for the two inhibitors it is obvious that haloxon is a better inhibitor than coumarin.

The constants K (S) characterize the enzyme-substrate reaction. For both enzymes and both substrates the experimentally obtained K (S) are larger than the corresponding Michaelis constants determined under the same experimental conditions (Table I). The difference between K (S) and K_m is parti-

cularly pronounced for acetylthiocholine where it amounts up to 21-fold. It follows from the large K (S) values and from the small slopes of the lines in Fig. 2, that large substrate concentrations are required to displace the inhibitor from the enzyme. The question consequently arises by what mechanism the two inhibitors react with the enzyme.

THEORY

Several theoretical considerations have been put forward for the mechanism of substrate inhibition in various enzymes (*cf.* refs.[7,8]). The majority is based on the assumption that inhibition occurs when more than one substrate molecule binds to the enzyme. Two theories on the mechanism of substrate inhibition in cholinesterases will be discussed here. One is by Krupka and Laidler[9] and the other by Aldridge and Reiner[1,3].

According to the Krupka and Laidler model substrates bind to the esteratic and anionic site of the catalytic centre, and hydrolysis occurs at the esteratic site. When the substrate concentration is large, one substrate binds to the anionic site only and prevents hydrolysis of the acetylated enzyme at the esteratic site; this is substrate inhibition. Consequently, by that theory, substrates bind to both, the free enzyme and the acetylated enzyme, and substrate inhibition occurs at the catalytic centre itself.

Contrary to that, Aldridge and Reiner have postulated that the enzyme has two binding sites, the catalytic centre and an allosteric site. Substrate hydrolysis occurs at the catalytic centre. The allosteric site is catalytically inactive, but it can reversibly bind the substrate. Both sites are independent as far as binding is concerned. However, the binding of the substrate to the allosteric site prevents hydrolysis of another substrate at the catalytic centre, and this is substrate inhibition. Consequently, the theory assumes that substrate inhibition is an allosteric reaction.

A postulate in common to both theories is the assumption that reversible inhibitors can react with enzymes in the same way as excess substrate. In the Krupka and Laidler model it means that inhibitors could bind to the free and acetylated enzyme, and in the Aldridge and Reiner model to the catalytic and allosteric site. The two models can be distinguished by kinetic equation. For the sake of comparison, all kinetic equations have to be derived in such way that they yield the same final form. The Hunter and Downs[10] way of presenting the experimental results is used in Fig. 2, and the theoretical equations will therefore be derived in that form.

According to the Krupka and Laidler theory the following reactions are possible:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_{+2}}{\rightarrow} EA \overset{k_{+3}}{\rightarrow} E + \text{products} \qquad (1)$$

$$EA + S \rightleftharpoons EAS \qquad (2)$$

$$E + I \rightleftharpoons EI \qquad (3)$$

$$EA + I \rightleftharpoons EAI \qquad (4)$$

where E, EA, S and I are free enzyme, acetylated enzyme, substrate and inhibitor resp. The Michaelis constant and the dissociation constants of the complexes EAS, EI and EAI are:

$$K_m = \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+3})} \tag{5}$$

$$K'_{ss} = \frac{[EA][S]}{[EAS]} \tag{6}$$

$$K'_a = \frac{[E][I]}{[EI]} \tag{7}$$

$$K'_i = \frac{[EA][I]}{[EAI]} \tag{8}$$

Four kinetic equations have to be considered depending on whether all or only some of the reactions (1) to (4) occur. When all four reactions occur, the substrate itself is an enzyme inhibitor and the inhibitor I binds to both, the free and acetylated enzyme. The total enzyme concentration $[E_o]$ equals:

$$[E_o] = [E] + [ES] + [EA] + [EAS] + [EI] + [EAI] \tag{9}$$

and the enzyme activity is:

$$v = k_{+3}[EA] =$$

$$= \frac{k_{+3}[E_o]}{1 + \frac{k_{+3}}{k_{+2}} + \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}k_{+2}[S]} + \frac{[S]}{K'_{ss}} + \frac{[I]k_{+3}(k_{-1} + k_{+2})}{K'_a k_{+1} k_{+2}[S]} + \frac{[I]}{K'_i}} \tag{10}$$

When no inhibitor I is present, the enzyme activity is:

$$v_o = \frac{k_{+3}[E_o]}{1 + \frac{k_{+3}}{k_{+2}} + \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}k_{+2}[S]} + \frac{[S]}{K'_{ss}}} \tag{11}$$

and the ratio equals:

$$\frac{v[I]}{v_o - v} = \frac{1 + \frac{k_{+3}}{k_{+2}} + \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}k_{+2}[S]} + \frac{[S]}{K'_{ss}}}{\frac{k_{+3}(k_{-1} + k_{+2})}{K'_a k_{+1} k_{+2}[S]} + \frac{1}{K'_i}} \tag{12}$$

The left-hand side of eqn. (12) is not a linear function of the substrate concentration.

When the substrate itself is an enzyme inhibitor, but the inhibitor I binds only to the free enzyime, reactions (1), (2) and (3) take place, and the corresponding equation derived like eqn. (12) is:

$$\frac{v[I]}{v_o - v} = \frac{1 + \frac{k_{+3}}{k_{+2}} + \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}k_{+2}[S]} + \frac{[S]}{K'_{ss}}}{\frac{k_{+3}(k_{-1} + k_{+2})}{K_a' k_{+1} k_{+2}[S]}} \tag{13}$$

Here again, the correlation between $v[I]/(v_o - v)$ and [S] is non-linear.

When the substrate itself in not an enzyme inhibitor, but the inhibitor I binds to the free and acetylated enzyme, reactions (1), (3) and (4) take place, and corresponding equation is:

$$\frac{v\,[\mathrm{I}]}{v_o - v} = \frac{1 + \dfrac{k_{+3}}{k_{+2}} + \dfrac{k_{+3}\,(k_{-1} + k_{+2})}{k_{+1}\,k_{+2}\,[\mathrm{S}]}}{\dfrac{k_{+3}\,(k_{-1} + k_{+2})}{K_a'\,k_{+1}\,k_{+2}\,[\mathrm{S}]} + \dfrac{1}{K'_i}} \tag{14}$$

Again, the left-hand side of the equation is a non-linear function of [S].

And finally, the simplest case is when substrate and inhibitor bind only to the free enzyme (reactions (1) and (3)). The corresponding equation equals:

$$\frac{v\,[\mathrm{I}]}{v_o - v} = \frac{\left(1 + \dfrac{k_{+3}}{k_{+2}}\right)[\mathrm{S}] + \dfrac{k_{+3}\,(k_{-1} + k_{+2})}{k_{+1}\,k_{+2}}}{\dfrac{k_{+3}\,(k_{-1} + k_{+2})}{K'_a\,k_{+1}\,k_{+2}}} \tag{15}$$

The correlation between $v[\mathrm{I}]/(v_o - v)$ and [S] is linear, and the line intersects the abscissa at $-[\mathrm{S}] = K_m$.

According to the Aldridge and Reiner theory the following reactions are possible:

$$\mathrm{E}< + \mathrm{S} \rightleftharpoons \mathrm{E}<^{\mathrm{S}} \rightarrow \mathrm{E}< + \text{products} \tag{16}$$

$$\mathrm{E}< + \mathrm{S} \rightleftharpoons \mathrm{E}<_{\mathrm{S}} \tag{17}$$

$$\mathrm{E}< + \mathrm{I} \rightleftharpoons \mathrm{E}<_{\mathrm{I}} \tag{18}$$

$$\mathrm{E}< + \mathrm{I} \rightleftharpoons \mathrm{E}<_{\mathrm{I}} \tag{19}$$

$$\mathrm{E}<^{\mathrm{S}} + \mathrm{S} \rightleftharpoons \mathrm{E}<^{\mathrm{S}}_{\mathrm{S}} \tag{20}$$

$$\mathrm{E}<^{\mathrm{S}} + \mathrm{I} \rightleftharpoons \mathrm{E}<^{\mathrm{S}}_{\mathrm{I}} \tag{21}$$

$$\mathrm{E}<_{\mathrm{S}} + \mathrm{S} \rightleftharpoons \mathrm{E}<^{\mathrm{S}}_{\mathrm{S}} \tag{22}$$

$$\mathrm{E}<_{\mathrm{S}} + \mathrm{I} \rightleftharpoons \mathrm{E}<^{\mathrm{I}}_{\mathrm{S}} \tag{23}$$

$$\mathrm{E}<^{\mathrm{I}} + \mathrm{S} \rightleftharpoons \mathrm{E}<^{\mathrm{I}}_{\mathrm{S}} \tag{24}$$

$$\mathrm{E}<^{\mathrm{I}} + \mathrm{I} \rightleftharpoons \mathrm{E}<^{\mathrm{I}}_{\mathrm{I}} \tag{25}$$

$$\mathrm{E}<_{\mathrm{I}} + \mathrm{S} \rightleftharpoons \mathrm{E}<^{\mathrm{S}}_{\mathrm{I}} \tag{26}$$

$$\mathrm{E}<_{\mathrm{I}} + \mathrm{I} \rightleftharpoons \mathrm{E}<^{\mathrm{I}}_{\mathrm{I}} \tag{27}$$

The symbol $\mathrm{E}<$ stands for the free enzyme; the upper line stands for the catalytic centre and the lower for the allosteric site. S and I are the substrate and inhibitor resp. As stated in the equations, it is assumed that the enzyme

is catalytically active only when the allosteric site is unoccupied, *i. e.* only reaction (16) yields products. It is assumed that the catalytic and allosteric site are independent as far as binding is concerned; therefore the dissociation constants of the reversible complexes are as follows:

$$K_s = \frac{[E<][S]}{[E<^{S}]} = \frac{[E<_{S}][S]}{[E<^{S}_{S}]} = \frac{[E<_{I}][S]}{[E<^{S}_{I}]} \tag{28}$$

$$K_{ss} = \frac{[E<][S]}{[E<^{S}]} = \frac{[E<_{S}][S]}{[E<^{S}_{S}]} = \frac{[E<_{I}][S]}{[E<^{I}_{S}]} \tag{29}$$

$$K_a = \frac{[E<][I]}{[E<^{I}]} = \frac{[E<_{S}][I]}{[E<^{I}_{S}]} = \frac{[E<_{I}][I]}{[E<^{I}_{I}]} \tag{30}$$

$$K_i = \frac{[E<][I]}{[E<^{I}]} = \frac{[E<_{S}][I]}{[E<^{S}_{I}]} = \frac{[E<_{I}][I]}{[E<^{I}_{I}]} \tag{31}$$

There are six theoretical possibilities in that model (*cf.* ref.[3]). When all reactions (16) to (27) take place, the corresponding equation is:

$$\frac{v\,[I]}{v_o - v} = \frac{\left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{[S]}{K_{ss}}\right)}{\frac{K_s}{[S]}\left(\frac{1}{K_a} + \frac{1}{K_i} + \frac{[I]}{K_a K_i}\right) + \frac{1}{K_i} + \frac{K_s}{K_a K_{ss}}} \tag{32}$$

At a given substrate concentration the ratio $v[I]/(v_o - v)$ is not constant, but depends on the inhibitor concentration. The ratio is a non-linear function of [S].

When the substrate reacts with the catalytic and allosteric site, and the inhibitor only with the allosteric site, the equation is:

$$\frac{v\,[I]}{v_o - v} = K_i + \frac{K_i}{K_{ss}}[S] \tag{33}$$

The left-hand side of the equation is linear with [S] and the line intersects the abscissa at $-[S] = K_{ss}$.

When the substrate reacts with the catalytic and allosteric site, and the inhibitor only with the catalytic, the equation is:

$$\frac{v\,[I]}{v_o - v} = \frac{K_a\left(1 + \frac{K_s}{[S]} + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}}\right)}{\frac{K_s}{[S]} + \frac{K_s}{K_{ss}}} \tag{34}$$

The left-hand side is not a linear function of [S].

When the substrate reacts only with the catalytic site, there are again three possiblities for the inhibitor to interact. One is with both, the catalytic and allosteric site the corresponding equation being:

$$\frac{v\,[I]}{v_o - v} = \frac{K_i\,K_a\,([S] + K_s)}{K_a\,([S] + K_s) + K_s\,([I] + K_i)} \tag{35}$$

The ratio $v[I]/(v_o - v)$ is non-linear with [S], and at a given [S], the ratio is not constant, but depends on [I].

The other possibility is that the inhibitor reacts only with the allosteric site:

$$\frac{v\,[I]}{v_o - v} = K_i \tag{36}$$

In that case the ratio is independent of the substrate concentration, *i. e.* the inhibition is non-competitive.

And finally the third possibility is for the substrate and inhibitor to react only with the catalytic site:

$$\frac{v\,[I]}{v_o - v} = K_a + \frac{K_a}{K_s}\,[S] \tag{37}$$

The left-hand side of the equation is linear with [S], and the line extrapolates to $-[S] = K_s$. When the equation is derived from steady-state kinetics, and not from equilibrium kinetics like all equations from (32) to (37), the extrapolation equals $-[S] = K_m$.

DISCUSSION

The kinetics of reversible inhibition of cholinesterase and acetylcholinesterase by haloxon and coumarin, measured with phenylacetate and acetylthiocholine as substrates, shows an apparently simple kinetics. The degree of inhibition, as expressed by the ratio $v[I]/(v_o - v)$ is a linear function of the substrate concentration (*cf.* Fig. 2), irrespective of whether the substrate itself is an enzyme inhibitor and also irrespective of the kinetics of the enzyme-substrate reaction in the absence of the inhibitor (*cf.* Fig. 1). This is not in agreement with the two theories on reversible inhibition which predict a different kinetics of inhibition when the substrate itself is an inhibitor than when it is not.

There are three reactions for which the above theories predict a linear correlation between $v[I]/(v_o - v)$ and [S] (eqn. (15) in the Krupka and Laidler model, and eqns. (33) and (37) in the Aldridge and Reiner model). Eqns. (15) and (37) should apply to phenylacetate as substrate, and the extrapolated line should intersect the abscissa at $-[S] = K_m$. In our experiments no line extrapolates to a value equal to K_m (*cf.* Table I). The third equation (eqn. 33) should apply to acetylcholinesterase as enzyme and acetylthiocholine as substrate; the equation predicts an intercept on the abscissa equal to the substrate inhibition constant K_{ss}. The experimental value of 2.3 mM (Table I) is smaller than the K_{ss} value which is 14 mM[6]. For the same enzyme (acetycholinesterase), but for phenylacetate as substrate, the theory of Aldridge and Reiner predicts a non-competitive inhibition (eqn. 36), and this was not observed experimentally (*cf.* Fig. 2).

Consequently, neither model agrees with the kinetics of the experimental results, because the reactions studied follow an apparently simpler kinetics than theoretically predicted. The allosteric model which fitted the reaction

acetylcholinesterase + haloxon/coumarin + acetylthiocholine/acetylcholine, cannot be expanded to the reaction of acetylcholinesterase + haloxon/coumarin + phenylacetate. The model also does not apply to the reaction of cholinesterase + coumarin + acetylthiocholine/phenylacetate. This disagreement excludes the kinetic postulates upon which the theoretical equations are based. However, it does not by itself exclude an allosteric mechanism, which is favoured by the evidence of binding sites outside the catalytic center.

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SAŽETAK

Kinetički studij efekta supstrata na reversibilnu inhibiciju kolinesteraze i acetilkolinesteraze dvama kumarinskim derivatima

Elsa Reiner i Vera Simeon

Istražena je reversibilna inhibicija kolinesteraze i acetilkolinesteraze kumarinom i haloksonom u nazočnosti supstrata fenilacetata i acetiltiokolina. Pokazalo se da je stupanj inhibicije, definiran izrazom $v\,[I]/(v_0 - v)$, linearna funkcija koncentracije supstrata i to bez obzira na kinetiku reakcije enzim—supstrat, inhibiciju enzima takvim supstratom, te bez obzira na to da li je inhibitor kumarin ili halokson. Kod dane koncentracije supstrata izraz $v\,[I]/(v_0 - v)$ konstantan je i neovisan o koncentraciji inhibitora. Odsječak na ordinati predstavlja konstantu disocijacije kompleksa enzim—inhibitor, K (I), a vrijednost te konstante nije ovisna o supstratu. Odsječak na apscisi K (S) karakterizira reakciju enzima i supstrata i njegova je vrijednost veća od Michaelisove konstante. Razmotrene su teorijske mogućnosti mjesta vezanja inhibitora na enzim kao i mjesta vezanja supstrata kada on inhibira enzim; izvedene su jednadžbe radi interpretacije konstante K (S), koja predstavlja vezanje inhibitora na katalitičko središte (prema teoriji Krupke i Laidlera) ili alosteričko mjesto enzima (prema teoriji Aldridge i Reiner). Niti jedna od iznesenih teorija i izvedenih jednadžbi ne odgovara eksperimentalno dobivenim rezultatima. Kinetika istraživanih reakcija pokazala se prividno jednostavnijom od teorijske.

LABORATORIJ ZA BIOKEMIJU,
INSTITUT ZA MEDICINSKA ISTRAŽIVANJA I
MEDICINU RADA
JUGOSLAVENSKE AKADEMIJE ZNANOSTI I UMJETNOSTI,
ZAGREB