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Transient Kinetic Approach to the Study of Acetylcholinesterase Reversible Inhibition by Eseroline

MARKO GOLIČNIK* and JURE STOJAN

Institute of Biochemistry, Medical Faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia

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The kinetic rate constants for interaction of (−)-eseroline-(3aS-cis)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo-[2,3-b]indol-5-ol with electric eel acetylcholinesterase (EC 3.1.1.7, acetylcholine acetylhydrolase) were measured at a low substrate concentration according to a transient kinetic approach by using a rapid experimental technique. The measurements were carried out on a stopped-flow apparatus where pre-incubated samples of enzyme with various inhibitor concentrations were diluted with a buffer solution containing the substrate. The experimental data in the form of sigmoid-shaped progress curves were analysed by applying an explicit progress curve equation that described the time dependence of product released during the reaction. The kinetic parameters were evaluated by non-linear regression treatment and the values of the corresponding constants showed approximately the equal affinities of eseroline and eserine (cf. Stojan, J. and Zorko, M. (1997) *Biochim. Biophys. Acta*, 1337, 75–84.) for binding into the active centre of the enzyme. On the other hand, the kinetic rates for association and dissociation of eseroline were two grades of magnitude higher than those of eserine. The explanation appears to be a substantionally impaired gliding of eserine into the active site gorge by the great mobility of the carbamoyl tail as well as by its numerous possible interactions with the residues lining the gorge. Additionally, a study of the dependence of the transition phase information on the inhibitor concentration was carried out using our experimental data.

Keywords: Enzyme kinetics; Progress curves; Acetylchloinesterase; Easeroline; Stopped-flow

INTRODUCTION

Eserine (physostigmine) is a natural carbamate isolated and purified from the Calabar bean. Its inhibitory action on acetylcholinesterase (AChE) was used in the treatment of glaucoma at the end of 19th century. Recently, long chain analogs of eserine have been studied as potential drugs against symptoms in Alzheimer's disease.[1,2] The three step reaction mechanism for the inhibition of AChE by eserine has been investigated for many years and the complete kinetic characterization of the interaction has recently been done by Stojan and Zorko.[3] It was found in that work that the submicromolar affinity of eserine is not so much a consequence of the fast initial complex formation but rather a consequence of its slow dissociation. A similar affinity was measured for the inhibition of AChE by eseroline[4] (Ese), a hydrolytic derivative of eserine devoided of its N-methyl carbamyl group. For this reason it seems that the interactions between the rigid tricyclic moiety of eserine and the enzyme active site are responsible for slow dissociation and consequently for high affinity of eseroline-based carbamates. According to our knowledge, the determination of the rate constants k_{on} and k_{off} for Ese had never been reported and thus the interesting question on the background of its efficient inhibition still remains. For this reason the comparison of kinetic constants for formation of non-covalent enzyme-inhibitor complexes of eserine and eseroline seemed meaningful.

There are two possible ways to determine the association and dissociation rate constants for fast equilibrating inhibitors, and both call for applying rapid experimental techniques. Direct methods are based on a system which contains only the enzyme and investigated ligand. The ligands are fluorescent probes and the rate constants are determined using a stopped-flow[5,6] or a temperature-jump[7] instrument

*Corresponding author. Tel.: +386-1-5437651. Fax: +386-1-5437641. E-mail: golicnik@ibmi.mf.uni-lj.si

Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine; DTNB, 5,5′-dithio-bis-nitrobenzoic acid; Ese, eseroline

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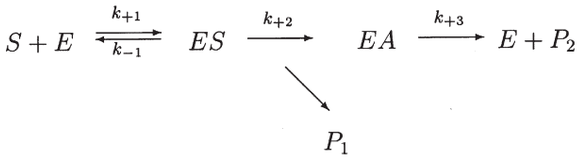
with fluorescence detection. However, such methods consume much enzyme as well as ligand. On the other hand, rapid hydrolysis of the substrate by enzymes like AChEs together with fast and powerful detection reactions, enables indirect absorbance based measurements using relatively small amounts of enzyme. The deviations in the shape of progress curves, obtained by the system containing enzyme, inhibitor, and the substrate, provide information on kinetic rates of individual processes occurring during the reaction. Such an indirect method has been examined and extensively analysed in this study.

THEORY

Electric eel acetylcholinesterase catalyses the hydrolysis of acetylcholine at one of the highest rates known.[8] It shows Michaelis–Menten like kinetic behaviour at substrate concentrations of up to 0.5 mM and negative pseudo-cooperativity above this concentration.[8] The kinetic model of the AChE reaction mechanism at low substrate concentrations has been described by the traditional reaction scheme[9]



SCHEME 1

where ES is the initial enzyme–substrate complex, EA is an acyl-enzyme intermediate, and P_1, P_2 are the two products of the reaction. Under classical steady-state conditions, the rate of hydrolysis v varies with $[S]$ according to the Michaelis–Menten equation[10]

$$v = \frac{d[P]}{dt} = \frac{V_{max}[S]}{[S] + K_{app}} \tag{1}$$

where

$$\frac{V_{max}}{[E]_T} = k_{cat} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}} \tag{2}$$

and

$$K_{app} = \frac{(k_{-1} + k_{+2})}{k_{+1}} \cdot \frac{k_{+3}}{(k_{+2} + k_{+3})} \tag{3}$$

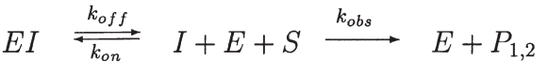
Integration of Equation (1) when K_{app} greatly exceeds $[S]$ give the following solution

$$[P]_t = [S]_0 \cdot (1 - e^{k_{obs} \cdot [E]_T \cdot t}) \tag{4}$$

where

$$k_{obs} = \frac{k_{cat}}{K_{app}} = \frac{k_{+1}k_{+2}}{k_{-1} + k_{+2}} \tag{5}$$

For acetylthiocholine, direct estimates of k_{+2} and k_{+3} for electric eel AChE, gave[11] a k_{+2}/k_{+3} ratio of 1.3. Inserting this ratio into Equation (2), where k_{cat} has been known for a long time,[8] gives an estimation of the k_{+2} constant higher than $3 \cdot 10^4\ s^{-1}$. This value also exceeds[8,12] k_{-1} and from this point of view it can be assumed that at low substrate concentrations ($[S] \ll K_{app}$), k_{obs} in Equation (4) is app. k_{+1} which means that the rate limiting step is the entry into the active center of AChE where every substrate molecule is also metabolized.[13] Following this explanation, the reaction mechanism for the inhibition of AChE by eseroline at sufficiently low substrate concentrations can be represented by Scheme (2)



SCHEME 2

where EI is an AChE-Ese reversible complex, I represents Ese, and both hydrolytic products are denoted as $P_{1,2}$. The differential equations of time-dependent quantities shown in Scheme 2 are as follows:

$$\frac{d[E]}{dt} = -k_{on} \cdot [I] \cdot [E] + k_{off} \cdot [EI]$$

$$= -k_{on} \cdot [I] \cdot [E] + k_{off} \cdot ([E]_T - [E]) \tag{6}$$

$$\frac{d[S]}{dt} = -k_{obs} \cdot [E] \cdot [S] \tag{7}$$

Equation (6) is a separable ordinary first-order linear differential equation and is therefore simply solved. It should be remembered that initial conditions are different (i) for case A where the reaction is started by the addition of enzyme into the mixture containing the substrate and the inhibitor and (ii) for case B where the enzyme is preincubated with the inhibitor and the reaction is started by the addition of substrate.

Case A

$$[E]_{t=0} = [E]_T \tag{8}$$

Case B

$$[E]_{t=0} = \frac{K_I[E]_T}{(K_I + R[I])} \tag{9}$$

where $[E]_T$ and $[I]$ are total final enzyme and inhibitor concentrations, respectively. K_I and R are the equilibrium constant (k_{off}/k_{on}) and dilution factor.

Solutions of Equation (6) for both cases are:

Case A

$$[E]_t = \frac{k_{off}[E]_T}{(k_{on}[I] + k_{off})}\left(1 + \frac{[I]}{K_I}e^{-(k_{on}[I]+k_{off})t}\right) \quad (10)$$

Case B

$$[E]_t = \frac{k_{off}[E]_T}{(k_{on}[I] + k_{off})}\left(1 + \frac{[I](1-R)}{(K_I + R[I])}e^{-(k_{on}[I]+k_{off})t}\right) \quad (11)$$

Inserting these two equations into Equation (7) gives final solutions describing the product formation during the reaction process

Case A

$$ln\left(1 - \frac{[P]_t}{[S]_0}\right) = -\frac{k_{obs}k_{off}[E]_T}{(k_{on}[I] + k_{off})} \times \left(t + \frac{[I]}{K_I(k_{on}[I] + k_{off})}(1 - e^{-(k_{on}[I]+k_{off})t})\right) \quad (12)$$

Case B

$$ln\left(1 - \frac{[P]_t}{[S]_0}\right) = -\frac{k_{obs}k_{off}[E]_T}{(k_{on}[I] + k_{off})} \times \left(t + \frac{[I](1-R)}{(K_I + R[I])(k_{on}[I] + k_{off})} \times \left(1 - e^{-(k_{on}[I]+k_{off})t}\right)\right) \quad (13)$$

For clarity, Equations (12) and (13) are given in logarithmic form. It should be emphasized that both equations hold only for classical inhibitors when free and total inhibitor concentrations are assumed to be equal. Progress curves for case A (Equation 12) are concave downward curves and often do not deviate obviously from the single exponential function (see Figure 1A). On the other hand, progress curves for case B (Equation 13) are sigmoid and thus provide more reliably the information on the early stages of the reaction. A special case is when $[I] = 0$ where progress curves described by both Equations (12) and (13) reduce to Equation (4). Information about the kinetic rates of inhibitor action on an enzyme catalysed reaction is included in the short period of time during the equilibration between the enzyme and inhibitor. Unfortunately, progress curves obtained at various inhibitor concentrations are not equally useful for the analysis. Sigmoidality is inhibitor-dependent (see Figure 1B). Consequently, an interesting question arises: what might be the criterion for the determination of the "quality" of transient phase information? In practice, the reliability of the analysis would increase if the time of the inflexion point (t^*) as well as the change of absorbance were well within the range of the detection method. Mathematically, the compromise between the two excluding demands can be achieved by maximizing a line integral L of the curvature κ_t multiplied by $[P]_t$, along the curve connecting origin ($t = 0$) and the inflexion point ($t = t^*$). The curvature of the progress curve at any point is a measure of the sigmoidality, while the amount of product formed ($[P]_t$) is responsible for efficient and reliable detection. Calculations of L can be obtained upon integration of $\kappa_t \cdot [P]_t$ along the path of integration (C):

$$L = \int_C \kappa_t \cdot [P]_t ds \quad (14)$$

where κ_t and $[P]_t$ are the curvature and the concentration of product at any time, while ds is the linear element of the curve (reference[14], see also Appendix).

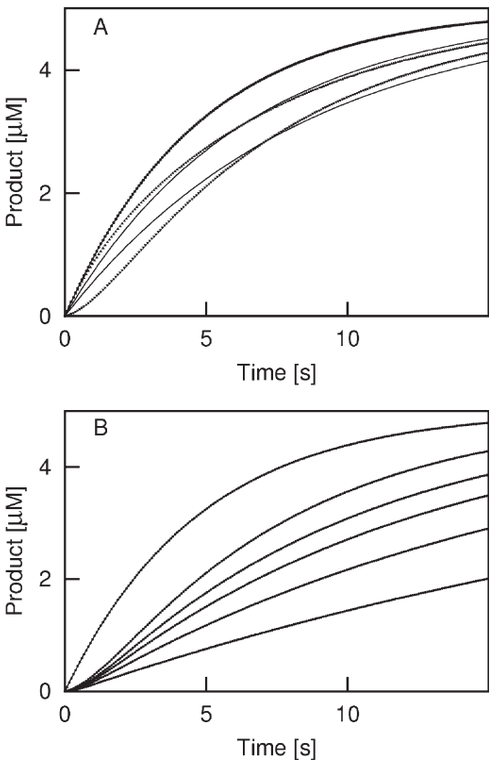


FIGURE 1 (A) Single exponential functions (lines) fitted on simulated progress curves calculated according to Equation (4), Equation (12) and Equation (13) (dots) ($E_T = 0.7\,nM$, $k_{obs} = 3{\cdot}10^8\,M^{-1}s^{-1}$, $k_{on} = 5{\cdot}10^6\,M^{-1}s^{-1}$, $k_{off} = 0.5\,s^{-1}$, $[I] = 0.5\,\mu M$). (B) Simulated progress curves calculated according to Equation (13) at various inhibitor concentrations (the same parameters as under (A) but $[I] = 0, 0.5, 1.0, 1.5, 2.5$ *and* $5.0\,\mu M$).

EXPERIMENTAL PROCEDURES

Chemicals

Acetylcholinesterase (AChE, EC 3.1.1.7) from *Electrophorus electricus*, acetylthiocholine (ATCh) and

5,5′-dithio-bis-nitrobenzoic acid (DTNB, Ellman's reagent) were purchased from Sigma Chemical Co. (St.Louis, U.S.A.). (−)-Eseroline-(3aS-cis)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo-[2,3-b]indol-5-ol (Ese) fumarate was from Research Biochemical International (Natick, U.S.A.).

Enzyme active sites concentration was independently estimated by pseudoirreversible titration, according to the procedure described previously.[3] For prevention of eseroline oxidation to the *o*-quinone derivative, rubreserine, the stock solution of the inhibitor was prepared freshly before the experiments.[4] All experiments were carried out at 25°C in a buffer solution prepared according to Britten and Robinson[15] (a mixture of 25 mM phosphoric, acetic and boric acids and 75 mM sodium hydroxide) with a pH of 8.0 and a total ionic strength of 0.2 *M*, obtained by the addition of NaCl. All reagents and salts were of analytical grade.

Kinetic Measurements

Rapid spectrophotometric measurements of acetylthiocholine hydrolysis catalysed by acetylcholinesterase were carried out on a Hi-Tech (Salisbury, UK) PQ/SF 53 stopped-flow apparatus connected to a SU-40 spectrophotometer and Apple E-II microcomputer. A theoretical dead time of 0.7 ms had been stated by the manufacturer for the mechanical part of the apparatus.

The k_{obs} value was independently determined by experiments done in the absence of Ese. Briefly, aliquots of two solutions, one containing the enzyme and the other containing ATCh and DTNB, were mixed together in the ratio 1:15 in the mixing chamber of the instrument. The absorbance of the reaction mixture was recorded according to Ellman *et al.*[16] at 412 *nm* in a sweep from zero to 20 *s* and from 20 *s* to 140 *s*. All progress curves reached the plateaus during that time. The time course of product formation was followed at various fixed final concentrations of the substrate ($1\,\mu M - 5\,\mu M$) at least 10 times lower than K_{app}.

For the determination of the kinetic constants k_{on} and k_{off}, the experiments were carried out by a similar procedure. The only difference was in the preincubation of the enzyme with various concentrations of Ese ($0.5\,\mu M - 5\,\mu M$) before mixing the solution with the substrate and DTNB. The latter measurements were done in the presence of a $5\,\mu M$ substrate concentration. Final concentrations of AChE active sites and DTNB were 0.69 *nM* and 1 *mM*, respectively.

Data Analysis

Evaluation of the corresponding kinetic parameters was carried out in two subsequent steps. (i) The determination of k_{obs} was done by fitting Equation (4) to the progress curves obtained at three different substrate concentrations in the absence of Ese. (ii) The progress curve data in the presence of Ese were used to evaluate the two rate constants k_{on} and k_{off} using Equation (15) (rearranged Equation 13, see Appendix). All computations were performed with a modified non-linear regression computer program originally written by R.G. Duggleby.[17] The values of evaluated kinetic parameters were crosschecked by a numerical integration treatment.[18]

The relationships between the kinetic constants, experimental conditions, and the quality of the information on the transient phase in each individual progress curve were also analysed. This was carried out to check the suitability of individual chosen experimental conditions ($[I]$) under which the progress curves were measured. The inhibitor concentration dependence of t^*, $[P]_{t=t^*}$ and L were calculated applying Equations (21), (15) (Appendix) and Equation (14), respectively. t^* and $[P]_{t=t^*}$ are explicit analytical functions of $[I]$, whereas the integral in Equation (14) has no general analytical solution. It was solved numerically using a *Mathematica* software package.[19]

RESULTS AND DISCUSSION

The time course of product formation in the hydrolysis of acetylthiocholine by AChE in the absence of eseroline is shown in Figure 2A. It should be emphasized that the measurements were performed at very low substrate concentrations ($1\,\mu M - 5\,\mu M$) while the concentration of DTNB was three times higher than in the original recipe for the colorimetric determination of acetylcholinesterase activity described by Ellman *et al.*[16] Under such conditions, the progress curves in the absence of inhibitor follow a single exponential function, as seen from the fitting of Equation (4) to these experimental data. On the basis of this information we can conclude the following: (i) the time course of product formation at lower substrate concentration represents the appropriate final portion of the curve obtained at the higher substrate concentration (using up to $5\,\mu M$ initial substrate); (ii) the fact that Equation (4) can be fitted to all curves in Figure 2A indicates that the intermediates *ES* and *EA* do not accumulate during the reaction with substrate concentrations of up to $5\,\mu M$. Scheme 2 is thus justified. (iii) The non-sigmoidal shape of progress curve(s) in the absence of inhibitor is evidence that the sigmoidality is introduced only by the addition of Ese and that the detection reaction does not distort the effect of the inhibitor.[20] The rate of the detection reaction is DTNB dependent and for this reason we performed the experiments at 1 *mM* DTNB

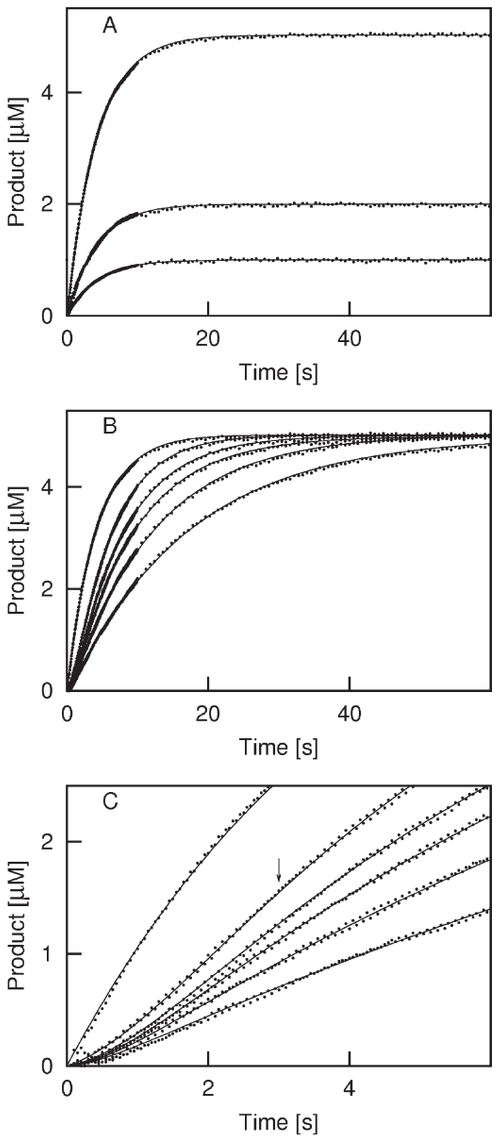


FIGURE 2 Progress curves for the hydrolysis of acetylthiocholine by electric eel AChE: (A) at various substrate concentrations ($1\,\mu M$–$5\,\mu M$) in the absence of eserolin, (B) at $5\,\mu M$ substrate concentration and various inhibitor concentrations (0,0.5,1.2,1.8,3.0 *and* $5.0\,\mu M$), (C) the same as under (B) but at lower time/product ranges.

concentrations. The value of the evaluated rate constant $k_{obs} = (3.43 \pm 0.04)\cdot 10^8\, M^{-1}\, s^{-1}$, obtained by the fitting of the data in Figure 2A to Equation (4) is in good agreement with the values found previously.[18,21,22]

Figures 2B,C show progress curves for the hydrolysis of $5\,\mu M$ ATCh in the presence of various Ese concentrations. They were all obtained after 16 fold dilution of Electric Eel AChE preincubated with 0–$5\,\mu M$ Ese. The uppermost, concave downward curve, is in fact the uppermost curve from Figure 2A without Ese, and the others were measured in the presence of increasing Ese concentrations. They are concave upwards up to the inflexion point and concave downwards after the inflexion (see also Experimental Procedures). As mentioned, the reason for the sigmoidal shape is not a slow detection reaction[20] but the rather slow approach of the enzyme-inhibitor system from an initial equilibrium to a new one. Fortunately, this re-equilibration is slow enough to be detected on a stopped-flow apparatus. The fitting of Equations (4) and (13) to all measured data yielded both characteristic rate constants from Scheme 2: an eseroline association rate constant $k_{on} = (5.17 \pm 0.03)\cdot 10^6\, M^{-1}\, s^{-1}$ and dissociation rate constant $k_{off} = (0.538 \pm 0.003)\, s^{-1}$. The good agreement of the theoretical curves with the experimental data also confirms the competition between the substrate and the inhibitor at the active site, as suggested by the model in Scheme 2. It is a reasonable conclusion supported by the molecular model of eserine docked in the active site of *Torpedo californica* AChE which shows that the bulky rings of eserine completely fill the space around the active center.[23] Nevertheless, we tried to fit our experimental data to other kinetic models proposing partial competition but they did not work.

The comparison of two rate constants with the values of the analogous constants for eserine reveals extremely interesting information: while the affinities of Electric Eel AChE for eseroline ($K_I = 0.10\,\mu M$) and eserine ($K_1 = 0.47\,\mu M$) are very similar, the rates of association and dissociation are some two grades of magnitude higher for eseroline than eserine ($k_{on}/k_{+1} = 135, k_{off}/k_{-1} = 30$). This calls for an answer to the following question: what might be the reason for the large difference in rate constants for eseroline and eserine but with only slight differences in the affinity constants for the two enzyme-inhibitor complexes? In order to put forward an adequate explanation, one must take into account two points. First, from the structural studies it is known that eseroline and carbamoyl-eseroline derivatives accomodate at the acylation site of cholinesterases;[24] additionally, the shape and the size of these ligands together with sub-micromolar affinities appear to be indirect evidence. Second, the very low ligand concentrations used in our experiments appear to exclude multiple binding in spite of relatively high ligand/enzyme ratios. All these would undermine the steric hindrance hypothesis similar to the mechanistic interpretation of AChE inhibition by peripheral site ligands suggested by Szegletes *et al.*[22] On the contrary, it seems that a small N-methyl-carbamyl group plays an important role in the gliding of eserine through the AChE's active site gorge and is only a minor obstacle in the aquisition of the Michaelis-Menten complex formation.[25,26] An only slightly decreased affinity in the case of eserine points to a minimal difference between the transition-state like binding pattern of eseroline and

the Michaelis–Menten complex in the case of eserine. On the other hand, gliding is substantially impaired by the great mobility of the carbamoyl tail (unpublished quantum mechanical computation data) as well as by its numerous possible interactions with the residues lining the gorge.

Finally, we would like to discuss the reliability of the evaluated kinetic parameters. Our analysis demonstrates that progress curve measurements at optimal conditions provide useful information on the transient phase, from which kinetic parameters can be evaluated. One of the most easily changed variables is the inhibitor concentration and it should therefore be chosen very carefully. Figure 3 shows various possible approaches for the optimization of the experimental design using the dependence of parameters t^*, $[P]_{t=t^*}$ and the line integral L (see Theory and Appendix) on the inhibitor concentration as possible criteria. Each of them might be the criterion for the reliability of progress curves in particular situations. It is obvious from Figure 3 that in the case of Electric Eel AChE and Ese, the normalized parameter t^* is poorly inhibitor concentration dependent. On the other hand, the normalized parameters $[P]_{t=t^*}$ and L possess more expressive peaks. These peaks are optima which indicate at which inhibitor concentration the progress curves have the most useful information of the transient phase. Indeed, the parameter L is more appropriate than $[P]_{t=t^*}$ but unfortunately cannot be expressed as an analytical mathematical function. The arrows in Figure 3 show the inhibitor concentrations performed in our study. It can be seen that the lowest inhibitor concentration used is also the optimal one. The same can also be observed by inspecting diagram C in Figure 2 where the progress curve marked by the arrow displays the most pronounced sigmoidal shape and consequently gives the deepest insight into the structure of the data.[27]

Finally, we must emphasize once again that our analysis is valid only for classical inhibitors ($[I]_t = [I]_T$) and non-cooperative substrates. If these basic assumptions are violated, as with very potent inhibitors, a different treatment must be applied.[28] For this reason the vertical line in Figure 3 indicates the inhibitor concentration where the ratio $[I]/[E]$ is 10. Above this ratio the inhibitor can be treated as classical.

The introduced method for determining kinetic rates of eseroline action on AChE seems perhaps complex in view of data analysis but it does retain some advantages in comparison to other direct methods that call for much higher enzyme concentrations.

Acknowledgements

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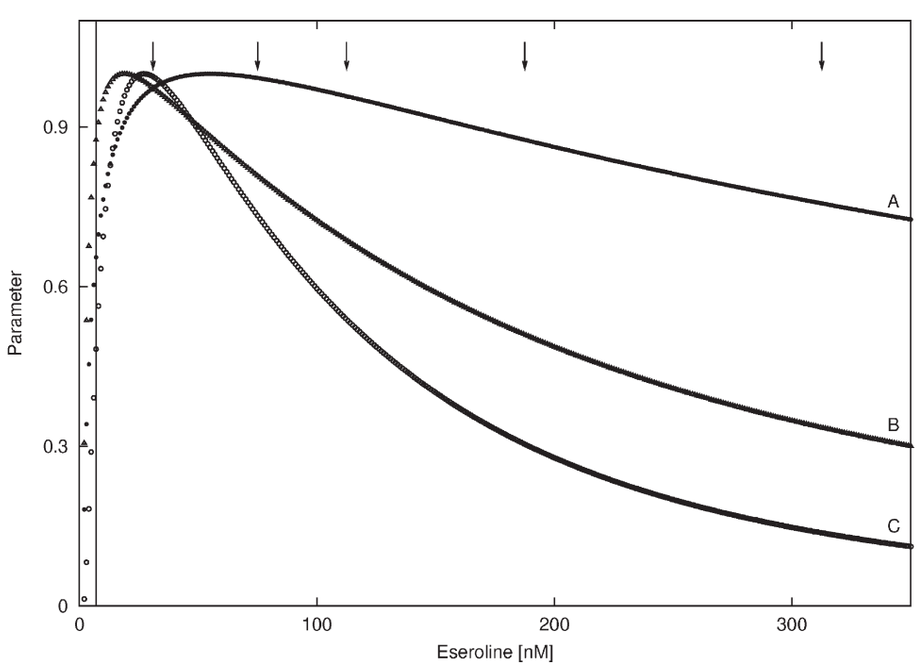

FIGURE 3 Inhibitor concentration dependence of normalized parameters (A) t^*/t^*_{max}, (B) $[P]_{t=t^*}/[P]_{t=t^*(max)}$ and (C) L/L_{max}. The arrows show inhibitor concentrations used in experiments. The vertical line inside the graph marks where the concentration equals $10{\cdot}[E]_T$.

APPENDIX

After the rearrangement of Equation (13), the following equation describes the time course of product formation:

$$[P]_t = [S]_0\left(1 - e^{-\frac{\alpha}{\omega}\left(t+\frac{\beta}{\omega}(1-e^{-\omega t})\right)}\right) \tag{15}$$

where

$$\alpha = k_{obs}k_{off}[E]_T \tag{16}$$

$$\beta = \frac{[I](1-R)}{(K_I + R[I])} \tag{17}$$

$$\omega = k_{on}[I] + k_{off} \tag{18}$$

It should be remembered again that Equation (15) holds only when the inhibitor acts by classical means.

The second derivative of function $[P]_t$ must be zero at the inflexion point. A rearranged equation $[P]''_t = 0$ gives the quadratic equation

$$\frac{\alpha^2}{\omega^2}(1+\beta x)^2 + \alpha\beta x = 0 \tag{19}$$

where $x = e^{-\omega t^*}$ and t^* is time at which the inflexion point occurs. Equation (19) gives two solutions but t^* is positive:

$$e^{-\omega t^*} = \frac{-(2\alpha+\omega^2) + \omega\sqrt{4\alpha+\omega^2}}{2\alpha\beta} \tag{20}$$

or

$$t^* = -\frac{ln\left(\frac{-(2\alpha+\omega^2)+\omega\sqrt{4\alpha+\omega^2}}{2\alpha\beta}\right)}{\omega} \tag{21}$$

The line integral given in Equation (14) can be written as

$$L = \int_0^{t^*} \kappa_t \cdot [P]_t \cdot \sqrt{1 + [P]_t'^2}\, dt \tag{22}$$

where κ_t is defined as

$$\kappa_t = \frac{[P]''_t}{(1 + [P]_t'^2)^{\frac{3}{2}}} \tag{23}$$

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