J. Enzyme Inhibition, 1997, Vol. 11, pp. 199–208
Reprints available directly from the publisher
Photocopying permitted by license only

© 1997 OPA (Overseas Publishers Association)
Amsterdam B.V. Published in The Netherlands
by Harwood Academic Publishers
Printed in Malaysia

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

MECHANISM OF ESERINE ACTION ON THE HYDROLYSIS OF BUTYRYLTHIOCHOLINE BY BUTYRYLCHOLINESTERASE

JURE STOJAN* and MILOŠ R. PAVLIČ

Institute of Biochemistry, Medical Faculty,
Vrazov trg 2, 1105 Ljubljana, Slovenia

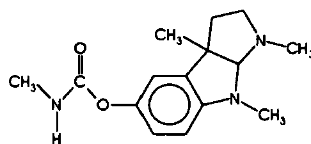
(Received 10 April 1996; in final form 15 July 1996)

The mechanism of the interaction of eserine with butyrylcholinesterase has been proposed only on the basis of analogy with acetylcholinesterase. Here the interactions was studied in detail and the results analysed by classical kinetic methods and by means of mathematical modelling. An appropriate kinetic scheme was developed, an adequate equation derived and the corresponding kinetic parameters evaluated. The findings suggest that a fast but relatively weak binding of eserine to the enzyme's active site is followed by a slow acylation step and by an even slower rate limiting deacylation step so misrepresenting eserine as an irreversible inhibitor. The proposed kinetic scheme also suggests that the reaction of eserine with a peripheral substrate site is unlikely as seen with the substrate, butyrylthiocholine.

Keywords: Butyrylcholinesterase; eserine; pre-steady state; stopped-flow; modelling.

INTRODUCTION

Eserine, primarily known as a potent competitive inhibitor of cholinesterases has also been considered a poor substrate of these enzymes and its interaction with cholinesterases was shown to obey classic Michaelis-Menten kinetics.[1–3]



Eserine

*Correspondence.

RIGHTSLINK

The rates of complex formation, acylation and deacylation of the enzyme however, are such that relevant kinetic data cannot be exactly determined by standard dilution experiments in which the remaining activity of cholinesterases, after various preincubation times with eserine, is determined by following the hydrolysis of a suitable substrate. In such dilution experiments, the required concentrations of eserine due to its apparently high affinity for the enzyme are so low that the depletion of eserine must also be considered. Such a mechanism which includes the depletion of not only the enzyme but also of the ligand is described by the system of non-linear differential equations for which no explicit solution could be obtained.[4] The depletion of eserine can be avoided, however, by using higher eserine concentrations. This leads to higher reaction rates, but by using a stopped-flow apparatus these high reaction rates can easily be followed in the presence of a suitable substrate.

The interaction of eserine with acetylcholinesterase has been elucidated and also quantitatively characterized to some extent[3,5] by classical dilution methods. The interaction with butyrylcholinesterase on the other hand has been proposed only on the basis of analogy between the two enzymes. The present paper is concerned with examining the interaction of eserine with butyrylcholinesterase.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

METHODS AND MATERIALS

The hydrolysis of butyrylthiocholine catalysed by butyrylcholinesterase was recorded spectrophotometrically, in the absence and presence of eserine, in a stopped-flow apparatus. Aliquots of two solutions, one containing the enzyme and the other containing the substrate, eserine and Ellman's reagent were mixed together in the mixing chamber of the apparatus. The absorbance of the reaction mixture was recorded according to Ellman *et al.*[6] in time-ranges from zero to 20 s and from zero to 1000 s. The time course of the product formation was followed, at various fixed concentrations of substrate (20 μm–5 mM) and at varied concentrations of eserine (0.2 μm–80 μm). This wide concentration ranges were chosen in order not to overlook a possible interaction of eserine with some other site on the enzyme, distinct from the catalytic site.

Since the progress curves in the presence of eserine reveal a concave downwards pattern with sloped asymptotes the general equation for a slow establishment of the steady-state between the enzyme and a slow binding inhibitor in the presence of substrate was applied:[7,8]

$$P_t = v_s t - \frac{[v_s - v_o]}{k} * \left[1 - e^{-kt}\right] \quad (1)$$

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

where P_t is the product at any time (t) , v_o the initial velocity, v_s the steady-state velocity and k the first-order rate constant for the transition from the initial velocity to the steady-state velocity.

This equation was fitted to the experimental data by means of a non-linear regression program developed by Duggleby.[9]

At high concentrations of eserine and low concentrations of the substrate, another type of progress curves was obtained: concave downwards curves with asymptotes parallel to the x-axis. Equation (1) is valid also for this kind of curves, with $v_s = 0$.

The progress curves obtained in the absence of eserine were straight lines; they were analysed on the basis of the model for the hydrolysis of butyrylthiocholine catalysed by butyrylcholinesterase at the catalytic site with substrate activation from a peripheral activation site.[8,10] In this case, $v_s = v_o$, and therefore Equation (1) becomes $P_t = v_o t$.

The fitting of Equation (1) to all experimentally determined progress curves resulted in three sets of values: one for v_o, one for v_s and the third one for k which were analysed as described previously.[8] The corresponding multistep analysis being dependent on the results of each particular subsequent step is elaborated in detail later (see Results and Discussion). On the basis of all these data, along with previously known facts,[1–3,5] the most adequate model for the inhibition of butyrylcholinesterase by eserine in the presence of the substrate was constructed. For this model, the equations expressing the significance of v_o, v_s and k in Equation (1) were derived. Finally, Equation (1), containing these derived expressions, was fitted to the experimental data. In this way, all relevant kinetic parameters for the model were determined.

Experiments were done at 25°C in 23.5 mM borax-HCl buffer solution (pH = 8.0), with total ionic strength of 0.2 M, obtained by addition of NaCl. The enzyme used was butyrylcholinesterase from horse serum, purchased from Worthington (9.3 U/mg); the concentration of the active sites in the reaction vessel, as estimated from the catalytic site activity (50000 min^{-1}, 1) and the specific activity (9.3 U/mg) was 2 nM. Butyrylthiocholine iodide, Ellman's reagent (5,5′-dithio-bis-nitro-benzoic acid) and eserine were purchased from BDH Biochemicals. All other materials were of reagent grade.

RESULTS AND DISCUSSION

Experiments in the absence and presence of eserine within the time ranges from 0 to 20 s and from 0 to 1000 s resulted, for each time range, in six sets of progress curves for six various concentrations of the substrate, each set containing the data in the absence of the inhibitor and the data for nine concentrations of eserine.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

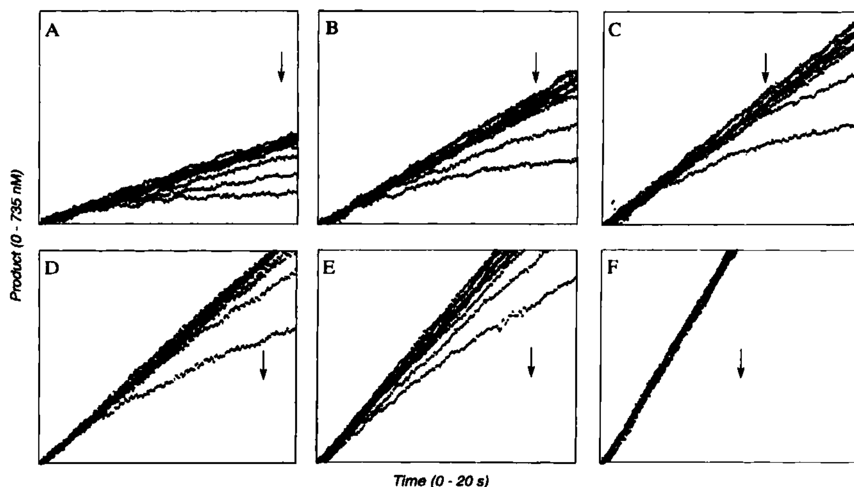


FIGURE 1 Progress curves for the hydrolysis of butyrylthiocholine catalysed by butyrylcholinesterase in the absence and presence of eserine. x-axis represents the time from 0 to 20 s and y-axis the concentration of product from zero to 735 nM (corresponding to the absorbance difference of 0.01 units). The final concentrations of eserine were: 0 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1 μM, 3 μM, 5 μM, 10 μM, 30 μM and 80 μM. The concentrations of butyrylthiocholine were A (50 μM), B (0.125 mM), C (0.25 mM), D (0.5 mM), E (1 mM) and in F (5 mM). The arrows in each picture show the direction of increase of eserine concentration. The drawings on the figure are reproductions from the original hardcopies performed by a stopped-flow apparatus.

Figure 1 shows the progress curves in the time range from 0 to 20 s for all six sets. It can be seen from this figure that curves representing the process in the presence of eserine lie under the corresponding curve in its absence. This indicates the existence of a fast phase in the inhibition of butyrylcholinesterase by eserine. Besides, with increasing concentrations of the substrate the initial velocities in the curves obtained in the presence of eserine gradually approach the lines obtained in its absence and finally become indistinguishable, suggesting a complete displacement of eserine by the substrate and so a competitive type of inhibition in this fast phase.

There is another clearly visible characteristic as seen in Figure 1: all curved lines are concave downwards. This finding indicates the beginning of another inhibition phase, a slow one.

Indeed, when the reaction process is followed at longer time intervals the slow phase becomes evident. Such a set of progress curves in the time range of 0 to 1000 s is shown in Figure 2, from which it can be seen that the curves, after a certain time period, become straight lines. The shapes of the progress curves in Figure 2 can be classified into three types: straight lines in the absence of eserine (the uppermost

RIGHTSLINK

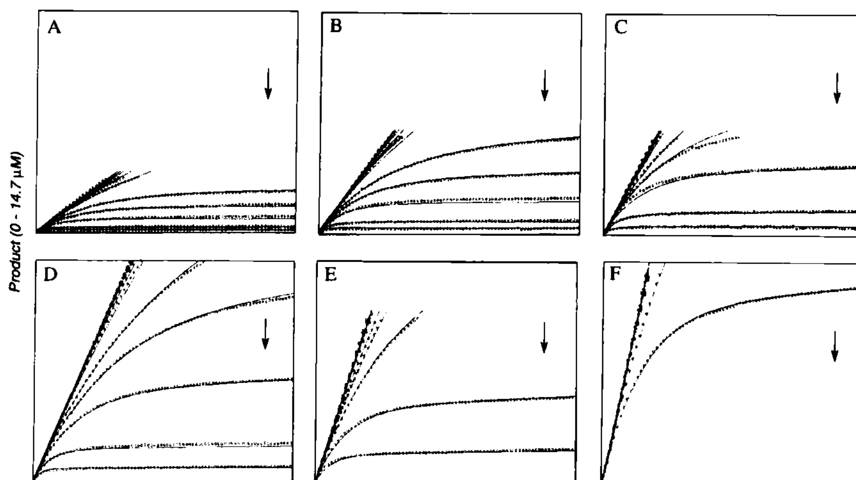


FIGURE 2 Progress curves for the hydrolysis of butyrylthiocholine catalysed by butyrylcholinesterase in the absence and presence of eserine. x-axis represents the time from 0 to 1000 s and y-axis the concentration of product from 0 to 14.7 μM (corresponding to the absorbance difference of 0.2 units). The final concentrations of eserine were: 0 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1 μM, 3 μM, 5 μM, 10 μM, 30 μM and 80 μM. The concentrations of butyrylthiocholine were: A (50 μM), B (0.125 mM), C (0.25 mM), D (0.5 mM), E (1 mM) and in F (5 mM). The arrows in each picture show the direction of increase of eserine concentration. Dotted curves in each picture are reproductions from the original hardcopies performed by a stopped-flow apparatus; solid curves are theoretical, representing Equation (1) in which the parameters v_o, v_s and k were substituted with Equations (2–4) respectively, and the corresponding constants taken from Table I. (For details see text).

curves in each drawing), concave downward curves with sloped asymptotes, and concave downwards curves with asymptotes parallel to the x-axis. The straight progress curves in the case without eserine are expected, since it is known that the formation of steady-state in the reaction between butyrylcholinesterase and butyrylthiocholine is too fast to be recorded on a stopped-flow apparatus.[8] In the concave downward progress curves the curvature represents a pre-steady-state while the slope of the asymptotes reflects the steady-state of the slow phase.[7] The existence of the curves with sloped asymptotes reflects the catalytic decomposition of eserine. On the other hand, the appearance of the curves with asymptotes parallel to the x-axis indicates a long lasting complete saturation of the enzyme with eserine at relatively high concentration of the inhibitor.

In order to quantify these findings, Equation (1) was fitted to the experimental data in the progress curves from Figure 2 (dotted curves). The results are given in Figure 3 which shows the dependence of the three parameters in Equation (1) (v_o, v_s and k) on the concentration of eserine at various concentrations of the substrate.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

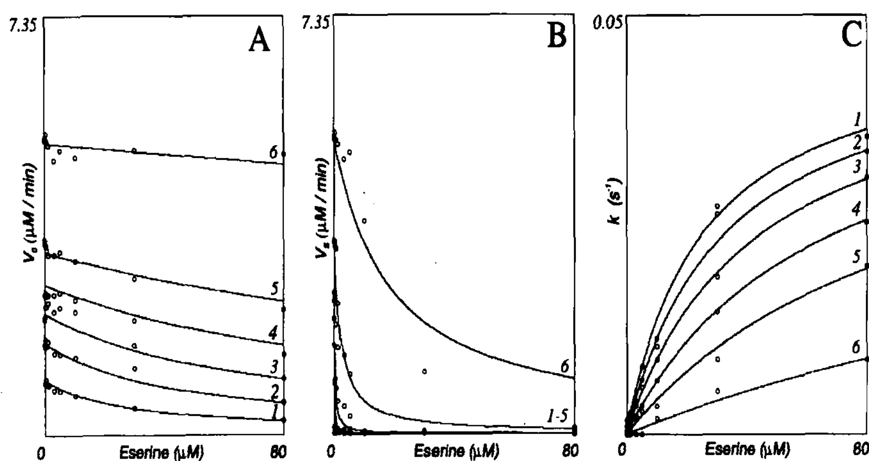


FIGURE 3 Dependence of the parameters v_o (A), v_s (B) and k (C) on the concentration of eserine at various concentrations of butyrylthiocholine: 1 (50 μM), 2 (0.125 mM), 3 (0.25 mM), 4 (0.5 mM), 5 (1 mM), 6 (5 mM). The points represent the values of the three parameters, obtained by fitting each individual progress curve in Figure 2 to Equation (1). The curves in each picture are theoretical; they were calculated. The curves from Equations (2–4) using the corresponding constants in Table I.

Referring to the fast inhibition phase (Figure 3A) a competition between butyrylthiocholine and eserine cannot be recognized as expected,[3] since none of the curves falls to zero. Experiments at higher eserine concentrations could provide a clearer picture but, unfortunately, the maximum solubility of eserine was reached in the experiments described here. However, the double reciprocal plots constructed from the same data are indicative (Figure 4). In accordance with the mechanism of substrate activation the plots are non-linear, but it can be seen that at high substrate concentrations the experimental points converge at one point on the y-axis and that at low substrate concentrations the extrapolations also give lines intersecting on the y-axis. This corresponds to a competitive type of inhibition in this fast phase.[8,10]

The steady-state velocity (v_S) is dependent on the concentration of eserine at various substrate concentrations since it can be seen that with increasing concentration of inhibitor the enzyme activity decreases to zero at all tested substrate concentrations (Figure 3B). This indicates a complete displacement of the substrate by eserine and suggests a competition between the two ligands at steady-state conditions.[8]

The diagnostic plots[11] (Figure 3C), shows concave downward dependence of the overall first order rate constant (k) on the concentration of eserine. Such a

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

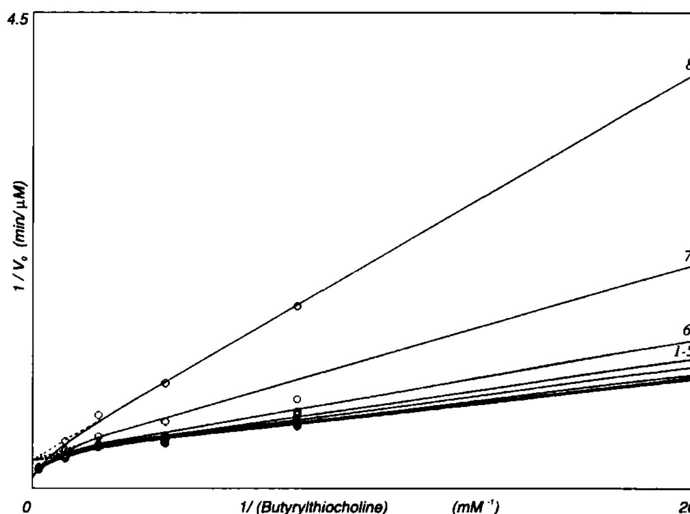
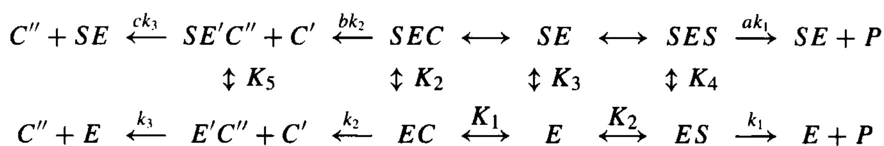


FIGURE 4 Double reciprocal plots for the instantaneous phase of the inhibition of cholinesterase by eserine. v_o represents the initial velocity of the product formation. The concentrations of eserine were 1 (0 μM), 2 (0.5 μM), 3 (1 μM), 4 (3 μM), 5 (5 μM), 6 (10 μM), 7 (30 μM) and 8 (80 μM). The curves in the drawing are theoretical; they were calculated from Equation (2) using the corresponding constants in Table I. (For details see text).

dependence is expected with the mechanisms where saturation of a ligand binding sites occurs.[12]

All these results can be associated into a mechanistic model which describes the investigated reaction between butyrylcholinesterase and eserine in the presence of substrate:[1,3]



$$
\begin{array}{ccccccccccccc}
C'' + SE & \xleftarrow{ck_3} & SE'C'' + C' & \xleftarrow{bk_2} & SEC & \longleftrightarrow & SE & \longleftrightarrow & SES & \xrightarrow{ak_1} & SE + P \\
 & & \updownarrow K_5 & & \updownarrow K_2 & & \updownarrow K_3 & & \updownarrow K_4 & & \\
C'' + E & \xleftarrow{k_3} & E'C'' + C' & \xleftarrow{k_2} & EC & \overset{K_1}{\longleftrightarrow} & E & \overset{K_2}{\longleftrightarrow} & ES & \xrightarrow{k_1} & E + P
\end{array}
$$

SCHEME 1

The symbols in this scheme represent: E the enzyme, ES the complex between the enzyme active site and the substrate, SE the complex between the enzyme peripheral site and the substrate, SES is the substrate-enzyme-substrate complex, P the

RIGHTSLINK

product, C eserine, EC the complex between the enzyme active site and eserine, EC' the acylated i.e. the carbamoylated enzyme, SEC the substrate-enzyme-eserine complex, SEC' the substrate-carbamoylated enzyme complex and C', C'' the hydrolytic products of eserine. K_s, K_1 to K_5 are the corresponding dissociation constants and k_1 to k_3 the corresponding rate constants; a, b and c are proportionality factors.

For this model, the quasi-equilibrium assumption for instantaneously forming complexes,[4,5,9,11,12] gives the following significance to the initial velocity, v_o, in Equation (1):

$$v_o = \frac{V_{\max} * (S) * \left[1 + a * \frac{(S)}{K_4}\right]}{K_s * \left[1 + \frac{(S)}{K_3} + \frac{(C)}{K_1} + \frac{(S)*(C)}{K_1 * K_2}\right] + (S) * \left[1 + \frac{(S)}{K_4}\right]} \tag{2}$$

where the symbols have the same meaning as explained above.

The combined quasi-equilibrium and steady-state assumptions[5,9,11,12] give the following expression for the significance of the steady-state velocity, v_s:

$$v_s = v_o * \frac{k_3\left[1 + c * \frac{(S)}{K_5}\right]}{k * \left[1 + \frac{(S)}{K_5}\right]} \tag{3}$$

In this equation, k represents the overall first order rate constant for the transition from the initial velocity to the steady-state velocity; its significance, is represented by the following equation (cf. references 5,11,12):

$$k = \frac{k_2 * (C) * \left[1 + b * \frac{(S)}{K_2}\right]}{K_1\left[1 + \frac{(S)}{K_s} + \frac{(S)}{K_3} + \frac{(S)^2}{K_s * K_4}\right] + (C) * \left[1 + \frac{(S)}{K_2}\right]} + \frac{k_3 * \left[1 + c * \frac{(S)}{K_5}\right]}{1 + \frac{(S)}{K_5}} \tag{4}$$

Substituting these three expressions (Equations (2–4)) for the parameters v_o, v_s and k in Equation (1), this equation becomes the specific equation for the model in Scheme 1. It describes the time course of product formation in the reaction between butyrylcholinesterase and butyrylthiocholine in the absence and presence of eserine.

If the model is appropriate, the specific equation with the corresponding kinetic constants and independent variables should be able to describe all progress curves in Figure 2 simultaneously. Therefore, the parameters of the specific equation were fitted to the data for each of the six sets of progress curves in Figure 2. Since there were eleven kinetic parameters to be determined, rather good first estimates were needed. So, the determination of the first estimates were performed in three

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

TABLE I Characteristics constants from the action of eserine on butyrylcholinesterase in the presence of butyrylthiocholine according to Scheme 1.

K_s	0.196 ± 0.012	mM
K_1	17.5 ± 1.9	μM
K_2	2.22 ± 0.66	mM
K_3	0.143 ± 0.03	mM
K_4	1.45 ± 0.25	mM
K_5	0.29 ± 0.15	mM
k_2	0.0482 ± 0.0057	1/s
k_3	0.000119 ± 0.000043	1/s
a	1.12 ± 0.07	
b	1.57 ± 0.35	
c	1.13 ± 0.20	

The values in the table are the means of the six representations for each constant, obtained by direct fitting of each of the six sets of progress curves in Figure 2 to Equation (1) which the parameters v_o, v_s and k were substituted with Equation (2–4), respectively. (For details see Results and Discussion).

consecutive steps, using previously determined kinetic constants as non-fitting parameters in the subsequent step:[8] the three equations for the dependence of the parameters v_o, v_s and k on the concentration of eserine (Equations (2–4)) were fitted to the corresponding points in Figure 3A–C.

The result of the complete procedure is presented in Table I, and in Figures 2–4 in the form of theoretical curves through experimental points. A comparison of the curves with the corresponding points along with small values of the standard deviations of all constants in Table I strongly indicate the consistency of the constructed model with performed experiments.

One could argue at this point that an interaction of eserine with a peripheral binding site on the enzyme is possible. Such an interaction cannot be ruled out, but is evidently not operative in our experiments, although they were performed with a very wide concentration range. Moreover, the simultaneous fit of the specific equation for the constructed model to all experimental data, and the agreement of the theoretical curves with these data, provides another evidence of the adequacy of the proposed model.[13]

Acknowledgements

This work was supported by Ministry of Science and Technology of the Republic Slovenia. The authors are indebted to Mrs. Nevenka Klenovšek-Špat for her valuable technical assistance.

RIGHTSLINK

References

[1] Usdin, E. (1970). In *Anticholinesterase Agents*, (Karcsmar, A.G.,(ed.)), pp. 63–185. Oxford: Pergamon Press.
[2] Main, A.R. (1976). In *Biology of Cholinergic Function*, (Goldberg, A.M. and Hanin, I. (eds.)), pp. 249–351.
[3] Wilson, I.B., Hatch, M.A. and Ginsburg, S. (1960). *J. Biol. Chem.*, **235**(8), 2312–2315.
[4] Williams, J.W., Morrison, J.F. and Duggleby, R.G. (1979). *Biochemistry*, **18**(12), 2567–2573.
[5] Marta, M., Gatta, F. and Pomponi, M. (1992). *Biochem, Biophys. Acta*, **1120**, 262–266.
[6] Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961). *Biochem. Pharmacol.*, **7**, 88–95.
[7] Morrison, J.F. and Walsh, C.T. (1988). *Adv. Enzymol.*, **61**, 201–301.
[8] Stojan, J. and Pavlič, M.R. (1991). *Biochim. Biophys. Acta*, **1079**, 96–102.
[9] Duggleby, R.G. (1984). *Comp. Biol. Med.*, **14**(4), 447–455.
[10] Cauet, G., Friboulet, A. and Thomas, D. (1987). *Biochem. Cell Biol.*, **65**, 529–535.
[11] Cha, S. (1968). *J. Biol. Chem.*, **243**(4), 820–825.
[12] Cha, S. (1975). *Biochem. Pharmacol.*, **24**, 2177–2185.
[13] Senear, D.F. and Bolen, D.W. (1995). *Meth. Enzymol.*, **210**, 463–485.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/17/11
For personal use only.

RIGHTSLINK