KINETICS OF THE INHIBITION OF ACETYLCHOLINESTERASE FROM DESERT COBRA (*WALTERINNESIA AEGYPTIA*) VENOM BY LOCAL ANESTHETICS: PROCAINE AND TETRACAINE

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The kinetic parameters of *W. aegyptia* venom acetylcholinesterase (AChE) inhibition by procaine and tetracaine hydrochloride were investigated in the present study. Procaine and tetracaine reversibly inhibited the AChE activity in a concentration-dependent manner, the IC_{50} being about 0.28 and 0.04 mM, respectively. The Michaelis-Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide was found to be 0.051 mM with V_{max} 10.2 μmole/min/mg protein. Both K_m and V_{max} were affected by procaine while only V_{max} decreased with tetracaine. A Lineweaver-Burk plot and its secondary replot indicated that the nature of the inhibition is of the linear mixed type for procaine which is considered to be a mixture of competitive and noncompetitive types while the inhibition was noncompetitive for tetracaine. The values of $K_{i(slope)}$ and $K_{I(intercept)}$ were estimated as 0.133 mM and 0.451 mM for procaine and 7.2×10^{-3} mM for tetracaine, respectively, by the secondary replots of the Lineweaver-Burk plot.

Keywords: Acetylcholinesterase; procaine; tetracaine; kinetics; inhibition; venom.
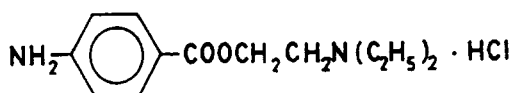
INTRODUCTION

In the central nervous system (CNS), acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7; AChE) is a pivotal enzyme responsible for the final stages of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (AChE).[1] It is also found in non-neuronal cells such as erythrocytes, lymphocytes, platelets, placenta and snake venom[2-6] of various species where
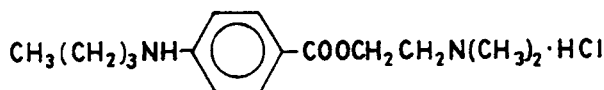
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Abbreviations: AChE – acetylcholinesterase; ASCh – acetylthiocholine; DTNB – 5,5'-dithiobis (2-nitro) benzoic acid.

its biological function is not clear. Snake venoms provide the only known source of the enzyme, other than the red cell, that does not require destruction of the source.[7] AChE from elapid venoms has very similar biochemical properties to its counterpart from membranous sources, including its stereo-specificity and substrate inhibition.[8–10] Recently, studies of the AChE in *W. aegyptia* in our laboratory have shown that AChE is present in large quantity in this source[6,11] and possesses structural as well as biochemical properties which are similar to those of the membranous enzyme;[12–14] these include the presence of isoforms with identical molecular weights and the nature of the inhibition by specific inhibitors. Effects of a number of tertiary amine local anesthetics on AChE activity from rabbit muscle and human serum,[15] rat brain synaptosome and erythrocyte,[16] eel electroplax and snake venom[10] as well as other hydrolyzing enzymes i.e. $Na^+.K^+$-ATPase and Mg^{2+}-ATPase[17] have been reported.

Within this framework, we studied the effects of two important local anesthetics drugs namely: procaine and tetracaine (Figure 1) on the activity of *W. aegyptia* venom AChE, investigating in particular the nature of the inhibition by these two drugs. This source was chosen because it has not been studied before except in our previous two reports.[6,11] However, in Saudi Arabia the desert cobra is a major problem, because it is distributed widely and its bites are frequently fatal.[18] In a previous report, we showed that the venom of the desert cobra, *W. aegyptia* contains a mixture of proteins, some of a lethal nature.[19]

$$NH_2 - \bigodot - COOCH_2CH_2N(C_2H_5)_2 \cdot HCl$$

procaine hydrochloride

$$CH_3(CH_2)_3NH - \bigodot - COOCH_2CH_2N(CH_3)_2 \cdot HCl$$

tetracaine hydrochloride

FIGURE 1 Chemical structures of the local anesthetics used in this study.

MATERIALS AND METHODS

Materials

Acetylthiocholine iodide, (ASCh), 5,5′-dithiobis-(2-nitro) benzoic acid (DTNB) and tetracaine hydrochloride were purchased from Sigma. Bovine serum albumin and procaine hydrochloride were obtained from Fluka (Switzerland). All other chemicals used were of analytical reagent grade.

Venom Preparation

W. aegyptia were captured in the desert of Saudi Arabia. They were kept in an animal house, maintained at $28 \pm 2°C$ and equipped with a diurnal cycle system of 17 h dark and 7 h of light. They were fed with one mouse (20–30 g) per snake every two weeks and water *ad libitum*. Venom from 3 desert Cobras (85 mg of protein/300 μl of venom fluid) was collected and dialyzed overnight in 50 mM NaCl. The venom was centrifuged at $5000 \times g$ for 15 min to remove any turbidity and before use diluted with sodium phosphate buffer,[6] pH 7.75.

Assay of AChE Activity

The AChE activity was determined according to the spectrophotometric method of Ellman *et al.*[20] at 25°C. The assay mixture (3 ml) contained 0.05 M sodium phosphate buffer (pH 7.75) 0.5 mM ASCh, 0.25 mM DTNB and 4 μg venom protein as the source of the enzyme. A blank was also run i.e. without enzyme (venom) to check the stability of ASCh and DTNB. One unit of enzyme activity is defined as the amount of the AChE that catalyzes the hydrolysis of 1 μmol of ASCh per min per mg of venom protein under standard conditions.

Estimation of IC$_{50}$

The transformed data $\log v/v_o - v$ (where v_o = velocity in the absence of procaine and tetracaine and v in the presence of procaine or tetracaine) versus log (procaine or tetracaine) were plotted for the determination of IC$_{50}$.[21]

Estimation of Kinetic Parameters

The Michaelis constant (K_m) was determined by means of a Lineweaver-Burk plot,[22] using initial velocities obtained over a substrate concentration range from

25–150 μM. The assay conditions for determining the residual activities in the presence of procaine and tetracaine were identical to the above assay procedure, except that a fixed concentration of procaine and tetracaine i.e. 0.05–0.40 mM and 0.01–0.04 mM respectively were used in the assay medium. The enzyme was preincubated with procaine or tetracaine for 5 min prior to addition of substrate.

Estimation of Protein

The protein content of the enzyme preparation was estimated according to the method of Lowry et al.,[23] using bovine serum albumin as standard.

Statistical Analysis

The graphs were plotted by using Grafitter program,[24] performed on a PC-486 Compaq; prolinea-466. The values of the correlation coefficient, slope and intercept were obtained by the linear regression analysis of the said program.

RESULTS

The transformed data for the inhibition of W. aegyptia venom AChE as a function of procaine and tetracaine concentration is presented in Figure 2 (a & b). The results show that procaine (0.0625–3.0 mM) and tetracaine (0.0125–0.3 mM) inhibits the AChE activity (9.7–83.26%) and (16.8–85.39%) in a concentration-dependent manner, with IC_{50} values of about 0.277 ± 0.015 mM and 0.037 ± 0.002 mM, respectively.

The data in Table Ia shows that procaine increases K_m with decreasing V_{max}. The nature of the inhibition was of the linear mixed type in this case. This mixed type system is considered to be a mixture of competitive and noncompetitive inhibitions. The K_m value of the W. aegyptia venom AChE was found to be 0.0509 ± 0.0018 mM from the Lineweaver-Burk plot. The value of K'_m calculated from the plot in Figure 3(a) was found as 0.196 ± 0.005 mM. K'_m represents the dissociation constant of AChE-ASCh-procaine complex into the AChE-procaine complex and ASCh. The inhibition constant K_i was estimated by secondary replots (slopes (Figure 3c) and intercepts ($1/V_{max}$), (Figure 3b) versus procaine concentrations) of data from the primary Lineweaver-Burk plot (Figure 3a).[25] The respective values were 0.133 ± 0.004 mM and 0.451 ± 0.014 mM.

The inhibition of W. aegyptia venom AChE activity by tetracaine was found to be non-competitive (Figure 4). Tetracaine decreased V_{max} (33.48–79.42%) without
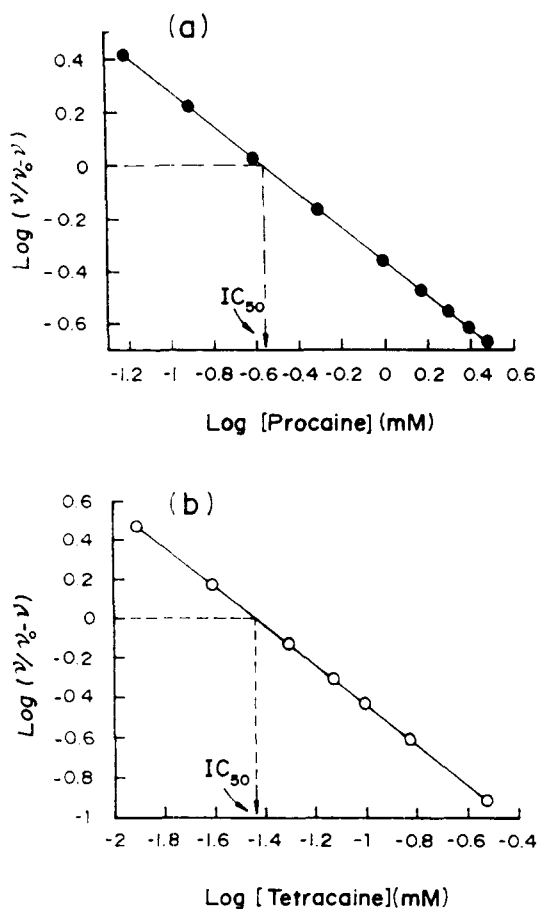
FIGURE 2 Transformed data for *W. aegyptia* AChE inhibition by procaine (a) and tetracaine (b) are presented in the form of a Hill plot, where v and v_o are the reaction rates for the experimental and control systems respectively. The correlation coefficient was 0.999 and 0.964 while the slope was -0.649 and -1.003 for procaine and tetracaine respectively. Each point is the mean of four independent determinations.

any appreciable change in K_m values (the K_m value for AChE of *W. aegyptia* venom at 25°C was found to be 0.051 ± 0.0043 mM, using acetylthiocholine iodide as substrate). The inhibition constant (K_i) was estimated by secondary replots (slope and intercepts i.e. $1/V_{max}$ versus tetracaine concentration) of the Lineweaver-Burk plot (Figure 4b), which yielded a K_i value of $7.2 \pm 0.0091 \times 10^{-3}$ mM.

TABLE Ia   Effect of procaine on kinetic parameters of W. aegyptia venom AChE.

[Procaine] (mM)	K_m (mM)	% increase	V_{max} (μmole/min/mg)	% decrease
0.00	0.051	0.00	10.17 ± 0.32	0.00
0.05	0.073	42.47	9.09 ± 0.08	10.61
0.1	0.093	83.41	7.88 ± 0.25	22.52
0.2	0.106	107.41	6.67 ± 0.31	34.42
0.4	0.125	145.77	5.33 ± 0.062	47.59

Each value shows the mean ± SD of triplicate determinations.

TABLE Ib   Effect of tetracaine on kinetic parameters of W. aegyptia venom AChE.

[Tetracaine] (mM)	V_{max} (μmole/min/mg)	% decrease
0.00	10.16 ± 0.17	0.00
0.01	6.76 ± 0.19	33.48
0.02	4.51 ± 0.24	55.62
0.03	3.13 ± 0.046	16.33
0.04	2.09 ± 0.02	79.42

Each value shows the mean ± SD of triplicate determinations.

DISCUSSION

The IC_{50} values for procaine and tetracaine in the present study of crude W. aegyptia venom AChE is 0.277 and 0.037 mM respectively. The comparative data in Table II, suggests that crude W. aegyptia venom AChE is more sensitive to procaine and tetracaine. Moreover, the IC_{50} values of tetracaine for human erythrocytes and W. aegyptia venom AChE show the closest similarity.

The K_m value of W. aegyptia venom AChE is closer to the K_m value of the shore pit viper (Trimeresurs purpureomaculatus) venom AChE and camel erythrocyte AChE than the AChE from the other sources (Table II), while sensitivity towards tetracaine resembles that of human origin,[21] nature of inhibition by procaine and tetracaine resembles the situation with pigeon and Eel electroplax, respectively.[10,26] The V_{max} value of W. aegyptia venom AChE, is 8.3, 29.1, 9.6, 72.6, 127.1, 36.3 and 169.5 times greater than that for human erythrocytes, camel erythrocytes, pigeon brain, chicken brain, rat erythrocytes, rat brain and the worm homogenate of adult Ascaridia galli AChE, as summarized in Table II.
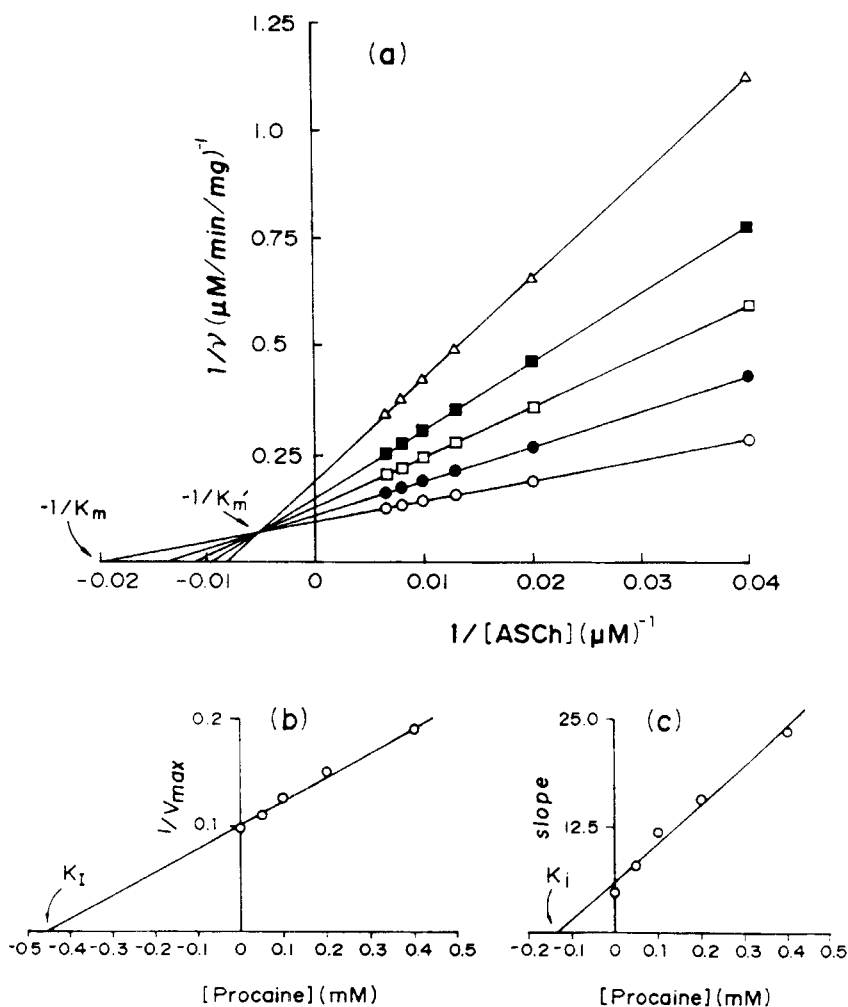
FIGURE 3 (a) Lineweaver-Burk plot of $1/v$ versus 1/ASCh in the absence (○) and presence of 0.05 mM
(●), 0.10 mM (□), 0.20 mM (■) and 0.40 mM (△) procaine. Each point is the mean value of three separate
determinations (correlation coefficients were 0.977, 0.993, 0.997, 0.999 and 0.993 for the lines for ○, ●, □,
■ and △ respectively. (b) Secondary replot of $1/V_{max}$ from primary double reciprocal plot versus procaine
concentration for the determination of inhibition constant K_I. Where correlation coefficient was 0.995.
(c) Secondary replot of slope from primary Lineweaver-Burk plot versus procaine concentration for the
determination of K_i where correlation coefficient and slope values were 0.991 and 45.27 respectively.

TABLE II Kinetic parameters (IC_{50}, V_{max} and K_m) of AChE from various sources.

| Source of AChE | IC_{50} | | V_{max} | K_m | Reference |
	Procaine	Tetracaine	($\mu mole/min/mg$)	(mM)	
venom(p. W. a.)[a]	0.194	0.044	588.24	0.054	11
human erythrocytes	0.4	0.05	1.23	0.1	21, 28
pigeon brain	0.38	–	1.06	0.15	26
rat brain	0.71	0.18	–	–	17
camel erythrocyte	–	0.12	0.35	0.07	27
Eel electroplax	–	0.07	–	–	10
Naja naja siamensis	–	0.18	–	–	10
chicken brain	–	–	0.14	0.13	29
rat erythrocytes	–	–	0.08	0.69	16
rat brain	–	–	0.28	2.4	16
worm (A.g.)[b]	–	–	0.06	4.0	30
(T.p.)[c] venom	–	–	–	0.04	35

[a](p. W.a.) purified W. aegyptia; [b](A.g.) Ascardia galli; [c](T.p.) shore pit viper (Trimeresurs purpureomaculatus).

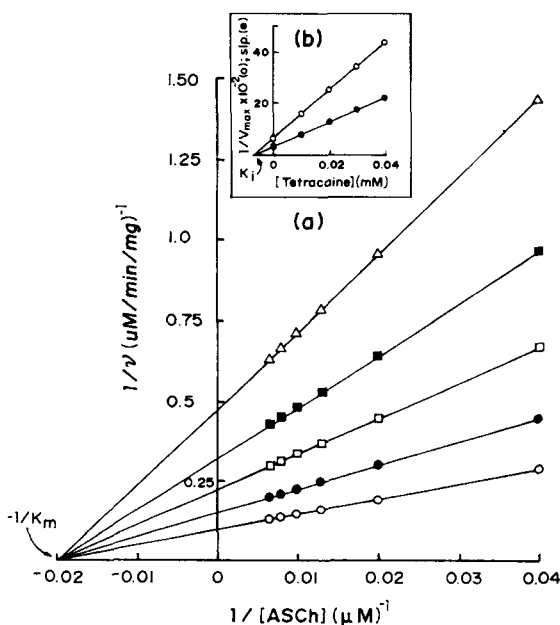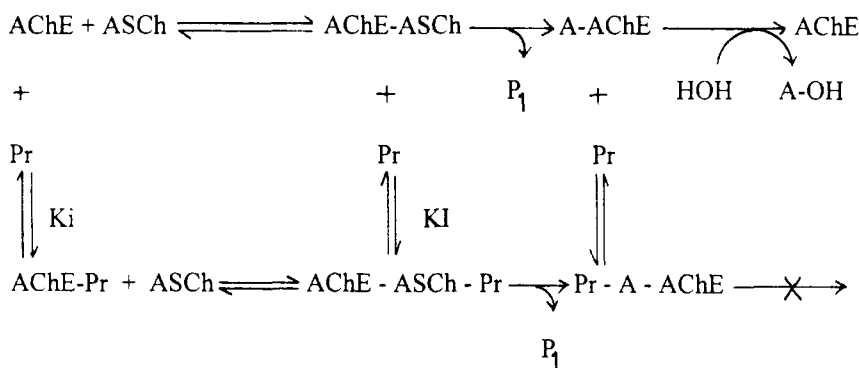
FIGURE 4 (a) Lineweaver-Burk plot of $1/v$ versus 1/ASCh in the absence (o) and presence of 0.01 mM (•), 0.020 mM (□), 0.03 mM (■) and 0.04 mM (△) tetracaine. Each point is the mean value of three separate determinations (correlation coefficients were 0.975, 0.982, 0.962, 0.975 and 0.983 for the lines for o, •, □, ■ and △ respectively). (b) Secondary replot of $1/V_{max}$(o) and slope (•) from primary double reciprocal plot versus tetracaine concentration for the determination of inhibition constant K_i. The correlation coefficient was 0.976 and 0.977 for $1/V_{max}$, and slope respectively.

AChE + ASCh $\rightleftharpoons$ AChE-ASCh $\longrightarrow$ A-AChE $\longrightarrow$ AChE

$+$ $+$ P_1 $+$ HOH   A-OH

Pr   Pr   Pr

$\Big\updownarrow$ Ki $\Big\updownarrow$ KI $\Big\updownarrow$

AChE-Pr $+$ ASCh $\rightleftharpoons$ AChE - ASCh - Pr $\longrightarrow$ Pr - A - AChE $\longrightarrow\!\!\times\!\!\longrightarrow$

P_1

SCHEME 1 Where Pr, A-AChE, A-OH and P_1 denote procaine, acetylated AChE, acetic acid (second hydrolyzing product) and thiocholine (first hydrolyzing product).

The results of the Lineweaver-Burk plot indicated that procaine inhibited AChE in a linear mixed fashion. According to the hydrolysis scheme for a typical substrate by AChE,[31] there are several patterns by which procaine inhibits AChE i.e. either to combine with free AChE or with the AChE-ASCh complex or acylated AChE as indicated in Scheme 1. If we consider the free AChE and low [ASCh] stage, procaine competes with ASCh for binding at the anionic substrate binding site of AChE, due to its diethylamine group (tertiary nitrogen) (Figure 1) which can be converted into a quaternary nitrogen with a positive charge on protonation and resembles the quaternary ammonium of the choline portion of the acetylcholine substrate. While the benzoate ester (-C = O - O) of procaine has some resemblance with the acetate (CH_3 - C = O - O) of the substrate for binding at the esteratic site. However, these two resemblances are not sufficient to give pure competitive inhibition by competition with ASCh. Therefore, procaine shows a mixed pattern of inhibition as very clearly indicated in Figure 3a. In the case of mixed type inhibition there are two types: partial and linear. These two types can be distinguished on the basis of the shape of a double reciprocal plot; if it is curved it means a partial mixed type inhibition system is present. In the present study, the Lineweaver-Burk plot was linear (Figure 3a), indicating a linear mixed-type inhibition system. The linear mixed-type inhibition system has further categories into two main types: one in which $\alpha > 1$ i.e, the family of straight lines of the Lineweaver-Burk plot, intersect above the horizontal axis while in the second case $\alpha < 1$, the family of straight lines (control and "plus inhibitor") in Lineweaver-Burk, as well as Dixon plots, intersect below the X-axis. In the present study intersection occured above the

X-axis which indicates a mixture of competitive and noncompetitive components of the mixed-type inhibition system.

The competitive type has also two subtypes: partial and pure. We found the pure one, because $1/v$ - axis intercept i.e. $1/V_{max}$ versus (Procaine) was found linear (Figure 3b), instead of hyperbolic as in the case of partial competitive inhibition.[25]

As for the competitive type of inhibition, the noncompetitive type of inhibition also has two sub-types: partial and pure, which can be distinguished on the basis of replots of slope$_{1/ASCh}$ and $1/V_{max}$ versus (Procaine). In this study we found linear plots (Figures 3c and 3b) which indicate pure non-competitive, instead of hyperbolic, as in the case of the partial noncompetitive subtype.

If procaine fails to compete with ASCh, as 100% pure competitive inhibitor, then one question arises that how the formation of the AChE-procaine complex is possible as indicated in Scheme 1. We believe that there are other binding sites having some similarities with the catalytic and choline binding sites of AChE. The location of these pseudoactive sites may be very close to the true one so that when procaine fails to bind with the true site it affiliates itself to the pseudo site. On the basis of this hypothesized site procaine also shows pure noncompetitive pattern along with competitive one. The pockets of the pseudo subsites may be more suitable in size and charge for the benzoate and the diethylamine groups of procaine. It can also be assumed that this pseudo site may have some aromatic region, where the pi (π) electrons are involved in the binding of the procaine through the diethylamine group. The electrons of the aromatic ring of the benzoate part of the procaine may also be involved in binding with some positive charged group of the pseudo cationic subsite. The existence of this type of binding is suggested as an aromatic cation binding site has been reported elsewhere.[32,33]

There are two other possibilities for the interaction of procaine with AChE; either in the AChE-ASCh complex form or the acylated AChE form (acyAChE) to produce an AChE-ASCh-Procaine or an acyAChE-Procaine complex, respectively. The first possibility is the more likely one since if procaine was bound to acyAChE, thiocholine (P_1), the first product of the reaction, would not be affected, yet our results show that it is decreased by increasing the concentration of procaine. This suggests that procaine does not bind with acyAChE and instead binds to the AChE-ASCh complex. In the case of AChE-ASCh complex, the anionic substrate binding site is occupied by the β substituent of ASCh (quaternary ammonium ligand of the choline part of ASCh) and thus is not available to a second ligand. Therefore, procaine must bind with some other site such as the peripheral binding site (PBS), which is supported by the low value of the K_I/K_i ratio (3.39).[34]

The nature of inhibition of human erythrocytes and pigeon brain AChE by procaine was reported as competitive[21] and mixed type.[26] The K_i values for Eel electroplax AChE was reported as 0.78 mM[10] while in the case of pigeon brain

AChE, the K_I and K_i were reported as 0.22 and 0.14 mM, respectively. These results show that the mechanism of procaine interaction with *W. aegyptia* venom AChE is different from its interaction with human erythrocyte membrane bound AChE but similar to pigeon brain AChE.

In the case of tetracaine the results of the Lineweaver-Burk analysis indicates that tetracaine at different concentrations of 0.01–0.04 mM, inhibits AChE activity noncompetitively (Figure 3). The decrease in V_{max} in the presence of tetracaine without any change in K_m of AChE suggests that tetracaine produces a conformational change of the enzyme.[29] Moreover, these results indicate that tetracaine binds to either AChE-ASCh (E.S) to form an AChE-ASCh-Tetracaine complex (E.S.I), or to a regulatory site of the free AChE to form an AChE-Tetracaine complex (E.I). This would inhibit acylation as well as deacylation, since the concentration of thiocholine decreased with increase in tetracaine concentration (0.01–0.04 mM). It was also confirmed that tetracaine binds at the peripheral or regulatory site of AChE because in the case of the E.S. complex, the anionic site is occupied by the β-substituent of ASCh and thus is not available to a second ligand (tetracaine), while it is exposed and accessible in acyAChE, although tetracaine did not bind to it. If tetracaine was bound to acyAChE, then P_1 would not be affected in that case.

The nature of inhibition of AChE from human erythrocyte,[21] rat brain,[17] Eel electroplax and *Naja naja siamensis*[10] by tetracaine was reported as competitive, competitive, noncompetitive and mixed type, respectively. The K_i values for Eel electroplax and *Naja naja siamensis* AChE were reported as 0.32 and 0.12 mM respectively.[10] In the light of the literature, it can be calculated that *W. aegyptia* venom AChE has 5.9 times more affinity for procaine towards its peripheral binding site than Eel electroplax AChE while 44.4 and 16.7 times more affinity for tetracaine than Eel electroplax and *Naja naja siamensis* AChE[10] respectively. These results show that the mechanism of tetracaine interaction with *W. aegyptia* venom AChE is different from its interaction with human erythrocyte membrane bound AChE, rat brain and *Naja naja siamensis* but similar to Eel electroplax AChE.

It is apparent from the results reported in this investigation that *W. aegyptia* venom AChE has unique kinetic characteristics and higher AChE activity (even in crude form) than the mentioned sources. The *W. aegyptia* venom AChE could therefore serve as a unique and a convenient model for kinetic and pharmokinetical study of various chemicals and drugs.

Acknowledgements

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