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Centro de Ciências Naturais e Exatas

Programa de Pós-Graduação em Ciências Biológicas:Bioquímica Toxicológica

CARACTERIZAÇÃO DA ATIVIDADE DA ENZIMA ACETILCOLINESTERASE DE VENENO DE *Bungarus sindanus*: ESTUDOS COMPARATIVOS

Tese de Doutorado

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KRAIT VENOM ACETYLCHOLINESTERASE IS A SUITABLE CANDIDATE FOR BIOCHEMICAL ANALYSIS: CHARACTERIZATION AND COMPARATIVE INHIBITORY STUDIES

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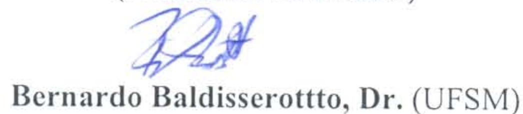

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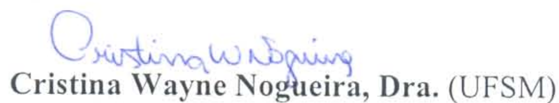

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DEDICATION

This thesis is dedicated to my father Sher Bahadar Khan. I owe him for all my life and accomplishments. Without him it would not have been such a success story.

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RESUMO

Tese de Doutorado

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A enzima acetilcolinesterase (AChE, E.C. 3.1.1.7) faz parte de uma família distinta de serina hidrolases, sendo uma importante enzima regulatória, encontrada principalmente no encéfalo, músculos, eritrócitos, neurônios colinérgicos e também em veneno de cobra. Nas sinapses, a sua principal função é a hidrólise no neutrotransmissor acetilcolina (ACh), enquanto que em tecidos não-sinápticos a sua função é desconhecida. O veneno de cobra, principalmente da família Elapidae, é uma fonte não sináptica abundante de AChE. Por exemplo, a espécie *Bungarus sindanus*, possui um alto conteúdo de AChE. No entanto, na literatura não existem muitos relatos sobre o estudo desta enzima neste veneno. Sendo assim, o veneno de cobra da espécie *Bungarus sindanus*, foi selecionado comno a principal fonte de AChE neste estudo. A partir dele fez-se estudos de caracterização, comparação com outras fontes de colinesterases (soro eqüino, encéfalo de ratos e sangue humano) e estudos de inibição da enzima por diversos agentes (tacrina, malation, carbofuran, paraquat e

antidepressivos). Foi observado que a enzima de veneno de cobra possui um pH ótimo alcalino de pH 8.5 e uma temperatura ótima de ensaio de 45°C. A inibição por substrato, característica cinética da AChE, foi reduzida significativamente ao se usar um tampão com alta força iônica (10 mM PO_4 pH 7.5). A enzima possui estabilidade térmica a 45°C. A enzima perdeu somente 5% de sua atividade após 45 min de incubação à 45°C. A constante de Michaelis-Menten (Km) para a hidrólise de ACh foi de 0.052 mM com uma V_{max} de 530 µmoles/min/mg de proteína. Também, foi observado que a AChE de veneno da cobra *Bungarus sindanus* é inibida por $ZnCl_2$, $CdCl_2$ e $HgCl_2$. A enzima apresentou alta sensibilidade a tacrina, a aqual é sabidamente um inibidor da AChE sináptica. A inibição observada foi de tipo mista, tanto em veneno de cobra, tanto quanto em cholinesterase de soro humano (BChE). Também, a enzima apresentou padrão inibitório similar com a colinesterase de soro humano quando foram testados agentes pesticidas e herbicidas. A AChE de veneno de cobra exibiu uma inibição de tipo mista para os pesticidas malation e carbofuran e para o herbicida paraquat. A única diferença foi o padrão de inibição da BChE em relação ao carbofuran, que foi do tipo incompetitivo. A atividade da enzima AChE de veneno de cobra foi inibida, também, pelos antidepressivos paroxetina, imipramina, clomipramina e sertralina. A paroxetina e a sertralina causaram uma inibição de tipo mista, enquanto que a imipramina e a clomipramina exibiram um padrão de inibição competitivo. Também, o bem conhecido composto químico N,N,N´,N´-Tetramethylethylene diamine (TEMED), o qual é rotineiramente utilizado para iniciar processos de polimerização de géis de poliacrilamida causou uma inibição de tipo mista na Ache de veneno de cobra bem como em BChE de soro eqüino. Além disto, a inibição da AChE por TEMED foi observada em diferentes estruturas cerebrais de ratos, tais como estriato, hipocampo, cortex, hipotálamo e cerebelo. Estes resultados confirmam a hipótese de que o TEMED

é colinotóxico. Analisando, em conjunto, os resultados podemos concluir que a AChE do veneno da cobra *Bungarus Sindanus* apresentou padrão cinético inibitório quase similar a outras colinesterases para os compostos tacrina, malation, carbofuran, paraquat, antidepressivos e TEMED. Além disto, o veneno desta cobra possui altas quantidades de AChE o que o torna uma fonte valiosa para estudos bioquímicos e cinéticos desta enzima.

Palavras-chave: Acetilcolinesterase, veneno de cobra, *Bungarus sindanus*, inibidores.

ABSTRACT

PhD Thesis

Federal University of Santa Maria, RS, Brazil

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Acetylcholinesterase (AChE) belongs to a distinct family of serine hydrolases and is found in both synaptic and non-synaptic locations. At the synapses, it plays a major role in the hydrolysis of the neurotransmitter acetylcholine (ACh) while in non-synaptic tissue its function is unclear. Snake venom, particularly from the Elapidae family, is a common non-synaptic source of AChE. The venom of krait (*Bungarus sindanus*), an Elapidae snake, contained a high level of AChE activity. As there is no literature study about *Bungarus sindanus* venom AChE, it was selected as a main source of AChE activity. We characterized the venom AChE following comparative inhibitory studies with human, horse and rat cholinesterase using different ligands (tacrine, malathion, carbofuran, paraquat, antidepressants, TEMED). The enzyme of krait venom showed optimum activity at alkaline pH 8.5 with an optimal temperature of 45°C. We observed a significant reduction in substrate inhibition of krait venom AChE by using a high ionic strength buffer. With a low ionic strength buffer (10 mM PO_4 pH 7.5) the enzyme was inhibited by 1.5 mM AcSCh, while with a high ionic strength buffer (62 mM PO_4 pH 7.5) the enzyme was inhibited by 1mM AcSCh. Furthermore, we found that krait

venom acetylcholinesterase is thermally stable at 45^oC. The enzyme lost only 5% of its activity after incubation at 45^oC for 40 min. The Michaelis-Menten constant (Km) for the hydrolysis of acetylthiocholine iodide was found to be 0.052 mM. We noted that snake venom AChE was also inhibited by $ZnCl_2$, $CdCl_2$ and $HgCl_2$ in a concentration dependent manner. In addition, this enzyme showed high sensitivity to tacrine, which is known to inhibit synaptic AChE.

We observed that tacrine caused a mixed type of inhibition in krait venom as well as in human serum BChE. Snake venom AChE presents similar inhibitory behavior toward commonly used pesticides and herbicides as that of human serum BChE. The snake venom AChE exhibited a mixed type of inhibition for the pesticides malathion and carbofuran and the herbicide paraquat while human serum BChE presented a mixed inhibition for malathion and paraquat and an uncompetitive inhibition for carbofuran. The krait venom AChE was also affected by antidepressants such as paroxetine, imipramine, clomipramine and sertraline. Paroxetine and sertraline caused a mixed type of inhibition, while imipramine and clomipramine exhibited a competitive inhibition. Moreover, the well-known chemical N,N,N´,N´-tetramethylethylene diamine (TEMED) caused a mixed type of inhibition in snake (*Bungarus*) venom as well as in horse serum BChE. Futhermore, the inhibition of TEMED, was also confirmed from *in vivo* study in different structures of the brain, such as striatum, hippocampus, cortex, hypothalamus and cerebellum. Decrease in AChE activity was observed in all treated groups. The results suggest that TEMED exhibits toxic effect via inhibition of cholinesterase.

Taken together the krait venom AChE showed similar behavior towards different ligands (tacrine, malathion, carbofuran, paraquat, antidepressants, TEMED) like other sources of cholinestease. Furthermore, krait venom contains large amount of

acetylcholinesterase having highest catalytic activity and comparatively more stable than any other sources, making it more valuable for biochemical analysis.

Keywords: Snake venom, Acetylcholinesterase (AChE), Inhibitors.

List of abbreviations

Å	Angstroms
ACh	Acetylcholine
AChR	Acetylcholine Receptor
AChE	Acetylcholinesterase
Asp	Aspartatic acid
BChE	Butyrylcholinesterase
His	Histidine
Ser	Serine
Glu	Glutamic acid
kDa	kilo Dalton
Km	Michaelis-Menten constant
Kss	Substrate inhibition constant
Lys	Lysine
Met	Methionine
mRNA	Messenger Ribonucleic Acid
SAR	Structure Activity Relationship
Trp	Tryptophan
Tyr	Tyrosine
WT	Wild Type

1. INTRODUCTION

Acetylcholinesterase (AChE) is present in synaptic tissue of all vertebrates, particularly in the muscles and nervous tissues (Rotundo, 2003). The snake venom is a non-synaptic source of acetylcholinesterase (AChE) where it is present in very large quantity close to 8 mg/g of dried venom (0.8 % w/w). Its function in venom is unknown. However, it is clear that it is present in both poisonous and non-poisonous snake venom (Bawaskar and Bawaskar, 2004; Mackessy et al., 2006). A partial peptide sequence study showed that snake venom enzymes have a close homology with other AChE and have the same catalytic triad for substrate hydrolysis (Cousin et al., 1996). For comparative inhibitory studies serum butyrylcholinesterase (BChE) was select as the source. Its function is not well defined. However, AChE and BChE exhibit 51-54% amino acid identity, identical disulphide arrangement and use same "catalytic triad" of Ser, His and Glu in the active centre for substrate hydrolysis (MacPhee et al., 1986; Chatonnet and Lockridge, 1989). Surprisingly, the catalytic triad contains glutamate instead of aspartate, typically present in serine proteases and it is mirror image of the traditional one (Sussman et al., 1991). The classical inhibitors (eserine, decamethonium, fasciculin, BW284C51, propidium and edrophonium) have been reported as inhibitors of snake venom AChE (Frobert et al., 1997). All the above compounds showed similar sensitivity towards different snake venom AChEs, except fascilculin (a short chain peptide isolated from Mamba snake venom), for which large variation in the inhibitory effect was observed. Taken cognizance of the points raised above, we set the following objectives:

Main objective

- In snake venom (non-synaptic source) particularly, in Elapidae, the krait (*Bungarus*) genera contain large amount of AChE with high activity. Thus, the present study was conducted to characterize and to compares the kinetic parameters of acetylcholinesterase from krait (*Bungarus sindanus*) snake venom with other sources of cholinesterase by using different ligands.

Specific objectives

- To characterize krait (*Bungarus sindanus*) venom acetylcholinesterase in terms of thermal stability, presence of BChE, substrate specificity, substrate inhibition, ionic strength, pH, temperature and metals effect.
- To examine non specific property and mode of inhibition of krait venom acetylcholinesterase (AChE) by tacrine and to compare the kinetic behavior with serum butyrylcholinesterase (BChE) in order to reduce the deleterious effect of this drug.
- To determine whether the herbicide (paraquat) and the pesticides (malathion and carbofuran), which are classical inhibitors of AChE, also inhibit *Bungarus sindanus* AChE and human serum BChE.
- To establish via *in vitro*, whether commonly used antidepressants (paroxetine, imipramine, clomipramine and sertraline) can also be considered inhibitors of snake venom and human serum cholinesterase.
- To access *in vitro* and *vivo*, the effect of N,N,N´,N´-tetramethylethylene diamine (TEMED) as a potential anticholinesterasic agent.

2. LITERATURE REVISION

2.1. Cholinergic system

The cholinergic system consists of nerve cells that use acetylcholine (ACh) as its neurotransmitter, mainly found in vertebrates and arthropods. ACh is stored in the nerve terminals in structures called vesicles. The contents of these vesicles are released from the nerve endings when the nerve terminal is depolarised; ACh thus released enters the synapse and binds to the receptor. The muscranic and nicotinic receptor are sensitive for ACh (Watling, 1998). The muscarinic receptors are chiefly associated with the peripheral nervous system and with smooth and cardiac muscles. The binding of ACh with muscranic receptor stimulates parasympathetic nervous system which decreased heart rate and blood pressure, constriction of bronchi, increased salivation, promotion of digestion and increase in intestinal peristalsis, release of fluids from the bladder and accommodation of the eyes for near vision, with contraction of the pupils. The nicotinic receptors are found in the central nervous system (CNS) and in the motor end plates which are the synapses between nerves and skeletal muscle (Watling, 1998). In CNS, binding of ACh with nicotinic receptor causes stimulation which is associated with cognitive processes and memory, whilst in skeletal muscles it causes contraction (Hogg et al., 2003). Release of ACh after activation of the receptor is degraded by enzyme called acetylcholinesterase (AChE), leading to loss of stimulatory activity. As far as nervous transmission is concerned, the concept of inhibition of AChE has been employed in medicine for treating disease states associated with inadequate levels of ACh, and toxicologically to cause illness or death by means of excess cholinergic stimulation (Silman and Sussman, 2005). AChE has also been found to be involved in a number of other functions besides nerve transmission. These include a role as an

adhesion protein, a bone matrix protein, in neurite growth and in the production of amyloid fibrils, which are characteristically found in the brain cells of patients with Alzheimer's disease (Zhang et al., 2004).

2.2. Acetylcholine

Acetylcholine was the first chemical agent reported to establish a communication between two distinct mammalian cells and it acts by propagating an electrical stimulus across the synaptic junction. The structure is displayed in Figure 1. At the presynaptic or transmitting neuron end, an electrical impulse triggers the release of ACh, which accumulates in vesicles in the synaptic cleft via exocytosis. After its release into extracellular space, ACh binds to ACh receptors (AChR) on the postsynaptic or receiving neuron surface, and the ACh-AChR binding induces the emission of subsequent impulses to the postsynaptic neuron (Hartzell et al., 1977). Finally, ACh is rapidly degraded by the enzyme acetylcholinesterase (AChE) resulting in the formation of choline and acetic acid (Kelly et al., 1979; Dunant et al., 1980; MacIntosh, 1981) (Figure 2 and 3). Moreover, ACh hydrolysis can be carried out by a related, but less specific enzyme, butyrylcholinesterase (BChE), also called serum cholinesterase or pseudocholinesterase (Dave et al., 2000; Li et al., 2000).

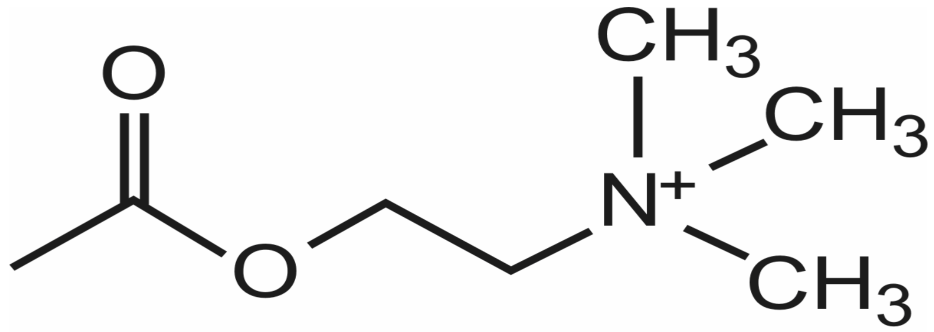

Figure 1. Chemical structure of acetylcholine

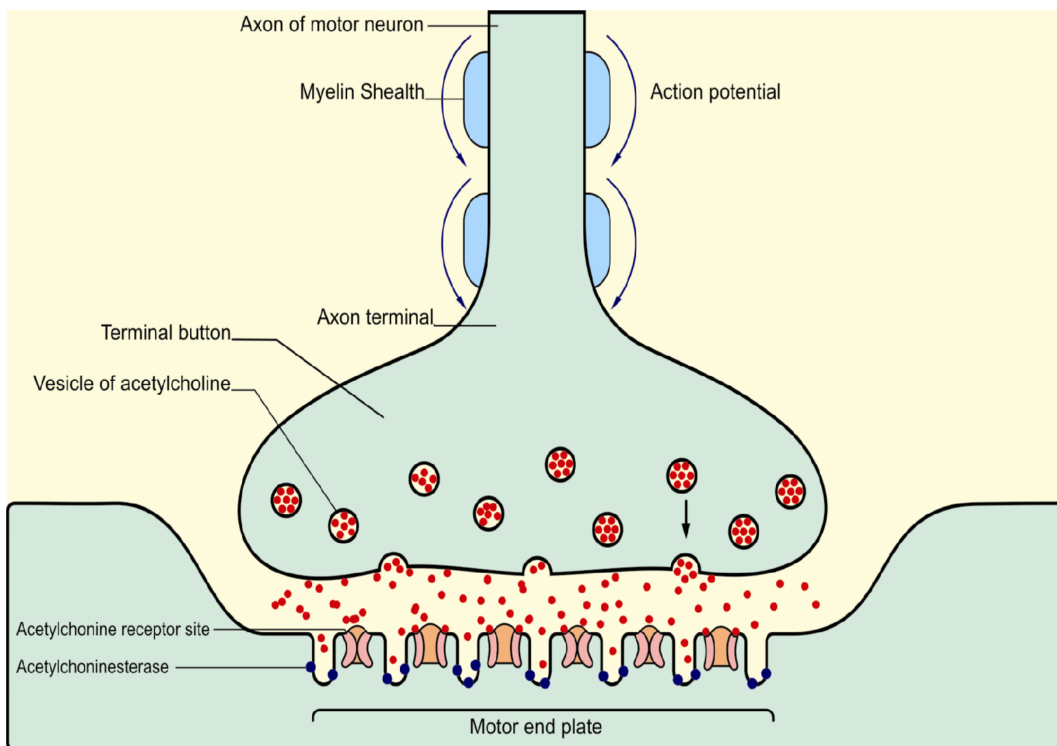

Figure 2. General components of the neuromuscular junction

2.3. Acetylcholinesterase

Acetylcholinesterase is present in all vertebrates, particularly in the muscle and nervous tissue, and plays a fundamental role in the hydrolysis of ACh (Figure 3). Structurally and functionally, both AChE and BChE are serine hydrolases that belong to the esterase family within the higher eukaryotes. The efficacy with which AChE controls neurotransmission life time in the synaptic cleft depends not only on its enzymatic activity but also on its density and location relative to acetylcholine receptors (Martinez-Pena et al., 2005). The enzyme is also found in non-synaptic locations, such as in the blood cells, notably erythrocytes and lymphocytes (Kawashima and Fujii, 2000; Thiermann et al., 2005). Different types of snake venom, for example, venom from the *Naja*, *Hemachatus* and *Ophiophagus* of the *Elapidae* family and mainly from the *Bungarus* genera are a common example of a nonsynaptic source of AChE (Iwanaga and Suzuki, 1979; Frobert et al., 1997) in which its function is unknown.

Figure 3. Reaction catalyzed by acetylcholinesterase

2.4. General Structure of AChE

AChE was discovered in the 1930's and it was found to be one of the most efficient enzymes with a high turnover number. Structure Activity Relationship (SAR)

studies have shown several major domains within the protein: a catalytic active site composed of two subsites, the aromatic gorge in which the catalytic active site lies, and a peripheral anionic site, distinct from the catalytic active site, which plays a role in the confirmation of the residues within the aromatic gorge and active site (Rosenberry et al., 2005). SAR studies have also ascertained that there are probably two binding domains in the active site: an ionic site containing a glutamate residue that can bind with the cationic head of ACh (van Der Waals interactions probably contribute significantly) and an esteratic site that contains active serine and histidine residues, which function as an acid/base catalyst domain (Kabachnik et al., 1970; Soreq and Seidman, 2001; Rosenberry et al., 2005).

The hydrolysis of ACh occurs at the serine containing esteratic site, which is functionally coupled to a substrate binding area containing a distinct anionic locus (Forede et al., 1971; Rosenberry et al., 1975; Quinn et al., 1987; Massoulié et al., 1993). The ester group of ACh allows hydrogen bonding with an asparagine residue of AChE. The hydrophobic area of AChE is responsible for binding the alkyl substituents of acetylcholine located close to the esteratic and anionic site (Rosenberry et al., 1975; Quinn et al., 1987; Massoulié et al., 1993). The negative charged group in the active site of cholinesterase, which is at a distance of 4.5 to 5 A° from the active-site serine residue, accommodates the quaternary ammonium moiety of the substrate through via ion pair formation between the cationic side chain of the substrate and the anionic amino acid side chain (Forede et al., 1971; Rosenberry et al., 1975; Quinn et al., 1987).

The most interesting aspect of this enzyme is the peripheral anionic site (PAS) on its surface. Site-directed labeling and mutagenesis studies situate the location of the PAS at the entrance of an active center gorge (Barak et al., 1994). This site has the

ability to bind diverse types of ligands. Six residues have established activity within this site: Trp-286, Tyr-72, Tyr-124, Glu-285, Asp-74 and Tyr-341, which are located on the opposite side of the gorge entrance. This arrangement of residues exhibits flexibility which accommodates many different ligands, and also implies their conformational mobility (Ordentlich et al., 1995).

The crystal structure of *Torpedo californica* AChE confirmed the previous conclusion about the structure and mechanism of the substrate hydrolysis via the catalytic triad (Sussman et al., 1991 and 1992). The esteratic serine (Ser-200) is assisted by His-440 and Glu-327 at an appropriate hydrogen binding distance located near the bottom of a 20 A° deep narrow gorge. Interestingly, the catalytic triad of AChE consists of glutamate rather than aspartate, which is the most important residue of the catalytic triad of serine protease. The three-dimensional structure of AChE indicates that the active site gorge contains negative ionic charges. Two anionic residues within the gorge, Glu-27 and Asp-443 are essential for the catalytic machinery of the enzyme. Glu-327 is a member of the catalytic triad while Asp-443 is involved in hydrogen bonding with the water molecule to keep the water molecule in a position suitable for the chemical reaction. Removal of the Asp-443 reduced the catalytic activity of the enzyme (Neville et al., 1992). A molecular modeling study revealed that ACh binds with some of 14 aromatic residues located near the active site gorge instead of binding to the negatively charged anionic site (Sussman et al., 1991; Radic et al., 1992).

It has been suggested that Glu-199 binds with catalytic essential water molecules (Sussman et al., 1991). It had been speculated that Glu-199 was involved in substrate inhibition but this speculation was proved incorrect when similar results were obtained from the replacement of Glu-199 by glutamine. Thus, it was shown that the presence of

an anionic charge at this location is not responsible for substrate inhibition (Radic et al., 1992). One important feature which is pointed out from the substitution of Trp-84 by alanine in human AChE is that it causes a 3000 fold decrease in the rate of hydrolysis of ACh (Soreq et al., 1992; Ordentlich et al., 1995). Additionally, an essential role for Trp-84 was confirmed from experiments with mice, where mutation of Trp-84 by alanine caused a 50 fold decrease in the hydrolysis of ACh (Radic et al., 1992).

2.5. Mechanism of acetylcholine hydrolysis

The catalytic triad of AChE consists of serine, histidine and glutamate. The serine and histidine residues at the catalytic site are involved in the hydrolysis of ACh (Figures 4 and 5).

The serine residue acts as a nucleophile while histidine acts as an acid/base catalyst in the hydrolysis process. Serine itself is unable to hydrolyze an ester but the dual function (acid/base catalyst) of histidine increases the reactivity or nucleophilicity of serine OH and overcomes this chemical impediment. The hydrolysis of the substrate (ACh) is very fast. Within 100 microseconds, AChE lyses ACh by passing through the following steps (Figure 6).

Step 1

The substrate acetylcholine binds at the active site through ionic and hydrogen bonding. The oxygen from the serine residue has one pair of electrons which acts as a nucleophile and forms a bond with the ester from ACh. Thus, the nucleophilic addition opens up the carbonyl group of the ester.

Step 2

Histidine acts as a base and removes the proton, making serine more nucleophilic.

Step 3

Histidine acts as an acid by protonating the "OR" making it a better leaving group

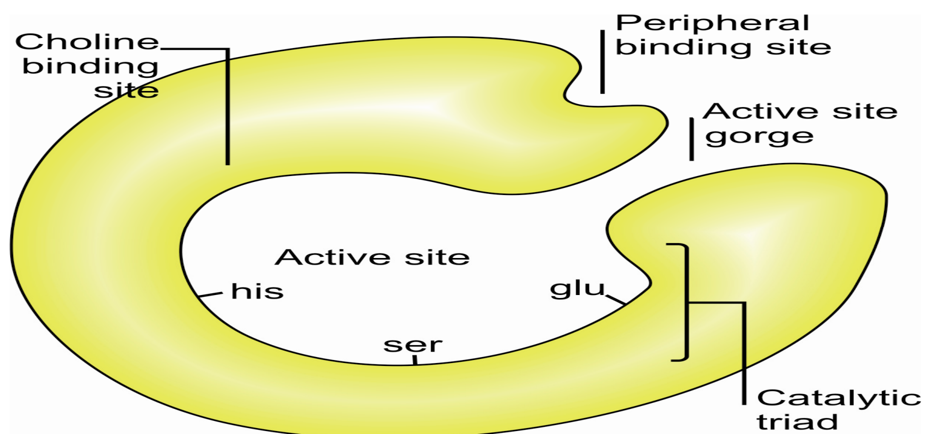

Figure 4. Structural features of acetylcholinesterase. (Adapted from Soreq and Seidman 2:294-302, 2001. Obtaining permission).

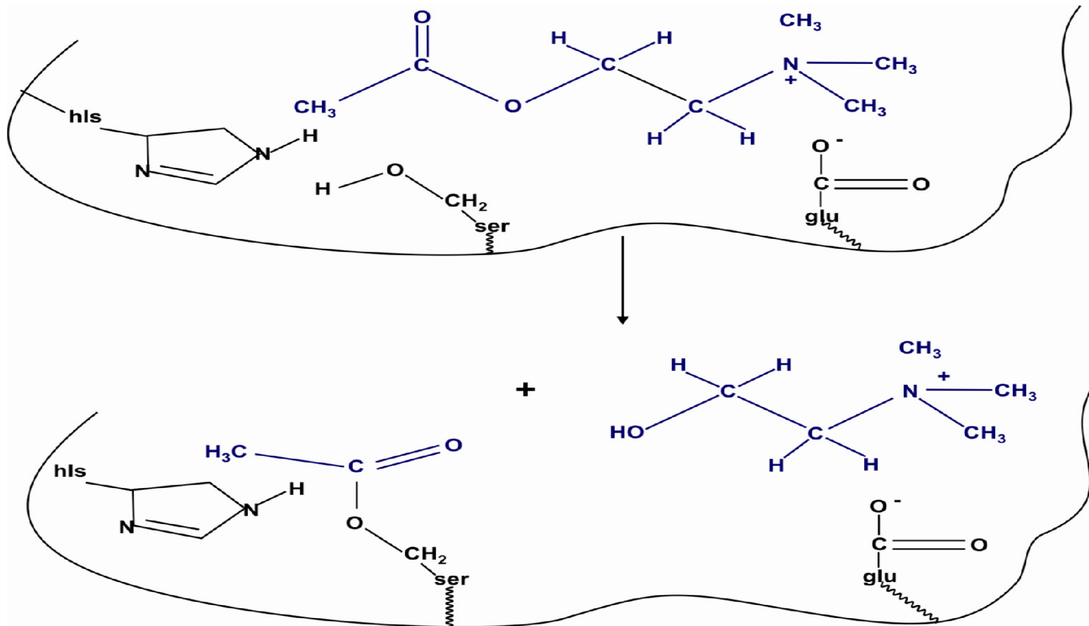

Figure 5. Binding of acetylcholine to the active center of acetylcholinesterase (Adapted from Soreq and Seidman 2:294-302, 2001. Obtaining permission).

Step 4

The carbonyl group reforms and repels the alcohol portion of the ester (choline)

Step 5

The choline leaves the active site and is replaced by a water molecule

Step 6

The water oxygen has a single pair of electrons and acts as a nucleophile by attacking the acyl group.

Step 7

Histidine acts as a base and removes the proton, which makes serine more nucleophilic.

Step 8

Histidine acts as an acid by protonating the intermediate, making it a better leaving group.

Step 9

The protonation causes the serine residue to release the acetic acid in which the carbonyl group is reformed.

Step 10

Acetic acid leaves the active site and the cycle can be repeated depending upon the availability of another molecule of acetylcholine.

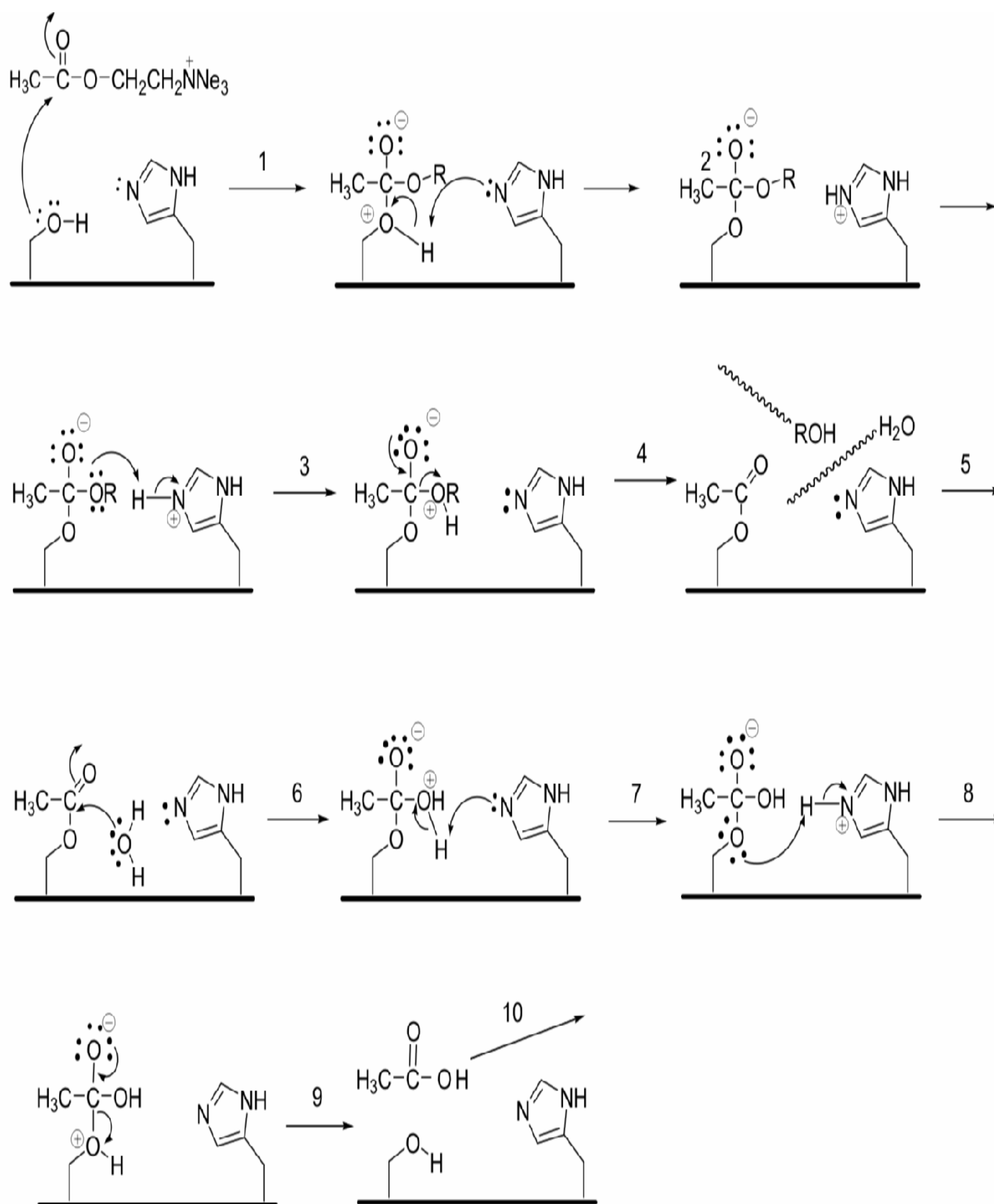

Figure 6. Mechanism steps of acetylcholine hydrolysys (Adapted from Patrick, G.L. second edition, page 471. Obtaining permission)

3. Krait Snake

The name krait represents any of the 12 species and 5 subspecies belong from *Bungarus* genera. *Bungarus* are venomous elapid snakes found in India, Pakistan and South-East Asia. Kraits (*Bungarus sindanus*) usually range between 1 to 1.5 m in length, although specimens as large as 2 m have been observed. They have shining scale and typical black and white bands (Boulenger, 1897).

3.1. Snake Venom composition

Generally the venom contains both organic and inorganic substances. In addition venom may contain lipids, carbohydrate, some biogenic amines and inorganic metals e.g. Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}, Fe^{+2}, Ni^{+2}, Co^{+2}, Cu^{+2}, Mn^{+2} etc (Russel and Gertsh, 1983). These metals play an important role in the action of enzyme activity such Ca^{+2} in phospholipase (Habermehl, 1981) Mg^{+2} in 5´-nucleotidase etc. The chemical composition of venom is given in the Figure 7.

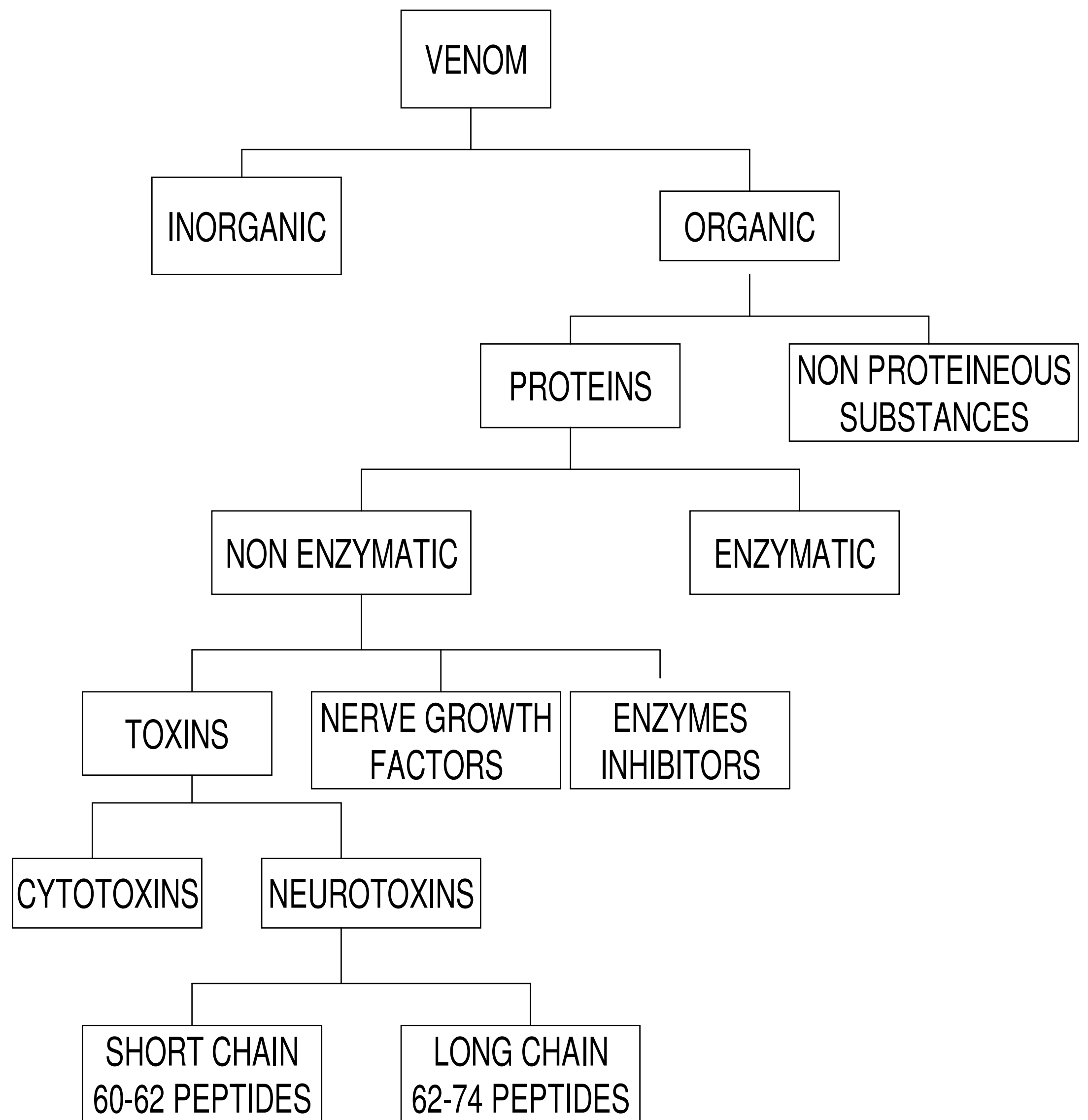

Figure 7. Chemical composition of snake venom

3.2. Snake Venom Enzymes

Snake venom contains more than twenty five different enzymes with various pharmacological activities. Among these phospholipase are common in almost all snake venom. These enzymes display number of pharmacological activities including lipid catabolism, neurotoxicity, cytotoxicity, mytotoxicity, anticoagulant, hypertensive and hemolytic effect (Kini and Evans, 1987). The different types of enzymes in snake venom are listed in Table. 1.

Table. 1 Enzymes of snake venom (Bailey, 1998)

L-Amino acid oxidase	Proteolyic enzymes
Arginine ester hydrolase	Hyaluronidase
Lactate dehydrogenase	Colagenase
Phosphomonoesterase	Phosphodiesterase
Phospholypase A2	Phospholypase B
Phospholypase C	NTPDase
Thrombin like enzymes	RNase
5´-Nucleotidase	NAD-nulcleotidase
Acetylcholinesterase	

3.2.1. Snake families that possess AChE

The presence of AChE in snake venoms is mysterious because limited literature evidence which is based on an artificial model have suggested that it is nontoxic byself and does not enhance the toxicity of other venomous components (Cousin et al., 1998). However, snake venoms are the richest source of AChE known, presenting only the soluble globular form of AChE (Cousin et al., 1998).

Approximately 2,500 different species of snakes are known. Of these, only 20 % of the total numbers of snake species are poisonous and these are divided into four families (Figure 8). Among these, the Elapidae family is the main family that possesses AChE activity. The *Crotolidae* and *Viperidae* families lack this enzyme (Frobert et al., 1997) and the availability of AChE is unknown in the *Hydrophidae* family. In the *Elapidae* family, all the snakes present AChE activity in their venom, excepting those from the *Dendroaspis* genus (Mambas). In the *Dendroaspis* genus, instead of AChE, its venom contains a very potent reversible inhibitor of AChE, known by the name of fasciculin, which binds at the peripheral site of the enzyme. The coral snakes' (Genus *Micrurus*) venom is also one of the most potent sources of AChE (Nget-Hong and Gnanajothy, 1992). The catalytic characterization of affinity-purified AChEs from the venoms of four elapid genera showed that they share features of other vertebrate AChEs such as inhibition by eserine, preferential hydrolysis of acetyl rather than of propionyl or butyryl esters and excess substrate inhibition (Frobert et al., 1997). Moreover, the presence of AChE activity was demonstrated in non-poisoning snakes of the *Colubridae* family, such as the Brown Treesnake *(Boiga irregularis)* (Mackessy et al., 2006) and the *Boiga blandingi* and *Boiga dendrophila* (Broaders and Ryan, 1997).

3.2.2. Molecular forms of snake cholinesterase

The *Bungarus* possesses a single AChE gene containing a novel alternative exon, “S” located downstream from the T-exon. In the *Torpedo* and mammalian AChE genes, the catalytic domains are coded by common exons, followed by alternative spliced sequences, encoding C-terminal peptide, characterized as

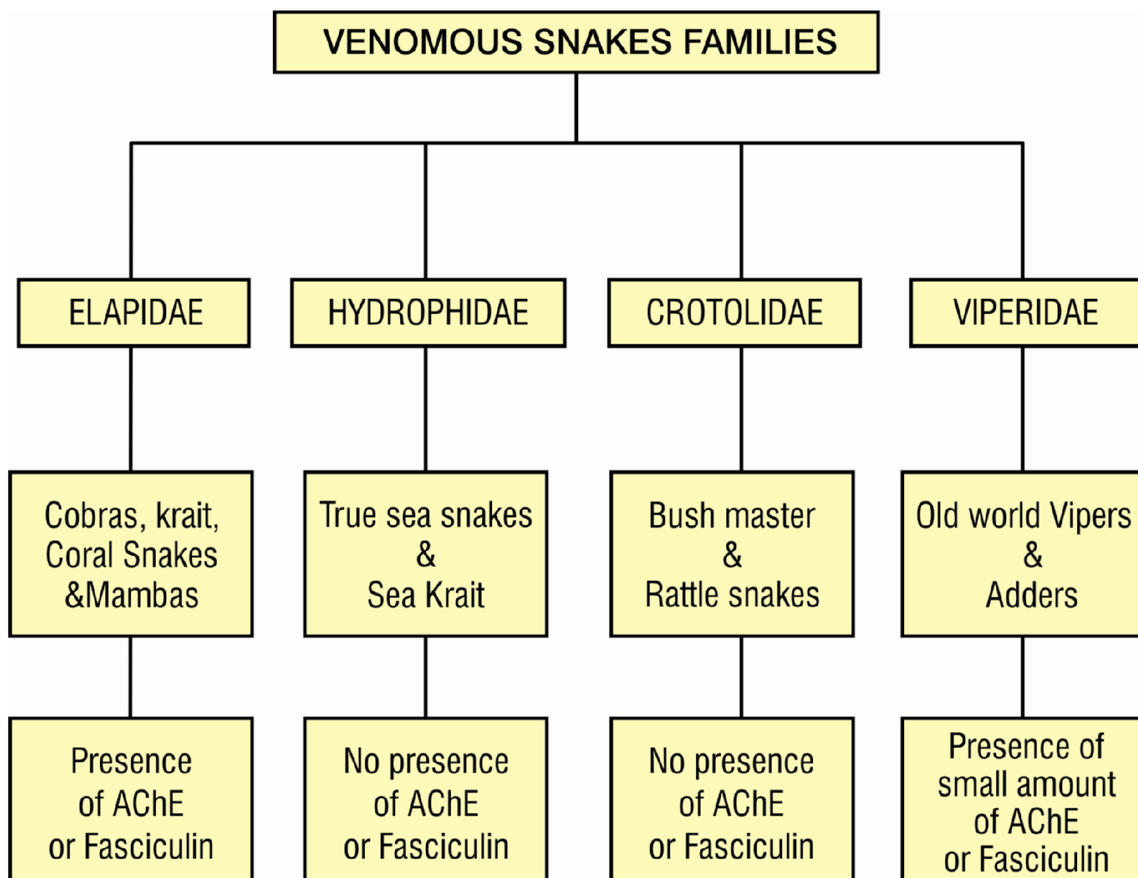

Figure 8. The presence of AChE or fasciculin in the venomous snake families

T (tailed), H (hydrophobic) and R (read through) subunits. H subunits generate amphiphilic glycolipid anchored dimmer while T subunits produce both amphiphilic (G1, G2 and G4 forms) and non amphiphilic asymmetric forms. Interestingly, all these subunits have the same catalytic property for the substrate hydrolysis but produce different molecular enzyme forms. A number of different forms, which are the oligomers of subunits H and T, have been reported (Massoulié et al., 1993). The snake AChE gene possesses an alternative spliced sequence of T and a new type of S-exon which encodes the C-terminal peptide. The H-exon, which is commonly found in mammalian and *Torpedo* AChE, is absent in snake AChE. The alternative splicing generates S-mRNAs in the venom gland while in the liver and muscle it produces both S-mRNAs and T-mRNA. In the *Bungarus* muscle, AChE, which is derived from an $AChE_T$ subunit, is mainly present in globular forms (G_1 and G_4) and in small amounts in asymmetric forms (A_8 and A_{12}). The snake liver possesses both AChE and BChE. In the liver, AChE is found in a nonamphiphilic monomeric form (G_1) while BChE is in a nonamphiphlic tetrameric form (G_4) (Massoulié et al., 1999).

An amino acid sequence study revealed that snake venom AChE is different from both human ($AChE_H$) and *Torpedo* ($AChE_T$) AChE basically due to the C-terminal peptide sequence. Venom AChE has the typical C-terminal sequence which is given the name of SARA due to the last four amino acids of the sequence, which have a hydrophilic characteristic (Massoulié et al., 1999). Furthermore, snake venom AChE has the same catalytic triad as that of other AChEs and hydrolyzes the substrate in the same way as shown in Figures 4 and 5.

3.2.3. Molecular weight and isoelectric points

In cobra (*Naja naja oxiana*) venom, AChE has a molecular weight ranging from 65 to 69 KDa. The different isoelectric points are caused by the difference in the number of free carboxyl groups of glutamic acid or the number of the enzyme's aspartic acid residues side chains. The formation of isoforms is caused by the post translational deamidation of asparagine and glutamine residues (Raba et al., 1982). AChE purified from snake venom consists of soluble, hydrophilic monomers. The molecular weight of desert cobra *(Walterinnesia aegyptia)* AChE is identical to that of the cobra (*Naja naja oxiana*), i.e 67±3 KDa. However, in other sources of AChE it is possible to find a number of subunits connected through disulphide and the enzyme presents a different molecular weight (Raba et al., 1979; Li and Bon, 1983; AI-Saleh et al., 1994). The isoelectric points of desert cobra AChE range between pH 7.4-7.9, which is different from other venom AChEs, where the isoeletric points range from pH 6.3-7.3 (Duhaiman et al., 1996). These data indicate that the desert cobra AChE isoform has more basic amino acid residues than does the enzyme from other snakes. A recent investigation on purified AChEs from the *Bungarus* genera revealed that snake venom contains a monomeric form of AChE which presents a molecular weight of 70 kDa in agreement with the reported monomeric structure of AChE from *Naja naja oxiama*. Furthermore, the isoelectric points of *Bungarus* AChE range from 5.2-5.8 (Cousion et al., 1996).

3.2.4. Enzymatic and biochemical properties

Elapid AChE exhibits similar biochemical properties to those of other membrane bound AChEs, both in terms of substrate hydrolysis and inhibition by excess of substrate (Kesvatera et al., 1979; Kumar and Elliot, 1975; Agbaji et al., 1984; Frobert et al., 1997). The elapid AChE enzyme is stereo specific in the hydrolysis of acetyl-β-

methylcholine and do not hydrolyse butyryl, propyonil or benzoylcholine (Frobert et al., 1997). Venom AChE differs from other vertebrate tissue AChES in that it is soluble and composed of non-amphiphilic monomers.

Snake venom AChE is more stable than other AChEs. Among snake venom AChEs, *Bungarus* genera AChE is very high compared with that of *Haemacatus, Ophiophagus* and *Naja* (Frobert et al., 1997). Rochu et al. (2001) studied the thermal stability of monomeric *Bungarus* AChE using capillary electrophoresis and observed that the wild-type is a stable enzyme under standard conditions.

3.2.4.1. Venom AChE turnover number

Snake venom AChE exhibits the highest turnover number for ACh, ranging from 6100 to 7800 s^{-1} (Table 2). These values are lower than that of *Electrophorus* AChE (Vigny et al., 1978). Interestingly, in the crude venom as well as in the purified form, the turnover number for AChE was the same, demonstrating that the presence of another serine hydrolase does not affect venom AChE (Frobert et al., 1997). Among the Elapidae, the *Bungarus* (krait) genera has a high content, perhaps the highest content of AChE (8 mg/per gram of dry venom), about 654000±120000 Ellman units / g ofdry venom, while the activity of the *Naja* cobra genera, about 150000±108000 Ellman units/g of dry venom (Frobert et al., 1997).

3.2.4.2. Toxicity

Elapidae venom exhibits AChE activity and the *Bungarus* genera contains 747,000 Ellman's units per g of dry venom of AChE-like activity, being one of the richest venoms with AChE activity. The cloned enzyme from *Bungarus fasciatus* is non-toxic even when tested at a high concentration (80 mg/kg, intravenously).

Furthermore, it does not affect the other components of the venom nor does it enhance their activities (Cousin et al., 1996). The existence of elevated amounts of AChE or fasciculin in snake venom may be related to an attempt to disrupt cholinergic transmission in the central nervous system and at the neuromuscular junction of the preys.

One hypothesis about its toxicity is that the poisons and toxins that attack AChE, such as fasciculin, cause acetylcholine to accumulate in the nerve synapse, affecting the muscle, particularly at the neuromuscular junction. On the other hand, in venoms where AChE is present, the reverse can be observed. In fact, the depletion or accumulation of ACh could be deleterious for the prey. Taking together these conditions we can say that these venoms function as cholinotoxins.

Table 2. List of the different snake venoms tested for measurement of AChE activity and turnover number (From Frobert et al., Biochem. Biophys. Acta 1339:258, 1997. With permission).

ELAPIDAE		
Venom	**Activity (ELL units/g)**	**Turnover number (s^{-1})**
Bungarus fasciatus	505000-890000	6130-6920
Bungarus multicinctus	666000	7570
Bungarus caeruleus	747000	7340
Dendroaspis	6	ND
Haemocatus haemacates	157000	6430
Naja haje	282000-331000	7470-7800
Naja kaouthia	72900-79700	7520-7770
Naja nigricolis	380-22000	ND-7550
Naja naja naja	89200	7410
Naja naja atra	82000	7690
Naja nivea	147000-238000	7600-7690
Ophiophagus hannah	41800-84800	4350-6960

CROTALIDAE AND VIPERIDAE	
Venom	**Activity (ELL units/g)**
Bitis gabonica	0
Bitis lachesis	6- 146
Bothrops atrox	0
Bothrops lanceolatus	0
Echis carinatus	0
Crotalus durissus terrificus	0-6
Vipera aspis	0-6
Vipera russeli	6
Vipera ammodytes	0

4. Sequence alignment study of snake venom AChE

Bungarus fasciatus venom AChE presents a very close resemblance to that of *Torpedo* and mammalian AChE. The catalytic domain shows more than 60% identity and 80% homology among these enzymes. *Bungarus* venom AChE has four *N*-glycosylation sites which correspond to the glycosylated position in *Torpedo* and mammalian AChEs. The six cysteine residues, which play an important role not only in the formation of intermolecular disulphide loops but also in the catalytic triad (Ser-200, Glu-327, and His 440) and the tryptophan residue (Trp-84), are present in all types of cholinesterase (Weise et al., 1990). The aromatic amino acid near the active gorge of *Torpedo* AChE (Sussaman et al., 1991) is conserved in the *Bungarus* AChE. The only differences observed were at the peripheral site, where tyrosine 70 was replaced by methionone and lysine 285 was replaced by aspartic/glutamic acid. The C-terminal region of *Bungarus* AChE has a short hydrophilic peptide of 15 residues which contains six arginine and two aspartic acid residues.

4.1. Role of 70 and 285 residues

Snake venom AChE is different from the AChEs from other sources in its sensitivity to peripheral site ligands. Labeling and mutagenesis studies have indicated that the peripheral site is located at the mouth of a catalytic gorge (Sussman et al., 1991) about 20 A^0 from the active site (Berman et al., 1980; Kreienkamp et al., 1991; Harel et al., 1995). Comparing the amino acid sequence of venom AChE with that of mammalian and *Torpedo* AChEs reveals that the difference is at positions 70 and 285 where there are methionine and lysine residues instead of tyrosine and glutamic/aspartic acid. The site-directed mutagenesis study indicated that any modification of one or both of these two residues changes the enzymatic properties at the peripheral site level of the

venom (*Bungarus*) AChE. Furthermore, these changes make the enzyme less sensitive to the ligands which bind at the peripheral site of *Bungarus* AChE such as propidium, gallamine and fasciculin (Cousin et al., 1996).

4.2. Comparative inhibitory study of venom AChE

AChE from venoms varies in terms of sensitivity to natural anticholinesterase inhibitors called fasciculins (FAS; 61 amino acid peptides) isolated from the venom of *Dendroaspis* snakes (Karlsson et al., 1984) *Ophiophagus* AChE is more sensitive to fasciculins ($IC_{50}=10^{-10}$ M) than are *Naja*, *Bungarus* and *Heamacatus* AChEs ($IC_{50}=10^{-6}$, $IC_{50}=10^{-8}$ and $IC50=10^{-6}$ M, respectively) (Table 3). The main mechanism of AChE inhibition by FAS is related to its binding to the peripheral binding site near the rim of the gorge, sterically occluding the ligand access to the active site and/or by allosteric influence, locking the enzyme in a closed conformation (Radic et al., 1994; 1995; 2005).

Table3- IC_{50} values (in Mole) of different inhibitors of AChE (From Frobert et al., Biochem. Biophys. Acta 1339:263, 1997, with permission).

	Fascilculin	**BW 284C51**	**Propidium**	**Tacrine**	**Edrophonium**	**Decamethonium**
Bungarus fasciatus (lot 6)	$1.2x10^{-8}$	$4x10^{-9}$	$3.6x10^{-5}$	$4.5x10^{-8}$	$2.3x10^{-6}$	$7x10^{-6}$
Bungarus fasciatus (lot 8)	$1.5x10^{-8}$	$3.6x10^{-9}$	$2.7x10^{-5}$	$5x10^{-8}$	$2.4x10^{-6}$	$7x10^{-6}$
Bungarus fasciatus (China1)	$1.6x10^{-8}$	$3.2x10^{-9}$	$2.6x10^{-5}$	$5x10^{-8}$	$2.2x10^{-6}$	$12x10^{-6}$
Bungarus multicinetus	$8.5x10^{-9}$	$4x10^{-9}$	$6.2x10^{-5}$	$5x10^{-8}$	$2.2x10^{-6}$	$13x10^{-6}$
Bungarus caeruleus	$1.3x10^{-8}$	$5.2x10^{-9}$	$3x10^{-5}$	$4x10^{-8}$	$2.7x10^{-6}$	$7x10^{-6}$
Haemacatus haemacates	$>>10^{-6}$	$7.3x10^{-9}$	$2.8x10^{-5}$	$4.6x10^{-8}$	$3.8x10^{-6}$	$18x10^{-6}$
Naja haje	$>>10^{-6}$	$7.5x10^{-9}$	$2.5x10^{-5}$	$7.3x10^{-8}$	$2x10^{-6}$	$15x10^{-6}$
Naja kaoutia	$>>10^{-6}$	$6.4x10^{-9}$	$1.9x10^{-5}$	$7x10^{-8}$	$3x10^{-6}$	$13x10^{-6}$
Naja naja	$>>10^{-6}$	$9x10^{-9}$	$2x10^{-5}$	$8x10^{-8}$	$3.5x10^{-6}$	$15x10^{-6}$
Naja nívea	$>>10^{-6}$	$6.7x10^{-9}$	$2.5x10^{-5}$	$8x10^{-8}$	$3.5x10^{-6}$	$13x10^{-6}$
Ophiophagus hannah	$5x10^{-11}$	$4.5x10^{-9}$	$4.5x10^{-5}$	$4.2x10^{-8}$	$2.7x10^{-6}$	$11x10^{-6}$

RESULT PRESENTATION

The results are presented in the form of published articles and submitted manuscripts. The materials and methods are stated in the articles.

1) Krait (*Bungarus sindanus*) snake venom acetylcholinesterase: biochemical and enzymatic characterization (submitted)

2) Inhibition of two different cholinesterases by tacrine (published)

3) Malathion, carbofuran and paraquat inhibit *Bungarus sindanus* (krait) venom acetylcholinesterase and human serum butyrylcholinesterase in vitro (published).

4) Comparative study of the inhibitory effect of antidepressants on cholinesterase activity in *Bungarus sindanus* (krait) venom, human serum and rat striatum (published)

5) Interaction of tetramethylethylene diamine (TEMED) with venom acetylcholinesterase and horse serum butyrylcholinesterase (submitted)

6) Anticholinesterase action of N,N,N´,N´- Tetramethylethylene diamine (TEMED) *in vitro* and in *vivo* in rats (submitted)

General Discussion of Results

General Conclusion

References from the introduction, literature revision and general discussion are presented together at the end of the thesis.

Chapter 1-Manuscript

Krait (*Bungarus sindanus*) snake venom acetylcholinesterase: biochemical and enzymatic characterization.

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(Submitted to the Protein Journal)

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Abstract

Elapidae venom is well-known due to its highest acetylcholinesterase (AChE) activity, close to 8 mg/g of dried venom (0.8 % w/w). No other tissue or biological fluid demonstrates such a high level of AChE activity. In the present study, the venom of *Bungarus sindanus*, an Elapidae snake, contained a high level of AChE activity. The enzyme showed optimum activity at alkaline pH 8.5 with an optimal temperature of 45°C. The snake venom AChE was inhibited by excess substrate. We observed a significant reduction in substrate inhibition of krait (*Bungarus sindanus*) venom AChE by using a high ionic strength buffer. With a low ionic strength buffer (10 mM PO_4 pH 7.5) the enzyme was inhibited by 1.5 mM AcSCh, while with a high ionic strength buffer (62 mM PO_4 pH 7.5) the enzyme was inhibited by 1 mM AcSCh. Furthermore we found that krait venom AChE is thermally stable at 45°C. The enzyme lost only 5% of its activity after incubation at 45°C for 40 min. The Michaelis-Menten constant (Km) for the hydrolysis of acetylthiocholine iodide was found to be 0.052 mM. We noted that snake venom (*Bungarus sindanus*) acetylcholinesterase was also inhibited by $ZnCl_2$, $CdCl_2$ and $HgCl_2$ in a concentration dependent manner. The present study was designed in an attempt to discover more about krait venom AChE, in terms of thermal stability, substrate specificity, substrate inhibition, ionic strength, pH, temperature and metals. Due to high level of catalytic activity and because it is more stable than any other sources, krait (*Bungarus sindanus)* venom is very valuable for the biochemical study of this enzyme.

Keywords: AcetylcholinesteraseIonic strength; Acetylthiocholine iodide

1. Introduction

Acetylcholinesterase is present in all vertebrates, particularly in the muscles and nervous tissues (Rotundo, 2003). Structurally and functionally, acetylcholinesterase is a serine hydrolase (AChE, acetylcholine hydrolase, EC. 3.1.1.7). The enzyme is found both in synaptic and non-synaptic tissues (Barnard, 1974; Quinn, 1987; Rotundo, 2003). In synaptic tissues, the major role of AChE is to hydrolyze the neurotransmitter acetylcholine (ACh). The catalytic activity of this enzyme is essential for normal cholinergic transmission and for neuromuscular function (Rosenberry, 1975). In non-synaptic contexts, AChE and BChE are expressed in early embryonic development and some time before the formation of cholinergic synapses (Drews, 1975; Layer et al., 1985), where their function is the formation and regulation of the acetylcholine gradient that guides the growth of nerve cells (Layer et al., 1988). A noteworthy amount of acetylcholinesterase is also present in the blood cells, particularly in erythrocytes and lymphocytes (Kumar and Elliott, 1973; Thiermann et al., 2005). The most common example of non-synaptic tissues containing AChE is snake venom, where its function is unknown (Iwanaga and Suzuki, 1979; Frobert et al., 1997). Acetylcholinesterase exists in all *Elapidae* venom except in mambas (Bawaskar and Bawaskar, 2004). A recent study indicated that acetylcholinesterase is also present in the *Crotiladae* family, which presents mostly non-poisonous venom (Mackessy et al., 2006). In the *Elapidae*, the *Bungarus* genera is well-known due to the presence of high a amount of acetylcholinesterase, about 8 mg/g of dried venom (0.8 % w/w), with activity (> 60,0000 Elman units/mg) (Frobert et al., 1997). The enzyme reaction catalysed by acetylcholinesterase is one of the most efficient reactions. A partial peptide sequence study showed that snake venom enzymes have a close homology with other acetylcholinesterases and have the same catalytic triad for substrate hydrolysis (Cousin

et al., 1996). The efficiency of substrate hydrolysis could be related to three-dimensional structures of the enzyme. The long narrow active site gorge is 20 Å deep, composed of two subsites for ligand interaction: an acylation site at the base of the gorge with a catalytic triad and peripheral site at its mouth, distinct from the catalytic active site (Szegletes et al., 1999; Rosenberry et al., 2005).

Previously, we found that snake venom (*Bungarus sindanus*) AChE is inhibited by commonly used pesticides and herbicides with similar inhibitory behavior as that of human serum butrylcholinesterase (BChE) (Ahmed et al., 2007). Furthermore, krait (*Bungarus sindanus*) venom AChE shows high sensitivity towards tacrine, commonly use for the treatment of Alzheimer's disease (Ahmed et al., 2006). However, snake venom AChE is different from other vertebrate tissues in the fact that it is present in a soluble, non-amphiphilic, monomeric form (Cousin et al., 1996). The present study was designed in an attempt to discover more about krait venom AChE, in terms of thermal stability, substrate specificity, substrate inhibition, ionic strength, pH, temperature and sensitivity to metals.

2. Materials and methods

2.1. Materials

Acetylthiocholine iodide, butyrylthiocholine iodide, DTNB [5,5´-dithiobis(2-nitro-benzoic acid)], bovine serum albumin, coomassive Brilliant blue R-250 and electric eel acetylcholinesterase were purchased from Sigma (St. Louis, MO, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Neon Comercial LTDA, Brasil. Tris (hydroxymethyl amino methane) from Vetec-Brazil. All other reagents used were of analytical grade.

2.2. Venom

Mature live Krait (*Bungarus sindanus*) snakes of both sexes were captured through special design stick by snake man. Venom of two to three drops was squeezed out manually from each snake, mixed, lyophilized immediately and stored at −20°C for further study.

2.3. Protein determination

Protein was assayed by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

2.4. Statistical Analysis

Statistical analysis was performed using one way ANOVA, which was followed by post-hoc analysis (Duncan multiple range test).

2.5. Cholinesterase assay

Cholinesterase activities were determined by the method of Ellman (Ellman et al., 1961) and modified by Rocha (Rocha et al., 1993). Hydrolysis rates (V) were measured at various acetylthiocholine (S) concentrations (0.01–3 mM) in 1 mL assay solutions with 10 mM or 62 mM phosphate buffer, pH 7.5, and 0.2 mM DTNB at 25°C. 40 µL snake venom (4 µg of protein) were added to the reaction mixture and pre-incubated for 30 min at 37°C. We did not find the presence of butyrylcholinesterase activity in the crude venom. Therefore we did not use ethopropazine (a specific inhibitor of butyrylcholinesterase) throughout the enzyme essays. The hydrolysis was monitored by verifying the formation of the thiolate dianion of DTNB at 412 nm at an interval of

15 seconds over 2–3 min using a Hitachi 2001 spectrophotometer. All samples were run in duplicate or triplicate.

2.6. K_m and V_{max} determination

K_m and V_{max} were determined by a double reciprocal of Lineweaver-Burk plot (Lineweaver and Burk 1934).

3. Results

To evaluate the specificity of substrate for snake venom acetylcholinesterase (AChE), we tested acetylthiocholine (AcSCh) and butyrylthiocholine (BuSCh) as a substrate, varying from 0.01 to 3 mM. Krait (*Bungaraus sindanus*) venom AChE only hydrolyzed acetylthiocholine (AcSCh) (Fig.1). The K_m and V_{max} for the substrate (acetylthiocholine) were calculated by a double reciprocal plot of Lineweaver-Burk (Lineweaver and Burk 1934) and the K_m was found to be 0.052 mM while the V_{max} was 530 µmole/min/mg protein (Fig.2). AChE was inhibited by the excess substrate. However, the inhibition of the substrate concentration varied in relation to the ionic strength of the buffer. We distinctly observed that with a low ionic strength buffer (10 mM PO_4 Buffer pH 7.5) the enzyme was inhibited by 1.5 mM substrate while with a high ionic strength buffer (62 mM mM PO_4 Buffer pH 7.5) AChE became more sensitive and was inhibited by 1 mM acetylthiocholine (Fig.3). The enzyme was found to be pH-sensitive and showed highest activity in alkaline media (pH 8.5) while in acid media (pH 4) the enzyme was not active (Fig.4). The enzyme became slightly more active at 45°C for substrate hydrolysis (Fig.5). Krait venom AChE retained 100% of its activity at 37°C after 40 min incubation (Fig.6A). Furthermore, this enzyme was thermally stable at 45°C. The enzyme lost only 5% of its activity after incubation at 45°C for 40 min (Fig.6A). In comparison, the electric eel acetylcholinesterase lost 69%

of its activity after 10 minutes at 45°C in relation to incubation at 37°C, while it lost 80% of its activity after 40 minutes at 45°C when compared with incubation at 37°C (Fig.6B). The *Bungaraus sindanus* venom AChE was also inhibited by $Zncl_2$, $CdCl_2$ and $HgCl_2$ in a concentration dependent manner (Fig.7). The inhibition of krait (*Bungarus sindanus*) venom AChE by metals was also confirmed by the electrometric method, using the substrate acetylcholine (Data not shown).

4. Discussion

In the present study, we show that the venom of *Bungarus sindanus* contains true AChE, which presents characteristic catalytic properties, as previously reported (Kumar and Elliott, 1973). Among the elapidae, the krait (*Bungarus sindanus)* are the richest source of AChE. The optimum substrate concentration was 1 mM using a medium of 10 mM PO_4, pH 7.5 (Fig.1). This concentration is much lower when compared to the desert cobra (*Walterinnesia aegyptia*), in which the optimum activity was observed at 3 mM (AlJafari, 1995). At a higher substrate concentration, above 1 mM, the hydrolytic property of krait venom AChE decreased. The same type of substrate inhibition has also been observed with other snake venom AChEs (Kreienkamp et al., 1995; AlJafari et al., 1995; Frobert et al., 1997). *Bungarus sindanus* AChE was specific for acetylthiocholine, as it did not hydrolyze butyrylthiocholine (Fig.1). Snake venom AChE from all other genera also lacks the property of hydrolyzing butyrylthiocholine (Frobert et al., 1997). The Michaelis-Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide was found to be 0.052 mM with a V_{max} of 530 µmole/min/mg protein (Fig.2). The V_{max} of the *Bungarus* genera is very high compared to that of other genera of Elapidae. We observed a significant reduction in substrate inhibition of krait venom AChE by using high ionic strength buffer. With a

low ionic strength buffer (10 mM PO_4 pH 7.5), the enzyme was inhibited by 1.5 mM AcSCh while with a high ionic strength buffer (62 mM PO_4 PH 7.5) the enzyme was inhibited by 1mM AcSCh. It is worth noting that a significant reduction in substrate inhibition with the use of a high ionic strength buffer was also mentioned by Frobert (Frobert et al., 1997). Furthermore, with a high ionic strength buffer (62 mM PO_4 PH 7.5), the enzyme showed higher activity when compared with a low ionic strength buffer (10 mM PO_4 pH 7.5) Fig.3. The *Bungarus sindnus* enzyme showed optimum activity at pH 8.5. The AChE from all other sources also shows higher activity in an alkaline media (Aliriz and Turkoglu, 2003). The optimal temperature was 45°C, which is higher than that for the desert cobra (*Walterinnesia aegyptia*), which shows its highest activity at 30°C (AlJafari et al., 1995). Furthermore we found that krait (*Bungarus sindnus*) venom AChE was thermally stable at 45°C. The enzyme lost only 5% of its activity after incubation at 45°C for 40 min (Fig.6A) while at 37°C, we did not find any loss in its activity (Fig.6A). In comparison, the AChE from the electric eel lost 69 and 80% of its activity at 45 ^{o}C after 10 and 40 min incubation repectively, compared with with incubation at 37°C (Fig.6B). Generally, snake venom AChE is more stable that from other sources. Among snake venoms, that of the *Bungarus* genera is more stable than that of the *Haemacatus, Ophiophagus* and *Naja* genera (Frobert et al., 1997). A thermal stability study of *Bungarus* AChE by capillary electrophoresis also supports that venom AChE is stable under standard conditions (Rochu et al., 2001). Furthermore, $ZnCl_2$ (A), $CdCl_2$ (B) and $HgCl_2$ (C) (Fig.7) can also be considered inhibitors of snake venom AChE. The concentration of these metals necessary to inhibit snake venom AChE is very high. This may be due to the absence of a free sulfhydryl group. According to Frasco (Frasco et al., 2007), when a free sulfhydryl group is absent in the enzyme (*Drosophila melonogaster* acetylcholinesterase and human serum

butyrycholinesterase) inhibition by mercury will occur in the millimolar range, while in the presence of a free sulfhydryl group (*Electrophorous electricus*), the inhibition will require a micromolar range concentration (Frasco et al., 2007). The inhibitory effect of metals on venom acetylcholinesterase may be due to formation of inactive aggregation of the enzyme (Lee and Singleton, 2004).

In conclusion, the *Bungarus* venom exhibits large amounts of AChE with the highest catalytic activity of all sources and it is comparatively more stable than any other source, making this source more valuable for biochemical study.

Acknowledgements

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References

Ahmed, M., Rocha, J.B.T., Correa, M., Mazzanti, C.M., Zanin, R.F., Morsch, A.L., Morsch, V.M., Schetinger, M.R.C. (2006) *Chem. Biol Interact* **162**(2):165-71

Ahmed, M., Rocha, J.B.T., Mazzanti, C.M., Morsch, A.L., Cargnelutti, D., Correa, M., Loro, V., Morsch, V.M., Schetinger, M.R.C. (2007) *Ecotoxicology* **16**(4):363-369

AlJafari, A.A., Kamal, M.A., Duhaiman, A.S., Alhomida, A.S. (1995) *Mol Cell Biochem* **151**(1):21-6

Aliriz, S., Turkoglu, V. (2003) *Prep Biochem Biotechnol* **33**:137-145

Barnard, E.A. (1974) (Hubbard, J.I., ed), *Plenum press*, New York, 201-224

Bawaskar, H.S., Bawaskar, P.H. (2004) *Wilderness Environ Med* **15**(4):257–266

Bradford, M.M. (1976) *Anal Biochem* **72**:248–254

Cousin, X., Créminon, C., Grassi, J., Méflah, K., Cornu, G., Saliou, B., Bon, S., Massouliém J., Bonm, C. (1996) *FEBS Lett* **387**:196-200

Drews, U. (1975) *Prog Histochem Cytochem* **7**(3):1–52

Ellman, G.L., Courtney, K.D., Andres, Jr.V. (1961) *Biochem Pharmacol* **7**:88–95

Frasco, M.F., Colletier, J-P., Weike, M., Carvalho, F., Guilhermino, L., Stojan, J., Fournier, D., (2007) *FEBS* **274**:1849-1861

Frobert, Y., Créminon, C., Cousin, X., Rémy, M., Chatel, J., Bom, S., Bom, C., Grassi, J. (1997) *Biochim Biophys Acta* **1339**(2):253–267

Iwanaga, S., Suzuki, T. (1979) *Hand book of Experimental Pharmacology* **52**:61-158

Kumar, V., Elliott, W.B. (1973) *Eur J Biochem* **34**:586-592

Kreienkamp, H.J., Weise, C., Raba, R., Aaviksaar, A., Hucho, F. (1991) *Proc Natl Acad Sci USA* **14**:6117–6121

Layer, P.G., Girgert, R., Hahn, U. (1988) *Development* **102**:387-396

Layer, P.G., Girgert, R., Rommel, S., Spoms, O. (1985) *J Neurochem* **44**:129-131

Lee, A.M., Singleton, S.F. (2004) *J Inorg Biochem* **98**:1981–1986

Lineweaver, H., Burk, D. (1934) *J Am Chem Soc* **56**:658–666

Mackessy, S.P., Sixberry, N.M., Heyborne, W.H., Fritts, T. (2006) *Toxicon* **47**:537-548

Quinn, D.M. (1987) *Chem Rev* **87**:955–979

Rosenberry, T.L. (1975) *John Wiley & Sons*, New York. pp 43

Rotundo, R.L. (2003) *J Neurocyto* **32**:743–766

Rocha, J.B.T., Emanuelli, T., Pereira, M.E .(1993) *Acta Neurobiol Exp***53**: 431–437

Rosenberry, T.L., Johnson, J.L., Cusack, B., Thomas, J.L., Emani, S. (2005) *Chem Biol Interact.* **157–158**:181–189

Rochu, D., Georges, C., Répiton, J., Viguié, Daliou, N., Bon, C., Masson, P. (2001) *Biochim Biophys Acta* **1545**:216-226

Szegletes, T., Mallander, W.D., Thomas, P.J., Rosenberry, T.L. (1999) *Biochemistry* **38**:122–133

Thiermann, H., Szinicz, L., Eyer, P., Zilker, T., Worek, F. (2005) *Chem Biol Interact* **157**:345-347

LEGENDS OF THE FIGURES

Fig.1. Substrate specificity. Hydrolysis of acetylthiocholine (AcSCh) and butyrylthiocholine (BuSCh) by Krait (*Bungarus sindanus*) venom AChE. The venom protein (4µg) was pre-incubated for 10 min in 1 mL assay solutions with 10mM PO_4 Buffer, pH 7.5, and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)] before addition of substrate using from 0.05 to 3 mM. The result represents the mean of three different experiments.

Fig. 2. The Lineweaver-Burk plot, representing the reciprocal velocity of the initial enzyme versus AcSCh concentration for K_m determination. The K_m is 0.052 mM while V_{max} is 530 µmole/min/mg protein.

Fig. 3. Increase of substrate inhibition of enzyme in a high ionic strength buffer. Hydrolysis of acetylthiocholine in 10 mM PO_4 Buffer, pH 7.5, and 62 mM PO_4 buffer, pH 7.5, by Krait (*Bungarus sindanus*) venom AChE. The venom AChE was pre-incubated in the media for 10 minute at 37°C before the addition of different concentrations of substrate (0.01-3 mM).The results represent the mean of four different experiments done in duplicate.

Fig. 4. The effect of pH on the enzyme activity was analyzed by incubating the enzyme for 10 minute in different buffers; 10 mM sodium acetate buffer, pH 4 and 5.5, 10 mM PO_4 buffer, pH 6.5 and 7.5, 10 mM Tris-HCl buffer, pH 8.5-10.5, at 37°C before the addition of 1 mM acetylthiocholine as a substrate. The result represents the mean of three different experiments.

Fig. 5. The effect of temperature on the enzyme activity was analyzed by incubating the enzyme for 10 minute at different temperatures; 25, 37, 45, 50, and 60°C before the addition 0.2 mM DTNB and 1mM acetylthiocholine as substrate. The results represent the mean of four different experiments and similar results were obtained.

Fig. 6. Thermal stability was studied by incubating the Krait (*Bungarus sindanus*) venom AChE (A) and pure electric eel (B) at different temperatures (37 – 50)°C for different time intervals (10–40 minutes) before the addition of 0.2 mM DTNB and 1mM acetylthiocholine as substrate.

Fig.7. Inhibition of krait (*Bungarus sindanus*) venom AChE by metals. Various concentrations of $ZnCl_2$ (A), $CdCl_2$ (B) and $HgCl_2$ (C) were incubated at 37°C for 10 min in 10 mM Tris-HCl buffer, pH 8.5. The reaction was started by the addition of 1mM acetylcthiocholine (AcSCh) as substrate. $^*P < 0.029$; $ZnCl_2$ (A), $^*P < 0.00028$; $CdCl_2$ (B) and $^*P < 0.000128$ for $HgCl_2$ (C) when compared with the control. The results represent the mean of four different experiments done in duplicate.

FIGURES

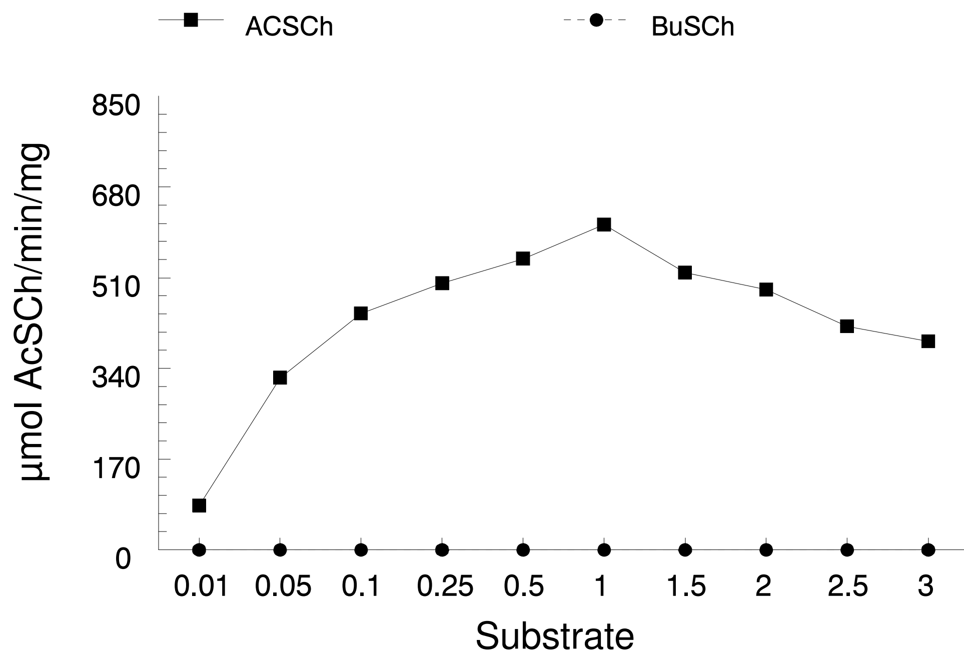
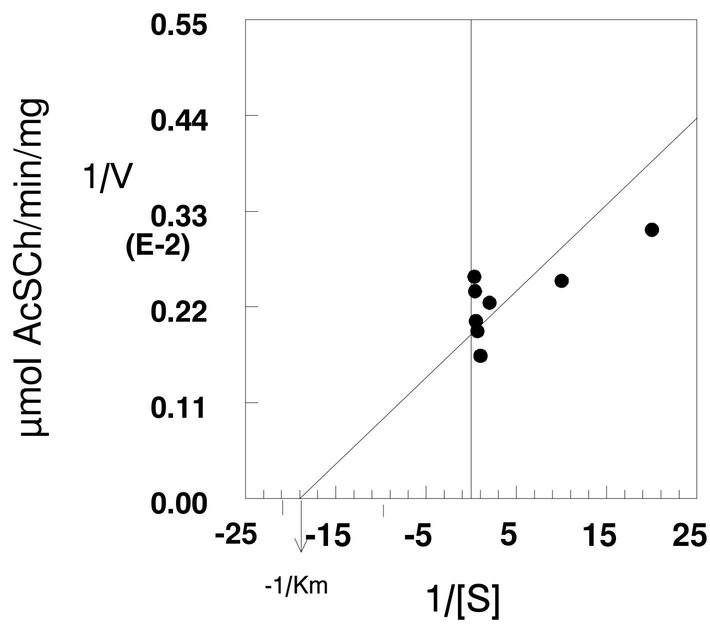


Fig.1



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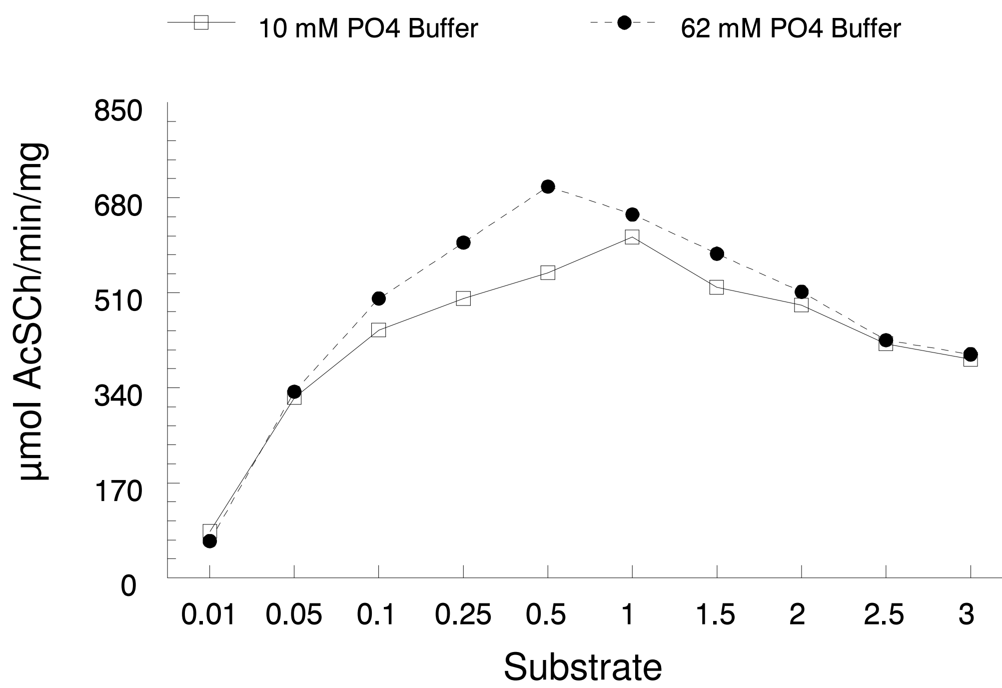

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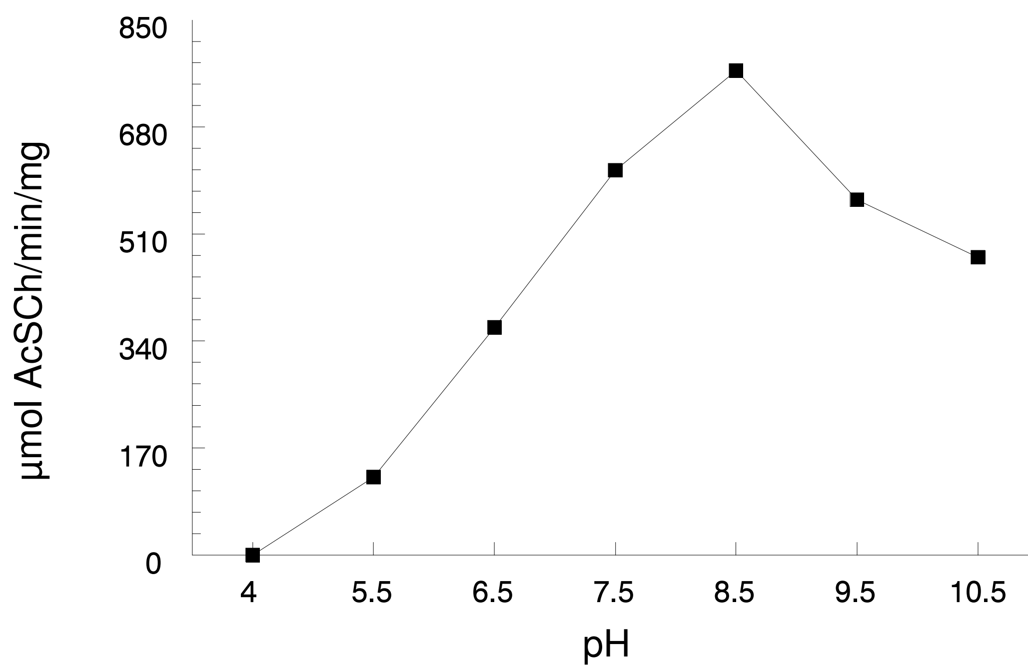

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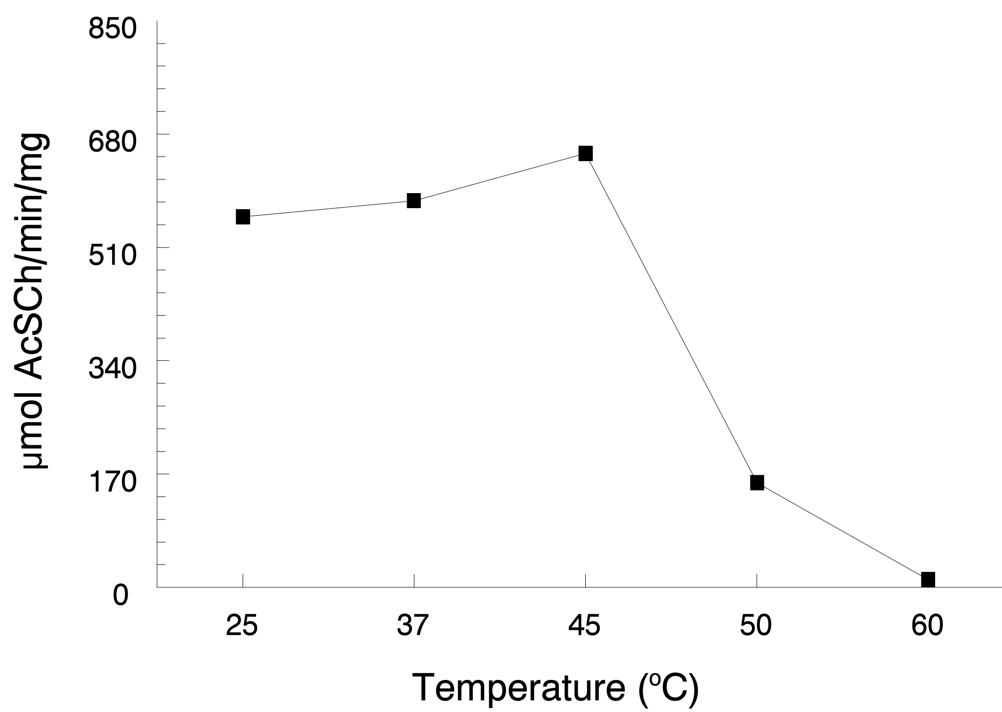
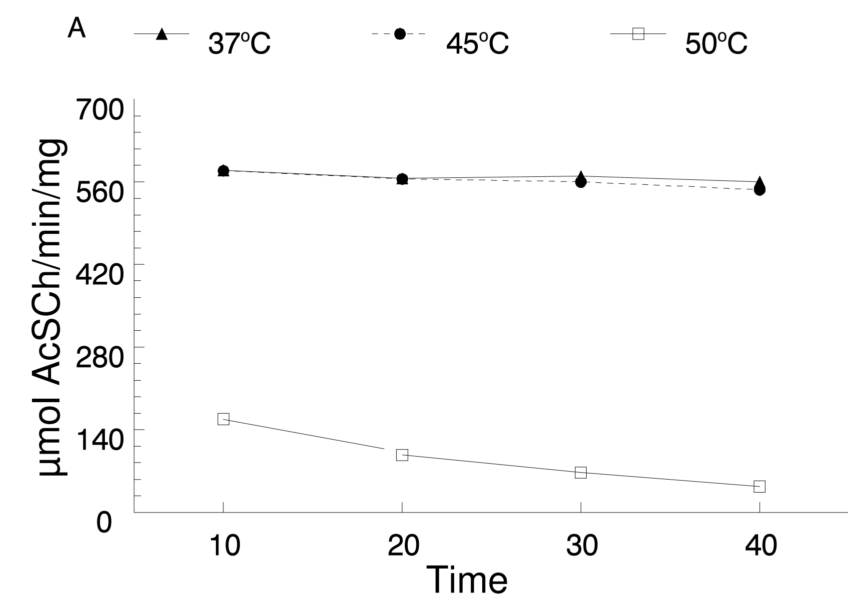


Fig.5



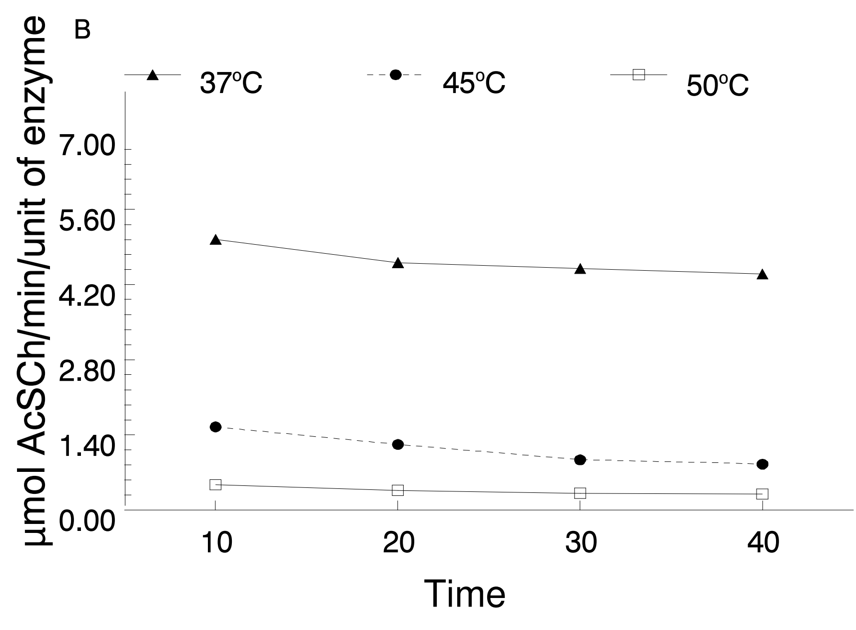


Fig.6

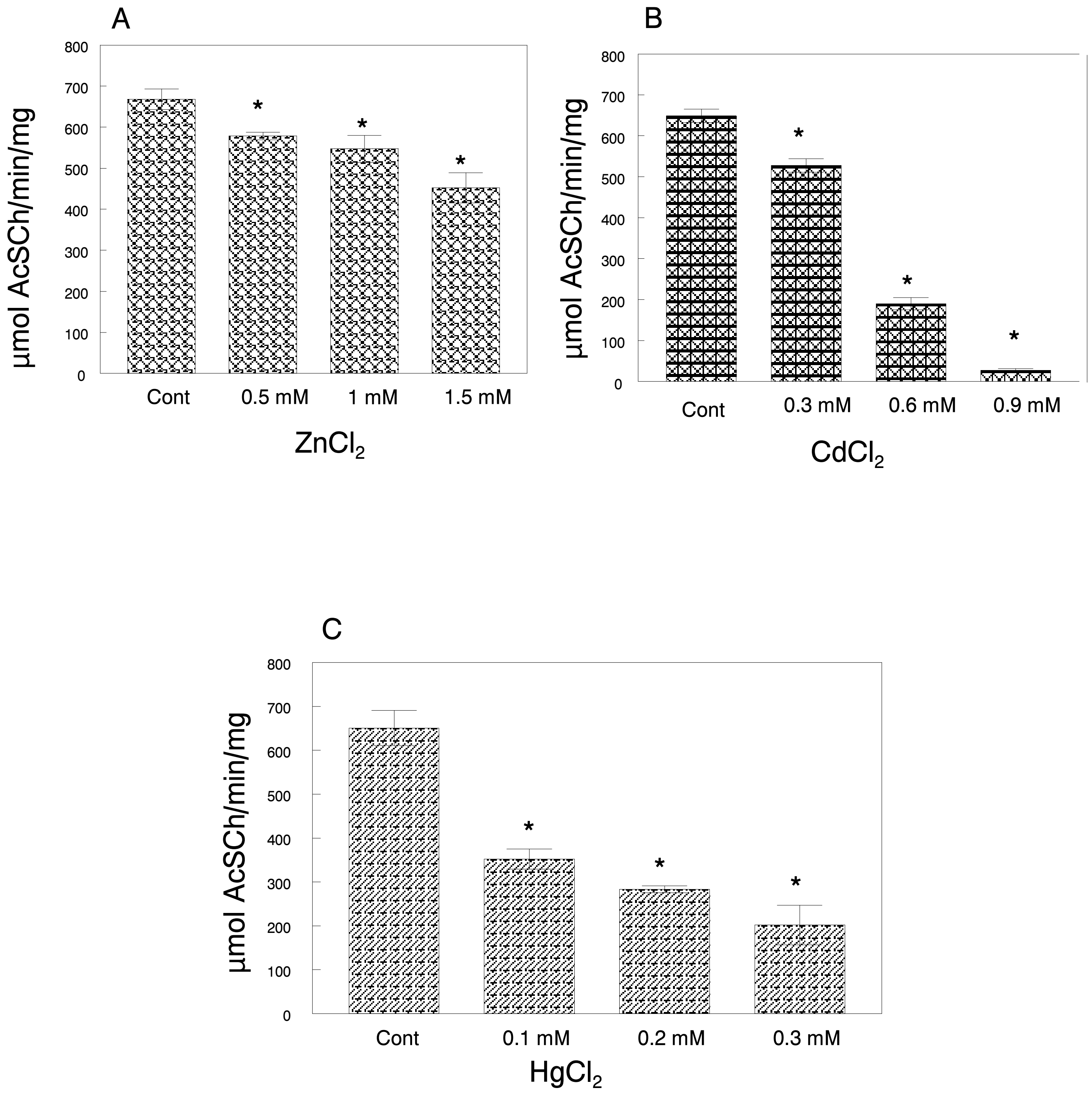


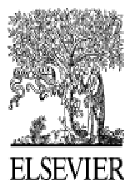
Fig.7

Chapter 2- Published Paper

Inhibition of two different cholinesterases by tacrine

Mushtaq Ahmed, João Batista T. Rocha, Maísa Corrêa, Cinthia M. Mazzanti, Rafael F. Zanin, André L. B. Morsch, Vera Maria Morsch, Maria R.C. Schetinger*

Chemico-Biological Interactions 2006; 162: 165-171.



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Inhibition of two different cholinesterases by tacrine

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Abstract

Kinetic parameters of the effect of tacrine as a cholinesterase inhibitor have been studied in two different sources: snake venom (*Bungarus sindanus*) acetylcholinesterase (AChE) and human serum butyrylcholinesterase (BChE). Tacrine inhibited both venom acetylcholinesterase (AChE) as well as human serum butyrylcholinesterase (BChE) in a concentration-dependent manner. Kinetic studies indicated that the nature of inhibition was mixed for both enzymes, i.e. K_m values increase and V_{max} decrease with the increase of the tacrine concentration. The calculated IC_{50} for snake venom and for human serum were 31 and 25.6 nM, respectively. K_i was observed to be 13 nM for venom acetylcholinesterase (AChE) and 12 nM for serum butyrylcholinesterase (BChE). K_I (constant of AChE–ASCh–tacrine complex into AChE–ASCh complex and tacrine) was estimated to be 20 nM for venom and 10 nM for serum butyrylcholinesterase (BChE), while the γK_m (dissociation constant of AChE–ASCh–tacrine complex into AChE–tacrine complex and ASCh) were 0.086 and 0.147 mM for snake venom AChE and serum BChE, respectively. The present results suggest that this therapeutic agent used for the treatment of Alzheimer's disease can also be considered an inhibitor of snake venom and human serum butyrylcholinesterase. Values of K_i and K_I show that tacrine had more affinity with these enzymes as compared with other cholinesterases from the literature.
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Keywords: Tacrine; Cholinesterase; Snake venom; Human serum

1. Introduction

In vertebrates, there are two types of cholinesterases which are distinguished on the basis of their substrate specificities, distribution in various tissues and sensitivity toward various inhibitors. They are acetylcholinesterae (AChE; E.C. 3.1.1.7) or true cholinesterase/specific cholinesterase and butyrylcholinesterase (BChE; E.C. 3.1.1.8) or pseudocholinesterase/non-specific cholinesterase.

Acetylcholinesterase is a membrane-bound enzyme mainly found in the brain, muscles, erythrocytes and cholinergic neurons. It plays a major role in the regulation of several physiological events [1,2] by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses [3,4]. BChE is found in the intestine, liver, kidney, heart, lung, and serum and plays a major role in the metabolism of ester containing compounds [5–7]. Butyrylcholinesterase can also take the place of AChE in acetylcholine (ACh) degradation when acetylcholinesterase is inhibited or absent [8,9].

Both AChE and BChE have some homology; 65% of their amino acid sequence is the same, and they have the

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"catalytic triad" for substrate hydrolysis [10]. Venom AChE was first reported as a monomeric form [11]. AChE occurs at high levels in Elapid snakes, except for Mambas. In comparison, all snakes belonging to Viperid or Colubrid families lack this type of enzyme in their venom [12]. *Elapidae* poisoning is well known due to its high toxicity and death is common in several parts of the world, particularly in Asia [13,14]. Probably, death caused by *Bungarus* poisoning can at least in part be related to the presence of high quantities of AChE in the venom.

Tacrine is a well-known drug used for the treatment of Alzheimer's disease (AD), which inhibits cholinesterase, increasing the concentration of acetylcholine in the brain [15–17]. This increase is believed to be responsible for the improvement in memory with the use of tacrine. In addition, tacrine is very effective to inhibit plasma BChE [18].

Normally, in the healthy brain AChE is predominant. However, in AD brain BChE activity rises while AChE activity remains unchanged or diminished [19]. Therefore, a drug inhibiting both AChE and BChE, could have additive and potential therapeutic benefits. Since krait (*Bungarus sindanus*) venom is rich in AChE and human serum have high amount of BChE, they were selected as sources of cholinesterases. Therefore, it was of interest, to examine non-specific property and mode of inhibition of venom AChE by tacrine and compare the kinetic behavior with serum BChE, and perhaps, to extend it to brain BChE.

2. Materials and methods

2.1. Materials

9-Amino-1,2,3,4-tetrahydroarcidine hydrochloride hydrate (tacrine), DTNB [5,5′-dithiobis (2-nitro-benzoic acid)], acetylthiocholine iodide (used as a substrate), bovine serum albumin and ethopropazine were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Neon Commercial LTDA, Brazil. All other reagents used were of analytical grade.

2.2. Human serum

Human serum was obtained from healthy volunteers (24–33 years old) of both sexes with written consent. The protocol was approved by the Human Ethic Committee of the Federal University of Santa Maria. None of the volunteers had a recurrent or a past history of psychiatric illness, any significant medical disorder, or drug, cigarette or alcohol abuse. None of them had been taking any previous medication for at least 30 days. From all participants, 5 ml of blood was collected in vacutainer tubes, centrifuged at 3000 rpm for 10 min and the serum was collected, aliquoted and stored at −20 °C for further use.

2.3. Venom

Venom from live *B. sindanus* snakes was squeezed out manually, lyophilized immediately, aliquoted and stored at −20 °C for further use.

2.4. Cholinesterase assay

Cholinesterase activities were determined by the method of Ellman [20] modified by Rocha [21]. Hydrolysis rates (*V*) were measured at various acetylthiocholine (*S*) concentrations (0.05–1 mM) in 1 ml assay solutions with 62 mM phosphate buffer, pH 7.5, and 0.2 mM DTNB at 25 °C. Forty microliters of human serum (700 μg of protein) and snake venom (4 μg of protein) were added to the reaction mixture and pre-incubated for 10 min at 37 °C. About 0.06 mM ethopropazine (a classic selective, potent inhibitor of BChE) was used in the AChE assay. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min at intervals of 10 s using a Hitachi 2001 spectrophotometer.

2.5. Protein determination

Protein was assayed by the method of Bradford using bovine serum albumin as a standard [22].

2.6. Kinetic determinations

The kinetic parameters of the interaction between tacrine and cholinesterase were determined using the Lineweaver and Burk [23] double reciprocal plot analyzed over a range of acetylthiocholine concentrations (0.05–1 mM) in the absence and in the presence of tacrine (12.5–37.5 nM). K_m values were obtained by two different estimations, $1/V$ versus $1/S$ [23] and V versus V/S [24,25]. The K_i values were obtained using Cornish–Bowden plots of S/V versus [I]. IC_{50} was determined by percentage residual activity versus concentration of tacrine. K_I and γK_m values were calculated by using Dixon and Lineweaver and Burk plots, respectively [26,23].

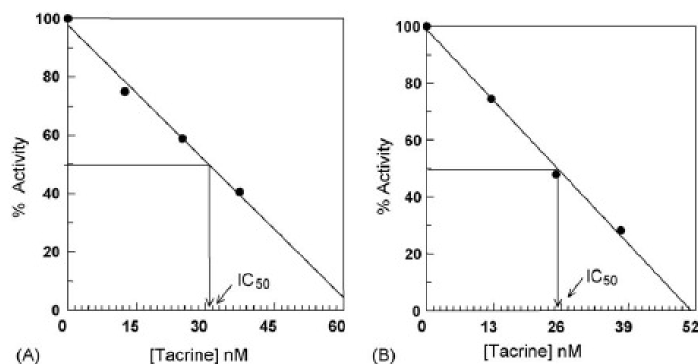

Fig. 1. A plot of the percentage residual activity vs. concentration of tacrine. Snake venom acetylcholinesterase (A) and human serum butyrylcholinesterase (B).

2.7. Statistical analysis

Statistical analysis was performed using one-way ANOVA, which was followed by post hoc analysis (Duncan multiple range test).

3. Results

The results showed that tacrine (12.5–37.5 nM) inhibited venom acetylcholinesterase as well as human serum butyrylcholinesterase in a concentration-dependent manner. The IC_{50}, calculated by plotting the percentage of residual activity versus [I], was 31 nm (Fig. 1A) for snake venom AChE and 25.6 nM for human BChE (Fig. 1B). Tacrine inhibited both snake venom AChE and serum BChE (Fig. 2). Statistical analysis revealed a concentration-dependent inhibition of both enzymes.

K_m values for the hydrolysis of substrate (ASCh) by snake venom AChE and human serum BChE, calculated by using the Lineweaver and Burk plot, were 0.0537 mM (Fig. 3) and 0.0299 mM (Fig. 4), respectively. The V_{maxapp} and K_{Iapp} (dissociation constant of AChE–ASCh–tacrine complex into AChE–ASCh com-

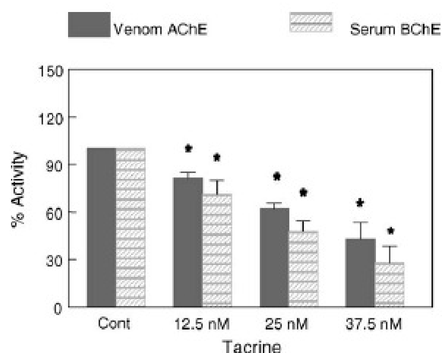

Fig. 2. Acetylcholinesterase experiments in the presence and absence of different concentrations of tacrine. Hydrolysis rates (V) were measured at 412 nm by using 0.4 mM substrate (S) concentration in 1 ml assay solutions with 62 mM phosphate buffer (pH 7.4) and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)] was pre-incubated for 10 min before addition of 0.5 mM substrate. All experiments were repeated at least three times and similar results were obtained. For venom $^*P < 0.01$ and for serum $^*P < 0.0003$ significantly different from control.

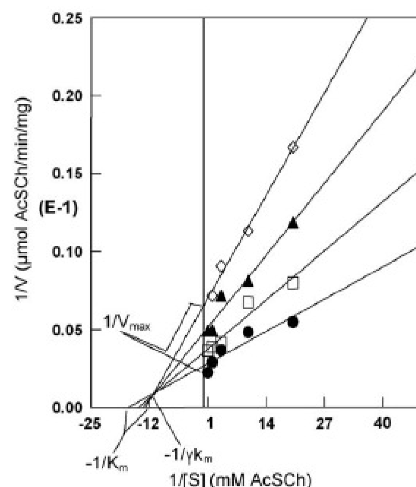

Fig. 3. Lineweaver and Burk plots representing reciprocals of initial snake venom (*Bungarus sindanus*) enzyme velocity vs. reciprocal of ASCh concentration in the absence and the presence of different concentrations of tacrine (0–37.5 nM). (●) 0 nM; (□) 12.5 nM; (▲) 25 nM; (◇) 37.5 nM.

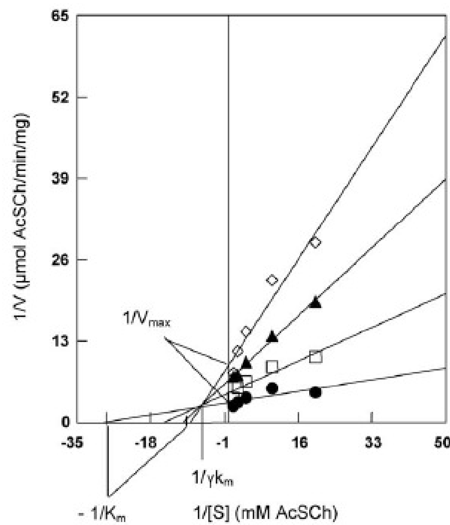

Fig. 4. Lineweaver and Burk plots representing reciprocals of initial human serum enzyme velocity vs. reciprocal of ASCh concentration in the absence and the presence of different concentrations of tacrine (0–37.5 nM). (●) 0 nM; (□) 12.5 nM; (▲) 25 nM; (◊) 37.5 nM.

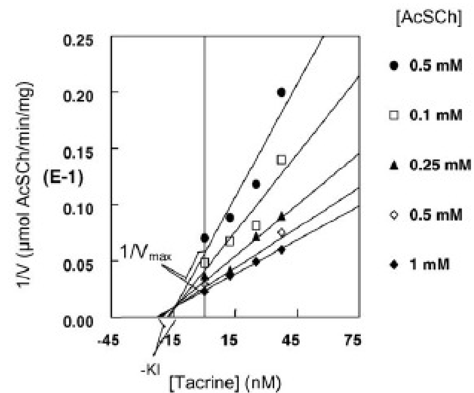

Fig. 5. Dixon plot for snake venom (*Bungarus sindanus*) AChE representing reciprocal of initial enzyme (AChE) velocity vs. different concentrations of tacrine (0–37.5 nM) at five fixed concentrations of substrate as shown in the legend.

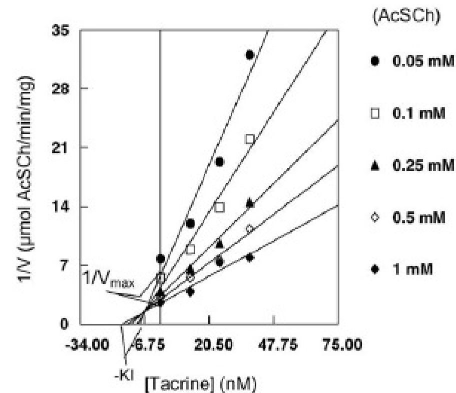

Fig. 6. Dixon plot for human serum BChE representing reciprocal of initial enzyme (BChE) velocity vs. different concentrations of tacrine (0–37.5 nM) at five fixed concentrations of substrate as shown in the legend.

plex and tacrine) were estimated for snake venom AChE (Fig. 5) and for serum BChE (Fig. 6) with the help of the Dixon plot. V_{maxapp} and K_{Iapp} were increased by 36.73–142.17% and 5.88–35.39%, respectively, for AChE values are presented in Table 1. While for BChE, V_{maxapp} from 31.79 to 128.66% and K_{Iapp} were increased 11.1 to 80.77%, values are presented in Table 2.

4. Discussion

Two ligand interaction sites have been identified in cholinesterase: an acylation or A-site and a peripheral binding or p-site. The acylation or A-site is at the base of the active site gorge and the p-site is at its mouth [27]. The substrate transiently binds to the p-site and forms the ES-complex at the active site, due to its low affinity with the p-site, contributing to catalytic processes by insuring that all substrate molecules, which transiently bind to the p-site will proceed toward the A-site [27]. Binding of tacrine to the p-site changes the conformation of the enzyme and sterically blocks the active site or A-site, substantially reducing the rate at which other molecules

Table 1
Effect of tacrine on K_{Iapp} and V_{maxapp} of *Bungarus sindanus* venom AChE

[ASCh] (mM)	K_{Iapp} (nM)	% increase	V_{maxapp} (µmol/min per mg)	% increase
0.05	17	0	177.99	0
0.1	18	5.88	243.36	36.73
0.25	20	17.64	317.29	78.24
0.5	21	23.53	388.70	118.38
1.0	23	35.29	431.034	142.17

Table 2
Effect of tacrine on K_{Iapp} and V_{maxapp} of human serum BChE

[ASCh] (mM)	K_{Iapp} (nM)	% increase	V_{maxapp} (μmol/min per mg)	% increase
0.05	9	0	0.173	0
0.1	10	11.1	0.228	31.79
0.25	12.4	37.77	0.289	67.10
0.5	13.9	54.44	0.338	95.30
1.0	16.27	80.77	0.396	128.66

enter or exist in the active site. The increase in K_{mapp} values indicates that tacrine reduces the affinity of the substrate with the active site of the enzyme. In the case of the ChE–substrate complex, the active site is already occupied, therefore, in this case, as well, tacrine will bind at another site, rather than at the active site, the p-site. For snake venom $K_I/K_i = 1.538$ and for human serum $K_I/k_i = 0.833$. The low values of the above ratios also support the binding of tacrine at the p-site in the case of the enzyme–substrate complex.

The values of various constants calculated for BChE, such as K_I, K_i, IC_{50}, γK_m, K_m, are presented in Table 3, which are lower than the values calculated for snake venom AChE.

In contrast, the mode of inhibition of AChE and BChE by tacrine was found to be similar, i.e. linear mixed type. Others have also reported a mixed type of inhibition by using tacrine as an inhibitor of cholinesterase (ChE) from different origins [28,29]. The IC_{50} values show that venom acetylcholinesterase (AChE) and human serum butyrylcholinesterase (BChE) are more sensitive toward tacrine than other acetylcholinesterases except bovine retinal (AChE) [17,29] (Table 3). γK_m value in the present study for venom AChE was found to be lower than the values calculated for other cholinesterases [16,28,29] (Table 3). Values of K_I and K_i show that tacrine demonstrates greater affinity toward snake venom acetylcholinesterase (AChE) and for human serum butyrylcholinesterase (BChE) when compared with other cholinesterases (Table 3). In fact, in these two differences sources the inhibition of acetylcholine hydrolysis by tacrine can promote an elevation in the acetylcholine levels and in its actions.

In brain, BChE plays a very important role by act as supporting enzyme to AChE to cleave ACh [19,30–37]. Furthermore, cholinesterases have non-classical actions unrelated to their enzymatic function, such as neurotogenesis, synaptogenesis, glial activation, cell adhesion, cerebral blood flow, the amyloid cascade and tau phosphorolylation [34,35]. In fact, in AD brain the coordinated depletion of ACh and AChE occur as much as 85%, contrasting with the progressive rise in BChE that accompanies an increase in glial cell number [36–38].

It is known that these actions of cholinesterases could affect the etio-pathogenesis of AD [34,35]. Perhaps, kinetic studies with unselective cholinesterase inhibitors could help to seek for novel pharmacological and biochemical approaches. In this regard, comparative kinetic analysis of the action of the unselective cholinesterase inhibitors, in a rich source of AChE, such as snake venom, and in a rich source of BChE, such as human serum, could be very useful for these purposes.

In conclusion, a comparison between human serum and snake venom cholinesterase, human serum butyryl-

Table 3
Comparative study of kinetic parameters of cholinesterase (from different origins) inhibition by tacrine

Parameter	SV AChE[a]	HS BChE[b]	HR AChE[c]	CR AChE[d]	BR AChE[e]
K_i (nM)	13	12	37.76	68	4.5
K_I (nM)	20	10	64.36	181	8.5
IC_{50} (nM)	31	25.6	44.6	230	8.07
γK_m (mM)	0.086	0.147	0.231	0.207	0.131
K_m (mM)	0.0537	0.0299	0.120	0.0644	0.061

K_i, inhibition constant; K_I, dissociation constant of the AChE–ASCh–tacrine complex into the AChE–ASCh complex and free tacrine; γK_m (dissociation constant of AChE–ASCh–tacrine complex into AChE–tacrine complex and ASCh) and K_m, Michaelis–Menten constant.

[a] Snake venom (*Bungarus sindanus*) AChE.

[b] Human serum BChE.

[c] Human retinal AChE [28].

[d] Camel retinal AChE [29].

[e] Bovine retinal AChE [16].

cholinesterase (BChE) are more sensitive to tacrine than snake venom acetylcholinesterase (AChE). We suppose that this inhibitory potency of tacrine on serum BChE could be extended to brain BChE, once they are very similar. These findings may help in the understanding of mechanism of actions of the unselective cholinesterase inhibitors, and perhaps, it can be used in the biochemical studies with other compounds.

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References

[1] D. Milatovic, W.D. Dettbarn, Modification of acetylcholinesterase during adaptation to chronic, subacute paraoxon application in rat, Toxicol. Appl. Pharmacol. 136 (1996) 20–28.
[2] M.R.C. Schetinger, N.M. Porto, M.B. Moretto, V.M. Morsch, J.B.T. Rocha, V. Vieira, F. Moro, R.T. Neis, S. Bittencourt, H.G. Bonacorso, N. Zanatta, New benzodiazepines alter acetylcholinesterase and ATPase activities, Neurochem. Res. 25 (2000) 949–955.
[3] J. Massoulié, L. Pezzementi, S. Bom, E. Krejci, F.M. Vallette, Molecular and cellular biology of cholinesterases, Prog. Neurobiol. 41 (1993) 31–91.
[4] J. Silman, L. Sussman, Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology, Curr. Opin. Pharmacol. 5 (2005) 293–302.
[5] K.R. Dave, A.R. Syal, S.S. Katyare, Tissue cholinesterases. A comparative study of their kinetic properties, Z. Naturforsch. 55 (2000) 100–108.
[6] C.A. Prody, D. Zevin-Sonkin, A. Gnatt, O. Goldberg, H. Soreq, Isolation and characterization of full length cDNA clones coding for cholinesterase from fetal human tissues, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 3555–3559.
[7] D.J. Ecobicon, A.M. Corneau, Pseudocholinesterase of mammalian plasma: physiochemical properties and organophosphate inhibition in eleven species, Toxicol. Appl. Pharmacol. 24 (1973) 29–100.
[8] B. Li, J.A. Stribley, A. Ticu, W. Xie, L.M. Schopfer, P. Hammond, S. Brimijoin, S.H. Hinrichs, O. Lockridge, Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse, J. Neurochem. 75 (2000) 1320–1331.
[9] F. Chatonnet, E. Boudinot, A. Chatonnet, L. Taysse, S. Daulon, J. Champagnat, A.S. Foutz, Respiratory survival mechanism in acetylcholinesterase knockout mouse, Eur. J. Neurosci. 18 (2003) 1419–1427.
[10] V. Chhaliani, D. Deer, B. Earles, E. Schmell, T. August, Purification and partial amino acid sequence analysis of human erythrocyte acetylcholinesterase, FEBS. Lett. 247 (2) (1989) 279–282.
[11] X. Cousin, Créminon, J. Grassi, K. Méflah, G. Cornu, B. Saliou, S. Bon, J. Massoulié, C. Bon, Acetylcholinesterase from *Bungarus* venom: a monomeric species, FEBS. Lett. 387 (1996) 196–200.
[12] X. Cousin, C. Bon, Acetylcholinesterase from snake venom, C. R. Seances Soc. Biol. Fil. 191 (1997) 381–400.
[13] H.S. Bawaskar, P.H. Bawaskar, Envenoming by the common krait (*Bungarus caeruleus*) and Asian cobra (Naja naja): clinical manifestations and their management in a rural setting, Wilderness Environ. Med. 15 (2004) 257–266.
[14] Y. Frobert, C. Créminon, X. Cousin, M. Rémy, J. Chatel, S. Bon, C. Bom, J. Grassi, Acetylcholinesterases from *Elapidae* snake venoms: biochemical, immunological and enzymatic characterization, Biochim. Biophys. Acta 23 (1997) 253–267.
[15] S. Eagger, N. Morant, R. Levyl, B. Sahakian, Tacrine in Alzheimer's disease. Time course of changes in cognitive function and practice effect, Br. J. Psychol. 160 (1992) 36–40.
[16] A.A. Al-Jafari, M.A. Kamal, A.S. Alhomida, Sensitivity of bovine retinal acetylcholinesterase (E.C. 3.1.1.7) toward tacrine. Kinetic characterization, J. Biochem. Mol. Toxicol. 12 (1998) 245–251.
[17] H.A. Berman, K. Leonard, Interaction of tetrahydroaminoacridine with acetylcholinesterase and butyrylcholinesterase, Mol. Pharmacol. 41 (1992) 412–418.
[18] D.H. Cheng, X.C. Tang, Comparative studies of huperzine A, E2020, and tacrine on behavior and cholinesterase activity, Pharmacol. Biochem. Behav. 60 (2) (1998) 377–386.
[19] N.H. Greig, T. Utsuki, Q.-S. Yu, X. Zhu, H.W. Holloway, T. Perry, B. Lee, D.K. Ingram, D.K. Lahiri, A new therapeutic target in Alzheimer's disease treatment: attention to butyrylcholinesterase, Curr. Med. Res. Opin. 3 (23) (2001) 159–165.
[20] G.L. Ellman, K.D. Courtney Jr., V.R.M. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[21] J.B.T. Rocha, T. Emanuelli, M.E. Pereira, Effects of early under nutrition kinetic parameters of brain acetylcholinesterase from adult rats, Acta Neurobiol. Exp. 53 (1993) 431–437.
[22] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.
[23] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, J. Am. Chem. Soc. 56 (1934) 658–666.
[24] B.H. Hofstee, On the evaluation of the constant V_m and constant K_m enzyme reactions, Science 116 (1952) 329–331.
[25] J.E. Dowd, D.S. Riggs, A comparison of estimates of Michaelis Menten kinetic constants from various linear transformations, J. Biol. Chem. 240 (1965) 863–869.
[26] M. Dixon, E.C. Webb, Enzyme Kinetics, 2nd ed., Longman, London, UK, 1964, p. 54.
[27] T.L. Rosenberry, J.L. Johnson, B. Cusack, J.L. Thomas, S. Emani, K.S. Venkatasubban, Interaction between the peripheral site and the acylation site in acetylcholinesterase, Chem. Biol. Interact. 157/158 (2005) 181–189.
[28] A.S. Alhomida, A.A. Al-Rajhi, M.A. Kamal, A.A. Al-Jafari, Kinetic analysis of the toxicology effect of tacrine (Cognex) on human retinal acetylcholinesterase activity, Toxicology 147 (2000) 33–39.
[29] A.A. Al-Jafari, The nature of the inhibition of camel retina acetylcholinesterase (E.C. 3.1.1.7) activity by tetrahydroacridine, J. Ocul. Pharmacol. Ther. 12 (1996) 503–514.
[30] N. Greig, T. Utsuki, D. Ingram, Y. Wang, G. Pepeu, C. Scali, Q.-S. Yu, J. Mamczarz, H.W. Holloway, T. Giordano, D. Chen, K. Furukawa, K. Sambamurti, A. Brossi, D. Lahiri, Selective butyryl-

Chapter 3- Published Paper

Malathion, carbofuran and paraquat inhibit Bungarus sindanus (krait) venom acetylcholinesterase and human serum butyrylcholinesterase in vitro

Mushtaq Ahmed, João Batista T. Rocha, Cinthia M. Mazzanti, André L. B. Morsch, Denise Cargneultti, Maísa Corrêa, Vania Loro, Vera Maria Morsch, Maria R.C. Schetinger*

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Malathion, carbofuran and paraquat inhibit *Bungarus sindanus* (krait) venom acetylcholinesterase and human serum butyrylcholinesterase in vitro

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Abstract Carbofuran and malathion, well known pesticides, and paraquat, a world widely used herbicide, were tested on acetylcholinesterase (AChE) from *Bungarus sindanus* venom and butyrylcholinesterase (BChE) from human serum. The calculated IC_{50} values for inhibition of venom enzyme by malathion, carbofuran and paraquat were 2.5, 0.14, and 0.16 μM, respectively. The values for inhibition of serum butyrylcholinesterase (BChE) were 3.5, 0.09 and 0.18 μM, respectively. Analysis of kinetic data indicated that the inhibition caused by malathion, carbofuran and paraquat was mixed for venom AChE. For BChE from human serum, the inhibition caused by malathion and paraquat was mixed and for carbofuran it was uncompetitive. The present results suggest a commercial paraquat preparation (a popular herbicide) inhibits cholinesterases with similar or higher potency than classical pesticide inhibitors. Furthermore, this inhibition was observed both in human serum and snake venom, a newly studied source of AChE.

Keywords Pesticides · Herbicides · Cholinesterase · Snake venom · Human serum

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Introduction

Cholinesterases are classified into two major groups, depending on their substrate specificity. Acetylcholinesterase (AChE; E.C.3.1.1.7) is a membrane bound enzyme present in cholinergic neurons and it plays an essential role in controlling the transmission of nerve impulses across cholinergic synapses and at the neuromuscular junction (Chatonnet and Lockridge 1989; Milatovic and Dettbarn 1996; Schetinger et al. 2000). Butyrylcholinesterase (BChE; E.C.3.1.1.8) is known as a non-specific cholinesterase, pseudo cholinesterase or simply cholinesterase. Physiologically, BChE modulates the levels of cholinergic agents formed during the metabolism of lipids and also regulates free cholines in the plasma (Dave et al. 2000). In mammals, AChE is abundant in the brain, muscles and erythrocyte membranes, whereas BChE is predominantly found in the liver, intestine, heart, kidney and serum (Ecobicon and Corneau 1973; Prody et al. 1987; Dave et al. 2000).

The venom of *Elapidae* snakes possesses several poisons which are highly toxic to man, causing death in several parts of the world, particularly in Asia (Bawaskar and Bawaskar 2004). *Bungarus* venom is one of the richest source of actylcholinesterase, containing 747000 Ellman's units per g of dry venom of AChE-like activity (Frobert et al. 1997).

The study of the amino acid sequence shows that both vertebrate AChE and mammalian BChE have some homology. They share 65% of the amino acid sequence and their substrate hydrolysis is carried out by the ''catalytic triad'' of Ser, His and Glu in the active center (Chhajlani et al. 1989). Moreover, our previous study indicated that they can differ in their sensitivity toward inhibitors such as tacrine (Ahmed et al. 2006).

Thus, the first aim of the present study was to find out in vitro, whether the herbicide paraquat and the widely used pesticides malathion and carbofuran, which are classical inhibitors of AChE in the central cholinergic system and in neuromuscular junctions (Ansari and Kumar 1984; Hassal 1990; Gupta 1994; Bretaud et al. 2000; Miron et al. 2005), also inhibit *Bungarus sindanus* AChE. The second aim was to compare the inhibitory potency of these compounds using these different sources of enzymes (snake venom and human serum).

Materials and methods

Materials

Acetylthiocholine iodide, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], carbofuran (99%; 2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate) ethopropazine and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Malathion (95%; [(dimethoxyphosphinoth-ioyl) thio] butanedioic acid diethyl ester), and paraquat (*N*,*N*′-Dimethyl-44′-bipyridinium dichloride) were kindly donated by FMC Quimica do Brasil LTDA. Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Neon Comercial LTDA (Brasil). All other reagents used were of analytical grade.

Human serum

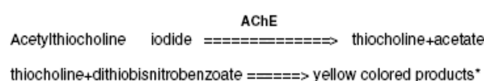
Human serum (without any anticoagulant) was obtained from healthy volunteers [24–33 years old from both sexes (Males = 5 (50%) and Females = 5 (50%)] from the University Hospital from Santa Maria, with written consent. The protocol was approved by the Human Ethics Committee of the University Hospital from Santa Maria (Protocol number: 23081.006134/2006-81). None of the volunteers had a recurrent or a past history of psychiatric illness, significant medical disorder, or drug, cigarette or alcohol abuse. None of them had been taking any medication for at least the past 30 days. From all participants, 5 ml of blood was collected in vacutainer tubes, centrifuged at 3,000 rpm (1,000 *g*) for 10 min and the serum was stored at –20°C for further use.

Venom

Venom from fifteen live adult *Bungarus sindanus* snakes was milked manually, mixed up randomly to give 5 different samples ($N = 5$), immediately lyophilized and stored at –20°C. All the snakes were captured from wild and identified by zoologists.

Cholinesterase assay

Cholinesterase activities were determined by the method of Ellman et al. (1961) as modified by Rocha et al. (1993). BChE was determined in a medium containing 0.05–1 mM acetylthiocholine iodide, 62 mM phosphate buffer, pH 7.5, 0.2 mM DTNB and 40 µl of human serum [700 µg of protein, 17.5 µg/µl (w/v)]; while for AChE 40 µl snake venom [4 µg of protein, 0.1 µg/µl (w/v)] were added to the reaction mixture and pre-incubated for 30 min at 37°C. About 60 µM of ethopropazine (a potent and selective inhibitor of BChE) was used in AChE assay in order to inhibit the presence of BChE in the sample. This method employs acetylthiocholine iodide (AcSCh) as a synthetic substrate for cholinesterases. The substrate is hydrolyzed to thiocholine and acetate by AChE. Thiocholine reacts with dithiobisnitrobenzoate (DTNB) to produce a yellow color (Scheme 1). That is directly proportional to cholinesterase activity. The yellow color was monitored at 412 nm at every 10 s during 2–3 min using a Hitachi 2001 spectrophotometer. All samples were run in duplicate or triplicate. The enzyme activity was expressed as µmol AcSCh hydrolyzed/min/mg of protein.



Scheme 1 Products of the reaction are 2-nitrobenzoate-5 mercapto-thiocholine and 5-thio-2-nitrobenzoate (the latter is the yellow colored product)

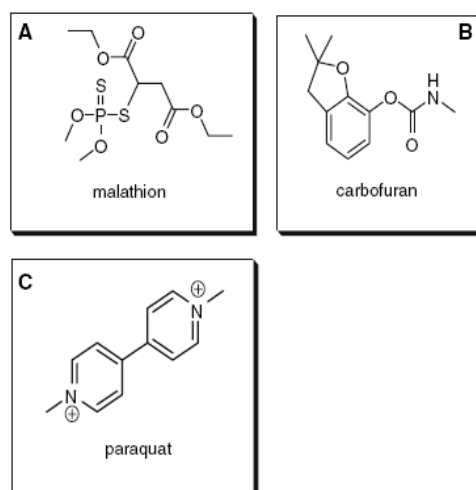


Fig. 1 Chemical structure of (**A**) malathion (**B**) carbofuran) and (**C**) paraquat

Protein determination

Protein was assayed by the method of Bradford (1976) using bovine serum albumin as standard.

Kinetic determinations

The kinetic of the interaction of the pesticides and herbicide with cholinesterase was determined using the Lineweaver and Burk (1934), double reciprocal plot. The *x*-axis represent inverse of acetylthiocholine concentration (1/*S*) and *y*-axis represents the inverse of the enzyme activity (1/*V*). Substrate concentrations ranged from 0.05 mM to 1 mM in the absence and in the presence of malathion (1.2–6 μM), carbofuran (0.07–0.2 nM) and paraquat (0.12–0.36 μM). K_m values were obtained by two different estimations, 1/*V* vs. 1/*S* (Lineweaver and Burk 1934) and *V* vs. *V*/*S* (Hofstee 1952; Dowd and Riggs 1965). The K_i values were obtained using Cornish–Bowden plots of *S*/*V* vs. [*I*]. IC_{50} was calculated according to the Dixon and Webb (1964) plot using 1/*V* vs. [*I*].

Statistical analysis

Statistical analysis was performed using one way ANOVA, which was followed by post-hoc analysis (Duncan multiple range test) using the Statistica software package (Stat Soft®, TULSA, OK, USA).

Results

Malathion (Fig. 2), carbofuran (Fig. 3) and paraquat (Fig. 4) inhibited snake venom and human serum cholinesterase activities. Statistical analysis revealed a concentration dependent inhibition in the two different sources of enzyme. For human serum BChE, kinetic analysis indicated that the inhibition caused by malathion (Fig. 5A) and paraquat (Fig. 5B) was mixed, while carbofuran (Fig. 5C) caused an uncompetitive type of inhibition. For snake venom AChE, kinetic data indicated that the inhibition caused by malathion (Fig. 6A), carbofuran (Fig. 6B) and paraquat (Fig. 6C) was of the mixed type. In fact, K_m values increased and the V_{max} decreased as the concentration of the inhibitors increased. The calculated IC_{50} values for inhibition of snake venom AChE were 2.5 μM, 0.14 nM and 0.16 μM for malathion, carbofuran and paraquat, repectively (Table 1). For human serum (BChE), the calculated IC_{50} values were 3.5, 0.09 and 0.18 μM for malathion, carbofuran and paraquat, respectively (Table 2). The K_i calculated for snake venom AChE and for human serum BChE were obtained by using Cornish–Bowden plots of *S*/*V* vs. [*I*]. The K_i values for snake venom AChE was 0.73, 0.017 and 0.35 μM for malathion, carbofuran and paraquat, respectively (Table 1) while for human serum it was 1.6, 0.012 and 0.1 μM for malathion, carbofuran and paraquat, respectively (Table 2).

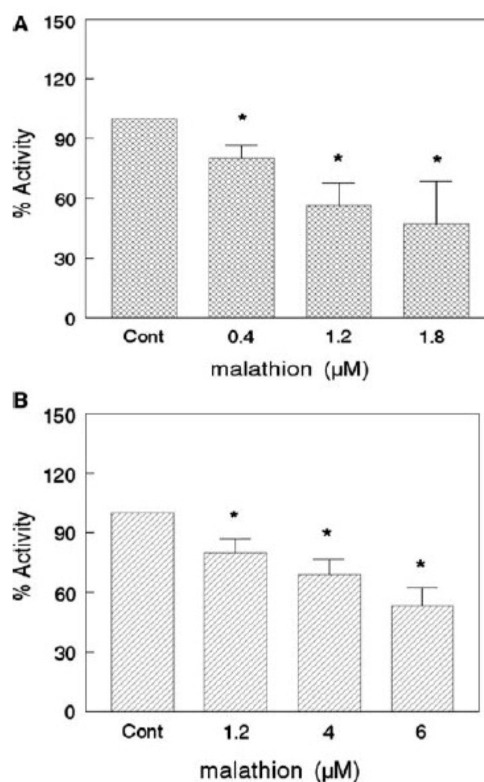


Fig. 2 Snake venom acetylcholinesterase (**A**) and human serum butyrylcholinesterase (**B**) activities in the presence and absence of different concentrations of malathion. Hydrolysis rates v were measured at 412 nm by using 0.4 mM substrate (S) concentration in 1 ml assay solutions with 62 mM phosphate buffer (pH 7.4) and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)] which was pre incubated for 30 min before 0.4 mM substrate addition. All experiments were repeated at least three times and similar results were obtained. * $P < 0.002$ for (**A**) and * $P < 0.0002$ for (**B**) significantly different from control

Discussion

The pesticides (malathion and carbofuran) and herbicide (paraquat) inhibited cholinesterase in *Bungarus sindanus* (Krait) venom and human serum using acetylthiocholine as a substrate (Fig. 1). The inhibitory potency of the pesticides and herbicide was different depending on the nature

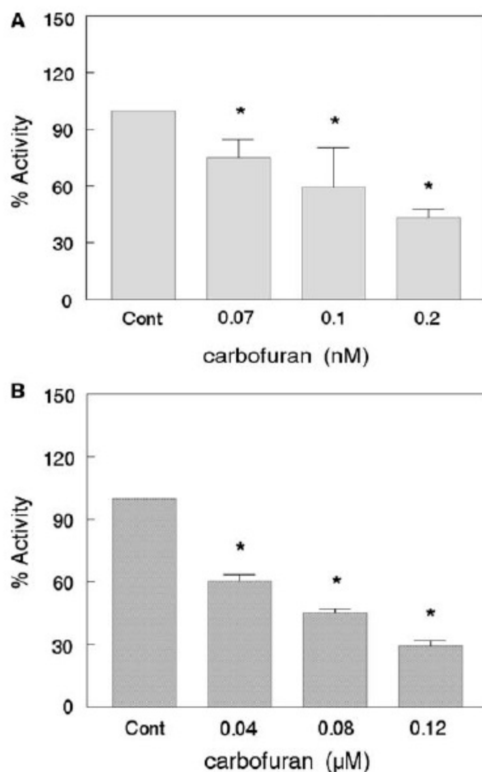


Fig. 3 Snake venom acetylcholinesterase (**A**) and human serum butyrylcholinesterase (**B**) experiments in the presence and absence of different concentrations of carbofuran. Hydrolysis rates v were measured at 412 nm by using 0.4 mM substrate (S) concentration in 1 ml assay solutions with 62 mM phosphate buffer (pH 7.4) and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)], which was pre incubated for 30 min before 0.4 mM substrate addition. All experiments were repeated at least three times and similar results were obtained. * $P < 0.0004$ and * $P < 0.005$ for (**A**) and (**B**) respectively, significantly different from control

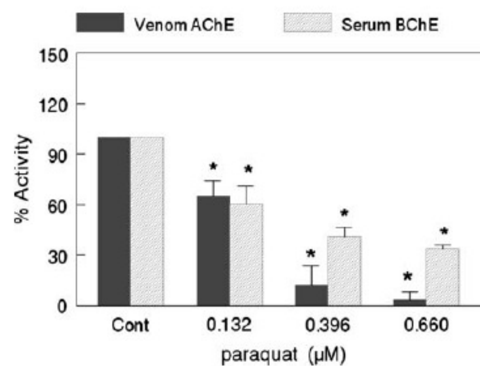


Fig. 4 Cholinesterase activities in the presence and absence of different concentrations of paraquat. Hydrolysis rates v were measured at 412 nm by using 0.4 mM substrate (S) concentration in 1 ml assay solutions with 62 mM phosphate buffer (pH 7.4) and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)] which was pre incubated for 30 min before 0.4 mM substrate addition. All experiments were repeated at least three times and similar results were obtained. For venom * $P < 0.0002$ and for serum * $P < 0.0002$ significantly different from control

of the enzyme source (Figs. 2, 3, and 4), which indicates different types of interaction between inhibitors and the two classes of cholinesterases. In addition, structural differences between the enzymes tested, mainly peculiarities in the primary and tertiary enzyme structure, are present.

The results of Lineweaver–Burk analysis indicate that pesticides (malathion, carbofuran) and herbicide (paraquat) inhibited human serum (Fig. 5) and snake venom acetylcholinesterase (Fig. 6) in a concentration dependent manner. The nature of the inhibition caused by malathion and paraquat were mixed type with both type of enzymes. It was clearly observed that K_m values increased and V_{max} decreased with increase concentration of pesticides. For the case of carbofuran, a mixed type of inhibition was found with snake venom AChE (Fig. 6C), while with human serum BChE activity it was found to be of an uncompetitive type (Fig. 5C). The decrease in V_{max} suggests that the toxicant produce conformational changes in the enzymes. The enzymes have two sites: an acylation site or A-site and peripheral site or P-site (Rosenberry et al. 2005). 3-D structure of AChE shows that the A-site is below the active site residue, while the P-site is at its mouth (Harel et al. 1993; Szegletes et al. 1999). Toxicants (pesticides and herbicide) could interact with ChE at either the ChE-AcSCh complex stage or at regulatory site of the free ChE. In the case of free enzyme, the toxicant would form a complex with ChE i.e., toxicant-ChE that would thereby decreased both acylation and deacylation (Kreienkamp et al. 1991; Bourne et al., 1995). In line with this, thiocholine formation, the first product of the hydrolysis of AcSCh, was decreased with increasing concentrations of toxicant. In the case of AChE–AcSCh complex, the anionic subsite of ChE is occupied by the choline moiety of the substrate AcSCh and is not available for the toxicant; therefore toxicant will prefer to bind other than active site (A-site) i.e., peripheral site or P-site. When the toxicant binds to the P-site, conformational changes of the enzyme can occur and sterically block the active site of the gorge, reducing the rate at which other molecules enter or exit the A-site. If the toxicant remains associated with the enzyme–product complex at the bottom of the active site gorge, the

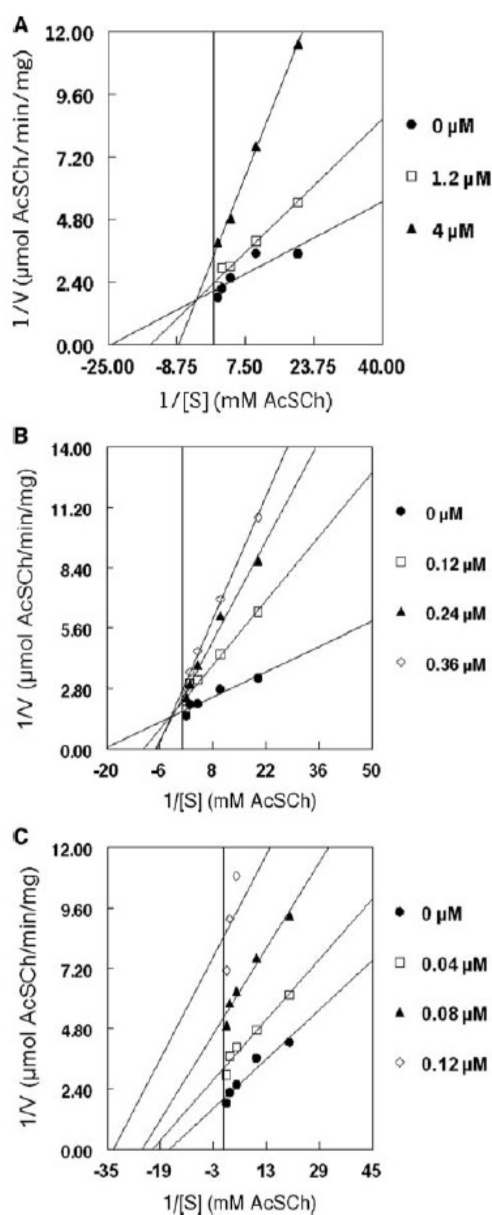


Fig. 5 Kinetic analysis of the inhibition of human serum butyrylcholinesterase by malathion (**A**), paraquat (**B**) and carbofuran (**C**). Data show double reciprocal plots of the butyrylcholinesterase experiments in the absence and in the presence of the different concentration of pesticides and herbicide. Hydrolysis rates v were measured at 412 nm by using various substrate (S) concentrations (0.05–1 mM) in 1 ml assay solutions with 62 mM phosphate buffer (pH 7.4) and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)]. They were pre-incubated for 30 min before substrate addition. All experiments were repeated at least three times and similar results were obtained

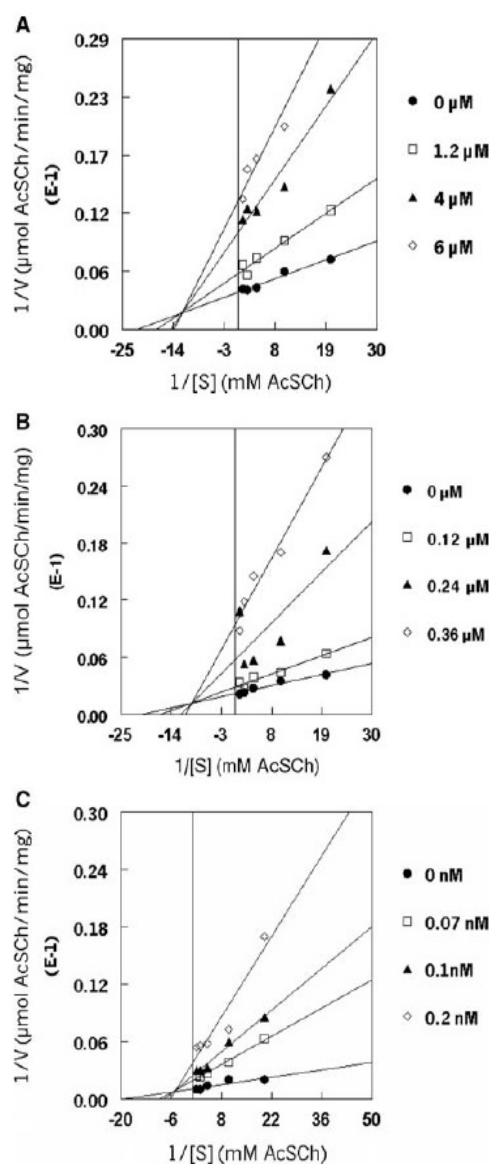


Fig. 6 Kinetic analysis of the inhibition of acetylcholinesterase by malathion (**A**), paraquat (**B**) and carbofuran (**C**) in the snake venom. Data show double reciprocal plots of the acetylcholinesterase experiments in the absence and in the presence of the toxicant. Hydrolysis rates v were measured at 412 nm by using various substrate (S) concentrations (0.05–1 mM) in 1 ml assay solutions with 62 mM phosphate buffer (pH 7.4) and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)]. They were pre-incubated for 30 min before substrate addition. All experiments were repeated at least three times and similar results were obtained

Table 1 K_i and IC_{50} values for venom acetylcholinesterase

	IC_{50}	K_i
Malathion	2.5 μM	0.73 μM
Carbofuran	0.14 nM	0.017 nM
Paraquat	0.16 μM	0.35 μM

The K_i values were obtained using Cornish–Bowden plots of S/V vs. $[I]$ and IC50 was calculated according to the Dixon and Webb plot using $1/V$ vs. $[I]$. All experiments were repeated at least three times and similar results were obtained

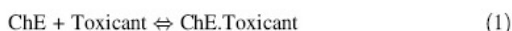
Table 2 K_i and IC_{50} values for human serum butyrylcholinesterase

	IC_{50}	K_i
Malathion	3.5 μM	1.6 μM
Carbofuran	0.09 μM	0.012 μM
Paraquat	0.18 μM	0.1 μM

The K_i values were obtained using Cornish–Bowden plots of S/V vs. $[I]$ and IC_{50} was calculated according to the Dixon and Webb plot using $1/V$ vs. $[I]$. All experiments were repeated at least three times and similar results were obtained

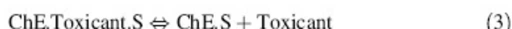
toxicant can cause a decrease in the rate of product dissociation from the catalytic site. In this case, the complex ChE.Toxicant.S is converted to ChE.Toxicant.P and the presence of the toxicant at the bottom of the active site delays the dissociation of the product.

We propose the following scheme for the interactions between the toxicant with the snake venom AChE.



ChE + Toxicant ⇔ ChE.Toxicant (1)

ChE.Toxicant + S ⇔ ChE.Toxicant.S (2)



ChE.Toxicant.S ⇔ ChE.S + Toxicant (3)

ChE.S ⇔ ChE.P (4)

ChE.P ⇔ ChE + P (5)

Both binary (ChE.Toxicant or ChE.S) and ternary (ChE.Toxicant.S) complexes could be formed. When the ternary complexes ChE.Toxicant.S are formed, the product is liberated at a lower rate, decreasing the V_{max} in the case of the mixed type of inhibition.

In the present study, the IC_{50} values calculated for snake venom AChE and for human serum BChE are very close to values for other cholinesterases (from different origins) inhibited by organophosphorus compounds (Jamal et al. 2001). The pesticides and herbicide presented a high affinity toward venom AChE, when compared to human serum BChE, and this may be due to the structural difference between the two different sources of enzymes. Surprisingly, the calculated IC_{50} values calculated for AChE and BuChE inhibition by paraquat were less than malathion, which can be due to impurities present in the commercial paraquat. These data show that both the pesticides and herbicide, which are commonly used in agriculture, inhibited AChE in snake venom and had toxicological effects on human serum BChE.

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References

Ansari BA, Kumar K (1984) Malathion toxicity: in vivo inhibition of acetylcholinesterase in the fish *Brachydanio rerio* (Cyprinidae). Toxicol Lett 20:283–287

Ahmed M, Rocha JBT, Correa M, Mazzanti CM, Zanin RF, Morsch VM, Schetinger MRC (2006) Inhibition of two different cholinesterases by tacrine. Chem Biol Interact 162:165–171

Bawaskar HS, Bawaskar PH (2004) Envenoming by the common krait (*Bungarus sindanus*) and Asian cobra (*Naja naja*): clinical manifestations and their management in a rural setting. Wilderness Environ Med 15(4):257–266

Bourne Y, Taylor P, Marchot P (1995) Acetylcholinesterase inhibition by fasciculin, crystal structure of the complex. Cell 83:503–512

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Bretaud S, Bretaud JP, Toutant, Saglio P (2000) Effects of carbofuran, diuron and nicosulfuron on acetylcholinesterase activity in goldfish (*Carassius auratus*). Ecotoxicol Environ Saf 47:117–124

Chatonnet A, Lockridge O (1989) Comparision of butyrylcholinestrase and acetylcholinestrase. J Biochem 260:625–634

Chhajlani V, Derr D, Earls B, Schmell E, August T (1989) Purification and partial amino acid sequence analysis of human erythrocyte acetylcholinesterase. FEBS Lett 24 247(2):279–282

Dave KR, Syal AR, Katyare SS (2000) Tissue cholinesterases. A comparative study of their kinetic properties. Z. Naturforsch 55:100–108

Dixon M, Webb EC (1964) Enzyme kinetics, 2nd edn. Longman, London, UK, p 54

Dowd JE, Riggs DS (1965) A comparison of estimates of Michaelis menten kinetic constants from various linear transformations. J Biol Chem 240:863–869

Ecobicon DJ, Corneau AM (1973) Pseudocholinestarase of mammalian plasma: Physiochemical properties and organophosphate inhibition in eleven species. Toxicol Appl Pharmacol 24:29–100

Ellman GL, Courtney KD, Andres JrV, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Frobert Y, Créminon C, Cousin X, Rémy M, Chatel J, Bon S, Bom C, Grassi J (1997) Acetylcholinesterases from *Elapidae* snake venoms: biochemical, immunological and enzymatic characterization. Biochim Biophys Acta 23 1339(2):253–267

Gupta RC (1994) Carbofuran toxicity. J Toxicol Environ Health 43:383–418

Hassal KA (1990) The biochemistry and uses of pesticides, 2nd edn. MacMillan Press Ltd., London, pp 140–144

Harel M, Schalk I, Ehret-Sabatie L, Bouet F, Goeldner M, Hirth C, Axelsen P, Silman I, Sussman JL (1993) Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. Proc Natl Acad Sci USA 90:9031–9035

Hofstee BH (1952) on the evaluation of the constant V_m and constant K_m enzyme reactions. Science 116:329–331

Jamal EIY, Shashidhar NR, Adrea M, William Jr S (2001) Prediction of organophosphorus acetylcholinesterase inhibition using three-dimensional quantitative structure-activity relationship (3D-QSAR) methods. Toxicol Sci 63:223–232

Kreienkamp HJ, Weise C, Raba R, Aaviksaar A, Hucho F (1991) Anionic subsites of the catalytic center of acetylcholinesterase from Torpedo and from cobra venom. Proc Natl Acad Sci USA 14:6117–6121

Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658–666

Milatovic D, Dettbarn WD (1996) Modification of acetylcholinesterase during adaptation to chronic, subacute paraoxon application in rat. Toxicol Appl Pharmacol 136:20–28

Miron DS, Crestani M, Schetinger MR, Morsch VM, Baldiserotto B, Tierno MA., Moraes G, Vieira VLP (2005) Effects of the herbicides clomazone, quinclorac, and metsulfuron methyl on acetylcholinesterase activity in the silver catfish (*Rhamdia quelen)* (Heptapteridae). Ecotoxicol Environ Saf 61:398–403

Prody CA, Zevin-Sonkin D, Gnatt A, Goldberg O, Soreq H (1987) Isolation and characterization of full length cDNA clones coding for cholinesterase from fetal human tissues. Proc Natl Acad Sci USA 84:3555–3559

Rocha JBT, Emanuelli T, Pereira ME (1993) Effects of early undernu- tritionon kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol Exp 53:431–437

Rosenberry TL, Johnson JL, Cusack B, Thomas JL, Emani S, Venkatasubban KS (2005) Interaction between the peripheral site and the acylation site in acetylcholinesterase. Chem Biol Interact 157–158:181–189

Schetinger MRC, Porto NM, Moretto MB, Morsch VM, Rocha JBT, Vieira V, Moro F, Neis RT, Bittencourt S, Bonacorso HG, Zanatta N (2000) New benzodiazepines alter acetylcholinesterase and ATPase activities. Neurochem Res 25:949–955

Szegletes T, Mallander WD, Thomas PJ, Rosenberry TL (1999) Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis: substrate inhibition arises as a secondary effect. Biochemistry 38:122–133

Chapter 4- Published Paper

Comparative study of the inhibitory effect of antidepressants on cholinesterase activity in *Bungarus sindanus* (krait) venom, human serum and rat striatum

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Comparative study of the inhibitory effect of antidepressants on cholinesterase activity in *Bungarus sindanus* (krait) venom, human serum and rat striatum

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20

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Abstract

25 Cholinesterases are divided into two classes based on differences in their substrate specificity and tissue distribution: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). These enzymes may be inhibited by several compounds, such as antidepressants. The antidepressants paroxetine, imipramine, clomipramine and sertraline inhibited both venom AChE as well as human serum BChE in a concentration-dependent manner but had no effect on AChE in the rat brain striatum. The IC_{50} of venom calculated for imipramine was 0.3 mM, paroxetine 0.38 mM, clomipramine 0.34 mM and sertraline 0.35 mM. Analysis of kinetic data indicated that the inhibition caused by sertraline and paroxetine was mixed, i.e. K_m values increased and
30 V_{max} decreased in a concentration dependent manner. Imipramine and clomipramine exhibited competitive inhibition, i.e. K_m values increased and V_{max} remained constant. The present results suggest that these therapeutic agents used for depression can also be considered as inhibitors of snake venom and human serum cholinesterase.

Keywords: *Antidepressan't, cholinesterase, snake Venom, human Serum, rat striatum, inhibitors*

35

Introduction

Cholinesterases are divided into two classes based on differences in their substrate specificity; acetylcholin-
40 esterase or "true cholinesterase" (AChE; acetylcholine acetyl hydrolase, E.C.3.1.1.7) and butyrylcholinesterase (BChE; acyl choline acylhydrolase, E.C.3.1.1.8). BChE is also known as pseudo cholinesterase, non specific cholinesterase or simply cholinesterase. AChE
45 hydrolyses acetylcholine faster than other cholinesterases and is very less active on butyrylcholine [1,2]. The hydrolysis of the neurotransmitter acetylcholine (ACh) by AChE in the nervous system is known to be one of the most efficient enzyme catalytic reactions.
50 Ligand-binding studies suggest that the active centre of cholinesterase is composed of a cationic esteratic subsite containing the active serine, an anionic site which accommodate the choline moiety of ACh and a peripheral anionic site [3,4]

In mammals, AChE is abundant in the brain, muscles and erythrocyte membranes, whereas BChE has higher activity in the liver, intestine, heart, kidney and lung [5–7]. The physiologic function of human BChE is still unknown; but it can take the place of AChE in acetylcholine (ACh) degradation when AChE is inhibited or absent [8]. AChE and BChE share 65% amino acid sequence homology and have a similar molecular form and their substrate hydrolysis is carried out by the "catalytic triad" of Ser, His and Glu in the active centre of both types of enzymes [9]. The 3-D structure of the enzyme showed the long and narrow active site gorge is about 20 A° deep and

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includes two sites of ligand interaction: an acylation site at the base of the gorge with the catalytic triad and a peripheral site in its mouth. Some ligands can bind specifically to the acylation or to the peripheral site, and ternary complexes with different ligands bound to each site can be formed [10–12].

Elapidae snake is found in several parts of the world [13]. *Bungarus* venom contains 747000 Ellman's units per g of dry venom of AChE-like activity, being one of the richest venoms in this activity [14]. It is non toxic to mice even at very high doses, and does not reinforce the toxicity of other venom components, thus venom enzyme provides an excellent model for analyzing catalytic mechanism of AChE [15]. In the literature there are reports indicating that antidepressants are inhibitors of cholinesterases from different sources [16,17]. The kinetic and specific fluorescent probe propidium study indicate that the tricyclic anti depressants drugs (TCA) amitriptyline and nortriptyline interact at the peripheral anionic site of E*lectrophorus electricus* AChE [18].

In the present study three different classes of antidepressants were selected. Imipramine and clomipramine are tricyclic antidepressants. During depression, it occurs a decrease in the amount of the chemicals noradrenaline and serotonin released from nerve cells in the brain. The release of these chemicals results in a mood exciting effect. Imipramine and clomipramine both pharmacologically act by stopping these chemicals from going back into the nerve cells. Paroxetine (a phenylpiperidine derivative) and sertraline are selective serotonin reuptake inhibitor (SSRIs), commonly used to treat the symptoms of depression [19].

The interaction of antidepressants with the cholinergic system is poorly related in the literature. It is known that, krait venom and rat brain striatum are rich source of AChE and human serum have high amount of BChE. In view of this, we selected three different rich sources of cholinesterase to study the interaction of these enzymes with the commonly used antidepressants, imipramine, clomipramine, paroxetine and sertraline.

Materials and methods

Materials

Acetylthiocholine iodide, DTNB [5,5′-dithiobis(2-nitro-benzoic acid)], ethopropazine, imipramine, were purchased from Sigma Chemical Co. (St.Louis, MO,USA). Sertraline, clomipramine and paroxetine were obtained from Galena (Campinas, SP, Brazil).

Animals

Male Wistar 3 to 6-month-old rats with a body weight of 300–400 g were kept in separate animal cages, on a 12hr light/dark cycle, at a temperature of 22°C, with free access to food and water. All animal procedures were approved by the Institutional Commission of the Federal University of Santa Maria.

Human serum

Human serum was obtained from healthy volunteers, 20–28 years old, from both sexes from the University Hospital from Santa Maria, with written consent. The Human Ethics Committee of the Federal University of Santa Maria approved the protocol (Protocol number: 23081.006134/2006-81). None of the volunteers had a recurrent or past history of psychiatric illness, any significant medical disorder nor history of drug, cigarette or alcohol abuse. None of them had been taking any medication for at least one month. 6mL of blood was collected from all participants in vaccutainer tubes, centrifuged at 3000 rpm for 10 min and the serum was collected and used for the enzyme assays.

Venom

Mature Krait (*Bungarus sindanus*) snakes (1–1.5 meter length) of both sexes were captured from wild by snake man with the help of specific capturing stick. The snakes were kept in separate animal cages, with free access to soil and water. Venom of two to three drops (300–350 microliters) was squeezed out manually from each snake, mixed, lyophilized immediately and stored at −20°C for further use.

Cholinesterase assay

AChE and BChE activities were determined by the method of Ellman [20] modified by Rocha [21] using a Hitachi 2001 spectrophotometer. Hydrolysis rates (V) were measured at various acetylthiocholine (S) concentrations (0.01–1 mM) in 1 mL assay mixture with 62 mM phosphate buffer, pH 7.5, and 0.2 mM DTNB at 25°C. Twenty microliters of human serum (350 μg of protein), snake venom (2 μg of protein) and 50 μL of rat brain striatum (75 μg of protein) were added to the reaction mixture and pre-incubated for 30 min at 37°C. The experiments were performed separately with each source of AChE. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 10 s). For AChE activity, 0.06 mM ethopropazine (specific inhibitor of BChE) was used to inhibit the activity of BChE in snake venom as well as in rat brain striatum. All samples were run in duplicate or triplicate.

Protein determination

The protein content of the enzyme preparation was assayed by the method of Bradford [22] using bovine serum albumin as a standard.

Kinetic determinations

The interaction of antidepressants and cholinesterase was determined using the Lineweaver–Burk [23] double reciprocal plot, by plotting 1/V against 1/S analysed over a range of acetylthiocholine concentrations (0.01–1 mM) in the absence and in the presence of imipramine (0.3–0.5 mM), paroxetine (0.3–0.7 mM), clomipramine (0.3–0.7 mM), and sertraline (0.3–0.7 mM). Michaelis constants (K_m) were determined by two different plots of 1/V vs. 1/S and V vs. V/S [24,25] The values of inhibition constant (Ki) were obtained using Cornish-Bowden plots of S/V vs. [I]. IC_{50}, was estimated at fixed substrate concentration (0.5 mM), according to the Dixon and Webb [26] plot using 1/V vs. [I].

Statistical analysis

Statistical analysis was performed using one-way ANOVA, which was followed by post-hoc analysis (Duncan multiple range test). The difference was considered to be significant for $P < 0.05$.

Results

To compare the inhibitory potency of the different class of antidepressant against different sources of cholinesterase, 500 µM concentration of antidepressants imipramine, paroxetine, clomipramine and sertraline (Figure 1) were observed to modify the activity of snake venom as well as human serum cholinesterase (Figure 2) when using 0.4 mM substrate (a saturated concentration) in 1 ml assay mixture. In contrast, these antidepressants had no effect on rat brain striatum AChE (Figure 3) in the same conditions. The rate constants (K_m) and V_{max} for ACh hydrolysis versus substrate concentration were measured in the absence and the presence of antidepressants. Analysis of kinetic

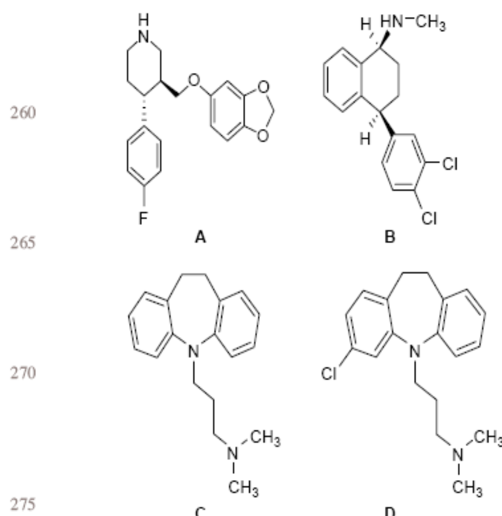


Figure 1. Chemical structure of paroxetine (A), sertraline (B), imipramine (C) and clomipramine (D).

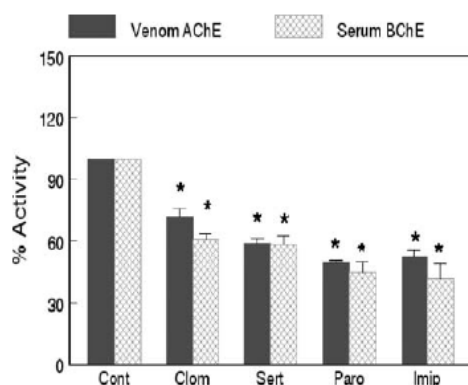


Figure 2. Acetylcholinesterase (AChE) and butrylcholinesterase (BChE) experiments in the presence of 0.5 mM of antidepressants (Clom; clomipramide, Sert; sertraline, Paro; paroxetine and Imip; imipramine). Hydrolysis rates were measured at 412 nm by using 0.4 mM substrate in 1 mL assay solutions with 62 mM phosphate buffer (pH 7.5) and 1 mM DTNB [5,5-dithiobis(2-nitronenzoic acid)]. Enzyme was pre incubated for 30 min before 0.4 mM substrate addition. For AChE activity, 0.06 mM ethopropazine (specific inhibitor of BChE) was used to inhibit the activity of BChE in snake venom. All experiments were repeated at least three times and similar results were obtained. For snake venom AChE ⋆ $P < 0.005$ and for human serum BChE ⋆$P < 0.038$. Significantly different from control.

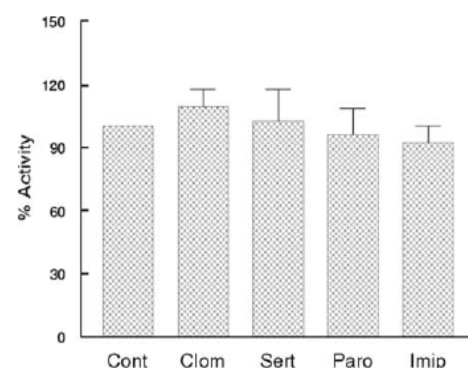


Figure 3. The graphs show rat brain striatum acetylcholinesterase experiments in the presence of 0.5 mM different antidepressants (Clom; clomipramide, Sert; sertraline, Paro; paroxetine and Imip; imipramine). Hydrolysis rates were measured at 412 nm by using 0.4 mM substrate in 1 mL assay solutions with 62 mM phosphate buffer (pH 7.5), and 1 mM DTNB [5,5-dithiobis(2-nitronenzoic acid)]. Enzyme was preincubated for 30 min before 0.4 mM substrate addition. 0.06 mM ethopropazine (specific inhibitor of BChE) was used to inhibit BChE activity. All experiments were repeated at least three times and similar results were obtained.

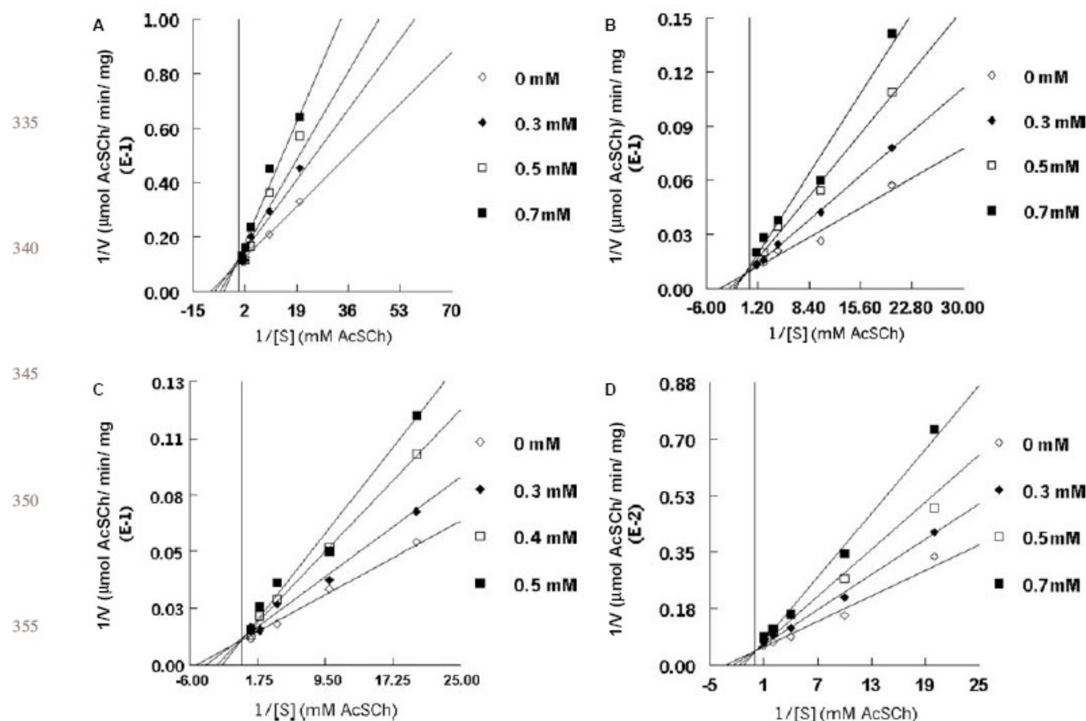


Figure 4. Kinetic analysis of inhibition of snake venom acetylcholinesterase by Paroxetine (A) Sertraline (B) Imipramine (C) and Clomipramine (D).

data indicated that the inhibition caused by paroxetine (Figure 4A) and sertraline (Figure 4B) in snake venom AChE was mixed, where the K_m values increased and the V_{max} decreased in a concentration dependent manner. Imipramine (Figure 4C) and clomipramine (Figure 4D) exhibited competitive inhibition, where the K_m values increased and the V_{max} remained the same. The concentration of antidepressants that inhibits 50% of enzymatic activity, IC_{50}, was estimated at fixed substrate concentration (0.5 mM), the values of imipramine range from 0.3–0.5 mM while paroxetine, clomipramine and sertraline range from 0.3 to 0.7 mM. The concentrations required to inhibit 50% of AChE activity calculated by using the Dixon and Webb [25] for venom AChE are depicted in Table I. The values of inhibition constant (Ki) calculated by using Cornish-Bowden plots of S/V vs. [I] are also listed in Table I.

Discussion

The major role of AChE is the rapid hydrolysis of acetylcholine after it release at the cholinergic synapses and a large number of drugs inhibit AChE activity prolonging the synaptic action of acetylcholine. Recently, we verified the inhibition of AChE from krait (*Bungarus sindanus*) venom and BChE from human serum by malathion, carbofuran, paraquat [27] and tacrine [28]. The three commonly prescribed compounds donepezil, rivastigmine and galantamine are used for the treatment of Alzheimer's disease. All these compound inhibit AChE while rivastigmine additionally inhibit BChE [29,30]. Furthermore, fasciculin, BW284C51, propidium, and decamethonium are potent reversible inhibitors of AChE. These compounds prefer to bind at the peripheral site of the enzyme [14].

Table I. K_i and IC_{50} values for venom acetylcholinesterase.

	Imipramine	Paroxetine	Clomipramine	Sertraline
$IC_{50(mM)}$	0.3	0.38	0.34	0.35
$Ki_{(mM)}$	0.35	0.41	0.38	0.4

The K_i values were obtained using Cornish-Bowden plots of S/V vs. [I] and IC_{50} was calculated according to the Dixon and Webb plot using 1/V vs. [I]. The values were obtained for at least three experiments with standard deviation lower than 10%.

The antidepressants drugs which exhibit anticholinesterase properties have great medical application [31]. In the present study, different classes of antidepressants (Figure 1) inhibited cholinesterase in *Bungarus sindanus* venom as well as human serum (Figure 2) but had no effect on rat brain striatum AChE (Figure 3). It may be due to different forms in which cholinesterase is found: ChEs may be in globular soluble forms, but can also be anchored to the neuronal membrane. These two main types of cholinesterasic forms may exhibit distinct hydrolytic profiles, and this fact may be the basis of the differences observed. The inhibitory potency of the antidepressants was imipramine > clomipramine > sertraline > depending on the nature of the enzyme source as well as the structure of compounds, which resulted in the diverse interactions with cholinesterase (BChE–serum or AChE–snake venom and rat brain striatum), mainly for primary and tertiary enzyme structure peculiarities. In the present study competitive inhibition was observed with venom AChE for imipramine (Figure 4C) and clomipramine (Figure 4D), with different inhibition constant (Table I), in this case the K_m increased and the V_{max} was unchanged. The kinetic analysis indicated a mixed inhibition for paroxetine (Figure 4A) and sertraline (Figure 4B), it was clearly observed that the k_m values increased and V_{max} decreased with increase of inhibitor concentration. Cholinesterase has two sites for substrate binding an acylation site or A-site and peripheral binding site or P-site [32]. Recent literature from NMR spectroscopy and molecular dynamic study with some antidepressants revealed that these compounds prefer conformations in which the side chain is folded over the phenyl rings in a "scorpion-like fashion [33] due to which conformation of the active site of cholinesterase is effected by occupation of peripheral anionic site, it is possible that sertraline and paroxetine act in a scorpion-like fashion, in order to bind with peripheral site or P-site and block the entry of substrate toward the active site of the enzyme. This is supported by the study of Harel [34] which demonstrated that the crystal structure of tacrine (an inhibitor of AChE with a structure similar to that of sertraline) in AChE binds at the bottom of the active site gorge. In cholinesterase the substrate momentarily binds to the p-site and forms the ES-complex at the active site, due to its low affinity with the p-site, this fact contributes to catalytic processes by insuring that all substrate molecules which transiently bind to the p-site will proceed toward the A- site. The presence of any toxicant at peripheral site would block the access of substrate toward the active site [35]. Imipramine (Figure 4C) and clomipramine (Figure 4D) compete with substrate to bind at the active site. As these antidepressants inhibited venom AChE competitively, only a binary (ChE.Drug or ChE.S) complex could be formed. The complex ChE.S liberates the product at a higher rate, while V_{max} remains unchanged. We propose the following scheme for such type of interaction.

$$\text{ChE} + \text{Drug} \Leftrightarrow \text{ChE.Drug} \quad (1)$$

$$\text{ChE.Drug} + \text{S} \Leftrightarrow \text{ChE.S} + \text{Drug} \quad (2)$$

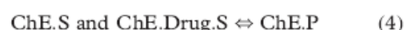
$$\text{ChE.S} \Leftrightarrow \text{ChE} + \text{P} \quad (3)$$

Paroxetine (Figure 4A) and sertraline (Figure 4B) bind other site than the active site i.e peripheral binding site or P-site. So, we propose the following scheme for the interactions between the sertraline and paroxetine with snake venom AChE.

$$\text{ChE} + \text{Drug} \Leftrightarrow \text{ChE.Drug} \quad (1)$$

$$\text{ChE.Drug} + \text{S} \Leftrightarrow \text{ChE.Drug.S} \quad (2)$$

$$\text{ChE.Drug.S} \Leftrightarrow \text{ChE.S} + \text{Drug} \quad (3)$$



$$\text{ChE.S and ChE.Drug.S} \Leftrightarrow \text{ChE.P} \quad (4)$$

$$\text{ChE.P} \Leftrightarrow \text{ChE} + \text{P} \quad (5)$$

Both binary (ChE.Drug or ChE.S) and ternary (ChE.Drug.S) complexes could be formed. The complex ChE.Drug.S liberates the product at a lower rate, decreasing the V_{max} in the case of the mixed inhibition type.

In conclusion, the commonly used antidepressants (imipramine, paroxetine, clomipramine and sertraline) inhibited AChE of *Bungarus sindanus* venom and human serum BChE. However, these compounds did not inhibit AChE activity from the rat brain striatum, which may be related to subtle differences in the primary structure of the enzymes. Furthermore, the different cholinesterasic forms may exhibit distinct hydrolytic profiles, and this fact may be the basis of the differences observed. The venom of the *Bungarus* genera is the richest source of AChE in nature [14] and, of medical significance an excellent model for analyzing catalytic mechanism of AChE. In this vein, kinetic studies with antidepressants, as cholinesterase inhibitors, could be useful for novel neuropharmacological purposes.

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References

[1] Chatonnet A, Lockridge O. Comparision of butyrylcholinestrase and acetylcholinestrase. J Biochem 1989;260:625–634.

[2] Ekholm M. Predicting relative binding free energies as substrate and inhibitors of acetyl and butyrylcholinestrase. J Mol Structure 2001;572:25–34.

[3] Changeux JP. Response of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. Mol Pharmacol 1966;2:369–392.

[4] Epstein DJ, Berman HA, Taylor P. Ligand-induced conformational changes in acetylcholinesterase investigated with fluorescence phosphonates. Biochemistry 1979;18:4749–4754.

[5] Dave KR, Syal AR, Katyare SS. Tissue cholinesterases. A comparative study of their kinetic properties. Z Naturforsch 2000;55:100–108.

[6] Prody CA, Zevin-Sonkin D, Gnatt A, Goldberg O, Soreq H. Isolation and characterization of full lenght cDNA clones coding for cholinestrase from fetal human tissues. Proc Natl Acad Sci USA 1987;84:3555–3559.

[7] Ecobicon DJ, Corneau AM. Pseudocholinestarase of mammalian plasma: Physichemical properties and organophosphate inhibition in eleven species. Toxicol Appl Pharmacol 1973;24:29–100.

[8] Li B, Stribley JA, Ticu A, Xie W, Schopfer LM, Hammond P, Brimijoin S, Hinrichs SH, Lockridge O. Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. J Neurochem 2000;75:1320–1331.

[9] Chaliani V, Derr D, Earls B, Schmell E, August T. Purification and partial amino acid sequence analysis of human erythrocyte acetylcholinesterase. FEBS Lett 1989;247(2):279–282.

[10] Szegletes T, Mallander WD, Thomas PJ, Rosenberry TL. Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis: Substrate inhibition arises as a secondary effect. Biochemistry 1999;38:122–133.

[11] Benzi G, Moretti A. Is there a rationale for the use of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease? Eur J Pharmacol 1998;346:1–13.

[12] Bourne Y, Taylor P, Marchot P. Acetylcholinesterase inhibition by fasciculin, crystal structure of the complex. Cell 1995; 83:503–512.

[13] Bawaskar HS, Bawaskar PH. Envenoming by the common krait (*Bungarus caeruleus*) and Asian cobra (*Naja naja*): Clinical manifestations and their management in a rural setting. Wilderness Environ Med 2004;15(4):257–266.

[14] Frobert Y, Créminon C, Cousin X, Rémy M, Chatel J, Bon S, Bom C, Grassi J. Acetylcholinesterases from *Elapidae* snake venoms: Biochemical, immunological and enzymatic characterization. Biochim Biophys Acta 1997;1339:253–267.

[15] Cousin X, Créminon, Grassi J, Méflah K, Cornu G, Saliou B, Bon S, Massoulié J, Bon C. Acetylcholinesterase from *Bungarus* venom: A monomeric species. FEBS Lett 1996; 387:196–200.

[16] Barcellos CK, Schetinger MRC, Dias RD, Sarkis JJF. *In vitro* effect of central nervous system active drugs on the ATPase—ADPase activity and acetylcholinesterase activity from cerebral cortex of adult rats. Gen Pharmacol 1998;31:563–567.

[17] Müller TC, Rocha JBT, Morsch VM, Neis RT, Schetinger MRC. Antidepressants inhibit human acetylcholinesterase and butyrylcholinesterase activity. Biochim Biophys Acta 2002;1587:92–98.

[18] Nunes-Tavares N, Matta AN, Silva CMB, Araújo GMN, Louro SRW, Hassón-Voloch A. Inhibition of acetylcholinesterase from *Electrophorus electricus* by tricyclic antidepressants. Int J Biochem Cell Biol 2002;34:1071–1079.

[19] Bourin M, Chue P, Guillon C. Paroxetine. a review. CNS Drug Rev 2001;7:25–47.

[20] Ellman GL, Courtney KD, Andres Fe, JrV, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

[21] Rocha JBT, Emanuelli T, Pereira ME. Effects of early under nutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol Exp 1993;53:431–437.

[22] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–254.

[23] Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 1934;56:658–666.

[24] Hofstee BH. On the evaluation of the constant Vm and constant Km enzyme reactions. Science 1952;116:329–331.

[25] Dowd JE, Riggs DS. A comparison of estimates of Michaelis menten kinetic constants from various linear transformations. J Biol Chem 1965;240:863–869.

[26] Dixon M, Webb EC. Enzyme Kinetics. 2nd ed. London UK: Longman; 1964. p 54.

[27] Ahmed M, Rocha JBT, Mazzanti CM, Morsch AL, Cargnelutti D, Corrêa M, Loro V, Morsch VM, Schetinger MRC. Malathion, carbofuran and paraquat inhibit Bungarus sindanus (krait) venom acetylcholinesterase and human serum butyrylcholinesterase *in vitro*. Ecotoxicology 2007;16(4):363–369.

[28] Ahmed M, Rocham JBT, Corrêa M, Mazzanti CM, Zanin RF, Morsch AL, Morsch VM, Schetinger MRC. Inhibition of two different cholinesterases by tacrine. Chem Biol Interact 2006;162:165–171.

[29] Lahiri DK, Rogers JT, Sambamurti K, Greig NH. Rationale for the development of cholinesterase inhibitors as anti-Alzheimer agents. Curr Pharm Des 2004;10:3111–3119.

[30] O'Brien JT, Ballard CG. Drugs for Alzheimer's disease. Br Med J 2001;323:123–124.

[31] Snyder SH, Yamamura HI. Antidepressants and the muscarinic acetylcholine receptor. Arch Gen Psychiatry 1977;34:236–239.

[32] Rosenberry TL, Johnson JL, Cusack B, Thomas JL, Emani S, Venkatasubban KS. Interaction between the peripheral site and the acylation site in acetylcholinesterase. Chem Biol Interact 2005;157–158:181–189.

[33] Marone S, Rozas I, Weaver DF. Theoretical structuralanalyses of tricyclic neuroactive drugs: Quantum pharmacologic descriptors for clustering anticonvulsant, antidepressant, and antipsychotic activities. J Mol Struct 1999;467:25–30.

[34] Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, Hirth C, Axelsen P, Silman I, Sussman JL. Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. Proc Natl Acad Sci USA 1993;9031:9035.

[35] Massoulié J, Pezzementi L, Bom S, Krejci E, Vallette EF. Molecular and cellular biology of cholinesterases. Prog Neurobiol Rev 1993;41:31–91.

Chapter 5-Manuscript

Interaction of tetramethylethylene diamine (TEMED) with venom acetylcholinesterase and horse serum butyrylcholinesterase

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Abstract

The present work examines tetramethyethylene diamine (TEMED) as a cholinesterase inhibitor against snake venom (*Bungarus sindanus*) acetylcholinesterase (AChE) and horse serum butyrylcholinesterase (BuChE). Kinetic studies indicated that the inhibition caused by TEMED was of mixed type, i.e., K_m increased and V_{max} decreased in a concentration dependent manner. γK_m (dissociation constant of ChE-SCh-TEMED complex into ChE-TEMED complex and SCh) was 0.143 mM and 0.454 mM for snake venom AChE and horse serum BChE, respectively. K_I (constant of ChE-SCh-TEMED complex breakdown into ChE-SCh complex and TEMED) was estimated to be 2.044 mM for venom AChE and 0.448 mM for horse serum BChE. The calculated IC_{50} for snake venom and horse serum cholinesterases were 2.49 mM and 0.67 mM respectively. Our results indicate that TEMED inhibits venom AChE and horse serum BChE. However, its affinity was higher for horse serum BChE when compared to snake venom AChE.

***Keywords*:** Tetramethylethylene diamine; TEMED; Acetylcholinesterase; Snake venom; Horse Serum; Butyrylcholinesterase.

1. Introduction

Acetylcholinesterase, or "true cholinesterase", (AChE - acetylcholine acetyl hydrolase, E.C.3.1.1.7) and its related enzyme butyrylcholinesterase, or pseudo cholinesterase (BChE - acyl choline acylhydrolase, E.C.3.1.1.8) are serine hydrolases that hydrolyze choline esters [1]. An amino acid sequence study shows that the two enzymes exhibit 51-54% amino acid identity and identical disulphide arrangement [2, 3]. Furthermore, these two classes of enzyme use the same "catalytic triad" of Ser, His and Glu in the active centre for the substrate hydrolysis [4, 5]. In spite of these similarities, AChE and BChE differ with respect to inhibition caused by the excess of substrate, tissue localization and sensitivity to inhibitors [6, 7]. The enzyme reaction catalyzed by AChE is one of the most efficient reactions known. This efficiency is related to the three-dimensional structure of the enzyme. The three-dimensional structure of *Torpedo* AChE revealed several important features about the catalytic site. The long and narrow active site gorge linked with aromatic residues is about 20 A° deep and includes two sites for ligand interaction: an acylation site, or A-site, at the base of the gorge with the catalytic triad and a peripheral site in its mouth [5]. The venom of *Bungarus sindanus*, an *Elapidae* snake, contains a high level of AChE (Cousin and Bon, 1997) and was firstly described as a non amphiphilic dimer. However, recent investigations revealed that it is a non-amphiphilic monomer like the enzyme from *Naja oxiana* venom [8-10]. According to Frobert the venom of *Bungarus* contain up to 8 mg of acetylcholinesterase per gram of dried venom, have turnovers from 6000 to 8000 s^{-1} which is very high from any other source except that of *Electrophorus* [11]. Furthermore, partial peptide sequencing revealed that acetylcholinesterase from *Bungarus* genera is closely homologous to other cholinesterases [9]. Previously, we have shown that venom AChE from *Bungarus sindanus* (krait) and human serum BChE

respond in a similar way to the inhibitory effects of pesticides and herbicides [12]. In addition, this enzyme showed high sensitivity to tacrine [13], which is known to inhibit synaptic AChE.

N,N,N,N-Tetramethylethylene diamine (TEMED) is a chemical commonly used for initiating polymerization of polyacrylamide gel for electrophoresis. TEMED is also used as a base and has two tertiary amino groups. In the present study, we examined *in vitro* effect of TEMED as a potential anticholinesterasic agent, using two different sources of cholinesterase. In order to clarify the type of inhibition caused by TEMED, kinetic parameters for AChE (snake venom) and BChE (horse serum) were investigated. This *in vitro* study can provide some indications whether TEMED could be considered as an inhibitor of cholinesterase.

2. Materials and methods

2.1 Materials

N,N,N',N'-Tetramethylethylene diamine (TEMED) was purchased from VETEC Química Fina LTDA-Brazil. Ethopropazine, horse serum butyrylcholinesterase, butytylthiocholine iodide (used as substrate), DTNB [5, 5´-dithiobis (2-nitro-benzoic acid)], acetylthiocholine iodide (used as substrate) and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO, USA). Sodium di hydrogen phosphate and di sodium hydrogen phosphate were purchased from Neon Commercial LTDA-Brazil. All other reagents used were of analytical grade.

2.2. Venom

Mature live Krait (*Bungarus sindanus*) snakes of both sexes were captured through specially designed stick by a snake man. Venoms of two to three drops were squeezed out manually from each snake, mixed, lyophilized immediately and stored at −20°C [12] for further studies.

2.3. Cholinesterase assay

AChE and BChE activities were measured spectrophotometrically, using a Hitachi 2001 spectrophotometer by the method of Ellman [14] as described by Rocha et al [15]. The assay mixture (1 mL) contained 0.05 to 1 mM AcSCh or BuSCh as substrate and 0.2 mM DTNB as chromogen in 62 mM phosphate buffer (pH 7.4). All concentrations refer to final concentrations. The snake venom AChE 40 microliters (4 µg of protein), pure horse serum butyrylcholinesterase 40 microliters (0.1 unit) were pre-incubated with different TEMED concentrations (1.11 to 3.3 mM) and (0.37 to 1.11 mM), respectively for 10 min at 37°C. Ethopropazine (0.06 mM) a classic selective potent inhibitor of BChE was used in the AChE assay in order to inhibit the presence of any BChE in snake venom. The method is based on the formation of the yellow anion, 4.4′-dithio-bis-acid-nitrobenzoic measured by absorbance at 412 nm at 25°C for 2–3 min at intervals of 10 sec. Specific activity was defined as µmol of AcSCh or BuSCh hydrolyzed per min per mg of protein or per min per unit of enzyme.

2.4. Protein determination

Protein was assayed by the method of Bradford using bovine serum albumin as a standard [16].

2.5. Kinetic determinations

The enzymes (4 µg snake venom and 0.1 unit of horse serum BChE) was pre-incubated at 37°C for 10 min in the presence of various concentrations of TEMED 1.11---3.33 mM and 0.37---1.11 mM for venom AChE and horse serum BChE, respectively, in 1 mL phosphate buffer containing 0.2 mM DTNB and 0.5 mM AcSCh (or 0.5 mM BuSCh) for the measurement of residual activity. The concentration of TEMED that inhibits 50% of the enzymatic activity, i.e., IC_{50}, was estimated at fixed substrate concentration (0.5 mM) by plotting percentage of residual activity and percentage inhibition versus concentration of TEMED. The kinetic parameters of the interaction between TEMED and cholinesterase were determined using the Lineweaver- Burk double reciprocal plot [17] analyzed over a range of acetylthiocholine or butyrylthiocholine concentrations (0.05–1 mM) in the absence and in the presence of TEMED (1.11---3.33 mM) for venom AChE and (0.37---1.11 mM) for horse serum. K_I (constant of ChE-SCh-TEMED complex into ChE-SCh complex and TEMED) was calculated by using Dixon plot [18] and γK_m (dissociation constant of ChE-SCh-TEMED complex into ChE-TEMED complex and SCh) was calculated by using Lineweaver-Burk plot [17]. The K_i values were obtained using Cornish–Bowden plots of S/V versus [I] [19].

2.6. Statistical Analysis

Statistical analysis was performed using one way ANOVA, which was followed by post-hoc analysis (Duncan multiple range test).

3. Results

TEMED inhibited venom AChE (Fig. 1A) and pure horse serum BChE (Fig. 1B) in a concentration dependent manner. The concentrations of TEMED required to inhibit 50% (IC_{50}) of venom AChE and horse serum BChE activities were calculated by plotting the percentage of residual activity, and the percentages of inhibition vs [I]. IC_{50} values for snake venom AChE and horse serum BChE were 2.49 mM (Fig. 2A), 0.67 mM (Fig. 2B), respectively.

K_m values (calculated by Lineweaver-Burk plot) for the hydrolysis of the substrate (AcSCh/BuSCh) by snake venom AChE and horse serum BChE (Lineweaver-Burk plot) were 0.0765 mM (Fig. 3A) and 0.168 mM (Fig. 3B), respectively. V_{maxapp} and K_{Iapp} (dissociation constant of ChE-SCh-TEMED complex into ChE-SCh complex and TEMED) were estimated for snake venom AChE (Fig. 4) and for horse serum BChE (Fig. 5) by using Dixon plot [18] of $1/V$ versus [I]. V_{maxapp} *and* K_{Iapp} were increased from 49.5 to 262.2% and from 7.9 to 40.6%, respectively (for venom AChE values are presented in Table 1). Besides, for horse serum BChE, V_{maxapp} and K_{Iapp} increased from 66.5 to 333.3% and from 12.7 to 88.5%, respectively (Table 2). In snake venom AChE, K_m values were increased from 13.9 to 43%, while v_{max} decreased from 18.1 to 51.4% (Table 3) by increasing TEMED concentration (1.1--3.3 mM). In horse serum BChE, the K_m values increased from 79.4 to 183.3% and v_{max} decreased from 36.9 to 96.4% (Table 4) by increasing of TEMED concentration (0.37—1.11 mM). The γK_m values (dissociation constant of ChE-SCh-TEMED complex into ChE-TEMED complex and SCh) for snake venom AChE and horse serum BChE were 0.143 mM and 0.454 mM (Table 5), respectively. The K_i value for snake venom AChE was 0.924 mM and for horse serum BChE it was 0.63 mM (Table 5).

4. Discussion

TEMED inhibits snake venom AChE (Fig. 1A) and horse serum (Fig. 1B) BChE in a concentration dependent manner. In cholinesterase for substrate hydrolysis there are two sites, i.e., ionic site (A) and esteratic site (B). The active site residue glutamic acid is present in the ionic site while serine and histidine are located in the esteratic site of the cholinesterase [5]. Substrate hydrolysis takes place at the serine containing esteratic site, which is functionally coupled to the substrate binding area containing a distinct anionic locus [20-23]. The hydrophobic region in cholinesterase is responsible for binding the alkyl group of the substrate located around the ionic and esteratic sites [22-24]. Based on kinetic parameter of ChE inhibition, we presume that TEMED can bind either to enzyme-substrate complex (ES) to form the enzyme-substrate-toxicant (EST) or to a regulatory site of the free enzyme to form an enzyme-toxicant complex**.** In case of free AChE and low concentration of substrate (AcSCh or BuSCh), TEMED competes with substrate for binding at the anionic substrate binding site of the enzyme, a possible reason for TEMED showing partial competitive inhibition. This assumption was confirmed from the Dixon plot (Fig 4 and 5) as well as from Lineweaver-Burk plot (Fig 3)**.** We propose that TEMED inhibit ChEs through either acylation or deacylation steps, because of the decrease in production of thiocholine by TEMED. In the case of high concentration of substrate (AcSCh or BuSCh) and free enzyme, TEMED has little chance of getting access to the active site hence it prefers to bind at the secondary binding site i.e peripheral binding site. This interpretation is supported by the decrease in V_{max} and increase in K_{mapp} with increase of TEMED concentration (Fig 3). The toxicant therefore binds to peripheral binding site, located at the mouth of enzyme, triggering conformational changes and sterically hindering the entrance of substrate toward the active site [25]. Thus the rate of hydrolysis of substrate becomes slow.

The γK_m (dissociation constant of ChE-SCh-TEMED complex into ChE-TEMED complex and SCh) and IC_{50} values (Fig. 2) showed that BChE was sensitive to TEMED inhibition than venom AChE. This is supported by the value of the inhibitory constant (Ki), which indicated that TEMED has more affinity towards horse serum BChE (Table 5). K_m values calculated for venom AChE increased from 13.9 to 43% while V_{max} decreased from 18.1 to 51.4% (Table 3) by the increase of the TEMED concentration from 1.1 to 3.3 mM when compared to the values obtained in the absence of inhibitor (TEMED). In horse serum BChE K_m vaules increased from 79.4 to 183.3% and V_{max} decreased from 36.9 to 96.4% (Table 4) by the increase of the TEMED concentration (0.37—1.11 mM). The IC_{50} values in mM range suggest the low effect of TEMED on snake venom and horse serum cholinesterase when compared with the classical inhibitor of acetylcholinesterase [13, 26].

In conclusion, the results of the present study indicate that TEMED can be considered as an inhibitor of cholinesterase.

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References

[1] Wilson IB, Cabib E. Acetylcholinesterase: enthalpies and entropies of activation. J. Am Chem Soc 1956;78:202 -207.

[2] MacPhee-Quigley K, Vedvick,T, Taylor P, Taylor S. Profile of disulfide bonds on acetylcholinesterase. J Biol Chem 1986;261:13565-13570.

[3] Chatonnet A, Lockridge O. Comparision of butyrylcholinestrase and acetylcholinestrase. J Biochem 1989;260:625–634.

[4] Chhaliani V, Deer D, Earles B, Schmell E, August T. Purification and partial amino acid sequence analysis of human erythrocyte acetylcholinesterase. FEBS Lett 1989;247:279-82.

[5] Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. Science 1991;253:872–879.

[6] Silver A. The Biology of the Cholinesterases. North Holland, Amsterdam; 1974.

[7] Marcel V, Gagnoux-Palacios L, Pertuy C, Masson P, Fournier D. Two invertebrate acetylcholinesterases show activation followed by inhibition with substrate concentration. J Biochem 1998;329:329-334.

[8] Raba R, Aaviksaar A, Raba M, Siigur J. Cobra venom acetylcholinesterase. Purification and molecular properties. Eur J Biochem 1979;96:151-158.

[9] Cousin X, Cre´minon, Grassi J, Me´flah K, Cornu G, Saliou B, Bon S, Massoulie´ J, Bon C. Acetylcholinesterase from *Bungarus* venom: A monomeric species. FEBS Lett 1996; 387:196–200.

[10] Cousin X, Bon C. Acetylcholinesterase from snake venom. C R Seances Soc Biol Fil 1997;191:381-400.

[11] Frobert Y, Cre´minon C, Cousin X, Re´my M, Chatel J, Bon S, Bom C, Grassi J. Acetylcholinesterases from Elapidae snake venoms: Biochemical, immunological and enzymatic characterization. Biochim Biophys Acta 1997;1339:253–267.

[12] Ahmed M, Rocha JBT, Mazzanti CM, Morsch AL, Cargnelutti D, Corrêa M, Loro V, Morsch VM, Schetinger MRC. Malathion, carbofuran and paraquat inhibit *Bungarus sindanus* (krait) venom acetylcholinesterase and human serum butyrylcholinesterase in vitro. Ecotoxicology 2007;16(4):363–369.

[13] Ahmed M, Rocham JBT, Corrêa M,Mazzanti CM, Zanin RF, Morsch AL, Morsch VM, Schetinger MRC. Inhibition of two different cholinesterases by tacrine. Chem Biol Interact 2006;162:165–171.

[14] Ellman GL, Courtney KD, Andres Fe, JrV, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.

[15] Rocha JBT, Emanuelli T, Pereira ME. Effects of early under nutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol Exp 1993;53:431–437.

[16] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–254.

[17] Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 1934;56:658–666.

[18] Dixon M, Webb EC. Enzyme Kinetics. 2nd ed. London UK: Longman; 1964. p 54.

[19] Cornish-Bowden, A., 1995. Analysis of Enzyme Kinetic Data, Oxford University Press, Oxford.

[20] Kabachnik MI, Brestkin AP, Godovikov NN, Michel-son MJ, Rozengart EV, Rozengart VI. Hydrophobic areas of the active surface of cholinesterase. Pharmacol Rev 1970;22:355-388.

[21] Alhomida AS, Al-Rajhi AA, Kamal MA, Al-Jafari AA. Kinetic analysis of the toxicological effect of tacrine (Cognex) on human retinal acetylcholinesterase activity. Toxicology 2000;147(1):33-39.

[22] Quinn DM. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition States. Chem Rev 1987; 87:955-979.

[23] Massoulie´ J, Pezzementi L, Bom S, Krejci E, Vallette EF. Molecular and cellular biology of cholinesterases. Prog Neurobiol Rev 1993;41:31–91.

[24] Rosenberry TL, Johnson JL, Cusack B, Thomas JL, Emani S, Venkatasubban KS. Interaction between the peripheral site and the acylation site in acetylcholinesterase. Chem Biol Interact 2005;157–158:181–189.

[26] Kamal MA, Al-Jafari AA, Yu QS. Greig NH. Kinetic analysis of the inhibition of human butyrylcholinesterase with cymserine. Biochim Biophys Acta 2006;1760(2):200-6.

LEGENDS OF THE FIGURES

Fig.1. Concentration dependent inhibition of snake venom AChE (Fig. 1A) and horse serum BChE (Fig. 1B) after 10 minutes pre-incubation with TEMED at 37°C. All experiments were repeated at least three times and similar results were obtained. For venom $*P < 0.003$ and for horse serum $*P < 0.00026$ significantly different from control. The results represent the mean of three different experiments.

Fig.2. A plot of the percentage residual activity and the percentage inhibition versus concentration of TEMED. 0.5 mM AcSCh or 0.5 mM BuSCh were used as a substrate for snake venom AChE (Fig. 2A) and pure horse serum BChE (Fig. 2B), respectively. The results represent the mean of three different experiments.

Fig. 3. Mixed type of inhibition of snake venom AChE (A) and pure horse serum BChE (B) by TEMED. Data is expressed in the form of Lineweaver–Burk (reciprocal of enzyme velocity versus reciprocal of AcSCh/BuSCh) plot. The results represent the mean of three different experiments done in duplicate by using different concentration of TEMED as shown in the legend boxes.

Fig. 4. Dixon plot (reciprocal of enzyme velocity versus concentration of TEMED) for snake venom AChE at five fixed concentrations of substrate (AcSCh) as shown in the legend box. The values were used to determine V_{max} and Ki presented in Table.1.

Fig. 5. Dixon plot (reciprocal of enzyme velocity versus inhibitor concentration) for pure horse serum BChE at five fixed concentrations of substrate (BuSCh) as shown in the legend box. The values were used to determine V_{max} and Ki presented in Table.2.

FIGURES

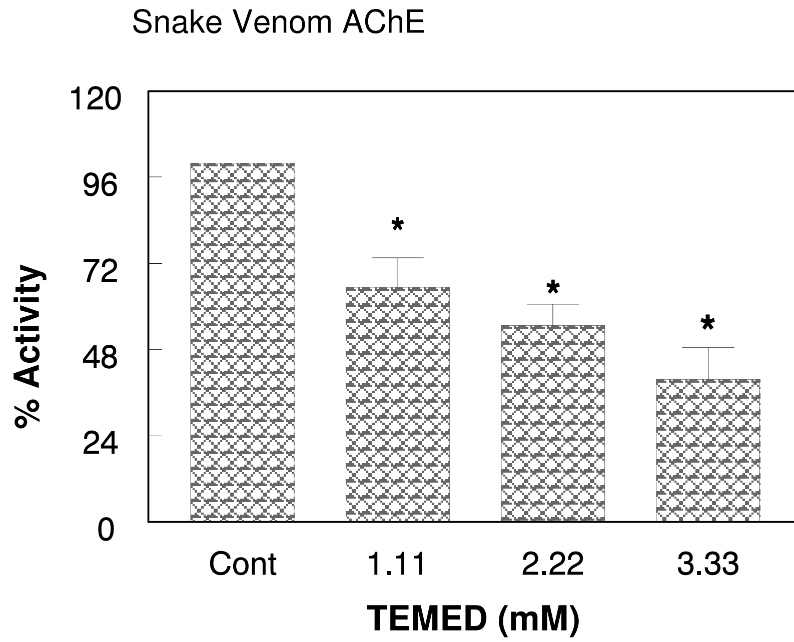
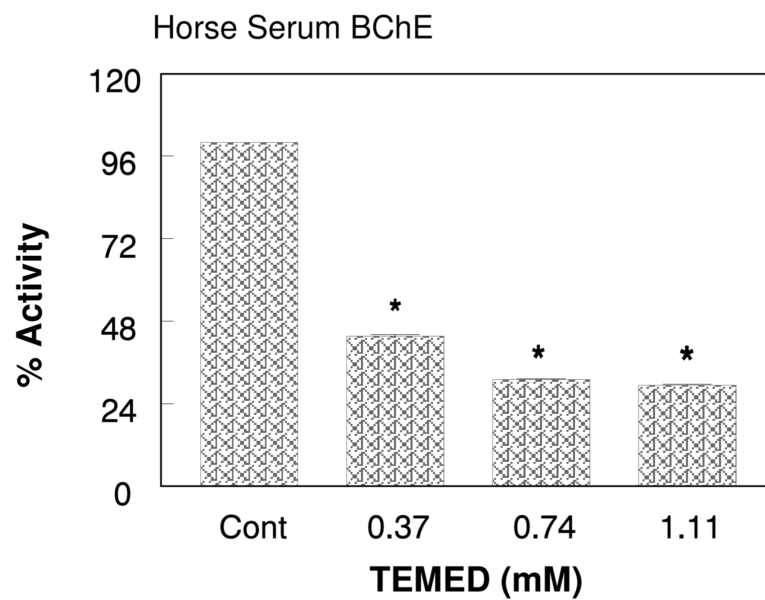


Fig.1A



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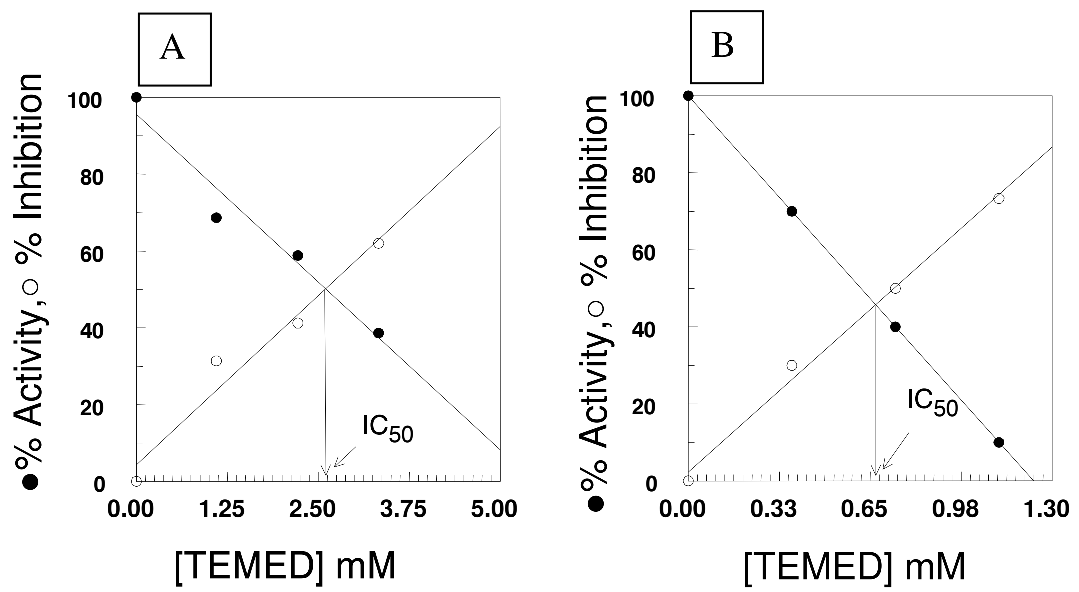
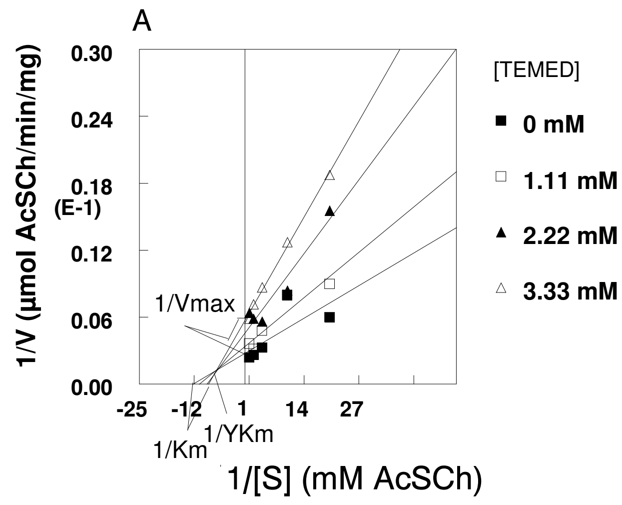


Fig. 2



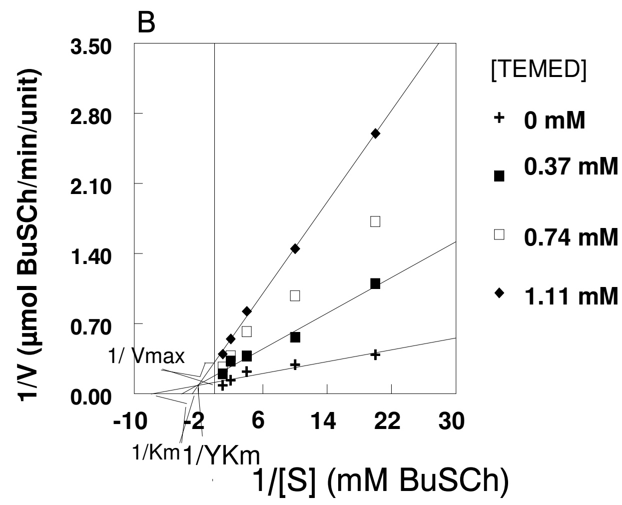


Fig.3

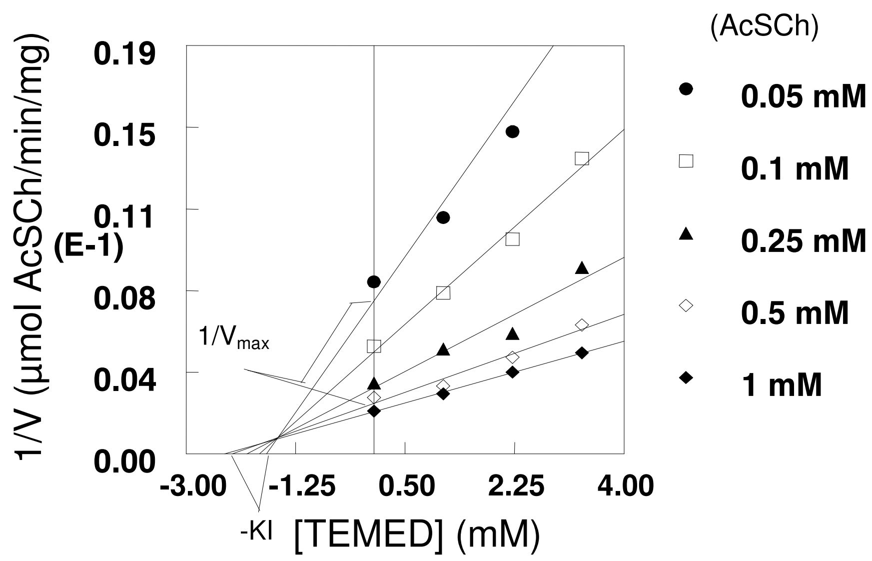


Fig. 4

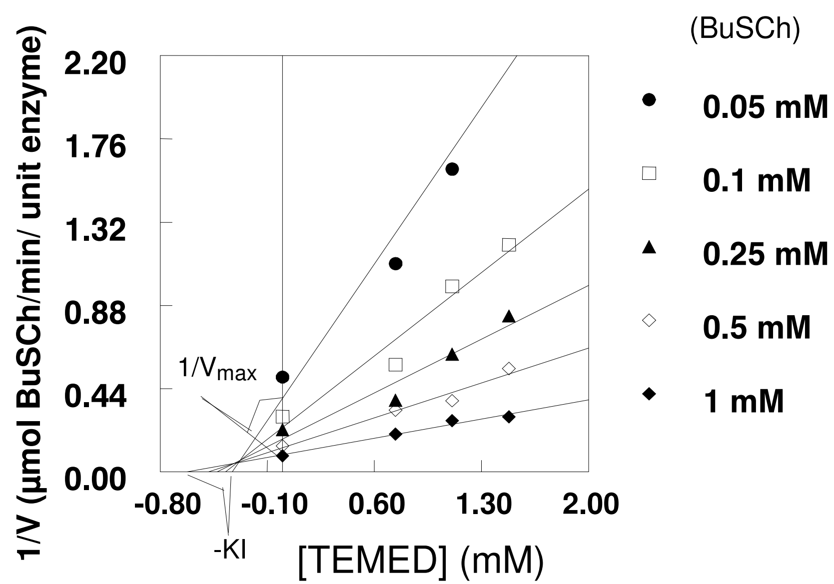


Fig. 5

Table 1. Effect of TEMED on K_{Iapp} and $V_{maxiapp}$ of *Bungarus sindanus* (Krait) venom AChE.

[ASCh] (mM)	K_{Iapp} (mM)	% Increase	$V_{maxiapp}$ (µmol / min per mg)	% Increase
0.05	1.7	0	140.845	0
0.1	1.834	7.9	210.526	49.5
0.25	2.032	19.5	324.149	130.2
0.5	2.266	33.2	426.25	202.6
1.0	2.39	40.6	510.204	262.2

The $V_{maxiapp}$ and K_{Iapp} were determined from Dixon plot (Fig. 4). The $V_{maxiapp}$ is equal to the reciprocal of y-axis intersection of each line for each AcSCh concentration while K_{Iapp} is equal to the x-axis intersection in Dixon plot.

Table 2. Effect of TEMED on K_{Iapp} and $V_{maxiapp}$ of pure horse serum BChE.

[BuSCh] (mM)	K_{Iapp} (mM)	% Increase	$V_{maxiapp}$ (µmol / min per unit)	% Increase
0.05	0.331	0	2.555	0
0.1	0.373	12.7	4.253	66.5
0.25	0.427	29.0	5.767	125.7
0.5	0.485	46.5	7.842	207.0
1.0	0.624	88.5	33.071	333.3

The $V_{maxiapp}$ and K_{Iapp} were determined from Dixon plot (Fig. 5). The $V_{maxiapp}$ is equal to the reciprocal of y-axis intersection of each line for each BuSCh concentration while K_{Iapp} is equal to the x-axis intersection in Dixon plot.

Table 3. Effect of TEMED on K_m and V_{max} of snake venom AChE.

TEMED(mM)	K_m (mM)	% Increase	V_{max}	% Decrease
0	0.0807	0	358.68	0
1.1	0.0919	13.9	293.858	18.1
2.2	0.1107	37.2	217.485	39.2
3.3	0.1154	43	174.367	51.4

The $V_{maxiapp}$ and k_m were determined by their regression equation (Fig. 3A).

Table 4. Effect of TEMED on K_m and V_{max} of horse serum BChE.

TEMED (mM)	K_m (mM)	% Increase	V_max	% Decrease
0	0.126	0	8.615	0
0.37	0.226	79.4	5.438	36.9
0.74	0.304	141.4	4.095	52.5
1.11	0.357	183.3	3.134	96.4

The $V_{maxiapp}$ and k_m were determined by their regression (Fig. 3B).

Table 5. Comparison of kinetic parameters for Inhibition of pure horse serum BChE and snake venom AChE by TEMED.

Parameters	Horse serum BChE	Krait Venom AChE
K_i (mM)	0.63	0.924
K_I (mM)	0.448	2.044
IC_{50}(mM)	0.67	2.49
γK_m (mM)	0.454	0.143
K_m(mM)	0.168	0.0765

K_i, inhibition constant; K_I, dissociation constant of the AChE–ASCh–TEMED complex into the AChE–ASCh complex and free TEMED; γK_m, (dissociation constant of AChE-ASCh-TEMED complex into AChE-TEMED complex and ASCh) and K_m, Michaelis–Menten constant.

Chapter 6-Manuscript

Anticholinesterase action of N,N,N´,N´- Tetramethylethylene diamine (TEMED) *in vitro* and *in vivo* in rats

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Abstract

N,N,N´,N´-tetramethylethylenediamine (TEMED) is a commonly used chemical in biochemistry laboratory for initiating polymerization of acrylamide and bis-acrylamide gel for electrophoresis. The present study evaluate the toxicological effect of TEMED on cholinesterase (ChE) activity in different region of rats brain *in vivo* as well as *in vitro*. TEMED solution in water was given to the rats by gavage for seven consecutive days (2 ml/kg body weight). Animal were divided into four groups. Control group received tap water while treated groups received 30, 60 and 90 mg/kg of TEMED solution. AChE activity was determined in the striatum (ST), hippocampus (HC), cortex (CX), hypothalamus (HT) and cerebellum (CB). Decrease in AChE activity was observed in all treated groups. Moreover, the above concentration also inhibits serum butyrylcholinesterase (BChE) in a dose dependent manner. *In vitro* kinetic study reveals that TEMED caused mixed type of inhibition i.e. a combination of competitive and non competitive inhibition in striatum, cortex, hypothalamus and cerebellum. Taken together, the present results suggest that acute treatment (30-90 mg/kg) with TEMED produce toxicological effect via inhibition of cholinesterase.

Keywords: Rats; N,N,N´,N´- Tetramethylenediamine; Gavage: Cholinesterase; Serum.

1. Introduction

Cholinergic neurons are widely distributed in mammalian central nervous system. They exist as both projection neurons, interneurons and play an important role in the regulation of various functions, such as learning, memory, cortical organization of movement and the control of cerebral blood flow [1-3]. Two main functions have been ascribed to cholinesterases. At cholinergic synapses, acetylcholinesterase (EC 3.1.1.7) is responsible for the termination of nerve impulse transmission; by rapid hydrolysis of the neurotransmitter acetylcholine [4-6]. This role is vital, as it allows restoration of neuronal excitability in cholinergic neuron networks. In non-cholinergic tissues, butyrylcholinesterase (E.C 3.11.8) belongs to the group of the scavenger proteins which are responsible for the degradation of xenobiotics, e.g. succinylcholine or cocaine [7-9].

Apart from its hydrolytic property, AChE has an important role on neurite extension, cellular adhesion and participates in the structural regulation of postsynaptic differentiation [10-13]. In healthy human brain, AChE predominates over BChE activity and histochemically localized mainly in neurons. In contrast, BChE is associated primarily with glial cells and endothelial cells [14-16]. In fact, AChE has become the target for some compounds. These compounds inhibit acetylcholinesterase and promote the cholinergic function via blocking the ACh hydrolysis thus, improving the cognitive deficit in Alzheimer's patient. In healthy person an absence or inhibition of this enzyme may alter the normal function of brain and other organs of the body causing death of the individual [17-19].

N,N,N´,N´-tetramethylethylenediamine (TEMED) is a chemical which is commonly used in protein chemistry lab. It plays an important role in the formation of free radicals by ammonium persulfate and accelerates the polymerization of acrylamide and bisacrylamide. Structurally, it has two tertiary amino groups which are expected to play

similar role as other tertiary and quaternary ions, hence they inhibit cholinesterase [20]. In literature there are reports on oral toxicity of TEMED on rats (LD50: 268 mg/kg). Therefore, the present study was conducted with very low concentration (30-90 mg/kg) compared with LD50 to investigate the toxicological effect of TEMED on serum butyrylcholinesterase and acetylcholinesterase from different parts of brain region of the rat *in vivo* as well as "*in vitro*", in order to extend it to human health.

2. Materials and methods

2.1 Chemicals

DTNB [5, 5´-dithiobis (2-nitro-benzoic acid)], acetylthiocholine iodide, bovine serum albumin and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical (St. Louis, MO, USA) N,N,N',N'-tetramethylethylene diamine (TEMED) was purchased from VETEC Química Fina LTDA (Brazil). Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Neon Commercial LTDA (Brazil). All other reagents used were of analytical grade.

2.2 Animals

Adult female Wistar rats (80–120 days; 170–260g) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1°C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Institutional Commission of the Federal University of Santa Maria and were in agreement with the International Council.

2.3 Treatment

The animals were exposed to TEMED by gavage for seven consecutive days. Gavage was performed using a syringe with a modified steel point to introduce the solution into the rat's stomach without injuring the tissue. Rats were divided into four groups (n=10 for all groups): (1) control animals that received tap water (2 ml/kg); (2) animals treated with 30, (3) 60 and (4) 90 mg/kg TEMED solution prepared in de-ionized water. The behavior of the animals before and after dosed was observed daily. The animals were euthanized 24 h after the last dose.

2.4 Brain tissue preparation

Brain structures were immediately removed and separated into cerebral cortex (CC), striatum (ST), hippocampus (HP), cerebellum (CB) and hypothalamus (HY). Structures were homogenized with a glass potter (1 w/v) in cold 10 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 1500 g for 10 min. Aliquots of resulting slow speed brain supernatants were stored at −20°C until utilization. Protein was adjusted before enzyme assay depending on the structure as follow: ST (0.4 mg/ml), HP (0.8 mg/ml), CC (0.7 mg/ml), CB (0.6 mg/ml), and HY (0.6 mg/ml).

2.5 Rat Serum

From all groups, 5 ml of blood was collected in vacutainer tubes, centrifuged at 3000 rpm for 10 min and the serum was collected individually, stored at −20°C for further use.

2.6 Protein estimation

Protein was assayed by the method of Bradford using bovine serum albumin as a standard [21].

2.7. Cholinesterase assay

Cholinesterase activities were determined by the method of Ellman [22] with some modification [23]. The assay medium (2 ml final volume) contained 100 mM K^+-phosphate buffer, pH 7.5, 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB) and hundred micro-liter enzyme of different concentration (ST (0.4 mg/ml), HP (0.8 mg/ml), CC (0.7 mg/ml), CB (0.6 mg/ml), and HY (0.6 mg/ml)). The reaction was started by the addition of 0.8 mM substrate (ATC or BTC) at 25^oC. *In vitro*, for kinetic analysis, hydrolysis rate v of the enzyme was measured at 25^oC at various substrate (s) concentrations (0.05-1.0 mM) after 10 min pre-incubation of enzyme at 37^oC with TEMED (0.85-1.48 mM). All samples were run in duplicate or triplicate. The method is based on the formation of the yellow anion, 4.4′-dithio-bis-acid-nitrobenzoic measured by absorbance at 412 nm at 25^oC for 2–3 min at intervals of 10 sec. The enzyme activity was expressed in µmoles SCh/h/mg of protein. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 mM for 2-3 min (interval of 15 second) using a Hitachi 2001 spectrometer.

2.8. Kinetic determinations

The kinetic parameters, k_m and v_{max} of the interaction between TEMED and cholinesterase were determined using the Lineweaver Burk double reciprocal plot [24] analyzed over a range of acetylthiocholine concentrations (0.05–1 mM) in the absence and in the presence of TEMED (0.85–1.48 mM). The concentration of TEMED that

inhibits 50% of enzymatic activity, IC_{50}, was estimated by plotting Dixon plot (1/V vs. [I]) [25] and Ki values were calculated by using Cornish Bowden plot (S/V vs. [I]) [26].

2.9 Statistical analysis

One-way ANOVA followed by Duncan's multiple range tests, when appropriate was used to compare groups. *$P < 0.05$ was considered significantly different.

Results

All the animals treated with 30 mg/kg of TEMED solution showed no sign of toxicity (Table 2) while two and four animals from the groups treated with 60 and 90 mg/kg, repectively died (Table 1). Furthermore, generally, we observed sluggishness, fasciculation, abnormal breathing, red tearing, feeling problem in breathing, bleeding from nose in the goups treated with 60 and 90 mg/kg TEMED solution while all the rats from the group treated with 30 mg/kg were healthy similar to control group. Statistical analysis showed that in all treated groups AChE activity was inhibited by TEMED, in the Striatum, Cortex, Hypothalamus, Hyppocampus, and Cerebellum ($p < 0.05$), when compared to the control (Fig.1). We did not find any significant difference among the groups treated with TEMED (Fig.1). Furthermore, in the same *in vivo* experiment the doses (30-90 mg/kg) also caused the inhibition of serum butyrylcholinesterase (Fig.2) which was significant among all the treated groups (Fig.2).

In vitro, kinetic study indicate that TEMED inhibited AChE activity in a dose dependent manner (Fig.3) and caused mixed type inhibition i.e. K_m increases and V_{max} decreases with increase of TEMED concentration in all region of the brain (ST, CX, CB

and HT, Fig. 4). The calculated values of IC_{50} and K_i are given in Table 2. The IC_{50} values calculated for different regions of brain AChE revealed that striatum (ST) and cortex (CC) have higher affinity toward TEMED compared to cerebellum (CB) and hypothalamus (HT) (Table 2).

Discussion

In healthy brain acethylcholinesterase (AChE) is the most important enzyme regulating the level of ACh while butyrylcholinesterase (BChE) plays a minor role. The hydrolysis of neurotransmitter acetylcthiocholine (ACh) by acetylcholinesterase is an essential process for normal body function. The inhibition of acetylcholinesterase in healthy person causes disturbance of numerous body functions [27]. The present results indicate that TEMED inhibit acetylcholinesterase from different regions of the brain (Fig.3).
During seven days treatment, we strictly observed the toxicological effect of TEMED in all treated groups. It was demonstrated that doses of 60 and 90 mg/kg caused overt sign of toxicity. Generally, we observed sluggishness, fasciculation, abnormal breathing, red tearing, feeling problem in breathing, bleeding from nose, tonic convulsions followed by death, that's why four animal from group doses 90 mg/kg and two from group doses 60 mg/kg died after last day dosage (Table 1).
The data showed that the administration of TEMED by gavage caused inhibition of AChE activity (Fig.1) and butyrylcholinesterase (Fig.2).Two ligand interaction sites have been reported in cholinesterase ; (1) an active site or A-site and (2) peripheral site or P-site, located close to the active site gorge of the enzyme [28]. The peripheral anionic side of cholinesterase plays an important role in the hydrolysis of substrate by insuring that all substrate molecules which bind at peripheral side will move toward the

active site of the enzymes therefore, binding of any toxicant at peripheral side will decrease the tendency of substrate toward the active site. The studies suggest that TEMED binding site is located both at the peripheral anionic site as well as in A-site of different region of brain acetylcholinesterase. When enzyme-substrate complex is formed at active site or A-site during hydrolysis then TEMED tends to bind at the peripheral anionic site of the different region of brain acetylcholinesterase (Fig.4). The *in vitro* study indicates linear mixed type inhibition of acetylcholinesterase (AChE) in different region of brain by using TEMED as an anticholinesterase agent. We strictly observed that K_m increases and V_{max} decreases with the increase of TEMED concentration. Our results are in agreement with previous study of Al-Jafari [29] in which he also observed mixed type inhibition *in vitro* with water soluble and membrane bound acetylcholinesterase. The IC_{50} values calculated for different regions of brain AChE reveals that striatum (ST) and cortex (CC) AChE have higher affinity toward TEMED compared to cerebellum (CB) and hypothalamus (HT) (Table 2) which may be due to different structure of the enzyme. In the present study the inhibition of serum butyrylcholinesterase was found to be dose dependent (Fig.2) while, no such difference was observed among the groups in the inhibition of different regions of brain acetylcholinesterase (Fig.1).

In conclusion, acute treatment (30-90 mg/kg) of TEMED inhibits AChE activity in all brain region as well as BChE in serum. The inhibitory effect of TEMED toward butyrycholinesterase was higher than that of acetylcholinesterase. Taken together, the results showed that ingestion of TEMED is toxic via inhibition of cholinesterase. Therefore, proper precaution should be used during its handling.

Acknowledgements

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References

[1] M.M Mesulam, A. Guillozet, P. Shaw, A. Levey, E.G. Duysen, O. Lockridge, Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience. 110 (4) (2002) 627–639.

[2] D.K. Lahiri, J.T. Rogers, K. Sambamurti, N.H. Greig, Rationale for the development of cholinesterase inhibitors as anti-Alzheimer agents. Curr. Pharm. Des. 10 (2004) 3111 – 3119.

[3] C.R. Houser, Cholinergic synapses in the central nervous system: Studies of the immunocytochemical localization of choline acetyltransferase. J. Electron. Microsc. Tech.15 (2005) 2-19.

[4] D. M. Quinn, acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. Chem. Rev. 87 (1987) 955-979.

[5] P. Taylor, Z. Radic, The cholinesterases: from genes to proteins. Annu. Rev. Pharmacol. Toxicol. 34 (1994) 281-320.

[6] A. Das, M. Dikshit, C. Nath, Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. Life Sci. 68 (2001) 1545–55.

[7] C. Mattes, R. Bradley, E. Slaughter, S. Browne, Cocaine and butyrylcholinesterase (BChE): determination of enzymatic parameters. Life Sci. 58 (1996) 257-261.

[8] D.E. Lenz, D.M Maxwel, I. Koplovitz, C.R Clark, B.R. Capacio, D.M. Cerasoli, J.M Federko, C. Luo, A. Saxena, B.P. Doctor, C. Olson, Protection against soman or VX poisoning by human butyrylcholinesterase in guinea pigs and cynomolgus monkeys. Chem. Biol. Interact. 15(2005) 205-10.

[9] D.M. Cerasoli, E.M. Griffiths, B.P. Doctor, A. Saxena, J.M. Fedorko, N.H. Greig, Q.S. Yu, Y. Huang, H. Wilgus, C.N. Karatzas, I. Koplovitz, D.E. Lenz, In vitro and in vivo characterization of recombinant human butyrylcholinesterase (Protexia) as a potential nerve agent bioscavenger. Chem. Biol. Interact. 15 (2005) 363-5.

[10] H. Tago, T. Maeda, P.L. McGeer, H. Kimura, Butyrylcholinesterase-rich neurons in rat brain demonstrated by a sensitive histochemical method. J. Comp. Neurol. 325 (1992) 301–312.

[11] P.G. Layer, T. Weikert, R. Alber. Cholinesterase regulate neurite growth of chick nerve in vitro by means of a non-enzymatic mechanism. Cell. Tissue. Res. 273 (1993) 219–226.

[12] G. Johonson, S.W. Moore, The adhesion function on acetylcholinesterase is located at peripheral anionic site. Biochem. Bioph. Res. Co. 258 (1999) 758–762.

[13] H. Soreq, S. Seidman, Acetylcholinesterase-new roles for an old actor. Nat. Rev. Neurosci. 2 (2001) 294–302.

[14] E. Giacobini, Cholinesterases: new roles in brain function and in Alzheimer's disease. Neurochem. Res. 28 (2003) 515–522.

[15] S. Darvesh, D.A. Hopkins , C. Geula, Neurobiology of butyrylcholinesterase. Nat. Rev. Neurosci. 4(2) (2003) 131-8.

[16] A. Chatonnet, O. Lockridge, Comparision of butyrylcholinestrase and acetylcholinestrase. J. Biochem. 260 (1989) 625-634.

[17] N.H. Greig, X.F. Pei, T. Soncrant, D. Ingram, A. Brossi, Phenserine and ring-C hetero-analogues: drug candidates for the treatment of Alzheimer's disease. Med. Chem. Rev. 15(1995) 3– 31.

[18] D.k. Lahiri, M.R Farlow, N.H Greig, K. Sambamurti, Current drug targets for Alzheimer's disease treatment. Drug. Devel. Res. 56 (2002) 267–281.

[19] L. Lopez-Carillo, M. Lopez-Cervantes, Effect of exposure to organophosphate pesticides on serum cholinesterase levels. Arch. Environ. Health. 48 (1993) 359-63.

[20] P.K. Mukherjee, V. Kumar, M. Mal, P.J. Houghton, Acetylcholinesterase inhibitors from plants. Phytomedicine**.** 14 (2007) 289-300.

[21] M.M Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72 (1976) 248–254.

[22] G.L. Ellman, K.D. Courtney, Jr.V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7(1961) 88–95.

[23] J.B.T. Rocha, T. Emanuelli, M.E. Pereira, Effects of early under nutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta. Neurobiol. Exp. 53 (1993) 431– 437.

[24] H. Lineweaver, D.Burk, The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56 (1934) 658– 666.

[25] M. Dixon, E.C. Webb, *Enzyme Kinetics*, 2nd ed., Longman, London, UK, (1964) p. 54.

[26] A. Cornish-Bowden, Analysis of Enzyme Kinetic Data, *Oxford Univ. Press, Oxford* (1999).

[27] M. Eddleston, F. Mohamed, J. O Davies, P. Eyer, F. Worek, M.H. Sheriff, N.A. Buckley, Respiratory failure in acute organophosphorus pesticide self-poisoning. Q. J. Med. 99 (2006) 513–522.

[28] T.L. Rosenberry, J.L. Johnson, B. Cusack, J.L. Thomas, S. Emani, K.S. Venkatasubban, Interaction between the peripheral site and the acylation site in acetylcholinesterase. Chem. Biol. Interact. 157–158 (2005) 181–189.

[29] A. A Al-Jafari, The inhibitory effect of tetramethylethylene diamine on water soluble and membrane bound acetylcholinesterase activity. Int. J. Biochem. 25(3) (1993) 319-24.

LEGENDS OF THE FIGURES

Fig.1. *In vivo*, effect of on female Wistar rats, (A);Striatum (ST), (B); Cerebral Cortex (CX), (C); Hypothalamus (HT), (D); Hyppocampus (HC) and (E); Cerebellum (CB), acetylcholinesterase (AChE) treated with TEMED (30, 60 and 90 mg/kg orally) for seven consecutive days. The results are presented as mean S.E.M. as compared to controls (one-way ANOVA, followed by Duncan's multiple range tests. $P < 0.05$)

Fig.2. Effect on female Wistar rats serum butyrylcholinesterase (BChE) treated with same as (Fig. 1.) TEMED (30, 60 and 90 mg/kg orally). The results are presented as mean S.E.M as compared to controls. The groups not sharing the same letter are different at $P < 0.05$ (one-way ANOVA, followed by Duncan's multiple range tests).

Fig.3. *In vitro,* Inhibition of Striatum (ST), Cortex (CX), Cerebellum (CB) and Hypothalamus (HT) acetylcholinesterase (AChE) in the presence of different concentrations of TEMED. Hydrolysis rates V were measured at 412 nm by using fixed 0.8 mM substrate (AcSCh) in 2 ml assay solutions with 100 mM phosphate buffer (pH 7.4) and 1 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)] was pre incubated for 10 min at 37°C before addition of substrate. ($n = 4$), $p < 0.05$.

Fig.4. Kinetic analysis by Lineweaver–Burk representation of Striatum (A), Cortex(B), Cerebellum(C) and Hypothalamus(D); acetylcholinesterase (AChE) inhibition by TEMED using acetylthiocholine as substrate. Double reciprocal plot was constructed by

plotting $1/V$ against $1/S$ analyzed over a range of substrate concentrations (0.05–1 mM) in the absence and in the presence of TEMED as shown in the legend box. The plot represents the means of three experiments ($n = 3$), S.D. < 10%, $p < 0.05$.

FIGURES

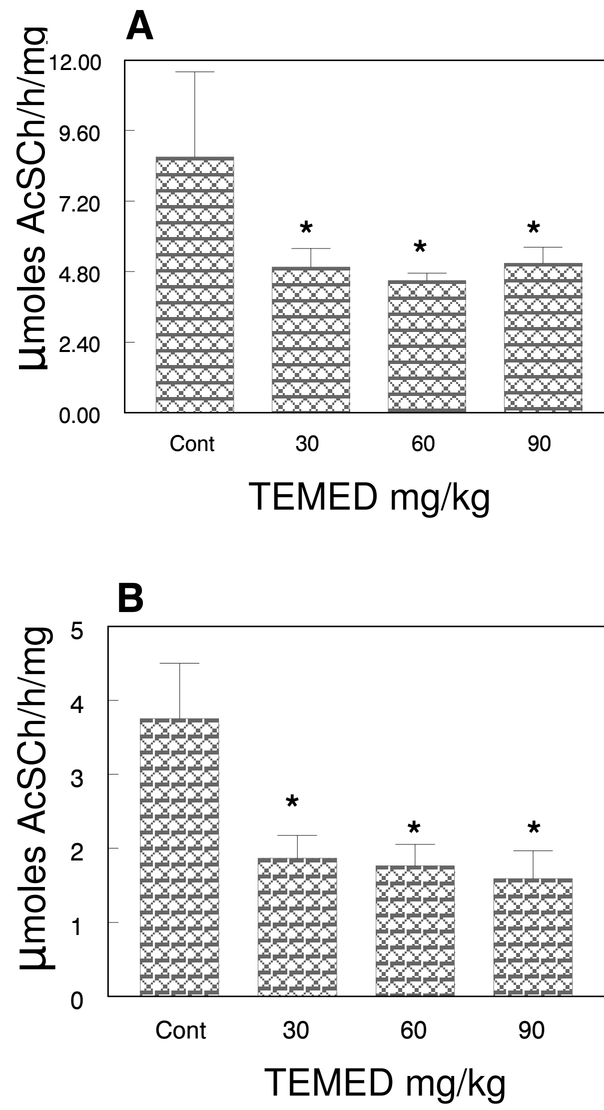
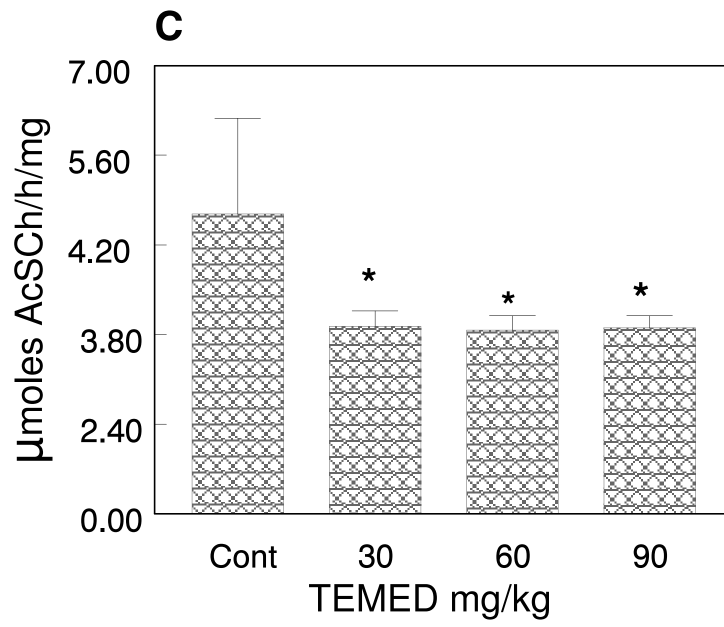
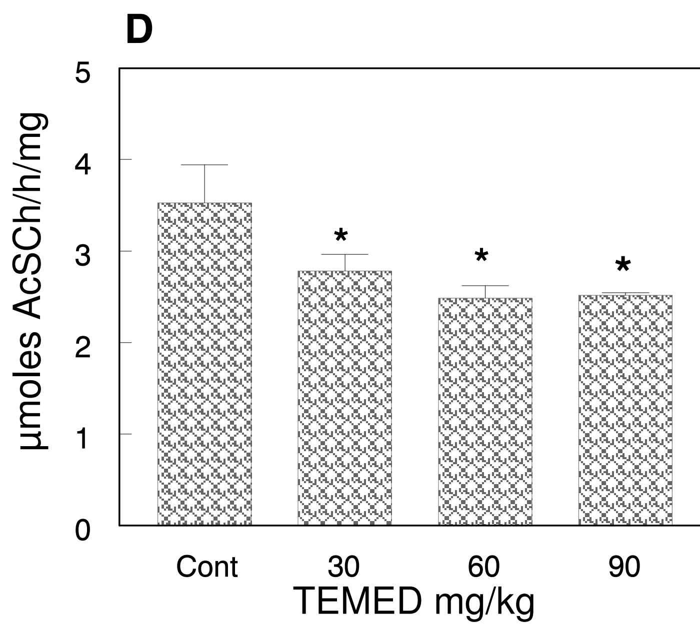


Fig.1





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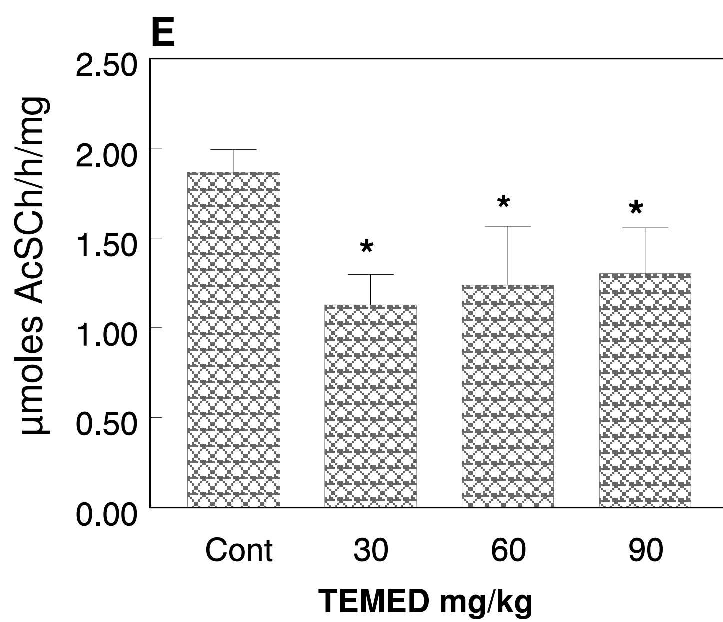

Fig.1

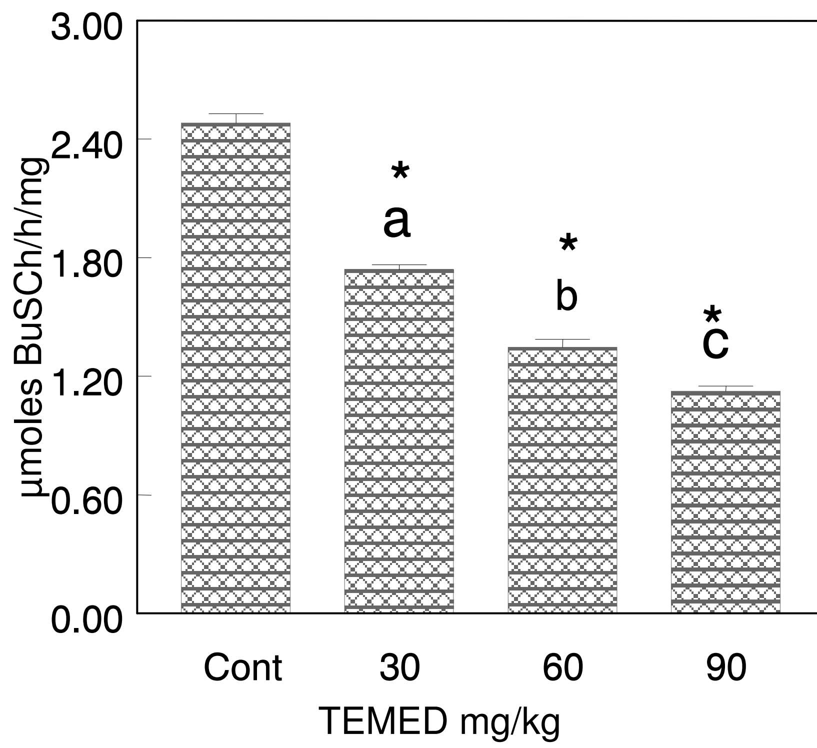

Fig.2

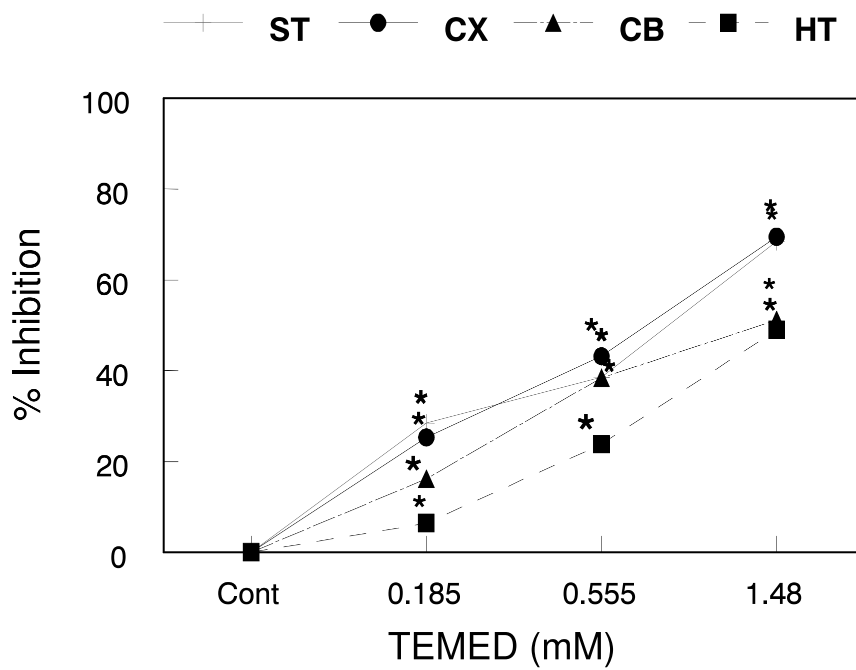

Fig.3

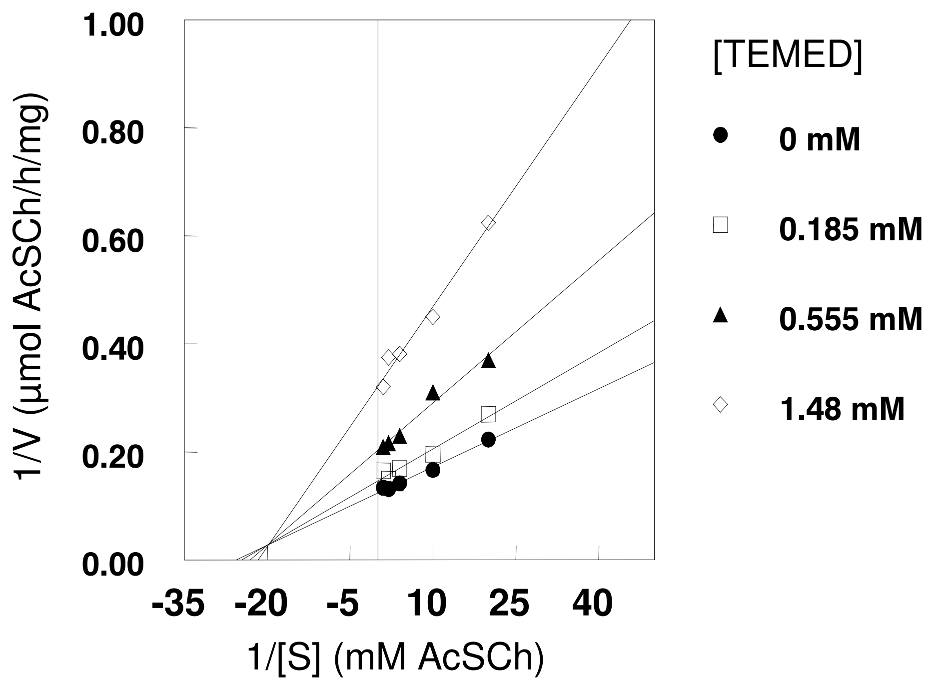

Fig.4A.

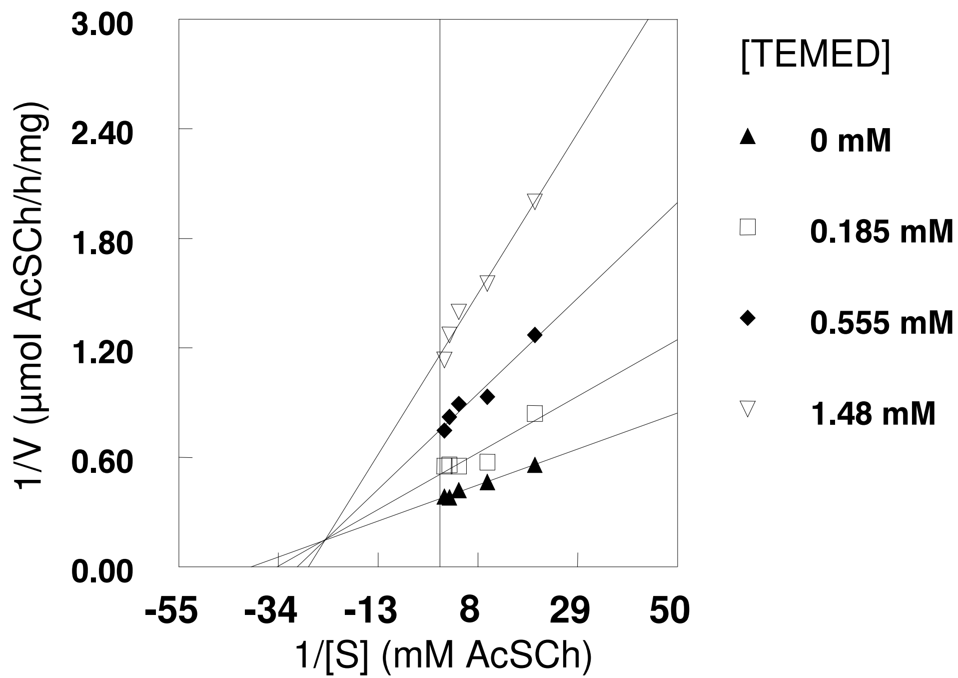

Fig.4B

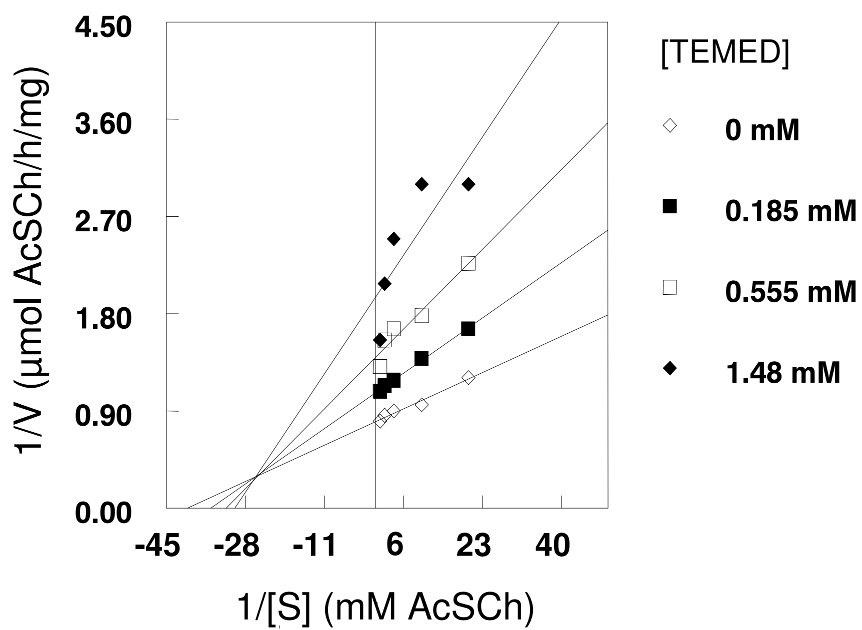

Fig.4C.

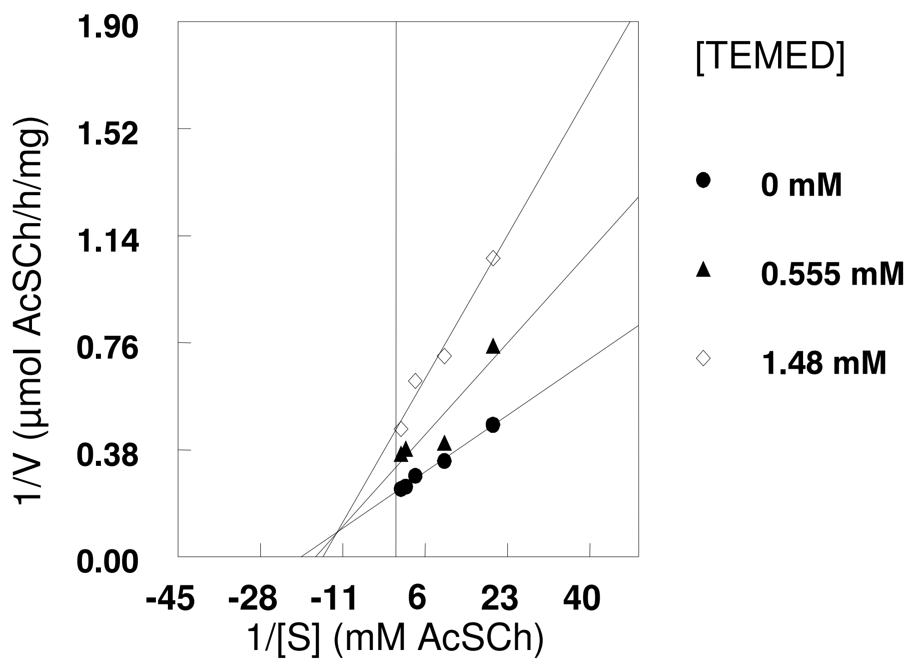

Fig.4D.

Table.1. Toxicological effect of N,N,N´,N´- Tetramethylethylene diamine (TEMED) on female rats after seven days treatment.

	Number of animals	Weight (g)	Dose (mg/kg)	Died	Survivals
Control	10	174-250	----------	--------	10
Group (1)	10	188-214	30	--------	10
Group (2)	10	170-252	60	2	8
Group (3)	10	186-242	90	4	6

Table.2. Kinetic parameters of AChE (from different regions of the brain) inhibited by TEMED.

Parameters	Striatum	Cortex	Cerebellum	Hypothalamus
IC_{50} (mM)	0.92	0.92	1.44	1.42
K_i (mM)	1.24	1.4	1.45	1.47

Discussion

Among Elipadae venoms, the *Bungarus* genera are marked very high in terms of acetylcholinesterase content. In the present study we find that krait (*Bungarus sindanus*) venom contains very high amount of acetylcholinesterase like other species (Frobert et al., 1997). Generally the venom acetylcholinesterase exists in monomeric form while in other sources it is present in multimeric form (Cousin et al., 1996). In snake venom the monomeric form is actually in globular form while in other sources it exists both in globular as well as in asymmetric forms (Cousin et al., 1996). Like other sources krait venom also shows the highest activity at alkaline pH (Aliriz and Turkoglu, 2003). In krait venom the optimal temperature was 45°C, which is higher than that for the desert cobra (*Walterinnesia aegyptia*), which shows its highest activity at 30°C (AlJafari et al., 1995). In the present study we found that krait (*Bungarus sindanus*) venom acetylcholinesterase is thermally stable at 45°C. The venom acetylcholinesterase lost only 5% of its activity after incubating the enzyme at 45°C for 40 minute while at 37°C we did not find any loss in its activity (Manuscript 1, Fig.6A). In comparison the electric eel lost 69% of its activity at 45°C after 10 min incubation while after 40 minutes it lost 80% of its activity (Manuscript 1, Fig.6B). Generally, snake venom AChE is more stable compared to other sources. Among snake venom, the *Bungarus* genus is more stable than that of the *Haemacatus, Ophiophagus* and *Naja* genera (Frobert et al., 1997). A thermal stability study of *Bungarus* AChE by capillary electrophoresis also supports that venom AChE is stable under standard conditions (Rochu et al., 2001).

The krait *(Bungararus sindanus)* venom contains true AChE, which presents characteristic catalytic properties, as previously reported (Kumar and Elliott, 1973). For the krait *(Bungararus sindanus)* venom acetylcholinesterase optimum substrate

concentration was 1 mM using a medium of 10 mM PO_4, pH 7.5 (Manuscript 1, Fig.1) but this concentration is very low when compared to the desert cobra (*Walterinnesia aegyptia*), in which the optimum activity was observed at 3 mM (AlJafari, 1995). At highest substrate concentration the hydrolytic properties of krait *(Bungarus sindanus)* venom AChE was decreased. Such type of behavior was also observed in other snake venom AChEs (Kreienkamp et al., 1995; AlJafari et al., 1995; Frobert et al., 1997). The krait *(Bungararus sindanus)* venom acetylcholinesterase hydrolyses only acetylcholine like other source of acetylcholinesterase as it did not hydrolyze butyrylthiocholine (Manuscript 1, Fig.1). It also shows that krait venom lacks butrylcholinesterase on crude venom testing. Furthermore, we observed a significant reduction in substrate inhibition of krait venom AChE by using high ionic strength buffer (Manuscript 1, Fig. 3). Such type of reduction in substrate inhibition was also observed by Frobert (Frobert et al., 1997). Krait venom acetylcholinesterase was inhibited by metals ($ZnCl_2$, $CdCl_2$ and $HgCl_2$) (Fig.7). The concentration of the metals to inhibit venom acetylcholinesterase was very high; it may be due to absence of free sulfhydryl group. According to Frasco et al.(2007), when a free sulfhydryl group is absent in the enzyme (*Drosophila melonogaster* acetylcholinesterase and human serum butrycholinesterase) inhibition by mercury will occur in the millimolar range, while in the presence of a free sulfhydryl group (*Electrophorous electricus*), the inhibition will require a micromolar range concentration (Frasco et al., 2007). The inhibitory effect of metals on venom acetylcholinesterase may be due to formation of inactive aggregation of the enzyme (Lee and Singleton 2004).

Tacrine is a well known drug used for the treatment of Alzheimer's disease which inhibits AChE, increasing the concentration of acetylcholine in the brain (Eagger et al., 1992). This increase is believed to be responsible for the improvement in memory with

the use of tacrine. In the present study (Paper 2) tacrine was found to be very effective against krait venom acetylcholinesterase as well as human serum butyrylcholinesterase. Kinetic studies indicated that the nature of inhibition was mixed for both enzymes, i.e. K_m values increase and V_{max} decrease with the increase of the tacrine concentration (Paper 1, Fig. 3 and 4). The IC_{50} values calculated for krait snake venom and human serum cholinesterase were 31 and 25.6 mM, respectively. These values are very low when compared with human retinal, human erythrocyte and camel retinal AChE (Soreq and Zakut, 1993; Al-Jafari, 1996; Alhomida, 2000). In comparison with bovine retinal AChE these values are very high (Al-Jafari, 1998). Comparing between human serum and snake venom cholinesterase, human serum butyrylcholinesterase (BChE) is more sensitive to tacrine than snake venom acetylcholinesterase (AChE) (Paper 2, Table. 3).

A comparative study of the commonly used pesticides (malathion and carbofuran) and the herbicide (paraquat) showed inhibition of Krait (*Bungarus sindanus) venom* as well as human serum butrylcholinesterase. However, these compounds did not alter the rat brain striatum acetylcholinesterase, may be due to the structural difference between the different sources of enzymes. The nature of the inhibition by malathion and paraquat were mixed type for both types of enzymes. In case of carbofuran mixed type inhibition was found with snake venom AChE (Paper 3, Fig. 6C) while with human serum BChE activity it was found to be of an uncompetitive type (Paper, Fig.5C). The IC_{50} values calculated for snake venom AChE and for human serum BChE are very close to values described for other cholinesterases (from different origins) inhibited by organophosphorus compounds (Jamal et al., 2001). The IC_{50} values calculated for paraquat were lower than malathion, perhaps due to certain component present in the commercial paraquat, may be it enhance the inhibitory potency (Paper 3, Table 2 and 3). Furthermore, we found that krait venom as well as horse serum cholinesterase are

sensitive towards N,N,N,N-tetramethylethylene diamine (TEMED: commonly used for initiating polymerization of acrylamide and bisacrylamide gel for electrophoresis). The IC_{50} values (manuscript 5, Fig. 2) showed that BChE was more sensitive to TEMED than venom AChE. This is supported by the value of the inhibitory constant (*Ki*), which indicated that TEMED has more affinity towards horse serum BChE (manuscript 5, Table 5). Kinetic studies indicated that the inhibition caused by TEMED was of mixed type, i.e., K_m increased and V_{max} decreased in a concentration dependent manner. Our results are in agreement with previous study of Al-Jafari (Al-Jafari, 1993) in which he also observed mixed type inhibition *in vitro* with water soluble and membrane bound acetylcholinesterase by using TEMED as an anticholinesterase. Additionally, we found the same type of inhibition in different regions of rat brain (ST, CX, CB and HT) acetylcholinesterase (manuscript 6, Fig. 4). Additionally, this compound also causes inhibition of cholinesterase *in vivo* (manuscript 6, Fig.1). Taken together, all the results indicate that krait venom (non-synaptic tissue) acetylcholinesterase exhibits similar properties toward different ligands like other source of cholinesterase.

Conclusion

1) The results indicate that krait venom AChE exhibits highest catalytic activity of all sources and is comparatively more stable than any other source. Furthermore, inorganic metals ($ZnCl_2$, $CdCl_2$ and $HgCl_2$) can be considered as inhibitors of snake venom AChE. Thus krait venom AChE can be used for biochemical studies.

2) The results presented suggest that tacrine can also be considered an inhibitor of snake venom and human serum butyrylcholinesterase. Thus snake venom AChE shows similar behaviour toward tacrine like other sources.

3) The results indicate that herbicide (paraquat) and the pesticides (malathion and carbofuran) can inhibit the non synaptic AChE in snake venom. Furthermore, these compounds can also change the human serum BChE. Thus both sources have a common feature i.e they are sensitive toward herbicide and pesticides.

4) The results suggest that antidepressants (paroxetine, imipramine, clomipramine and sertraline) can be used as inhibitors of krait (*Bungarus sindanus*) venom AChE. Futhermore, the study also emphasis that antidepressant have toxicological effect on human health via inhibition of serum BChE.

5) The results indicate that TEMED inhibits venom AChE and horse serum BChE. However, its affinity was higher for horse serum BChE when compared to snake venom acetylcholinesterase. Decrease in AChE activity was observed in the rat brain striatum (ST), hippocampus (HC), cortex (CX), hypothalamus (HT) and cerebellum (CB) when the animals were exposed to TEMED by gavage for seven consecutive days. Thus

TEMED has toxic effect on both AChE and BChE. This compound could have a negative effect on human health hence care should be handled carefully.

References

Agbaji, A.S., K.Gerassimidis and R.C. Hider. 1984. A comparison of electroplax and snake venom acetylcholinesterase. *Comp. Biochem. Physiol* 178C*:* 211-216.

Aliriz, S and V. Turkoglu. 2003. Purification and characterization of acetylcholinesterase from the Lake Van fish (*Chalcalburnus tarichii* Pallas, 1811). *Prep. Biochem. Biotechnol* 33:137-145.

Al-Jafari, A.A., M.A. Kamal and A.S. Alhomida. 1998. Sensitivity of bovine retinal acetylcholinesterase (E.C 3.1.1.7) toward tacrine. Kinetic characterization. *J. Biochem. Mol. Toxicol* 12:245-251.

Al-Jafari, A.A. 1996. The nature of the inhibition of camel retina acetylcholinesterase (E.C 3.1.1.7) activity by tetrahydroacridine. *J. Ocul. Pharmacol.Ther* 12:503-514

Al-Jafari, A.A., M.A. Kamal., A.S. Duhaiman and A.S. Alhomida. 1995. Acetylcholinesterase from desert cobra (Walterinnesia aegyptia) venom. optimization and kinetics study. *Mol. Cell. Biochem* 151(1):2-6.

Al-Jafari, A.A. 1993. The inhibitory effect of tetramethylethylene diamine on water soluble and membrane bound acetylcholinesterase activity. *Int. J. Biochem* 25(3):319-24.

Alhomida, A.S., A.A. Al-Rajhi., M.A. Kamal and A.A. Al-Jafari. 2000. Kinetic analysis of the toxicology effect of tacrine (Cognex) on human retinal acetylcholinesterase activity. *Toxicology* 147: 33-39.

AI-Saleh, S.S., N. Rabbani, M.K. AI-Sadoon, A.A. AIJafari and A.S. Duhaiman. 1994. A rapid fractionation method for the desert cobra venom *(Walterinnesia aegyptia). Med. Sci. Res* 22:659-660.

Barak, D., C. Kronman, A. Ordentlich, N. Ariel, A. Bromberg, D. Marcus, A. Lazar, B. Velan and A. Shafferman. 1994. Acetylcholinesterase peripheral anionic site degeneracy conferred by amino acid arrays sharing a common core. *J. Biol. Chem* 269(9):6296-305.

Bawaskar, H.S and P.H. Bawaskar. 2004. Envenoming by the common krait (*Bungarus sindanus*) and Asian cobra (*Naja naja*): clinical manifestations and their management in a rural setting. *Wilderness. Environ. Med* 15(4): 257-66.

Berman, H.A., J. Yguerabide and P. Taylor. 1980. Fluorescence energy transfer on acetylcholinesterase: spatial relationship between peripheral site and active center. *Biochemistry* 19:2226-2235.

Broaders, M. and M.F. Ryan. 1997. Enzymatic properties of the duvernoy's secretion of blanding's treesnake (*Boiga blandingi*) and of the mangrove snake (*Boiga dendrophila*). *Toxicon* 35:1143-1148.

Boulenger, G.A. 1897. A new krait from Sind (*Bungarus sindanus*). *J. Bombay Nat. Hist. Soc.* 11: 73-74

Bourne, Y., P. Taylor and P. Marchot. 1995. Acetylcholinesterase inhibition by fasciculin: crystal structure of the complex. *Cell* 83(3):503–512.

Chatonnet, A and O. Lockridge, 1989. Comparison of butyrylcholinesterase and acetylcholinesterase. *J. Biochem* 260:625–634.

Cousin, X., S. Bon, N. Duval, J. Massoulié and C. Bon. 1996. Cloning and expression of acetylcholinesterase from *Bungarus fasciatus* venom. *J. Biol. Chem* 271**:**15099–15108.

Cousin, X., C. Créminon, J. Grassi, K. Méflah, G. Cornu, B. Saliou, S. Bon, J. Massoulié and C. Bon. 1996. Acetylcholinesterase from *Bungarus* venom: a monomeric species. *FEBS. Lett* 387:196-200.

Cousin X., S. Bon, J. Massoulié and C. Bon. 1998. Identification of a novel type of alternatively spliced exon from the acetylcholinesterase gene of *Bungarus fasciatus. J. Biol. Chem* 273(16):9812-9820.

Dave, K.R., A.R. Syal and S.S. Katyare. 2000. Tissue cholinesterases, A comparative study of their kinetic properties. *Z. Naturforsch* 55:100-108.

Duhaiman, A.S., A.S. Alhomida, N. Rabbani, M.A. Kamal and A.A al-Jafari. 1996. Purification and characterization of acetylcholinesterase from desert cobra (*Walterinnesia aegyptia*) venom. *Biochimie* 78(1):46-50.

Dunant, Y., E. Babel-Guerin and D. Droz. 1980. Calcium metabolism and acetylcholine release at the nerve-electroplaque junction. *J. Physiol* 76**:** 471–478.

Eagger, S., N. Morant., R. Levyl and B. Sahakian. 1992. Tacrine in Alzhemer`s disease. Time course of changes in cognitive function and practice effect. *Br. J. Psychol* 160:36-40.

Frasco, M.F., J-P. Colletier., M. Weike., F. Carvalho., L. Guilhermino, J. Stojan and D. Fournier. 2007. Mechanisms of cholinesterase inhibition by inorganic mercury. *FEBS* 274:1849-1861.

Frobert, Y., C. Creminon, X. Cousin, M.H. Remy, J.M. Chatel, S. Bon, C. Bon and J. Grassi.1997. Acetylcholinesterases from *Elapidae* snake venoms: biochemical, immunological and enzymatic characterization. *Biochim. Biophys. Acta* 1339:253–267.

Forede, H.C and I.B. Wilson. 1971. In: P.D. Boyer, Editor, *Enzymes*, Academic Press, New York: Boyer, 87–114 p.

Habermehl, G.G. (1981). Venomous animals and their toxins. *Springer Verlag, Berlin*

Harel, M., G.J. Kleyweght, R.B.G Ravelli, I. Silman, and J.L. Sussman. 1995. Crystal structure of an acetylcholinesterase–fasciculin complex: interaction of a three-fingered toxin from snake venom with its target. *Structure* 3:1355-1366.

Hartzell, H.C., S.W. Kuffler, R. Stickgold and D.Yoshikami. 1977. Synaptic excitation and inhibition resulting from direct action of acetylcholine on two types of chemoreceptors on individual amphibian parasympathetic neurones. *J. Physiol.* 271: 817-846.

Hogg, R.C., M. Raggenbass and D. Bertrand. 2003. Nicotinic acetylcholine receptors: from structure to brain function. *Rev. Physiol. Biochem. Pharmacol* 147:1-46.

Iwanaga, S. and T. Suzuki. 1979. Enzymes in snake venom. *Hand. Exp. Pharmacol* 52:61-158.

Jamal, E.I.Y., N.R. Shashidhar., M. Adrea and SJr. William. 2001. Prediction of organophosphorus acetylcholinesterase inhibition using three-dimensional quantitative structure-activity relationship (3D-QSAR) methods. *Toxicol. Sci* 63:223-232.

Kabachnik, M.I., A.P. Brestkin, N.N. Godovikov, M.J. Michel-son, E.V. Rozengart, V.I. Rozengart. 1970. Hydrophobic areas of the active surface of cholinesterase. *Pharmacol. Rev* 22:355-388.

Karlsson, E., P.M. Mbugua and D. Rodriguez-Ithurralde. 1984. Fasciculins, anticholinesterase toxins from the venom of the green mamba (*Dendroaspis angusticeps). J. Physiol. (Paris)* 79(4):232-40.

Kawashima, K. and T. Fujii. 2000. Extraneural cholinergic system in lymphocytes. *Pharmacol. Therap* 86:29-48.

Kesvatera, T., M. Ustar and A. Aviksaar. 1979. Cobra venom acetylcholinesterase. II. Substrate specificity. *Comp. Biochem. Physiol* 162C: 193-197.

Kelly, R.B., J.W. Deutsch, S.S. Carlson and J.A. Wagner. 1979. Biochemistry of neurotransmitter release. *Annu. Rev. Neurosci* 2**:** 399–466.

Kini, R.M and H.J. Evans. 1987. Structure function relationship of phospholipase.The anticoagulent region of phospholypase A2. *J. Biol chem* 262:14402-14407.

Kreienkamp, H.S., C. Weise, R. Raba, A. Aaviksaar and F. Hucho. 1991. Anionic subsites of the catalytic center of acetylcholinesterase from *Torpedo* and from cobra venom. *Proc. Natl. Acad. Sci. U. S. A* 88: 6117-6121.

Kumar, V. and W.B Elliot. 1975. Acetylcholinesterase from *Bungarus fasciatus* venom I-substrate specificity. *Comp. Biochem. Physiol. Part C* IC : 249-253.

Kumar, V and W.B. Elliott. 1973. The acetylcholinesterase of Bungarus fasciatus venom. *Eur. J. Biochem* 34:586-592.

Lee, A.M and S. F. Singleton. 2004. Inhibition of the *Escherichia coli* RecA protein: zinc(II), copper(II) and mercury(II) trap RecA as inactive aggregates. *J. Inorg. Biochem* 98:1981–1986.

Li, B., J.A, Stribley, A. Ticu, W. Xie, L.M. Schopfer, P. Hammond, S. Brimijoin, S.H. Hinrichs and O. Lockridge. 2000. Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. *J. Neurochem* 75:1320–1331.

Li, Z-Y. and C. Bon. 1983. Presence of membrane-bound acetylcholinesterase form in a preparation of nerve endings from *Torpedo marmorata* electric organ. *J. Neurochem* 40:338-349.

Mackessy, S.P., N.M. Sixberry, W.H. Heyborne and T. Fritts. 2006. Venom of the Brown Treesnake, *Boiga irregularis*: Ontogenetic shifts and taxa-specific toxicity. *Toxicon* 47: 537-548.

MacIntosh, F.C**.** 1981. Acetylcholine. Basic Neurochemistry. Little Brown and Co., Boston, 183–204 pp.

MacPhee-Quigley, K., T. Vedvick., P. Taylor and S. Taylor. 1986. Profile of disulfide bonds on acetylcholinesterase. *J. Biol. Chem* 261:13565-13570.

Martinez-Pena y Valenzuela I., R.I. Hume, E. Krejci and M. Akaaboune. 2005. In vivo regulation of acetylcholinesterase insertion at the neuromuscular junction. *J. Biol. Chem* 280(36) 31801-31808.

Massoulié, J., L. Pezzementi, S. Bon, E. Krejci, and F. M. Vallette. 1993. Molecular and cellular biology of cholinesterases. *Prog. Neurobiol* 42:31–91.

Massoulié, J., A. Anselmet, S. Bon, E. Krejci, C. Legay, N. Morel and S. Simon. 1999. The polymorphism of acetylcholinesterase: post-translational processing, quaternary associations and localization. *Chem. Biol. Interact* 119:29-42.

Müller, T.C., J.B.T. Rocha, V.M. Morch, R.T. Neis and M.R.C. Schetinger. 2002. Antidepressants inhibit human acetylcholinesterase and butrylcholinesterase activity. *Biochim. Biophys. Acta* 1587:92-98.

Neville, L.F., A. Ganatt, Y. Loewenstein, S. Seidman, G.Enrlich and H.Soreq. 1992. Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BChE. *EMBO J* 11:1641-9

Nget-Hong, T. and P. Gnanajothy.1992. The biological properties of venoms of some american coral snakes (Genus *Micrurus*). *Comp. Biochem. Physiol. Part B* 110: 471-474.

Ordentlich, A., D. Barak, C. Kronman, N. Ariel, Y. Segall, B. Velan and A. Shafferman. 1995. Contribution of aromatic moieties of tyrosine 133 and of the anionic

subsite tryptophan 86 to catalytic efficiency and allosteric modulation of acetylcholinesterase. *J. Biol. Chem* 270(5):2082-91.

Patrick, G.L. 2001. An introduction to Medicinal Chemistry. Cholinergics, anticholinergics, and anticholinesterases.Oxford: Oxford University Press, 471 p.

Quinn, D.M. 1987. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition state. *Chem. Rev* 87: 955–979.

Raba, R., A. Aaviksaar, M. Raba and J. Siigur. 1979. Cobra venom acetylcholinesterase. Purification and molecular properties. *Eur. J. Biochem* 96:151-158.

Raba, R. and A. Aaviksaar. 1982. Cobra venom acetylcholinesterase: nature of charge isoforms. *Eur. J. Biochem* 127:507-512.

Radic Z., R. Duran, D.C. Vellom, Y. Li, C. Cervenansky and P. Taylor. 1994. Site of fasciculin interaction with acetylcholinesterase. *J. Biol. Chem* 269(15): 11233-11239 .

Radic, Z., D.M. Quinn, D.C. Vellom, S. Camp and P. Taylor. 1995. Allosteric control of acetylcholinesterase catalysis by fasciculin. *J. Biol. Chem* 270:20391-20399.

Radic, Z., R. Manetsch, A. Krasinski, J. Raushel, J. Yamauchi, C. Garcia, H. Kolb. K.B. Sharpless and P. Taylor. 2005. Molecular basis of interactions of cholinesterases with tight binding inhibitors. *Chem. Biol. Interac* 157-158: 133-141.

Radic, Z., G. Gibney, S. Kawamoto, K. MacPhee-Quigley, C. Bongiorno and P. Taylor. 1992. Expression of recombinant acetylcholinesterase in a baculovirus system: kinetic properties of glutamate 199 mutants. *Biochemistry* 31:9760–9767.

Rochu, D., C. Georges, J. Répiton, N. Viguié, B. Daliou, C. Bon and P. Masson. 2001. Thermal stability of acetylcholinesterase from *Bungarus fasciatus* venom as investigated by capillary electrophoresis. *Biochim. Biophys. Acta* 1545:216-226.

Rosenberry, T.L. J.L. Johnson, B. Cusack, J.L. Thomas, S. Emani and K.S. Venkatasubban. 2005 Interaction between the peripheral site and the acylation site in acetylcholinesterase, *Chem. Biol. Interact* 157–158:181–189.

Rosenberry, T.L.1975**.** Acetylcholinesterase. *Adv. Enzymol* 43:103-218.

Russel, F.E and W.J. Gertsh. 1983. Arthropode bites. Letter to the editor. *Toxicon* 21, 237.

Silman, I and J.L Sussman. 2005. Acetylcholinesterase: ‘classical’ and ‘non-classical’ functions and pharmacology. *Curr. Opin. Pharmacol* 5:293-302.

Soreq, H and S. Seidman. 2001. Acetylcholinesterase--new roles for an old actor. *Nat. Rev. Neurosci* 2(4):294-302.

Soreq, K and H. Zakut. 1993. Human Cholinesterase and Anti-cholinesterase. Academic Press, New York.

Soreq, H., A. Gnatt, Y. Loewenstein, Y. and L.F. Neville. 1992. Excavations into the active-site gorge of cholinesterases. *Trends Biochem. Sci* 17:353-358**.**

Sussman, J. L., M. Harel and I. Silman. 1992. Multidisciplinary Approaches to Cholinesterase Functions. New York: Plenum, 95-107.

Sussman, J.L., M. Harel, F. Frolow, C. Oefner, A.Goldman, L.Toker and I. Silman. 1991. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* 253:872-879.

Thiermann, H., L. Szinicz, P. Eyer, T. Zilker and F. Worek. 2005. Correlation between red blood cell acetylcholinesterase and neuromuscular transmission in organophosphate poisoning. *Chem- Biol. Interact* 157:345-347.

Vigny, M., S. Bon, J. Massoulié and F. Leterrier. 1978. Active-site catalytic efficiency of acetylcholinesterase molecular forms in *Electrophorus*, *Torpedo*, rat and chicken. *Eur. J. Biochem* 17:317–323.

Watling, K.J. 1998. The RBI Handbook of Receptor Classification and Signal Transduction. *3rd Edition.*

Weise, C., H.J. Kreienkamp, R. Raba, A. Pedak, A. Aviksaar and F. Hucho. 1990. Anionic subsites of the acetylcholinesterase from *Torpedo californica*: affinity labelling with the cationic reagent N, N-dimethyl-2-phenyl-aziridinium**.** *EMBO. J* 9:3885-3888.

Zhang X. 2004. Cholinergic activity and amyloid precursor protein processing in aging and alzheimer's disease. *Curr. Drug Targets: CNS Neurol. Disord* 3:137–152.