Chapter I
CHAPTER -I

GENERAL INTRODUCTION

1. HISTORICAL BACKGROUND:

The existence of an enzyme involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) was predicted in 1914 by Dale [1] based on the short-lived action of ACh on the heart of frog. In 1920s, Loewi and Navafil [2] showed that ACh was produced by stimulating the vagus nerve of frog, and the choline ester was destroyed by an enzyme, present in the heart muscle extracts. The enzyme was inhibited by the natural calabar bean alkaloid eserine [physostigmine; 1’-methylpyrrolidino (2':3':2:3)1,3-dimethylindolin-5-yl N-methyl carbamate]. In 1932 Stedman et al., purified for the first time, an enzyme present in horse serum which catalysed the hydrolysis of ACh [3]. They called this enzyme “choline esterase”. By 1940, the existence of two major forms of cholinesterase (ChE) was discovered by Allis and Hawes. The enzyme from human serum and red The cholinesterases of human serum and erythrocyte correspond to AChE and BChE respectively.

acetylcholine acylhydrolase; EC 3.1.1.7) and butyrylcholinesterase (BChE; acetylcholine acylhydrolase; EC 3.1.1.8) respectively. Both enzymes are type B carboxylesterases by virtue of their biochemical properties. These two enzymes exist in multiple molecular forms, although AChE displays greater
diversity. Both degrade the neurotransmitter ACh rapidly [5-7]. AChE is more tissue specific than BChE. Momentum in ChE research has been and continues to be facilitated by two intrinsic attributes of the enzyme. One is its high catalytic capacity (turnover number for ACh >10^4 sec^-1) and the other is its susceptibility to a plethora of natural and man-made inhibitors.

The high substrate turnover by ChEs enabled measurements in heterogeneous systems [8] and permitted the identification of enzyme isoforms based on their hydrodynamic properties [9]. The susceptibility of ChEs to inhibitors allowed the study of mechanism of catalysis through the use of hemisubstrates and reactivators. Furthermore, the inhibitors of ChEs were shown to have applications in therapeutics and toxicology.

In the post-World War era, the focus was directed to the specificity and pharmacological actions of ChE inhibitors. Shortly thereafter, important developments occurred in histochemical techniques and in the methodology to differentiate between AChE and BChE. Wilson in 1950’s contributed to ChE research by way of site directed inhibitor studies, delineating individual steps involved in the reaction mechanisms and also developed affinity based methods of enzyme purification [10]. The discovery of the asymmetric, collagen-tailed AChE by Massoulie and Rieger in 1969 gave a new dimension to the study of ChE molecular polymorphism [9].

Recombinant DNA technology was a natural direction for ChE research in 1980’s. This was followed by the crystallographic era with the
deciphering of the crystal structure of *Torpedo* AChE in the early 1990's. The crystal structure and results from site-directed mutagenesis revealed a vast array of information on the three-dimensional structure, active site regions and inhibitor interactions of ChEs.

2. PUTATIVE BIOLOGICAL ROLES OF BChE:

In vertebrates, the major form of BChE is produced in the liver and secreted into the circulation. In addition it is also present in adipose tissue, intestine, smooth muscle cells, white matter of the brain and many other tissues [11]. However, the tissue specific enzymes, other than liver origin, are not secreted into the blood. There is no evidence to indicate that tissue enzymes are derived from the plasma. In spite of its ubiquitous distribution, the biological role of BChE is still uncertain [12]. Among several proposals made to describe the biological role of BChE, only some have relevance to cholinergic functions.

2.1 BChE AS AN ELEMENT IN CHOLINERGIC SIGNALLING :-

Termination of signal transmission by hydrolysis of ACh is an essential part of cholinergic signalling. The importance of ChEs in this process implies that their turnover number should be high and indeed it is as high as $10^4$ ACh molecules per second by a single catalytic site [5, 6, 13]. This evidence suggests that the actual concentration of the catalytically active ChEs at cholinergic synapse and neuromuscular junction should be very low, and so is the case [14]. In the central nervous system (CNS),
AChE is the predominant enzyme terminating the cholinergic signalling which hydrolyses ACh much faster than BChE [15, 16]. However, certain neurons appear to contain only BChE [17, 18]. Further, AChE but not BChE is inhibited by excess amount of substrate ACh (3mM). This substrate inhibition may affect the residence time of ACh in the synapse, provided that i) the released ACh in the synapse, in the vicinity of AChE approaches concentrations above 3mM and ii) the amount of BChE in that synapse is too low to perform catalysis instead of AChE. Estimates of synaptic concentrations of ACh are in the range of 0.3 mM [19]. However, as diffusion of ACh occurs from vesicles, its transient concentration at discrete release site may be higher than 3 mM. In the absence of BChE, the substrate inhibition effect can prolong the residual time of ACh in the synapse, and facilitate further interaction with receptors on post synaptic membrane [20]. This cascade of events may be pronounced in the neuromuscular junction than in brain synapse, since in the former much of the synaptic AChE is localised in the basal lamina, closer to the sites of ACh release than the receptor molecule positioned on the plasma membrane of the acceptor muscle cells [21]. Thus BChE is generally viewed as a backup enzyme of AChE.

AChE and BChE serve a pivotal role in terminating muscle contraction by rapidly hydrolysing ACh in neuromuscular junctions. Though AChE appears to be the predominant enzyme, BChE has been
shown to be an adequate substitute for neuromuscular AChE in certain cases, as is exemplified by the sole presence of BChE in the heart muscles of *Torpedo marmorata* [22]. In humans, BChE may be perhaps substituted by AChE, since no physiological dysfunction has been found to date in people with the “silent variant” (inactive serum BChE) type.

### 2.2 BChE AS A SCAVENGER

One major physiological role proposed for BChE is to function as a scavenger of anti-ChE agents, thus protecting the AChE from inactivation at the neuromuscular junctions and other cholinergic sites [23]. This fact is deduced from the observation that BChE interacts with a wider range of anti-ChE agents [24] and in certain cases (e.g., diisopropyl phosphofluoridate [DFP] and many carbamates), the rate of its inactivation is considerably faster than that of AChE [25]. Accordingly, there must be an evolutionary pressure that accounts for the need for a scavenger of anti-ChE agents. This is evident by the presence of natural ChE inhibitors in the environment, including glycoalkaloids from solanaceous plants [26], fungal antibiotics like puromycin [27] and its analogs, cocaine derivatives [28], poisons from several species such as oysters, organophosphate (OP) from cyanobacteria and polypeptides from snakes [29-31], metals such as aluminium, mercury, scandium [32-34] and the carbamate of calabar bean, physostigmine [11]. Individuals who carry a variant BCHE allele, one of the genes that codes for catalytically inactive BChE, have an increased
sensitivity to these inhibitors [35]. Further evidence for the scavenging role of BChE comes from the observation that de novo amplification of an aberrant BCHE gene in humans subjected to chronic exposure to the agricultural insecticide parathion [35].

BChE has been shown to hydrolyse the methyl ester bond of cocaine and its derivatives in vitro [28, 37, 38]. Cytochrome P450 catalysed destruction of cocaine generates the hepatotoxic narcocaine nitroxide. In contrast, BChE catalysed hydrolysis of cocaine results in the formation of less harmful products. The serum level of BChE, similar to its modulation by ChE inhibitors, are also correlated with the serum levels of cocaine related narcotics [39, 40]. Further, exogenous human BChE has been shown to confer protection against cocaine toxicity in rats, when given either prophylactically or therapeutically [41]. Cocaine based local anaesthetics are also hydrolysed by BChE [42]. Thus BChE plays an active role in the metabolism of cocaine and related narcotics such as heroin. BChE also exerts a key clinical role in degrading drugs such as succinylcholine, an inhibitor of AChE, which is commonly used as a muscle relaxant. However, individuals with BChE mutant (the Asp70Gly variant) are unable to hydrolyse succinylcholine, causing prolonged apnea [36].

2.3 BChE IN LIPID METABOLISM:-

In 1963, Clithrow et al., proposed a role for BChE in fatty acid metabolism. These investigators suggested that butyrylcholine formed as an
intermediate during lipid metabolism was toxic and has to be removed from
the system; BChE was proposed to play a role in such a detoxification
process [43]. Another suggestion was that serum BChE participates in
choline homeostasis and thus controls the synthesis of acetylcholine [44].
These proposals could not be substantiated because there was no
accumulation or even presence of such choline esters in serum/liver/urine of
rats treated with BChE specific inhibitor tetraisopropyl pyrophosphoramide
(iso-OMPA) for prolonged period [45].

Immunoelectrophoresis of purified serum β-lipoprotein demonstrated
that serum BChE could be sequestered with β-lipoprotein in blood in vivo
[46]. Many patients with hyperlipoproteinemia, including nephrotic
syndrome, had both elevated levels of serum BChE and low density
lipoprotein (LDL) [47]. Further more, a patient who was poisoned by an
organophosphate had extremely low levels of serum BChE and LDL.
However, when the patient recovered, both his serum BChE and LDL levels
increased concomitantly [48]. Similar results were also obtained with
experimental models treated with anti-ChE agents [49]. Except these
observations no explanation was provided regarding the role of BChE in
lipid metabolism. This is because only about 10% of serum BChE activity
can be recovered in LDL fraction [50]. Nevertheless, one consistent
observation is that many patients who are either hyperlipoproteinemic,
diabetic or obese always showed an increased serum BChE activity [50,
The common characteristics of these patients are elevated serum triglycerides as well as very-low density lipoprotein.

**2.4 ARYL ACYLAMIDASE (AAA) ACTIVITY OF BChE AND ITS POSSIBLE BIOLOGICAL ROLES:**

Human serum BChE exhibits an AAA activity capable of hydrolysing the acylamide bond of the synthetic substrate o-nitroacetanilide [52]. This AAA activity is strongly inhibited by classical ChE inhibitors. In addition, it is susceptible to inhibition by 5-hydroxytryptamine (serotonin) and several fold activation by tyramine (Table 1) [52]. The aryl amide bond cleaved by AAA is similar to those found in pain-relieving drugs such as paracetamol and phenacetin. It has been suggested that, if AAA cleaves any such synthetic drug, then serotonin, by inhibiting AAA, may help in prolonging the life of such drugs [53]. It has been speculated that, tyramine activates the AAA activity on circulating BChE. This would lead to increased destruction of an endogenous acylamide compound. These series of events result in derangement of a natural pain-relief mechanism, there by triggering migraine [53]. Another important facet of AAA on BChE is the C-terminal deamination of the neuropeptide Substance P, there by initiating the metabolism of this compound. Since both cholinergic and serotonergic drugs modulate the AAA activity, this activity may represent an interface between these two-neurotransmitter systems [54].
### Table 1. The characteristics of aryl acylamidase activity associated with BChE$^{53}$

<table>
<thead>
<tr>
<th>Property</th>
<th>AAA associated with BChE</th>
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<tbody>
<tr>
<td>Amine sensitivity</td>
<td>Inhibited by 5-hydroxytryptamine and stimulated by tyramine</td>
</tr>
<tr>
<td>Sensitivity to choline esters</td>
<td>Inhibited by succinylcholine and other choline esters</td>
</tr>
<tr>
<td>Sensitivity to cholinesterase inhibitors</td>
<td>Inhibited by eserine, neostigmine and the BChE specific inhibitor iso-OMPA</td>
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</table>
Besides the AAA activity, two different types of peptidase activities have been described for BChE [57-58]. The first has the specificity of dipeptidylaminopeptidase and the second is a carboxypeptidase like activity. The functional significance of these peptidases remains to be explored. Unlike the AAA activity, the presence of peptidase activities has been subjected to divergent observations from different laboratories [53,59].

2.5 ROLE FOR BChE IN DEVELOPMENT:-

The early expression of BChE during embryogenesis and cell proliferation [62-64] and amplification of BCHE gene in hemopoietic disorders [65] are all suggestive of a developmental role for this enzyme. Since BChE in the neural tube of chick embryo is closely associated with cell proliferation, this enzyme has been suggested to have a role in cell growth [67]. Addition of purified BChE preparations have been found to exert a mitogenic action on various neural cells in vitro, as measured by an increase in [³H]-thymidine uptake. This stimulatory effect was found to be due to the esterolytic activity of BChE, since pre-treatment of BChE by DFP reduced its stimulatory potential. Ethopropazine and iso-OMPA, two specific inhibitors of BChE diminish the uptake of [³H]-thymidine, again supporting a stimulatory role of BChE in cell proliferation. In contrast, BW 284c51, a specific inhibitor of AChE showed no effect on the cell proliferation [72].
BChE expression precedes that of AChE in time and space during development [71]. The expression of AChE itself is regulated by BChE activity. Three-dimensional retinal cell reaggregates were used to demonstrate the regulatory role of BChE activity on AChE expression. Addition of the irreversible BChE inhibitor iso-OMPA to reaggregating retinal cells not only completely suppresses BChE expression, but also AChE expression. Also, the release of AChE into the media of tectal cell cultures is inhibited by iso-OMPA to less than 20% [71]. During early development in rabbit, the BChE transcripts appear before AChE transcript [73]. These findings confirm that BChE is expressed transiently during late stage of cell proliferation, whereas AChE expression is associated with differentiating cells [71].

AChE and BChE have been found to share amino acid sequence homology with cell adhesion molecules such as neurotactin and glutatin of Drosophila [74-76]. These data suggest that ChEs may also possess properties similar to cell adhesion molecule. The adhesion functions of cell adhesion molecules belonging to the immunoglobulin superfamily is because these molecules exhibit Human Natural Killer cell type 1(HNK-1) carbohydrate epitope on their surface [77, 78]. In a histochemical study, the expression of AChE, BChE and HNK-1 have been compared during cranial nerve growth [79]. A meshwork of HNK-1 was noted spatio-temporally, closely matching BChE expression. In addition, migrating AChE- positive
cells become decorated by HNK-1 when approaching their target. This indicated that the HNK-1 epitope on ChEs are highly regulated, and the HNK-1 matrices help directing neuronal cells and growing neurites to their targets. Of the five different types of BChE investigated in chicken, four have been shown to express the HNK-1 epitope [80]. The major inactive form of BChE from adult chicken, BChE75, belongs to the rare o-glycosylated type of proteins. This BChE75 binds strongly to HNK-1 IgM and after treatment with neuraminidase, the BChE75 effectively binds to peanut lectin, presenting a chemical paradox. With the HNK-1 and peanut agglutinin (PNA) receptor on it, this molecule could have both growth supportive and suppressive functions [81].

2.6 ROLE OF BChE IN TUMORIGENESIS:

High levels of ChE activity have been reported in primary brain tumours [60], ovarian tumours [66] and in the sera of patients with various types of primary carcinoma [61]. Experiments have provided compelling evidence that CHE genes are abnormally expressed in many types of human tumours [82]. The aberrant expression of these genes can be manifested at the DNA, mRNA or protein level (Table 2). Many tumour cells that display no detectable electrical response to ACh have been shown to express ChEs and ACh receptors [62], suggesting that cholinergic signalling may be functionally important in their growth.
Table 2. Aberrations in ChE genes and their products in various tumours

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Aberration</th>
</tr>
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<tbody>
<tr>
<td>Leukaemia</td>
<td>ACHE, BCHE gene amplification</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>ACHE, BCHE gene amplification, high levels of ACHE, BCHE mRNA</td>
</tr>
<tr>
<td>Neuroblastoma, glioblastoma</td>
<td>High frequency of mutants in BCHE gene, Asp70→Gly; Ser425→Pro substitutions in BChE protein</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>BCHE mRNA of abnormal size, high levels of BChE monomers and dimers</td>
</tr>
<tr>
<td>Meningioma</td>
<td>High levels of ACHE, BCHE mRNA, high levels of BChE monomers and dimers</td>
</tr>
<tr>
<td>Breast, bladder carcinoma</td>
<td>Abnormal pattern of enzyme inhibition</td>
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Several lines of evidence suggest that the activation of cholinergic signalling pathway is directly correlated with cell proliferation. For example, treatment of *Xenopus* oocytes with ACh or its analogue carbamylcholine enhanced phosphoinositide metabolism releasing the oocytes from their meiotic block [84]. This enhancement in phosphoinositide metabolism has been correlated with developmentally induced alterations in the phosphorylation status of substrates of the cdc2 family of protein kinases, the universal controller of cell division [85-89]. Both AChE and BChE contains the consensus peptide moiety Ser/Thr-Pro-X-Z (where X is a polar amino acid and Z is a basic amino acid) which is found in many substrates of cdc2-related protein kinases. The cdc2-related protein kinase(s) thus could modulate the level of ACh through phosphorylation/dephosphorylation of ChEs and this may be the molecular mechanism linking ChEs with tumour cell proliferation. Conceivably, AChE and BChE may be substrates of distinct cdc2-related kinases that, for example, are specific to particular cell types and/or cell differentiation stages. This notion is strengthened by the identification of a novel cdc-like kinases, CHED, whose expression, along with the amplification of BCHE gene is required for megakaryocytopoiesis [90]. Thus it is likely that ChEs influence tumorigenesis through their ability to hydrolyse ACh. Studies by Williams and Lennon have shown that small-cell lung carcinoma possess
nicotinic receptors, the stimulation of which caused enhanced cell proliferation [91].

Cytotoxic inhibitors have been shown to cause amplification of their target protein’s gene. Thus, OP poisoning may also induce ChE genes to amplify. A considerable amount of evidence has accumulated over the years, demonstrating cytotoxic and mitogenic effects of OP poisons. Using cultured tumour cells pesticide induced DNA damage and subsequent DNA repair processes were shown by several research groups [92-94]. An epidemiology study revealed an increased risk of developing leukaemia and CNS tumours for agricultural workers exposed to OP insecticides [95]. Figure 1, shows a hypothetical scheme of the various routes through which changes in the ChE genes and their protein product may contribute to tumorigenesis [90].

2.7 ROLE OF BChE IN ALZHEIMER DISEASE (AD):

Neurofibrillary tangles and amyloid plaques are the major histopathological hallmark of AD. Light and electron microscopic and immunohistochemical studies have demonstrated that these structures contain altered forms of AChE and BChE. These AD-related ChEs (ADChEs) have different histochemical and biochemical properties when compared to ChEs of normal neuronal cell bodies/axons (Table 3) [96-104]. The ADChEs do not appear to originate from neurons. Histochemical pattern and enzymic properties of glial ChEs parallel that of ADChEs [105].
Figure 1. Hypothetical scheme showing the various ways in which aberrant expression of cholinesterase genes and their protein products may contribute to tumorigenesis. 

- Tumor growth
- Tumor-associated chromosome breakage
- Accelerated rate of cell division
- Mutability and/or amplification of cholinesterase genes
- Exposure to cholinesterase inhibitors
- Changes in activities of cdc2-like kinases
- Defective cholinergic signalling

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In normal brain, the ratio of BChE to AChE positive glia is higher in entorhinal and interotemporal cortex, two regions that are highly susceptible to the pathology of AD than the primary somatosensory and visual cortex. In comparison to normal elderly individuals, AD brains displayed significantly higher density of BChE-positive glia and lower density of AChE-positive glia in the entorhinal and interotemporal cortex, but not in the primary somatosensory and visual cortex [105]. These observations indicate that glia are a likely source of ADChEs, and particularly the BChE, associated with the pathological lesions of AD. It has been suggested that the high BChE- to AChE-positive glia may play a permissive or causative role in the neuropathology of AD [106].

Although a matter of controversy, ChEs have been shown to display protease/peptidase activity [107, 109]. In particular, purified ChEs display a specific AAA activity that is sensitive to inhibition by indolamines [52, 109]. The observation that ADChEs are also inhibited by indolamines, as well as certain protease inhibitors suggests that these enzymes might be more closely associated with amidase- or peptidase-like activities, and might be involved in the proteolytic processing of amyloid precursor protein leading to AD [101].

The molecular pattern of ADChEs parallel that of ChEs found in embryonic brain [110]. This resemblance of ChEs in AD with embryonic forms has been suggested to reflect the activation of a restorative
Table 3. Enzymic properties of ChEs associated with pathological and normal elements in AD brain¹⁰⁶

<table>
<thead>
<tr>
<th>Type of ChE</th>
<th>pH</th>
<th>Conventional inhibitors</th>
<th>Indolamines and protease inhibitors</th>
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<tbody>
<tr>
<td>AChE in normal neurons and axons</td>
<td>Stained best at pH 8.0</td>
<td>Completely inhibited by low (10⁻⁷-10⁻⁶ M) concentration</td>
<td>Not affected by concentrations as high as 10⁻³ M</td>
</tr>
<tr>
<td>ChEs in plaques tangles and amyloid angiopathy</td>
<td>Stained best at pH 6.8</td>
<td>Inhibited by high (10⁻⁵-10⁻³ M) Concentration</td>
<td>Completely inhibited by 5 x 10⁻⁴ - 10⁻⁵ M</td>
</tr>
<tr>
<td>ChEs in glial cells</td>
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programme in AD, so that the embryonic functioning of ChEs, such as regulation of cell growth/cell adhesion is part of a complex neurotrophic repair mechanism [111].

3. STRUCTURE AND ACTIVE SITE OF BChE:

Human serum BChE belongs to the α/β hydrolase family of protein, whose constituents evolutionarily diverged from a common ancestor [112]. Other members of this family include acetylcholinesterase, lipase, carboxylesterases, cholesterol esterases, carboxypeptidase II, dinelactone hydrolase and haloalkane dehydrogenase [113-118]. The core structure of each enzyme is similar: α/β sheet of eight β-strands connected by α-helices. The catalytic apparatus of these enzymes is composed of Ser-His-Glu/Asp triad. Apart from the hydrolytic enzymes, there are also other proteins with domains that clearly belong to this family, but devoid of the hydrolytic activity, e.g., neurotactin, thyroglobulin [74-76]. The amino acid identity within the α/β hydrolase family of proteins varies from ≈16% for closely related proteins, to 97% for distantly related proteins [119].

3.1 SUBUNIT ORGANISATION OF HUMAN SERUM BChE:-

Human serum BChE exists predominantly as a soluble, hydrophilic, globular tetrameric (G₄) form. The G₄ is a 340 kDa homotetramer arranged as dimers of dimer [120]. It appears to be an asymmetric enzyme in which the two dimers are associated in a quasi-linear fashion [121, 122]. A single disulfide bridge located at Cys571, near the C-terminus links the two
subunits in each dimer [123]. Strong hydrophobic bond contributed by the conserved aromatic amino acids of the C-terminus holds the four subunits together [22,7]. The molecule remains to be a tetramer even after the interchain disulfide bonds are reduced [120].

Other molecular forms of serum BChE include the G₁ and G₂ forms, representing the monomeric and dimeric form of the enzyme [121, 124]. These two forms may be the precursors and/or degradation products of G₄. Apart from these two size-isomers, two other molecular forms, designated as C₂ and C₅ have been identified (Figure 2) [125-127]. The C₂ form has been characterised as a G₁-albumin conjugate [125], but the composition of C₅ is not known, except that it is a conjugate of G₄ with an unknown 60 kDa protein [128]. Other than the hydrophilic G₄ form, minor amphiphilic G₄ forms have been characterised from human brain [129], mucosal cells of rat intestine [130], serum of mouse [131] and Torpedo heart [132].

3.2 STRUCTURE OF HUMAN SERUM BChE SUBUNIT:-

The complete amino acid sequence of human serum BChE was determined by Edman degradation method of over-lapping peptides [123] and latter confirmed by cDNA [133-135]. Each subunit is made up of 574 amino acids with 9 asparagine linked carbohydrate chains. The carbohydrates linked to Asn residues are at position 17, 57, 106, 241, 256, 341, 455, 481 and 486. Of the total weight of 85,534 per subunit, 20,442 is
Figure 2. Molecular forms of BChE and their relationship. $G_1$ (monomer); $G_2$ (dimer); $G_4$ (tetramer); $C_2$ (hybrid of $G_1$ and albumin); $C_5$ (hybrid of $G_4$ and a 60 kDa protein). Since the pathways leading to the formation of $C_2$ & $C_5$; and the nature of the 60 kDa factor associated with $C_5$ are unknown, they are denoted by a ‘?’.
contributed by the carbohydrates. Each subunit contains 3 internal disulfide 
bonds between Cys65-Cys92, Cys252-Cys 263 and Cys400-Cys519 [123].

The polypeptide of BChE includes the loops A, B and C, governed 
by the Cys-Cys bonds. There is synergistic interactions between loop A 
(i.e., Asp70) and loop C constituents (such as Pro425). The C loop has been 
further subdivided into C₁ (Cys400 to Met437) and C₂ (His438 and Cys519) 
peptide domains. Assuming a stem structure for the peptide domain, which 
starts with the key His438 residue and ends at Glu443, a two-dimensional 
structure has been modelled by Neville et al., (Figure 3)[140].

Human BChE displays higher level of homology to Torpedo AChE 
than to human AChE. AChE from the electric organ of Torpedo has 575 
amino acids per subunit [137, 138], a number almost identical to the 574 of 
human BChE. The two sequences contain 309 amino acids located exactly 
in the same position. The positions of the intrachain disulfides are precisely 
in the same positions in both protein and contain 27, 11, and 119 amino 
acids within the three disulfide loops. Torpedo AChE has an interchain 
disulfide near its carboxy terminus at Cys572, a location similar to that of 
human BChE at Cys571. The active site serine is in position 200 in Torpedo 
and 198 in human BChE. Their hydropathy profiles are highly similar 
suggesting that protein folding in Torpedo AChE and human BChE are 
highly similar [139]. One significant difference is most Torpedo AChE has
Figure 3. Schematic two-dimensional model for human serum BChE. The peptide domains that are identical in human AChE are presented in black letters over white background, whereas peptide domains unique to human serum BChE are drawn in white letters over black background. Sites of natural or site-directed mutability are marked by dots; cysteine loops are labelled with connected dots; the active site serine is marked with an asterisk\textsuperscript{21}
only four asparagine linked carbohydrate chains, while human BChE has nine.

3.3 ACTIVE SITE OF HUMAN SERUM BChE

The catalytic efficiency of BChE and its high reactivity towards a variety of covalent and non-covalent inhibitors seem to originate from the unique architecture of the active centre. Complete amino acid sequencing of human BChE [133-135], X-ray crystallography of Torpedo AChE [113] and biochemical analysis of natural and site directed mutants of BChE [140, 142, 143] revealed the active site of BChE to be within a 20 Å deep gorge which penetrates halfway into the enzyme and contains the “catalytic triad” at about 4 Å from the base of the gorge (Figure 4) [113]. Several functional subsites within the active site gorge were identified, including the catalytic triad in the acylation site. The “acylation site” promotes the acyl transfer reaction and consists of Ser198(200)*, His438(440) and Glu325(327), which form a planar array that resembles the catalytic triad of chymotrypsin and other serine proteases [144-146]. There are however two differences, i) ChEs contain Glu instead Asp in the catalytic triad [147] and ii) the triad is of the opposite “handedness” to that of chymotrypsin [123]. Nevertheless the three residues occur within highly conserved regions of the sequence, and is typical of active sites of α/β proteins [148].

* Numbers in parenthesis indicate the position of the respective amino acids in Torpedo AChE
Figure 4. Schematic representation of BChE active site. Active site of BChE showing the catalytic triad, functional subsites and positioning of key amino acid residues (denoted by single letter abbreviation; D - Asp; E - Glu; F - Phe, H - His; M - Met; S - Ser; W - Trp; Y - Tyr) [21]
The presence of Trp in the active site of BChE was predicted by chemical modification studies [149]. The elucidation of the X-ray crystallographic structure of *Torpedo* AChE [113] has in fact identified Trp82(84) to be at the “anionic subsite” or the “choline binding pocket”. The Trp82(84) stabilises the charged moieties of substrates and other charged ligands at the active site by cation-π interaction. The choline binding subsite is characterised by the presence of Ala328(Phe330) apart from the conserved Trp82(84) and Glu197(199) [144, 145]. The presence of Ala allows BChE to interact with substituted tricyclic inhibitors such as ethopropazine [145]. In contrast, human AChE has a Tyr in this position which sterically occludes the binding of the substituted tricyclic inhibitor to it [144, 145].

The “acyl pocket” of BChE includes the residues Leu286 and Val288, which is in contrast to the presence of Phe288 and Phe290 in *Torpedo* AChE [144, 145]. Thus, the presence of aliphatic residues in BChE allows for catalysis of larger substrates and accommodates the BChE selective alkyl phosphate, iso-OMPA [144, 145]. The difference in substrate specificity and inhibitor sensitivity between BChE and AChE has shown to be mainly due to the differences in the acyl pocket structure of the two enzymes.

The “hydrophobic subsites” accommodates the alcohol portion of the covalent adduct of the substrates (tetrahedral intermediate) that includes
residues Trp82(84), Tyr334(336) and Phe329(337), which operate through non-polar and/or stacking interactions depending on the substrate [150-152].

Another important functional architectural element of the BChE active centre is the arrangement of hydrogen bond donor amino acids that can stabilise the tetrahedral transition enzyme-substrate complex by accommodating the negatively charged carbonyl oxygen [153]. Structural and modelling studies revealed a three-pronged “oxyanion hole” formed by peptidic NH-groups Gly116(118), Gly117(119) and Ala199(201) [154]. Apart from its role in accommodating the oxyanion, this structure is one of the important determinants of the active site geometry. This has been supported by the X-ray structure of the huperzine A-Torpedo AChE adduct [155].

Before crystal structure of Torpedo AChE was available, Asp70(72) was thought to be the anionic site [142, 156], defined as the site that stabilises the positively charged choline moiety of substrates in the active site. However, it is now clear that the anionic site is actually Trp82(84). The Asp70(72), at the rim of the active site gorge is too far away (1.1963 nm) from the active site Ser198(200), located at the bottom of the gorge, to bind the substrate molecule while it is under going bond breaking. In Torpedo AChE the amino acid residue Asp72 has been found to be a component of the “peripheral anionic site” (PAS). One of the proposed
functions of the PAS is to transmit a signal from the top of the gorge to its bottom, resulting in a conformational change [152, 158, 159]. In AChE several amino acids participate in the functioning of PAS, but only two amino acids, Trp279 and Asp72 constitute the core of the PAS. The other amino acids in the AChE PAS, Tyr70, Tyr121, Glu278 and Tyr334 may or may not participate in binding, depending on the structure of the ligand with which it interacts [158]. Since Asp72 is an essential component of AChE PAS, and since the residue is located at the mouth of the gorge in both AChE and BChE, it follows that Asp70 is the PAS of BChE. Site-directed mutation analysis has indeed revealed Asp70 to be the PAS of BChE [157]. The function of PAS in BChE is to facilitate transfer of substrate to the active site. The PAS of BChE has been shown to have all features of AChE PAS, except those that rely on aromatic amino acids [157].

The disulfide loop Cys65-Cys92 is a typical “omega loop”, which is a structural element conserved throughout the esterase/lipase family of hydrolytic enzymes sharing the α/β hydrolase fold. The loop is characterised by its important function of orienting Asp70 and Trp82 [160]. Molecular dynamics suggests that the omega-loop is flexible in BChE, changing conformation upon binding of the bis-quaternary substrate such as succinylcholine [161]. The conformational change in the omega loop of BChE is also supported by the stopped-flow analysis of the reaction of horse serum BChE with D-tubocurarine [162].
3.4 CATALYTIC MECHANISM OF BChE

3.4.1 Catalytic mechanism

The general features of BChE catalysis are outlined in Figure 5. The first step is the nucleophilic attack by Ser198(200), and is assisted by general-base catalysis by His438(440) to produce a tetrahedral intermediate. This intermediate collapses to the acyl-enzyme by general-acid catalysed expulsion of choline by His438(440). The next step of deacylation follows a similar series of events, with the expulsion of acyl acid from the tetrahedral intermediate of the active site Ser198(200) [5, 136, 163]. Glu325(327) functions with His438(440) in a proton relay network [164].

The information that Asp70 is located at the entrance of the active site gorge required the cationic substrates to bind with BChE in at least a two-step process. The first step is the binding of the substrate with Asp70 on the rim of the gorge, followed by re-equilibration of the substrate down into the catalytic site at the bottom of the gorge. However this model still did not provide a basis for rationalising the differential effect of the Asp70Gly mutation on mono- and bis-quaternary substrates. Hence, a third binding step was introduced [161].

The three-step model is schematically represented in Figure 6. The first complex ES1 involves a charge-charge interaction between choline
Figure 5. Mechanism of BChE catalysis of butyrylcholine. (E - Asp; H - His; S - Ser)
Figure 6. Three step model for substrate binding to BChE\textsuperscript{161}
group and Asp70(72). The second intermediate ES2 finds the substrate positioned vertically in the gorge between Asp70(72) and Trp82(84). In the third complex ES3, the substrate is positioned horizontally at the bottom of the gorge, in the catalytic site [161]. The following scheme depicts a mechanism with three enzyme-substrate complexes:

\[
E + S \xrightleftharpoons{K_s} ES_1 \xrightleftharpoons{K_1} ES_2 \xrightarrow{K_{II}} ES_3 \rightarrow EA + P_1 \rightarrow E + P_2
\]

E is enzyme, S is substrate, ES1, ES2 and ES3 are enzyme-substrate complexes. EA is the acylated enzyme intermediate. P1 and P2 are products. The dissociation constant of ES1 complex is \(K_s\). The isomerisation constant of the ES2 complex is \(k_I\) and for ES3 is \(k_{II}\).

### 3.4.2 Substrate activation and inhibition

Substrate activation is a characteristic feature of BChE and is observed in the range of 0.4 - 40 mM butyrylthiocholine iodide (BTCI) in 0.1 M potassium phosphate buffer, pH 7.0. The extrapolated \(V_{\text{max}}\) determined from this range of BTCI concentration is 3-fold higher than that of the \(V_{\text{max}}\) calculated with low BTCI concentrations from 0.01-0.1 mM [157, 165]. Substrate activation in BChE is seen with acetylthiocholine iodide, propionylthiocholine iodide and BTCI [166-170], but not with benzoyl choline, succinylcholine or \(o\)-nitrophenylbutyrate. Masson \textit{et al.}, proposed that a second BTCI binds in the gorge between Asp70 and Trp82 to cause activation [161]. The second substrate molecule in this position
induces a conformational change in the protein, which involves the omega loop and at least one catalytically important group, His438. Substrate activation is a consequence of this induced conformational change. Such a mechanism could explain substrate inhibition, as seen with benzoyl choline, since different substrates could induce different conformations [161]. Mutants Asp70Gly and Asp70Lys showed complete loss of both substrate activation and inhibition, suggesting Asp70 to be the critical residue for these phenomena. Also Trp82Ala mutants showed no activation or inhibition by substrate, indicating the role of Trp82 [161]. Since Asp70 and Trp82 are part of the omega loop, activation/inhibition may result from a conformational change involving this loop.

3.5 ARYL ACYLAMIDASE ACTIVITY OF BChE:-

Butyrylcholinesterase exhibits an aryl acylamidase (AAA) activity (arylacylamide amino hydrolase, EC 3.5.1.13) capable of hydrolysing the acylamide bond of the synthetic substrate o-nitroacetanilide releasing acetate and o-nitroaniline [52, 171, 172]. This activity is strongly inhibited by classical cholinesterase inhibitors such as iso-OMPA, eserine and by substrates of esterase activity like butyrylcholine and acetylcholine. In addition, the AAA on BChE activity is also sensitive to inhibition by 5-hydroxy tryptamine (serotonin). An exclusive feature of AAA activity on human serum BChE is its many fold activation by tyramine [52, 171, 172]. Only monkey serum BChE shares this unique property along with human
BChE. Chemical modification studies have indicated overlapping active sites for esterase and AAA activities of human serum BChE [149]. Further, limited protease digestion of human serum BChE protein has helped to locate the AAA and esterase activities to be within a 20 kDa fragment of the protein [173]. However, the actual location of AAA activity within the active site of BChE, the amino acids involved in the mechanism of catalysis have not been elucidated.

4. INHIBITORS:

From a mechanistic point of view, inhibitors of ChEs can be regarded as poor substrates, which react with the enzyme, forming the enzyme-inhibitor complexes, which interfere with the reaction velocity [174]. The point of attachment of the inhibitor can be at the esteratic/anionic/allosteric sites such as PAS or to a hydrophobic area [175]. Inhibitors of ChEs are traditionally considered as acting reversibly or irreversibly, and the type of bond that they form with the enzyme distinguishes the two classes. Reversible inhibitors form only non-covalent bonds. Irreversible inhibitors are held by covalent as well as non-covalent binding at the active site, and it is the covalent bonding that causes irreversibility [175].
**4.1 REVERSIBLE INHIBITORS:**

A general mechanism of inhibition involving a substrate (S), a reversible inhibitor (I), with the enzyme (E) is depicted as follows:

![Diagram of enzyme inhibition](image)

In the absence of inhibitor, only the enzyme-substrate complex, ES is formed, while with inhibitor, two new complexes, EI and ESI are formed [174]. Reversible inhibitors may then affect the substrate reaction in several ways: (i) by competing with the substrate for free enzyme, (ii) by binding to the acylated enzyme thereby decreasing the normal deacylation rate, (iii) by further altering the deacylation rate in the event the acylated-enzyme-inhibitor complex yields product and (iv) by competing with substrate for the acylated enzyme. The binding of inhibitors to the enzyme may involve one or more of the non-covalent bonds such as hydrogen bonds, ionic interactions, ion-dipole couplets or van der Wall’s forces [175].

Almost all reversible inhibitors contain at least one positively charged nitrogen group. This group is evidently attracted to the choline binding pocket of the ChEs. There they are bound by cation-π interaction, which may be augmented by van der Wall’s attraction of alkyl groups to the hydrophobic area near the choline binding pocket [175].
Primary and secondary amines are not good inhibitors of ChEs. The positively charged nitrogen atom must be either a protonated tertiary amine or a fully substituted quaternary ammonium group [174]. Being a strong base, the quaternary ammonium group is charged at all values of pH, in contrast to the tertiary nitrogen group, whose charge is pH dependent [175]. The positively charged nitrogen may be an aliphatic or aromatic. Compounds with two or three quaternary ammonium groups are also good inhibitors of ChEs. The best reversible inhibitors invariably contain an aromatic ring in addition to quaternary nitrogen group.

4.1.1 Aliphatic ammonium ions:-

Studies using series of methyl substituted ammonium ions indicated that binding increased as the number of methyl groups increased up to three. The increase in binding energy is by about 1 to 2 kCal per methyl group [175]. This suggests that binding to the choline binding pocket involves both cation-π interaction and van der Wall’s forces. Tetra-n-butyl ammonium ion binds 42 time better than tetramethyl ammonium ion and gives the best binding in the substituted series, methyl to pentyl. However, analogs of ACh, in which N-methyl groups are substituted in part with ethyl, n-propyl and n-butyl groups, are not good substrates. A good substrate thus must have a quaternary ammonium group that readily leaves the choline binding pocket after it has promoted acylation, while an inhibitor must have a quaternary ammonium group that lingers to the
choline binding pocket. The quaternary ammonium group of inhibitors/substrates interacts with Trp82 of the choline binding pocket via cation-π interaction. The alkyl group of the ligands stabilises the complex by forming van der Wall’s bond with the adjacent hydrophobic region [113, 176].

4.1.2 Bis-quaternary inhibitors:-

Inhibitors that contain two positively charged nitrogen are also good inhibitors of ChEs in general. These bis-quaternary compounds tend to inhibit AChE better than monoquaternary compounds, provided the distance between the two charged groups of the inhibitors is 14Å [175]. Many of the best reversible inhibitors of AChE are bis-quaternary compounds, including the AChE specific BW 284c51 (Figure 7). Nevertheless, BChE do not appear to exhibit this bis-quaternary effect [177].

4.1.3 Tricyclic inhibitors:-

The acridine derivatives N-methylacrydinium, 9-aminoacridine and tacrine (Figure 7) show higher affinities for ChEs than the parent acridine ring. Moreover, they show higher affinities for BChE than AChE [168]. Similarly, in the case of phenothiazines, the longer the side chain substituents, as in ethopropazine (Figure 7), higher the affinity for BChE [168]. This high affinity of BChE for the ligands is mediated by the presence of Ala328 in the choline binding pocket of BChE. Presence of Phe330 in Torpedo AChE and Tyr337 in human AChE at this position is the
Figure 7. Structure of some reversible inhibitors of cholinesterases
reason for their low affinity towards the substituted acridines and phenothiazines [168].

4.2 IRREVERSIBLE INHIBITORS:-

Irreversible inhibitors are esteratic site inhibitors which, like true substrates, react with the hydroxyl group of Ser at the catalytic site. Such inhibitors, also called as acid-transferring inhibitors, include the organophosphates, the carbamates and organosulfonates. All irreversible inhibitors form acyl-enzyme intermediates that are relatively stable to hydrolysis. Indeed, the phosphorylated enzyme intermediates have half-lives from a few hours to several days [180], whereas the sulfonated and carbamylated enzyme complex have much shorter half-lives -several minutes to few hours. The formation of an acyl-enzyme intermediate have been proved by the isolation of phosphorylated Ser from hydrolysis of horse BChE [181] and sulfonation of BChE by methanesulfonyl fluoride in the presence of tubocurarine and eserine [182, 183].

4.2.1 Carbamates:-

Carbamates were considered to react reversibly with ChEs [184]. But experiments by Myers and Kemp, Myers and Wilson et al., [185-187] confirmed that carbamate inhibition is a true irreversible one which involves the formation of a carbamylated enzyme intermediate as depicted by the following scheme:
Where CX is the carbamate, ECX is the Michaelis-Menten complex intermediate, EC is the carbamylated enzyme. The carbamylated enzyme has a short half-life of several minutes to few hours [180, 187]. Three key structural features of carbamate were identified, based on the structure of the naturally occurring carbamate, eserine: the carbamate group, the benzene ring and the tertiary nitrogen group in the pyrollidine rings [188, 189]. The subsequent synthetic carbamates were designed to contain one or more of these features. Simple carbamates were not active, but phenyl substituted carbamates were active. Substitution of charged nitrogen group in the meta position on the benzene ring resulted in the most potent inhibitor.

Eserine and its derivative carbamate (Figure 8) are pharmacologically important as they are used to treat disease with deficiencies in cholinergic systems such as myasthenia gravis and Alzheimer’s disease [190, 191]. These include eptastigmine and the recently developed MF268 (Figure 8). These lipotrophic derivatives are characterised by their reduced toxicity and increased bioavailability [191].
Figure 8. Structure of some important carbamate compounds

**PHARMACOLOGICAL CARBAMATES**

**Physostigmine**

**Neostigmine**

**Pyridostigmine**

**MF268 (Heptylphysostigmine)**

**INSECTICIDAL CARBAMATES**

**Carbaryl**

**Aldicarb**
In contrast to the pharmacological carbamates, the insecticidal carbamates are devoid of charged nitrogen groups. It was observed that the dimethyl carbamates of alicyclic and hetrocyclic enols were highly toxic to house flies and aphids [192]. One of the most successful insecticidal carbamate is 1-napthyl-N-methyl carbamate (Carbryl). In addition to the aryl carbamates, the oxime carbamates that include Aldicarb are also effective insecticides (Figure 8).

4.2.2 Organophosphate inhibitors:

Organophosphates are organic derivatives of phosphorus containing acids and have the following general (structural) formulas,

\[
\begin{aligned}
\text{X} \\
R_1 \quad \text{P} &=\text{O} \\
R_2
\end{aligned}
\]

where X is the group that leaves the OP molecule during its reaction with ChEs. It is acidic in character, and OP inhibitors therefore tend to be acid anhydrides insofar as the P-X bond is concerned. Typical X-radical includes the aldoximes, phenoxy and thiophenoxy groups, sulfhydral, carboxylates, halides, cyanides, cyanoates and thiocyanates [175]. R1 and R2 are capable of infinite variations [193], but they tend to be associated with relatively small alkyl groups which are attached either directly to P by a P-C bond or they are linked to P by an O or N atom (Table 4).
Table 4. Structure of some organophosphates.

<table>
<thead>
<tr>
<th>Type</th>
<th>General Structure</th>
<th>Example</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphates</td>
<td>( {\text{RO}} - \text{P} - \text{OR} )</td>
<td>Dichlorvos</td>
<td>( \text{H}_3\text{C} \cdot \text{O} - \text{P} - \text{OCH}_2 = \text{CCl}_2 )</td>
</tr>
<tr>
<td>Phosphonates</td>
<td>( {\text{RO}} - \text{P} - \text{R} )</td>
<td>Dipterex</td>
<td>( \text{H}_3\text{C} \cdot \text{O} - \text{P} - \text{CH}_2 \text{CCl}_3 )</td>
</tr>
<tr>
<td>Phosphorothioates</td>
<td>( {\text{RO}} - \text{P} - \text{OR} )</td>
<td>Parathion</td>
<td>( \text{H}_2\text{C}_2 \cdot \text{O} - \text{P} - \text{S} - \text{O} - \text{NO}_2 )</td>
</tr>
<tr>
<td>Phosphorothioate</td>
<td>( {\text{RO}} - \text{P} - \text{OR} )</td>
<td>Echothiophate</td>
<td>( \text{H}_2\text{C}_2 \cdot \text{O} - \text{P} - \text{S} - \text{(CH}_2)_2 - \text{N(CH}_3)_2 )</td>
</tr>
<tr>
<td>Phosphono fluoride</td>
<td>( {\text{RO}} - \text{P} - \text{F} )</td>
<td>DFP</td>
<td>( \text{H}_7\text{C}_3 \cdot \text{O} - \text{P} - \text{F} )</td>
</tr>
<tr>
<td>Phosphono fluoride</td>
<td>( {\text{RO}} - \text{P} - \text{F} )</td>
<td>Soman</td>
<td>( \text{CH}_3 - \text{C} - \text{CH} - \text{O} - \text{P} - \text{F} )</td>
</tr>
</tbody>
</table>
Organophosphates, mainly man-made, but also in at least one example, occurring naturally in cyanobacteria [30], acts as hemi-substrates of ChEs, specifically phosphorylates the active site Ser.

$$EOH + XP(RO)\_2O \rightleftharpoons EOH.XP(RO)\_2O \rightarrow EO-P(RO)\_2O + HX$$

where, EOX is free enzyme, XP(RO)\_2O is an OP with leaving group X, EOX.XP(RO)\_2O is the reversible complex, EO-P(RO)\_2O is the phosphorylated enzyme.

Compounds that structurally resemble the substrates are usually very good inhibitors of BChE. Thus, OPs modelled on ACh are powerful inhibitors of the enzyme [174]. Quaternary aminophenylphosphates and quaternary quinolylphosphates are more potent inhibitors of ChEs than their corresponding tertiary compounds [178, 179], and replacement of a quaternary nitrogen by a sulfonium ion results in a strong inhibitor [180]. The major requirement for potent inhibition thus appears to lie with the structural orientation of the substituted groups of the inhibitor rather than the positive charge of the tertiary nitrogen itself [174].

Insecticide organophosphates are in general characterised by the thionophosphate structure,
Such compounds do not inhibit ChEs directly. They are converted *in vivo* to the oxy analogues, which are the active inhibitors. A classical example is parathion, which is converted to paraxon.

\[
\begin{align*}
\text{Parathion (inactive)} & : & \text{Paraxon (active)} \\
\text{S} & O, N \rightarrow O-P & O-C_2H_5 \\
O_2N & O-C_2H_5 & O-C_2H_5
\end{align*}
\]

OP compounds have agricultural, military and medical applications. Over 50,000 OP compounds have been synthesised and screened for insecticidal potency, and nearly 200 of them have therapeutic uses, including the treatment of glaucoma, myasthenia gravis and AD [190]. The highly toxic OP agents soman, tabun and sarin were developed as chemical warfare agents during World War II.

4.2.3 *Ageing and regeneration:* -

Anti-ChEs act by generating a carbamylated or phosphorylated enzyme, instead of the acylated enzyme which is formed during substrate hydrolysis. These adducts prevent substrate binding and are hydrolysed more slowly than the acyl-enzyme. This slow hydrolysis has been explained by steric exclusion; the active site His is not positioned to carry a water molecule to the correct face of the phosphorus required for nucleophilic attack [194, 195]. It has also been proposed that the active site
His is rendered ineffective as a general base because the imidazolium forms an unproductive H-bond with an oxygen atom of the OP [196].

The X-ray crystallographic study of phosphorylated *Torpedo* AChE [197] has revealed that the acyl pocket of ChEs provides another barrier to reactivation of the enzyme. The ‘dry’ hydrophobic path in BChE is formed by Leu286, Val288, Trp231 and Gly117, completely surrounds the OP alkyl (or alkoxy) group. This could limit the dephosphorylation by blocking access of attacking water molecules to the correct face of the phosphorus. The dry path also provides stabilising non-bonded contacts to the OPs such as sarin and soman. The Gly117(C119) Cα is only about 4.3 Å away from the phosphorus and is almost on-line with the phosphorus Ser198(200) Oγ bond. Placing an imidazole side chain specifically at this position in human BChE confers OP hydrolase activity by increasing spontaneous dephosphorylation [198, 199].

After phosphorylation of the active site Ser, some OP-ChE conjugates undergo post-inhibition reactions, what is thought to be a conformational change, rendering the enzyme-inhibitor complex extremely resistant to lysis by water or reactivators. This phenomenon is collectively called “ageing” which results in the true irreversible enzyme inhibitor adduct [200]. Wilson found that hydroxylamine could reactivate phosphorylated enzyme adducts by releasing the phosphoryl group attached to the Ser residue more rapidly than water [201]. However, hydroxylamine
was toxic at the concentrations required for this reactivation. The search for more effective reactivators led to the discovery of pyridinium-2-aldoxime (2-PAM; pralidoxime) by Wilson and Ginsberg in 1953 [202]. The quaternary group in the pyridine ring of 2-PAM orients it in such a way as to exert a nucleophilic attack on the phosphorus of the inhibited enzyme, transferring the phosphate from the active site of the enzyme to the oxime (Figure 9-A). Pralidoxime reactivates OP-inhibited AChE at one million times faster the rate of hydroxylamine [203]. However, its therapeutic effectiveness has been limited because, i) it cannot cross the blood-brain barrier and hence cannot reverse the inhibition of brain AChE and ii) it is not an efficient reactivator of soman-inhibited AChE.

Certain symmetric bisquaternary compounds such as obidoxime, TMB-4 and MMB-4 were shown to be more potent reactivators than 2-PAM [204-206]. These compounds were more toxic than 2-PAM and did also not prove effective against the rapidly ageing chemical warfare agent, soman. A series of bis-pyridinium monooximes were synthesised by Hagedron and co-workers [207]. One of the most potent members of this class, HI-6 (Figure 9-B), has been shown to be efficient against soman-induced toxicity [208]. Apart from reactivation of soman-inhibited unaged AChE, other direct effects of HI-6 on the central nervous system also seems to play an important role in the efficacy of this compound.
Figure 9-A. Schematic representation of the process of “ageing” and reactivation of phosphorylated enzyme by oxime. 9-B. Structure of some reactivators.
The inability of oximes to provide adequate protection against the
toxicity of rapidly ageing OP compounds stimulated the development of
carbamate pre-treatment regimen in which carbamylation of AChE
effectively protects it against inhibition by OP compounds [209].
Behavioural side effects from carbamate pre-treatment in the absence of
exposure to OP compounds have been avoided by the use of cationic
carbamates such as pyridostigmine [210]. Although these regimens were
effective in preventing lethality, their restriction to periphery led to
postexposure incapacitation in experimental animals [211]. This has lead to
the use of enzyme scavenger approach. Both AChE and BChE were tried as
scavengers and studies indicated that BChE is most effective against rapidly
ageing OP compounds [212-215].

4.3 EFFECT OF AMPHIPHILES AND HYDROPHOBIC
COMPOUNDS:-

Primary amino acid sequencing and molecular modelling of BChE
based on the crystal structure of Torpedo AChE has revealed the importance
of hydrophobic amino acids in BChE catalysis. The hydrophobic subsites
within the active site are indeed the chief determinants of its substrate
specificity and inhibitor sensitivity. Hence, a vast array of compounds that
are amphiphilic/hydrophobic in nature could interact with BChE, there by
modulating its catalytic function.
Horse serum BChE shows a biphasic change in activity in the presence of n-butanol with benzoylcholine as substrate. Lower concentrations of n-butanol activated the enzyme, while higher concentrations caused inhibition of the activity [216]. In contrast, with ACh as substrate, the enzyme was inhibited by n-butanol. AChEs from human erythrocytes and rat brain were activated by n-butanol similar to BChE with ACh as substrate. On the contrary, AChEs from bovine erythrocytes and electric eel were irreversibly inactivated by aliphatic and aromatic alkanols [217-219].

Increasing concentrations of alkanols were found to activate human BChE and this activation increases in magnitude as the chain length of the alkyl group increased [220]. Higher concentrations of alkanols caused irreversible inactivation due to denaturation of the enzyme protein.

Aromatic hydrocarbons such as benzene, toluene, naphthalene, anthracene, biphenyl inhibited horse serum BChE reversibly [221]. The inhibition is brought about by binding of these compounds with the hydrophobic subsites of the active site of BChE. Similarly, several polyaromatic hydrocarbons were also shown to inhibit electric eel AChE in a competitive manner [222]. Horse serum BChE has been shown to be inhibited by a variety of aryl and arene boronic acids reversibly [223].

Several anionic surfactants, at low concentrations, strongly inhibited horse serum BChE, whereas non-ionic surfactants are without effect with
acetyl- and propionylthiocholine as substrate [224]. Low concentrations of hemolysin strongly inhibited human BChE activity with ACh as substrate [225]. The zwitterionic detergent 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate behaves as a reversible inhibitor of ChEs from several sources. Human and horse serum BChEs were more sensitive to inhibition by the detergent than AChE from human erythrocytes or electric eel [226].