Acetylcholinesterase Activity and Isozyme Pattern in Normal and Lithium-treated Developing Chick Brain.

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C E R T I F I C A T E

This is to certify that the undersigned candidate Ms. Anahita Gouri has completed the Literature Survey and Project Work for Master of Science (II), in Life Sciences (Neurobiology), for the year 2003-2004.

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A C K N O W L E D G E M E N T S

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C O N T E N T S

❖ **Project Report: -** AChE activity and Isozyme Pattern in Normal and Lithium-treated Developing Chick Brain.

❖ **Bibliography**

❖ **List Of Tables**

❖ **List Of Figures**

Literature Survey: -

A Review on Invertebrate and Vertebrate Acetylcholinesterase

A B S T R A C T

Acetylcholinesterase [AChE] is an enzyme that terminates Acetylcholine [ACh] mediated neurotransmission. Its wide neuronal and non-neuronal cellular distribution has made it the focus of intense research. The data on tissue and species specific AChE expression, the detection of its various isoforms and its cholinergic and non-cholinergic functions has been accumulated and reviewed from a range of evolutionary diverse vertebrates and invertebrates that include insects, nematodes, fish, reptiles, birds and several mammals, among them man.

Chapter 1: -

Acetylcholinesterase- History, function, structure and molecular diversity

1.1 Introduction: -

In the last 25 years of neuroscience research, the main aspects of the cholinergic neurotransmission, including proteins responsible for the synthesis, storage, release and degradation of Acetylcholine (ACh), have been elucidated. In this chapter, the lytic enzyme of the cholinergic system, Acetylcholinesterase (AChE), has been discussed with reference to its history, function, structure and molecular diversity, in order to provide a general overview and understanding of some of its basic attributes before discussing its more complex roles.

1.2 Historical outline of Cholinesterases (ChEs)

Dale postulated the existence of ChEs in 1914. The first experimental evidence came from the observation that the horse serum had a ‘splitting’ activity on ACh. Then, in 1932, Stedman and Colleagues successfully prepared and studied crude extracts of esterase from whole blood and showed that serum contains 2 enzymes capable of hydrolyzing ACh. A little later, Nachmasohn and Lederer (1939) reported esterases not only in blood, but also in the nervous and muscular tissues. A year later, Alleb and Hawes

(1940) reported, that the erythrocyte esterase showed optimum substrate concentration for rate of ACh hydrolysis. They also reported, that the esterase present in the erythrocytes was similar to the one present in the brain tissue. Augustinson and Nachmansohn introduced the term ‘Acetylcholinesterase’ in 1949, for the esterase capable of hydrolyzing Acetylcholine faster than other choline esters. In 1964, the enzyme commission recommended [Acetylcholine Acetylhydrolase; E.C.3.1.1.7.], Acetylcholinesterase (AChE) for a ‘true’ and ‘specific’ cholinesterase. The other cholinesterases were collectively termed as ‘pseudo’ [Acylcholine Acylhydrolose; E.C.3.1.1.8.]. The pseudocholinesterases included Butyrylcholinesterase (BChE) [defined by its capacity of hydrolyzing butyrylcholine faster than any other choline esters] and Propionylcholinesterase [defined by its capacity of hydrolyzing propionylcholine faster than any other choline esters]. Hence on the basis of substrate specificity and inhibitor sensitivity [Table 1], it is possible to differentiate between the ‘true’ and ‘pseudo’ forms of cholinesterase. Sequence analysis of AChE and BChE reveals a strong homology [53% identity] between these two enzymes and mutagenesis of only a few amino acids can convert AChE into a BChE-like enzyme.

[Legay C., 2000][Ashwani V., 1992]

Table 1.1: - Cholinesterases

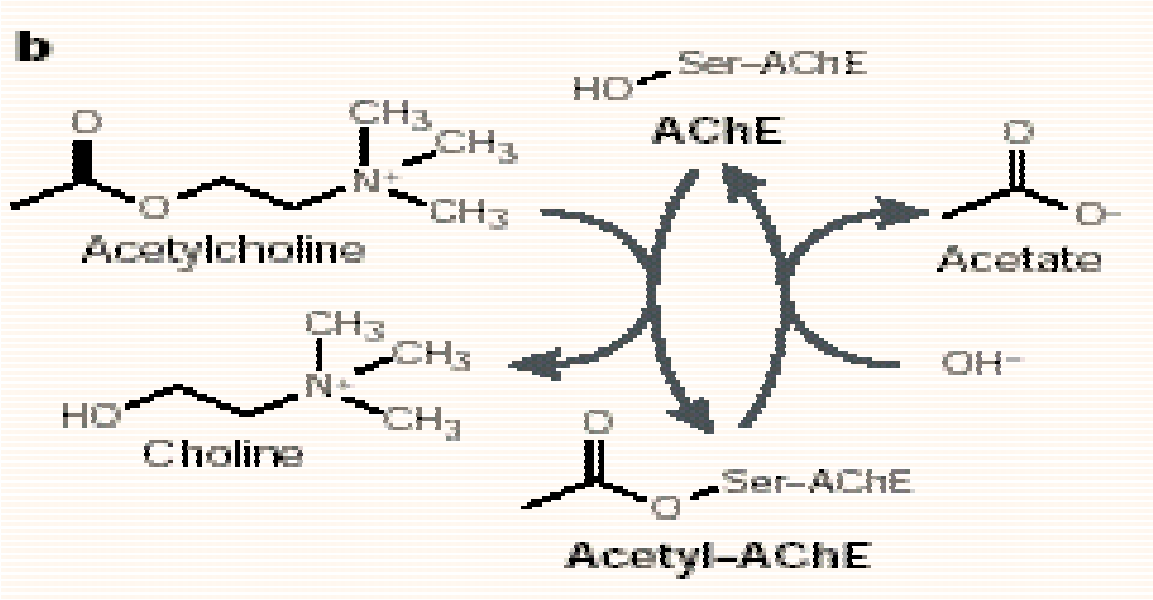
	ACETYLCHOLINESTERASE (ACHE)	PSEUDOCHOLINESTERASE
	E.C. 3.1.1.7	E.C. 3.1.1.8.
Preferred Substrate	Acetylcholine	Butyrylcholine or Propionylcholine
Specific Substrate	D-acetyl-B Methyl choline	Butyrylcholine or Benzoylcholine
Specific Inhibitor	BW284C51 [1,5 bis {4-allyldimethyl ammonium phenyl} pentane-3-one- dlibromide)	Iso-OMPA, DFP
Inhibited by High concentration Of Ach [Substrate Inhibition]	Yes	No

[Ashwani V., 1992]

1.3 Physiological function of Cholinesterases: -

1.3a Physiological function of AChE: - AChE catalyses the hydrolysis of the neurotransmitter ACh in the following ways: -

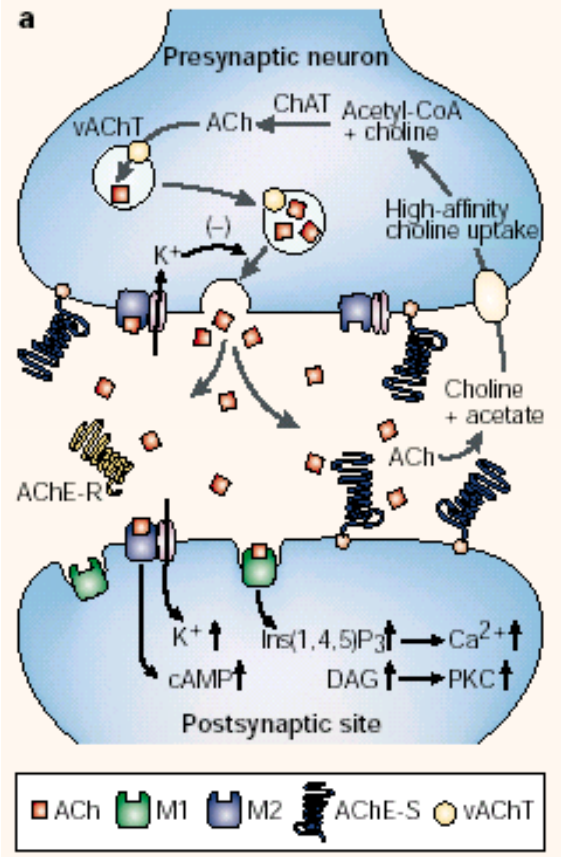
Reaction: -



[Soreq H and Seidman S., 2001]

As seen in the above AChE promotes ACh hydrolysis by forming an acetyl-AChE intermediate with the release of choline, and the subsequent hydrolysis of the intermediate to release acetate. The catalytic turnover rate of AChE is very high. The choline released is recycled in the pre-synaptic nerve cell. Thus AChE is a key enzyme for cholinergic transmission and plays a vital role in the nervous system of both the vertebrates and the invertebrates and in the neuromuscular junction (NMJ) of vertebrates and some invertebrates. [Massoulie J., et al., 1999]

Figure 1.1: - The Cholinergic Synapse



In the pre-synaptic neuron, cholineacetyltransferase (ChAT) catalyses the synthesis of acetylcholine (ACh) from choline and acetyl-coenzyme A. ACh is packaged in synaptic vesicles via a vesicular ACh transporter (vAChT). Action potentials trigger the release of ACh into the synaptic cleft, where ACh can bind to muscarinic receptors located on the pre- and postsynaptic membrane. Muscarinic M2 receptors (M2) on the pre-synaptic membrane regulate ACh release via a negative feedback response. At the postsynaptic site, M1 receptors transduce signals through a pathway involving diacylglycerol (DAG), inositol-1, 4,5-trisphosphate (Ins (1,4,5) P3) and a Ca2+- dependent protein kinase (PKC). In the hippocampus, most of the postsynaptic receptors are of the M1 subtype; in the cortex M2 receptors might also be located on the postsynaptic membrane. ACh is hydrolysed in the synaptic cleft by AChE-S tetramers, which are indirectly attached to the neuromuscular junction by a collagen-like tail, or by another structural subunit to brain synapses. AChE-R monomers would remain soluble within the synaptic cleft. A high affinity choline-uptake mechanism returns choline to the pre-synaptic neuron. Brain distribution of AChE includes both acetylcholine releasing and cholinoceptive neurons [Soreq H and Seidman S., 2001]

1.3b Physiological function of BChE: -

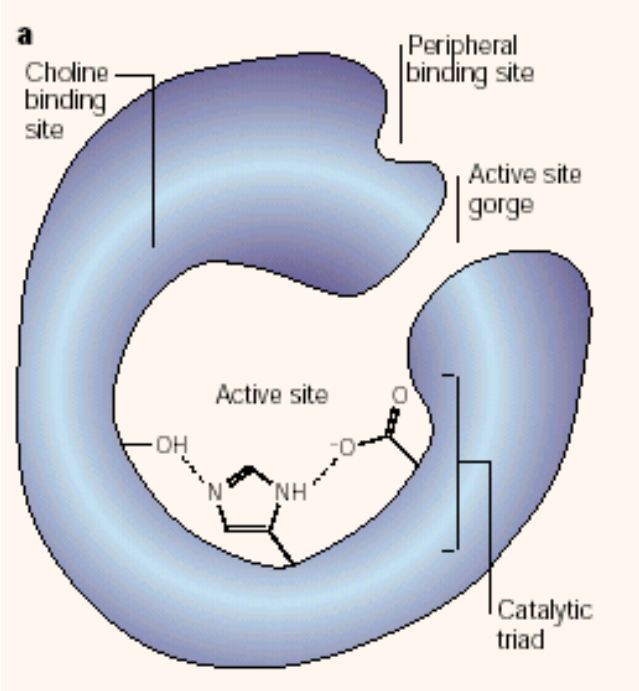
BChE is present in non-neuronal tissues like liver, lung, plasma etc. The function of BChE in higher vertebrates is unclear. Humans lacking BChE activity do not show any pathology, except patients undergoing surgery when exposed to succinylcholine as a curarizing agent, fail to recover breathing at the end of anaesthesia. BChE present as a soluble form, in the plasma of mammals, is suggested to serve as a safeguard against the diffusion of ACh into the blood stream and/or against orally ingested toxic compounds, since this enzyme has a broad range of substrates including plant alkaloids such as cocaine. [Massoulie J., et al., 2002]

1.4 Structure of AChE: -

The 3-D structure of AChE obtained by X-ray crystallography in Torpedo, mammals and Drosophila showed that the catalytic domain of the enzyme is organized as a globular assembly of β sheets and α helices [α/β fold]. Since it is known, that AChE has a very high catalytic turnover rate, it was very surprising to find, the 'active site' of the enzyme located in a cavity, only accessible through a deep and narrow 'catalytic gorge'. The active serine belongs to the catalytic triad Glu-His-Ser. The substrate ACh is oriented in the active site by cation-π interaction of its quaternary ammonium group with a tryptophan group. The polarized distribution of the charged residues generates an electrostatic dipole, which may attract cationic substrates towards the active site, but this needs further investigations. Moreover, the other aromatic residues in the AChE structure form an acyl pocket, which

determines its specificity towards different choline esters and also its sensitivity to active site inhibitors. It is also a possibility, that the peripheral site located at the entrance of the catalytic gorge serves as the first binding site for the positively charged substrates, on their way to the active site. The catalytic domain of AChE consists of N-terminal and C-terminal sub-domain, which do not interpenetrate but establish a close contact at the peripheral site. The general stability and flexibility of enzyme depends on the residues localized in this region. This suggests, that the catalytic and the substrate specificity of AChE depends on the dynamic movements within the protein structure, and that the reaction products, exit the active site of the enzyme via an alternative route, called the 'back door' avoiding trafficking problems with the substrate molecules entering via the catalytic gorge.
[Massoulie J., et al., 2002]

Figure 1.2: - Structural Features of the enzyme AChE.



X- ray crystallography has identified an active site at the bottom of a narrow gorge, lined with hydrophobic amino acid side chains. The catalytic triad is GLU-HIS-SER. A choline-binding site featured hydrophobic tryptophan residues and a peripheral binding site has also been identified by site-directed mutagenesis. [Soreq H and Seidman S., 2001]

1.5 Isoenzymes / Isozymes: -

They are variable forms of the same enzyme. Isozymes are highly homologous to each other but contain small differences in amino acid sequence. The term isozyme is not a precise one and can refer to different gene products, or different products of the same gene with alternative splicing. The term "isoenzyme" or "isozyme" should apply only to those multiple forms of enzymes arising from genetically determined differences in primary structure and not to those derived by modification of the same primary sequence. Groups 1 to 3 of Table 1.2 represent enzymes having isozymes. The term "multiple forms of the enzyme " should be used as a broad term covering all proteins catalyzing the same reaction and occurring naturally in a single species, for instance groups 4 to 6 of Table 1.2.
[Micales A.J and Bonde R.M., 1995]

TABLE 1.2: - Multiple Forms of Enzymes

GROUP	REASON OF MULTIPLICITY	EXAMPLE
1	Genetically independent proteins	Malate dehydrogenase in mitochondria and cytosol
2	Heteropolymers (hybrids) of two or more polypeptide chains, noncovalently bound	Hybrid forms of lactate dehydrogenases
3	Genetic variants (allelozymes)	Glucose-6-phosphate dehydrogenases in man
4	Conjugated or derived proteins	
	a. Proteins conjugated with other groups	Phosphorylase b, glycogen synthase a
	b. Proteins derived from single polypeptide chains	The family of chymotrypsins arising from chymotrypsinogen
5	Polymers of a single subunit	Glutamate dehydrogenase of molecular weight 1,000,000 and 250,000
6	Conformationally different forms	All allosteric modifications of enzymes

[Micales A.J and Bonde R.M., 1995]

1.5a Isoenzymes of AChE

AChE exists in a large variety of molecular forms. There are designated as ‘G’ (globular forms) and ‘A’ (asymmetric collagen-tailed forms). All the molecular forms posses the same catalytic domain but distinct C-terminal peptides [$AChE_R$, $AChE_H$, $AChE_T$, $AChE_S$]. They are as follows: -

Figure 1.3: - Isoenzymes of Acetylcholinesterase

$AChE_R$: - where R stands for 'readthrough'

Readthrough or the 'R' transcripts or subunits of type R correspond to the hypothetical products of 'readthrough' transcripts, which retain the 'intronic' region that forms the last exon encoding the catalytic domain. $AChE_R$ molecular forms remain soluble and monomeric and are found only in vertebrates. [Massoulie J., et al., 1999, 2002]

$AChE_H$: - where H stands for 'hydrophobic'.

'Hydrophobic' or H transcripts or subunits of type H, are called so, because the C-terminal regions of these subunits are characterized by hydrophobic sequences. These hydrophobic regions consist of one or two cysteine residues, which establish disulphide bonds & result in a dimeric mature protein. The hydrophobic region majorly corresponds to a signal for a glycophosphotidyl (GPI) anchor. Hence these molecular forms are GPI-anchored dimmers and are studied in both vertebrates and invertebrates. [Massoulie J., et al., 1999, 2002]

$AChE_T$: - wherein T stands for 'Tailed' forms

$AChE_T$ is the only type of catalytic subunit that exists in all vertebrates producing major AChE forms in the brain and the muscle. $AChE_T$ generates multiple structures, ranging from monomers, dimmers to collagen- tailed and hydrophobic tailed forms. These tailed forms are catalytic tetramers associated with anchoring proteins, which help them attach to the basal lamina of NMJ or to the cell membrane. The monomeric G_1 form is the first translational product and more complex forms like G_2, G_4 are sequentially derived from it. Hence the order of formation and appearance is G_1->G_2->G_4->A_{12}. This order also involves subdivisions of the major forms, like G_4

AChE is subdivided into a secreted form, hydrophobic tailed form and an incipient collagen tailed asymmetric form. In the collagen tailed forms, $AChE_T$ subunits are associated with a specific collagen, ColQ which contains a short peptide motif, the proline- rich attachment domain [PRAD], that triggers the formation of different heteromeric forms, which contain one, two or three catalytic tetramers [A_4, A_8, A_{12}] from monomers and dimmers. Forms such as A_4 and A_8 AChE are intermediates, existing in appreciable amounts only when the production and breakdown of A_{12} AChE is rapid. The collagen-tailed forms are predominant at the neuromuscular junction (NMJ). The $AChE_T$ subunits are also found to be associated with a hydrophobic glycoprotein of about 20 KD. This protein contains a signal peptide, an extracellular domain, which includes a proline rich motif and an N-glycosylation site, a transmembrane domain and a cytoplasmic domain. The proline rich domain present is responsible for the formation of membrane bound G_4 tetramers. This transmembrane glycoprotein, which constitutes the membrane anchor of AChE, is called the Proline-Rich Membrane Anchor [PRiMA]. $AChE_T$ subunits associated with PRiMA predominantly exist in the brain. Although careful biochemical analysis of muscle sections have shown, that this form is also located in the ‘peri-junctional’ zone surrounding the NMJ and is physiologically regulated [increases or decreases] depending on the type of muscle activity/exercise. Hence it is suggested, that the physiological role of $AChE_T$ subunits associated with PRiMA in muscles is to control the diffusion of ACh around the junction on repetitive stimulation of the muscle. These findings need to be further investigated. New developments in the intracellular pathways, AChE transport, mode of association and regulation of the catalytic subunit & their interactions with the structural subunits can emerge from the cloning

of the PRiMA. The $AChE_T$ subunits are studied in both vertebrates and invertebrates. [Massoulie J., et al., 1999, 2002]

$AChE_S$: - wherein S means 'Soluble'. These $AChE_S$ subunits comprise of soluble monomeric forms of the enzyme and have been studied only in vertebrates. [Massoulie J., et al., 1999, 2002]

In Vertebrates a single gene encodes for AChE. The following 3 processes generate this panoply of AChE molecules.
[1] Alternate splicing of the AChE gene.
[2] Oligomerisation of the catalytic subunits.
[3] Associations with non-catalytic subunits.
Vertebrate AChE molecular forms are further discussed in Chapter 3.

Unlike Vertebrates, invertebrates posses variable number of AChE genes. Moreover the process of alternate splicing has never been suggested for the generation of invertebrate AChE molecular forms. The molecular forms of invertebrates and the genes encoding them have been discussed in Chapter 2.

1.6 Conclusion: -

The enzyme history and it's various attributes like basic structure, functions and molecular diversity, has enabled biologists in understanding, interpreting and analyzing the complex role played by this multifaceted enzyme in different invertebrates (Chapter 2) and vertebrates (Chapter 3).

Chapter 2: -

Role of Acetylcholinesterase in Invertebrates

2.1 Introduction: -

AChE in invertebrates, has been and still is the focus of research for various reasons. In some invertebrates (e.g.: - sea urchins, oysters etc) it's potential as a biochemical marker for environmental toxicity has been explored, in others (e.g.: - Protozoans, Aplysia etc) it's non-cholinergic functions in processes like cell-adhesion and neuritogenesis have been elucidated; whereas the multiplicity and the variable number of genes encoding invertebrate AChE (e.g.: - four genes in nematodes, one gene in Drosophila) are being interpreted to derive a phylogeny providing an insight in it's evolution and it's sensitivity towards various antiparasitic drugs and insecticides being continuously understood in order to prevent infection by worms and destruction of crops by insects respectively. Hence, in order to provide a better understanding of all the above functions of invertebrate AChE, the study of it's expression, function, structure and molecular diversity within each phylum was undertaken and explained in this chapter.

2.2 Acetylcholinesterase in Invertebrates: -

Invertebrates are those animals that do not possess a vertebral column.

2.2a Phylum Protozoa: -Protozoans are unicellular Eukaryotes, now often included in a separate kingdom together with fungal protists and algae.

i) Sarcodina

Acetylcholinesterase [AChE] was detected in Protozoans. Work done on Dictyostelium discoideum showed its serine esterase to have a strong sequence similarity with AChE of Torpedo. This led to further investigations on characterization and possible function of cholinesterases in protozoan. It appeared that histochemically, biochemically and electrophoretically AChE activity of amoeboid cells was similar to that of Electrophorus Electricus. Possibilities of using this Protozoan as an efficient bio-indicator were investigated. Dictyostelium Discoideum used in bioassays for pre-chemical screening of both moist environments and fresh waters in relation to neurotoxic organophosphorus compounds like Basudin, showed a significant dose dependant inhibition of Propionylcholine but the effect on AChE was insignificant. [Falugi C., et al 2002]

ii) Ciliophora

Work done on another protozoan Paramecium primourelia indicates the role of AChE in cell-to-cell adhesion in the process of conjugation. In immature cells, choline acetyl transferase (ChAT) activity was completely absent. But AChE was seen in the cytoplasm. The activity in the cytoplasm is due to the 260-Kda molecular form of AChE. This form is found in the membrane protein fraction of competent mating cell along with the presence of other molecules of the cholinergic system. The AChE form present in this

protozoan shows similarity to the membrane bound tetrameric form present in human erythrocytes. By inhibiting AChE activity in competent mating cells, a significant reduction in mating cell pairing was observed. Hence the role of AChE in cell-to-cell adhesion for the process of conjugation was elucidated. [Corrado M.U.D, et al., 2001]

2.2b Metazoa: -Metazoans are multicellular mitochondrial eukaryotes.

i) Phylum: - Echinodermata: Echinderms, are marine deuterostome organisms' characterized by tube feet which form part of water vascular system, thought to have a common ancestry with the chordates.

The cholinergic system is formed by a set of molecules, they are, the signal molecule ACh [acetylcholine]; the biosynthetic enzyme ChAT [cholineacetyltransferase E.C.2.3.1.6.]; the lytic enzyme Acetylcholinesterase [E.C.3.1.1.7.] and two classes of receptors Nicotinic and Muscarinic. [Harrison K.P., et al 2002]. Detection of the Cholinergic System in the male gametes of vertebrates and invertebrates lead to questioning the possible role of this signaling system in regulating intracellular ion exchange, functional to sperm mobility and its probable interaction with the egg. When sea urchin eggs (species: - Paracentrotus lividus) were used for this study, the presence of AChE molecules performing AChE activity detected by Immunofluorescence suggested a signaling pattern. [Piomboni P., et al 2001] Furthermore work done on sea urchin larvae suggested the presence of molecules of the cholinergic system since early developmental stages. With the progress in development, a change in localization and hence function of the molecules was detected by

histochemical, immuno-histochemical and confocal laser scanning microscopy. [Falugi C., et al., 2002]. Like in the Neuromuscular Endplates of higher organisms, AChE in sea urchin larvae was generally distributed in motile structures. In Starfish, AChE activity is enhanced with increasing motility [Semenova M.N., 2000]. Along with other molecules of the cholinergic system AChE was found to be associated with the ciliary structures, which modulate ciliary movement for feeding. The pattern in which the molecules of AChE are present in the larval body wall muscles and along the arms contributes in understanding its role and organization. Also a sensitive function for AChE is suggested since it is found localized at the tip of the spines in the larvae. The mature molecular form of the enzyme is present since early larval stages as indicated by electrophoretic studies. [Falugi C., et al., 2001]. In another electrophoretic study on sea urchins, Strongylotrotus drobachiensis and S. intermedius and their hybrids, two AChE fractions were found. These fractions differed in their thermostability and are also developmentally regulated such as the thermo-labile form is predominant at the Gastrula stage whereas the thermo-stable one predominates the mid-plutus stage. [Ivanenkov V.V., et al., 1976] Hence in sea urchins AChE has been detected in sperm cells, eggs, Primary Mesenchye Cells [suggesting a role in developmental cell interaction] and during larval growth and metamorphosis. Since several structures with “Embryonic” cholinergic molecules modulated by AChE activity are present from the early stages of sea urchin development, which indeed is sensitive to a variety of pollutants and hence is extensively being used as a model system in assessing the effects of neurotoxic pesticides. The Effects of Basudin [an organophosphate compound containing 20% of a thionophosphate Diazinon, irreversibly links the AChE molecule by a phosphate group] and carbonates

[which compete with the substrate ACh] were studied. Results indicated concentration dependant AChE inhibition during early development of sea urchin. These effects revealed at molecular level of AChE activity were more sensitive and reveal anomalies at chronic exposure concentrations. [Pesando D., 2003]. Hence determining AChE activity and using this biomarker as an early warning signal of exposure and/or adverse effects can also be used in the future to study the spatial and temporal trends in the quality of coastal waters. [den Besten P.J., et al., 2001]

ii) Phylum: - Platyhelminthes: - Flatworms, acoelmate animals of uncertain origin.

AChE is very broadly distributed in many species including parasites. It is present in all helmintic invertebrates, including trematodes and nematodes. In parasitic studies, the mechanism in which the parasite evades the immune defense mechanism of the host is puzzling; and hence functional proteins like AChE that might elicit protective immunity against infection are being identified. The biological role of AChE in parasites is been constantly understood in context with the development of antihelmintic drugs. For instance various AChE inhibitors were shown to significantly decrease the amplitude of muscle contractions in various parasites, such as F.hepatica and S.mansoni; and are being used as antihelmintic drugs. Furthermore, levels of parasitic AChE in the serum, is assayed after immunoprecipitation and is used to monitor circulating filarial parasites both in human and experimental animals. [Arnon R., et al., 1999]

Example: - Echinococcus Granulosus

Though little information is available on the neuromuscular system of cestoda, it was known that ACh exists as an inhibitory neurotransmitter. PG et.al. (2000) detected the presence of AChE spectrophotometrically and electrophoretically. It is known that on ingestion by a definitive host Echinococcus granulosa protoscolices become adults and their interest may be related to dissemination following cyst rupture. It is hypothesized that Echinococcus granulosa protoscolices could release AChE in hydrated cyst. The released enzyme could to pass through the germinal layer and travel to the host tissues, destroying the host ACh. Hence AChE present in this tapeworm could be an attractive target for chemotherapeutic or immunological intervention in hydatid disease. In future, a combination of compounds could be used, which would inhibit AChE thereby allowing ACh to accumulate and cause abnormally high levels of inactivation and a total blockage of the nerve function. Hence AChE activity would be considered as a possible target in chemotherapy. [Gimenez-Pardo C., et al., 2000]

Example: - Fasciola hepatica

Several putative neurotransmitters have been identified in the primitive nervous system of parasitic flatworms. But the mechanism of neurotransmission and kinds of neurotransmitters remain unclear. Acetylcholine [ACh] has been identified as an inhibitory neurotransmitter responsible for muscle contractions. Its receptors are the mixed muscarinic and nicotinic type and hence pharmacologically primitive. The worms are capable of synthesizing and degrading endogenous ACh. Although this data throws some light on the role of ACh as a neurotransmitter in the worms, it remains inconclusive. In order to provide concrete evidence for the same, it

is necessary to demonstrate the localization of ACh in the appropriate sites of the nervous system. To do so, an indirect method was adopted wherein the lytic enzyme of the cholinergic system, AChE was localized histochemically and this localization of AChE was then linked with the functional localization of ACh. Histochemical studies performed on Trematoda Fasciola hepatica demonstrated AChE activity in the neural cell bodies and extracellularly in the neuropile of the cerebral ganglia. This ultrastructural localization of AChE activity provided strong evidence of ACh as a neurotransmitter. However the patterns and location of AChE in the nervous system of F.hepatica were found to be very similar to those seen in higher invertebrates and vertebrates suggesting that the basic mechanisms of neurotransmission in higher invertebrates and vertebrates are already present in this primitive phylum. [Sukhdeo S.C., et al., 11988]

Example: - Schistoma

A lot of groups have worked on AChE of Trematoda Blood fluke Schistoma. By 1994 it was known that AChE was present at all stages of Schistoma Life Cycle. AChE was found on the parasitic surface and in the muscles. Comacho and Colleagues [1994] worked on 3 species of Schistoma {S.mansoni, S.haematobium and S.bovis} and found G_2 as the dominant molecular form, majorly located on the tegument, in all the 3 species. But they also noticed, that the AChE activity differed from species to species. They found, at the adult stage, the major difference between species lay in the relative amounts of AChE activity in their teguments; S.haematobium teguments carried 20 times and S.bovis 6.9 times the activity present on S.mansoni teguments. This explained the species-specific susceptibility to AChE inhibitor drugs like Metrifonate. [Camacho M., et al., 1994]

In S.mansoni, AChE was first demonstrated in adult worms by Bueding [1952] and was partially characterized both histochemically and biochemically. Early studies also suggest involvement of AChE in the motor activity of S.mansoni by demonstrating anticholinestrase-induced paralysis in worms. Schistosomal AChE is the target for several antiparasitic drugs, including hycanthon, lucanthone, metrifonate and phosphonium compounds. Extensive biochemical and immunological investigations were carried out in Ruth Arnon's laboratory on the enzyme AChE of S.mansoni. On extensive analysis of these investigations it was shown that the enzyme AChE was concentrated up to 350 times more in the outer membranes of the worms, in comparison to the rest of the worm. This AChE present in the outer membranes of the worm, is probably expressed at the surface of the parasite attached to the tegumental membrane via a covalently linked GPI anchor, was suggested to possess a non-cholinergic function and also serve as an effective immunological target. Furthermore their investigation showed that AChE in S.mansoni appears in 2 principal molecular forms both globular, with sedimentation coefficient of ~6.5S and ~8S. The 2 forms of AChE were found to differ in their heparin binding and immunological specificity. The 8S form binds to heparin, the 6.5S form did not and the 2 forms were selectively recognized by different monoclonal antibodies. Polyclonal antibodies raised against S.mansoni AChE purified by affinity chromatography were specific for the parasitic AChE, reacting with both molecular forms, but do not recognize AChE from the other species. They interact with the surface localized enzyme on the intact organism and produce almost total compliment dependent killing of the parasite S.mansoni. AChE is thus demonstrated to be a functional protein, in

multifaceted activities, which can serve as a suitable candidate for diagnostic purposes, vaccine development and drug design. [Arnon R., et al., 1999]

The presence of AChE in bacteria, plants, non-excitable tissues of complex organisms suggests it has a wide range of alternative non-cholinergic functions. Similarly the AChE on the parasitic surface is known to be involved in non-cholinergic functions like glucose scavenging from the host blood stream. The glucose is transported through the membrane with the help of transporters, but the exact mechanism underlying the link of non-cholinergic system present on the parasitic surface to glucose transport is not yet known. AChE on the schistome surface is the target of organophosphorous drugs like Metrifonate. But this drug is not widely used, since it cannot differentiate between host AChE and parasitic AChE and thus is toxic. On elucidating the structure of Parasitic AChE, it was known that the several functionally important regions of schistome AChE are sufficiently different from human AChE. Hence, now it is possible to design drugs and vaccines, which would specifically inhibit only the parasitic AChE. [Jones A.K., et al., 2002]

iii) Phylum: - Aschelminthes – includes Nematodes, roundworms, threadworms [some], whipworms, lungworms, hookworms, eelworms, a pseudocoelamate phylum with both parasitic and free-living representatives, exist in very large numbers.

Class Nematoda: -

Example: - C.elegans

Nematoda is one of the rare phyla, wherein multiple “Ace” genes encoding for AChE have been identified. For instance in Nematoda C.elegans, four genes [Ace-1, Ace-2, Ace-3 and Ace-4] encoding for AChE have been identified. Amongst the four genes, Ace-1 and Ace-2 encode the 2 major forms of AChE, which show a 35% identity in their coding regions, but differ in the pharmacological properties and tissue repartition. Ace-1 expression is observed throughout the body wall and vulval muscle cells of the nematode C.elegans. Moreover, further studies revealed that the C.terminus of Ace-1 was homologous to that of the T subunits present in vertebrate AChE’s and that Ace-1 oligomerized into amphiphilic tetramers. Whereas Ace-2 is exclusively expressed in neurons and has a hydrophilic C terminus of $AChE_H$ type. It associates into glycolipid-anchored dimmers. Ace-3 and Ace-4 show 54% identity, as they lay in close proximity to each other on the same chromosome. Ace-3 represents a mere 5% of the total AChE activity. They are known to hydrolyze the substrate butyrylcholine faster than acetylcholine. Ace-3 encodes H subunit catalytic subunits and are resistant to usual cholinesterase inhibitors. The mRNA level of Ace-4 is extremely low, and hence, even though it is transcribed, no enzyme activity has been detected for this form of AChE till date. This distinct tissue-specific expression of Ace-1, Ace-2 and Ace-3 is indicative of the fact, that these genes are not redundant. [Combes D., et al 2003],[Combes D., et al.,2001],[Combes D., et al 2000]. The enzyme AChE expressed by these genes has been recognized in the crude extracts and in the excretion secretion products of the nematodes. The amount of AChE present varies from species-to-species, from stage to stage and in between sexes. This

variation in the amount of AChE present is responsible for sex-, species-, stages- and strain specific behavior of the nematode. AChE plays an important role in the nematode neuromuscular system and in the host parasite relationship and has been associated with the modulation of several host mechanisms like gastrointestinal motility, cell membrane permeability, anti-coagulant processes, glycogenesis, acetate and choline metabolism, antihelmintic resistance, immune and anti-inflammatory responses. Hence AChE of the nematode poses as the priority target for several drugs been tested to protect against nematode infection.[Ros-Moreno R.M., et al., 2002]

iv) Phylum: - Annelida: - Annelids, Segmental Coelamate worms with Chitonous Bristles.

Example: - Leech

Two forms of AChE, one the "spontaneously-soluble" form i.e. SS-AChE and the other the "detergent-soluble" form i.e. DS-AChE were studied in medicinal leech "Hirudo medicinalis". The 2 forms of AChE were found to differ in their substrate and inhibitor specificities. The spontaneously soluble form was recovered easily from the hemolymph and tissues of leech in a low salt buffer solution and was suggested to represent the hydrophilic monomeric [G_1] form of AChE. Whereas the detergent-soluble form was extracted from the tissues of leech in low salt buffer solution containing 1% Triton-X and was suggested to represent an amphiphilic glycolipid anchored dimeric molecular form of AChE. [Talesa V., et al., 1995] Early studies on leech suggested that AChE was a key enzyme marker in the leech cholinergic neuron as it was demonstrated that the cholinergic neuron

contained 3 to 24 folds higher AChE than non-cholinergic neurons.[Wallace B.G., et al., 1982]

v) Phylum: - Rotifer [-Rotatoria] rotifers, “wheel animals”, named for rotating ring of cilia; a pseudocoelamate phylum.

In rotifers, AChE activity was localized in the neurons and muscular tissues, in sensory organs, in all the ciliated cells, secretory cells (sub cerebral, saliva and pedal glands) and gonad cells (nuclei of the syncytial vitellaruim and follicular layer, oocytes and eggs)[Raineri M 1984]

vi) Phylum Mollusca: -Molluscs, soft bodied animals, mostly with an internal or external calcareous shell.

Soluble and membrane bound AChE was detected in the bivalve mollusc Mytillus gallprovincialis [Pelecypoda: -filibranchia] collected from the northern Adriatic Sea and in the Benthic mollusc Scaphara inaequivalvis which was collected in spring 1999 from 3 areas of northern Adriatic Sea. [V Talesa et.al., 2001,2002]. Amongst the 3 forms of AChE purified in this mollusc, 2 were the spontaneously soluble forms and one was the membrane bound form. The two spontaneously soluble forms [A and B] occurred in the hemolymph and accounted for 80% of the total AChE activity. They represented the globular tetrameric [12S] and the globular dimeric [6S] molecular forms of AChE respectively. Whereas the third membrane bound, detergent soluble form of AChE represents the GP1 anchored amphiphilic globular dimeric form of AChE. All the 3 forms were inhibited by

BW284C51 a specific inhibitor for AChE, indicating that they represent “true” AChE forms. [Talesa V., et al., 2001]

In the gills of oysters, 2 forms of AChE [Form A and Form B] were detected. Form A is a glycolipid anchored and non-glycosylated molecular form of AChE sensitive to organophosphorus and carbamate insecticides. Whereas form B is a hydrophilic and glycosylated molecular form of AChE insensitive to organophosphorus and carbamate insecticides. Oysters are widely used as bio-indicators and AChE activity as a Biomarker. Hence elucidating the sensitivity of each AChE molecular form present in the gills of oysters would help improving the techniques used to monitor the marine environment and report accurate results. [Bocquene G., et al., 1997]

AChE is also present in significantly high amounts in the haemolymph of Aplysia. Haemolymh in mature Aplysia is neurotropic. Hence the possibility of AChE playing a neurotropic factor in haemolymph was investigated. When Dopaminergic neurons from Pedal ganglia were cultured in a medium containing haemolymph for 24 hrs, it was observed, that the neurons were well attached to the substratum and exhibited multiple neuritis. Similar observations were reported when the culture medium was supplemented with AChE purified from the haemolymph. But in medium with no haemolymph the neurons showed poor attachment to the substratum and only one or two short neuritis were exhibited, indicating that AChE circulating in the haemolymph promotes neurite growth of adult neurons. Similar reduction in neurite growth was observed, when its specific inhibitor BW284C51 inhibited both, the catalytic and the peripheral site of the enzyme AChE, in haemolymph. But only if the catalytic site of the enzyme

was inhibited by Echothiophate, the enzyme does not loose it's function as a neurotropic factor, indicating that only the peripheral site of the enzyme AChE is responsible for Neurite growth. [Srivatsan M., et al 1997] It is known that the decrease in the AChE levels signals the onset of aging in Aplysia. This process coincides with impaired neural function. It was observed a specific sensory input like chronically applied sensory stimulation [CSS] could upregulate enzyme activity [Peretz B., et al 1996]

vii) Phylum Arthropoda: -arthropods, 'jointed legged animals' characterized by segmented bodies and jointed appendages; have gills or tracheae; easily the largest phylum of all animals & of great economic importance.

Example: - Insects

Acetylcholinesterase is an important enzyme of the insect nervous system. For several years it was and still remains to be the priority target of insecticides. Hence extensive work done in this area led to the bio-chemical characterization of this enzyme in more than 20 insect species. Moreover, molecular analysis of its gene structure was also completed in some insect species like [1] fruit fly [Drosophila melanogaster] [2] yellow fever mosquito [Aedes aegyypti] [3] Colorado potato beetle [Leptinotarsa decemlineata], housefly [Musca domestica], green rice leafhopper [Nephotettix cincticeps] and Austrailian sheep blow fly [Lucilio cuprina]. Recent developments in this area elucidate the 3D structure of the AChE enzyme in insects. It was then learnt, that although the insect AChE showed relatively low amino acid sequence identity [36%] to that of vertebrates it showed similar folding and close over-lapping of the active sites. Further on

the basis of several other studies in Lepidotera, Diptera, Orthoptera and Coleoptera it was learnt that AChE in insects exhibited polymorphism and is majorly present as a glycolipid anchored amphiphilic dimer [G2a], with variable amounts of hydrophilic dimmer [G2h] and sometimes traces of hydrophilic and amphiphilic monomers [G1a and G1h] all possessing the same catalytic properties and belonging to a single gene. These findings were further confirmed with the detection of a single Ace locus in Drosophila and with the cloning of a single gene in Anopheles stephensi and Aedes aegypti. The only exception to this was Culex pipens, wherein Bourget and Colleagues were successful in detecting not one but two AChEs [AChE 1 and AChE 2] differing in their substrate and inhibitor specificities. This finding is under speculation and is being investigated. [Bourguet D., et al., 1997] [Gao J.R., et al., 2002]

2.3 Conclusion: -

This survey helps understand the cholinergic and non-cholinergic essence of AChE, a well established enzyme, of the invertebrate nervous system and muscles; and throws light on it’s high potential in being used as a biomarker of environmental toxicity. It also builds up a need, to acquire more knowledge about this interesting multifaceted enzyme in vertebrates (Chapter3) for the sake of comparison and better understanding.

Chapter 3:-

Role of Acetylcholinesterase in Chordates

3.1 Introduction: -

Extensive research conducted on vertebrates (Torpedo, electric eel, chick, rat etc) for several years has generated a sea of information related to all aspects concerning the AChE enzyme. Some of these aspects include expression, structure, cholinergic functions, non-cholinergic functions, gene regulation, isoforms, implications in neurodegeneration, post translational modifications etc. A sincere effort has been put in accumulating as much as information possible and surveying it with reference to each phylum in vertebrates.

3.2 AChE in Chordates: -

Chordates are characterized by having a single dorsal nerve cord, a notochord and pharyngeal gill slits at some stage of their life cycle.

i) Subphylum Cephalochordata:

Cephalochordate amphioxus possesses two ChE genes of which only one represents a ‘true’ AChE. Phylogenetic sequence analysis of these genes indicated that the two genes were the result of a duplication event in the lineage leading to amphioxus. This duplication event is distinct from the event that gave rise to AChE gene in vertebrates, because Agnatha [hagfishes], which represent the most primitive extinct vertebrates, seem to possess a single AChE gene and exhibit both globular and asymmetric forms of the enzyme AChE. AChE of ‘Myxine glutinosa’ was found to possess an

acyl-binding site, typical of other vertebrates. These studies added to our better understanding on evolution of AChE in Chordates.
[Sutherland D., et al., 1997]

3.3 AChE in Vertebrates: -

Vertebrata: Vertebrates have backbones, group indicates lampreys and all jawed vertebrates.

3.3a Chondrichthyes: -

Example: - Torpedo

A single AChE gene, in Torpedo, undergoes alternative splicing of its H and T exons, to generate $AChE_H$ and $AChE_T$ subunits. In addition to $AChE_H$ and $AChE_{T,}$ R transcripts [i.e. 'readthrough' which are not spliced after the last catalytic exon, but appear to be mature i.e. polyadenylated] have been characterized. The GPI anchored dimeric molecular form derived from $AChE_H$ subunit is the only form expressed in the Torpedo skeletal muscle. This form has also been characterized in other tissues, like the electric organs [derived from modified muscles] and the electric lobes [which is the region of the CNS that commands electric discharges]. It is also known that this form, shows dramatic increase in its level, right from the first electric discharges, that is from their origin in the Electric Lobes to their entry in the electric organs. The soluble tetrameric G_4 form derived from $AChE_T$ subunits is maintained at a uniform level in the electromotor nerves. Whereas the collagen tailed AChE molecular forms are present in the electric organs. It has been shown, that the presence of the AChE isoforms in

embryonic electric organs coincides with the establishment of contacts between the nerve terminals and the electrocytes. The expression of AChE molecular forms, in Torpedo, was observed to be tissue-specific suggesting that polymorphism exhibited by AChE is dependant on a number of factors including [1] amount of ACh released [2] size of the synaptic space and/or [3] temporal course of the physiological stimulation [Massolie J., et.al., 1999]. In developing electric organ of torpedo, earliest AChE expression was observed at the 35mm stage of the embryo. Both asymmetric as well as G_2 isoforms of AChE were present at this stage. The G_1 isoforms of AChE become prominent later, at the 85mm stage of the embryo, and remains more abundant than G_2 through adulthood. Hence the pattern of expression of AChE isoforms in a developing electric organ of Torpedo reveals less about the production and inter-conversion of AChE forms, but nevertheless indicates maturation of specific structures with which these particular forms are associated, like the appearance of asymmetric forms might be associated synaptogenesis and laying down of the basal lamina [Brimijoin S., 1982]

3.3b Aves: - Birds have feathers, no teeth, modified forelimbs [wings], can regulate their body temperature and have land adapted eggs with shells.

Birds possess a single AChE gene. This gene contains only one exon encoding C- terminal region of type T and does not contain any other alternative exons [Massoulie J., et al., 1999] Extensive work been carried out on AChE of the developing chick brain indicates, that the ganglia of 3.5-4 day old embryo shows the earliest expression of AChE. Further, intense AChE activity is evident on embryonic day 6, as the structures expressing AChE increase in number. [Tarrao S.A., et al., 2000] The lighter isoforms

like G_1 appear first during the early embryonic stages and pose as biosynthetic precursors of more complex forms like G_4, which appears a little later and gradually increases until maturity. [Salceda R., et al 1992] Though the hydrophobic G_4 form predominates in the brain, the A_{12} form of AChE has also been detected in some structures like the mesencephalon [1%], retina [7%] and the ciliary ganglion [10%]. The latter is a parasympathetic [cholinergic] structure, which along with A_{12} AChE [10%] at the nerve endings also expresses G_1 AChE [7%], G_2 AChE [43%] in the cell body and G_4 AChE [40%] in the axons of the pre-synaptic and post-synaptic nerves. This structure-specific expression of AChE isoforms, elucidated by Courad and Colleagues [1980], suggested structure related functions for these isoforms. Like for instance, the A_{12} AChE form is known to be densely packed with catalytic sites and hence its concentration at the nerve endings increases efficiency of the cholinergic transmission. [Brimijoin S., 1983]. Early embryonic expression of AChE is not only observed in neuronal [e.g.: - ciliary ganglion] but also in non-neuronal tissues [e.g.: - retinal pigment epithelial cells]. Furthermore, AChE activity in these tissues; show a parallel increase with development of the chick brain. These findings strongly suggested some non-cholinergic functions for AChE apart from its novel role in neurotransmission. In vitro studies on chick retinal cultures exposed to an AChE inhibitor showed cell size reduction whereas, studies on an AChE rich medium stimulated neurite growth. [Tarrao S.A., et al., 2000] In another study, chick retinal pigment epithelial cells in culture were reported to synthesize AChE and express A_{12}, G_1, G_2, and G_4 molecular forms of the enzyme. The synthesized AChE either remained associated to the cell, expressing A_{12} in a one day old culture but equivalent levels of G_2, G_4 and A_{12} in an 4-day old culture or the synthesized

AChE was released in the growth medium expressing higher levels of G_4 AChE in the latter stages and lower levels of G_2 and G_1 AChE in the earlier stages. Hence the process of cell differentiation is accompanied by an increase in the cell-associated as well as released AChE along with an alteration in their isozyme pattern with respect to the age of the culture. The released AChE reported higher activity than cell associated AChE and was implicated in non-cholinergic function of cell differentiation. [Sakeda R., et al., 1992] These in vivo and in vitro studies in the central nervous system [CNS] suggested that the developmentally regulated AChE enzyme played a role in non-cholinergic functions like morphometric processes, cell differentiation and synaptogenesis along the nervous system. [Tarrao S.A., et al., 2000] In the peripheral nervous system [PNS], AChE was studied in dystrophic chicken muscles, wherin it showed increased activity per unit weight of the tissue and an altered isozyme pattern depending on the severity of the disease. Avian dystrophy primarily affects fast-twitch glycolytic fibers. Hence it was observed that the AChE isozyme pattern was remarkably altered in muscles rich in fast-twitch fibers [e.g.: -pectoral muscles]. The isozyme patterns of a dystrophic chicken muscle and a dennervated avian muscle, showed no resemblance, and it was observed, that a dystrophic muscle just like a normal muscle could respond to a neural response and loose its asymmetric AChE on denervation. Hence it was concluded that the avian dystrophy in no ways resembled the process of denervation. [Brimijoin S., 1988] Studies done on dystrophic chickens, in order to analyze potential abnormality in the axonal transport of AChE forms surprisingly yielded negative results wherein despite large increases in the activity of G_1 and G_2 forms in limbs and pectoral muscle, no change was observed in the basal content or the rate of accumulation of any AChE form

in ligated branchial and sciatic nerves. But experiments done on chickens with peripheral neuropathy induced by acrylamide revealed specific abnormalities in the axonal transport of AChE forms. It was observed that in sciatic nerves, the A_{12} AChE form was 5 times that of normal and in ligated nerves, the rate of accumulation of A_{12} was less than half of normal as well as the G_4 AChE was reduced by 20%. These abnormalities were accompanied by alteration in the isozyme pattern of AChE, in the hind limb muscle and resembled effects of denervation. It was hence suggested, that, the degenerative changes induced in muscles of animals with acrylamide neuropathy is due to defective fast axonal transport of proteins like AChE. Hence AChE and its isozymes are also implicated in peripheral neuropathies. [Brimijoin S., 1983]

3.3c Reptilia:- reptiles have scales and an amniote egg adapted to survival out of water, cold blooded mostly, well adapted to life on land, contains many fossil groups including dinosaurs.

A single AChE gene in the snake Bungarus fasciatus undergoes alternative splicing its 'S' and 'T' exons to generate $AChE_S$ and $AChE_T$ subunits.
A short, highly charged peptide region, which does not contain any cysteine, characterizes the C-terminal region encoded by the $AChE_S$ subunit and hence the resultant form of AChE is soluble and monomeric. This form is exclusively present in Venom glands, liver and muscle of elapid snakes. Venoms of Mambas [e.g.: - the green mamba, Dendroaspis viridus] are an exception, as they do not contain AChE but contain a peptide toxin fasciculin that inhibits mammalian AChE with high affinity. The possibility that AChE plays a role in imparting toxicity to venom, where it is found in

high levels, has been ruled out, by the fact, that neither AChE itself is toxic, nor does it reinforce toxicity of venom toxins. Hence the pathological significance of this form of AChE in venom remains unclear, however it was suggested that in muscles, the soluble monomeric form of AChE co-exists with the molecular forms encoded by $AChE_T$ subunits and participates in the functional hydrolysis of ACh. [Cousin X., et.al.;1998]

3.3d Mammalia: - Mammals can regulate their body temperature, generally have hair, bear live young ones and nourish them with milk produced by mammary glands.

Mammals possess a single AChE gene, which undergoes alternative splicing of its H and T exons to generate $AChE_H$ and $AChE_T$ subunits. Any defect in this process of alternate splicing generates R transcripts.

The AChE H subunit generates GPI-anchored dimmers in mammals. This isoform was also detected in Torpedo and Drosophila. However the H peptide encoding the H exon in mammals, showed no sequence homology to the H peptides of Torpedo or Drosophila. Hence even though these H peptides were found to be functionally equivalent, their exons appeared independent of each other, during evolution. This isoform of AChE is expressed in mammalian blood cells [erythrocytes and lymphocytes] embryonic muscles and liver. In mammalian blood cells, AChE expression demonstrated by immunochemical and immunocytochemical techniques in both T and B-lymphocytes, suggested a putative role for the cholinergic system as regulators of immune function. [Tayebati K.S., et al., 2002].

The AChE T subunit generates amphiphilic monomers [G_1^a] amphiphilic dimmers [G_2^a], hydrophobic tailed tetramer [G_4^a] soluble tetramers [G_4] and

collagen tailed forms amongst these isoforms, the G_1^a and G_2^a are intercellularly present biosynthetic precursors of functional hydrophobic tailed tetramers, soluble tetramers, and collagen- tailed forms of AChE. The soluble tetrameric form of AChE constitutes the majority of the plasma AChE [56%] and the cerebro spinal fluid [CSF] AChE [82% in lumbar CSF and [69%] inventricular CSF] and hence AChE activity measured in these body fluids in a number of neurological and neuropsychiatric disorders involving central or peripheral cholinergic neuropathy, reflects the pathological changes in the structures of the CNS or PNS from where these enzymes originate. Like for instance AChE and it's isoforms in the CSF are prognostic markers for Alzheimer's disease. [Atack R.J., et al., 1987]. The hydrophobic tailed tetramers, mainly anchors AChE in the mammalian brain membranes, where it constitutes 80% - 90% of the total AChE activity and is apt for efficient cholinergic transmission in the small synaptic spaces of the Central Nervous System [CNS]. This form of AChE is also implicated in cell adhesion and neurodegeneration. It was demonstrated, that the membrane-associated G_4 form of AChE expressed in neuroblastoma cells, consisted of the HNK-1 epitope, which functions as a mediator in cell adhesion. Hence this form of AChE functions as a cell adhesion molecule binding laminin and other extra cellular matrix components. These cell adhesion molecules in the CNS might play an important non-cholinergic role in long term storage of information as the HNK-1 present on the isoform might be involved in memory formation. Hence the significant loss of hydrophobic membrane- associated tetrameric isoforms may lead to dementia as seen in neurodegenerative diseases like Alzheimer's. [Johnson G and Moore W.S., 2001]. The hydrophobic tailed tetramers were also found to be located at the 'peri-junctional zone' that surrounds the NMJ controlling

the diffusion of AChE around the junction, on repetitive stimulation of the muscles. Hence regulation of this isoform in the peripheral nervous system [PNS] depends on the kind of muscle expressing it and the type of activity exhibited by that muscle. The collagen-tailed isoforms generated by associations of AChE T subunits with a special collagen called ColQ, is mainly expressed at the NMJ. The collagen ColQ encoded by a single gene producing multiple variants in mammals is of great physiological significance, since the levels of AChE and ColQ multiple variants could describe the kinds of isoform present in a particular type of muscle and help the muscle in adapting to various stimulations. For instance, fast muscles in rat exclusively express A_{12} isoform, as ColQ is only expressed at the junction and its level is comparatively low compared to AChE, so all the three ColQ chains are associated with AChE tetramers, giving rise to the A_{12} AChE. Whereas slow soleus muscles express both A_4 and A_8 AChE as ColQ is expressed uniformly in extra-junctional and junctional regions and AChE is expressed at a lower level, so significant fractions of ColQ chains are not occupied by AChE tetramers resulting in A_4 and A_8 AChE. Hence collagen-tailed isoforms are controlled by innervations and by the type of muscle. Moreover, a defect in the ColQ gene, in humans, results in a rare congenital myasthenia syndrome, classified as CMS-1c, wherin due to lack of collagen-tailed AChE, abnormal accumulation of AChE at NMJ leads to receptor desensitization resulting in muscle weakness and fatigability.

The ‘R’ transcripts generating soluble monomers, result from errors during the process of alternate splicing. The C-terminal peptide of $AChE_R$ subunits is called ARP. The ‘R’ transcripts [mRNAs] are expressed in embryonic tissues during development, in cell cultures and in mouse erythroid cells. They are usually not expressed in adult mice, unless induced by stress or

intoxication by anti-cholinesterase drugs, in order to modulate hematopoietic differentiation and immunological responses. The best evidence for these isoforms is also derived from experiments using the brain of a stress induced mice, wherin an increase in the mRNA level was followed by specific pattern of migration in native gels. These soluble monomers have never been characterized in vivo and hence lack direct evidence of existence. [Massoulie J., 2002] [Massoulie J., et al., 1999]

Acetylcholinesterase in mammals is also implicated in morphogenesis and neurodegeneration [e.g.: - Alzheimer's disease]. It's role in insulin-dependent diabetes mellitus, a neurological disease, was best studied by Gustavo S.C and Rocio S [1995-2001]. Diabetes mellitus is characterized by increase in blood glucose level and by other complications, like retinopathy. AChE activity was reported to significantly increase [100%] in the cerebral cortex and [55%] in the serum whereas decrease [30%-40%] in the erythrocytes and [30-40%] in all the molecular forms of the retinal pigment epithelium [RPE], but did not deviate from its normal activity in the retina, liver and muscle of a streptozotocin induced diabetic rat. This change in AChE activity was not evident and significant until 7 days after streptozotocin treatment. Hence, not only hyperglycemia, but also, differential regulation of the enzyme in different tissues by insulin was suggested to affect enzyme activity. Moreover the decrease in activity of AChE molecular forms in RPE may alter the RPE environment resulting in abnormalities observed in diabetic rat retinopathy. These changes in AChE activity reflect impairment in biosynthesis; degradation or insertion into the plasma membrane as diabetic state is known to cause membrane alterations affecting kinetic properties of membrane bound AChE. [Sanchez-Chavez G., et al., 1995; 2000; 2001]

3.4 Conclusion: -

The sea of information generated from the extensive research on acetylcholinesterase in vertebrates represents “ONE Enzyme – MULTIPLE Functions” scenario. Acetylcholinesterase, the lytic enzyme of the cholinergic system, is developmentally regulated, involved in synaptogenesis, morphogenesis and is also implicated in central nervous system and peripheral nervous system neuropathy.

On having surveyed acetylcholinesterase in both invertebrates and vertebrates, it is now possible to make comparisons and derive a phylogeny, giving an insight into acetyl cholinesterase’s evolution.

Although the information obtained from molecular biology techniques for invertebrate acetylcholinesterase is limited, it has provided an insight, indicating, that the invertebrate acetylcholinesterase is not a typical vertebrate acetylcholinesterase, but exhibits properties (kinetics and sequence analysis) intermediate to that of vertebrate acetylcholinesterase and butyrylcholinesterase. This is particularly evident on comparing the structures of vertebrate and invertebrate cholinesterases. The catalytic gorge of vertebrate acetylcholinesterase is lined with fourteen aromatic amino acids. These aromatic amino acids interact with the cholinergic ligands like acetylcholine, as they are associated with the anionic or choline-binding subsite of the active site, the acyl-binding pocket and the peripheral anionic site. Two bulky side chains of phenylalanine residues, present at the acyl-binding pocket, sterically inhibit the binding of butyrylcholinesterase with

large acyl groups, accommodate small substrates like acetylcholine and hence are essential in determining the substrate specificity of cholinesterases. In vertebrate butyrylcholinesterase, six of the fourteen aromatic amino acids, including both the phenylalanines of the acyl pocket are replaced with small non-aromatic amino acids. These small non-aromatic amino acids might play an important role in functionally differentiating butyrylcholinesterase from acetylcholinesterase. It has been suggested, that the duplication of the acetylcholinesterase gene and its subsequent replacement with small non-aromatic amino acids in an ancestor, is responsible for the emergence of vertebrate butyrylcholinesterase. The invertebrate acetylcholinesterase on the other hand, posses only a single phenylalanine residue at the acyl-binding pocket and hence expresses a deletion, relative to both vertebrate acetylcholinesterase and butyrylcholinesterase, thereby exhibiting intermediate substrate specificity. This finding, also suggested, that the insertion of a second phenylalanine residue in an ancestral invertebrate cholinesterase gene may be responsible for the origin of vertebrate acetylcholinesterase. [Sutherland D., et.al.,1997] Further research on acetylcholinesterase of invertebrates and chordates, will result in better comparisons and a clearer picture on the evolution of cholinesterases.

Project Report: -

Acetylcholinesterase [AChE] Activity and Isozyme Pattern in Normal and Lithium-treated Developing Chick Brain.

A B S T R A C T

Acetylcholinesterase [AChE], the lytic enzyme of the cholinergic system, functions in hydrolyzing the neurotransmitter Acetylcholine [ACh] and hence is used as a marker for cholinergic function. In vertebrates the protein is synthesized by a single gene and undergoes alternative splicing to give several isoforms. This enzyme and its isoforms are also involved in synaptogenesis, modulated by stages of development and differentially distributed in the brain. Not only is it known to be a marker for the developing chick brain, but is also implicated in neurodegenerative diseases like Alzheimer's Disease. Isozyme Pattern of AChE is suggested to serve as a useful prognostic marker in neuronal degeneration.

Lithium, a well-known teratogen, has been shown in our laboratory to induce apoptosis in a developing chick brain. Understanding the dynamics of AChE isoform pattern in lithium induced neural tissue damage would help elucidating the role of these isoforms in a degenerating system and add to our understanding of neurodegenerative diseases.

We have therefore studied activity and isozyme pattern of AChE in lithium-treated and control 7-day old developing chick brain and report the same.

A] Introduction: -

AChE, the lytic enzyme of the cholinergic system functions in hydrolyzing and thus terminating the action of neurotransmitter ACh. Since it plays an important role in cholinergic transmission, it is used as a marker for cholinergic functions. This enzyme exhibits polymorphism. In vertebrates AChE is synthesized by a single gene, which undergoes alternative splicing to yield several isoforms, which are basically of two types [1] Globular [G]: - G_1, G_2 and G_4 which are readily extractable in low ionic strength buffer or tightly bound to membranes, requiring detergent for solubilisation [2] Asymmetric forms [A]: - A_4, A_8 and A_{12}. They are collagen tailed, do not interact with detergent but are released in buffer with high salt concentration [Sanchez-Chavez G., et al., 1995]. All the molecular forms posses the same catalytic domain but distinct C-terminal peptides [$AChE_R$, $AChE_H$, $AChE_T$, $AChE_S$] [Massoulie J., et al., 1999]. They are as follows: -

Figure A: - Isoenzymes of Acetylcholinesterase

$AChE_R$: - where R stands for 'readthrough'

Readthrough or the 'R' transcripts or subunits of type R correspond to the hypothetical products of 'readthrough' transcripts, which retain the 'intronic' region that forms the last exon encoding the catalytic domain. $AChE_R$ molecular forms remain soluble and monomeric and are found only in vertebrates [Massoulie J., et al., 1999, 2002].

$AChE_H$: - where H stands for 'hydrophobic'.

'Hydrophobic' or H transcripts or subunits of type H, are called so, because the C-terminal regions of these subunits are characterized by hydrophobic sequences. These hydrophobic regions consist of one or two cysteine residues, which establish disulphide bonds & result in a dimeric mature protein. The hydrophobic region majorly corresponds to a signal for a glycophosphotidyl (GPI) anchor. Hence these molecular forms are GPI-anchored dimmers [Massoulie J., et al., 1999, 2002].

$AChE_T$: - wherein T stands for 'Tailed' forms

$AChE_T$ is the only type of catalytic subunit that exists in all vertebrates producing major AChE forms in the brain and the muscle. $AChE_T$ generates multiple structures, ranging from monomers, dimmers to collagen- tailed and hydrophobic tailed forms. These tailed forms are catalytic tetramers associated with anchoring proteins, which help them attach to the basal lamina of NMJ or to the cell membrane. The monomeric G_1 form is the first translational product and more complex forms like G_2, G_4 are sequentially derived from it. Hence the order of formation and appearance is G_1->G_2->G_4->A_{12}. This order also involves subdivisions of the major forms like G_4 AChE is subdivided into a secreted form, hydrophobic tailed form and an

incipient collagen tailed asymmetric form. In the collagen tailed forms, $AChE_T$ subunits are associated with a specific collagen, Col Q which contains a short peptide motif, the proline- rich attachment domain [PRAD], that triggers the formation of different heteromeric forms, which contain one, two or three catalytic tetramers [A_4, A_8, A_{12}] from monomers and dimmers. The collagen-tailed forms are predominant at the NMJ. The $AChE_T$ subunits are also found to be associated with a hydrophobic glycoprotein of about 20 KDa. This protein contains a signal peptide, an extracellular domain, which includes a proline rich motif and an N-glycosylation site, a transmembrane domain and a cytoplasmic domain. The proline rich domain present is responsible for the formation of membrane bound G_4 tetramers. This transmembrane glycoprotein, which constitutes the membrane anchor of AChE, is called the Proline-Rich Membrane Anchor [PRiMA]. $AChE_T$ subunits associated with PRiMA predominantly exist in the brain. Although careful biochemical analysis of muscle sections have shown, that this form is also located in the ‘peri-junctional’ zone surrounding the NMJ and is physiologically regulated [increases or decreases] depending on the type of muscle activity/exercise. Hence it is suggested, that the physiological role of $AChE_T$ subunits associated with PRiMA in muscles is to control the diffusion of ACh around the junction on repetitive stimulation of the muscle. These findings need to be further investigated. New developments in the intracellular pathways, AChE transport, mode of association and regulation of the catalytic subunit & their interactions with the structural subunits can emerge from the cloning of this PriMA [Massoulie J., et al., 1999, 2002].

$AChE_S$: - wherein S means 'Soluble'. These $AChE_S$ subunits comprise of soluble monomeric forms of the enzyme and have been studied only in vertebrates [Massoulie J., et al., 1999, 2002].

In Vertebrates a single gene encodes for AChE. The following 3 processes generate this panoply of AChE molecules.

[1] Alternate splicing of the AChE gene.

[2] Oligomerisation of the catalytic subunits.

[3] Associations with non-catalytic subunits.

AChE is an interesting enzyme, because on one hand it is involved in non-cholinergic functions like neuritogenesis [Srivatsan M., et al 1997] and on the other hand it is implicated in neurodegenerative diseases [Atack R.J., et al., 1987].

Lithium a clinically effective and well established drug, in spite of it's side effects, toxicity and narrow therapeutic range, is in use for bipolar disorders for several years [Janka Z., et al., 1979]. Interestingly, lithium is also known as a teratogen causing apoptosis in embryonic nervous system and affecting development of a number of organisms like Dictyostelium, Xenopus, Mice etc [Kao R K., et al., 1998]. Another important attribute of lithium is its differential action on immature and mature neurons. On one hand, in vitro studies showed, that, treatment of immature neurons with lithium at a particular concentration, results in neuronal cell death [Janka Z., et al., 1979] whereas on the other hand lithium at the same concentration imparts neuroprotection in a low potassium environment resulting in survival of mature neurons.

The developing chick has been established in our laboratory as an in vivo model to study the effects of lithium on developing nervous system. The advantage of using chick embryo, as an experimental system is that it's developing plan has been extensively studied. Besides this, the system is easy to handle, easily available in large numbers and economically feasible. Birds possess a single AChE gene. This gene contains only one exon encoding C- terminal region of type T and does not contain any other alternative exons [Massoulie J., et al., 1999]. Extensive work been carried out on AChE of the developing chick brain indicates, that the ganglia of 3.5-4 day old embryo shows the earliest expression of AChE. Further, intense AChE activity is evident on embryonic day 6, as the structures expressing AChE increase in number [Tarrao S.A., et al., 2000]. The lighter isoforms like G_1 appear first during the early embryonic stages and pose as biosynthetic precursors of more complex forms like G_4, which appears a little later and gradually increases until maturity. [Sakeda R., et al 1992] Though the hydrophobic G_4 form predominates in the brain, the A_{12} form of AChE has also been detected in some structures like the mesencephalon [1%], retina [7%] and the ciliary ganglion [10%]. The latter is a parasympathetic [cholinergic] structure, which along with A_{12} AChE [10%] at the nerve endings also expresses G_1 AChE [7%], G_2 AChE [43%] in the cell body and G_4 AChE [40%] in the axons of the pre-synaptic and post-synaptic nerves. This structure-specific expression of AChE isoforms, elucidated by Courad and Colleagues [1980], suggested structure related functions for these isoforms. Like for instance, the A_{12} AChE form is densely packed with catalytic sites and hence its concentration at the nerve endings increases efficiency of the cholinergic transmission. [Brimijoin S., 1983] Early embryonic expression of AChE is not only observed in neuronal

[e.g.: - ciliary ganglion] but also in non-neuronal tissues [e.g.: - retinal pigment epithelial cells (RPE)]. Furthermore, AChE activity in these tissues; show a parallel increase with development of the chick brain. These findings strongly suggested some non-cholinergic functions for AChE apart from its novel role in neurotransmission. In vitro studies on chick retinal cultures exposed to an AChE inhibitor showed cell size reduction whereas, studies on an AChE rich medium stimulated neurite growth. [Tarrao S.A., et al., 2000] In another study, chick retinal pigment epithelial (RPE) cells in culture were reported to synthesize AChE and express A_{12}, G_1, G_2, and G_4 molecular forms of the enzyme. The synthesized AChE either remained associated to the cell, expressing A_{12} in a one day old culture but equivalent levels of G_2, G_4 and A_{12} in an 4-day old culture or the synthesized AChE was released in the growth medium expressing higher levels of G_4 AChE in the latter stages and lower levels of G_2 and G_1 AChE in the earlier stages. Hence the process of cell differentiation is accompanied by an increase in the cell-associated as well as released AChE along with an alteration in their isozyme pattern with respect to the age of the culture. The released AChE reported higher activity than cell associated AChE and was implicated in non-cholinergic function of cell differentiation [Sakeda R., et al., 1992]. These in vivo and in vitro studies in the CNS suggested that the developmentally regulated AChE enzyme played a role in non-cholinergic functions like morphometric processes, cell differentiation and synaptogenesis along the nervous system [Tarrao S.A., et al., 2000]. In the PNS, AChE was studied in dystrophic chicken muscles, wherin it showed increased activity per unit weight of the tissue and an altered isozyme pattern depending on the severity of the disease. Avian dystrophy primarily affects fast-twitch glycolytic fibers. Hence it was observed that the AChE isozyme pattern was remarkably

altered in muscles rich in fast-twitch fibers [e.g.: -pectoral muscles]. The isozyme patterns of a dystrophic chicken muscle and a dennervated avian muscle, showed no resemblance, and it was observed, that a dystrophic muscle just like a normal muscle could respond to a neural response and loose its asymmetric AChE on denervation. Hence it was concluded that the avian dystrophy in no ways resembled the process of denervation [Brimijoin S., 1988]. Studies done on dystrophic chickens, in order to analyze potential abnormality in the axonal transport of AChE forms surprisingly yielded negative results wherin despite large increases in the activity of G_1 and G_2 forms in limbs and pectoral muscle, no change was observed in the basal content or the rate of accumulation of any AChE form in ligated branchial and sciatic nerves. But experiments done on chickens with peripheral neuropathy induced by acrylamide revealed specific abnormalities in the axonal transport of AChE forms. It was observed that in sciatic nerves, the A_{12} AChE form was 5 times that of normal and in ligated nerves, the rate of accumulation of A_{12} was less than half of normal as well as the G_4 AChE was reduced by 20%. These abnormalities were accompanied by alteration in the isozyme pattern of AChE, in the hind limb muscle and resembled effects of denervation. It was hence suggested, that, the degenerative changes induced in muscles of animals with acrylamide neuropathy is due to defective fast axonal transport of proteins like AChE. Hence AChE and its isozymes are also implicated in peripheral neuropathies. [Brimijoin S., 1983].

Therefore activity and isozyme pattern of AChE has been monitored in the brain of lithium treated and control (non–lithium treated) embryos, to check if lithium has any effect on this developmentally important enzyme.

B] Materials and Methods: -

1) Procuring and Inoculation of the Egg: -
   a) The eggs of Gallus *domesticus* of the white leghorn species variety were procured from Central Poultry Breeding Farm, Aarey Milk Colony, Goregaon, Mumbai.
   b) The eggs were surface-cleaned with alcohol, labeled/marked and incubated at 37°C 2°C in the incubator. A bowl filled with water was also kept in the incubator to provide moisture.
   c) After 24 hours of incubation, the eggs were removed from the incubator and inoculated in the following way: -
      - Using a sterile needle a hole was punctured at the broad end (air-sac region) of the egg.
      - 0.125 ml of either of the LiCl solutions (0.06M, 0.125M) was inoculated through this hole using a disposable 1ml insulin syringe so as to maintain lithium concentration in the albumin within the therapeutic range (2mM-1mM).
      - The hole was sealed with paraffin wax; the egg was rolled between the palms and kept at room temperature for 10 minutes to allow the LiCl to diffuse into the albumin.
      - Unopened eggs were kept as controls. The eggs were kept back in the incubator and incubated at 37°C 2°C for 6 more days. Moisture bowls were provided in the incubator to maintain humidity.
      - The eggs were harvested on day 7 of incubation and body weights and brain weights of embryos were noted to ascertain lithium action. Only reduced embryos were taken for further studies.

2) Determination of AChE activity: -

A variety of techniques have been used to measure enzyme activity. These include electrometric, titrimetric, manometric, colorimetric, radiometric and potentiometric methods. In this work, AChE activity in the developing chick brain, was determined colorimetrically by Ellman's Method, which is the most popular method as it is sensitive for kinetic and enzymological studies. Ellman showed that kinetic constants obtained with acetythiocholine are similar to those obtained with acetylcholine, it is also most favoured assay used routinely for toxicological studies, as it is precise, cheap and quick. [Ashwani V., 1992] [Methods of Enzymatic Analysis, Volume IV]

Principle: - ACh is hydrolyzed by AChE to acetic acid and thiocholine.

Reaction: -

A)

The catalytic activity is measured by following the increase of the yellow anion, 5-thio-2-nitrobenzoate, produced from thiocholine when it reacts with DTNB.

Reaction: -

B)

Materials: -

Sr. No	Names of the chemicals: -
1.	Na_2HPO_4
2.	DTNB (colour developer)
3.	Na_2CO_3
4.	Acetlythiocholine Iodide (specific substrate for AChE)
5.	BW284C51 (selective inhibitor for AChE)

Reagents: -

a) Phosphate Buffer: - (100mmol/litre); pH 8.0 dissolve 15.6 gms of $Na_2HOPO_4.2H_2O$ in 750 ml of water. Adjust pH 8.0 by adding NaOH solution (100mmol/l) or HCl (100mmol/l). Check pH at 25 C. Make up to 1litre with distill water.

b) Phosphate Buffer: - (100 mmol/litre); pH 7.0 dissolve 15.6 gms of $Na_2HOPO_4.2H_2O$ in 750 ml of water. Adjust pH 7.0 by adding NaOH solution (100 mmol/l) or HCl (100 mmol/l). Check pH at 25 C. Make up to 1litre with distill water.

c) Buffered Ellman's Reagent: - DTNB (DTNB 10 mmol/litre; NaHCO3, 17.85 mmol/litre) dissolve 39.6 mg DTNB in 10 ml phosphate Buffer pH 7.0 and add 15 mg sodium bicarbonate.

d) Acetylthiocholine Iodide: - (75 mmol/litre), dissolve 108.35 mg of Acetlythiocholine iodide was dissolved in 5ml of distill water.

Equipment: -

- ELISA Reader to measure absorbance at 405nm

Glassware: -

1. Test tubes, inorder to store the prepared DTNB and Acetylthiocholine Iodide, in the fridge, till used.
2. Stopper Bottles, to store the prepared Phosphate buffer, in the fridge, till used.
3. Measuring Flask
4. Pipettes, 25ml, 10ml and 5ml
5. Micropipettes, 40 ul – 200 ul and 1ul - 10 ul
6. Ependauf and Micro pestle

Miscellaneous: -

1. Weighing Machine
2. Stop watch
3. Ice
4. Dissection Box
5. Filter papers

Precautions: -

1. AChE activity is maximum between pH 7.5 to pH 9. At high pH considerable non-enzymatic hydrolysis of the substrate acetylcholine takes place, which yields a high blank in the assay procedure.
2. It is customary to use phosphate buffer at pH 8.0 for the assay.
3. Reagent Blank is essential.

4. In some studies it may be desirable, to lower the pH to reduce blank to negligible proportions. In such cases it is necessary to confirm a linear relationship of the assay procedure at the selected temperature.
5. Cholinesterases also hydrolyze acetylcholine, although at a slower rate and so it is customary to include a specific inhibitor for this enzyme in the assay.
6. This assay is valid at 20°C, 25°C, 30°C and 37°C.
7. Analytical grade reagents must be used.
8. All reagents must be stored in glass bottles.
9. Acetylcholine Iodide powder must be stored in a desiccator.
10. All solutions must be prepared in distill water.
11. Store all solutions at 4C.
12. Phosphate Buffers are stable as long as no microbial contamination occurs.
13. DTNB more stable at pH 7 than pH 8.
14. DTNB is stable for 4 weeks if stored in dark bottle.
15. Acetylcholine Iodide solution must not be kept for more than 7 days.
16. Tissue should be thoroughly homogenized.
17. A blank reaction must be setup to estimate non-enzymatic hydrolysis of substrate, substituting phosphate buffer pH 8.0 in place of sample.

Procedure: -

- Extraction of Brain: - Eggs were harvested on day 7 of incubation and body, head and brain weights were measured.
- Homogenization of the Brain: - Brain was put in an Ependauf and was thoroughly homogenized with the help of a micro-pestle in chilling conditions. Adding appropriate volume of phosphate buffer pH8.0, 2% of brain homogenate prepared. This homogenate was then centrifuged at 11,500 rpm for 5 mins. The debris settles and the supernatant was used for further analysis.
- Assay: - The assay for AChE activity was carried out in the following way: -
    a) With the help of a micropipette, 151ul of phosphate buffer pH 8.0 was directly put in ELISA control wells in triplicates and 131ul of phosphate buffer pH 8.0 was directly put in ELISA activity wells in triplicates.
    b) Then to the control and activity wells, 1ul of substrate acetylcholine iodide was added.
    c) Followed by 5ul of DTNB in both the control and activity wells
    d) This was incubated at 25°C for 10 mins
    e) Absorbance measured at 405nm. This is 'Blank' reading.
    f) Now 20ul of enzyme (brain homogenate) was added in Activity ELISA wells ONLY.
    g) Absorbance was measured at 405nm every 2 minutes. 12 readings were taken.

h) Values obtained were analyzed, Blank reading was subtracted from the Sample Readings and the Mean for triplicates calculated. Absorbance VS Time graph was plotted.

i) Change in OD / minute calculated.

j) Follin-Lowry, mentioned below, was performed to find out Protein Concentration. Dilutions were prepared in the test tubes and then with the help of a micropipette 150ul transferred in the ELISA wells. Absorbance was measured at 630nm.

k) The Specific Activity of Enzyme AChE was calculated as follows: -

Calculations:

$$\text{Specific Activity (Rs)} = \frac{[A] \times [\text{Total Volume in cuvette (ul)}]}{[\text{Molar extinction coefficient of DTNB}] \times [\text{Volume of Brain Homogenate (ul)}] \times [\text{Protein Concentration (mg/ml)}]}$$

Where: -

Rs = moles of substrate hydrolyzed / minute / mg of protein

A = change in O.D. per minute = Slope

Molar extinction coefficient of DTNB = 1.36×10^4

Volume of 2% Brain homogenate = 20ul

Volume in Cuvette = 20ul [vol. Of 2% brain homogenate] +
131ul [vol. Of phosphate buffer pH 8.0] +
5ul of DTNB +
1ul of Acetylthiocholine Iodide
= 157 ul

- **Protein Estimation By Follin Lowry Method**

Principle: -Proteins reacts with the Follin Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline Copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity depends on the amount of these aromatic amino acids present and will thus vary for different proteins. The standard graph is obtained from B.S.A because it has the reactive amino acids tyrosine and tryptophan.

Materials: -

Sr. No	Names of the chemicals: -
1.	Sodium Potassium Tartarate
2.	Sodium Carbonate
3.	Copper Sulphate (hydrated)
4.	Sodium Hydroxide
5.	F.C Reagent
6.	BSA

Reagents: -

1. Std BSA stock (200 ug/ml) – 20 mg Bovine Serum Albumin in 100 ml double distill water. Add d. D/W to the BSA and gently stir.
2. Solution A: - 2% Na_2CO_3 in 0.1 N NaOH – 2g of Na_2CO_3 to 100ml of 0.1N NaOH prepared in d.d/w
3. Solution B – 0.5% $CuSO_4$ in 1% Na-K Tartarate – 50 mg $CuSO_4$ in 10 ml of 1% Na-K Tartarate prepared in d.d/w

4. Reagent C – Mix 50 ml of Solution A + 1ml of solution B fresh on the day of use.
5. Follin Ciocalteau Reagent – Dilution 1:1 with d.d/w fresh on day of use.

Method: -

Vol. Of BSA (ml)	Conc. Of BSA (ug/ml)	D/w (ml)	Reagent C	Incubate at room temp for 20 mins	F.C. Reagent	Incubate at room temp for 30 mins	Absorbance at 630 nm
Blank	-	1.0	5 ml		0.5 ml		
0.1	20	0.9	5 ml		0.5 ml		
0.2	40	0.8	5 ml		0.5 ml		
0.3	60	0.7	5 ml		0.5 ml		
0.4	80	0.6	5 ml		0.5 ml		
0.5	100	0.5	5 ml		0.5 ml		
0.6	120	0.4	5 ml		0.5 ml		
Sample (2 ul if 100% homogenate)		-	5 ml		0.5 ml		
Sample2 (15 ul if 2% homogenate)		-	5ml		0.5ml		

3) Electrophoresis: -

Inorder to standardize the method of electrophoresis, the following protocols were carried out in the laboratory.

Protocol 1: -

[Manjrekar J and K.S Krishnan, 1986]

Procedure: -

- Extraction of Brain: - Eggs are harvested on day 7 of incubation and body, head and brain weights are measured.
- Homogenization of the Brain: - Brain was put in an Ependauf and was thoroughly homogenized with the help of a micro-pestle in chilling conditions. Adding appropriate volume of Buffer (10mM Tris-chloride – 0.32M sucrose (pH7.4) [Manjrekar J and K.S Krishnan, 1986], 100% of brain homogenate prepared. This homogenate was then centrifuged at 11,500 rpm for 5 mins. The debris settles and the 50 ul of the supernatant was used for further analysis.
- Analytical PAGE

Chemicals: -

Sr. No	Names of the chemicals
1	Tris
2	Glycine
3	Hydrocholric Acid
4	Sodium Phosphate
5	Acrylaminde
6	N, N' – Methylene- Bis Acrylamide

7	Ammonium Persulphate
8	Bromophenol Blue
9	Glycerol
10	TEMED

Reagents: -

1. Tank Buffer in the cathode compartment: -

   (37.6mM Tris, 40mM Glycine pH 8.3)

   Tris = 2.277g

   Glycine = 1.5g

   D/w = 500ml

2. Tank Buffer in anodic compartment: -

   (62mM Tris, 50mM HCl pH 8.3)

   Tris = 3.755 g

   HCl = 0.912 ml

   D/w = 500 ml

3. Separating Gel Buffer: -

   (340mM Tris, 290mM HCl, pH 8.8, final concentrations in polymerized gel pH 8.8)

   Gel Buffer (5X) => Tris = 20.595 g

   HCl = 5.3 ml

   D/W = 100ml

4. Stacking Gel Buffer: -

   (32mM Tris, 97mM Sodium Phosphate final concentrations on polymerized gel pH 6.8)

   Gel Buffer (5X)

   Tris = 1.94g

   Sodium Phosphate = 7.565g

   D/w = 100ml

5. Monomer: -

   (30% T, 2.7% C)

   Acrylamide = 2.919 g

   Bis = 0.081 g

   D/w = 10ml

6. Catalyst: - (Ammonium per Sulphate 10%)

   Ammonium per Sulphate = 1 g

   D/w = 10ml

7. Gel Loading Buffer (Bromophenol Blue dye):-

   Glycerol = 3ml

   Bromophenol Blue = 25 mg

   D/w = make volume up to 10 ml

   Sterilize the above by autoclaving.

Method

- Setting Up the mini electrophoresis apparatus: -

The apparatus is washed. The plates are washed and cleant with alcohol to make them grease free. Then the spacers and gas kits are placed on the plate attached to the unit. Followed by the second plate on top of it and this is clamped tightly. The unit is placed in the anodic tank. Hot melted 1% agar is

poured in the anodic tank. Agar seeps in between the two plates and solidifies. Thus unit is sealed from the bottom.

Diagram: -

The gel is prepared as follows: -

Separating Gel (7% T, 2.7% C)

Chemicals	Volume
Monomer	5ml
Gel Buffer	4ml
D/w	10.3ml
Catalyst	0.7ml
TEMED	35ul

Gel contents mixed well and poured in between plates, till 3/4th of the height of the plate. After the separating gel polymerizes, the stacking gel is added.

Stacking Gel (5% T, 2.7% C)

Chemicals	Volume
Monomer	0.834ml
Gel Buffer	1ml
D/w	2.81ml
Catalyst	0.35ml
TEMED	17ul

Gel contents mixed well and poured in between plates, till the top. The comb is then inserted, taking care that no air bubbles are formed below the teeth of the comb. After the gel is polymerized, the comb is removed straight in upward direction, and care is taken not to disturb the wells. The upper and the lower tanks are filled with their respective cathode and anodic buffers Samples and the tracking dye are mixed in the proportion (5:1) respectively, on a paraffin strip. With the help of a micropipette the sample is loaded in the well. The protein concentration of all the samples loaded is kept constant. The cables are connected to the mini electrophoresis unit and the power pack. The power pack is connected to a stabilizer. The current switched on, the gel is run at 30mA constant current for 5 hours. When the tracking dye has run $3/4^{th}$ of the gel, the power is switched off. The plates are opened with the help of a spatula and the gel is then stained for AChE activity and proteins.

- Localization of AChE activity after PAGE: -

Materials: -

Sr. No	Chemicals
1	Sodium Citrate
2	Potassium Ferricyanide
3	Sodium phosphate
4	Acetlythiocholine Iodide
5	Acetic Acid

Reagents: -

a) Pre – staining Solution: -

(20mM tri-sodium citrate, 2mM Potassium Ferricyanide)

Tri-sodium Citrate: - 0.59g

Potassium Ferricyanide: - 0.07g

D/w = 100 ml

b) Staining Solution: -

(7mM tri-sodium citrate, 0.7mM Potassium Ferricyanide, 65mM sodium phosphate (ph 6.0), 0.25mg/ml Acetylthiocholine iodide)

Sodium Phosphate = 1.014g

Tri Sodium Citrate = 0.205g

Potassium Ferricyanide = 0.023g

Acetylthiocholine iodide = 0.025 g

D/w = 100 ml

Method: -

Gels were removed and placed in pre-staining solution for one hour at 4˚C. The Gel was then transferred to staining solution and agitated on shaker in dark for 4 hours at 25˚C. Placing gels in 7% acetic acid can stop reaction.

Protocol 2: -

[Ashwani V., 1992]

Procedure: -

- Extraction of Brain: - Eggs are harvested on day 7 of incubation and body, head and brain weights are measured.
- Homogenization of the Brain: - Brain was put in an Ependauf and was thoroughly homogenized with the help of a micro-pestle in chilling conditions. Adding appropriate volume of Buffer (0.32M sucrose, 50mM Sodium Phosphate Buffer pH7.5, 1mM EDTA), 100% of brain homogenate prepared. This homogenate was then centrifuged at 11,500 rpm for 5 mins. The debris settles and the 50 ul of the supernatant was used for further analysis. 1mM-2.5mM of EDTA in the homogenizing buffer provided optimum solubilisation of brain AChE. To the rest of the supernatant 0.1% (v/v) of TritonX-100 is added and mixed thoroughly. It is then centrifuged at 11,500 rpm for 5 mins. The debris settles and supernatant used for further analysis. TritonX-100 (Polyoxyethyle ene glycol (9,10) p-t-octyl phenol) is a non-ionic detergent, which is commonly used to solubilize AChE. Unlike other detergents, it does not denature protein and selectively solubilizes AChE. In this study, TritonX-100 was used to extract membrane form of the enzyme.

- Analytical PAGE

Sr. No	Chemicals
1	HCL
2	Glycine
3	Phosphoric Acid
4	Acrylamide
5	N, N' – Methylene- Bis Acrylamide
6	Ammonium Persulphate
7	Bromophenol Blue
8	Glycerol
9	Riboflavin
10	TEMED
11	Tris

Reagents: -

a) Tank Buffer

(27.5mM Tris, 192mM Glycine, pH 8.3, 0.1% Triton X)

Tris = 3.3310g

Glycine = 18.6336g

Triton X-100 = 1.0 ml

D/w = 1L

b) Separating Gel Buffer 4X (0.375M Tris-Cl, pH 8.8, 0.1% Triton X- 100, final concentration in polymerized gel)

Tris = 18.165g

HCL = 1N to bring pH 8.8

Triton X-100 = 0.4ml, D/w = 100ml

c) Stacking Gel Buffer 4X (0.125M Tris-Cl, pH 6.8, 0.1% Triton X- 100, final concentration in polymerized gel)

Tris = 6.055g

HCL = 1N to bring pH 6.8

Triton X-100 = 0.4ml

D/w = 100ml

d) Monomer solution for Separating Gel (40%T, 5%C)

Acrylamide = 38g

Bis = 2g

D/w = 100ml

e) Monomer solution for Stacking Gel (6.25%T, 20%C)

Acrylamide = 5g

Bis = 1.25g

D/w = 100ml

f) Catalyst

(0.006% APS, 0.002% riboflavin)

Ammonium per Sulphate = 60mg

Riboflavin = 2 mg

D/w = 100ml

g) Gel Loading Buffer (Bromophenol Blue dye):-

Glycerol = 3ml

Bromophenol Blue = 25 mg

D/w = make volume up to 10 ml

Sterilize the above by autoclaving.

Method

- Setting Up the mini electrophoresis apparatus: - as mentioned above

The gel is prepared as follows: -

Separating Gel (7.5% T, 5% C)

Chemicals	Volume
Monomer	3.75 ml
Gel Buffer	7.5 ml
D/w	15 ml
Catalyst	3.75 ml
TEMED	60ul

Gel contents mixed well and poured in between plates, till 3/4th of the height of the plate. After the separating gel polymerizes, the stacking gel is added.

Stacking Gel (3.125% T, 20% C)

Chemicals	Volume
Monomer	10 ml
Gel Buffer	5 ml
D/w	-
Catalyst	5ml
TEMED	10ul

Gel contents mixed well and poured in between plates, till the top. The comb is then inserted, taking care that no air bubbles are formed below the teeth of the comb. After the gel is polymerized, the comb is removed straight in upward direction, and care is taken not to disturb the wells. The upper and the lower tanks are filled with buffers. Samples and the tracking dye are mixed in the proportion (5:1) respectively, on a paraffin strip. With the help

of a micropipette the sample is loaded in the well. The protein concentration of all the samples loaded is kept constant. The cables are connected to the mini electrophoresis unit and the power pack. The power pack is connected to a stabilizer. The current switched on, the gel is run at 15mA for 30 minutes and then increased to 22.5 mA, constant current for 5 hours. When the tracking dye has run 3/4th of the gel, the power is switched off. The plates are opened with the help of a spatula and the gel is then stained for AChE activity and proteins.

- Localization of AChE activity after PAGE: -

Materials: -

Sr. No	Chemicals
1	Sodium Citrate
2	Copper Sulphate
3	Potassium Ferricyanide
4	Di-sodium hydrogen phosphate
5	Acetlythiocholine Iodide
6	Acetic Acid

Reagents

1) Phosphate Buffer: - (100 mmol/litre); pH 7.0 dissolve 15.6 gms of Na2HOPO4.2H2O in 750 ml of water. Adjust pH 7.0 by adding NaOH solution (100 mmol/l) or HCl (100 mmol/l). Check pH at 25 C. Make up to 1litre with distill water.
2) Citrate (0.1M): - 2.941 g of tri sodium citrate in 100ml D/w

3) Copper Sulphate (30mM)= 0.74907g in 100 D/w
4) Potassium-Ferricyanide (5mm)= 0.1646g in 100ml D/w
5) Solution. A (freshly prepared) = Citrate: CuSO4: D/w: K3 [Fe (CN) [1 : 2 : 2 : 2]
6) Solution. B = Acetylthiocholine Iodide 28.918 mgs in 65ml phosphate buffer, 0.1M pH 7.0
7) Incubation Mixture (freshly prepared)= Solution. A [35ml] + Solution. B [65ml].

Method: -

Gels were removed and placed in phosphate buffer (0.1 M, pH 7.0) for ½ hr on shaker to equilibrate gel to the optimum pH for subsequent localization reaction. The Gel was then transferred to incubation mix and agitated on shaker in dark until bands appeared. (4 hours). Bands appeared as brown zones. Placing gels in 7% acetic acid can stop reaction.

Protocol 3: -

Combining two protocols (K.S.Krishnan and V.Ashwani), standardized in our laboratory, electrophoresis was carried out for AChE Isozyme Pattern in the following ways: -

Procedure: -

- Protocol 2 was followed to carry out the extraction and homogenization of the chick brain.
- Analytical PAGE

Chemicals: - From Protocol 1

Reagents: - From Protocol 1

Method

- Setting Up the mini electrophoresis apparatus: -

  As mentioned above

The gel is prepared as follows: -

GEL (6% T, 2.7% C)

Chemicals	Volume
Monomer	5ml
Gel Buffer	5ml
D/w	14.3ml
Catalyst	0.7ml
TEMED	35ul

Gel contents mixed well and poured in between plates, till the top. The comb is then inserted, taking care that no air bubbles are formed below the teeth of the comb. After the gel is polymerized, the comb is removed straight in upward direction, and care is taken not to disturb the wells. The upper and the lower tanks are filled with their respective cathode and anodic buffers. Samples and the tracking dye are mixed in the proportion (5:1) respectively, on a paraffin strip. With the help of a micropipette the sample is loaded in the well. The protein concentration of all the samples loaded is kept constant. The cables are connected to the mini electrophoresis unit and the power pack. The power pack is connected to a stabilizer. The current switched on, the gel is run at 30mA constant current for **2** hours. When the tracking dye has run 3/4th of the gel, the power is switched off. The plates are opened with the help of a spatula and the gel is then stained for AChE activity and proteins.

- Localization of AChE activity after PAGE: -

Materials: - From Protocol 2
Reagents: - From Protocol 2
Method: - From Protocol 2

- **Protein staining: -**

Materials: -

Sr. No	Chemicals
1.	Commasie Blue powder
2.	Methanol
3.	Acetic Acid

Reagents: -

1) Commasie Blue Stain

   Commasie Blue = 0.125 gms

   Methanol = 50 ml

   Acetic Acid = 10 ml

   D/w = make vol. 100ml

2) De staining solution

   Methanol 50ml = 50ml

   Acetic acid 10ml = 10ml

   D/w = make to 100ml

Method: -

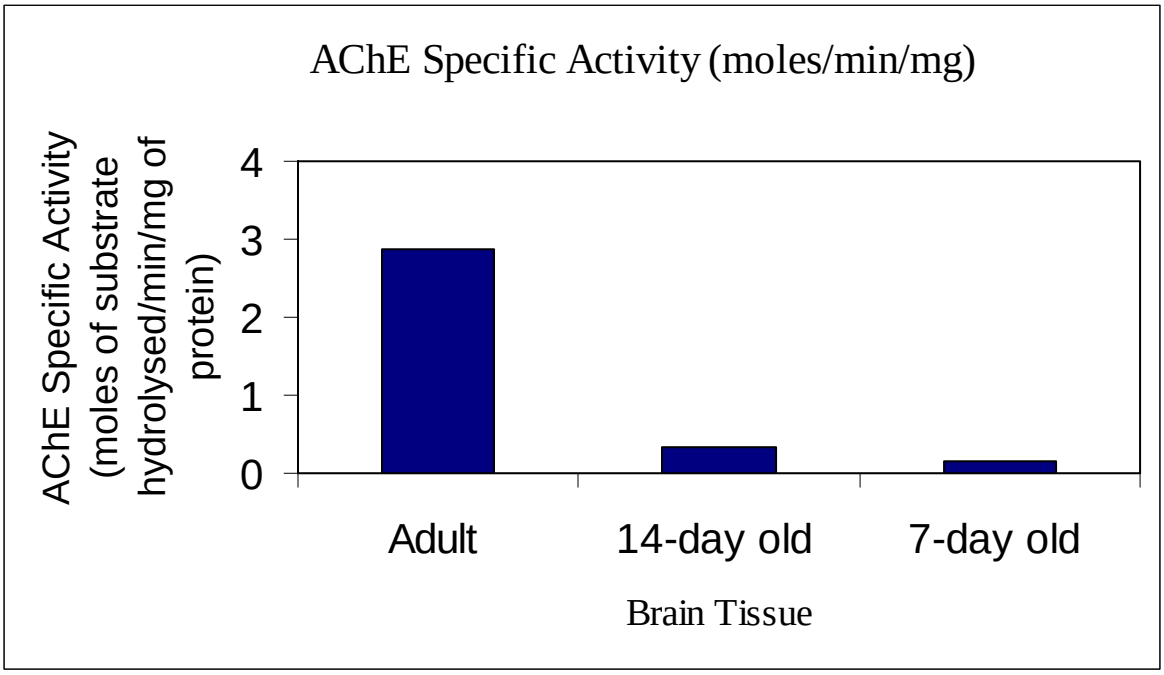
The gel was placed in staining solution overnight. The next day it is de-stained with the de-staining solution on the shaker. The de-staining solution is periodically changed. The gel is de-stained till bands can be clearly seen.

- Gel scanning and Packing: -

The gels were then scanned on the Gel Doc. The Gel Doc image saved on a floppy and then fed into the Gel Doc software (photo-capt) loaded in the life sciences computer, to analyze and get the profile of each band. Prints taken out. The gels were packed in between two gelatin sheets. On a glass plate, a little water was poured and then the gelatin sheet cut according to the size of the glass plate was placed on top the glass plate taking all care to avoid air bubbles. The gel was placed on top of this gelatin sheet and another gelatin sheet also cut according to the size of the glass plate was placed on top of the gel avoiding air bubbles. This was kept in the oven at room temperature overnight. On drying, it was cut with the help of a blade from the plate and hence preserved.

C) Results: -

1. The specific activity of AChE in the chick brain increases significantly with age.



2. Specific activity (moles/min/mg) of AChE in the centrifuged and non-centrifuged fractions of the 7 – day old embryonic chick brain showed statistically no significant difference.

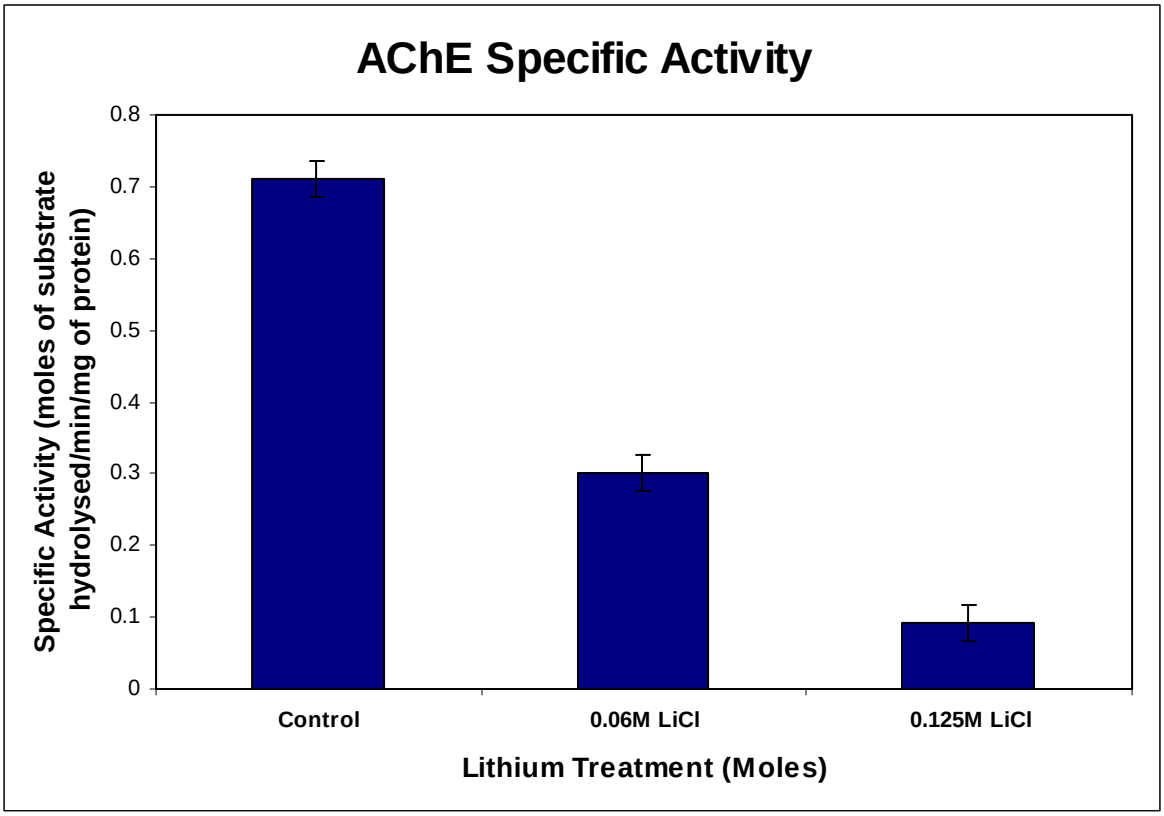
SR. NO	WHOLE BRAIN (CENTRIFUGED FRACTION)	WHOLE BRAIN (NON-CENTRIFUGED FRACTION)
1	0.1237 X 10-4	0.1399 X 10-4
2	0.1502 X 10-4	0.1651 X 10-4
3	0.1584 X 10-4	0.1636 X 10-4
Mean	0.1441 X 10-4	0.1562 X 10-4
SD	0.0181 X 10-4	0.0141 X 10-4

2. Specific activity of AChE in the cortex and whole brain fractions of the 7-day-old embryonic chick showed statistically no significant difference.

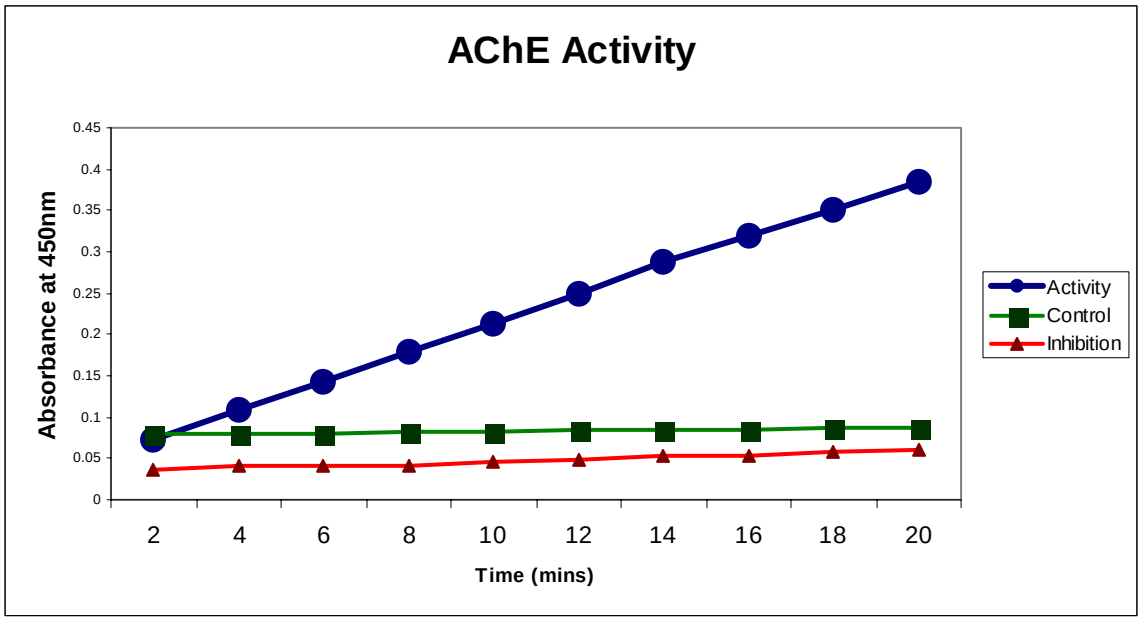
Specific Activity of AChE (moles/min/mg)

SR. NO	CORTEX	WHOLE BRAIN
1	0.1642×10^{-4}	0.1237×10^{-4}
2	0.1176×10^{-4}	0.1502×10^{-4}
3	0.16×10^{-4}	0.1584×10^{-4}
Mean	0.1473×10^{-4}	0.1441×10^{-4}
SD	0.0258×10^{-4}	0.018×10^{-4}

3. Specific Activity in control and lithium treated embryos showed a dose-dependant statistically significant reduction.



4. Activity of AChE was inhibited completely with specific inhibitor BW284C51



5. No Bands were observed with the staining procedure of Protocol 1. Whereas dark brown bands, as expected, showed up after activity staining, performed by procedure suggested by Protocol2.

	Electrophoresis carried out by following Protocol [1]. The Gel was cut in 2 parts. One part of the gel was stained according to Protocol [1] while the other part of the gel was stained according to Protocol [2]. Protocol [1] staining, failed to yield observable bands. Whereas the adjoining gel is a result of Protocol [2] staining.

6.

a) Protein Staining By Commasie Blue	b) Staining for AChE activity, Protocol [2], in 7-day old chick brain	c) Specific Inhibition of AChE activity by specific inhibitor BW284C51

7. Gel obtained from Protocol [3].

8. The Isozyme Pattern of AChE was altered in Lithium-treated 7-day old chick brain.

-ve Triton CONTROL	+ve Triton CONTROL
0.06M LiCl	0.06M LiCl
0.125M LiCl	0.125M LiCl

D) Discussion: -

- The developing chick has been established in our laboratory as an in vivo model system, to study the effects of lithium on developing nervous system. The advantage of using chick embryo, as an experimental system is that it's developing plan has been extensively studied. Besides this, the system is easy to handle, easily available in large numbers and economically feasible. The incubation time period (24hrs) prior to inoculation and the concentration of LiCl (0.06M and 0.125M) that brings about effective alterations in body and brain weights has been standardized in our laboratory. The inoculated doses not only maintain the lithium concentration in the albumin within the therapeutic range (0.2mM-1mM) but also are effective enough in bringing about generalized growth retardation.

- The development of the chick embryo is described in terms of length of time of incubation. The Hamilton-Hamberger Classification, a widely accepted classification, covers the entire period of incubation. A chick emerges after a brief three weeks of incubation. Soon after incubation is begun, a pointed thickened layer of cells becomes visible in the caudal end of the embryo. This pointed area is the primitive streak and is the longitudinal axis of the embryo. Before the first day of incubation is through, many new organs are forming. The head of the embryo becomes distinguishable; a precursor of the digestive tract, the foregut, is formed; blood islands appear and will develop later into the vascular or blood system; the neural fold forms and will develop into the neural groove; and the eye begins. On the second day of

incubation, the blood islands begin linking and form a vascular system, while the heart is being formed elsewhere. By the 44th hour of incubation, the heart and vascular systems join, and the heart begins beating. The neural groove forms and the head portion develop into the parts of the brain. The ears begin development, and the lenses in the eyes are forming. At the end of the third day of incubation, the beak begins developing and limb buds for the wings and legs are seen. By the end of the fourth day of incubation, the embryo has all organs needed to sustain life after hatching, and most of the embryo's parts can be identified. The embryo grows and develops rapidly. By the seventh day, the heart is completely enclosed in the thoracic cavity, the optic vesicles have developed into the eyes while the various regions of the brain such as the cerebellum, the cerebrum and the brainstem can be distinctly identified. (Hamilton H.L., 1965)

- AChE, the lytic enzyme of the cholinergic system functions in hydrolyzing and thus terminating the action of neurotransmitter ACh. Since it plays an important role in cholinergic transmission, it is used as a marker for cholinergic functions [Massoulie J., et al., 1999]. In the present study the specific activity of AChE was calculated, in a 7-day-old chick brain, 14-day-old chick brain and an adult chicken brain. An age-dependant increase in AChE specific activity is reported. This result is in accordance with the finding of Tarrao S.A., et al., [2000] who state that AChE activity intenses with age as the structures expressing AChE increase in number. Early embryonic expression of AChE is not only observed in neuronal [e.g.: - ciliary ganglion] but also in non-neuronal tissues [e.g.: - retinal pigment

epithelial cells (RPE)]. Furthermore, AChE activity in these tissues; show a parallel increase with development of the chick brain. These findings strongly suggested that the developmentally regulated AChE enzyme played a role in non-cholinergic functions like morphometric processes, cell differentiation and synaptogenesis along the nervous system. [Tarrao S.A., et al., 2000], apart from its novel role in neurotransmission.

- In an attempt to standardize the protocol, for extraction of the enzyme from the brain, the AChE specific activity was calculated in both the centrifuged and non-centrifuged brain homogenate fractions of 7-day-old chick embryos. The results obtained were statistically insignificant. Hence only centrifuged fractions having less debris were used for further assays.

- A histological study carried out in our laboratory [Radhika L.K., 1997] indicates that the cerebral cortex of a lithium treated chick embryo brain shows reduction in cell number and density when compared to the controls. Hence in the present study, assays were performed, inorder to compare the specific activity in the whole brain and the cortex of the 7-day-old controls. The results obtained were statistically insignificant. Hence centrifuged homogenate of a 7-day old chick brain was used for further assays.

- Lithium a clinically effective and well established drug, in spite of it's side effects, toxicity and narrow therapeutic range, is in use for bipolar disorders for several years [Janka Z., et al., 1979].

Interestingly, lithium is also known as a teratogen causing apoptosis in embryonic nervous system and affecting development of a number of organisms like Dictyostelium, Xenopus, Mice, etc [Kao R K., et al., 1998]. Primary cultures of 7-day old chick embryo brains exposed to LiCl, show a dose-dependant decrease in total protein, profound reduction in the length of neural processes and in the number of neuronal cell bodies, suggesting, that the ultra structurally swollen and degenerating neural processes observed, are sensitive to the lithium ion. In another study on the rat brain, acute lithium intoxication imparts ultra structural changes in the neurocytes, increase in number and fine structural alterations in the mitochondria, changes in the Golgi complex and in the synaptic vesicles. [Janka Z., et al., 1979]. Lithium is also known to modulate cytoskeletal elements. A study on cultured chick dorsal root ganglia neurons wherein lithium inhibits phosphorylation of newly synthesized middle molecular mass neurofilament polypeptide and also rapidly degrades Tubulin synthesized in it's presence shows Li(+)-induced metabolic abnormalities that are accompanied by alterations in cellular and cytoskeletal morphology [Hollander B.A.,et.al.,1991]. In the present study a dose dependent statistically significant reduction in AChE specific activity was observed in the brain of lithium treated 7-day-old chick embryos as compared to controls. These results were further confirmed by using a specific inhibitor for AChE in the assay.

- AChE exhibits polymorphism. In vertebrates the protein is synthesized by a single gene, which undergoes alternative splicing to give several isoforms. AChE isoforms are basically of two types [1]

Globular [G]: - G_1, G_2 and G_4 which are readily extractable in low ionic strength buffer or tightly bound to membranes, requiring detergent for solubilisation [2] Asymmetric forms [A]: - A_4, A_8 and A_{12}. They are collagen tailed, do not interact with detergent but are released in buffer with high salt concentration [Sanchez-Chavez G., et al., 1995]. All the molecular forms posses the same catalytic domain but distinct C-terminal peptides [$AChE_R$, $AChE_H$, $AChE_T$, $AChE_S$] [Massoulie J., et al., 1999]. This enzyme and its isoforms are also involved in synaptogenesis, modulated by stages of development and differentially distributed in the brain. Not only is it known to be a marker for the developing chick brain [Tarrao S.A., et al., 2000], but is also implicated in neurodegenerative diseases like Alzheimer's Disease. Isozyme Pattern of AChE is suggested to serve as a useful prognostic marker in neuronal degeneration [Atack R.J., et al., 1987].

- In chick a single gene synthesizes the enzyme AChE. This gene contains only one exon encoding C- terminal region of type T and does not contain any other alternative exons. The lighter isoforms like G_1 appear first during the early embryonic stages and pose as biosynthetic precursors of more complex forms like G_4, which appears a little later and gradually increases until maturity. The hydrophobic G_4 form predominates in the brain whereas the A_{12} form of AChE has also been detected in some structures like the mesencephalon [1%], retina [7%] and the ciliary ganglion [10%] and hence contributes 1% - 10% of the brain AChE. The asymmetric forms can be extracted in a high salt buffer or by collagenase treatment. [Brimijoin S., 1983].

- Inorder to monitor the isozyme pattern of AChE in the developing brain of lithium treated and control (non–lithium treated) chick embryo, different protocols for electrophoresis and localization of AChE activity were carried out in our laboratory. The staining procedure suggested by protocol [1], failed to yield any observable bands. On the other hand, dark brown bands of AChE were observed when electrophoresis was performed by protocol [2]. Inorder to get the best observable resolution, a combination of the two protocols was implemented. Using protocol [3], The isozyme pattern of AChE was monitored in the brain of 7-day-old lithium treated and control (non–lithium treated) chick embryos, to check the effect of lithium on this developmentally important enzyme. An alteration in the AChE isozyme pattern of lithium treated 7-day old chick embryo was observed. We report 3 bands (R_{V1}=1cm (G_4), R_{V2}=3cm (G_2), R_{V3} =5.4cm (G_1); where Rv = distance of band from the well) representing the isoforms of AChE in normal embryonic 7-day-old chick brain. This result is in accordance with Wilson W.B et al [1969] who have also reported 3 AChE bands in 20-day old embryonic chick brain. E1 appears to be the membrane bound enzyme (G_4), which is solubilized by triton and is reflected as increased proportion in triton containing assay. Lithium appears to increase the proportion of this isoform E1 (Rv=2.1). Since lithium is known to modulate cytoskeletal elements, the preliminary data suggests that this increase in relative proportion of E1 is because lithium inhibits membrane binding of this isoform. The change in isoform pattern is probably because of poor anchoring of E1 in the cell membrane and needs to be further investigated.

Hence in the present study

1. The specific activity of AChE in the chick brain increases significantly with age.
2. Specific activity of AChE in the centrifuged and non-centrifuged fractions of the 7–day-old embryonic chick brain shows statistically insignificant difference.
3. Specific Activity of AChE in the cortex and whole brain fractions of the 7-day-old embryonic chick shows statistically insignificant difference.
4. Specific Activity in control and lithium treated 7-day-old chick embryos showed a dose-dependant statistically significant reduction.
5. Activity of AChE was inhibited completely with specific inhibitor BW284C51.
6. The isozyme pattern of AChE was altered in lithium treated 7-day-old chick embryo.

E) Future Direction: -

- To investigate the AChE activity and isozyme profile in Lithium-treated and non-lithium treated controls of 7-day-old embryonic chick brain, in order to confirm the above-obtained results.

- To investigate the AChE activity and isozyme profile in Lithium-treated and non-lithium treated controls of 14-day-old embryonic chick brain, in order to check for restoration of altered activity and isozyme profile to normal levels by compensatory mechanisms.

- A further expansion of this project would include investigation of AChE activity and isozyme profile in embryonic chick brain, subjected to Glutamate toxicity. Earlier studies in our laboratory have shown that, lithium offers neuroprotection against Glutamate excitotoxicity as it reduces glutamate induced cytotoxicity in astrocyte culture. Hence, it has been proposed to further investigate whether Lithium rescues or suppresses chick embryo damage due to Glutamate toxicity and whether its action is reflected in restoration of disturbed AChE activity and Isozyme Profile. Such investigations would not only establish AChE as a marker for Glutamate toxicity but also provide an insight into molecular mechanics of Lithium action as a neuro-protective agent. Both these aspects are of immense clinical relevance.

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XXVII ALL INDIA CELL BIOLOGY CONFERENCE

EFFECT OF LITHIUM ON ACETYLCHOLINE ESTERASE ACTIVITY, AND ISOZYME PATTERN IN DEVELOPING CHICK BRAIN.

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Acetylcholine Esterase is an enzyme, which hydrolyses acetylcholine and is used as a marker for cholinergic neural function. It is known to be involved in synaptogenesis.

While on one hand it is known to be a marker for the developing chick brain it is also implicated in neurodegenerative diseases. In vertebrates the protein is synthesized by a single gene and undergoes alternative splicing to give 6-8 isoforms. Isozyme patterns of acetylcholine esterase have been suggested to be useful prognostic markers of neuronal degeneration.

Lithium a well-known teratogen is known to induce apoptosis in the developing chick brain. Understanding the dynamics of acetylcholine esterase isoform pattern in lithium induced neural tissue damage would help elucidating the role of these isoforms in frank neurodegenerative diseases. We have therefore studied activity and isozyme pattern of acetylcholine esterase in lithium treated and control 7 day old developing chick brain and report the same.