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Enzyme Cholinesterases. A Review

[1]**Santosh Kumar ,**[2]**Madhavi Gaur and** [3]**Urmila Saluja**
[1]Former Vice Chancellor, Dr. H.S.Gour University, Sagar (M.P),
[2] Sant Hirdaram Girl's College, Hirdaram Nagar, Bhopal (M.P) and
[3] Govt.P.G. College, Sehore (M.P)

Cholinesterases:-

The enzyme, cholinesterase belongs to esterases, a subgroup of hydrolytic enzymes which are found in many tissues throughout the body. An organic ester is the result of action between an organic acid and an alcohol to yield an inorganic salt. Similarly, ester can also be formed due to action between an inorganic acid and an alcohol, for example, between phosphoric acid and alcohol. The main characteristic feature is that the group of esterases hydrolyzes choline esters at a higher rate than other forms of esters (Augustinsson, 1948, 1949,1950, 1963 and 1974). The esterases can be classified into three main groups as under: -

1. Lipases, 2. Specific esterases, 3. Nonspecific esterases. They are not synthesized in the body.

Both enzymes hydrolyse ACh to choline and acetic acid. However, butyrylcholinesterase (BuChE) hydrolyzing butyrylcholine (BuCh) more rapidly than ACh (Rosenberry, 1975, 1979; Rosenberry and Scoggin.,1984; Rosenberry et al.,2005; Parveen and Kumar., 2005). It is important to know that these two enzymes differ in distribution, function and substrate specificity.

The term serum cholinesterase is generally used with reference to a clinical test and reflects the occurrence of levels of both enzymes in blood. Importance of serum cholinesterase in health diseases has been described in detail about six decades earlier by Vorhaus and Kark (1953) and Vorhaus et al. (1950, 1951,1952). The cholinesterase of RBCs in the peripheral blood is an indication of hematopoietic activity. Generally, a low amount of cholinesterase is found in patients suffering from liver disease, malnutrition, and chronic debilitating and acute infectious diseases causing anemia (Voss and Sachsse, 1970). Contrarily, a high level occurs in nephritic syndrome. Many drugs temporally depress cholinesterase activity, as do some insecticides. The tests for the activity of this enzyme in blood plasma and body tissues may be useful in detecting over exposure to these agents. In fish, Km of AChE increases in injured myocardium (Gaur and Kumar, 1993).

The enzyme butyrylcholinesterase is studied by pharmacologists because it is responsible for the hydrolysis of succinylcholine, a drug used in surgery as a short-acting blocker of the acetylcholine receptor. Some patients experience prolonged apnea due to slow hydrolysis of succinylcholine which can be related to a genetic variation of the enzyme (Kalow and Davies, 1958; Silman and Futerman, 1987; Grippo and Heath, 2003).

AChE is found in red blood cells, cholinergic fibers and muscle (motor- end-plates). Comparison of AChE and BChE shows extensive similarities in protein sequences and in molecular forms. Cholinesterases replaces each other during development, suggesting a complementarily roles for AChE and BChE (Chatonnet and Lockridget, 1989).

The main function of AChE is to terminate the action of acetylcholine (ACh) a neurotransmitter at the post-synaptic membrane at the neuromuscular junction into choline and acetic acid, while butyrylcholinesterase acts on synthetic substrate as the butyrylcholine, does not occur naturally in the body. It also acts on additional ester, such as procaine, succinylcholine and propanidid.

Structure of AChE:-

The structure and mechanism of action of AChE have been elucidated through Crystallography (Sussman *et al.,* 1991 & 1993). AChE is serine proteases. It has a gorge about 20Å deep long which penetrates half way through the enzyme (Fig.1).

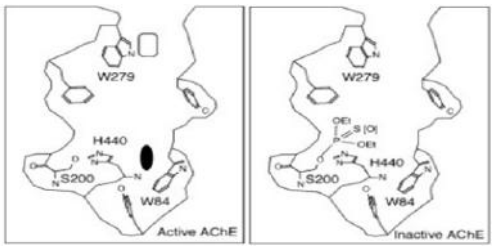

Fig. 1. Structure of AChE showing active and inactive sites.

It is lined by 14 aromatic amino acids which are highly conserved across different species. Among the aromatic amino acids, tryptophan 84 is critical and its substitution with alanine results in a 3000-fold decrease in reactivity (Touge, 2001). The active site is located 4 Å from the bottom of the molecule (Harel *et al.,* 1993, 1996). The esteric subsite, where acetylcholine is hydrolysed to acetate and choline, contains the catalytic triad of three amino acids: serine (s) 203, histidine (H) 447 and glutamate (E) 327 (Vellom *et al.,* 1993). These three amino acids are similar to triad in other serine proteases except that the glutamate (E) at third number is replaced by aspartate (D). The hydrolysis reaction of the carboxyl ester leads to the formation of an acyl-enzyme and free choline.

Then, the acyl-enzyme undergoes nucleophilic attack by a water molecule, assisted by histidine 447 group, liberating acetic acid and regenerating the free enzyme. For example, Phenylalanine 295 and Phenylalanine 297 form that largely dictates "aceyl pocket" a group specificity of a substrate and glycine 121 and glycine 122 contribute to an oxyanion the hole that interact with substrate at carbonyl group (Ordetlich *et al*., 1996 , 1998 a,b).

AChE is a glycoprotein and has six main forms designated as G_1, G_2, G_4, A_4, A_8 and A_{12} (Anglister and Silman, 1978; Brimijoin, 1983, Kerkut, 1984). A 7th form of AChE has also been reported at the nerve muscle junction. Unlike the other forms, it is not extractable. The monomer of AChE can be converted into a dimer via the S-S single chain disulphide bond. A dimer can form a tetramer. The tetramer can join to a three-stranded tail with each strand connected to the tetramer via the S-S bond (Kumar *et al*., 1993). The tail helps to attach the enzyme to membranes at synapses and at the neuromuscular junction. A_4 has one tetramer attached to the tail, A_8 two tetramers and A_{12} three (Fig. 2).

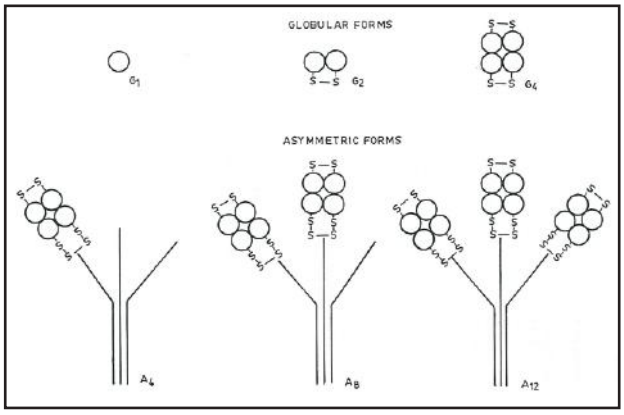

Fig.2. Quaternary structure of the six main forms of acetylcholinesterase, Globular forms are represented by G and asymmetrical form by A (Brimijoin,1983)

The sedimentation coefficient of some forms of AChE has been investigated. They are as follows:

1. G_1-4s

2. G_4-10s

3. A_{12}-16s

AChE has an optimum pH of 8.6; the stability of the enzyme at room temperature is about 7 days, under refrigeration about 14 days and in a deep freeze about 3 months.

The membrane-bound globular AChE forms have hydrophobic domains that anchor them in the membrane made up of phospholipid bi-layers. Two different amphiphilic forms, G2 and G4, are attached to two different hydrophobic anchors (*Chatonnet* and Lockridget, 1989). AChE has been found in mammalian erythrocytes (Rosenberry,1975), platelets and sheep basal ganglia (Majumdar and Balasubramanian, 1982), *Drosophila* head (Fournier *et al*.,1987, 1988; Toutant *et al.,* 1988) and *Torpedo* electric organ (Silman, & Futerman., 1987;, Bon & Massoulie, 1976).

The AChE acts on the neurotransmitter, acetylcholine. ACh plays role in the propagation of action potential. This potential is transferred across the synapse by the release of acetylcholine. AChE prevents excessive accumulation of ACh at the cholinergic synapse and at the neuromuscular junction. Cholinergic presynaptic terminals release the neurotransmitter acetylcholine (ACh), which is to be hydrolysed to choline and acetate by the enzyme acetylcholinesterase (AChE) present at the surface of the post synaptic membrane. The choline is reabsorbed by the presynaptic endings, where it combines with acetyl CoA to form a new molecule of ACh.

Active site of AChE.

The active site of AChE consists of two sub-sites (Fig.3); a negatively charged, anionic site, to which the positively charge quaternary nitrogen moiety bind and another is estertic sub-site containing the actual catalytic residues containing triad of Asp-His-Ser (Nachmansohn and Wilson, 1951). The estertic site is weak and undergoes spontaneous hydrolysis during recovery stage of the enzyme regenerating the serine hydroxyl and thus the active form of the enzyme. The nucleophile is assumed to be serine residue, while a histidine residue enhancing its nucleophilicity (Cunninghan, 1957).

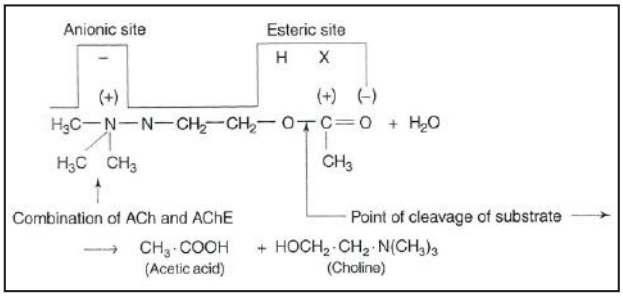

Fig.3. Reaction between ACh and AChE resulting in the formation of acetic acid and choline. The figure. also shows anionic and esteric site of the enzyme.

Inhibitors of AChE and its importance in Medicine.

Eserine (physostigmine) is a natural carbonate isolated and purified from the Calabar beans and is considered as best inhibitor for AChE. Other AChE groups inhibitors are BW284C51(1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide and paraoxon. Three step reaction mechanisms for the inhibition of AChE by serine have been investigated for many years and complete kinetic characterization of the interaction has been done by Golicnikm and Stojan (2005). On the other hand, butrylcholinesterases is inhibited by quinidine, ethapropazine and iso-OMPA (tetraisopro pylpyrosphor-amide).

Inhibition of AChE leads to accumulation of ACh in the synaptic cleft and results in impeded neurotransmitter (Kumar *et al.*,1999, Kumar and Gupta., 2003, Gupta *et al.,* 2011). There are two types of inhibition, irreversible and reversible.

Irreversible inhibitors may lead to accumulation of ACh at the neuromuscular junction causing paralysis of muscle, convulsion, bronchial constriction, and death by asphyxiation. Excessive accumulation of ACh causes excessive mitosis, sweating and more secretion in upper respiratory tract, discomfort in chest, nausea, vomiting, diarrhea, headache, giddiness and fasciculation (Sharma, 1993) in mammals. Irreversible AChE inhibitors have been used in insecticides. malathion/ dimethoate/ chlorpyrifos) and nerve gas gases for chemical war fare. Some carbonates particularly, esters of N-methyl carbamic acid are AChE inhibitors that hydrolyze in hours and have been used for medical purposes. Physostigmine is used for the treatment of glaucoma. Rivastigmine is used to treat Alzheimer and Lewy body dementia and pyridostigmine bromide is used to treat Myasthenia gravis.

Methods for Quantitative Estimation of AChE Activity

To measure the quantity of the enzyme in a sample of tissue extract or other biological fluid, the rate of the reaction catalyzed by the enzyme in the sample is measured under appropriate conditions; the measured rate is proportionate to the quantity of enzyme present. Enzyme units are best expressed in micromoles (µmol, 10^{-6}mol), nano moles (nmol; 10^{-9}mol) or picomole (picomol; 10^{-12}mol) of substrate reacting or product produced per minute.

For the biochemical estimation of AChE two methods are used extensively, proposed by Hestrin (1949) and modified by Metcalf (1951) and the other by Ellman *et al*. (1961).

1.Hestrin (1949) technique is a sensitive chemical method for determination of unreacted ACh. Metcalf (1951) used a principle similar to that of Hestrin with slight modification for its determination. In both methods AChI is used as substrate and the tissue homogenate was prepared in sucrose (0.25M) solution. The principle is that the ester reacts with hydroxylamine to form acethydroxamic acid. The hydroxamic acid forms a soluble red purple complex with ferric ions in acid solution. Absorbance of red purple complex is measured at 540 nm in a spectrophotometer. The intensity of color is proportional to the concentration of ACh present.

2. Ellman *et al.* (1961) developed an extremely sensitive spectrophotometric method for the determination of low concentration of AChE in tissue extract, homogenate cell suspension. It has been widely used by investigators as a rapid colorimetric determination of AChE. The principle involves measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. The released thiocholine upon reacting with dithiobis - nitro benzoate (DTNB) produces a yellow color.

The rate of color production is measured at 412 nm by spectrophotometer. The reaction with thiol is sufficiently rapid and not rate limiting in enzyme measurement. Moreover, its concentration does not inhibit the reaction. Modifications of the Ellman method have been proposed by numerous investigators. All investigators used ATChI and DTNB, however, modifications constitute changes in concentration of DTNB, incubation time etc.

Alzheimer's disease and cholinergic neurons:-

Alzheimer's disease (AD) is a progressive irreversible neurodegenerative disorder on account of the death or atrophy of the basal cholinergic neurons present in the basal forebrain, neocortex and hippocampus, amygdale and basal nucleus in the brain resulting loss of cholinergic function (Geula & Mesulam,1994; Dolezal and Kasparova, 2003). The “amyloid hypothesis” is also been accepted as a cause of AD. The amyloid hypothesis is a pathological model. According to this theory, the overproduction and accumulation of amyloid-beta (Aβ) peptides present in the neurotic plagues along with the changes due to hyperphosphorylation and intracellular aggregation of microtubules associated protein Tau to form neurofibrillary tangle as downstream phenomenon (Rosenberry *et al.,*2005 and Aprahamia *et al* ., 2013). In fact, the only treatment of AD systems has been the use of the cholinesterase inhibitors,

Alzheimer' s disease is classified either sporadic or familial**.** Sporadic Alzheimer's disease can affect adults at any age, but commonly occurs after 40- 65 years while Familial Alzheimer's disease is a genetic disorder caused by a mutation in one of the several genes. In 1906 it was designated as Alois Alzheimer.

More than three million people suffer with disease in USA (Hebert *et al*., 2003). The features of this disease are with senile dementia, cognitive dysfunction, altered behavior and progressive decline of language function and cardinal failure (Selkloe, 2001., Marin *et al*., 1997). Epidemiological studies conducted in India between 1996 and 2006 showed that dementia affects 2.7% of the population (Aprahamia *et al* ., 2013). In fact, the only consistent treatment for AD symptoms has been the use of cholinesterase inhibitors (Inestrosa and Colomres., 2005).

Varez and Inestrosa (2005) described the role of acetycholinesterase in Alzheimers disease with reference to its molecular interactions with amyloid peptide. According to them, the main pathological feature is the formation and accumulation of amyloid formation resulting mental failure in humans. Amyloidal accumulation has been found in brain tissue of senile AD patients in as diffused deposits as plaques consisting of 40-42 residues of amyloid- ß. There are also anomalies in the generation/handling of Aß peptides. It is also suggested that its assembly in fibrils or protofibril initiate a series of downstream neurotoxin events. Keeping this point into consideration a few investigators studied the involvement

of Aß monomers in AD. The Aß-AChE complexes exhibit higher neurotoxicity than Aß fibril alone (Varez and Inestrosa., 2005). Most cases of AD are idiopathic (disease whose cause is unknown) and inherited used $_{\epsilon}4$ allele of apolipoprotein E gene (ApoE) is considered the main risk factor. In small % of the cases it is reported that is inherited due to Mendelian autosomal dominant gene.

Heart and its innervation of vertebrates

The heart of vertebrates is richly innervated and influenced by both parasympathetic and sympathetic nerve fibers. In homeothermal vertebrates the sympathetic fibers and parasympathetic reach the heart separately. In fishes and amphibians, the heart is innervated by a pair of cardiac branch of vagosympathetic trunk (Nicol,1952; Bhatnagar and Kumar,1969, 1973), except in myxinoid heart, which receive no extrinsic innervations (Davies and Francis,1941.Fange,1972;Fange *et al.*, 1963, Randall,1970, Gupta *et al.*, 2011).

For many years the influenced of the right and left sympathetic cardiac nerves was considered to be accelerator (chronotropic) in effect that is, having a primary effect of increasing the heart rate when stimulated (Shipley and Gregg, 1945). However, many investigators noticed another response, the augmenter (inotropic), which is manifested primarily by releasing catecholamine. Although, primary manifestation of augmenter influences on myocardial contractility and force the contraction of cardiac contraction, it has many hemodynamic and myocardial implications too.

In all vertebrates, the heart is profusely innervated by cholinergic, adrenergic and the post ganglionic nerve fibers of intracardiac ganglia. After penetrating the heart the nerve fibers divide and re-divide to form neuromuscular contact in the myocardial muscles and in specialized tissue (Burnstocks & Robinson, 1967 Bhatnagar & Nair 1973). The total density of nerves has been studied by silver impregnation method (Fig.4) while the cholinergic and adrenergic components were studied with the help of acetylcholinesterase histochemistry and fluorescence histochemistry respectively.

Mapping of cholinergic innervations has also been done by immunohistochemistry for high-affinity choline transporter (CHT), which is a new antibody to the human and high-affinity for choline transporter present in cholinergic nerves. AChE positive nerves are more in the atrium than in the ventricle. They are present in the sinuatrial node, atrioventricular node, and in Bundle of His and its branches (Hoover *et al*.,2003) . The cardiac conducting system is also well developed in *Columba livia* due to flying habit of the animal**.**

Central and peripheral neurons, including intrinsic cardiac neurons located on the surface of the mammalian heart express both BuChE and AChE activity. On the basis of degenerative studies, two different populations of ganglion cells can be identified as argentophilic and argentophobic.

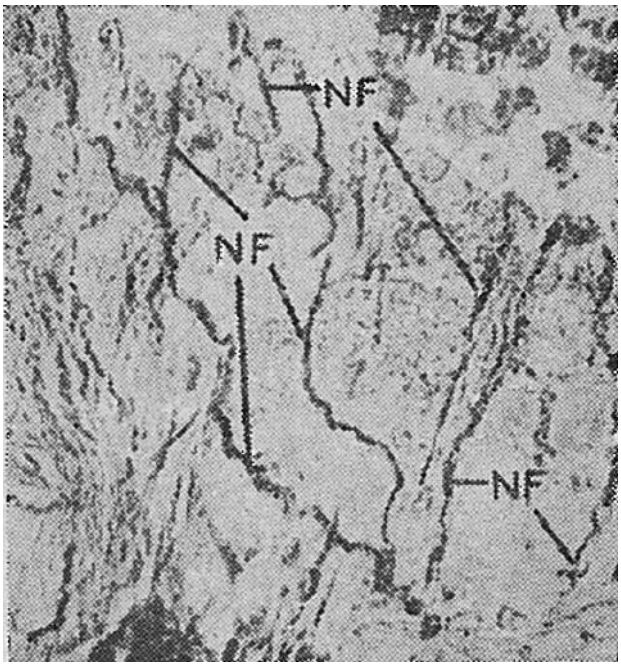

Fig.4 Photomicrograph of the heart showing nerve plexus in the atrium. Transfer section, 480 NF, nerve fibre. (Kumar 1975)

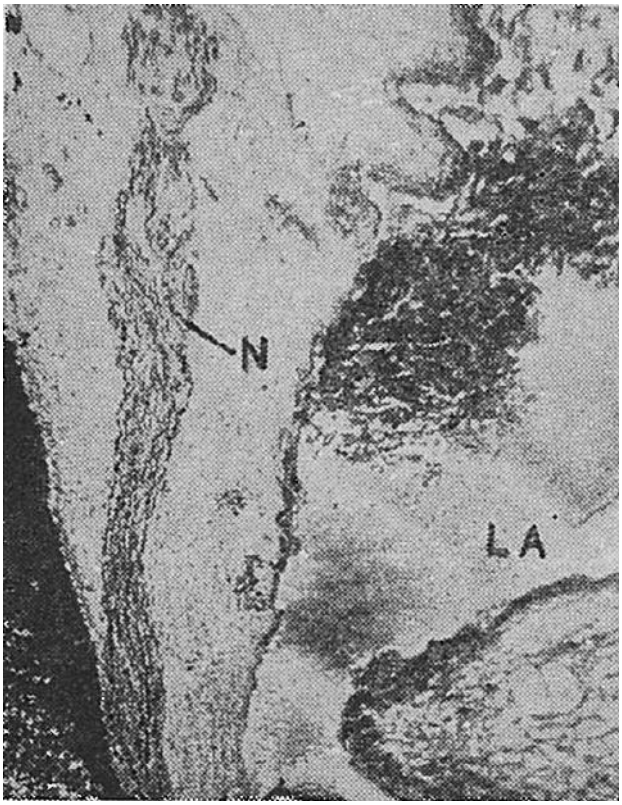

Fig.5 Photomicrograph of the heart showing a large nerve present in the myocardium. Transfer section x 320. LA, left atrium and N, nerve. (Kumar, 1974)

The former takes a darker stain while the latter takes a light color. Argentophilic types of ganglion cells are sympathetic (Corzo,1956). The neurons are also classified as cholinergic and adrenergic ganglion cells. The multicellular ganglion cells are observed in atria, ventricle and in cells at the atrioventricular sulcus has been noticed, which is innervating the AVN, AVB and in Purkinje Fibers in higher vertebrates (Fig.5&6). In the early stages of development the nerve cells are generally small, round or oval with a faintly granular cytoplasm (Prakash.,1953, 1954., Prakash and Kumar,1981).

In amphibian heart, Remaks ganglia is present at the sinuatrial junction and two Bidders ganglia which are situated at the caudal end of interatrial septum at the atrioventricular junction (Abraham,1969; Kumar and Tembhre,1996, Kumar & Bhatnagar 1969, Kumar and Jain 2003) Neurophysiological studies showed that acetylcholine and butyrylcholine increase or decrease the spontaneous activity of the intrinsic cardiac neurons. About 43,000 intrinsic neurons in adult heart, while in young heart of fetuses, neonates and children contains approximately 94,000 neurons ((Pauza *et al.,*2002).

The different types of nerve endings have been described in the cardiac tissue by Miller and Kasahara (1964) and Kumar (1971 and 1976), Kumar & Singh 1990, Kumar *et al.* 2003. The encapsulated endings are situated in the deeper tissue and vary in complexity of structures from relatively simple branches to multi-branched (Fig. 7).

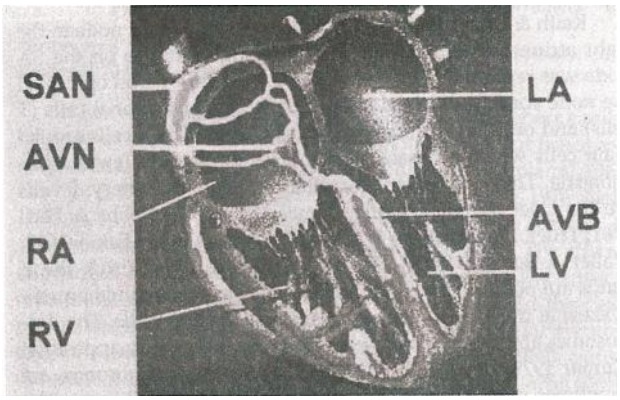

Fig.6 Diagrammatic sketch of the heart showing various components of the conducting system/ connecting system in the heart.

AVB, Atrioventricular bundle.

AVN, Atrioventricular node

LA, Left atrium

LV, Left ventricle

RA, Right atrium

RV, Right ventricle

SAN, Sinuatrial node.

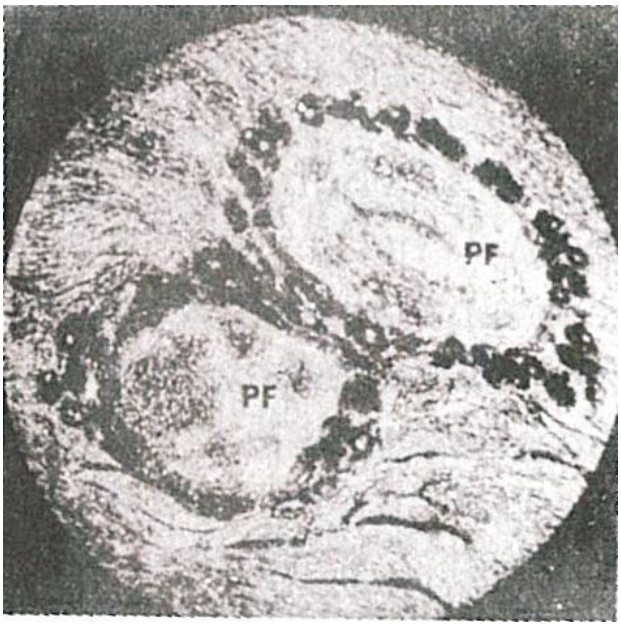

Fig.7 Microphotograph of the heart showing Purkinje fibres in the myocardium of ventricle in mammalian heart. Transfer section x 400.
PF, Purkinje fibres.

Distribution of cholinesterase in the heart muscles of camel has been described in detail by Fatani *et al*. (1987). They found numerous cholinesterase positive nerve fibers in the pericardium, atria and both ventricles. They reported that the density of cholinergic nerves was more in atria than in the ventricle. In conformity to the findings of earlier investigators they also noticed fine cholinergic nerve fibers in the components of cardiac conducting system (Field,1951, Bhatnagar,1959;Bhatnager and Qayyum, 1972; Copenhaver and Truex, 1952; Qayyum, ,1969 a,b, & 1970, 1972; Robbs,1956; James,1964; Yousuf, 1965).

Kinetic constants (Km & Vmax) of Enzyme (AChE) in cardiac tissue

Dimethoate/incision/MI.

The percentage of inhibition of AChE is depended on the quality and quantity of pesticides (organochlorine, organophosphate and carbamate). Pesticide do effect on the inhibition of AChE, 30 to 90 mg/kg dose of dichorovos causes 55.6-88.9 % inhibition along with changes in ECG (Q wave) and heart rate (Naidu *et al.,* 1987 and Praveen and Kumar ,1994).

There is an increase of Km value, in the artificially produced MI and in MI patient serum. It is suggested that these parameters are important in the diagnosis as well as for predicting prognosis in case of MI in addition to tissue specific CK (MB) or troponin - t (Gaur *et al*.,1999).

The kinetic constants, Km and V max, for AChE were calculated by the double reciprocal method of Lineweaver and Burk [1934] in our laboratory, other method such as

Eadie and Hofstee graph has also been studied. LC_{50} was calculated according to the Dixon and Webb [1964] plot using 1/v vs.1/s with saturate substrate concentration. Ki was calculated by Dixon's plot. The basic theory is that if enzyme, AChE is inhibited, the ACh (substrate/ neurtransmitter) will be accommodated at the neuromuscular junction because ACh could not be hydrolyzed into choline and acetic acid due to inhibition of AChE as stated earlier. Presence of AChE in heart muscles is unanimously accepted on account of the occurrence of profusely cholinergic innervation (Abraham,1969 and Kumar, 1971,1973,1975,1976 &1978).

The toxic effect of pesticides has been less reported on cardiac muscles of vertebrates. Moreover, the % of AChE inhibition and quantity of ACh in the normal myocardium and infarction cardiac muscle has not been thoroughly investigated in vertebrates including man. Regarding the contents of ACh, AChE and inhibitory constants (Km/Vmax and Ki) of the heart of vertebrates on the basis of existing literature has been reviewed as under.

ACh contents:-

Pesticides and injured myocardium cause an accumulation of ACh due to inhibition of AChE. During chronic exposure of 1 to 3 mg/kg dose of Dichloro dimethyl vinyl phosphate (DDVP) ACh increases in heart muscles. The ACh contents in control rats heart was 70.05 µmoles/gm weight of tissue. With 1 mg/kg it was elevated to 99.6µm and with 3mg/kg it becomes 105.21µm (Praveen & Kumar, 2001).

The ACh contents in normal cardiac tissue of *Clarias batrichus* (Fish) was 14.49 µM and was raised to 25.00 µM in ischemic cardiac tissue. ACh contents in normal cardiac tissue of *Rana tigrina* (Amphibian) were 23.57µM and were increased to 36.45µM in ischemic cardiac tissue. Similar raised % was noticed in the heart tissues of *Calotes versicolor* (Reptilies), *Colomba livia* (Bird) and *Rattus norvegicus* (Mammal). The ACh contents were also increased in the serum of *Rana tigrina*, *Columba livia* and *Rattus norvegicus.* The ACh in normal human serum were 2.00 µM while in MI patients' serum, it was 3.75 µM (Gaur & Kumar,1993, 2003, a, b & Gaur *et al*,1999). The dose of drug, duration of exposure, and their comparison with human serum were given in (Table1 & 2).

Table-1. Acetylcholine content (ACh) (µ moles/g wet weight tissue) of heart of vertebrate series of normal and exposed to isoproterenol hydrochloride for 48 hrs. Each value is the mean ± S.D. of five individual observations.

Experimental animals	Control/Isoproterenol hydrochloride treated	48 hours exposure	
		ACh content	% Increase
Clarias batrachus	Control	14.49 ± 1.54	-
	85 mg/kg body wt.	25.00 ± 1.87	72.53
Rana tigrina	Control	23.57 ± 1.52	-
	85 mg/kg body wt.	36.45 ± 1.72	54.64
Calotes versicolor	Control	56.84 ± 1.74	-
	85 mg/kg body wt.	69.46 ± 1.81	22.20
Columba livia	Control	2.66 ± 0.264	-
	85 mg/kg body wt.	3.42 ± 0.988	28.57
Rattus norvegicus	Control	4.50 ± 0.787	-
	85 mg/kg body wt.	6.50 ± 1.37	44.44

Table-2. Acetylcholine content (ACh) (µ moles/ml serum) of *Rana tigrina*, *Columba livia, Rattus norvegicus* of normal and exposed to isoproterenol hydrochloride for 48 hrs and compared it with the human serum of myocardial infarction patients. Each value is the mean ± S.D. of five individual observations.

Experimental animals	Control/Isoproterenol hydrochloride treated	48 hours exposure	
		ACh content	% Increase
Rana tigrina	Control	7.50 ± 1.536	-
	85 mg/kg body wt.	12.16 ± 1.841	62.13
Columba livia	Control	3.00 ± 0.714	-
	85 mg/kg body wt.	6.50 ± 0.888	116.6
Rattus norvegicus	Control	2.75 ± 1.070	-
	85 mg/kg body wt.	5.65 ± 0.892	105.4
Homo sapiens (Human)	Control	2.00 ± 0.547	-
	Myocardial infarction patients	3.75 ± 0.836	87.5

Constants of inhibition(Km,Vmax,Ki):

The enzyme kinetics (Km and Vmax) of AChE have been studied in various organs of fishes on account of pesticides toxicity. They were unanimously reported depletion in AChE activity in comparision to control (Ratha and Ramanujam.,1986; Rao *et al*., (1984); Ahammad *et al*., 1980; Subburaju and Selvarajan,1988; Nemcsok *et al*., 1985; Benke and Murphy ,1974;).

Keeping this into consideration MI was artificially produced by drug, isoproterenol hydrochloride and injury to ventricular myocardium was done with giving incision. In fish, *Channa punctatus,* in artificially produced cut in the ventricular myocardium, the Km became $2.78x10^{-3}$M in comparison to normal, which is $1.87x10^{-3}$M. However, when heart was treated with 2ppm dimethoate, Km became $3.30x10^{-3}$ M. Further, when incised heart is treated with 2ppm diamethote, Km further change to $4.07x\ 0^{-3}$M (Gaur and Kumar, 1993). The slope obtained, intersected at one point on ordinate indicating competitive inhibition which is further confirmed by computed value of constant Vmax (0.74) in all experiments. (Fig.8)

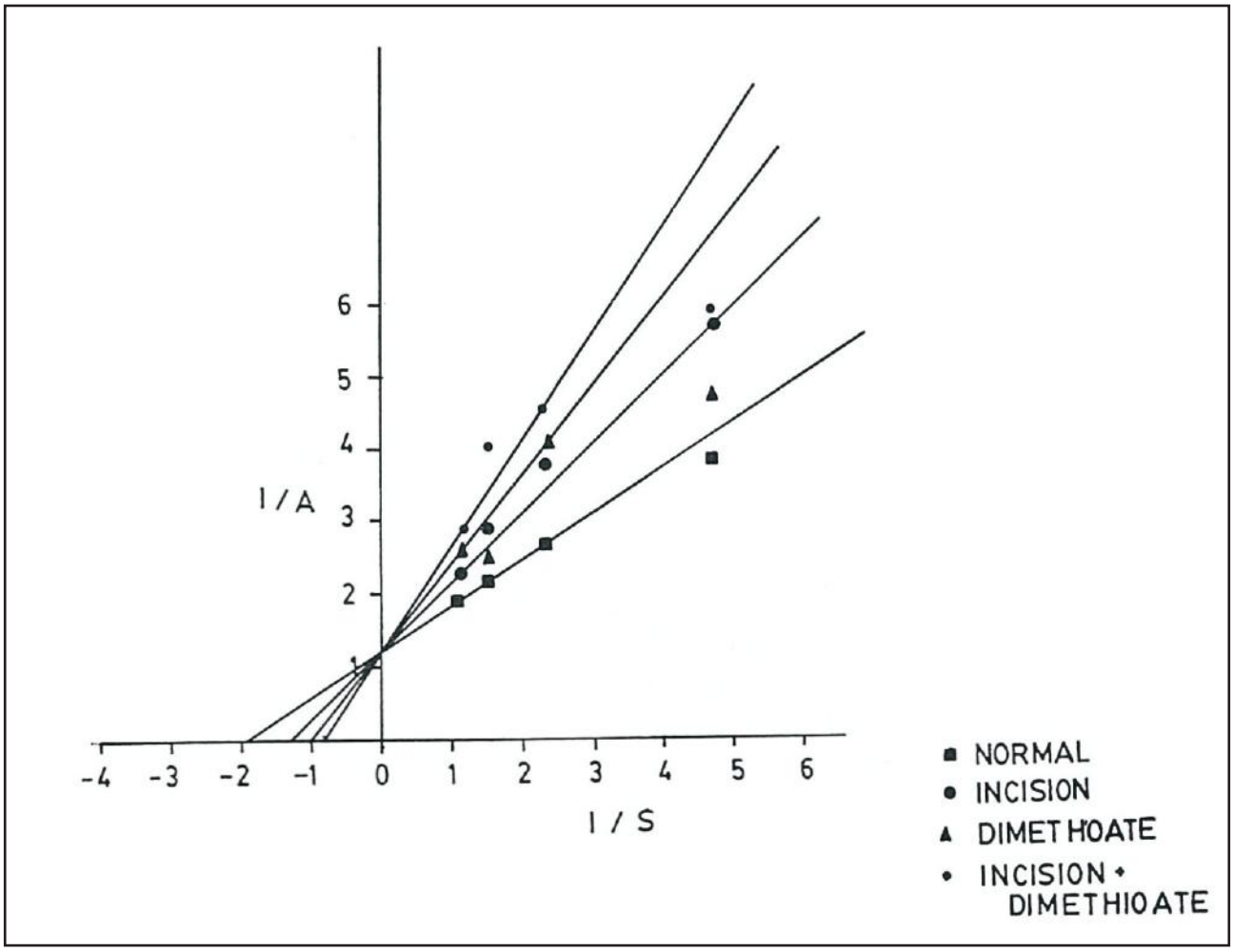

Fig.8 Lineweaver- Burk plot of inhibition of heart AChE. in incision, dimethoate and incision with dimethoate in *Channa punctatus.*

The Km of AChE in cardiac tissue of control and ISO-HCl treated *Clarias batrachus* were 3.78×10^{-3} M and $6.0x10^{-3}$M respectively. The Vmax was found to be constant *i.e.,* 1.66A/mg protein/30 minutes showing that inhibition is competitive in nature (Gaur and Singh 2004). In serum and heart tissue of *Rana tigrina* the Km value value was $2.88x10^{-3}$M and $3.66 x10^{-3}$M respectively, due to accumulation of ACh. It is also noticed that in ISO-HCl treated animals, Km value both in serum and heart tissue increased to $3.99 x10^{-3}$M and $5.1 x10^{-3}$M respectively (Fig. 9, 10)

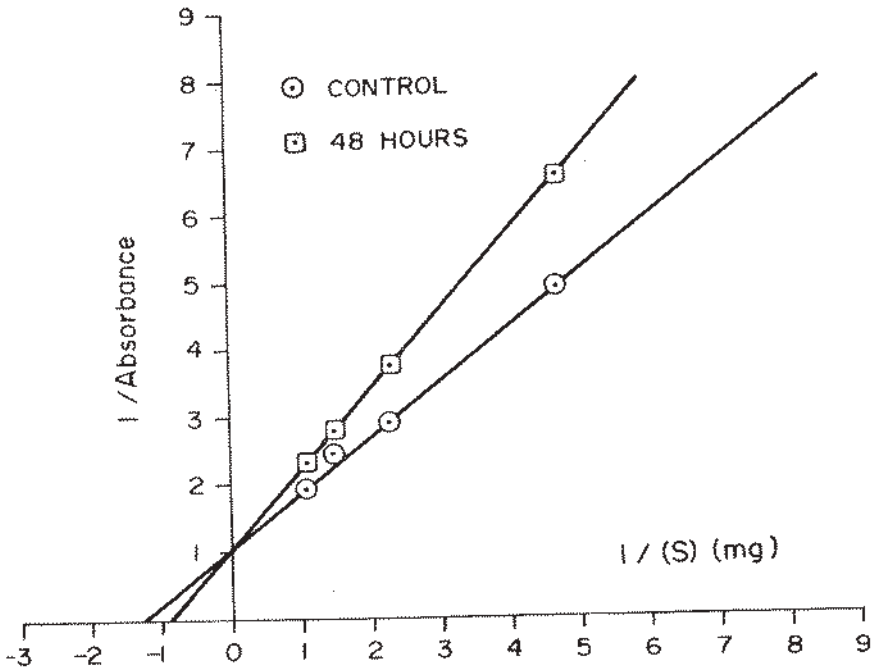

Fig.9. Lineweaver Burk plot showing inhibitory effect of ISO-HCl on AChE (*Rana tigrina* serum). S is the substrate concentration of AChl.

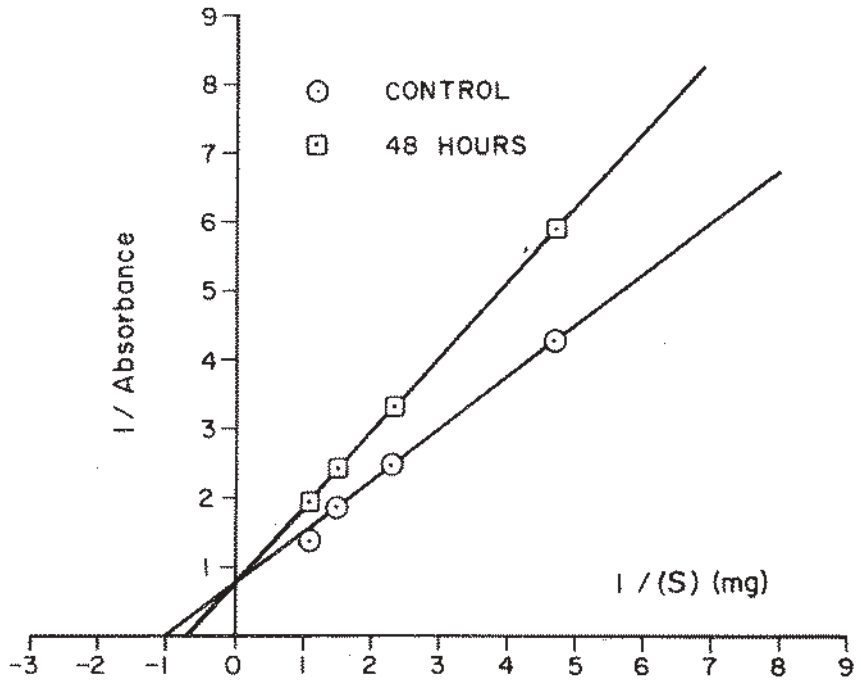

Fig.10. Lineweaver Burk plot showing inhibitory effect of ISO-HCl on AChE (*Rana tigrina* cardiac tissue). S is the substrate concentration of AChl.

In *Calotes versicolor,* MI was produced by administration of ISO-HCl, the Km (in tissue) was raised to 4,5x 10^{-3}M against control value of Km 3.1 x 10^{-3}M, the V max value remained constant i.e., 1.11A/mg protein/30 minutes.

The Km and Vmax of AChE, in serum of control and MI in *Colombia livia were* 3.78x10^{-3}M and 5.11x10^{-3}M respectively*, the Vmax value was constant i.e* 1.0 A/mg protein/30 minutes.

However, in serum of control and experimental *Rattus norvegicus* Km value was 3.2x10^{-3}M and 4.5x10^{-3}M. minutes respectively. The Vmax value was constant in both the cases i.e 2.0 A/mg protein/30 indicating that ISO-HCl has inhibitory effect on AChE.

It is interesting to note that in serum of MI patients, , Km value was also raised to 4.5 2x10^{-3}M as compared to control value 2.762x10^{-3}M. The Vmax value remain constant i.e., 1.0 A/mg protein/30 minutes (Fig 11). Thus it is concluded that the enzyme (AChE) in the heart of vertebrates t is competitive in nature.

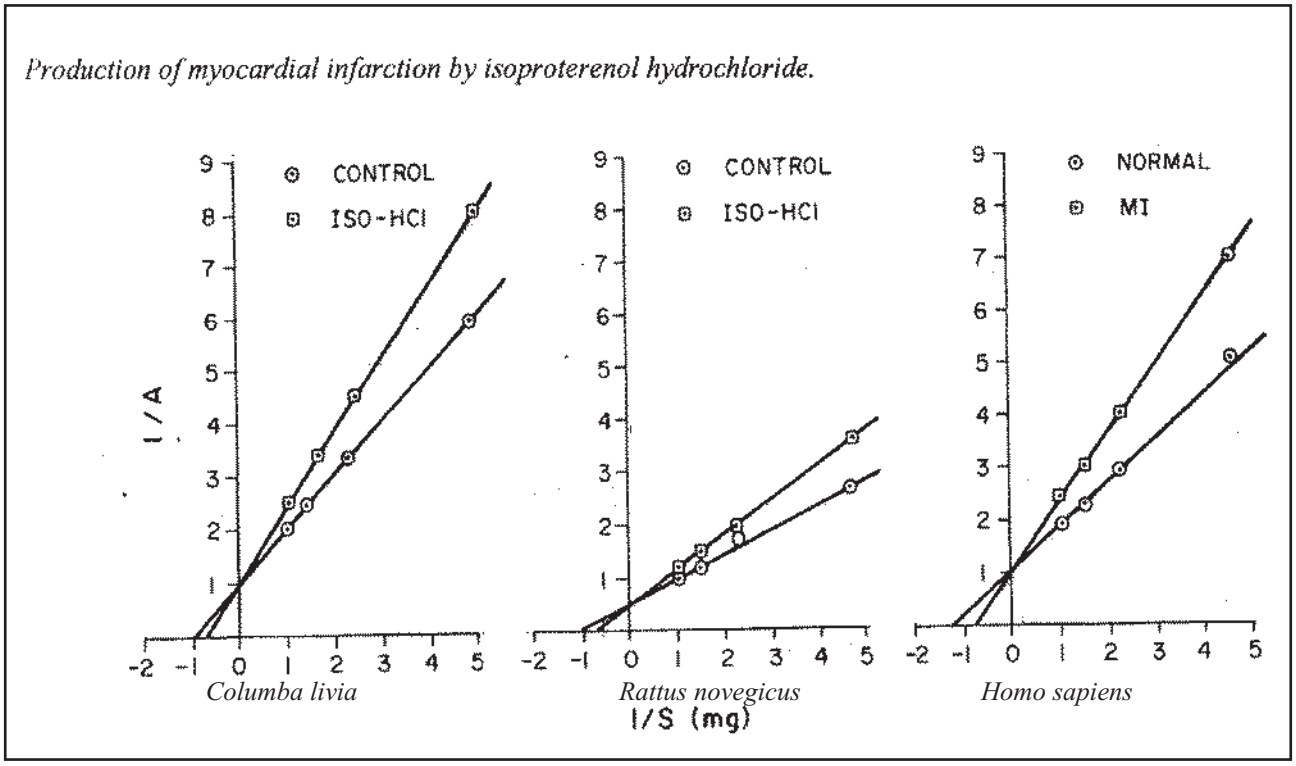

Fig.11 Lineweaver-Burk plot showing inhibitory effect of ISO- HCl on AChE in the serum. S is the substrate concenteration of AChI.

Digestive System (Inhibitory Constants):-

Inhibition, recovery and enzyme kinetics (Km and Vmax) of AChE have been studied in digestive organs of fishes and rats to know the effects of pesticides toxicity. The previous investigators were unanimously observed depletion in AChE activity in comparison to control. The inhibition % ranges between 10 to 89% (Ratha and Ramanujam.,1986; Rao *et al*., (1984); Ahammad *et al*., 1980; Subburaju and Selvarajan,1988; Nemcsok *et al*., 1985; Benke and Murphy ,1974; Parveen,1997; Parveen and Kumar, 1994, 2001,2005;`Kumar *et al*., 1995 ,1999, Gaur and Kumar,1993, 2003 a,b & Gaur *et al*,1999, , Tembhre and Kumar,1994, 1995 a,b,1997; Hassanein, 1991,2002,2005; Golicnik and Stojan,2005; Demble *et al.,* 2000, Abou-Donia and Menze,1967; Kozlovskaya *et al*.,1993; Turdean *et al*.,2000,2002, Turdean,2005).

Km of the gut AChE in fish**,** *Cyprinus carpio* subjected to various concentrations of dimethoate cause increase to 1.5 x 10^{-3}M and 2.6 x 10^{-3}M from the control which is 1.2 x 10^{-3} M with treatment of 2,3.5, and 4.5 ppm dimethoate for 96 hrs. Vmax was constant at 0.83 x 10^{-3}M (Fig.12)**.**

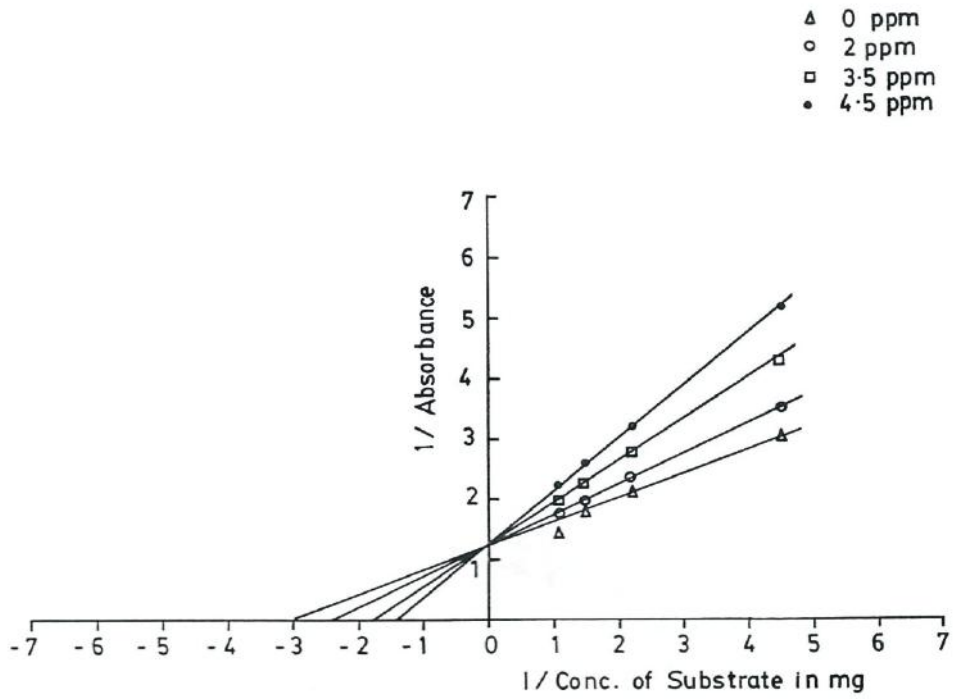

Fig.12. Lineweaver – Burk plot showing inhibition of AChE in the gut of *Cyprinus carpio* by dimethoate (pesticide), 96 hours. S is the substrate concentration of AChI. Each point is the mean of five assays (Tembhre & Kumar, 2002)

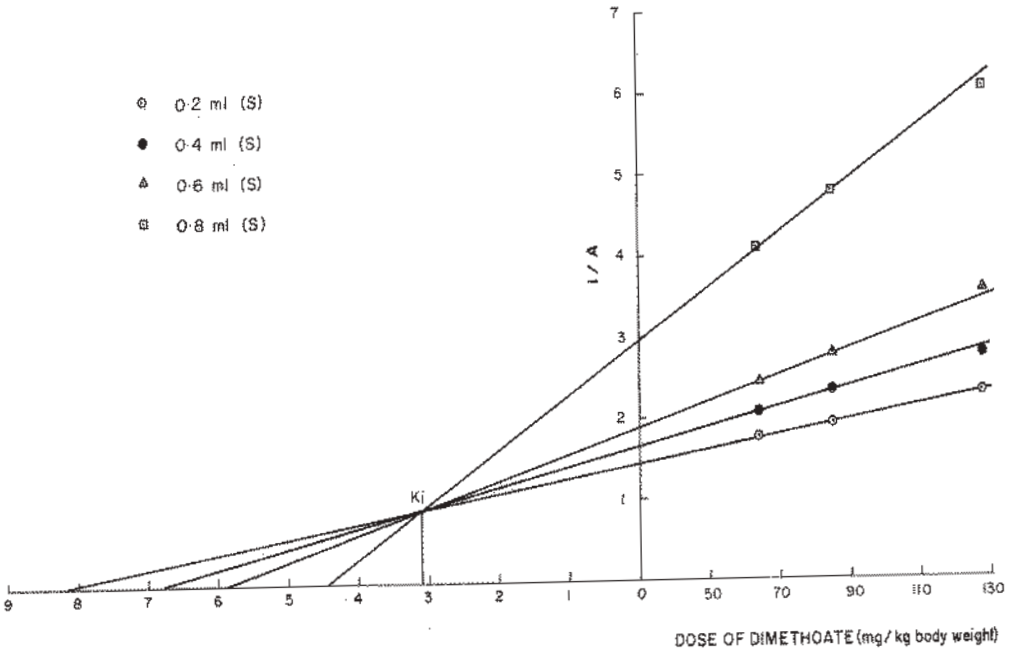

Fig. 13 Dixon plot of the inhibition of AChE of Duodenum of *Rattus norvegicus* by dimethoate for four concentration of acetylcholine iodide.

In another study, Tembhre and Kumar (1995 b) reported carp gut AChE kinetic effected by 96hrs exposure to methylamine of 2,5 and 7 ppm causes an increase trend of Km to1.57 x 10^{-3}M, 2.60 x10^{-3}M and 4.03 x 10^{-3}M against control Km of 1.06x10^{-3}M.The significance of methylamines is important as it had been the exposed at the Bhopal Gas Tragedy.

Saluja and Kumar (2004 a, b) studied the acute toxicity of dimethote on acetyl-cholinesterae activity in the oesophagus, stomach and duodenum of *Rattus norvegicus.* According to them, dimethoate significantly inhibited the acetylcholoinesterase activity in a dose dependent manner. They noticed gradual increase of Km and constant value of Vmax with every dose revealed competitive nature of inhibition both in oesophagus and stomach and duodenum which is they confirmed by Dixon's plot too. Recovery of test compounds were also worked out by cessation of treatment for 15 days after 15 days exposure (Fig.13).

Copper Sulphate (Heavy metal)

The effect of copper sulphate, a heavy metal, also shows similar behavior with regard to Km and Vmax of AChE. The long term experiments were conducted for 15 & 30 days to study the chronic toxicity of copper sulphate. Recovery of test compounds were also worked out by cessation of treatment for 15 days after 15 days exposure. In *Rattus norvegicus*, chronic toxicity of sublethal concentration of copper sulphate on the activity of AChE has raised the Km to 2.49 X10^{-3}M after 15 days and 3.04 x 10^{-3}M after 30 days from control value 1.65 x 10^{-3}M. The Vmaxwas found to be dercreased from the contol value of 1.03 A/mgprotein/30 minutes to 0.86 nd 0.7A/mg protein/30 min. after 15 and 30 days respectively. It is concluded that copper sulphate inhibit the AChE activity in a mixed way i.e., competitive and noncompetitive as reported by earlier workers. (Saluja & Kumar, 2004 a,b &2005). (Fig.14 & 15) Nemcsok *et al*. (1984) showed that 7.19 x 10^{-4} copper sulphate produce 50% inhibition of AChE in the serum of *Cyprinus carpio*. Tembhre and Kumar (1995 a) investigated acute and chronic effect of $CuSO_4$ on the gut AChE in carps. According to them the normal Km was 0.84x 10^{-3}M, which gradually increased to 1.06 x 10^{x-3}M, 1.35x10^{-3}M, 1.83 x10^{-3}M with 2,3 and 4 ppm. copper sulphate respectively. With chronic exposure for 30 days, 2ppm. copper sulphate resulted in 1.90 x 10^{-3} M Km. Vmax reduces to 0.90,0.83 and 0.76 (against 1.00) with 2,3 and 4 ppm. copper sulphate. They have also concluded that copper sulphate yielded mixed inhibition in gut AChE. The inhibition pattern was competitive non-competitive as shown by increase Km and reduced Vmax values (Fig. 14&15).

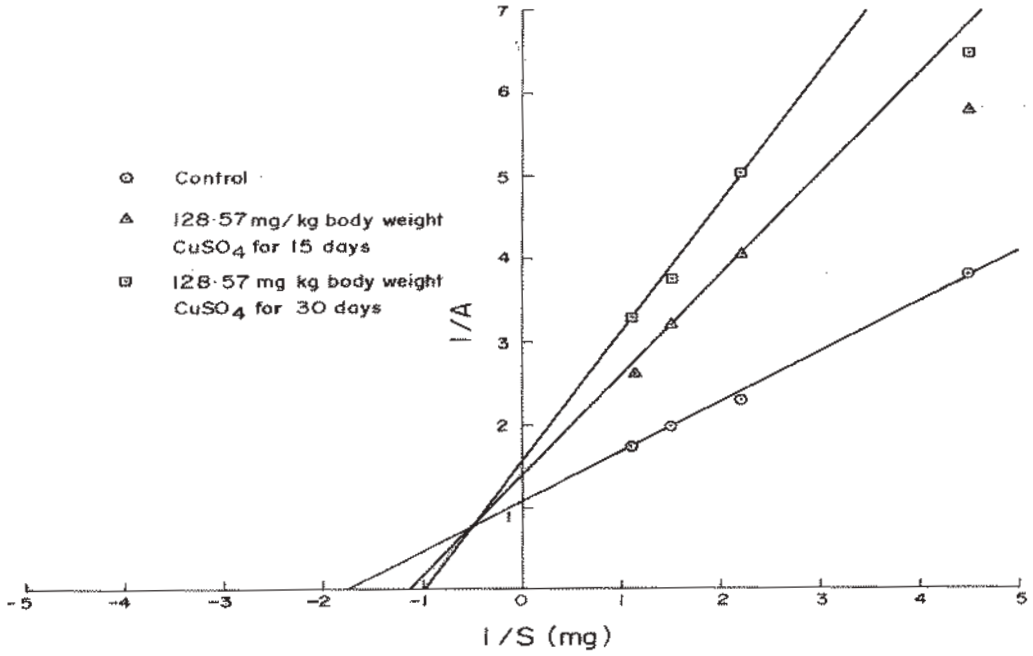

Fig. 14 Line weaver - Burk plot of the inhibition of AChE of oesophagus of *Rattus norvegicus* by copper sulphate for 15 and 30 days.

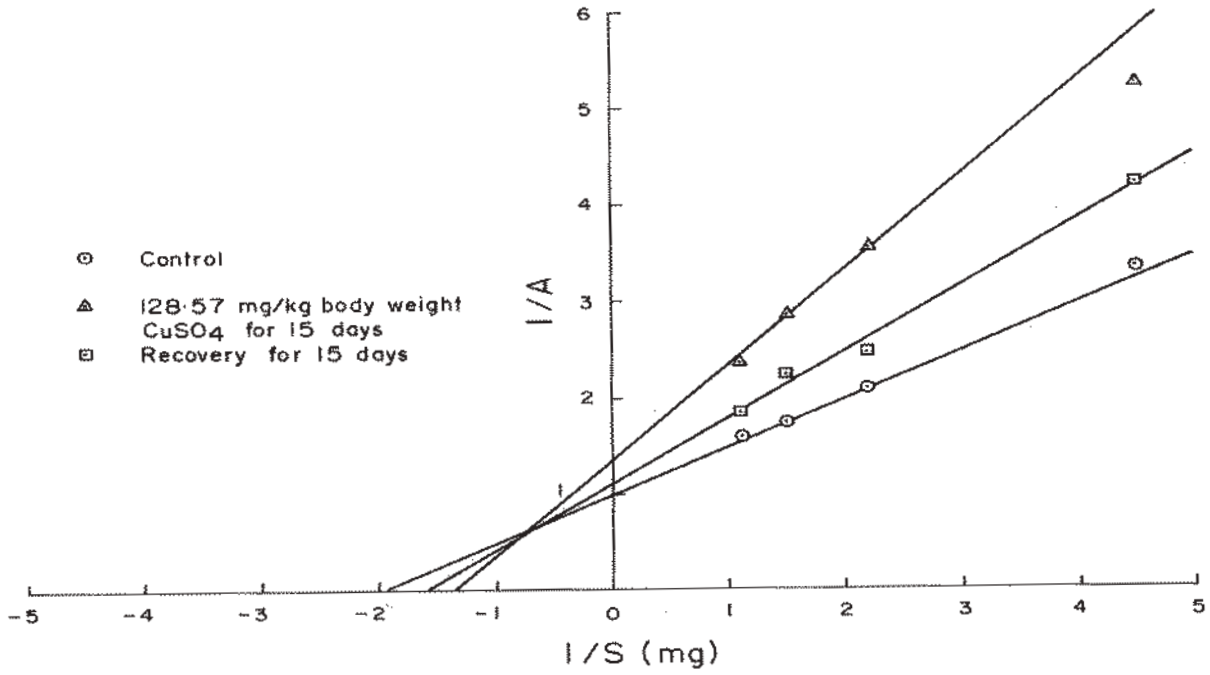

Fig. 15 Line weaver - Burk plot of the recovery of 15 days after exposer of 128.57 mg / kg body weight of copper sulphate on the duodenum AChE of *Rattus norvegicus* for 15 days.

Brain:-

The acetylcholinesterase was present in nuclei and the tracts of spinal cord and medulla oblongata of bat. Bhatnagar and Tewari (1988) observed AChE in nuclei and fiber tracts in forebrain, telencephalon and diencephalon of neonatal rat. They distinguished three groups of nuclei in accordance to intensity of AChE, nuclei which are moderately positive for AChE, another group of nuclei with intense cytoplasmic reaction which is mainly present in neuron cell bodies and thirdly the nuclei which do not show any AChE reactivity (Bhatnagar,1988 and 1993). Ependymal cell layer, caudate putman, globus pallidus, habenular nuclei and ventromedial thalamic nuclei reveal intense AChE reactivity. The fiber tracts were mostly devoid of enzyme reactivity**.**

Panday *et al*. (1989) studied butyrylcholinesterase (BChE. EC 3.1.1.8) activity in adrenal cortex and medulla in control mice and in DL amphetamine treated mice. 6mg/kg BW dose of amphetamine enhance the enzyme activity in adrenal medulla. In cortical layers, the effect was different for various layers; the enzyme activity decreased in the zona glomerulosa and in zona reticularis, but remained unaltered in zona fasciculate. They suggested that the difference in effect of amphetamine on activity of BChE activity in various parts of gland is due to neuronal and nonneuronal origin and functions of both adrenal medulla and cortex. .

A comparative analyses of distributive patterns of Nissl substance (NS) and acetylcholinesterase present in the neuron cell bodies of various hypothalamic and thalamic nuclei was studied by Bhatnagar *et al*., (1990). Study demonstrated that there is a close parallelism in distribution of NS and AChE was noticed in most of the neuron cell bodies of nuclei studied. This parallelism is important from evolutionary point of view as such parallelism was noticed in mammals alone but not reported in birds and reptiles**.**

Dietary exposure to cadmium, even at lower doses can lead to free radical induced neurotoxicity, neurobehavioural changes and alteration in neurotransmitters (ACh). Such changes are likely to be more pronounced in the developing brain due to incompleteness of the blood brain barrier.

Hippocampus has a role in learning and cognitive behavior and any damage to this structure during development may result in neurodegenerative changes in later life. Thus, fetal and neonatal exposure of cadmium was achieved by exposing pregnant dams of swiss albino mice throughout the gestation and following parturition up till 5th day post partum through drinking water (3ppm/animal/day) the neonates were sacrificed on day 6th and indices of oxidative stress, levels of trace elements and changes in cholinergic system were evaluated in the hippocampus. Increased accumulation of cadmium, differential alterations in trace elements and decreased activity of AChE were the features

observed. Simultaneous administration of melatonin to cadmium challenged animals offset these detrimental changes. The result suggest that melatonin co-adminstration can effectively protect against the adverse affects of cadmium (Bhatnagar and Panday,1988, Bhatnagar and Tiwari,1980, 1985; Mukherji *et al*., 2010)

Inhibitory effects of a fresh leaf juice of *Withania somnifera* (Ws) on the acetylcholinesterase and nicotinamide adenine dinucleotide diaphorase (NADPH-d) were studied in mice brain using histochemical and biochemical approaches. The results obtained show significant reduction in the number and reaction intensity in AChE and NADPH-d positive neurons within selected areas of the brain of treated mice. Localization of AChE and NADPH-d also showed significant reduction in the number of cells positive for both the enzymes after Ws treatment as compared to cells with isolated staining. Biochemical estimation of these enzymes in brain tissue also showed significant dose dependent inhibition of the activity of both enzymes after Ws treatment. In conclusion, our study allows us to suggest that Ws significantly inhibit both the enzymes. Inhibition of AChE is direct while NADPH-d is indirect (Bhatnagar *et al* ., 2012**).**

Hippocampus in birds is divided into fields. The pyramidal neurons are the main subtype of neuronal classes in the medial hippocampus of birds (Srivastava.,*et al* 2007)

The histologically, cerebellum of teleosts consists of three parts, the vestibulolateralis lobe, the corpus cerebelli and valvula cerebelli. The histopathological changes were evaluated in the brain tissue of Indian major carp*, Catla catla* after chronic exposure to an organopshophate pesticide, the dimethoate. The experimental group was exposed to sublethal dose of dimethoate (0.001 ppm) for four weeks. For the study of histology of nerve tissue silver impregnation technique was employed. Nerve cells showed necrosis, vacuolation, loss of cytoplasm and Nissl's bodies. The nerve bundles become loose and nerve fibrils dispersed in all parts of the brain. The severe damage was noticed in the optic tectum but less damage was noticed in the organelles of the Purkinje cells and also in the granular layer of cerebellum. The necrosis was present in the axons of Mauthner cells as well as in medullary region.

Dimethoate even in 0.001concentraion is highly toxic to brain of the carp fingerlings and may lead to subsequent killing of fish (Meetheissen & R,1982; Singh and Kumar, 2013).

AChE showed mild or no AChE activity in Purkinje cells (Clemente *et al.,*2004*)*.

The corpus cerebelli shows that the outermost thick molecular layer (ML) showed fainted activity for AChE. The inner granular layer (GL) also demonstrated intense reaction to AChE. the Purkinje cells (PC) present in the intermediate layer exhibit strong cholinesterase positive The cell bodies exhibit deep strong reaction while the inner granular layer (GL) also showed intense reaction for AChE (Contestebile & Zanoni, 1975,Contestebile *et al.*, 1977, 2004; Ekstrom, 1987; Brantley and Bass, 1988; Adrio *et al*., 2000; Anadon *et al*., 2000; Perez *et al*., 2000 and Rehman *et al*., 2013;Singh and Kumar,2013). Satyadevan *et al.* (1993) and Kumar *et al* (1995) investigated dimethoate as a competitive inhibitor of Brain AChE in *Cyprinus carpio.* The Km in absence of dimethoate wass 0.62×10^{-3}M. However, in the presence of 2,3.5 and 4.5 ppm dimethoate, the Km raised to 0.73×10^{-3}M, 1.1×10^{-3}M and 2.0×10^{-3}M and respectively. The Vmax was found to be constant *i.e.*, 1.5 A/mg protein/30 min.

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