Chapter 4

Discussion
DISCUSSION

4.1 Characterization of Acetylcholine - sterases

Anticholinesterase compounds, including organophosphates and organochlorine enjoy a broad commercial market both as insecticidal and therapeutic agents. In estimating the risks associated with incidental environmental exposures to non-target organisms, the assessor is plagued by the extremely unpredictable species selectivity of anticholinesterase poisons as reviewed by comparative toxicity testing (Murphy, 1966; Murphy et al., 1968; Benke et al., 1974). Several investigators have concluded that species related differences in either metabolic activation or detoxification do not adequately account for the species selectivity of P-esterase insensitive organophosphorous insecticides (Murphy, 1966; Benke et al., 1974).

According to Florking and Stotz (1965) Cholinesterases are basically of two types viz.: 1) Those using acetyl-choline esters as substrate (acetyl cholinesterase = acetyl choline hydrolases )
2) Those using other esters of choline as substrates ( Pseudocholinesterase = acylcholine hydrolases )

The former, namely the acetylcholinesterase is numbered EC 3.1.1.7 and second namely pseudocholin esterase is numbered EC 3.1.1.8 (Pilz, 1074). The enzyme acetylcholinesterase (AChE) has been found in virtually all the classes of vertebrates (Silver, 1974). Reviews have discussed biological aspects of these forms in detail (Massoulie, 1980; Massoulie and Bon, 1982; Brimijoin, 1983; Kerkut, 1984; Malu, 1993). Further more, the proceedings of second international meeting on cholinesterases have now been published and provide concise update account research laboratories (Brzin et al., 1984). There is considerable confusion in literature concerning the naming of the molecular forms of AChE. In some cases the nomenclature is based on solubility of the particular form (Futerman et al., 1984; Plummer et al., 1984). While in others the sedimentation coefficients are used to
identify the forms.

Extensive literature is available on the methods of the extraction, purification and characterization of AChE from electric organs of fishes and many other tissues and organs of various animals. (Nachmansohn and Lederer, 1939; Dudai et al, 1972; Taylor et al., 1974). Varela (1975) reported the presence of a soluble (0.9% NaCl) and a detergent extractable fraction of AChE from rat brain. Later on the same method was employed to extract the soluble and the membrane bound AChE from fishes. (Pradhan, 1982; Hande and Pradhan, 1990) and amphibians (Sharma, 1986). All the experiments carried out in present investigation for extraction and characterization of AChE from the plasma, liver and brain of mice Viz.: Substrate specificity, use foethopropazine hydrochloride confirmed the presence of AChE in the plasma, liver and brain of the experimental mice. Thus present findings are in correlation with finding of Dudai et al., (1972); Taylor et al., (1974); Varela, (1975); Pradhan, (1982); Hande and Pradhan (1990); Malu, (1993); Charjan, (1997).

4.1.1 Standardization of Optimum Parameters.

Enzyme activity is influenced by many parameters viz: hydrogen ion concentration, assay temperature, protein concentration, substrate concentration etc., Therefore optimum levels of above parameters were standardized.

Maximum activity of AChE from all tissues was observed between pH-7 to 9. Therefore in all experiments of present investigation pH of the enzyme assay was maintained between 7 to 9.

Maximum activity of AChE from all tissues was observed between incubation period of 7 to 10 minutes. Therefore in all experiments of present investigation incubation period was 8 min, 7 min and 10 min for plasma, liver and brain.

AChE from plasma, liver and brain exhibited maximum activity at 30°C assay temperature. Thus in all further experiments of present investigation for both fractions of AChE the assay temperature was maintained at 30°C.
Acetylcholinesterase from plasma, liver and brain showed linear activity up to 0.13 ml of enzyme extracts. Therefore in all further experiments, the protein concentration was maintained close to 0.13, 0.14 and 0.15 mg corresponding to 0.05 ml of enzyme extract from brain, liver and plasma respectively.

4.1.2 Effect of Pesticides on AChE

This study indicates that both organophosphates (malathion) and organochlorine (dieldrin) inhibited the AChE activity in plasma, liver and brain. Several investigators had given the comprehensive list of anti-cholinesterase agents (Heath, 1961; O'Brien, 1967). The interaction between the substrate and AChE enzyme mostly involves production of an acetylated form of enzyme through rapid hydrolysis of enzyme substrate complex and reusable enzyme can be reformed. Anticholinesterase compound binds with anionic or esteratic site of enzyme and the substrate (Silver, 1974). It has reported that the rate of penetration of a charged inhibitor through blood-brain barrier in the central nervous system may vary from area to area. Philip et. al., (1985) studied the effect of malathion on AChE and carboxylesterase. He demonstrated tolerance to the cholinergic toxicity of malathion after repeated dosing. In the present study, the recovery of AChE after the long-term dosing of the malathion to mice was observed. This represented an interesting observation since the ability of the organophosphate insecticides malathion to induce cellular tolerance was in doubt because its enzyme inhibition is reversible.

Cholinergic tolerance to “irreversible” organophosphates ChE inhibitors is thought to be a cellular type of tolerance. (Philip.et.al.,1985). This study demonstrated that the tolerance induced by repeated exposure to malathion was also of the cellular type and not of the metabolic type. This is supported by the observations that the tissue esterases were significantly inhibited throughout the dosing period.

Our results with malathion supported the generally accepted hypothesis that repeated exposure to organophosphates and organochlorine resulted in prolonged AChE inhibition and a cellular
type of tolerance.

The mode of action of organophosphate insecticides in vertebrates is generally regarded as disruption of nerve impulse transmission in the central and peripheral nervous system by inhibition of AChE (EC 3.1.1.7), the enzyme that modulate the amount of neurotransmitter acetylcholine. (Schaffer et al., 1954; O'Brien 1960, 1967, 1969; Heath, 1961; Koelle 1963; Aldridge, 1971). It has been shown with radio-actively labelled organophosphate acetylcholinesterse agents in vitro, that the actual toxic agents are deacylated metabolites which “irreversibly phosphorylate” U-serine in the esterate site of “purified” cholinesterase from several sources, including teleost fish Electrophorus eletricus and that further alteration of the organophosphate may occur by dealkylation.

There is a strong evidence that malathion is metabolically altered before it inhibits AChE in vivo. Murphy (1966) and Murphy et al., (1968) have shown that malathion has little or no direct capacity to inhibit AChE but is converted to active inhibitor in liver in vitro. This active inhibitor is believed to be malaoxon, the oxygen analog of malathion created by desulfuration CP = S ---- P =O., which reacts with AChE to form dimethyl phosphorylated enzyme. The metabolic conversion of 99.5 % pure malathion is resulted in more than 1000 fold increase in (fish) brain AChE inhibitory potency in vitro (Murphy et al., 1968).

The inhibiting power of organophosphate insecticides is governed by the affinity of insecticides for the enzyme active site and for by the rate of phosphorylation (Main, 1964). Main and Iverson (1966) and Aldridge (1953) proposed that differences in the inhibitory power of organophosphate compounds might be the result of differences in their affinity to the enzyme’s active site. They observed similar phosphorylation rates for diisopropyl fluorophosphate (DFP) and malaoxon.

Davies et al., (1979) reported that measurement of dialkyl phosphate in human insecticidal poisonings was a highly sensitive indicator of recent exposure to
organophosphate insecticides. Thompson et al., (1983) observed that cholinesterase depression was recognizable in goats in the low and high 7-day treatment of organophosphate IMIDAN group. Cholinesterase depression had been used for determination of anticholinesterase exposure (Anderson, 1971; Carson and Furr, 1976; Wills, 1972). A dose effect was observed between the low and high 7-day treatment of organophosphate IMIDAN groups comparing the RBC and plasma ChE activities. Cholinesterase depression was dramatic in the acute single-dose goat (92.8 and 94.1%) depression of RBC ChE and plasma ChE respectively, 3 h. after treatment. Recovery of the plasma and red-blood cell cholinesterase activity was greater than 60% in the 5 day period. This rapid recovery may be related to the 2-PAM therapy which was given 14 hr after exposure (O'Brien, 1967) and to the activated charcoal which had also been shown to improve therapeutic response in ruminants (Furr and Carson, 1975).

Detection of dialkyl phosphates is a sensitive indicator of exposure and has been shown superior to cholinesterase measurement (Shafik et al., 1973; Bradway et al., 1977).

Corresponding to maximal inhibition of AChE activity, acetyl-choline (ACh) content also showed corresponding increase. Thus the inhibition of the enzyme activity resulted in the accumulation of acetyl-choline. As the activity of AChE was restoring, the level of acetyl-choline content was also lowering. Since acetyl-choline is a substrate for the AChE, the change observed in its content in brain at different exposure period was compatible with alteration in the enzyme activity.

Koelle, (1953) and Karczmar et al., (1970) had observed that depression in AChE activity may in itself cause physiological and behavioural modifications that ultimately reduced animal survival ability during organophosphate insecticide exposure even if lethal poisoning did not occur during that exposure was not worthy. (Malu, 1993).

The rate of reactivation of AChE is again of discussion. Aldridge and Davison (1953) suggested that the rate of
reactivation of erythrocyte AChE was influenced by the characteristics of the alkoxy substituents, with the dimethyl phosphorylated enzyme.

Although the reactivation of AChE is temperature dependent (Aldridge, 1953; Lanks and Seleznick, 1981; Reiner and Aldridge, 1967), difference in reaction temperature did not entirely account for significant difference in Kr for paraoxon inhibited AChE observed between rats and fathead minnows. In contrast, difference between rats and minnows for the reactivation of the malaoxon inhibited enzyme could be attributed solely to the temperature dependence of the reaction. The aging of phosphorylated AChE, which represents an irreversible process, is also influenced by temperature. (Davis and Green, 1956) as reflected by the decrease in K2 for the rat enzyme, when the temperature was lowered from 37 to 25 degree centigrade.

However, this decrease was not statistically significant for either inhibitor. It was found that, in plasma taken from rats dosed with carbaryl, cholinesterase reactivation at 37°C was approximately two fold faster than the same sample at 23°C.

The rate of reactivation (decarbamylation), in addition to being related to incubation temperature and dilution factors, may be expected to vary with tissue type. It has been shown that the rate of reactivation may vary in the same tissues collected from different species. Another variable determining the rate of reactivation is the chemical structure of carbamate inhibitor. It has been noted that the decarbamylation rate is related primarily to the structure of acylside group of the carbamate (Reiner, 1971).

Carbamates are esters of carbamic acid that may be considered synthetic analogs of acetyl-choline, and their complex-formation with acetyl-cholinesterase is dependent on the similarity of “fit” upon the enzyme surface. Complex formation is followed by the carbamylation of serine hydroxyl at the active site. Inhibition results because the carbamylated enzyme, EC is several orders of magnitude more stable than the acetylated enzyme formed in normal acetyl choline hydroly-
sis. Inhibition of acetyl-cholinesterase in vitro by carbamate inhibitor is progressive but level off before completion, in contrast to the process for organophosphate inhibitors (O'Brien et al., 1966; and Coppage, 1977). Because the decarbamylation rate was relatively faster than the dephosphorylation rate (Reiner, 1971).

The large AChE inhibitions (59-65%) caused by "sublethal" exposure of aquatic animals to 1,2, Dibromo2-2 dichloroethyl Dimethyl phosphate (Naled) indicate pollution & can be readily detected, possibly before acute poisoning occurs. However, even if acute lethal poisoning did not occur during organophosphate insecticide exposure, depression of AChE in vertebrates may cause physiological and behavioral modifications (Koelle, 1963; Karczmar et al., 1970) that reduce animal survival ability. Cumulative reduction of AChE by repetitive exposure to some organophosphate pesticides had been demonstrated in some vertebrates (Heath, 1961; Koelle, 1963; Karczmar, 1970).

Detection of dialkyl phosphate is a sensitive indicator of exposure and has been shown superior to cholinesterase measurement. (Shafik et al, 1973; Bradway et al, 1977). Thompson et. al., (1984) studied that O,O-dimethyl phosphorodithioate (DMDTP) appeared in the urine of goat immediately following exposure. In contrast, cholinesterase depression was significantly present only after seven days of continuous treatment in the low seven day treatment group.

Fernandez and et al. (1974) showed that several anti-histaminic compounds having a \[ R_1-\text{CH}_2\text{CH}_2\text{N}^{\text{R}_2}\text{R}_3 \] portion in their molecules inhibit plasma ChE from several sources (man, rat, or horse). They inhibit ChE competitively and reversibly. The interaction between the enzyme and a antihistaminics seems to involve the basic aminoportion of their molecules and a proton donating group from the enzyme molecule. (Zawoiski 1974).

A very important features of the inhibition of AChE by antihistaminies in that even though it is competitive with re-
pect to the substrate ACh, as is also the inhibition by organophosphate, these compounds do not compete with each other in inhibiting the enzyme. Thus, antihistaminics must bind to some group important to substrate processing or important to substrate binding to ChE since their inhibitory action is competitive with it. That group does not belong to either the esteratic site of the enzyme (since antihistamines do not delay organophosphate inhibition of ChE) or to the anionic site (Since the quaternary compound diphenylhydramine methiodide does not compete with diphenylhydramine). That is in the enzyme-inhibitor complex resulting from the interaction of antihistaminic with ChE, the processing of approach of the substrate (which interact with both the anionic and the esteratic site simultaneously, Engelhard et al 1967) is hindered, while the approach or interaction of compounds acting only at the esteratic or the anionic site is not impeded (Fernandez et al., 1974).

Soman given in an acute non-lethal dose (100 µg/Kg.) produced typical signs of anticholinesterase toxicity within a few minutes with a preponderance of central activity as evidenced by posturing and body shaking. Fasciculations superimposed upon this activity were less prominent than the signs of gross motor unit activity. In constrast, the use of DFP (Gupta et al., 1985,1986) caused greater peripheral activity with fasciculations and fewer central toxicity sings. This difference between these two inhibitors could be due to a) non-specific effects, independent of inhibition of AChE activity b) differences in affinities of central and peripheral AChE for the inhibitors, c) Variations in transport rate across the blood-brain barrier and d) differences in rates of degradation of DFP or soman resulting indifferent times to achieve sufficient concentrations in the CNS to produce CNS effects. All these factors with the exception of a] determine the speed with which AChE is critically inhibited inspecific brain areas. When enzyme activity was reduced slowly over a period of hours to a critical level, signs of toxicity were less severe than when AChE activity was critically reduced within minutes. The possible adaptation mecha-
nisms such as changes in the release of acetyl-choline (AChE) and receptor desensitization attenuated the sings of toxicity (Gupta et al, 1986) difference in rate of inhibiton at shorter intervals after soman may also explain the observed variation in toxicity seen among individual animals that have similar levels of AChE inhibition at the end of 24 hr. There was no difference between soman and DFP, however in the degree of AChE inhibition in various brain regions when measured after 1 hr. It is of course possible that there may be different rates of inhibition when activity is measured at still shorter time after administration (Mitchell and Petersen 1986).

The quantitative necrotic changes in skeletal muscle correlate well with the observed biochemical changes, that was the faster the rate of the AChE inactivation, the higher the number of necrotic lesions found (Wecker et al, 1978). Thus, the EDL muscle with the slowest rate of AChE inhibition showed the lowest number of necrotic lesions. Soleus muscle, with the fastest rate of AChE inhibitions, exhibited the greatest number of lesions. The detailed examination of molecular forms of AChE especially of the 16 s form, further confirms this observation (Mitchell and Petersen 1986).

In constrast to soman, other inhibitors such as paraoxon and DFP (Gupta et al., 1985, 1986) caused the slowest rate of AChE inhibiton and the lowest number of necrotic lesions in soleus. These include pharmacokinetic variables which influence the delivery of a particular organophosphates, variations in location of AChE in soleus and EDL (Dettbarn, 1981; 1984; Groswald and Dettbarn, 1983). or changes in ACh release due to different firing patterns. Peripherally generated short high frequency bursts up to 800 discharges / sec from single fibers with long silent periods between bursts were prevalent with DFP. Soman produced a greater overall frequency of discharge mainly as motor unit potentials, but at a much lower frequency than the high frequency discharge seen with DFP this suggested a cause effect relationship between the high frequency discharges and muscle lesions.
The hepatic AChE could be inhibited only to 40-60% of control activity from which it did not recover during 48 h. On the other hand, Butyrylcholinesterase rose rapidly to about 70% of control activity in 24 h. In lungs, AChE recovered significantly only at 20°C during the first 24 hours. BuChE showed, however, only marginal regain of activity in rats and mice responded differently to cold exposure. Despite the lower absolute dose, pulmonary AChE was constantly lower in mice at 5°C than at 20°C (P<0.05). In rats pulmonary BuChE behaved similarly. In mice, blood ChE, corelated to the degree of ChE inhibition in brain and other tissues. The measurement of whole blood ChE might not always adequately describe the inhibition of ChEs in tissues.

Johnson et al., (1986) showed that rodent brain AChE was more sensitive to inhibition by paraoxon and malaoxon than was fish brain AChE, however the species releated differences were much greater for paraoxon than for malaoxon. The greater toxicity of parathion and paraoxon in rodents than in fish corresponds with and may be due primarily to differences in the kinetics of AChE inactivation. In contrast the greater toxicity of malathion and malaoxon in fish than in rodents could not be completely explained by differences in the kinetics of inhibition by AChE.

In a report by Sasinovich (1968) the group of rats were exposed daily to dichlorvos (0.11 to 1.1 mg/ m³) for 4 hr. per day for 4 months caused signs of intoxication exposure to 5.2 mg/ m³ resulted in marked depression of ChE activity and impairment in blood suger curve. Stevenson and Blair (1977) reported that mice became distressed at concentration above 50 mg/ m³ and prolonged exposure to 80 mg/ m³ was frequently lethal. In another study, monkeys were exposed to dichlorvos (0.05 mg/m³) for 3 months showed no adverse effects except plasma ChE was inhibited (up to 27%) while the greatest erythrocyte ChE depression was 36% (Who 1989). Main and Iverson (1966) and Aldridge(1953) proposed that the inhibitory power of organophosphate insecticides is governed by the affinity of insecticides for the enzyme active site and for
by the rate of phosphorylation. Earlier studies were centered around the conclusion that death occurs when brain AChE activity was reduced to 40-70% of normal. But exceptions were found in *Lepomis macrochirus* (Gibson et al., 1969); *Lagodon rhombodies* (Coppage and Mathews, 1974); *A. testudineus* (Bakthavath salam and Ready 1982). The difference in inhibitory effect probably might be due to the duration of exposure to the pesticide. Gibson et al., (1969) also observed higher inhibition of AChE with sub-lethal concentration in parathion exposed blugill *Lepomis macrochirus*, which was attributed to the longer duration of exposure. Though acute lethal poisoning may not occur during exposure to 120 h. LC₅₀ disyston, depression of brain and muscle AChE may possibly bring in physiological and behavioural modifications. (Richmonds and Dutta, 1989, 1992). The perusal of the data from this study reveals that both pesticides viz, malathion (organophosphate) and dieldrin (organochlorine) inhibited the brain AChE activity. The inhibition was more when exposed to dieldrin than malathion.

For plasma, liver and brain, AChE the degree of AChE activity inhibition was increased up to four days of exposure period. Further exposure to either pesticide the inhibition was in the following decreasing order: 4 days > 8 days > 15 days > 30 days. These findings indicated that the continuous exposure of body organs to the same pesticide resulted in the recovery of AChE activity. Such a recovery in the enzyme activity may be due to product of degradation or elimination which is yet to be confirmed. The partial recovery of brain AChE suggests a possible damage in the central and peripheral neurotransmitter.

Dieldrin (organochlorine) is a nerve poison insecticide reduce food intake, depletes fat reserves and may have some cancer producing effects. (unpublished data of the present study)

4.2 Characterization of Carboxylesterases

Carboxylesterases play a major role in the metabolism, detoxification, and elimination of esters encountered in the diet.
or administered as drugs (Sone and Wang, 1997; Satoh and Hosokawa, 1998). The family of carboxylesterase enzymes has a common mechanism of hydrolysis involving a catalytic triad composed of a serine that is acylated by the substrate ester plus a base (usually histidine) and an acid that activate catalytic serine. These esterases, which include acetylcholinesterases, cholesterol esterases, and lipases, all have a characteristic "carboxylesterase fold" consisting of 8 to 11 sheet structures (usually parallel) that are connected by -helix (or less frequently loop) structures. These enzymes catalyze the hydrolysis of various carboxylic ester, thioester, or amide groups. They generally are expressed in high amounts in liver. Their role in xenobiotic metabolism is to convert the apolar esters or amides to the more soluble acid, alcohol, or amine metabolites for elimination. (Sone and Wang, 1997; Satoh and Hosokawa, 1998)

4.2.1 Standardization of optimum parameters:

Carboxylesterase activity was influenced by many parameters eg. viz. hydrogen ion concentration, assay temperature, substrate concentration, etc. Therefore optimum levels of above parameters were standardised.

Maximum activity of CE from all tissues was observed between pH 7.5 to 9.5. Therefore in all experiments of present investigation pH of the enzyme assay was maintained between pH 7.5 to 9.5.

Maximum activity of CE from all tissues was observed between incubation period of 7 to 10 minutes. Therefore in all experiments of present investigation incubation period was 10 min, 8 min and 7.5 min for plasma, liver and brain.

Carboxylesterases from plasma, liver and brain used exhibited maximum activity at 30°C assay temperature. Thus in all experiments of present investigations for CE, the assay temperature was maintained at 30°C.

Carboxylesterase show linear activity upto 0.15 mg of enzyme extracts. Therefore in all further experiments, the protein concentration was maintained close to 0.11, 0.13 and 0.15 mg corresponding to
0.05 ml of enzyme extract, from plasma, liver and brain, respectively.

4.2.2 Effect of Pesticides on Carboxylesterases:

Enzyme chemistry has expanded rapidly in recent years and has now reached a stage when it is possible to think in more precise terms about the chemical groups and the physical forces which contribute to the binding of the substrate to the enzyme and its subsequent transformation. The concept of "active center" is assuming reality in terms of amino-acid sequences and electric forces, particularly in case of cholinesterase (ChE) and related enzyme with carboxylic esterase activity. Progress has been greatly aided by the discovery of substances of widely varying chemical structure, which are highly specific in their inhibitory action towards these enzymes. Their use has permitted the identification of amino-acids near the active centers and has also provided much indirect evidence on the nature of the enzyme active surfaces.

It is now clear that when esterases are inhibited by organophosphorus compounds, the enzyme is phosphorylated at or near the active centre. These phosphorylated enzymes are stable and can be isolated in crystalline form but can be degraded by acids, alkalis, or enzymic digestion. Plasma carboxylester hydrolase (Ec 3.1.1.1) is very important detoxification route for the toxic stereoisomers of soman (Clement, 1984). Detoxification is meant in the sense that if soman was bound to carboxylic esters - hydrolase it was not free to inhibit acetyl cholinesterase. Clement (1984) demonstrated that (BDP (2-(3-methylphenoxy)-4H-1,3,2,-benzodioxaphosphorin 2-oxide) pretreatment, which inhibited irreversibly carboxyl ester hydrolase activity without inhibiting phosphoryl-phophatase activity potentiated the toxicity of soman in mice.

A wide range of drugs and other xenobiotics are found to alter the expression of carboxylesterases in animals, but the inducibility is minimal in most species compared with cytochrome P-450 (CYP)3 enzymes (Morgan et al., 1994; Parkinson, 1995; Satoh and Hosokawa, 1998). In rats, phenobarbital and clorfibrate, two
potent CYP inducers, cause a small increase (15-30 %) of activity in hydrolyzing para-nitrophenylacetate (Morgan et al., 1994). The mechanism of the lack of inducibility was unclear, probably due to the fact that carboxylesterase genes are constitutively expressed at a relatively high level (Morgan et al., 1994). In contrast to induction, suppression of carboxylesterase expression was profound by many chemicals (Morgan et al., 1994; Watson et al., 1994; Satoh and Hosokawa, 1998). Treatment of mature rats with dexamethasome and nahthoflavone causes as much as an 80 % decrease in hydrolytic activity towards para-nitrophenylacetate and the corresponding immunoreactive proteins of hydrolase A, B, C and S (Morgan et al., 1994; Yan et al., 1995).

Malathion is widely used pesticide. The compound is much less toxic to mammals than to its target pests (Dauterman and Main, 1966) because of the predominance of a detoxifying hydrolytic pathway for malathion and its toxic metabolic malaoxon (Main and Braid, 1962). The enzyme which catalyze this hydrolysis are the carboxylesterases including organophosphorous insecticides malathion, soman and sarin. Dauterman and main, 1966; Fonnum et al., 1985; Talcott et al., 1979).

Malathion is an effective insecticide, and its selectivity, low mammalian toxicity and propensity to the development of resistance are related to its metabolism by carboxylesterase Kao et al., (1985). In addition , Talcott et al., (1982) suggested that hepato-cellular damage and malathion carboxylesterase solubilization are related and they explored the use of diethylsuccinate as a substrate to assay serum ethyl esterases as a prospective liver function test.

Clorfibrate is a hypolipidemic drug which causes an increase in hepatic and renal peroxisomes and in the content of liver smooth endoplasmic reticulum as well. Induction of these organelles is accompanied by increases in peroxisome and microsome associated enzymes (Hammock and Ota, 1983; Reddy and Lalwani, 1983; Berge and Aarsland, 1985). Induction of smooth endoplasmic reticulum suggested
that microsomal carboxylesterase activities may also be affected by peroxisome proliferators.

The response of xenobiotic-metabolizing carboxylesterases to peroxisome proliferators has been limited to a very recent study of Metlein et al (1986). WHO reported that the specific activities of acetanilide carboxylesterase and decanoyl-DL-carnitine hydrolase increased more than three fold in clorfibrate treated rat liver microsomes while the specific clorfibrate hydrolase activity remained unchanged. Ashour et al., (1987) revealed that carboxylesterase activities, on the substrate used in liver, kidney, and testis microsomes, from both mouse and rat are significantly affected by clorfibrate treatment; however these effects are species, tissue and substrate dependent. The induction was higher in mouse than rat.

Su et al., (1971) fed rats 18 different organophosphates for month and found that for all compounds tested, liver hydrolysis of both diethylsuccinate and tributyrin was more sensitive to inhibition than was brain cholinesterase activity. Parathion inhibited liver carboxyl esterase activity at non-cholinesterase-inhibiting doses it did not potentiate malathion toxicity in mice. (Cohen and Murphy, 1971).

The susceptibility of liver malathion carboxylesterases to inactivation may vary among different species such that potentiators of malathion toxicity in one species may not be active in another. (Talcott et al., 1979).

Effect of dieldrin on rat esterases are complex and depend on the type of esterases, duration of exposure, and sex of the animal. A single oral dose of dieldrin produced modest elevations both plasma and hepatic carboxylesterase activities.

For the laboratory rat, malathion is one of the least toxic commercial pesticides having an acute LD_{50} perhaps as high as 129/kg (Umetsu et al., 1977). The extent to which the response of the rat to malathion is typical of other mammals is not known. Rat is particularly well equipped to detoxify malathion, with active malathion carboxylesterase distributed among its circulatory, digestive and excretory systems. In the malathion poisoned rat
the serum carboxylesterase would be expected to reduce the amount of absorbed dose that reaches the activating enzymes of the liver. (Talcott. 1979). However, in humans, the serum carboxylesterase is apparently lacking (Seume and O'Brien, 1960) and the greater fraction of the absorbed dose may be susceptible to metabolic activation in liver. It may be more appropriate to study malathion toxicity in animals with lower extrahepatic detoxification potential. The study of malathion carboxylesterase activity in the rat indicated that these enzymes are distributed among several tissues. The largest share of the total activity as contributed by the enzymes localized in the liver. The specific carboxylesterase activity (in n moles malathion hydrolyzed /minute/ milligram protein) was highest in serum and in agreement with a previous report (Seume and O'Brien, 1960). It exceeded the specific activity in liver homogenate by over two fold (Tolcott 1979).

The apparent kinetic properties of the soluble brain esterase activity for the hydrolysis of trans permethrin are also considerably different from those reported for the hydrolysis of this substrate by liver microsomal esterases. Soluble brain esterases also differ considerably from hepatic microsomal esterases in their substrate specificity for pyrethroid esters. Liver esterases preferentially hydrolyze trans-substituted cyclopropane carboxylates of primary alcohols(Abernanthy et al., 1973). Brain esterases hydrolyze trans-permethrin more rapidly than cis-permethrin. Brain esterases also hydrolyze fenvalerate and fluvalinate at rates similar to that measured for trans-permethrin, despite the fact that these are esters on non-cyclopropane acids and a secondary alcohol.

4.3 POLYMORPHIC FORMS OF AChE.

The esterase activity is found largely on or in the membranes (Chaveau, et al., 1962). Lengsfed et al., 1973 found that triton X-100 penetrate between the protein layers of the membrane changing the structure and thus enabling the purification of membrane bound enzymes. Wright and
Plummer (1972) suggested that acetylcholine esterase in erythrocytes appears to be bound to the membrane primarily by the hydrophobic interaction and that weakening of electrostatic bonding is necessary for maximum solubilization. One of the agents that makes this possible is Triton X-100. Barrow and Holt (1971) pointed out that the release of esterases by deoxycholate implies that the enzyme is bound to microsomes through lipid components of their membranes.

4.3.1 Effect of pesticides on Polymorphoic forms of AChE:

The observation that brain ChE from various species is composed of two molecular forms is in accordance with the results of other workers. (Rieger and Vigny 1976; Rieger et al., 1976; Henderson, 1977; Marchand et al., 1977; Andersen and Mikalsen, 1978-1979). Polymorphic forms of ChE in brain were demonstrated in frog earlier by Andersen and Mikalsen, (1979). It appeared that the heavier ChE form always was solubilized in the highest proportion regardless of the detergent concentration. The lighter ChE form was however, solubilized in the highest proportion during the beginning of the ChE recovery time following soman inhibition of the chicken. At later stages, the heavier ChE form predominated. The lighter ChE form was the first one to be synthesized after irreversible ChE inhibition.

The development and maturation into functional forms of ChE following conception have previously been studied in chicken brain by Marchand et al., (1977) and in rat brain by Rieger and Vigny (1976). These authors found the higher proportions in earlier stages for both species on various cell and tissue cultures also indicated that the pattern of ChE isozymes progressively shifted from a preponderance of lower to higher molecular weight forms following irreversible ChE inhibition (Wilson and Walker., 1974, Rieger et al., 1976), These results compared to those of the present work may therefore indicated a general mechanism by which ChE is synthesized and incorporated into its functional membrane form in such a way that this form is preceded by a form of lower mol. wt. This
form may be freely present in the plasma and more easily extractable in earlier stages in the membrane incorporating process. (Mikalsen et al., 1981). Data shown by Mikalsen et al., (1981) stated that in chicken and rat soluble blood plasma ChE is not identical to this precursor form. This can be deduced from the differences in sedimentation constant observed for the ChE forms. Substrate specificities and inhibition constants of the blood plasma ChE and the membrane-bound ChE forms are also different. This is shown by the data given by Andersen and Mikalsen (1978).

The brain of mice investigated by Sorensen (1985) contained the three major forms of AChE. The vast majority comprises a detergent extractable form known to be a tetrameric enzyme. (Sorensen et al., 1982; Laudauer et al., 1983). It is an amphipathic membrane-bound protein that in the presence of micellar concentrations of triton x-100 sediments at 10s with a small amount at 5s and that in the absence of detergent formed polydisperse aggregates. (Sorensen et al., 1985).

This is in contrast to the behaviour of the pure human and the pure bovine enzyme, which in the absence of detergent formed defined aggregates (Sorensen et al., 1982; Landauer et al., 1983). About 10-20% of extractable activity comprise and salt-soluble AChE which is distributed between two forms sedimenting at 5 and 10s. The former most likely is a monomer and the latter a tetramer (Sorensen et al., 1982; Gennari and Brodbeck, 1985). The yield and the speed of solubilization of 5s-AChE from brain was superior in buffer containing 1 m NaCl (higher salt condition).

4.4 Enzyme kinetics

The majority of enzyme kinetics studies are carried out by the conventional steady state method. In these methods it is important to evaluate the effect of varying concentration of substrate on the v (initial velocity) and thereby compute the kinetic indices like km (Michaelis Constant) and V max (Maximum velocity) from the progress of curves obtained from these experiments. The relation between the initial velocity (v) Michaelis constant (Km)
and maximum velocity (V max) is given by the expression.

\[ v = \frac{V_{\text{max}} \cdot S_0}{S_0 + K_m} \]

Where \( S_0 \) = initial substrate concentration. It can be seen from the above equation that there is an inverse relationship between \( K_m \) and initial velocity. In fact, \( K_m \), which represents the extent of affinity between the enzyme, the substrate and \( V_{\text{max}} \) together reflect the efficiency of an enzyme (Price and Stevens, 1982).

In present investigation these kinetic indices were established for AChE, CE and polymorphic forms of AChE from plasma, liver and brain of mice. The data were analysed by Michaelis-Menten method (M.M. method) and Eisenthal Cornish-Bowden method (E.C.B. Method).

Another observation was that AChE of brain had higher efficiency than the liver and plasma AChE.

It was observed that the AChE of brain, which was involved in neuromuscular functions, is more efficient. On the other hand, lowest \( V_{\text{max}}/K_m \) ratios was found in plasma.

Lowest \( V_{\text{max}}/K_m \) ratio was found in brain while studying the kinetics for CE. Lowest \( V_{\text{max}}/K_m \) ratio was found in plasma while studying the kinetics for polymorphic forms of AChE.