SECTION - II

STUDIES ON NEUROTRANSMISSION

1. Cholinergic transmission during methyl parathion induced toxicosis
Many neurotoxic compounds have been shown to interfere with neurotransmission both *in vivo* and *in vitro* following acute and chronic administration (Costa, 1988). Majority of insecticides act as poisons to the nervous system and most of them attack the target, acetyl cholinesterase (AChE), which is one of the regulatory proteins involved in cholinergic transmission (Eldefrawi *et al.* 1986). The function of the enzyme AChE is primarily the termination of cholinergic synaptic transmission by rapid hydrolysis of the neurotransmitter Acetylcholine (ACh) (Marquis and Judith, 1986). The presence of ACh and AChE in all the invertebrates and vertebrates is well documented (Nachmansohn, 1963). The significance of AChE at post synaptic membranes has been demonstrated by the prolonged duration of ACh effects seen after application of esterase inhibitors (Katz, 1966). Subcellular fractionation studies (Whittaker, 1969) and histochemical evidence (Lewis and Shate, 1966; Brzin *et al.*, 1966) indicate that this hydrolytic enzyme is localized in the external membrane of the nerve terminal. A detailed discussion on AChE has been reviewed by several investigators (Triggle, 1965; Engelhard *et al.*, 1967) and changes in AChE in various parts of the brain during ageing has been reported by Aprison and Himwich (1954).
Cholinergic transmission is characterised by the release of ACh from the terminal regions of electrically depolarised cholinergic neurons. These terminal regions have been shown to contain high concentrations of ACh and its synthetic enzyme, choline acetyltransferase (ChAT) (Hebb and Whittaker, 1958). Thus ACh is a transmitter operating in the cholinergic synapses, which include the synapses of the CNS, the neuromuscular junctions of motor nerves, sensory nerve endings, ganglionic synapses of both sympathetic and para sympathetic nerves, all post ganglionic para sympathetic nerve terminals and sympathetic nerve terminals on the sweat glands, blood vessels and the adrenal gland (Eto, 1974). ACh is removed from its site of release partly by diffusion and mainly due to the hydrolysis of ACh by AChE (Klee et al., 1982) which catalyses the rapid breakdown of ACh to acetic acid and choline.

Acetylcholine is widely distributed in the animal kingdom. It is a characteristic constituent of the vertebrate nervous system and its presence is also reported in invertebrate nervous tissues (Baeq, 1947). The distribution of ACh throughout the CNS with high concentrations in the cerebral cortex, thalamus and various nuclei in the basal fore brain has been found. (Dowdall and Zimmermann, 1974).
The interest in ACh as a transmitter compound led directly to studies on its biosynthetic enzyme choline acetyltransferase (ChAT)/choline acetylase (ChA) (Hebbe, 1963). According to Hebb and Smallman (1956) knowledge of the intracellular distribution of ChAT is important for the understanding of the chemical transmission of nerve effects.

Choline acetyltransferase is widely, but selectively distributed in the nervous system (Nachmanson et al., 1974) and its presence is predominant in presynaptic nerve terminals (Tonge et al., 1975). The activity of this enzyme has been shown to increase at autonomic nerve terminals during hyperactivity of synapses (Goldberg and Welch, 1972; Oesch and Thoenen, 1973). ChAT is a characteristic constituent of the vertebrate nervous system and its presence is also reported in invertebrates (Smallman, 1956). ChAT has been shown to exist in soluble as well as particulate form (De Robertis et al., 1962). It catalyses the transfer of acetyl groups from acetyl CoA to choline and it is possible to measure its activity independently by the synthesis of acetyl CoA (Smallman, 1956).

AChE has a somewhat wider distribution in the nervous system than ACh or ChAT. AChE is characteristically a membrane bound enzyme and this enzyme has, had special attention because a number of drugs, insecticides and nerve
gases act by inhibiting it (Pauling, 1974). The importance of studying AChE function in the development of strategies for understanding and treating insecticide toxicity is well established. Organophosphate and carbamate insecticides, are highly potent cholinesterase inhibitors (Judith Marquis, 1986). Hopff et al. (1984) studied the inhibition of ACh synthesis by blocking its synthesizing enzyme ChAT using methyl methane thiol sulfonate (MMTS) in organophosphate poisoned rats. High affinity choline uptake and ChAT activity after injection of DDVP in the crude synaptosomal preparations of rat brain was studied (Hasuokobayashi et al., 1986). Baillie et al. (1975) investigated a variety of compounds mainly choline analogues as potential inhibitors of ChAT.

It is now becoming increasingly clear that toxic effect of a large number of organophosphorous compounds is due to their inhibitory action on AChE activity. This enzyme being responsible for the rapid inactivation of the neurotransmitter ACh, plays an important role in the regulation of cholinergic neurotransmission (Palaas, 1988).

Eto (1974) suggested that the principal lethal lesion caused by organophosphorous insecticides is due to the inhibition of AChE which disturbs the normal nervous function leading to severe and often lethal damage in the
organism finally resulting in death. Based on the reaction of organophosphorous compounds Holmes and Masters (1967) classified esterases into four main categories carboxylesterases, arylesterases, acetyl esterases and cholinesterases. Except arylesterases, all the other types of esterases are reactive to organophosphorous insecticides, whereas acetyl esterases neither hydrolyse by organophosphate insecticides nor are inhibit by them (Eto, 1974).

Cholinesterases are classified into two types viz., Acetyl cholinesterases (acetyl choline acetyl hydrolase) which hydrolyse most rapidly its natural substrate ACh. Cholinesterase (acetyl choline acyl hydrolases) also called as pseudo cholinesterase, non-specific cholinesterase or butyryl cholinesterase, on the other hand its presence can be seen chiefly in serum and some organs like pancreas, heart and liver etc. and it is reported that these pseudo cholinesterases do not participate in nerve functioning (Eto, 1974).

Jamieson (1963) on the other hand, reported that acetyl choline-acetyl hydrolase normally degrades neurogenic and non neurogenic acetyl choline in the ileum of vertebrates (rats, guinea pigs), while acyl choline-acylhydrolase is capable only of hydrolysing non-neurogenic acetylcholine in the intestine.
Majority of vertebrate AChE are highly specific for ACh and have a little activity against butyl choline as a substrate, but some insects AChEs' (Brady, 1970) hydrolyze both substrates rapidly while others are like the vertebrate AChEs' (Lee, et al., 1974). The kinetics of AChE inhibition by various inhibitors have been studied in detail and effective reactivators of AChE have successfully been developed as a result of such studies (Koelle, 1970).

Organophosphates and Carbamates inhibit AChE, causing hyperstimulation of the cholinergic system by endogenous accumulation of ACh. (Costa et al., 1987). It has been reported that ACh levels may rise to 260% of normal, when insects are treated with organophosphorous insecticides (Smallman and Fisher, 1958). Application of various organophosphates to cockroach nerve cord, prolonged end plate potential, presumably due to the persistence of ACh in the synapse (Narahashi, 1971) eventually causing a block in nervous transmission. Administration of malathion to developing Philosamia ricini larvae induces accumulation of ACh, marked inhibition of AChE activity, depletion of all nutrients, heavy weight loss and high mortality (Radhapant and Ramana, 1989). Generally death is due to respiratory failure because of effects on central respiratory neurons and/or paralysis of respiratory muscle aggravated by the effects on the lungs and bronchi (Doull, 1976). Failure at
the respiratory neuromuscular junction is due to the nicotine effects of ACh where by low doses stimulate and high doses block junctional transmission because of persistent depolarization of the post synaptic membrane (Dolivia and Koelle, 1970).

The inhibitory activity of organophosphorous esters against cholinesterase was found in 1941 by Adrian et al. (1947). Balls (1949) pointed out that the inhibition was attributable to the phosphorylation of the esteratic site. The inhibitory effect of other compounds on AChE have been investigated by many investigators. Inhibition of AChE by pulegone-1-2-epoxide (insect neurotoxin) was studied by Dawn and Cecil (1985) in eels, madagascar roachs, in horse serum ChE and house fly AChE.

Esterases have been found to be a sensitive indicator of organophosphorous poisoning. In vitro inhibition of goat cerebellum AChE by DFP has been reported by Guhathakurta and Bhattacharya (1989). Inhibition of eel AChE and bovine erythrocyte AChE by 4-nitrophenyl esters of methyl, ethyl and isopropyl (phenyl) phosphoric acid (MPP, EPP and IPP) has been reported (Leiske et al., 1982). Multiple intraperitontial administration of hostathion and decamethrin on AChE activity in selected brain and spinal cord areas of albino rats was studied by Ahmed et al. (1987). According to
them the manual decrease in AChE activity obtained by the intraperitoneal injection of $1/4 \text{LD}_{50}$ of hostathion was recorded on 6th day in all the areas studied except cerebrum, whereas $1/4 \text{LD}_{50}$ of decamethrin caused an inhibition of AChE activity which was maximal on the third day in cerebellum, on the fourth day in the mid brain, on the 5th day in the cerebellum and on 8th day in all its spinal cord areas, as well as the pons/plus medulla.

Several salmonoid fish species were subjected to malathion exposure in water under controlled conditions to study the behavioral effect by correlating mortality with brain AChE activity and physical stamina by Post and Leasure (1974). They reported 50% decrease in stamina, was accompanied by an approximately 50% decrement in normal brain ChE activity. Jennings et al. (1975) reported 60-90% inhibition in the brain AChE activity by carbophenothion in different wild and domestic birds like Canada geese, Pigion and Japanese quil. Fish brain AChE studies showed similar results as those of birds. Coppage (1972) reported that death occurs when brain AChE activity falls by more than 84% but a 13% or greater fall is necessary to indicate organophosphate pesticide exposure. According to Weiss (1958) inhibition of AChE ranging from 40-90% is deadly. Since both mortality and brain ChE inhibition are dose related, Tucker and Leitzke (1979) have constructed dose
response regression lines for both mortality and AChE inhibition. Ludke et al. (1975) derived a very similar relationship between brain ChE inhibition and death among birds fed with AChE inhibitors. The inhibition of AChE by organophosphorous compounds in selected ectothermic vertebrates was investigated by Michael and Janice (1985).

The biological activity of organophosphorous atom to phosphorylate the active site of the enzyme cholinesterase has been reported (Michael and Janice, 1985). The phosphorylated enzyme is irreversibly inhibited and is no longer able to carry out its normal functions of rapid removal and destruction of neurohormone (ACh) from the nervous synapse. This results in an accumulation of ACh and consequent disruption of the normal functioning of the nervous system. The inhibited cholinesterase changes gradually into a non-reactivable form on storage. This phenomenon is called aging, which is due to dealkylation of the dialkxyoxyphosphinyl enzyme (Oosterbaan and Jansz, 1965). The existence of the same mechanism for the aging of rat brain AChE inhibited in vivo with Sarin was also confirmed (Harris et al., 1966; Bruce Clothier et al. 1980). It was proposed that both inhibition of neurotoxic esterase of nervous tissue and subsequent aging of the inhibited esters are necessary events in the pathogenesis of organophosphorous induced delayed neuropathy (Johnson, 1974).
Histochemical investigations on the cholinesterase of insects treated with organophosphate insecticide have shown that the peripheral area of the thoracic ganglion of houseflies and the brain and nerve-cord in crickets are the vital organs of inhibition (Booth and Lee, 1971). Rats injected with non lethal acute dose organophosphate insecticide soman, showed comparatively greater involvement of cerebral cholinergic system than peripheral neuromuscular effects. During peak toxicity all the brain regions tested showed more than 95% inhibition of AChE activity and cortex area affected maximum (99%) (Gupta et al., 1987).

The mammalian and insect cholinesterase inhibitory ratio is frequently used as a measure of the relative affinities of a toxicant for different enzymes. This selectivity between insects and mammals is found to be due to cholinesterase inhibitors that are highly ionized at physiological pH, for these are frequently highly toxic to mammals, and poorly toxic to insects (O'Brien, 1967).

Brownson et al. (1977) summarised histochemical and biochemical studies in their laboratory, which demonstrated that after parathion administration to 15 and 30 day old rats inhibited AChE activity in cell bodies and synapses of brain stem neurons and in myoneural junction had recovered by 24hrs.
AChE exists as several isozymes with different turnover rates (Davis and Agranoff, 1968) and different isozymes predominate in neural versus non-neural tissues. Following inhibition of retinal AChE with DFP in adult male rats, a potential difference in the physiological importance of AChE isozymes was indicated (Davis and Agranoff, 1968). Differential inhibition of AChE isozymes of head and thorax of house fly *Musca domestica* by pesticides like DDT, malaoxon, paraoxon, diazinan, dichlorvos and dimethoxon was reported by several workers (Tripati and O'Brain 1972; Huang *et al.*, 1974; Zetter and Brady, 1975). However the studies of Steele and Maneekyee (1979) suggested that the preferential inhibition of thoracic AChE by the pesticide was not due to the biochemical difference between the enzymes of the two regions.

*In vitro* studies revealed that the isozymes of AChE differed measurably in their sensitivity to inhibition by anticholinesterases. By comparing the sensitivities of the four different organophosphorous insecticides it was concluded that inhibition of thoracic isozymes was the most significant, in poisoning by inhibitors (O'Brien, 1967).

Thus, from the review presented above, it is clear that in spite of the established importance of the nervous system as a target site very little information is available on the
impact of pesticides on developing CNS in mammals. Since nervous system is one of the most susceptible and vulnerable portions of the body of highly developed organisms, poisoning the nervous system is the quickest and surest way of chemically upsetting the regular body mechanisms (O'Brien, 1967). An ability to measure the effect of neurotoxicants during critical period of CNS development is to establish the precise mechanism by which they act leading to neuronal inefficiency. Hence the present study has been proposed and the impact of sublethal concentration of the organophosphorous insecticide, methyl parathion has been followed on the developing CNS in a mammal during the critical stage of brain development.

MATERIALS AND METHODS

Developing albino rat pups (2nd and 7th day-old) were injected with sublethal doses of methyl parathion. They were decapitated 48hrs after insecticide administration and discrete areas of brain and spinal cord were separated for analysis at 0°C as described in general materials and methods.

Acetyl cholinesterase activity (Acetyl choline acetyl hydrolase, AChE, E.C. 3.1.1.7)

Discrete areas of brain and spinal cord were separated out and homogenates (10% w/v) were prepared in 0.02M sodium-
potassium phosphate buffer (pH 7.0). The activity levels of AChE in the uncentrifuged samples of brain and spinal cord of control and methyl parathion administered rat pups were measured following the colorimetric method of Hestrin (1949).

The incubation mixture consisted of 0.1ml of 10% homogenate, 0.9ml of sodium-potassium phosphate buffer (pH 7.0), 1ml of buffered substrate, 2ml of hydroxylamine hydrochloride, 1ml of 4N HCl and 1ml of 0.37M FeCl₃ in 0.1N HCl (All in the final volume of 6ml).

ACh content was determined by the colorimetric procedure (Hestrin, 1949).

Isozymes of AChE

The molecular heterogeneity and sub-unit pattern of AChE was detected in different parts of brain and spinal cord of control and methyl parathion administered 7-day-old rat pups. using 7.5% polyacrylamide gel electrophoresis developed by Davis (1964) and Ornstein (1964). 25% (w/v) homogenates of the tissues were prepared in deionised distilled water and centrifuged at 7,000rpm for 1hr at 4°C and supernatants were used for electrophoresis.

Gels were cast in running tubes by polymerisation of monomers as suggested by Ornstein (1964). Raymond's buffer
system (of pH 8.5) consisting of 5.5g of Tris (hydroxymethyl aminomethane), 2.5g of boric acid, 1g of EDTA in 1 litre with deionised distilled water was used (Ismail Sudderuddin, 1973). Prior to use, the gels were allowed to mature for two hrs (Ismail Sudderuddin, 1973).

10μl (consists of 100μg of protein) of the samples were pipetted on to folded filter papers (0.5x0.25mm) in each tube. The remaining space was filled with buffer (Ismail Sudderuddin, 1973). The tubes were run at 6mA/tube (DC power supply Type CM07/02 Sr.No. 111; Toshniwal) for 2hrs, using Raymonds buffer system (of pH 8.5).

At the end of the electrophoretic run, the gels were removed from the glass tubes and incubated in Raymonds buffer for 10min. After 10min the gels were washed twice with double distilled water and were stained with staining solution for 30min, as described by Gomeri (1952).

The staining solution consisted of 5mg of acetylthiocholine iodide, 0.5ml of 100mM sodium citrate buffer, 1ml of 30mM CuSO₄ and 1ml of 5mM Potassium ferricyanide in a total volume of 10ml of Phosphate buffer (pH 7.0). The incubation carried out at 37°C for 30min. After incubation gels were rinsed twice in distilled water and incubated once again in diluted yellow ammonium sulfide solution till clear appearance of bands.
The destained gels were passed under gel densitometer (Biochem Instrument, India) using green filter. The readings were recorded for every mm of the gel distance moved and the graphs were prepared.

Assay of choline acetyltransferase activity
(ChAC/ChAT, acetyl-CoA; Choline-o-acetyltransferase E.C. 2.3.1.6)

Choline acetyltransferase activity was determined by the procedure described by Morris (1967).

Tissues were extracted with 0.05M thioglycolytic acid neutralised to pH 6.5 with 1N KOH in 0.154M NaCl and 10% homogenate was prepared. A final volume of 1ml of incubation medium consisted of choline chloride (12mM), NaCl or KCl (300mM), eserine sulphate (0.26mM), phosphate citrate buffer pH 7.5, 40mM, Acetyl CoA 0.35mM, and the homogenate. The incubation medium was adjusted to pH 7.5 with 0.3N NaOH and warmed in a 37°C water-bath for 2min before addition of CoA. After the period of incubation for 15min at 37°C the ACh content was estimated by the colorimetric method of Hestrin (1949).

Isozymes of ChAT

The multiple molecular forms of choline acetyltransferase in discrete regions of brain and spinal cord of
control and methyl parathion administered 2 and 7-day-old animals were resolved employing polyacrylamide gel electrophoresis developed by Ornstein (1964) and Davis (1964).

The gel matrix was prepared as described earlier. Twenty percent (w/v) homogenate of cortex, brain stem and spinal cord prepared in 0.05M, phosphate buffer, pH 7.1. The samples were centrifuged at 7,000rpm for 30min at 4°C. 100µl of each sample was applied to each gel for the separation of isozymes.

The electrophoresis was run in 0.05M tris-HCl buffer pH 8.9 at a voltage of 100v and 3mA current per tube (DC power supply type CM 07/02 Sr.No. 111, toshniwal). The electrophoresis was carried out at room temperature for about 4-5hrs.

After the run, the gels were removed from the glass tubes and washed in distilled water. Gels were then cut into 2mm bits and each bit was homogenised in 1ml of distilled water and centrifuged at 4,000rpm for 30min at 4°C. The supernatant was used to estimate the choline acetyltransferase activity as described earlier.

The data obtained is plotted graphically and isozymic profiles of control and experimental animals were compared.
RESULTS

The data after statistical analysis is presented in the tables (13-18) and figs. (11-15). From the data it is clear that remarkable changes in the activity levels of enzyme AChE and the content of neurotransmitter ACh was observed on administration of sublethal doses of organophosphorous pesticide methyl parathion in different regions of brain and spinal cord of 2nd and 7th day-old developing rat pups. Characteristically 2-day-old pups showed lower activity compared to 7 days old animals.

Table (13-14) gives the changes occurring in the activity levels of AChE in different regions of brain and spinal cord of control and methyl parathion administered 2 and 7-day-old rat pups respectively.

In 2-day-old rat pups, a remarkable decrease in AChE activity was observed in all the regions like cerebral cortex, brain stem and spinal cord, as a function of methyl parathion toxicity. The percent of change observed was -31.14, -39.99, and -24.80, respectively (Table 13). Even in 7-day-old animals the activity levels of AChE showed a decreased activity in Cortex (-44.88%), brain stem (-37.64%) and spinal cord (-31.86%) (Table 14).

A significant accumulation of ACh content in the CNS was found on methyl parathion administration in both age
groups studied. From the tables (15-16) it is clear that ACh content increased considerably. The percent increase in 2-day-old animals was +43.47, +36.11, and +33.33 for cortex, brain stem and spinal cord respectively (Table 15).

In 7 day-old animals the percent accumulation of ACh was comparatively high when compared to 2-day-old animals (Table 16). The percent increase in 7-day-old animals was +38.02 (cortex), +41.98 (brain stem), and +36.92 (spinal cord) respectively. Thus an inverse relationship exists between the content of ACh and AChE activity in the nervous tissues both in control and methyl parathion treated animals (Tables 13-16).

The isozymic profiles of AChE of control and methyl parathion treated animals are depicted in figs. (11-13). It is clear from the figs. (11-13) that a characteristic disruption in the isozymic spectrum of AChE has occurred on administration of sublethal concentrations of methyl parathion. It is also clear from the figs. (11-13) that a considerable inhibition in the activity of isozymes has occurred due to methyl parathion administration. However, the relative electromobility of the isozymes was not altered during pesticide poisoning.

In the cerebral cortex two isozymal forms showed complete abolition and the remaining isozymal forms showed
considerable inhibition due to methyl parathion toxicity (Fig.11).

In the brain stem, one isozymic form got abolished and remaining isozymal forms exhibited a characteristic inhibition during methyl parathion toxicity. The slowest moving isozymal form showed a remarkable inhibition in the activity level on methyl parathion administration (Fig. 12).

In the spinal cord one isozymal form was completely absent whereas the remaining isozymal forms were inhibited considerably due to methyl parathion toxicity (Fig.13).

Thus considerable disruptions of the isozymic spectrum of AChE activity was observed on administration of methyl parathion.

From the data presented in tables (17-18) it is clear that a significant decrease was observed in the activity levels of the enzyme choline acetyltransferase (ChAT) in cortex, brain stem and spinal cord.

In 2-day-old animals methyl parathion decreased considerably the activity of ChAT -24.42%, -22.60%, and -26.91% in cortex, brain stem and spinal cord respectively (Table 17). In 7-day-old animals the percent decrease in ChAT activity was -23.08 (in cortex), -21.59% (in brain stem) and -15.15% (in spinal cord).
The isozymic profiles of ChAT of the cortex, brain stem and spinal cord of control and methyl parathion administered developing rats are depicted in figs. (14-15). It is clear from the figs. that methyl parathion considerably affected the activity levels of isozymic spectrum (Figs. 14-15). Four isozymal forms of ChAT resolved in cortex and three isozymal forms in brain stem and spinal cord (Fig.14-15). It is clear from the figs. (14-15) that a profound suppression of the different forms of ChAT occurred as a consequence of methyl parathion exposure. The relative electromobility of the isozymes was also altered to some extent during pesticide poisoning (Fig. 14-15).

In the cerebral cortex of 2-day-old rat pups, one isozymal form got abolished completely and the fastest moving isozymal form showed considerable stimulation in the activity on methyl parathion administration (Fig.14A).

In the brain stem of 2-day-old rat pups, three isozymal forms could be resolved both in control and methyl parathion treated animals. One, out of the three isozymes, showed slight stimulation in the activity on methyl parathion administration and fast moving forms were considerably inhibited (Fig. 14B).

In the spinal cord of 2-day-old rat pups, induction of a new isozymal form and suppression in the activity of other
three isozymal forms was observed as a function of methyl parathion administration (Fig.14C).

In 7-day-old animals, methyl parathion affected considerably, the activity levels of the isozymes (Fig.15). In the cerebral cortex (Fig.15A), one isozymal form was abolished completely (fastest moving isozymal form) and the activity of other isozymal forms were stimulated on methyl parathion administration. The other two slow moving isozymal forms exhibited suppressed activity due to methyl parathion toxicity (Fig.15A).

In the brain stem of 7-day-old animals, one out of the four isozymes showed slight stimulation with altered electromobility, whereas the other fast and slow moving isozymal forms showed considerable inhibition in their activity on methyl parathion treatment (Fig.15B).

In the spinal cord of 7-day-old animals, only 2 isozymal forms could be resolved both in control and methyl parathion treated animals. On methyl parathion exposure both the isozymal forms showed inhibition in the activity (Fig. 15C).

Thus considerable disruptions of the isozymic pattern of ChAT was observed on inducing methyl parathion toxicity by sublethal concentration.
DISCUSSION

The present study clearly illustrates that the normal functioning of the CNS has been significantly altered on methyl parathion administration. This is evidenced by changes occurring in the level of ACh and activity levels of ChAT and AChE, in the developing CNS. Inhibition of target enzyme AChE disturbs the normal functioning of nervous system leading to severe damages in the organism (Eto, 1974). In the present investigation a significant decline in the activity level of AChE was observed in all the compartments of CNS studied. Earlier investigators also reported a similar decline in the activity level of AChE in the nervous tissues by methyl parathion in paeneid prawn (Srinivasalu Reddy et al., 1987). There is a strong evidence to show that methyl parathion is metabolically altered to a more active AChE inhibitor by oxidation of the thiosulphur atom (P=S) to an oxygen atom (P=O). The resulting oxygen analogue (methyl paraxon) is several times more potent in inhibiting AChE (Benke et al., 1974).

From the results obtained in the present study it is clear that methyl parathion caused a significant accumulation of ACh in all the regions of CNS (Tables 15-16). This can be attributed to the inhibition of AChE the enzyme responsible for the hydrolysis of ACh. Earlier
experiments showed that ACh levels may raise to 260% of the normal insects, when treated with organophosphorous insecticide (Smallman and Fisher, 1958). It is a well established fact that organophosphorous insecticides inhibit AChE causing less facilitation and blockage of cholinergic transmission due to accumulation of ACh (Narahashi, 1976). The phosphorus atom of methyl parathion phosphorylates the active site viz., the esteratic site of cholinesterase enzyme (Eto, 1974). This active site being surrounded by hydrophobic areas can interact with appropriate groups on the inhibitor. The phosphorylated enzyme is irreversibly inhibited (Eto, 1974). Hence there is an accumulation of ACh in the synapses resulting in the disruption of normal nervous functioning.

The present study also demonstrates that ChAT activity gets inhibited by methyl parathion in all the compartments of CNS studied (Tables 17-18).

It is clear from the data presented in tables (13-14) and figures (11-13) that remarkable inhibition of AChE occurred in cortex when compared to brain stem and spinal cord of the CNS. This suggests that various regions of the CNS may contain AChE forms with different degrees of sensitivity to methyl parathion (Maflah et al., 1984; Muller et al., 1984; Massoulia and Bon, 1972).
Thus the remarkable inhibition of AChE activity and a parallel accumulation of ACh observed during the present investigation may be responsible for the severe impairment of coordination and disruption of normal functioning during methyl parathion toxicosis. Earlier studies also indicated a substantial inhibition of the activity of AChE in different compartments of the human brain during parathion toxicity (Finkelstein et al., 1988). In vitro effects of malathion on sheep brain AChE revealed a mixed type of inhibition with a decreased catalytic efficiency of the enzyme AChE (Mohanachari et al., 1980).

The physiological significance of neuronal transmission is well known (Himwich and Agarwal, 1969). The activity level of AChE is related to the electrical activity and electrical activity to potentiality of cell response (Murali Krishnadas and Venkatachari, 1968). In view of this it can be said that methyl parathion administration has decreased the potentiality of cell response in developing rat pups. This in turn affects the synaptic activity leading to dysfunction of the nervous system.

The AChE and nervous tissue proteins are found to be involved in learning and memory processes (Karunakaran and Selvarajan, 1987). Studies on AChE activity and protein levels and their relationship would therefore reveal the
patterns of neuronal development, acquisition of abilities, storage of information and behavioural adaptations (Karunakaran and Selvarajan, 1987). In the present study on CNS the protein content was found to increase on methyl parathion exposure (Tables 10-11). Hence it can be said that methyl parathion administration brings about a stimulation of the protein synthetic machinery. It is probable that some of the new enzymes/isozymes (proteins) thus synthesized may have deleterious effects leading to decreased neuronal efficiency. The characteristic alterations in the heterogeneity of AChE observed on methyl parathion administration in the present study corroborates this.

Isozymes of AChE

The alterations found in isozymic profile of control and methyl parathion administered animals clearly points out to a characteristic disruption in the isozymic spectrum. It is clear that in general slow moving molecular forms got affected to a greater extent. The forms which were abolished were slow moving. Thus the isozymes which are inhibited considerably appear to be highly susceptible to the effect of methyl parathion. This indicates that methyl parathion is a potent inhibitor of the isozymes of AChE in the developing albino rats. This renders the animal physiologically less efficient.
In a series of elegant animal studies, Michaleck et al. (1982) demonstrated regional differences in the molecular forms of AChE in the brain and selective inhibition and recovery of soluble and membrane bound forms following intoxication by organophosphates. Michaleck et al. (1981) and Mineguz et al. (1981) measured the molecular forms in soluble fraction and in total homogenates of cerebral cortex, hippocampus and striatum, three brain regions with widely different total AChE activity. The overall extent of AChE inhibition by DFP and paraoxon (Px) was highly correlated with total enzyme activity. They found that the molecular forms were inhibited to different levels by both organophosphates. Selective inhibition and recovery of AChE molecular forms following organophosphate treatment has also been demonstrated in chick embryo muscle cells (Wilson and Walker, 1974), mouse neuroblastoma cells (Rieger et al., 1976) and rat retina (Davis and Agranoff, 1968). In the present study also methyl parathion was found to affect the AChE activity in the discrete areas of the CNS differentially. For instance, cortex was effected to a higher degree compared to brain stem and spinal cord.

The activity level of enzyme ChAT also decreased significantly in the CNS of developing rat pups on methyl parathion administration (Tables 17-18).
ChAT is involved in the transfer of acetyl groups from coenzyme A to choline. The two required substrates are choline and acetyl CoA. Investigations of Hoff et al. (1984) and Hasuokobayashi et al. (1988) suggest that ACh synthesis and high affinity choline may be in a suppressed state when ACh concentration increases and AChE activity decreases in the brain cholinergic system of DDVP poisoned rats. The high affinity choline uptake is generally thought to be a rate limiting step for ACh synthesis. This leads to accumulation of ACh. ACh thus accumulated can be envisaged to cause an eventual block in the neurotransmission.

In view of this it can be presumed that organophosphorous compounds act as nerve poisons by blocking synaptic transmission in cholinergic parts of the nervous system.

Isozymes of ChAT

The isozymic profile of ChAT of control and methyl parathion administered animals clearly showed a characteristic disruption (Figs.14-15). In general, four isozymal forms of ChAT in cortex and three isozymal forms each in brain stem and spinal cord were resolved. A characteristic abolition, induction, suppression and also stimulation of the functional isozymal forms were observed.
as a result of methyl parathion administration (Figs. 14-15). It is evident from figs. (14-15) that the electromobility of isozymes was not altered during methyl parathion poisoning. It appears that different brain regions have a specific pattern and distribution of isozymal forms of ChAT (Figs. 14-15).

Thus the alterations observed in the isozymic spectrum of ChAT on methyl parathion injection indicate a characteristic disruption in the isozymic spectrum as a function of methyl parathion administration. In general, a remarkable and characteristic suppression of the isozymic profiles was found in all the compartments of CNS studied on methyl parathion exposure. It is probable that the decline in the total activity level of ChAT found (Tables 17-18) is due to the inhibition of the activity of all the isozymes. This renders the animal physiologically less efficient as cholinergic transmission is profoundly affected in the cortex, brain stem and spinal cord during methyl parathion toxicosis.

Thus the present investigation clearly depicts a correlation between the altered functional dynamics of the CNS and extent of toxicity induced by methyl parathion. This may lead to the functional debility during the critical state of CNS development.
The disruption in the cholinergic transmission mechanism found as a function of methyl parathion administration in the present study appears to be the causing factor in inducing biochemical lesions in the developing mammalian brain.
Table 13: Changes in the activity levels of acetyl cholinesterase (AChE) in different regions of brain and spinal cord of control and methyl parathion treated (MPT) 2-day-old rats pups

<table>
<thead>
<tr>
<th>Region of the tissue</th>
<th>Control</th>
<th>MPT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>29.83±2.87</td>
<td>20.54±1.02</td>
<td>-31.14 *</td>
</tr>
<tr>
<td>Brain stem</td>
<td>36.96±1.97</td>
<td>22.18±1.25</td>
<td>-39.99 *</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>17.74±1.13</td>
<td>13.34±1.76</td>
<td>-24.80 *</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D. of 6 observations.

Values are expressed as μmoles of ACh hydrolysed/mg of protein/min.

Sign '-' indicates decrease over controls.

* P > 0.001.
Table 14: Changes in the activity levels of acetyl cholinesterase (AChE) in different regions of brain and spinal cord of control and methyl parathion treated 7-day-old rat pups

<table>
<thead>
<tr>
<th>Region of the tissue</th>
<th>Control</th>
<th>MPT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>39.42±1.21</td>
<td>21.73±2.12</td>
<td>-44.88 *</td>
</tr>
<tr>
<td>Brain stem</td>
<td>56.45±2.89</td>
<td>35.20±2.64</td>
<td>-37.64 *</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>27.9±1.16</td>
<td>19.01±1.50</td>
<td>-31.86 *</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D. of 6 observations.
Values are expressed as µmoles of ACh hydrolysed/mg of protein/min.
Sign '-' indicates decrease over the controls.
* P > 0.001.
Table 15: Changes in the levels of acetyl choline (ACh) in different regions of brain and spinal cord of control and methyl parathion treated 2-day-old rat pups

<table>
<thead>
<tr>
<th>Region of the tissue</th>
<th>Control</th>
<th>MPT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>0.69±0.07</td>
<td>0.99±0.04</td>
<td>+43.47 *</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.72±0.09</td>
<td>0.98±0.07</td>
<td>+36.11 @</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.51±0.05</td>
<td>0.68±0.02</td>
<td>+33.33 @</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D. of 6 observations.

Values are expressed as μmoles of ACh/gm wet wt. of tissue.

Sign '+' indicates increase over the controls.

* P < 0.05;  @ P < 0.01.
Table 16: Changes in the levels of acetyl choline (ACh) in different regions of brain and spinal cord of control and methyl parathion treated 7-day-old rat pups

<table>
<thead>
<tr>
<th>Region of the tissue</th>
<th>Control</th>
<th>MPT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>0.71+0.02</td>
<td>0.98+0.06</td>
<td>+38.02 #</td>
</tr>
<tr>
<td>Brain stem</td>
<td>1.31+0.09</td>
<td>1.86+0.08</td>
<td>+41.98 #</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.65+0.02</td>
<td>0.89+0.04</td>
<td>+36.92 #</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D. of 6 observations.

Values are expressed as μmoles of ACh/gm wet wt. of tissue.

Sign '+' indicates an increase over the controls.

# P < 0.05.
Plate 1A: Discontinuous polyacrylamide gel electrophoresis of Acetyl cholinesterase (AChE) isozymes in discrete areas of central nervous system.

Photograph of stained gels representing the isozymic pattern of AChE in cortex, brain stem and spinal cord of control and methyl parathion treated rat pups.

A. Control cortex
B. MPT cortex
C. Control brain stem
D. MPT brain stem
E. Control spinal cord
F. MPT spinal cord
Fig. 11: Polyacrylamide gel electrophoresis for the isozymes of Acetyl cholinesterase in Raymonds buffer, pH 8.5 in the cerebral cortex of 7-day-old rat pups.

Lines with closed circles represent the electrophoretic pattern of AChE in the control cerebral cortex.

Lines with open circles represent the electrophoretic pattern of AChE in the methyl parathion treated cerebral cortex.

Representation of the gel is semidiagramatic.
FIGURE 11

Absorbancy

Distance in mm

C

MPT

10  20  30  40  70

FIGURE 11
Fig. 12: Polyacrylamide gel electrophoresis for the isozymes of Acetyl cholinesterase in Raymonds buffer, pH 8.5 in the brain stem of 7-day-old rat pups.

Lines with closed circles represent the electrophoretic pattern of AChE in the control brain stem.

Lines with open circles represent the electrophoretic pattern of AChE in the methyl parathion treated brain stem.

Representation of the gel is semidiagramatic.
FIGURE 12

Distance in mm

Absorbancy

Distance in mm

C

MPT

FIGURE 12
Fig. 13: Polyacrylamide gel electrophoresis for the isozymes of Acetyl cholinesterase in Raymonds buffer, pH 8.5 in the spinal cord of 7-day-old rat pups.

Lines with closed circles represent the electrophoretic pattern of AChE in the control spinal cord.

Lines with open circles represent the electrophoretic pattern of AChE in the methyl parathion treated spinal cord.

Representation of the gel is semidiagramatic.
FIGURE 13
Table 17: Changes in the activity levels of ChAT in different regions of brain and spinal cord of control and methyl parathion treated 2-day-old rat pups

<table>
<thead>
<tr>
<th>Region of the tissue</th>
<th>Control</th>
<th>MPT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>6.84±0.20</td>
<td>5.17±0.31</td>
<td>-24.42 #</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.15±0.31</td>
<td>4.76±0.18</td>
<td>-22.60 #</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>6.39±0.57</td>
<td>4.67±0.16</td>
<td>-26.91 #</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D. of 6 observations.

Values are expressed as μmoles of the ACh/gm wet wt. of tissue/hr.

Sign '-' indicates decreases over controls.

# P < 0.005
Table 18: Changes in the activity levels of ChAT in different regions of brain and spinal cord of control and methyl parathion treated 7-day-old rat pups

<table>
<thead>
<tr>
<th>Region of the tissue</th>
<th>Control</th>
<th>MPT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>9.92 ± 0.16</td>
<td>7.63 ± 0.31</td>
<td>-23.08 *</td>
</tr>
<tr>
<td>Brain stem</td>
<td>8.94 ± 0.31</td>
<td>7.01 ± 0.16</td>
<td>-21.59 @</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>8.12 ± 0.39</td>
<td>0.89 ± 0.23</td>
<td>-15.15 @</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D. of 6 observations.
Values are expressed as μmoles of ACh/gm wet wt. of tissue/hr.
Sign '-' indicates decrease over the controls.
* P > 0.001;  @ P < 0.005.
Fig. 14A: Polyacrylamide gel electrophoresis for the isozymes of choline acetyl transferase (ChAT) in 0.05M Tris-HCl buffer, pH 8.9 in the cerebral cortex of 2-day-old rat pups.

Solid lines with closed circles represent the electrophoretic pattern of ChAT in the control cerebral cortex.

Broken lines with open circles represent the electrophoretic pattern of ChAT in the methyl parathion treated cerebral cortex.
FIG. 14A

CONTROL

EXPERIMENTAL

ACTIVITY

DISTANCE (mm)
Fig. 14B: Polyacrylamide gel electrophoresis for the isozymes of choline acetyl transferase (ChAT) in 0.05M Tris-HCl buffer, pH 8.9 in the brain stem of 2-day-old rat pups.

Solid lines with closed circles represent the electrophoretic pattern of ChAT in the control brain stem.

Broken lines with open circles represent the electrophoretic pattern of ChAT in the methyl parathion treated brain stem.
FIG. 14B

- - - - CONTROL

○ - - ○ EXPERIMENTAL

ACTIVITY

DISTANCE (mm)
Fig.14C: Polyacrylamide gel electrophoresis for the isozymes of choline acetyl transferase (ChAT) in 0.05M Tris-HCl buffer, pH 8.9 in the spinal cord of 2-day-old rat pups.

Solid lines with closed circles represent the electrophoretic pattern of ChAT in the control spinal cord.

Broken lines with open circles represent the electrophoretic pattern of ChAT in the methyl parathion treated spinal cord.
Fig. 15B: Polyacrylamide gel electrophoresis for the isozymes of choline acetyl transferase (ChAT) in 0.05M Tris-HCl buffer, pH 8.9 in the brain stem of 7-day-old rat pups.

Solid lines with closed circles represent the electrophoretic pattern of ChAT in the control brain stem.

Broken lines with open circles represent the electrophoretic pattern of ChAT in the methyl parathion treated brain stem.
Fig. 15C: Polyacrylamide gel electrophoresis for the isozymes of choline acetyl transferase (ChAT) in 0.05M Tris-HCl buffer, pH 8.9 in the spinal cord of 7-day-old rat pups.

Solid lines with closed circles represent the electrophoretic pattern of ChAT in the control spinal cord.

Broken lines with open circles represent the electrophoretic pattern of ChAT in the methyl parathion treated spinal cord.