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METHYL PARATHION INDUCED REGIONAL ALTERATIONS IN THE REGULATORY PROTEINS DURING CRITICAL STAGE OF CENTRAL NERVOUS SYSTEM DEVELOPMENT IN ALBINO RAT PUPS

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Abstract : Sublethal doses of methyl parathion (O,O-dimethyl-O-nitrophenyl-thiophosphate) injected intraperitoneally to 15 and 21 day old rat pups induced regional alterations in the central nervous system (CNS) in the levels of total RNA, total proteins, modulatory protein Calmodulin (CaM), in the activity levels of memberane bound enzyme Ca^{2+} - ATPase and phospholipids. Levels of RNA and total proteins increased considerably in 15 days old methyl parathion treated (MPT) rat pups. Contrary to this the RNA and total protein content exhibited remarkable decrease in 21 day old melthyl parathion treated animals.

Calmodulin level showed an increase in cerebral cortex and brain stem and decrease in cerebellum and spinal cord in 15 day old methyl parathion treated rat pups. Whereas the level of Calmodulin decreased in cerebral cortex and cerebellum and increased in brain stem and spinal cord in 21 day old methyl parathion treated rat pups. Activity levels of calcium dependent ATPase showed significant inhibition in all the regions of Central Nervous System (CNS) of 15 and 21 day old methyl parathion treated rat pups. Phospholipids showed a general increase in all the regions of Central Nervous System on methyl parathion exposure. In the light of these observations, it has been suggested that the molecular regulatory mechanisms involving Ca^{2+}/CaM are rendered inefficient due to toxic impact of methyl parathion.

Key words : methyl parathion regulatory protein calmodulin Ca^{2+}-ATPase cerebral cortex brain stem crebellum spinal cord molecular regulatory mechanism neurotransmitter release

INTRODUCTION

Organophosphorous insecticides (OP) have been shown to affect protein metabolism in addition to their specific inhibition of cholinesterase enzyme (1). In the literature surveyed so far pesticides were shown to either decrease or increase the protein synthetic machinery. Shakuntala and Srihari (2) found a decrease in total protein levels in livers of vacor treated Bandicota bengalensis. Malinska *et al* (3) reported decreased protein synthesis in the brain of dicholorvos (DDVP) treated suckling rabbits. Contrary to the decrease in protein synthesis cited in the above references Siva Prasad Rao and Ramana Rao (4) reported an increase in the total proteins and free amino acid content in muscle, gill and liver tissue of fish, Tilapia mossambica on methyl parathion exposure. Naiyara Yasmeen (5) also reported a similar increase in protein synthesis in the brain of developing amphibian tadpoles exposed to methyl parathion. From this short review it is evident that the response of proteins to insecticide stress has not been clearly elucidated.

Brain proteins are known to exist in a dynamic state (6). Hence it appears to be a challenge to study the effect of an OP insecticide, methyl parathion, particularly during the critical stage of Central Nervous System (CNS) development. Hence the present study was proposed in view of the increasing evidence of pesticide-protein interaction and its relevance to the mode of action of insecticides (7). The effect of methyl parathion administration on Calcium binding regulatory protein, Calmodulin (CaM) and on the activity level of membrane bound enzyme Ca^{2+}-ATPase was followed, keeping in view, the involvement of calmodulin in the neurotransmitter release and

cellular regulation (8). The role of Ca^{2+}- ATPase in ionic transport (9) has been followed in view of the Ca^{2+}/CaM regulation of this enzyme. Alterations in phospholipids on methyl parathion treatment have been followed in view of the importance of phospholipids for maintaining structural integrity of the membrane.

METHODS

Developing albino rat pups (15 and 21 days post natal) were used for the study. Sublethal doses (0.66 mg/kg and 1 mg/kg body weight, respectively for 15 and 21 days old pups) of methyl parathion (0-0-dimethyl-0-nitrophenyl-thiophosphate, EC. 50%, Bayer Ltd., India) was administered intraperitoneally to experimental animals. Controls were injected with distilled water. Animals were decapitated 48 hrs after the respective injections. Different regions of the Central Nervous System (CNS) viz., Cerebral cortex, Brain stem, Cerebellum and Spinal cord were dissected and separated at 0°C and were used immediately for the study. Total proteins and calmodulin levels were estimated colorimetrically by the method of Lowry et al (10). Total Ribonucleic Acid (RNA) was extracted by employing the method of Schmidt-Thanhauser- Schneider (11) and estimated by Orcinol colour reaction following the colorimetric procedure as described by Glick (12). Calmodulin (CaM) was extracted following the procedure of Watterson et al (13) and was resolved by polyacrylamide gel electrophoresis (PAGE). One gram of tissue was homogenized in 2 vols of 0.1M Sodium acetate buffer having 0.001M 2-meracaptoethanol and 0-001M EDTA (pH 7.2) and centrifuged at 10,000 rpm for 1 hour at 4°C. The supernatant fraction was heated in boiling water bath, the temperature was quickly brought to 95°C. After 5 min, the solution was cooled in an ice slurry. The coagulated proteins were removed by centrifugation at 10,000 rpm. The supernatant and standard calmodulin solution (Sigma Chemical Company) was subjected to PAGE (12.5%). Electrophoresis was performed in the presence of 8M urea to get a defined single calmodulin band. The gels were stained with 0.25% (W/V) Coomassie brilliant blue R250 in 50% (V/V) methanol and 10% (V/V) glacial acetic acid for 1 hour at 37°C and destained in 7.5% (V/V) acetic acid and 5% (V/V) methanol at 37°C. The gels were scanned under a transmission of densitometer and readings were presented graphically. Ca^{2+}-ATPase activity was determined by the method of Matsumara and Narahashi (14) as modified by Yamaguchi et al (15). The incubation medium (14) as modified by Yamaguchi et al (15) was used. The incubation medium for the assy included 2mM $CaCl_2$, 25mM Tris-HCl (pH 7.4), 0.2M sucrose and 0.1mM Ouabain. The final vol of incubation medium was 1.5 ml and included approximately 500 µg of homogenate and 3mM Tris-ATP. Phospholipids were determined by the method given by Zilversmit and Davis (16) and the colour developed was measured following the method of Fiske and Subbarow (17).

Total RNA, total proteins and phospholipids were expressed as mg/gm weight tissue. Calmodulin is expressed as mgm/gm protein. Ca^{2+}-ATPase activity is expressed as µmoles pi liberated/mg protein/hr.

RESULTS AND DISCUSSION

The data after statistical analyses are presented in Tables (I-V) and figures 1 and 2.

Figures 1 and 2 represent the electrophoretic and densitometric profiles of Calmodulin resolved on PAGE (12.5%) in control and methyl parathion administered developing rats. Tables (I, II, III, IV, V) show the regional alterations of total RNA, total proteins, modulatory protein, calmodulin (CaM), activity levels of membrane bound enzyme

TABLE I : Alterations in the levels of total Ribonucleic Acid (RNA) in discrete regions of CNS, control and MPT 15-day and 21 day old rat pups.

Regions	*Control*	*MPT*	*%Change*	*Control*	*MPT*	*%Change*
	15 days			*21 days*		
Cerebral cortex	1.7 ±0.067	1.8 ±0.0836	+9.6 P<0.1	1.8 ±0.1	1.6 ±0.098	−11.1 P<0.1
Brain stem	1.4 ±0.096	1.7 ±0.107	+22.5 P<0.05	1.5 ±0.117	1.3 ±0.096	−13.3 P<0.1
Cerebellum	1.5 ±0.10	1.9 ±0.067	+22.1 P<0.05	1.7 ±0.104	1.5 ±0.087	−11.8 P<0.1
Spinal cord	1.4 ±0.1	1.6 ±0.067	+18.1 P<0.05	1.5 ±0.1	1.1 ±0.108	−26.2 P<0.05

Values (Mean ± S.D of 5 observations) are expressed as mgm/gm wet wt. Sign + indicates increase and sign - indicates decrease over the control.

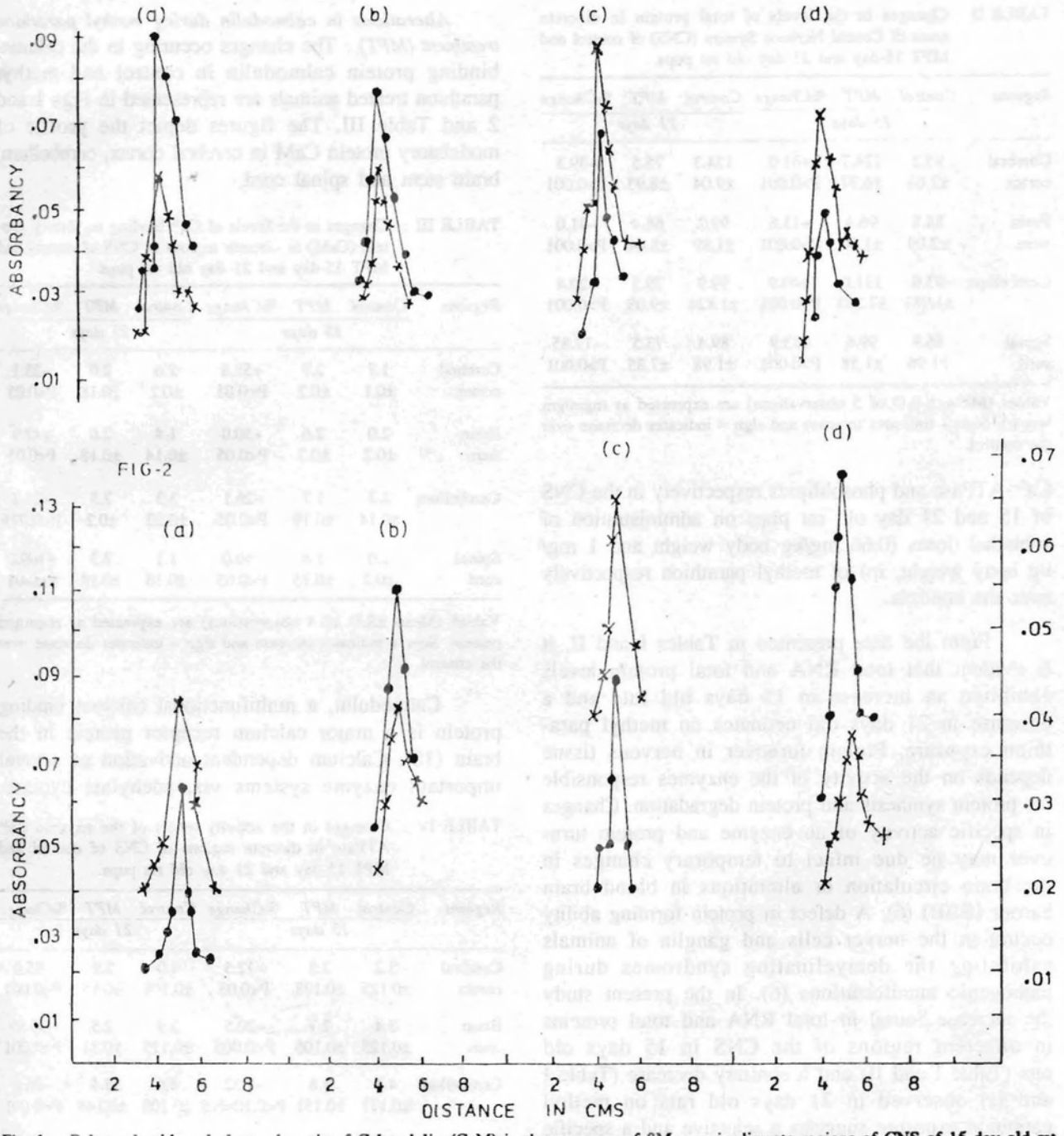

Fig. 1 : Polyacrylamide gel electrophoresis of Calmodulin (CaM) in the presence of 8M urea in discrete regions of CNS of 15 day old rat pups.

Fig. 2 : Polyacrylamide gel electrophoresis of Calmodulin (CaM) in the presence of 8M urea in discrete regions of CNS of 21 day old rat pups.

(a) Cerebral cortex, (b) Brain stem, (c) Cerebellum, (d) Spinal cord

Lines with crosses represent the electrophoretic pattern of CaM in the control rat pups.

Lines with closed circles represent the electrophoretic pattern of CaM in the Methyl Parathion treated (MPT) rat pups.

TABLE II : Changes in the levels of total protein in discrete areas of Central Nervous System (CNS) of control and MPT 15-day and 21 day old rat pups.

Regions	*Control*	*MPT*	*%Change*	*Control*	*MPT*	*%Change*
	15 days			*21 days*		
Corebral cortex	95.2 ±2.68	124.7 ±6.77	+31.0 P>0.001	124.3 ±9.04	75.5 ±8.93	-39.3 P>0.001
Brain stem	84.8 ±2.09	96.4 ±1.89	+13.6 P>0.001	99.2 ±1.89	68.4 ±8.64	-31.0 P>0.001
Cerebellum	93.0 ±1.981	131.0 ±7.363	+40.9 P>0.001	99.9 ±1.824	79.5 ±9.08	-20.4 P>0.001
Spinal cord	86.9 ±1.96	99.6 ±1.58	+13.9 P>0.001	89.4 ±1.98	73.5 ±7.85	-17.85 P>0.001

Values (Mean ± S.D of 5 observations) are expressed as mgm/gm wet wt. Sign + indicates increase and sign – indicates decrease over the control.

Ca^{2+}-ATPase and phosphlipids respectively in the CNS of 15 and 21 day old rat pups on administration of sublethal doses (0.66 mg/kg body weight and 1 mg/kg body weight, ip) of methyl parathion respectively over the controls.

From the data presented in Tables I and II, it is evident that total RNA and total protein levels exhibited an increase in 15 days old rats and a decrease in 21 days old neonates on methyl parathion exposure. Protein turnover in nervous tissue depends on the activity of the enzymes responsible for protein synthesis and protein degradation. Changes in specific activity of an enzyme and protein turnover may be due infact to temporary changes in the brain circulation or alterations in blood brain barrier (BBB) (6). A defect in protein forming ability occurs in the nerver cells and ganglia of animals exhibiting the demyelinating syndromes during pathogenic manifestations (6). In the present study the increase found in total RNA and total proteins in different regions of the CNS in 15 days old rats (Table I and II) and a contrary decrease (Table I and II) observed in 21 days old rats on methyl parathion exposure suggests a selective and a specific response of BBB of these regions to methyl parathion administration in the 2nd and 3rd week (postnatal) of neonatal development caused by the toxic impact of methyl parathion in the developing mamalian brain.

Alterations in calmodulin during methyl parathion treatment (MPT) : The changes occuring in the calcium binding protein calmodulin in control and methyl parathion treated animals are represented in Figs 1 and 2 and Table III. The figures depict the profile of modulatory protein CaM in cerebral cortex, cerebellum, brain stem and spinal cord.

TABLE III : Changes in the levels of Ca^{2+} binding regulatory protein (CaM) in discrete regions of CNS of control and MPT 15-day and 21 day old rat pups.

Regions	*Control*	*MPT*	*%Change*	*Control*	*MPT*	*%Change*
	15 days			*21 days*		
Cerebral cortex	1.7 ±0.1	2.7 ±0.2	+58.8 P<0.01	2.6 ±0.2	2.0 ±0.18	-23.1 P<0.05
Brain stem	2.0 ±0.2	2.6 ±0.3	+30.0 P<0.05	1.4 ±0.14	2.0 ±0.18	+42.9 P<0.05
Cerebellum	2.3 ±0.14	1.7 ±0.19	-26.1 P<0.05	3.3 ±0.23	2.5 ±0.2	-24.2 P<0.025
Spinal cord	2.0 ±0.2	1.4 ±0.15	-40.0 P<0.05	1.1 ±0.18	2.3 ±0.14	+109.0 P<0.01

Values (Mean ±S.D of 4 observations) are expressed as mgm/gm protein. Sign + indicates increase and sign – indicates decrease over the control.

Calmodulin, a multifunctional calcium binding protein is a major calcium receptor protein in the brain (18). Calcium dependent activation of several important enzyme systems viz, adenylate cyclase,

TABLE IV : Changes in the activity levels of the enzyme Ca^{2+} ATPase in discrete regions of CNS of control and MPT 15-day and 21 day old rat pups.

Regions	*Control*	*MPT*	*%Change*	*Control*	*MPT*	*%Change*
	15 days			*21 days*		
Cerebral cortex	3.2 ±0.125	2.8 ±0.178	-12.5 P<0.05	4.0 ±0.108	2.6 ±0.15	-35.0 P<0.001
Brain stem	3.4 ±0.125	2.7 ±0.106	-20.5 P<0.005	3.9 ±0.125	2.5 ±0.11	-35.89 P<0.001
Cerebellum	4.1 ±0.171	3.8 ±0.151	-7.32 P<0.10=NS	4.6 ±0.108	3.4 ±0.148	-26.8 P<0.001
Spinal cord	2.7 ±0.108	2.3 ±0.098	-14.8 P<0.05	3.4 ±0.298	2.5 ±0.3	-26.4 P<0.001

Values (Mean ± S.D of 5 observations) are expressed as µmoles of Pi liberated/mg protein/hr. Sign + indicates increase and sign – indicates decrease over the control.
NS = Not significant

cyclic nucleotide phosphodiesterase, Ca^{2+}–ATPase, myosin light chain kinase, glycogen synthetase (19) in brain are known to require calmodulin. In the present study calmodulin exhibited a general decline in the discrete regions of CNS on melthyl parathion treatment. It is found that the cerebral cortex suffers the brunt of the insecticide impact on 21st day of postnatal life when the level of CaM declined considerably (Fig. 2 and Table III). However, the cerebellum with its inhibitory purkinje fibre systems suffered the impact of methyl parathion profoundly and exhibited a decline in CaM both in 15th and 21st days of postal natal life (Fig. 1 and 2, Table III). Since Ca^{2+}–ATPase exhibited a significant inhibition (Table IV) and phospholipids an increase (Table V) respectively in this region it can be envisaged that calcium-calmodulin

TABLE V : Alterations of Phospholipids in discrete regions of CNS, control and MPT 15-day and 21 day old rat pups.

Regions	*Control*	*MPT*	*%Change*	*Control*	*MPT*	*%Change*
	15 days			*21 days*		
Cerebral cortex	14.5 ±3.27	18.0 ±1.43	+24.1 P<0.05	24.8 ±1.14	27.6 ±2.29	+11.3 P<0.025
Brain stem	23.9 ±4.06	30.6 ±3.83	+29.7 P<0.005	32.7 ±5.62	41.1 ±7.43	+25.7 P<0.005
Cerebellum	17.9 ±3.22	23.8 ±4.56	+33.0 P<0.01	26.0 ±3.41	31.2 ±3.98	+20.0 P<0.005
Spinal cord	39.3 ±3.89	54.8 ±6.53	+39.7 P<0.001	50.7 ±9.14	68.8 ±8.53	+35.7 P<0.001

Values (Mean ± S.D of 4 observations) are expressed as mgm/gm wet wt. Sign + indicates increase over the control.

complex, the ionic transport and the structural integrity of the membrane in the cerebellum were affected by methyl parathion toxicosis.

In brain stem, the calmodulin exhibited an increase on methyl parathion administration both in 15 and 21 days old rat pups (Figs. 1, 2 and Table III). The significance of this is not known. However, it can be said that the brain stem faces the brunt of injury due to methyl parathion toxicity and in order to maintain homeostasis during toxic stress resorts to synthesis of the regulatory protein calmodulin.

The spinal cord exhibited significant decline in the level of CaM in 15 days old neonatal pups on methyl parathion exposure. This indicates that 2 weeks after birth the spinal neurons suffered the impact of organophosphorous compounds and faced a decline in the cellular regulation. However, it may have acted as a prelude for, the defence mechanism appeared to have been triggered in 21 days old pups and hence on 21st day, methyl parathion exposure caused a remarkable (109%) increase in calmodulin level (Table III and Fig. 2).

Earielr studies of Nayeemunnisa and Naiyara (20) reported a remarkable decline in the level of

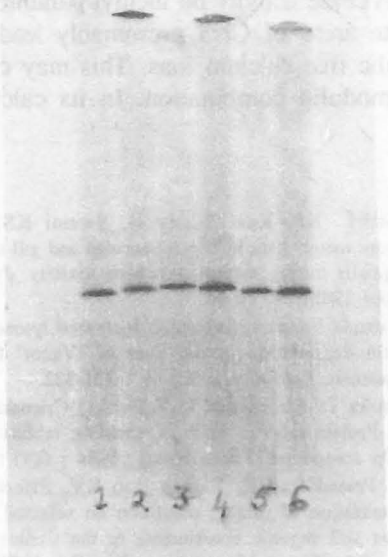

Fig. 2-A : **Polyacrylamide gel elctrophoresis of calmodulin (CaM)** in the presence of 8 M urea in discrete regions of CNS of 21 day old rat pups.
Gel-1 represents the electrophoretic pattern of CaM in the cerebral cortex of 21 day old controlled rat pups.
Gel-2 represents the electrophoretic pattern of CaM in the cerebral cortex of 21 day old methyl parathion treated (MPT) rat pups.
Gel-3 represents the electrophoretic pattern of CaM in the brainstem of 21 day old control rat pups.
Gel-4 represents the electrophoretic pattern of CaM in the brain-stem of 21 day old MPT rat pups.
Gel-5 represents the electrophoretic pattern of CaM in the spinal cord of 21 day old control rat pups.
Gel-6 represents the electrophoretic pattern of CaM in the spinal cord of 21 day old MPT rat pups.

calmodulin in the developing brain of amphibian tadpoles during methyl parathion toxicosis. Basing on this, the authors suggested a disruption in the regulatory molecular mechanisms during methyl parathion treatment. The increase and decrease found in the calmodulin levels in the levels in the present study (Table II, Fig. 1 & 2) on methyl parathion administration also indicate a specific disruption occuring in the physiologically efficient biomolecules. The molecular regulatory mechanisms involving CaM, in general, are rendered inefficient due to toxic impact of methyl parathion. It has been reported that calmodulin affects the neurotransmitter release (8) and has a significant role to play in junctional transmission of nerve impulse. In view of this, it can be said that the inhibition found in Ca^{2+}-ATPase activity on methyl parathion exposure in discrete areas of CNS presumably leads to a rise in cytosolic free calcium ions. This may cause a calcium-calmodulin combination. In its calcium bound form CaM binds with different affinities to specific proteins and alters their functions (21). It has been suggested that affinities for Ca^{2+}/CaM vary widely among its target proteins (21). Calcinurin and the Ca^{2+}-ATPase have a relatively high affinity for Ca^{2+}/CaM (21). It is therefore probable that Ca^{2+}/CaM variations caused by methyl parathion treatment in the present study has lead to inhibition of Ca^{2+}-ATPase activity in discrete areas of the CNS in developing rat pups. (See Table IV). Since Ca^{2+}-ATPase is a membrane bound enzyme and phospholipids are involved intimately in the maintenance of liquid crystalline state and structural integrity of the neuronal membranes, it can be envisaged that the organophosphorous insecticide, methyl parathion has disrupted the functional integrity of the neuronal membranes in the developing CNS and hence the dynamics and equilibrium of the membranes have changed as reflected by inhibition of Ca2+-ATPase and increase in phospholipids (See Table IV and V).

REFERENCES

1. Moorthy KS, Kasi Reddy B, Swami KS, Chetty CS. Glucose metabolism in hepatopancreas and gill of Lamellidens marginalis during methyl parathion toxicity. *Pestic Biochem Physiol* 1985; 24: 40-44.
2. Shakuntala Sridhara, Srihari K. Increased lysosomal mediated protein degradation in the liver of 'Vacor' fed Bandicota bengalensis. *Curr Sci* 1980; 49 : 331-332.
3. Malinska D, Strosnyder T, Zolewska; Crleuski P. Phospholipid Protein ratio in brain of suckling rabbits treated with an Op compound. *Tissue React* 1984 ; 6(4) : 317-322.
4. Siva Prasad Rao K, Raman Rao KV. Effect of sublethal concentration of methyl parathion on selected oxidative enzymes and organic constituents in the tissues of the Fresh water fish, Tilapia Mossambica. *Curr Sci* 1979; 48: 526-528.
5. Naiyara Yasmeen. Physiological and Biochemical studies on the effects of pesticides on the developing vertebrate brain. Ph.D. thesis, University of Bangalore, 1991.
6. Lajtha A. In : Protein metabolism of the Nervous System, Plenum Press, New York, 1970; pp.
7. Casida JE, Gammon DW, Glickman AH and Lawrence LJ. Mechanism of selective action of pyrethroid insecticides. *Ann Rev Pharmacol Toxicol* 1983; 23: 413.
8. De Lorenzo RJ , Freedman SD, Yoha WB, Maurer SC. Stimulation of Calcium dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by Calmodulin and calmodulin like protein isolated from synaptic vescles. *Proc Natl Acad Sci USA* 1979; 76: 1838-1842.
9. Skou JC. The infjluence of some cations on an ATPase from peripheral nerves. *Biochem Biophys Acta* 1957; 23: 394-401.
10. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951; 193-265.
11. Schmidt-Thanhausser-Schneider (1957) as cited by Glick (1964).
12. Glick. In : Methods of Biochemical Analysis. Inter Science Publisher, Inc., New York. 1964.
13. Watterson DM, Sharief F, Vanaman JC. The complete amino acid sequence of the calcium dependent modulator protein (calmodulin) of bovine brain. *J Biol Chem* 1980; 255: 962-975.
14. Matsumura F Narahashi T. As cited by Narahashi T. In the Future for Insecticides: Needs and Prospects 1976; P. 373. *Biochem Pharmacol* 1971; 20: 825.
15. Yamaguchi I, Matsumura F, Kadous AA. Inhibition of synaptic ATPase by Heptachlorepoxide in Rat Brain. *Int J Pestic Biochem Physiol* 1979 11: 285-293.
16. Zilversmit DB, Davis BS. *J Lab Clin Med* 1950; 35: 155. (As cited by Geetha KS).
    Geetha KS. Studies on some affects of OP insecticides on the developing vertebrate nervous system Ph.D Thesis, Univ. of Bangalore, 1991.
17. Fiske CH, Subbarow. *J Biol chem* 1925; 66: 375. As cited by Oser BL, 1976. In: Hawks Physiological Chemistry, 14th edn. Tata Mc Graw Hill Publishing Co, Ltd, New Delhi.
18. Cheung WY. Calmodulin plays a pivotal role in cellular regulation. *Sci* 1980; 207: 19.
19. Chafouleas JG, Dedman JR, Munjaal RP, Means AR. Calmodulin: Development and application of sensitive radio immuno assay. *J Biol Chem* 1979; 254: 10262.
20. Nayeemunnisa, Naiyaa Yasmeen. (Unpublished observation) 1991.
21. Mary B Kennedy. Regulation of neuronal function by Calcium. *TINS* 1989; 12: 417-420.