Acetylcholinesterase activity in the plasma and brain of the frog, *Rana tigrina* and the toad, *Bufo melanostictus* following the treatment with carbaryl & methyl parathion

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Acetylcholinesterase (EC 3.1.1.7) activity is a well-established biomarker to monitor environmental pollution caused by neurotoxic compounds such as carbamate and organophosphate (methyl parathion) pesticides. The presence of these compounds results in a measurable inhibition of the enzyme. To assess the effects of carbamate and methyl parathion on acetylcholinesterase, the frog, *Rana tigrina* and the toad, *Bufo melanostictus* have been selected because frogs and toads are significant predators in the ecosystems keeping insects into control. Frogs and toads are silent sentinels and keep watch on world's environmental health and also they are good indicators of habitat diversity. The neurobehavioural toxicity of the pesticide carbaryl (sevin) and methyl parathion have been studied in the present investigation. AchE activity in both plasma and brain decreased significantly. The inhibition of AchE in the brain of frog is greater than in the toad suggesting lesser sustainability of pesticidal stress in the frog than in the toad. *Bufo melanostiscus*, a terrestrial species is better adapted for pesticidal stress than *Rana tigrina*, a semi-aquatic species.

Key words: *acetylcholinesterase, brain, carbamate, methyl parathion, plasma.*

Активность ацетилхолинэстеразы в плазме и мозге лягушки, *Rana tigrina* и жабы, *Bufo melanostictus* после обработки карбарил- и метилпаратионом

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Активность ацетилхолинэстеразы (КФ 3.1.1.7) является хорошим биомаркером для мониторинга загрязнения окружающей среды нейротоксическими соединениями, такими как карбаматные и органофосфатные (метилпаратион) пестициды. Присутствие этих соединений приводит к ингибированию данного фермента. Для оценки эффектов действия карбамата и метилпаратиона на ацетилхолинэстеразу были выбраны лягушка, *Rana tigrina* и жаба, *Bufo melanostictus,* поскольку лягушки и жабы являются важными хищниками, контролирующими численность насекомых в экосистемах. Лягушки и жабы – это «безмолвные стражи», следящие за состоянием окружающей среды на глобальном уровне, а также являются хорошими индикаторами разнообразия местообитаний. В настоящем исследовании было изучено токсическое действие пестицидов карабарила (севин) и метилпаратиона на нервную систему и поведение. Активность ацетилхолинэстеразы значительно уменьшалась и в плазме, и в мозге. Ингибирование фермента в мозге у лягушки более значительное, чем у жабы, что означает меньшую устойчивость лягушки к пестицидному стрессу, по сравнению с жабой. *Bufo melanostiscus*, являющийся наземным видом, лучше адаптируется к пестицидному стрессу, чем *Rana tigrina*, являющийся полуводным видом.

Ключевые слова: *ацетилхолинэстераза, мозг, карбамат, метилпаратион, плазма.*

Introduction

The global decline in amphibians came to the world's attention in the beginning of last decade and was vigorously debated (Pechmann et al., 1991; Blaustein et al., 1994; Pechmann, Wilbur, 1994; Fisher, Shaffer, 1996; Wake, 1998). Since that time, surveys of natural populations have become more extensive and the general consensus is that the frog's population is declining. In tune with the global decline of amphibian population even in Patna (25°37'N 85°12'E) where frogs and toads were found in abundance during monsoon of year 2005, it was difficult to find frogs and toads even during the monsoon season probably due to very little rain and very few insects during the lighting festival last year and also due to indiscriminate use of pesticides (Kumari, Sinha, 2006a). Pesticides are receiving increased attention as a potential cause of amphibian decline. Amphibians are an important component of the food chain and are good bio-indicators of environmental pollution due to their susceptibility to chemicals during their freshwater cycles. The effects of environmental pollution together with changes in human activity and climate have contributed to the reduction of amphibian population. However, toxicological research on amphibians has been rather scarce compared with that on other vertebrates.

The poisonous effects of organophosphorous (methyl parathion) and carbamate pesticides come about through the inhibition of cholinesterase, an enzyme produced in the liver. Acetylcholinesterase can be found at the neurosynaptic junctions while another, butarylcholinesterase is primarily located in the plasma. This enzyme is present from insects to mammals.

The signs and symptoms are similar for carbamates and organophosphates poisonings. Both the pesticides combine with cholinesterase at nerve endings in the brain and in the tissues of the body, thereby

permitting the accumulation of acetylcholine. The occurrence of symptoms is primarily dependent upon the rate of cholinesterase decline. Most differences are due to the fact that cholinesterase reactivation is much more rapid after carbamate exposure than it is after organophosphate exposure.

It is apparent from the literature cited above that very few reports have been made on the effects of pesticides in frogs and toads. Therefore, it was considered of interest to study the effect of carbamate (sevin) and methyl parathion, an organophosphate on the frog, *Rana tigrina* and the toad, *Bufo melanostictus* in the laboratory as it is well known that frogs & toads are silent sentinels of the environment.

Materials & methods

Healthy frogs, *Rana tigrina* and toads, *Bufo melanostictus* were collected locally in and around Patna (25°37'N 85°12'E) and kept in large aquarium jars. They were kept in a well-ventilated place at room temperature under natural photoperiod (Sinha, 1983). The frogs were treated with methyl parathion sub-lethal dose 13.3 mg/kg body wt., carbaryl (sevin) dose 113.3 mg/kg body wt. intra-peritoneally and parallel control was run similarly. All readings were made within 24 hrs. of treatment because of short half life of acetylcholinesterase. Blood samples were collected by aortic puncture and collected in a watch glass containing anticoagulant. A known volume of blood was taken and centrifuged at 10000 X g in Remi Centrifuge (model C-30) at 4°C for 15 minutes and plasma was taken for analysis. Brain was dissected out and known weight (45 mg) of brain was homogenized in 10 ml of phosphate buffer (pH – 7.4) and assayed for acetylcholinesterase. 10 µl of plasma was taken for the assay of AchE activity.

Assay of acetylcholinesterase. The enzyme assay was done by the spectrophotometric method of (Ellman et al., 1961) using DTNB [dithiobisnitrobenzoic acid] as chromogen and acetylthiocholine iodide as the substrate.

Results and discussion

Effects of carbaryl and methyl parathion showed the following symptoms after treatment:

Symptoms	Carbaryl	Methyl parathion
Loss of muscular co-ordination and twitching of muscle	+++	+
Produced general prostration and flaccid paralysis	+++	-
Oro-nasal secretions	+++	+
Color of the skin of the hind limbs changed from light yellowish to bluish colour	+++	+

+++ (high), + (less), - (not observed)

Acetylcholine is synthesized in neuronal terminals by transfer of an acetyl group from acetyl CoA to choline, a reaction catalyzed by the enzyme choline acetyltransferase. The presence of acetylcholine appears to depend on several factors: transport of acetyl CoA from mitochondria where it is synthesized from pyruvate during glucose oxidation and the rate of uptake of choline into the nerve terminal.

Acetylcholine is metabolized to choline and acetate by the enzyme AchE found bound to the post-synaptic membrane. AchE therefore regulates the level of Ach at cholinergic synapses. The choline produced from the extra-cellular metabolism of acetylcholine by AchE is brought back into presynaptic cholinergic terminal by a high affinity of receptive process.

Results (Table 1) show that following the treatment of frogs and toads with carbaryl and methyl parathion there was almost a similar pattern in the decrease in AchE activity in both animals except in the intensity of inhibition and its regeneration. It was observed that following the treatment with carbamate, the death rate of the frogs was more than following the treatment with methyl parathion. Following the treatment of the frogs and toads with carbaryl the decrease in AchE activity in plasma was 62% and 47% respectively whereas in the methyl parathion treated frogs and toads the decrease in plasma AchE activity was 65.4% and 38.9%. In the brain AchE inhibition due to carbaryl in the frog and toad was 24.5% and 43%; in methyl parathion treated frogs and toads the inhibition was 13.9% and 22.6% respectively (Table 1). Thus, it is evident from the result above that carbaryl and methyl parathion block the AchE activity due to widespread distribution of cholinergic neurons both peripherally and centrally.

Both the animals under study when treated with the pesticides undergo physical stresses. Physical stress enhances the inhibition of AchE in skeletal muscles (Ott, 1985). Exposures to carbaryl and methyl parathion have been shown to alter respiration, oxygen consumption and catabolic effects in the central and peripheral tissues leading to oxidative stress (Fenichel et al., 1993).

Table 1.

AchE activity in blood & brain of the frog, *Rana tigrina* and the toad, *Bufo melanostictus.* Values are Mean ± S.D. Number in parenthesis are the sample sizes

Tissue	Animal	Control A	Sevin treated B	Parathion treated C	P value A vs. B	P value A vs. C
Plasma (IU/L)	Frog	270.074 ± 84.238 (5)	102.884 ± 21.5 (5)	93.31 ± 6.45 (5)	P<0.015 (-62%)	P<0.015 (-65.44%)
	Toad	344.16 ± 48.83 (5)	182.4 ± 57.23 (5)	210.35 ± 28.87 (5)	P<0.002 (-47%)	P<0.011 (-38.9%)
Brain µmol/min	Frog	127.028 ± 17.61 (5)	95.902 ± 20.54 (5)	109.36 ± 9.24 (5)	P<0.015 (-24.5%)	P<0.120 (-13.9%)
	Toad	108.21 ± 11.98 (5)	61.682 ± 11.68 (5)	83.74 ± 11.90 (5)	P<0.003 (-43%)	P<0.008 (-22.6%)

The loss of muscular co-ordination, muscle twitching and flaccid paralysis observed in the present investigations is due to the nicotinic effects through the inhibition of AchE. The presence of cholinesterase inhibiting chemicals prevents the breakdown of Ach and there is a build-up of Ach causing a “jam” in the nervous system. This inhibition involves the formation of an enzyme-inhibitor complex followed by the reaction of carbaryl and methyl parathion at the active site of enzyme which generates carbamylated or phosphorylated enzyme depending upon the dose. Phosphorylated AchE is relatively stable and the inhibition is virtually irreversible. However, carbamylated enzyme is sufficiently stable to disrupt cholinergic AchE which is rapidly regenerated following sub-lethal doses. The carbaryl-serine bond undergoes spontaneous hydrolysis with the regeneration of enzyme activity (18–24 hrs.). The occurrence of symptoms is primarily dependent upon the rate of cholinesterase decline. The differences are due to the fact that cholinesterase reactivation is much more rapid after carbamate exposure than it is for methyl parathion treatment. Repeated and unchecked firing of electrical signals causes uncontrolled, rapid twitching of muscles, paralyzed breathing, convulsions and in extreme cases death. It has been observed in the present investigation that initially the death rate of the frogs were much higher in the carbaryl treated frogs than the methyl parathion. This could be due to the animal going under stress condition. The general manifestations of the stress are called “General Adaptation Syndrome” which is divided into 3 stages as reported by Kumari and Sinha (2006b).

Thus, the higher death rates of the frogs following the treatment with carbaryl may be due to the fact that initially carbamate exerts greater stress during alarm reaction (1^{st} stage of stress) in which the frog is not able to sustain the stress effect. This could be explained due to the inhibition of brain AchE activity wherein the inhibition is 24.5% in carbamate treated frogs as against 13.9% in methyl parathion treated frogs. Since, the inhibition of AchE by carbamate in the brain of the treated frog is about two times higher than methyl parathion, the consequential nicotinic effects would be greater. The flaccid paralysis observed immediately after the treatment with carbaryl is due to the inhibition of AchE but once the cholinergic imbalance is corrected, the neurological signs and symptoms disappear. The oro-nasal secretions greater in the carbaryl treated frogs than in methyl parathion treated could be due to the pulmonary edema because of greater fluid accumulation in the lungs of carbaryl treated frogs (Kumari, Sinha, 2006a). The color of the hind limbs of frog changed from light yellow to bluish color in the carbaryl treated frogs which is due to cyanosis resulting from the decreased oxygen capacity and methaemoglobinaemia because it is known that Met Hb is caused by a number of drugs and chemicals, especially pesticides like carbamate (Kumari, Sinha, 2006a).

Plasma cholinesterase inhibition represents an indirect indicator of adverse effect on the nervous system. While blood measures of AchE inhibition are not adverse in themselves, they are generally the only available estimation of AchE inhibition potential in the peripheral nervous system, since data on AchE inhibition in peripheral nervous tissues or target organs are rarely available especially of humans. This is the reason why plasma measures of ChE should be considered as an appropriate end point of derivation of reference doses as a matter of prudent science policy. Pharmacokinetically both blood and peripheral nervous system are outside the CNS. Hence pesticides with limited penetration of the blood barrier, the blood ChE measures may be much better indicators of peripheral nervous system (PNS) cholinesterase inhibitor (ChE I) activity than brain measures.

The rapid decrease in plasma AchE activity shows that the inhibition in the plasma AchE is much higher than in the brain suggesting a differential response in the plasma and the membrane bound brain

cells, because of the limited penetrations of the pesticides in the blood-brain barrier. It has been reported by Sinha and Singh (1989), that ascorbic acid content is higher in the brain of the toad (76.7 mg/100 mg tissue) than in the in the frog, *Rana tigrina* (26.03 mg/100 mg tissue). Ascorbic acid has the capability of entering all cells. Under normal circumstances, its presence is beneficial, however, when the cell has been invaded by foreign substances like the pesticides under study (carbaryl and methyl parathion); ascorbic acid may be acting as an endogenous antioxidant preventing the propagation of lipid peroxidation or removing free radicals. It is suggested that ascorbic acid plays a role in inhibiting lipid peroxidase, thus resulting in lower AchE activity in the brain than in the blood.

It has also been observed that the recovery period of stress in the toad was 48 hrs in contrast to 72 hrs in the frogs. The pesticidal stress in toad is less than in the frog because of higher lipid and ascorbic acid content in the tissues under study (Kumari, Sinha, 2006b).

It is concluded that the toad, *Bufo melanostiscus,* a terrestrial species which relies more on aerobic metabolism is better adapted for pesticidal stress than the frog, *Rana tigrina,* a semi-aquatic species which relies more on anaerobic metabolism. The pesticides do not penetrate the blood-brain barrier and therefore cholinergic effects are predominantly peripheral in origin. The AchE inhibition by carbamate is reversible whereas in methyl parathion is irreversible. Since the global decline of amphibians is of paramount importance to scientists and general public, the indiscriminate use of pesticides should be restricted to conserve the sentinels of environment.

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