Biochemical changes in the toad, *Bufo melanostictus* as a function of methyl parathion: ascorbic acid as a biomarker of oxidative stress

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Amphibians may signal environmental stress earlier than most organisms and may serve as critical bio-indicators of the health of ecosystem. Amphibians provide an extremely visible indicator of pollution and hence are called the “sentinels of the environment”. The liver and muscle glycogen of the toad *B. melanostictus* decreases significantly in 48 and 72 hrs following the treatment by methyl parathion, whereas the liver and muscle proteins decrease significantly in 24, 48 and 72 hrs indicating proteolysis. Increase in plasma glutamate pyruvate transaminase is indicative of liver damage and increase in glutamate oxaloacetate transaminase is indicative of heart and skeletal muscle dysfunction. There is a significant increase in glutamate pyruvate transaminase in 48 hrs but the increase in glutamate oxaloacetate transaminase is not significant following the treatment. It is interesting to note that liver ascorbate decreases significantly in 24, 48 and 72 hrs whereas muscle ascorbate decreases significantly in 48 & 72 hrs following the treatment. The triglycerides and cholesterol increase though not significantly following the treatment. Lactic dehydrogenase activity in the muscle increases significantly in 48 hrs whereas succinic dehydrogenase activity in the liver decreases significantly in 24 hrs following the treatment. The physiological significance of these observations is discussed herein.

Key words: *methyl parathion, glycogen, protein, ascorbic acid, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase, succinic dehydrogenase, toads.*

Биохимические изменения у жабы, *Bufo melanostictus,* при действии метилпаратиона: аскорбиновая кислота как биомаркер оксидативного стресса

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Амфибии могут указывать на нарушения состояния среды ранее, чем другие организмы, и могут быть эффективными индикаторами состояния экосистемы. Амфибии обеспечивают исключительно отчетливую индикацию загрязнения и поэтому заслуживают название «стражей окружающей среды». Содержание гликогена в печени и мышцах жабы *B. melanostictus* значительно снижается через 48 и 72 ч после воздействия метилпаратиона. В то же время содержание белка в печени и мышцах значительно снижается через 24, 48 и 72 ч после воздействия, что указывает на протеолиз. Повышение содержания глутамат-пируват-трансаминазы в плазме указывает на повреждение печени, а повышение содержания глутамат-оксалоацетат-трансаминазы свидетельствует о дисфункции сердечной и скелетных мышц. Через 48 ч после действия метилпаратиона наблюдается значительное повышение содержания глутамат-пируват-трансаминазы, однако повышение уровня глутамат-оксалоацетат-трансаминазы после воздействия незначительно. Интересно отметить, что содержание аскорбата в печени существенно уменьшается через 24, 48 и 72 ч после воздействия, в то время как содержание аскорбата в мышцах снижается через 48 и 72 ч после обработки. После воздействия отмечается незначительный рост уровня триглицеридов и холестерола. Активность лактатдегидрогеназы в мышцах значительно повышается через 48 ч после действия метилпаратиона, в то время как активность сукцинатдегидрогеназы в печени существенно снижается через 24 ч после воздействия. Обсуждается физиологическое значение указанных наблюдений.

Ключевые слова: *метилпаратион, гликоген, белок, аскорбиновая кислота, глутамат-пируват-трансаминаза, глутамат-оксалоацетат-трансаминаза, лактатдегидрогеназа, сукцинатдегидрогеназа, жабы.*

Introduction

Amphibians may signal environmental stress earlier than most other organisms and may serve as critical bio-indicators of the ecosystem health. They may be sensitive to global environmental changes on the continental scale or they may be sensitive to local modifications of their environment. Amphibians provide extremely visible indicator of pollution. Amphibians as a group have only recently been included in routine toxicological assessment of environmental chemicals. Amphibians actually constitute the largest fraction of vertebrate biomass in some ecosystems, making them as important source of food for the higher vertebrates such as fish, birds, reptiles and mammals as well as important herbivores (tadpoles) and carnivores in these ecosystems (Blaustein et al., 1994). Coppo et al. (2002) reported the physiological variation of enzymatic

activities in the blood of bull frog, *Rana catesbeina*. Blood and urine physiological values in the captive frog, *Rana catesbeina* have also been reported by Coppo et al. (2005).

Most of the countries have diverse amphibian population but it is surprising that much attention has not been paid to the effects of environmental pollutants upon these animals. The largest single group of potential chemical pollutants that frogs and toads might encounter is various pesticides employed in agriculture and pest management. Most recent work examining the effects of pesticides on amphibians has concentrated on newer generation of pesticides such as methyl parathion although there has been resurgence of interest in older organochlorine (DDT) because of their persistence and possible links to amphibian decline. Methyl parathion has been used in the present investigation which is relatively insoluble in water and soluble in most organic solvents. Methyl parathion is readily absorbed through all routes (oral, dermal and inhalation) and is rapidly distributed in the body. The half life of methyl parathion is about 18 days but sun light can reduce it to 6.3 days. Signs and symptoms are those characteristic of poisoning by cholinesterase inhibiting organophosphates. They include peripheral and central cholinergic nervous manifestations (Kumari, Sinha, 2006a).

Methyl parathion is rapidly metabolized in most organisms resulting in low bio-concentration factor after acute exposure. Detoxification is achieved by degradation reaction, dimethylation or dearylation. These detoxifications are due to glutathione alkyl and aryl transferases; the reaction product is dimethyl phosphoric acid.

Ascorbic acid is a strong reducing agent which is a cofactor in several metabolic oxidation reduction reactions. Ascorbic acid is an antioxidant which should be present in large quantity for the survival of the toad undergoing pesticidal stress. The amphibians take necessary water and oxygen through their moist skin. This makes them susceptible to pesticides resulting in decline of population. The effects of environmental pollution together with changes in human activity and climate have contributed to the reduction in amphibian population over recent decades. However, toxicological research on amphibians has been rather scarce compared with that on other vertebrates.

The frogs and toads are called the sentinels of the environment. Presence of frogs and toads in one's backyard is an index of good environment. Therefore, the amphibians can be considered as an early indicator of a "global environmental disaster".

In light of the above, it was considered of interest to study the effect of pesticide, methyl parathion on some biochemical constituents of the liver, muscle, brain and blood of the toad, *Bufo melanostictus.*

Materials and methods

Healthy toads, *Bufo melanostictus* were collected locally in and around Patna (25°37'N, 85°12'E) and kept in large aquarium jars. They were maintained and kept in a well ventilated place at room temperature under natural photoperiod (Sinha, 1983). The toads were removed from the aquarium with minimum disturbance.

The toads were treated by methyl parathion with 13.33 mg/kg body wt., intraperitoneally. All readings were made after 24, 48 and 72 hrs of treatment. 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.

Glycogen was estimated according to the method of Kemp and Andrienne (1954) as modified by Sinha and Kanungo (1967).

The method adopted for the determination of protein content of the tissue was that of Sutherland et al. (1949).

The method of extraction of ascorbic acid from the tissues was that of Kanungo & Patnaik (1964) and determined according to the 2,4-dinitrophenyl hydrazine method of Roe (1954).

Glutamate pyruvate transaminase (GPT) (EC. 2.6.1.2) and glutamate oxaloacetate transaminase (GOT) (EC. 2.6.1.1) were determined by the method of Reitman and Frankel (1957).

Plasma cholesterol was determined by the CHOD–PAP method of Trinder et al. (1969). Plasma triglycerides were determined by the enzymatic method of Rifai et al. (1999). HDL (High-density lipoprotein) cholesterol in the plasma was determined by the method of Friedewald et al. (1972). Lactic dehydrogenase (LDH) activity was determined by the method of Elliott & Wilkinson (1963). Succinic dehydrogenase (SDH) activity was determined by the method of Kun and Abood (1949). The red formozan formed during the incubation period was extracted in acetone and the OD was measured at 485 nm in U-V-vis Spectrophotometer and expressed as µg formozan per hour.

Results and discussion

Liver is the central organ of metabolism and acts as an organ for storage. Many potentially toxic substances are metabolized by the hepatic parenchyma cells that has been regarded as an important

defense system against toxicants and the transformations involved are termed detoxification. The role of liver in metabolic conversion is due to the susceptibility to chemical injury (Zimmerman, 1974). Stores of readily available glucose to supply the tissues with an oxidizable energy source are found principally in the liver, as glycogen. A second major source of stored glucose is glycogen in the muscle.

The results presented in tab. 1 show that the liver glycogen decreases in 48 and 72 hrs following the treatment by methyl parathion. But in contrast to the liver, the muscle glycogen significantly decreases ($p<0.07$) only in 48 hrs and significantly increases ($p<0.03$) in 72 hrs. The treated toads undergo stressed condition and respond to stressors by increasing plasma levels of corticosterone (Guillette et al., 1995; Lance, 1990; Tyrell, Cree, 1998). These differences in adrenocortical responsiveness reflect a change in the sensitivity of the hypothalamo-pituitary adrenocortical (HPA) axis to stressors and all often are termed as adrenocortical modulation (Wingfield, Romero, 2001; Kumari, Sinha, 2006b). The chemical transmitter between preganglionic fibres and the adrenal cells is acetylcholine. As such, when acetylcholine is inhibited by methyl parathion, the secretion of adrenaline becomes higher thus enhancing glycogenolysis in the liver resulting in the decrease of glycogen (tab. 1). Reduction of glycogen level is supposed to be as a result of greater stress the liver experiences during the process of detoxification of active moieties and their metabolites. Decrease in liver glycogen might be due to enhanced breakdown of glucose through glycogenolysis to meet high energy demands during pesticidal stress (Kumari, Sinha, 2006b). Similar findings have been reported by Bhagyalakshmi (1981) in crabs exposed to sumithion and Dubhat and Bapat (1984) in fish, *Chana orientalis* exposed to ekalux. This alteration in glycogen content indicates its utilization for countering pesticidal stress suggesting prevalence of anaerobic conditions such as hypoxia (Mayes, 1977). Hypoxia causes an increase in carbohydrate consumption. Depletion of glycogen also indicates a shift towards anaerobic metabolism to maintain metabolic balance during pesticidal stress which is corroborated by significant increase in LDH activity (tab. 1).

Table 1.

Effect of methyl parathion on the different biochemical parameters in the toad *B. melanostictus*

Parameters	Tissue	Time	Control A	24 hrs B	48 hrs C	72 hrs D	p< value		
							A vs. B	A vs. C	A vs. D
Glycogen (mg/g tissue)	Liver	Mean, S.D.	91.4 ±1.82	86.3 ±5.1	45.6 ±2.07	48.2 ±5.5	0.15	0.001	0.001
		Range	89–94	80–93	43–48	40–55			
		C.V.	0.02	0.06	0.045	0.11			
	Muscle	Mean, S.D.	3.34 ±0.35	2.84 ±0.51	2.84 ±0.51	4.49 ±0.77	0.13	0.07	0.03
		Range	2.92–3.8	2.4–3.4	2.2–3.5	3.3–5.2			
		C.V.	0.105	0.18	0.18	0.17			
Protein (mg/g tissue)	Liver	Mean, S.D.	112.77 ±7.93	85.8 ±9.5	75.22 ±12.49	57.20 ±6.85	0.004	0.001	0.001
		Range	100.7–121.9	73.12–99.12	61.75–91	48.7–65			
		C.V.	0.07	0.11	0.17	0.12			
	Muscle	Mean, S.D.	84.5 ±8.78	66.3 ±7.74	53.25 ± 7.08	27.62 ±2.46	0.003	0.001	0.001
		Range	66.62–89.37	60.12–74.75	45.5–61.75	24.4–29.3			
		C.V.	0.10	0.12	0.13	0.09			
Ascorbic acid (mg/g tissue)	Liver	Mean, S.D.	0.86 ±0.033	0.40 ±0.06	0.32 ±0.08	0.42 ±0.09	0.001	0.001	0.001
		Range	0.8–0.88	0.34–0.50	0.25–0.44	0.31–0.55			
		C.V.	0.039	0.15	0.25	0.23			
	Muscle	Mean, S.D.	0.39 ±0.09	0.43 ±0.043	0.21 ±0.013	0.24 ±0.06	0.33	0.01	0.06

Table 1 continued.

		Range	0.26–0.47	0.38–0.49	0.20–0.23	0.20–0.34			
		C.V.	0.23	0.10	0.06	0.24			
Lipid fractions (mg/dl)	Cholesterol	Mean, S.D.	91.67 ±4.08	95.99 ±15.4	104.33 ±16.9	–	0.46	0.22	–
		Range	88.33–98.33	86.66–123.33	85–126.7	–			
		C.V.	0.045	0.16	0.16	–			
	Triglycerides	Mean, S.D.	87.33 ±9.09	96.24 ±16.94	93.35 ±20.87	–	0.33	0.58	–
		Range	79.2–99.99	79.21–122.76	72.7–122.76	–			
		C.V.	0.10	0.18	0.22	–			
	HDL	Mean, S.D.	42.78 ±3.61	40.45 ±6.71	40.90 ±13.1	–	0.57	0.74	–
		Range	36.66–45.45	29.54–47.73	29.5–61.36	–			
		C.V.	0.08	0.17	0.32	–			
	LDL	Mean, S.D.	37.14 ±9.33	38.56 ±21.01	36.16 ±7.26	–	0.91	0.86	–
		Range	29.87–53.12	21.9–73.59	24.6–42.61	–			
		C.V.	0.25	0.54	0.20	–			
	VLDL	Mean, S.D.	17.46 ±1.81	19.23 ±3.40	21.27 ±5.76	–	0.33	0.19	–
		Range	15.84–19.99	15.8–24.55	14.5–28.91	–			
		C.V.	0.10	0.18	0.27	–			
LDH (iu/l)	Muscle	Mean, S.D.	81.3 ±25.27	106.31 ±42.6	116.02 ±36.19	96.12 ±42.37	0.16	0.09	0.62
		Range	68.4–126.4	53.4–152	71–152	45.4–138			
		C.V.	0.31	0.40	0.31	0.44			
SDH (µg of formozan/hr.)	Liver	Mean, S.D.	312.37 ±8.03	230.20 ±30.6	299.88 ±67.65	297 ±62.7	0.006	0.71	0.59
		Range	301.35–318.5	203.35–279.3	216.8–389.5	216–367			
		C.V.	0.026	0.13	0.23	0.21			
GPT (iu/l)	Plasma	Mean, S.D.	25.8 ±4.82	28.2 ±2.68	51 ±6.56	26 ±2.0	0.11	0.002	0.93
		Range	20–32	26–32	46–60	24–28			
		C.V.	0.19	0.09	0.13	0.08			
GOT (iu/l)	Plasma	Mean, S.D.	59.4 ±3.85	63.2 ±4.14	60.4 ±4.34	59.6 ±12.28	0.22	0.47	0.98
		Range	56–66	58–68	56–66	50–74			
		C.V.	0.065	0.066	0.072	0.21			

Note: values are mean, S.D., number of sample used is 5.

The general manifestations of the stress are called “General Adaptation Syndrome” which is divided into 3 stages:

1. The alarm reaction which occur before the adaptation has occurred (1st stage)
2. The stage of resistance in which the adaptation is optimal (2nd stage)
3. The stage of exhaustion in which acquired adaptation is lost (3rd stage).

The adaptation is mediated largely through HPA axis (Kumari, Sinha, 2006b).

The muscle glycogen decreases in 24 and 48 hrs though not significantly except in 48 hrs in treated toads. The decrease could be due to the direct action of pesticides with the hormone or enzyme responsible

for glycogenolysis. Since muscular activity needs energy in the form of ATP generated through oxidative phosphorylation of glucose, increase in glycogen contents in 72 hrs may be linked to decreased muscular activity that could be from the inhibitory effect of pesticides on enzymes involved in the oxidation of glucose. Thus, the depletion of glycogen observed in the liver and muscle is an indication of typical stress related response of the animals with the pesticide (fig. 1).

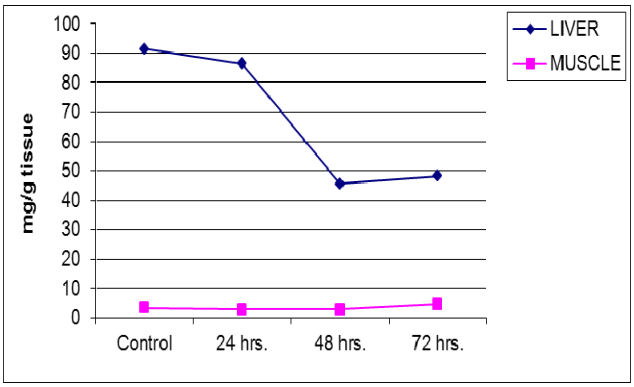

Fig. 1. Effect of methyl parathion on glycogen content

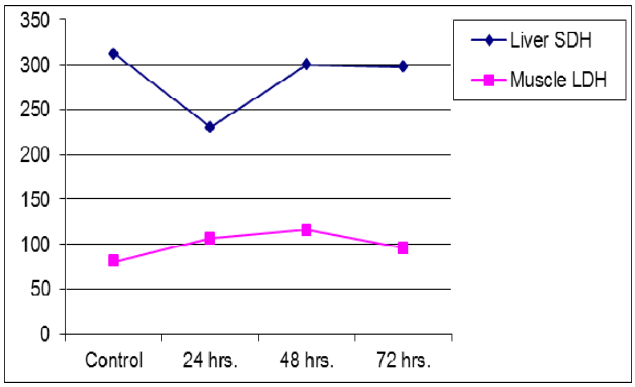

Fig. 2. Effect of methyl parathion on SDH (µg of formozan/hr.) & LDH (IU/L) activity

LDH is a hydrogen transfer enzyme that catalyzes the oxidation of L-lactate to pyruvate with the mediation of NAD^+ as hydrogen acceptor. The toad, *Bufo melanostictus* moves slowly, forms little lactate and remains aerobic. Following the treatment by methyl parathion significant increase in LDH activity in 48 hrs has been showed. LDH level indicates the energy demands are met by anaerobic respiration through increase in LDH activity. As mentioned earlier, the toads underwent pesticidal stress and thereafter to meet the energy demand, LDH activity increased. On the contrary, SDH activity decreased in the liver. The decrease in the liver SDH activity suggests that anaerobic metabolism is favoured over aerobic oxidation of glucose through Krebs cycle in order to mitigate the energy crisis for survival. The decrease in SDH activity indicates inhibition of SDH at mitochondrial level and enhancement of alternative pathway of carbohydrate metabolism via HMP shunt or pentose pathway as biochemical adaptation to overcome the toxic stress (fig. 2).

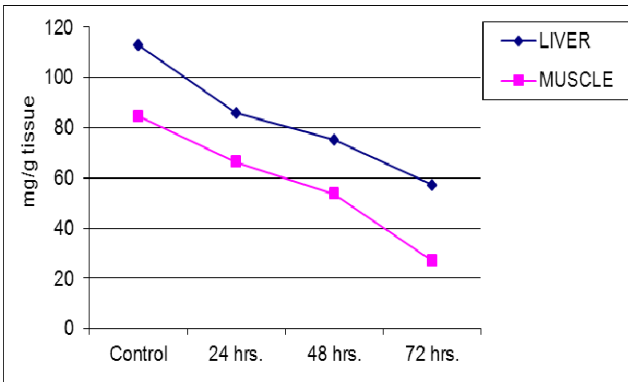

Fig. 3. Effect of methyl parathion on protein

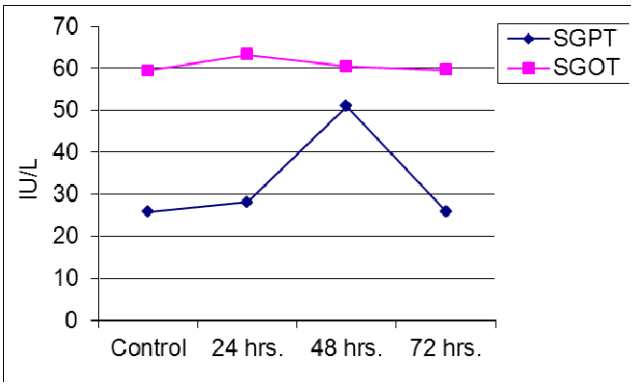

Fig. 4. Effect of methyl parathion on plasma GPT and GOT

In the present study, the liver and muscle proteins decrease significantly in 24, 48 and 72 hrs following the treatment by methyl parathion (fig. 3) which may be due to the greater need of energy to meet the energy demand during the initial stressed conditions (alarming stage – 1st stage) in general adaptation syndrome. This energy may be obtained from carbohydrates, proteins or lipids. The significant decline in proteins in both tissues is suggestive of an increased proteolysis and utilization of the products of their degradation for metabolic purposes. Reduction in total proteins in both tissues suggests that they may be channelized into the tricarboxylic acid cycle through aminotransferase system to cope up with the excess demand of energy

during toxic stress. It is well known that transaminases stand at a potential junction between protein and carbohydrate metabolism and directly are concerned with interconversions necessary for the production of amino acids required for various animal tissues. The increase in GPT and GOT (fig. 4) after the treatment by methyl parathion is for keeping pace with increasing demand of keto acids for gluconeogenesis to meet the energy demand during pesticidal stress (Kumari, Sinha, 2006b).

Ascorbic acid is a strong antioxidant and free scavenger that provides first line of defense against oxidant damage that is vital for aerobic life. Oxygen in its molecular state is essential for many metabolic processes. Aerobic organisms can not exist without O_2, which nevertheless is inherently dangerous to their lives. Like all aerobic organisms, toad is also susceptible to the effects of reactive oxygen and has inherent and effective antioxidant defenses like high content of ascorbic acid in the liver and brain. It has been observed that the recovery period from pesticidal stress in the toad is quicker than in the frog which could be due to higher content of ascorbic acid in the liver and brain of toads than of frogs (Kumari, 2006). Thus, ascorbic acid could act as a biomarker of oxidative stress. It was interesting to note that the lipid content per unit of tissue weight of the toad was higher than in the frog (Kumari, 2006). Hence, the higher lipid content could be a safety measure because methyl parathion is lipophilic and the pesticide is deposited in the fat which results in protection of other vital organ from the pesticide. It is suggested that the toad could withstand more oxidative stress than the frog due to two reasons: (1) higher ascorbic acid content in the liver and brain and (2) higher lipid content. In the liver and muscle, ascorbic acid content decreases significantly following the treatment by methyl parathion (fig. 5). It follows more or less similar pattern to that of glycogen after the treatment by pesticides suggesting dependence of ascorbic acid on hexoses (Kumari, 2006).

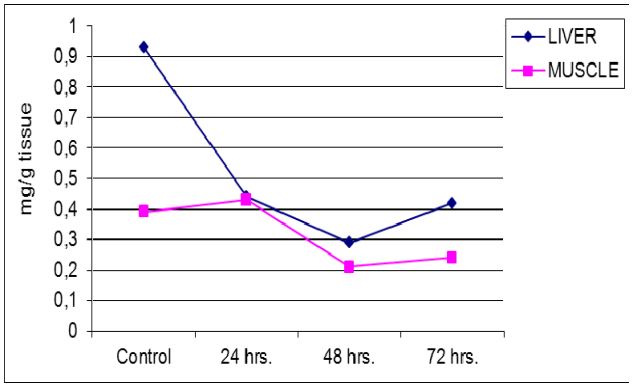

Fig. 5. Effect of methyl parathion on ascorbic acid

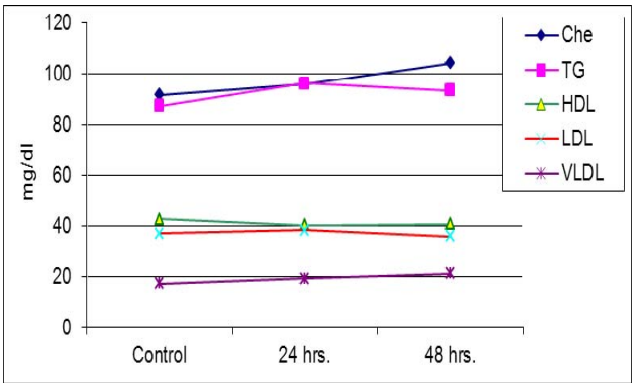

Fig. 6. Effect of methyl parathion on lipid fractions

Amphibians utilize lipids principally triglycerides stored in abdominal fat bodies and for metabolic maintenance during dormancy (Singh, Sinha, 1989). In the present study blood cholesterol (Che), triglycerides (TG), HDL (high-density lipoprotein), LDL (low-density lipoprotein) and VLDL (very-low-density lipoprotein) of toads following the treatment by methyl parathion have been investigated till 48 hrs because they were no significant changes within 48 hrs (tab. 1). The toads undergo oxidative stress following the treatment by methyl parathion. It is known that hypoxia elicits many physiological responses because of the increase in the secretions of catecholamines and cortisol in response to stress.

Free fatty acids are considered more dynamic form of lipid transport from storage to oxidation site in different tissues. Triglycerides in the blood are considered as principal form of transport to the storage site in the adipose tissues. The fatty acids diffuse in the plasma and bind to the plasma proteins and from there they diffuse into different cell types for oxidation. The last step of transport involves esterification with CoA and transport into mitochondria, CoA ester is regenerated from carnitine ester, and then broken down by ß-oxidation (Kumari, Sinha, 2006b).

Following the treatment by methyl parathion there was increase in triglycerides and cholesterol (fig. 6), but the increase was not significant. However, the slight increase after the treatment by methyl parathion was due to the inhibition of ß-oxidation. Catecholamines are usually released under stressed conditions including hypoxia. Under hypoxia, lipid mobilization is, however, not useful because ß-oxidation is impaired due to oxygen shortage. The combination of phospholipids hydrolysis, inhibition of ß-oxidation and lipolytic action of catecholamines caused increase in triglycerides and cholesterol (tab. 1). Further, glycogenolysis-glycolysis has an inhibitory effect on fat oxidation. The effect appears to be mediated by an increase in malonyl CoA which inhibits the enzyme responsible for transporting long chain fatty acids with mitochondrial

matrix, palmityl carnitine transferase (Lehninger, 1978). It is concluded that methyl parathion suppresses lipid metabolism. Similar reports have been made by Hall and Swineford (1981) and Indira et al. (1997).

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