DISCUSSION

Data presented in chapter 4 clearly demonstrate that the two pesticides, nuvacron and carbaryl, caused qualitative and quantitative alterations in the structural as well as functional aspects of the frog *Euphlyctis hexadactylus*. All the changes recorded were concentration and time dependent.

Behavioral changes are easily detectable compared to complicated and complex process underlying them. With this view behavioral changes were observed along with toxicity studies. Earlier studies reveal that pesticides are being absorbed by frogs in both aquatic and terrestrial systems, and are suppressing the activity of the enzyme called cholinesterase, which is essential for the proper functioning of the nervous system and thereby disrupting nervous system activity. In the present study also there were signs of nervous system disruptions as indicated by the behavioral abnormalities. There were reduced activity levels, reduction in pigmentation of the skin, loss of response to touch and sound, loss of righting reflex and excessive secretion of mucus.

These behavioral observations are not surprising because most pesticides are neurotoxic. Loss of equilibrium was reported also in bull frog tadpoles treated with malathion (Fordham *et al.*, 2001) and *Hyla arborea* tadpoles exposed to dimethoate (Sayim and Kaya, 2006). Bridges and Christine (1997) studied general activity and swimming performance of *Rana blairi* tadpoles after acute exposure to three sublethal concentrations of carbaryl and reported that there is a reduction in both tadpole activity and swimming performance even at 24\textsuperscript{th} hour of exposure. Vasait and Patil (2005) observed inclined body posture, change of color to paler shade and opened mouth in the fish *Nemacheilus botia* exposed to monocrotophos. Excessive secretion of mucus has been reported in the fishes exposed to pollutants as a defending mechanism against the irritation caused by the toxicant (Mezin and Hale, 2000). Loss of
equilibrium posture could affect the chances of predation, and since activity is closely associated with feeding, it may result in slowed growth leading to a prolonged larval stage and an indirect reduction in adult fitness. Excessive secretion of mucus may be due to the animal’s defending mechanism to escape from stressors.

5.1. HISTOPATHOLOGICAL CHANGES

Tissue changes in test organisms exposed to experimental concentrations of toxicants are functional responses that provide information on the mode of action of the toxicant on them. Exposure to sublethal and median lethal concentrations of nuvacron and carbaryl induced remarkable changes in the tissue architecture of liver, kidney and gonads of the frog *Euphlyctis hexadactylus*. The histopathological changes in the organs of treated frogs intensified with increased exposure and increasing concentration of the pesticide.

5.1.1 Liver

Hepatic cells have many vital functions. Besides the secretion of bile, they play an important role in protein, lipid and carbohydrate metabolism and serve as a storage site for many of the nutrients. They are the chief sites of detoxification and metabolism of pollutants. Hence, these pollutants or their toxic metabolites may get concentrated in hepatic tissue leading to its damage.

In the present study sublethal concentration of nuvacron (19.5 ppm) caused notable changes in the liver tissue after 15 days of exposure. On the fifteenth day exposure onwards there were mild pathological alterations - swelling of hepatocytes, extravasations of RBC, hemorrhage and hyalinization. Exposure for 30 days revealed more severe changes like liver cord disarray, nuclear atrophy and clumping of hepatocytes. In median lethal concentration (97.5 ppm) nuvacron caused pathological alterations on the tenth day of exposure itself. There were inflammation
of hepatocytes and degeneration of their cytoplasm, hemorrhage, sinusoidal dilations, atrophy in hepatic lobules and formation of vacuoles and rupture of blood vessels. From twentieth day onwards the changes became more prominent. There were liver cord disarray, massive congestion of hepatic tissue, nuclear atrophy and necrosis, presence of clumped RBCs and loss of cellular structure of the sinusoids. Extensive hemorrhage may have resulted in the presence of clumped RBCs. On the thirtieth day the original shape of the hepatocytes was completely lost and lymphocytes appeared in enormous numbers, which may be due to the animal’s defense mechanism to counter the pesticide molecules or its metabolites.

Sublethal concentration of carbaryl (20.5 ppm) also caused pathological alterations from fifteenth day onwards. There were slight inflammation and enlargement of hepatocytes, lysis of cell walls, extravasations of RBC, hemorrhage, clumping of RBCs and cloudy swelling of hepatocytes. In median lethal concentration (102.5 ppm) changes appeared on the tenth day of exposure. There were lysis of cell walls, extravasations of RBC, clumping of hepatic cells, vacuolation, rupture of basement membrane, loss of original shape of hepatic cells, necrosis of hepatic cells, pyknotic nuclei, occurrence of lesions and severe damage to basement membranes of sinusoids. On the thirtieth day there was complete loss of liver architecture showing cloudy masses of tissue without cell boundaries, considerable amount of clumped RBCs and large number of lymphocytes.

In higher concentrations (median lethal concentrations) the degeneration of the tissue was severe and rapid with both the pesticides. The difference in the architecture of liver of these animals, exposed to higher concentrations of pesticides, was pronounced when compared with the control. The tissue was less homogeneous, more irregular and disorderly in appearance.
The findings of a number of researchers are in conformity with the above observations. The chronic toxicity of methoxychlor to South African clawed frog *Xenopus tropicalis* was studied by Fort *et al.* (2004) and they reported a concentration dependent increase in external malformations and internal abnormalities of the liver exposed for 90 days. Histological changes associated with pesticides in fishes have been studied by many authors, and they reported swelling of hepatocytes with diffuse necrosis, cordal disarrangement, individualization of hepatocytes, hyperplasia, disintegration of hepatic mass, focal coagulative necrosis, atrophy, appearance of blood streaks among hepatocytes, formation of vacuoles and rupture in blood vessels (Radhaiah and Rao, 1992; Das *et al.*, 2000 a; Rodrigues *et al.*, 2001; Rana and Yeragi, 2002; Susan and Tilak, 2003; Gul *et al.*, 2004; Sarkar *et al.*, 2005; Tilak *et al.*, 2005 a; and Tilak *et al.*, 2005 b). Srivastava and Srivastava (1993) reported varying degree of necrosis and cirrhosis with cytoplasmic vacuolation giving the liver a ‘Swiss Cheese’ like appearance at sixty days after exposure to subacute level of chlordecon in the catfish *Heteropneustes fossilis* indicating heavy damage with longer duration of exposure.

In the liver of house sparrow exposed to sub lethal concentrations of furadan SP50, Nigam and Bakhre (1991) observed vacuolization, enlarged nuclei, karyorrhexis, pyknosis, karyolysis, lymphocytic infiltration, acidophilic cytoplasm and thickening of endothelial lining of portal vein. Rats, exposed to different pesticides and pesticide combinations, revealed hepatotoxicity as reported as mononuclear cell infiltration, hepatocellular damage, hydropic degeneration, enlargement of sinusoids, increase in number of Kupffer cells, sinusoidal dilation, pyknotic nuclei, cytoplasmic degranulation and various nuclear aberrations (Selmanoglu and Akay, 2000; Selmanoglu *et al.*, 2001; Choudhary *et al.*, 2003).
The great susceptibility of liver to damage by chemical agents may be a consequence of its primary role in metabolism of foreign substances. From the results obtained in the present study it is clear that both nuvacron and carbaryl at sublethal and median lethal concentrations caused dose and duration dependent damages to liver tissue, and the changes were similar to the findings of other workers.

5.1.2 Kidney

Renal excretion is one of the ways of eliminating toxicant molecules from the body. These molecules could cause several pathological changes to the kidney. Like liver, renal tissue of *Euphlyctis hexadactylus* also experienced many pathological changes under the stress of both nuvacron and carbaryl. But in contrast to the liver in which changes appeared earlier, renal tissue showed pathological symptoms at later stages of exposure. The sublethal concentrations caused no changes up to twenty days in both the pesticides. This may be due to the primary role of the liver in the detoxification process. But with increased dose and duration liver might have failed to eliminate or detoxify the toxicants resulting in renal excretion of them causing damage to the kidneys.

On twenty fifth and thirtieth days there were inflammations, hemorrhage and damage in tubular epithelium in kidneys of frogs exposed to sublethal concentration of nuvacron (19.5 ppm). Median lethal concentration (97.5 ppm) of nuvacron caused damage to renal tissue from fifteenth day onwards. There were inflammations, hemorrhage, dilation of tubules, destruction of tubular epithelium, focal collections of inflammatory cells, extravasations of RBC, widening of tubules due to flattening of tubular epithelium and hyalinization after 20 days of exposure.

As stated above, the renal tissue appeared normal up to twenty days of exposure to sublethal concentration (20.5 ppm) of carbaryl. On twenty
fifth and thirtieth days there were dilation of tubules, hemorrhage and focal collection of inflammatory cells. Median lethal concentration (102.5 ppm) of carbaryl was found to affect renal histology from twenty days onwards. There were damage to tubular epithelium, hemorrhage in haemopoietic tissue, lymphocytic infiltration, focal collection of inflammatory cells and destruction of tubular epithelium.

The renal tissue was subjected to damages by the two pesticides even though to a lesser extent, and these changes were concentration and time dependent. Studies on the effect of pesticides on the kidney of anurans are lacking. However, several reports are available on nephrotoxicity of pesticides on other vertebrates. The gold fish *Carassius auratus* after sublethal exposure to hexachlorobutadiene at 24 hour exhibited cytoplasmic vacuolation and necrosis in renal tubules (Reimschuessel et al., 1989). Das and Mukherjee (2000 a) reported distention in kidney tubules and necrosis in posterior kidney in the fish *Labeo rohita* exposed to hexachlorocyclohexane. Male albino rats, exposed to a combination of technical grade endosulfan, dimethoate and carbaryl, revealed a reduction in weight of the kidney and histopathological changes such as mononuclear cell infiltration, congestion in the cortex and medulla and glomerular and tubular degeneration (Selmanoglu and Akay, 2000). Carbendazim, a carbamate fungicide, in higher dozes caused congestion, mononuclear cell infiltration, tubular degeneration and fibrosis in kidneys of male rats (Selmanoglu et al., 2001). According to Veiga et al. (2002), renal tissue may suffer high structural variation when exposed to specific situations on reorganization attempt, as evidenced by glomerular expansion in the fish *Prochilodus lineatus* exposed to sublethal concentrations of trichlorofon. Renal haemopoietic tissue of *Cirrhinus mrigala* and *Catla catla* was also subjected to damage under pesticide stress as reported by Susan and Tilak (2003) and Tilak et al. (2005 a&b). They reported degenerative changes like severe necrosis, formation of large vacuoles, hemorrhage, cellular hypertrophy and granular cytoplasm
in renal haemopoietic tissue of fishes exposed to experimental concentrations of pesticides. The results of the present study are in agreement with the reports of these workers.

Once absorbed, toxicants are transported by blood circulation to liver for transformation and/or for storage (Das and Mukherjee, 2000a). If stored or accumulated, it may cause damage to liver tissue and if transformed or not accumulated in the liver, they may be excreted through the bile or passed back into blood for possible excretion by kidneys. Hence, during elimination they may cause damage to the renal tissue as evidenced from the present study as well as from the reports of many other workers.

5.1.3 Gonads

Both the pesticides caused profound pathological changes in the gonads of *Euphlyctis hexadactylus*. Here also the changes were time and concentration dependent.

**Testis.** The testis did not show any pathological change up to ten days of exposure to sublethal concentration of nuvaceron. On fifteenth day there were mild degenerative changes like breaking of cyst walls and scattering of sperm bundles in some tubules. As days proceeded scattering of sperm bundles became more prominent, and there were clumping of secondary spermatocytes and necrosis of Sertoli cells. The compact nature of the testis was found lost and there were scattered masses of disintegrating sperm bundles in frogs exposed for thirty days. In median lethal concentration nuvaceron caused more serious changes to the testis architecture. After ten days exposure itself there were scattering of sperm bundles and mild degenerative changes. From fifteenth day onwards there were more severe changes such as extravasations of RBC, vacuolation in tubules, clumping of secondary spermatocytes and loosening of connective tissue. On thirtieth day of exposure there were
exfoliation and agglutination of sperm bundles and severe necrosis of Sertoli cells.

Carbaryl caused degenerative changes in the testis of the test animals after exposure to sublethal concentration on the fifteenth day. There were slight disorganization of sperm bundles and vacuolation of cytoplasm at some places. After twenty days of exposure there were clumping of spermatocytes and extravasations of RBC. The sperm bundles became scattered due to damage in tubules. On twenty fifth and thirtieth days there were complete scattering of sperm bundles, severe damage to the interstitial tissue and necrosis of Sertoli cells. In median lethal concentration carbaryl induced marked changes in testicular architecture from tenth day onwards. There were pathological alterations like scattering of sperm bundles and the original shape of the interstitial cells was lost showing roughly polygonal shape, and they became clumped at some regions. Other changes noted were vacuolation of cytoplasm, congestion in interstitial tissue, shrunken tubules and reduction in sperm numbers. On the twenty fifth day there was exfoliation of sperms, and the spermatids were found degenerated. The testis section appeared as an irregular mass of tissue and the different cellular elements became unidentifiable after 30 days of exposure.

Results obtained from the histopathological studies on the testes of different vertebrates under pesticide stress also agree with the present observations. Pathological symptoms like reduction in GSI, atrophy, loss of structure and shape of interstitial cells, vacuolation, degeneration of sperms, connective tissue splintering, a decrease in the diameter of primary spermatogonia and a reduction in the number of Leydig cells were reported in the testes of fishes exposed to different pesticides by various authors (Lakhani and Pandey, 1985; Jyothi and Narayan, 1996; Zutshi and Murthy, 2001; Dutta et al., 2006). Birds treated with pesticides showed changes in the structure of testis like congestion in the
tunica albuginea, cellular debris in the lumina of seminiferous tubules, cytoplasmic swelling, vacuolation in cytoplasm, pyknosis, reduction in spermatogonial cells, decrease in seminiferous tubule diameter, degeneration in tubular epithelium, exfoliation and vacuolation of germ cells and gradual loss of healthy sperms in tubules (Bakre et al., 1989; Sarkar and Maitra, 1992).

In the frog *Rana cyanophlyctis* treated with the mercurial fungicide, emisan, the testes exhibited a reduction in GSI, diameter of seminiferous tubules and Leydig cell nuclei, number of secondary spermatocytes and spermatids (Kanmadi and Saidapur, 1992). Histopathological changes in the testes of *Rana hexadactyla* exposed to endosulfan were examined by George and Andrews (1994). There were rupture of basement membranes, degeneration of tubules, size increase in primary spermatocytes, hyperplasia of spermatogenic cells, loss of cystic appearance of various stages, disintegration of Leydig cells and scattered spermatids. This study also revealed that the effects of pesticides on the testes of the frog were dose and duration dependent. The toad *Bufo melanostictus* on exposure to sublethal concentrations of the pesticides, endosulfan and hinosan revealed several pathological alterations in the tissue architecture of the testis like breaking of basement membranes of seminiferous tubules, scattering of sperm bundles, reduction in the number of germ cells, congestion in the interstitial tissue, clumping of chromatin materials in primary spermatocytes, necrosis, degeneration in secondary spermatocytes and loss of cystic nature of the spermatogenic stages (Mathew, 1999). Orton et al. (2006) reported treatment related pathological changes in the testes of the frog *Rana pipiens* exposed to experimental concentrations of the fungicide, atrazine.

As evidenced from the results obtained in the present study it is clear that both the pesticides, even in their low concentrations, cause several
pathological alterations in the testes of frogs. These kinds of damages adversely affect spermatogenesis and male fertility.

**Ovary.** The ovaries were also not affected by sublethal concentration of nuvaceron up to ten days. From fifteenth day onwards there were pathological symptoms like increase in the number of atretic follicles that showed an increasing trend with increase in concentration as well as time. Other changes noted were shrinkage in follicles, vacuolation in the stroma, reduction in the number of oocytes, increased oocyte immaturity, retarded growth of previtellogenic oocytes, large number of atretic follicles, edema in stroma and presence of denatured yolk. Exposure to median lethal concentration for ten days revealed an increased number of atretic follicles which continued to increase throughout the experiment. In addition to this, there were shrinkage of yolky material in yolky oocytes, increased oocyte immaturity, retarded growth of vitellogenic oocytes, improper incorporation of yolk in the SGP oocytes, reduction in the number of SGP oocytes, edema in stroma and reduction in the total number of mature oocytes.

In sublethal concentration carbaryl also caused no major changes to the ovary up to ten days. An increasing number of follicular atretia was a prominent feature in carbaryl toxicity also. Besides this, there were vacuolation in primary oocytes, an observable reduction in ova diameter, reduction in the number of SGP oocytes, edema in stroma and extensive vacuolation in all types of oocytes. In median lethal concentration changes appeared from tenth day onwards. In addition to an increasing number of atresia, there were reduction in the diameter of all types of oocytes, improper incorporation of yolk elements, edema in stroma, vacuolation in oocytes, reduction in the number of SGP oocytes and total degeneration and necrosis.

Similar results were obtained and reported by several authors on the effects of pesticides on the female reproductive organs of different
animals. Pathological conditions, caused by experimental concentrations of various pesticides, in different fish species has been reported by several authors (Kaur and Virk, 1983; Shukla et al., 1984; Srivastava, 1989; Jyothi and Narayan, 1996; Chatterjee et al., 1997; Ghosh et al., 1999; Mohan, 2000; Kaur and Saxena, 2002). A study on carbamate pesticide furadan administered to Passer domesticus at sublethal and lethal concentrations showed pathological alterations such as vacuolation, atresia, deposition of fat and dissociation of granulosa layer in the ovary (Wadhwa et al., 1988).

Hussain and Saidapur (1983) reported a reduction in GSI, a reduction in the mean diameter of oocytes and an increase in the number of atretic follicles in the frog Rana cyanophyctis, exposed to methallibure. Histopathological alterations in the ovary of the frog Rana hexadactyla exposed to sublethal concentrations of endosulfan were studied by George and Andrews (1994). The most important effect of endosulfan to the ovary was the increased frequency of atretic follicles which were present from fifth day onwards for all treatments (0.35 and 0.70 ppm). There was a marked reduction in the gonadosomatic index also. Exposure to sublethal concentrations of the pesticides, endosulfan and hinosan, caused pathological alterations in the ovary of the toad Bufo melanostictus. Both the pesticides caused increased percentage of follicular atresia, necrosis, thickening of peritoneal covering and cytoplasmic vacuolation in treated frogs (Mathew, 1999). Pickford and Morris (1999) reported that methoxychlor, an endocrine disrupting pesticide, is a potent inhibitor of progesterone induced oocyte maturation at median inhibitive concentration (72nM) in the frog Xenopus laevis. This pesticide also caused developmental and reproductive toxicity in the ovaries of the frog Xenopus tropicalis also (Fort et al., 2004). Recently Orton et al. (2006) studied the effects of nitrate and atrazine on larval development and sexual differentiation in the northern leopard frog, Rana pipiens. Both the compounds showed no significant changes individually but in
combinations they showed increased size of mature ovarian follicles and a significant variation in the diameter of immature and mature follicles.

In the present study the most notable changes that appeared in the pesticide treated ovaries were increased number of follicular atresia, improper incorporation of yolky materials in mature oocytes and reduction in the number of SGP oocytes. Occurrence of atretic follicles is a common phenomenon in amphibian ovaries (Saidapur et al., 1982). But their presence in enormous numbers is reported to be a histopathological response to toxicants (George and Andrews, 1994).

In vertebrates, the regulation of gonadal functions is mediated through gonadotropic hormone and gonadotropin releasing hormone. Ghosh et al. (1990) recorded a lower level of gonadotropic hormone in Channa punctatus exposed to sublethal doses of metacid-50 and carbaryl, and also clearly established the mediation of the pituitary and hypothalamic hormones in affecting gonadal function of pesticide treated fish. Here it is worthy to mention that WWF has included carbaryl in the list of pesticides reported to have reproductive and/or endocrine disrupting effects (Lyons, 1999) The present study reveals that nuvacron also imposed the same type of effect as that of carbaryl. Pickford and Morris (1999, 2003) also reported that reproductive and endocrine dysfunction can occur in adult amphibia exposed to environmental toxicants with endocrine disrupting activity. In the present study reduction in the number of SGP oocytes in treated animals when compared with control animals indicates a disruption in the oocyte maturation process imposed by the pesticides. Since oocyte maturation is a prerequisite for the subsequent fertilization of the released ova, the disruption of this process has considerable potential to impair with female amphibian reproduction.

In amphibians yolk precursors are produced in the liver and then transported to oocytes (Pramoda and Saidapur, 1986; Wallace and
Selman, 1989). Present study showed that the two pesticides can cause a marked damage in the hepatic cells. The improper incorporation of yolk materials in the SGP oocytes exposed to median lethal concentrations of both the pesticides can be attributed to this.

5.2. BIOCHEMICAL CHANGES

In addition to neurotoxic and reproductive effects, organophosphate and carbamate pesticides are known to influence the chemistry of several tissues in animals. Present study also revealed alterations in the biochemical parameters in different tissues examined, in response to sublethal and median lethal concentrations of nuvacron and carbaryl.

5.2.1. Carbohydrate metabolism

Present study showed an initial hyperglycemia and later hypoglycemia along with a sharp and continuous reduction in liver and muscle glycogen levels in experimental animals treated with sublethal and median lethal concentrations of the pesticides, nuvacron and carbaryl. The changes observed were statistically significant.

The blood glucose level can be an indicator of biological stress caused by pollutants such as pesticides and metals (Silbergeld, 1974). Elevation in serum glucose level of *Euphlyctis hexadactylus* under the effect of nuvacron and carbaryl may be due to increased glycogenolysis; this was further supported by decreased glycogen content in the liver and muscle. Later hypoglycemia may be due to an increased utilization of glucose produced or may be due to a shift in animal’s carbohydrate metabolism. Pesticides are reported to affect carbohydrate metabolism in different animals under various experimental conditions. Sampath *et al.* (1992) reported an increase in blood glucose level and a reduction in the glycogen content in the liver, kidney, muscle and intestine of the frog *Rana tigrina* exposed to experimental concentrations of carbaryl. A dose
dependent augmentation of blood glucose level and a significant reduction in liver glycogen were reported in the Indian weaver bird, *Ploceus philippinus philippinus* following treatment with quinalphos (Anam and Maitra, 1992). Begum and Vijayaraghavan (1995) reported that the carbohydrate metabolism in the liver was adversely affected in the cat fish, *Clarias batrachus* exposed to the organophosphate insecticide, dimethoate. Increased blood glucose and decreased brain glycogen content in rainbow trout in response to acute exposure to lindane was reported by Soengas et al. (1997). In the European eel, *Anguilla anguilla* there was no significant change in liver glycogen content but there was marked hyperglycemia when the fish was exposed to an organophosphate pesticide, fenitrothion for 96 hours (Sancho et al., 1997). Increase in the blood glucose level was also reported in the fish, *Oreochromis mossambicus* exposed to lethal and sublethal doses of agrofen (Verma and Panigrahi, 1998). Decrease in the serum glucose level was reported by Rahman and Siddiqui (2006) in rats exposed for 90 days to subchronic doses of phosphorothionate. These reports indicate that hyperglycemia resulted with shorter durations of exposure, whereas longer exposures resulted in hypoglycemia as was noticed in the present study. Since blood glucose forms the immediate source of energy, particularly during energy crisis, the animal may have depended more on blood glucose resulting in its depletion under longer periods of stress.

The reduction in glycogen content, noticed in the liver and muscle tissues of the experimental frogs in both sublethal and median lethal concentrations of the two pesticides may be an indication of increased rate of glycogenolysis. It is well known that stored glycogen content depend on both the glucose concentration and the capacity for glycogen synthesis (Gimeno et al., 1995). Therefore, it is reasonable to assume that the glycogen content of the liver and muscle run parallel to the blood glucose levels. Decreased muscle glycogen content is also an indication of increased glycogenolysis at tissue level, as a part of animal’s struggle to
meet the energy crisis. Increased glycogenolysis in muscles can be assumed to be due to the increased energy demand created by muscular activities exhibited by the animal during pesticide stress. Sastry and Siddiqui (1983) reported pesticide induced muscular excitement in the fish, *Channa punctatus* exposed to sublethal concentrations of endosulfan. Niveditha et al. (1998) observed an elevation in serum glucose level of *Sarotherodon mossambicus* under the effect of a carbamate fungicide, ziram, due to increased glycogenolysis; also there was a decrease in glycogen content in the liver under pesticide stress. Breakdown of hepatic glycogen upon stress caused by an organophosphate pesticide, fenitrothion, was also reported in *Sarotherodon mossambicus* by Koudinya and Ramamurthy (1979). Muscle glycogen levels decreased significantly in the fish *Anguilla anguilla* exposed to a high sublethal dose of lindane (Ferrando and Andreu-Moliner, 1991). Similar results were obtained by many, who have studied the effect of experimental concentrations of pesticides on carbohydrate metabolism in different tissues of test animals (Sastry and Siddiqui, 1983; Vasanthi and Ramaswami, 1987; Medda et al., 1992; Kaur et al., 1998; Ramani et al., 2002). All these reports are in agreement with the results obtained in the present study.

Another possible reason for decreased amount of liver and muscle glycogen in treated frogs is the stress produced indirectly by the toxicant through its primary effect on the endocrine glands, exciting them to release large amounts of hormones, such as catecholamines and corticosteroids, which are active in carbohydrate metabolism. The secretion of glucocorticoids is a classic endocrine response to stress (Sapolsky et al., 2000). Singh et al. (1996) reported muscle and liver glycogenolysis in the fish, *Heteropneustes fossilis* exposed to experimental concentrations of aldrin, and suggested that this may be due to increased secretion of catecholamines in response to pesticide
The carbamate insecticide carbofuran caused an increase in the brain catecholamines and plasma glucose and a reduction in hepatic glycogen in the fish Carassius auratus on exposure for 48 hours (Bretaud et al., 2002). Das and Mukherjee (2001) also suggested that all types of stress can elevate the secretion of catecholamines, which in turn can increase the break down of glycogen.

The present study also revealed a severe damage to the hepatocytes by the two pesticides and it is noteworthy that hepatic damage was severe with extended duration and greater concentrations of the pesticides. Since decline in liver glycogen content also was intense in later stages and in higher concentrations, it can be assumed that the tissue damage in liver may also have contributed to the decreased amount of glycogen in the liver.

### 5.2.2. Protein metabolism.

The protein level of blood, liver and muscle in Euphlyctis hexadactylus exposed to sublethal and median lethal concentrations of pesticides, nuvacron and carbaryl showed gradual decrease, which was statistically significant. The decline was more pronounced in median lethal treatments in all the tissues indicating a dose dependent effect.

The decrease in protein content in the various tissues of the treated Euphlyctis hexadactylus also indicates the physiological adaptability of the animal to compensate for pesticide stress. To overcome such stress, animals require high energy. This energy demand might have led to stimulation of protein catabolism. Protein content in tissue is dependent on the dynamic equilibrium between the rates of its synthesis and degradation. The reduction in protein content might be because of increased utilization of protein to meet the energy demand when the frog was under toxic stress. The pesticide exposure might have accelerated proteolytic process to liberate aminoacids to overcome the metabolic
stress, which in turn has reduced the protein level in the tissues. A significantly lower level of total protein was reported in the fish, *Cyprinus carpio* when exposed to experimental concentrations of diazinon, an organophosphate pesticide (Luskova et al., 2002). Pan and Dutta (2000) also reported diazinon induced decline in serum protein fractions in the fish, *Micropterus salmoides*. A reduction in blood protein level was recorded in *Oreochromis mossambicus* exposed to lethal and sublethal concentrations of agrofen (Verma and Panigrahi, 1998). Das and Mukherjee (2001) investigated the effect of an organophosphate pesticide nуван on the blood biochemistry of the fish *Labeo rohita* and reported that there is a reduction in the blood protein content. The protein level was depressed in the blood of male and female rats when exposed to subchronic doses of phosphorothionate (Rahman and Siddiqui, 2006). Sampath et al. (1995) reported that the proteins after being synthesized from aminoacids are deposited in the cytoplasm and are rapidly used up to replace the loss of protein or aminoacids occurring during physiological demand. Naveed et al. (2004) reported a decrease in the protein content and an increase in the free aminoacids in the liver, brain and gill tissues of the fish, *Clarias batrachus* exposed to sublethal concentrations of endosulfan and kelthane, and suggested that the main source of energy for the exposed fish came from hydrolysis of proteins into aminoacids.

The quantity of protein is also dependent on the synthesis of RNA, which plays the key role in protein synthesis. According to Singh et al. (1996), the reduction in protein content of the exposed animal may be either through the inhibition of RNA synthesis at the transcriptional level or due to impaired incorporation of aminoacids into polypeptide chains. Significant decline in the nucleic acid content has also been reported in *Channa punctatus* subjected to dimethoate, an organophosphate (Tripathy et al., 2003). Maruthanayakam and Sharmila (2004) reported that when the fish *Cyprinus carpio* was exposed to sublethal concentration of monocrotophos for fifteen days, there was a significant
reduction in the concentration of DNA and RNA and this decrease in RNA might indicate a reduced protein synthesis.

Fall in tissue protein content might be attributed to utilization of aminoacids to meet the impending energy demands when the animal is under stress or stress induced enzyme activity alterations (Kumar and Saradhamani, 2004). An increase in the acid phosphatase activity recorded in the present study also supports the inference of enhanced protein catabolism. Shaikila et al. (1993) reported that the increased amount of acid phosphatase in tissues might be responsible for the severe drop of protein and RNA content in the fish, *Sarotherodon mossambicus* under pesticide stress.

Das and Mukherjee (2000) reported that histopathological changes in the kidney of experimental fish *Labeo rohita* could also cause considerable loss of blood proteins by renal excretion further augmenting its depletion in the blood. Present study also revealed considerable damage to the kidney in later stages of exposure to the pesticides. It is therefore logical to assume that prolonged and continued exposure to the pesticides has deleterious effects on protein synthesis and kidney function, which account for the progressive reduction in the concentration of total serum protein.

5.2.3. Lipid metabolism

Lipids also serve as energy reserves to meet the metabolic demand for more energy and mitigate toxic stress. Since they form the energy rich reserves whose calorific value is twice that of carbohydrates or proteins, the mobilization of lipid reserves indicate higher energy demands, which generally occur during severe stress. In the present study, a decrease in the total lipid content of liver and muscle and an increase in serum cholesterol in the median lethal concentrations suggest that lipids might have undergone lipolysis leading to energy production under severe
stress. Earlier studies also showed utilization of lipids in various animals under toxic stress. Parvathy et al. (2000) reported a reduction in liver total lipid in *Tilapia mossambica* exposed to phosalone for 150 days. The fish, *Glossogobius giuris* exposed to fenthion showed an increase in cholesterol content of the testis (Zutshi, 2003).

It is interesting to note that in the present study, lipid reserves started declining in the tissues slowly. In sublethal concentrations of both the pesticides there were no major changes in lipid concentration in liver as well as muscle. In median lethal concentrations, both the pesticides caused reduction in lipid content from twentieth day onwards. In median lethal concentration the pesticides induced an increase in blood cholesterol level also, whereas there was no significant change in sublethal concentrations. But in the case of glycogen and protein there was considerable decrease in their concentrations in sublethal as well as median lethal concentrations in all the tissues examined. Hence, it can be assumed that the animal primarily might have depended upon glycogen and protein reserves, and later due to severe stress caused by the pesticides under higher concentrations in longer duration of exposure, had relied on the lipid reserves also. A short-term exposure of 96 hours to the pesticides, cythion and hexadrin in their safe concentrations and median lethal concentrations caused no change in the blood cholesterol and liver lipid but there was an increase in ovarian lipid in the fish *Heteropneustes fossilis* (Singh and Singh, 1980). In the anuran *Rana tigrina*, Sampath and Elango (1997) reported a decrease in lipid content of liver, kidney, muscle and intestine and an increase in blood cholesterol when exposed to sublethal and median lethal concentrations of carbaryl. In their study, these changes were more severe in median lethal concentration. Their observations are in close agreement with the present study.
Increased blood cholesterol in frogs treated with median lethal concentrations of the pesticides, nuvacron and carbaryl, suggests stress induced hyper metabolic state of the animal, mobilization and utilization of cholesterol through blood and also the damage in tissue architecture of liver. Garg et al. (1989) suggested that the increase in cholesterol level under toxic stress might be an indication of liver malfunction. Here it is interesting to note that present study also revealed hepatic tissue damage under pesticide exposure.

An alteration in the blood cholesterol level was reported in a number of animals exposed to different concentrations of various pesticides. Ceron et al. (1996) reported a decrease in blood cholesterol level in the eel, Anguilla anguilla exposed to sublethal concentrations of diazinon for 96 hours. Similarly, a reduction in cholesterol content of the cardiac muscles was reported by Venkataraman et al. (2006) in the fish Glossogobius giuris exposed to sublethal concentrations of malathion. Here also the observations were made only up to 96 hours. An increase in blood cholesterol in the fish Oreochromis mossambica exposed to sublethal concentrations of agrofen for 60 days has been found by Verma and Panigrahi (1998). Carbaryl and fenthoate, in their sublethal concentrations, caused an elevation of blood cholesterol for 60 days of exposure (Rao et al., 1985). Selmanoglu et al. (2001) reported an increase in serum cholesterol level in male rats Rattus rattus exposed to carbendazim for 15 weeks. Hence, when the exposure period was short the blood cholesterol was either not affected or there was a slight decrease. But when the animals were exposed for longer duration the blood cholesterol level increased significantly.

These observations are in line with the fact that lipids are high energy reserve molecules and will be metabolized only when there was a substantial reduction in glycogen and protein reserves. Hence, from the
present study it can be inferred that both the pesticides are highly toxic to *Euphlyctis hexadactylus* as indicated by the decline in lipid reserves.

### 5.2.4. Enzymes

The enzymes are indicative of various aspects of metabolism, and they have been used to evaluate the physiological, biochemical and metabolic defects in various tissues of animals. It is well known that acid and alkaline phosphatases play a significant part in various metabolic processes, especially protein and carbohydrate metabolism. They are the enzymes concerned with the biosynthesis of fibrous protein (Johnson and Mc Minn, 1958) and deposition of calcium in bones (Duthie and Barker, 1955). These phosphatases can transfer the phosphate from the nucleotides and further lead to biosynthesis of chemical energy to overcome the toxic stress (Naveed et al., 2004).

**Acid Phosphatase.** The acid phosphatase activity in the blood, liver and muscle of *Euphlyctis hexadactylus* was found to increase with exposure to sublethal and median lethal concentrations of the pesticides. The increase was concentration and time dependent, and the data obtained are statistically significant. These enzymatic changes are indicative of the cellular toxicity and tissue damage induced by these pesticides in frog, probably by imposing an alteration in the specific metabolic pathways.

Phosphatases are involved in many different processes that require mobilization of phosphate ions or dephosphorelation as part of anabolic, catabolic or transfer processes. They act by hydrolyzing phosphomonoester bonds and liberating free inorganic phosphate groups. Rise in the activities of this enzyme along with a reduction in protein content in the different tissues suggests enhanced protein catabolism to meet energy demands. This inference is further supported by the findings
of Shaikila et al. (1993), who opined that the increased amount of acid phosphatase in tissues might be the reason for the severe drop of protein and RNA content in the fish *Sarotherodon mossambicus* under pesticide stress. Das and Mukherjee (2000 c) also reported an increase in acid phosphatase activity and a decrease in protein and RNA levels in *Labeo rohita* fingerlings exposed to quinalphos. Hence, the increased level of acid phosphatase in blood, liver and muscle of treated frogs indicates enhanced metabolic activity, perhaps to meet the stress induced by the pesticides.

An increase in acid phosphatase content of the liver and blood can also be attributed to the hepatocellular damage recorded in the present study. As this enzyme is associated with lysosomal activity, its increase may be due to a decrease in the stability or rupture of liver lysosomal membranes resulting in the release of this enzyme into surrounding tissues. In rats, CCl₄ induced liver injury caused a rise in acid phosphatase concentration in the blood (Abraham, 2004). As liver injury progressed the activities of the acid phosphatase also increased progressively. The author suggested that this may be due to the leakage of lysosomal enzymes into the blood. Sharma (1999) reported a rise in acid phosphatase activity in the fish *Clarias batrachus* exposed to experimental concentrations of carbaryl. According to him, this rise in acid phosphatase activity due to pesticide intoxication suggests an enhanced protein catabolism and probable hepatocellular damage in the organism. Dalela et al. (1978) also reported an increase in acid phosphatase activity in liver, muscle and kidneys of *Channa gachua*, treated with lethal and sublethal doses of rogor and thiodon, and suggested that this may be due to the strong toxic effect of the toxicant on the cellular and lysosomal membranes resulting in their rupture. This suggestion was also supported by the findings of Dubale and Aswathy (1982) who studied acid phosphatase content of the kidney in the fish *Heteropneustes fossilis* exposed to rogor. Further, Nigam and Bakhre (1991) suggested an alteration in the membrane permeability of
lysosomes and a resultant increase in acid phosphatase concentration due to toxicant induced anaerobic conditions.

Similar findings were reported by several authors. There was a significant increase in acid phosphatase activity in plasma and a marginal increase in liver of female rats exposed to different organophosphorus pesticides (Kaur and Dhanju, 2004). A marked increase in acid phosphatase activity was reported in the liver of *Heteropneustes fossilis* exposed to the pesticides, phosphamidon, endrin and sevin (Thomas and Murthy, 1976). In *Bufo melanostictus* exposed to dimethoate for a period of seven days acid phosphatase activity increased in plasma and kidney but decreased in liver and testis (Pradhan and Dasgupta, 1991). These authors suggested that long term exposure to dimethoate may have affected functions of liver, kidney and testis. Hence, it can be assumed that the increased acid phosphatase activity in blood, liver and muscle of the exposed frogs may be due to the stress induced protein catabolism in these tissues and may be also due to damage in the liver architecture.

**Alkaline Phosphatase.** In all the tissues except blood there was a reduction in alkaline phosphatase activity for sublethal and median lethal concentrations of the two pesticides. In blood enzyme level increased slightly but significantly for ten days in sublethal concentrations of both the pesticides. Thereafter, it recorded a fall till the end. In median lethal concentration both the pesticides caused reduction in alkaline phosphatase activity in the blood, liver and muscle.

The initial increase in blood alkaline phosphatase indicates enhanced metabolic activity perhaps due to animal’s immediate struggle to compensate the energy loss owing to the stress induced by the pesticides. An initial hyperglycemia also was noted in the present study in sublethal
concentrations. Increased secretory activity of the sensitive endocrine glands, such as the pituitary and adrenal is a common physiological response to acute stress. It results in increased levels of plasma corticosterone and consequently, increased activity of glucocorticoid responsive enzymes such as alkaline phosphatase (Ozmen and Akay, 1993). It is well known that alkaline phosphatase plays a significant role in carbohydrate metabolism (Singh, 1977). Increased serum alkaline phosphatase was reported by Kaur et al. (1998) in rats exposed to carbaryl for fifteen days.

In the present study, the general effect of the pesticides on the alkaline phosphatase activity was a decline in its concentration. This can be associated with the damage in the hepatic tissue architecture and an imbalance between degradation and synthesis of the enzyme (Mazur and Harrow, 1971). Since liver form a major site of its synthesis, severe damage of the liver in later stages of exposure to pesticides might have reduced the concentration of this enzyme in the tissues. By studying the functions of alkaline phosphatase in rat liver Alvaro et al. (2000) reported that the intrahepatic biliary epithelium plays a role in the biliary secretion of serum alkaline phosphatase. It is a membrane bound enzyme found at bile pole of hepatocytes and also found in pinocytic vesicle and Golgi complex. It is present in all cell membranes where active transport occurs, and hydrolase and transphosphorylase in function. Severe drop in tissue alkaline phosphatase could be adaptive for the frog to meet the energy demand aiding anaerobic break down of glycogen in liver and muscle under pesticide toxicity. Al- Attar (2004) in his studies on the frog Rana ridibunda treated with DMBA, a cytotoxic and immunosuppressive chemical agent, reported that the decrease in alkaline phosphatase activity can be taken as an index of hepatic parenchymal damage and hepatic necrosis. A decrease in the concentration of alkaline phosphatase in the liver of house sparrow when treated with furadan SP50 at
sublethal and LD$_{50}$ doses has been found by Nigam and Bakhre (1991). Das and Mukherjee (2000 c) also reported depletion in alkaline phosphatase activity in the brain, liver and kidney of *Labeo rohita* exposed to quinalphos, an organophosphorus pesticide. Shaikila et al. (1993) observed similar results in the fish *Sarotherodon mossambicus* under sevin toxicity. Verma et al. (1981) also reported reduction in gill, liver and kidney alkaline phosphatase activity of *Mystus vittatus* exposed to a pesticide thiotox.

There was a decrease in the activity of alkaline phosphatase in the muscle and gill of *Heteropneustes fossilis* exposed to experimental concentrations of Rogor (Borah and Yadav, 1996). This fall in the activity of the enzyme may be correlated with its function in the supply of inorganic phosphates for DNA synthesis. Ajayi and Ajimoko (2005) observed a reduction in alkaline phosphatase activity in the heart and brain of albino rats exposed to low doses of actellic – 20, an organophosphorous pesticide. According to them, this decline may be due to a disruption in the plasma membranes of the cells, leading to the leakage of this enzyme into extracellular fluids, which could grossly affect the rate of hydrolysis of phosphoric group of esters and hence production of energy in the form of ATP. Velisek et al. (2006) reported reduced serum alkaline phosphatase value in the rain bow trout, *Oncorhynchus mykiss* as a result of stress induced by low doses of cypermethrin, a widely used pesticide.

Severe drop in tissue alkaline phosphatase could be adaptive for the frog to meet the energy demand aiding anaerobic break down of glycogen in liver and muscle under pesticide toxicity. This finding is in close agreement with that of earlier workers.
5.3. REFERENCES


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