DISCUSSION
Studies on the effect of organophosphates on vertebrates are described in considerable details but less information is available on organophosphate toxicity in different regions of fish brain. Since the brain is a heterogenous organ, in some ways, it is more a collection of disparate organs than a single entity (Hertz, 1969). This heterogeneity is of great importance in the evaluation and interpretation of the biochemical, histochemical and ultrastructural findings. Therefore, in the present study, following exposure to three different concentrations of organophosphate dichlorvos, lipid profiles, nucleic acids, glycogen, -SH group and electron microscopic alterations were determined in different regions of the brain and spinal cord of the fish H. fossilis.

4.1 Lipid profiles

The lipids in the nervous system form an important part of neurochemical investigations. Myelin sheath and neuropil of gray matter account for much of the total lipid contents of brain tissue (Schimizu, 1965). Myelin metabolism may be affected by a variety of toxic agents.

In the present study, total lipids showed dose-dependent elevation in all the components of brain and spinal cord of H. fossilis after dichlorvos intoxication. These observations are in concordance with the histochemical findings. A report on the effect of three potent anticholinesterases containing phosphorus, diisopropylfluorophosphonate (DFP), triorthocresylphosphate (TOCP) and bismonoisopropylaminofluorophosphine oxide (Mipafox) indicates that anticholinesterases affect lipid metabolism in the nervous system (Majno and Karnovsky, 1961). Changes in the lipid content
and lipid composition must be due to changes in rates of anabolism, catabolism or both. These processes are controlled by the activities of appropriate enzymes. Hazzard et al. (1969) and Ham and Rose (1969) have suggested that a reduced lipoprotein lipase activity may be a factor contributing to the increased plasma lipid. Furthermore, the increase in total lipid contents in different regions of brain and spinal cord, irrespective of their regional variations, can be explained on the basis of the observation of Caley and Jenson (1973) who detected inhibition of lipase activity following organophosphate administration. Tayyaba and Hasan (1980) have also observed increased concentration of total lipids after organophosphate administration to rats. Hasan and Khan (1985) demonstrated similar results with methyl parathion.

Phospholipids play a significant role in metabolism, due to their share in composition of the membrane. Moreover, changes in phospholipid composition may result in alteration of membrane structure. The effect of drugs on phospholipid metabolism is of great importance.

In this study, significant decrement in phospholipid level has been observed biochemically as well as histochemically in fish brain following the exposure of the drug dichlorvos. The decrement in the level of phospholipid can be explained on the basis of the findings of Nelson and Barnum (1960), who reported an effect on brain phosphatidylecholine metabolism, occurring a very short time after the injection on 2.5 mg/kg body weight of DFP. Transfer of P$^{32}$ from the acid-soluble pool to the phospholipids was significantly reduced by determining the
specific radioactivities of the phosphoryleholine and the phosphotidylcholine at three different times after injection of $^{32}$P orthophosphate. They concluded that there was a reduction in the rate of transfer of phosphoryleholine to phosphotidylcholine by Kennedy pathway. It is likely that the reduction in the rate of transfer of phosphorylcholine was a genuine one. This inhibition of phospholipid biosynthesis at a point subsequent to the phosphorylation of the base is a feature exhibited by some other drugs that inhibit phospholipid metabolism.

Earlier, Austin (1957) observed decreased incorporation of $^{32}$P into total phospholipids after administration of DFP (diisopropylphosphorofluoridate) to mice. Rao and Rao (1984) also reported decline in phospholipid content after sublethal stress of methyl parathion for 48 hrs to the fish, Oreochromis mossambicus. Previous reports from this laboratory also embody similar findings after organophosphate intoxication (Tayyaba and Hasan, 1980; Islam et al., 1983; Tayyaba and Hasan, 1985; Vadhva and Hasan, 1986 and Naqvi et al. 1988).

In the present investigation, the increase noticed in brain cholesterol level after dichlorvos treatment suggests diversion of acetyl CoA to acetoacetate for the synthesis of cholesterol. The administration of organophosphorus insecticides has previously been shown to increase acetoacetate and $\beta$-hydroxybutyrate levels (Domschke et al., 1971). Since TCA cycle enzymes are inhibited during organophosphate stress (Rao and Rao, 1979), the accumulation of acetyl CoA is likely to cause this diversion. The acetyl CoA produced through augmented $\beta$ oxidation
and glycolysis (Rao and Rao, 1983) will produce increased amounts of ketone bodies, particularly acetoacetate, which then acts as precursor for the synthesis of cholesterol. The organophosphorus insecticides are known to inhibit the metabolism of steroids (Kupfer, 1969). Thus, the increase in cholesterol content might also be due to its non-utilization for the synthesis of steroidal hormones.

Previous studies with dichlorvos (Tayyaba and Hasan, 1980), sumithion (Nag and Ghosh, 1984) and methyl parathion (Rao and Rao, 1984 and Hasan and Khan, 1985), have indicated similar increase in the cholesterol content in rats and fishes.

Dose-dependent increment of esterified fatty acids was noted in all the regions of CNS following exposure to dichlorvos. The tendency of increase in esterified fatty acid as triglyceride concentration may be due to inhibition of lipase activity (Ryhanen et al., 1984). The concentration of esterified fatty acids depends upon the content of the fatty acids present in triglyceride and phospholipids (Stern and Shapiro, 1953). It has been reported that brain contains minor amounts of triglycerides (Rowe, 1969; Cook, 1981 and Horrocks and Harder, 1983). An enzymatic system present in the brain catalyzes the acylation of endogenous and exogenous diglycerides by long chain acyl CoAs to triglycerides.

Gangliosides are ubiquitously distributed in neural tissues of vertebrates. They act as receptor sites for neurotoxins, and hence are involved in the nerve impulse conduction (Van Heyningen, 1959
and North et al., 1961). It has recently been demonstrated that gangliosides modulate phosphorylation system (Agnati et al., 1984; 1985; Bremer et al., 1984, 1986 and Tsujii et al., 1985). Biosynthesis of brain gangliosides occurs by sequential addition of monosaccharides or N-acetyleneuraminic acid to the carbohydrate chain, starting from ceramide. Degradation of brain gangliosides proceeds by sequential removal of monosaccharide and Neu NAc (N-acetyleneuraminic acid) by glycosidases and neuraminidases. According to Irwin and Samson (1971) certain types of behavioural stimulations (stress, exercise, sensory stimulation and learning) seems to be accompanied with alteration of ganglioside metabolism. In the present study, the depletion of ganglioside levels can be explained on the basis of the recent findings of Khan and Hasan (1988), who have observed reduction in gangliosides concentration in different regions of CNS following the administration of organophosphate methyl parathion. Earlier, Tayyaba and Hasan (1985) and Islam et al. (1983) have also observed depletion of gangliosides after metasystox administration. Whatever may be the specific role or roles of gangliosides in the brain, it is clearly evident by now that they serve an active rather than passive function.

4.2 Lipid peroxidation

Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated lipids. The reaction is initiated by free radicals and the lipids are oxidized to hydroperoxides (Tappel, 1973). It is postulated that these hydroperoxides are further metabolized to malonaldehyde and the latter then reacts with amino groups of other biological molecules.
to form Schiff-base class compounds known as lipofuscin pigments, which are believed to be metabolic end products of the lipid peroxidation processes.

It has been reported that tissues most susceptible to lipid peroxidation appear to be those with low mitotic rate such as brain (Barber and Wilbur, 1959). Kartha and Krishnamurthy (1978) showed that among the various organs, the brain showed a considerably high degree of peroxidation. This might be because brain has the largest amount of lipid of all body organs. The brain homogenate has apparently the necessary unsaturated fatty acids and the catalysts for peroxidation in the architecture of the cell itself which are readily available for reaction with molecular oxygen to undergo lipid peroxidation. Biomembranes and subcellular organelles are the major sites of lipid peroxidation damage (Tappel, 1970). The peroxidative changes triggered by free radicals in brain fatty acids and phospholipids may be of importance in the development of brain cell damage. Free radicals i.e. highly reactive molecules with an unpaired electron in an outer orbital, are constantly being formed in various reactions essential for aerobic life. The best studied effect of free radical attack is the one causing lipid peroxidation i.e., oxidation of \( \alpha \)-methylene bridges of unsaturated fatty acids, resulting in the formation of lipid peroxides and hydroperoxides, leading finally to fragmentation of lipids. At least two systems are important in the animal body to protect against membrane damage resulting from uncontrolled lipid peroxidation. These systems rely on selenium and vitamin E, respectively, and form the basis for the hypothesis concerning
the antioxidant functions of these nutrients (Combs et al., 1975). Hoekstra (1975) has shown that the organophosphate, tri-o-cresyl phosphate, interferes with selenium and glutathione peroxidase. It is likely that a similar mechanism operates in the dichlorvos-induced increase in the rate of lipid peroxidation observed in the present investigation.

The induction of massive accumulation of cytoplasmic dense bodies (lipofuscin pigment) has been shown following organophosphate pesticide (Hasan and Ali, 1980). The increased incidence of electron dense bodies appears to be the end result of lipid peroxidation. Spoerri and Glees (1979) have reported similar increase of electron dense bodies, labelled by them as lipofuscin, following intoxication with TOCP (triothrocresyl phosphate) in the hen.

Recently, Tayyaba and Hasan (1985) have reported organophosphate metasystox induced increase in the rate of lipid peroxidation. Hence, in the present investigation it would not be unreasonable to conclude that the exposure of fish to DDVP causes increased rate of lipid peroxidation.

4.3 Lipase activity

In the brain, lipase activity is concentrated more in gray than in white matter (Gozzano, 1934). Triglycerols and diacylglycerols of brain are very active metabolically and are hydrolyzed by lipases (Mizobuchi et al., 1981 and Vyvoda and Rowe, 1973). Rates of lipase reaction can, therefore, be measured by determining either the rate
of disappearance of the substrate or the rate of production of the fatty acids. No report on the dose-dependent effect of organophosphate dichlorvos on the activity of lipase in various regions of fish CNS is available in the literature. Recently, Islam et al. (1983) and Tayyaba and Hasan (1985) have studied the effect of metasystox on rat brain lipids and lipase activity. In the present study, DDVP exposure was observed to cause significant dose-dependent depletion of lipase activity in different regions of fish brain and spinal cord. It has earlier been demonstrated that cholinesterases, chymotrypsin, trypsin, esterases, thrombin and lipases are inhibited as a result of reaction with DFP-diisopropylfluorophosphate (Webb, 1948 and Mounter et al., 1957). Depletion of lipase activity after parathion administration has been observed by Caley and Jenson (1973). Christensen and Riedel (1981) have reported decrement in lipase activity following organophosphate intoxication. The decrement of lipase activity noted in the present study points to the inactivation of this enzyme. This inactivation of enzyme activity may be attributed to the disruption of the three dimensional structure of protein which is responsible for the activity of the enzyme. Another possibility may be of an interaction between the amino acid present on the active site of the enzyme with the organophosphate. The function of phospholipase is to hydrolyse triglycerides. A decrease in the enzyme activity could result in accumulation of triglyceride.

4.4 Glycogen

The content of glycogen was found to decrease with all the three dose-treatments in the different regions of fish brain following
dichlorvos toxicosis. In fish, during actual or potential stress, catecholamines are secreted in increased amounts which deplete glycogen reserves (Nakano and Toulinson, 1967 and Larsson, 1973). The marked glycogenolysis in brain regions after treatment with dichlorvos was possibly caused by a stress-induced increase in circulating catecholamines. Studies in the killifish (Fundulus heteroclitus) and the brown bullhead (Ictalurus nobulosus) have indicated that epinephrine induced hepatic glycogenolysis with concomitant increase in specific activity of hepatic total glycogen phosphorylase assayed with AMP (Umminger and Bair, 1973 and Umminger and Benziger, 1975). Koundinya and Ramamurthy (1979) also reported increase in muscle and liver glycogen phosphorylase activity and depletion of hepatic content in Tilapia mossambica exposed to sumithion.

Hyperglycemia develops in fish during acute exposure to organophosphorus pesticides (Koundinya and Ramamurthy, 1979; Srivastava and Singh, 1980, 1981 and Verma et al., 1983). Husain and Matin (1986) and Matin and Husain (1987a) reported hyperglycaemia accompanied by depletion of glycogen in rat brain after malathion administration. The range and variety of agents active in affecting glycogen metabolism may be taken as a general indication that glycogen is important to brain function. Little information is available on the mechanism of anticholinesterase action of organophosphate pesticides on carbohydrate metabolism in fish. Nevertheless, organophosphate pesticides cause accumulation of acetylcholine with a simultaneous increase in the secretion of catecholamines in mammals (Brzezinski and Ludwicki, 1973). Further, exogenous acetylcholine in fish leads to an increased secretion of catecholamines
(Nilsson et al., 1976). Hence, the latter may induce glycogenolysis and hyperglycemia through the involvement of adenylcyclase (Sutherland, 1972).

Evidently, the hyperglycemic response noted in the present study was found to be induced by dichlorvos exposure which resulted in mobilization of glycogen reserves. An alternative mechanism for the development of hyperglycemia could be due to cholinesterase inhibitors blocking the glucose receptors of pancreatic \( \beta \) cells, resulting in their non-reactivity to the raised glucose level followed by reduction in glycogen content.

4.5 Nucleic acids

Dichlorvos-intoxication lowered the levels of DNA in all the regions of fish brain. Higher decreasing trend was found to be associated with higher dose levels. Since DNA is the chemical estimate of cell number, its decline also suggested some loss of cells. The loss of DNA content of fish shows the possible interference of dichlorvos with nucleic acid synthesis.

It has been shown that different regions of the brain have different DNA concentrations, with the maximum in cerebellum. The findings are in agreement with the observations of May and Grenell (1959). Greater amount of DNA in cerebellum reflects the extreme cell density of cerebellar granular layer. This observation finds support of the earlier report of Tayyaba et al. (1981). Among the regions analysed, the maximum (43.40\%) depletion of DNA due to dichlorvos intoxication was found to occur in the fore brain, whereas the optic lobe was the
least (-16%) affected region. This indicates that each region of the brain has its own vulnerability towards dichlorvos toxicity.

Different organophosphates are known to reduce DNA level of various brain regions. Paolo and Fini (1980) and Rath and Misra (1980) have reported diminution in the DNA content after dichlorvos intoxication. One of the reasons for this diminution is the degeneration of neurons and nerve fibres as reported in organophosphate toxicosis (Hasan et al., 1979) in connection with increased lipid peroxidation (Hasan and Ali, 1980). It has also been reported that organophosphates chromatolyse the neurons in chick (Janzik and Glees, 1966). In addition to this, the reduction in DNA level may be attributed to the increased DNase activity, in the brain regions of rat treated with metasystox, as suggested by Tayyaba et al. (1981). Thus, the reduction in the level of DNA in all the regions of the brain may be due to the disturbances caused by dichlorvos in the normal synthesis and turnover rate of DNA, in addition to degenerative changes.

The response of RNA to dichlorvos intoxication, on the other hand, was different from that of DNA. The RNA concentration was found to increase in all the regions of fish brain following dichlorvos exposure. Thus the effect of dichlorvos on RNA cannot be compared with DNA and this may be due to the changes in the activity of enzymes which controls the synthesis and turnover of nucleic acids.

It has been shown that demyelination is a feature of poisoning by many organophosphate pesticides, leading to increased production
of RNA (Heath, 1961). McIlwain and Bachelard (1971) have reported that RNA synthesis increased due to damage of neurons. In metasystox treated rat brain, the increase of RNA concentration paralleled with RNAse activity (Tayyaba et al., 1981). Furthermore, the increment of RNA level in the brain of dieldrin fed rats has also been reported by Bergen (1972). The elevation in the level of RNA is generally associated with improvement in protein nutrition or tissue function (Bergen et al., 1974). Hence, the enhanced level of RNA content in different regions of the brain suggests to a possible selectivity in protein synthesis under stress condition (Prosser, 1969).

4.6 Proteins

The changes in the neuronal activity are accompanied by measurable changes in macromolecules like protein in brain cell. However, it has also been reported that the increased neuronal activity decreases or inhibits the synthesis of proteins (Hyden and Lange, 1972). The specific neuronal functions, such as conduction of action potentials and synaptic transmission, are known to be mediated by protein (Bock, 1978). It has been demonstrated that many environmental and nutritional factors may perturb the brain protein (McIlwain and Bachelard, 1971). Furthermore, Arnaiz et al. (1975) have reported that inhibition of protein level might be an indication of a disequilibrium of the normal energy-yielding metabolism. In the present study, the decrement of protein contents in all the regions of brain can be explained on the basis of the findings of Ahmed et al. (1978) who reported that the decrement
of protein is due to increased proteolytic activity necessitated by greater energy demands under toxic stress. Evidence of a reduction in protein level was also obtained in metasystox treated rats (Tayyaba et al., 1981). Sub-lethal studies in fish, *Tilapia mossambica*, with dichlorvos has also revealed significant decrease in protein levels (Rath and Misra, 1980). The RNA content indicates the intensity of the protein synthesis in a tissue (Brachet, 1955). Hence, the increased level of RNA in the present investigation suggests the non-interference of dichlorvos directly in the protein synthesis. Despite the fact that the synthesis of protein is not inhibited, the decrement of protein suggests its higher rate of degradation due to dichlorvos toxicosis. Free amino acids in brain are known to be involved in a number of metabolic processes (Neame, 1968). Hence, demand for amino acids may possibly induce the breakdown of proteins enabling the animal to withstand the toxic stress by performing normal neurochemical functions.

4.7 Sulphydryl groups

To date, no report is available on the quantitative estimation of sulphydryl groups in different regions of fish brain following DDVP intoxication. Sulphydryl groups are known to act as active enzymatic sites (Hoch and Vallee, 1959). Sulphydryl enzymes have been observed to be the most susceptible to lipid peroxidation induced inactivation (Chio and Tappel, 1969). Several investigators have thought that glutathione plays specific roles in mitotic cells and may, therefore, be essential for repairing systems. It is further reported that glutathione and
sulfhydryl groups play a protective role against free radical mediated or peroxidative damage (Stokinger and Coffin, 1968; DeLucia et al., 1972; Chow and Tappel, 1972 and Little and O'Brien, 1968). In the present investigation dichlorvos exposure caused significant dose-related depletion of the total sulfhydryl groups and glutathion (Free-SH) in different regions of the fish CNS. This deficiency of total and free sulfhydryl groups may lead to the deficient degradation of lipid peroxides to hydroxy acids, as shown by Tappel (1970), leading to the accumulation of the former in various regions of the brain.

4.8 Electron microscopy

No comparable electron microscopic report on alterations in the different regions of the fish brain following dichlorvos treatment is available. The most significant finding of the present study was the demonstration of vacuolar spaces with multilamellar membranous whorls. Ahmed and Glees (1971) reported increased occurrence of laminated cytoplasmic inclusion bodies after organophosphate administration. They postulated that phospholipids become unmasked during a degenerative process and arrange themselves in the form of laminated dense bodies. It is conceivable that the cytoplasmic multilamellar membranous whorls observed in this study might represent a disturbance in phospholipids. Additionally, electron-dense bodies and compressed arcuate saccules of Golgi zone was observed in neuronal perikarya after dichlorvos intoxication. According to Kerencyi et al. (1968) these pigment masses are irreversibly injured lysosomes and are liable to develop under the influence
of specific stress on the nervous system. Glees and Hasan (1976), on the basis of available experimental evidence, concluded that neurons subjected to enhanced or reduced metabolic activity responded in final stages by lipofuscin/ceroid formation. On the other hand, according to Novikoff (1967), lipofuscin pigment may be formed as a result of a chain of activities in Golgi apparatus, endoplasmic reticulum and lysosome. In light of the above mentioned observations, it appears likely that the dichlorvos-intoxication, acting as a specific stress on the nervous system, triggers off a chain of activities in the Golgi apparatus-endoplasmic reticulum-lysosomes and mitochondria, thereby culminating in the deposition of pigment in neuronal perikarya. Remarkable increase in electron density in many neurons was also reported after organophosphate administration (Hasan et al., 1979a; Hasan et al., 1979b; Hasan et al., 1979c and Hasan and Ali, 1980). The evidence of edema and disruption of synaptic vesicles following dichlorvos intoxication was the remarkable feature of the present investigation and support the observations of several authors (Hasan et al., 1979a; Hasan et al., 1979b and Hasan et al., 1979c).