Sublethal Toxic Effects of Gusathion-A on the Morphology of the Hepatocytes of *Cyprinus carpio*

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ABSTRACT

Cyprinus carpio *fingerlings were exposed to sublethal levels of 0.05 ppm and 0.1 ppm Gusathion-A for 30 days.*

Cell disarray, hepatocyte necrosis and vacuolation were observed. Ultrastructural change in the hepatocytes was loss of structural integrity of the plasma membrane, nuclear membrane, endoplasmic reticulum and mitochondria and other cell structures. Glycogen deposits were depleted.

INTRODUCTION

Pesticides have been extensively used to increase food production to minimize the incidence of plant diseases since they were first introduced in the 1940's. Their widespread use however, has caused damage to many organisms, even to beneficial ones, resulting in the disruption of the ecological balance. Pesticides are known to enter the aquatic ecosystem by direct application, spray drift, aerial spraying, washing from the atmosphere by precipitation, erosion and run-off from agricultural land, and discharge of effluents from factories and sewage.

Pollution of aquatic ecosystem is widespread, sometimes so bad that no fish can live in the polluted waters (1). Pesticides are one of a wide variety of organic pollutants contributing to this situation. They cause pollution due to their stability and persistence in aquatic ecosystems, high toxicity to aquatic fauna and flora, low water solubility, potential for uptake and bioconcentration in aquatic organisms, and heavy usage. Because of these, pesticides cause impairments in

the development of organs, biochemical and physiological processes and histopathological changes in non-target organisms (2).

Among the different classes of pesticides such as chlorinated hydrocarbons (organochlorines), organophosphates, carbamates and chlorophenoxy acid esters and salts, the first three are the most commonly used. Organophosphates have been favored due to their greater effectiveness against some species, the development of resistance of some insects toward organochlorines, and the presumed environmental safety of the organophosphates by virtue of their more rapid degradation (3). Parathion, synthesized by Schrader in 1944, has a high mammalian toxicity. Further efforts to find less hazardous compounds led to the synthesis of malathion and other less persistent insecticides. At the time of this study, Azinphos-methyl (guthion) was one of the commonly used organophosphates in several countries including the Philippines. It was synthesized by Lorenz in 1953 for the control of insect and mite species (4). It was widely used here and abroad for controlling agricultural pests. In Chile, it has been used for the past 12 years as the standard insecticide for the control of rangeland pests (5). Its diethyl ester, azinphos-ethyl was commercially produced and sold in the Philippines under six brand names namely; Gusathion-A, Azinos, Bionex, Cotnion-ethyl, Crysthion, and Ethyl guthion.

The widespread use of Gusathion-A as a broad spectrum insecticide for the control of biting and sucking pests made possible its entry into the aquatic environment through both direct spraying and run-off.

Gusathion was reported to be toxic to fishes and also caused acetylcholinesterase inhibition in *Cyprinodon variegatus* (7). Though Gusathion-A is a widely used pesticide abroad, studies on its histological effects on fishes are limited.

This investigation aims to determine whether sublethal levels of Gusathion-A have toxic effects on the morphology of the hepatocytes of *Cyprinus carpio*, a freshwater fish of economic importance in the Philippines. The common carp was used as the test organism because of its availability, ease in maintenance in the laboratory and tolerance to a wide range of temperature (8).

MATERIALS AND METHODS

Chemical

The pesticide used was Gusathion-A ([0,0 -diethyl-S-(4-oxo-3H-1,2,3-benzotriazine-3yl) methyl]-dithiophosphate), commercial grade, 40 E.C. manufactured locally by Bayer Philippines.

Organism

The common carp, *Cyprinus carpio*, a commercially desirable and popularly cultured species in Asia and the Indo-Pacific region was used as the test organism.

Determination of 96h-LC_{50}

Prior to actual experimentation, the 96h-LC_{50} was determined by conducting assays on three replicates of 10 *Cyprinus carpio* each. They were exposed to six concentrations of Gusathion-A: 0.1 ppm, 0.2 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm and 1.0 ppm in 5.0 liters of test solutions. These are the ranges of 96h-LC_{50} in teleosts.

The 96h-LC_{50} (0.30 ppm) was determined and computed using two methods :1) the graphical method of Finney wherein the percent mortality of fish in different concentrations of Gusathion-A in 96h was plotted against Gusathion-A concentration and 2) the Dragstedt-Behrens method wherein the percent mortality was calculated from the cumulative mortality.

Exposure to Gusathion-A

Cyprinus carpio fingerlings (2 months old) obtained from Tanay Research Station, Bureau of Fisheries and Aquatic Resources, Tanay, Rizal were used in the study. They were acclimated at the Institute of Biology laboratory for one week prior to initial exposure to Gusathion-A. Twenty *Cyprinus carpio* were placed in each 20-liter aquarium with aerated aged tap water, and Gusathion-A was added to attain a concentration of 0.05 ppm and 0.1 ppm. Each treatment was done in triplicate. Twenty fishes per aquarium were used for the control group, also in triplicate. Commercial fish flakes were given to the fish twice a day. The aquarium medium was changed every 48 hours to replenish the Gusathion-A concentration and to reduce the build-up of waste products.

The study was conducted for 30 days employing a static method of organophosphate exposure.

Histologic Procedure

Ten fishes from each control and experimental group were sacrificed after 15- and 30-day exposure for identification of histological changes that may have occured in the developing liver.

Resin Sections

Liver tissue of five fishes from each experimental and control group were removed immediately and processed using the standard resin technique.

Observations were done using the Bausch and Lomb light binocular microscope and JEOL JEM 100U electron microscope. Photomicrographs and electron micrographs were prepared.

RESULTS AND DISCUSSION

Morphology of control and Gusathion A-treated hepatocytes of day 90 *Cyprinus carpio* fingerlings.

1. Light microscopy

a. Control hepatocytes

Light micrograph of a section of control liver tissues of day 90 *Cyprinus carpio* fingerlings has the features of the typical vertebrate liver (Figs. 1, 4).

b. Gusathion-A treated hepatocytes

Cyprinus carpio fingerlings exposed to 0.05 ppm Gusathion-A for 30 days show less intense pathological lesions of the hepatocytes than those exposed to 0.1 mg/L of Gusathion-A . Hepatocytes are disorganized resulting in the loss of the normal cord pattern (Figs. 2,5). There is severe hepatic vacuolation (Fig. 2), such that nuclei are displaced from a central location within the hepatocytes (Fig.5).

Pathological lesions are aggravated in the hepatocytes of day 90 *Cyprinus carpio* after exposure to 0.1 ppm Gusathion-A for 30 days. Plate disarray and vacuolation are intensified and sinusoids are dilated (Fig. 3). The most pronounced lesion

observed is necrosis leading to loss of normal liver appearance. Cell membrane disruption is evident producing atypical hepatocytes (Fig. 6).

2. Electron microscopy

a. Control hepatocytes

Electron micrographs of ultra thin sections of control liver tissue of day 90 *Cyprinus carpio* reveal the typical fine structure of hepatocytes (Fig. 7). It is similar to the cytoarchitecture of typical vertebrate hepatocytes.

b. Gusathion A- treated hepatocytes

Several ultrastructure changes are observed in the hepatocytes of day 90 *Cyprinus carpio* exposed to 0.05 ppm Gusathion-A for 30 days. As seen in Fig. 8, there is a change from the spherical shape of the nucleus to an irregular form with an indentation. The nuclear envelope is distinct. Mitochondria are swollen and have indistinct cristae and disintegrated outer membrane. In general, organelles lose their typical architecture (Fig. 10).

Electron micrographs of several sections of the liver tissue after 30 days of exposure to 0.1 ppm Gusathion-A reveal more serious histopathological changes in the hepatocytes of day 90 *Cyprinus carpio* (Figs. 9, 11, 12). The nuclear envelope appears intact in some hepatocytes but some manifest an irregular outline (Figs. 9, 12). Chromatin material is either clumped (heterochromatin) or dispersed (euchromatin). Numerous vacuoles of varied sizes become prominent feature of the cells (Fig. 9). Mitochondria are swollen and cristae are unrecognizable (Fig. 11). Some cisternae of the endoplasmic reticulum are broken and reorganized into sinuous and circular profiles (Fig. 12). The plasma membrane is severely disrupted just like in the hepatocytes exposed to 0.05 ppm Gusathion-A. Glycogen granules appear depleted.

As a metabolic centre, the liver is particularly exposed to harmful substances received via the food and, especially in the case of aquatic organisms, to environmental influences. In histochemical tests on Gusathion-A exposed *Cyprinus carpio* (9), there is an initial increase in certain enzymes in the liver indicating higher enzyme activity. This shows detoxification of Gusathion-A in the hepatocytes of the carp. However, as the exposure period is lengthened, the enzyme activity becomes lower. It is not surprising therefore that lesions have been reported in the livers of numerous fishes in response to any form of water pollutants (2). The lesions associated with Gusathion-A are non-specific in nature and have been reported in *Cyprinus carpio* fishes exposed to other pesticides (10). The extent

of liver damage observed in the present investigation indicates that chronic exposure causes changes to the architecture of the liver tissue. Since liver is involved in the detoxification of pesticides (11), it is susceptible to greater degree of disruption in its structural organization due to toxic stress.

Liver disarray and vacuolation were the initial toxic effects of Gusathion-A on the hepatocytes of *Cyprinus carpio* fingerlings. Increasing the concentration of the pesticide intensified the lesions observed. Cytoplasmic and nuclear degeneration had been reported also on mammalian and fish livers exposed to thiodan and agallol '3' MEMC (12), diazinon, methyl parathion and dimethoate (13).

Vacuolation is a dominant feature of the Gusathion-A exposed carp hepatocytes. This is a very common hepatic response to toxic agents such as CCl (14) and various pesticides (15). Vacuolation could be due to progressive dilation and distention of the cytoplasmic membranes believed to be caused by intracellular edema (16). Similar mechanism may work for Gusathion-A treated hepatocytes of carp. The vacuolation observed in the hepatocytes could be due to the failure of the ATPase system at the plasma membrane level. This disruption of the ATPase system can cause disturbance in fluid balance and bring about increased movement of water into the hepatocytes thus altering the hydrophobic core of the lipid bilayer. Certain components of the cell membrane, most notably phospholipid and cholesterol, are modified by exposure to organophosphate compounds thus membrane fluidity is dramatically altered.

Vacuolation could also be due to accumulation of fat. Hepatic injury characterized by cellular lipid accumulation is a common hepatic response to toxic substances. Various mechanisms may cause lipid accumulation in the liver. They include disturbances of the hepatocellular granular endoplasmic reticulum (18); increased lipid mobilization from peripheral tissue (19), and impaired release of lipoprotein from the liver cell (14). In this study no test was made to determine if fat accumulation was the cause of the extensive vacuolation observed.

Vacuolation is also associated with mitochondrial injury which impairs the function of citrate cycle causing diminished availability of ATP. This affects the fatty acid oxidation mechanism and causes the enhanced influence of free fatty acids to the liver and triglyceride accumulation. Acephate , an organophosphate inhibits the release of very low density lipoprotein (VLDL) from the liver and it has been proposed that it is due to a disturbance in the hepatic synthesis of

lipoproteins (20). There could also be mobilization of energy-rich lipids for production of energy during toxic stress caused by pesticides (21).

Generally, under any type of stress condition, the animals are bound to add extra energy to overcome the stress through the oxidation of either carbohydrates or proteins or lipid constituents (21). The increase in total lipids correlates with the increased activity levels of the enzyme lipase, responsible for the breakdown of lipids into free fatty acids and glycerol. The free fatty acids and glycerol formed might be diverted to yield energy to mitigate the toxic stress due to Gusathion-A. The mobilization of lipid reserves testifies the imposition of high energy demand under pesticide toxicity.

Massive swelling of mitochondria was similarly observed in the livers exposed to CCl, lindane and bacterial toxins (22). Mitochondrial swelling may be due to an increase in mitochondrial membrane permeability caused by an alteration of the ATPase membrane system thereby allowing the influx of Na ions and water (22). When the permeability of the inner mitochondrial membrane increases, the outer membrane is interrupted causing further expansion. Mitochondrial damage indicates change in the rate of respiration, phosphorylation and increase ion uptake in the fish.

Structural changes in the granular endoplasmic reticulum such as cisternal dilation and reorganization into sinuous and circular profiles may be considered functional changes (15). However, several studies attributed such changes to the interaction of the pesticides with lipid membranes (23). The insecticides modify the basic membrane mechanisms, eg. permeability to non-electrolytes and transport of cations mediated by ionophores which is similar to the mechanism that explains the vacuolation observed in the hepatocytes.

The depletion of glycogen was noted in the liver of rats subjected to fasting and refeeding (24) and *Chanos chanos* subjected to starvation (25). Glycogen depletion was also noted in pesticide-exposed fish livers (26). The reduction of liver glycogen would seem to be due to an effect of Gusathion-A on carbonate metabolism. The amount of glycogen depends on the nutritional state of the animal. Liver glycogen is a depot for glucose and is modified if the blood glucose falls. This is related to the low blood glucose level due to the damage done by Gusathion-A to the intestinal epithelium affecting digestion and absorption.

It has been suggested that the increase in the liver glycogen is presumably due to the depression of the glucokinase activity resulting in the reduction of G-6-PO4 which diminished the quantity of hexosephosphate, an intermediate

compound essential for the synthesis of glycogen (27). Another possible mechanism is that, the effect of pesticides involves some sort of activation on the sympathetic nervous system resulting in the release of adrenalin from the adrenal medulla which stimulates the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH). ACTH then activates the adrenal cortex to produce more glucocorticoid hormone (GCH) which stimulates hepatic glucose production from hepatic glycogen (28).

Organophosphates like Gusathion-A may exert their toxic effects to non-target organisms in two ways: 1) by altering the structure, function and activity of biological membranes and enzymes; and 2) by inhibiting the active transport systems which function to maintain the cellular level of inorganic electrolytes and fluids and the correct osmotic pressure within cells. The permeability of the lipid membranes is said to be modified by organophosphates by altering the hydrophobic core of the lipid bilayer. The impairment of the active transport systems is made possible by inhibiting electron transport in the mitochondria and altering the configuration of the ATPase molecule.

Liver lesions in teleost indicate the need for toxicological studies since these hepatic alterations have been linked to deterioration of the aquatic environment.

The decision of the Fertilizer and Pesticide Authority to finally ban the use of Gusathion-A is laudable.

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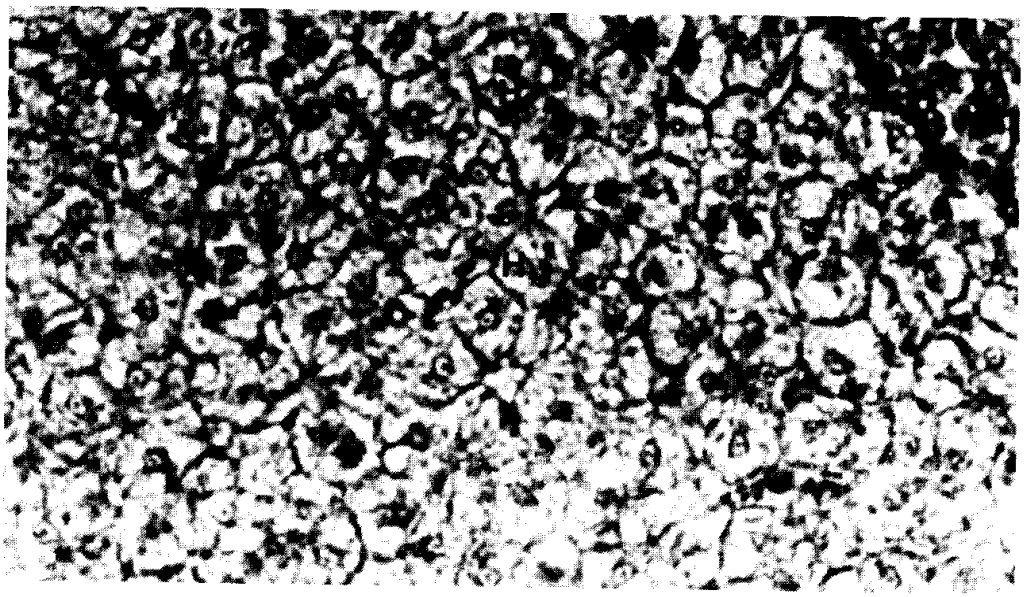

Figure 1. Control hepatocytes of day 90 *Cyprinus carpio* fingerlings in a cordal array. The hepatocyte (H) has an irregular polyhedral shape.

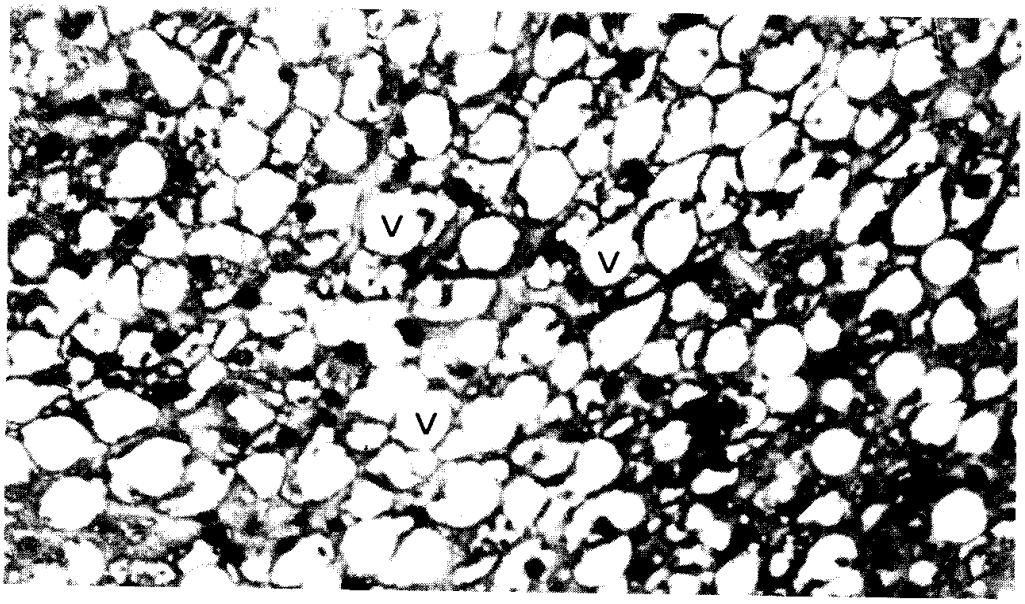

Figure 2. Hepatocytes of day 90 *Cyprinus carpio* fingerlings after exposure to 0.05 ppm Gusathion-A for 30 days showing severe vacuolation (V).

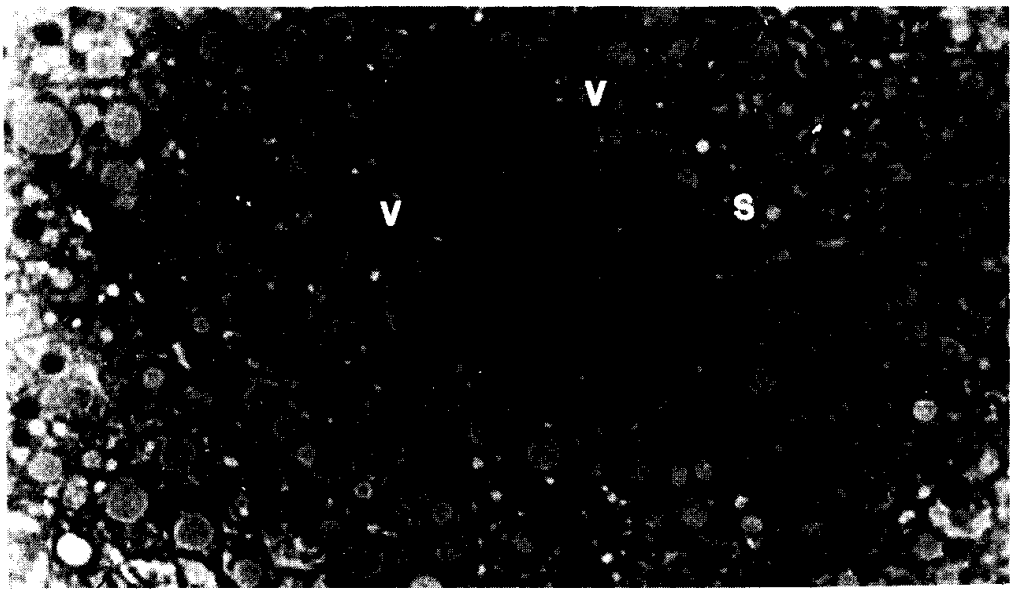

Figure 3. Hepatocytes of the day 90 *Cyprinus carpio* fingerlings after exposure to 0.1 ppm Gusathion-A showing plate disarray, extensive vacuolation (V), and dilated sinusoids (S).

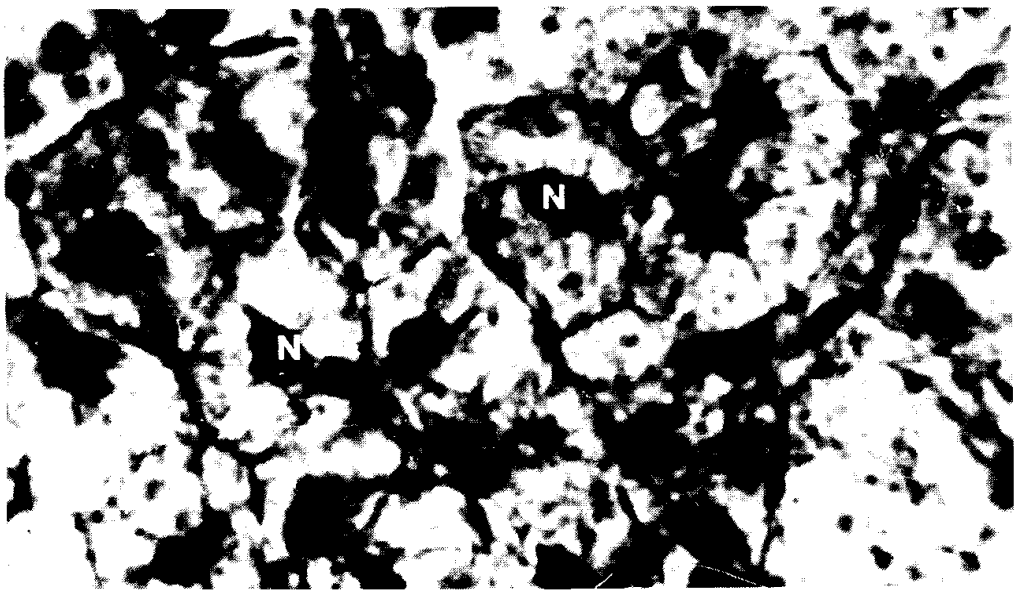

Figure 4. Control hepatocytes of day 90 *Cyprinus carpio* fingerlings displaying polyhedral forms with a more or less centrally located nucleus (N).

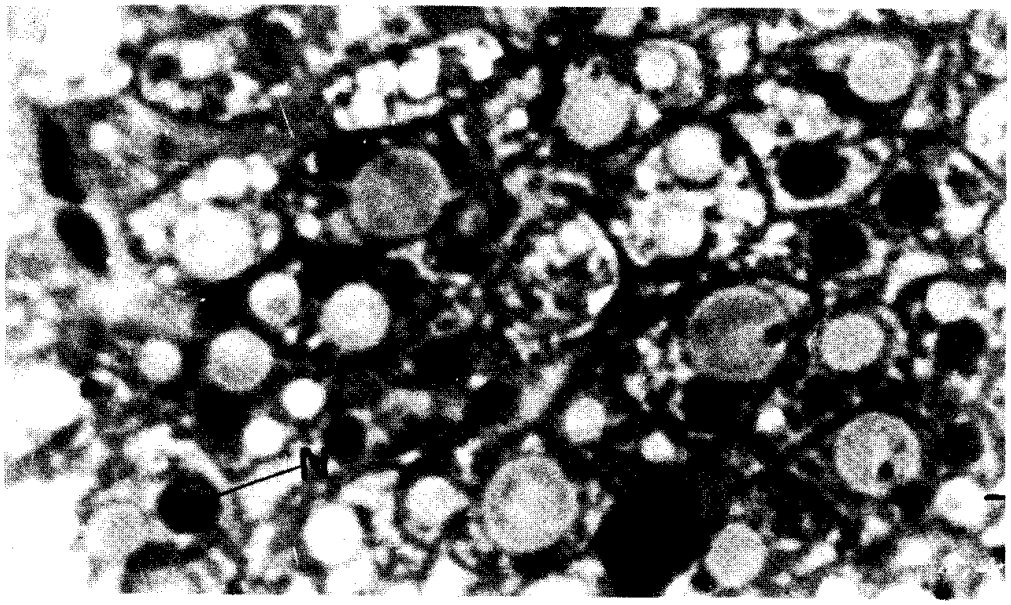

Figure 5. Hepatocytes of day 90 *Cyprinus carpio* fingerlings after exposure to 0.05 ppm Gusathion-A for 30 days showing loss of normal pattern and displaced nucleus (N).

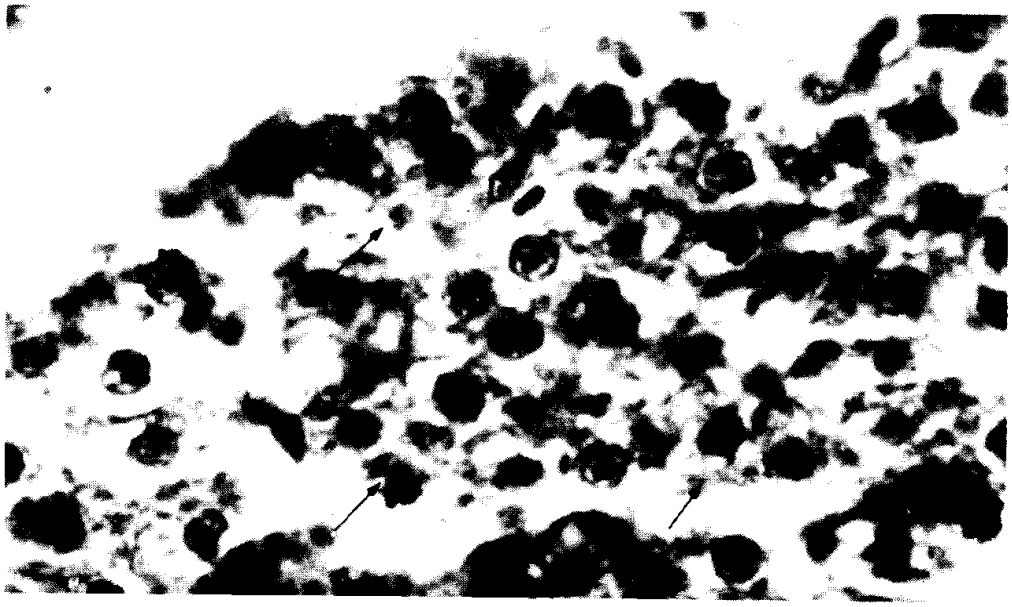

Figure 6. Hepatocytes of day 90 *Cyprinus carpio* fingerlings after exposure to 0.1 ppm Gusathion-A for 30 days. Advanced degenerative changes are observed (arrows)including cell membrane disruption.

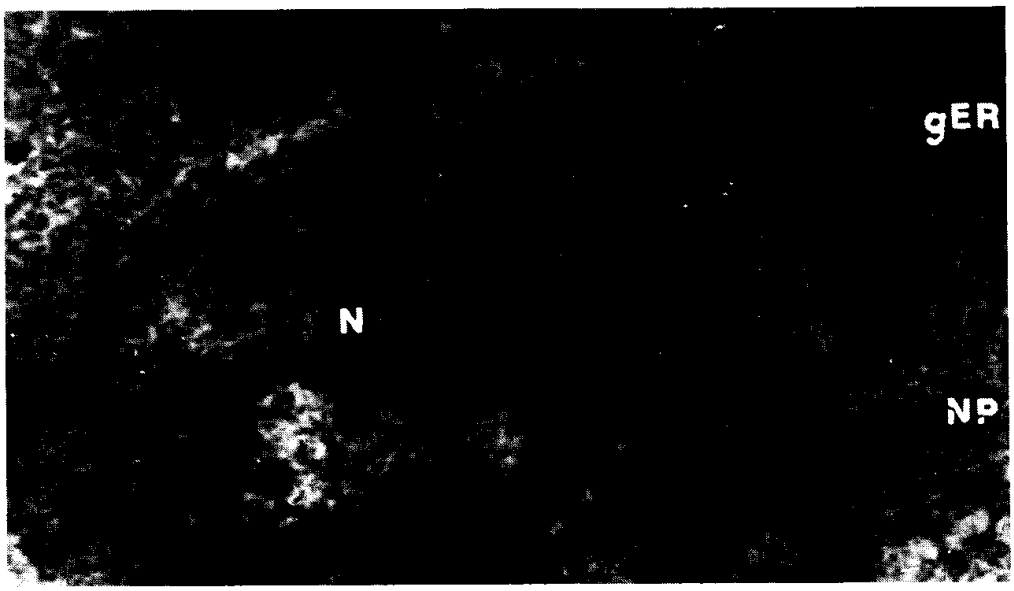

Figure 7. Electron micrograph of a portion of a control hepatocyte of day 90 *Cyprinus carpio* showing a part of the nucleus (N) with several nuclear pores (NP). Granular endoplasmic reticulum (gER) is seen composed of parallel stacks of cisternae studded with ribosomes.

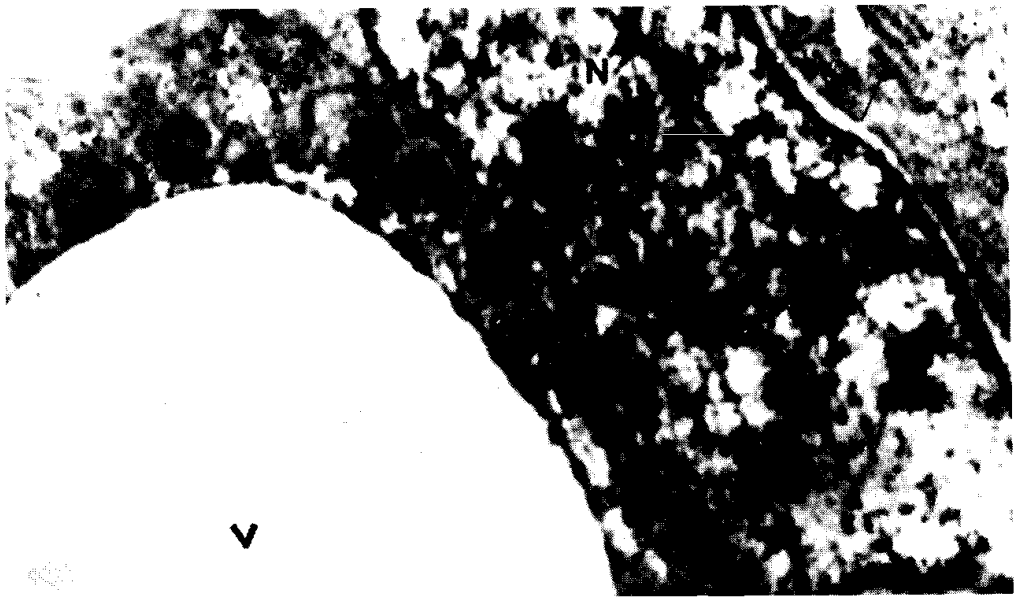

Figure 8. Electron micrograph of day 90 *Cyprinus carpio* hepatocyte after exposure to 0.05 ppm Gusathion-A for 30 days. Endoplasmic reticulum and mitochondria are not distinct structures. A nucleus (N) with an irregular outline is shown. V = vacuole.

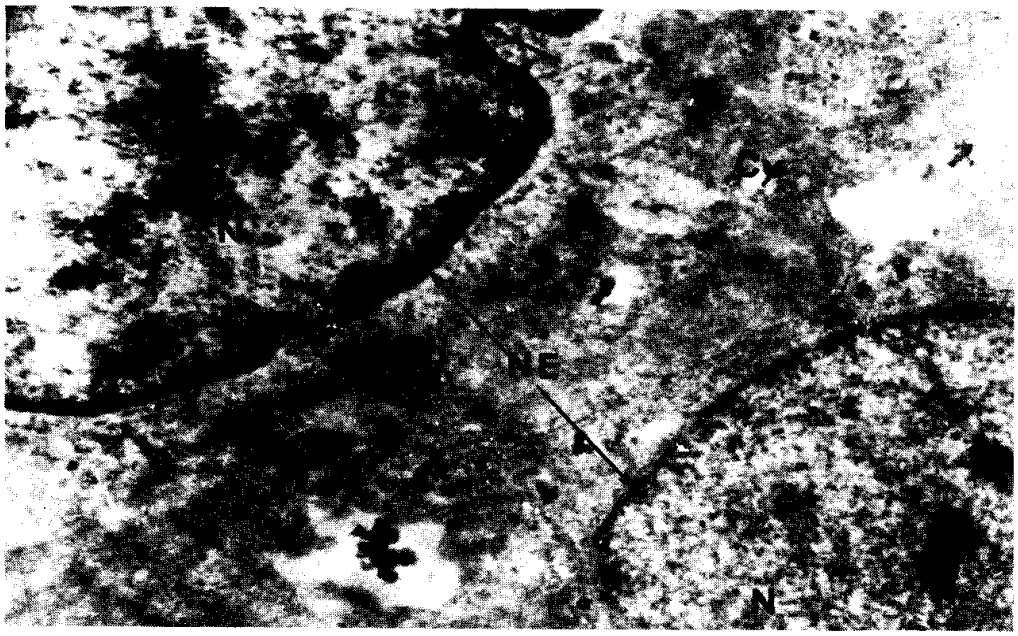

Figure 9. Electron micrograph of day 90 *Cyprinus carpio* hepatocyte after exposure to 0.1 ppm Gusathion-A for 30 days. Endoplasmic reticulum and mitochondria are hardly recognizable. A large vacuole (V) larger than the nucleus is shown. The nucleus has lost its spherical shape. Cy-cytoplasm, NE-nuclear envelope.

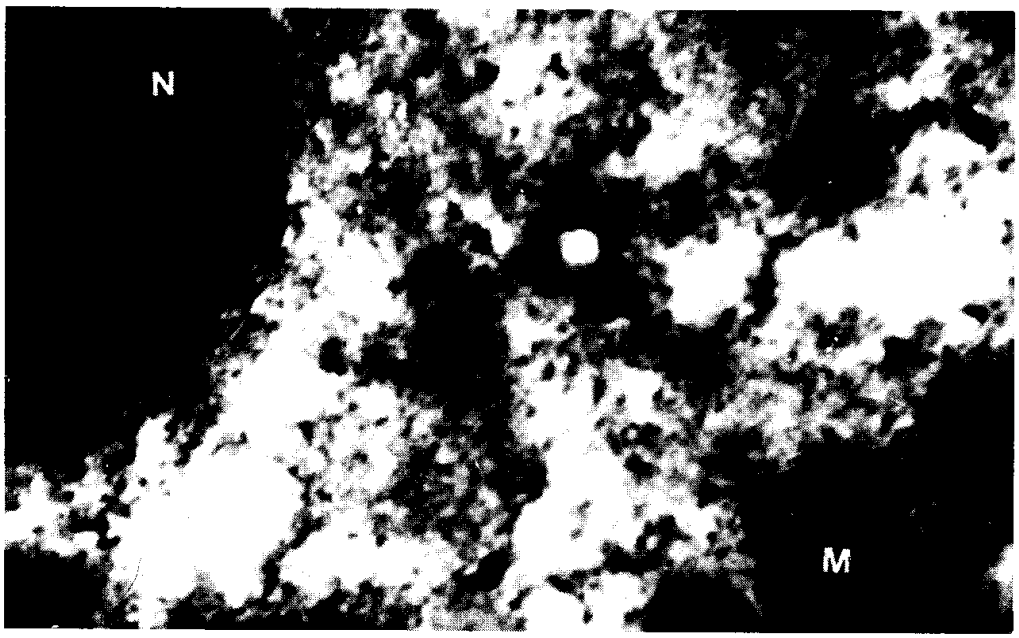

Figure 10. Electron micrograph of day 90 *Cyprinus carpio* hepatocyte after exposure to 0.05 ppm Gusathion-A for 30 days. The nucleus (N) appears intact but the mitochondria (M) appears abnormal.

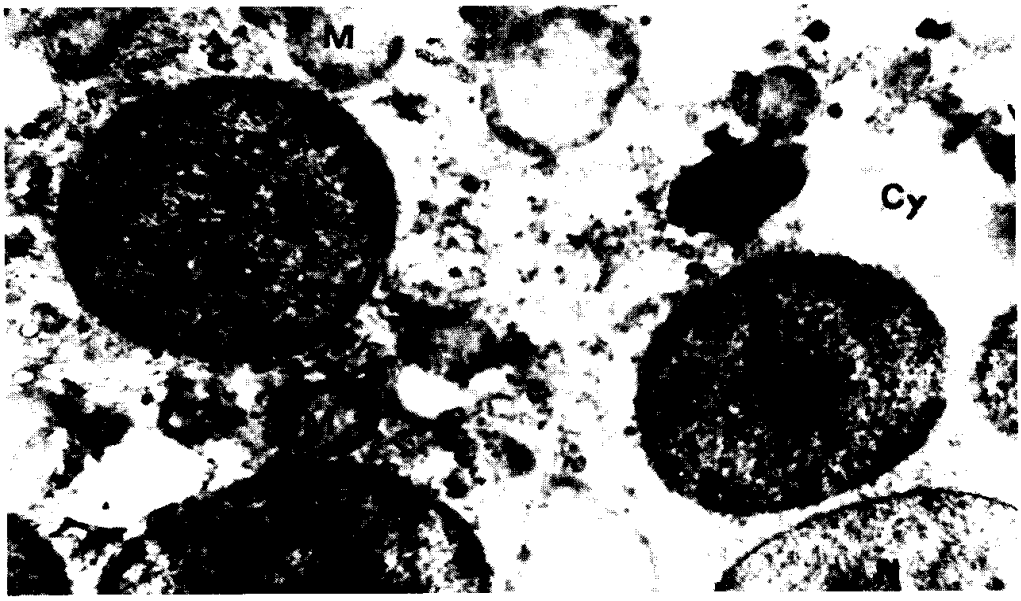

Figure 11. Electron micrograph of day 90 *Cyprinus carpio* hepatocyte after exposure to 0.1 ppm Gusathion-A for 30 days. The endoplasmic reticulum is not distinct and the plasma membrane is absent. The nuclei (N) are necrotic. M-mitochondria; Cy-cytoplasm.

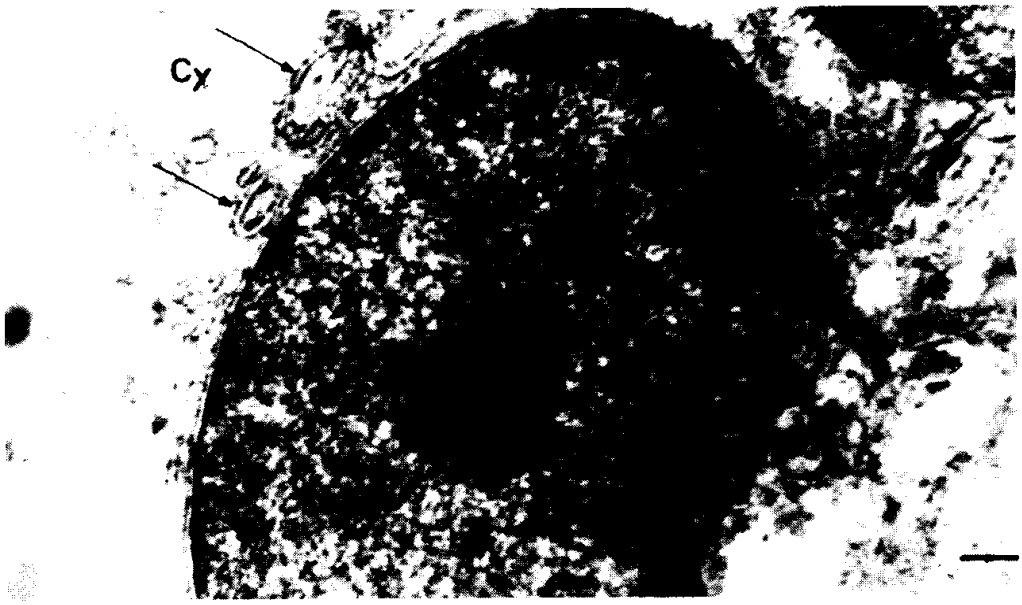

Figure 12. Electron micrograph of day 90 *Cyprinus carpio* hepatocyte after exposure to 0.1 ppm Gusathion-A for 30 days. The endoplasmic reticulum are reorganized into sinuous and circular profiles (arrows). Mitochondria are hardly recognizable. Glycogen granules appear depleted. Cy-cytoplasm