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# ELECTRONMICROSCOPIC ANALYSIS OF THE CYTOPATHOLOGICAL EFFECT OF PESTICIDES IN THE LIVER, KIDNEY AND GILL TISSUES OF CARP

I. BENEDECZKY, J. NEMCSÓK
and K. HALASY

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## Abstract

The cytopathological effect of the sublethal doses of paraquat, Ultracid 40 WP and copper sulphate was studied in the liver, kidney and gill tissues of carp with the help of electronmicroscopic method. After two weeks the Ultracid 40 WP produced expansive cell damage in the liver parenchyma cells, the paraquat did not produce significant cytopathological effect, however, the copper sulphate resulted a decrease in the heterochromatin substance of the nuclei. In the majority of the cell organelles the paraquat caused expansive degenerative alterations in the exocrine pancreas. All three agents displayed cell-damaging effect in the kidney tissue, contrary to this, alterations referring only to slight cell damage developed in the gill. Our observations call attention to the fact that pesticides and their residues found in our fish-ponds and natural waters imply potential toxic danger on the fish of the waters.
*Key words*: fish, cytopathology, pesticides

## Introduction

In Hungary 20–30 times severe fish perish occur annually as the consequence of water pollution. About one third of the water pollutions is due to chemicals used in agriculture (SZAKOLCZAI and MOLNÁR, 1978). Despite the fact that the pesticides dangerous to fish can be used within 200–500 meters from the banks of living waters only with permission of sanitary authorities, unfortunately the frequency of fish perish has not decreased in the recent years. Although the cause of the fish perish cannot always be traced back to a single factor (NEMCSÓK et al. 1981; NEMCSÓK, 1983), from the factors the toxic effect of pesticides almost always plays a significant role, the presentation of which is often due to the fact that certain tissues of fish accumulate the pesticides and their residues to an enormous degree (REICHENBACH-KLINKE, 1972; SALÁNKI et al. 1982). For this very reason the water chemical measurement data which prove that actually there are no pesticides present in higher concentrations than permissible in a given water are not reassuring in spite of this, as the consequence of the coincidence of certain unfavourable environmental factors (continuos warming up, lack of wind, etc.) fish perish may occur (NEMCSÓK, 1983). A further potential danger is that occasionally rainfalls may wash a large amount of pesticide into the waters from the plough lands. In such case temporarily a pesticide amount producing toxic effect may even get into the waters, however, the toxic values are generally not detected due to the rare water samplings.

All these circumstances propound the necessity to follow the „sanitary conditions" of the fish living in our waters with even greater attention, and to take the necessary measures in due time. At present the „arsenal" of fish hygiene is rather scant in Hungary. Practically, there is no regular veterinary control anywhere; recently a histopathological survey started at the Lake Balaton and occasional biochemical studies were also performed (BENEDECZKY et al. 1984; HORVÁTH and STAMMER, 1979; NEMCSÓK et al. 1981; SALÁNKI et al. 1982). In situ studies can well be complemented by laboratory experiments where besides the determination of the Lc50 values, the effect of the sublethal doses of pesticides can also be well studied in respect to the various physiological processes of the fish, furthermore, with which biochemical or histopathological parameters these patho-physiological alterations can be characterized. Starting from tnese possibilities the objective of the present study aimed at investigating the cytopathological alterations produced by the sublethal doses of paraquat, Ultracid 40WP and copper sulphate in the certain vital organs of carp (gill, liver, kidney) following 2 weeks' exposure, with the help of electronmicroscopy.

## Materials and Methods

Our experiments were performed on lacustrian carp (*Cyprinus carpio* L.), originating from the breed of the Fish-Hatchery Research Institute in Szarvas. The fish weighed 850–1000 g. Prior to the experiment the fish were habituated to aquarium for 7–14 days. Three individuals were kept in a 100 l sized aquarium. The water temperature was 10 °C all throughout the experiment. The animals were not fed during the two weeks' treatment period. Treatment with pesticides was carried out by dissolving the chemicals in the aquarium water. The final concentration of the effective agents was set to 5 mg/l of paraquat, 2 mg/l of Ultracid 40 WP and 5 mg/l of copper sulphate. During the course of the experiment the water of the aquaria was freshened by air insufflation.

For the purpose of electronmicroscopic study tissue samples were taken following an exposure of 2 weeks. The fish were numbed by blows on the head, then the abdominal cavity opened and 1 $mm^3$ pieces cut from the liver, kidney and gill using a sharp safety razor. The specimens were fixed in 2.5% glutaraldehyde for 2–24 hours at 4 °C . The pH of the fixative was set to 7.3 with cacodylate buffer. Following fixation the samples were washed for 5 minutes in 7.5% saccharose-ontaining cacodylate buffer. Then the tissue blocks were further fixed in 2% $OsO_4$ solution for 2 hours in dark, at 4 °C . The pH of the $OsO_4$solution was buffered to pH: 7.3 value with s-collidine. The dehydration was performed on ascending ethyl alcohol series. The blocks were further contrasted in 75% ethanol with saturated uranyl acetate solution for 30 minutes in dark. The samples were embedded in Durcupan ACM. The ultrathin sections were only „stained" with lead citrate according to the method of REYNOLDS. The electronmicroscopic pictures were prepared on TESLA BS 500 electron microscope.

## Results

### ELECTRON MICROSCOPIC STRUCTURE OF THE UNTREATED CONTROL CARP LIVER, KIDNEY AND GILL

LIVER: A large nucleolus of loose structure is detectable in the light karyoplasm of the nucleus, poor in heterochromatin (Fig.1). In the cytoplasm of the hepatocytes a great number of moderately electron dense mitochondria can be seen, being in

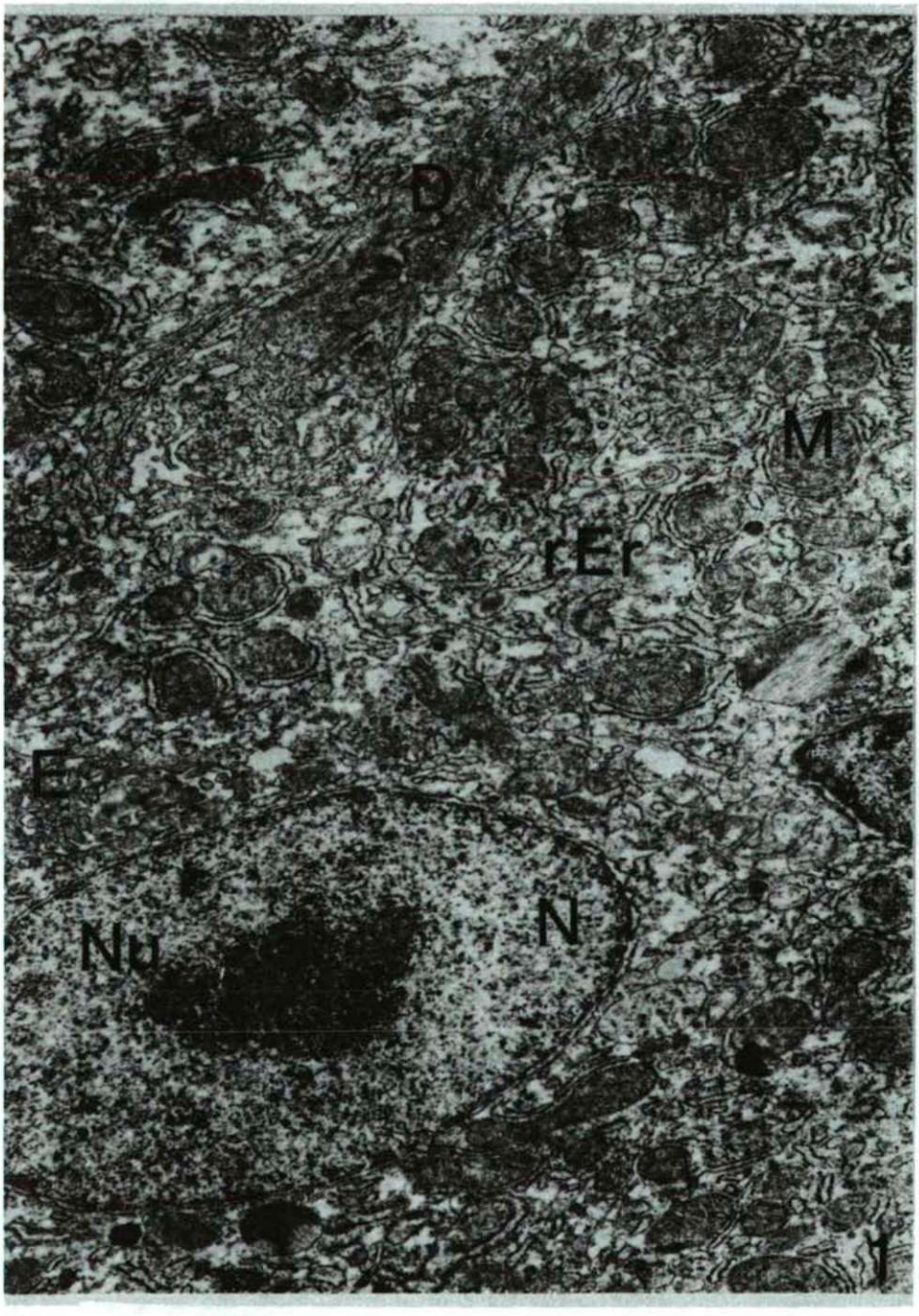


Fig. 1.
Detail of hepatocyte from the liver tissue of untreated carp. Large nucleolus (Nu) and few chromatins are observable in the nucleus (N). The cytoplasm contains many mitochondria (M) and abundant rough surfaced endoplasmic reticulum (rEr) substance. E = bile canaliculus; D = bile duct.
x 18.000

tight morphological contact with the slightly dilated rough surfaced endoplasmic reticulum. A bile canaliculus is situated in the neighbourhood of the nucleus. Few lysosomes and bile pigments also occur in the cytoplasm of the parenchyma cells.
KIDNEY: Microvilli of regular arrangement form the brush-border at the apical pole of the epithelial cells of the kidney tubuli. Cilia cross-sections are also observable in the lumen of the primary convoluted renal tubules (Fig.2). A rather high amount of electron dense endocytotic vesicles can be observed at the apical pole of the tubular epithelial cells. Lysosomes of varying shape and electron density can be found below them. The nucleus has basal location, its karyoplasm contains a significant amount of heterochromatin. A large number of mitochondria and a few lipid droplets can also be detected in the cytoplasm of the epithelial cells.
GILL: Moderately electron dense nucleus can be found in the pillar cells of the branchial lamellae (Fig. 3). Mitochondria, rough surfaced endoplasmic reticulum tubuli and some lysosomes are observable in the relatively narrow cytoplasm. Generally a high amount of erythrocytes occupy the capillary lumen. The pillar cells are limited by broad basal lamina.

## ELECTRONMICROSCOPIC STRUCTURE OF CARP LIVER, KIDNEY AND GILL TISSUES AFTER TREATMENT WITH PESTICIDES

LIVER: No striking ultrastructural changes were observed in the structure of the nucleus following paraquat-treatment and the structure of the mitochondria as well as the rough surfaced endoplasmic reticulum substance were similarly well preserved, too (Fig. 4).

The cytoplasm contained a considerable amount of bile pigments besides the high number of glycogen granules. Both filamentous substances and electron dense granular material were present in the matrix of the bile pigments (Fig. 4). Other alterations referring to cell damage were not observed in the liver tissue. On the contrary, focal cell damage was detected in the tissue of the exocrine pancreas situated in the direct neighbourhood of the liver tissue (Fig. 5). Large myelin figures developed in the glandular cells. The tubules of the rough surfaced endoplasmic reticulum became dilated as cisternae. The cytoplasmic vacuoles occurring in large numbers could also be evaluated as a degenerative alteration. The shape, size and inner electron density of the zymogen granules were rather divergent as well, even in the case of one and the same glandular cell (Fig. 5).

Following treatment with Ultracid 40WP striking ultrastructural alterations developed in the hepatocytes. The most obvious was the formation of light and dark cells, in each of which cytopatological alterations were detectable. Similary to the paraquat, ultrastructural alterations referring to the effect of the agent were not observable in the nucleus. Large autophage vacuoles were frequently seen in the cytoplasm (Fig. 6), and relatively intact mitochondria circumscribed by membranes were found in them. Numerous ribosomes could be observed on certain intravacuolar membranes, referring to the early stage of autophagy. At the same time it was striking that the majority of the mitochondria had well preserved structure in these

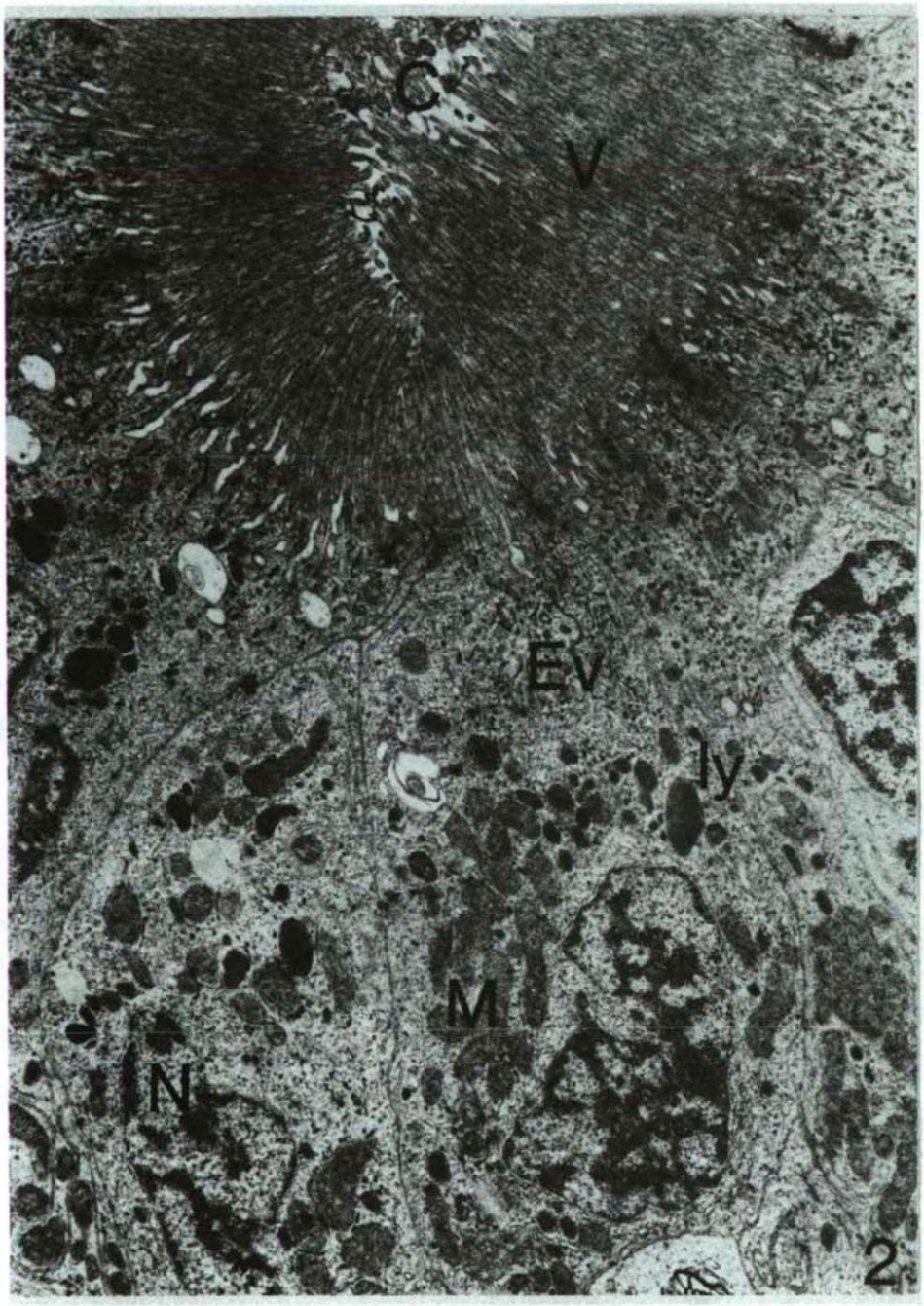


Fig. 2. Untreated carp kidney, primary convoluted renal tubules. Microvilli of regular structure (V) and some cilia (C) cross-sections can be seen on the tubular epithelial cell surfaces. Many endocytotic vesicules (Ev) and lysosomes (ly) occur in the parenchyma cells. N: nucleus; M: mitochondrium.
x 12.000

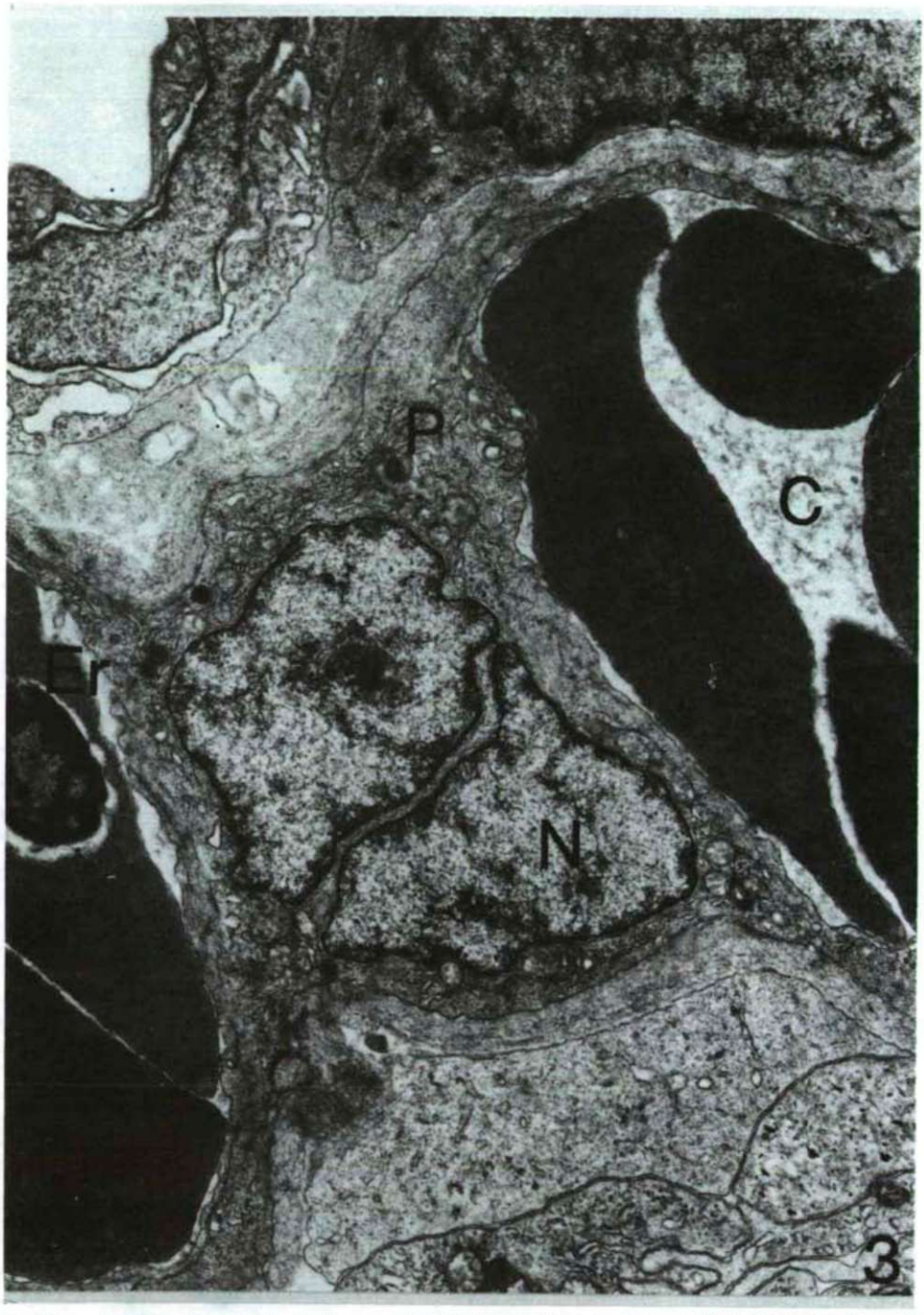


Fig. 3. Untreated carp gill. Erythrocytes (Er) fill in the lumen of the capillaries (C). P: pillar cell; N: nucleus.
x 12.000

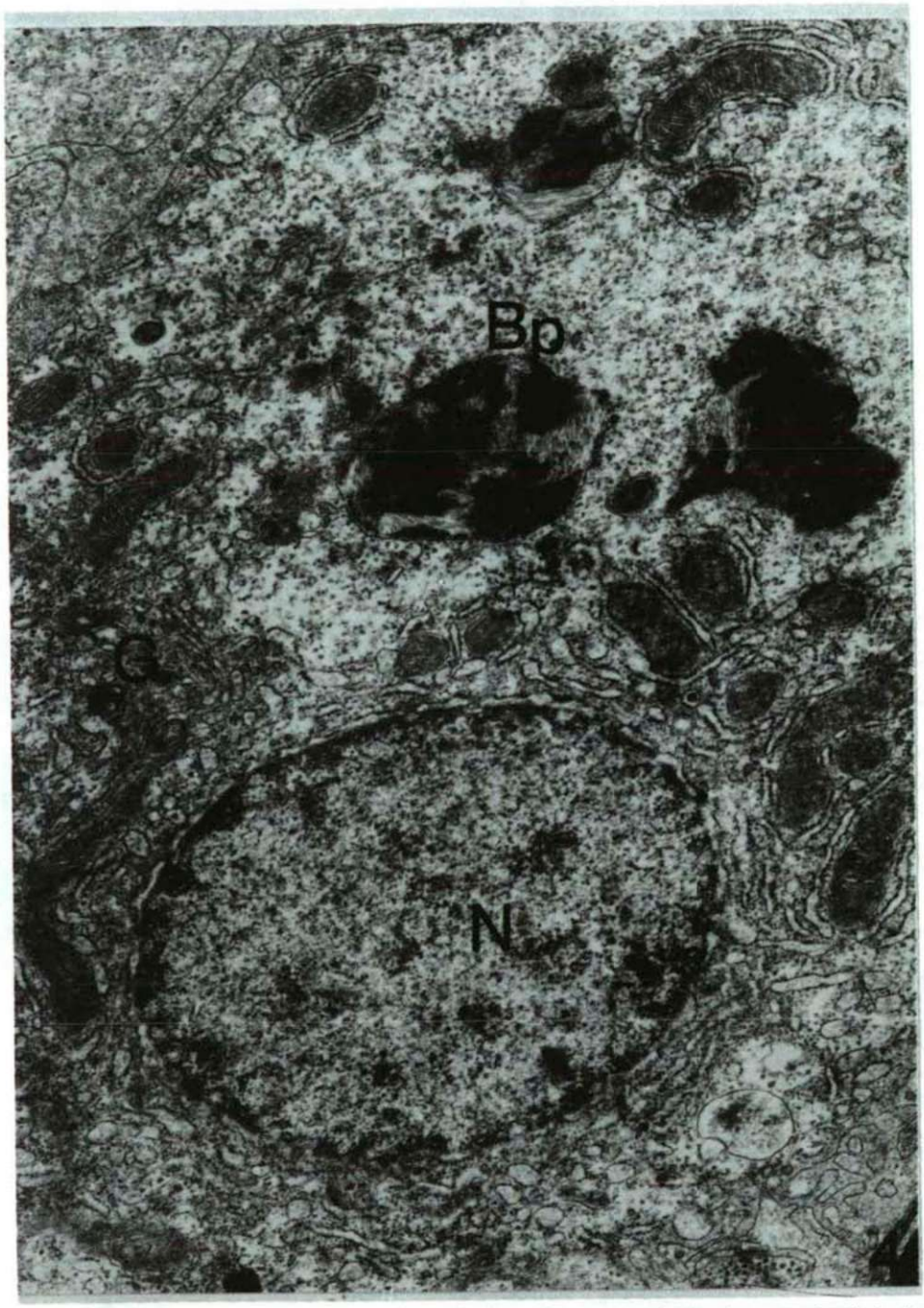


Fig. 4. Hepatocyte of paraquat-treated carp 14 days after treatment. Bile pigments of various size (Bp) have accumulated in the cytoplasm. N: nucleus; G: Golgi-apparatus.
x 18.000

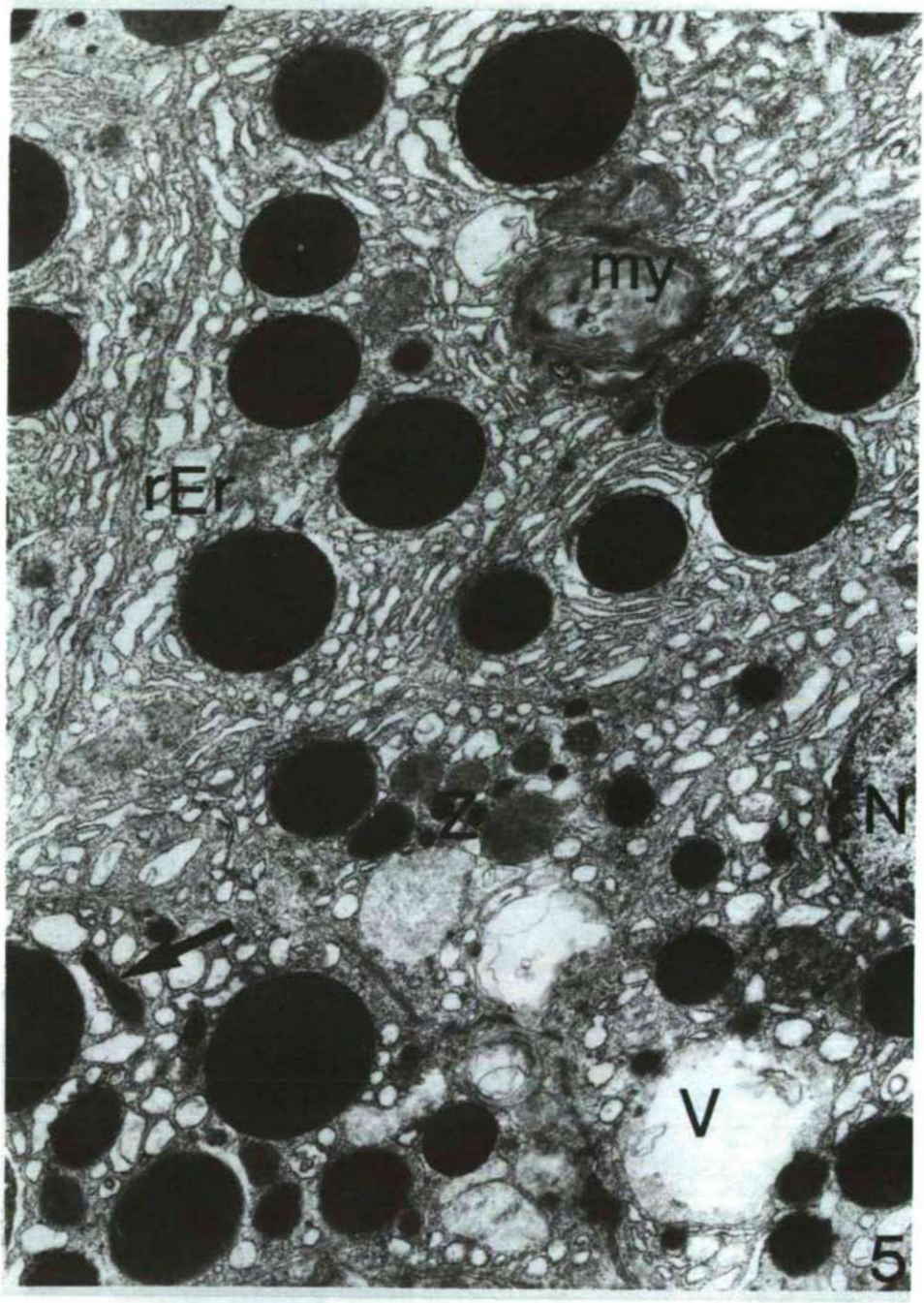


Fig. 5. Detail of exocrine pancreas in paraquat-treated carp. One part of the zymogen granules (z) have irregular shape and small size (arrow). The rough surfaced endoplasmic reticulum cavities are strongly dilated (rEr). Large, empty vacuoles (V) and myelin figures (my) are observable in the damaged cells. N: nucleus.
x 18.000

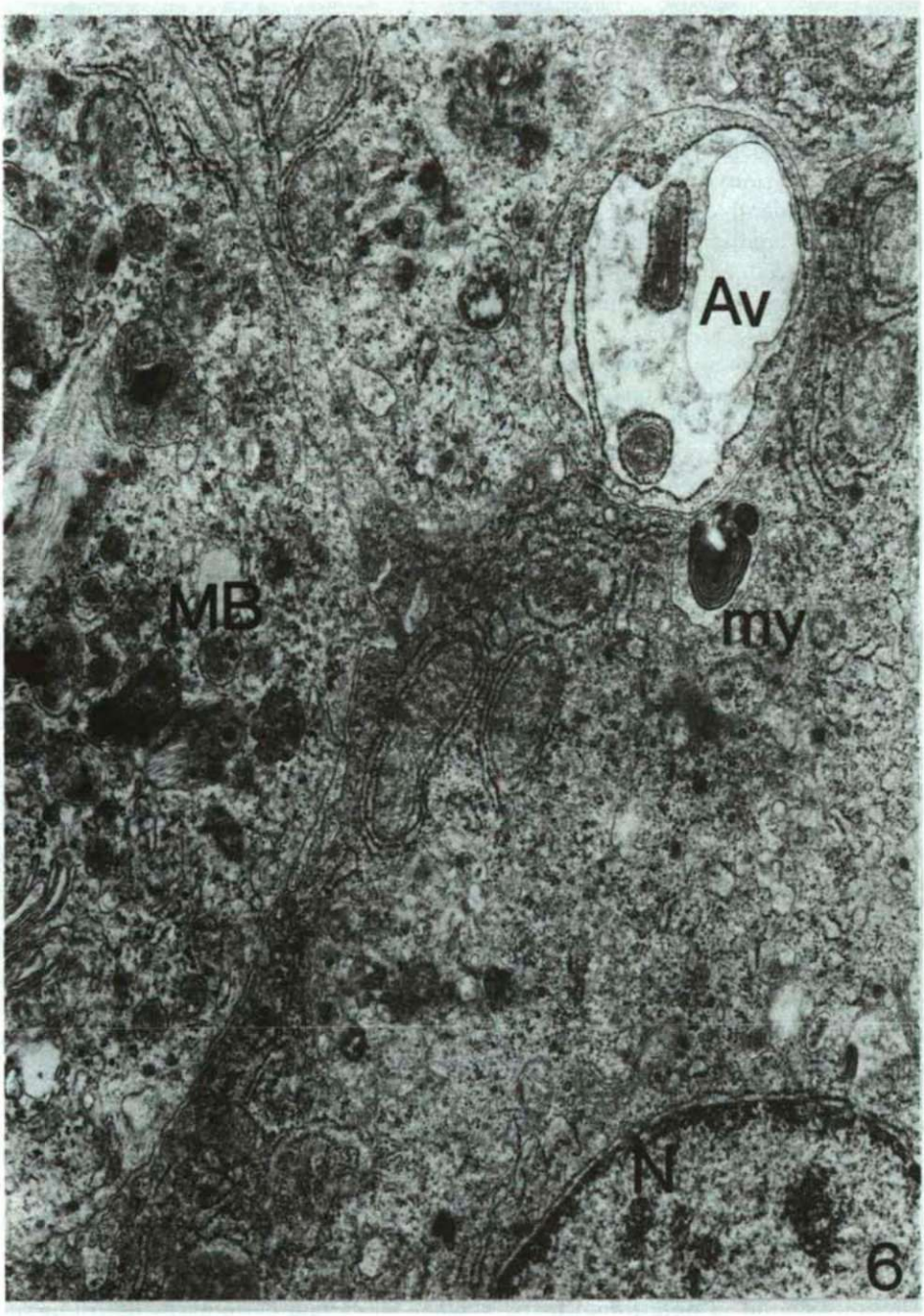


Fig. 6. Liver of carp treated with Ultracid 40 WP two weeks after treatment. The occurrence of large autophagic vacuole (Av) and numerous multivesicular bodies (MB) is characteristic of the hepatocytes. my: myelin figure; N: nucleus.
x 18.000

hepatocytes, too. Smaller-larger myelin figures, generally encapsulated in vacuoles, were observable in the Ultracid-treated hepatocytes. The number of multivesicular bodies — mainly in the neighbourhood of the Golgi-apparatuses — was strikingly high (Fig. 6). The electron density of certain vesicles was expressed in the multivesicular bodies very much.

In conformity with the condition subsequent to the paraquat treatment, there was a considerable increase in the number of bile pigments (Fig. 7). Mostly the filamentous components dominated in the inner substance of the bile pigments. The rough surfaced endoplasmic reticulum tubuli showed cisterna-like dilatation at places and fine granular, moderately electron dense material accumulated in their lumens (Figs. 7, 8). The substance in the rough surfaced endoplasmic reticulum cisternae occasionally showed regular arrangement. These are the so-called intracisternal paraprotein crystals which have probably accumulated as the consequence of the disturbed protein-transport (Fig. 8). In the neighbourhood of the rEr cisternae light mitochondria with swollen matrix could also be frequently observed. The majority of the mitochondria in the cytoplasm of the hepatocytes did not seem to be damaged and structural damage was not observed in the fine structure of the bile calaliculi either (Fig. 8).

Following copper sulphate treatment the previously described cytoplasmic alterations — thus e.g. increase in the amount of bile pigments, rEr-dilatation, mitochondrium swelling — were less expressed than in the case of the paraquat and Ultracid 40 WP. Nevertheless, the decreased electron density of the nuclei, as well as the appearance of myelin figures both in the karyoplasm and the cytoplasm could be regarded as new alterations. Lipid droplets were also detectable in higher amount in the hepatocytes after copper sulphate treatment than in the case of the previous two agents (Fig. 9).

KIDNEY: On the effect of paraquat-treatment the regular arrangement of the microvilli of the tubular epithelial cells disappeared, especially on the surface of the damaged cells (Fig. 10). Relatively intact cells also occurred besides the damaged tubular epithelial cells and focal, severely altered cells. The damaged cells were often of the „light" cell type, with strongly swollen mitochondria, picnotic nucleus, varying sized vacuoles and strongly electron dense, amorphus materialheaps were observable in their cytoplasm. Apart from mitochondrium-damages, a decrease in the amount of mitochondria was also manifested in the cytoplasm. Considerable cell damage could not be detected in the kidney glomeruli.

After treatment with Ultracid 40 WP, strikingly light nucleoli, poor in chromatin, were found in certain tubular epithelial cells (Fig. 11). A large number of phagolysosomes and myelin figures was observed in the cytoplasm. The presence of amorphus, electron dense material was observed in the phagolysosomes. There was a striking increase in the amount of the irregular shaped vacuoles, too, in the epithelial cells. The development of a part of the vacuoles was probably due to the fact that the ribosomes became detached from the cisternally dilated rEr-membranes. The mitrochondria were found to be swollen, in certain cases even the continuity of the outer membrane was found to be interrupted. The microvilli on the apical

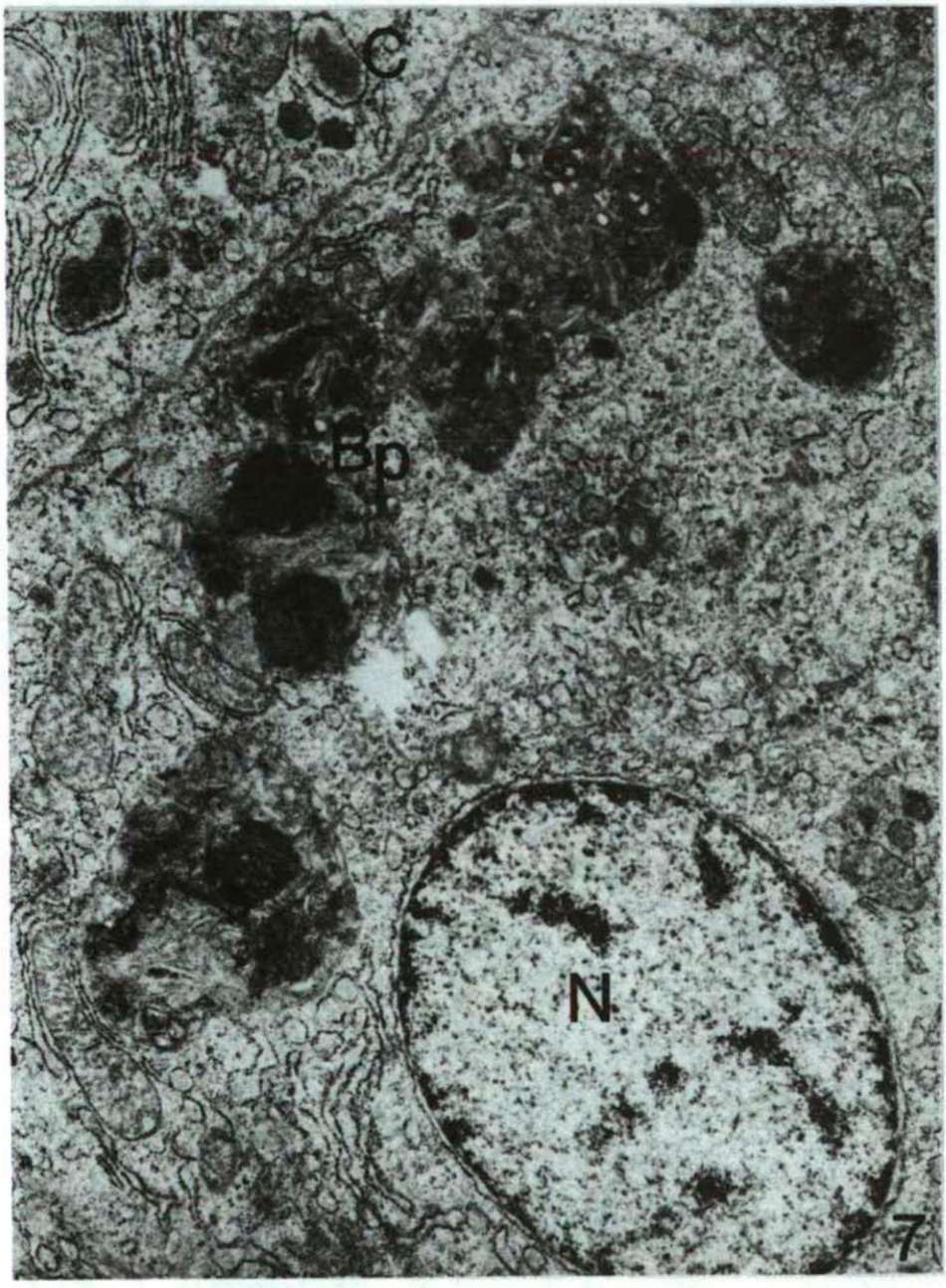


Fig. 7. The mass appearance of bile pigments (Bp) accompanied the Ultracid 40 WP treatment in the liver parenchyma cells, two weeks after treatment. N: nucleus; C: intracisternal accumulation of material.
x 18.000

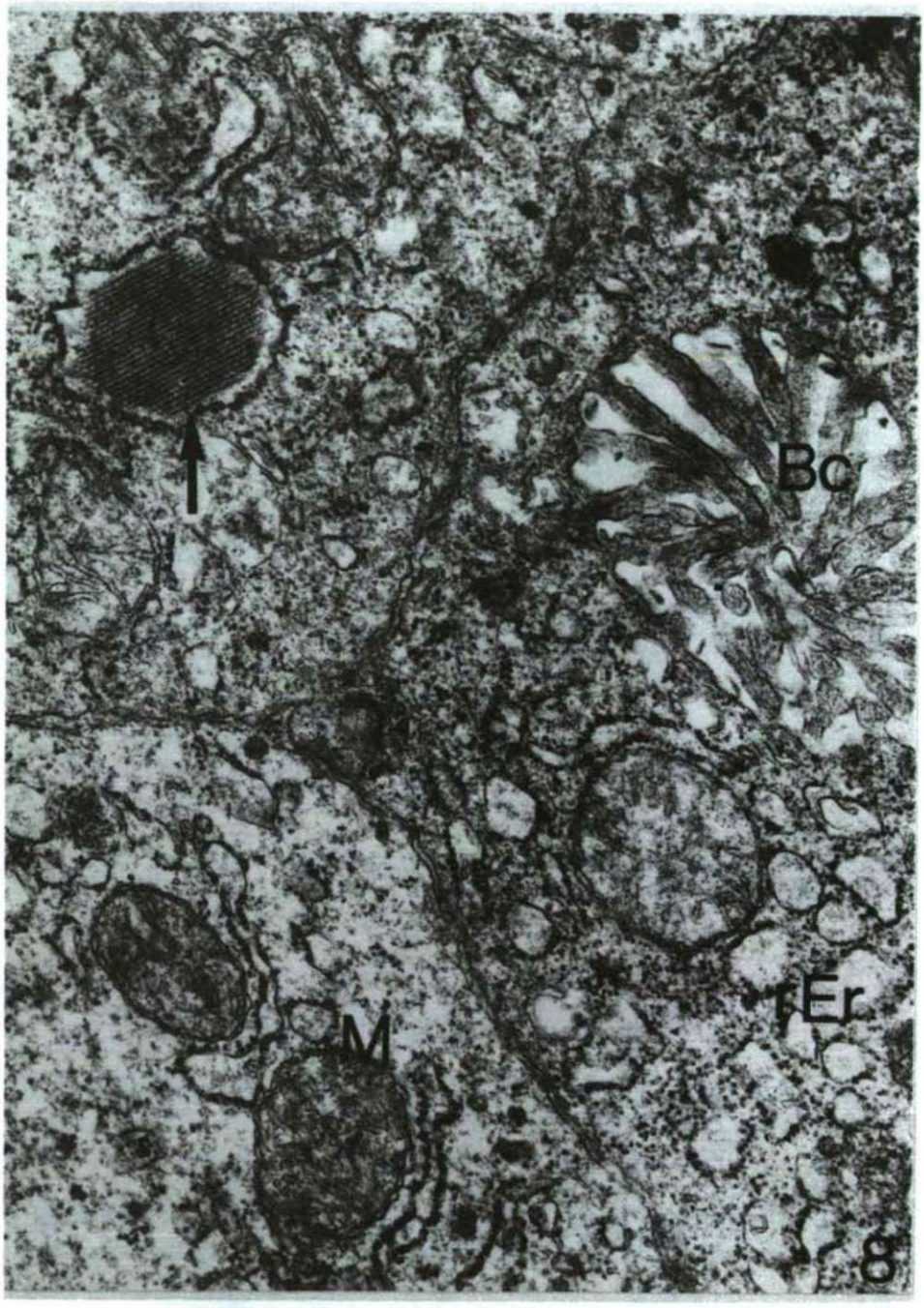


Fig. 8. Details of hepatocytes beside a bile canalicule (Bc). A paraprotein crystal showing parallel arrangement developed in the dilated rEr cisterna (arrow). M: mitochondrium
x 30.000

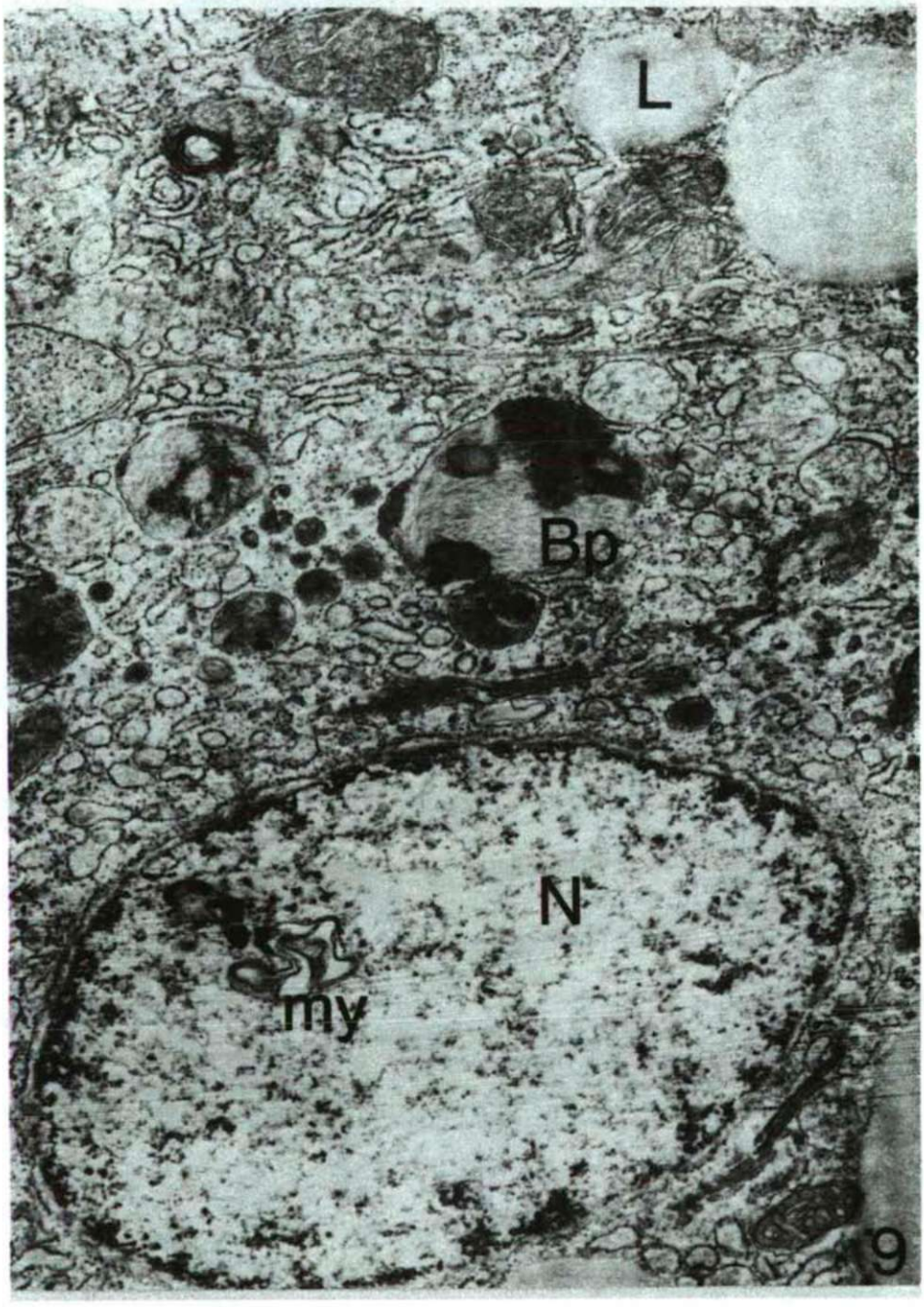


Fig. 9. Detail of carp hepatocyte 2 weeks after copper sulphate treatment. Myelin figures (my) appeared in the karyoplasm of the nucleus (N), poor in chromatin. Large lipid droplets (L) can be seen in the hepatocytes beside the bile pigments (Bp).
x 20.000

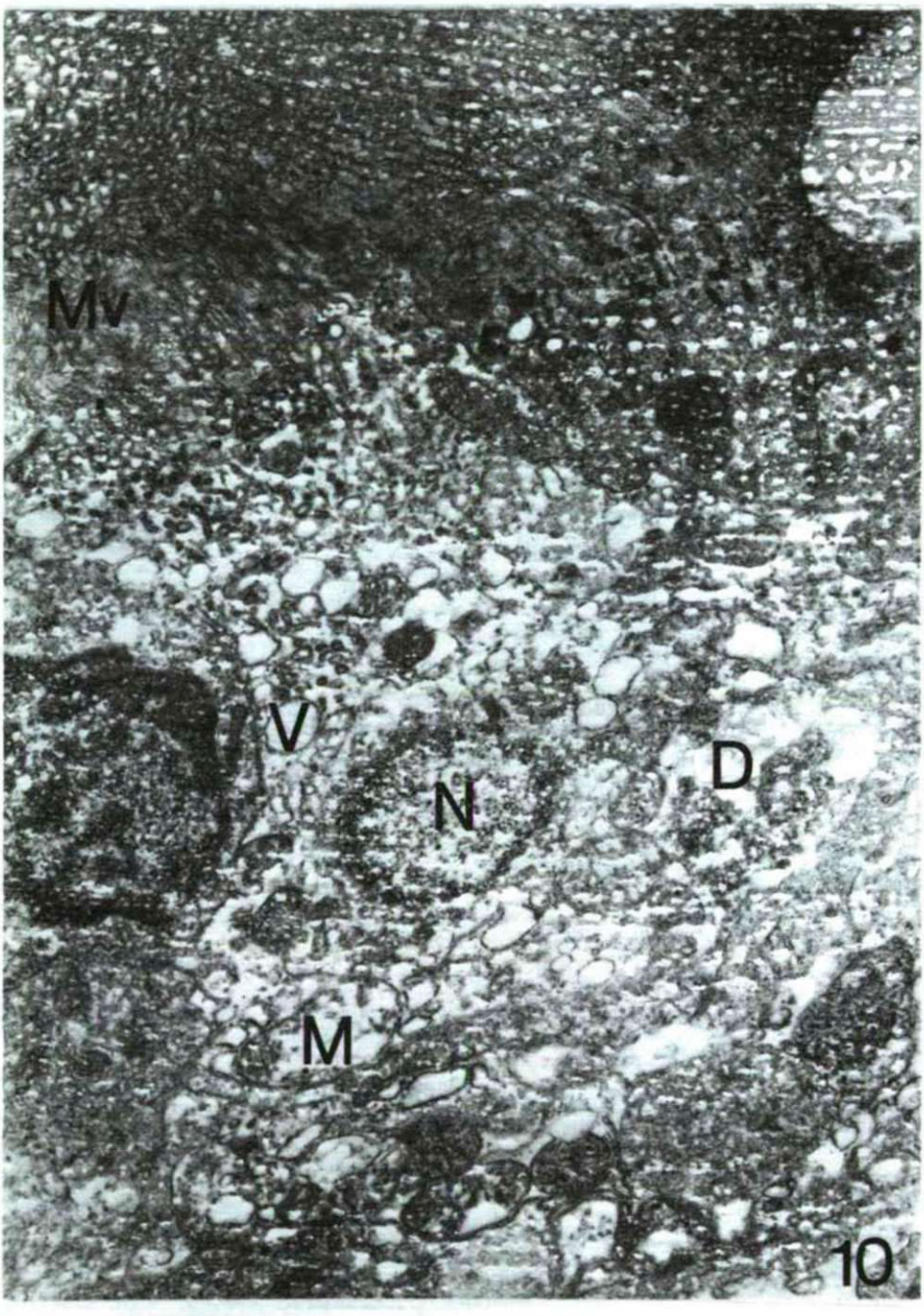


Fig. 10. Carp kidney, epithelial cells of primary convoluted renal tubule two weeks after paraquat treatment. The appearance of swollen mitochondria (M), vacuoles (V), cell detritus (D) are the signs of cell damage. The arrangement of the microvilli (Mv) has become disorganized. N: nucleus.
x 12.000

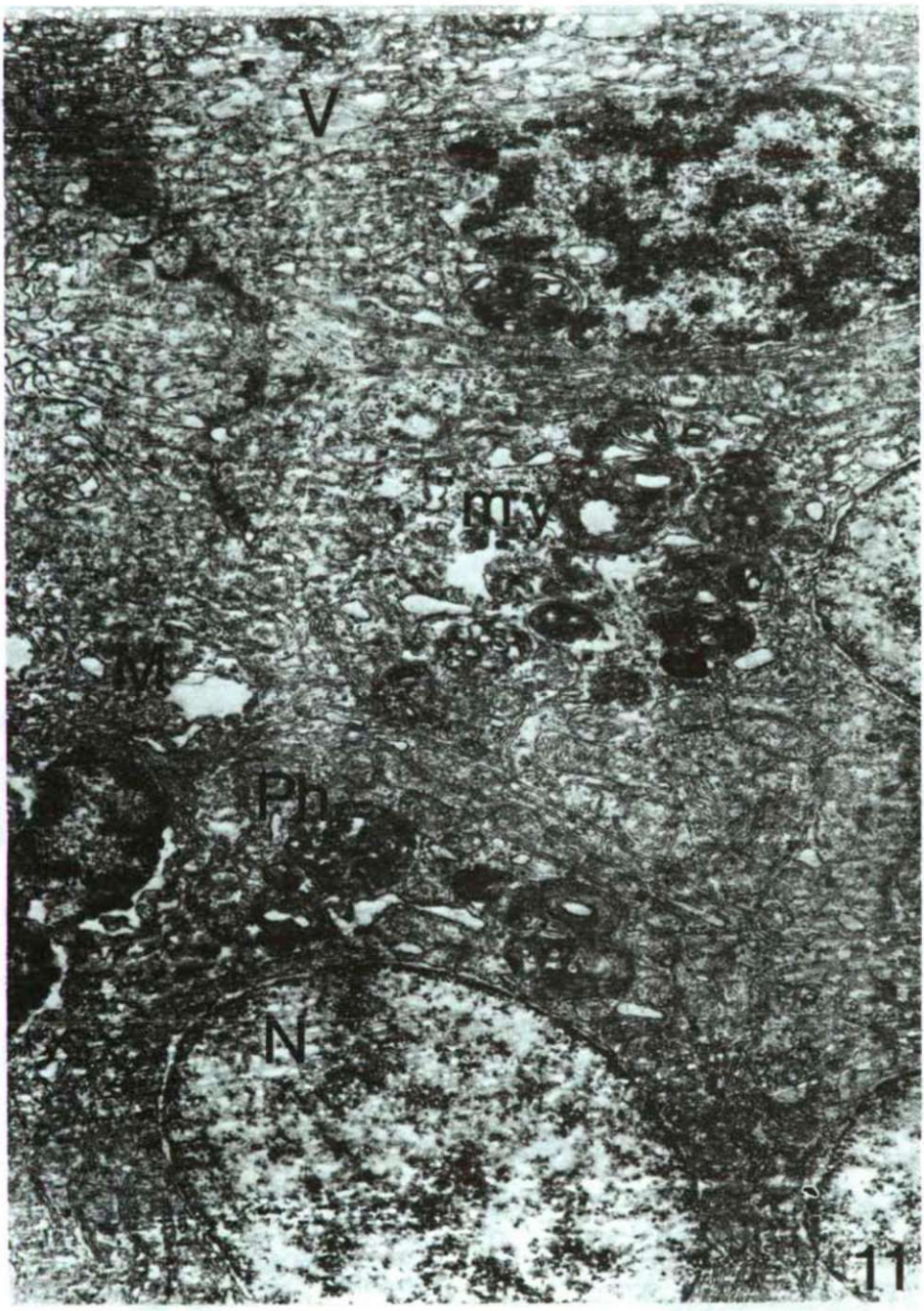


Fig. 11. Kidney of carp treated with Ultracid 40 WP, tubular epithelial ells. Chromatin-poor nucleus (N) and mass occurrence of myelin figures (my) is characteristic in certain cells. Ph: phagosome; V: vacuole; M: mitochondrium.
x 12.000

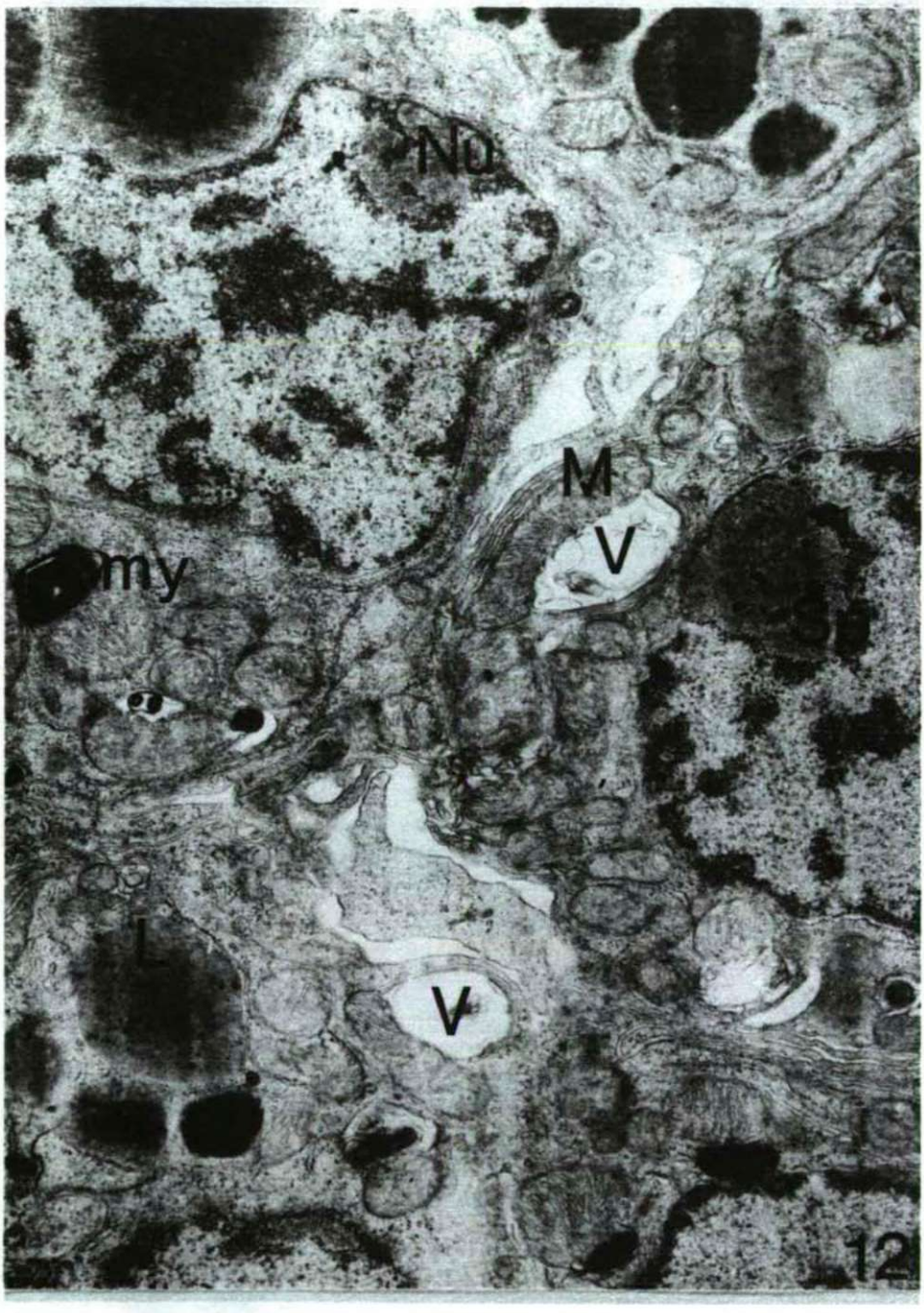


Fig. 12. **Kidney of carp treated with copper sulphate, 2 weeks after treatment.** Electron dense patches (Sp) are detectable in the clear substance of the nucleoli (Nu). Vacuoles (V) are frequent in the cytoplasm of the tubular epithelial cells, often in the neighbourhood of the damaged mitochondria (M). L: lipid droplet; my: myelin figure.
x 18.000

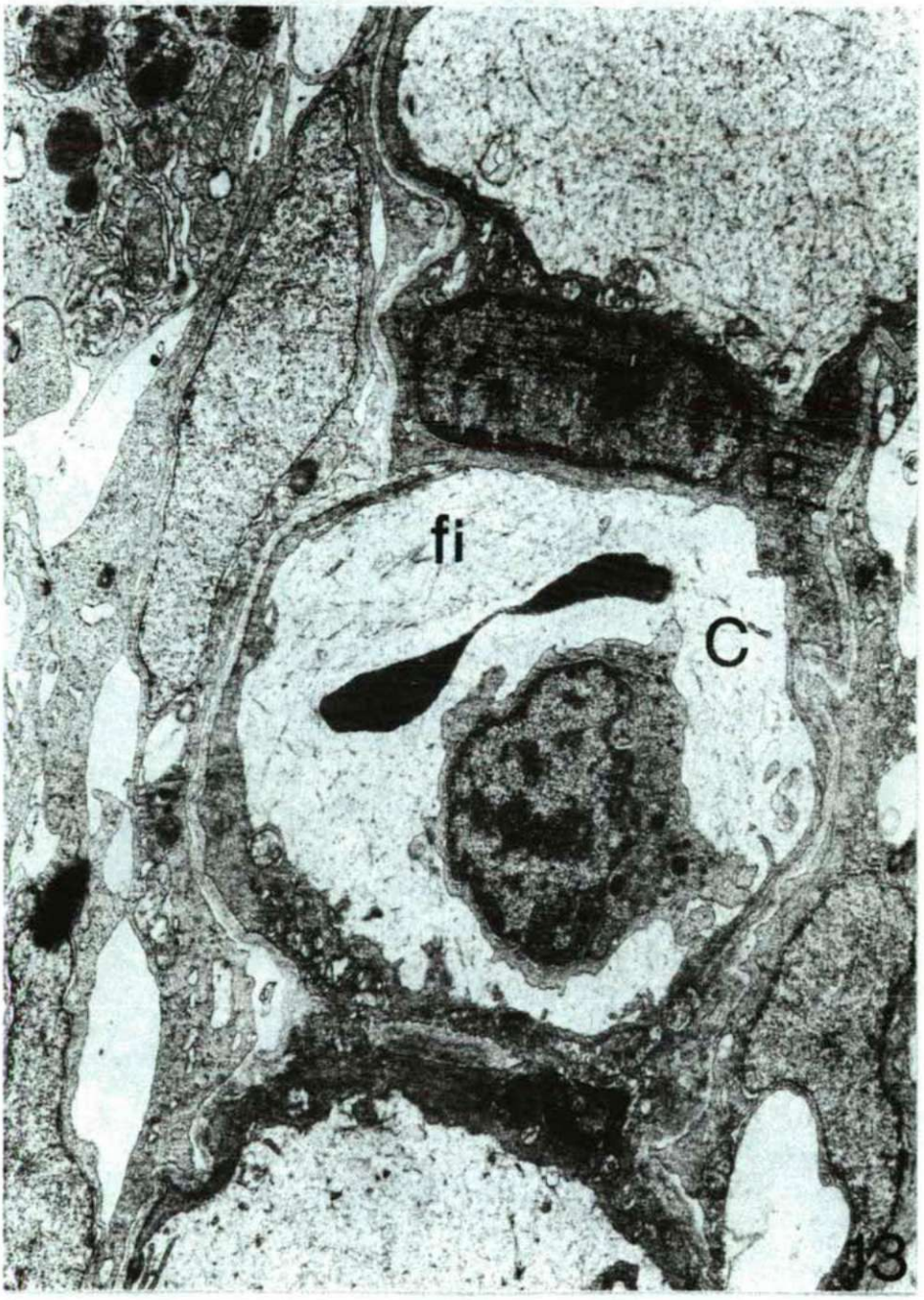


Fig. 13. Carp gill 2 weeks after paraquat treatment. Fine fibrous matter(fi) is observable in the lumen of the capillaries (C). P: pillar cell.
x 12.000

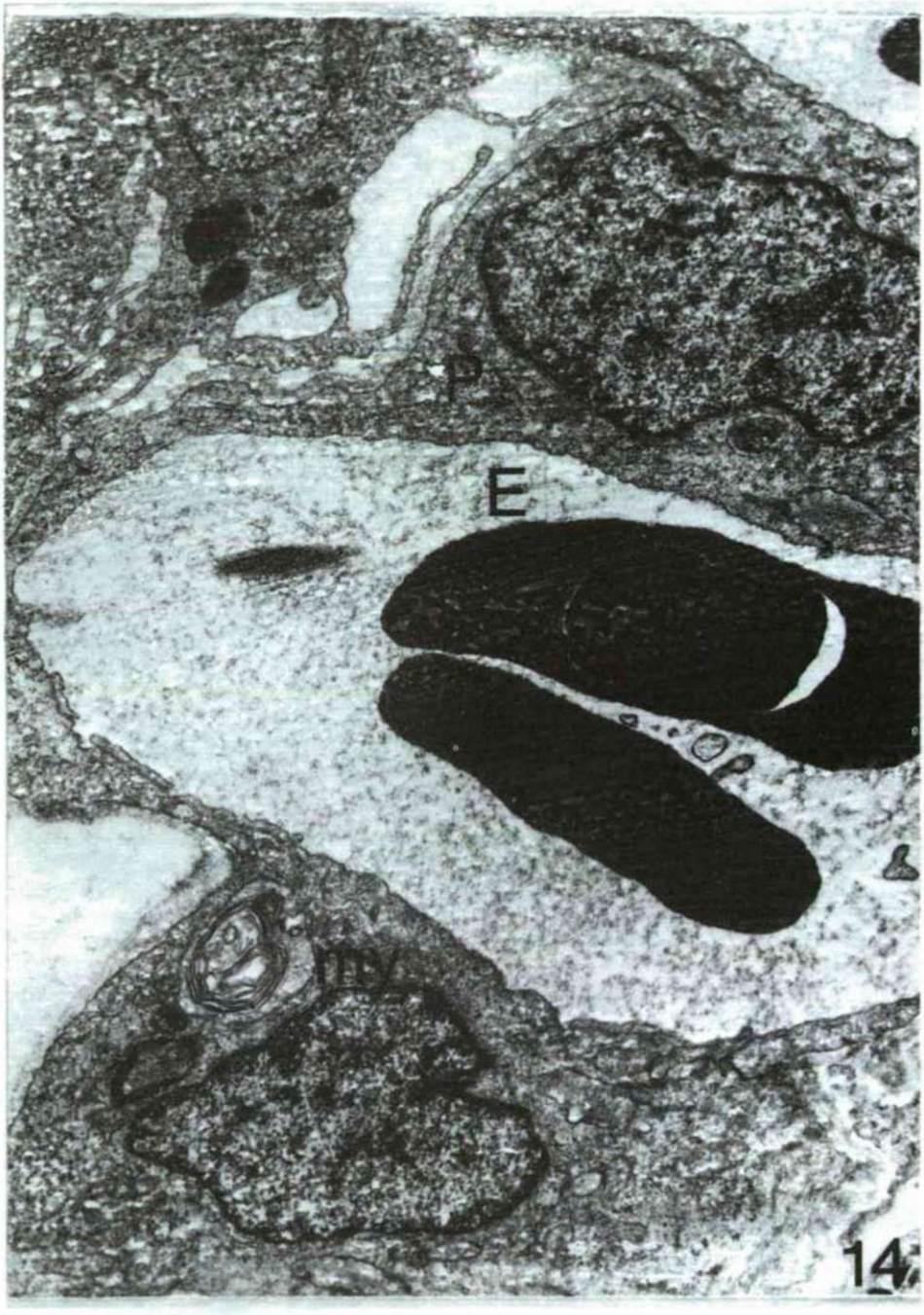


Fig. 14. Carp gill 2 weeks after Ultracid 40 WP treatment. Myelin figures (my) developed in the pillar cells (P). E: erythrocytes.
x 18.000

pole of the cells — similarly to the paraquat-treatment — lost their regular arrangement here, too. Contrary to the control, the presence of the electron dense endocytotic vesicles could hardly be observed on the apical pole of the tubular epithelial cells.

Following treatment with copper sulphate expansive cell damages were detectable in the renal tissue. Strikingly electron lucent nucleoli were found in the nuclei, in the matrix of which often electron dense patches (spotted nucleolus) appeared (Fig. 12). Large lipid droplets of varying density accumulated in the cytoplasm. The majority of the mitochondria were swollen, however, their crystae look relatively intact. Electron dense myelin figures were frequently detectable in the direct neighbourhood of the mitochondria. The presence of strikingly many, irregular vacuoles was also observable in the cytoplasm of the tubular epithelial cells. GILL: Following paraquat treatment, ultrastructural signs referring to cell damage could be observed neither in the pillar cells, nor in the respiratory epithelial cells, nevertheless, the accumulation of fine fibrous material was detectable in the capillary lumens (Fig. 13), which could be identified with fibrin on the basis of the ultrastructural characteristics.

Significant and expansive damage in the gill tissues was not caused by treatment with Ultracid 40 WP either, however, the occurrence of myelin figures was observed in the cytoplasm of certain pillar cells (Fig. 14).

The occurence of myelin figures was detected in small number following treatment with copper sulphate, too, mainly in the cytoplasm of the chloride cells.

## Discussion

Taking the present tendency into consideration, the environment-polluting and -deleterious effect of the chemical agents used in industry and agriculture remains a great problem in respect to environmental protection. Since the physiological spectrum of effect of a single chemical agent is rather wide, the estimation of the effect as well as the determination of the mechanism of effect require complex study methods. In Hungary, however, only few attempts have been made so far for the complex detection of the harmful effects of the various pesticides exerted on fish (NEMCSÓK et al. 1981; BENEDECZKY et al. 1984). Following two hours of treatment with pesticide ROJIK et al. (1983) had observed severe cell damage in the liver, kidney and gill of carp. According to the results of the simultaneously performed biochemical studies (in conformity with the above observed cell damages) the serum transaminase enzyme activity had also shown a considerable increase. Even these acute experiments have given important evidence that pesticides reaching the water may produce severe damage to the vital organs of fish. Under natural circumstances, however, the pesticides firstly affect the fish organisms in low concentration, but durably (chronically), therefore the effect of low (sublethal) pesticide concentrations and longer (2 weeks) pesticide exposure was examined in our present studies.

According to expectations, serious and expansive cell — and tissue damages, resp., were not detected during the course of our present studies in the case of either

pesticide. The circumstance that after two weeks of paraquat treatment there were hardly any alterations referring to cell damage in the hepatocytes (only the amount of bile pigments increased) proves the strongly dose-dependent cell-damaging effect of the agent. Following application of double dose (10 mg/litre) Nemcsók et al. had experienced expansive and severe cell damage 2 hours after treatment (NEMCSÓK and BOROSS, 1982; ROJIK et al. 1983). The fact, however, that focal cell necrosis was experienced in the pancreas located in the direct neighbourhood of the liver, furthermore, expansive cell-organelle damage to certain cells, points out that the tissues of certain organs are more sensitive to the chemical impacts. Such organs as the liver, where the detoxication of various chemical agents takes place and for which the „drug metabolising" enzyme (or enzyme system) is given, are capable of preserving their structural and functional integrity (SIMON et al. 1983; 1984), other organs, thus the above-mentioned pancreas, become significantly damaged by the same dose. This fact is noteworthy as the frequent and severe damage of the pancreas has been well-known for a long time from the fish-breeding practice, which may be caused by a number of external factors — infection, intoxication (SCHÄPERCLAUS, 1954). The damaging effect of the various pesticides exerted on the same target organ can be rather divergent. The paraquat did not produce considerable alteration in the hepatocytes, at the same time the Ultracid 40 WP resulted a whole series of cell damages (increase in bile pigment, the appearance of a large number of myelin figures and autophage vacuoles, rEr dilatation, etc.). The paraprotein crystals appearing in the dilated rEr cisternae refer to the disturbance in the transport-process of the transportable proteins. This assumption is also supported by the circumstance that the damaged mitochondria were the most common next to the rEr cisternae. The development of the paraprotein crystals may be the consequence of the mitochondrial damages: if there is a lack of sufficient energy the rEr-transport becomes hindered, the proteins accumulate in the lumen of the cisternae. Similar phenomenon was observed in Dikonirt-treated liver tissues as well (BENEDECZKY et al. 1984). Neither the paraquat, nor the Ultracid treatment caused alterations in the nucleus of the hepatocytes. The copper sulphate treatment, however, resulted in the development of chromatin-poor nuclei, and even led to the formation of myelin figures in certain nuclei. It is noteworthy that the damage of the karyoplasm was not accompanied by the simultaneous and severe damage to the cytoplasmatic cell organelles, as observed in the case of Ultracid. Therefore, that the primary target of copper sulphate may be the nucleus. ROJIK et al. (1983) detected a considerable decrease in the chromatin substance as early as two hours following a 10 mg/litre concentration of copper sulphate. In our present experiments not a single animal survived the 10 mg/litre concentration of copper sulphate, therefore the pesticide concentration had to be reduced to one half. The mechanism by which copper sulphate brings forth a decrease in the chromatin substance cannot be answered without biochemical studies. The copper can be assumed to incorporate into the chromatin substance, through the supplantation of the $Mg^{2+}$ inhibiting in such way the well-known condensation of heterochromatin in the karyoplasm.

The degenerative alterations were found to be more serious in the pancreas

than in the liver. The accumulation of the detritus representing focal cell necrosis points to lipid peroxidation (BLOCK, 1979), the membrane-damaging effect of which probably caused the complete decomposition of certain cells. This is also supported by the appearance of the large myelin figures, which are well-known to be products of decomposition (DE DUVE and WATTEAUX, 1966). The cisternal dilatation and sporadical degranulation of the rough surfaced endoplasmic reticulum tubuli prove that the protein-synthetizing system in the pancreas exocrine cells was damaged by the paraquat treatment. It is known that the longitudinal arrangement of the rEr tubuli is often discontinued in the case of insufficient energy supply (SCHAFF and LAPIS, 1979). Paraquat is known to be capable of the disjunction of the mitochondrial electrontransfer (OGATA and HASEGAWA, 1978). This agent seems to exert its damaging effect on the elements of the protein-synthetizing system (rEr) through the mitochondrial system in this case, too.

The focal cell necrosis observed in the pancreas did not develop in the renal tissues even following pesticide treatment either. From the three agents, only the copper sulphate and the Ultracid caused damage to the nucleus, the paraquat did not. Apart from the damage of the chromatin substance the appearance of spotted nucleoli is noteworthy, referring to the damage of the RNA–metabolism according to several authors (LAPIS and BENEDECZKY, 1966; STENRAM, 1969; KOPPER et al. 1969). All three agents produced the same cytoplasmic damages (swelling of the mitochondrium, appearance of myelin figures, lipid droplets, vacuoles and cell detritus), as they were observed in the liver tissues. „Kidney-specific" ultrastructural alterations could not be detected. This partly relates to the facts that the kidney glomeruli did not show pathomorphological alterations, and that as a parenchymatic tissue, the resorption epithelium of the convoluted renal tubules reacts to the external effects in many respects in similar way as also parenchymal liver tissue itself. Cell damage appearing expansively furnished unambiguous proofs that in the given sublethal dose all three agents induced pathological alterations in the renal tissue. The alterations in the glomeruli were not definite and were only of focal character in the epithelium of the convoluted renal tubules, too. Their significance can be summed up in that both the function and structure of a vital organ can be damaged even after a relatively short exposure time (2 weeks), which may lead to irreversible pathological alterations in the case of unfavourable external conditions (e.g. anoxia, high water temperature, infection, etc.).

It is striking that the slightest ultrastuctural alterations were experienced in the gill tissue (though this tissue is in the most direct contact with the harmful agents). Apart from the rare appearance of the myelin figures, fibrin filaments were observable as well in the capillary lumens following paraquat-treatment. In respect to the relative lack of the cytopathological alterations there are two concepcions:

1. The paraquat is depleted from the gill tissue quickly, and so it does not cause cell damage to a significant degree.
2. There may have been pathological alterations in the early stage of the treatment (see ROJIK et al. 1983), but the cells became regenerated by the end of the two weeks' treatment period.

Since the biochemical measurements of NEMCSÓK (1983) manifested the maximal transaminase and LDH activity values after the first week, and then these values gradually decreased, it is presumable that the tissue of the gill — due to its great ability of regeneration — had already restituted the transitional cell damages; therefore ultrastructural signs referring to cell damage could only sporadically be found at the end of the 2 weeks' treatment.

Summarizing our results we can conclude that with the help of the electron microscopic cytopathological method early cellular and subcellular alterations can be detected in the carp liver, kidney and gill tissues produced by the sublethal concentrations of pesticides and insecticides. Our observations supply important data for the agricultural practice and the veterinary organizations in view of both the prevention of the catastrophic perish of fish as well as the control of the chemical pollution of our water.

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Addresses of the authors:
I. BENEDECZKY
K. HALASY
Department of Zoology
H-6701 Szeged
P.O. Box 659
Hungary
J. NEMCSÓK
Department of Biochemistry
Attila József University
H-6701 Szeged
P.O. Box 533
Hungary