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THE EFFECT OF 2,4-D-CONTAINING HERBICIDE (DIKONIRT) ON THE ULTRASTRUCTURE OF CARP *(CYPRINUS CARPIO)* LIVER CELLS

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Abstract

The effect of sublethal concentrations (5, and 10 mg/l,) of 2,4-D-containing Dikonirt on the liver cells of carp, was studied by electronmicroscopy during an exposure of 6 months. Two months after treatment light swelling of the mitochondria, and fragmentation and loss of crystae were observed in the cytoplasm of the hepatocytes. A large number of inclusions containing electron dense bile pigment were detectable in the cytoplasm of the hepatocytes, and fine granular bile pigment appeared in the lumen of the bile canaliculi. The bile pigment being present simultaneously in the ductuli of the bile duct epithelial cells indicated that cholestasis was developing. The rough-surfaced endoplasmic reticulum tubuli displayed ring-formed fusion and paraprotein crystals were detected in certain dilated rEr cisternae. Later (after 3, 4, 5 months of exposure) inclusions similar to cholesterin crystals appeared in the cytoplasm, besides the cell alterations mentioned above; still later (in the 6th month) an increase in rough-surfaced endoplasmic reticulum and an appearance of fingerprints referred to an alteration of the protein-synthesizing system.

Key-words: Effect of 2.4-D on carp liver.

Introduction

The fish-ponds and natural waters of Hungary are loaded with communal and industrial sewages, chemical fertilizers, pesticides and detergents. The detection of the toxic agents and the diagnosis of the damaging effects caused by them on the aquatic organisms exposed to the increasing environmental pollution gain more and more emphasis. Many methods are at disposal for the demonstration of the damages developing in the aquatic organisms. Besides the classic toxicological studies, histopathological, biochemical and physiological methods are becoming all the more wide-spread. Thus, for example, the "normal" activity of various enzymes has been studied in the tissues, blood serum and plasma of certain fish species and their changes on environmental effects have been demonstrated (SIEBERT et al., 1964; MCBEAN et al., 1966; PEQUIN et al., 1970; GAUDET et al., 1975; MARQUEZ, 1976; SAURER and HAIDER, 1977, 1979). It has been unambiguously proved that even in the presence of low concentrations of toxic substances there is an increase in the activity of enzymes playing a role in the metabolism and the decomposition of toxic agents (BUHLER and BENVILLE, 1969; LANE and SCURA, 1970; RACICOT et al., 1975). Apart from the quantitative assays, not less significant are the histopathologic methods providing qualitative information, which supply important data to the localization and expansion of the tissue damages.

However, the light-microscopic histopathological alterations usually reflect the

"final result" of an irreversible vital process without giving adequate information in respect of the patho-mechanism. A further disadvantage of the light microscopic examination is the fact that it is only capable of detecting lethal (or perhaps sublethal) alterations caused by large doses; though, in the practice harmful effects of chemical substances being present in low concentration, but for long duration, (throughout months or years) are to be estimated.

In the viewpoint of attaining this object, besides the biochemical assays, the modern morphological methods, first of all electron microscopy, are becoming more and more significant. Electron microscopy is able to show, among others, incipient damages of cell organelles in due a time, when with the help of the biochemical assays no pathological alterations are demonstrable, e.g. in the blood. The acute effect of 2,4-D Na-salt on the early developmental stages of the bleak *(Alburnus alburnus)* has been reported elsewhere (BIRÓ, 1979).

In the present study, we wished to determine the effect of sublethal doses of the 2,4-D-containing herbicide Dikonirt on the ultrastructural organization of the hepatocytes of carps exposed for 6 months.

Materials and methods

Second-summer carps with 12.5–19.5 cm trunk lengths weighting 60–200 g, were used. The fish originating from fish-ponds were habituated to aquarium for a period of 4 months, and on one or two occasions weekly they received malachite green treatment until their susceptibility to Ichthyophthyrius infection ceased. Then the fish were divided into groups of 10 and kept in 100-1-sized glass aquariums in Dikonirt solutions of final concentrations 5 or 10 mg/l from February 14, 1980, till August 12, 1980 (2 parallel + 2 control groups). The water temperature was 15–17 °C. The fish were fed with living *Tubifex* and carp aliment. The water of the aquariums was strained with EHEIM filters filled with wad-strainer and the water was changed weekly. The water of the basins was ventilated with condensed air.

Five specimens were examined in each month. The animals were decapitated, their abdominal cavities opened, small slices were cut from various areas of the livers with a sharp safety razor, and the slices were immediately placed into ice-cold fixative. 2.5 % glutaraldehyde was used for prefixation, and that was buffered to pH 7.3 with cacodylate buffer. Following 2 h of prefixation the samples were fixed in 2 % OsO_4 solution for further 2 h. After dehydration in graded alcohol the samples were embedded in Spurr synthetic resin. The ultrathin sections were contrasted with lead and studied in the electronmicroscope.

Results

During the course of the 6 months' exposure no fish decay was observed either in the control group, or in those treated with Dikonirt.

Untreated control: On the basis of the overall view of low magnification (Fig. 1) spherical or kidney-shaped cell nuclei, poor in chromatin, were seen in the majority of the hepatocytes. The electron dense nucleolus was striking in the light karyoplasm. The cytoplasm of the liver parenchyma cells was moderately electron dense, many mitochondria, large amounts of glycogen granules, here and there lipid droplets and lysosomes, were observed in it. The rough-surfaced endoplasmic reticulum (rEr) was found mainly in lamellar form in large quantities in the hepatocytes (Fig. 1). In Fig. 2, of higher magnification, it can be seen that highly electron dense associated chromatin surrounded the nucleoli. The Golgi apparatus of the liver parenchyma cells was well-developed, with many vesicles, numerous granules and a few lysosomes in its area. Due to the double fixation, the glycogen granules occurring in the form

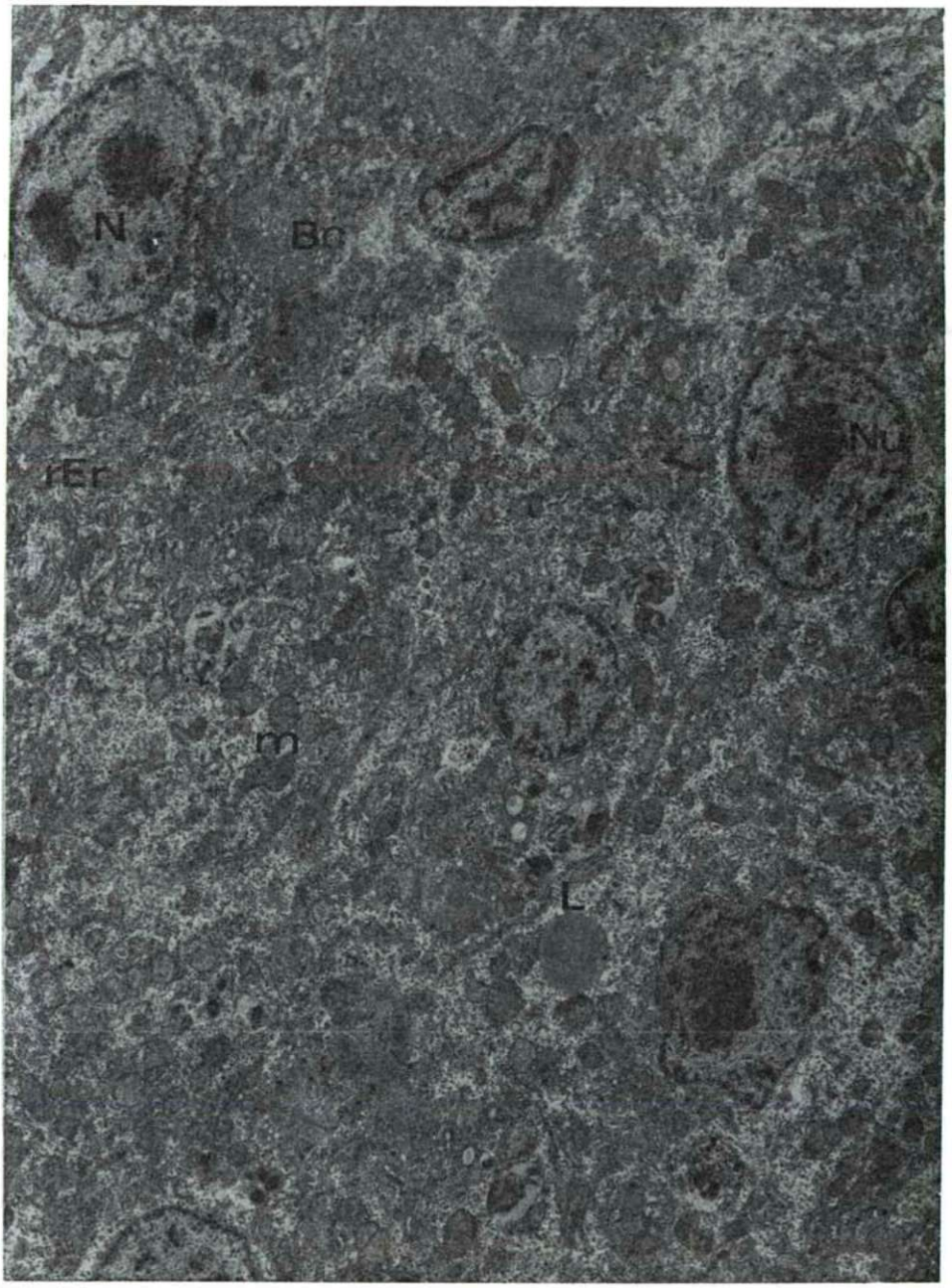

Fig. 1. Overall view of the liver parenchyma cells of an untreated carp. The nucleus (N) of the hepatocytes is poor in chromatin, the nucleolus (Nu) is striking. Many mitochondria (m), abundant rEr, moderate amount of glycogen granules can be found in the cytoplasms. L = lipid, Bc = bile canaliculus x 4400

of both monoparticles and rosettes appeared moderately electron dense (Fig. 2). The rEr tubuli were sometimes parallely arranged; they frequently formed shorter and small fragments and they were found all over the cytoplasm in an even distribution (Fig. 2). Tightly attached microvilli were found situated in the cavity of the bile canaliculi. A few cytoplasmatic inclusions were also detected in hepatocytes. A characteristic association of fine fibrous structures and electron dense amorphous tissue bundles was detected in these (Fig. 2).

Livers of fish exposed to 2,4-D (Dikonirt): The first sampling was performed two months following treatment. Macroscopically no striking alteration was seen in the livers. Light microscopically neither necrosis nor inflammation could be detected in the liver tissue. Ultrastructural alterations referring to the effect of the agent could not be detected in the cell nuclei in the electron micrographs, in the cytoplasm, however, ultrastructural signs referring to cell damage were observed in numerous cell organelles. Certain mitochondria, appeared to be light and swollen and crystae were fragmented or disappeared (Figs 3, 5, 6). In some areas mitochondria were encapsulated in autophage vacuoles (Fig. 3).

The cytoplasmic inclusions described in the normal liver, significantly increased in the hepatocytes (Fig. 4). The inclusions varied from 1 to 3 μm in diameter. Mostly filamentous substances accumulated in the inclusions, however, highly electron dense details were also regularly found in smaller numbers. The accumulation of electron dense matter was frequently observed in the wide lumen of bile canaliculi. In semithin sections this occurred in the form of a greenish-blue substance and corresponded to bile pigment, which indicated developing cholestasis (Fig. 7). Striking amounts of glycogen granules were found in certain hepatocytes, in which the amount of cell organelles decreased (Fig. 8). A frequent phenomenon was the ring-formed fusion of certain rEr tubuli as well as the presence of isolated cytoplasm detail and or a mitochondrion, in the lamella of the ring (Figs 3, 5, 6). Some rEr tubuli showed cisterna-like dilatations and a material of crystalline structure accumulated in the cisternae (Figs 6, 7). The accumulation of large amounts of bile pigment was often detectable in the ductuli of the bile duct epithelial cells (Fig. 9). The fine-structural appearance of this was rather similar to those observed in the cytoplasmic inclusions (Fig. 9).

The second sampling was performed on May 19, in the 3rd month of the treatment. The mitochondrial damages observed at the time of the first studying were still detectable, and as a new change of giant, elongated mitochondria appeared. Electron dense cytoplasmic inclusions were invariably observed in many hepatocytes (Fig. 10). Some of the inclusions resembled cholesterin crystals. Hepatocytes abundant, in cytoplasmic glycogen granules and poor in cell organelles were present in increased numbers (Fig. 8). The frequency of accumulation of bile pigment in the ductuli of the bile duct epithelial cells remained unchanged. The appearance of so-called "dark" and light cells was a new phenomenon in this period. At the time of the 3rd sampling (8 June) no new alterations were detectable, however, the amount of rEr considerably increased by the time of the last sampling (Fig. 11). Parallel with each other, long tubuli bundles were observable around the cell nucleus or at the peripheral part of the cytoplasm (Fig. 11). Fingerprints also appeared frequently (Fig. 12). The surface of the fingerprint membranes was generally found to be free of ribosomes (Fig. 12). The intermembranous cytoplasmic substance contained glycogen granules (Fig. 12). Occasionally, myelinization of fingerprint membranes could also be detected (Fig. 12).

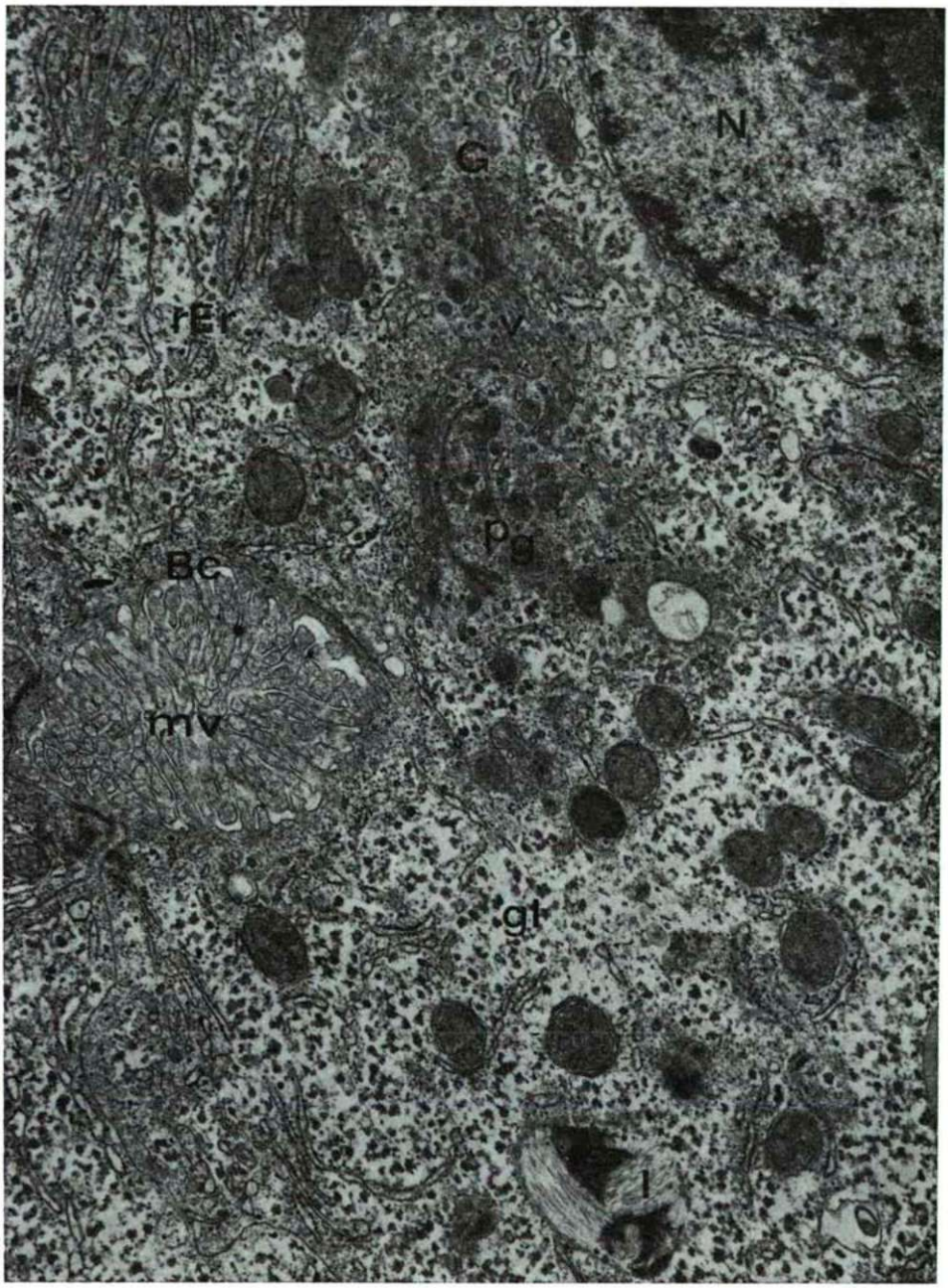

Fig. 2. Untreated control. Parenchymal cell. Well-developed Golgi apparatus (G) beside the cell nucleus (N). The Golgi area is rich in vesicular (v) elements and prosecretory granules (pg). The glycogen granules (gl) are evenly distributed in the cytoplasm. At places the tubuli of the rEr are slightly dilated. The lumen of the bile canaliculus (Bc) is abundant in microvilli (mv). I=inclusion x 12 000

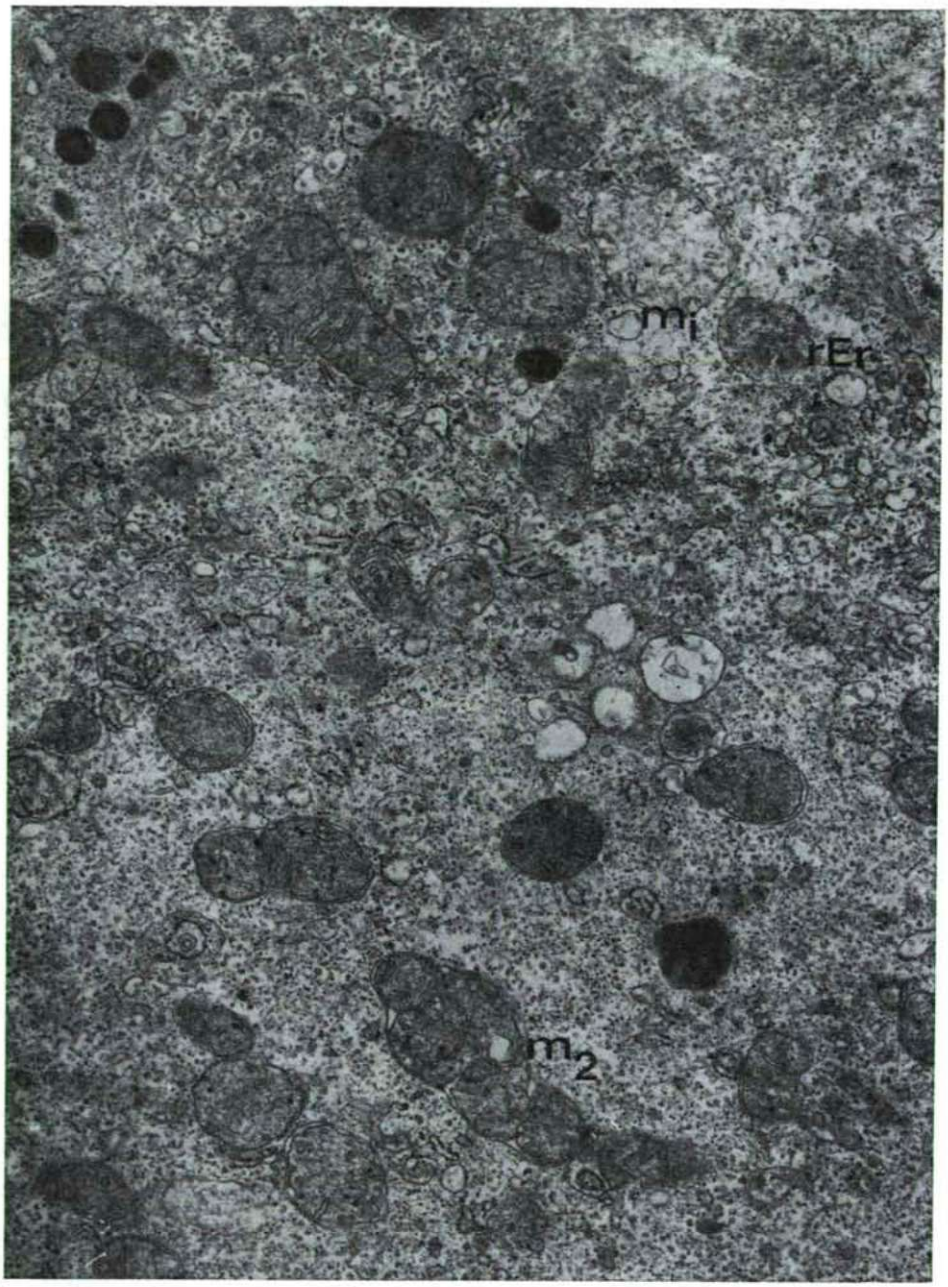

Fig. 3. Detail of a hepatocyte after 2 months of exposure to 2,4-D. Certain mitochondria (m_1) are strongly swollen, others (M_2) are closed in autophage vacuoles. The rEr is mainly present in vesicular form. x 13 000

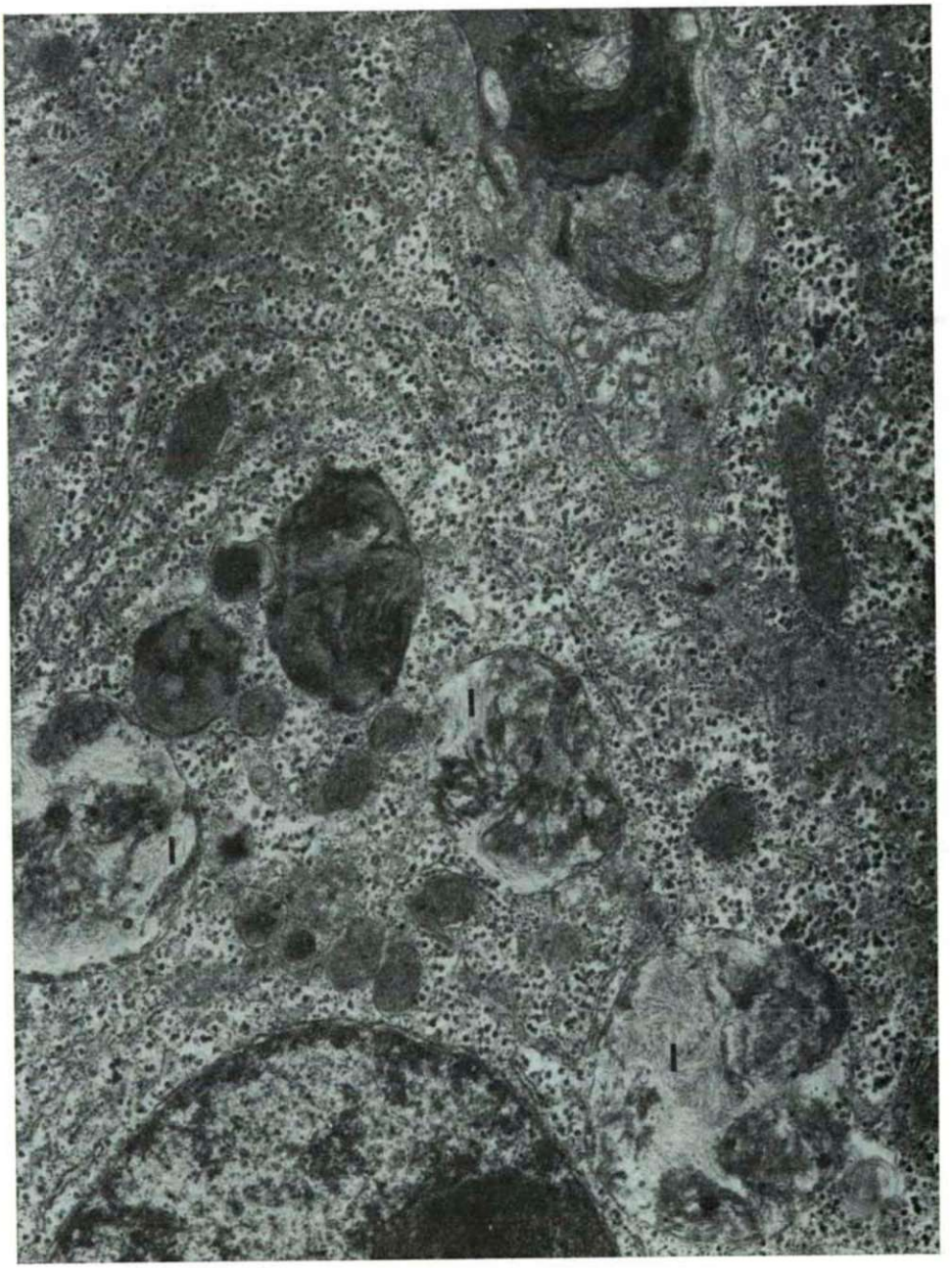

Fig. 4. Hepatocyte after 2 months of exposure to 2,4-D. Many inclusions (I) in the cytoplasm. The inclusions are spherical, their inner substance is made up of fine fibrous material of varying the electron density. Note the strongly electron dense amorphous material in the inclusions beside the fibrous matter. x 8000

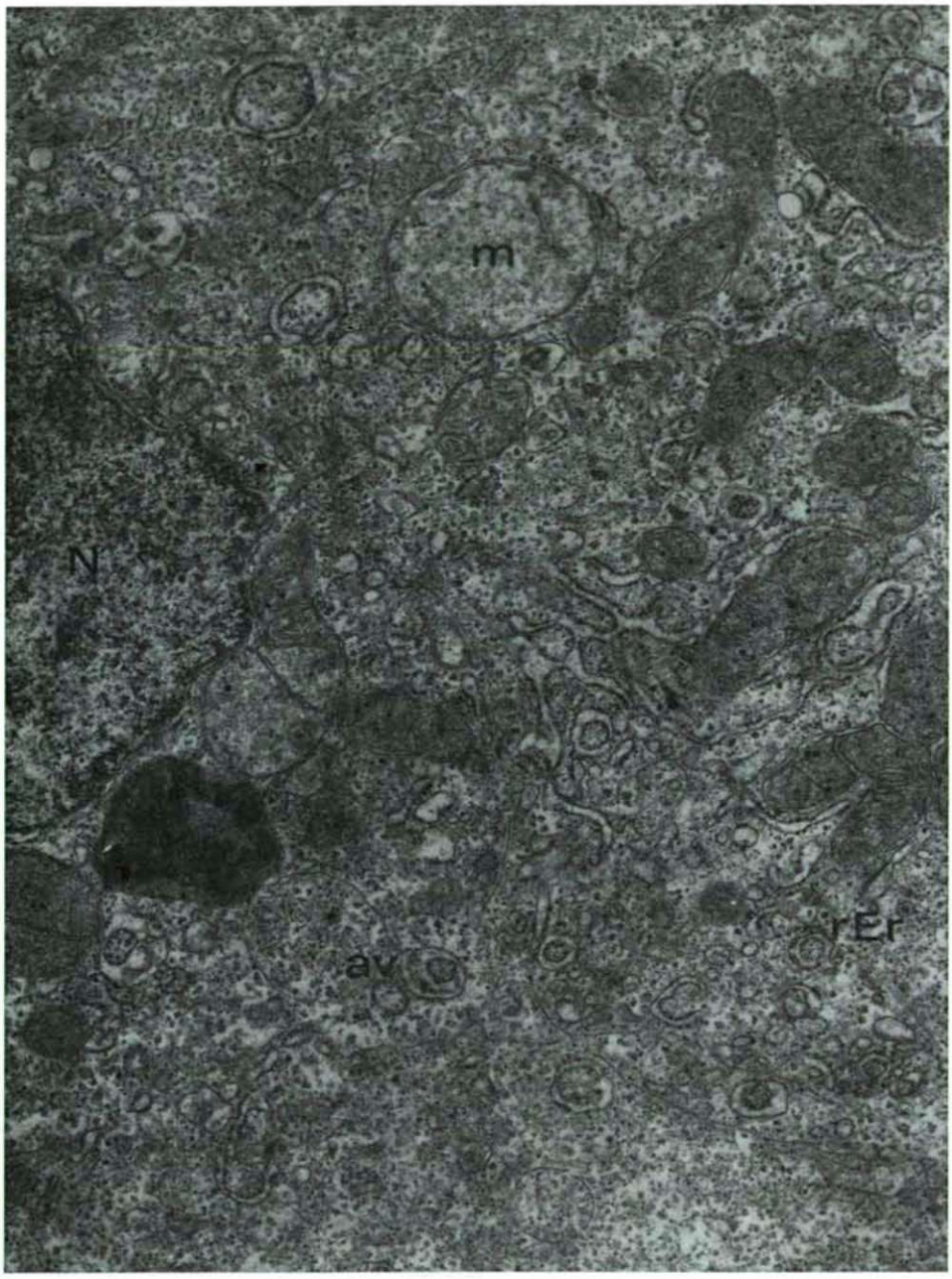

Fig. 5. Hepatocyte after 2 months of exposure to 2,4-D. Swollen mitochondria (m) and autophage vacuoles (av) limited by single or double membrane. The rEr has become transformed vesicularly. N=cell nucleus x 12 000

Fig. 6. Hepatocytes after 2 months of exposure to 2,4-D. Accumulation of crystalline substances in certain Er cisternae (→). m=swollen mitochondrium, N=cell nucleus x 20 000

Fig. 7. Hepatocytes after 2 months of exposure to 2,4-D. Accumulation of moderately electron dense material (bile pigment) in several bile canaliculi (Bc). The Golgi apparatus (G) has collapsed, no prosecretory granules can be found in its substance. rEr I = crystalline-like material in the Er cisternae x 10 000

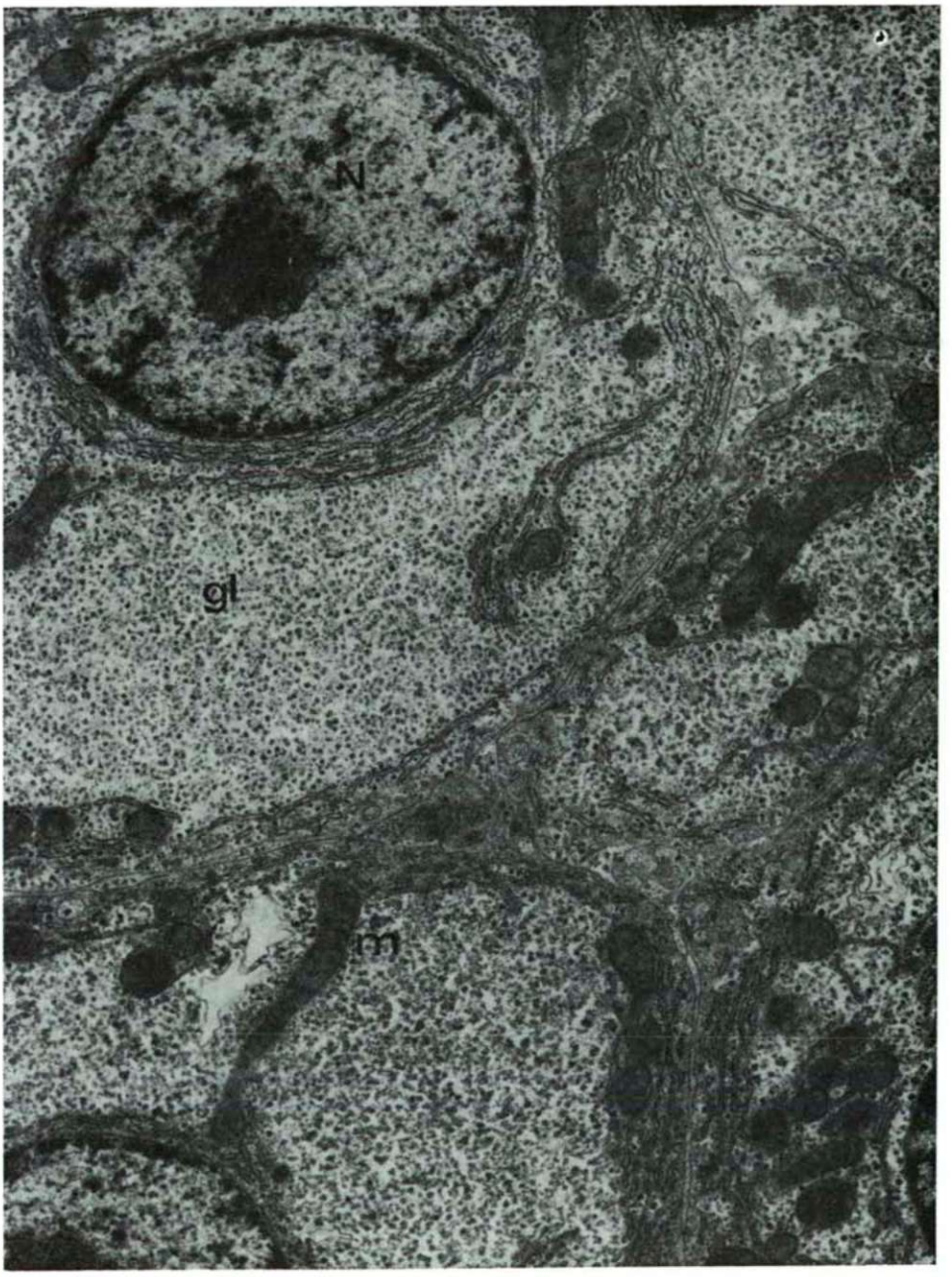

Fig. 8. Hepatocytes after 2 months of exposure to 2,4-D. Cell organelles have strikingly decreased in number in certain hepatocytes. The cytoplasm is mainly filled by glycogen (gl). N=cell nucleus, m=mitochondrion x 12 000

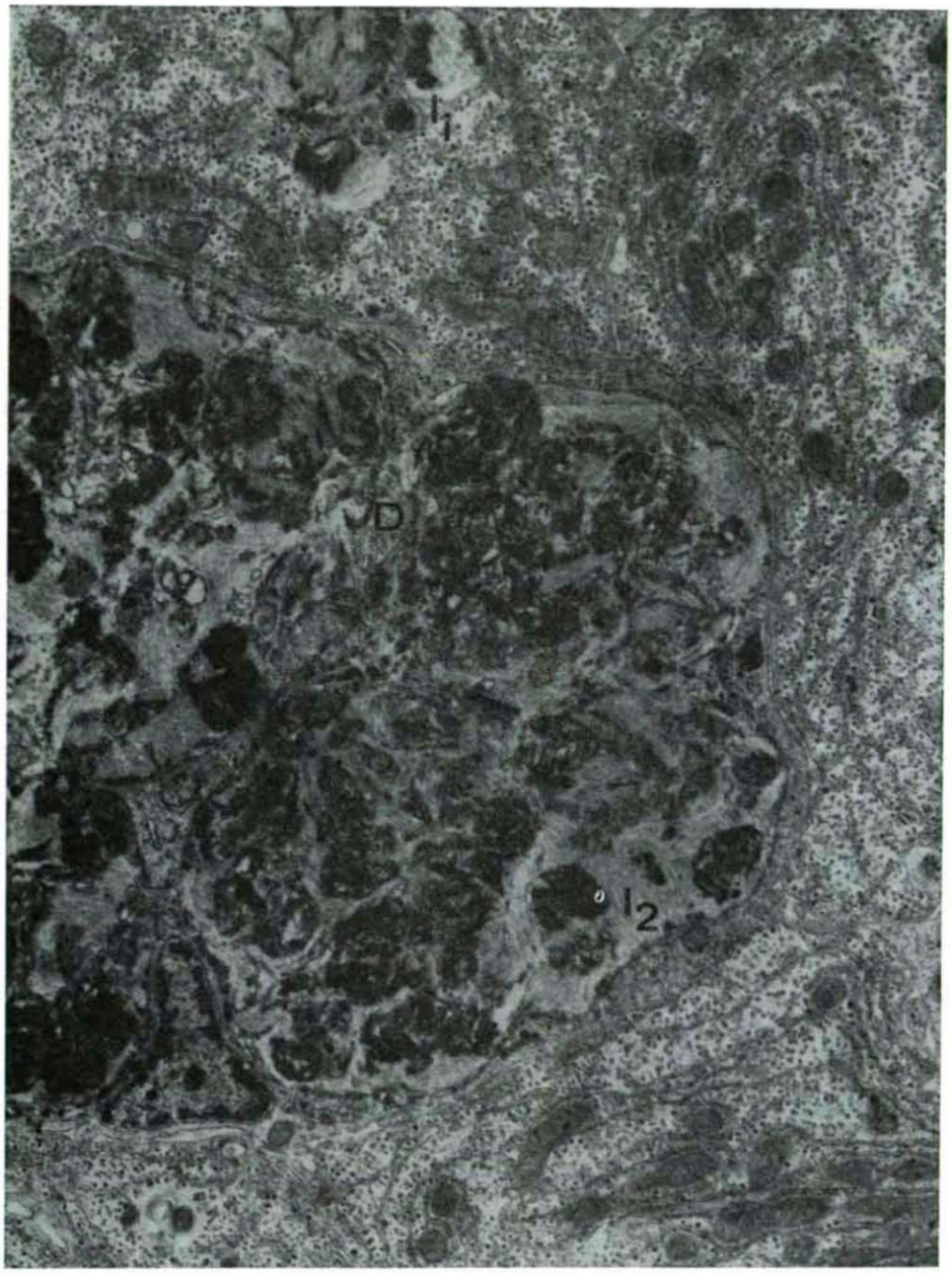

Fig. 9. Masses of inclusions (I_2), similar to those seen in the hepatocytes, frequently occurred in the bile duct (D). I = inclusion in the hepatocyte; 2 months after 2,4-D treatment x 8000

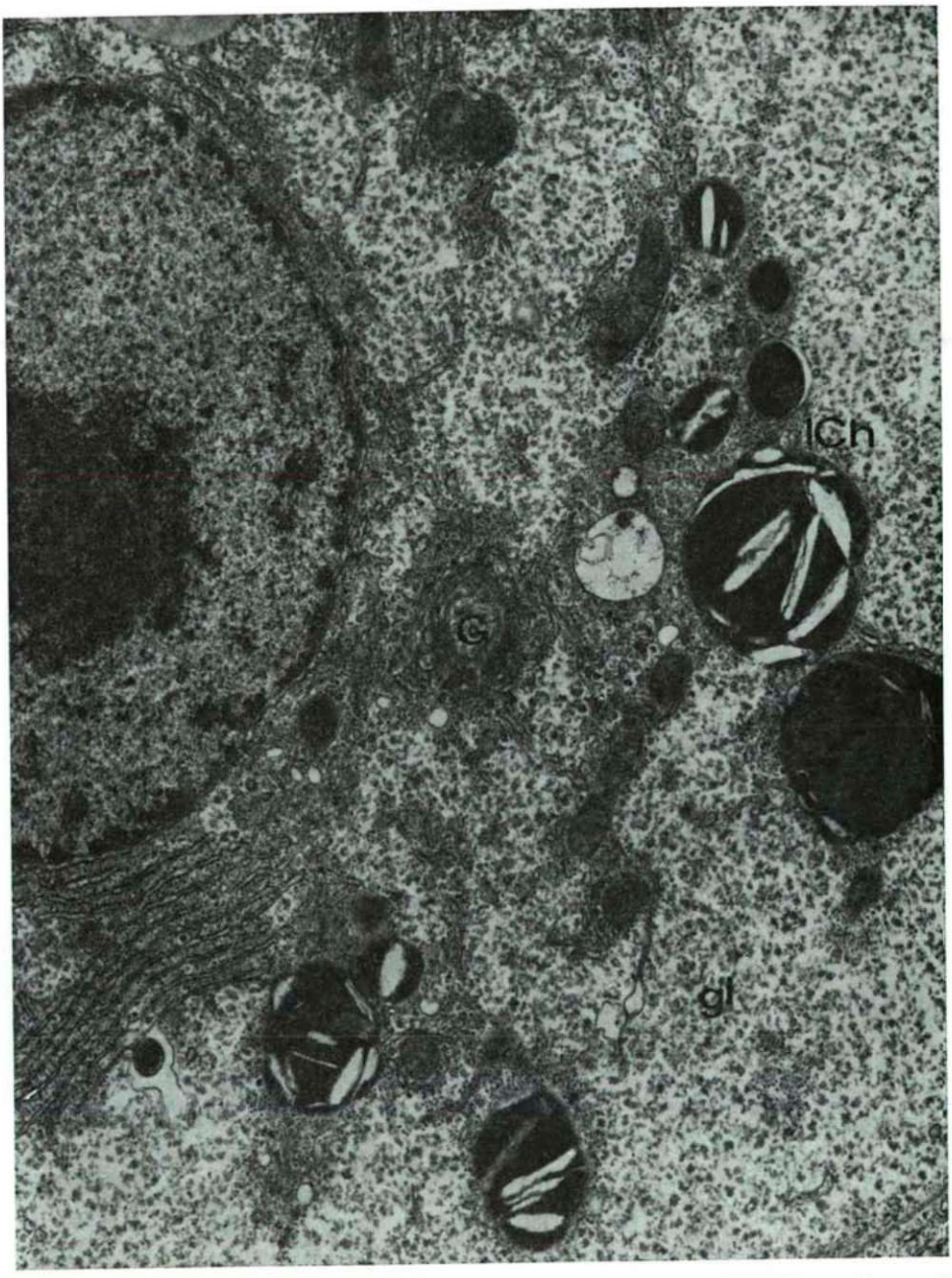

Fig. 10. Hepatocyte after 3 months of exposure to 2,4-D. Inclusions containing cholesterin crystals (ICh) in hepatocytes. The Golgi apparatus (G) has strikingly collapsed. The cytoplasm is rich in glycogen (gl). x 12 000

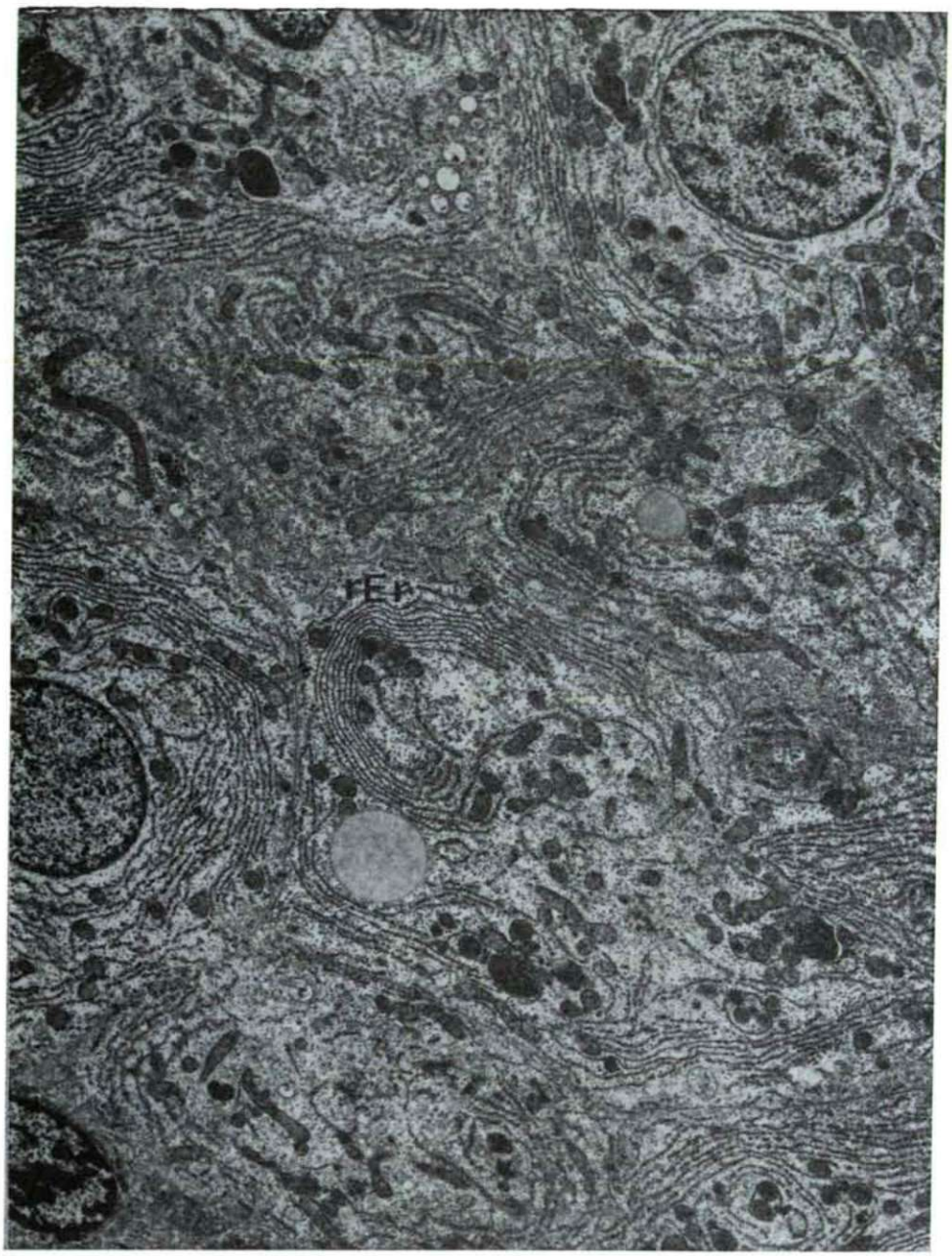

Fig. 11. Liver parenchyma cells after 6 months of exposure to 2,4-D. The cells are rather rich in rEr tubuli. 5–8 Er laminae (rEr) are frequently arranged as parallel bundles. x 4400

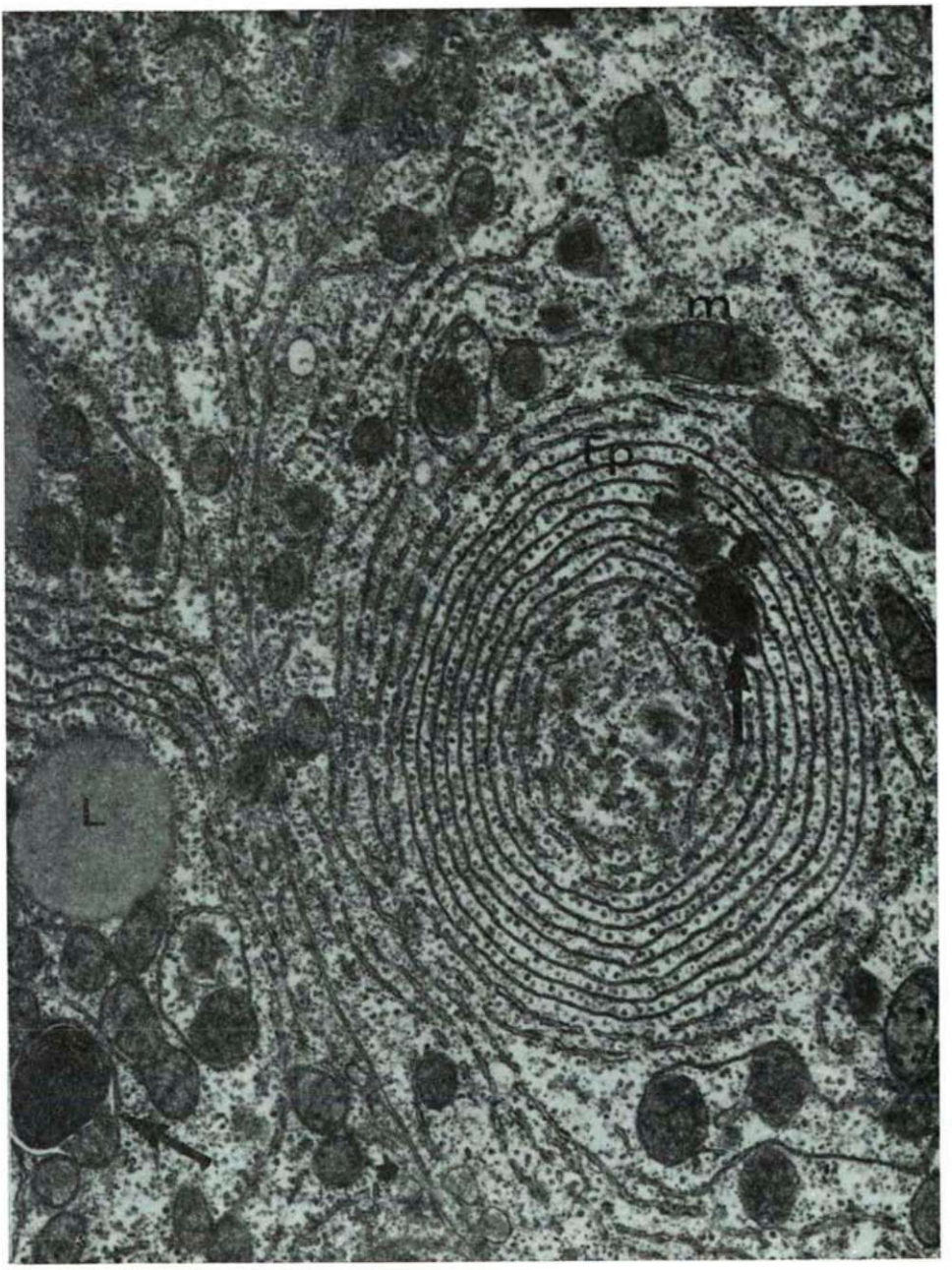

Fig. 12. Hepatocytes after 6 months of exposure to 2,4-D. The frequent appearance of "fingerprints" (Fp) was common in the hepatocytes. Note the myelinization of membranes in both the fingerprints (→) and certain mitochondria (m). L = lipid droplet x 12 000

Discussion

Salts and esters of 2,4-dichlorophenoxy acetic acid (2,4-D) have been utilized as herbicides in plant protection since long, and comprehensively (BORDÁS, 1967; BIRÓ, 1979; MCBRIDE et al., 1981).

In Hungary, a 2,4-D preparation named "Dikonirt" since the 'fifties it has been used regularly and in large amounts (BORDÁS, 1967) as selective herbicide.

In 1965, catastrophic fish decay was observed Lake Balaton (BARON et al., 1967). According to PÓNYI and PFEIFER (1979), it was caused by the chlorinated hydrocarbons demonstrated in the water. Although the cause of the fish decay in the Lake is still debated, there is no doubt that we must reckon with the occurrence of various contaminating chemical agents in natural waters and fish-ponds of Hungary. These agents may influence the metabolism of the flora and fauna of the waters even in low concentrations (TARZWELL, 1965; ASHLEY, 1972).

As to the occurrence of 2,4-D in Lake Balaton, PFEIFER et al. (1979) have shown that the agent is present from April to July generally in a concentration of 1—10 μg/l. At places even concentrations between 25 and 40 μg/l 2,4-D have been demonstrated in the Lake at places (PFEIFER et al. 1979). Since, due to occasional rain the concentration of 2,4-D may rise significantly in fish-ponds, the waters flowing into the Lake Balaton, and then in the Lake itself, it is important to estimate the effects of the sublethal doses of 2,4-D on the various aquatic organisms and the vital processes of the fish, in long-term experiments. It has been determined in regard to zooplankton that the amount of the most important filtering zooplankton of the Lake Balaton *(Eudiaptomus gracilis)* has considerably decreased compared to the 1930s (BANKÓS and PÓNYI, 1976; PÓNYI and PFEIFER 1979). PÓNYI and PFEIFER (1979) assume that the loading by 2,4-D-containing Dikonirt also has a role in the loss of the zooplankton. The acute toxic effect of the 2,4-D derivatives on the early developmental processes of fish has been reported by several authors (MOUNT and STEPHAN, 1967; KAMLER, 1972; BIRÓ, 1979). Sublethal doses of 2,4-D (5—50 mg/l) induced significant stress response in juvenile sockeye salmon (MCBRIDE et al., 1981). Histopathological alterations were observed by light microscopy in the branchiae, liver, and interrenal tissue of the kidney (MCBRIDE et al., 1981). Regarding the liver, MCBRIDE et al., (1981) determined that the tissue injury was focal, appearing in the form of vacuolar degeneration. Using second-summer carps, we failed to detect the alterations described by MCBRIDE et al., (1981) light microscopically; however, we were able to detect the presence of bile pigment proving the development of cholestasis, both in the cytoplasm of the liver cells and in bile ductuli. Cholestasis represents a severe disturbance of the bile secretory apparatus (DESMET, 1979), and this disturbance in liver function could be observed in the liver of the carps throughout the whole study period. Our electron microscopic studies support the diagnosis set up by light microscopy, since accumulation of electron dense bile pigment could be detected in both the hepatocytes and the lumen of the bile canaliculi. Accumulation of electron dense complex bile pigment was also observed by KENDREY (1980) in hepatocytes of a man working at a plant protection station. The development of paraprotein crystal-inclusions appearing in the rEr cisternae suggests that, apart from the damage of the bile-secretion, the transport of certain secretory proteins were also disturbed in the liver parenchyma cells of the fish exposed to 2,4-D. The development and persistence of the large amounts of cytoplasmic inclusions found in the hepatocytes can

practically be explained by an inhibited transport. The fine fibrous substance in the secondary lysosomes was structurally similar to the material found in the residual bodies accumulated in the bile ducts. Presumably, the accumulation of bile pigment in the cytoplasm of the cells and bile ducts resulted from disturbed transport processes, similarly to the human diseases accompanied by cholestasis (DESMET, 1979; SCHAFF and LAPIS, 1979).

Besides the accumulation of paraprotein crystals, further alterations occurred in the rEr. The vesicularization of the rEr tubuli, as well as their degranulation at places, also speaks in favour of the assumption that the exposure to 2,4-D considerably affected the protein synthesizing system, too. Since the rEr tubuli often cease being longitudinally arranged under energy-deficient circumstances (SCHAFF and LAPIS, 1979), it may be assumed that 2,4-D exerts its effect on the protein-synthesizing rEr elements through the mitochondrial system (ZVIRGZDS et al., 1971; KAMLER et al., 1974). We emphasize that the rEr was damaged throughout the whole period of exposure, nevertheless, the damage was more striking in the early and middle periods: in the last period, on the other hand, a significant rEr increase took place. The degenerative alterations were followed by a regenerative process, the cell-physiological background of which is unknown. The fact that "fingerprints" also appeared besides the increase in Er supports the view that 2,4-D affects the ribosome-Er-membrane relationship, i. e., it makes ribosomes detach from the Er-membrane, thus, affecting the process of protein synthesis (BUTLER, 1979; DAVIDSON, 1979).

A significant elevation of glutamate dehydrogenase activity was measured by BIRÓ in the blood of 2,4-D treated fish (BIRÓ, 1981, unpublished data). This increase in enzyme activity is consistent with the ultrastructural alterations referring to the mitochondrial damages (light swelling, disappearance of crystae, autophagy) detected by us. The enzyme may easily escape from the swollen, damaged mitochondria, and may become diffused in the blood in large quantities through the vascular pole of the hepatocytes. On the basis of the presence of damaged mitochondria, the consequence was drawn that the energy-supply of the cells was not satisfactory. In such a manner, the structural damage of the mitochondria may serve as an explanation also for the interpretation of the inhibited transport processes (cholestasis) discussed above.

In conclusion, exposure to 2,4-D for a period of 6 months produced ultrastructural cell damages in the hepatocytes suggestive of *(i)* a disturbance in the energy-supply of the cells, *(ii)* an inhibition of transport processes *(iii)* an alteration of the entire cell metabolism. There is no doubt that electronmicroscopic cytopathology can be applied successfully in the environment and nature conservancy research and in the elimination and prevention of the developed damages.

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