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Original Articles

Rat Liver Alterations After Chronic Treatment With Hexachlorobenzene

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Summary. Groups of female rats were treated orally with 0.5, 2.0, 8.0, and 32 mg/kg hexachlorobenzene twice a week for 203 days. The liver content of hexachlorobenzene was found to be dose-dependent. In the animals treated with the highest dose the concentration was 273 µg/g hexachlorobenzene. In the fresh and fixed hepatic tissue of the treated animals pink fluorescence was observed. Electron microscopy revealed a dose dependent enlargement of all hepatocytes due to proliferation of the SER in the centrolobular area or to increased glycogen deposits (β- or α-particles) and SER in the intermediary and periportal area. Numerous porphyrin deposits and siderosomes, intimate disorganisation and moderate dislocation of the RER and a moderate enlargement of bizarre-shaped mitochondria were recognized. The relationship between porphyrin crystals and mitochondria on the one hand and between SER and glycogen deposits on the other is discussed.

Key words: Hexachlorobenzene – Liver – Ultrastructure – Porphyrins – Smooth endoplasmic reticulum.

Introduction

Hexachlorobenzene (HCB) is not only a fungicidal agent, but also a by-product of industrial chlorination processes that may contaminate food and the environment. At prolonged moderate exposure it causes porphyria cutanea tarda in man (Cam and Nigogosyan, 1963). Long-term treatment of rats leads to increased synthesis of different porphyrins, cutaneous necrosis and neurological symptoms. Electron microscopic studies revealed enlargement of centrolobular hepatocytes due to extensive proliferation of the SER (Kuiper-Goodman et al., 1976), HCB-storing vacuoles, enlargement of mitochondria (Mollenhauer et al., 1975, 1976), fingerprints and "myelin-like" structures (Medline et al., 1973).

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In these studies the experimental animals received the substance via their food over a period of 9, 15 and 52 weeks, respectively. A correlation between the content of HCB in the tissues of the animals and its effects, however, has only been carried out by Kuiper-Goodman and her coworkers (1977). To contribute to the understanding of the effects on liver tissue of HCB, we made an attempt to find possible correlations between the concentration of HCB in the liver, the time of administration and the ultrastructural alterations observed.

Methods

1. Substance

HCB from Fluka AG (Switzerland) was purified by recrystallization from benzene. Its melting point was measured at 226–229° C (Dittmer, 1959: 229° C). Tests by means of gas chromatography revealed that the substance was of more than 99.8% chemical purity.

2. Animals and Treatment

36 female Wistar rats (Zentralinstitut für Versuchstierzucht, Hannover), weighing initially about 110 g, were housed in groups of three or four in wire bottom cages in order to prevent the intake of urine and feces. The animals had access to Altromin Standard Diet 1,324 pellets and drinking water.

HCB dissolved in olive oil DAB 7 was administered by means of a stomach tube every second and fifth day a week for 29 weeks. The doses administered were 0.5, 2.0, 8.0 or 32 mg/kg HCB. The concentrations were 0.01, 0.04, 0.16 and 0.64%, respectively, with respect to HCB in oil. The controls received 5 ml/kg olive oil under otherwise indentical conditions.

3. Analytical Procedures

At the end of the 29th week of treatment 3 animals of each dosing group were weighed, sacrificed and bled out. The liver was dissected, weighed and examined for its content of HCB. The determination of HCB compounds was performed by means of gas chromatography (Koss et al., 1976).

4. Morphological Examinations

All animals were killed between 9 to 11 a.m. Two untreated and 12 treated animals (2 rats treated with 32 mg/kg HCB, 5 rats treated with 8 mg/kg HCB, 3 rats treated with 2 mg/kg HCB, 2 rats treated with 0.5 mg/kg HCB) were perfused for 30–60″ under ether anesthesia by a tyrode-procain solution containing heparin (modified after Forssman et al., 1967). Thereafter they were perfused for 7–10′ by 2% glutaraldehyde (0.1 M phosphate buffer, pH 7.3, 360–380 mosm, perfusion pressure in the left ventricle 50 mm Hg). For electron microscopy hepatic tissue was cut into blocks of 1 mm^3, postfixed in glutaraldehyde, washed in 0.1 M cacodylate buffer and fixed in 1% OsO_4 (0.1 M cacodylate buffer). After dehydration they were embedded in Epon 812. Semithin sections of 1–2 μm thickness were stained by methylene-blue and azur II (Richardson, 1960). Ultrathin sections were stained with lead citrate (Venable and Coggeshall, 1965) or uranyl acetate (Watson, 1958) followed by lead citrate and were examined in a Siemens I electron microscope. Frozen sections or paraffin embedded material was stained by Sudan III, HE, the prussian-blue reaction, periodic acid Schiff and Best's carmine methods.

Table 1. Body weight, relative liver weight and the content of HCB in the liver of female rats at the end of the 29th week of treatment. The animals received oral doses between 0.5 and 32 mg/kg HCB dissolved in oil every second and 5th day a week for a period of 29 weeks. The controls received oil under otherwise identical conditions

Dose	Body weight (g)	Relative liver weight (%)	Content of HCB[a]
Oil	251.8 ± 13.1 (n = 3)	45 ± 0.12	0.1 ± 0.02
0.5 mg/kg	244.0 ± 26.5 (n = 3)	3.30 ± 0.25	3.5 ± 0.3
2 mg/kg	244.6 ± 33.0 (n = 3)	3.33 ± 0.32	11.4 ± 1.9
8 mg/kg	226.7 ± 8.3 (n = 3)	3.65 ± 0.73	49.4 ± 5.3
32 mg/kg	248.7 ± 14.2 (n = 3)	4.52 ± 0.27	273.0 ± 101.0

[a] Figures in μg/g wet liver tissue

Results

1. Determination of HCB

With the four-fold increase of the dose from one group of the animals to the other, an almost four-fold elevation of HCB-content in the liver of the animals was observed. In the liver of the animals treated with 32 mg/kg the content of HCB amounted to 273 μg/g wet tissue (Table 1).

2. Clinical Behavior

Between the 20th and 24th week 2 rats receiving 8.0 mg/kg HCB and oil, and 1 rat in each of the other groups died. The other animals treated with HCB or oil did not exhibit alterations in appearance or behavior when compared with untreated rats. Increase in body weight was the same in the HCB-treated animals as in those receiving oil only.

An increase in liver weight was observed in the rats treated with 32.0 mg/kg HCB (Table 1).

Before fixation it was noticed that the liver of the animals treated with 8.0 or 32 mg/kg HCB had a dark-brown colour and that the fixed hepatic tissue revealed red fluorescence under UV-light. This diminished with decreasing dose of HCB.

3. Light Microscopy

The lobular architecture remains intact. The portal tracts do not reveal fibrosis or inflammatory infiltrations. A dose-dependent response between the HCB

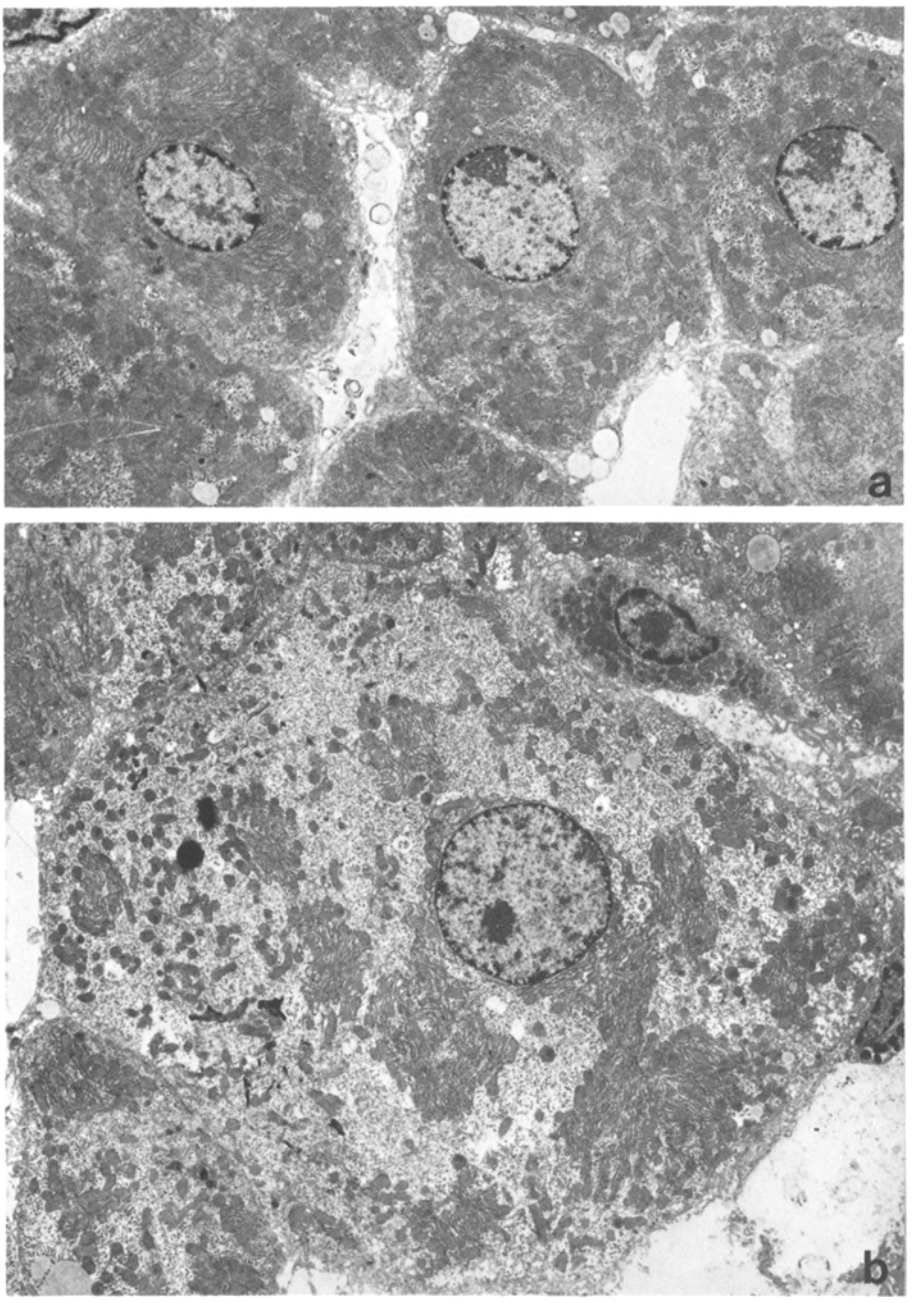

Fig. 1. a Centrolobular hepatocytes of an untreated animal (×2,070). **b** Hepatocyte in the periportal area of a rat treated with 32 mg/kg HCB. Proliferated SER and glycogen deposits are recognized as pale areas (×2,070)

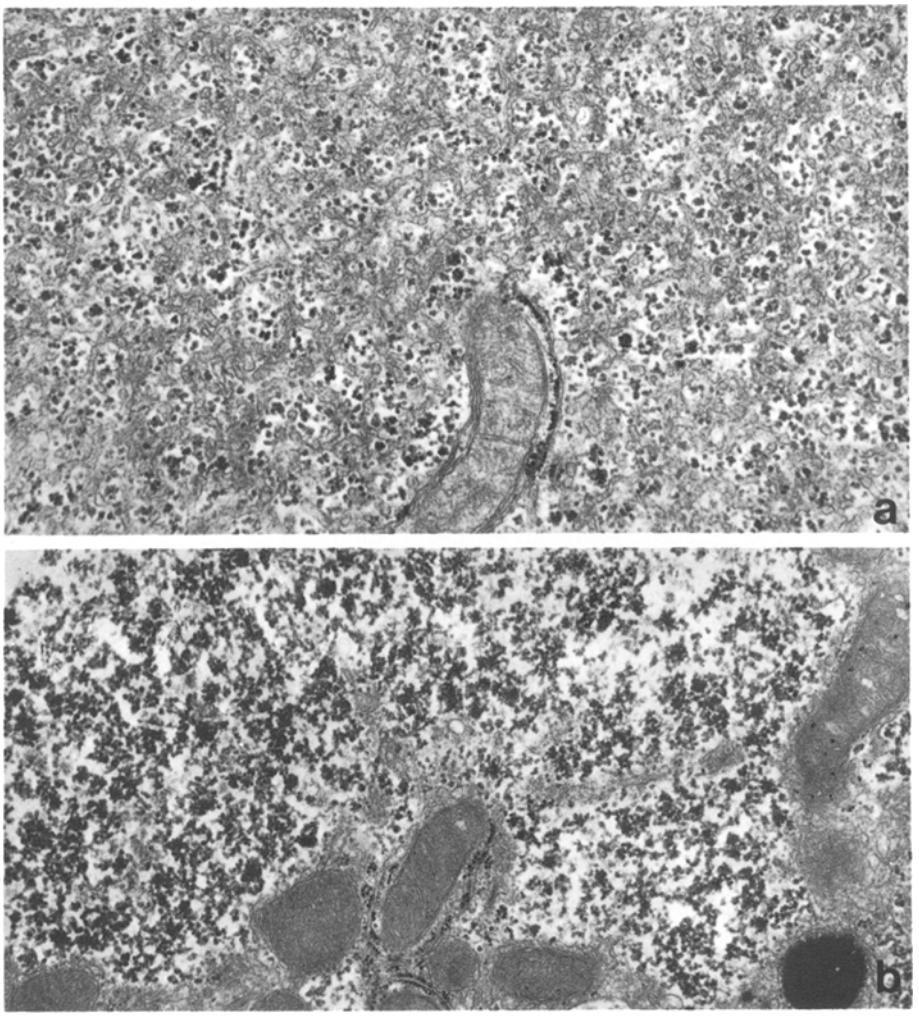

Fig. 2. a Smooth endoplasmic reticulum and a few monoparticulate glycogen deposits in a centrolobular hepatocyte of a rat treated with 32 mg/kg HCB (×25,000). **b** Monoparticulate glycogen deposits in a periportal hepatocyte of a rat treated with 32 mg/kg HCB (×20,000)

concentration and the morphological alterations can be observed. In the animals treated with 8.0 and 32 mg/kg HCB all hepatocytes are markedly enlarged while a moderate enlargement of the hepatocytes of the animals treated with 2.0 mg/kg HCB occurs, no enlarged hepatocytes can be seen in the animals dosed with 0.5 mg/kg when compared with the controls. The nuclei reveal a pronounced polymorphism and an increased number of binuclear hepatocytes can be seen. In the centrolobular area several very large oval nuclei occur, which contain up to 11 nucleoli. Mitosis occurs more frequently than in the control animals. Sections stained by Sudan III show one or more small lipid

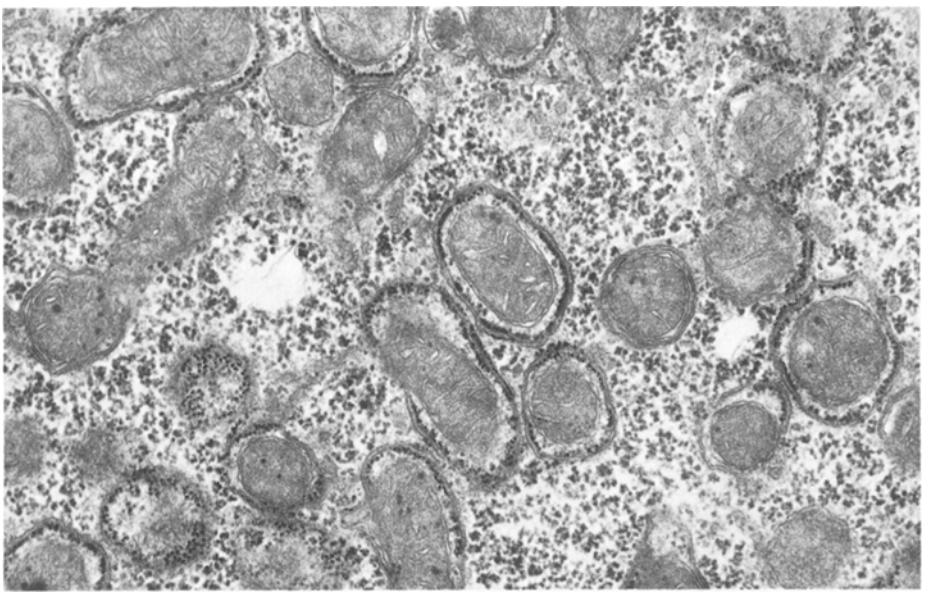

Fig. 3. Mitochondria are enveloped by partly degranulated RER. Centrolobular hepatocyte of a rat treated with 32 mg/kg HCB (×25,000)

droplets in enlarged nuclei. Several hepatocytes contain either numerous small or some large lipid droplets. Hepatocytes situated in the centrolobular area contain dusty iron deposits. About 80% of the cytoplasm of those hepatocytes, which are placed between the centrolobular and the intermediary area is represented by glycogen (identified by Best's carmine staining). In contrast hepatocytes in the centrolobular area do not contain any glycogen deposits at all.

Treated animals do not show an increase in the number of Kupffer cells or fat-storing-cells. Nevertheless the size of the fat-storing-cells is markedly increased by lipid droplets, especially in animals treated with 32 mg/kg HCB. Kupffer cells situated in the centrolobular area show distinct siderosis.

4. Electron Microscopy

Hepatic ultrastructure of each treated group and the control group was investigated. In the animals treated with 32 mg/kg HCB the diameter of the nuclei of the hepatocytes is enlarged by a factor of 1.3 ($n=77$) when compared with the controls. The size of hepatocytes is increased by two to two and a half fold which is thought to be due to a distinct proliferation of the smooth endoplasmic reticulum (SER) and to an increase of glycogen deposits (Fig. 1a and b). Tubular or vesicular SER is formed and occupies large cytoplasmic areas, especially in the centrolobular hepatocytes (Fig. 2a). Frequently communications between the SER and the rough endoplasmic reticulum (RER) can be seen. There appears to be a relationship between the SER and the amount of glycogen

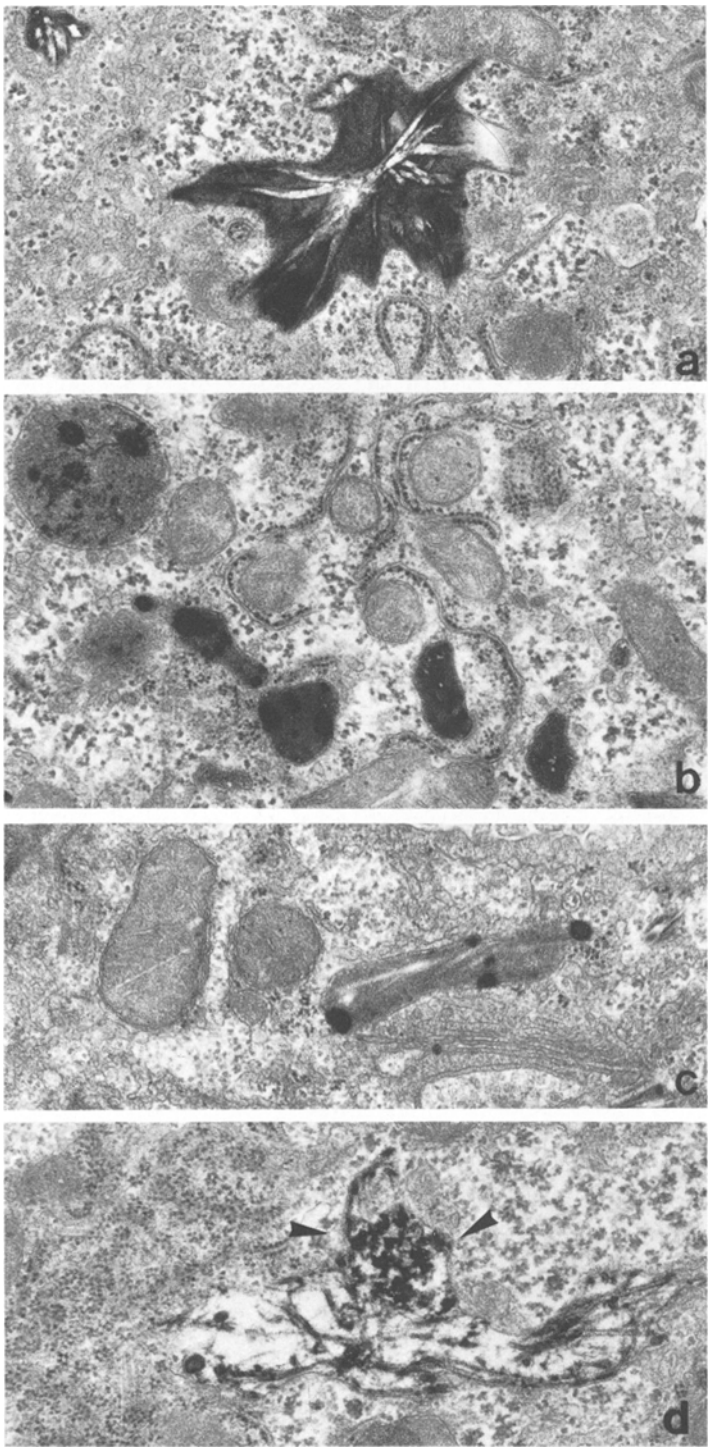

Fig. 4a–d. Porphyrin deposits. **a** Radially arranged crystalline structures embedded in an osmiophilic matrix. Centrolobular hepatocyte of a rat treated with 32 mg/kg HCB (×25,000). **b**, **c** Porphyrin deposits similar to mitochondria (×25,000). **d** The osmiophilic matrix is dissolved, crystalline structures are arranged in an angular fashion. Small granules resembling iron depotis (→) (×25,000).
Fig. 4b–d. Hepatocytes in the periportal area of rats treated with 32 mg/kg HCB

deposits. In particular hepatocytes situated in the intermediary area show moderate proliferation of the SER and extensive cytoplasm areas including numerous glycogen particles. They are present either as typical rosettes (α-particles about 1,300 Å in diameter) or as mono-particulate β-particles (200 Å in diameter) (Fig. 2b). In centrolobular hepatocytes proliferation of the SER is quite distinct but only a few mono-particulate glycogen deposits can be recognized. The cisternae of the RER in association with mitochondria are confined to some areas of the liver cells, as if they have been dislocated by proliferation of the SER or glycogen deposits. The cisternae of the RER mostly occur as closely packed stacks, but in the neighbourhood of the proliferated SER the RER is disorganized, being diffusely dispersed in the cytoplasm and coming into close contact with mitochondria by enveloping these organelles (Fig. 3). Atypical membrane complexes can be seen. Their arrangement resembles the SER, which is studded with ribosomes. In comparison with controls several liver cell mitochondria of the treated animals are moderately enlarged and of a bizarre shape.

With increasing dose of HCB slightly electron dense crystalline structures appear, which are arranged in more or less parallel, angular or radial (Fig. 4a) fashion and are embedded in an osmiophilic matrix. For the most they are membrane bound and look like mitochondria (Fig. 4b and c). The osmophilic matrix, which is sometimes seen to be dissolved (Fig. 4d), contains small osmiophilic droplets or electron dense granules resembling ferritin deposits. In the neighbourhood of bile canaliculi lipofuscin granules and phagolysosomes of different sizes are often recognized. The latter include flocculent or filamentous material, myelin figures and granular electron dense deposits which are supposed to be iron deposits, forming simple or complex siderosomes (Ghadially, 1975).

Cholestasis or alteration of microbodies and microvilli in the bile canaliculi and Disse space do not seem to be present.

Discussion

Porphyrin deposits, increase of glycogen and alteration of the endoplasmic reticulum prodominated among the ultrastructural alterations observed.

Waldo (1973) described porphyrin crystals as needle-like cytoplasmic deposits in the liver of patients suffering from porphyria cutanea tarda. Waterfield et al. (1969) observed porphyrin crystals in the neighbourhood of ruptured mitochondria after treatment with DDC. Timme et al. (1974) supposed that tadpole-shaped structures occuring after HCB-treatment were comparable with porphyrins. We found tubular crystalline structures embedded in an osmiophilic matrix and believe that crystalline porphyrins are stored in the mitochondria, that is, in organelles, which are involved in the synthesis of these substance (Granick, 1963; Scholnick and Marver, 1968). These altered mitochondria are situated in the close vicinity of the golgi complex, where they become autophagolysosomes. Timme et al. (1974) supposed that precipitation of porphyrins is possible after formation of complexes with iron ions. Perhaps this could be an explanation for the observation that the osmiophilic matrix was only dissolved sometimes, indicated by the pink colour of the fixative. It is presumed that the porphyrins

retained in the cells are responsible for the persistent fluorescence in the fixed hepatic tissue.

It may be possible that the chronic intoxication of rats with HCB leads to an "adaptation" of the hepatocytes in all lobular areas to the compound. Stereological analysis of the liver revealed an increase in hepatocyte volume in the intermediary and centrolobular area, a proliferation of the SER which occupied 53–77% of the cytoplasm (Kuiper-Goodman et al., 1976). An 103-day-treatment with 32 mg HCB/kg/d induced proliferation of nonfunctional SER with myelin figures in the liver cells and a centrolobular depletion of enzymes located on the membranes of the SER (Kuiper-Goodman et al., 1976, 1977). In our experiments rats received 32 mg/kg HCB by means of a stomach tube for 203 days twice a week, resulting in a hepatic HCB content of 273 μg/g. These figures were similar to the HCB levels in the liver (268 μg/g) measured by Kuiper-Goodman et al. (1977) although her animals received HCB every day, but for 103 days only. In the centrolobular area of the liver cells of our animals myelin figures were absent, periportal hepatocytes were enlarged and contained more SER than Kuiper-Goodman et al. (1976) had measured. Proliferation of the membranes gradually decreased from the centrolobular to the periportal area. It may be that both the levels of HCB in the tissues and the duration of exposure are responsible for the different patterns of structural lesions observed. It may also be that the differences which we observed are perhaps due to the higher purity of HCB in our study. We presume that chlorinated impurities of HCB which are more toxic than HCB itself (Villanueva et al., 1974) can be responsible for myelin figures in centrolobular hepatocytes.

In contrast to Kuiper-Goodman et al. (1974, 1977) and Timme et al. (1974) we saw disorganisation of the RER only in small cytoplasmic areas and we recognized only a few cases of degranulation of the RER. Although both alterations are the morphological equivalent of disturbed protein synthesis (Smuckler et al., 1962; Murhergee et al., 1963; Ashworth et al., 1965), the vital functions of the hepatocytes of our animals did not appear to be very much disturbed.

Glycogen deposits aggregate in the cytoplasm focally. The minor content of glycogen in centrolobular hepatocytes is perhaps due to inhibition of glycogen synthesis in this cell type (Bannasch, 1972). There is a remarkably close relationship between glycogen and SER which has been described both in cases of acute intermittent porphyria and porphyria cutanea tarda (Biempica et al., 1974) and in experimentally induced porphyria (Biempica et al., 1967). In comparison with our findings on HCB, the glycogen storage precedes the proliferation of the SER brought about by the carcinogenic agent N-nitrosomorpholine, and the hypertrophy of the SER spreads from the lobulus centre to the periphery (Theodossiou et al., 1971).

The monoparticulate "glycogen" deposits are presumed to be membrane elements composed of glycoliproteins, which can fuse to form smooth membranes (Löwe, 1969). It is possible that HCB-treatment also causes formation of membrane elements. At the time of sacrifice the membrane elements in the centrolobular hepatocytes were transformed to an extensive network of smooth membranes, in which few glycogen particles were interdispersed. The hepatocytes in the intermediary area had numerous monoparticulate deposits but less SER.

A further aspect might be discussed on the basis of investigations by Cabral et al. (1977). They observed that hamsters after treatment for their lifespan with 16 mg HCB/kg/d, developed hepatomas. It is conceivable that in our examination hepatocytes are in a very early precancerous stage and that numerous hepatocytes resemble x-cells or acidophilic cells, which are said to be precursors of hepatoma cells (Bannasch et al., 1972). In order to provide support for this hypothesis further long-term studies are necessary.

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