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Comparative Toxicity Study of 2,4,5,2′,4′,5′-Hexachlorobiphenyl and a Polychlorinated Biphenyl Mixture in Rabbits

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Comparative Toxicity Study of 2,4,5,2′,4′,5′-Hexachlorobiphenyl and a Polychlorinated Biphenyl Mixture in Rabbits. VOS, J. G. and NOTENBOOM-RAM, ERICA (1972). *Toxicol. Appl. Pharmacol.* **23**, 563–578. Three groups of rabbits were treated daily (5 times per week for 28 days) with 120 mg 2,4,5,2′,4′,5′-hexachlorobiphenyl, 120 mg polychlorinated biphenyl (PCB) mixture (Aroclor 1260®) and the solvent isopropanol, respectively. The dermal application resulted in early macroscopic skin lesions in the Aroclor group. The lesions in the 2,4,5,2′,4′,5′-hexachlorobiphenyl group appeared later on and were less severe. This difference was confirmed microscopically: hyperplasia and hyperkeratosis of the follicular and epidermal epithelium were more severe in the Aroclor® group. Fecal coproporphyrin levels were significantly increased in the experimental groups. Enhanced liver weights were found in both test groups. Liver injury, as judged by light microscopic lesions and elevated serum transaminase levels, was somewhat more severe in the hexachlorobiphenyl group when compared with the Aroclor® group, though the mean liver content was about the same (respectively, 239 and 236 ppm). Light microscopic findings included subcapsular necrosis, zonal necrosis, hydropic degeneration, as well as a peripheral and perinuclear shift of cell organelles, and focal cytoplasmic hyalin degeneration. In electron microscopy the shift was found to be due to a proliferation of smooth surfaced membranes of the endoplasmic reticulum (SER), resulting in a displacement of rough surfaced membranes (RER) and mitochondria. The hyalinized cytoplasm was recognized as tightly packed tubules of proliferated SER, that is considered as hypertrophic, hypoactive SER. The contribution of chlorinated dibenzofurans and pure PCB in the toxicity of crude PCB mixtures is discussed.

Polychlorinated biphenyls (PCB) are industrial chemicals with many applications. The toxicity of these compounds for industrial workers has been known for years, and especially the occupational disease known as chloracne (Jones and Alden, 1936; Schwartz, 1936; Meigs *et al.*, 1954). Recently, the consumption of rice bran oil accidentally contaminated by PCB resulted in many cases of chloracne among Japanese consumers (quoted by Crow, 1970); liver damage was also reported (quoted by Nishizumi, 1970). A critical report on the use, environmental contamination and the toxicity of PCB and other industrial halogenated hydrocarbons appeared recently

(Zitko and Choi, 1971). There is an additional danger for wildlife from the widespread environmental contamination with PCB through food chains, and this is, ultimately, also a menace to human health.

The purpose of the present study is to compare the toxicity of Aroclor 1260® with a single isomer 2,4,5,2′,4′,5′-hexachlorobiphenyl. The commercial Aroclor 1260® sample was found to be the least toxic in a comparative study using three PCB mixtures originating from different firms (Vos and Koeman, 1970). Chlorinated dibenzofurans, considered to be responsible for the higher toxicity of two PCB mixtures, were not found in the Aroclor 1260® sample (Vos *et al.*, 1970). To be more certain about the biological effect attributable to PCB itself, a pure compound, 2,4,5,2′,4′,5′-hexachlorobiphenyl, was used for comparison. This is also one step in the understanding of the toxicity of the different components of PCB mixtures.

Hepatic porphyria was also studied. Special attention was paid to PCB-induced liver damage, using light and electron microscopy for structural alterations and gas chromatography for the determination of liver residues.

METHODS

The technical PCB mixture, a viscous fluid with an average of 60% chlorine, was obtained from Monsanto in the United States (Aroclor 1260® Lot No. AK-3). 2,4,5,2′,4′,5′- Hexachlorobiphenyl, a crystalline compound, was synthetized according to the Ullman synthesis, using 2,4,5-trichloroiodobenzene and copper powder at 220°C (Tas and de Vos, 1971). Because of the chemical reaction, it is unlikely that this particular isomer contains chlorinated dibenzofurans as impurity. The Aroclor® sample was analyzed for the presence of chlorinated dibenzofuran and found to be free (limit of detection: 1 ppm).

Adult female New Zealand rabbits, 3.5 mo old and weighing 2.5–2.9 kg, were distributed at random into 3 groups of 4 animals. The performance of the dermal toxicity study was in general the same as described earlier (Vos and Beems, 1971). Because of the lower solubility of the hexachlorobiphenyl isomer when compared with the PCB mixture, 20 mg of the PCBs (instead of 120 mg in the prior study) were dissolved per milliliter of isopropanol. The PCB solutions were kept at body temperature in order to keep the PCB dissolved. Six milliliters of PCB solution (120 mg) or of isopropanol as a control was dropped daily, 5 times a week, on an area of 5 × 10 cm on the clipped and shaved backs. After a test period of 4 wk (20 applications of 120 mg PCB) the animals were killed.

The coproporphyrin and protoporphyrin analyses of feces from the cecum, the macroscopical examination of tissues in Wood's light as parameters for chemical porphyria, and the hematological examination were done according to a former study (Vos and Beems, 1971). At necropsy, body and organ weights were determined. The skin, liver and kidney were fixed in 10% buffered formalin. The spleen, thymus, axillary and mesenteric lymph node and appendix were fixed in Carnoy's fluid for 24 hr at 4°C and stained with methylgreen-pyronin according to Elias (1969). Paraplast® embedded sections were stained with hematoxylin and eosin. For detailed histology, selected sections were stained with Perls' iron stain and with Best's carmine. Moreover, cryostat sections of the livers were stained with Sudan black for the detection of lipids. Un-

stained Paraplast® embedded sections and unstained cryostat sections of liver, kidney and small intestine were studied in a fluorescence microscope for the detection of ceroid pigment and excess quantities of porpyrins, respectively.

For ultrastructural examination, small blocks of liver tissue were fixed in 0.1 M cacodylate-HCl buffered 5% glutaraldehyde (pH 7.0) for 5 hr. The liver tissue was postfixed two times for 45 min in 0.1 M phosphate buffered 2% osmium tetroxide

TABLE 1

COPROPORPHYRIN AND PROTOPORPHYRIN CONTENTS (μg/g DRY WEIGHT) OF FECES OF RABBITS TREATED WITH PCB FOR FOUR WEEKS[a]

	Coproporphyrin			Protoporphyrin		
	Hexachloro-biphenyl	Aroclor®	Control	Hexachloro-biphenyl	Aroclor®	Control
	45.0	24.1	4.5	88.3	35.1	17.8
	6.2	5.0	3.7	8.3	8.4	11.9
	20.8	29.4	3.6	51.2	65.1	15.6
	39.6	16.0	3.5	66.5	65.1	10.9
Mean:	27.9[b]	18.6[b]	3.8	53.6	43.4	14.0

[a] Figures are the contents of feces, collected from the cecum of the individual animals.
[b] Significantly different from controls, $p \leq 0.025$.

TABLE 2

ABSOLUTE AND RELATIVE LIVER WEIGHTS, MICROSCOPIC LIVER DAMAGE, LIVER RESIDUES AND TRANSAMINASE VALUES FOR RABBITS TREATED WITH PCB FOR FOUR WEEKS[a]

	Liver weight		Microscopic Liver damage[b]	Liver Content (ppm)	SGPT (U)	SGOT (U)
	g	g/100 g body weight				
Hexachloro-biphenyl	128	4.6	++	249	71	16
	132	4.2	+++	140	56	34
	146	4.8	++++	261	70	30
	126	5.1	+++	304	92	21
Mean:	133	4.7[c]		239	72[d]	25[d]
Aroclor®	141	5.0	++	198	64	29
	129	4.0	+++	248	32	10
	152	4.8	++++	317	53	13
	133	4.2	+	182	34	12
Mean:	139[c]	4.5		236	46	16
Control	131	4.3	–	0.8	43	9
	110	3.5	–	0.6	22	10
	91	2.9	–	0.6	20	11
	83	2.8	–	0.7	31	11
Mean:	104	3.4		0.7	29	10

[a] Values are given for the individual animals.
[b] + to ++++ = a comparative estimate of the liver damage.
[c] Significantly different from controls, $p \leq 0.05$.
[d] Significantly different from controls, $p \leq 0.025$.

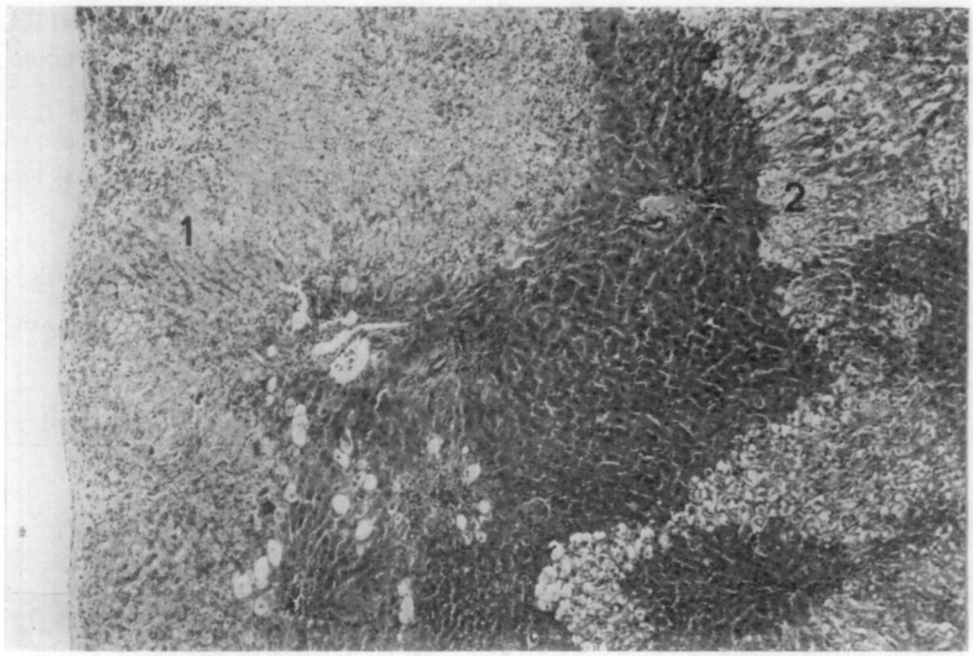

FIG. 1. Liver of a 2,4,5,2′,4′,5′-hexachlorobiphenyl treated rabbit. Subcapsular band of necrotic tissue (1) and area of hydropic degeneration (2). Hematoxylin and eosin. × 60.

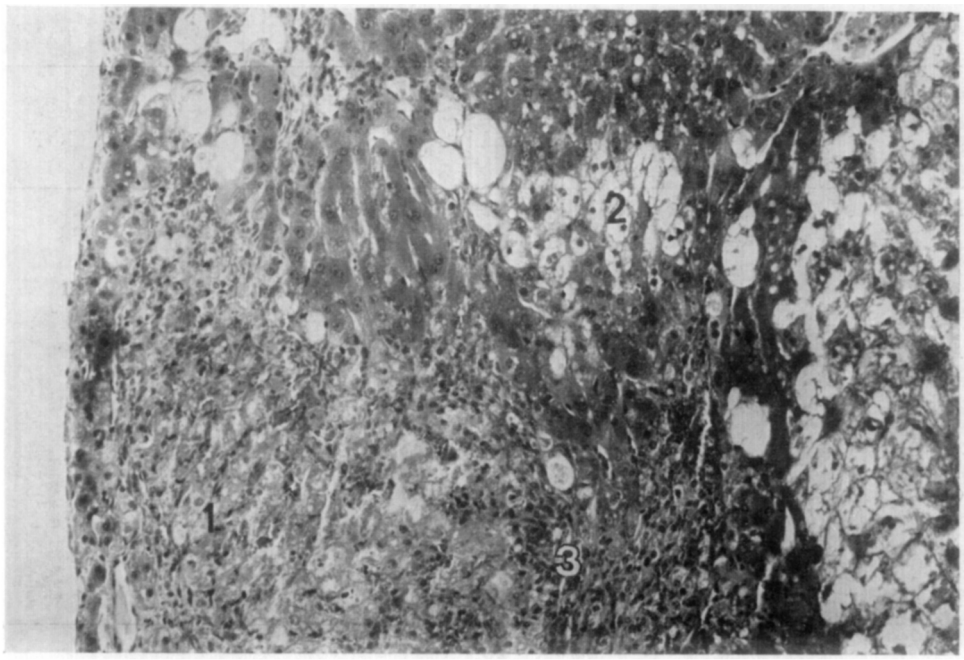

FIG. 2. Higher magnification of the subcapsular region of the liver seen in Fig. 1. Note the necrosis at 1, hydropic cells at 2 and proliferation of mesenchymal cells at 3. Hematoxylin and eosin. × 150.

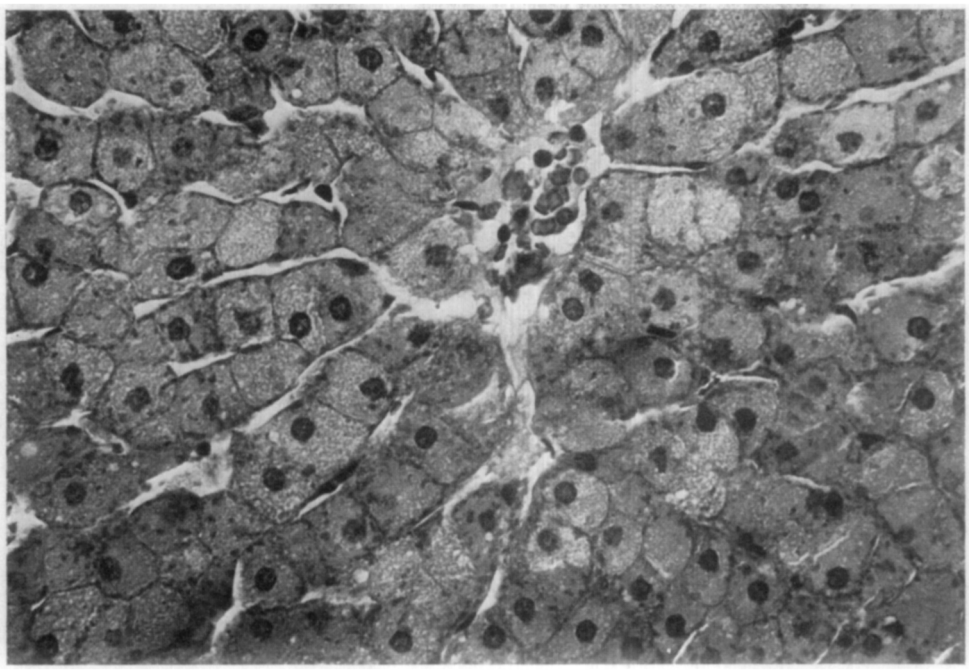

FIG. 3. Centrolobular area of an Aroclor® treated rabbit liver. A marked peripheral and perinuclear displacement of basophilic organelles can be seen. Hematoxylin and eosin. × 375.

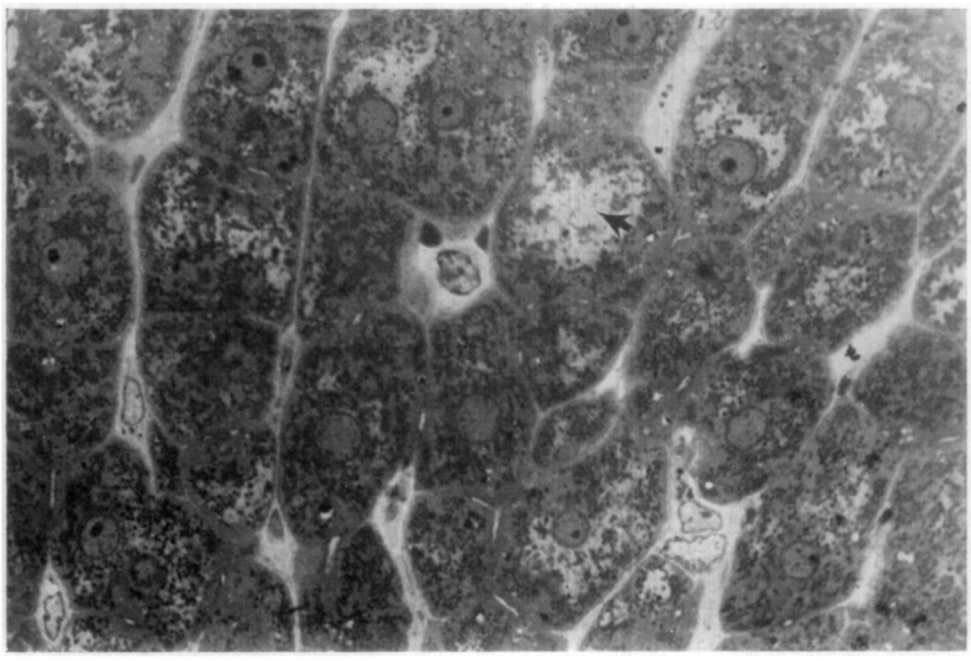

FIG. 4. Hepatocytes of a control animal. Clear areas (arrow) represent negative images of glycogen. Dense material representing cell organelles is scattered in the cytoplasm. Toluidine blue. × 940.

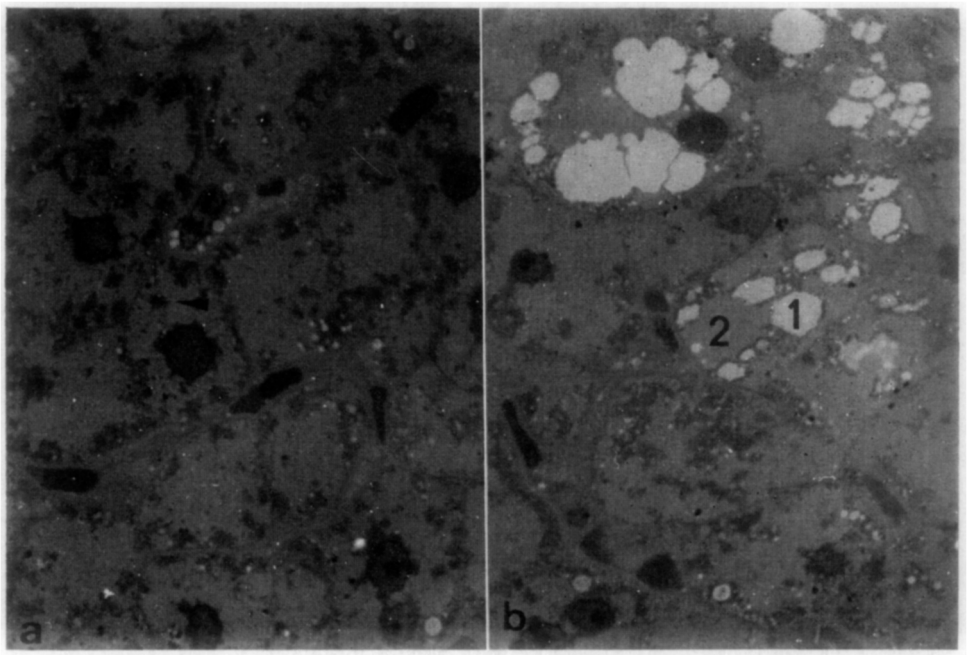

FIG. 5. Liver section of an Aroclor® treated rabbit. Toluidine blue. × 940. (a) Showing peripheral and perinuclear displacement of cell organelles; note also the foamy cytoplasm (arrow). (b) Hydropic cells showing vacuoles (1), that are separated from homogeneous hyalinized cytoplasm (2). Note the perivacuolar localization of densely staining organelles.

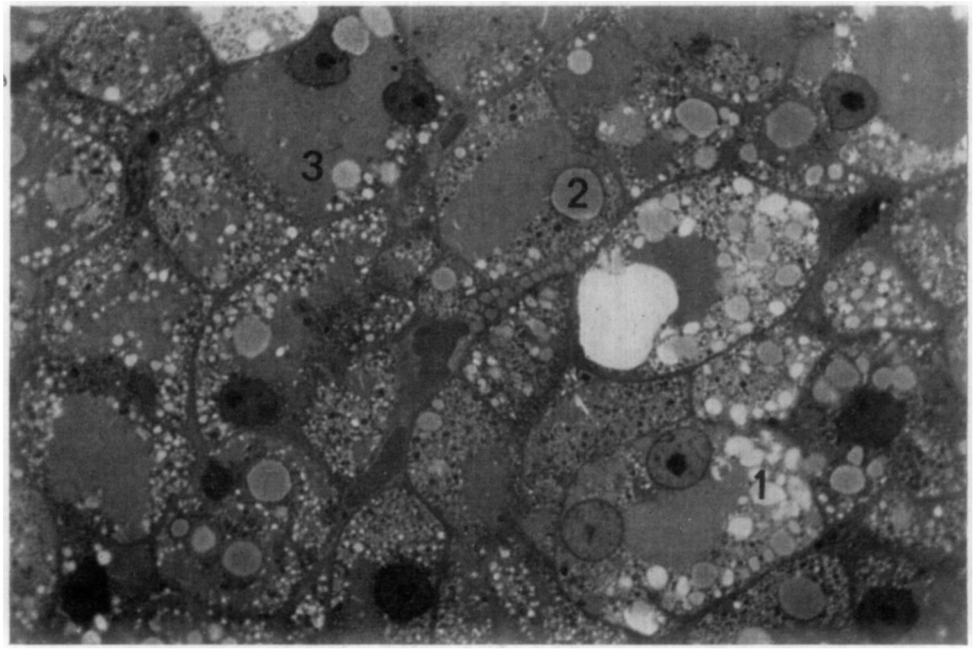

FIG. 6. Severely damaged hepatocytes of the same 2,4,5,2′,4′,5′-hexachlorobiphenyl-treated animal as shown in Figs. 1 and 2 showing hydropic changes (1), lipid droplets (2), and focal cytoplasmic hyalin degeneration (3). Note also the nuclear alterations such as the irregular outlines of the nuclei and the prominent nucleoli. Toluidine blue. × 940.

(pH 7.2). Block staining was carried out with uranyl acetate. The tissue was then dehydrated rapidly in ethanol and embedded in Dow epoxy resin (DER®) (Lockwood, 1964). Sections were cut with glass knives on a Reichert ultramicrotome. Thin (0.5–1.0 μ) sections for orientation and for light microscopic examination were stained with toluidine blue. Ultrathin sections poststained with lead citrate were examined by electron microscopy (Philips EM 100®) at 40 kV.

The PCB concentrations in the livers were determined by gas chromatography, as described earlier (Vos, *et al.*, 1971). The significance of difference between treated and control groups was determined on a one-tail significance level, using the Wilcoxon test for two unrelated samples (van der Waerden, 1957).

RESULTS

After 3 days of PCB treatment, the skin of rabbits in the Aroclor® group showed some redness. After 6 days, definite redness and some thickening of the skin was noted in this group. After 2 wk of treatment, hyperkeratosis and formation of transverse wrinkles were seen. At this time, some redness and slight hyperkeratosis, together with the presence of hexachlorobiphenyl crystals on the skin, was seen in the 2,4,5,2′,4′,5′-hexachlorobiphenyl treated group, although a slight hyperkeratosis was present in the

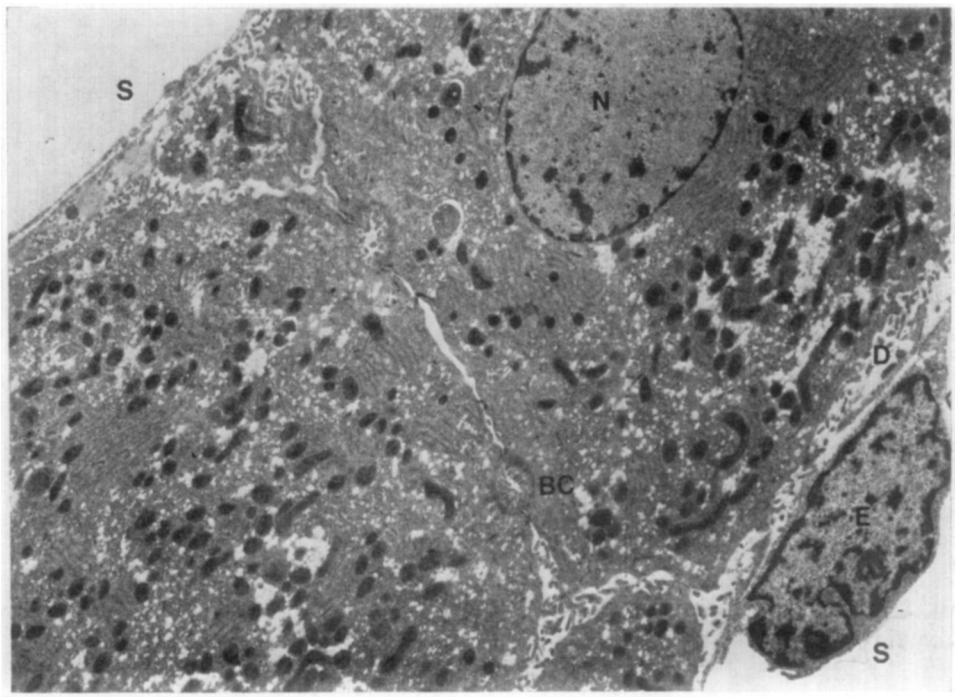

FIG. 7. Control liver with hepatocytes showing diffusely scattered mitochondria and network of the endoplasmic reticula of rough and smooth types. The clear areas may represent negative images of glycogen. The intercellular space between the bile canaliculi may be an artifact. Nucleus (N); bile canaliculus (BC); space of Disse (D); endothelial cell (E); and sinusoid (S). Uranyl acetate and lead citrate. × 4300.

control (isopropanol) group. Differences in the regrowth of hair between the two test groups were also noted. Regrowth was most reduced in the Aroclor® group and less in the hexachlorobiphenyl treated animals. During the course of the experiment, the lesions in the Aroclor group were clearly progressive, while the lesions in the hexachlorobiphenyl group increased rather slowly.

All animals, except 1 rabbit in the hexachlorobiphenyl group, showed a weight gain during the experimental period of 4 wk. Mean weight gain in the hexachlorobiphenyl animals was 142 g (ranging from −215 to 365 g), in the Aroclor® group 325 g (from 70 to 640) and in the control group 491 g (from 430 to 550).

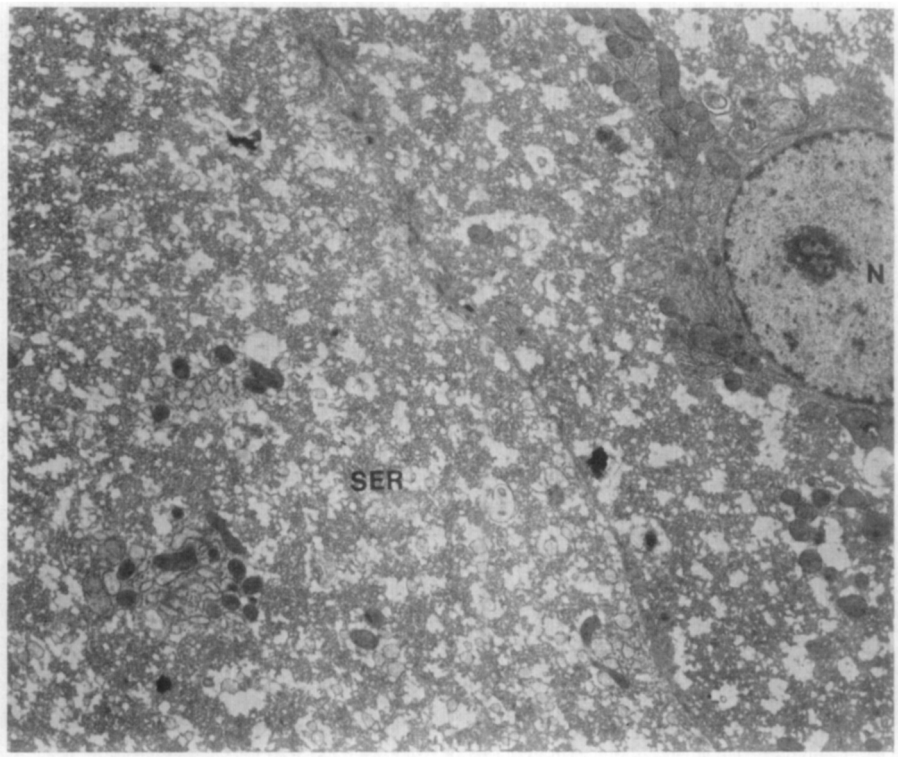

FIG. 8. Hepatocytes of the same liver of an Aroclor® treated rabbit as shown in Figs. 3 and 5a, showing foamy cytoplasm. The cytoplasm is mostly occupied by smooth endoplasmic reticulum (SER). Some perinuclear shift of mitochondria and RER can also be seen. N, nucleus. Uranyl acetate and lead citrate. × 4300.

Coproporphyrin levels in the feces, collected at necropsy from the cecum of individual animals, were significantly increased in the hexachlorobiphenyl as well as in the Aroclor® treated rabbits (Table 1). Protoporphyrin excretion was also increased, though not significantly. Macroscopically, fluorescence was found in the bile of 2 rabbits of both experimental groups. One animal of the Aroclor® group showed also fluorescence of liver and bile. Examination of cryostat sections of liver, kidney, and small intestine in the fluorescence microscope showed fluorescence in the livers of 7 out ot 8 PCB treated animals. This fluorescence was mostly located in the bile duct and sometimes diffusely

in periportally located hepatocytes. Some fluorescence was also noted in epithelial cells of the small intestine in 4 PCB treated animals. Severe abdominal edema was seen in an Aroclor® treated rabbit that showed also the highest liver content (317 ppm) and severe liver damage (Table 2).

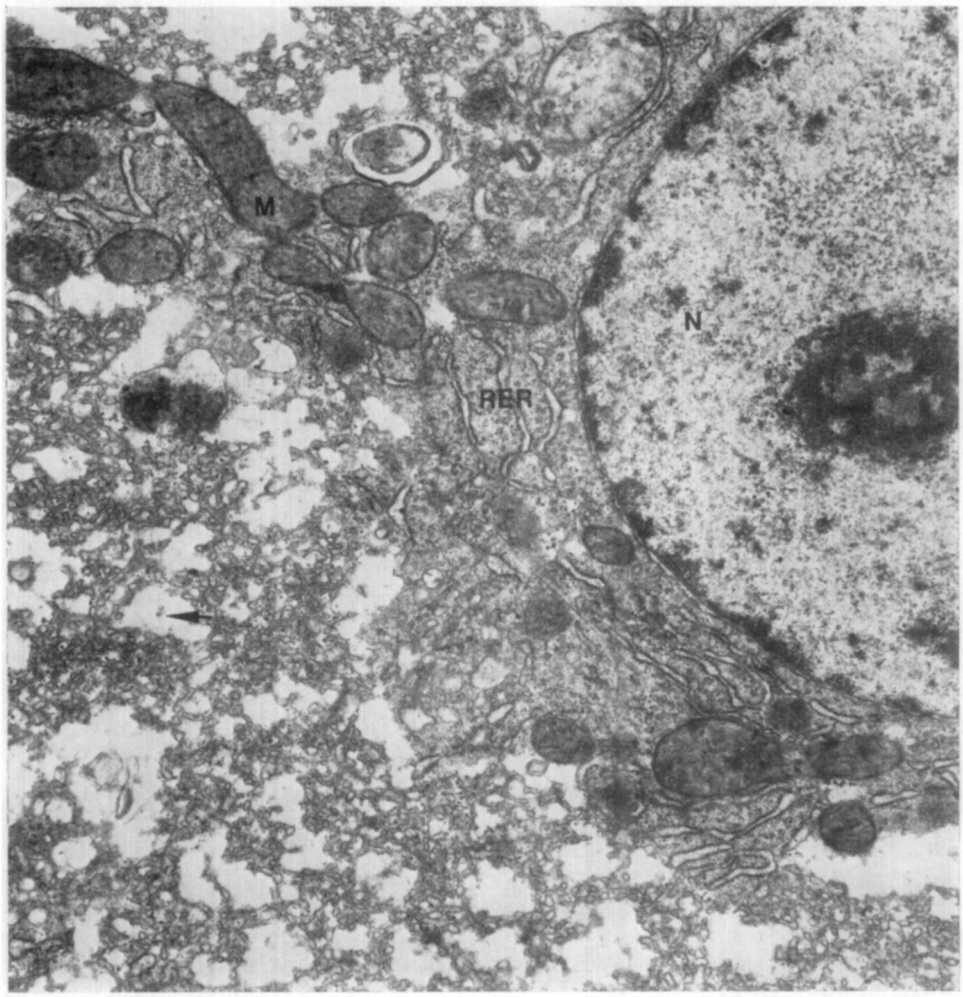

FIG. 9. Higher magnification of a part of Fig. 8. The proliferation of SER consists of vesicular and tubular membranes. Clear areas of the cytoplasm, associated with the SER, most likely represent negative images of glycogen (arrow). Note the dilatation and degranulation of the rough endoplasmic reticulum (RER). M, mitochondria; N, nucleus. Uranyl acetate and lead citrate. × 13,300.

No significant differences were found in absolute and relative organ weights, except for the liver, and in hematological findings, except for the serum transaminase levels. These values, together with the PCB concentrations in the livers and the comparative estimate of liver damage are given in Table 2. The SGPT and SGOT values were significantly increased in the hexachlorobiphenyl group. An increase was also noted in the Aroclor® group. Mean liver content of PCB was the same in the experimental

groups. The increase and severity of liver damage in the Aroclor® group agreed with the increase in liver residue.

At examination of skin sections of the Aroclor® group, hyperplasia and hyperkeratosis of the epidermal and follicular epithelium were found. The lesions were not yet so pronounced as in the prior study (Vos and Beems, 1971). Hexachlorobiphenyl induced skin damage, especially hyperplasia in the epidermal and follicular epithelium as well as follicular plugging, was clearly less when compared with the Aroclor® treated rabbits. A slight hyperkeratosis was found in some control animals. This could be due to the treatment with isopropanol (6 ml/day); it was not found in the prior study (Vos and Beems, 1971) using a lower dose (1 ml/day).

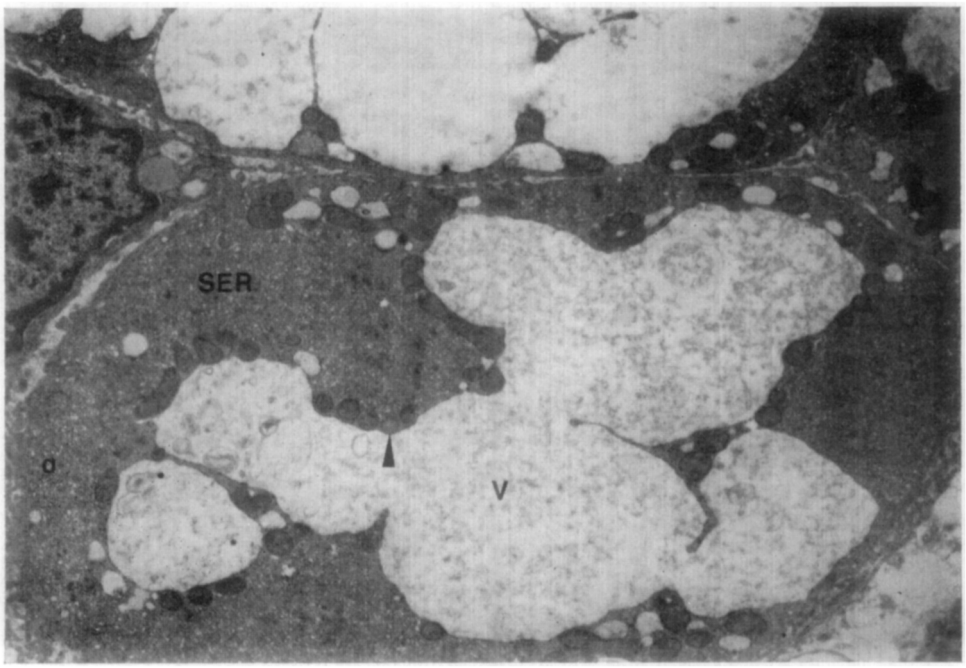

FIG. 10. Hepatocytes of an Aroclor® treated animal. Similar hydropic cells as seen in Fig. 5b, showing large confluent vacuoles with flocculent material within them (V). The hyalinized cytoplasm of hydropic hepatocytes shown in Fig. 5b consists of hypertrophied, densely packed SER. Note the arrangement of mitochondria around the vacuoles. (arrow). Uranyl acetate and lead citrate. × 5500.

When the lymphoid system was examined, no outstanding differences were seen in spleen, appendix and lymph nodes between control and treated groups. Some atrophy of the cortex, together with less pyroninophilic cells in the marrow, were found in the thymuses of rabbits in the PCB treated groups. The atrophy agrees with the lower absolute and relative (about 18%) thymus weights in these groups. No kidney damage was found, except for the presence of polyploid tubular epithelial cells in a hexachlorobiphenyl treated rabbit.

Pathologic changes in the livers of the treated animals were, in general, the same as described in a previous study (Vos and Beems, 1971). These findings included centrolobular degeneration and liver cell atrophy, focal hyalin degeneration of the cytoplasm

of hepatocytes, enlarged nuclei and loss of glycogen. As shown in Table 2, liver damage by hexachlorobiphenyl was somewhat more severe when compared with the Aroclor® induced lesions. However, in the present study, areas of hydropic, degenerated and necrotic cells dominated, often in the subcapsular region (Figs. 1 and 2), forming bands

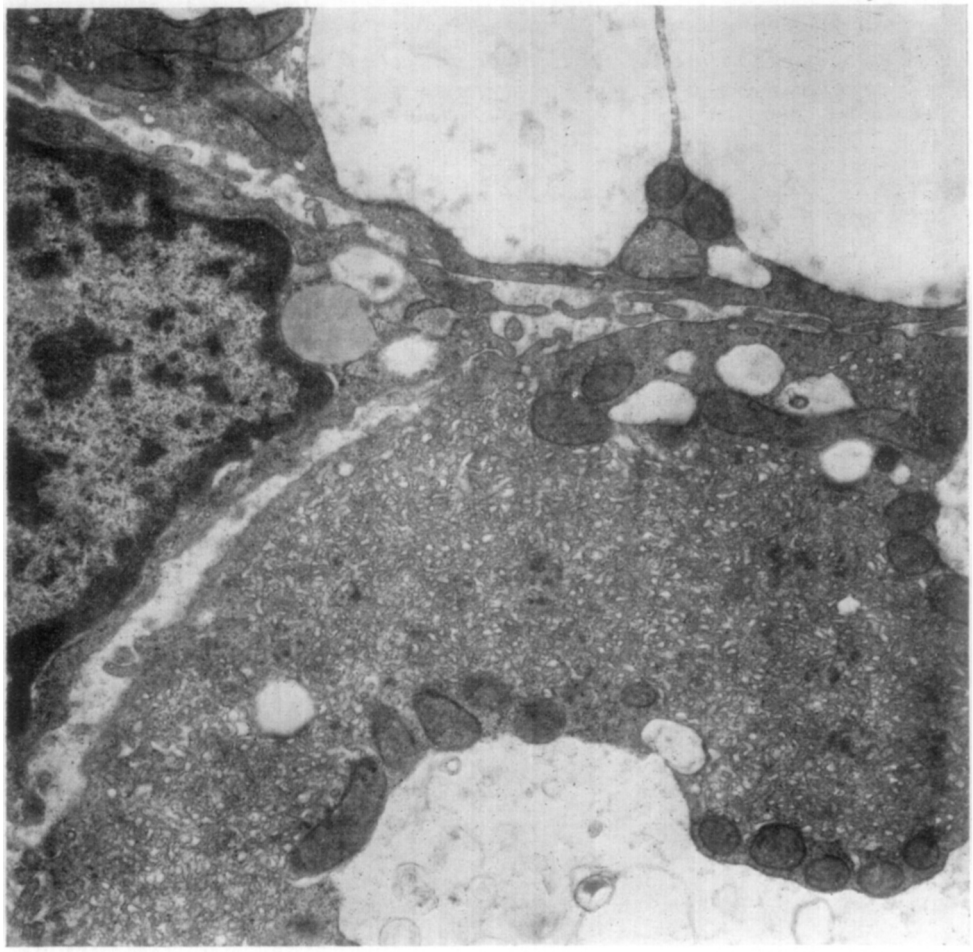

FIG. 11. Higher magnification of a part of Fig. 10 reveals the tubular structure of the densely hypertrophied smooth endoplasmic reticulum. Uranyl acetate and lead citrate. × 13,300.

of necrotic and degenerated cells, accompanied sometimes by proliferation of mesenchymal cells. Necrosis was usually accompanied by hemorrhage. Loss of glycogen was not limited to the degenerated, hydropic or necrotic areas, but was also seen in foci showing no alterations in hematoxylin-eosin sections. In the rest of the liver the glycogen content was also reduced when compared with the controls. The centrolobular damage was often zonal. This distribution can be explained by the concept of structural and functional hepatic unit (Rappaport *et al.*, 1954). The presence of Perls' positive material

and ceroid pigment in Kupffer and parenchymal cells (characterized by a yellow-brownish fluorescence) was evidently less than in the preceding study and was limited to some necrotic areas.

In both treated groups, numerous hepatocytes were seen with light staining cytoplasm. In these cells, the cell membrane and the nuclear membrane were emphasized by the presence of basophilic material (Fig. 3). When plastic embedded liver tissue was examined by light microscopy, these alterations could be observed more clearly (Figs. 4 and 5a). This was the general trend in the PCB treated animals. In electron microscopy, the most striking feature of these cells was the proliferation of smooth

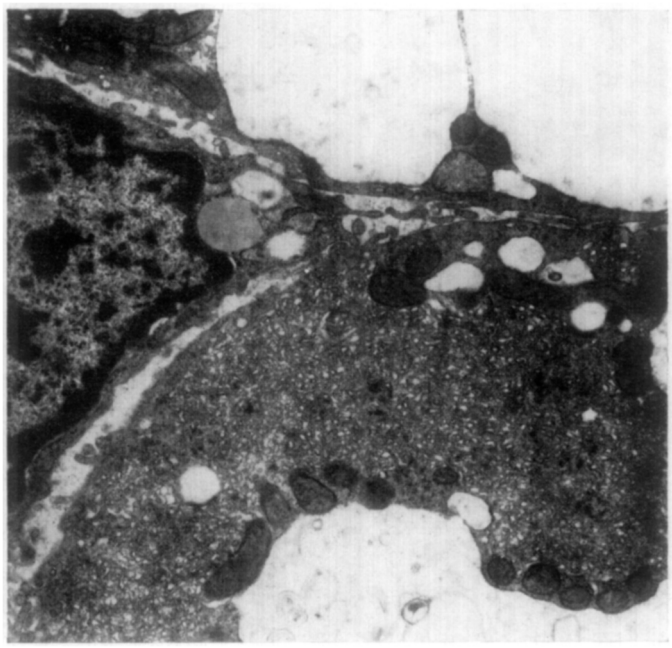

FIG. 12. Severely damaged hepatocyte of the same 2,4,5,2′,4′,5′-hexachlorobiphenyl treated animal as shown in Fig. 6. Photograph shows a cluster of tubular smooth endoplasmic reticulum (in light microscopy visible as focal hyaline degeneration), forming a dense, closely packed agglomeration (SER). Note also the lipid droplets (L), and hydropic swelling, as shown by a great number of small and large vesicles (V). Uranyl acetate and lead citrate. × 3000.

surfaced membranes of the endoplasmic reticulum (SER). As a result of the proliferation of SER, a perinuclear and peripheral displacement of mitochondria and rough surfaced membranes of the endoplasmic reticulum (RER) was apparent (Figs. 7 and 8). The proliferation of SER could well explain the accentuated linings of cell and nucleus in light microscopy and the increase in liver weight (Table 2) in the PCB treated groups. The RER showed some dilatation and degranulation (Fig. 9).

In addition to the proliferative changes of the SER, degenerative changes were also noted. Light microscopy showed the presence of hydropic cells (Fig. 2). Examination of plastic-embedded material under the light microscope showed some hydropic cells which consisted of large vacuoles separated from a homogeneous cytoplasm, inter-

preted as focal cytoplasmic degeneration. At the rim of the homogeneous cytoplasm cell organelles were visible (Fig. 5b). The focal hyalinized cytoplasm (Fig. 5b) was found by electron microscopy to consist of tightly packed tubules of proliferated SER (Figs. 10 and 11). The condensed SER was separated from large vacuoles, sometimes with flocculent material in it. The mitochondria were arranged around the vacuoles. Besides the hyalinized cytoplasm, lipid droplets and degenerative changes of the nucleus were found in the more severely affected areas (Figs. 6 and 12). The nuclear changes included irregular outlines of the nuclei and karyopycnosis. Myelin figures were found rarely in the cytoplasm. In the present study, no differences were noted in the hepatotoxic action between the polychlorinated biphenyl mixture and 2,4,5,2′,4′,5′-hexachlorobiphenyl.

DISCUSSION

From the observed acnelike lesions, both from the PCB mixture and 2,4,5,2′,4′,5′-hexachlorobiphenyl, and assuming that the hexachlorobiphenyl is free from contamination with chlorinated dibenzofuran, it can be concluded that this particular compound of the mixture PCB has a slight acnegenic action of itself. Considering the already mentioned dermal toxicity study, it is evident that the major acnegenic action of crude PCB mixtures comes from chlorinated dibenzofurans. In Table 1 it is shown that 2,4,5,2′,4′,5′-hexachlorobiphenyl is even more porphyrogenic than the PCB mixture. Thus it is probable that hepatic porphyria comes only from PCB itself.

In contrast with the prior dermal toxicity study, only slight effects were noted from PCB on the lymphatic tissue. This difference is explained by the longer experimental period in the former study (38 days) which resulted in a less healthy condition of the experimental animals. Stress (e.g., release of glucocorticoids) must be considered responsible for the major effects on the lymphatic tissue. However, using the sensitive fluorescent antibody technique in tetanus toxoid stimulated guinea pigs, immunosuppression was found (Vos and de Roij, 1972).

Liver damage was essentially the same after treatment with both the Aroclor® mixture and 2,4,5,2′,4′,5′-hexachlorobiphenyl. Liver damage caused by commercial PCB mixtures was due predominantly to the contaminants. This conclusion is based on the differences in liver toxicity between three PCB preparations (Vos and Koeman, 1970; Vos and Beems, 1971). The probable contribution of chlorinated dibenzofurans and uncontaminated PCB in the toxicity of crude PCB mixtures is summarized in Table 3.

TABLE 3

PROBABLE CONTRIBUTION OF CHLORINATED DIBENZOFURAN AND PURE POLYCHLORINATED BIPHENYL IN THE TOXICITY OF TECHNICAL PCB PREPARATIONS

	Chloracne	Edema formation	Liver damage	Hepatic porphyria
Chlorinated dibenzofuran	++	++	++	–
Polychlorinated biphenyl	+	–	+	++

Focal cytoplasmic hyalin degeneration, as well as ceroid pigment deposits in the hepatic cells, was more pronounced in the former dermal toxicity study (Vos and Beems, 1971), probably due to the longer experimental period. Hyalin inclusions were previously described after semichronic exposure to PCB in rat liver (Bennett *et al.*, 1938; Miller, 1944) and in mouse liver (Nishizumi, 1970). However, in the rabbit, the most important liver lesions described varied from fatty degeneration to marked hepatocytic degeneration and necrosis (von Wedel *et al.*, 1943; Miller, 1944). In this study, the presence of cells with proliferated SER, resulting in a perinuclear and peripheral shift of RER and mitochondria, was more frequently seen (Fig. 8). This proliferation, also found by Nishizumi (1970) in mouse and monkey liver, must be considered the structural indication for enhanced metabolism of foreign lipophilic compounds. Thus, the activity of aniline hydroxylase and aminopyrine *N*-demethylase (determined in vitro) was increased in the rabbit after administration of Aroclor® 1254 (Villeneuve *et al.*, 1971). A decrease in sleeping time after treatment with hexobarbital and enhanced in vitro rates of aniline hydroxylation and *p*-nitroanisole demethylation were demonstrated by Street *et al.*, (1969). They also found an increase of these effects with increasing chlorine content of different PCB preparations. Despite this increase in drug enzyme activity caused by the higher chlorinated mixtures, the lower chlorinated compounds are very probably metabolized to a greater extent (Grant *et al.*, 1971). On the other hand, the presence of focal cytoplasmic hyalin degeneration without glycogen (Figs. 5b and 6), in electron microscopy seen as densely packed agglomerations of SER, very probably represents hypertrophic, hypoactive SER. Hutterer *et al.* (1968) found reduced activity of microsomal oxidases (e.g. drug enzymes), while SER remained hypertrophic in livers of rats treated with high doses of dieldrin. As seen under the electron microscope, hypertrophic, hypoactive SER was recognized as tight clusters of tubular membranes, which could indicate a transition from adaptation to injury (Hutterer *et al.*, 1968, 1969). Liver damage was also suggested by elevated transaminase levels (Table 2). Myelin figures were seldom found in this study. Perhaps this depends on the animal species, since Nishizumi (1970) described myelin figures in mouse liver after PCB feeding, but not in monkey liver. The occurrence of SER proliferation and concentric membrane arrays is found in the livers of rats fed a chlorinated biphenyl diet (Norback and Allen, 1970) and toxic fat containing hexachlorodibenzo-*p*-dioxin, a compound related to chlorodibenzofuran (Norback and Allen, 1969). The results from the former study suggested that the concentric arrays of agranular membranes may be structural modifications of the endoplasmic reticulum having an enzymatic function similar to that associated with the SER.

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