RESPONSES OF RATS EXPOSED TO POLYCHLORINATED BIPHENYLS FOR FIFTY-TWO WEEKS I. COMPARISON OF TISSUE LEVELS OF PCB AND BIOLOGICAL CHANGES[1]

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Male Sprague-Dawley rats were fed a diet containing mixtures of polychlorinated biphenyls (PCB) isomers (Aroclor 1248, 1254 and 1262) at a concentration of 100 ppm in the diet for 52 weeks. During the subsequent 13 weeks the rats were placed on a control diet. Throughout the course of the experiment the animals ate well, were healthy in appearance and gained weight as rapidly as the control animals. Their hemograms were normal. In spite of the gross normalcy of these animals throughout the 52-week experimental period, clinical and morphologic examinations revealed distinct alterations. There was a decided increase in their total serum lipids and cholesterol and a transient increase in triglycerides accompanied by distinct morphological changes in the liver. Generalized liver hypertrophy and focal areas of hepatocellular degeneration were followed by a wide spectrum of repair processes. The tissue levels of PCB were greater in the animals receiving the higher chlorine mixtures. High levels persisted in these tissues even after the PCBs had been discontinued.

The polychlorinated biphenyls (PCBs) have become of worldwide health significance due to their persistence in the environment and the injurious effects on animals that result from exposure to these compounds. One of the primary concerns of scientists who are evaluating this problem is the effect of long term, low level exposure of the human population to these compounds as might occur following repeated consumption of contaminated foods. There are only a limited number of experiments that have attempted to clarify this question. Keplinger *et al.* (1971) fed rats PCB mixtures at levels of 100 ppm for two years and observed no gross injurious effects. The rats did show liver hypertrophy and fatty infiltration of the hepatic cells; however, liver function tests were normal. Kimbrough *et al.* (1972) fed levels ranging from 20 to 1,000 ppm for eight months producing liver hypertrophy at lower levels and adenofibrosis at higher dietary concentrations. Electron microscopic observations by these workers revealed lipid vacuoles surrounded by concentric membrane configurations at the higher dietary levels (100 to 1,000 ppm).

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Allen and Abrahamson (1973) fed rats 1,000 ppm of various mixtures of PCBs for six weeks. At this level the weight of the experimental animals was reduced, liver hypertrophy was marked, and widespread focal necrosis of the liver developed. Initially, in these rats there was a decided stimulation of hepatic microsomal enzyme activity followed by a rapid decline that was associated with degenerative hepatic changes. In order to further clarify the chronic toxic effects of PCBs, rats were fed mixtures of PCBs measuring 48%, 54%, and 62% chlorine by weight at a level that would allow normal growth rate and livability for 52 weeks. A portion of the animals was removed from the diet and evaluated for an additional 13 weeks in order to determine the residual effects of the PCBs. Distinct biochemical and morphological changes occurred, particularly in the liver of animals exposed to the PCB mixtures. These injurious effects did not abate after the PCBs were eliminated from the diet for 13 weeks.

Materials and Methods

Ninety-six male Sprague-Dawley rats weighing approximately 100 g (four weeks of age), were divided into four groups, three of which were fed a commercially prepared diet[2] to which 100 ppm of Aroclor 1248 (48% chlorine), 1254 (54% chlorine) or 1262 (62% chlorine)[3] were added. The fourth group was given an unmodified diet and served as the control group. After having been on these diets for 13, 26, 39 and 52 weeks, four rats from each group were weighed and sacrificed by decapitation. After 52 weeks, surviving animals were placed on a normal diet for an additional 13 weeks. Blood collected at the time of sacrifice was analyzed for total white cell count, hemoglobin, hematocrit, differential white count (Diggs 1971), and total serum protein (Werchselbaum 1946). Spectrophotometric determinations including serum lipid (Chabrol and Charonnat 1937), serum cholesterol (Schoenheimer and Sperry 1943) and serum triglycerides (Fletcher 1968) were also conducted. At necropsy all organs were examined and weighed. Small sections of the tissues were placed in 10% neutral formalin for 24 hr, embedded in paraffin, sectioned at five microns and stained with hematoxylin and eosin. For electron microscopy, the hepatic tissues were cut into small cubes, fixed in osmium tetroxide buffered with uranyl acetate (Caulfield 1957) for 1.5 hr and subsequently dehydrated through a graded series of ethanol and embedded in an epoxy resin mixture (Mollenhauer 1964). Sections of the tissues were cut on an ultramicrotome, placed on uncoated copper grids, stained with uranyl acetate and examined with an electron microscope.

Portions of the liver, fat, brain, and kidney were obtained at the time of sacrifice and analyzed for their PCB content. Liver, fat, and kidney were homogenized in hexane (500 mg:20 ml) and the brain in acetone (500 mg:20 ml) (Jennings 1968). The tissue extracts were subsequently evaporated under dry nitrogen in a 40°C water bath. Clean-up of the extracted material prior to analysis was carried out in a

[2]Ralston Purina Co., St. Louis, Missouri.
[3]Monsanto Company, St. Louis, Missouri.

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disposable pipet microcolumn (Curley *et al.* 1971) containing silica gel 60[4] (0.05-0.2 mm) by elution with a 1:1 benzene-hexane mixture. Levels of PCB were quantitated in a gas-liquid chromatograph[5] employing a ^{63}Ni electron capture detector. The pyrex glass column packed with Gaschrom Q (80–100 mesh) coated with 2% Apiezon L was operated at 220°C with argon-methane (95%-5%) as the carrier gas at a flow rate of 40 cc per min.

Results

Throughout the course of the experiment, the PCB-fed animals ate well, maintained a healthy appearance and gained weight as rapidly as did the controls (Table I). When the blood of the control and experimental animals was compared, there were no appreciable differences in the levels of hemoglobin, hematocrit, and white blood cells. The total protein and albumin to globulin ratios were also not affected by the ingestion of PCBs. There were, however, distinct modifications in the serum lipids. The total serum lipids were 120% that of the controls by the 13th week (control value 231.3 ± 64.9 mg%) and 160% by the 39th week (control value 235.0 ± 30.4 mg%) (Figure 1). Thereafter the levels declined to approximately 140% in the serum of the animals given Aroclor 1248 and 1262. However, the serum lipids of the Aroclor 1254 animals showed a decided increase during the terminal 13 weeks on the experimental diet. At 52 weeks, the mean serum lipid value of these animals was approximately 280% that of the controls. The increase in serum cholesterol (control values 57.5 ± 1.7 mg% for 13 weeks; 65.7 ± 4.0 mg% for 39 weeks) followed essentially the same course as did the total lipids, with the exception that no precipitous increase was recorded at 52 weeks in the animals given Aroclor 1254 (Figure 2). The serum triglyceride levels of the experimental animals were not altered appreciably in the groups receiving Aroclor 1248 and 1262 (Figure

Table I. *Body weights of rats fed 100 ppm of polychlorinated biphenyls for 52 weeks*

Weeks on trial	Control (g)	Aroclor 1248 (g)	Aroclor 1254 (g)	Aroclor 1262 (g)
0	137 ± 10[a]	129 ± 9	128 ± 8	131 ± 6
6	347 ± 32	310 ± 27	325 ± 18	342 ± 32
13	406 ± 23	386 ± 38	382 ± 20	412 ± 45
26	397 ± 39	367 ± 17	399 ± 19	390 ± 18
39	420 ± 53	415 ± 22	412 ± 31	430 ± 28
52	481 ± 50	491 ± 32	512 ± 50	469 ± 57
65	565 ± 61	490 ± 35	497 ± 33	563 ± 43

[a] Value indicates mean ± standard deviation.

[4] EM Laboratories Inc., 500 Exec. Blvd., Elmsford, N.Y. 10523

[5] Hewlett-Packard/Avondale Division, Route 41 and Starr Road, Avondale, Pennsylvania 19311

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390 ± 18
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563 ± 43

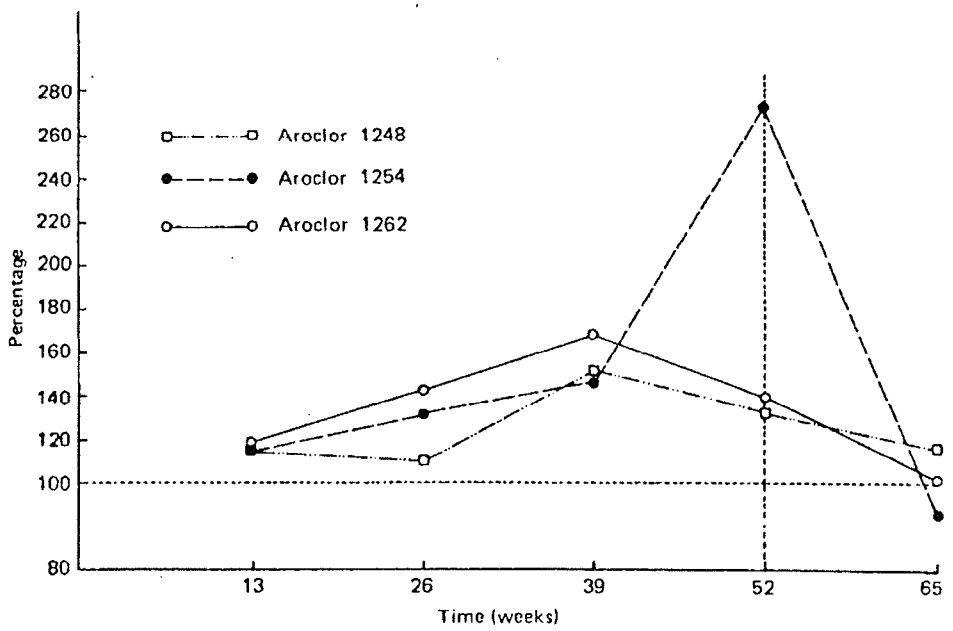


Fig. 1. Total serum lipids of rats fed polychlorinated biphenyls for fifty two weeks. Data expressed as percentage of control level. Rats taken off PCB diets at 52 weeks.

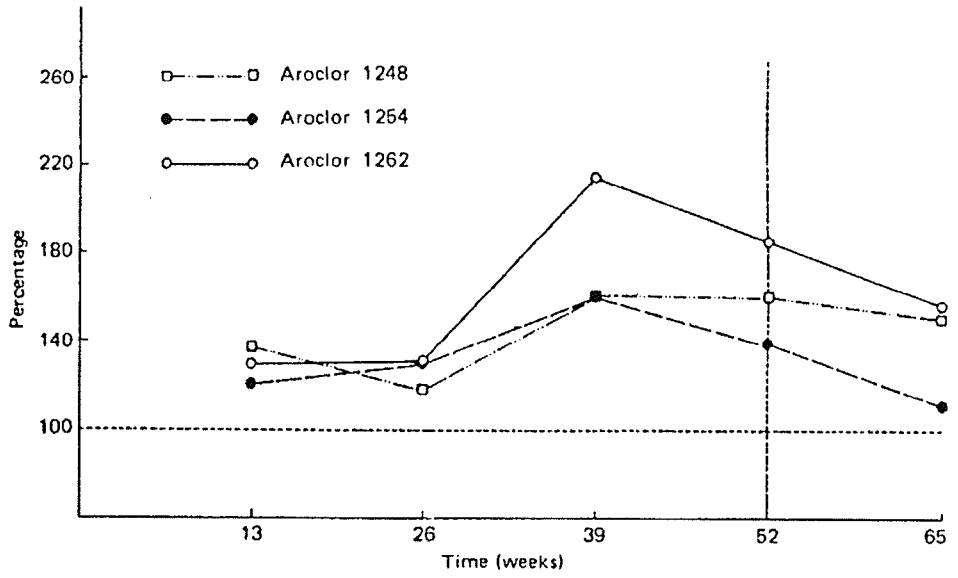


Fig. 2. Serum cholesterol levels of rats fed polychlorinated biphenyls for fifty two weeks. Data expressed as percentages of control levels. Rats taken off PCB diets at 52 weeks.

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3). However, the triglyceride levels in the serum of those rates given Aroclor 1254 increased markedly during the terminal 13 weeks on the experimental diet, reaching a concentration in excess of 500% that of the controls.

After removal of the experimental diet for 13 weeks, all of the experimental animals showed a decided decrease in the serum triglycerides, ranging from 20 to 40% below the control levels after the 13-week recovery period, while changes in total lipids and serum cholesterol were less worthy of comparison.

There were no appreciable differences in the organ weights of the experimental and control animals (lungs, heart, kidney, spleen, thymus, testes, and brain) that were sacrificed at 13, 26, 39, 52, and 65 weeks, with the exception of the liver. In all the examination periods, the livers were approximately one and one-half times as large in the experimental animals (Table II). When the above tissues were examined light and electron microscopically there were no major pathological alterations except for the focal lesions that were present in the liver. The latter organ had widely dispersed small focal areas of degeneration and necrosis. In the livers of the experimental animals sacrificed at 26, 39, and 52 weeks, degenerative changes assumed several distinct morphological patterns. Some were characterized by decided ballooning and rarification of the cytoplasm of the hepatic cells. In many instances portions of the plasma membranes of these cells had disappeared, thereby forming cystic spaces of variable size (Figure 4). Frequently these spaces became lined with endothelial cells and their lumen contained numerous blood cells (peliosis hepatis, not shown). Hepatocytes not within the immediate area appeared relatively

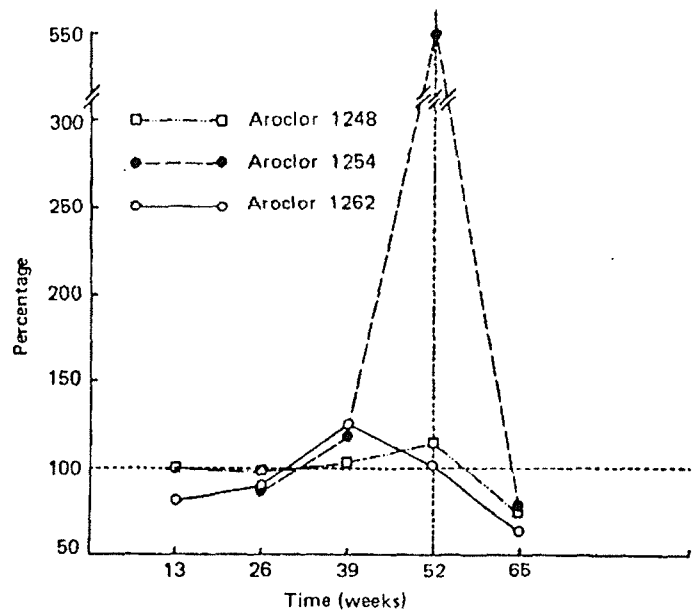


Fig. 3. Serum triglyceride levels of rats fed polychlorinated biphenyls for fifty two weeks. Data expressed as percentage of control level. Rats taken off PCB diets at 52 weeks.

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normal. In a second type of hepatic lesion there was shrinkage and hyalinization of the cytoplasm of a well circumscribed collection of hepatic cells. In most instances the nuclei of these cells had disappeared and the entire area had taken on the

Table II. *Liver weights of rats fed 100 ppm of polychlorinated biphenyls expressed as percentage of body weight*

Weeks on trial	Control (g)	Aroclor 1248 (g)	Aroclor 1254 (g)	Aroclor 1262 (g)
13	1.64 ± 0.10[a]	2.49 ± 0.26	2.83 ± 0.26	2.76 ± 0.21
26	1.87 ± 0.13	2.56 ± 0.14	2.78 ± 0.31	2.88 ± 0.47
39	1.74 ± 0.05	2.72 ± 0.28	3.13 ± 0.44	2.98 ± 0.41
52	1.66 ± 0.37	2.81 ± 0.24	2.60 ± 0.27	3.23 ± 0.60
65	2.72 ± 0.30	3.78 ± 0.55	3.52 ± 0.46	3.99 ± 0.62

[a] Value indicates mean ± standard deviation.



Fig. 4. Large widely dispersed cystic spaces developed in the liver of rats fed PCB. Note the remnants of the cell membrane and pyknotic nuclei within the space and the compressed hepatocytes that form its boundary. Large hepatocytes having abundant granular cytoplasm and numerous plump Kupffer cells surround the lesion. Tissue from rat given Aroclor 1248 for 39 weeks. Hematoxylin and eosin stain; X 420.

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appearance of a large structureless eosinophilic mass (Figure 5). Frequently these areas were surrounded and infiltrated by inflammatory cells and macrophages.

A third type of focal hepatic lesion was characterized by the presence of well circumscribed collections of closely packed enlarged hepatic cells with faintly staining cytoplasm which assumed a lacy appearance. Sinusoidal spaces were absent; however, abundant reticuloendothelial cells were present between the hepatocytes. Large lipid-laden cells also were randomly distributed throughout the area. The hepatocytes surrounding these areas were normal (Figure 6).

In a fourth type of focal hepatic degeneration the hepatocytes had disappeared and had been replaced primarily by reticuloendothelial cells interspersed with lymphocytes. Here, too, the lesions were small and the surrounding hepatocytes unaffected (Figure 7).

Particularly obvious as the experiment progressed was the variation in the morphological features of the cells in the centrilobular and periportal areas of the

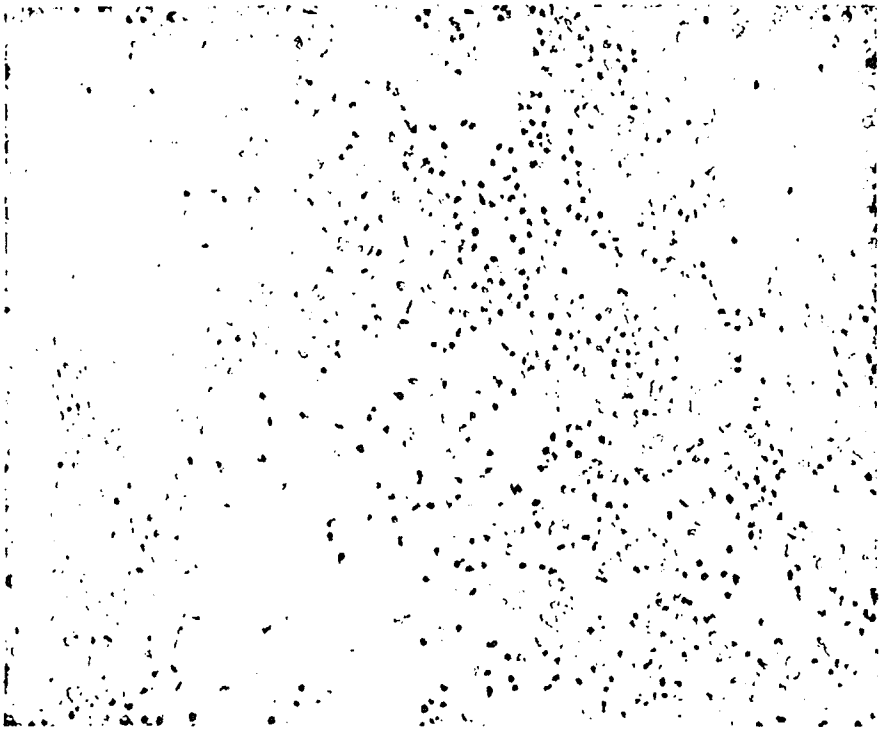


Fig. 5. Widely dispersed throughout the liver of PCB-fed rats were focal areas of necrosis that were characterized by clumps of closely packed eosinophilic cells devoid of nuclei and surrounded by polymorphonuclear leukocytes. Note the normal appearance of the hepatocytes that surround the lesion. Tissue from rat fed Aroclor 1254 for 39 weeks. Hematoxylin and eosin stain; X 167.

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liver. Those hepatocytes occupying the centrilobular and inner half of the midzonal area were large, closely packed and had abundant lightly staining cytoplasm. Obvious sinusoidal spaces were in most instances obliterated. However, the cells that comprised the outer midzonal and periportal areas were much smaller, contained less cytoplasm and the sinusoidal spaces that separated the liver cords were readily apparent (Figure 8).

The ultrastructural features of these focal hepatic lesions further clarified the changes that occurred within the affected hepatocytes. The large lucent hepatocytes that comprised one of the groups of altered cells contained abundant, opaque cytoplasm. The organelles were widely separated and swollen. Those nuclei that remained intact showed an irregularly distributed clumping of the chromatin. The plasma membranes of many of these cells had ruptured and the organelles and nuclear fragments were in the extracellular spaces or had been engulfed by Kupffer cells. In the more severely affected areas, a majority of the hepatocytes had disappeared and were replaced by cystic spaces filled with proteinaceous appearing material.

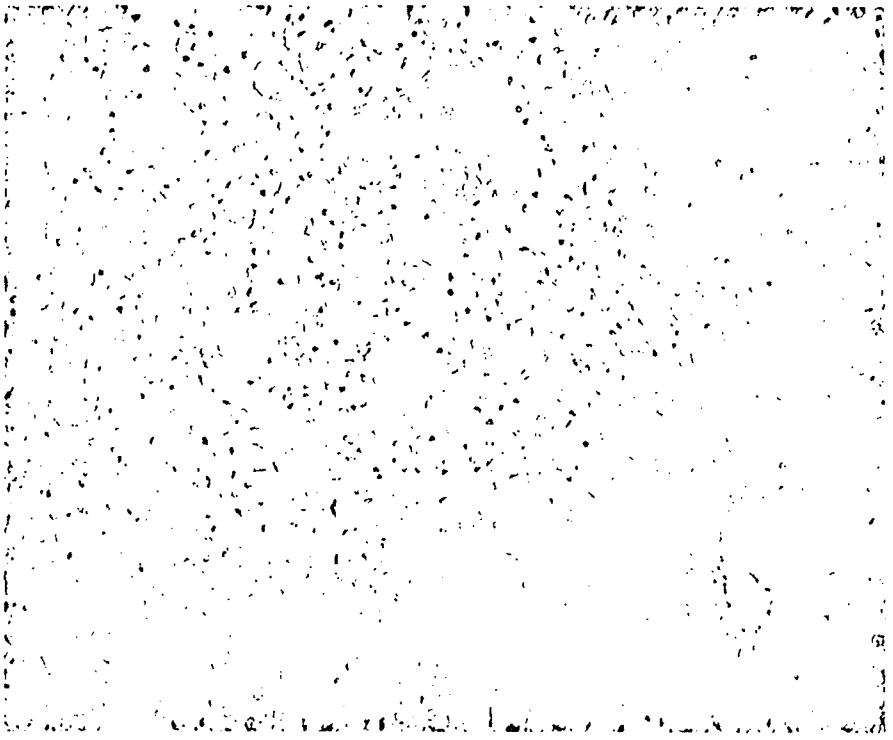


Fig. 6. In some well circumscribed areas within the livers of the PCB-fed rats there was a loss of the hepatic architectural pattern. The hepatic cells were large and their cytoplasm had a lacy appearance. Distinct sinusoids and cordlike arrangement of the hepatic cells were absent. Abundant Kupffer cells were dispersed among these closely associated hepatic cells. Hematoxylin and eosin stain; X 167.

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In the hyalinized areas, the individual hepatocytes appeared dehydrated with the organelles being closely packed in an extremely dense cytoplasmic matrix. Due to the loss of fluid, the shrunken dense mitochondria most frequently assumed an undulating profile. The cisternae of the endoplasmic reticulum and Golgi complex were compressed and devoid of proteinaceous material. The nuclear membranes of these cells were irregular and the chromatin was extremely dense. Cell fragments were observed in the sinusoids within the cytoplasm of the reticuloendothelial cells.

Except for moderate enlargement, most of the hepatocytes appeared normal microscopically. Ultrastructurally there was found to be a decided proliferation of the endoplasmic reticulum (ER) in all hepatocytes (Figure 9). The rough ER consisted primarily of lamellar arrays while the smooth ER was comprised of closely associated small vesicles. Continuous with the smooth and rough ER were closely packed concentric arrays of smooth membranes These arrays frequently encircled mitochondria and the moderately numerous lipid droplets. The other cytoplasmic organelles as well as the nuclei of these hepatocytes appeared normal.



Fig. 7. Small foci of hepatic cells were replaced primarily by proliferated reticuloendothelial cells and lymphocytes. Kupffer cells were numerous in the sinusoids adjacent to the proliferative area. Other than containing a moderate number of lipid droplets, the hepatocytes surrounding this lesion are normal. Tissue from a rat receiving Aroclor 1262 for 52 weeks and a control diet for 13 weeks. Hematoxylin and eosin stain; X 420.

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After having been off the experimental diet for 13 weeks, the individual hepatocytes continued to be enlarged. Their cytoplasm contained abundant smooth ER and lipid droplets. Focal areas of degeneration and necrosis that were observed earlier were even more abundant than previously recorded when the animals were on the experimental diet.

When the tissues were analyzed for their PCB content by gas liquid chromatography (GLC) it was determined that there was considerable variation of the tissue deposition of these three mixtures of PCB isomers. Those rats receiving Aroclor 1248 had the lowest concentration of PCB isomers in their tissues throughout the experiment (Table III). Considerably higher levels were present in those rats given Aroclor 1254 and 1262 (Tables IV and V). The PCB level in the adipose tissue of these animals was invariably higher than in the liver, kidneys, or brain. Relatively steady state PCB levels were established in these tissues by the 39th week. During the recovery period there was a decrease in the level of PCB isomers in the tissues of

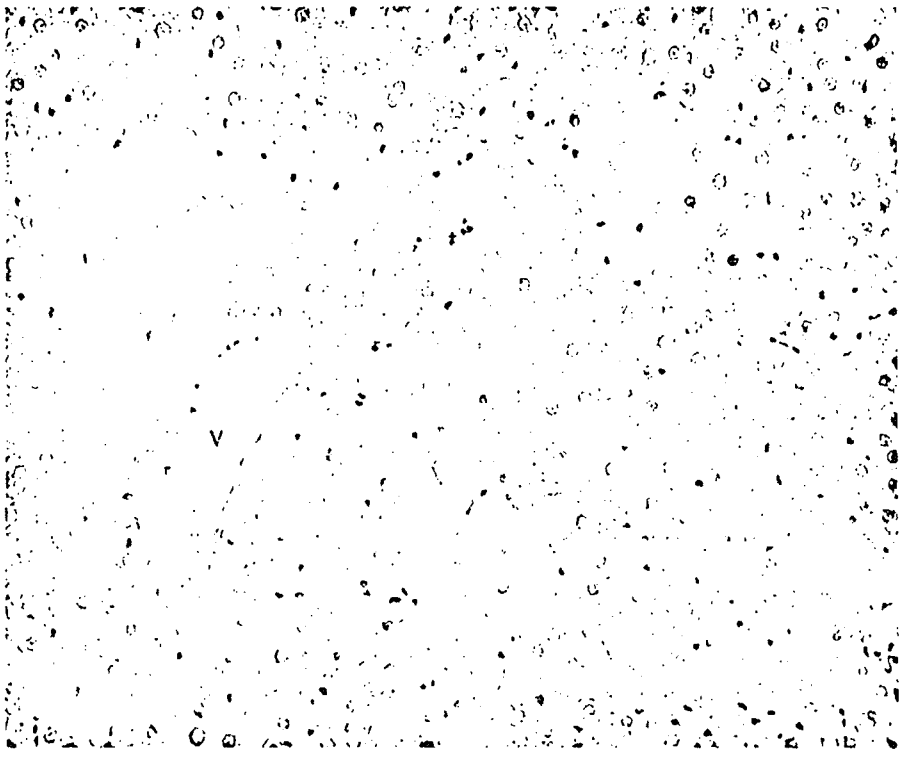


Fig. 8. Variation in the morphological features of hepatocytes in various areas of the liver lobules of rats fed PCB occurred. Note the large hepatocytes containing abundant lightly staining cytoplasm around the central vein (V). Encroachment of the hepatocytes on the sinusoids has markedly reduced their luminal size. Toward the periphery of the lobule the hepatocytes are smaller and the nuclei comprise a greater percentage of the cell profile. Tissue from rat receiving Aroclor 1262 for 52 weeks. Hematoxylin and eosin stain; X 167.

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the animals that had received Aroclor 1248; however, high levels persisted in the tissues of the animals receiving Aroclor 1254 and 1262 (Tables IV and V). As is shown in Figure 10 the PCBs that accumulate in the tissues are primarily those with longer retention times.

Discussion

It is evident from the present study that a thorough morphological and biochemical evaluation of the experimental animals is necessary to consider a given compound innocuous. If one were to consider some of the more routine evaluations, such as growth rate, percentage mortality and hematological studies, the experimental animals of this study would be classified as normal. However, as the evaluations become more detailed, it becomes quite obvious that these animals are not normal. The most interesting clinical data obtained on these animals was the effect upon the serum lipids. Increase in serum lipids occurred early and persisted throughout the period of study.

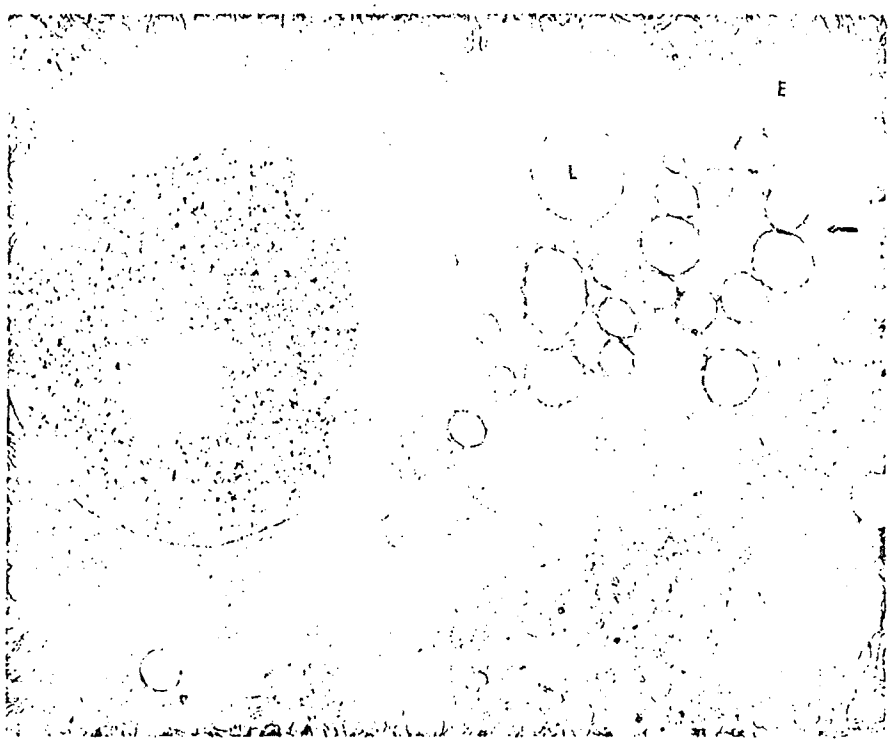


Fig. 9. Proliferation of the smooth ER (E), formation of membranous concentric array (→) and accumulation of lipid droplets (L) occurred in the cytoplasm of hepatic cells taken from rats that consumed PCB. Cells having these features predominated in livers of the PCB-fed rats. Tissue from rat given Aroclor 1254 for 13 weeks. Uranyl acetate stain; X 9,570.

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Table III. *Tissue levels of polychlorinated biphenyls in rats fed 100 ppm of Aroclor 1248 for 52 weeks*

	Time (weeks)				
Tissue	13	26	39	52	65[b]
Fat	228 ± 43[a,c]	262 ± 95	570 ± 192	529 ± 75	192 ± 122
Liver	9 ± 0.8	9 ± 6	19 ± 8	105 ± 23	15 ± 6
Kidney	—	6 ± 4	29 ± 9	65 ± 9	21 ± 19
Brain	—	—	8 ± 5	15 ± 9	—

[a]Data expressed as μg/g tissue.
[b]Rats were off PCB diet from 52nd to 65th week.
[c]Values expressed as mean ± standard deviation.

Table IV. *Tissue levels of polychlorinated biphenyls in rats fed 100 ppm of Aroclor 1254 for 52 weeks*

	Time (weeks)				
Tissue	13	26	39	52	65[b]
Fat	982 ± 212[a,c]	1170 ± 76	981 ± 120	1080 ± 440	1130 ± 590
Liver	30 ± 6	25 ± 17	86 ± 67	31 ± 18	35 ± 9
Kidney	40 ± 31	66 ± 43	75 ± 76	186 ± 160	16 ± 5
Brain	6 ± 0.8	3 ± 2	8 ± 3	60 ± 8	10 ± 2

[a]Data expressed as μg/g tissue.
[b]Rats off PCB diet from 52nd to 65th week.
[c]Values expressed as mean ± standard deviation.

Table V. *Tissue levels of polychlorinated biphenyls in rats fed 100 ppm of Aroclor 1262 for 52 weeks*

	Time (weeks)				
Tissue	13	26	39	52	65[b]
Fat	1450 ± 270[a,c]	1700 ± 470	3380 ± 1290	3140 ± 1150	3420 ± 1350
Liver	19 ± 8	16 ± 8	48 ± 18	51 ± 8	78 ± 66
Kidney	76 ± 22	122 ± 21	174 ± 40	151 ± 141	64 ± 24
Brain	8 ± 4	8 ± 6	27 ± 7	28 ± 12	25 ± 2

[a]Data expressed as μg/g tissue.
[b]Rats off PCB diet from 52nd to 65th week.
[c]Values expresses as mean ± standard deviation.

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Indications are that the effect of the PCBs or serum lipids is a rather consistent finding in man and lower animals. Rats fed 0.1 g of PCB showed a moderate increase in serum cholesterol and tenfold increase in triglycerides (Tanaka *et al.* 1969). However, Nagai *et al.* (1969) observed that 50 mg of PCB given to rats daily for 13 and 21 days caused an increase in cholesterol and phospholipids. In the 'Yusho'' outbreak of 1968, exposed persons developed a decided increase in serum lipids which was primarily due to the increase in triglyceride levels (Kuratsune 1969). Undoubtedly these changes in levels of serum lipids were related to the marked hypertrophy of the liver of these exposed animals. Due to the decided proliferation of the smooth ER in hepatic cells following chronic dietary stimulation with PCBs (Kimbrough *et al.* 1972), it seems reasonable to postulate that there is an increased synthesis of these lipids within these cytoplasmic membranes.

As has been reported previously from this laboratory as well as others the higher chlorine isomers are less readily metabolized and excreted. In this experiment, it was shown that less PCB was detectable in the tissues of the animals given Aroclor 1248 than either of the other Aroclors. Likewise, less PCB accumulated in tissues of

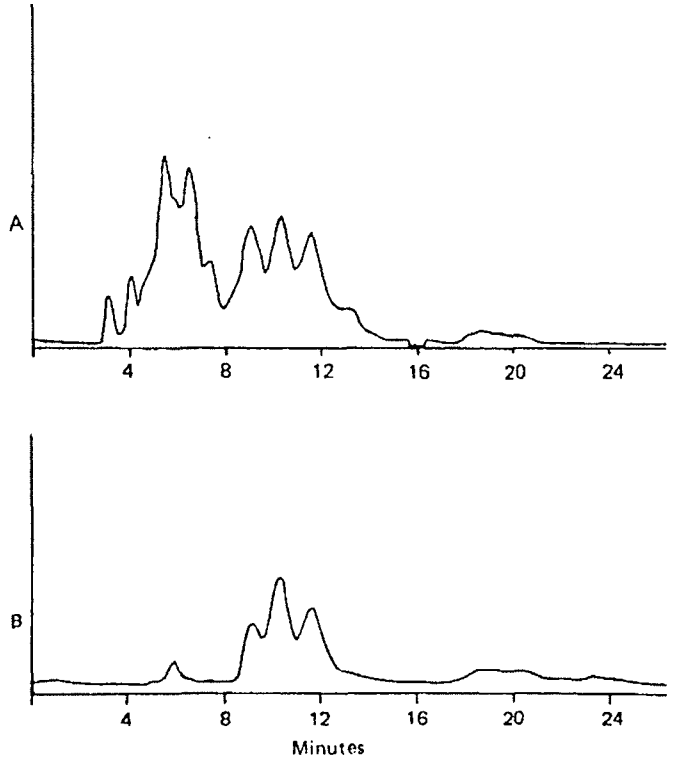


Fig. 10. GLC-EC tracing of (A) Aroclor 1254; (B) the residue in the fat after 52 weeks.

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the Aroclor 1254 fed animals than those given Aroclor 1262. The persistence of more highly chlorinated isomers in the tissues 13 weeks following discontinuation of exposure attest to their persistence in the body. Since the PCBs are deposited in the cell membranes and tissues throughout the body as well as the depot fat it is only as the membranes are replaced or the depot fat is utilized that the PCBs are metabolized and excreted.

Focal areas of hepatic degeneration and necrosis are common observations in the livers of rats and monkeys exposed to PCBs (Allen and Abrahamson 1973, Allen *et al.* 1974). Although the mechanism responsible for this focal liver necrosis is undetermined, it seems to be directly related to the PCB-induced changes that occur in these cells. Invariably the cells that undergo necrosis are those that have previously shown the most pronounced hyperplastic changes in their organelles. Following a period of hyperactivity as indicated by hyperplasia of the ER and increase in microsomal enzyme activity, these cells undergo degenerative changes that are characterized by clumping of the endoplasmic reticulum and decrease in microsomal enzyme activity, rarification of cytoplasm and an increase in the number of cytoplasmic lipid droplets. Fracture of their plasma membranes is followed by dehiscence of the cytoplasmic organelles.

Allen and Abrahamson (1972) have reported an increase in the phospholipid and cholesterol in microsomal membranes and have equated these changes to an increase in their ability to sequester PCBs. Allen *et al.*[6] have observed that the microsomal fraction of liver homogenates contained the highest levels of PCBs. When these data are considered along with the morphological and biochemical changes, it seems reasonable that up to a certain point, the PCBs stimulate membrane development. When this point is attained, either as a result of excessive PCB sequestration *per se* or as a result of altered membrane synthesis and degradation, the membranes no longer function adequately. Decreased enzyme activity and increased cell permeability attest to these membrane alterations. When these changes become sufficiently severe, cell death ensues.

The reason for the continued degenerative changes in the liver for 13 weeks following the discontinuation of exposure is at least partially explained by the maintenance of essentially the same level of PCB in hepatic tissue. Under normal circumstances there is a gradual movement of the PCBs, primarily from the depot fat into the liver, thus maintaining a relatively constant level as long as the storage areas are maintained. It has been shown that under adverse conditions, the food intake is reduced, such as would be experienced during illness, and sufficiently large amounts of PCBs migrate from depot fat into the liver to cause severe liver damage (Allen *et al.* 1974). Therefore it can be said unequivocally that the injurious effects of PCB exposure may last for lengthy periods after contact with the compound has been eliminated.

[6]To be discussed in greater detail in "Responses of Rats Exposed to Polychlorinated Biphenyls for Fifty-Two Weeks. II. Compositional and Enzymatic Changes in the Liver".

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