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POLYCHLORINATED BIPHENYL CONGENER 153-INDUCED ULTRASTRUCTURAL ALTERATIONS IN RAT HEPATOCYTES: A QUANTITATIVE STUDY

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Masters of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Jian Peng

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ABSTRACT

Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants and industrial chemicals that had been widely used. Concern over the PCB effects on human and animal health arose as a result of their bioaccumulation and biomagnification through the aquatic and terrestrial food web. Because of its primary role in the metabolism of xenobiotics, liver is the most significantly affected organ following exposure to PCBs. Alterations occurring in the liver of male and female rats fed PCB congener 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) at concentrations of 0.5, 5, or 50 ppm for 13 weeks were determined morphometrically. A dose-dependent increase of hepatocyte volume was detected in the animals. The cytoplasmic compartment contributed to the increase in cell volume in an overwhelming fashion; nuclear participation in the hypertrophy was slight. A marked increase of smooth endoplasmic reticulum (SER) volume and its surface area in hepatocytes was observed in 5 and 50 ppm groups of both genders; the organelle played the largest part in the increase of cytoplasm volume or hypertrophy of the cells. Rough endoplasmic reticulum (RER) alteration was shown to depend on gender, where its volume per hepatocyte augmented in females of 5 or 50 ppm groups, but decreased values were noted in males at the same dose concentrations. A significant decrease in normal mitochondrial volume at 50 ppm dose was observed, which may be a consequence of a transformation of mitochondria to abnormal types. Two types of abnormal mitochondria, type I and type II, were defined: type I comprised mitochondria that had cristae which lie parallel to long axis of the organelle; type II consisted of mitochondria that showed C- or ring-shaped profiles. Data revealed a trend toward an increase of abnormal mitochondria volume in the hepatocytes as congener concentration elevated. In addition, an increase in the volume of lysosomal elements per cell was noted in 50 ppm PCB-dosed rats of both genders; an increase in peroxisomal volume per cell of female rats was detected at a lower dose than those in male rats. However, PCB 153 effect on lipid droplets in centrilobular hepatocytes was not detected. In conclusion, the present study revealed that subchronic exposure to PCB 153 caused ultrastructural alterations in centrilobular hepatocytes of Sprague-Dawley rats. Cell hypertrophy, SER proliferation, RER volume alterations, mitochondrial abnormalities, and augmentation of peroxisomes and lysosomal elements were the most conspicuous changes. Alterations were dose-dependent and were most severe in 50 ppm groups. Based on data presented above, the no observable morphologic alteration level for PCB 153 was judged to be 0.5 ppm in diet or equivalent to 34 μg/kg bw/day.

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Finally, thanks go to the faculty members, staff and fellow students in Atlantic Veterinary College and researchers in Health Canada, Ottawa who helped me during this program.

DEDICATION

To my parents and my husband Jun

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ABBREVIATIONS

Abbreviation	Term
Ah	aryl hydrocarbon
AHH	aryl hydrocarbon hydroxylase
APDM	aminopyrine N-demethylase
ATP	adenosine triphosphate
AVC	Atlantic Veterinary College
BQ	Bioquant
bw	body weight
$CV_N(V)$	coefficient of variation of numeric distribution of particle volume
df	degrees of freedom
DiCB	dichlorobiphenyl
DNA	deoxyribonucleic acid
EM	electron microscopy
EROD	ethoxyresorufin-O-deethylase
GC/MS	gas chromatography/mass spectrometry
HeptaCB	heptachlorobiphenyl
HexaCB	hexachlorobiphenyl
IUPAC	International Union of Pure and Applied Chemists
MC	3-methylcholanthrene

NOAEL	no observable adverse effect level
OECD	Organization for Economic Co-operation and Development
PB	phenobarbital
PCB	Polychlorinated biphenyl
PentaCB	pentachlorobiphenyl
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
S_v	surface density
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TetraCB	tetrachlorobiphenyl
$\overline{V}_N$	number weighted mean particle volume
V_v	volume density
$\overline{V}_V$	volume weighted mean particle volume
w/w	weight/weight

Chapter 1. Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants, among the most abundant chlorinated hydrocarbon pollutants in the environment (Norback and Allen, 1972; Kasza et al., 1976). Potential health hazards exist for PCBs because of their long half-life and capacity to bioaccumulate and biomagnify in the aquatic and terrestrial food web (Safe, 1984,1994). The manufacture of PCBs ceased in 1978, however, the contaminant still persists in the environment due to its continuing use as dielectric fluid in older transformers and capacitors (Kimbrough, 1987). Numerous studies have been conducted on PCB mixtures, however, our comprehension of the adverse effects of individual PCB congeners remains limited.

1.1. Congeners of polychlorinated biphenyls

The chemical formula of PCBs is $C_{12}H_{10-n}Cl_n$ where n is 1-10. The structure of PCBs (Fig. 1.1) shows the compounds have multiple sites for chlorine substitution. Differences in substitution patterns and the degree of chlorination result in 209 possible PCB congeners (Safe, 1990). The number of PCB isomers is detailed in Table 1.1. To identify individual PCB compounds, a numbering system (Ballschmiter and Zell, 1980) has been designed that is consistent with the International Union of Pure and Applied Chemists' (IUPAC) nomenclature system for the biphenyls. For instance, the 42 HexaCB congeners have numbers 128-169; each number, in turn, is used as a synonym

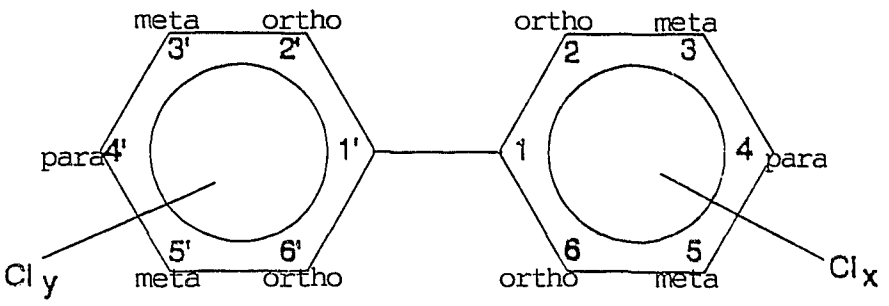


Figure 1.1. Structure of PCBs

Table 1.1. Number of possible isomers of PCBs

Chlorine substitution	Number of possible isomers
Mono-	3
Di-	2
Tri-	24
Tetra-	42
Penta-	46
Hexa-	42
Hepta-	24
Octa-	12
Nona-	3
Deca-	1
Total congeners	209

for a specific PCB compound, e.g., congener 153 is 2,2′,4,4′,5,5′-hexaCB.

1.2. Commercial PCB products

Commercial PCB products are composed of complex mixtures of 40-70 individual PCB congeners (De Voogt and Brinkman, 1989). Aroclor was the most common of the trade names and was produced by the Monsanto Chemical Company (U.S.A.). All Aroclor products are characterized by a four digit number; the first two digits represent the type of molecule (12=chlorinated biphenyl) and the last two digits give the weight percent of chlorine, for instance, Aroclor 1221, 1232, 1242, 1248, 1254, and 1260 are the chlorinated biphenyl mixtures with an average chlorine content of 21%, 32%, 42%, 48%, 54% and 60%, respectively. Similar commercial PCB mixtures have been produced by other manufacturers which include the Clophens (Bayer, Germany), Phenoclors (Prodelec, France), Fenclors (Caffaro, Italy), Delors (Chemko, Czechoslovakia) and Kaneclors (Kanegafuchi, Japan) (Safe, 1984).

1.3. Physical and chemical properties of PCBs

A pure PCB congener has the form of colourless crystals, although the commercial products with lower chlorine content (< 60%) are liquids due to melting-point depression in the mixtures. PCBs are highly lipophilic and the degree of lipophilicity is increased with increasing ring chlorination (Safe, 1990). The solubility of PCBs in water is low, especially for the higher chlorinated congeners, but they are soluble in organic solvents, oils, and fats. The compounds have a broad range of physicochemical

properties which include nonflammability, chemical and thermal stability, and miscibility with organic compounds (Safe et al., 1987). These favourable characteristics led to their extensive industrial applications (*vide infra*).

1.4. Uses of PCBs

It has been estimated that approximately 1.5 million metric tons of PCBs were produced worldwide during the 1930s-1970s (De Voogt and Brinkman, 1989). Prior to 1965, PCBs were widely used in industry as heat transfer fluids, plasticizers, pesticide extenders, lubricant, fire retardants, sealing liquids, hydraulic fluids, paint additives, organic diluents and dielectric fluids for transformers and capacitors (Safe, 1990). The detection of PCBs in the environment in 1966 resulted in a voluntary restriction on all "open" uses of these compounds whereas their use in closed systems (transformers and capacitors) was permitted until the late 1970s (Safe, 1984). The production of PCBs was stopped in 1978, but due to their persistence in the environment, and because significant quantities of the compounds are still in use as dielectric fluids in old transformers and capacitors, it will take considerable time before the levels of PCBs in the environment are reduced (Kimbrough, 1987).

1.5. PCBs in the environment

Serious environmental contamination with PCBs has been documented for industrialized areas such as the Great Lakes Basin, the Baltic Sea and Tokyo Bay. Moreover, contamination in the global ecosystem was shown by the presence of PCBs

in the samples of air, snow, ice, fish, birds and mammals obtained from the polar regions, in sediment, water and atmosphere of oceans, and in a wide range of plankton, fish, and marine and terrestrial mammals including humans (Safe, 1984; Waid, 1987). Probable sources of environmental contamination with PCBs include spillage and leaks from equipment containing large volumes of PCBs in service, in storage, or deposited in landfills. Other sources include emission from incinerators, engines and furnaces burning fuels contaminated with PCBs, and re-evaporation from water and land (Murphy et al., 1985). It seems that atmosphere and ocean currents are the most likely routes for the global transport of PCBs (MacClure, 1976; Safe, 1984).

PCBs tend to bioaccumulate and biomagnify through the food web because of their lipophilicity and chemical stability (Safe, 1984,1994). The concentration of PCBs in lower trophic levels of aquatic organisms such as plankton and benthic species depends primarily on the PCB concentration in water and sediments. PCBs build up to higher concentrations at each step in the food web reaching the highest concentrations at the top of the food web in fish-eating birds, where the factor of biomagnification becomes quite large. For example, PCBs measured in Lake Ontario water in 1986 were present in concentrations of 5 ppt; PCBs in phytoplankton, rainbow smelt (*Osmerus mordax*), and lake trout (*Salvelinus namaycush*) were 0.01, 1.7, and 5.6 ppm, respectively; PCBs in the eggs of herring gulls (*Larus argentatus*) have been measured greater than 60 ppm. Thus, there was a biomagnification factor of 10 million from water to herring gull eggs (Environment Canada, 1991).

1.6. PCBs in humans

1.6.1. Human exposure

Human populations have been exposed to PCBs via three major pathways: occupational, accidental, and environmental (Safe, 1984,1994).

Occupational exposure refers to the exposure of workers who produced PCBs or used PCB-containing products, but currently it is limited to the individuals who carry out disposal of PCB waste or maintain PCB-containing electrical equipment. The major sources of occupational exposure are through inhalation of the contaminated air and skin contact with PCB-containing products in the workplace (Kimbrough, 1987).

There have been two big accidental PCB-poisonings. The first episode occurred in western Japan in 1968 and led to the term Yusho patients for victims of this poisoning; the second occurred in central Taiwan in 1979 and victims were termed Yu-Cheng patients. Both incidents resulted from contamination of rice oil with PCBs that were used in the cooling process during the oil processing (Safe, 1984; Kimbrough, 1987).

The principal source of environmental exposure appears to be the ingestion of foods (particularly fish, meat, and dairy products) contaminated with PCBs. Direct

exposure to contaminated air, water, and dust have also contributed to the accumulation of PCB congeners in the body (Environment Canada, 1991).

1.6.2. PCB concentrations in body fluids and tissues

Polychlorinated biphenyls have been detected in human adipose tissue, liver, blood, and breast milk. For concentrations expressed as per gram fat, PCB distribution ratios show some preferential accumulation in the liver compared to that in the blood, milk, and adipose tissue compartments. But, unadjusted concentrations (on a wet weight basis) were highest in adipose tissue (Jensen, 1987).

Serum PCB levels in the general population were reported to be 2-5 ppb (Kreiss, 1985; Mussalo-Rauhamaa, 1991). Great Lake fish eaters had average serum PCB levels that were as high as 20 ppb (Hoving et al., 1992). Average serum PCB levels in occupationally exposed workers averaged from 30 to 500 ppb (Maroni et al., 1981a; Chase et al., 1982).

In general, breast milk samples from women in industrial areas had higher levels of PCBs than those from non-industrial areas (Schecter et al., 1989a). PCB concentrations in human milk from industrial areas were in the range of 0.5-1.5 ppm on a milk fat basis; on a whole milk basis the usual range was approximately 10-50 ppb (Jensen and Slorach, 1991). Women consuming large amounts of fish, seafood, or marine mammals had significantly higher PCB levels in their blood and milk (Jacobson

et al., 1984). Hara (1985) reported that PCB levels in milk from occupationally exposed women ranged from 30-200 ppb when measured on a whole milk basis.

PCBs accumulate in the adipose tissue of human body; the highly chlorinated congeners are more readily deposited in the adipose tissues because of their greater lipophilicity (Luotamo et al., 1991). PCB levels in adipose tissue and serum increase with age, but the concentrations are lower in the females of each age group (Mes et al., 1982; Kimbrough, 1987; Mes, 1990). The age-associated increase in PCB levels could be related to the long half-life of some PCB isomers that are preferentially retained in mammals (Matthews and Anderson, 1975). The gender-associated difference might be the result of the excretion of PCBs through placenta or milk secretion (Jacobson et al., 1990; Rogan and Gladen, 1991).

Environmental scientists have recognized that the precise congener composition of PCB extracts from biota samples varies and depends, in part, on which specific commercial PCB preparations were associated with a contaminated area (Safe, 1990). However, the PCBs found in the human milk samples were highly complex and the congener composition and their relative concentrations did not resemble any of the commercial PCB preparations (Safe, 1990). A reason for this discrepancy is that individual PCB congeners exhibit different physicochemical properties that influence their rates of partitioning, uptake, and metabolism in the body. Currently, most environmental standards for PCBs are derived from the results of animal studies using

commercial PCB mixtures. However, risk assessment of PCBs in food products or environmental samples should take into account the potential adverse impacts of the individual congeners (Safe, 1994).

1.7. Toxicity to humans

1.7.1. Occupational exposure

Effects of PCBs on occupationally-exposed humans vary, but the most noticeable and prevalent symptoms were related to skin, such as chloracne, skin rash, and burning sensation. The appearance of these complaints seemed to depend on the concentration of PCBs in the working environment (Fishbein et al., 1979; Taylor, 1979; Hara, 1985). A study of the relation of PCBs to birth weight and gestational age among the live offspring of women occupationally-exposed to PCBs indicated a significant correlation between increased serum PCB level and reduced gestational age and birth weight, but the decrease in birth weight probably resulted from a shortened gestation rather than a retardation of the intrauterine growth (Taylor et al., 1984,1989). Hepatic abnormalities which included hepatomegaly, increased serum enzymes and induced hepatic drug-metabolizing enzymes were also reported (Alvares et al., 1977; Maroni et al., 1981a,b). Some of the responses described above were reversible after the exposure ended (Fishbein et al., 1979; Hara, 1985).

1.7.2. Accidental exposure

The effects and symptoms found in Yusho and Yu-Cheng patients were similar, and included: severe and persistent dermal lesions such as chloracne, pigmentations and dermatitis; various ocular problems including hypersecretion, swelling of the upper eyelids, and narrow field of vision; a number of systemic disorders such as dullness, abnormal liver function, nervous and endocrinologic disturbances (Urade et al., 1979; Kimbrough, 1987). In addition, children born to Yusho mothers had reduced birth weight, lower overall intelligence scores, more behavioral problems, and some of the same symptoms such as dermal lesions that were observed in their mothers (Rogan et al., 1988). Compared to the occupationally exposed workers, Yusho and Yu-Cheng patients appeared to have more severe and persistent toxic symptoms. However, the serum levels of PCBs in industrial workers exposed to these compounds were either comparable to or higher than those in the Yusho and Yu-Cheng patients (Safe, 1984,1994). A plausible explanation for this variation is the presence of relatively high levels of the toxic polychlorinated dibenzofurans (PCDFs) in the contaminated rice oil. Moreover, analysis of adipose tissue and serum obtained from the Yusho and Yu-Cheng patients confirmed the presence of several PCDF residues (Safe, 1994).

1.7.3. Environmental exposure

Whether human health is affected by the continuing environmental exposure to PCBs is yet to be determined. Traditional measures of human mortality and disease incidence have not indicated any adverse effects (Cordle et al., 1982). On the other

hand, the health related issue of environmental exposure which has received the most attention focuses on developmental deficits in children exposed *in utero*. A study of the children of 242 women who consumed Lake Michigan fish over a six-year period has demonstrated the occurrence of several adverse effects. There was a correlation between umbilical cord serum PCB levels and several parameters such as reduced birth weight, reduced head circumference, and behavioral deficits including poor recognition memory in infants (Fein et al., 1984; Jacobson et al., 1985). At the age of four, those children were re-assessed. Prenatal exposure (indicated by umbilical cord serum PCB levels) but not breast-feeding exposure, correlated in a dose-dependent fashion with decreased short-term memory function on both verbal and quantitative tests (Jacobson et al., 1990). This finding corroborated previous findings with infants and indicated that memory deficit was persistent. Rogan and co-workers (1986, 1991) also showed a correlation between the levels of prenatal PCB exposure of North Carolina infants from the general population and similar neurodevelopmental deficits. However, in this study the deficits did not persist since the children tested normal by the time they were three years old.

1.7.4. Carcinogenicity

Data on carcinogenicity in humans are inadequate. Several epidemiologic studies on the carcinogenicity of PCBs in workers revealed no overall increase in the cancer-related mortality due to the occupational exposure to PCBs (Brown and Jones, 1981; Bertazzi et al., 1987; Brown, 1987). A statistically significant increase in mortality was

reported for male Yusho patients (Ikeda et al., 1986). However, due to the possibility that the patients consumed other potentially carcinogenic compounds including PCDFs, a definitive causal relationship between PCBs and carcinogenicity could not be made. Because there was limited evidence for carcinogenicity of PCBs in humans, but sufficient evidence in animals (See 1.8.5.), the U.S. Environmental Protection Agency has classified the PCBs as suspected human carcinogens (Stone, 1992).

1.8. Toxicity to animals

The toxic effects of PCBs have been extensively investigated with laboratory animals, fish and wildlife species. The symptoms after exposure to PCBs vary with the dose, the route and the duration of exposure, the animal species and strain, and the age and gender of the animal (Safe, 1994).

1.8.1. Dermal lesions

Rabbit skin is particularly sensitive to commercial PCB mixtures, typically producing hyperplasia and hyperkeratosis of the epidermal and follicular epithelium (Vos and Beems, 1971; Vos and Notenboom-Ram, 1972). Nonhuman primates also exhibit highly characteristic skin and ocular lesions after dietary exposure to PCBs (Allen et al., 1974; Altman et al., 1979; McConnell et al., 1979). Rhesus monkeys (*Macaca mulatta*) developed acne on the facial skin, alopecia, and facial edema especially of lips and eyelids. After topical applications of PCBs, hairless mice (Skh:HR-1) developed acne and other skin lesions, but rats (Sprague-Dawley) did not

exhibit any dermal lesions (Puhvel et al., 1982), and neither did mink (*Mustela vison*), which is a species highly susceptible to PCB toxicosis (Bleavins et al., 1982).

1.8.2. Immunotoxicity

PCB toxicosis is accompanied by atrophy of the lymphoid tissues in many animal species (Vos and Koeman, 1970; Vos and Beems, 1971; Vos and Rou, 1972; Thomas and Hinsdill, 1978; Smialowicz et al., 1989). Lymphopenia, atrophy of the thymic cortex and a reduction in the number of germinal centres in the spleen and lymph nodes of New Zealand rabbits occurred following dermal administration of PCBs (Vos and Beems, 1971). Decreased humoral immune response in the guinea pig was observed after exposure to PCB congeners (Vos and Rou, 1972). A wide spectrum of immunotoxic effects of PCBs in mice and rats has been reported (Silkworth and Loose, 1978; Smith et al., 1978; Thomas and Hinsdill, 1978; Smialowicz et al., 1989), including thymic and splenic atrophy, decreased resistance to infections, depression of natural killer cell and T cell activity, and suppression of antibody response.

1.8.3. Reproductive effects

Altman et al. (1979) reported an outbreak of PCB toxicity in rhesus monkeys. There was a high incidence of abortion in the pregnant monkeys and live offspring were small and weak contributing to a high infant mortality rate. The reproductive toxicity of PCBs has also been confirmed in several rat and mouse studies (Linder et al., 1974; Kihlstrom et al., 1975; Spencer, 1982). Some of these effects included a reduction in

the number of live births from PCB-fed rats and a decrease in birth weight and survival of offspring. The reproductive effects described above were thought to be related to changes in maternal steroid hormones (Allen et al., 1979).

1.8.4. Neurotoxicity

Mice exposed to 3,3',4,4'-TCB *in utero* exhibited signs of behavioral and neurological effects (Tilson et al., 1979). The resultant syndromes consisted of increased motor activity and rotational movements, muscular weakness, and significantly elevated avoidance latencies which were indicative of impaired acquisition ability. Allen et al. (1979) reported hyperactivity and learning deficits in rhesus monkeys prenatally exposed to PCB mixtures. Recently, Cooke and Hanson (cited in Stone, 1995) have shown that offspring of Sprague-Dawley rats exposed to PCBs suffer a low-frequency hearing loss that was thought to be linked to hypothyroidism.

1.8.5. Carcinogenicity

Several studies indicated that mixtures of PCBs induced preneoplastic lesions and hepatocellular carcinomas in animals when given at appropriate doses for extended periods of time. These responses were observed primarily in studies with Aroclor 1260 and Clophen A60 (Kimbrough et al., 1975; Schaeffer et al., 1984; Norback and Weltman, 1985; Rao and Banerji, 1988).

1.8.6. Hepatotoxicity

Liver is the most significantly affected organ following PCB exposure due to its primary role in the metabolism of xenobiotics (Fishbein, 1974; Kasza et al., 1978; Lin et al., 1979). Various hepatotoxic effects have been observed in different animal species. Moderate to severe hepatic damage was detected in rats, mice, rabbits, and chickens, whereas minimal liver damage occurred in monkeys (Nishizumi et al., 1970; Safe, 1984).

1.8.6.1. Morphologic alterations

Based on short- and long-term feeding experiments using commercial mixtures of PCBs (Kimbrough et al., 1972; Allen and Abrahamson, 1973; Greene et al., 1973; Kasza et al., 1978; Jonsson et al., 1981) and individual PCB congeners (Hansell and Ecobichon, 1974; MacLellan et al., 1994a,b,c), morphologic alterations were detected in the rat and mouse liver. Grossly, there was an increase in liver weight and in liver-body weight ratios. Light microscopic findings indicated hypertrophy of hepatocytes, cytoplasm vacuolation, eosinophilic cytoplasmic inclusions, hepatocyte necrosis and pigment in hepatocytes and Kupffer cells. Electron microscopic studies revealed proliferation of smooth endoplasmic reticulum (SER), increase of lipid droplets, abnormal mitochondria and membranous whorls in the liver parenchymal cells. The severity of the morphologic changes was dose-related (Kasza et al., 1978; MacLellan et al., 1994a,b,c) and the alterations were more pronounced in the female animals than in the males (Kimbrough et al., 1972; MacLellan et al., 1994a). However, some of the

morphologic alterations were not consistent. Greene et al. (1973) reported a decrease in microbody population in the liver of mice exposed to Aroclor 1242 or 1254 in diet for 60 days, but Nishizumi (1970) found a marked increase in hepatic microbodies in mice fed relatively large amounts of PCBs for 13 to 26 weeks. In several reports, a proliferation of SER concomitant with decrease of rough endoplasmic reticulum (RER) in rodents was described (Nishizumi, 1970; Greene et al., 1973; Jonson et al., 1981); however, studies conducted by MacLellan et al. (1994a,b,c) did not report the RER decrease. The variations in the results may be explained by differences in strain or age of animals, type and amount of PCB administered, and duration of treatment. Furthermore, the above reports were mostly based on qualitative studies. Quantitative investigation of PCB effects on liver morphology has been limited to a single report (Gillette et al., 1987), where only SER alteration was subjected to morphometry; data on the changes of other organelles are unavailable. The present study was therefore initiated to quantify the ultrastructural alterations in congener-exposed livers.

1.8.6.2. Induction of drug-metabolizing enzymes

PCBs are potent inducers of hepatic drug-metabolizing enzymes, namely, cytochrome P-450-dependent monooxygenases, epoxide hydrolase, glutathione S-transferase, and glucuronosyl transferase (Safe, 1984). Classically, microsomal enzyme inducers can be divided into two main groups: phenobarbital (PB)-type inducers and 3-methylcholanthrene (MC)-type inducers. Both induce hepatic Phase I (microsomal) and Phase II (cytosolic and microsomal) drug-metabolizing enzymes. Phase I enzymes, i.e.,

cytochrome P-450-dependent monooxygenase enzymes, include cytochrome P-450 and NADPH-cytochrome P-450 reductase. Phase II enzymes are composed of epoxide hydrolase, glutathione S-transferase, and glucuronosyl transferase. In Phase I metabolism the PCB molecule is modified by an oxidative process, and the derivative so formed is conjugated in the Phase II reaction with polar groups, notably glucuronide, water, and glutathione (Safe, 1984; Jakoby, 1994; Ziegler, 1994). Unlike PCBs, such conjugates are hydrophilic and rapidly excreted. Although PB- and MC-type inducers can produce a similar spectrum of enzyme activities, there are major differences in substrate and/or oxidation site specificities between them. The multiplicity of cytochrome P-450 isozymes has been demonstrated; differences between PB- and MC-type inducers are parallel to their induction of different cytochrome P-450 isozymes. PB-type inducers induce rat hepatic cytochromes P-450IIB1, P-450IIB2, and P-450IIA1, whereas MC-type inducers induce P-450IA1, P-450IA2, and P-450IIA1 (Parkinson et al., 1980; Safe, 1994). Aldrin epoxidation (Wolf et al., 1979) and N-demethylation reactions (Denomme et al., 1983) are catalyzed by PB-type inducers, whereas aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-o-deethylase (EROD) (Burke et al., 1985) activities are induced by MC-type inducers. Adminstration of commercial PCB mixtures to rats results in both PB- and MC-like induction. Individual PCB congeners are found to be either PB-type inducers, MC-type inducers or both (mixed-type inducers) (Safe, 1994).

Drug-metabolizing enzymes are primarily located on the hepatocyte SER. A correlation between proliferation of SER and the induction of drug-metabolizing enzymes has been established (Weibel et al., 1969; Safe, 1984; Cullen and Ruebner, 1991).

1.9. Mechanism of action

Although a great deal is known about the various biological and toxicological effects of PCBs in experimental animals, the mechanisms underlying these effects are not well understood. Nebert and Gonzalez (1987) have postulated a mechanism to explain the inductive response of drug-metabolizing enzymes via receptor-mediation. According to this model, highly hydrophobic xenobiotics enter cells via diffusion or via the normal uptake process of lipoproteins and plasma proteins. Once inside the cell, the chemical agent may be able to compete successfully with the normal cellular ligand for the aryl hydrocarbon receptor protein, designated the Ah-receptor. The outcome of this competition may result in the formation of a ligand-receptor complex in the cytosol. This complex enters into the nucleus, leading to the transcription of specific mRNAs which, after translation on ribosomes, ultimately induce forms of cytoplasm P-450 and thus enhance metabolism of xenobiotics. The resulting metabolism may lead to the formation of reactive intermediates that are capable of binding to critical molecules in the cytosol or the nucleus, thereby initiating toxicity and perhaps leading to cell necrosis. Nebert and Gonzalez (1987) also suggested that the toxicity could be

explained by the inability of a normal cellular ligand to bind to the Ah-receptor which is required to maintain normal cell functions.

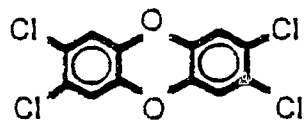
MC-type PCB congeners have shown high affinity to the Ah-receptor. A good correlation exits between the affinity with which a given congener binds to the receptor and its potency to induce AHH activity. Furthermore, toxic potency and ability to induce AHH activity are directly related (Safe, 1990). However, the PB-type PCB congeners have no affinity to this receptor protein although they have the capacity to increase the activity of certain enzymes in mammalian cells. The mechanism by which these PB-type inducers elicit their effect is unknown (Safe, 1990).

1.10. Structure activity relations for PCBs

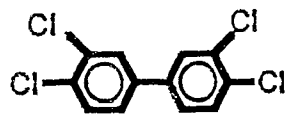
The biologic and toxic effects of individual PCB congeners are dependent on their structures (Parkinson et al., 1980; Bandiera et al., 1982; Safe, 1984; Leece et al., 1985).

1.10.1. Coplanar PCBs

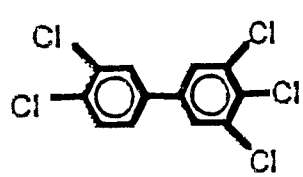
The most toxic group of PCB congeners are found in the coplanar sections, namely, 3,3′,4,4′-tetrachlorobiphenyl (TetraCB), 3,3′,4,4′,5-PentaCB and 3,3′,4,4′,5,5′-HexaCB (Fig. 1.2). These three compounds with Cl-substitution on both para and at least one meta position of both phenyl rings and without any ortho chloro substituent assume a coplanar conformation which approximates 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic halogenated aromatic hydrocarbon with the highest affinity to Ah-receptor protein (Safe, 1984). The potent MC-type inducers are 3,3′,4,4′-TetraCB, 3,3′,4,4′,5-PentaCB and 3,3′,4,4′,5,5′-HexaCB. They exhibit the highest affinity for the Ah-receptor protein and have the highest capacity to induce AHH and EROD activities among PCBs (Bandiera et al., 1982; Safe, 1984; Leece et al., 1985). Besides inductions of drug-metabolizing enzymes, treatment of rats with 3,3′,4,4′-TetraCB, 3,3′,4,4′,5-PentaCB, and 3,3′,4,4′,5,5′-HexaCB results in growth suppression, hepatomegaly, severe hepatic damage, marked increases in liver lipids, immunosuppresion, thymic atrophy, reproductive and developmental toxicity (Silkworth et al., 1984; Leece et al., 1985; Chu et al., 1994).



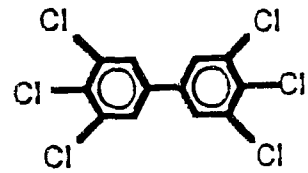
2,3,7,8-TCDD



3,3',4,4'- tetraCB



3,3',4,4',5 - pentaCB



3,3',4,4',5,5'- hexaCB

Figure 1.2. Structure of coplanar PCB congeners and 2,3,7,8-TCDD
Copied from Safe S. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. CRC Crit Rev Toxicol 1994. p. 104.

1.10.2. Mono-ortho analogues of the coplanar PCBs

The introduction of a single ortho chloro substituent into a biphenyl ring results in decreased coplanarity between the two phenyl rings due to steric interactions between the bulky ortho chloro and hydrogen substituents (Fig. 1.3). In contrast to what was initially assumed, this reduction in coplanarity did not prevent binding of mono-ortho coplanar PCBs to the Ah-receptor, but the affinity decreases in these PCBs compared to the non-ortho coplanar types. All the mono-ortho analogues of the coplanar PCBs (except those of 4,4'-DiCB) were found to be the mixed-type inducers (Parkinson et al., 1980; Safe, 1994). The biological and toxic responses observed for the mono-ortho coplanar PCBs include induction of cytochromes P-450IA1, P-450IA2, P-450IIB1, P-450IIB2 and P-450IIA1, inhibition of body weight gain, immunosuppressive effects, thymic atrophy, hepatotoxicity, and reproductive and developmental toxicity (Safe, 1984,1994).

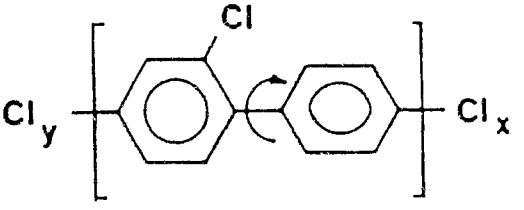


Figure 1.3. Structure of mono-ortho PCB congeners

1.10.3. Di-ortho analogues of the coplanar PCBs

The steric interactions of two ortho chloro substituents inhibit biphenyl ring coplanarity (Fig. 1.4) and presumably decrease binding to the Ah-receptor. The di-ortho substituted PCB congeners are divided into two sub-groups; one is composed of mixed-type inducers such as 2,2',3,3',4,4'-, 2,2',3,4,4',5'-, 2,3,3'4,4',6-, 2,3,4,4',5,6-HexaCB and 2,2',3,3',4,4',5-HeptaCB which exhibit weak Ah-receptor affinity, the other consists of PB-type inducers: for example, 2,2'4,4',5,5'-, 2,2',4,4',5,6'-HexaCB, 2,2',3,4,4',5,5'-, and 2,2',3,4,4',5',6-HeptaCB. The PB-type group has shown no Ah-receptor avidity and related enzyme (AHH and EROD) inducing ability. In a study based on thymus and spleen weight depression in mice, 2,2'4,4',5,5'-HexaCB was approximately 100 fold less toxic than 3,3',4,4',5,5'-HexaCB (Kerkvliet et al., 1990). Recent interest in this sub-toxic group of PCB congeners stems from the fact that they occur frequently in commercial products, the environment, and biological samples; in addition, these isomers are generally found in higher concentrations, relative to other PCB congeners. For example, 2,2'4,4',5,5'-HexaCB is the dominating congener (9.6%) in Aroclor 1260 as well as in human milk, blood, and adipose tissue (Schecter et al., 1989a,b; Luotama et al., 1991; Safe et al., 1994).

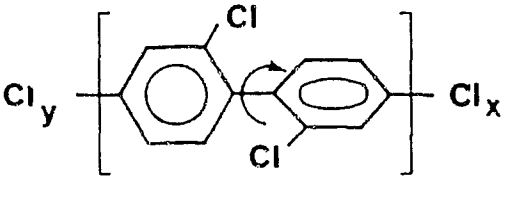


Figure 1.4. Structure of di-ortho substituted PCB congeners

1.11. Morphometry of liver

Morphometric analysis has become valuable for quantitative studies of fine structural alterations, especially in relationship to functional or biochemical changes. Morphometry has been used in the studies of many tissues, including lung (Weibel, 1973a,b; Michel and Cruz-Orive, 1988), pancreas (Bolender, 1974), muscle (Eisenberg and Kuda, 1975,1976; Mathieu et al., 1982), intestine (Hecker et al., 1974; Baddeley et al., 1986), kidney (Gundersen and Jensen, 1985; McDonald-Taylor et al., 1992), and liver (Loud, 1968; Weibel et al., 1969; Bolender and Weibel, 1973; Gillette et al., 1987). Because liver is the most important organ for detoxification of xenobiotics, morphologic effects of drugs, for example, phenobarbital on this organ have been quantified (Stäubli et al., 1969; Bolender and Weibel, 1973). Stäubli and co-workers (1969) reported that the increase in SER accounted for more than half the increase in cytoplasmic volume of hepatocyte after treatment of rats with phenobarbital for five days. Bolender and Weibel described the SER membranes induced by phenobarbital were removed within five days after the end of the treatment period. However, no account was taken of the localization of the hepatocytes within the lobule in the two phenobarbital studies.

1.11.1. *Variations within the lobule*

Anatomically, hepatocytes are distributed in three areas of a classical liver lobule: periportal, midzonal, and centrilobular. Functionally, hepatocytes are considered to reside in acini composed of three metabolic zones (Fig. 1.5). Zone 1 (periportal area) reflects proximity to blood that contains the greatest supply of substrates and oxygen. Zones 2 and 3 (midzonal and centrilobular areas, respectively) represent proximity to vascular supply with decreasing proportions of substrates and oxygen (Rappaport, 1963). The differences in anatomic distribution and metabolic function result in variations in cellular structure, as well as enzymes within the lobule. For instance, the centrilobular hepatocytes contain relatively more SER and more drug-metabolizing enzymes in comparison with those of the periportal hepatocytes (Loud, 1969; Farber and Fisher, 1979). In order to reduce the error due to the differences in hepatocyte structure, a morphometric study should concentrate on a specific area of a lobule. The centrilobular zone is the most likely site of the lesions, because abundant drug-metabolizing enzymes are located in the hepatocytes of this zone (Farber and Fisher, 1979; Cullen and Ruebner, 1991). The detoxification of xenobiotics is more active in the cells of zone 3; also the toxic effects of a compound or its metabolites are more evident in zone 3 (Cullen and Ruebner, 1991; Elangbam et al., 1991).

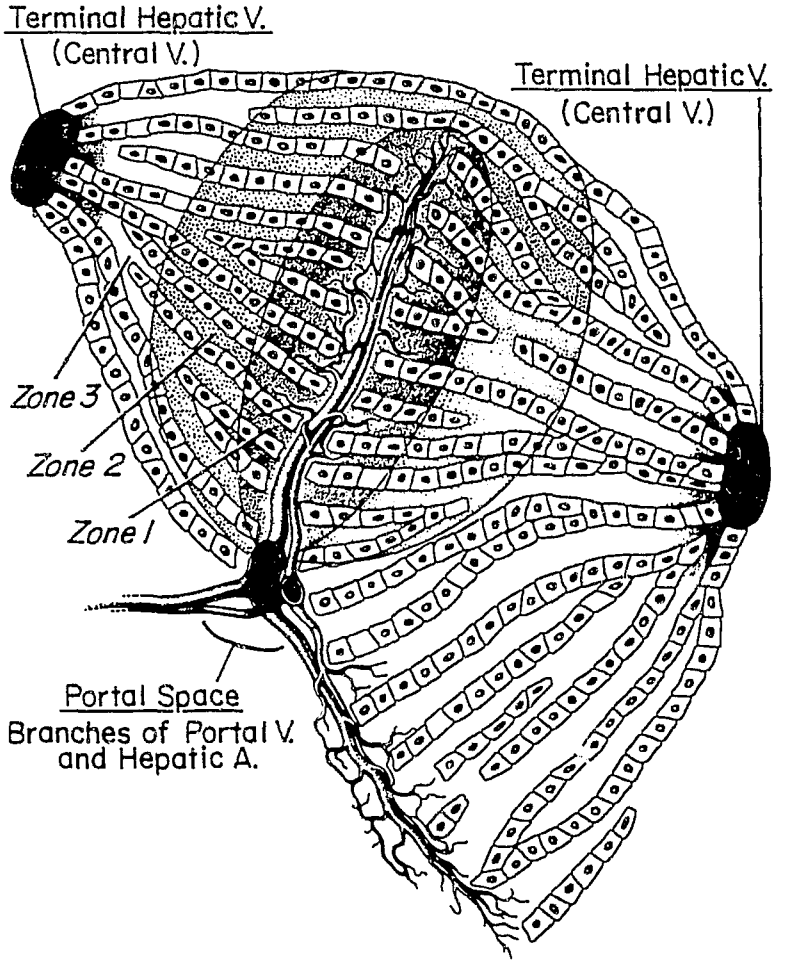


Figure 1.5. Diagram of the hepatic acinus. Three zones are designated, indicating the relative position of the cells in the acinus with respect to a gradient in oxygen and nutrient concentration of blood flowing from branches of the hepatic artery and portal vein.
Copied from Fawcett DW. Bloom and Fawcett A Textbook of Histology 12th ed. New York: Chapman and Hall. 1994. p. 663.

1.11.2. Stereologic principles

The basic idea of stereology is that quantitative information about the composition of three-dimensional tissues can be derived from two-dimensional samples, such as tissue sections or micrographs. Density is the fundamental quantitative descriptor in stereology; volume density (V_v) is defined as the volume of a component per unit volume of the containing tissue; surface density (S_v) expresses the amount of surface area of a component in the unit volume of the containing tissue (Weibel, 1979). It can be easily conceived that a change in volume density of a certain organelle may be due as well to a change in the volume of the organelles as to a change in the volume of the containing tissue since density is a relative value. For example, if the volume density of mitochondria in hepatocyte cytoplasm decreases, this cannot be interpreted as a true decrease in mitochondrial volume, unless it is confirmed that the volume of hepatocyte cytoplasm has not increased. Therefore, it is necessary to determine the cytoplasmic volume of a hepatocyte to calculate the true volume or surface area of an organelle in the cell.

Cytoplasmic volume is related to cell volume and nucleus volume. Cells and nuclei are considered particles in stereology (Weibel, 1979; Cruz-Orive, 1983). The mean particle volume is usually the parameter of interest. Of a particle population, the mean volume is defined as the expected value of random variable (particle volume) and expressed as:

$$E(X) = \sum p_i \times x_i \qquad (1)$$

where E(X) is the expected value (mean) of X, X is a random variable (particle volume) with possible values $x_1,..., x_n,...$ and p is the probability that X takes on each particular value x_i (Meyer, 1970). The mean particle volume can be estimated from either numeric distribution or volume-weighted distribution of particle volume (Gundersen and Jensen, 1983). The numeric distribution of particle volume is described as the fraction of the total number of particles which have a certain volume or which have volume within a certain narrow range (Fig. 1.6). In this distribution, each particle is given the same weight, and the mean particle volume of the distribution is denoted number-weighted mean particle volume, $\overline{V}_N$. The volume-weighted distribution of particle volume reports how large a fraction of the total volume of all particles belongs to particles with volume within a certain narrow range. Each particle in this distribution is given a weight, viz., the volume percent of the total volume contributed by particles of that volume, and the mean particle volume of the distribution is denoted the volume-weighted mean particle volume, $\overline{V}_V$. The relation between $\overline{V}_V$ and $\overline{V}_N$ is:

$$\overline{V}_V = \overline{V}_N \times (1 + CV^2_N(V)) \qquad (2)$$

where $CV^2_N(V)$ is the squared coefficient of variation of the numeric distribution of particle volume: $CV^2_N(V) = Var_N(V) / (\overline{V}_N)^2$. It follows that $\overline{V}_V \geq \overline{V}_N$, and the equality applies when all particles have the same volume (Gundersen and Jensen, 1985)

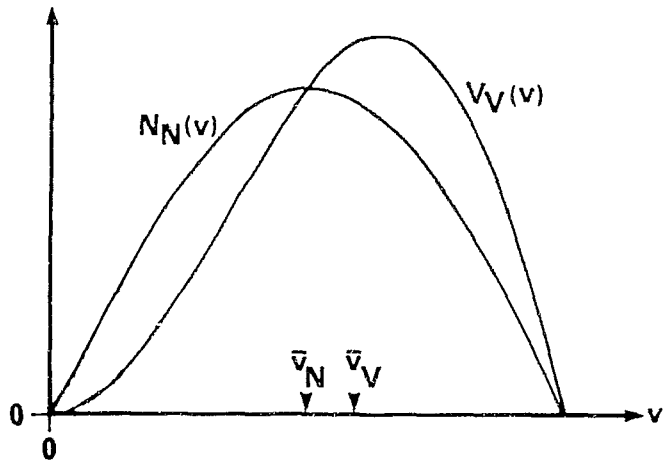


Figure 1.6. Numeric distribution $N_N(v)$ of particle volume and volume-weighted distribution $V_V(v)$ of particle volume, showing the fraction of the total particle volume that belongs to particles of a certain volume. Corresponding mean volumes $\bar{V}_N$ and $\bar{V}_V$ are indicated.
Modified from Gundersen HJG and Jensen EB. Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. J Micros 1985. p. 128.

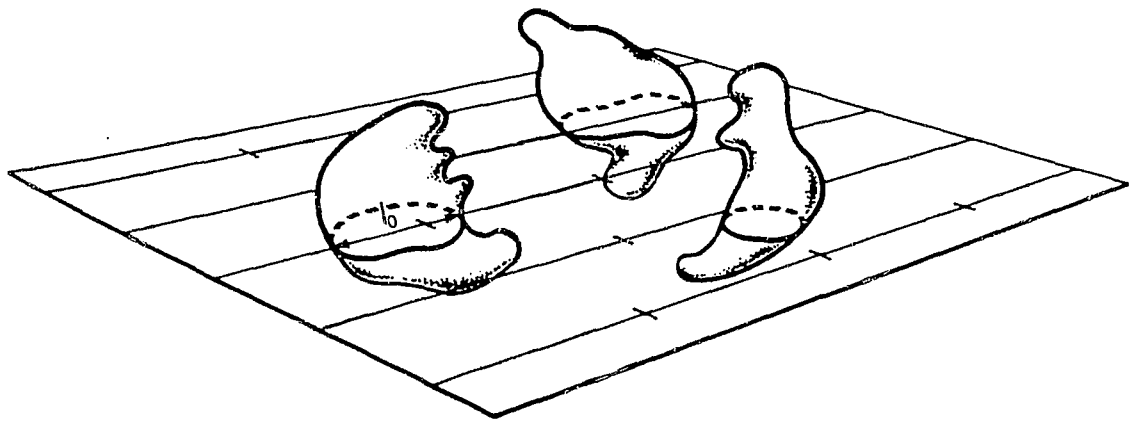


Figure 1.7. Particles hit by a section plane with a set of test-points and associated directions. Only particles hit by test-points are sampled. l_0 denotes the length of an intercept in the associated direction through the sampled particle.
Modified from Gundersen HJG and Jensen EB. Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. J Micros 1985. p. 129.

The classical method to estimate $\overline{V}_N$ (Wicksell, 1925) requires an assumption about the particle shape, which most often gives biased estimates because the assumption is seldom realized. However, by the introduction of a three-dimensional probe, the Disector (Sterio, 1984), direct estimators of number-weighted mean particle volume have become available without assumptions concerning particle shape. But in order to use the Disector it is necessary to measure precisely the actual distance between two parallel section planes and align them. This technique is rather difficult to follow in practice.

In terms of efficiency and practical limitations, the estimated $\overline{V}_V$ is generally more appropriate (Gundersen and Jensen, 1985). The approach involves measuring point-sampled intercept lengths on random, single, two-dimensional tissue sections. By this method, the probability that a particle will be measured is its volume density in the tissue. The sampling and measuring protocol is shown in Fig. 1.7. The test system is a set of test-points and associated directions indicated by lines. Only particles hit by test-points are sampled and the length of an intercept in the associated direction through the particle is denoted l_0. If all particles are convex, there is always one and only one such intercept and the volume-weighted mean particle volume is calculated by the following formula:

$$\overline{V}_V = \frac{\pi}{3} \times \overline{l_0^3} \qquad (3)$$

where l_0^3 is the average of the third power of the point-sampled intercept lengths.

It should be kept in mind that $\overline{V}_V$ is a biased estimator of $\overline{V}_N$ since $\overline{V}_V$ is larger than $\overline{V}_N$ when the particles vary in volume. However, the difference may not necessarily be of much importance in many applications. For instance, if in the numeric distribution of particle size the coefficient of variation $CV_N(V)$ is ≤ 0.33, the relative difference between $\overline{V}_V$ and $\overline{V}_N$, $(\overline{V}_V - \overline{V}_N)/\overline{V}_N$, is less than or equal to 10%.

1.12. Rationale and objectives

Based on information available in the literature, morphologic alterations occur in the liver of rats after exposure to PCBs. However, previous results were obtained from qualitative studies. The present project was therefore conducted to assess quantitative morphologic changes in the liver of rats exposed to PCB 153. Specific objectives included:

1. To quantify the morphologic alterations in the liver of rats after subchronic exposure to PCB congener 153.

2. To determine the 'no observable adverse effect level' (NOAEL) for the congener.

3. To investigate gender differences, if any, in the congener-exposed livers of the rats.

Such experiments would build a bridge between the classical qualitative and quantitative morphology studies, and bring into focus analyses of the xenobiotic effects that are needed for decision making concerning environmental problems.

Chapter 2. Materials and Methods

2.1. PCB #156 (2,3,3',4,4',5-hexachlorobiphenyl)

The animal exposure was performed at the Health Canada laboratories in Ottawa in 1993.

2.1.1. Compound

The congener, PCB 156, was synthesized by Dr. A. Bergman, Department of Environmental Chemistry, University of Stockholm, Stockholm, Sweden and had a stated purity of > 99%, which was confirmed by gas chromatographic/mass spectrometric (GC/MS) analyses.

2.1.2. Animals and treatment

Weanling Sprague-Dawley rats (40-50 g bw) were purchased from Charles River Laboratories (St. Constante, Quebec), and were randomly distributed into five groups of male and five groups of female, each comprising 10 animals. Randomization of animals was done by using a computer program written in-house. This program randomly selects and groups animals so that each group of animals of the same gender has the same mean body weight at the start of the study, and the animals with too high and too low body weights are rejected from the study. The animals were housed individually in stainless steel mesh cages at Animal Resources Division of Health Protection Branch in Ottawa, and were given Rodent Chow (Ralston Purina Company,

St. Louis, MO) and water, *ad libitum*. Housing was maintained at a temperature of 21±3 ^{0}C (relative humidity 50±20%), with alternate 12-hour light/dark cycles. After an acclimatization period of two weeks, eight groups (4 groups of female and 4 groups of male) were each administered PCB 156 that was incorporated in the diet at levels of 0.01, 0.1, 1.0 and 10.0 ppm concentration for 13 weeks, the standard period defined by Organization for Economic Co-operation and Development (OECD) for subchronic toxicity studies. Corn oil (Mazola) in 4% w/w of the diet was used to dissolve the congener to facilitate mixing with Chow. Animals in two untreated control groups (10 females and 10 males) received the diet containing an equivalent amount of corn oil only. At the termination of the study, the animals were anaesthetized with Equethesin™ (Appendix A) at a dose of 3.5 ml/kg bw, and were exsanguinated via the abdominal aorta. Regulations of the Canadian Council of Animal Care Committee were followed in conducting the experiments described here.

2.1.3. Specimen collection and processing

Liver samples were harvested randomly from five selected animals of each group. The left lobe of the liver was removed and placed on a cutting board and flooded with 2% glutaraldehyde (0.1 M, 436 mOsM, phosphate-buffered, pH 7.3) at room temperature (22 ^{0}C). A tissue strip was cut from the central region of the lobe and diced into 1-2 mm^3 pieces. Ten pieces were randomly selected from each sample and placed in vials containing the glutaraldehyde solution for three minutes after which the solution was discarded. The samples were then kept in fresh fixative for two hours at

room temperature. After washing three times in the above buffer at 22 ^{0}C, tissue samples were post-fixed for two hours in 1% osmium tetroxide prepared in the same buffer at 4 ^{0}C. Samples were then washed in the buffer once and stored in fresh buffer at 4 ^{0}C for one week (buffer was changed every two days). All samples were transported to AVC, and were further processed in the Electron Microscopy (EM) suite. Samples were dehydrated in 50, 75, 95, and 100% ethanol, cleared in propylene oxide, infiltrated with Epon 812/Araldite 502 resin (Marivac Ltd., Halifax, NS) in 50:50, 25:75 and 0:100 propylene:resin ratios, respectively. Following overnight resin infiltration in a desiccator under vacuum, individual tissue pieces were embedded in fresh resin. Polymerization of the resin into blocks was carried out in a 60 ^{0}C oven for approximately eight hours. Five blocks were produced per animal.

2.1.4. Section preparation

Thick sections, 0.85 μm, were cut on a Reichert-Jung Ultracut E ultramicrotome (Cambridge Instruments Company, Cambridge, MA) using glass knives, mounted on glass slides and stained with 1% toluidine blue made in a 1% sodium borate solution. After viewing all sections using a compound microscope, a block that contained a terminal hepatic venule was chosen from each animal for thin sections. Periacinar or centrilobular areas were trimmed so that they included all or part of a venule. Thin sections were cut at approximately 70-90 nm (silver-gray interference colour) with glass knives and collected on 200 mesh, uncoated, copper grids. The sections were double contrasted with uranyl acetate and lead stain (Sato, 1968), then were examined and

photographed using a Hitachi H-7000 electron microscope (Nissel Sangyo, Rexdale, ON) operated at 75 kV.

2.1.5. Preliminary observations

Examination of the tissue using the electron microscope revealed loss of membrane structures making it difficult to define organelle outlines. The morphometry study could not be done with these samples. The project, however, continued using known, properly-fixed samples of another congener study.

2.2. Fixation experiment

An experiment was performed to compare the fixation quality of tissues processed according to the conventional protocol used in the EM suite (see Appendix B) with that of the protocol used in fixing the liver from PCB 156 experiment.

2.2.1. Animals

Four female Sprague-Dawley rats (250-300 g bw), purchased from Charles River Laboratories (St. Constante, Quebec) were placed in two groups of two rats each. Rats were anaesthetized with Equethesin™ at a dose of 3.5 ml/kg bw, and exsanguinated via the abdominal aorta. Liver samples from each group were processed following either conventional protocol or the protocol of congener 156 experiment.

2.2.2. PCB 156 experiment protocol

See 2.1.3.

2.2.3. Conventional protocol

Most of the steps were the same as the protocol of PCB 156 experiment except that the tissue samples were not left in the buffer for one week (Appendix B). After being fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide, liver samples were washed in buffer for 5 minutes, followed immediately by dehydration, infiltration and embedding as described in section 2.1.3.

2.2.4. Section preparation

See 2.1.4.

2.2.5. Comparison of fixation quality

Thin sections prepared from the tissue obtained from the conventionally fixed specimens appeared normal by electron microscopy. However, the sections obtained from the buffer-held specimens were poorly-fixed, and appeared similar to sections from congener 156 exposed tissues.

2.3. PCB #153 (2,2',4,4'5,5'-Hexachlorobiphenyl)

The animal exposure was performed at the Health Canada laboratories in 1991.

2.3.1. Compound

PCB 153 was synthesized according to a method previously described (Cadogan, 1962) and had a purity of > 99%, as determinated by GC/MS.

2.3.2. Animals and treatment

See 2.1.2. for the details. Concentrations of PCB 153 in the diet were 0.05, 0.5, 5.0, and 50.0 ppm.

2.3.3. Tissue collection and processing

Liver samples were taken randomly from three animals of each group. Ten tissue samples of approximately 2-4 mm^3 were cut from the central region of the left lobe and immersed immediately into a large volume of 2% glutaraldehyde. After three minutes of immersion the samples were removed from the fixative, trimmed to 1-2 mm^3 and immersed in fresh fixative, and sent to AVC for further processing. Tissues remained in the fixative for approximately 20 hours and were further processed. The details of post-fixation, dehydration, clearing, infiltration and embedding were similar to those for the conventional protocol used in the EM suite (Appendix B). About 10-12 blocks were produced per animal.

2.3.4. Section preparation

Details for preparing thick and thin sections are included in 2.1.4. Three blocks, from each animal, which contained the terminal hepatic venules in thick sections were chosen for electron microscopy.

2.3.5. Morphometry

Three treatments (0.5, 5.0, and 50.0 ppm) and the control groups of both genders were subjected to a morphometric study.

2.3.5.1. Light microscopy

The thick sections that were obtained by a procedure described in 2.1.4. were used to quantify the average hepatocyte and nucleus size using a "Bioquant" computerized quantification system (BQ system) (R & M Biometrics, Nashville, TN). The method proposed by Gundersen and Jensen (1983,1985), described in 1.11.2., was used to perform this task.

2.3.5.1.1. BQ system

The BQ system included both hard- and software (Fig. 2.1). The BQ software was supplemented with hardware that included a television camera (JE3012/A, Javelin Electronics), which received information from a light microscope (Carl Zeiss, Canada Ltd., Don Mills, ON) and projected its image onto a computer monitor screen (Zenith-Model #ZVM -135, IL). The information from a digitizing pad (Hipad Digitizer -

Model #Dt11, Houston Instruments, Austin, TX) was superimposed onto the monitor. By using a mouse and digitizing pad, images of the tissue section were measured.

2.3.5.1.2. Sampling and measuring

Three sections which contained terminal hepatic venule from three different blocks per animal were used for this study. The range of the centrilobular zone was defined as up to six cell diameters from the venule (Loud, 1968). A magnification of 400 diameters (40 X 10) at the light microscope level was chosen, but the actual magnification (2,593x) of the image on the monitor screen was obtained by calibration, using a stage micrometer. About 4-5 microscopic fields from the zone were evaluated for each section. A double square lattice of length **d** was superimposed on the image screen (Fig. 2.2). To randomize position of the lattice, the upper left coarse point was placed within a square of side length **d** (itself superimposed at the upper left corner of the image field) by means of two random numbers (Mathieu et al., 1981). The nuclei hit by the points and the hepatocytes hit only by the coarse points were sampled. The intercept length of the horizontal line through each sampled hepatocyte or nucleus was measured individually.

2.3.5.2. Electron microscopy

Electron micrographs of the thin sections were prepared at two magnifications: 5,400x and 11,500x. Six micrographs from each section were recorded at each

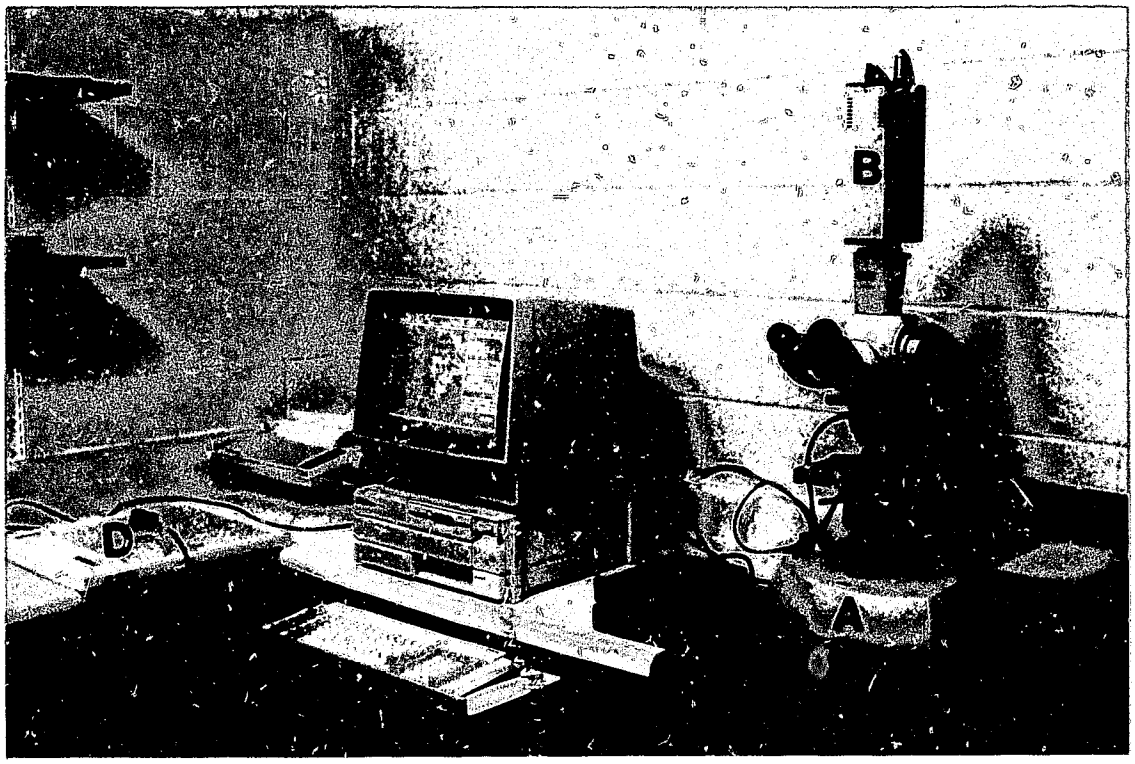


Figure 2.1. Hardware of Bioquant system: light microscope (A), television camera (B), computer monitor screen (C), and digitizing pad (D).

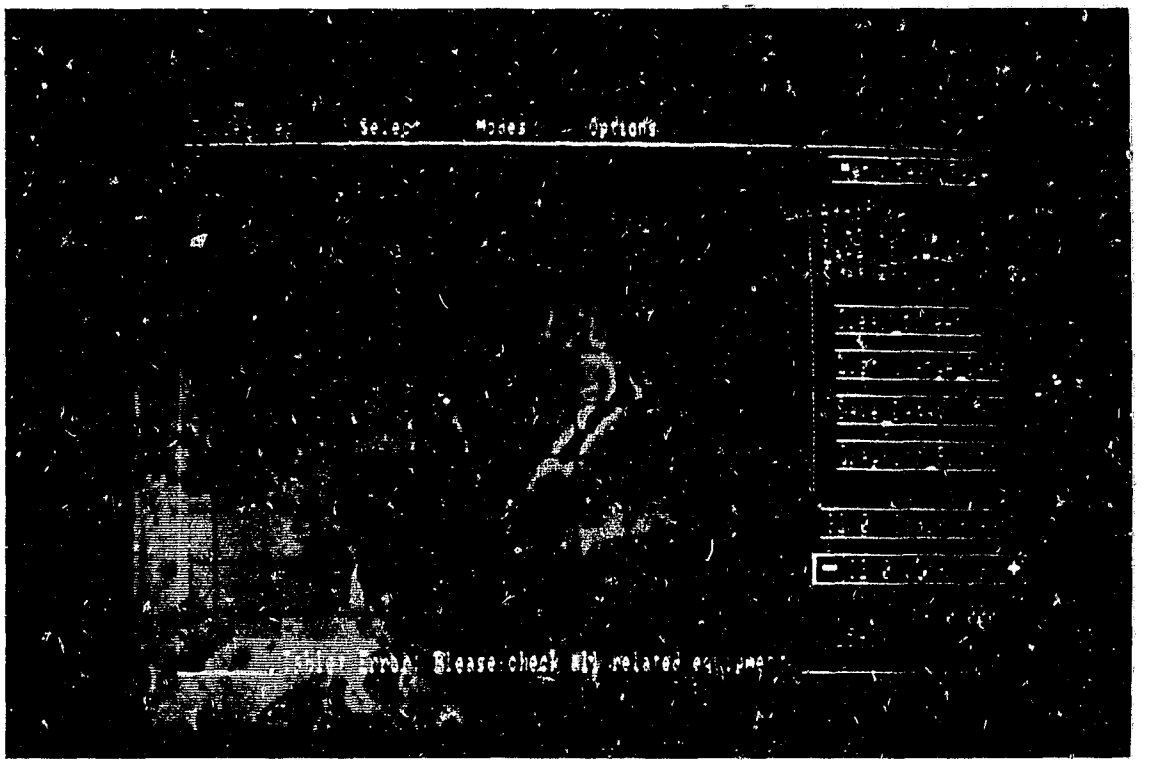


Figure 2.2. Sampling screen: the upper left coarse point (arrow head) of a double square lattice of length d (distance between two coarse points) is arranged in a regular square lattice of side length d (itself superimpose at the upper left corner of the image field) by means of two independent random numbers (3 and 4 in the figure). The nuclei hit by the points and the hepatocytes hit only by the coarse points were sampled. l_C and l_N denote the intercept length of the horizontal line through the sampled hepatocyte and nucleus, respectively.

magnification. Since three blocks and, subsequently, three sections were prepared from each animal, a total of 18 pictures per animal, and 54 pictures per group at each of the two sampling magnification were obtained.

2.3.5.2.1. Sampling and measuring

The principle for recording the micrographs was similar to the one proposed by Loud (1968). Six micrographs at each magnification were taken from C-1 to C-6 cells; C-1 cells were those located next to the venule, C-2 cells were one cell removed, and so forth. Each microscopic field for recording was randomized within the cell according to the following procedure: 1. random X value of coordinate within the cell 2. random Y value of coordinate within the cell 3. centre of the recording field was made to coincide with the point (X,Y) 4. a micrograph was taken if more than 60% of the recording field filled with the image of the selected cell, otherwise the point (X,Y) was reset.

Measurements were carried out on 8x10 inch enlarged prints which yield a 2.5-fold secondary magnification. Thus the final magnifications were 13,500x and 28,750x.

In the lower magnification, estimates were made for the volume density (V_v) of mitochondria, abnormal mitochondria type I and II, SER, RER, lipid droplets, peroxisome, and lysosomal elements in hepatocyte cytoplasm. These structure were identified on the basis of the criteria defined in the succeeding section. A simple square

lattice which contained 336 points on a field of 336 cm^2 was superimposed on each micrograph (Fig. 2.3). Points hitting on profiles of different organelles and the points enclosing in cytoplasm were counted, respectively.

The higher magnification was used to estimate the surface area of SER per volume of cytoplasm or surface density (S_v). The multipurpose test system which contained 18 lines of length 3 cm was superimposed on each micrograph (Fig. 2.4). The number of intersections between the test lines and the SER profiles was counted and the points (end points of the lines) included in the cytoplasm were recorded.

2.3.5.2.2. Morphological criteria used to identify hepatocyte compartments

Mitochondria: round or oblong profiles that are bounded by two membranes with cristae that pursue generally a transverse course to the longitudinal axis of the organelle (Ghadially, 1988).

Abnormal mitochondria type I: longitudinally orientated cristae that lie parallel to the long axis of the organelle.

Abnormal mitochondria type II: C- or ring-shaped mitochondria.

RER: group of cytoplasmic membranes with attached ribosomes, arranged in the form of cisternae. Membrane profiles lacking ribosomes within the cisternae stacks, the "intercalated areas", are counted as the cisternae (Bolender and Weibel, 1973).

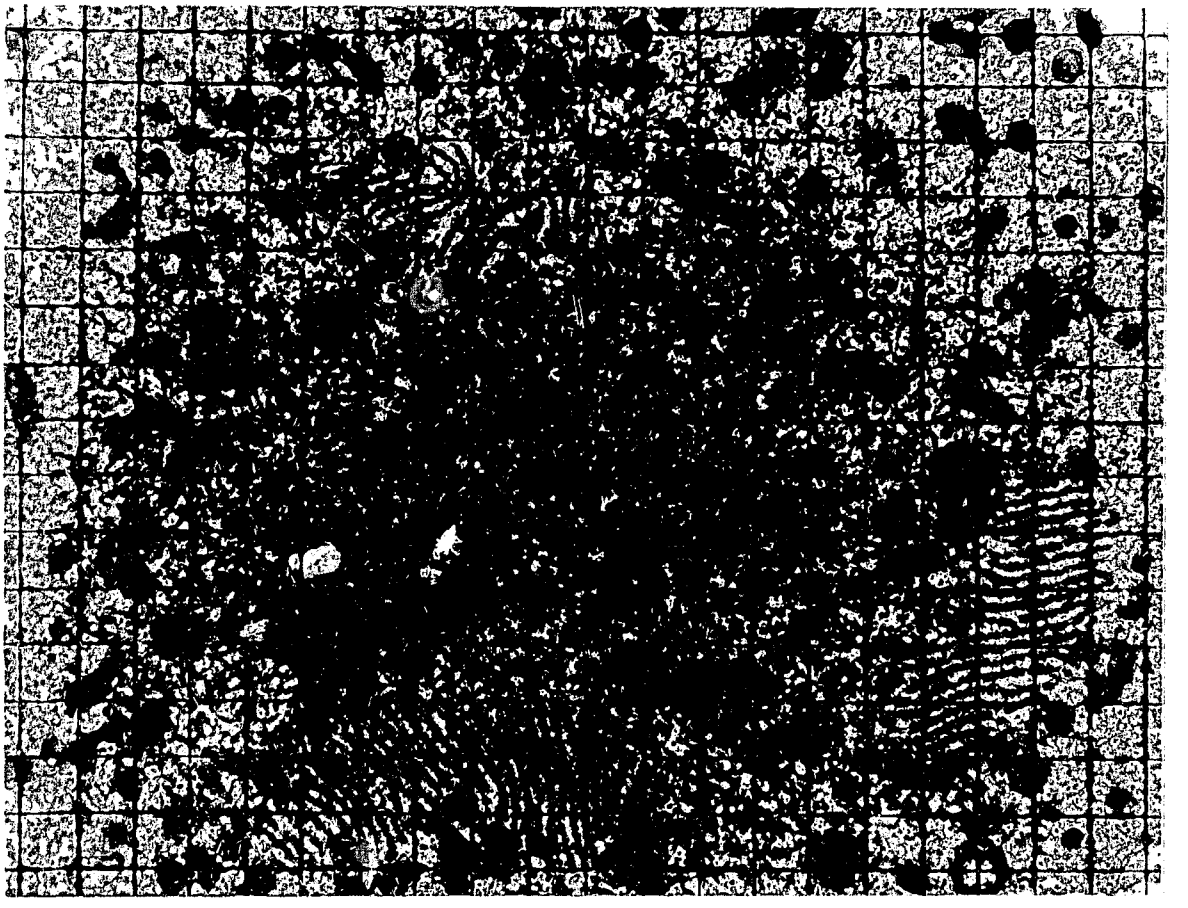


Figure 2.3. Electron micrograph from a female rat receiving 50 ppm PCB has a superimposed grid of simple square lattice with total 336 points on a field of 336 cm^2, which is used for estimation of volume density of different organelles in cytoplasm. The figure is 70% reproduction of electron micrograph at original magnification of 5,400 x.

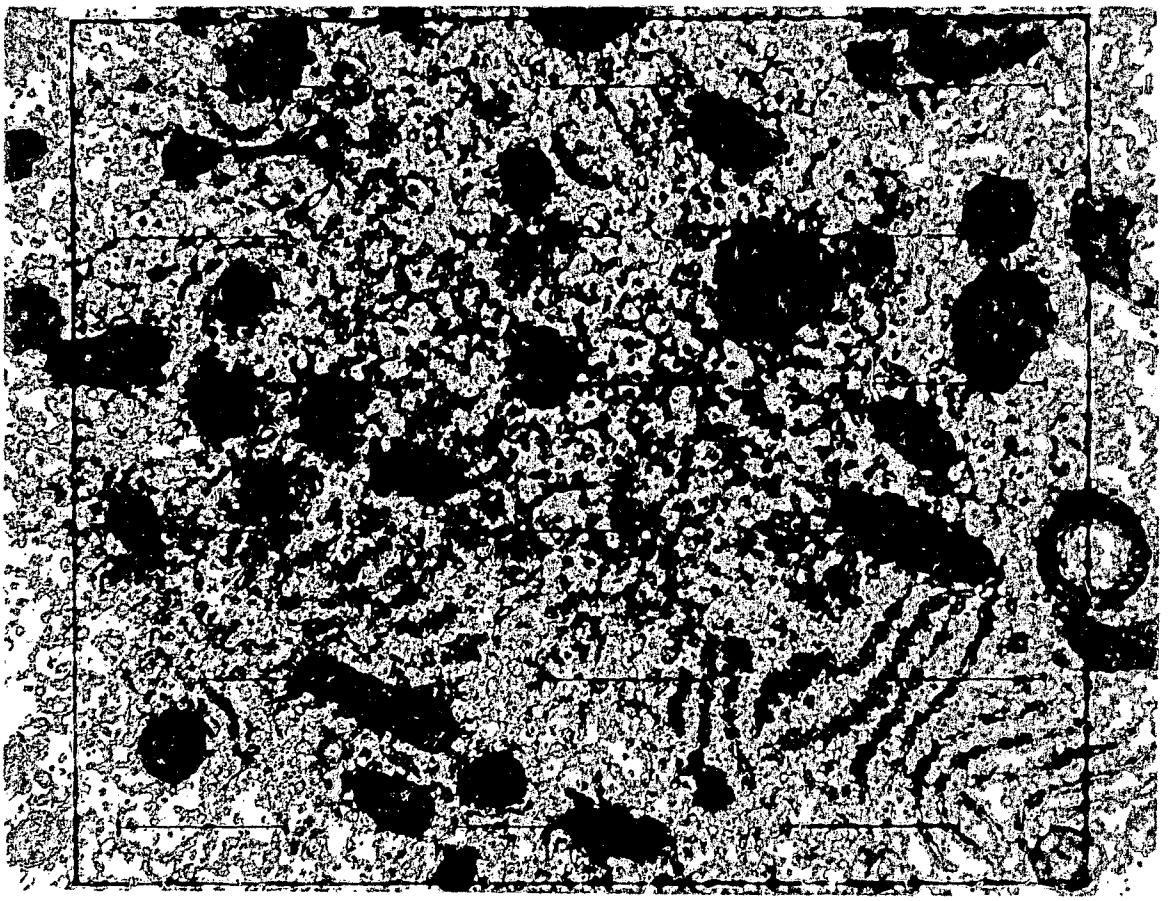


Figure 2.4. Electron micrograph from a female rat receiving 50 ppm PCB has a superimposed grid of multipurpose test system with 18 test lines of lenght 3 cm and 36 end points, which is used to estimate surface density of SER. Figure is 70% reproduction of the micrograph, at original magnification of 11,500 x.

SER: smooth-surfaced tubules or vesicles smaller than approximately 120 nm diameter.

Peroxisome: structure surrounded by a single membrane, and displaying a crystalloid.

Lipid droplet: a non-membrane-bound spherical structure, but an osmiophilic "ring" is sometimes seen around it (Ghadially, 1988).

Lysosomal elements: the single, membrane-bound bodies of different shape or size containing contents of variable density.

2.3.5.2.3. Calibration

The magnification of electron micrographs and the length of test line were calibrated by a carbon grating replica (Marivac Limited, Halifax, NS) which had 2160 lines/mm.

2.3.5.3. Parameter calculation and statistical analysis

Average volume of hepatocyte or nucleus was calculated by the formula (3) (in section 1.11.2) of Gundersen and Jensen (1983). The average cytoplasm volume per hepatocyte (volume-weighted) was the mean hepatocyte volume (volume-weighted) minus the mean nucleus volume (volume-weighted).

Volume density of different organelles and surface density of SER were calculated by pooling data of six micrographs from each section. The formula for calculation of V_v and S_v was as follow:

$$V_v = \sum_{i=1}^{6} P_a(i) / \sum_{i=1}^{6} P_c(i) \quad (4)$$

$$S_v = 4 \times \sum_{i=1}^{6} I(i) / [\, d \times \sum_{i=1}^{6} P_c(i) \,] \quad (5)$$

($\mathbf{P_a}$ **(i)**: points hitting in organelle **a** on micrograph **i**; **I (i)**: number of intersections between test lines and SER profiles of micrograph **i**; $\mathbf{P_c}$ **(i)**: points enclosing in cytoplasm of hepatocytes on micrograph **i**; **d**: test line length) (Weibel, 1979).

Surface area of SER per volume of SER is estimated by the ratio of SER surface density to its volume density in cytoplasm.

The specific values which relate volume and surface area per hepatocyte were calculated as below:

$$V = V_v \times \overline{V}_{V(cyt)} \quad (6)$$

$$S = S_v \times \overline{V}_{V(cyt)} \quad (7)$$

where $\overline{V}_{V(cyt)}$ is volume-weighted mean volume of cytoplasm per hepatocyte.

Analyses of data were conducted using two-way analysis of variance through SAS ANOVA program. The dose, gender, and dose by gender interaction effects were evaluated. Animal (dose by gender) mean square was used as an error term (df=16). If there was only significant difference between dosed groups, the significance was explored further by Duncan's multiple range test. If dose by gender interaction effects was significant, separate analyses were done on data from each gender and animal (dose) mean square was used as an error term (df=8).

Chapter 3. Results

3.1. Evaluation of fixation quality

The ultrastructure of hepatocytes in the buffer-held specimens from the animals in PCB 156 and in "Fixation" experiments is illustrated in Figure 3.1 and 3.2. Revealed in the micrographs is a loss of membrane structure making it difficult to discern organelle outlines, especially those of SER and Golgi complex. Cytoplasmic inclusions such as glycogen granules are lost resulting in electron-lucent spaces in the cells. These fixation artifacts are not seen in thin sections prepared from the conventionally fixed specimens (Fig. 3.3). Tubular profiles of the SER are dispersed in the cytoplasm; RER consists of stacked cisternae; mitochondria are bounded by inner and outer membranes and appear in rounded or elongated profiles; the Golgi complex is detected infrequently but is clearly defined by membranes; peroxisomes are single-membrane-limited, round organelles with crystalloid, and the glycogen granules are seen as electron-dense particles that are distributed randomly in the cytoplasm.

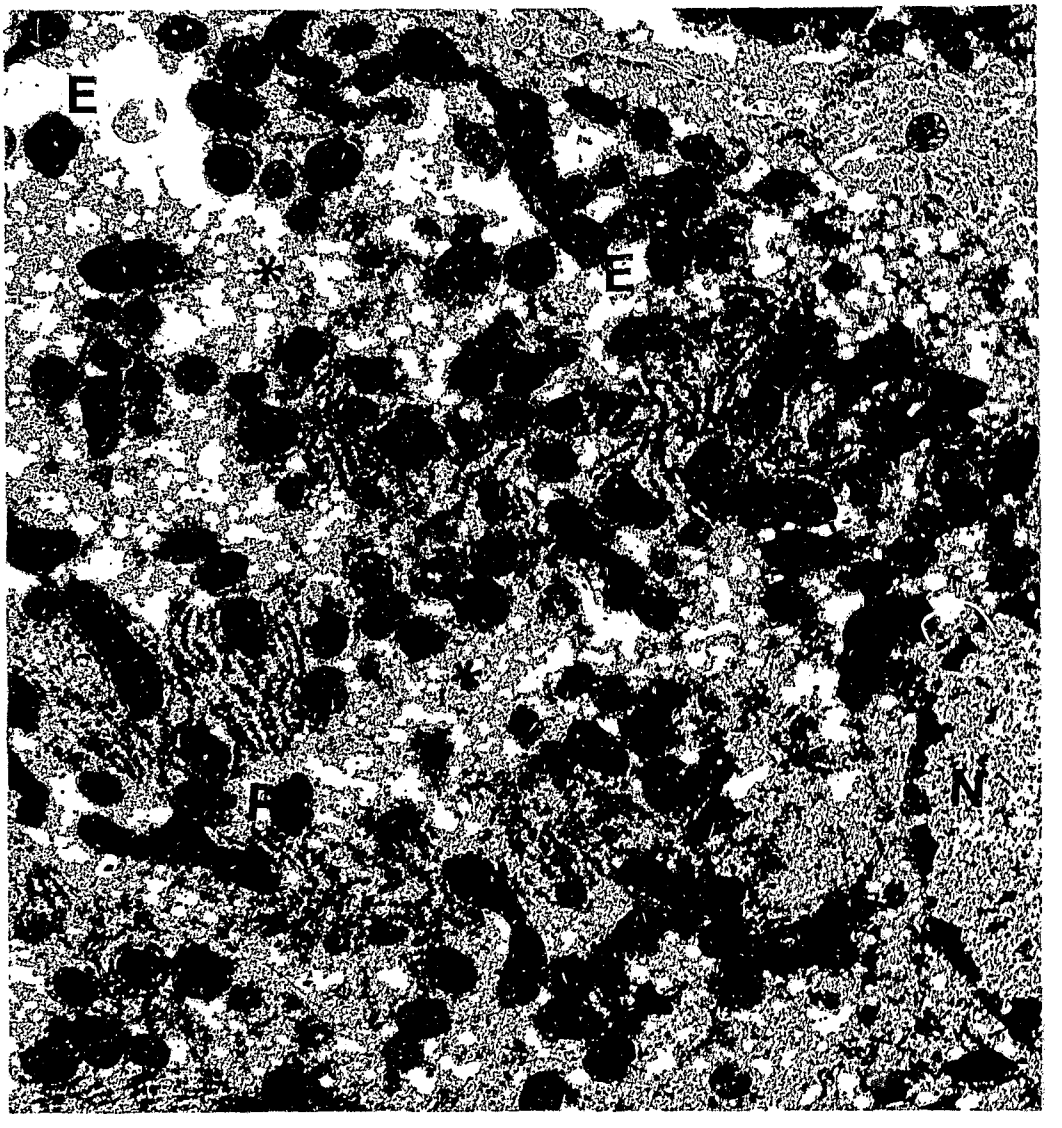


Figure 3.1. Electron micrograph of portions of hepatocytes from a female rat of control group in PCB 156 experiment. Smooth reticulum profiles (*) are unresolved. Electron-lucent spaces (E) indicate loss of cytoplasmic elements. M: Mitochondria; N: Nucleus; P: Peroxisome. x 5,000

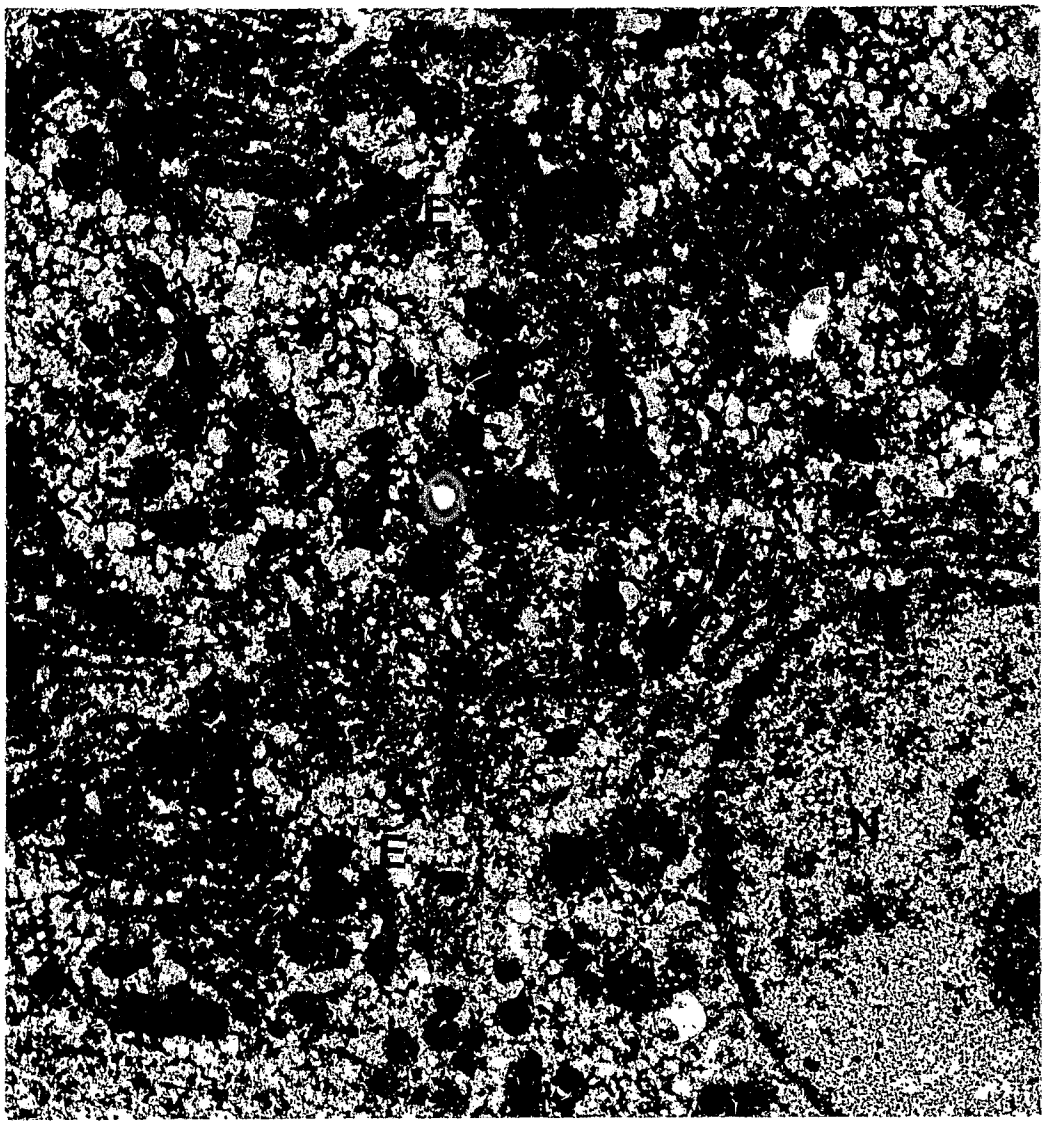


Figure 3.2. Electron micrograph of a portion of a hepatocyte from a buffer-held specimen of a female rat in "Fixation" experiment. Unresolved smooth reticulum (*) and electron-lucent spaces (E) are strikingly similar to that depicted in preceding illustration. L: Lysosomal elements; N: Nucleus; P: Peroxisome. x 5,000

Figure 3.3. Electron micrograph of a portion of a hepatocyte from a specimen fixed by conventional method. Characteristic profiles of rough (R) and smooth (S) endoplasmic reticulum, Golgi complex (Gc), and mitochondria (M) are shown. Gl: Glycogen granules; L: Lysosomal element; P: Peroxisome. x 6,000

3.2. Morphometry of liver after PCB exposure

3.2.1. Hepatocyte

A dose-dependent increase of hepatocyte volume (volume weighted mean volume) in male and female animal groups was detected (Table 3.1; Fig. 3.4). Values in 5 and 50 ppm PCB-treated groups of both genders were significantly greater than those in control and 0.5 ppm groups; values at 50 ppm for both genders were significantly higher than those at 5 ppm. The cytoplasmic compartment was a larger contributor to the hepatocyte hypertrophy than the nucleus (Fig. 3.4). In comparison with the cells from controls, the cytoplasm was significantly enlarged at 5 ppm and was further enlarged at the highest dose in all animals. The cells in females had larger cytoplasmic volume than in males, especially at doses of 0.5 and 5 ppm (Table 3.1).

In comparison with those in the controls, volume of the hepatocyte nuclei was significantly elevated only at 50 ppm (Table 3.1). However, its contribution to the increase of hepatocyte volume was small. Increase in nuclear volume contributed less than 2% of cell volume increase. Because the increase of nuclear volume was not proportional to the increase in cytoplasmic volume, the actual volume fraction of nuclei in the hepatocyte decreased.

Table 3.1: Mean volume of hepatocyte, nuclei, and cytoplasm

Volume (μm^3)	Gender	Dose			
		Control Mean ± SE	0.5 ppm Mean ± SE	5.0 ppm Mean ± SE	50 ppm Mean ± SE
Cell	♂	5482 ± 115 ^{A}	5837 ± 75 $^{A}{}_{a}$	6597 ± 140 $^{B}{}_{a}$	9219 ± 152 ^{C}
	♀	5785 ± 54 ^{A}	6293 ± 47 $^{A}{}_{b}$	7785 ± 200 $^{B}{}_{b}$	9240 ± 146 ^{C}
Nucleus	♂	292.1 ± 24.7 ^{A}	310.4 ± 4.8 ^{A}	306.9 ± 1.1 ^{A}	357.6 ± 5.7 ^{B}
	♀	296.5 ± 2.9 ^{A}	309.8 ± 7.1 ^{A}	311.7 ± 2.2 ^{A}	357.4 ± 4.3 ^{B}
Cytoplasm	♂	5190 ± 97 ^{A}	5526 ± 76 $^{A}{}_{a}$	6290 ± 140 $^{B}{}_{a}$	8861 ± 155 ^{C}
	♀	5489 ± 57 ^{A}	5984 ± 49 $^{A}{}_{b}$	7473 ± 202 $^{B}{}_{b}$	8883 ± 148 ^{C}

SE: Standard error n=3
Superscript letters represent differences among treatment groups within a gender.
Subscript letters represent differences between genders within a treatment dose.
Numbers with different letters are significantly different ($p<0.05$).
Numbers with no letters or the same letters are not significantly different ($p<0.05$).

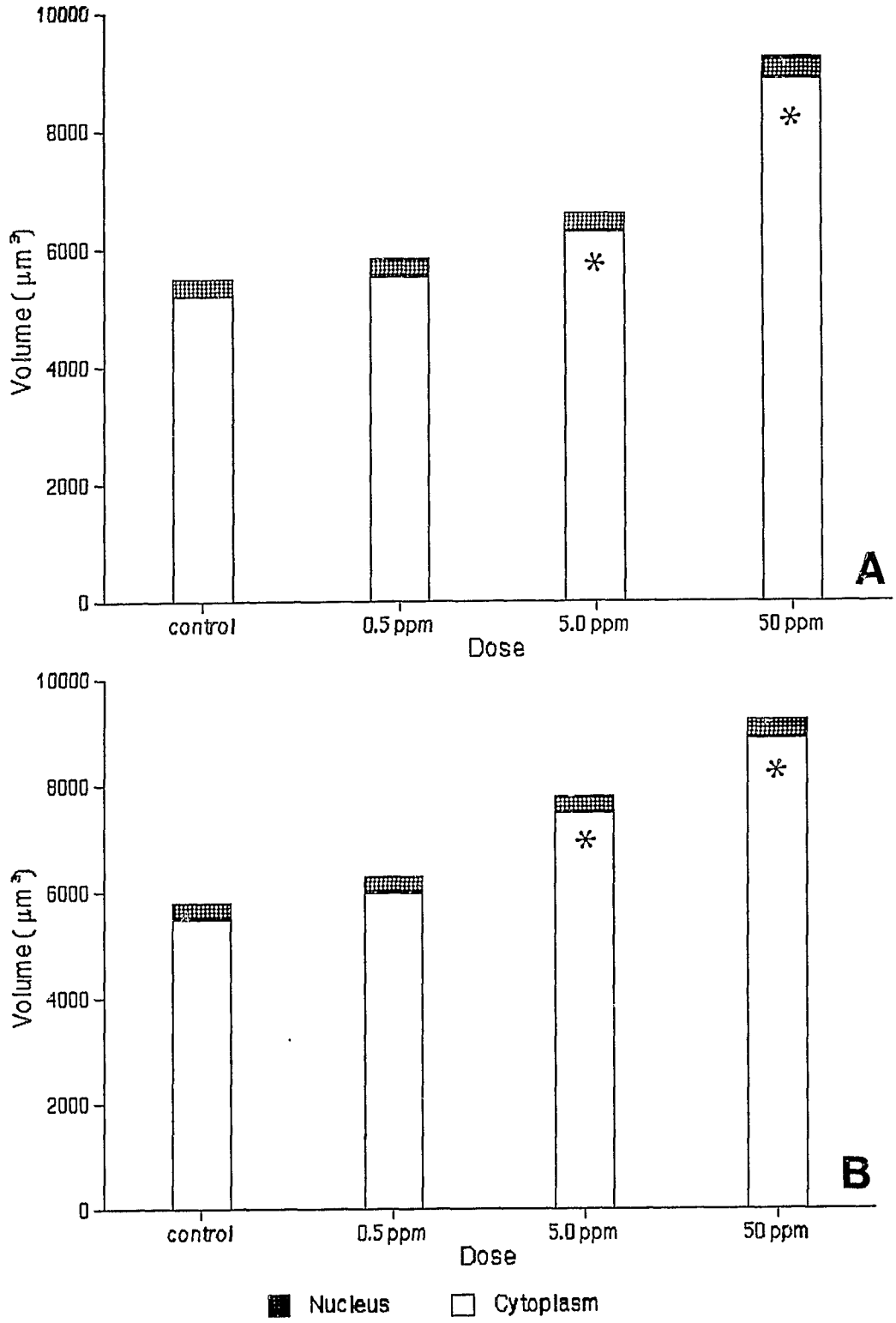


Figure 3.4. Cytoplasmic and nuclear volume after treatment with PCB in male (A) and female (B) rats. Asterisks indicate volume of cytoplasmic space that is significantly greater than those in the lower dose(s) and control in both genders (Duncan, df=8, $p<0.05$).

3.2.2. Cytoplasmic components

3.2.2.1. Smooth endoplasmic reticulum

The SER volume per hepatocyte augmented as the PCB dose increased. Significant increases above the control were seen in 5 and 50 ppm groups (Fig. 3.5). Also, a dose-dependent elevation of the organelle volume density was observed where more than 40% of the cytoplasm was occupied by SER at the highest dose (Fig. 3.6). Hypertrophy of SER in the centrilobular hepatocytes from 5 and 50 ppm PCB-treated rats appeared as a tubular network that was not usually associated with glycogen granules (Fig. 3.7-3.9) as in the cells from rats in control groups (Fig. 3.10).

Compared to those in controls, significant increases of total surface area of SER per hepatocyte and surface density of SER in cytoplasm were detected in 5 and 50 ppm group rats (Fig. 3.11 and 3.12), however, the surface of organelle to volume of organelle ratio did not change (Fig. 3.13).

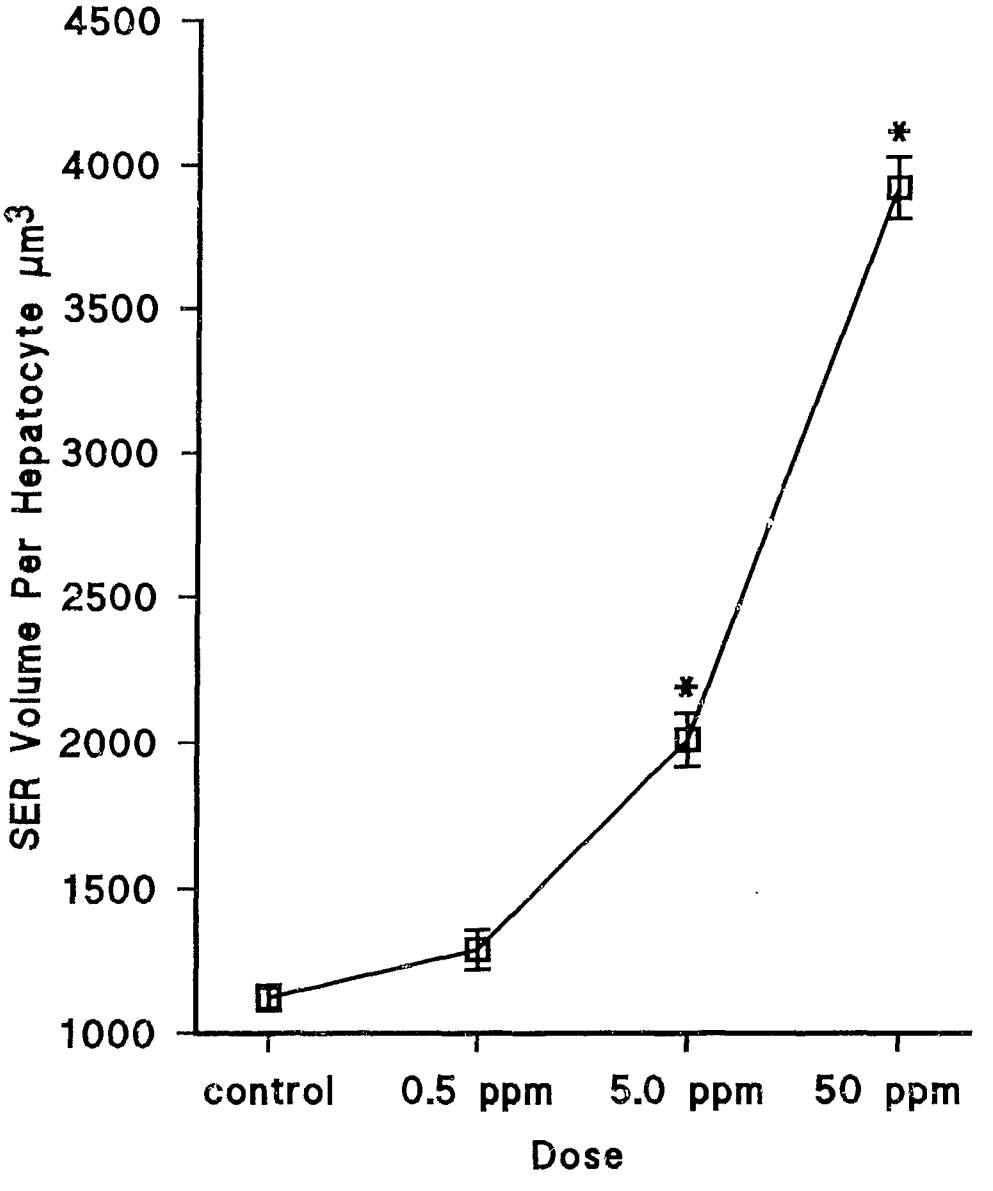


Figure 3.5. Mean volume of SER per hepatocyte. Asterisks indicate values that are significantly greater than those of lower dose(s) and control (Duncan, df=16, $p<0.05$). Data (□) from males and females are pooled at each dose because there is neither a gender difference nor a gender by treatment interaction.

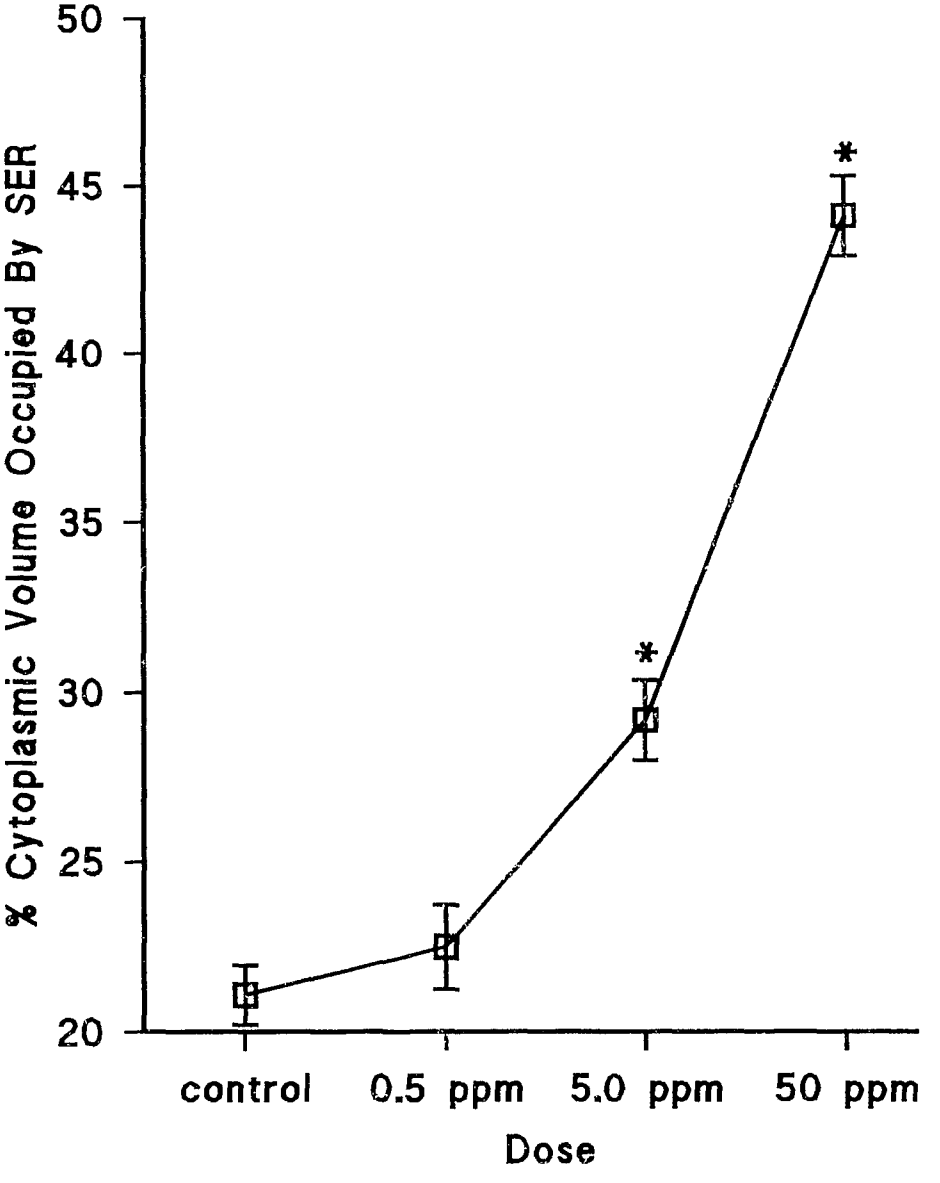


Figure 3.6. Volume density of SER in cytoplasm. Asterisks indicate the values that are significantly greater than those of lower dose(s) and control (Duncan, df=16, $p<0.05$). Data (□) from males and females are pooled at each dose because there is neither a gender difference nor a gender by treatment interaction.

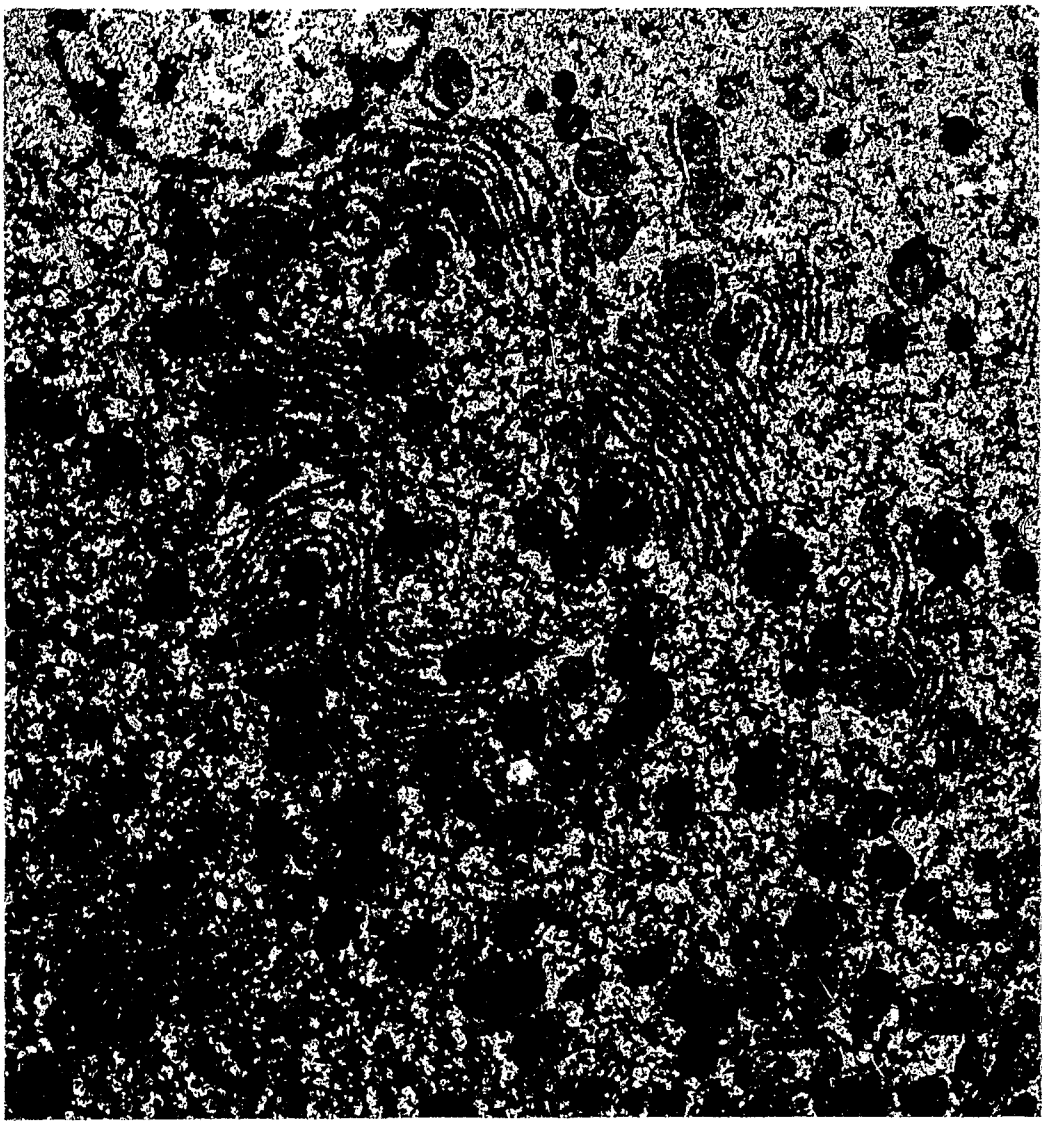


Figure 3.7. Electron micrograph of portion of a centrilobular hepatocyte from a female rat of 5 ppm group. An apparent increase of SER (S) and RER (R) profiles is demonstrated. The SER has a typical tubular network appearance and is not associated with glycogen granules. M: Mitochondria; P: Peroxisome. x 5,400

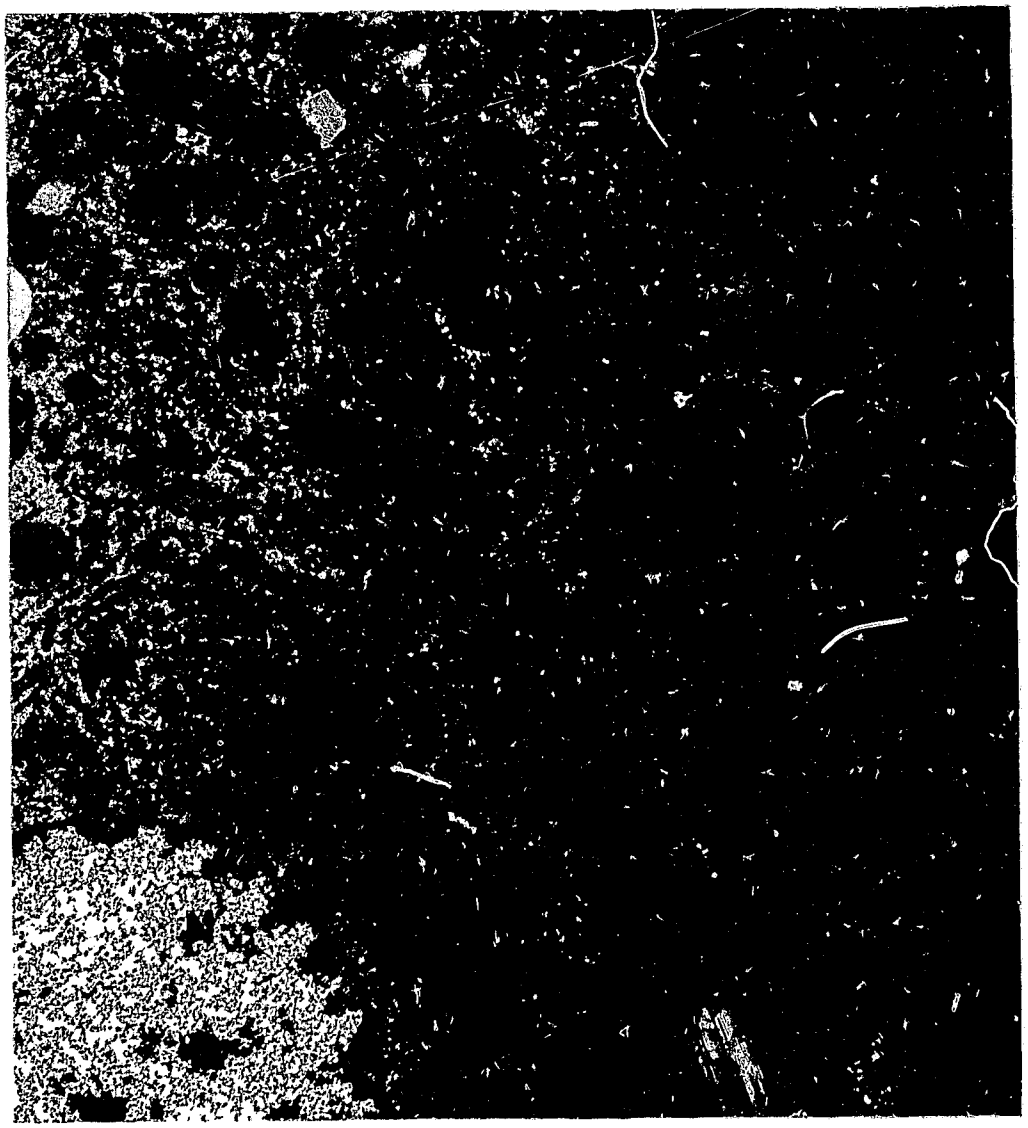


Figure 3.8. Electron micrograph of portion of a centrilobular hepatocyte from a male rat of 5 ppm group. Increased SER profiles (S) are clearly visible but cisternae of RER (R) are apparently shortened. Compare RER profiles in this illustration with those in the preceding. M: Mitochondria; N: Nucleus. x 5,400

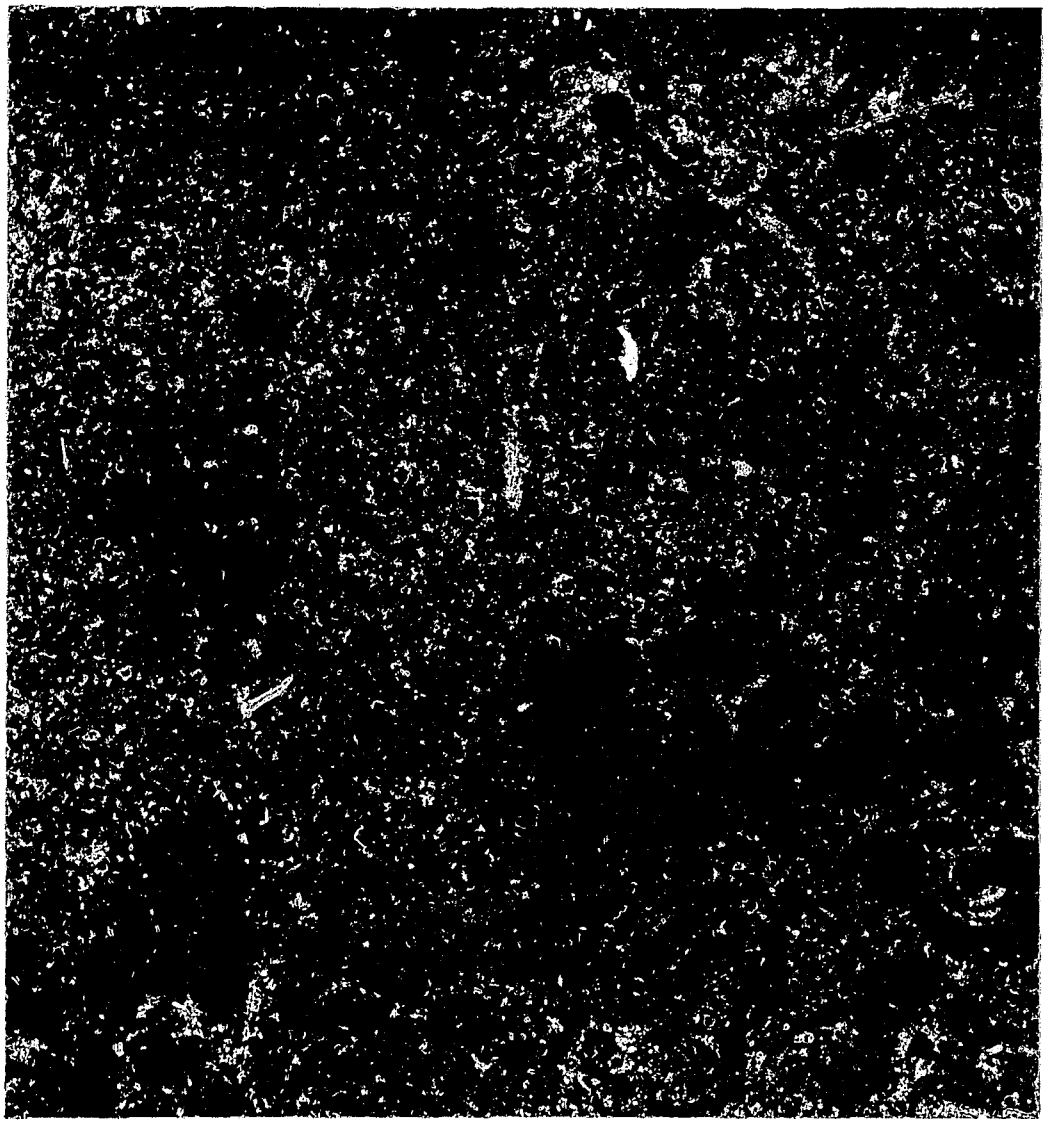


Figure 3.9. Electron micrograph of portion of a centrilobular hepatocyte from a male rat of 50 ppm group. A striking proliferation of SER profiles (S) with few cisternae of RER (R) is depicted. Gc: Golgi complex; L: Lysosomal element; P: Peroxisome. x 5,400

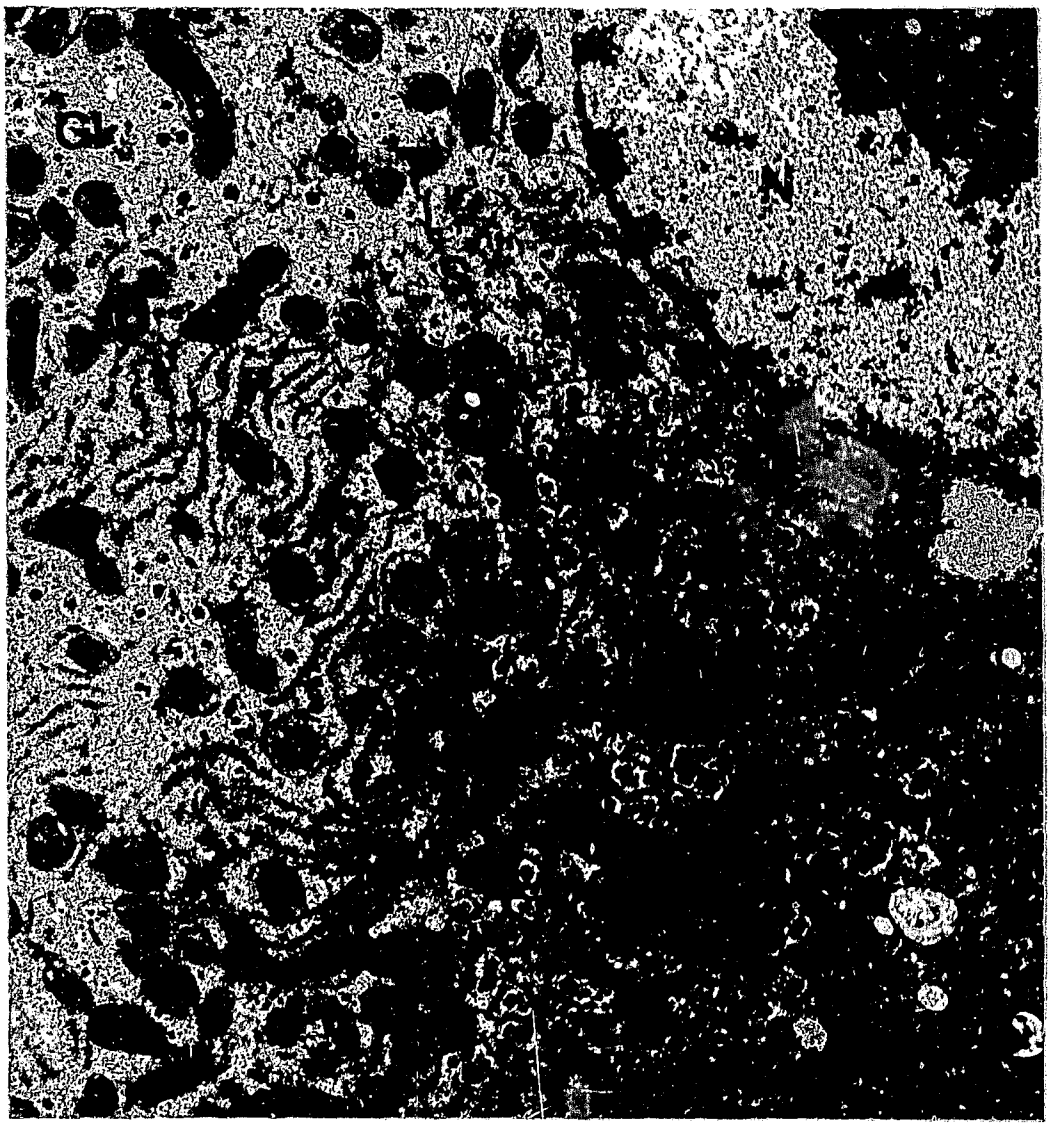


Figure 3.10. Electron micrograph of portion of a centrilobular hepatocyte from a female rat of control group. Note the characteristic form and distribution of rough endoplasmic reticulum (R) and smooth endoplasmic reticulum (S), Golgi complex (Gc), mitochondria (M) and glycogen granules (Gl). L: Lysosomal element; N: Nucleus; P: Peroxisome. x 5,400

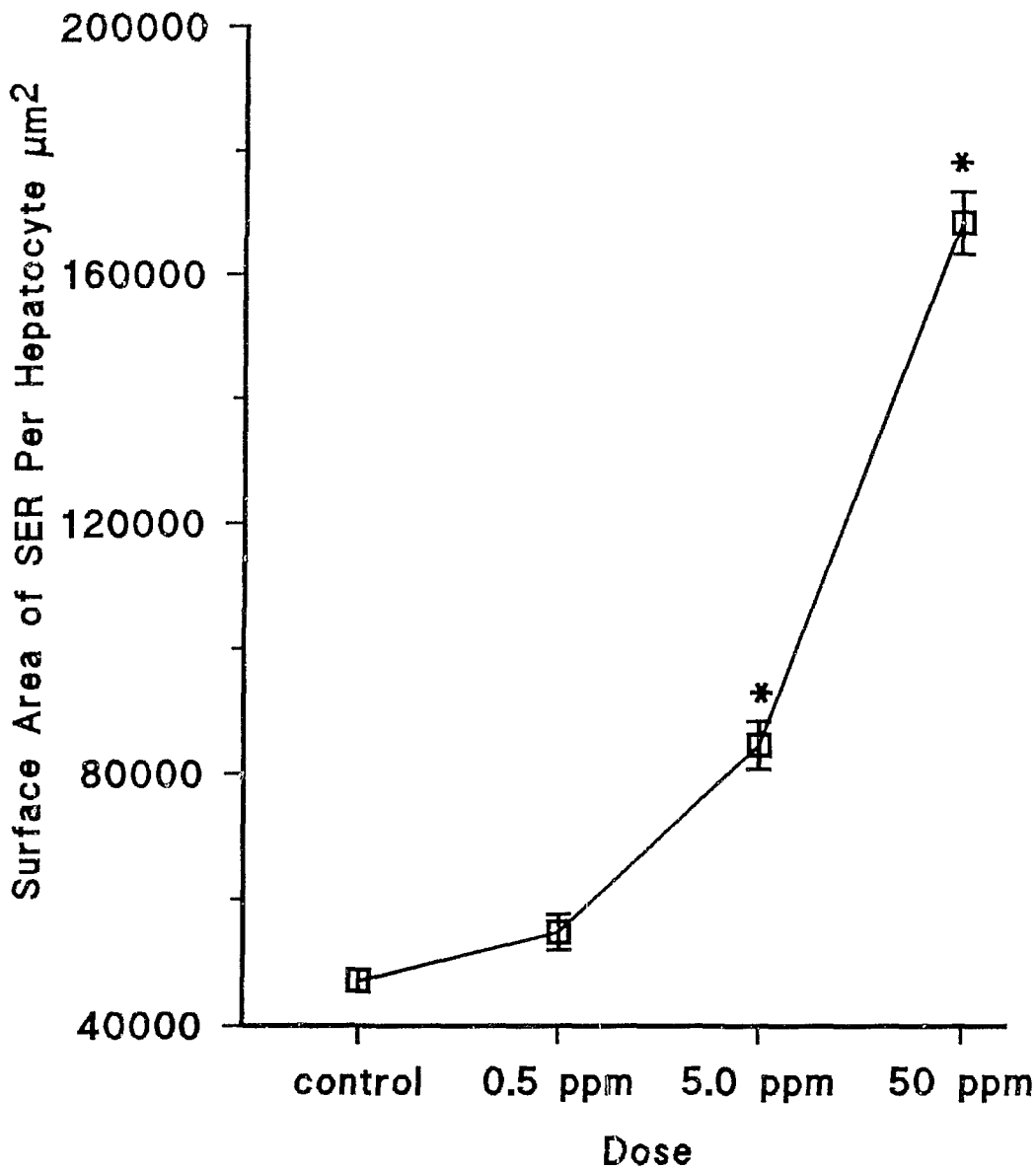


Figure 3.11. Mean surface area of SER per hepatocyte. Asterisks indicate values that are significantly greater than those of lower dose(s) and control (Duncan, df=16, p<0.05). Data (□) from males and females are pooled at each dose because there is neither a gender difference nor a gender by treatment interaction.

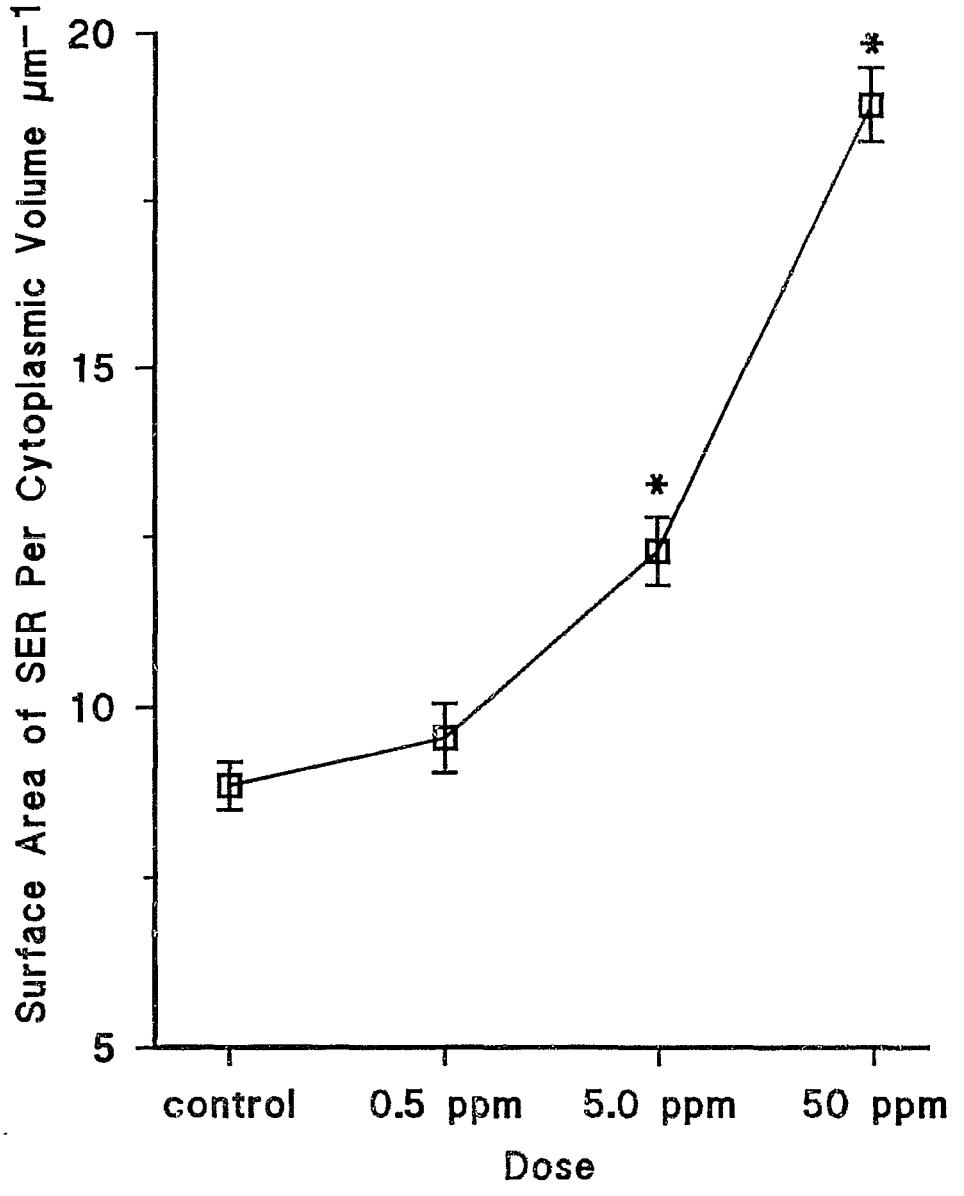


Figure 3.12. Surface density of SER in cytoplasm. Asterisks indicate values that are significantly greater than those of lower dose(s) and control (Duncan, df=16, $p<0.05$). Data (□) from males and females are pooled at each dose because there is neither a gender difference nor a gender by treatment interaction.

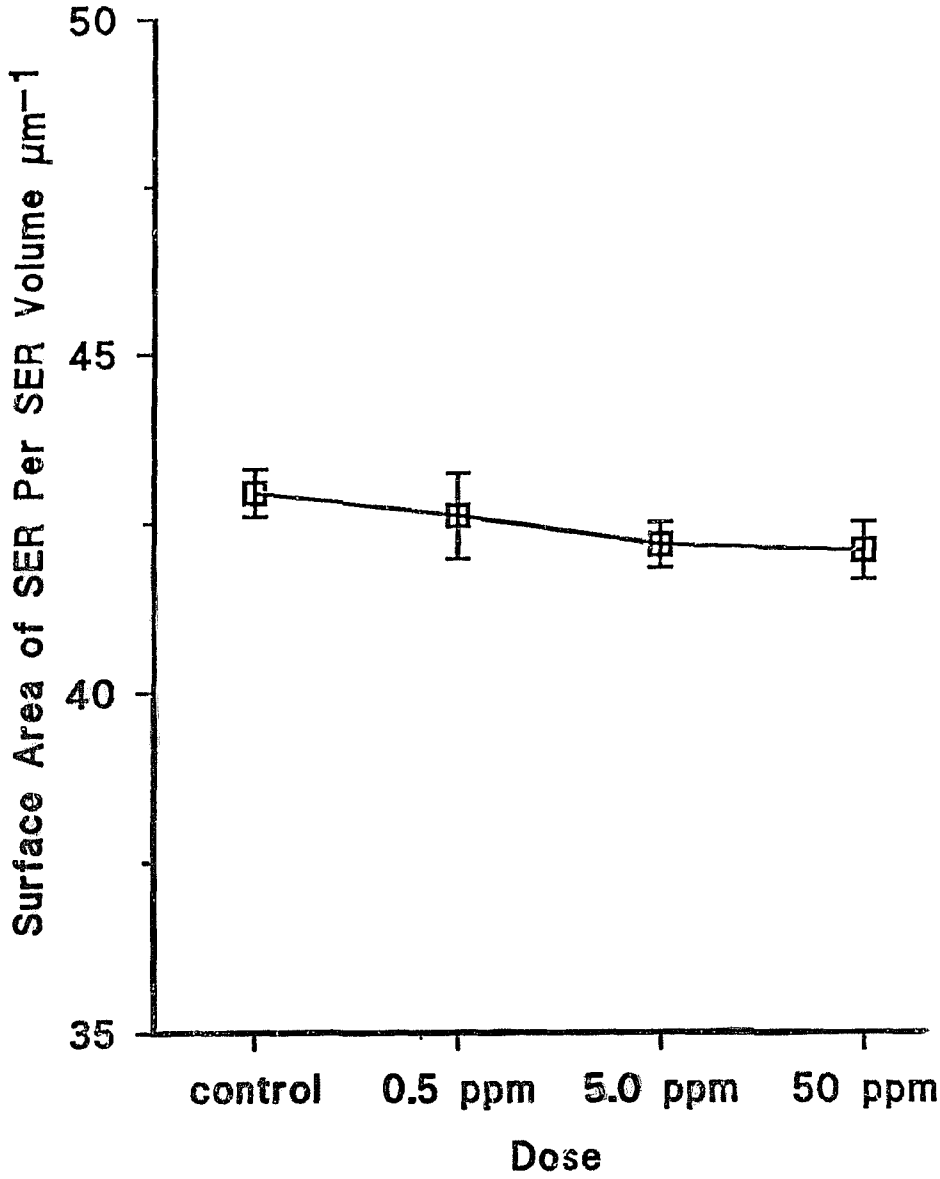


Figure 3.13. Surface area of SER per volume of SER. Values are maintained at approximately 42 μm^{-1} among treated groups. Data (□) from males and females are pooled at each dose because there is neither a gender difference nor a gender by treatment interaction.

3.2.2.2. Rough endoplasmic reticulum

The changes of RER volume in the hepatocyte differed between male and female animals after exposure to PCB 153 (Fig. 3.14). For the female, there were significant increases in RER volume in 5 and 50 ppm groups; but in the male, significant decreases were observed at the same doses. In addition, in male animals volume density of RER decreased from 12.9% in control to 7.6% in 5 ppm, and to 6% in 50 ppm groups whereas for females the value was maintained at approximately 10% (Fig. 3.15). Figures 3.7, 3.8 and 3.9 depict RER alterations; most of centrilobular hepatocytes of PCB-treated female rats show the wide, parallel stacks of RER cisternae (Fig. 3.7), however, the cisternae in many cells from treated male animals appeared shortened (Fig. 3.8), single or randomly oriented (Fig. 3.9).

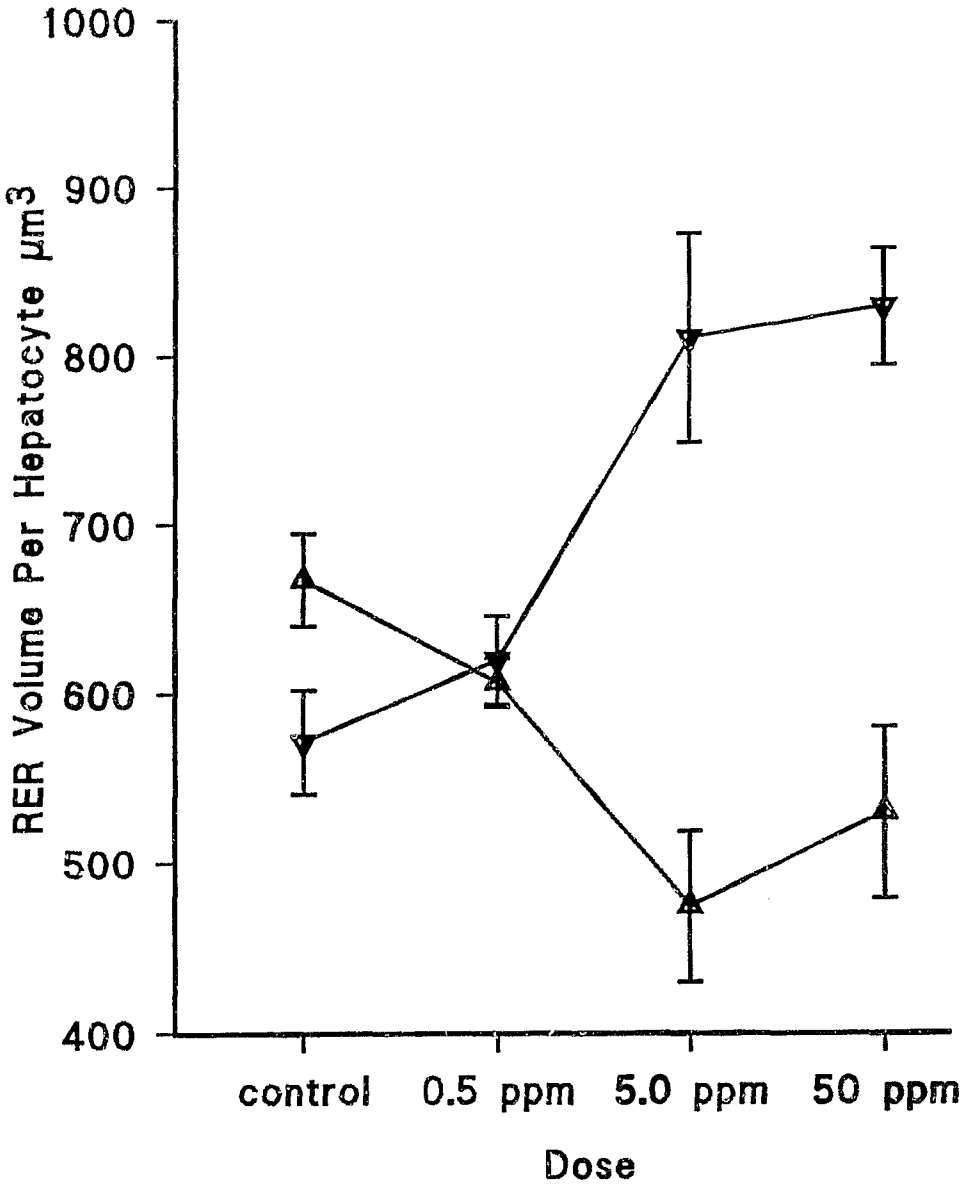


Figure 3.14. Mean volume of RER per hepatocyte. In males (△), RER volume decreases significantly at doses of 5 and 50 ppm (Duncan, df=8, $p<0.05$); in females (▽), this value significantly increases at the same doses (Duncan, df=8, $p<0.05$).

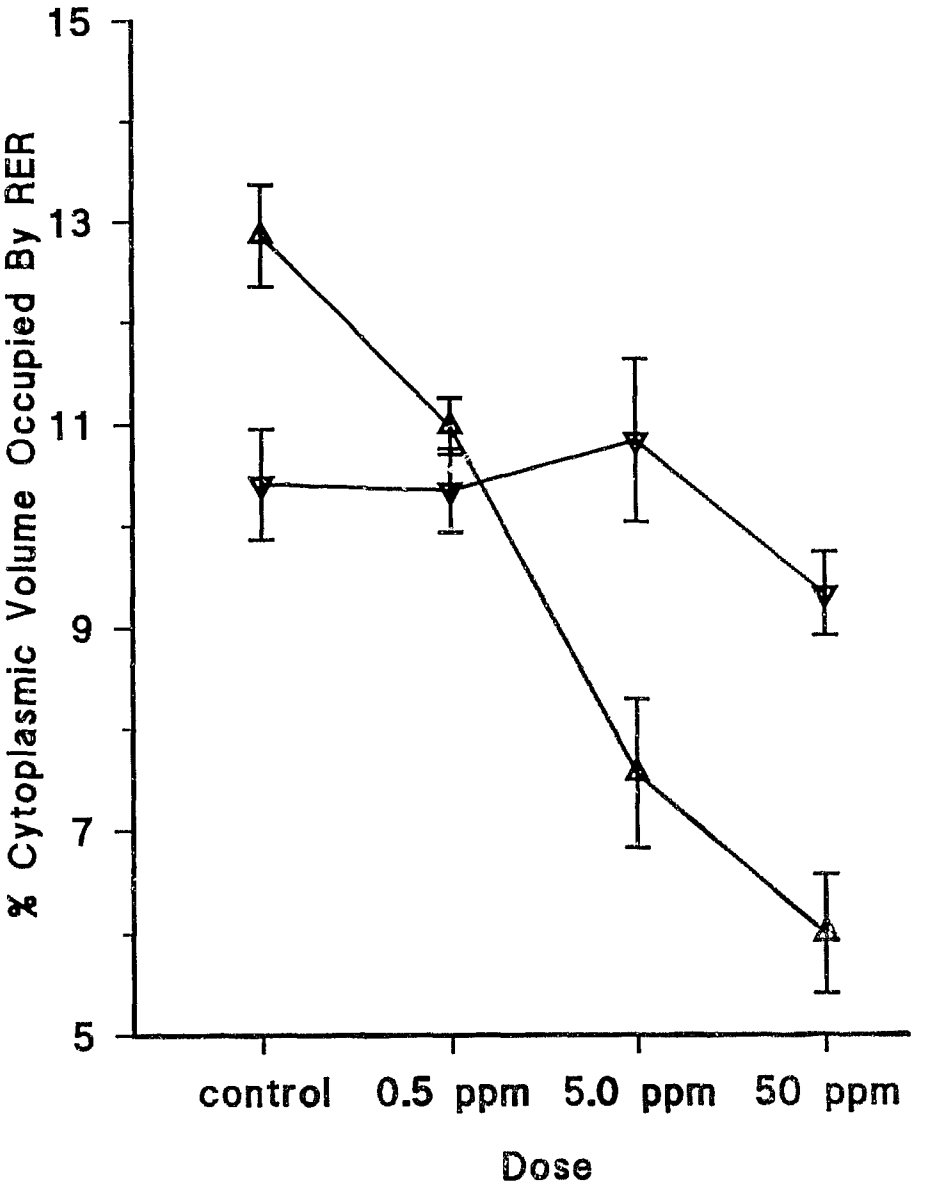


Figure 3.15. Volume density of RER in cytoplasm. Values in males (△) significantly decrease with increasing dose (Duncan, df=8, $p<0.05$). For females (▽), significant difference is not detected (Duncan, df=8, $p<0.05$).

3.2.2.3. Mitochondria

A significant decrease of the mitochondrial volume in hepatocytes of rats fed the 50 ppm PCB diet was detected (Fig. 3.16). Volume density of mitochondria decreased significantly at 5 and 50 ppm doses (Fig. 3.17).

3.2.2.4. Abnormal mitochondria type I

There was a trend towards an increase in volume of abnormal mitochondria type I (those with the longitudinally oriented cristae) in the hepatocytes of both male and female animals with elevated congener concentrations. However, a significant increase was only detected in female rats at 5 ppm (Fig. 3.18). Figure 3.19 illustrates several mitochondria that have parallel cristae in a hepatocyte from a treated female rat.

3.2.2.5. Abnormal mitochondria type II

As concentration of PCB increased, the volume of abnormal mitochondria type II, which included c- and ring-shapes, augmented in animals of both genders (Fig. 3.20). In comparison with that in the control, significant increases were found in those from 5 and 50 ppm PCB-treated groups of males, but not in females although the latter showed a high value at 50 ppm (Fig. 3.20). Figure 3.21 depicts c- or ring-shape mitochondria that are abundant in the hepatocyte.

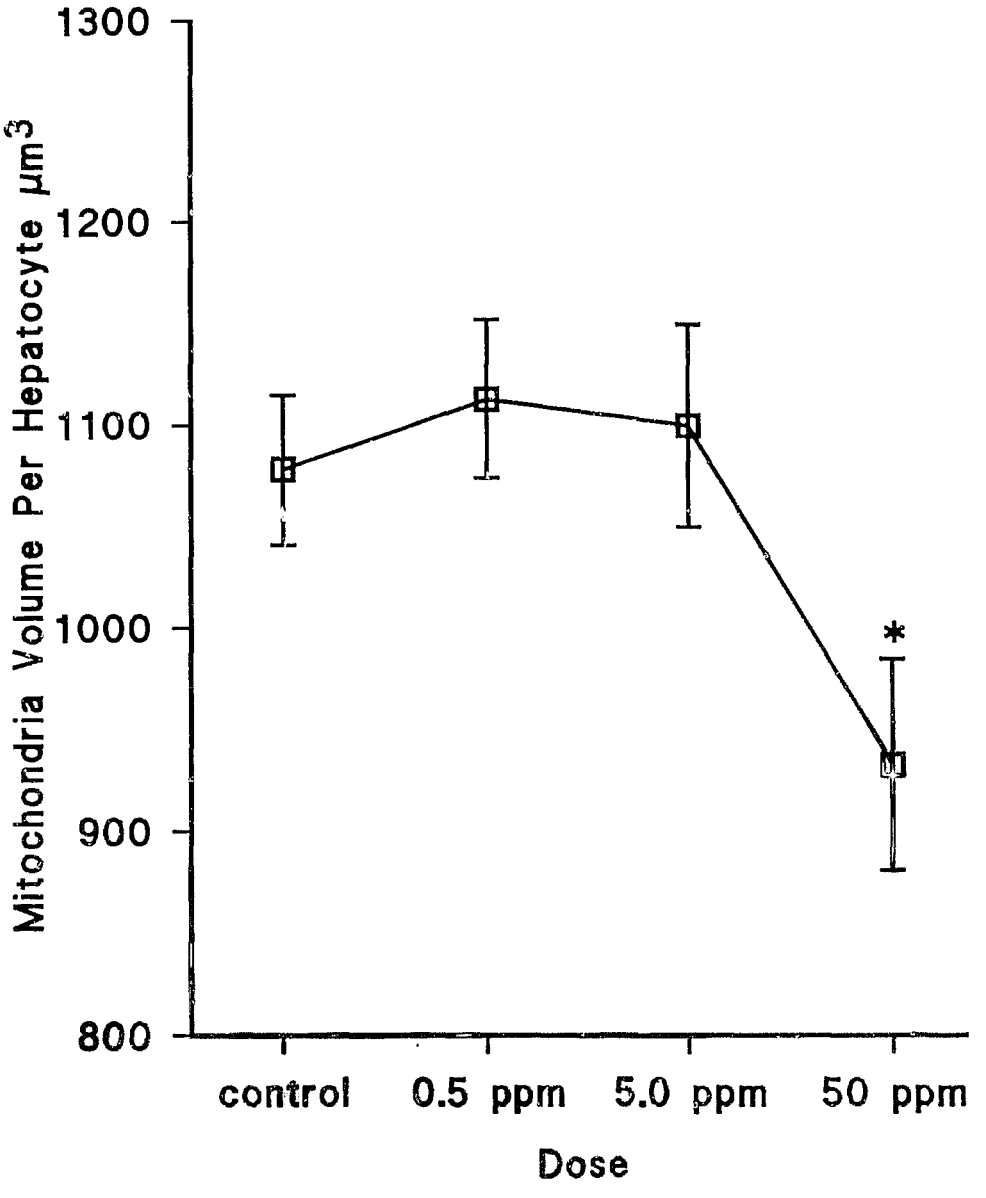


Figure 3.16. Mean volume of mitochondria per hepatocyte. Asterisk indicates the value that is significantly less than those of the lower doses and the control (Duncan, df=16, $p<0.05$). Data (□) from males and females are pooled at each dose because there is neither a gender difference nor a gender by treatment interaction.

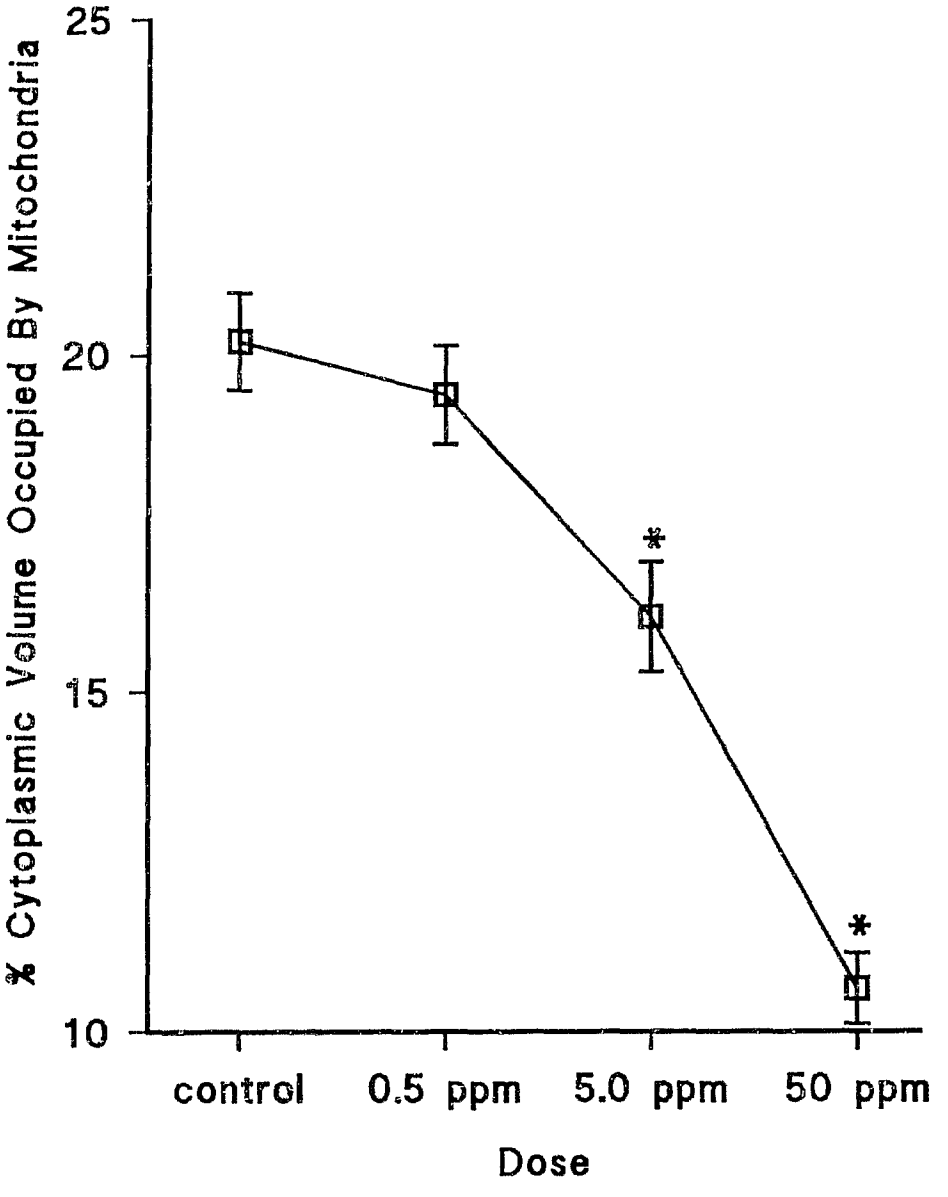


Figure 3.17. Volume density of the mitochondria in cytoplasm. Asterisks indicate values that are significantly less than those of the lower dose(s) and control (Duncan, df=16, $p<0.05$). Data (□) from males and females are pooled at each treated dose because there is neither a gender difference nor a gender by treatment interaction.

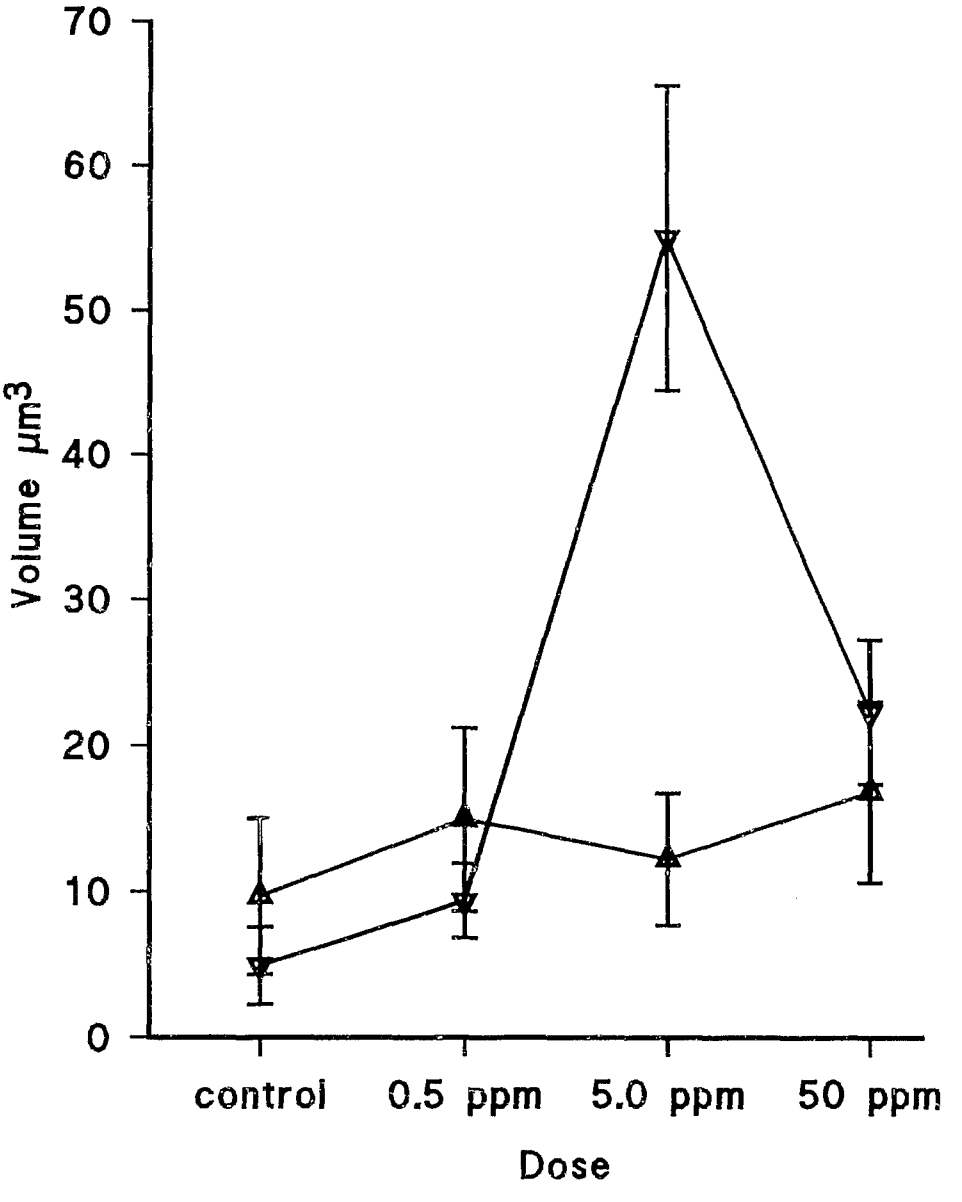


Figure 3.18. Mean volume of abnormal mitochondria type I. Value in females (▽) is significantly increased at 5 ppm compared to that of control (Duncan, df=8, $p<0.05$). Also, at 5 ppm, value in females is significantly higher than in males (△) (Duncan, df=16, $p<0.05$).

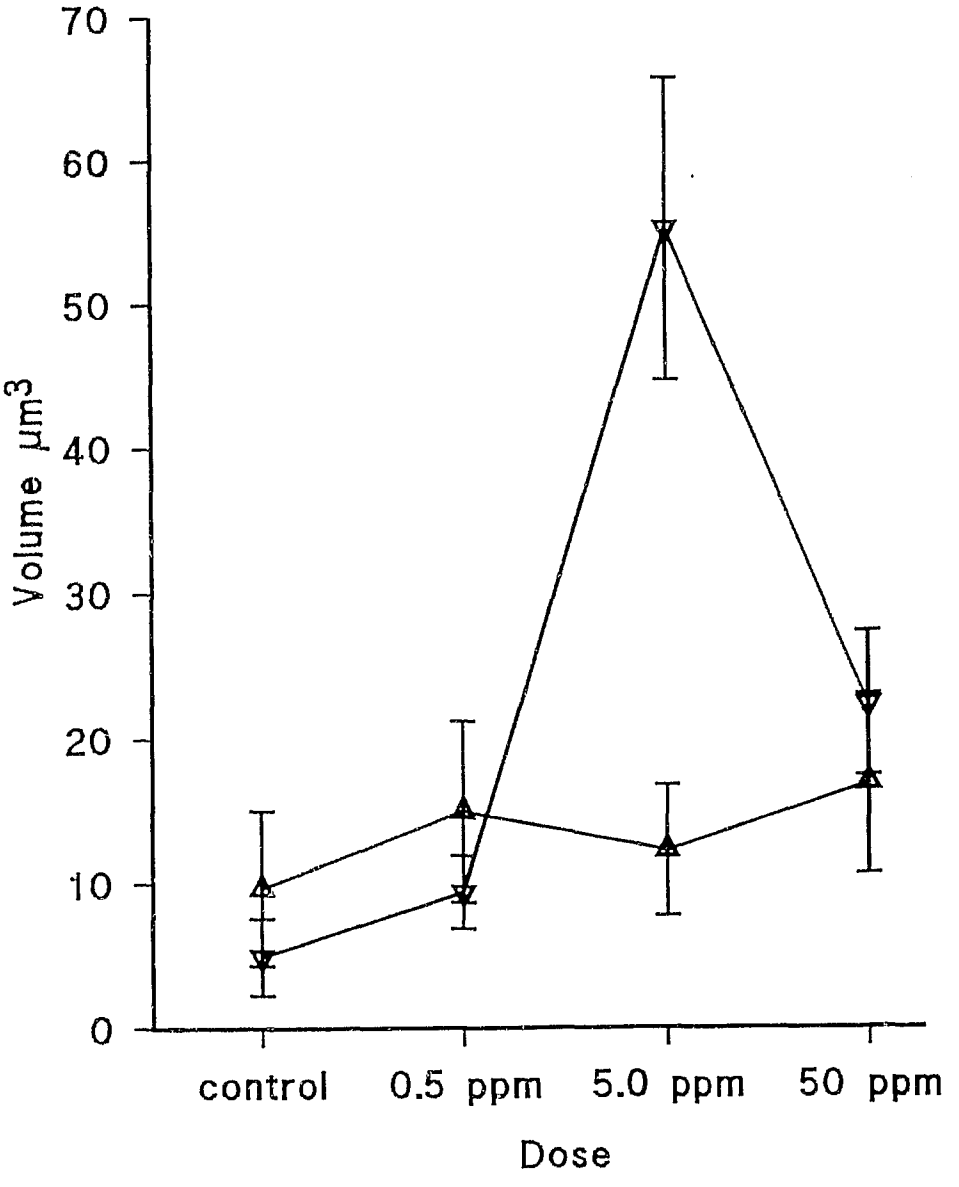


Figure 3.18. Mean volume of abnormal mitochondria type I. The value in females (▽) is significantly increased at 5 ppm compared to that of control (Duncan, df=8, $p<0.05$). Also, at 5 ppm, value in females is significantly higher than in males (△) (Duncan, df=16, $p<0.05$).

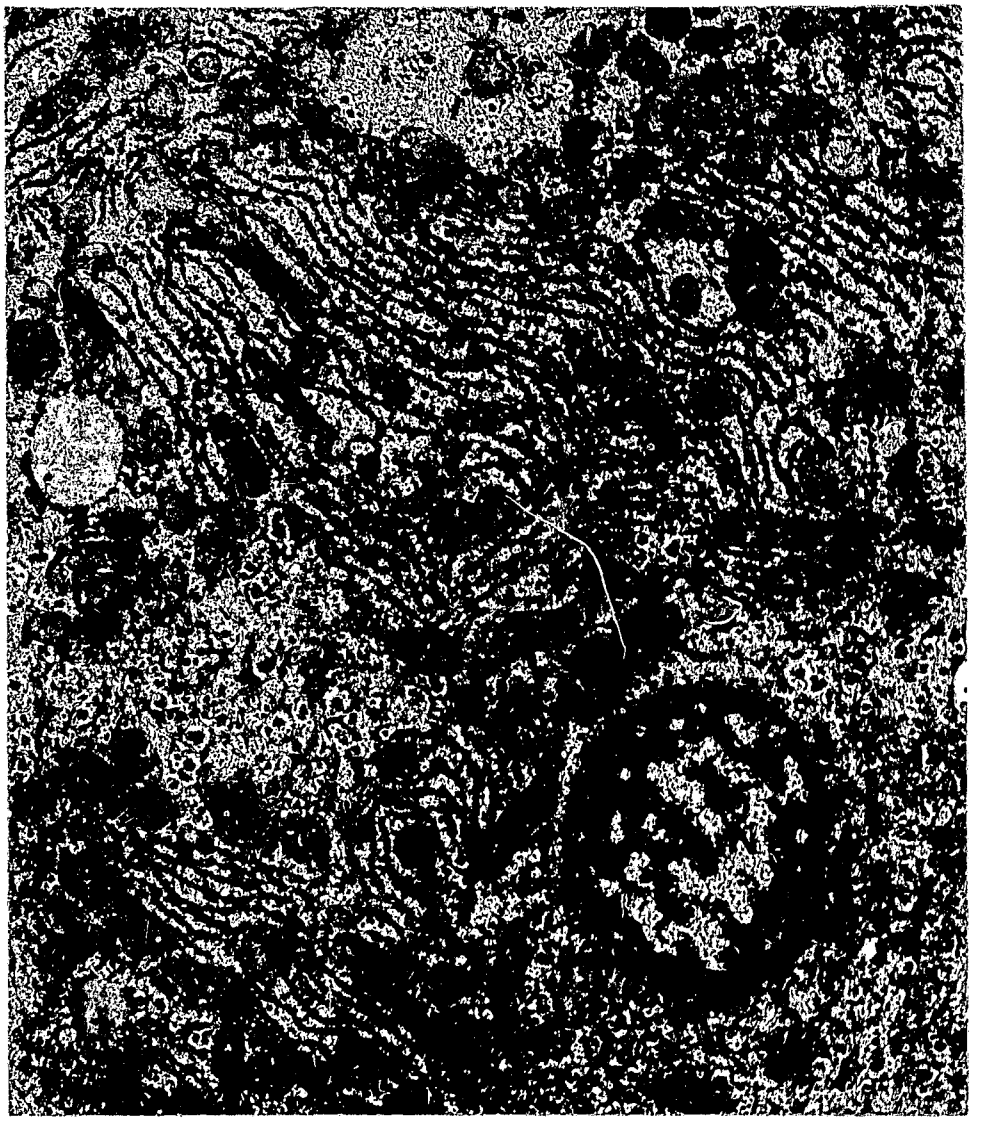


Figure 3.19. Electron micrograph of portion of a centrilobular hepatocyte from a female rat of 5 ppm group. Note mitochondria with longitudinally-oriented cristae (arrows). N: Nucleus; R: RER; S: SER. x 5,400

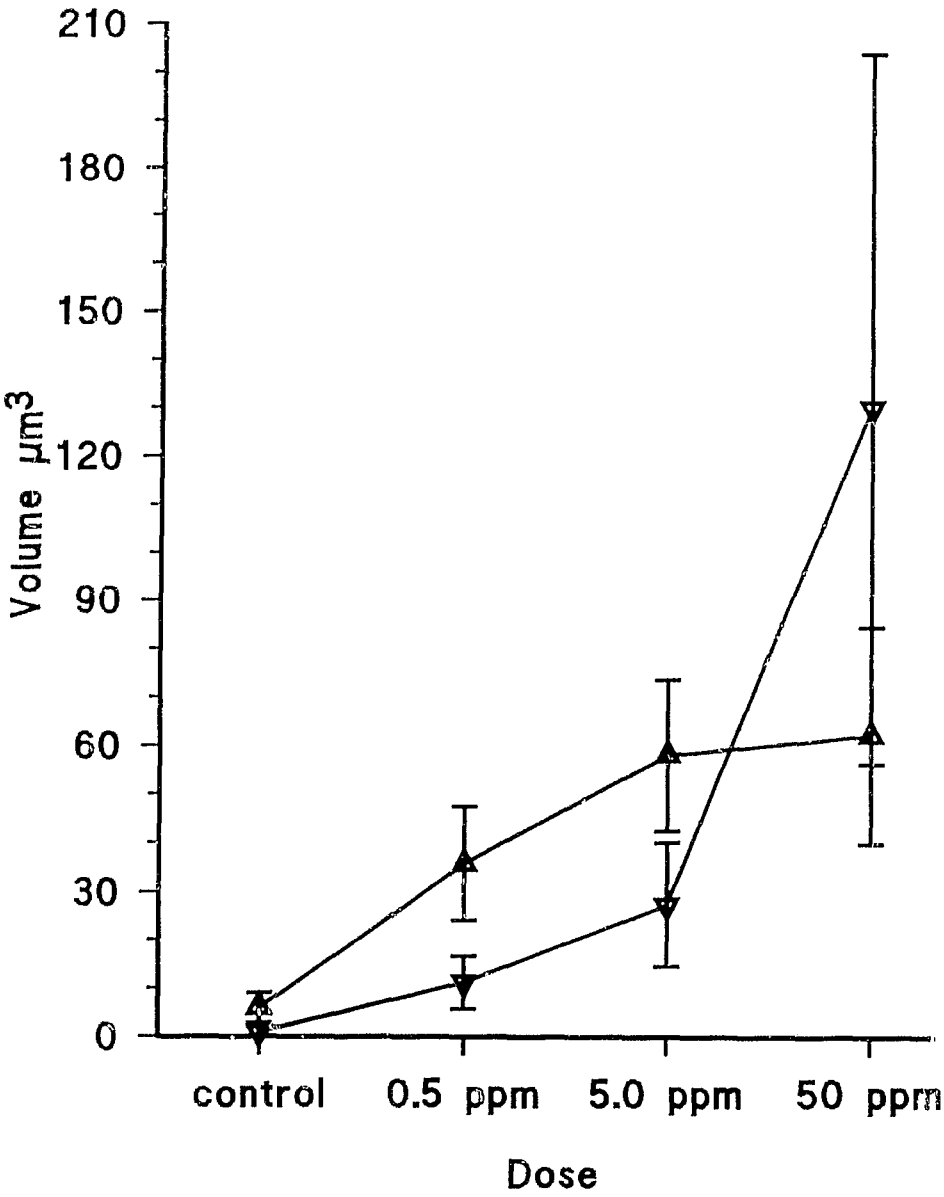


Figure 3.20. Mean volume of abnormal mitochondria type II. Values for 5 and 50 ppm groups of males (△) are significantly greater than those in controls (Duncan, df=8, $p<0.05$). Significant differences are not detected in female groups (▽) (Duncan, df=8, $p<0.05$).

Figure 3.21. Electron micrograph of portion of a centrilobular hepatocyte from female rat of 50 ppm group. Many c- (arrowhead) or ring-shape mitochondria (arrows) and peroxisome (P) are depicted in this image. Letters S and R denote regions of proliferated SER and RER, respectively. x 5,400

3.2.2.6. Lipid droplets

An effect of PCB exposure on lipid droplets in liver was not detected, however, a gender difference was seen (Fig. 3.22-3.23). For example, in control group, mean volume of lipid droplets per hepatocyte from the female measured 19.3 μm^3 that occupied only 0.35% of the cytoplasm; for the male, this value was as high as approximately 180 μm^3, occupying 3.45% of the cytoplasm volume.

3.2.2.7. Lysosomal elements

There was a significant increase of mean volume of lysosomal elements per hepatocyte in 50 ppm PCB-treated rats (Fig. 3.24). These alterations were not significant in the cells from animals of the remaining groups. The observed increase in lysosomal elements in hepatocytes from 50 ppm PCB-treated rats is illustrated in Fig. 3.25.

3.2.2.8. Peroxisomes

Volume of peroxisomes per hepatocyte in female animals showed a significant increase at 5 and 50 ppm. for males a significant increase was detected only in 50 ppm group (Fig. 3.26). Generally, peroxisome volume per cell was higher in female rats than in the males (Fig.3.26).

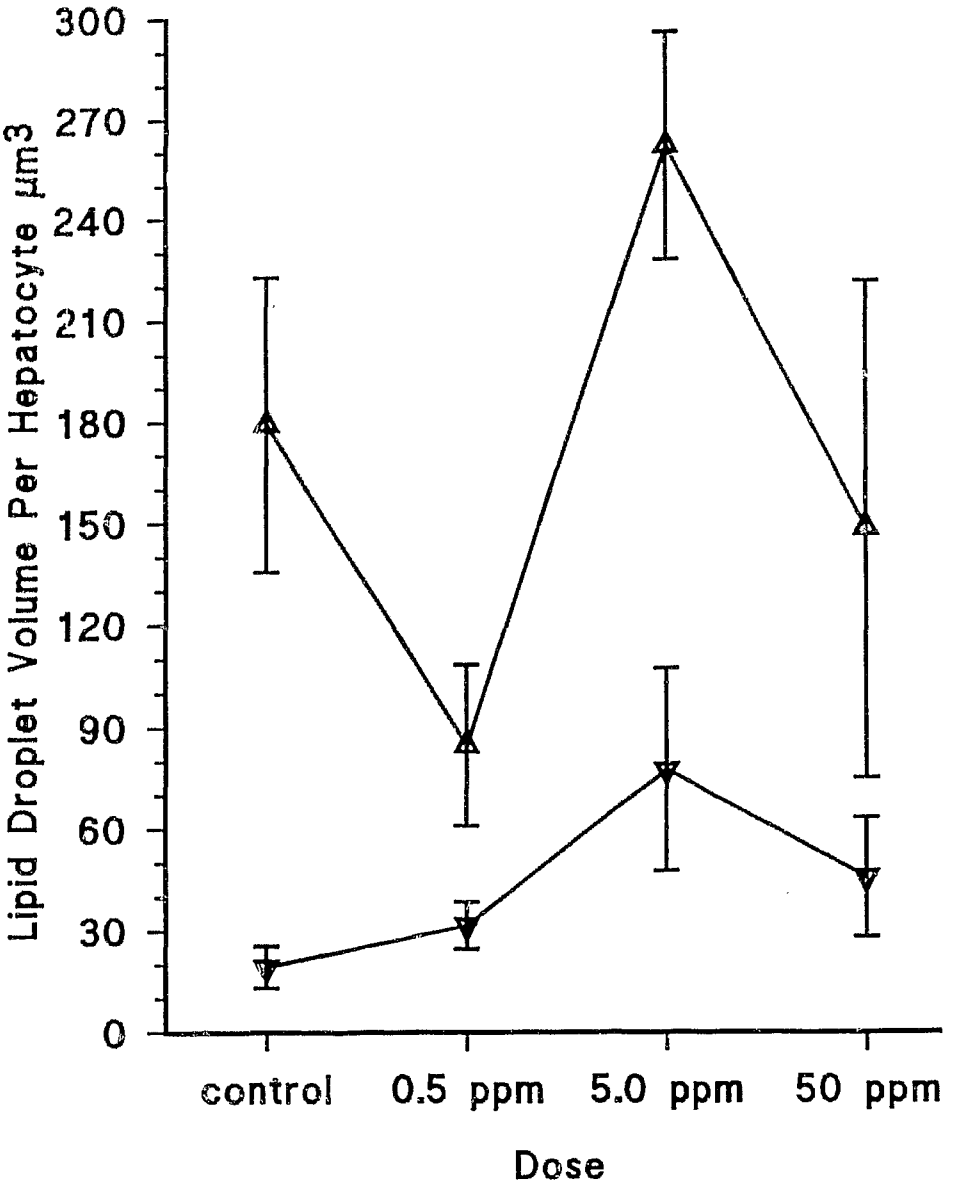


Figure 3.22. Mean volume of lipid droplets per hepatocyte. The value is significantly higher in males (△) than in females (▽) (Duncan, df=16, $p<0.05$).

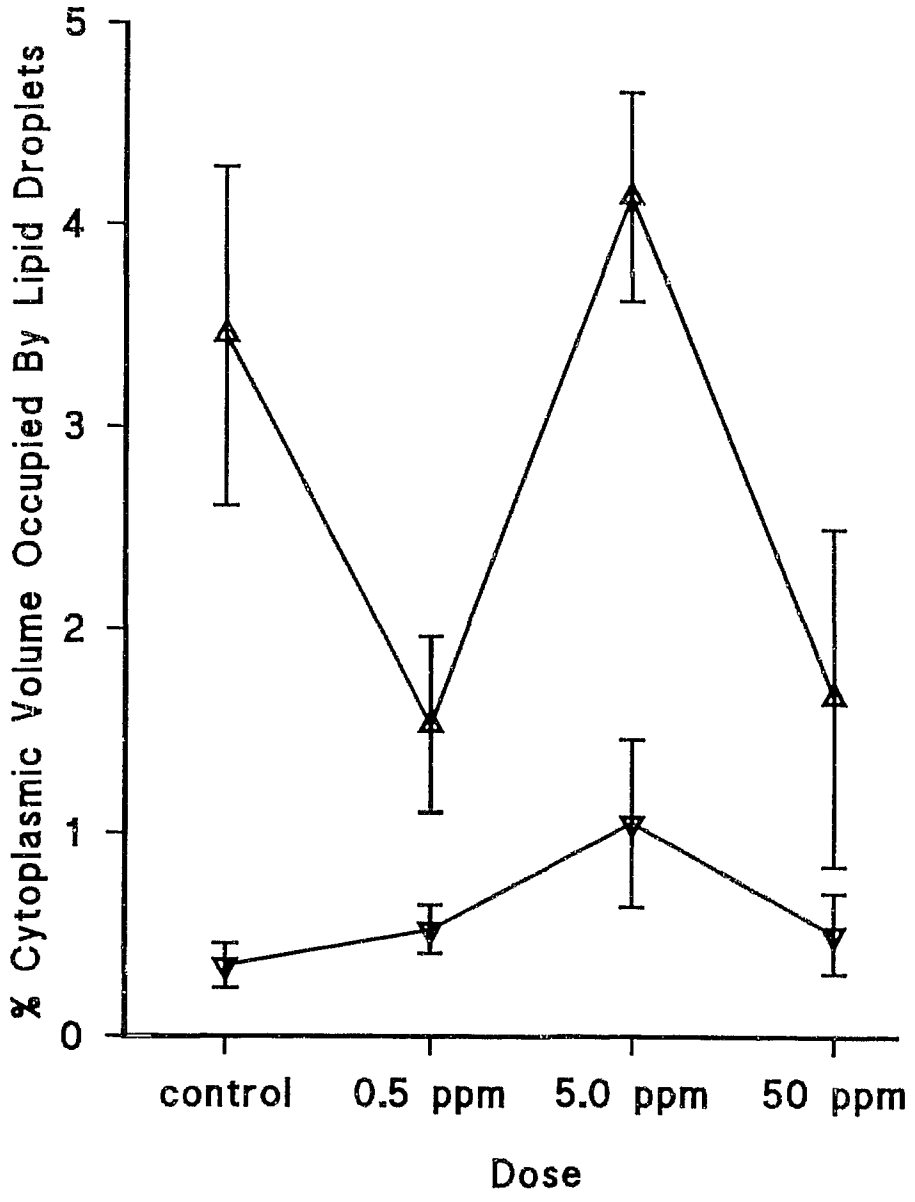


Figure 3.23. Volume density of lipid droplets in cytoplasm. The value is significantly higher in males (△) than in females (▽) (Duncan, df=16, $p<0.05$).

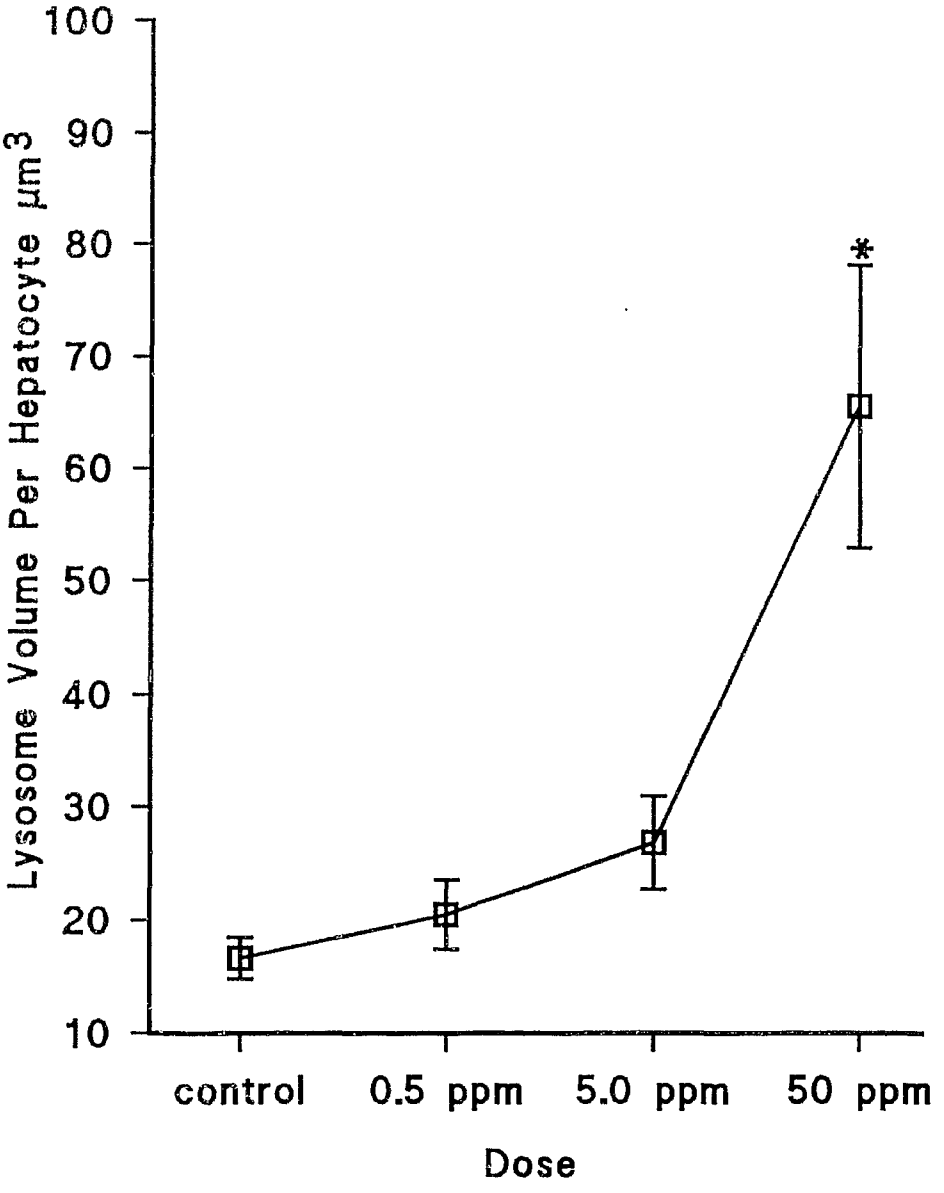


Figure 3.24. Mean volume of lysosomal elements per hepatocyte. Asterisk indicates the value that is significantly higher than those in lower doses and control (Duncan, df=16, $p<0.05$). Data (□) from males and females are pooled at each treated dose because there is neither a gender difference nor a gender by treatment interaction.

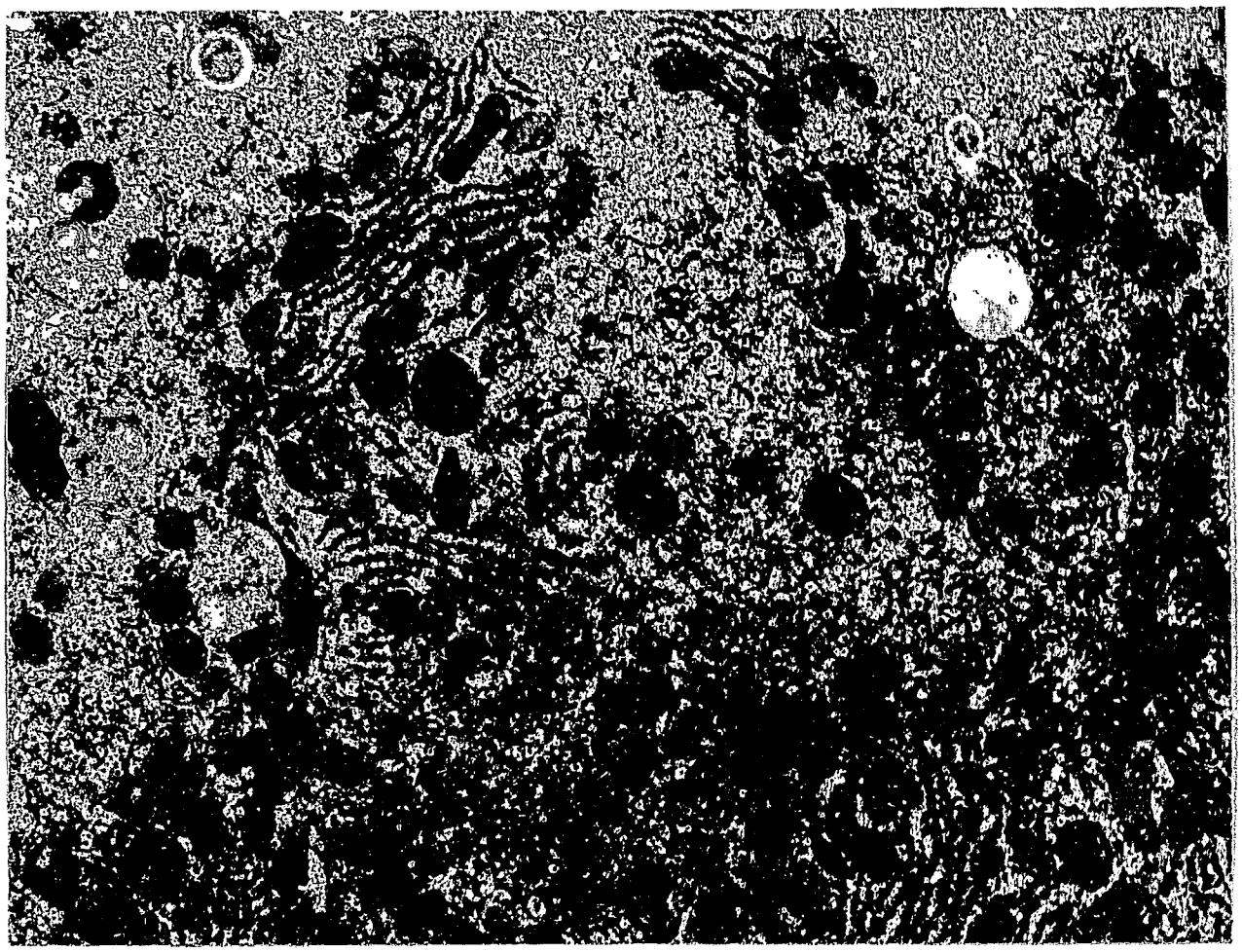


Figure 3.25. Electron micrograph of portion of a centrilobular hepatocyte from a female rat of 50 ppm group. The number of lysosomal elements (L) appears augmented. M: Mitochondria; R: RER; S: SER. x 5,400

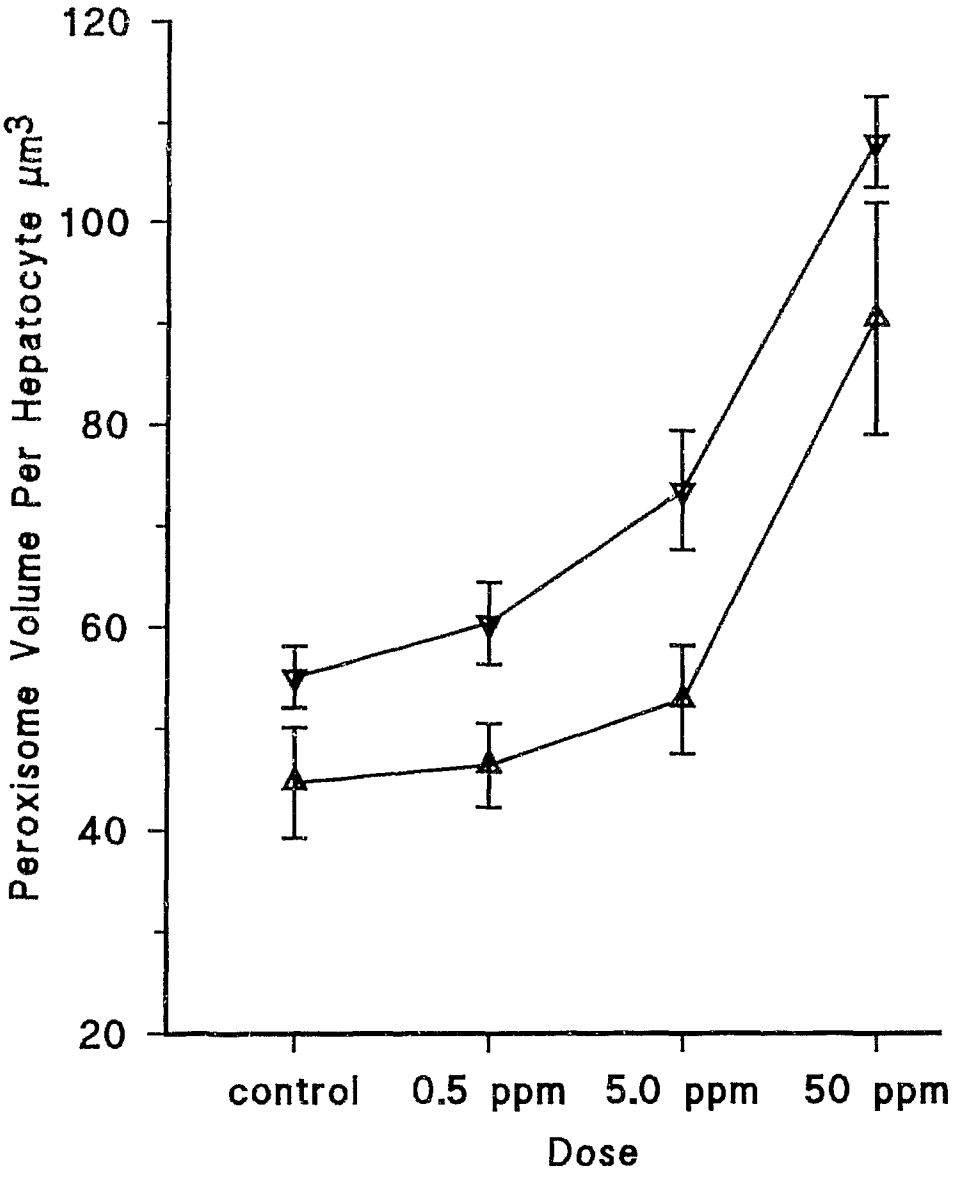


Figure 3.26. Mean volume of peroxisomes per hepatocyte. In females (▽), values are significantly higher at 5 and 50 ppm than in control; in males (△), a significant increase of peroxisome volume per hepatocyte in comparison with those in control is detected at 50 ppm (Duncan, df=8, $p<0.05$). Generally, values are greater in females than in males (Duncan, df=16, $p<0.05$).

3.2.3. Summary

The ultrastructure alterations of hepatocytes after administration of PCB to rats are summarized in Table 3.2.

Table 3.2: Summary of ultrastructure alterations

Component Volume	Dose effect	Gender effect	Interaction (Dose by Gender)
Hepatocyte	↑ (5,		↑ in ♀ compared to ♂ at 0.5, 5 ppm
Nucleus	↑ (50 ppm)		
Cytoplasm	↑ (5, 50 ppm)	♀ > ♂	↑ in ♀ compared to ♂ at 0.5, 5 ppm
SER	↑ (5, 50 ppm)		
RER		♀ > ♂	↑ in ♀ but ↓ in ♂ at 5, 50 ppm
Mitochondria	↓ (50 ppm)		
Type I[a]	↑ (5 ppm)		↑ in ♀ compared to ♂ at 5 ppm
Type II[b]			↑ is only in ♂ at 5, 50 ppm
Lipid droplet		♂ > ♀	
Lysosome	↑ (50 ppm)		
Peroxisome	↑ (50 ppm)	♀ > ♂	

↑: significant increase; ↓: significant decrease at indicated doses
♀ > ♂: the value in females is significantly greater than in males
♂ > ♀: the value in males is significantly greater than in females
a: abnormal mitochondria type I b: abnormal mitochondria type II

Chapter 4. Discussion and Conclusion

Hypertrophy of hepatocytes, increased SER profiles, RER alteration, abnormal mitochondria, and augmentation of peroxisomes and lysosomal elements were revealed in liver of rats treated with PCB 153 for 13 weeks. These observations were subjected to morphometric analyses, and were determined to be dose-dependent.

4.1. Fixation artifacts

A loss of membrane structures and cytoplasmic inclusions such as hepatocyte glycogen granules was observed in 1-week-buffer-held specimens from animals fed PCB 156, and in "Fixation" experiments. These artifacts likely appeared because of prolonged stay of specimens in a buffer solution after fixing in glutaraldehyde and post-fixing in osmium tetroxide solutions. Glutaraldehyde's attribute as a fixative is in its ability to cross-link protein. Osmium tetroxide works as a fixative by reacting primarily with lipid moieties. Application of glutaraldehyde followed by osmium tetroxide is considered a standard fixation protocol, capable of stabilizing a maximum number of different cell components (Bozzola and Russell, 1992). Poor fixation may be a consequence of the fact that some of the cross-links introduced by glutaraldehyde are potentially reversible (Hayat, 1989). The present study revealed that the fixation was partially reversible because keeping specimens in a buffer for one week (as a result of limited facilities and time for further processing the specimens) produced undesirable

effects as described above in "Results". Specimens should not be held in a buffer solution for a period longer than that stated in an estabilished protocol.

4.2. Morphometry

In the present study, ultrastructure alterations of centrilobular hepatocytes were investigated morphometrically after administration of PCB 153 in the diet of weanling Sprague-Dawley rats for 13 weeks. Cell hypertrophy, SER proliferation, RER alterations, mitochondrial abnormalities, and augmentations of peroxisomes and lysosomal elements were the most conspicuous changes in hepatocytes. Alterations were dose-dependent and were most severe in 50 ppm groups. Before discussing the significance of morphologic alterations, possible sources of systematic errors in the measurements will be addressed.

4.2.1. Systematic errors of measurement

Three basic sources of systematic error are expected that affect data: tissue shrinkage during processing, section thickness, and compression in tissue sectioning. Compression causes a reduction in section length of the block face in the direction of the cutting stroke, while the width remains unchanged (Weibel, 1979). If all components in a tissue are about equally affected upon sectioning, volu density estimate should not be in error, however, surface density will be overestimated (Weibel, 1979). The effect of tissue shrinkage is in some way similar to that of compression, although it acts, not in one, but in three dimensions. Volume density is not affected by

shrinkage and surface density is overestimated (Weibel, 1979). According to Weibel (1979), volume density and surface density will be overestimated because of section thickness. This is termed the Holmes effect. However, membrane structures such as SER are difficult to recognize when sectioned at an angle of less than 45^0 (Loud, 1967). This effect causes underestimation of the above two parameters and is an essential compensation for the Holmes effect (Weibel et al., 1969). The data from the present study were not corrected because it is not known which correction factors are relevant. Moreover, for comparative work such a correction is unnecessary because it can be assumed that all experimental groups are affected equally by the same degree of systematic errors.

4.2.2. Morphologic alterations

4.2.2.1. Hepatocyte

Exposure of Sprague-Dawley rats to PCB 153 via the diet for 13 weeks resulted in an increase of the relative liver weight (as % bw) of male and female rats at the highest dose (Appendix C). Hypertrophy of centrilobular hepatocytes was, in part, responsible for the liver enlargement. Cytoplasm of the cells contributed to the increase in cell volume in an overwhelming fashion. Although the volume of the nucleus underwent a significant increase at 50 ppm, its participation in the cell hypertrophy was regarded as slight.

4.2.2.2. Cytoplasmic organelles

4.2.2.2.1. Smooth endoplasmic reticulum

Exposure to the PCB resulted in a marked increase of SER volume and surface area in hepatocytes, especially in the 5 and 50 ppm groups of both genders in the present study. Our data indicate SER played the largest part in the increase of cytoplasm volume or hypertrophy of the cells because the increase in SER volume accounted for approximately 60- and 80% of the increase in cytoplasmic volume at 5 and 50 ppm, respectively. Proliferation of SER is a non-specific and reversible response to a variety of xenobiotics including halogenated hydrocarbons and is considered to be a structural reflection of enhanced metabolism of foreign lipophilic compounds (Hansell and Ecobichon, 1974). Drug-metabolizing enzymes are known to be located on SER membranes, therefore, proliferation of SER can be related to increased amounts of drug-metabolizing enzymes resulting in enhanced enzyme activity in the liver (Vos and Notenboom-Ram, 1972; Farber and Fisher, 1979; Render et al., 1982). This fact was confirmed in a biochemical study which showed increased hepatic aminopyrine N-demethylase (APDM) activity induced by the congerner (Chu et al., in press). Literature is replete with reports of SER proliferation after administration of PCBs (Kimbrough et al., 1972; Hansell and Ecobichon, 1974; Kasza et al., 1978; Harris and Bradshaw, 1984; Gillette et al., 1987; MacLellan et al., 1994 a,b,c), tetrachlorodibenzodioxin (Schecter et al., 1984, 1985), dichlorodiphenyltrichloroethane (Jonsson et al., 1981), and chlorinated diphenyl ethers (Chui et al., 1985). The SER augmentation after PCB exposure was closely related to the degree of chlorination and

to type of drug-metabolizing enzymes induced (Hansell and Ecobichon, 1974; Harris and Bradshaw, 1984; Gillette et al., 1987). The more chlorinated a compound is, greater is the effect on SER because metabolic studies have shown that lower chlorinated analogs are eliminated from tissues more rapidly than the highly chlorinated compounds (Grant et al., 1971; Bickers et al., 1972; Hansell and Ecobichon, 1974). PCB 153 is known as a typical PB-type inducer (Goldstein et al., 1977; Parkinson et al., 1983; Graves et al., 1990; Van Der Kolk et al., 1992). The PB-type inducers such as 2,2′,4,4′-tetraCB that can induce APDM caused a dramatic increase in volume density of SER in the hepatocyte, however, 3-MC-type inducers like 3,3′,4,4′-tetraCB which can induce AHH or EROD did not stimulate SER proliferation in rats (Hodgson, 1980; Gillette et al., 1987; Peng et al., 1995). Why this proliferation occurs in response to only certain inducers is not clear (DePierre et al., 1988). Results from the present study are supported by previous investigations in that PB-type inducers can cause proliferation of SER in rats.

Although surface area of SER per hepatocyte increased as the dose augmented, SER surface-to-volume ratio was maintained at approximately 42 μm^{-1} for the control and treated groups in the present study. According to Weibel (1969), multiplying 4 by the volume to surface ratio (a reciprocal of the surface to volume ratio) allows estimation of average width of SER tubules, SER profiles had a diameter of approximately 96 nm [4 × (1/42) μm] in our control and treated animals. Therefore, proliferated smooth membranes induced by PCB congener maintained the same shape

as those in the control. Amagase (1975) reported that glucose-6-phosphatase activity was similar in SER of normal liver cells and in the proliferated profiles induced by PCBs indicating that the normal and proliferated smooth membranes were similar in their enzymatic characterizations for glucose-6-phosphatase.

The mechanism of SER proliferation induced by chemical agents may be due to the induction of membrane biogenesis and to reduction in the membrane degradation (DePierre et al., 1988). Not only increased synthesis but also decreased breakdown of microsomal membrane protein occurred after exposure to PCB was shown by Hinton et al. (1978). In addition, these authors stated that phospholipid synthesis did not change after PCB treatment; instead, an increase of the half-life of phospholipids resulting in decreasing their turnover rate was detected.

4.2.2.2.2. Rough endoplasmic reticulum

After exposure to the PCB at 5 or 50 ppm, an increase of RER volume per hepatocyte in female rats was revealed in the present study, but in the males this volume decreased significantly. A diversity of opinions exists on the effect of PCBs on hepatocyte RER as reported in descriptive works. Some authors (Nishizumi, 1970; Greene et al., 1973; Jonsson et al., 1981) observed a reduction of RER profiles in the cells after administration of PCBs to female mice or rats, however, MacLellan et al. (1994a,b,c) did not detect this alteration in either male or female rats. Differences in strain or age of animals, duration of PCB administration, and type of PCB (commercial

product or purified congener) used would account for the different observations. In a morphometric study on the effects of phenobarbital on the hepatocytes of male rats, Stäubli et al. (1969) noted an increase in RER profiles 16 hours after initial dosing which returned to control values between two and five days later despite further administration of the drug. Howard et al. (1991) did not find RER alteration in centrilobular hepatocytes in male rats dosed with the same drug for either three days or 14 days. However, Massey and Butler (1979) cited a measurable RER proliferation in centrilobular hepatocytes after administration of phenobarbital to male rats for 24 days. Thus, a fluctuation of RER volume that depended on the duration of dosing occurred in male rats treated with phenobarbital. But the data on RER alteration induced by the drug in female rats was not available. In the current work, only a single dosing time, 13 weeks, was subjected to study and gender-related RER alterations were observed. But a sequential examination at earlier and later times than 13 weeks treatment needs to be conducted to provide further information on RER alteration patterns in male and female rats.

4.2.2.2.3. Mitochondria

The total normal mitochondrial volume per hepatocyte decreased significantly at 50 ppm dose in the present study. The decrease may be a consequence of an increased transformation of mitochondria to abnormal types, resulting in the numerical reduction of normal mitochondria in the cell. It is recognized that mitochondria are the major source of cellular ATP (Weiss, 1988). Moreover, it is widely held that there is

a positive correlation between the metabolic activity of a tissue and the number and size of mitochondria, and the number, size and surface area of cristae (Ghadially, 1988). Therefore, reduced normal mitochondria volume in the cells may result in decreased energy and metabolic activity, which, in turn, may not meet the energy requirements to support enhanced drug-metabolizing capacity of the cell.

Two types of mitochondrial transformation, type I and type II, were observed in this study. Type I comprised mitochondria that have cristae which lie parallel to long axis of the organelle; type II consisted of mitochondria that show C- or ring-shaped profiles. The latter, according to Ghadially (1988), represent sections in various plane through cup-shaped organelles. Mitochondria in hepatocytes are known to contain cristae that are normally arranged in a plane transverse to the longitudinal axis of the organelle (Ghadially, 1988; Fawcett, 1994). Data from our investigation revealed a trend towards an increase of mitochondrial transformations in the rats as congener concentrations elevated. However, the significance of such transformations is obscure (Ghadially, 1988). The possibility that mitochondrial membranes may be the site of toxic effects is suspected for PCBs as these compounds may act at the membrane level because of their lipophilic character (Nishihara, 1985). The mitochondrial inner membrane possesses the energy transducing function, and mitochondrial oxidative phosphorylation is responsible for supplying over 95% of the total cellular ATP requirement (Weiss, 1988). Thus, damage to the mitochondrial energy-transducing functions causes deleterious effects on cellular activities (Nishihara and Utsumi, 1986).

Nishihara (1985) showed that biphenyl and PCBs (Kanechlor-400) dissipated the membrane potential by increasing the permeability of ions as evidenced by release of K^+ from mitochondria. Nishihara and Utsumi (1986) reported that 2,2',5,5'-tetraCB inhibited electron transport and impaired Ca^{+2} sequestration by mitochondria through the inhibition of Ca^{+2} accumulation and the releasing of Ca^{+2} from mitochondrial stores. Stotz and Greichus (1978) observed swollen mitochondria in hepatocytes of white pelican treated with PCBs. Our finding of mitochondria with altered cristae are in accord with results of Kimbrough et al. (1971), Turner and Collins (1983), and MacLellan et al. (1994 a,b,c). An altered orientation of mitochondrial cristae would presumably change and/or diminish enzyme activity, and consequently interfere with the production of ATP (Schecter et al., 1984). Cup-shaped mitochondria observed in our work were also seen in the liver after administration of various toxic chemicals, namely carbon tetrachloride and alcohol (Reynolds, 1960; Koch et al., 1968), and, at times, the organelle proceeded to the formation of membranous whorls and myelin figures. Thus, cup-shaped mitochondria may represent a stage in degeneration of the organelle (Ghadially, 1988). As to PCB exposure, cup-shaped mitochondria were numerically abundant in the present study in comparison to those described in other reports (Nishizuchi, 1970; Allen and Abrahamson, 1973; Greene et al., 1973).

4.2.2.2.4. Lipid droplets

Because of previous reports of increased detoxification in this particular zone, our study investigated the effects of PCB 153 on centrilobular hepatocytes (Cullen and

Ruebner, 1991; Elangbam et al., 1991). However, the effect of dose on lipid droplets in centrilobular hepatocytes was not detected in the current study. Kasza et al. (1976), using light and electron microscopy, reported ingestion of Aroclor 1254 at 50 or 500 ppm for four weeks resulted in increased lipid accumulation initially in midzone but subsequently it spread to the centrilobular and periportal zones in livers of Osborne-Mendal rats. Type of PCB administered, duration of the treatment and species may cause variation in the location of lipid accumulation within the hepatic lobule. In the present study only the centrilobular zone was examined. It is possible that lipid accumulation occurred in midzonal or periportal cells. Triglycerides of fatty acids are the most common form of lipid inclusion in animal cells (Lodish et al., 1995). Under different physiological and pathological conditions, the size and number of lipid droplets vary markedly in liver cells. A few lipid droplets are considered normal inclusions of cytoplasm and represent a potential source of energy and short carbon chains for the synthesis of membranes and other lipid-associated cell products. However, enhanced accumulations of lipid droplets in parenchymal cells are pathological (Ghadially, 1988; Cheville, 1994). According to Hinton et al. (1978), PCB-induced fatty liver resulted from an increased half-life but not increased synthesis of triglycerides in hepatocytes; moreover, increased half-life of the lipids produced by PCBs was due to a partial block of intracellular migration of lipoprotein particles in rat liver (Hinton et al., 1978; Sanberg and Glauman, 1980; Mendoza-Figueroa et al., 1989). Accumulation of lipid droplets after exposure to PCBs points to a disturbance of lipid metabolism in hepatocytes, a suggestion supported by Mendoza-Figueroa et al. (1989).

Although the effect of PCB on lipid droplets was not observed in our study, nevertheless, a gender difference occurred in rats of the control groups in that the male generally had higher lipid droplet volume in hepatocytes than their female counterpart. The result was consistent with a biochemical assay in which the lipid content was 5.4 ± 0.6% liver wet weight for males and 4.3 ± 0.5% for females in control groups (Poon R, unpublished data). MacLellan et al. (1994a) also showed higher lipid content in livers of male rats (Sprague-Daley) compared to that in females in PCB 126 study. We speculate that the differences in genetics of this inbred rat strain may be a reason for higher lipid content in the livers of the males.

4.2.2.2.5. Lysosomal elements

In the current study, an increase in the volume of lysosomal elements of hepatocytes was noted in 50 ppm PCB-dosed male and female rats. Lysosomes are believed to be the main component of the intracellular digestive system that is capable of digesting or degrading a variety of endogenous and exogenous substances because of a large complement of hydrolytic enzyme contents (Ghadially, 1988; Lodish et al., 1995). Lysosomes comprise heterogeneous structures in hepatocytes and other cells (Ross et al., 1995). In the present study, morphologic criteria to identify this organelle was similar to that described by Rouiller (1954) for peribiliary dense bodies in rat liver (cited in Ghadially, 1988; Sahagian and Novikoff, 1994). An increase in number or size of this organelle is related to accelerated intracellular breakdown of structures (Stäubli et al., 1969; Seglen and Bohley, 1992). As described above, there were

increases in volume of SER and abnormal mitochondria at 50 ppm dose. Therefore, abnormal amounts of membranous structures needed to be removed from the cell, which, in turn, would require extra hydrolytic enzymes. This augmented need for hydrolytic enzymes coincides with an increase of lysosomal elements in the cells. Results from our investigation were in agreement with that reported by Nishizumi (1970) in a study using PCB mixtures, and by MacLellan et al. (1994a) in a study of PCB 126; an exception was a gender-related effect on lysosomal elements noted with PCB 126, where the increase in lysosomal elements was more pronounced in the females than in the males. Work on the toxicity of chlorinated biphenyls in human beings by Hirayama et al. (1969) (cited by Tanikawa, 1979) has shown an increased number of lipofuscin granules in hepatocytes which are believed to be terminal stages of lysosomal digestion. Stäubli et al. (1969) reported a heightening of dense body volume in rat hepatocytes for a 5-day exposure period with phenobarbital. Deter (1971) concluded that the main source for the lysosomal hydrolases that appeared following glucagon administration to rats originated from dense bodies in liver.

4.2.2.2.6. Peroxisomes

Total volume of peroxisomes per hepatocyte was calculated at different concentrations of PCB 153 in our work, which doubled in male and female rats dosed at 50 ppm in comparison with those in controls. Peroxisomes are especially important in oxidation and detoxification reactions (Cheville, 1994). At least 40 enzymes associated with peroxisomes have been identified, which include oxidases, catalase,

hydrolases and enzymes that participate in β-oxidation of long-chain fatty acids (Rao and Reddy, 1991). In hepatocytes, catalase constitutes about 15% of the total peroxisomal proteins. Catalase functions as a protective enzyme for cells; it oxidizes hydrogen peroxide produced during cell injury (De Duve, 1983). Epoxides are formed during biotransformation of many xenobiotics including PCBs (Parkinson et al., 1983; Safe, 1984). They react with nucleophilic groups in nucleic acids and proteins, which, according to DePierre and Ernster (1978), results in mutagenicity, carcinogenicity and cytoxicity. Epoxide hydrolases that deactivate epoxides via hydration to diols are present in SER, cytosol and peroxisomes of hepatocytes (Guenthner and Oesch, 1983; Moody et al., 1986; Patel et al., 1986). Peroxisomes may contribute to the deactivation of epoxides because the non-selective permeability of their membrane could allow the diffusion of epoxide hydrolase out of peroxisomes or diffusion of epoxide into peroxisomes (Mannaerts and Van Veldhoven, 1992). Numerical increase in hepatic peroxisomes was reported in rats fed chlorinated biphenyls with the greatest response coming from substances composed of highly chlorinated chemicals and those possessing a chlorine in 4 and/or 4′ position (Hansell and Ecobichon, 1974), similar to that as in PCB 153. Proliferation of peroxisomes was also described in the liver of rodents administrated high-fat diets (Christiansen et al., 1981; De Craemer et al., 1994), or treated with hypolipidemic drugs such as clofibrate, nafenopin and SaH 42-348, which are designated as peroxisome proliferators (Hess et al., 1965; Reddy et al., 1974; Moody and Reddy, 1976). However, alterations of hepatocytes after exposure to the proliferators are different from those detected in the present study. Liver enlargement

caused by proliferators is principally due to increase in number and size of peroxisomes; SER augments only to a mild degree (Rao and Reddy, 1991). In the current study, SER played the greatest role in the cell hypertrophy; contribution of peroxisomes to the increase of cell volume was small. A difference in mechanism of action of PCBs and peroxisome proliferators would account for the variation. A gender difference in the peroxisomal alteration was also noted in the current study. A significant increase of peroxisomal volume was detected in female rats at 5 ppm dose, but not in the male counterparts. Peroxisome biosynthesis, however, does not provide a clue to the gender differences revealed in our work. The organelle, according to latest concept, is synthesized by posttranslational incorporation of new matrix and membrane proteins into preexisting peroxisomes, which first increases in size, and then divides to form daughter peroxisomes (Lazarow and Fujiki, 1985; Lodish et al., 1995; Masters and Crane, 1995). Ghadially (1988), based upon a finding that clofibrate caused alterations in male rat hepatocytes whereas hepatocytes from females receiving similar dose were unaffected, states that the alterations in peroxisome are sex and species specific. The gender-related effects have also been noted previously in the studies on PCBs in the rat using Aroclor (Kimbrough et al., 1972), PCB 118 and 126 (MacLellan et al., 1994a), where females were more sensitive to the compound than the males. Sugiyama et al. (1994) had pointed out that gender difference in clofibrate induction of peroxisomal beta-oxidation in rats was because of testosterone that is involved in regulating the responsiveness. Insofar as PCB is concerned, we suggest that gender-related effects may be a consequence of estrogen involvement.

4.2.3. Conclusion

The present study revealed that subchronic exposure to PCB 153 causes ultrastructural changes in centrilobular hepatocytes of Sprague-Dawley rats. The alterations included cell hypertrophy, SER proliferation, RER alterations, mitochondrial abnormalities, and augmentations of peroxisome and lysosomal element volume. Hepatocytes from animals in the 50 ppm (highest dose) groups had the most severe changes. Except for the fact that increase in lysosomal elements was detected only at 50 ppm, the rest of the morphological alterations occurred at 5 ppm concentration. Gender-related PCB effects occurred in peroxisomes and RER profiles. An increase in peroxisomal volume per hepatocyte of female rats was detected at a lower dose than those in male rats. The RER increased in volume in females at 5 and 50 ppm; in contrast, it was puzzling to detect decreased values in males at the same doses. Because the morphologic alterations described above would represent an adaptive response rather than pathologic lesions, the NOAEL for PCB 153 was not obtained, instead, the no observable morphologic alteration level for the congener was judged to be 0.5 ppm in diet or equivalent to 34 μg/kg bw/day.

Although there has been one quantitative study of the changes in SER induced by PCBs, the present study quantifies, for the first time, all of the morphological alterations observed in centrilobular hepatocytes of rats given a PCB congener. Knowledge gained from the present work would provide a foundation for comparative studies using other PCB pollutants. It is not known whether the morphologic changes in hepatocytes

observed in this study have an impact on hepatocellular function. Nevertheless, in conjunction with other facets of a comprehensive PCB project at Health Protection Branch, Ottawa, the present work will add another set of baseline data for regulation by Health Canada designed to protect human and animal populations from exposure to environmental contaminants.

The present study concentrated on the centrilobular zone of the liver lobule, however, most of the studies on the effects of PCBs on hepatocyte morphology reported in the literature have not considered the zonal heterogeneity. There is little information available on the response of hepatocytes in the other two zones to PCB congener. More studies specifically focusing on the hepatocytes of the midzone and periportal zone are required to fill this gap in the literature.

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Appendix A

Equethesin formulation:

Chloral hydrate	40.4 mg
Pentobarbital	9.7 mg
Magnesium sulphate heptahydrate	21.3 mg
Ethanol (95%)	148.5 mg
Propylene glycol	400.0 mg
Water to make	1.0 ml

Appendix B

Fixation and Processing Protocol for Transmission Electron Microscope

Fix:	Place specimens in 2% glutaraldehyde, phosphate buffered, for 1-2 hours at room temperature	
Wash:	In 0.1 M phosphate buffer, 2-5 times, each for 10 min on rotator	
Post Fix:	In 1% OsO_4, phosphate buffered, for 1-2 hours in refrigerator	
Wash:	In distilled water or 0.1 M phosphate buffer for 5-10 min	
Dehydrate:	50% Ethanol	5-10 min
	70% Ethanol	10 min twice
	95% ethanol	10 min twice
	100% ethanol	15 min twice
Clear:	Propylene Oxide (PO)	10 min on rotator
		10 min not on rotator
Infiltrate:	50:50 Epon mixture to PO	1 hr
	75:25 Epon mixture to PO	1 hr
	Pure Epon mixture	overnight in vacuum desiccator
Embed:	Place tissue in labelled moulds, fill 2/3 with Epon mixture	
Polymerize:	In vacuum oven overnight at 65-70^{0}C	

Appendix C

PCB Intake, Body Weight and Relative Liver Weight of Rats dosed with PCB 153

Treatment (ppm PCB in diet)	n[a]	Calculated Amount[b] of PCB Intake (μg/kg bw/day)	Final Body Weight (g)	Liver (% bw)
		Male		
Control 0	10	0	485 ± 52	3.1 ± 0.3
0.05	10	3.6	499 ± 43	3.2 ± 0.2
0.5	10	34	505 ± 44	3.1 ± 0.3
5.0	10	346	527 ± 42	3.2 ± 0.2
50.0	10	3,534	511 ± 36	4.2 ± 0.5*
		Female		
Control 0	10	0	272 ± 26	3.1 ± 0.2
0.05	10	4.2	275 ± 27	3.0 ± 0.3
0.5	10	42	280 ± 25	3.1 ± 0.2
5.0	10	428	274 ± 24	3.1 ± 0.2
50.0	10	4,125	267 ± 22	3.6 ± 0.5*

[a] Number of animals; data denote means ± S.D.
[b] The dose in μg/kg bw/day was calculated using average body weight and food
* Significantly different from control groups ($p \leq 0.05$)consumption measured weekly over a 13-week period