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Metabolic and Toxicologic Evaluation of 2,3,4,3',4'-Pentachlorobiphenyl in Rats and Mice

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When 2,3,4,3',4'-pentachlorobiphenyl (PenCB), a component of Kanechlor 400, was administered orally at a single dose of 150 mg/kg or 60 mg/kg to 3 rats, all died in 8 days or 9 to 11 days, after treatment, respectively, with approximately a 30% decrease in body weight. Marked disappearance of fat from adipose tissues and severe fatty change in the liver were observed within 3 days after treatment, and became enhanced after 7 days. No metabolites possessing phenolic nature could be detected in the feces, urine or tissues, although about 34% of the dose was recovered unchanged in the feces collected for 8 days following administration. Treatment with 2,4,3',4'-tetrachlorobiphenyl (2,4,3',4'-TCB) did not induce either a decrease in body weight nor damage of any tissues of the rat. The lethality of PenCB in mice, on the other hand, was much weaker than in rats, because the 14-day *i.p.* LD_{50} was determined to be 0.40 g/kg. This value is about 5 times as high as that of 2,4,3',4'-TCB in mice (*i.e.*, LD_{50}, 2.15 g/kg).

Kanechlor 400 (KC-400), the commercial preparation of polychlorinated biphenyls (PCBs) with about 48% chlorine content, is now well known as the material that caused accidental PCB poisoning (so-called Yusho) in southwest Japan in 1968.[2] Since then, a series of metabolic studies of KC-400[3] and its components[4-8] has been conducted in this laboratory toward the view of obtaining a clue to the mechanism. In these studies it was established that 2,4,3',4'-tetrachlorobiphenyl (2,4,3',4'-TCB), the most abundant component of KC-400, was converted mainly to the 5-hydroxy metabolite in rats[5,6] and mice,[8] which was about 5 times higher in acute toxicity than 2,4,3',4'-TCB.[6] Similarly, 3,4,3',4'-tetrachlorobiphenyl (3,4,3',4'-TCB) was found to be metabolized to 5- or 2-hydroxy-3,4,3',4'-TCB in rats.[4,7]

Recent studies in other laboratories also provide evidence that TCBs are metabolized essentially by an hydroxylation reaction in animals, although the metabolic rate and excretion route of the metabolites is different from each other among individual isomers and animal species.[9-12] Quite recently, Jensen and Sundström[13] found that in rats and mice even

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2) H. Tsukamoto, *et al.*, *Fukuoka Acta Med.*, **60**, 496 (1969).
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2,4,5,2′,4′,5′-hexachlorobiphenyl, a chlorobiphenyl containing only isolated unsubstituted positions, was very slowly metabolized to the monohydroxy derivative and excreted in the feces. The excretion rate of this metabolite in the feces of rats was determined to be about 1.3% of the dose during 7 days after oral administration of 2,4,5,2′,4′,5′-hexachlorobiphenyl.

2,3,4,3′,4′-Pentachlorobiphenyl (PenCB) is one of the components of KC-400.[14] This isomer is calculated by gas chromatographic analysis to be about 2% in KC-400. This paper will describe the metabolic fate and toxicological evaluation of this particular pentachlorobiphenyl in rats and mice, comparing it with those of other components in KC-400.

Materials and Methods

Materials——2,4,3′,4′-TCB (mp 123—124°), 3,4,3′,4′-TCB (mp 172—173°) and PenCB (mp 115—116°) were prepared according to the method described in the previous paper.[14] The last compound (PenCB) was also synthesized by the Gomberg-Hey reaction[15] in which diazotized 3,4-dichloroaniline was allowed to react with a large excess of 1,2,3-trichlorobenzene in the presence of sodium acetate. The crude reaction products obtained by above synthetic methods were exhaustively purified by repeating column and preparative thin-layer chromatographies using silica gel until the samples were proved to be pure by gas chromatographic analysis described below. Mass spectra, obtained for above samples, confirmed the corresponding molecular weights. KC-400 was supplied by the Ministry of Health and Welfare of Japan. The rats were males of the Wistar-King strain, weighing about 160 g and the mice were males of the CF-1 strain, weighing about 24 g or 20 g.

Administration of PCBs——For the growth experiment, 2,4,3′,4′-TCB, 3,4,3′,4′-TCB and KC-400 were dissolved in soybean oil to make a 2.5% (w/v) solution. One ml of each solution containing 25 mg of each PCB was orally administered to each rat (150 mg/kg). In the case of PenCB, 0.2, 1.0 and 2.5% (w/v) solutions were made, and one ml containing 2, 10 or 25 mg of PenCB, respectively, was administered orally (12, 60 or 150 mg/kg, respectively).

For metabolic study, each rat was housed in an individual cage and given free access to food and water. The feces and urine of those administered PenCB (150 mg/kg) were collected daily for 8 days and body weight was measured until death. For morphological study, a single oral dose of 2,4,3′,4′-TCB (30 or 150 mg/kg) and PenCB (30 or 150 mg/kg) was given to rats as described above. Each experiment was conducted with 3 rats. The animals were fed a solid type feed, MF, (Oriental Yeast Co., Ltd., Tokyo), which in the subacute experiment on mice described below was ground once, mixed with PCB, and reformed as a solid type.

For the subacute experiment, mice weighing about 24 g, were given the diet containing about 200 ppm of 2,4,3′,4′-TCB, 3,4,3′,4′-TCB, KC-400, or PenCB, and body weights were determined daily for 25 days. Twenty mice were used for this experiment which were divided into 4 groups. For acute toxicity evaluation of PenCB, 48 mice, weighing about 20 g, were divided into 6 groups of 8 mice each. PenCB was dissolved in 0.2—0.5 ml of soybean oil and injected intraperitoneally at doses of 0.1, 0.4, 0.6, 0.7, 0.8 or 1.0 g/kg.

Extraction Procedure of Metabolites——Urine samples from rats described above were adjusted to pH 2.0 with HCl and shaken three times with ethylacetate for 15 min. The extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The resulting residue was dissolved in dry pyridine and trimethylsilylated with N,O-bis(trimethylsilyl)acetamide (BSA) for gas chromatographic analysis. Concentrated H_2SO_4 was added to the urine that remained after ethylacetate extraction to make a 4 N H_2SO_4 concentration, which was heated for one hr on a boiling water bath. The hydrolyzed urine was adjusted to pH 2.0 with 10 N NaOH and shaken three times for 15 min each with ethylacetate. The resulting extract was treated as above and analyzed by gas liquid chromatography (GLC).

After being dried in a desiccator (P_2O_5) and ground with a mortar, samples of feces were extracted three times for 15 min each with hexane and then with ethylacetate by mechanical shaking. The residue that remained after extraction was heated with 4 N H_2SO_4 for one hr in a boiling water bath. The hydrolyzed sample was adjusted to pH 2.0 with 10 N NaOH, shaken three times for 15 min each with ethylacetate and analyzed by GLC after trimethylsilylated with BSA in dry pyridine.

Gas Liquid Chromatography (GLC)——The instrument used was a Shimadzu GC-3AE gas chromatograph equipped with an electron capture detector. The column was a glass spiral tube (4 mm × 2.5 m) and the column packing was 1.5% SE-30 on Chromosorb W (60—80 mesh). The column temperature was maintained at 200°. Nitrogen was used as a carrier gas with a flow rate of 60 ml/min (1.5 kg/cm²). Amounts of PenCB in fecal extracts were calculated from the standard curve by measuring their peak areas. The recovery test of this method including extraction procedure was shown to be almost quantitative.

Morphological Effect of PenCB on Rat Liver——Small pieces of the liver tissue were removed from rats 3 and 7 days after oral administration of single doses of 30 or 150 mg/kg of 2,4,3′,4′-TCB or PenCB. They

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15) O. Hutzinger, S. Safe, and V. Zitko, *Bull. Environ. Contam. Toxicol.*, **6**, 209 (1971).

were fixed in 3% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.4 for 2 hr and postfixed in ice cold 1% osmic acid in the same buffer for one hr. After dehydration in ethanol, the specimens were embedded in epoxy resin. Thick sections (one μ) for light microscopy and thin sections (0.08 μ) for electron microscopy were cut with glass knives on a Porter-Blum microtome. The thick sections were stained with 1% toluidine blue and examined in an ordinary light microscope. Thin sections were stained with lead tartrate solution and examined in a JEOL JEM 100B or Hitachi HU 12A electron microscope.

Results

Excretion of Unchanged PenCB in Rat Feces

The excretion rate of unchanged compound in rat feces, determined by GLC during 8 days after oral administration of PenCB at a single dose of 150 mg/kg, is depicted in Table I. About one third of the dose was excreted as unchanged compound during 8 days post treatment, most of which occurred in the first day. However, small amounts of unchanged compound could still be detected in excreta 8 days later.

Detection of Metabolites in Rat Urine and Feces

The ethylacetate extracts of urine samples and hexane and ethylacetate extracts of feces samples collected everyday for 8 days after administration of PenCB were submitted to GLC according to the procedure described above. Gas chromatograms were compared with those of respective control samples collected before treatment. No difference could be observed between the gas chromatograms of urine from test and control rats, indicating that neither unchanged PenCB nor its metabolites were excreted into rat urine. In addition, no evidence was obtained for the excretion of conjugated metabolites by examination of hydrolyzed urine samples.

The results obtained from fecal samples indicated that these extracts contained only unchanged PenCB (retention time: 17 min), but no hydroxylated metabolites.

Influence of PenCB on Growth and Organ Weight of Rats

Growth curves of rats over a period of 25 days after administration of a single dose of PenCB (12, 60 or 150 mg/kg), 2,4,3′,4′-TCB (150 mg/kg), 3,4,3′,4′-TCB (150 mg/kg) and KC-400 (150 mg/kg) are presented in Fig. 1.

Table I. Excretion of Unchanged 2,3,4,3′,4′-Pentachlorobiphenyl in Rat Feces

Days after administration	Excretion rate (% of dose)
1	32.3
2	0.8
3	0.3
4	0.3
5	0.2
6	0.1
7—8	0.3

A single dose (150 mg/kg) of 2,3,4,3′,4′-pentachlorobiphenyl which was dissolved in soybean oil was orally administered to male rats of Wistar King strain weighing about 160 g. Each value represents a mean of 3 experiments.

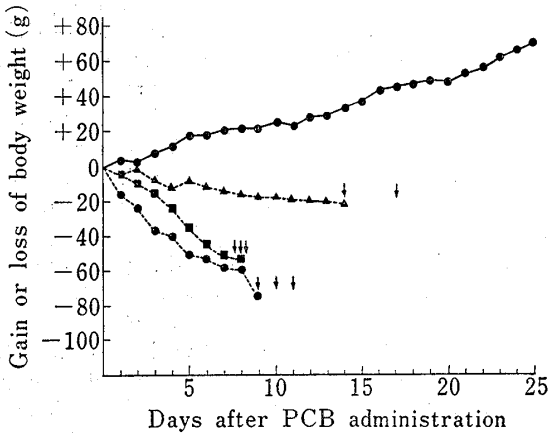

Fig. 1. Growth Curves of Rats after a Single Oral Dose of 2,3,4,3′,4′-Pentachlorobiphenyl, 2,4,3′,4′-Tetrachlorobiphenyl, 3,4,-3′,4′-Tetrachlorobiphenyl and Kanechlor 400

Each value represents a mean of 3 rats; The arrows show time of death of animals. —●—: Control; 2,4,-3′,4′-TCB (150 mg/kg); 3,4,3′,4′-TCB (150 mg/kg); KC-400 (150 mg/kg); ---▲---: PenCB (12 mg/kg); ---●---: PenCB (60 mg/kg); ---■---: PenCB (150 mg/kg)

The body weight of rats given 2,4,3′,4′-TCB, 3,4,3′,4′-TCB or KC-400 increased about 40% during the 25 day treatment period which was almost equal to that of the controls. On the contrary, PenCB caused a marked decrease of body weight and after a single dose of 150 or 60 mg/kg, all rats died on the 8th day or between the 9th and the 11th day, respectively. A single dose of 12 mg/kg caused death of two of three rats on the 14th and the 17th day with accompanying weight loss of about 15%. Very interesting finding was marked disappearance of fat from adipose tissues which was observed within 3 days after treatment with PenCB. It became enhanced after 7 days and in larger dose levels. On the other hand, any differences were not observable in the appearance of adipose tissues between the rats treated with 2,4,3′,4′-TCB, 3,4,3′,4′-TCB or KC-400 and the control rats.

The organ weight of rats that died 8 days after treatment with a single dose of 150 mg/kg of PenCB is shown in Table II.

Table II. Influence of 2,3,4,3′,4′-Pentachlorobiphenyl on the Weight of Wet Organs of Rats 8 Days after a Single Oral Dose of 150 mg/kg

Organ	Organ weight			
	Control		Test	
	(g)	(g/100 g B.W.)	(g)	(g/100 g B.W.)
Liver	10.7	5.9	5.8	5.4
Spleen	0.3	0.2	0.1	0.09
Testes	2.0	1.1	1.7	1.6
Kidneys	1.7	0.9	1.5	1.4
Lungs	1.1	0.6	1.3	1.2
Heart	0.6	0.3	0.5	0.5
Brain	1.0	0.6	1.0	0.9

Each value represents a mean of 3 experiments.
B.W.: body weight

The spleen weight of treated rats was markedly decreased, as described already in chicks fed Aroclor 1260 by Vos and Koeman,[16] compared with the controls. The liver was also quite small in the test group than in the controls. This is very interesting because 2,4,3′,4′-TCB and other PCB isomers (68 mg/kg/day×3, *p.o.*) were reported to increase the liver weight of rats about 50%.[17] Taking the relative organ weight (g/100 g body weight) in consideration, all the organs except spleen and liver increased the values after PenCB treatment. On the contrary, relative spleen and liver weights were reduced or not increased, compared with the controls. These findings strongly suggested that PenCB was severely toxic to the liver and spleen.

Morphological Changes of Rat Liver

The liver of rats that received 2,4,3′,4′-TCB (a single dose of 30 or 150 mg/kg) did not show any structural changes that were detectable under the light microscope (Fig. 2). In contrast, in rats given PenCB (a single dose of 30 or 150 mg/kg) lipid droplets of variable size were observed in hepatocytes (Fig. 3). The droplets were more numerous after 7 days than after 3 days. The affected liver cells were distributed evenly throughout the lobule. No other significant hepatic changes were found.

At the electron microscopic level, liver cells at 3 days after treatment with 2,4,3′,4′-TCB (a single dose of 30 or 150 mg/kg) did not show any unusual appearance. After 7 days, however, slight proliferation of the smooth endoplasmic reticulum (SER) was observed in rats given

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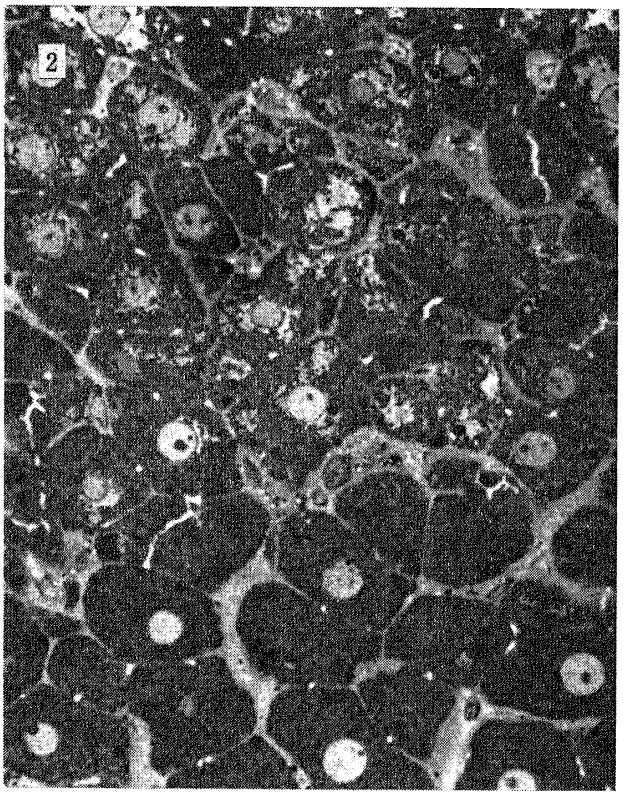

Fig. 2. Light Micrograph of the Liver from a Rat 7 Days after Administration of 2,4,3′,4′-Tetrachlorobiphenyl (a single dose of 150 mg/kg)

No structural change is noticed. 700×

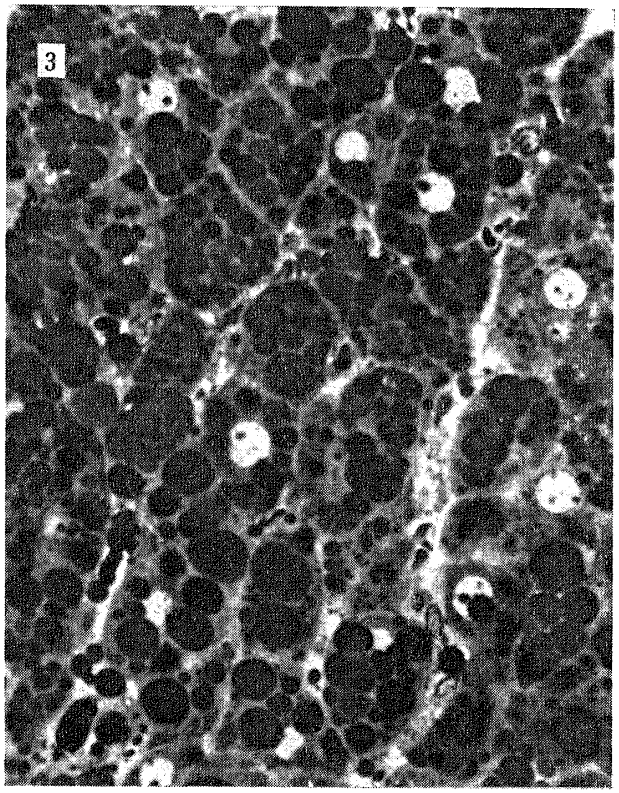

Fig. 3. Light Micrograph of the Liver from a Rat 7 Days after Administration of 2,3,4,3′,4′-Pentachlorobiphenyl (a single dose of 150 mg/kg)

Liver cells show lipid droplets without necrosis. 700×

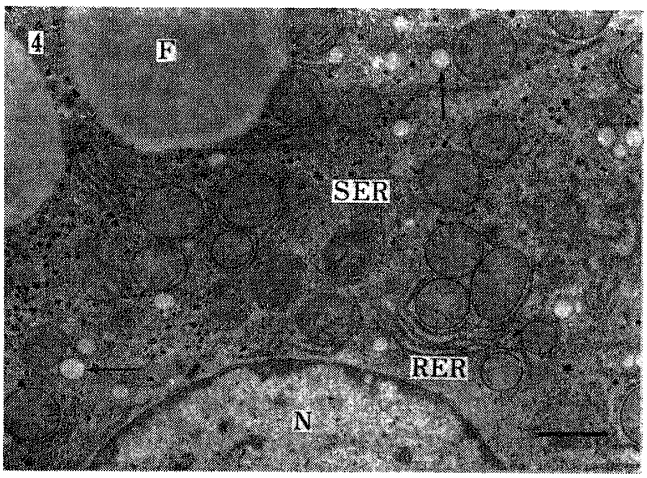

Fig. 4. Electron Micrograph Showing Part of the Liver Cell from a Rat 3 Days after Administration of 2,3,4,3′,4′-Pentachlorobiphenyl (a single dose of 150 mg/kg)

Note lipid particles bounded by a single membrane (arrow) and large fat droplets (F) in the cytoplasm. N: Nucleus, RER: Rough endoplasmic reticum, SER: Smooth endoplasmic reticulum. 8000×

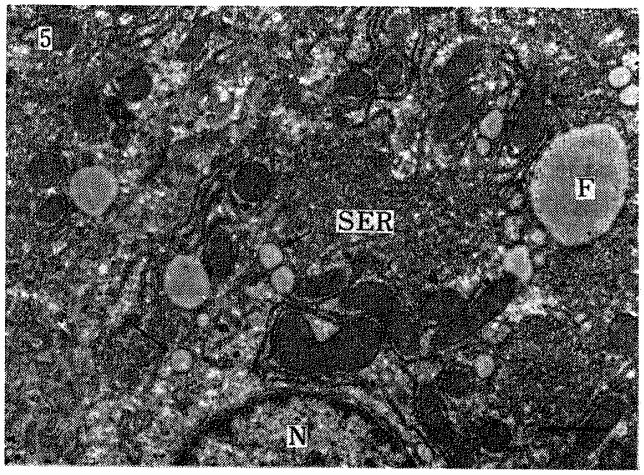

Fig. 5. Electron Micrograph Showing Proliferation of the Smooth Endoplasmic Reticulum (SER) and Lipid Particles (arrow) Bounded by a Single Membrane in the Liver Cell from a Rat 7 Days after Administration of 2,3,4,3′,4′-Pentachlorobiphenyl (a single dose 150 mg/kg)

N: Nucleus, F: Fat droplets. 8000×

TCB (a single dose of 150 mg/kg). By contrast, the rats that received PenCB (a single dose of 30 or 150 mg/kg) exhibited proliferation of the SER, occurrence of lipid particles bounded by a single membrane and accumulation of fat droplets lacking a limiting membrane (Fig. 4 and 5). Proliferation of SER and deposition of fat droplets were enhanced in rats given a dose of 150 mg/kg of PenCB than in those given 30 mg/kg and, similarly, in rats at 7 days than at 3 days post treatment. Within 3 days after treatment with PenCB (150 mg/kg), fat droplets were observed in the cytoplasmic matrix and gradually increased thereafter both in size and number and finally occupied a major portion of the cytoplasm in 7 days. Lipid particles bounded by a single membrane that appeared in the endoplasmic vesicles were similar in appearance to chylomicrons found in intestinal lacteals.

Toxicity of PenCB to Mice

The growth pattern of mice was determined over a period of 25 days by giving feed containing about 200 ppm of PenCB, 2,4,3′,4′-TCB, 3,4,3′,4′-TCB or KC-400. Mice fed 2,4,3′,4′-TCB or 3,4,3′,4′-TCB showed increased body weight similar to the controls, whereas the growth rate for the KC-400 group was slightly less than controls. The group fed PenCB had reduced body weight (12% decrease in 17 days) and three among five died after 18, 20 and 24 days, respectively. One mouse that died on the 24th day had marked edema and ascites. The amount of feed containing PenCB taken by a mouse was determined to be about 3.5 g per day on an average and therefore, the drug intake in this group was calculated to be about 12 mg of PenCB in the period of 17 days.

The *i.p.* 14 day LD_{50} of PenCB in male mice of the CF-1, estimated according to the method of Litchfield-Wilcoxon,[18] was calculated to be 400 mg/kg.

Discussion

From previous metabolic studies on 2,4,3′,4′-TCB[5,6] and 3,4,3′,4′-TCB,[3,7] both of which are the representative components of KC-400, it was found that, although the metabolic rates were different from each other, their metabolites in rats were all monohydroxylated derivatives and detectable only in the feces. Jensen and Sundström[13] reported recently that 2,4,5,2′,4′,5′-hexachlorobiphenyl, administered orally to rats, was metabolized to a lesser extent to the monohydroxylated derivative which was also excreted into the feces. From these findings it was assumed that PenCB might not be readily metabolized but that some should be excreted in the feces of rats as the hydroxylated metabolite(s). Surprisingly, rats given PenCB did not produce any phenolic metabolites, as indicated by GLC of the ethylacetate extract of urine, feces and tissues.

As shown in Table I, elimination of unchanged compound was highly concentrated in feces on the first day, suggesting unabsorbed material. If true, the absorption rate of PenCB from the gastrointestinal tract would be calculated to be 65% of the dose. Since the excretion rates of 2,4,3′,4′-TCB[5] and 3,4,3′,4′-TCB[7] in the first day feces of rats were about 37% and 60% of the dose, respectively, the present results support the proposition that gastrointestinal absorption rate of each component of PCBs is different from each other as well as their metabolic rate. In addition, the data indicated that unchanged PenCB continues to be excreted for 8 days after a single oral administration. This phenomenon could be explained as a gradual excretion through the small intestinal wall, as was established in a previous study.[19]

The acute toxicity of PCBs so far reported was not especially high. For example, the LD_{50} (*i.p.*, mice) of 2,4,3′,4′-TCB[6] and that (*p.o.*, mice) of KC-400[20] were both reported to be about 2 g/kg. On the other hand, the LD_{50} (*i.p.*, mice) of PenCB was determined to be

18) J.T. Litchfield, Jr. and F. Wilcoxon, *J. Pharmacol. Exptl. Therap.*, **96**, 99 (1949).
19) H. Yoshimura and H. Yamamoto, *Bull. Environ. Contam. Toxicol.*, **13**, 681 (1975).
20) K. Tanaka, S. Fujita, F. Komatsu, and N. Tamura, *Fukuoka Acta Med.*, **60**, 544 (1969).

0.4 g/kg in our studies. Thus, the acute toxicity of PenCB in mice is about 5 times as high as that of 2,4,3′,4′-TCB. Furthermore, PenCB seemed more toxic to rats than to mice, because two of three rats that were given PenCB at a single dose as low as 12 mg/kg died within 17 days after treatment. Although available amounts of PenCB were not enough to determine the LD_{50} (*p.o.*) to rats, it could be assumed that this value should be less than 12 mg/kg from above result.

It is known that PCBs induce a proliferation of SER and, simultaneously, the activation of drug-metabolizing enzymes in mammals.[21] The present study also demonstrates the proliferation of SER in the rat liver by PenCB or 2,4,3′,4′-TCB, although the former revealed greater activity than the latter.

Of particular interest was the observation that PenCB caused severe fatty change of the liver, whereas 2,4,3′,4′-TCB did not. Since this fatty change was characterized by the accumulation of fat droplets in the cytoplasmic matrix of the liver cells, and the occurrence of lipid particles bounded by a single membrane, it might be postulated that PenCB acts to raise the rate of triglyceride synthesis, coupled with the proliferation of the SER, and/or to inhibit the synthesis of protein necessary for the export of lipid from liver cells. However, further studies are necessary, of course, in order to clarify the mechanism.

Although the purity of PenCB has been established by GLC, unusually high toxicity of this sample in comparison to that of other PCBs may raise a question about some contamination with polychlorodibenzofurans (PCDF) or polychlorodibenzo-*p*-dioxins (PCDD), both of which are known to be extremely toxic.[22] This could, however, be ruled out by the fact that neither 2,4,3′,4′-TCB nor 3,4,3′,4′-TCB revealed high toxicity as PenCB, although these three were synthesized by the analogous method. Dissimilarity can be also seen in their toxic property among PenCB, PCDF and PCDD. The latter two usually caused a severe liver necrosis in the rabbit after a single dose of 0.5—1.0 or 0.05—0.1 mg/kg, respectively, but PenCB did not induce such necrosis in the rat. In conclusion, the present study indicated that in some component(s) the toxicity is much higher than expected from that of PCBs mixtures.

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