



## Enhancement in fecal excretion of dioxin isomer in mice by several dietary fibers

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### Abstract

The effect of increased nutrients (protein, lipid, vitamins and minerals) on dioxin-induced toxic manifestations such as immune suppression, hepatic hypertrophy, splenic atrophy and enzyme induction was investigated in mice after oral administration of 1,2,3,4,7,8-HxCDD (HxCDD) as one of a representative compound of dioxin isomers. Consequently, it appeared that increased minerals and vitamins in the diet prevented immune suppression by HxCDD. In addition, to clarify the additive effect of nutrients and the ability to hasten the excretion of dioxins by dietary fiber, the adsorbing of dioxins by 16 dietary fibers was investigated by *in vitro* experiment. Among 16 dietary fibers, locust bean gum, pectin, alginic acid, guar gum, chitin and cellulose were effective in binding dioxin isomers. These dietary fibers also enhanced the fecal excretion of HxCDD in mice. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** HxCDD; Nutrient; Toxicity; Mice; Prevention

### 1. Introduction

Our bodies are continuously exposed to toxic dioxins (PCDDs, PCDFs and Co-PCBs) through food, ambient air, water and soil. Biologic half-lives for most of the extremely toxic 2,3,7,8-chlorine-substituted isomers have been estimated to range from 3 to 15 years (Flesch-Janys et al., 1996). Therefore, dioxins accumulate in the human body transfer from the mother to the baby, and alter thyroid hormone regulation and immune function in babies who seem more sensitive to these compounds than adults (Nagayama, 1998). Moreover, pollution of the human body by these compounds has attracted attention as a factor in the incidence of atopy in babies

and the rapidly increasing incidence of endometriosis in women. To maintain our health, it is necessary to prevent possible adverse effects of extremely toxic and highly persistent dioxins.

Previous studies have shown that adding activated charcoal or cholic acids to chow hastened the elimination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from the tissues of mice (Coccia et al., 1981; Manara et al., 1982). Moreover, chow containing activated charcoal or cholic acids reduced mortalities in rodents treated with a single lethal dose of TCDD (Manara et al., 1984). However, in humans, it is difficult to ingest both compounds continuously. Recently, as an effective means of hastening the excretion of dioxins, ingesting dietary fibers capable of adsorbing mutagens and carcinogens has been reported (Kada et al., 1984; Morita et al., 1997). The dietary fiber extracted from vegetables and cereals increased fecal excretion in rats fed rice-bran containing dioxins. Among the fibers examined, spinach

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fiber and rice-bran fiber showed high potency in augmenting excretion. The results indicated that the ability to promote the excretion of dioxins depends on the composition of dietary fibers contained in food.

In the present study, the potency of adsorbing dioxins by 16 dietary fibers was investigated in an *in vitro* experiment for the selection of dietary fibers having the highest ability to absorb dioxin isomer. The selected dietary fibers showed the ability to improve fecal excretion of dioxins in mice. In addition, the effect of increased nutrient (protein, lipid, vitamins and minerals) absorption on dioxin-induced toxic manifestations such as immune suppression, hepatic hypertrophy, splenic atrophy and enzyme induction in mice after oral administration of a dioxin isomer was investigated to clarify the additive effect of nutrients and the ability of dietary fiber to hasten dioxin excretion.

## 2. Experimental conditions

### 2.1. Preventing HxCDD-induced toxicity in mice by increasing dietary nutrients

Female C57BL/6 mice, weighing 20–25 g, were obtained from Nihon SLC, Shizuoka, Japan. Six groups of 10-week-old mice (four or five mice per group) were used for this experiment. Basal diet and four high nutrient diets rich in protein, lipids, minerals and vitamins shown in Table 1 were, respectively, given to five groups of mice for 14 days. The minerals and vitamins were the same as those in the commercial diet AIN-76. All five groups were treated with a single oral administration of HxCDD dissolved in ethanol:Tween 20:saline (1:10:89) at a dose of 10 µg/kg body weight on day 8. A dosage of 10 µg was selected for toxic manifestations such as immune suppression, enzyme induction, hepatic hypertrophy and splenic atrophy observed in mice orally treated with HxCDD dose of 0.1–100 µg/kg. An additional group (control animals) was maintained on the basal diet for 14 days and received only vehicle on day 8. All the mice were challenged with antigen (DNP-dextran) one day following the HxCDD administration. On day 14, all mice were sacrificed, and whole blood was col-

lected. The anti-DNP-IgM antibody in the separated serum was determined using an enzyme-linked immunosorbent assay. The liver and spleen were removed and weighed. Hepatic microsomes were prepared from the liver and EROD activity was measured fluorometrically according to the procedures described by Pohl and Fouts (1980).

### 2.2. Adsorption of dioxin isomer in an *in vitro* experiment

Methanolic solution (3.0 ml) containing 10–50 ng of three dioxin isomers (TCDD, HxCDD and OCDD) and 30 mg dietary fiber were added to 0.1 M buffer pH 7.6 (3.0 ml). The solution was incubated at 37°C for 30 min and centrifuged at 3000 rpm for 10 min. On centrifugation, the solution deposited dietary fiber and dioxins binding to fiber. The <sup>13</sup>C-labeled dioxin internal standards (each 500 pg) were spiked to the supernatant, then the free dioxins were extracted from the supernatant by mechanical shaking with *n*-hexane (5 ml). The *n*-hexane layer was cleaned using a multi-layer column containing, from the bottom, 2% KOH-silica (3.0 g), silica (0.9 g), 44% H<sub>2</sub>SO<sub>4</sub>-silica (4.5 g), 22% H<sub>2</sub>SO<sub>4</sub>-silica (6.0 g), silica (0.9 g) and 10% AgNO<sub>3</sub>-silica (3.0 g). The *n*-hexane layer was transferred to the top of the multi-layer column, then eluted with a 150 ml of *n*-hexane. The purified eluate was concentrated, allowed to stand at room temperature for complete evaporation of solvent, then dissolved with 50 µl of *n*-decane. Analysis of three dioxin isomers was performed using a Hewlett Packard 6890 gas chromatograph-JEOL JMS700 mass spectrometer in EI-SIM mode at a resolution of 10000. A Supelco SPB-5 (30 m × 0.32 mm I.D., 0.25 µm film thickness) capillary column was used for analysis of tetra-through octachlorinated congeners, and the temperature was programmed as follows: 180°C isothermal for 1 min, 20°C per min to 310°C, and held for 3.5 min.

### 2.3. Enhanced fecal excretion of HxCDD in mice with increased intake of dietary fiber

Six groups of 10-week-old C57BL/6 female mice (four or five per group) were used for this experiment. One group was fed the basal fiber-free diet shown in

Table 1

Compositions of basal diet, test diets containing high nutrients and basal fiber-free diet (g)<sup>a</sup>

Constituent	Basal	High protein	High lipid	High mineral	High vitamin	Basal fiber-free
Casein	25	50	25	25	25	25
Oil, soy	6.0	6.0	30	6.0	6.0	6.0
Mineral mix.	3.5	3.5	3.5	17.5	3.5	6.0
Vitamin mix	1.0	1.0	1.0	1.0	5.0	2.0
Starch, corn	41.5	41.5	41.5	41.5	41.5	41.5
Granulated sugar	5.0	5.0	5.0	5.0	5.0	5.0

<sup>a</sup> Mineral and vitamin mixture were identical with AIN-76 in the basal diet.

Table 1 and other five groups were, respectively, given diets containing 10% of the following dietary fibers ad libitum for 10 days: cellulose, chitin, pectin, locust bean gum, guar gum. These dietary fibers were selected on the basis of the adsorption rate for three dioxin isomers shown in *in vitro* experiments. All six groups were treated with a single oral administration of HxCDD dissolved in ethanol: Tween 20:saline (1:10:89) at a dose of 10  $\mu\text{g}/\text{kg}$  body weight on day 4. Feces were collected every day for three days after exposure. 1–10 g of the collected feces was extracted with 100 ml of methanol/chloroform (1:2) for 5 h under reflex after spiking with internal standard (500 pg of  $^{13}\text{C}$ -HxCDD). The extract was replaced with 5 ml of hexane and cleaned up on a multi-layer column, then analyzed by GC–MS according to the procedures described in Section 2.2.

### 3. Results and discussion

#### 3.1. Preventing HxCDD-induced toxicity in mice by increasing dietary nutrient

The oral administration of dioxins to mice resulted in toxic effects such as reduction of antibody level in serum, the decreased ratio of spleen versus body weight and increase in liver weight, and the biological effects such as an elevation in hepatic microsomal EROD activity (Vos et al., 1974; Peterson et al., 1984). Positive close correlation has been confirmed between the EROD activity induction and toxic effects of dioxins such as the lethal effect and the inhibition weight gain in rodents (Safe, 1987). The oral dosage of a dioxin isomer used in this study was determined by the findings of the toxic manifestations described above. In this study, 1,2,3,4,7,8-HxCDD (HxCDD) was given to mice as a representative compound among dioxin isomers. Congener-specific differences have been observed in gastrointestinal adsorption and degree of toxicity among 2,3,7,8-substituted PCDDs. The toxicity of TCDD is the highest among dioxins, and degree toxicity among 2,3,7,8-PCDDs gradually decreases with increasing chlorine content. In contrast, highly chlorinated isomers show a slow elimination rate from the tissue of experimental animals. Both the toxicity and pharmacokinetic behavior of HxCDD are considered intermediate among seven 2,3,7,8-PCDDs.

Fig. 1 shows a comparison of dose–response curves for the serum anti-DNP-IgM antibody level, the hepatic microsomal EROD activity and the ratios of tissues/body weight in the mice. The mice were maintained on the basal fiber-free diet, and were given HxCDD on day 8. On day 6 after HxCDD treatment, the liver weight was increased in mice treated with an oral dose of 0.1  $\mu\text{g}/\text{kg}$  in an early stage of dosage compared to that in the vehicle-treated control group. The serum anti-DNP-IgM

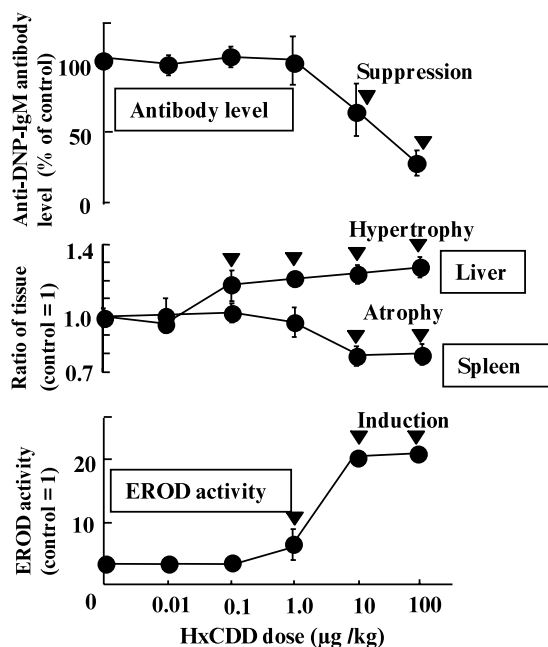


Fig. 1. Anti-DNP-IgM antibody level, EROD activity and ratios of tissues/weight in mice treated with HxCDD.

antibody level was decreased at a dose of 10  $\mu\text{g}/\text{kg}$  body weight. In addition, HxCDD treatment at a dose of 10  $\mu\text{g}/\text{kg}$  significantly increased the ratio of liver/body weight, reduced the ratio of spleen and elevated EROD activity. From these results, a dosage of 10  $\mu\text{g}/\text{kg}$  was confirmed to be suitable for this experiment, because this amount-induced immune suppression as well as hepatic hypertrophy, splenic atrophy and induction of enzymes.

Fig. 2 shows the effects of increased dietary nutrients on anti-DNP-IgM antibody levels in mice treated with HxCDD. Mice in the control group were maintained on

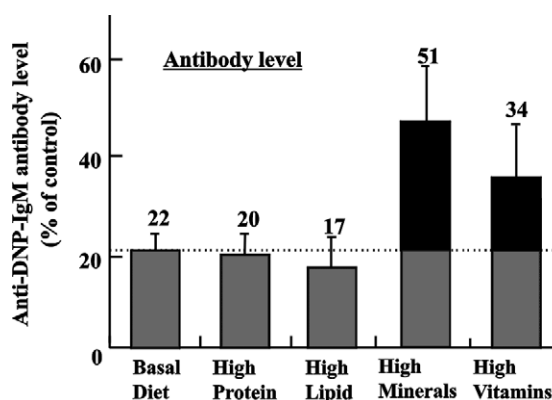


Fig. 2. Effects of high nutrients on anti-DNP-IgM antibody levels in mice treated with a single oral dose of HxCDD at 10  $\mu\text{g}/\text{kg}$ .

the basal diet, which contained less vitamins and minerals than the basal fiber-free diet, to clarify the effect of the four nutrients added to the diet. The amounts of protein, lipid, vitamins and minerals contained in the four test diets were two-fold to five-fold higher than the amount of nutrients in the basal diet as shown in Table 1. HxCDD administration decreased the serum anti-DNP-IgM antibody level to 22% relative to that in mice treated without HxCDD. When mice were maintained on a high mineral diet containing a five-fold increase in mineral mixture for 14 days, there was a 51% decrease in the anti-DNP-IgM antibody level. Intake of a high vitamin diet caused a 34% reduction in the antibody level. These results suggest that increased minerals and vitamins in the diet prevent immune suppression by HxCDD. There were no significant differences between the values in mice fed basal diet and tests diet containing high protein and high lipids, respectively. It was expected that a high lipid diet reduces the gastrointestinal absorption of liposoluble dioxins and decreases immune suppression by dioxins. However, the gastrointestinal absorption of HxCDD was slightly increased by the high lipid diet, and no recovery of immune suppression was observed.

Mohammad et al. (1985) investigated the effects of vitamins A and E on TCDD-induced lipid peroxidation and other biochemical changes in the rat. Vitamin E markedly inhibited microsomal lipid peroxidation, and did not prevent the reduction of body and liver weights. Furthermore, vitamin E had little effect on TCDD-induced lethality. Vitamin A inhibited lipid peroxidation and provided a TCDD-induced decrease in glutathione concentration in the liver.

On the other hand, it has been reported that increasing the calcium level in the diet increased fecal dry weight and decreased the gastrointestinal transit time. This result suggests that high dietary calcium inhibits the gastrointestinal adsorption of HxCDD. In addition, the lack of some minerals causes suppression of humoral immunity. Therefore, the supply of these components with high mineral diet might lead to the recovery of immune suppression. These results indicate that vitamin intake reduces the dioxin-induced toxic manifestations and biological change. From these results, in the following experiment, the enhancement of fecal HxCDD excretion by adding dietary fiber to diet was investigated using basal fiber-free diet containing a two-fold increase in mineral and vitamin concentrations compared to those in basal diet, in order to clarify the additive effect of increased nutrients and the ability of dietary fiber to hasten dioxin excretion.

### 3.2. Adsorption of three dioxin isomers in *in vitro* experiment

The potency in promoting fecal excretion of dioxin by dietary fiber depended on the ability of dioxin ad-

sorption. It was necessary to select the dietary fiber that demonstrated a high adsorption ability in the *in vitro* experiment. To assess the potency of several dietary fibers in adsorbing dioxin using an *in vitro* experiment, the amount of dioxin adsorbed by cellulose was determined as the standard because cellulose is a typical dietary fiber contained in numerous plants. Three dioxin isomers (TCDD, HxCDD and OCDD) were, respectively, incubated for 30 min at 37°C in the presence of 30 mg cellulose. Fig. 3 shows each adsorption rate (%) in 5, 10 and 50 ng of three dioxin isomers by cellulose, respectively. The data showed amounts of dioxin isomers extracted from the supernatant of samples incubated with cellulose as a percentage versus that of dioxin isomers in controls incubated without cellulose. Each 5 ng of TCDD and HxCDD were absorbed by added cellulose at rates of 8% and 10%, respectively. Compared to TCDD and HxCDD, the adsorption rates of OCDD were increased to 52%. High rates of OCDD adsorption were also observed for 10 and 50 ng of the three dioxin isomers. However, the level in 50 ng of TCDD was significantly decreased compared to those of 5 and 10 ng. This result indicated that 50 ng of TCDD might exceed the adsorption potency of cellulose. A similar pattern was also seen in HxCDD. Therefore, 5 ng of dioxin was selected for the following *in vitro* experiment, because this amount was absorbed at a rate similar to that of 10 ng and was the smallest amount possible for stable measurement.

Table 2 shows the adsorption rates of three dioxin isomers by 16 dietary fibers in the *in vitro* experiment. Locust bean gum was maximum effective in binding three dioxin isomers among 16 dietary fibers tested, and the bound percentages of dioxin isomers were increased from 47% for TCDD, 64% for HxCDD and 76% for OCDD. In addition, pectin also bound large amount of the three isomers in a range from 37% to

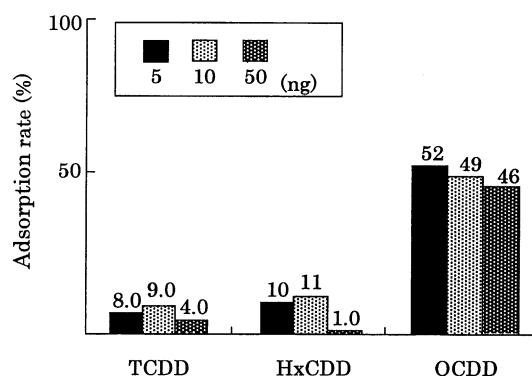


Fig. 3. Adsorption rates (%) of TCDD, HxCDD and OCDD by cellulose.

Table 2  
Adsorption rates (%) of dioxins by 16 dietary fibers in in vitro experiment<sup>a</sup>

Dietary fiber	Adsorption rate (%)			Water solubility
	TCDD	HxCDD	OCDD	
<b>Heteroglucan</b>				
Gum, Locust Bean	47	64	76	Soluble
Gum guar	23	44	58	Soluble
Gllucomannan from konjac	8	30	36	Soluble
<b>Glucronan</b>				
Pectin	37	60	79	Soluble
Alginic acid	27	42	58	Soluble
Gum karaya	11	23	35	Soluble
<b>Aromatic hydrocarbon polymer</b>				
Lignin	10	29	34	Soluble
<b>Homoglucan</b>				
Chitin	13	21	55	Insoluble
Cellulose	8	10	52	Insoluble
Mannan	0	4	22	Insoluble
Xylan	0	0	39	Soluble

<sup>a</sup> Levan, inulin, gum arabic, L-arabingalactan, agar < 20%.

79%. High adsorption rates were also seen in gum guar and alginic acid. The adsorption rates of the three isomers were elevated with increased chlorination of the isomer. These four dietary fibers are water soluble. Chitin and cellulose, which are water insoluble dietary fibers, also absorbed high rates of OCDD, showing 55% for chitin and 52% for cellulose. The adsorption rates of OCDD by chitin and cellulose showed levels similar to those of guar gum (58%) and alginic acid (58%). In contrast, the adsorption rates of TCDD and HxCDD were lower in chitin and cellulose (8–21%) than in pectin and alginic acid (23–44%). Chitin and cellulose, which are insoluble dietary fibers, absorbed OCDD with high chlorination at an especially high rate compared to soluble dietary fiber such as guar gum and alginic acid. Among the six dietary fibers showing high absorbing potency, there were significant differences in contributions to the adsorption rate for three dioxin isomers between water-soluble dietary fibers and insoluble fibers. However, in all the 16 dietary fibers, the adsorption rate of the three isomers was elevated by increased chlorination of the isomers. Increased adsorption activity of dietary fiber for less polar highly chlorinated isomers was observed compared to that of polar isomer, suggesting that the adsorption process is hydrophobic in nature.

Birkner and Kern (1974) investigated in vitro adsorption of bile salts to dietary fibers such as nondigestible food residues and hemicellulose. The adsorption of bile salts to food residues was greater for less polar bile salts. Consequently, in bile salts as well as in dioxin isomers, dietary fibers demonstrated greater adsorption potency for less polar compounds.

### 3.3. Enhanced fecal excretion of HxCDD in mice by increased intake of dietary fiber

Fig. 4 shows the effects of five selected dietary fibers on fecal excretion for three days in mice treated orally with HxCDD at 10 µg/kg. As shown in Fig 1, the dosage of 10 µg/kg was confirmed to be suitable for this experiment, because this amount-induced immune suppression as well as hepatic hypertrophy, splenic atrophy and induction of enzymes. The following dietary fibers were selected on the base of adsorption rate for HxCDD shown in an in vitro experiment: cellulose, chitin, locust

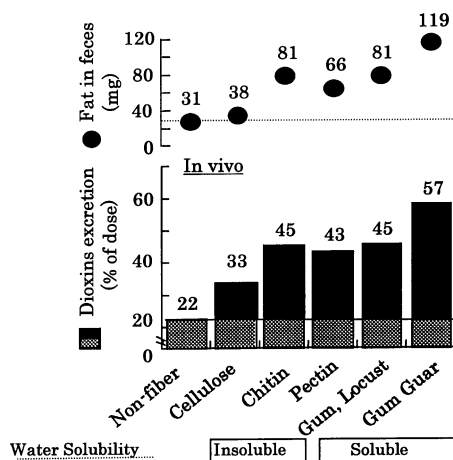


Fig. 4. Effect of dietary fiber intake on fecal dioxins excretion, fat in feces and feces in mice treated orally with HxCDD dose of 10 µg/kg, and adsorption rate in in vitro experiment.

bean gum, pectin, and guar gum. Each dietary fiber was mixed with the diet at a 10% ratio. The five mixed diets increased the excretion of HxCDD in a range from 33% to 57% compared to that in mice eating basal fiber-free diet (22%). The maximal level (57%) of fecal excretion was observed in mice given a guar gum diet which showed the highest potency in adsorbing dioxin isomers among the five fibers tested in the in vitro experiment. From these results, it appears that the intake of guar gum is most effective in fecal dioxin excretion in mice.

Morita et al. (1997) reported the effects of 12 dietary fibers extracted from vegetables and cereals on the fecal excretion of PCDD congeners in rats. The fecal excretion of 1,2,3,6,7,8-HxCDD in the group fed a non-fiber diet was 21% of the dose. Fecal excretion in the group fed 10% rice-bran fiber and spinach fiber was, respectively, promoted to 44% and 45%. The rate of fecal excretion of 1,2,3,6,7,8-HxCDD (21%) in the group fed a non-fiber diet was close to that of 1,2,3,4,7,8-HxCDD (22%) in our study, and enhancement of excretion by rice-bran fiber and spinach fiber was similar to that obtained in mice fed chitin, pectin and guar gum.

The fat in the feces of the group fed guar gum, which showed the highest ability to promote fecal excretion, was clearly elevated (119 mg) compared to that in the group given other dietary fibers (38–81 mg). The elevation of fecal fat level after cellulose intake was minor along with HxCDD excretion. The enhanced fecal excretion rate for HxCDD by dietary fiber was closely related to fecal fat content. This result indicates that enhancement of fecal dioxin isomer excretion by dietary fiber is associated with lipophilic compositions such as lipids and lipophilic vitamins.

## References

- Birkner, H.J., Kern, F., 1974. In vitro adsorption of bile salts to food residues, salicylazosulfapyridine, and hemicellulose. *Gastroenterology* 67, 237–244.
- Coccia, P., Croci, T., Manara, L., 1981. Less TCDD persists in liver 2 weeks after a single dose to mice fed chow with added charcoal or cholic acid. *J. Pharmacol.* 72, 181.
- Flesch-Janys, D., Becher, H., Gurn, P., Jung, D., Konietzko, J., Manz, A., Päpka, O., 1996. Elimination of polychlorinated dibenzo-*p*-dioxins and dibenzofurans in occupationally exposed persons. *J. Toxicol. Environ. Health* 47, 363–378.
- Kada, T., Kato, M., Aikawa, K., Kiriya, S., 1984. Adsorption of pyrolysate mutagens by vegetable fibers. *Mutation Res.* 141, 149–152.
- Manara, L., Coccia, P., Croci, T., 1982. Persistent tissue levels of TCDD in the mouse and their reduction as related to prevention of toxicity. *Drug Metab. Rev.* 13, 423.
- Manara, L., Coccia, P., Croci, T., 1984. Prevention of TCDD toxicity in laboratory rodents by addition of charcoal or cholic acids to chow. *Fd Chem. Toxic.* 22 (10), 815–818.
- Mohammad, Q.H., Stohos, S.J., Murray, W.J., 1985. Effects of vitamins E and A on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced lipid peroxidation and other biochemical changes in the rats. *Arch. Environ. Contam. Toxicol.* 14, 437–442.
- Morita, K., Matsueda, T., Iida, T., 1997. Effect of dietary fiber on polychlorinated dibenzo-*p*-dioxins in rats. *Jpn. J. Toxicol. Environ. Health* 43 (1), 35–41.
- Nagayama, J., et al., 1998. Perinatal exposure to polychlorinated biphenyl on lymphocyte subpopulations and thyroid hormone status in Japanese breast-fed infants. *Organohalogen Comp.* 37, 163–167.
- Peterson, R.E., et al., 1984. Wasting syndrome in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity: basic features and their interpretation. In: *Banbury Report: Biological Mechanisms of Dioxin Action*, vol.18.
- Pohl, R.J., Fouts, J.R., 1980. A rapid method for assaying the metabolism of 7-ethoxyresolufin by microsomal subcellular fractions. *Anal. Biochem.* 107, 150–155.
- Safe, S., 1987. Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs). *Chemosphere* 16, 791.
- Vos, J.G., Moore, J.A., Zinkl, J.G., 1974. Toxicity of 2,3,7,8-TCDD (TCDD) in C57BL mice. *Toxicol. Appl. Pharmacol.* 29, 229–242.