Influence of Dietary Antioxidants on Polychlorinated Biphenyls (PCB)-Induced Hepatic Lipid Peroxide Formation and Vitamin A Reduction in Rats\textsuperscript{1}

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Summary This paper describes experiments designed to characterize the effect of dietary antioxidants on lipid peroxide formation and vitamin A reduction in the liver of rats fed on diets containing polychlorinated biphenyls (PCB). Rats were given 0.025% PCB diets supplemented with dietary antioxidants, for 2 weeks. The antioxidants used were as follows: 10 mg\% (basal and usual level), 50 mg\% and 100 mg\% of vitamin E, and 50 mg\% of DPPD and tinoridine respectively. A marked liver enlargement and a significant increase of total liver lipid content were observed in the PCB-fed groups irrespective of the levels of vitamin E and kinds of antioxidants, suggesting that antioxidants were ineffective in preventing the development of fatty liver. Endogeneous lipid peroxide contents in the liver of rats receiving the diets containing 10 mg\% vitamin E, DPPD, and tinoridine with PCB increased significantly, whereas no increase was found with the 50 and 100 mg\% vitamin E diets with PCB. Hepatic glutathione peroxidase activity was unaffected by PCB and dietary antioxidants. No increase in hepatic vitamin E content occurred in the PCB groups with the addition of 10 mg\% vitamin E, DPPD, and tinoridine. However, dietary supplementation of vitamin E at higher levels caused an elevation of hepatic vitamin E content and a further increase was observed on the addition of PCB. These results suggest that a sufficiently high level of vitamin E suppresses the increment of the endogeneous lipid peroxide content in the liver of rats fed PCB. On the other hand, the administration of PCB to rats resulted in a significant decrease in hepatic vitamin A content regardless of the levels of

\textsuperscript{1} Polychlorinated biphenyls toxicity and nutrition. XIV. Polychlorinated biphenyls toxicity and vitamin A (7).

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Abbreviations used: PCB, polychlorinated biphenyls; DPPD, \textit{N},\textit{N}'-diphenyl-\textit{p}-phenylenediamine.

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vitamin E and kinds of dietary antioxidants. The antioxidants used in this experiment failed to suppress the hepatic vitamin A reduction caused by PCB administration. In addition, an absence of a significant difference in hepatic vitamin A contents among PCB-fed groups was observed. This suggests that the hepatic vitamin A level was independent of PCB-induced lipid peroxidation. Thus lipid peroxidation probably did not mediate the hepatic vitamin A reduction caused by PCB, especially in vivo.

Key Words PCB, lipid peroxide formation, lipid peroxidation, antioxidants, vitamin E, DPPD, tinoridine, vitamin A

Previous studies, in our group and in others, have shown that animals with ingested polychlorinated biphenyls (PCB) exhibit not only increased lipid peroxide formation (1-6) but also reduction of vitamin A content (2, 7-11) in the liver; however, at the present time we still have only minimal insight into the detailed mechanisms thereof, especially in vivo.

Combs and Scott (4) reported that dietary PCB enhanced lipid peroxidation in hepatic microsomes of chicks through the decrease of biological utilization of dietary selenium. Ito and Murata (5) showed that the administration of a PCB diet to carp caused increased lipid peroxide formation, decreased amounts of glutathione and vitamin E, and elevated glutathione peroxidase activity in the hepatopancreas. But in our previous papers (2, 3), increase of lipid peroxide and vitamin E contents, and decrease or no change of glutathione peroxidase activity were observed in the liver of rats fed on PCB diet.

On the other hand, Villeneuve et al. (9) reported that vitamin A concentration in the liver was lower in rabbits given PCB (Aroclor 1254) than in controls. The present authors have previously reported that 0.1% PCB diet given to rats causes a significant decrease of vitamin A in the liver within 2 weeks and of retinol binding protein in the serum to one-half that of the control group on the 10th day (7). Roberts and DeLuca (12) found that decarboxylation of retinoic acid in vitro was initiated by a free radical mechanism resembling that functioning in lipid peroxidation. However, we observed that the decrease of vitamin A content did not coincide with the level of lipid peroxide formation in the liver of rats administered PCB, casting doubt on the participation of lipid peroxidation in vitamin A reduction (13).

The present experiments were carried out to ascertain the effects of antioxidants on the formation of lipid peroxide and its scavenging components in the liver of rats given PCB, and further, to determine the mechanism of lipid peroxide formation by PCB. Furthermore, this study deals with the interaction between lipid peroxidation and vitamin A reduction in the liver of rats given PCB.

MATERIALS AND METHODS

Animals and diets. Weanling male rats of the Sprague-Dawley strain weighing
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an average of 65 g (ranging 57–73 g, 3 weeks of age) were housed individually in wire cages in an animal laboratory with a 12-hr cycle of light (07:00–19:00 hr) and dark. Room temperature in the laboratory was kept at 22 ± 1°C. The basal diet was composed of 63.0% sucrose, 20.0% milk casein, 9.0% soybean oil, 1.0% vitamin oil (one gram of this oil contains 300 IU of vitamin A palmitate, 30 IU of vitamin D₂ and 10 mg of dl-α-tocopheryl acetate dissolved in soybean oil), 0.85% vitamin mixture (14), 0.15% choline chloride, 4.0% mineral mixture (14) and 2.0% cellulose powder. The experimental diets with or without the addition of 0.025% PCB contained several kinds and levels of antioxidants as follows: 10 mg\% (basal and usual level), 50 mg\% and 100 mg\% of vitamin E (dl-α-tocopheryl acetate), 50 mg\% of DPPD (N,N'-diphenyl-p-phenylenediamine), and 50 mg\% of tinoridine (2-amino-3-ethoxy-carbonyl-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine) hydrochloride respectively. DPPD or tinoridine hydrochloride was added to the diet with 10 mg\% of vitamin E. After being fed on a basal diet for three days, six rats of each group were maintained on the experimental diets for two weeks. All diets and water were supplied ad libitum throughout the experiment. Food consumption and body weight were recorded 4 times a week. At the end of the experimental period, the food cups were removed at 7:00 a.m. After starving rats for 7 to 8 hr, the animals were sacrificed by decapitation. The liver was quickly removed and weighed and immediately analyzed for endogeneous lipid peroxide content. The other part of the liver was stored in a freezer maintained at −20°C until the performance of determinations.

Analytical methods. Liver lipids were extracted by the Bligh and Dyer method (16) and determined gravimetrically. Endogeneous lipid peroxide content was determined by the Thiobarbituric Acid (TBA) method (15), and was expressed in terms of malondialdehyde formed. Hepatic vitamin E (α-tocopherol) was assayed as described in the previous paper (3) in which a high-speed liquid chromatograph (HSLC) with an attached fluorometer (Ex. 298 nm, Em. 325 nm), and 2,2,5,7,8-pentamethyl-6-hydroxychroman as an internal standard, were used. Hepatic vitamin A (as retinol) was assayed by virtually the same method as for vitamin E in which the liver was extracted with n-hexane after saponification by potassium hydroxide and the extract subjected to HSLC with a fluorometer (Ex. 340 nm, Em. 460 nm), using Anthracene as an internal standard (13). Glutathione peroxidase activity in the liver was determined according to the method of Noguchi et al. (17) except for the concentration of liver homogenate, from 2.0% to 1.67%, and hydrogen peroxide as substrate, from 4.17 mM to 1.25 mM.

Chemicals. The PCB used in this experiment were purchased from Wako Pure Chemical Inc. They consisted of a mixture of the isomer, the dominant component of which was tetrachloride. dl-α-Tocopheryl acetate was obtained from Sigma Chemical Co., and DPPD from Wako Pure Chemical Inc. Tinoridine hydrochloride was a gift from Yoshitomi Pharmaceutical Ind. Other reagents used in this experiment were of analytical grade.

Statistical analysis. Student’s t-test was used to determine significant differ-
Table 1. Effects of dietary antioxidants on food intake, body weight gain and liver weight in rats given diets with and without PCB.  

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>V.E ²</th>
<th>DPPD³</th>
<th>Tinoridine³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>level (mg%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCB⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antioxidants</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Food intake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/day)</td>
<td>±0.6⁵,ac</td>
<td>±0.6⁵ec</td>
<td>±0.6⁵b</td>
</tr>
<tr>
<td>Body weight</td>
<td>90.6</td>
<td>79.6</td>
<td>93.3</td>
</tr>
<tr>
<td>gain (g/14 days)</td>
<td>±6.0ad</td>
<td>±4.6abc</td>
<td>±5.9a</td>
</tr>
<tr>
<td>Liver weight</td>
<td>4.61</td>
<td>6.63</td>
<td>5.15</td>
</tr>
<tr>
<td>per 100 g</td>
<td>±0.08a</td>
<td>±0.19b</td>
<td>±0.20abc</td>
</tr>
<tr>
<td>body weight</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(g)</td>
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</tbody>
</table>

¹ Six rats were used for each group. ² d-α-Tocopheryl acetate was used as vitamin E. ³ Fifty mg % of DPPD or tinoridine was added to the diet containing 10 mg % of vitamin E. ⁴ PCB made up 0.025% of the diet. ⁵ Mean ± SEM. Means not sharing a common superscript letter are significantly different (p < 0.05).
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enences between treatment means. The level of significance chosen was $p<0.05$.

RESULTS AND DISCUSSION

Effects of antioxidants on food intake, body weight gain, liver weight and total liver lipid content in rats given PCB

Changes in food intake, body weight gain and liver weight for the rats on the various dietary treatments are given in Table 1. Food intake and body weight gain in the rats given PCB were not different from respective control values. A marked liver enlargement was observed in the PCB-fed groups irrespective of the levels of vitamin E and kinds of dietary antioxidants. A significant increase of total liver lipid content was also found in the PCB-fed groups regardless of excess administration of dietary antioxidants (Fig. 1). Therefore, an enlargement of the liver caused by PCB was thought to be due to the accumulation of lipids in the liver, suggesting that dietary antioxidants were ineffective in modifying the PCB-induced fatty liver.

Effects of antioxidants on PCB-induced endogeneous hepatic lipid peroxide formation

As shown in Fig. 2, the endogeneous lipid peroxide content of the liver of rats receiving PCB increased significantly at the level of 10 mg% vitamin E, but not at two higher levels of 50 or 100 mg% as compared with that of the respective control rats. There were no differences in hepatic lipid peroxide contents between 50 and 100 mg% levels of vitamin E in both the control and PCB groups. It is obvious that a 50 mg% dietary level of vitamin E is sufficient for suppressing the hepatic lipid

Fig. 1. Effects of dietary antioxidants on total liver lipid contents in rats given diets with and without PCB. PCB made up 0.025% of the diet. Each bar represents mean ± SEM for 6 rats. Bars not sharing a common letter are significantly different ($p<0.05$).

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peroxide production caused by a 0.025% PCB level. In contrast, DPPD and tinoridine failed to suppress the raised hepatic lipid peroxide contents caused by PCB administration to the respective control levels, suggesting that further addition of dietary DPPD or tinoridine is required to prevent accelerated lipid peroxide formation. However, the hepatic lipid peroxide level in the group ingesting the DPPD diet with PCB was significantly lowered as compared to the group given the PCB-containing diet with 10 mg% of vitamin E, and tended to be suppressed in the group fed on the PCB- and tinoridine-containing diet. Shimada and Yasuda (18) reported that the anti-peroxidative activity of tinoridine was about 50 times greater than that of α-tocopherol in vitro, but a lower activity of tinoridine in vivo was observed. In our experiment, although either DPPD or tinoridine was added to the diet containing 10 mg% of vitamin E, no additive or synergic action with vitamin E on the anti-peroxidative activity was observed.

Di Luzio (19, 20) demonstrated that treatment with antioxidants such as α-tocopherol acetate and DPPD inhibited ethanol- or carbon tetrachloride-induced fatty liver and proposed the participation of free radical-induced peroxidation of lipids in the development of the fatty liver. However, the antioxidants used in this experiment failed to prevent the development of PCB-induced fatty liver, and even in such a case, higher levels of vitamin E suppressed PCB-induced lipid peroxide formation at the same levels as in the control group, clearly demonstrating that the lipid peroxidation caused by PCB was not associated with PCB-induced fatty liver. The development of fatty liver caused by PCB administration has ascribed to the stimulation of liver triglyceride, cholesterol and phospholipid synthetases (1, 21, 22), and to the depression of triglyceride transport from liver into blood (22).
addition, it has also been reported (21) that PCB-induced fatty liver is attendant to the increment of unsaturated fatty acids. Therefore, it is significant to note that the increase of unsaturated fatty acids participates in the stimulation of lipid peroxide formation caused by PCB administration.

**Effects of antioxidants on hepatic glutathione peroxidase activity in rats given PCB**

Glutathione peroxidase activity in the liver of rats is given in Fig. 3. While the

![Fig. 3. Effects of dietary antioxidants on hepatic glutathione peroxidase activities in rats given diets with and without PCB. PCB made up 0.025% of the diet. Each bar represents mean ± SEM for 6 rats. Bars not sharing a common letter are significantly different (p<0.05).](image)

![Fig. 4. Effects of dietary antioxidants on hepatic vitamin E contents in rats given diets with and without PCB. PCB made up 0.025% of the diet. Each bar represents mean ± SEM for 6 rats. Bars not sharing a common letter are significantly different (p<0.05).](image)
activities tended to decline on PCB ingestion as compared with the respective controls, no significant differences were observed. We did not observe an elevation of glutathione peroxidase activity caused by PCB administration as reported by Ito and Murata (5). Our and Ito's findings were obviously different from those of Combs and Scott (4) who stated that dietary PCB enhanced lipid peroxidation through the decrease of biological utilization of dietary selenium. Furthermore, our result agrees with the results reported by Porta et al. (23) and Chow et al. (24) that dietary supplements of vitamin E had no significant effect on hepatic glutathione peroxidase activity.

**Effects of antioxidants on hepatic vitamin E content in rats given PCB**

Hepatic vitamin E content of rats is presented in Fig. 4. There was no difference between the two groups given the respective 10 mg% vitamin E diets with and without PCB. Higher levels of dietary vitamin E caused a profound elevation of vitamin E content and the addition of PCB increased it more markedly. However, the hepatic vitamin E content in the 50 mg% vitamin E-supplemented group was not different from that in the 100 mg% group both with and without PCB. We found that the elevation of vitamin E content in the liver of rats caused by PCB administration differed from the result observed in carp by Ito and Murata (5). This suggests a rise in the requirement of the rats for vitamin E and also an antitoxic activity against PCB toxicity. Combs et al. (25) found that chicks reared on PCB diet deficient in vitamin E and supplemented with a marginal level of selenium showed an increase of susceptibility to vitamin E-selenium deficiency, as measured by the incidence of exudative diathesis. This phenomenon, in agreement with our present observation on vitamin E content and glutathione peroxidase activity in rats, shows that PCB increases the apparent requirements of chicks for vitamin E and selenium for prevention of exudative diathesis. On the other hand, there were no differences in the hepatic vitamin E contents between the control and PCB groups on the DPPD and tinoridine diets, and the levels thereof were nearly the same as those of the 10 mg% vitamin E diet groups.

In view of the results presented above, as no elevation of glutathione peroxidase activity caused by PCB administration was recognized and the higher levels of dietary vitamin E (50 and 100 mg%) suppressed the PCB-induced hepatic lipid peroxide formation, the increment of lipid peroxide formation by PCB administration in rats fed on diet with 10 mg% vitamin E seemed to result from the insufficiency of lipid peroxide scavenging components in the liver of rats.

**Effects of antioxidants on hepatic vitamin A reduction caused by PCB administration**

Vitamin A content of the liver is indicated in Fig. 5. The administration of PCB as previously observed (2, 7, 8) resulted in a significant decrease in vitamin A contents irrespective of the levels of vitamin E and kinds of dietary antioxidants. Though the dat is not shown, a significant decrease was recognized in the total vitamin A content of the liver. On the other hand, there were no significant
differences in hepatic vitamin A contents among the PCB-fed groups. It is concluded that dietary antioxidants used here had no significant effect on the prevention of reduction of hepatic vitamin A caused by PCB.

It was found that liver storage of vitamin A increased in vitamin E- and DPPD-treated rats (26, 27), although neither of these reports distinguished protective effects in the diet from those occurring in vivo. In contrast to these reports, Green et al. (28) presented the case that under conditions ruling out interaction in the diet, vitamin E had no effect on the rate of depletion of vitamin A from the liver. Since we have already showed that vitamin A is not specifically destroyed in the copresence of PCB in the diet (7), the antioxidants used in this experiment have no protective effect on the reduction of vitamin A in the liver caused by PCB.

Relationship between PCB-induced lipid peroxidation and vitamin A reduction in the liver

It can be seen from Fig. 2 that PCB-induced lipid peroxide formation in the liver was suppressed by higher levels of vitamin E in the diet (50 and 100 mg%) and also by DPPD (50 mg%) as compared with the 10 mg% vitamin E diet with PCB. However, no significant differences in hepatic vitamin A contents were observed among these four PCB-fed groups, and the hepatic vitamin A level was not parallel to the degree of PCB-induced lipid peroxidation. Therefore, this suggests that lipid peroxidation was not involved in the reduction of hepatic vitamin A caused by PCB administration, especially in vivo.

It is quite readily accepted that retinol is normally metabolized to retinal (29, 30) and further to retinoic acid (31, 32). Moreover, Roberts and DeLuca (12, 33) demonstrated that decarboxylation was a significant process in the
catabolism of retinoic acid \textit{in vitro} in slices of tissue from rat liver and kidney as well as in the microsomal fraction of these same tissues, and that it occurred by a free-radical mechanism similar to lipid peroxidation and was prevented by antioxidants such as DPPD and vitamin E. In addition, in contrast to that occurring \textit{in vitro}, decarboxylation \textit{in vivo} was not inhibited by vitamin E (33). This observation essentially agrees with our finding that vitamin E failed to suppress the reduction of hepatic vitamin A content caused by PCB. However, Roberts \textit{et al.} also indicated in the same paper that DPPD showed a slight but significant decrease in the extent of decarboxylation \textit{in vivo}, and concluded that only a part of decarboxylation \textit{in vivo} proceeded by the same mechanism as \textit{in vitro}. As regards the lack of an effect of vitamin E on decarboxylation \textit{in vivo}, they guessed that as an antioxidant, DPPD had a stronger more direct effect than had vitamin E (33). However, our experiment showed that hepatic vitamin A content in the DPPD group significantly decreased on PCB administration. So, DPPD would have no protective effect for the reduction of hepatic vitamin A caused by PCB.

It is well known that organochlorine compounds including PCB cause the reduction of hepatic vitamin A content (7, 9, 10, 34) and also that these compounds induce the operation of the hepatic microsomal mixed function oxidase system as a common action. Therefore, we presumed that microsomal mixed function oxidases might be associated with the reduction of hepatic vitamin A caused by PCB administration, that is, vitamin A would be metabolized to inactive metabolites by the oxidases, resulting in the decrease of hepatic vitamin A content (7). However, since the level of induction of cytochrome P-450 caused by PCB administration was not necessarily parallel to the degree of reduction of hepatic vitamin A content (2, 13), it appeared that microsomal mixed function oxidases were possibly not directly involved in vitamin A metabolism including catabolism.

Over the past several years, Roberts \textit{et al.} (35–37) have revealed the reaction pathway for retinoic acid metabolism (deactivation pathway) in hamster liver microsomes, two steps of which are highly responsive to retinoic acid and slightly so to phenobarbital and 3-methylcholanthrene, require oxygen and NADPH, and are carbon monoxide-sensitive. The reaction of these steps, therefore, is similar to that for monoxygenase-type hepatic microsomal mixed function oxidases (cytochrome P-450s). On the other hand, since PCB induces the oxidases (38), it might appear possible that PCB also induces the retinoic acid-inducible monoxygenase-type enzymes which metabolize retinoic acid to deactivated metabolites, resulting in the reduction of hepatic vitamin A content on PCB administration. Future investigations will therefore center around this possibility.

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