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# Effects of Biphenyl Ethers on the Hepatic Mixed-Function Oxidase (MFO) Systems and on the Conversion of Procarcinogens to Mutagens

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Biphenyl ethers have been used widely as herbicides all over the world and regarded as environmental pollutants(Gretch et al.,1979; Yamagishi et al.,1979; Suzuki et al.,1983). Their toxicologic mode of action is thought to be similar because of having a biologically active nitro group and lipophilic chlorine substituents, in addition to a biologically stable ether linkage in their molecules. Methemoglobin formation (Miyauchi et al.,1981), mutagenicity (Miyauchi et al.,1983, 1984; Draper and Casida,1983) teratogenicity (Gray et al.,1982) and carcinogenicity (Milman et al.,1978) by biphenyl ethers have been reported. These toxicities are thought to be caused by an activated nitro group. In view of the lipophilic chlorine substituents like PCBs, biphenyl ethers are suspected to induce the hepatic mixed-function oxidase (MFO) systems. Indeed, one of the biphenyl ethers used in this study, 2',4'-dichloro 4-nitrobiphenyl ether (2,4-$DCNO_2$) induced the hepatic MFO systems in mammals (Burke Hurt et al.,1983). It has been known that treatment of animals with suitable inducers of hepatic MFO systems accerelates chemical metabolism and alters metabolic activation and biological activity(Conney,1967). To investigate the effect of hepatic MFO inducers on the metabolic way of chemicals, particulally procarcinogens is a subject of absorbing interest. The purpose of this study is to determine whether treatment of rats with biphenyl ethers induce the hepatic MFO systems and influences the conversion of procarcinogens to mutagens in the Salmonella mutation assay (Ames et al.,1975).

## MATERIALS AND METHODS

Biphenyl ethers(4-nitrobiphenyl ether(4-$NO_2$),4'-chloro(4-$CNO_2$), 2',4'-dichloro(2,4-$DCNO_2$) and 2',4',6'-trichloro(2,4,6-$TCNO_2$)4-nitrobiphenyl ethers) were prepared by procedures described in the previous paper

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(Miyauchi et al., 1981). D-Glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase, MW markar (cytochromes C from horse heart), NADPH and bovine serum albumin were obtained from Oriental Yeast Co., (Tokyo, Japan). Cytochrome C reductase (from horse heart, type III) was from Sigma Chemical Co., St. Louis, Missouri. All other chemicals used were of greagent grade were from Wako Chemicals Industry Co., Ltd. Osaka, Japan.

Male Sprague-Dawley rats weighing 150-200g were used. They were injected i.p. with biphenyl ethers (0.93 mmole/Kg) dissolved in olive oil for 3 days, and as positive controls, sodium phenobarbital (PB) (50 mg/Kg, for 4 days), 3-methylchlanthrene (3-MC) (80 mg/Kg, 24 hr prior to sacrifice) or Kaneclor-500 (500 mg/Kg, 5 days prior to sacrifice) were used. Control rats received an equivalent volume of olive oil or water. After treatment, rats were killed and their livers were perfused in situ, removed and weighed. The S-9 fractions were prepared according to Ames et al. (1975) for mutation assay and microsomal fractions were for levels of hepatic MFO systems and SDS-PAGE electrophoresis by the $Ca^{2+}$-bound method ( Aitio and Vainio, 1976). Protein contents of these fractions were determeined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

The amount of cytochrome P-450 or P-448 was measured according to Omura and Sato (1964), and the activity of cytochrome C reductase, aniline hydroxylase and aminopyrine N-demethylase was measured according to the methods described by Mazel (1972). The O-demethylation of 4-nitroanisol was determined as previously described (Forlin and Lidman, 1979). Benzo(a)pyrene hydroxylase was measured by the method of Nebert and Gelboin (1968), and in this study, activity was expressed as units (one unit was equal to the fluorescence intensity of 0.3 ppm of qunine sulfate in 0.1N sulfric acid at ex. 365 nm and em. 520 nm).

SDS-PAGE electrophoresis was performed in 7.5% acrylamide gels by the method of Laemmli (1970). The procedure was carried out in a Atto slab gel apparatus (15 cm length, 1.0 mm gel thickness). The protein in gels was fixed in a solution of 25% isopropanol and 10% acetic acid, stained in the same solution containing 0.05% Coomassie brilliant blue R 250, and destained in 10% isopropanol and 10% acetic acid. Standard proteins and their accepted MW were cytochrome C monomer (12,400), dimer (24,800), trimer (37,200), tetramer (49,600) and hexamer (74,400).

Salmonella/microsome mutation assay was carried out mainly according to the Ames method as modified by Yahagi (1975) with tester strain TA 98. In this study, 2-aminoanthracene (2-AAT), 2-acethylaminofluorene

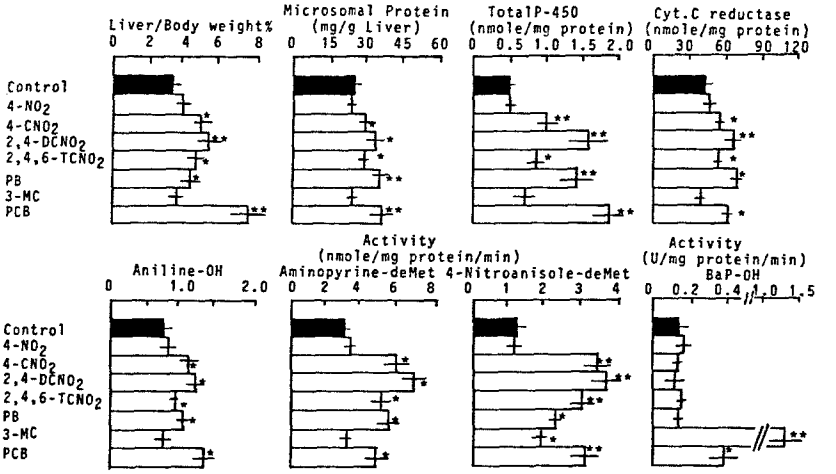


Figure 1. Effects of biphenyl ethers on the hepatic mixed-function oxidase (MFO) systems of rats. Five rats were used in each group. Bars are the mean ±S.E.
Statistical significance at *P<0.05 and **P<0.01 by t-test.

(2-AAF) and benzo(a)pyrene(BaP) were used as procarcinogens. Titrations were performed with three concentrations of each of three procarcinogens(1, 5 and 10 μg/plate for 2-AAT and BaP; 10, 25 and 50 μg/plate for 2-AAF) and with three separate protein concentrations (0.5, 2.0 and 5.0 mg/plate) for each of the three procarcinogen concentrations. Each determination was carried out in parallel on three or four different plates.

## RESULTS AND DISCUSSION

The effects of biphenyl ethers and positive controls(PB, 3-MC and PCB) on the hepatic MFO systems are given in Fig. 1. All biphenyl ethers exept for 4-$NO_2$ had the capacity to increase the levels of liver weight/body weight, microsomal protein, cytochrome P-450, cytochrome C reductase, aniline hydroxylase, aminopyrine N-demethylase and 4-nitroanisole O-demethylase. However, all biphenyl ethers had no capacity to increase the level of BaP hydroxylase. Compounds having the capacity to induce the hepatic MFO systems are of at least two types, exemplified by PB and 3-MC. PB-type compounds increase the levels of liver somatic index, microsomal protein, cytochrome P-450, cytochrome C reductase, aminopyrine N-demethylase, etc., and 3-MC-type compounds cause the formation of new cytochrome P-450(P-448) and increase the level of arylhydrocarbon hydroxylase(Conney, 1967; Alvares et al., 1968). PCBs(Kaneclor-500) having about 50% chlorine content are the mixed type inducer with characteristics of both PB and 3-MC. Judging from the results in Fig. 1, biphenyl ethers are

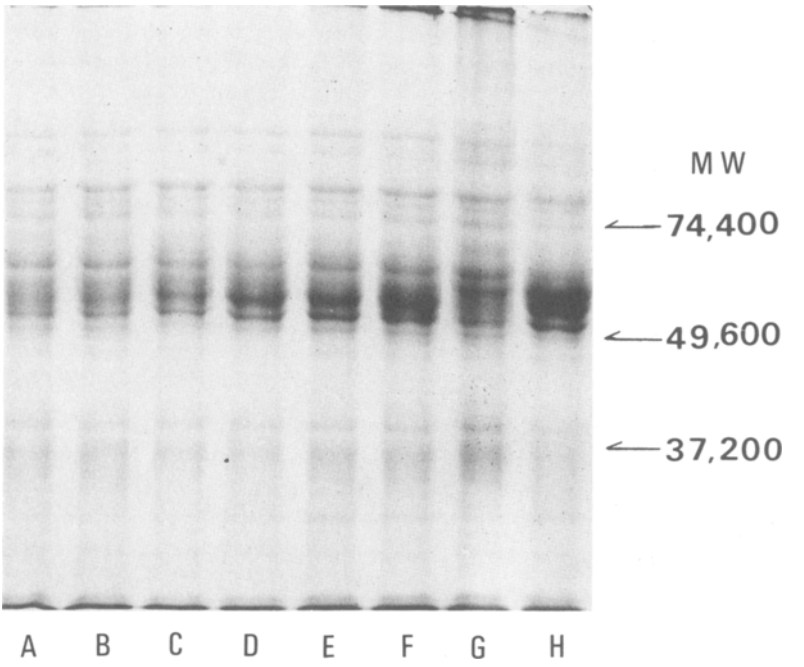


Figure 2. SDS-PAGE electrophoresis of liver microsomes from variously treated rats. A, Control; B, 4-$NO_2$; C, 4-$CNO_2$; D, 2,4-$DCNO_2$; E, 2,4,6-$TCNO_2$; F, PB; G, 3-MC; H, PCB

thought to be classified into PB-type inducers. Moreover, the peak of carbon monoxide-cytochrome P-450 complex of biphenyl ethers-treated rats was at 450 nm as same as that of PB-treated rats, in contrast that of 3-MC-treated rats was at 448 nm (data not shown). Among biphenyl ethers, 2,4-$DCNO_2$ was the most effective on the hepatic MFO systems followed by 4-$CNO_2$ and then 2,4,6-$TCNO_2$. The failure of 4-$NO_2$ to induce the hepatic MFO systems is perhaps derived from having no chlorine and easy metabolizing by rat liver enzymes. The less effect of 2,4,6-$TCNO_2$ is thought to be caused by the low absorption and/or the structure with relative planarity (2',4'and 6'-positions of one phenyl are chlorinated and 2-position of other phenyl is not substituted) which is convenient to induce cytochrome P-448 rather than cytochrome P-450 (Goldstein et al., 1977).

SDS-PAGE electrophoresis of liver microsomes from control rat and rats treated with biphenyl ethers and positive controls is shown in Fig. 2. Treatment of rat with 4-$NO_2$ resulted in microsomal protein profiles

identical to those seen with control rat. While, treatments of rats with 4-$CNO_2$, 2,4-$DCNO_2$ and 2,4,6-$TCNO_2$ resulted in a marked increase of the protein band of 53,000 molecular weight and an increase of this band was observed when rats were treated with PB and PCB. A new protein band of 56,000 molecular weight was observed in microsomes of 3-MC and PCB-treated rats. From the fact that various forms of cytochrome P-450 exist in the 44,000-60,000 moleculer weight region (Rikans et al., 1978), these two proteins of 53,000 and 56,000 molecular weights, respectively, are suggesting possible correspondence to P-450b and P-450c of Ryan et al. (1979), P-450 and P-448 of Masuda-Mikawa et al. (1979), and P-$450_{PB}$ and P-$450_{MC}$ of Harada and Omura(1980).
These results in SDS-PAGE elestrophoresis supported that biphenyl ethers exept for 4-$NO_2$ were not 3-MC or PCB-type inducer but PB-type inducer.

Large numbers of environmental pollutants are known to stimulate their own metabolism or the metabolism of other compounds. The relationship between environmental pollutants having the capacity to induce the hepatic MFO systems and the metabolism of procarcinogens must be discussed. The Salmonella/microsome mutation assay is one of the methods for detection of mutagenic intermediates through the

Table 1. Effects of biphenyl ethers on conversion of procarcinogens to mutagens.

| | Procarcinogens | | |
|---|---|---|---|
| Treatment | 2-AAT(10$\mu$g) | 2-AAF(50$\mu$g) | BaP(10$\mu$g) |
| Control | 54" | 55" | 1.9' |
| 4-$NO_2$ | 58" | 46" | 1.5' |
| 4-$CNO_2$ | 71" | 91" | 1.6' |
| 2,4-$DCNO_2$ | 88" | 101" | 2.0' |
| 2,4,6-$TCNO_2$ | 67" | 84" | 1.4' |
| PB | 75" | 105" | 2.1' |
| 3-MC | 73" | 85" | 9.9" |
| PCB | 66" | 96" | 6.5" |

Values in the table are ratios of numbers of revertant colonies observed in the test plate to the number of revertant colonies appearing on the corresponding control (enzyme blank) plate, i.e., ratio of test to background. Statistical significance toward to the enzyme blank at '$P<0.01$ and "$P<0.001$ by t-test.

metabolism of procarcinogens (Ames et al., 1975). The effects of biphenyl ethers on conversion of procarcinogens (2-AAT, 2-AAF and BaP) to mutagenic intermediates are given in Table 1. All the S-9 fractions from rats treated with biphenyl ethers except for 4-$NO_2$ caused about 1.5-fold and about 2.0-fold increases in the mutagenicities of 2-AAT and 2-AAF, respectively, but caused no increase in the mutagenicity of BaP compared with the S-9 fraction from control rat. The S-9 fraction from rat treated with 4-$NO_2$ had no increase in the mutagenicity of any procarcinogens. Among biphenyl ethers, 2,4-$DCNO_2$ was the most effective on enhancement of mutagenicities of 2-AAT and 2-AAF followed by 4-$CNO_2$ and then 2,4,6-$TCNO_2$. This order of biphenyl ethers in the mutation assay was the same as the order of them in the experiment on hepatic MFO systems (Fig. 1), indicating that the enhancement of mutagenicities of 2-AAT and 2-AAF was drived from the increased levels of hepatic MFO systems by biphenyl ethers. No enhancement of mutagenicity of BaP by the S-9 fractions from rats treated with biphenyl ethers verifies the classification of biphenyl ethers into PB-type inducers as shown in Figs. 1 and 2. According to Ames et al. (1975), the mutagenicities of polycyclic hydrocarbons and aromatic amines and amides are enhanced by treatments with 3-MC and PB, respectively, and PCB enhances the mutagenicities of both polycyclic hydrocarbons and aromatic amines and amides. The results of positive controls (PB, 3-MC and PCB) in Table 1 were agreement with those by Ames et al.

From the results through this study, it was revealed that biphenyl ethers except for 4-$NO_2$ were PB-type inducers and accerelated the metabolism of procarcinogens (2-AAT and 2-AAF) to mutagenic intermediates. Among biphenyl ethers, 2,4-$DCNO_2$ and 2,4,6-$TCNO_2$ are widely used as herbicides and are pollutants in the environment and animals. These herbicides are known to be dechlorinated in the environment by various factors, such as the sunlight (Nakagawa and Crosby, 1974). When animals or man were polluted with biphenyl ethers and encountered procarcinogens(aromatic amines and amides), the risk of carcinogenicity would increase.

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