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STUDIES ON THE TOXICITY OF CHLORINATED *p*-NITROBIPHENYL ETHER

I—METHEMOGLOBIN FORMATION *IN VITRO* AND *IN VIVO* INDUCED BY NITROSO AND AMINO DERIVATIVES OF CHLORINATED BIPHENYL ETHER

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Abstract—Methemoglobin formation by nitroso and amino derivatives of chlorinated biphenyl ether was investigated. All of the nitroso derivatives used induced methemoglobin formation in suspensions of human erythrocytes. Ability to induce methemoglobin formation among the nitroso derivatives was the highest for nitrosobenzene, followed by *p*-nitrosobiphenyl ether, 4-chloro *p*-nitrosobiphenyl ether, 2,4-dichloro *p*-nitrosobiphenyl ether and then 2,4,6-trichloro *p*-nitrosobiphenyl ether. Three monochloro *p*-nitrosobiphenyl ethers showed all the same activity. Therefore, the ability to induce methemoglobin formation depended on the number of chlorines rather than on the position of chlorine substitution on the phenoxy group in the molecule. Since lactate decreased but glucose and xylitol increased the methemoglobin level produced by the nitroso compounds, this reaction would appear to require an NADPH-dependent enzyme system in erythrocytes. All of the amino derivatives used induced methemoglobin formation in suspensions of human erythrocytes with rat liver homogenates. Ability to induce methemoglobin formation among the amino derivatives was the highest for *p*-aminobiphenyl ether, followed by 4-chloro *p*-aminobiphenyl ether, 2,4-dichloro *p*-aminobiphenyl ether, 2,4,6-trichloro *p*-aminobiphenyl ether, and then aniline. Methemoglobin formation by chlorinated *p*-aminobiphenyl ether was examined by intraperitoneal and oral administration to rats. After i.p. injection, methemoglobinemia was induced by *p*-aminobiphenyl ether, aniline, and 2,4-dichloro *p*-aminobiphenyl ether but not by 2,4,6-trichloro *p*-aminobiphenyl ether over the period tested. The order of methemoglobin formation was *p*-aminobiphenyl ether ≧ 4-chloro *p*-aminobiphenyl ether > aniline ≧ 2,4-dichloro *p*-aminobiphenyl ether. Oral administration gave almost the same result as i.p. injection. The maximum methemoglobin levels produced by the amino derivatives were highest for 4-chloro *p*-aminobiphenyl ether, followed by *p*-aminobiphenyl ether, 2,4-dichloro *p*-aminobiphenyl ether, aniline, and then 2,4,6-trichloro *p*-aminobiphenyl ether. However, the methemoglobin level produced by 2,4,6-trichloro *p*-aminobiphenyl ether was very low and not significantly greater than the background level.

Chlorinated *p*-nitrobiphenyl ethers are used increasingly for both pre- and post-emergence weed control in such major crops as rice and sugar beets all over the world. However, chlorinated *p*-nitrobiphenyl ethers are thought to have various toxicities in common because of having a biologically active nitro group and lipophilic chlorine substituents, in addition to a biologically stable ether linkage in their molecules. These compounds have been shown to be easily converted to the corresponding amines in the environment [1–3]. However, their metabolic fate *in vivo* has received little attention [4]. Recently, it was reported that one of them, 2,4-dichloro *p*-aminobiphenyl ether, was carcinogenic [5, 6]. This adverse effect is believed to depend on the metabolic conversion to active metabolites.

The aromatic nitro structure is known to cause methemoglobinemia, and to exhibit mutagenicity and carcinogenicity after metabolic activation [7–10]. The reduction of aromatic nitro compounds has long been recognized as a common metabolic pathway both *in vivo* and *in vitro* [11–13]. The reductive reaction is presently thought to proceed via the formation of nitroso and hydroxylamino intermediates to corresponding amines [14–16]. The aromatic amines, too, cause methemoglobinemia, and exhibit mutagenicity and carcinogenicity after metabolic activation [17, 18]. The ultimate metabolite responsible for these toxic effects by both the aromatic nitro and amino compounds is regarded as the same intermediate, i.e. the aromatic hydroxylamine [19].

Since methemoglobin formation is regarded as an indicator of hydroxylamine production [20], the levels of methemoglobin induced by the amino and nitroso derivatives of chlorinated *p*-nitrobiphenyl ether may show a positive correlation with their various toxicities.

The purpose of this study was to determine whether the nitroso and amino derivatives of chlorinated *p*-nitrobiphenyl ether have an ability to form methemoglobin. In this experiment, the nitroso derivatives were synthesized and used instead of the hydroxylamino derivatives because the latter materials are very unstable, whereas the former are

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rapidly reduced to the latter in erythrocytes and can therefore be regarded as equally effective.

EXPERIMENTAL

Chemicals. Inorganic salts, D-glucose, xylitol and L-lactic acid were all of analytical quality and were purchased from Wako (Osaka, Japan). D-Glucose-6-phosphate and NADP were obtained from Oriental Yeast (Tokyo, Japan).

Preparation of herbicides and their related compounds. 2,4-Dichloro *p*-nitrobiphenyl ether was isolated from the commercial NIP concentrate, which was obtained from Nippon Noyaku (Tokyo, Japan), and was recrystallized from 90% methanol to give pale yellow needles of m.p. 70.5–71.0° [21]. 2,4,6-Trichloro *p*-nitrobiphenyl ether was isolated from the commercial MO granules obtained from Hokko Kagaku (Tokyo, Japan) and was recrystallized from a solvent mixture of ethanol and ethylene chloride to give brown needles of m.p. 106–109° [22]. *p*-Nitrobiphenyl ether and 2-, 3-, and 4-chloro *p*-nitrobiphenyl ethers were synthesized according to Morgan *et al.* [23].

Preparation of the nitroso derivatives. The nitroso derivatives were synthesized as follows. To a mixture of 3 g of the parent compound, 150 ml of acetone and 50 ml of 1% ammonium chloride solution was added 3 g of zinc dust in small portions with vigorous stirring over a period of 5 min. Thirty minutes after the addition of zinc, the reaction mixture was filtered and the precipitate was washed on the filter with 2 × 10 ml of acetone. The filtrate and washings were combined and the solvent was removed *in vacuo* below 45°. The obtained aqueous solution was extracted with 3 × 50 ml of ethanol-free chloroform. To the combined chloroform extract was added 20 g of active manganese dioxide prepared by the method of Papadoupoulos *et al.* [24]. After stirring for 12 hr at 0°, the reaction mixture was filtered to remove the solids. The filtrate was evaporated *in vacuo* to a syrup. The syrup was dissolved in 5 ml of chloroform and mixed with 2 g of silica gel. The chloroform was removed by evaporation. Hexane was added and the slurry was added to a silica gel column (2.0 × 35 cm, 50 ml, packed with hexane). The column was developed with hexane and a green colored fraction was collected, evaporated *in vacuo*, and then solidified in a freezer. Yield, melting point, mass and u.v. spectra, and elemental analysis: [*p*-nitrosobiphenyl ether] 11.3%, 30–32°, *m/e* 199 (M^+), λ_{max} (EtOH) 338 nm (ε18,500) and 239 nm (ε8,400). Calcd. for $C_{12}H_9NO_2$: C, 72.35; H, 4.55; N, 7.03. Found: C, 71.98; H, 4.47; N, 7.05. [2-Chloro *p*-nitrosobiphenyl ether] 10.0%, 34–36°, *m/e* 233 (M^+), λ_{max} (EtOH) 332 nm (ε16,900) and 236 nm (ε7,900). Calcd. for $C_{12}H_8NO_2Cl$: C, 61.69; H, 3.45; N, 5.99. Found: C, 61.89; H 3.45; N, 5.97. [3-Chloro *p*-nitrosobiphenyl ether] 11.5%, liquid at room temp., *m/e* 233 (M^+), λ_{max} (EtOH) 334 nm (ε15,600). Found: C, 61.66; H, 3.43; N, 5.80. [4-Chloro *p*-nitrosobiphenyl ether] 14.7%, 55–56°, *m/e* 233 (M^+), λ_{max} (EtOH) 335 nm (ε16,800) and 226 nm (ε13,000). Found: C, 61.66; H, 3.45; N, 6.07. [2,4-Dichloro *p*-nitrosobiphenyl ether] 20.0%, 60–62°, *m/e* 267 (M^+), λ_{max} (EtOH) 330 nm (ε16.800). Calcd. for $C_{12}H_7NO_2Cl_2$: C, 53.76; H, 2.63; N, 5.22. Found: C, 53.72; H, 2.53; N, 5.24. [2,4,6-Trichloro *p*-nitrosobiphenyl ether] 33.0%, 86–88°, *m/e* 301 (M^+), λ_{max} (EtOH) 327 nm (ε14,600). Calcd. for $C_{12}H_6NO_2Cl_3$: C, 47.64; H, 1.99; N, 4.63. Found: C, 47.35; H, 1.97; N, 4.59. Nitrosobenzene (NOB) was prepared from nitrobenzene according to Coleman *et al.* [25].

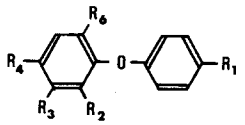
Preparation of the amino derivatives. The amino derivatives were synthesized from the parent compounds by reduction with activated iron according to Hazlet and Dornfeld [26]. They were isolated as hydrochlorides by passing hydrochloride gas into benzene solution and then recrystallized from *n*-propanol–3 N HCl as colorless needles. Yield, melting point, mass and u.v. spectra, and elemental analysis: [*p*-aminobiphenyl ether hydrochloride] 80.0%, 200–205°, *m/e* 185 (M^+), λ_{max} (EtOH) 234 nm (ε8,400). Calcd. for $C_{12}H_{11}NO \cdot HCl \cdot 1/4H_2O$: C, 63.72; H, 5.57; N, 6.19. Found: C, 63.71; H, 5.50; N, 5.97. [2-Chloro *p*-aminobiphenyl ether hydrochloride] 56.0%, 194–196°, *m/e* 219 (M^+), λ_{max} (EtOH) 241 nm (ε11,000). Calcd. for $C_{12}H_{10}NOCl \cdot HCl$: C, 56.27; H, 4.33; N, 5.54. Found: C, 56.47; H, 4.25; N, 5.46. [3-Chloro *p*-aminobiphenyl ether hydrochloride] 70.0%, 169–172°, *m/e* 219 (M^+), λ_{max} (EtOH) 242 nm (ε12,300). Found: C, 56.29; H, 4.31; N, 5.45. [4-Chloro *p*-aminobiphenyl ether hydrochloride] 70.0%, 185°, *m/e* 219 (M^+), λ_{max} (EtOH) 241 nm (ε15,100). Found: C, 56.31; H, 4.21; N, 5.23. [2,4-Dichloro *p*-aminobiphenyl ether hydrochloride] 67.0%, 154–155°, *m/e* 253 (M^+), λ_{max} (EtOH) 241 nm (ε13,300). Calcd. for $C_{12}H_9NOCl_2 \cdot HCl$: C, 49.60; H, 3.47; N, 4.82. Found: C, 49.46; H, 3.47; N, 4.78. [2,4,6-Trichloro *p*-aminobiphenyl ether hydrochloride] 75.0%, 200°, *m/e* 287 (M^+), λ_{max} (EtOH) 227 nm (ε18,800). Calcd. for $C_{12}H_8NOCl_3 \cdot HCl$: C, 44.35; H, 2.79; N, 4.31. Found: C, 44.40; H, 2.71; N, 4.08. Mass and u.v. spectra were determined by a Hitachi gas mass spectrometer M-60 and a Shimazu double beam spectrophotometer UV-200S respectively.

Animals. Male Wistar rats weighing 200–250 g were used in this study. They were maintained on a commercial diet and tap water *ad lib.*

Preparation of human erythrocytes. Human blood was obtained from the Japan Red Cross Center. Blood samples were centrifuged for 5 min to separate cells and plasma. Plasma and buffy coat were removed by suction. The erythrocytes were washed successively with 0.9% NaCl and twice with Krebs–Ringer solution. Erythrocytes thus obtained were suspended in an incubation medium.

Preparation of rat liver homogenates. Rats were fasted for a day before killing. They were anesthetized by ethyl ether and livers were removed. Livers were perfused with 0.9% NaCl *in situ* and homogenized with a Potter–Elvehjem glass–Teflon homogenizer in 2 vol. of cold 1.15% KCl. The homogenates were centrifuged at 9000 *g* for 20 min in a Hitachi 65-P centrifuge below 4°. The post-mitochondrial supernatant fraction was used in this study.

Incubation medium. In the case of methemoglobin measurement in suspensions of human erythrocytes, incubation medium, in a test tube, contained 0.1 ml dimethylsulfoxide (containing the nitroso derivative at a final concentration of 0.5 mM), 0.8 ml human



				Abbreviation		
				R_1		
R_2	R_3	R_4	R_6	NO_2	NO	NH_2
H	H	H	H	p-NO_2	p-NO	p-NH_2
Cl	H	H	H	2-CNO_2	2-CNO	2-CNH_2
H	Cl	H	H	3-CNO_2	3-CNO	3-CNH_2
H	H	Cl	H	4-CNO_2	4-CNO	4-CNH_2
Cl	H	Cl	H	2,4-$DCNO_2$	2,4-DCNO	2,4-$DCNH_2$
Cl	H	Cl	Cl	2,4,6-$TCNO_2$	2,4,6-TCNO	2,4,6-$TCNH_2$

Fig. 1. Structures and abbreviations of chlorinated biphenyl ether derivatives and related compounds.

erythrocytes and 1.1 ml Krebs–Ringer phosphate buffer, pH 7.4 [27]. Incubations were carried out aerobically in a shaking water bath at 37°. For observation of the effects of D-glucose, xylitol and L-lactate on methemoglobin formation by the nitroso derivatives, these additives were added to the medium at zero time at a concentration of 0.04 mM. Methemoglobin was determined in aliquots taken out at the indicated times by the procedure of Evelyn and Malloy [28]. A control was run in the absence of the nitroso derivatives and its absorbance was subtracted from the observed values. Methemoglobin was expressed as percent methemoglobin. In the case of methemoglobin measurement in human erythrocytes with rat liver homogenates, incubation medium, in a test tube, contained 0.1 ml dimethylsulfoxide (containing the amino derivative at a final concentration of 0.5 mM), 0.1 ml $MgCl_2$ (60 μmoles), 0.1 ml nicotinamide (120 μmoles), 0.1 ml D-glucose-6-phosphate (25 μmoles), 0.1 ml NADP (1.8 μmoles), 1.0 ml 33% rat liver homogenate (equivalent to 330 mg fresh liver), 1.3 ml human erythrocytes and an appropriate volume of 0.1 M phosphate buffer, pH 7.4 (KH_2PO_4–NaOH) to make a final incubation volume of 3.1 ml. Appropriate tissue blanks (containing all the constituents except the substrate) and blood blanks (containing all the constitutents except liver homogenate) were incubated in all these assays, and their absorbances were subtracted from the observed values.

Methemoglobin measurement in vivo *in rats.* The amino derivatives were dissolved in olive oil and administered intraperitoneally and orally at a volume of 0.4 ml/rat (0.77 mmole/kg of body weight). At the indicated times, 50 μl of blood was removed from the tail vein and treated as described before.

Structures and abbreviations of chlorinated biphenyl ether derivatives and their related compounds are shown in Fig. 1.

RESULTS AND DISCUSSION

Methemoglobin formation by the nitroso derivatives in suspensions of human erythrocytes. Figure 2 shows the rates of methemoglobin formation by the nitroso derivatives in suspensions of human erythrocytes. All of the nitroso derivatives used induced methemoglobin formation. The order of ability to induce methemoglobin formation was the highest for NOB followed by *p*-NO, 4-CNO, 2,4-DCNO and then 2,4,6-TCNO. This result indicates that more highly chlorine substituted compounds induced less methemoglobin formation. Among 2-, 3- and 4-CNOs, there were no significant differences in the rate of methemoglobin formation. From this observation, it is evident that all of the tested nitrosobiphenyl ether derivatives which can be considered as NOB derivatives substituted with varied phenoxy groups in the para position in NOB possess less methemoglobinemic activity compared with that of NOB, and this lesser activity is related to the numbers of chlorines, rather than the position of substitution of the halogen on the phenoxy group.

These findings might reflect the following factors: (1) the relative susceptibility of the nitroso derivatives to conversion to the hydroxylamines by an NADPH-dependent enzyme system in the red cell, although it has been said that aromatic nitroso compounds and corresponding hydroxylamines are equally effective because of rapid reduction of the former to the latter [29]; (2) the relative difference in nitrogen–hydrogen, or oxygen–hydrogen, bond

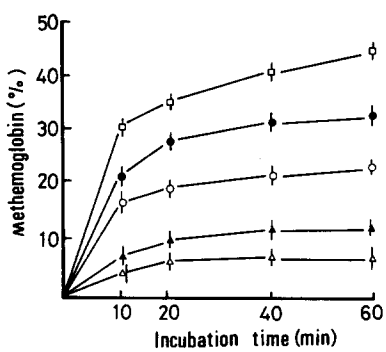


Fig. 2. Methemoglobin formation in suspensions of human erythrocytes by NOB (□), *p*-NO (●), 4-CNO (○), 2,4-DCNO(▲) and 2,4,6-TCNO (△). The incubation medium is described in the Experimental section; reactions were run for 60 min at 37°. Each point is the mean ± S.E. for five experiments.

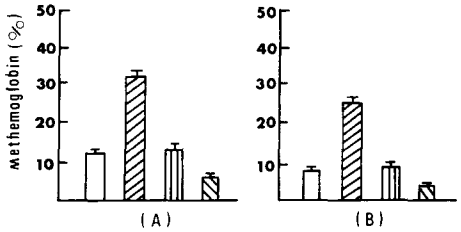


Fig. 3. Effect of D-glucose, xylitol and L-lactate on methemoglobin formation in suspensions of human erythrocytes exposed to 2,4-DCNO (A) and 2,4,6-TCNO (B). The incubation medium is described in the Experimental section, and reactions were run for 40 min at 37°. Bars represent the mean ±S.E. for five experiments. Key: control with no addition (□); 0.04 mM D-glucose (▨); 0.04 mM xylitol (▥); and 0.04 mM L-lactate (▧).

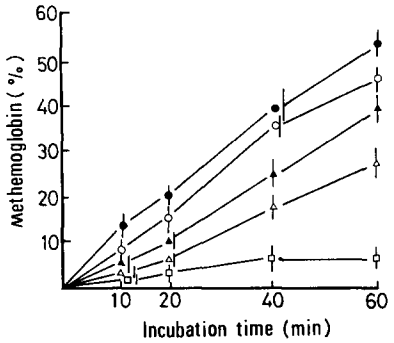


Fig. 4. Methemoglobin formation by the metabolites of *p*-NH_2 (●), 4-CNH_2 (○), 2,4-$DCNH_2$ (▲), 2,4,6-$TCNH_2$ (△) and aniline (□) by rat liver homogenates. The incubation medium is described in the Experimental section, and reactions were run for 60 min at 37°. Each point is the mean ± S.E. for five rat livers.

dissociation energy in the formed hydroxylamine [30]; and (3) the relative rate of the subsequent metabolism of the hydroxylamines to non-methemoglobin forms in the red cell [29].

Effects of D-*glucose, of xylitol and* L-*lactate on methemoglobin formation in suspensions of human erythrocytes exposed to* 2,4-*DCNO and* 2,4,6-*TCNO.* The effects of D-glucose, xylitol and L-lactate on methemoglobin formation are shown in Fig. 3. D-Glucose greatly enhanced methemoglobin formation while xylitol increased it slightly. Lactate, however, yielded a smaller amount of methemoglobin. Methemoglobin formation depends on the relative rates of oxidation of hemoglobin and its subsequent reduction by NADH-dependent methemoglobin reductase, back to hemoglobin [31]. In the case of D-glucose and xylitol, both NADH and NADPH are produced through glycolysis and the pentose phosphate shunt, respectively, in erythrocytes, while in the case of lactate, only NADH is produced by the conversion of it to pyruvate, catalyzed by lactate dehydrogenase in the red cell. There is another methemoglobin reductase system in the red cell which is NADPH-dependent. However, this system is not operative under normal conditions. From the results shown in Fig. 3, it was obvious that the reduction of the nitroso derivatives to the hydroxylamines by an NADPH-dependent enzyme system in erythrocytes led to the methemoglobin formation and, once formed, methemoglobin was reduced to hemoglobin by an NADH-dependent methemoglobin reductase system. These results are in agreement with the mechanism of methemoglobin formation by the other type of nitroso compound, established by Kiese [17].

Methemoglobin formation by the amino derivatives with rat liver homogenates in suspensions of human erythrocytes. The mechanism of methemoglobin formation by aromatic amines is well established [17, 18]. Aromatic amines form no methemoglobin in suspensions of erythrocytes alone, but do so after activation by liver homogenate. Aromatic amines are generally metabolized to aromatic hydroxylamines (*N*-hydroxylation) and/or aminophenols (*C*-hydroxylation). Although it is well known that aminophenols can induce methemoglobin formation, the rate of methemoglobin formation by them is very minor. The ultimately effective metabolite of aromatic amine in inducing methemoglobin formation is the hydroxylamine. Oxidation of the latter to the nitroso compound is coupled to the oxidation of Fe^{2+} to Fe^{3+} in hemoglobin. The nitroso compounds is reduced rapidly to the hydroxylamine by an NADPH-dependent enzyme in erythrocytes. Therefore, the methemoglobin formation by aromatic amine is closely related to *N*-hydroxylation. The methemoglobin formation by the amino derivatives with rat liver homogenates is shown in Fig. 4. All of the amino compounds used induced methemoglobin formation; the order of ability to induce methemoglobin formation by them was highest for *p*-NH_2, followed by 4-CNH_2, 2,4-$DCNH_2$, 2,4,6-$TCNH_2$ and then aniline. The results indicated that the amino derivatives were obviously converted to the methemoglobin-forming metabolites, namely the hydroxylamines, by rat liver homogenates because they formed no methemoglobin in the absence of the homogenate. This order, with the exception of aniline, was the same as that of the corresponding nitroso derivatives described before. It is said that the rate of methemoglobin formation reflects the extent of *N*-hydroxylation of the aromatic amine by the liver microsomal oxidation system. The fact that aniline showed the least activity among them while the corresponding NOB showed the highest activity is probably due to slower conversion of the less lipid soluble aniline by the microsomes to phenylhydroxylamine and/or the higher metabolic rate of aniline to non-methemoglobin formers by the liver homogenate, compared with that for aminobiphenyl ether derivatives.

Recently, there have been reports [31, 32] that a chlorine substitution in the para position in aniline enhanced the activity because of diminished *C*-hydroxylation in the amino group. Since *p*-aminobiphenyl ethers are considered as aniline derivatives having phenoxy substitution in the para position in aniline, our observation is consistent with these results. Among monochloro *p*-aminobiphenyl ethers, the rate of methemoglobin formation by 2- and 3-CNH_2s was similar. However, the rate of meth-

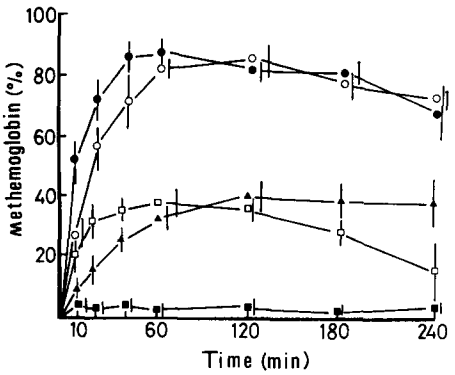


Fig. 5. Concentration of methemoglobin in the blood of rats after the intraperitoneal administration of 0.77 mmole/kg of p-NH_2 (●), 4-CNH_2 (○), 2,4-$DCNH_2$(▲), aniline (□), and no compound (■). Each point is the mean ± S.E. for five rats.

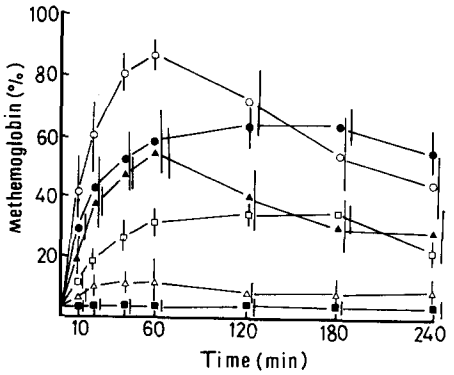


Fig. 6. Concentration of methemoglobin in the blood of rats after the oral administration of 0.77 mmole/kg of p-NH_2 (●), 4-CNH_2 (○), 2,4-$DCNH_2$ (▲), 2,4,6-$TCNH_2$ (△), aniline (□), and no compound (■). Each point is the mean ± S.E. for four rats.

emoglobin formation by 4-CNH_2 was lower than that by 2- and 3-CNH_2s. This indicated that *N*-hydroxylation by rat liver homogenate was affected by the position of substitution of chlorine on the phenoxy group. The rate of *N*-hydroxylation of the compound with a *para*-chlorine substituent was lower than that of *ortho*- and *meta*-substituted compounds although there were no significant differences in the rate of methemoglobin formation by their corresponding nitroso derivatives.

Methemoglobin formation in rats. The concentration of methemoglobin in the blood of rats after intraperitoneal injection of the amino derivatives is shown in Fig. 5. The trichloro compound, 2,4,6-$TCNH_2$, formed no methemoglobin in rats. Methemoglobin formation by the other amino derivatives up to about 40 min was the highest for p-NH_2, followed by 4-CNH_2, aniline, and then 2,4-$DCNH_2$. After 40 min, the methemoglobin levels with p-NH_2 were the same as those obtained with 4-CNH_2, and those with 2,4-$DCNH_2$ were the same as those obtained with aniline. The decline in the aniline curve by 4 hr may be experimental error as seen by the wide standard deviations. Among monochloro p-aminobiphenyl ethers, the methemoglobin levels induced by 3- and 4-CNH_2s were the same and were higher than that induced by 2-CNH_2, with respect to their maximum level. This is different from the result of the liver homogenate experiment described before, in which 2-CNH_2 showed activity higher than that of 4-CNH_2. It is obvious that this was due to utilization of different metabolic pathways qualitatively and/or quantitatively *in vivo* and *in vitro*. The concentration of methemoglobin in the blood of rats after the oral administration of the amino derivatives is shown in Fig. 6. Methemoglobin formation by oral administration was almost the same as that by intraperitoneal injection. Although 2,4,6-$TCNH_2$ formed methemoglobin, there was no statistically significant difference from background. From these results, there were several similarities between i.p. and oral administration. The only possible difference between i.p. and oral administration was the rate of maximum formation of methemoglobin. p-NH_2 gave the highest maximum levels, followed by 4-CNH_2, aniline, 2,4-$DCNH_2$ and then 2,4,6-$TCNH_2$.

From these results, all of the amino derivatives studied are *N*-hydroxylated to the hydroxylamines in rat as indicated by methemoglobin formation. It is well known that, as a consequence of chronic exposure to aromatic amines, tumors are formed [15, 18]. Therefore, the biological formation of the hydroxylamine derivatives through *N*-hydroxylation of the chlorinated p-aminobiphenyl ethers would give a potential hazard. A great many chlorinated nitrobiphenyl ethers have been used as herbicides; 2,4-$DCNO_2$ especially is used all over the world. The herbicides are decomposed by environmental factors. Environmental decomposition can lead to reduction of these compounds. For example, 2,4-$DCNO_2$ is reduced easily to 2,4-$DCNH_2$ by sunlight [3] and in the rumen fluid of the dairy cow [4]. Additionally, many nitroaromatic compounds have been known to be reduced by various intestinal microorganisms [33]. It is thought that there are many chances for chlorinated nitrobiphenyl ethers to be reduced to the amine form. Therefore, study on the binding to cellular macromolecules of metabolites of the chlorinated nitrobiphenyl ether is indicated. Additionally, there are many questions to be elucidated for the chlorinated nitrobiphenyl ethers, e.g. accumulation in the adipose tissues, and the induction of hepatic microsome enzyme systems owing to their high lipophilic nature as observed with polychlorinated biphenyls (PCB), benzene hexachloride and DDE [34, 35].

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