Studies on Toxicities of Biphenyl Ether Herbicides and Their Analogues to Fish—II

Production of Methemoglobin-Forming Metabolites of the Amino-Derivatives by White-Spotted Char, *Salvelinus leucomaenis*, Liver Homogenate^{*1,*2}

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(Received July 31, 1979)

The activities of liver homogenates from white-spotted char and rat, to metabolize the amino-derivatives of biphenyl ether herbicides (2,4-dichloro *p*-nitrobiphenyl ether and 2,4,6-trichloro *p*-nitrobiphenyl ether) and their analogues to methemoglobin-forming metabolites were compared by incubating liver homogenates with the amino-derivatives, a NADPH-generating system and white-spotted char, rat or human erythrocytes. The results indicated that white-spotted char liver homogenates had the ability to metabolize *p*-aminobiphenyl ether to methemoglobin-forming metabolites although the activity was very low; while rat liver homogenates had the ability to metabolize all the amino-derivatives to methemoglobin-forming metabolites and the rates of methemoglobin formation among the amino-derivatives were the highest in *p*-aminobiphenyl ether followed by 4-chloro *p*-aminobiphenyl ether, 2,4-dichloro *p*-aminobiphenyl ether and then 2,4,6-trichloro *p*-aminobiphenyl ether. The sensitivity of erythrocytes to the methemoglobin-forming metabolites were the highest in human followed by rat and then white-spotted char erythrocytes.

Aromatic nitro compounds have been known to be reduced to corresponding aromatic amino compounds in animals by nitro reductase¹⁻³⁾ or intestinal microorganisms.⁴⁾ And even by environmental factors, this reaction proceeds, e. g., one of the biphenyl ether herbicides, 2,4-dichloro *p*-nitrobiphenyl ether is known to be reduced to the corresponding amino-derivative by the sun light,⁵⁾ the rumen fluid of the dairy cow⁶⁾ or the chemical and microbial process in soil.⁷⁾ And 2,4,6-trichloro *p*-nitrobiphenyl ether, one of the biphenyl ether herbicides, is known to be detected in soil and river as the amino-derivative and to be concentrated in carp.⁸⁾ The amino-derivatives thus formed in environment may be regarded as pollutants including the parent compounds.

Aromatic amines are well known to cause methemoglobin formation, mutagenesis and carcinogenesis by metabolic activation.^{9,10)} The ultimate metabolite in the appearance of these toxic

effects by aromatic amino compounds is regarded as aromatic hydroxylamine-derivatives.⁹⁻¹¹⁾ In the previous paper,^{*5} we have reported that all of the amino-derivatives of the biphenyl ether herbicides and their analogues used, induced the methemoglobin formation in rats *in vivo* and in rat liver homogenates *in vitro* and that the metabolites of the amino-derivatives were thought to be *N*-hydroxylated metabolites, i. e., the hydroxylamine-derivatives. Therefore the biological formation of aromatic hydroxylamines is of considerable importance to animals including fish.

Little attention has been paid to fish about metabolism of xenobiotics because fish is thought to excrete them without metabolizing.^{12,13)} However there are reports that fish has mechanisms to metabolize pentachlorophenol (PCP) and 2-amino-4-phenylthiazole, known as a herbicide and an anesthetic of fish, respectively.^{14,15)} Therefore it is of interest to know if fish is able to metabolize

*1 A part of this study was presented at the autumn meeting of the Japan. Soc. Sci. Fish. on Oct. 8, 1978, Shimizu.

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the amino-derivatives of biphenyl ether herbicides and their analogues to methemoglobin-forming metabolites.

The present paper reports a convenient assay method to determine the activity of liver homogenate to metabolize the amino-derivatives to methemoglobin-forming metabolites. Using this method, the relative activities of the liver from white-spotted char and rat were compared.

Fig. 1. Structures of the amino-derivatives of biphenyl ether herbicides and their analogues used.

Materials and Methods

Chemicals

Inorganic salts were all of analytical quality and purchased from Wako Chemical Industry Co., Tokyo, Japan. D-Glucose-6-phosphate and NADP were from Oriental Yeast Co., Tokyo, Japan. The procedure of synthesis of *p*-NH, 4-CN, 2, 4-DCN and 2, 4, 6-TCN was as described in the previous paper.^{*5}

Animals

The white-spotted char, *Salvelinus leucomaenis*, were obtained as described in the previous paper.¹⁶⁾ The male Wistar rats (body weight, 200–250 g) were used and they were maintained on a commercial diet obtained from Nippon Clea Co., Tokyo, Japan and on tap water *ad libitum*.

Preparation of Erythrocytes

Erythrocytes from white-spotted char and human were prepared as described in the previous paper.¹⁶⁾ Erythrocytes from rat were prepared as follows; rats were sacrificed by decapitation and exsanguination. Blood samples were collected in a heparinized tube and centrifuged for 5 min in order to separate cells and plasma. Plasma and buffy coat were removed by suction. After one washing with 0.9% NaCl for 5 min, washing with Krebs-Ringer solution, pH 7.4 for 15 min was repeated. The erythrocytes obtained were suspended in an

incubation medium given below.

Preparation of Liver Homogenates

Liver samples from white-spotted char and rat were perfused with 0.9% NaCl and homogenized in a Potter-Elvehjem glass homogenizer with a Teflon Pestle, using sufficient cold 1.15% KCl to produce a final homogenate concentration of 33%. After the liver homogenates were centrifuged at 4°C and 9,000 × g for 20 min, the post-mitochondrial supernatant was used.

Incubation Medium

The incubation medium contained 0.1 ml dimethyl sulfoxide (containing the amino-derivative as a final concentration of 0.5 mM), 0.1 ml MgCl₂·6H₂O (60 μmol), 0.1 ml nicotinamide (120 μmol), 0.1 ml D-glucose-6-phosphate (25 μmol), 0.1 ml NADP (1.8 μmol), 1.3 ml white-spotted char, rat or human erythrocytes and an appropriate volume of 0.1 M phosphate buffer, pH 7.4 (KH₂PO₄-NaOH) to make a final incubation volume of 3.1 ml. Appropriate tissue blanks that contained all the constituents except the amino-derivative and blood blanks that contained all the constituents except liver homogenates were incubated in each assay and their absorbances were subtracted from the observed values. All the incubation was performed aerobically at 25°C in a shaking water bath.

Methemoglobin Measurement

Methemoglobin was measured in aliquots taken out of the incubation medium at different times by the procedure of Evelyn and Malloy.¹⁷⁾

Results and Discussion

Activities of Livers and Sensitivities of Erythrocytes

For comparison of the activities of liver homogenates from white-spotted char and rat to metabolize the amino-derivatives to methemoglobin-forming metabolites and the sensitivities of erythrocytes from white-spotted char, rat and human to the methemoglobin-forming metabolites, the following combinations were made: I, white-spotted char liver and erythrocytes; II, white-spotted char liver and rat erythrocytes; III, white-spotted char liver and human erythrocytes; IV, rat liver and white-spotted char erythrocytes; V, rat liver and erythrocytes; VI, rat liver and human erythrocytes. And *p*-NH of which metabolite was the most powerful methemoglobin-former as reported in the previous paper^{*6} was incubated with a NADPH-

generating system in these combinations. Fig. 2 shows the relative activities of liver homogenates from white-spotted char and rat in the presence of human erythrocytes to produce the methemoglobin-forming metabolite of *p*-NH (the combinations III and VI). Fig. 3 shows the relative sensitivities of erythrocytes from white-spotted char, rat and human in the presence of rat liver homogenates to the methemoglobin-forming metabolite of *p*-NH (the combinations IV, V and VI). No methemoglobin was formed in the combinations I and II. From these results it was concluded that rat liver had a fairly higher activity than white-spotted char liver to produce the methemoglobin-forming metabolite of *p*-NH and that human erythrocytes were the most sensitive to the methemoglobin-forming metabolite of *p*-NH followed by rat and then white-spotted char ones. No methemoglobin formation in the combination I was obviously due to the very low activity of white-spotted char liver to produce the methemoglobin-forming metabolite of *p*-NH and the low sensitivities of white-spotted char erythrocytes to the methemoglobin-forming metabolite of *p*-NH. No methemoglobin formation in the combination II was due most probably to the low activity of white-spotted char liver. The methemoglobin levels depend on the relative rates between oxidation of hemoglobin and its subsequent reduction by methemoglobin reductase, back to hemoglobin.¹⁸⁾ The low sensitivities of white-spotted char erythrocytes might be due to their high activities of me-

Fig. 2. Rates of production of a methemoglobin-forming metabolite of *p*-NH in the presence of human erythrocytes by liver homogenates from white-spotted char (○) III and rat (●) VI with *p*-NH as substrate. Points represent the mean $\pm$ S.E. from assays on the livers from five animals.

Fig. 3. Rates of methemoglobin formation by rat liver homogenates with *p*-NH in the presence of erythrocytes from white-spotted char (○) IV, rat (●) V and human (Δ) VI. Points represent the mean $\pm$ S.E. from assays on five rat livers.

themoglobin reduction as reported in the previous paper.¹⁶⁾ According to Brodie *et al.*, fish have no activity to metabolize drugs.¹²⁾ However, present data showed that methemoglobin was formed at the low rate when *p*-NH was incubated with white-spotted char liver and the most sensitive human erythrocytes as shown in Fig. 2. This indicates that fish do have the activity to metabolize drugs although the activity is very low.

The Activity of Liver to Produce the Methemoglobin-Forming Metabolites of Other Amino-Derivatives

In this test, white-spotted char liver was omitted because of its very low activity to produce the

Fig. 4. Rates of methemoglobin formation by rat liver homogenates with *p*-NH (■), 4-CN (Δ) and 2, 4-DCNH (▲) in the presence of erythrocytes from rat. Points represent the mean $\pm$ S.E. from assays on five rat livers.

Fig. 5. Rates of methemoglobin formation by rat liver homogenates with *p*-NH (■), 4-CNH (△), 2,4-DCNH (▲), 2,4,6-TCNH (○) and ANL (●) in the presence of human erythrocytes. Points represent the mean $\pm$ S.E. from assays on five rat livers.

methemoglobin-forming metabolite of *p*-NH. All of the other amino-derivatives in the combination IV formed no methemoglobin. In the combination V, methemoglobin was formed with 4-CNH and 2,4-DCNH as shown in Fig. 4, where the result on *p*-NH is also given. In the combination VI, methemoglobin was formed with all of the amino-derivatives used, and aniline (ANL) as shown in Fig. 5, where the result on *p*-NH is also given. These results indicated that the combination VI was the best among all the combinations tested to measure the activity of liver to produce the methemoglobin-forming metabolites of amino-derivatives. In the combination VI, the rate of methemoglobin formation among the amino-derivatives was the highest in *p*-NH followed by 4-CNH, 2,4-DCNH, 2,4,6-TCNH and then ANL. It has been reported that the rate of methemoglobin formation by aromatic amines reflects on the degree of reception of *N*-hydroxylation by the liver¹⁹⁾ and that a chlorine substitution in the para position in aniline enhanced the activity because of diminished *C*-hydroxylation on the ring and of higher *N*-hydroxylation in the amino-group.^{20, 21)} The result that all of the *p*-aminobiphenyl ethers used showed the higher activity than aniline was in agreement with their results, since *p*-aminobiphenyl ethers are considered as a kind of aniline derivatives having a phenoxy substituent in the para position in aniline. It is of interest that more chlorine substitutions on the phenoxy ring in *p*-aminobiphenyl ether induced less activity. This is prob-

ably due to lower biotransformation of more chlorinated molecules to the corresponding hydroxylamines and or lower reactivity of resultant hydroxylamine to hemoglobin. Aromatic amines are first metabolized to aminophenols (*C*-hydroxylation) or aromatic hydroxylamines (*N*-hydroxylation). Although it has been known that aminophenols induce methemoglobin formation, the rate of methemoglobin formation by them is very minor.⁹⁾ The hydroxylation of aromatic amines in various position is not due to the action of a single enzyme.⁹⁾ The species differences in the activity of the liver to produce the methemoglobin-forming metabolites from aromatic amines are probably due to the different activity of enzyme concerning with *N*-hydroxylation of aromatic amines. In this experiment, the activity of white-spotted char liver to produce the methemoglobin-forming metabolites of *p*-NH was very low. This might be thought that the activity of white-spotted char liver was due to not only the low activity to metabolize xenobiotics but also the low activity of enzyme concerning with *N*-hydroxylation.

Acknowledgement

The authors thank Assistant Professor, Dr. T. Kubo in Nanae Fish Culture Experimental Station for gifting of the white-spotted char.

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