Reductive Dechlorination of *p,p'*-DDT Mediated by Hemoproteins in the Hepatopancreas and Blood of Goldfish, *Carassius auratus*

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The *in vitro* metabolism of *p,p'*-DDT (*p,p'*-dichlorodiphenyltrichloroethane), an important environmental pollutant, was examined in fish, focusing on reductive dechlorination. When *p,p'*-DDT was incubated with hepatopancreas microsomes or the blood of goldfish, *Carassius auratus*, in the presence of both a reduced pyridine nucleotide and FMN, a dechlorinated metabolite, *p,p'*-DDD (*p,p'*-dichlorodiphenyldichloroethane) was formed under anaerobic conditions. These reductase activities were inhibited by carbon monoxide. Although the microsomes or blood was boiled, the dechlorinating activity was not abolished. Hemoglobin and hematin exhibited the reductase activity toward *p,p'*-DDT with NADH and FMN. The activity of hematin was also exhibited with $FMNH_2$. The reductive dechlorination appears to proceed nonenzymatically by the reduced flavin, catalyzed by the heme group of hemoproteins.

Key words —— *p,p'*-DDT, reductive dechlorination, goldfish, blood, hemoglobin, hepatopancreas microsome

INTRODUCTION

p,p'-DDT (*p,p'*-dichlorodiphenyltrichloroethane) is a potent insecticide, which is still used in large quantities, mainly in developing countries, and is extremely persistent in the environment.[1] *p,p'*-DDT and its related compounds, many of which are carcinogenic and mutagenic, are also known as environmental estrogens. It is postulated that these compounds act as mimic hormones and bind the estrogen receptor.[2–4] *p,p'*-DDT and its metabolites accumulate in animal tissues and induce hepatic microsomal enzymes.[5,6] In order to assess the possible risks associated with human exposure to the pesticide, it is essential to thoroughly elucidate metabolism in mammalian species, marine and freshwater species. *p,p'*-DDT is converted by a reductive dechlorination to yield *p,p'*-DDD (*p,p'*-dichlorodiphenyldichloroethane). The dechlorination is the first and limiting step in the metabolism in mammals and microorganism, and therefore is of considerable significance. Several reports have indicated that *p,p'*-DDT is mainly metabolized by reductive dechlorination in mammalian species and microorganisms (Fig. 1).[7] However, no report is available concerning the metabolism of *p,p'*-DDT in fish.

p,p'-DDT *p,p'*-DDD

Fig. 1. Reductive Dechlorination of *p,p'*-DDT to *p,p'*-DDD

In the present study, the *in vitro* metabolism of *p,p'*-DDT by the hepatopancreas and blood of goldfish was examined, focusing on its reductive dechlorination to *p,p'*-DDD. The livers of some fish, such as carp and goldfish, exist as hepatopancreas in the body. The hepatopancreas is reported to be an important tissue for drug metabolism in fish.[8]

MATERIALS AND METHODS

Fish —— Goldfish (*C. auratus*, a kind of red crucian carp, 9—12 cm length, 12—15 g), which were commercially available, were used.

Chemicals —— *p,p'*-DDT and *p,p'*-DDD were purchased from Tokyo Chemical Industry Co., Ltd. NADPH and NADH were from Oriental Yeast Co., and FMN, hemoglobin and hematin (Fe^{3+}) from Sigma Chemical Co. Reduced FMN was prepared from FMN photochemically by the method of Yubisui *et al.*[9]

Preparation of Hepatopancreas Microsomes —— Fish hepato-pancreases were homogenized in 3 vol-

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umes of 1.15% KCl. The homogenate was centrifuged for 20 min at 9000×*g*, and the supernatant fraction was separated into microsomes and cytosol by its centrifugation for 60 min at 105000×*g*. The microsomes were washed by resuspension in 2 volumes of the KCl solution and by resedimentation for 60 min at 105000×*g*.

Preparation of Blood and Erythrocytes —— Blood samples were collected from the goldfish. Blood was put into polystyrene tubes containing heparin as an anticoagulant and centrifuged for 5 min at 4℃ at 3000×*g*. The plasma was removed, and the pellet (erythrocyte fraction) was washed with 2 volumes of cold isotonic saline. Erythrocytes were suspended in one volume of 0.01 M K,Na-phosphate buffer (pH 7.4).

Assay Method —— The incubation mixture consisted of 0.2 μmol of *p,p'*-DDT, 1 μmol of NADPH or NADH, 0.1 μmol of FMN, and hepatopancreas microsomes, blood, hemoglobin or hematin in a final volume of 1 ml of 0.01 M K,Na-phosphate buffer (pH 7.4). The incubation was performed using a Thunberg tube under anaerobic conditions. The side arm contained NADPH or NADH, and the body contained all the other components. The tube was gassed for 3 min with nitrogen, evacuated with an aspirator for 5 min and again gassed with nitrogen. The reaction was started by mixing the components of the side arm and the body together, and this was continued for 30 min at 37℃. The mixture, after adding 1 μg of phenothiazine as an internal standard, was extracted once with 5 ml of ethyl ether and the extract was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 ml of methanol and then subjected to high performance liquid chromatography (HPLC).

HPLC —— HPLC was performed in a Hitachi L-6000 high performance liquid chromatograph equipped with an ultraviolet absorption detector. The instrument was fitted with a 4 × 125 mm LiChrospher RP-Select B (5 μm) (Merck). The mobile phase was acetonitrile-H_2O (7 : 3). The chromatograph was operated at a flow rate of 0.5 ml/min at ambient temperature and at a wavelength of 254 nm. Elution times of *p,p'*-DDT and *p,p'*-DDD were 23.6 and 14.2 min, respectively. The amount of *p,p'*-DDD formed was determined from its peak area.

Measurement of Protein Content —— Protein content was determined by the method of Lowry *et al.*[10] with bovine serum albumin as a standard protein.

RESULTS

The hepatopancreas microsomes of goldfish catalyzed the reductive dechlorination of *p,p'*-DDT to *p,p'*-DDD in the presence of NADH or NADPH under anaerobic conditions. By the addition of FMN, these activities were enhanced. Although the microsomal fraction was boiled, the activities with both NAD(P)H and FMN were not abolished. The activity of microsomes was inhibited by carbon monoxide (Fig. 2). When FMN was replaced with FAD or riboflavin, a similar result was obtained in the dechlorination of *p,p'*-DDT (data not shown).

The blood of goldfish also catalyzed the reductive dechlorination of *p,p'*-DDT to *p,p'*-DDD, when supplemented with both NADPH and FMN, or NADH and FMN. Although the blood was boiled, these activities were not abolished, but were enhanced to some extent. The activity was sensitive to inhibition by carbon monoxide (Fig. 3). The erythrocytes also exhibited a significant dechlorinating activity in the presence of both NADH and FMN, but not plasma (data not

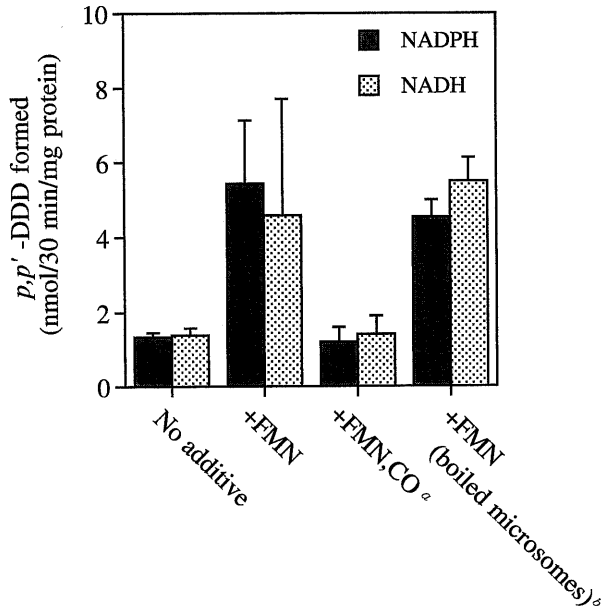

Fig. 2. Dechlorination of *p,p'*-DDT by Hepatopancreas Microsomes of Goldfish in the Presence of NAD(P)H and FMN

Each bar represents the mean±S.D. of three experiments. Incubation was performed at 37℃ for 30 min with 200 μl of hepatopancreas microsomes of goldfish (about 2.5 mg protein) under anaerobic conditions. The *p,p'*-DDD formed was determined by HPLC. Other details are described in Materials and Methods. [a]The assay was performed under an atmosphere of carbon monoxide. [b]Microsomes were boiled for 5 min.

shown). These facts suggested that hemoglobin is involved in the dechlorination of *p,p'*-DDT in the blood of goldfish.

When hemoglobin was used instead of blood, the dechlorinating activity, which was sensitive to carbon monoxide, was observed in the presence of NADH and FMN. Boiled hemoglobin with both NADH and FMN also exhibited the dechlorinating activity (Fig. 4). Furthermore, hematin revealed significant dechlorinating activity in the presence of both NADH and FMN (9.6 μmol/30 min/mg). The dechlorination of *p,p'*-DDT by hematin was also observed in the presence of the photochemically reduced form of FMN (16.5 μmol/30 min/mg).

These results led us to conclude that *p,p'*-DDT can be reduced to *p,p'*-DDD nonenzymatically by catalytic action of the heme group of hemoproteins in the hepatopancreas microsomes and blood of goldfish in the presence of both a flavin and a reduced pyridine nucleotide, which can be replaced with a reduced flavin.

DISCUSSION

Drug metabolism in mammalian species has been well investigated and comprehensive literature exists. However, the metabolic pathways in fish species have received considerably less attention. The present paper is the first description of the metabolic conversion of *p,p'*-DDT to *p,p'*-DDD in fish. Previously, a similar result was obtained in studies of the metabolism of *p,p'*-DDT in mammalian species.[7,11] This fact suggests the similarity of drug metabolism between fish and mammals. This assumption is also supported by the metabolism of sodium nifurstyrenate or furazolidone, which is a veterinary antimicrobial nitrofuran,[12,13] and 1-nitropyrene, which is a new class of environmental pollutant in automobile exhaust, in the urban atmosphere and so on.[14,15] Fish enzymes responsible for the reduction of a variety of xenobiotics are now under investigation in this laboratory.

It is known that halogenated hydrocarbons are metabolized to dehalogenated metabolites by reductive dehalogenation. Previous studies showed that the dehalogenation of carbon tetra-

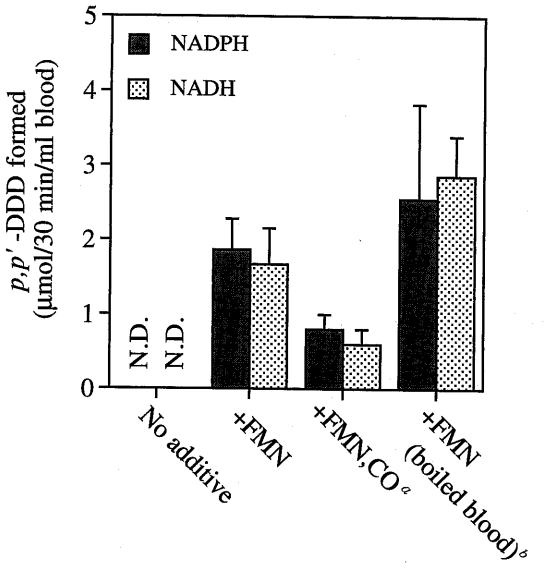

Fig. 3. Dechlorination of *p,p'*-DDT by the Blood of Goldfish in the Presence of NAD(P)H and FMN

Each bar represents the mean±S.D. of three experiments. ND: not detected. Incubation was performed at 37℃ for 30 min with 20 μl of goldfish blood (about 1.2 mg protein) under anaerobic conditions. The *p,p'*-DDD formed was determined by HPLC. Other details are described in Materials and Methods. [a]The assay was performed under an atmosphere of carbon monoxide. [b]Blood was boiled for 5 min.

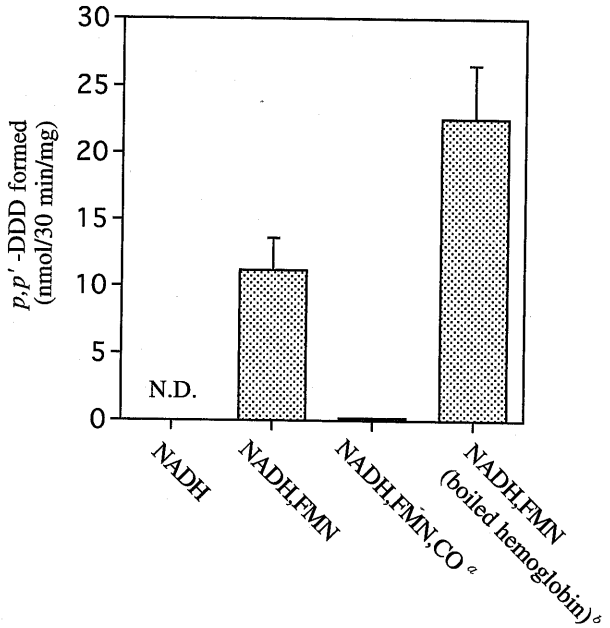

Fig. 4. Dechlorination of *p,p'*-DDT by Hemoglobin in the Presence of NADH and FMN

Each bar represents the mean±S.D. of three experiments. ND: not detected. Incubation was performed at 37℃ for 30 min with 1 mg of hemoglobin under anaerobic conditions. The *p,p'*-DDD formed was determined by HPLC. Other details are described in Materials and Methods. [a]The assay was performed under an atmosphere of carbon monoxide. [b]Hemoglobin was boiled for 5 min.

chloride, halothane or α-(bromoisovaleryl)urea is limited in the liver microsomal fraction of mammalian species, and that cytochrome P450 is involved in the microsomal dehalogenation.[16–18] Recent experiments have demonstrated that rat liver microsomes can directly transform *p,p'*-DDT to *p,p'*-DDD in a reductive reaction involving the formation of a *p,p'*-DDT free radical intermediate.[19] The microsomal dechlorinative activity is inhibited by carbon monoxide. A definite role for cytochrome P450 in the microsomal reduction has been established.[7,20] In this study, the dechlorination of *p,p'*-DDT by the hepatopancreas microsomes and blood of goldfish is proposed as a new type of reduction: a nonenzymatic dechlorination catalyzed by heme.

The reductive metabolism of xenobiotics by blood or its component was reported in *N*-oxide reduction. These include the reduction of imipramine *N*-oxide by boiled blood, of indicine *N*-oxide by denatured hemoglobin, of trimethylamine *N*-oxide by hemoglobin in the presence of cysteine, and of 4-bromo-*N*,*N*-dimethylaniline *N*-oxide by ferrihemoglobin.[21–24] Recently, we also showed quinone-dependent tertiary amine *N*-oxide reduction in the blood of rats. This reduction was catalyzed by hemoglobin in the presence of diols formed from various quinones by quinone reductase(s) in blood.[25]

We cannot deny the enzymatic reduction of *p,p'*-DDT by the hepatopancreas microsomes or blood of goldfish. The microsomal NAD(P)H-dependent reduction appears to proceed enzymatically. However, when a flavin was added to the mixture, most of the reducing activity was exhibited nonenzymatically by microsomes or blood. The dechlorination of *p,p'*-DDT presented in this study appears to proceed in two steps: The first step is the reduction of a flavin by a reduced pyridine nucleotide. In native microsomes, a flavin is reduced by NADPH-cytochrome P450 reductase with NADPH or NADH-cytochrome b_5 reductase with NADH, and in native blood, a flavin is mainly reduced by NADPH-flavin reductase with NADPH or NADH-cytochrome b_5 reductase with NADH, as reported by mammalian species,[26,27] while in boiled microsomes or blood, the flavin appears to be reduced nonenzymatically by NADPH or NADH as reported by Singer and Kearney.[28] The second step is the nonenzymatic reduction of *p,p'*-DDT to *p,p'*-DDD by the reduced flavin, as catalyzed by the heme group of hemoproteins such as cytochrome P450 and hemoglobin. In boiled microsomes or blood, the activity was increased, possibly because the substrate and cofactor have easy access to the heme moiety of denatured hemoproteins.

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