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1-Nitropyrene-Metabolizing Activities of Fish Liver Preparations

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Nitropolycyclic aromatic hydrocarbons, which are a new class of carcinogenic environmental pollutants, enter waterways by release of urban wastewater into the environment and by atmospheric fallout of airborne particles associated with smog (Wang *et al.* 1980; Rosenkranz and Mermelstein 1983; Manabe *et al.* 1984; Hayakawa *et al.* 1995). It is important to examine their metabolism not only in mammalian species but also in fish species for assessment of possible risk associated with human exposure to the pollutants.

Recently, we examined the *in vivo* metabolism of 1-nitropyrene, a typical nitropolycyclic aromatic hydrocarbon, in fish focusing on nitroreduction and acylation (Kitamura and Tatsumi 1996). When goldfish were bathed in a solution of 1-nitropyrene or its reduction product l-aminopyrne, one or two metabolites were isolated from the solution, respectively. The former metabolite was identified as l-aminopyrene and the latter two metabolites as l-acetylaminopyrene and 1-formylaminopyrene by comparing their mass and UV spectra, and behaviors in HPLC and TLC with those of authentic samples. In mammalian species, nitro-reduction followed by *N*-acetylation and *N*-formulation of nitropolycyclic aromatic hydrocarbons have been demonstrated with their liver preparations (Tatsumi and Amano 1987; Tatsumi *et al.* 1989). To our knowledge, such metabolic reactions of nitropolycyclic aromatic hydrocarbons have not been studied with fish liver preparations.

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In the present study, nitroreductase activity toward 1-nitropyrene, and *N*-acetylating and *N*-formulating activities toward its reduction product, l-aminopyrene, were examined using liver preparations from sea breams and carps.

MATERIALS AND METHODS

Sea bream livers (18 - 26 g) and black carp livers (15 - 23 g) were kindly supplied from a fresh fish shop.

1-Nitropyrene, 1-aminopyrene and 2-hydroxypyrimidine were purchased from Tokyo Chemical Industry Co., Ltd. Menadione and xanthine were obtained from Nacalai Tesque, Inc. NADPH and NADH were obtained from Oriental Yeast Co. Acetyl-CoA and *N*-formyl-L-kynurenine were purchased from Sigma Chemical Co. l-Acetylaminopyrene and *o*-dinitrobenzene were obtained from Aldrich Chemical Co. and Wako Pure Chemical Industries, Ltd., respectively. l-Formylaminopyrene was prepared as described previously (Tatsumi and Amano 1987).

Fish livers were homogenized in 3 volumes of 1.15% KCl. The homogenate was centrifuged for 20 min at 9,000*xg*, and the supernatant fraction was separated to microsomes and cytosol by its centrifugation for 60 min at 105,000*xg*. The microsomes were washed by resuspension in 2 volumes of the KCl solution and by resedimentation for 60 min at 105,000*xg*.

Silica gel plates (Kieselgel 60 GF_{254}, Merck; 0.25 mm thick) were developed in benzene - acetone (7:3, v/v). Spots were visualized under UV light (254 nm). Rf values of authentic 1-nitropyrene, 1-aminopyrene, l-acetylaminopyrene and 1-formylaminopyrene were 0.64, 0.55, 0.35 and 0.39, respectively.

HPLC was performed in a Hitachi L-6000 chromatograph fitted with a 130 x 4 mm column of LiChrosphere 100 RP-8(e). The mobile phase was CH_3CN - H_2O (1:1, v/v). The chromatograph was operated at a flow rate of 1 mL/min at ambient temperature and at a wave length of 254 nm. Elution times (min) of authentic l-nitropyrene, l-aminopyrene, 1-acetylaminopyrene and 1-formylaminopyrene were 31.8, 10.3, 3.9 and 4.8, respectively.

The incubation mixture consisted of 0.1 µmol of l-nitropyrene,

0.5 μmol of an electron donor, and a liver preparation equivalent to 0.25 g of liver in a final volume of 2 mL of 0.1 *M* K, Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 30 min in nitrogen or in air. In control experiments, boiled liver preparations were used. After incubation, 30 μg of *o*-dinitrobenzene was added to the mixture as an internal standard, and then the mixture was extracted twice with 5 mL each of ethyl acetate. The combined ethyl acetate extract was evaporated to dryness *in vacuo* and the residue was subjected to HPLC. 1-Aminopyrene formed was determined from its peak area.

In the assay of *N*-acetylating activity, the incubation mixture consisted of 0.1 μmol of 1-aminopyrene, 0.5 μmol of acetyl-CoA and sea bream liver cytosol equivalent to 0.25 g of liver in a final volume of 2 mL of 0.1 *M* Tris-HCl buffer (pH 7.4). In the assay of *N*-formulating activity, acetyl-CoA was replaced with *N*-formyl-L-kynurenine as a donor of the formyl group. The incubation was performed at 37°C in air. In control experiments, boiled cytosols were used. After incubation, *o*-dinitrobenzene was added to the mixture, and then the mixture was extracted with ethyl acetate in the same manner as described above. The ethyl acetate extract, after removal of solvent, was subjected to HPLC. l-Acetylaminopyrene or 1-formylaminopyrene formed was determined from its peak area.

The incubation mixture consisted of 0.1 μmol of 1-acetylamino-pyrene or 1-formylaminopyrene, and sea bream liver cytosol equivalent to 0.25 g of liver in a final volume of 2 mL of 0.1 *M* or 0.2 *M* Tris-HCl buffer (pH 7.4). The incubation was performed at 37°C in air. In control experiments, boiled cytosols were used. After incubation, *o*-dinitrobenzene was added to the mixture and then the mixture was extracted with ethyl acetate as described above. The residue from the ethyl acetate extract was subjected to HPLC. 1-Aminopyrene formed was determined from its peak area.

In all cases, the metabolites formed were identified by comparing their behaviors in TLC and HPLC with those of authentic samples.

RESULTS AND DISCUSSION

The comparative ability of fish liver microsomes and cytosols to reduce l-nitropyrene to 1-aminopyrene was examined. As shown in Table 1, sea bream and carp liver microsomes exhibited an NADPH-dependent reductase activity under anaerobic conditions. The NADPH-dependent activity was inhibited by carbon monoxide. In this case, NADH was less effective compared to NADPH. On the other hand, when 2-hydroxypyrimidine, which is an electron donor of aldehyde oxidase, was added, the liver cytosols from both species exhibited a significant reductase activity toward the nitro compound under anaerobic conditions. The 2-hydroxypyrimidine-dependent activity was markedly inhibited by menadione, an inhibitor of aldehyde oxidase. The cytosolic activity was much higher than the NADPH-dependent microsomal activity. In the cytosolic reduction, xanthine, NADPH and NADH were much less effective compared to 2-hydroxypyrimidine. When the liver microsomes and cytosols were boiled, their reductase activities were completely abolished (data not shown).

In mammals, our previous studies showed that a liver microsomal cytochrome P450 system and liver cytosolic aldehyde oxidase catalyze the reduction of 1-nitropyrene and 2-nitrofluorene to the corresponding amines (Kitamura *et al.* 1983; Tatsumi *et al.* 1986). The participation of cytochrome P450 in the nitroreduction was confirmed with a reconstituted cytochrome P450 system from rat liver as reported by Saito *et al.* (1984).

In fish, an early study by Hitchcock and Murphy (1966) showed that under appropriate anaerobic conditions, liver preparations could reduce the nitro group of parathion to give the less toxic amino compound. In addition, nitroreductase activity toward *p*-nitrobenzoic acid was found by Buhler and Rasmussen (1968), and by Pedersen *et al.* (1976) in liver preparations from several fish species. However, no information was available concerning fish liver enzymes responsible for the reduction of aromatic nitro compounds. The present study suggested for the first time that in fish species as well as mammalian species a liver microsomal P450 system and liver cytosolic aldehyde oxidase are mainly involved in the nitroreduction by liver preparations.

Table 1. Reduction of 1-nitropyrene by liver preparations of carps and sea breams

Fraction	1-Aminopyrene formed (nmol / 30 min / g liver)			
	Sea bream		Carp	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Microsomes	0.1	0.7	0.2	0.8
+NADPH	2.1	4.5	0.8	5.4
+NADH	0.5	0.8	0.6	2.5
+NADPH, CO	—[a]	0.8	—[a]	2.2
Cytosol	3.3	8.7	4.2	7.3
+2-Hydroxypyrimidine	6.0	64.9	4.5	81.4
+Xanthine	4.1	11.2	2.9	16.6
+NADPH	4.4	4.0	7.3	18.3
+NADH	2.1	3.1	2.8	13.2
+2-Hydroxypyrimidine, menadione	—[a]	15.7	—[a]	4.9

Each value represents the mean of four fish.
[a] not determined

The reduction product, 1-aminopyrene, was further metabolized to l-acetylaminopyrene or l-formylaminopyrene, when incubated with sea bream liver cytosol in the presence of acetyl-CoA or *N*-formyl-L-kynurenine. The amount of 1-acetylaminopyrene formed increased depending on incubation time up to 20 min and then decreased gradually. On the other hand, the amount of 1-formylaminopyrene formed increased up to 3 min and then decreased sharply (Figure 1). *N*-deacylation of the acylaminopyrenes to 1-aminopyrene by sea bream liver cytosol was shown in Figure 2. 1-Formyl-aminopyrene was *N*-deformylated rapidly, whereas l-acetyl-aminopyrene was resistant to its *N*-deacetylation. This result accounted for the data shown in Figure 1.

In mammals, there is ample evidence of metabolic *N*-acetylation, whereas there are relatively few reports on the *in vivo* formation of *N*-formyl derivatives of aromatic amino compounds, or the *in vitro* *N*-formylation of the amino group (Weber 1985; Tatsumi *et al.* 1989). Previously, we showed that

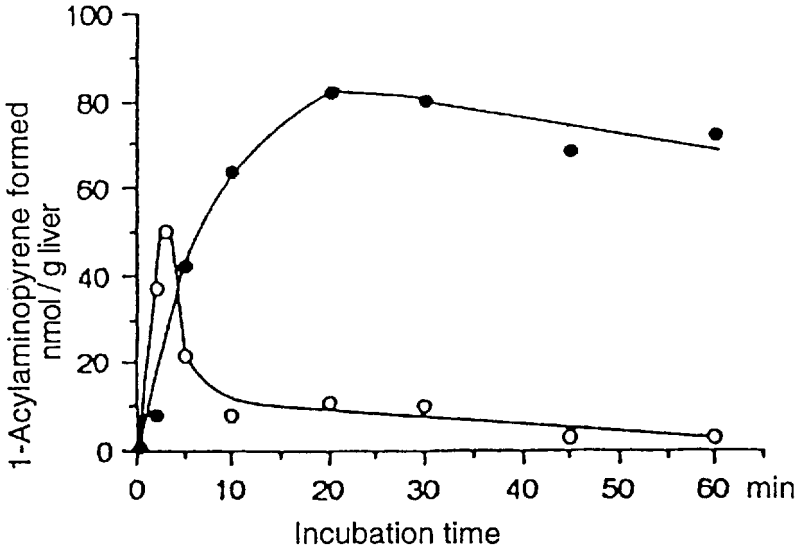
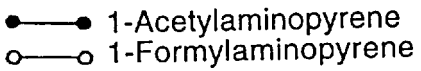


Figure 1. Time-courses of *N*-acetylation and *N*-formylation of l-aminopyrene by sea bream liver cytosol



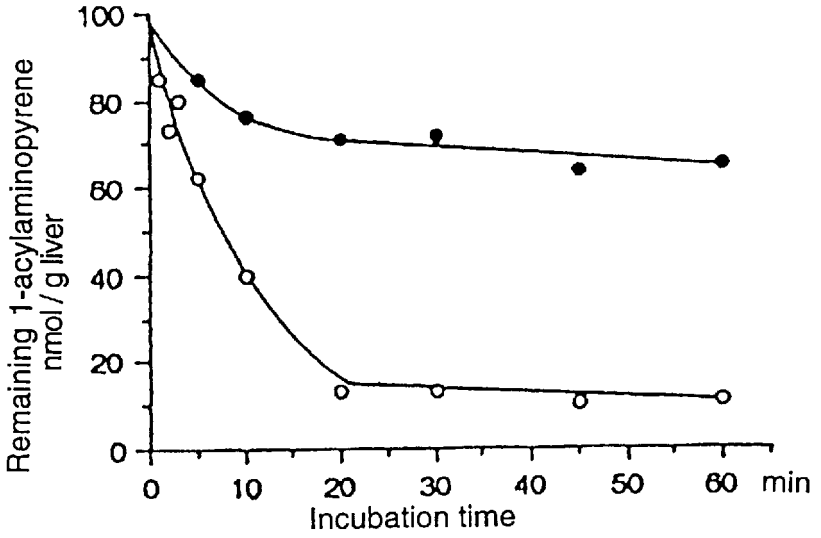


Figure 2. Time-courses of *N*-deacetylation of l-acetylamino-pyrene and *N*-deformylation of l-formylamino-pyrene by sea bream liver cytosol

the *N*-formylation as well as *N*-acetylation occurs as a common reaction in the metabolism *in vivo* of 1-aminopyrene, 2-aminofluorene, 4-aminobiphenyl and 2-aminonaphthalene in mammalian species (Tatsumi *et al.* 1989). Liver cytosols from several mammalian species exhibited a significant *N*-formylating activity toward aromatic amino compounds in the presence of *N*-formyl-L-kynurenine and *N*-acetylating activity in the presence of acetyl-CoA. The liver cytosolic *N*-formylating and *N*-acetylating activities are due to formamidase and arylamine acetyltransferase, respectively.

In fish, Huang and Collins (1962) found that 2-, 3- and 4-aminobenzoic acids were partly acetylated and excreted in urine as the *N*-acetyl derivatives. To our knowledge, *N*-acylating pathways have not been extensively studied in aquatic species either *in vivo* or *in vitro*. The present study strongly suggested that there are formamidase and arylamine acetyltransferase in fish livers similar to mammalian livers, which catalyze *N*-formylation and *N*-acetylation of aromatic amino compounds, respectively.

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