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Metabolism of 2-Nitrofluorene, an Environmental Pollutant, and 2-Acetylaminofluorene in Fish

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Nitropolycyclic aromatic hydrocarbons (nitro-PAHs) have been found in diesel engine exhaust, urban pollution sources, wastewater from gasoline stations, cigarette smoke among others (Rosenkranz and Mermelstein 1983; Hayakawa *et al.* 1995). These pollutants, many of which are carcinogenic and mutagenic, enter waterways through atmospheric fallout of airborne particles associated with smog and by release of urban wastewater into the environment. In order to assess the possible risks arising from these pollutants, it is essential to elucidate thoroughly their metabolism. 2-Nitrofluorene (NF) is a typical carcinogenic nitro-PAH and a potent mutagen (Möller *et al.* 1989). NF was detected in diesel exhaust particles as a major component, together with nitropyrenes. We previously examined the *in vitro* metabolism of NF in mammalian species, focusing on reduction of nitro group (Tatsumi *et al.* 1986). When the nitro compound was incubated with liver cytosol or microsomes of rabbits, its reduction product, 2-aminofluorene (AF), was isolated from the mixture. We presented evidence that liver aldehyde oxidase and cytochrome P450 catalyze this nitro reduction.

Fish have the ability to metabolize a variety of compounds such as pesticides and environmental contaminants (Stegeman 1981; Schlenk and Buhler 1991; Livingstone 1998). It has provided evidence that drug-metabolizing enzymes such as cytochrome P450 system, flavin-containing monooxygenase, quinone oxidoreductase and phase II enzymes exist in fish, as in mammalian species (Schlenk and Buhler 1991; Livingstone 1998). We reported that nitro compounds such as 1-nitropyrene, and sodium nifurstyrenate, a veterinary antimicrobial nitrofuran, were reduced to amino derivatives in goldfish,

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Carassius auratus, and sea bream, *Pagrus major* (Tatsumi *et al.* 1992; Kitamura and Tatsumi 1996; 1997). The reductive metabolism of xenobiotics in fish is an important pathway, as it is in mammalian species. However, no report is available concerning the metabolism of NF in fish. The present study was designed to elucidate the *in vivo* metabolism of NF in goldfish, focusing on biotransformation to the corresponding amino, acylamino and hydroxylated compounds.

MATERIALS AND METHODS

NF (98 % pure), AF (>98 %) and 2-acetylaminofluorene (AAF, >98%) were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). β-Glucuronidase (100,000 Fishman units/ mL)/ arylsulfatase (800,000 Roy units/mL) from *Helix pomatia* was obtained from Roche Diagnostics (Mannheim, Germany). 2-Formylaminofluorene (FAF) was prepared as described previously (Tatsumi *et al.* 1989). 7-Hydroxy-2-nitrofluorene (7-OH-NF), 7-hydroxy-2-aminofluorene (7-OH-AF) and 7-OH-AAF were prepared by the method of Bielschowsky (1945). 5-Hydroxy-2-acetylaminofluorene (5-OH-AAF) and 5-hydroxy-2-nitrofluorene (5-OH-NF) were prepared by the method of Weisburger and Weisburger (1955). Goldfish (5-8 g), *Carassius auratus*, were obtained commercially.

Thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60 GF_{254}, 0.25 mm thick; E. Merck) using benzene-acetone (7 : 3, v/v) as developing solvent. Spots were visualized under UV light (254 nm). The *Rf* values of authentic samples were as follows: 7-OH-AAF 0.17, 5-OH-AAF 0.23, AAF 0.45, FAF 0.48, AF 0.64 and NF 0.78. High performance liquid chromatography (HPLC) was performed using a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a 125 x 4 mm LiChrosorb RP-18 column (E. Merck, Darmstadt, Germany). The mobile phase was acetonitrile-H_2O (3:7, v/v) for 30 min, acetonitrile-H_2O (linear gradient from 3:7 to 7:3, v/v) for 30 min and acetonitrile-H_2O (7:3, v/v) for 20 min. The flow rate was 0.6 mL/min, and effluent monitored at 280 nm. The elution times of NF and its metabolites were as follows: 7-OH-AAF 15.4, 7-OH-AF 16.2, 5-OH-AAF 22.3, FAF 45.5, AAF 46.9, AF 50.3, diethylstilbestrol (DES, an internal standard) 56.2 and NF 63.7 min.

Twenty goldfish were kept for two day in 10 L of distilled water containing NF or AAF (5 mg/L). The tank water was extracted twice with equal volumes of ethyl acetate. The extracts were evaporated to dryness *in vacuo*. The metabolites were purified by preparative TLC and HPLC. In the control experiments, goldfish were kept in water without NF or AAF.

After the 2-day period, the tank water was collected. Ten mL of the water was incubated with β-glucuronidase 3,000 units/arylsulfatase 24,000 units in 20 mL of 0.1 *M* citrate-phosphate buffer (pH 6.0) at 37°C for 16 hr. The incubation mixture, after addition of 10 μg of DES, was extracted once with 2 volumes of ethyl acetate by shaking. The extract was evaporated to dryness *in vacuo*, and the residue was dissolved in a small amount of acetonitrile and subjected to HPLC.

RESULTS AND DISCUSSION

After goldfish had been kept in the tank water containing NF for 2 days, NF metabolites were extracted with ethyl acetate, and the extract was subjected to HPLC as described in Materials and Methods. Five peaks due to NF metabolites were detected together with the peak due to unchanged NF (Fig. 1). These metabolite peaks were not detected in the HPLC chromatogram of the extract of the control experiment. Five metabolites detected at 15.4, 16.2, 45.5, 46.9 and 50.3 min were designated NF-1, NF-2, NF-3, NF-4 and NF-5, respectively. The retention times of NF-1, 2, 3, 4 and 5 corresponded to those of authentic 7-OH-AAF, 7-OH-AF, FAF, AAF and AF, respectively. These metabolites were isolated from the extract by TLC and HPLC as described in Materials and Methods. Mass and UV spectral data of these metabolites are shown in Table 1. The mass spectrum of NF-1 showed the molecular ion at *m/z* 239, and fragment ions at *m/z* 197 ($M^+ - CH_2CO$) and 181 (197-OH), suggesting that the metabolite is hydroxylated AAF. The mass spectrum of NF-2 showed the molecular ion at *m/z* 197, and fragment ion at *m/z* 181 ($M^+ - OH$), suggesting that the metabolite is hydroxylated AF. The mass spectra of NF-3, NF-4 and NF-5 showed that these metabolites were FAF, AAF and AF, respectively. The mass and UV spectra, and the Rf values in TLC, as well as the elution times in HPLC, of the metabolites were identical with those of authentic samples. However, we could not detect 7-OH-NF or 5-OH-NF in the extract of the tank water. Based on these

observations, we concluded that NF was metabolized to the reduced and acylated metabolites, AF, AAF and FAF, and the hydroxylated metabolites, 7-OH-AF and 7-OH-AAF.

Quantitative determination of these metabolites showed that AF, AAF, FAF, 7-OH-AF and 7-OH-AAF in the tank water after 48 hr amounted to about 0.5, 6.2, 0.4, 2.2 and 7.3 % of the initial NF, respectively.

Furthermore, the *in vivo* metabolism of AAF, detected as a metabolite of NF in fish, was examined. In this case, four metabolites (AAF-1 - 4) were detected in an HPLC chromatogram of the extract of the tank water. The retention times of AAF-1, 2, 3 and 4 corresponded to those of authentic 5-OH-AAF, 7-OH-AAF, FAF and AF. These metabolites isolated by TLC and HPLC were identified by comparison with authentic samples, as in the case of NF metabolites. Mass and UV spectral data of AAF-1 are shown in Table 1. The amounts of these metabolites excreted in the tank water are shown in Fig. 2. When tank water was treated with β-glucuronidase/ arylsulfatase, the amounts of 7-OH-AAF and 5-OH-AAF were markedly increased compared with those in the tank water before the treatment. In these metabolites, 7-OH-AAF was the major metabolite, followed by 5-OH-AAF, with FAF and AF as minor metabolites.

Our results suggest that NF is predominantly converted to the amino and acylamino derivatives, which are probably more toxic, by fish. Tatsumi *et al.* (1986) indicated that the reduction of nitro-PAHs is mediated by the cytochrome P450 system and aldehyde oxidase in mammalian species. Furthermore, amino compounds are acylated to the acetylamino and formylamino compounds by arylamine acetyltransferase and formamidase, respectively, in mammals (Tatsumi *et al.* 1989). In our preliminary study, we demonstrated that the liver microsomes and cytosol of goldfish exhibit reductase activity toward NF, in the presence of NADPH or 2-hydroxypyrimidine, an electron donor to cytochrome P450 or aldehyde oxidase, respectively. These facts suggest that there are marked similarities between fish and mammals with respect to the qualitative metabolism of these compounds.

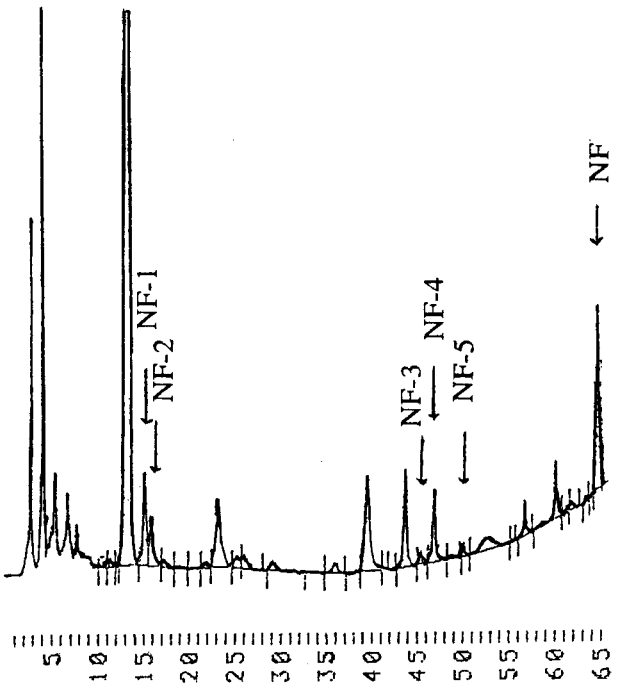

Figure 1. HPLC of the metabolites (NF-1 - 5) of 2-nitrofluorene (NF) in goldfish. The absorbance (full scale=0.016 AU) was measured at 280 nm.

Table 1. Mass and UV spectra of the metabolites of 2-nitrofluorene and 2-acetylaminofluorene in goldfish

Metabolite	Mass spectral peak (m/z)	UV spectral peak (nm)
NF-1	239 (M^+), 197, 181	276 (S), 291, 320 (S)
NF-2	197 (M^+), 181	286, 326 (S)
NF-3	209 (M^+), 181, 165	278, 288, 300 (S), 313 (S)
NF-4	223 (M^+), 181, 165	276, 286, 300 (S), 313 (S)
NF-5	181 (M^+), 165, 152	288, 314 (S)
AAF-1	239 (M^+), 197, 181	272 (S), 283, 299, 309 (S)

S : shoulder , NF-1 - NF-5 : metabolites of 2-nitrofluorene in goldfish , AAF-1 : a metabolite of 2-acetylaminofluorene in goldfish.

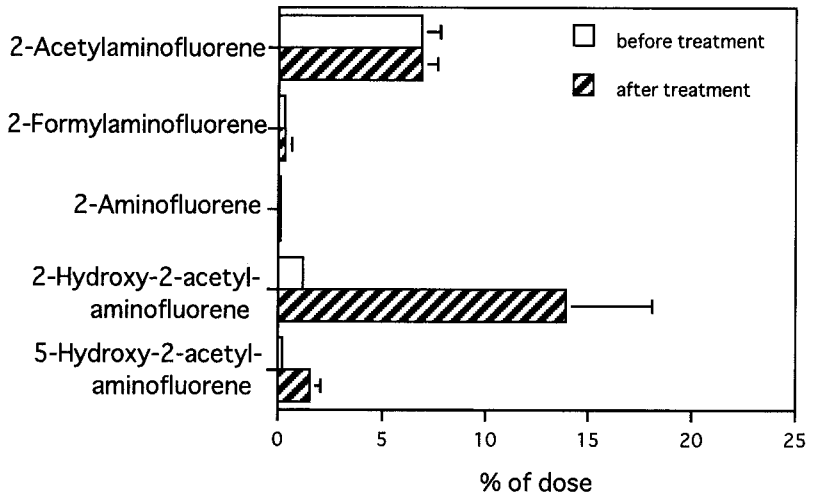

Figure 2. Excretion of the metabolites of 2-acetylaminofluorene (AAF) in goldfish.
Each bar represents the mean ± SD of three experiments. The metabolites were extracted from AAF-containing tank water in which goldfish had been kept, before or after treatment with β-glucuronidase/arylsulfatase.

We propose that the metabolic pathway of NF in fish is as shown in Fig. 3. In this study, we have demonstrated that NF in fish is predominantly metabolized to, and excreted as AF and its acylated metabolites, but not hydroxylated metabolites of NF. Therefore, in goldfish, the metabolism of NF is mainly reductive. It is likely that the hydroxylamino derivative, probably the most toxic compound, is formed as an intermediate of NF reduction. Fu (1990) suggested that hydroxylamino derivatives of nitro PAHs bind to DNA. We found that, after the treatment of the tank water with β-glucuronidase/arylsulfatase, the majority of the metabolites could be extracted with ethyl acetate, as compared with only 10 % of the metabolites before the treatment. This suggests that the hydroxylated metabolites of AF, AAF or FAF are excreted largely as glucuronic or sulfuric acid conjugates. In contrast, we found that FAF was excreted as a metabolite of AAF, suggesting that AAF and FAF are interconverted, perhaps *via* AF, in fish *in vivo*.

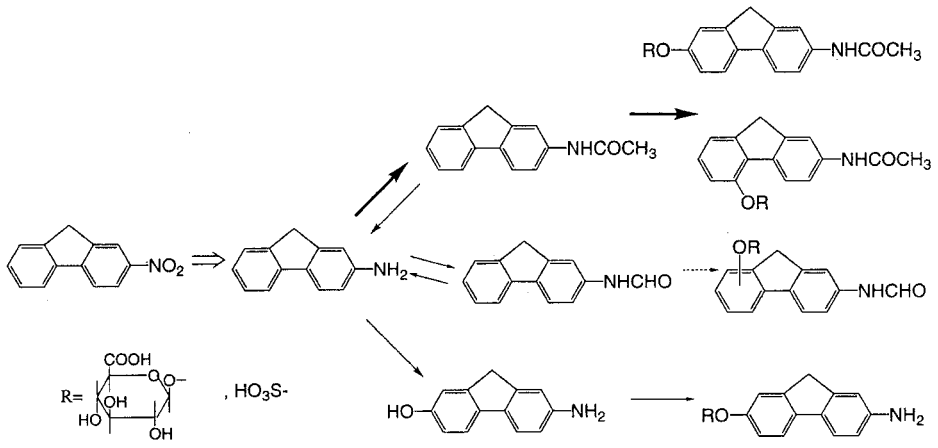

Figure 3. Postulated metabolic pathways of 2-nitrofluorene in fish.

We previously reported that various types of arylamines were acylated to the acetylamino and formylamino derivatives in rabbits (Tatsumi *et al.* 1989). In this study, we also demonstrated that AF formed from NF is acylated to acetylamino and formylamino derivatives in fish. In a preliminary experiment, the acetylating activity of liver cytosol toward AF was stimulated by the addition of acetyl CoA, which is an acetyl donor to mammalian arylamine acetyltransferase. Formylation of AF was enhanced by *N*-formyl-L-kynurenine, which is a formyl donor to mammalian formamidase. Further studies on the enzyme systems in fish responsible for the metabolism of NF are under way.

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