Metabolism *in Vivo* of 1-Nitropyrene, an Environmental Pollutant, in Fish

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The *in vivo* metabolism of 1-nitropyrene, an important environmental pollutant, was examined in fish, focusing on nitroreduction followed by acylation. When goldfish were bathed in a solution of 1-nitropyrene or its reduction product, 1-aminopyrene, one or two metabolites were isolated from the solution, respectively. The former metabolite was identified as 1-aminopyrene and the latter two metabolites as 1-acetylaminopyrene and 1-formylaminopyrene by comparing their mass and UV spectra, and their behaviors in HPLC and TLC, with those of authentic samples.

Key words 1-nitropyrene; *in vivo* metabolism; fish; 1-aminopyrene; 1-acetylaminopyrene; 1-formylaminopyrene

Nitropolycyclic aromatic hydrocarbons have been identified as a new class of important environmental pollutants in automobile exhaust, the urban atmosphere, wastewater from gasoline stations, cigarette smoke and so on.^{1–12)} The pollutants, many of which are carcinogenic and mutagenic, enter waterways by the atmospheric fallout of airborne particles associated with smog and by the release of urban wastewater into the environment.^{12–20)} In order to assess the possible risks associated with human exposure to the pollutants, it is essential to elucidate thoroughly their metabolism in mammalian species, marine and freshwater species, and microorganisms. Several reports have indicated that 1-nitropyrene, which is a typical nitropolycyclic aromatic hydrocarbon, is mainly metabolized by nitroreduction, ring hydroxylation, acylation and conjugation in mammalian species.^{21–29)} The metabolism of 1-nitropyrene by nitroreduction and acylation was also demonstrated with bacteria.^{13,30–32)} However, no report is available concerning the metabolism of 1-nitropyrene and other nitropolycyclic aromatic hydrocarbons in fish.

In the present study, the *in vivo* metabolism of 1-nitropyrene in goldfish was examined focusing on its biotransformation to corresponding amino and acylamino compounds.

MATERIALS AND METHODS

Fish Goldfish (a kind of red crucian carp, 6–7 cm length), which were commercially available, were used.

Chemicals 1-Nitropyrene and 1-aminopyrene were purchased from Tokyo Chemical Industry Co., Ltd. 1-Acetylaminopyrene was obtained from Aldrich Chemical Co. 1-Formylaminopyrene was prepared as described previously.²⁸⁾

TLC Silica gel plates (Kieselgel 60 GF₂₅₄, Merck; 0.25 mm thick for analytical TLC and 0.4 mm thick for preparative TLC) were developed in benzene–acetone (7:3, v/v). Spots were visualized under UV light (254 nm). *R_f* values of authentic 1-nitropyrene, 1-aminopyrene, 1-acetylaminopyrene and 1-formylaminopyrene were 0.64, 0.55, 0.35 and 0.39, respectively.

HPLC HPLC was performed in a Hitachi L-6000 chromatograph fitted with a 130 × 4 mm column of Li-Chrosphere 100 RP-8(e). The mobile phase was CH₃CN–H₂O (1:1, v/v). The chromatograph was

operated at a flow rate of 1 ml/min at ambient temperature and at a wavelength of 254 nm. Retention times (min) of authentic 1-nitropyrene, 1-aminopyrene, 1-acetylaminopyrene and 1-formylaminopyrene were 31.8, 10.3, 3.9 and 4.8, respectively. *o*-Dinitrobenzene was used as an internal standard.

Analytical Procedures Mass spectra were taken with a Japan Electron Optics (JEOL) JMS-SX 102 GCG mass spectrometer. UV spectra were recorded with a Hitachi 320 spectrophotometer in ethanol.

Isolation of *in Vivo* Metabolites 1) Two hundred goldfish were divided into four groups. The fifty fish in each group were bathed for 48 h at a temperature of 18 °C in the dark in 3 l of 8.3 ppm 1-nitropyrene solution which was prepared by mixing 2 ml acetone solution of the nitro compound with distilled water. After bathing, the solution was extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extract was dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*. When the residue was subjected to TLC, three metabolites (NPM-1, NPM-2 and NPM-3) of 1-nitropyrene, which had the same *R_f* values as those of authentic 1-aminopyrene, 1-acetylaminopyrene and 1-formylaminopyrene, respectively, were detected together with the parent compound. Among these metabolites, NPM-1, which was a major metabolite, was further purified to a single spot by TLC. 2) Similarly, two hundred goldfish were divided into four groups and the fish in each group were bathed for 48 h in 3 l of 8.3 ppm 1-aminopyrene solution, which was prepared by mixing 2 ml acetone solution of 1-aminopyrene with distilled water. After bathing, the solution was extracted with ethyl acetate and the extract, after removal of the solvent, was subjected to TLC. Two metabolites (APM-1 and APM-2) of 1-aminopyrene, which had the same *R_f* values as those of authentic 1-acetylaminopyrene and 1-formylaminopyrene, respectively, were detected together with the parent compound. These two metabolites were further purified to a single spot by TLC, respectively. 3) In control experiments, fifty goldfish were kept in 3 l of distilled water for 48 h and then the fish were taken out from a fishbowl. The remaining distilled water was mixed with the acetone solution of 1-nitropyrene or 1-aminopyrene (its final concentration, 8.3 ppm), and the mixed solution was allowed to stand for 48 h at a water temperature of 18 °C. The solution was extracted with ethyl acetate and

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the extract, after removal of the solvent, was subjected to HPLC as described above. In this case, only the unchanged compound of 1-nitropyrene or 1-aminopyrene was detected in TLC. The result excluded the possible role of environmental or fecal microorganisms in the reduction of 1-nitropyrene and in the acylation of its reduction product, 1-aminopyrene, under the present experimental conditions.

Determination of *in Vivo* Metabolites Fifty gold fish were bathed for 48 h in 3 l of the 1-nitropyrene solution as described above. An aliquot (200 ml) of the solution was taken out and extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extract was evaporated to dryness *in vacuo* and the residue was subjected to HPLC. The metabolites, NPM-1, NPM-2 and NPM-3, were determined from their peak areas.

RESULTS

Goldfish were bathed for 48 h in the 1-nitropyrene solution, the solution was extracted with ethyl acetate, then the ethyl acetate extract was subjected to HPLC as described in Materials and Methods. Three peaks due to 1-nitropyrene metabolites (NPM-1, NPM-2 and NPM-3) were detected, together with a peak due to unchanged 1-nitropyrene (data not shown). Among these metabolites, NPM-1 was isolated from the solution. The metabolite showed a mass spectrum with a molecular ion at m/z 217, accompanying a fragment ion at m/z 189 ($M^+ - 28$), as shown in Fig. 1. The UV spectrum gave absorption maxima at 242, 281 and 346 nm. The mass and UV spectra, as well as the R_f value in TLC and the retention time in HPLC of the metabolite, were identical with those of authentic 1-aminopyrene. The results led us to conclude that NPM-1 was 1-aminopyrene.

Purification of two other metabolites, NPM-2 and NPM-3, was difficult because of their small quantities. These metabolites appeared to be formed from the parent compound *via* its nitroreduction product, NPM-1. Therefore, the *in vivo* metabolism of 1-aminopyrene in gold-

fish was also examined. When goldfish were bathed in a solution of 1-aminopyrene, then APM-1 and APM-2, which had the same R_f values as NPM-2 and NPM-3 in TLC, respectively, were isolated as metabolites of the amino compound, as described in Materials and Methods. The mass spectrum of APM-1 gave a molecular ion at m/z 259, and fragment ions at m/z 217 ($M^+ - 42$) and 189 ($M^+ - 70$), as shown in Fig. 2. The UV spectrum showed absorption maxima at 245, 278 and 342 nm. The mass spectrum of APM-2 gave a molecular ion at m/z 245, and fragment ions at m/z 217 ($M^+ - 28$) and 189 ($M^+ - 56$), as shown in Fig. 2. The UV spectrum showed absorption maxima at 243, 282 and 343 nm, and the behaviors in TLC and HPLC of these metabolites were identical with those of authentic 1-acetylaminopyrene and 1-formylaminopyrene. Based on these facts, we concluded that APM-1 and APM-2, or NPM-2 and NPM-3 were 1-acetylaminopyrene and 1-formylaminopyrene, respectively.

Fig. 1. Electron Impact Mass Spectra of the *in Vivo* Metabolite of 1-Nitropyrene in Goldfish, and Authentic 1-Aminopyrene

NPM-1, 1-nitropyrene metabolite 1.

Fig. 2. Electron Impact Mass Spectra of the *in Vivo* Metabolite of 1-Aminopyrene in Goldfish, and Authentic 1-Acetylaminopyrene and 1-Formylaminopyrene

APM-1, 1-aminopyrene metabolite 1; APM-2, 1-aminopyrene metabolite 2.

Results of the quantitative determination of these 1-nitropyrene metabolites showed that 1-aminopyrene, 1-acetylaminopyrene and 1-formylaminopyrene formed from 1-nitropyrene after 48 h bathing were 10.5, 2.3 and 0.6 nmol/50 goldfish, respectively.

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 in fish of a nitropolycyclic aromatic hydrocarbon to the corresponding amino compound which is further metabolized to the acetylamino and formylamino compounds. Previously, a similar result was obtained in a study on the *in vivo* metabolism of nitropolycyclic hydrocarbons such as 1-nitropyrene and 2-nitrofluorene in mammalian species.²⁸⁾ These facts suggest similarities between fish and mammals with respect to the qualitative metabolism of drugs, although pronounced differences also occur. The assumption is supported by the fact that when rats were orally given sodium nifurstyrenate or furazolidone, which is a veterinary antimicrobial nitrofur, the corresponding acetamidofuran and the furan ring opening compound were identified as its *in vivo* metabolite, whereas when goldfish were bathed in the nitrofur solution, only the furan ring opening compound was identified as its metabolite.^{33,34)} Fish enzymes responsible for such metabolic reactions of nitropolycyclic aromatic hydrocarbons are now under investigation in this laboratory.

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