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Short Communications

Separation of the Different Classes of Conjugates Formed by Metabolism of Benzo[*a*]pyrene in the Northern Pike *(Esox lucius)* *

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There is now a growing awareness concerning the fate of polycyclic hydrocarbons and the many other xenobiotics with which we pollute our environment. Many of these substances find their way sooner or later into our aquatic environment. For instance, polycyclic hydrocarbons, including benzo[*a*]pyrene, reach our rivers, lakes and seas in the form of spillages of crude and refined petroleum products, in industrial and domestic effluents, in run-off water from the land, and by dry or wet precipitation from the atmosphere. (The recent article by Neff[1] provides a good review of polycyclic hydrocarbons in the aquatic environment.) It seems likely that the growing frequency of tumors in fish that live in polluted waters is closely related to increasing aquatic pollution.[2]

In our laboratory we have recently begun intensive investigations on the fate of xenobiotics in the Northern pike (*Esox lucius*). First, we have characterized different drug-metabolizing systems using subcellular fractions from the liver of this fish.[3] Subsequently, we have complemented these studies with an investigation on the bioaccumulation of benzo[*a*]pyrene in Northern pike from the surrounding water.[4] The factor by which benzo[*a*]pyrene is concentrated from the water varies widely for different organs, but the highest concentrations are recovered in the liver–bile–intestine and the kidney–urine systems.

The present experiments represent therefore a continuation of our earlier studies. Here we have attempted to use a published procedure – namely, chromatography on a column of alumina oxide[5] – to separate the different classes of conjugates (sulfates, glucuronides, glutathione conjugates) formed during the metabolism of benzo[*a*]pyrene in the Northern pike. Such separation is an important first step in characterizing all the different products resulting from *in vivo* metabolism of benzo[*a*]pyrene and other xenobiotics.

The fish used in this study were Northern pike purchased from a local hatchery in the larval stage. The fish were maintained in 100-l glass aquaria with continuously circulating top water (*circa* 1 l/min) and fed commercial trout pellets. The fish were starved during the period of exposure, so that possible adsorption to food and feces should not disturb the distribution of benzo[*a*]pyrene between the water and the fish. However, a starvation period of 4.5 days is not uncommon for the pike in its natural environment. The fish were used when they were between 20–30 g in weight and approximately 10 cm long. Both sexes were used without discrimination. The fish were subjected to a continuous 12 h light–12 h dark cycle. At no time did any fish show symptoms of illness or bad health.

The solution of [G-^{3}H]-benzo[*a*]pyrene (purchased from the Radiochemical Centre, Amersham, England) was evaporated to dryness and the benzo[*a*]pyrene subsequently dissolved in hexane and purified with 8 extractions using alcoholic NaOH.[6] Benzo[*a*]pyrene purified according to this method is about 99.85 % pure. The benzo[*a*]pyrene was then dissolved in 25 μl acetone and added to 10-l aquaria with continuous stirring to obtain a homogeneous solution-suspension containing 87 ±10 ng/l (which corresponds to mildly polluted water[1]).

If the entire amount of benzo[*a*]pyrene added to one aquarium was taken up by one fish, this would give a dose of approximately 50 μg/kg body weight. Judging from experiments with mammals[7] this dose is far too low to induce benzo[*a*]pyrene monooxygenase activity. However, a dose-response study for induction with 3-methylcholanthrene has not yet been performed in fish.

One fish was placed in each aquarium and exposed for 4.5 days. After immobilization with the

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anesthetic MS-222 (tricaine methanesulfonate) the livers and gall bladders (containing bile) were removed from 3 fish and pooled. These organs were homogenized separately in 70% ethanol using a glass-glass homogenizer and approximately 15 up-and-down hand-driven strokes. The sample was then centrifuged at 2250 g for 15 min in a desk centrifuge and the clear supernatant was collected using a Pasteur pipette. The pellet was resuspended in 70% ethanol, homogenized, and centrifuged a total of three additional times and the four different supernatants were pooled.

The separation of benzo[*a*]pyrene itself, metabolites and water-soluble conjugates was accomplished by chromatography on a column of alumina oxide (150 × 15 mm; Aluminium oxid 90 aktiv neutral (*aktivitätsstufe* I) grain size 0.063–0.200 mm (70–230 mesh ASTM) essentially according to the published procedure for separation of the water-soluble metabolites of benzo[*a*]pyrene produced by cultured human colon.[5] The column was eluted with 150 ml absolute ethanol, 150 ml water, 150 ml 0.05 M ammonium phosphate, pH 3.0 and 150 ml 25% formic acid, in that order, at a flow rate of 2 ml/min and 5 ml fractions were collected. 0.2 ml aliquots of each fraction were submitted to scintillation counting and the external standard procedure was used to correct for quenching. Approximately 85% of the total radioactivity applied to the column was recovered and we are presently trying to determine what happens with the other 15%.

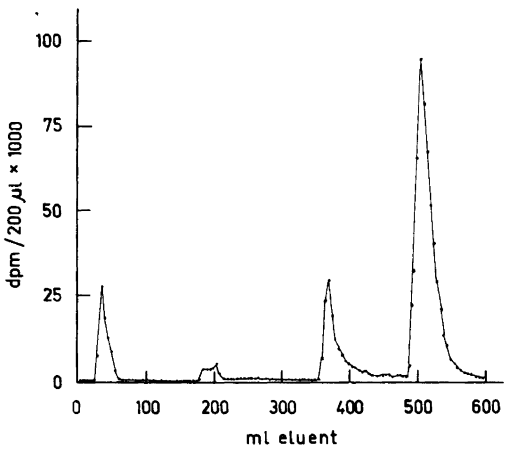

Fig. 1. Separation of benzo[*a*]pyrene and its metabolites recovered from the liver on an alumina oxide column. The experimental procedure is discussed in the text. The presumptive identity of the peaks is, from left to right, (*e.g.*, in order of elution from the column), benzo[*a*]pyrene + unconjugated metabolites, sulfate conjugates, glucuronides and glutathione conjugates.

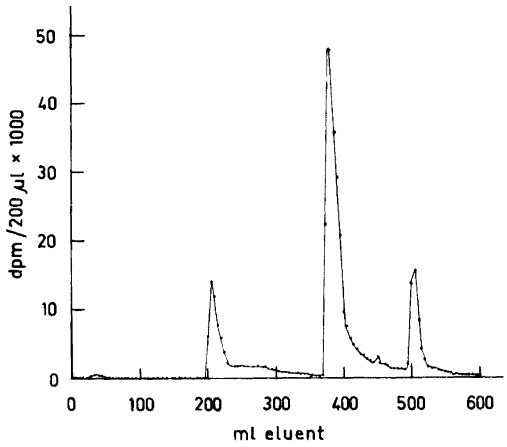

Fig. 2. Separation of benzo[*a*]pyrene and its metabolites recovered in the bile on an alumina oxide column. See legend to Fig. 1.

Figs. 1 and 2 illustrate the elution profiles obtained from liver and bile, respectively. According to the earlier study,[5] the peaks contaim, in order of elution from the column, benzo[*a*]pyrene and unconjugated metabolites; sulfate conjugates; glucuronides; and glutathione conjugates. It is not yet clear where mercapturic acids and amino acid conjugates would be eluted from such a column, a question which we are presently investigating. We are also in the process of reconfirming the identity of the sulfate, glucuronide, and glutathione conjugate peaks.

With these reservations in mind, certain observations can be made about the formation of benzo[*a*]pyrene conjugates in the liver and secretion of such conjugates into the bile of the Northern pike. It is immediately apparent that the conjugate pattern from these two body compartments is quite different. About 75% of the conjugates present in the liver are glutathione conjugates, most of the remaining are glucuronides, and only very small amounts of sulfate conjugates are recovered from this organ.

On the other hand about 60% of the conjugates recovered from bile are glucuronides, while the remaining 40% is approximately equally divided between sulfate and glutathione conjugates. In addition the bile contains virtually no benzo[*a*]pyrene and conjugated metabolites, while the liver apparently contains significant amounts of both the parent hydrocarbon and of unconjugated metabolites. These findings suggest that glucuronides and sulfates of benzo[*a*]pyrene are

selectively secreted into the bile and, consequently, that glutathione conjugates must be selectively secreted into the blood and excreted, either in unchanged form or after conversion to mercapturic acids, in the urine. For the reasons discussed above, these conclusions are at present preliminary and our investigations continue.

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