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In Vivo Metabolism of Pentachlorophenol and Aniline in Arctic Charr (*Salvelinus alpinus L.*) Larvae

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As biotransformation of xenobiotics to more polar and readily eliminated products usually serves as detoxification process, the high persistence and/or toxicity of some chemicals in fish has been related to insufficient metabolic rate in these animal species (Glickman *et al.* 1982; Binder *et al.* 1984; Coats *et al.* 1989). Fish larvae generally have been reported to be more sensitive than adults to pollutant toxicity (McKim 1977). The capability of these stages to metabolize foreign compounds may play an important role in determining their tolerance to environmental contaminants. However, few studies have been published on the metabolism of xenobiotics in early life stages of fish.

Within the group of pesticides, pentachlorophenol is a widely used fungicide and herbicide which has been increasingly found as an environmental contaminant. It is highly toxic to a range of aquatic organisms (Spehar *et al.* 1985), refractory to degradation, and easily concentrated in fish (Gluth *et al.* 1985; Kobayashi 1979; Stehly and Hayton 1989). Aniline is widely used in the dye, rubber, pharmaceutical, agricultural and tanning industries, and is introduced into the aquatic environment directly through industrial discharge. Although it is moderately toxic to rainbow trout, aniline may be metabolized to yield reactive metabolites such as N-hydroxyarylamines (Dady *et al.* 1991).

The present investigation examined the *in vivo* metabolism of aniline and pentachlorophenol in arctic charr *(Salvelinus alpinus)* larvae exposed to contaminated water. Metabolites from oxidation, acetylation and conjugation pathways were identified and quantitated after a 48-hr static exposure period.

MATERIELS AND METHODS

Uniformly ring labeled [^{14}C]-pentachlorophenol (PCP, 215 MBq/mmol, Sigma chimie, Saint Quentin Fallavier, France) and [^{14}C]-aniline hydrochloride (2.3 GBq/mmol, Sigma) with a radiopurity greater than

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Throughout the static exposure period (48 hr) water temperature was held constant at 6°C ± 0.5°C. After exposure, the larvae were removed from the medium, killed; frozen at -20°C and stored at this temperature approximatly 1 mon at this temperature until radioactivity measurement.

PCP and metabolites were isolated from the medium by lyophilization of the water and subsequent solubilization of residues in methanol. This technique resulted in recoveries of about 95 % as estimated by radioactivity measurements in samples taken before and after freeze-drying.

PCP and aniline metabolites were identified by HPLC and TLC and quantified using ^{14}C activity monitoring. Peaks which could not be identified by co-chromatography using reference substances were subjected to enzymic cleavage and identified by co-chromatography of the labeled portion. Incubations with β-glucuronidase and sulphatase were done as indicated by the supplier except that 20 µL 10% D-saccharic acid-1,4-lactone was added during incubation with sulfatase to inhibit possible β-glucuronidase activity.

RESULTS AND DISCUSSION

No mortality occurred during the experiment in both PCP and aniline exposed larvae. Based on breathing and swimming activity observation, neither the confinement nor the presence of pollutants seemed to stress the larvae. The oxygen content in water measured in exposure tubes during the study (*ca.* 5 ppm) was markedly above the LC50 obtained at 6°C for arctic charr eleutheroembryos (0.5 ppm, *C. Gillet personal communication*). However, in comparison with field conditions, it cannot be excluded that our experimental conditions could affect the metabolism of the xenobiotics tested.

At the end of the 48-hr exposure to aniline, 12.9% of the radioactivity was present in the larvae and 66.1% was dissolved in water (Table 1). The remaining 21% was unaccounted for. Since only 77% of the radioactivity added to the blank tube was found after 48 hr (data not shown) and no ^{14}C was absorbed to walls, it may be presumed that this loss could be due to a partial volatilization of aniline. For PCP, the major part of the radioactivity was concentrated into the fry and no loss of radioactivity was observed (Table 1).

Table 1. Distribution of radioactivity after 48-hr exposure of charr larvae to [^{14}C]-PCP and [^{14}C]-aniline as a percentage of dose. [^{14}C] applied to the water. Values are means ± SD from 4 experiments.

	Water	Larvae	Total
PCP	18.3 ± 9.4	78.6 ± 12.0	96.9 ± 11.6
Aniline	66.1 ± 8.3	12.9 ± 4.0	79.0 ± 5.8

98 % were used in this study. Acetanilide, p-aminophenol, β-glucuronidase from bovine liver type B-1, sulfatase from *Aerobacter aerogenes* type VI and D-saccharic acid 1,4-lactone were from Sigma. All solvents were of analytical grade of HPLC grade.

Aliquots of water were counted in a Packard Tricarb 4430 liquid scintillation counter (Packard Instruments, Downers Grove, Illinois) by using Packard Ultima Gold scintillation cocktail. Whole body total radioactivity was measured after combustion of each larva in a Packard oxidizer model 306.

HPLC analyses were carried out on a 250 mm x 4.6 mm I.D. ODS2, 5-μm column (SFCC, Eragny, France). The HPLC system was composed of the following Spectraphysics Analytical (Santa Clara, California) equipment: model P 4000 gradient pump, model 1000 UV detector set at 280 nm and a Chromjet integrator. Fractions (0.5mL) were collected in disposable 3-mL scintillation counting tubes with a microcollector (model 202 Gilson, Middleton, Wisconsin). After addition of 2.2 mL of the fluor mixture (Quickszint flow 202, Zinsser analytic, Frankfurt, Germany), each fraction was counted in the scintillation spectrometer.

The metabolites of PCP were separated at ambient temperature at a flow rate of 1 mL/min by a step gradient elution with 70 % 20 mM potassium phosphate buffer (pH 5.5) and 30 % acetonitrile for 20 min, then 30% 20 mM potassium phosphate buffer (pH 5.5) and 70% acetonitrile for 15 min. HPLC separation of aniline metabolites was performed using methanol-water buffered with 0.26 M amonium acetate and 0.02 M nickel acetate as described by Sternson and Dewitte (1977).

TLC was carried out with plates coated with 0.2-mm thin layers of silica gel F 254 from Merck (Darmstadt, Germany). The solvent systems were those described by Kasokat *et al.* (1987) for PCP and by Sternson and Dewitte (1977) for aniline. Radioactivity was detected using a Berthold LB 2760 TLC scanner (Wilbad, Germany).

The arctic charr larvae were obtained from the Institut National de la Recherche Agronomique experimental pisciculture at Thonon-les Bains (France) and originate from Lake Geneva strain. The larvae were exposed to labeled compounds as follows : 80 charr eleutheroembryos (end of yolk sac resorption, 50-100 mg) were randomly divided into 8 groups of 10 fish. These groups were placed in 50-mL disposable tubes containing 20 mL water obtained directly from lake Geneva. The water temperature was maintained at 6°C ± 0.5°C, hardness averaged 150 mg/L as $CaCO_3$ and pH was 7.9. Dissolved oxygen concentration was *ca.* 5 ppm and remained stable during the experiment. [^{14}C]-PCP (0.5 μg, 0.41 KBq) was added to four of the eight tubes in 10 μL ethanol. As a blank, the same dose was added to a tube containing 20 mL water and no larva. [^{14}C]-aniline hydrochloride (2μg, 35.6 KBq) was added in the four remaining tubes in 10 μL ethanol. The same dose was added to a tube containing only 20 mL water.

Two days after exposure, parent compound accounted for less than 15% of the total ^{14}C found in water for aniline (Table 2), indicating an extensive metabolism of the chemical. In the case of PCP, *ca* 19% of the ^{14}C dissolved in water was associated with the parent compound. However, because the low percentage of radioactivity present in water (Table 1), excreted metabolites corresponded to only 13.7% of the administered radioactivity (in comparison with 51.9% for aniline).

The HPLC profiles obtained from PCP exposure resulted in three separated peaks. After β-glucuronidase incubation of the concentrated medium and subsequent HPLC analysis, the more polar compound (RT 4.5 min.) completely disappeared yielding a corresponding increase of free PCP. This finding suggests that the more polar compound corresponds to the glucuronide conjugate of PCP.
Hydrolysis with sulfatase completely removed the major peak (RT 15.5) which was converted into free PCP, indicating the presence of PCP-sulfate. The glucuronide and sulfate conjugates comprised 24.2 and 49.4%, respectively of the ^{14}C excreted in water.
During aniline treatment, 76.4% of the total ^{14}C at 48hr consisted of the N-acetyl metabolite while a minor polar metabolite was identified as 4-aminophenol by co-chromatography with authentic standard.
For aniline as for PCP experiments, no trace of degradation products was found in blank assays and no mortality was observed among exposed larvae.

Table 2. Metabolites excreted in water during 48-hr exposure of fish to [^{14}C]-PCP (A) and [^{14}C]-aniline (B).

	Metabolite	RF	RT (min)	% of ^{14}C found in water [a]
	PCP-glucuronide	0.42	4.5	24.2 ± 5.5
A	PCP-sulfate	0.81	15.5	49.4 ± 11.6
	parent compound	0.86	29.5	18.3 ± 5.6
Total				91.9 ± 9.1
	p-aminophenol	0.65	5.5	2.1 ± 0.4
B	acetanilide	0.90	31.0	76.4 ± 7.0
	parent compound	0.83	16.0	13.8 ± 2.6
Total				92.3 ± 5.7

a Values are means ± SD from 4 experiments.

The fate of PCP has been studied *in vivo* in several fish species (Glickman *et al.* 1976; Stehly and Hayton 1989; Gates and Tjeerdema 1993). The bioconcentration factor for rainbow trout was found to range between 193 and 460 (McKim *et al.* 1986; Stehly and Hayton 1989). Although the amount of ^{14}C in larvae cannot be taken as true

true steady-state tissue concentration, our results are in good agreement with those observed in adults salmonids and suggest that bioconcentration is limited by metabolism.

PCP glucuronide and sulfate conjugates were detected as excreted metabolites in addition to unchanged parent compound in different fish species (Kobayashi 1979; Kasokat *et al.* 1987; Stehly and Hayton 1989). In addition to these conjugates, Gates and Tjeederma (1993) demonstrated the presence of tetrachloro-p-hydroquinone as a minor metabolite of PCP in the striped bass. In the present study we confirm the formation of pentachlorophenyl-glucuronide and pentachlorophenyl-sulfate in charr larvae exposed to PCP, but no trace of chlorinated hydroquinone was detected. Moreover, further studies are necessary to examine the identity of radioactive compound retained in the larvae.

The main metabolic pathway of aniline in larvae was N-acetylation since after 48hr exposure more than 75% of the total radioactivity in water was found as acetanilide. In addition to this major compound, a minor metabolite was identified as 4-aminophenol. Although there is limited information on the metabolism of aniline in fish, Bradbury *et al.* (1993) demonstrated that N-acetylation was an important biotransformation route for this primary aromatic amine in medaka whereas no indication of ring hydroxylation was previously observed *in vivo* in fish. N-Hydroxylamine, a metabolite from aniline formed in trout hepatic microsomal incubation (Dady *et al.,* 1991) was not detected in our study.

In addition, to demonstrating that *Salvelinus alpinus* larvae may be a useful model for *in vivo* xenobiotic biotransformation studies, our results provide evidence that salmonids are able to biotransform chemicals during early life stages by several metabolic reactions such as hydroxylation, N-acetylation, glucuronidation and sulfation. These biotransformations involve various enzyme systems including cytochrome P450 dependent monooxygenases which had been partly characterized *in vitro* in salmonid larvae (Binder and Stegeman, 1983).

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REFERENCES

Binder RL, Melancon MJ, Lech JJ (1984) Factors influencing the persistence and metabolism of chemicals in fish. Drug Metab Rev 15:697-724

Binder RL, Stegeman JJ (1983) Basal levels and induction of hepatic aryl hydrocarbon hydroxylase activity during the embryonic period of development on brook trout. Biochem Pharmacol 32: 1324-1327

Bradbury SP, Dady JM, Fitzsimmons PN, Voit MM, Hammermeister DE, Erikson RJ (1993) Toxicokinetics and metabolism of aniline and 4-chloroaniline in medaka (*Oryzias latipes).* Toxicol Appl Pharmacol 118:205-214

Coats JR, Symonik DM, Bradbury SP, Dyer SD, Timson LK, Atchison GJ (1989) Toxicology of synthetic pyrethroids in aquatic organisms: an overview. Environ Toxicol Chem 8: 671-679

Dady JM, Bradbury SP, Hoffman AD, Voit MM, Olson DL (1991) Hepatic microsomal N-hydroxylation of aniline and 4-chloroaniline by rainbow trout (*Onchorhyncus mykiss).* Xenobiotica 21: 1605-1620

Gates VL, Tjeerdema RS (1993) Disposition and biotransformation of pentachlorophenol in the striped bass (*Morone saxatilis).* Pestic Biochem Physiol 46:161-170

Glickman AH, Statham CN, Wu A, Lech JJ (1977) Studies on the uptake, metabolism and disposition of pentachlorophenol and pentachloroanisole in rainbow trout. Toxicol Appl Pharmacol 41: 649-658

Glickman AH, Weitman SD, Lech JJ (1982) Differential toxicity of trans-Permethrin in rainbow trout and mice. I. Role of biotransformation. Toxicol Appl Pharmacol 66:153-161

Gluth G, Freitag D, Hanke W, Korte F (1985) Accumulation of pollutants in fish. Comp Biochem Physiol 81C:273-277

Kasokat T, Nagel R, Urich K (1987) The metabolism of phenol and substituted phenols in zebra fish. Xenobiotica 17:1215-1221

Kobayashi K (1979) Metabolism of pentachlorophenol in fish. In: Khan MAQ, Lech JJ (eds) Pesticide and xenobiotic metabolism in aquatic organisms. American Chemical Society, Washington DCD. p 131

McKim JM (1977) Evalutation of tests with early life stages of fish for predicting long-term toxicity. J Fish Res Board Canada 34: 1148-1154

McKim JM, Schmieder PK, Erickson RJ (1986) Toxicokinetic modeling of [^{14}C]-pentachlorophenol in the rainbow trout (*Salmo gairdneri*).

Spehar RL, Nelson HP, Swanson MJ, Renoos JW (1985) Pentachlorophenol toxicity to amphipods and fathead minnows at different test pH's. Environ Toxicol Chem 4:389-398

Stehly GR, Hayton WL (1989) Disposition of pentachlorophenol in rainbow trout (*Salmo gairdneri*): effect of inhibition of metabolism. Aquat Toxicol 14:131-148

Sternson LA, Dewitte WJ (1977) High pressure liquid chromatographic analysis of aniline and its metabolites. J Chrom 137:305-314