Bull. Environ. Contam. Toxicol. (1989) 42:254–261

Environmental Contamination and Toxicology

Metabolism of Chlorobenzene and Hexachlorobenzene by the Zebra Fish, *Brachydanio rerio*

Thomas Kasokat, Roland Nagel, and Klaus Urich

Department of Zoology, Johannes Gutenberg—University Mainz, Saarstraße 21, D-6500 Mainz, Federal Republic of Germany

It is now becoming evident that in many cases metabolites have a greater toxic potential than the parent xenobiotic exposed. For this reason, the knowledge of biotransformation pathways of xenobiotics plays a substantial role in environmental monitoring programs.

The zebra fish (Brachydanio rerio, Hamilton-Buchanan) has received increased attention as a model species for freshwater fish in environmental surveillance programs to evaluate potential health and impacts of anthropogenic chemicals (Bresch et al. 1986; Nagel 1986). The metabolism of phenol and various substituted phenols in zebra fish has recently been studied (Kasokat et al. 1987). The purpose of the present study was to examine the metabolism of chlorobenzene and hexachlorobenzene (HCB), by the zebra fish following in vivo exposure of sublethal concentrations in the water.

MATERIALS AND METHODS

[U-C14]-Hexachlorobenzene (0.37 mCi/mg) and [U-C14]-chlorobenzene (0.27 mCi/mg) were commercially obtained from The Radiochemical Centre Amersham (Bucks, Great Britain). The radiochemical purity was examined by h.p.l.c. and t.l.c. and was found to be >99% in both instances. Unlabeled hexachlorobenzene (Oekanal reference standard) was purchased from Riedel de Häen (Hannover, FR Germany), unlabeled chlorobenzene was supplied from EGA-Chemie (Steinheim, FR Germany). The following unlabeled reference compounds were used in h.p.l.c. analysis: pentachlorophenol, phenol and 4-chlorophenol (Oekanal reference standards, Riedel de Häen, Hannover, FR Germany), 2-chlorophenol, 3-chlorophenol and benzene (Merck-Schuchardt, Hohenbrunn, FR Germany). ß-glucuronidase from E. coli (200 U/mL) was purchased from Boehringer Mannheim GmbH (Mannheim, FR Germany), aryl sulphatase/ß-glucuronidase from Helix pomatia (2990 U/mL), D-saccharic acid-1,4-lactone from Sigma (Taufkirchen, FR Germany), and streptomycin sulphate from Serva (Heidelberg, FR Germany). All chemicals utilized were of the highest commercially obtainable grade of purity.

Send reprint requests to Thomas Kasokat at the above address.

Adult zebra fish (Brachydanio rerio, Hamilton-Buchanan) were purchased from West-Aquarium (Bad Lauterberg, FR Germany). The fish were acclimated in our laboratory for at least 2 weeks prior to the start of the experiments. They were kept in aerated tap water at 26°C and fed with Tetramin (Tetra-Werke, Melle, FR Germany) three times a day. The photoperiod changed with a 12-hr interval. The body weight of the fish averaged 380 mg.

The static test apparatus used has recently been described (Kasokat et al. 1987). The medium consisted of 3 L charcoal-filtered tap water. Prior to the administration of test substances, the antibiotic streptomycin sulphate was added at a concentration of 100 mg/L in the medium to eliminate the possibility of bacterial degradation of test compounds and of deconjugation of fish metabolites, respectively. The above mentioned dose of the antibiotic drug was chosen on the basis of favorable results obtained in an initially performed dose-ranging study which showed that 100 mg/L streptomycin sulphate effectively suppressed bacterial growth without affecting the fish.

The administration of the test compounds occured at a sublethal dose in the tank-water by means of solution carriers under stirring to achieve a homogeneous distribution of the xenobiotics. 4-chlorobenzol (0.1 mg/L) was administered as an ethanolic solution (final concentration of ethanol was 0.01%, v/v). Hexachlorobenzene was applied as a toluenic solution (final concentration of toluene: 0.007%, v/v) in a concentration of 0.005 mg/L because of its higher acute toxicity. The radioactivity amounted to 20 µCi for each test substance.

20 female adult zebra fish were randomly taken from the common fish stock and placed into the test chamber containing the test compounds at concentrations as described above. A small glass pipet ventilated the system via a suction pump. The temperature of the water was 26°C. The fish were not fed during the experiment.

By 48 hr, the metabolites were isolated from the medium as follows: the tank-water was extracted continuously with diethyl ether (pH 6.5, 30°C) according to Nagel and Urich (1983). After separating the different phases, the ether extract, containing nonpolar metabolites and the unchanged parent compound, was concentrated under nitrogen, cooled in ice. The aqueous phase with the polar metabolites was concentrated by a rotary evaporator operating at 40°C. Both concentrates were taken for h.p.l.c. analysis to separate and to identify the metabolites. In the case of the aqueous concentrates, aliquots were subjected to enzymatic cleavage prior to h.p.l.c. analysis. Metabolites which could not directly be identified by co-chromatography using unlabeled reference substances were subjected to enzymatic cleavage by ß-glucuronidase and aryl sulphatase and identified by co-chromatography of the released aglycone residue. The enzymatic cleavage was judged by the disappearance of radioactive peaks in the h.p.l.c. elution profile and the concomitant appearance of the deconjugated labeled por-

tion. Enzyme preparations and incubation conditions used have been described recently (Kasokat et al. 1987).

In the experiment with hexachlorobenzene, we additionally tried to isolate metabolites from fish by extraction. After decapitation, the fish were extracted twice in 10 volumes of ethanol using an Ultra-Turrax TP 18-10 (Janke & Kunkel, Staufen, FR Germany) as homogenizer, operating at half-maximal speed for 2 min under cooling in ice. After centrifugation at 3000 rpm for 10 min at room temperature (BHG 800 Properus, B. Hermle GmbH, St. Leon-Rot, FR Germany), the supernatants were pooled. The total radioactivity was measured and the pooled ethanolic extracts were concentrated and analyzed further by h.p.l.c. for the determination of metabolites. The pellets were dried and taken for combustion in a Tri-Carb Sample Oxidizer 306 (Packard Instruments, Downers Grove, IL, USA) to determine the amount of unextractable radioactivity.

H.p.l.c. was carried out with a Knauer device (Berlin, FR Germany) on a reversed-phase column (LiChrosorb RP 18, 10 µm, 4.6 x 250 mm) using a flow rate of 2 mL/min. The eluents used and the retention times measured are listed in Table 1. The determination of unlabeled reference compounds was performed photometrically at 254 nm. For the determination of radioactivity, the mobile phase was fractionated into 15-sec portions in scintillation vials. The radioactivity in liquid samples was measured in a Packard Tri-Carb 300C Liquid Scintillation Counter with automatic quench correction using Monophase 40 Plus (Packard) as scintillator.

RESULTS AND DISCUSSION

In the experiment with chlorobenzene, polar and nonpolar metabolites were detected in the tank-water at trace amounts (Fig. 1). In the ether extract of the medium, 4 radioactive peaks appeared in the h.p.l.c. elution profile (Fig. 1b). Peak B could not be identified by means of co-chromatography. Peak C showed the same retention time as authentic 2-chlorophenol, peak D corresponded to 3- and/or 4-chlorophenol. Unfortunately, the isomers 3- and 4-chlorophenol could not be separated in spite of numerous variations of the chromatographic conditions involving the composition of the mobile phase and the flow rate, respectively. Peak E represented unmetabolized chlorobenzene.

Chromatographing the aqueous phase of the medium with a more polar eluent (see Table 1), the radioactivity was separated into 4 peaks (not shown). Peak I and peak II could not be identified. In an earlier experiment we examined the metabolism of 4-chlorophenol by zebra fish (Kasokat et al. 1987). The results previously obtained and the appearance of isomeric chlorophenols in the etheral phase of the present experiment promted us to the assumption that the polar metabolites in part might be represented by chlorophenol conjugates. By comparison, peak III behaved chromatographically as 4-chlorophenol glucuronide, while peak IV had a retention time similar to that of 4-chlorophenol sulphate (not shown). This conjecture was further confirmed by chromatographing the enzymatical-

Table 1. HPLC conditions and retention times for chlorobenzene and hexachlorobenzene (HCB), their metabolites, and reference compounds.*

Test compound	Reference compound or metabolite	Eluent	Retention time (min)
Chlorobenzene		0.2 M Ammonium acetate buffer (pH 7.0) - methanol (1:1, v/v)	19.0
	Phenol		3.5
	Unidentified B		4.0
	2-Chlorophenol		5.5
	3- and 4-Chlorophenol		7.3
	Benzene		9.5
		10 mM Potassium phosphate buffer (pH 5.5) - methanol (8:2, v/v)	
	Unidentified I		1.75
	Unidentified II		3.25
	3-/4-Chlorophenyl-glucuronid		4.75
	3-/4-Chlorophenyl-sulfat		7.25
HCB		Methanol - Aqua pura (9:1, v/v)	9.5
	Pentachlorophenol		2.0

*(LiChrosorb RP 18, 10 µm, 4.6 x 250 mm; 2 mL/min)

ly treated aqueous residue of the tank-water. After incubation with ß-glucuronidase, peak II and peak III vanished compared with the untreated medium. Additionally, 2 new-coming peaks were detectable, corresponding to the unidentified metabolite (peak B in the ether extract) and to 3- and/or 4-chlorophenol (Fig. 1c). After treatment with aryl sulphatase, peak IV disappeared and liberated 3- and/or 4-chlorophenol were/was found (Fig. 1d).

In summary, isomeric chlorophenols as phase I products and the corresponding glucuronide and sulphate conjugates as phase products were identified as metabolites of chlorobenzene. This result indicates that Brachydanio rerio is able to hydroxylate aromatic compounds like other freshwater fish species (Nagel 1983; Nagel and Urich 1983). On the other hand, there was no hint of dechlorination reactions since possible dehalogenated products, phenol, e.g., were not found similar to the findings

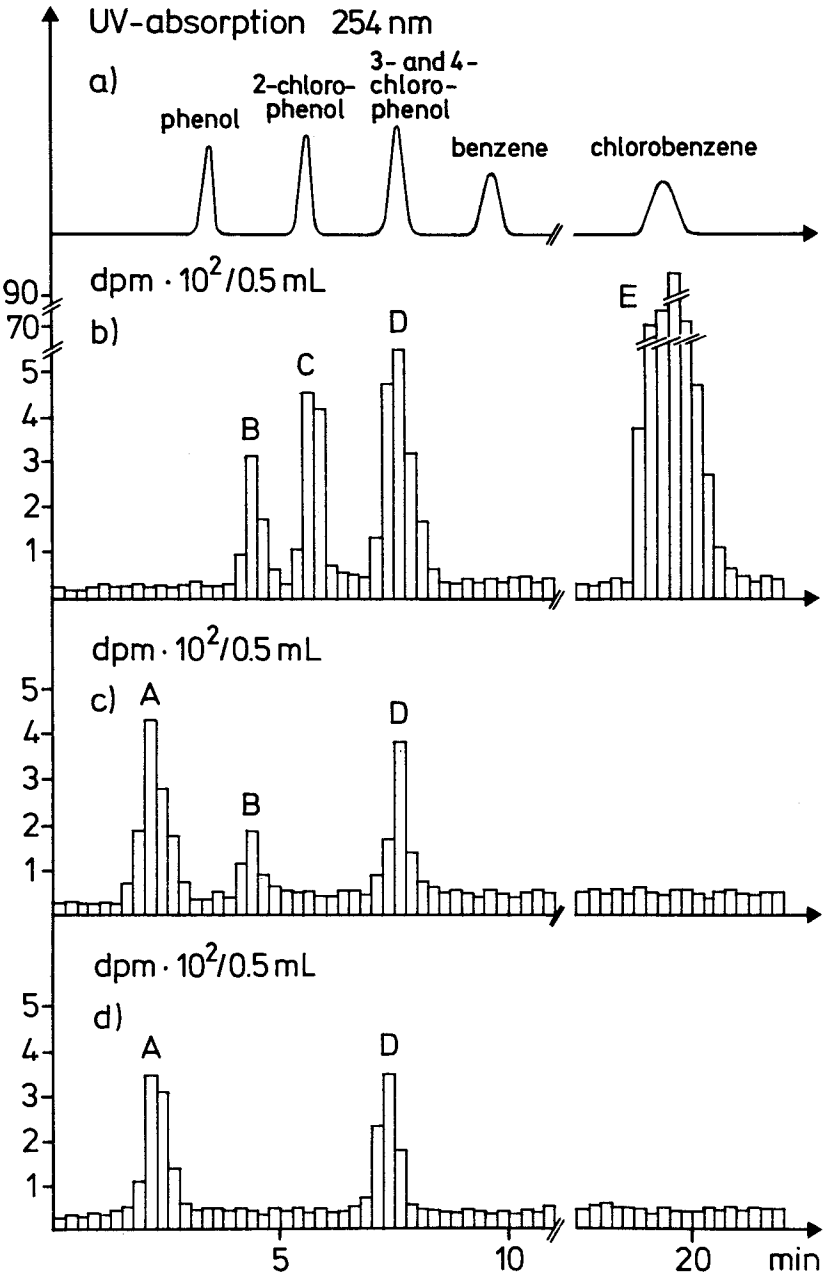

Figure 1. HPLC elution profile of reference compounds (a) and the C14-radioactivity (b-d) after a 48-hr exposure of C14-chlorobenzene (0.1 mg/L) in the medium of 20 female zebra fish: ether fraction (b) and aqueous residue of the medium after treatment with ß-glucuronidase (c) and aryl sulphatase (d) (LiChrosorb RP 18, 10 µm, 4.6 x 250 mm, 0.2 M ammonium acetate buffer (pH 7.0) - methanol (1:1, v/v), 2 mL/min).

obtained using 4-chlorophenol and pentachlorophenol as test compounds (Kasokat et al. 1987).

It is still necessary to make a small comment concerning the prospective chemical constitution of the ether-extractable unidentified metabolite, which was also found as a glucuronide conjugate. This substance may be attributed to a dihydrodiol compound like 4-bromocatechol which was described as a metabolite of the analogous bromobenzene in rats (Monks and Lau 1984). Despite our general lack of unlabeled dihydrodiol reference compounds, there was a plausible explanation that may slightly support this assumption as derived from the chromatographic behavior which is determined by the physico-chemical properties: the substance seemed to be more polar than the mono-hydroxylated chlorophenols because of its shorter retention time as observed in our reversed-phase h.p.l.c. system.

It is important to emphasize, however, that both, the phase I and phase II metabolites, accounted for only a very small portion of the total radioactivity chromatographed as compared to that of the unmetabolized parent compound. All metabolites formed were below the 1%-level of the administered dose. These numerical realities should be kept in mind with regard to the evaluation of potential environmental impacts of chlorobenzene.

Turning to HCB, no metabolites could be detected under the experimental conditions used. 48 hours after administration, only the unchanged parent compound was recorded in the concentrated ether extract of the medium. Furthermore, there was no evidence for polar metabolites in the aqueous phase of the tank-water. Examining the concentrated ethanolic fish extracts, the total radioactivity was represented by unmetabolized HCB. Determinating the degree of extraction we could state that the two-fold extraction occurred quantitatively since no significant amounts of unextractable radioactivity were found in the pellets after combustion.

Our finding was in contrast to published work concerning the metabolism of HCB in fish. For example, Koss et al. (1977) isolated pentachlorophenol (PCP) as a metabolite of HCB from the rainbow trout (Salmo gairdneri). Also, Sanborn and co-workers (1977) specified the same metabolite in all organs examined of the Green Sunfish (Lepomis cyanellus Raf.) after oral administration of HCB-contaminated food. In addition, the ratio of PCP/HCB increased as a function of time in all tissues except for the skeletal muscle during the experiment lasting for 28 days. Compared with our experiment, the exposure time was only 48 hr to obtain comparable results with respect to our previously performed studies with phenolic compounds (Kasokat et al. 1987). In summary, these earlier studies have indicated that no substantial differences do exist between Brachydanio rerio and related freshwater fish species in the metabolic handling of the phenols examined. Quantitatively, however, the oxidation rate of Brachydanio seemed to be lower since the relative contributions of the hydroxylation products formed were smaller. The central question concerning the

transformation of HCB to PCP is the dechlorination as a key reaction prior to hydroxylation. With this regard, it is premature to decide whether the lack of PCP formation in the present study is caused by an inhererent inability of Brachydanio or by our experimental conditions selected.

Concerning the first difference to Sanborn and colleagues (1977), a grossly longer exposure time may lead to an induction and hence metabolism of the administered drug. As recently reviewed (Kleinow et al. 1987), exposure of fish to low levels of certain compounds may result in an induction of specific P-450-dependent monooxygenase activities. These enzyme systems may favor oxidative dechlorination reactions since the mechanism of reductive dechlorination is not involved in the conversion of HCB to PCP.

With respect to the second difference to the above cited study (Sanborn et al. 1977), we suggest that the mode of exposure may profoundly affect the biotransformation of xenobiotics in fish. As previously reported (Nagel 1983), the route of administration influenced the rate of elimination, the extent of metabolism, and the quantitative metabolite pattern of phenol in goldfish: following dietary exposure, phenol was more slowly eliminated, and the amount of conjugates found in the tank-water was greater after uptake from the medium or intraperitoneal injection. Additionally, the percentage of the oxidation product quinol sulphate was much higher.

Considering these facts, we cannot state with certainty that Brachydanio is not able to convert HCB to PCP, but we can exclude the potential influence of bacteria on the biotransformation of HCB in our experiment by the co-administration of streptomycin sulphate which strikingly avoided bacterial growth. Nevertheless, additional experiments with HCB after oral administration or intraperitoneal injection in conjunction with longer exposure times will be required to clarify this point. As an alternative approach, in vitro experiments would provide the basis for further useful information.

Acknowledgments. This work was supported by the Bundesministerium für Forschung und Technologie (BMFT).

REFERENCES

Bresch H, Gode P, Hamburger B, Hansen PD, Juhnke I, Krebs F, Lillelund K, Markert M, Munk R, Nagel R, Nusch EA, Scheubel J, Spieser OH (1986) Deliberations and investigations of the DIN working group "fish test" on a long-term test according to the German Chemicals Act. Zeitschrift für Wasser-Abwasser-Forschung 19:47-52

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

Kleinow KM, Melancon MJ, Lech JJ (1987) Biotransformation and induction: implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environm Health Per-

spectives 71:105-119
Koss G, Strik JJ, Kan CA (1977) Metabolites of hexachlorobenzene in the excreta of different animal species. Abstract presented at the International Conference on in vivo aspects of biotransformation and toxicity of industrial and environmental xenobiotics. Prague, Sep 13-15
Monks TJ, Lech JJ (1980) Activation and detoxification of bromobenzene in extrahepatic tissue. Life Sciences 35:561-565
Nagel R (1983) Species differences, influence of dose and application on biotransformation of phenol in fish. Xenobiotica 13: 101-106
Nagel R, URICH K (1983) Quinol sulphate, a new conjugate of phenol in goldfish. Xenobiotica 13:97-100
Nagel R (1986) Untersuchungen zur Eiproduktion beim Zebrabärbling (Brachydanio rerio, Ham.-Buch.). J Appl Ichthyol 2:173-181
Sanborn JR, Childers WF, Hansen LG (1977) Uptake and elimination of C14-hexachlorobenzene (HCB) by the green sunfish, Lepomis cyanellus Raf., after feeding contaminated food. J Agric Food Chem 25:551-553

Received February 19, 1988; accepted July 30, 1988.