Bull. Environ. Contam. Toxicol. (1987) 39:182–187

Environmental Contamination and Toxicology

Metabolism of n-butyl, n-hexyl, and n-octyl Benzene in the Bile of Rainbow Trout

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Exposure of fish to petroleum can occur directly in the aquatic environment, via the water, or indirectly through the diet (Corner et al. 1976). In vertebrates, metabolic transformations have been shown to take place in the liver and terpenoid and aromatic type molecules to concentrate in the gall bladder bile before elimination (Klaassen 1975).

In our previous investigations of petroleum metabolites extracted from the bile of fish, the mixture of metabolites was examined by ^{13}C NMR spectroscopy (Hellou et al. 1986), and by GLC-MS together with ^{1}H NMR spectroscopy (Hellou and Payne, accepted in Environ. Toxicol. and Chem.). ^{13}C NMR analysis indicated the presence of β-glucuronides enriched with unsaturated vs saturated carbons, when comparing the metabolites mixture to No. 2 fuel oil. The naphthenic carbons represented a significant fraction of the total carbons and were observed at different chemical shifts than in the petroleum oil, strongly indicating that metabolic oxidations took place on the aliphatic carbons.

Mass spectra analysis of the hydrolyzed metabolites isolated from the bile of trout exposed to No. 2 fuel oil, using β-glucuronidase, enabled the identification of at least eight metabolites (Hellou and Payne, accepted in Environment Toxicol. and Chem.). These two- and three-ring aromatic alcohols had side chains of two to six carbons.

In order to answer some of the questions raised from the above observations, the metabolism of n-butyl benzene, n-hexyl benzene and n-octyl benzene was considered. The present study was undertaken in order to answer three questions: (1) For a given

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ring system, does the number of carbons in the alkyl side chain influence the rate of hydrocarbon uptake? (2) For n-alkyl aromatic molecules, are primary, secondary or aromatic alcohols formed preferentially? (3) Are long alkyl side chains attached to aromatic rings degraded to a relatively shorter number of carbon atoms prior to excretion? To our knowledge, these questions have not been previously considered in the study of fish metabolites.

MATERIALS AND METHODS

Rainbow trout, Salmo gairdneri were acclimated to 12°C water for two weeks prior to exposure and not fed during that period. Trout ranging in weight between 100 and 200 g were injected through the mouth with 0.5 ml of a 5% solution of either n-butyl benzene, n-hexyl benzene or n-octyl benzene (Aldrich) in olive oil. Another exposure used a solution containing 5% of each of the above n-alkyl benzenes, while control fish were exposed to olive oil only. Fish were killed with a blow on the head, 120 h after the beginning of the experiment.

Solvents used were HPLC or spectral grade. GLC analyses were performed on a Varian 3700, equipped with a DB-1 capillary column (30 m x 0.25 mm I.D.) and the following temperature programming: initial temperature of 100°C maintained for 1 min, increasing at a rate of 5°/min, up to 250°C, maintained for 15 min. Combined GLC-EI-MS was performed on a Finnigan 4021 mass spectrometer equipped with an INCOS Data System, using the same type of column and temperature programming described above.

A 100 µl sample of bile, from each exposed fish, was processed as follows. Water (1900 µl) was added to each fraction and these were extracted with 2 ml of CH_3OH: CH_2Cl_2 (1:1 v/v). After centrifugation, the organic layer was discarded and extraction was repeated twice with 2 ml of CH_2Cl_2. The aqueous methanol layer was evaporated and redissolved in 1 ml of H_2O. Two milliliters of a solution containing 4 mg of β-glucuronidase (from limpets, 1,480,000 units/g) per milliliter of acetate buffer (0.3 M, pH 4.8) were added to each fraction. Enzymatic hydrolysis took place at 37°C for 24 h. The samples were then acidified and extracted with CH_2Cl_2. The organic layer was evaporated, the samples were redissolved in 10 µl of CH_2Cl_2 and analyzed by GLC. After GLC analysis, the hydrolyzed samples obtained from each type of exposure were separately pooled and analyzed by GLC-MS.

RESULTS AND DISCUSSION

We have previously reported the composition of No. 2 fuel oil, after separation by column chromatography, of the saturates fraction from the aromatics and volatiles (Hellou et al. 1986); and we have also published the ^{13}C NMR spectra of these two fractions. The quantitative analysis of certain polyaromatic components of No. 2 fuel oil is presented in Table 1. Further qualitative analysis, by GLC-MS, of the monoaromatics fraction separated from the oil (Altgelt and Gouw, 1975), indicated the presence of several isomers of the C-2 to (at least) C-8 alkyl benzenes. These results indicate that the three alkyl benzenes chosen in this study are true representatives of the components present in this refined oil.

The GLC-MS analysis of the hydrolyzed bile samples after exposure of the fish to n-butyl benzene revealed the presence of one xenobiotic in the bile. This constituent was identified as 1-phenyl-1-butanol.

After exposure to n-hexyl benzene, four new peaks were detected in the GLC-MS chromatogram. These have been assigned, in order of their increasing retention times on the GLC as: 4-phenyl-1-butanol, 6-phenyl-1-hexanol, 1-phenyl-1-hexanol and 6-phenylhexanoic acid. The major peak is due to 6-phenyl-1-hexanol, while 4-phenyl-1-butanol is very minor.

Exposure to n-octyl benzene produced four new detectable products after hydrolysis of the bile samples. These were identified, in order of increasing retention times, present in decreasing amounts, as: 6-phenyl-1-hexanol, 8-phenyl-1-octanol, 1-phenyl-1-octanol and 1-phenyl octanoic acid.

In one experiment, fish were exposed to a mixture containing equal amounts of n-butyl, n-hexyl and n-octyl benzene, to see if one of these hydrocarbons would be taken up preferentially to the others. The bile from each exposed fish was individually analyzed by GLC and these samples were then pooled and re-analyzed to see the average relative percentage of the metabolites in the mixture. Examination of the chromatograms indicated a steady increase in the peak heights with increasing retention times. There are about three and four times more, hexyl and octyl metabolites respectively, than butyl metabolites. Bearing in mind that the butyl alcohols seem to derive from exposure to n-butyl or n-hexyl benzene, and that the hexyl metabolites can originate from an exposure to n-hexyl or n-octyl benzene, a definite preference in the concentration of long chain metabolites vs short chain ones is indicated.

Table 1. Concentration (μg/g) of polyaromatic hydrocarbons in no. 2 fuel oil.

C-2 benzene	11000
C-3 benzene	5000
C-4 benzene	1700
C-5 benzene	3600
C-0 naphthalene	1500
C-1 naphthalene	6400
C-2 naphthalene	10000
C-3 naphthalene	11000
C-4 naphthalene	2100
C-0 fluorene	200
C-1 fluorene	530
C-2 fluorene	600
C-0 dibenzothiophene	180
C-1 dibenzothiophene	620
C-2 dibenzothiophene	750
C-3 dibenzothiophene	160
C-4 dibenzothiophene	31
C-0 phenanthrene/anthracene	260
C-1 phenanthrene/anthracene	1100
C-2 phenanthrene/anthracene	1000
C-3 phenanthrene/anthracene	470
C-4 phenanthrene/anthracene	180

The above components add up to 58380 μg of polyaromatic hydrocarbons in 1 g of oil, which represents around 27% of the aromatics fraction.

Considering the questions raised in the introduction and our previous results, it would seem that, in the case of mono-aromatic molecules with a single aliphatic unbranched side chain, the molecule with the longest hydrocarbon chain will be preferentially concentrated. This new result is in agreement with studies on aromatic molecules such as benzene, naphthalene and anthracene, which demonstrated that molecules with a larger number of rings, (i.e. molecules with higher molecular weight), are preferentially concentrated in the gall bladder (Roubal et al., 1977). It would appear that not only are the number of rings important, but the size of the aliphatic moiety is also important.

The present results also indicate that primary and secondary alcohols are formed rather than aromatic ones, with oxidation taking place at either end of the n-alkyl side chain. When considering the metabolism of n-hexyl benzene and n-octyl benzene,

the major metabolite formed is the primary alcohol and the carboxylic acid is obtained in a relatively smaller amount. The absence of phenolic type metabolites was confirmed by UV analysis. No bathochromic shift was observed in the UV maximum, going from a neutral to an alkaline solution, which indicates the absence of a phenol-phenoxide type moiety in the extract.

The preferential formation of alcohols on the alkyl side chain rather than the aromatic ring was also observed by Kojimo et al (1982) in carp exposed to 2,6-diisopropyhnapl thalene. However, Melancon et al. (1985) identified considerably smaller amounts of methyl oxidation products than dihydrodiols in trout cytochromes P-450 exposed to 2-methylnaphthalene.

Degradation of the side chain took place after exposure to the two hydrocarbons with higher molecular weights. Although the degradation of n-hexyl benzene afforded a very minor metabolite, the degradation of n-octyl benzene gave a major metabolite. This result might indicate that there is a lower limit for the number of side chain carbons enzymatically degraded. The higher rate of aromatic to aliphatic carbons observed by ^{13}C NMR (Hellou et al., 1986) for the fish metabolites vs the petroleum hydrocarbons could reflect such a process. Another possibility would involve the preferential concentration of molecules with a larger number of rings vs smaller ones, given the same number of carbons in the side chain. There could also be a preference in the concentration of short substituted side chains vs n-alkyl side chains.

The purity of the aromatic hydrocarbons used in the present study was checked by GLC. Although the purchased products were indicated as 99%, 97% and 99% pure, the GLC spectra did not show any detectable amount of shorter chain aromatics in any of the samples. Therefore, the degraded alcohols observed after hydrolysis of the bile metabolites cannot be considered as artifacts due to impurities present in the hydrocarbons used to inject the fish.

Acknowledgments. The authors would like to thank Dr. J. H. Banoub for his help in the preparation of this manuscript.

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Table 2. Metabolites identified after exposure of fish to n-alkyl aromatic molecules.

alkyl benzenes	ratio*	metabolites
n-butyl benzene		1-phenyl-1-butanol
n-hexyl benzene	1	4-phenyl-1-butanol
	7	6-phenyl-1-hexanol
	3	1-phenyl-1-hexanol
	3	6-phenylhexanoic acid
N-octyl benzene	5	6-phenyl-1-hexanol
	3	8-phenyl-1-octanol
	2	1-phenyl-1-octanol
	1	1-phenyloctanoic acid

* average ratio observed for the identified metabolites.

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Received January 25, 1987; accepted March 17, 1987