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Metabolites of Three Structural Isomers of Butylbenzene in the Bile of Rainbow Trout

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In an ongoing study of the fate of petroleum hydrocarbons in fish, we have examined the petroleum derived metabolites which concentrate in the gall bladder bile of cunners, Tautogolabrus adspersus, (Hellou et al. 1986) and rainbow trout, Salmo gairdneri (Hellou and Payne 1987). ^{13}C NMR analysis of the conjugate metabolites isolated from the bile of cunners exposed to No. 2 fuel oil indicated the presence of unsaturated and saturated carbons (Hellou et al. 1986). Although the saturated carbons were present in a smaller amount in the metabolites mixture vs the petroleum oil, they represented a significant fraction of the total carbon atoms present in the glucuronides. The observed chemical shifts suggested that functionalization had taken place on the aliphatic side chain of the aromatic molecules prior to conjugation to glucuronic acid.

The metabolites obtained from the bile of rainbow trout exposed to a petroleum oil have been analyzed by GLC-MS (Hellou and Payne 1987), and several two- and three-ring aromatic alcohols were identified after hydrolysis with β-glucuronidase. With the help of ^{1}H NMR and single ion monitoring (GLC-MS), a possible average structure was proposed for the mixture of metabolites.

Another approach toward the elucidation of the structure of metabolites derived from No. 2 fuel oil relied on chemical synthesis (Hellou et al. 1987). Several aromatic and aliphatic glucuronides were prepared. The chemical shifts of the anomeric carbon C-1 and that of the glycosylated aglyconic carbon atom C-1′, of the synthetic glucuronides were compared to those isolated from the bile of fish. This study demonstrated that no primary n-alkyl alcohol moiety could be detected in the ^{13}C NMR of the isolated metabolites mixture.

In another series of experiments (Hellou and King, 1987), we investigated the type of metabolites obtained in the bile of trout exposed to three n-alkylbenzenes. Phenolic type products were not detected, while primary and secondary alcohols were observed in various amounts. Exposure of fish to a mixture containing equal

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amounts of these three n-alkylbenzenes showed their presence in the bile, in a ratio of 1:3:4, for n-butylbenzene, n-hexylbenzene and n-octylbenzene metabolites respectively.

The present exposure was undertaken to determine if, given aromatic hydrocarbons of same molecular weight and ring structure, a relationship could be found between the type of alkyl side chain and metabolites formed; and the influence of the amount of hydrocarbon administered during an exposure on the type of glucuronides formed. Three structural isomers of butylbenzene were chosen for the present experiment: n-butylbenzene, sec-butylbenzene and tert- butylbenzene. These were chosen because they are commercially available and because several isomers of a C-4 benzene were detected in the GLC of the monoaromatics fraction of No. 2 fuel oil. Therefore, these molecules are probably true constituents of the petroleum oil.

MATERIALS AND METHODS

Trout, Salmo gairdneri were accommodated to 7°C water for at least two weeks prior to exposure and were not fed during that period. Trout ranging in weight between 100 and 200 g were intubed through the mouth with 0.5 ml of a 5% solution of either n-butyl benzene, sec-butyl benzene or tert-butyl benzene in olive oil. An exposure to a 0.5% and 0.1% sec-butyl benzene solution was also undertaken. Another exposure used a solution containing 5% of each of the above butyl benzene isomers. Control fish were exposed to olive oil only. Ten to twelve fish were used for each exposure. After 120 h the fish were killed, weighed, measured and their sex determined. The gall bladder was then removed, weighed and the bile stored at -40°C until needed.

Solvents used were HPLC or spectral grade. GLC analyses were performed on a Varian 3700 instrument 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 5°/min, up to 250°C and maintained there for 10 min. Combined GLC-EI-MS was performed on a Finnigan 4021 instrument equipped with an INCOS Data System, using the same type of column and temperature programming described.

A 100 µl sample of bile from each fish was processed as follows. Water (1900 µl) was added to each fraction, followed by extraction with CH_3OH:CH_2Cl_2 (2 ml : 2 ml) and by two more extractions with CH_2Cl_2 (2 ml). The aqueous methanol layer was separated and redissolved in water (2 ml). A solution containing 8 mg of β-glucuronidase (Sigma, from Limpets 1,480,000 units/g) in 4 ml of acetate buffer (0.3 M, pH = 4.8) was added to each fraction and the samples were hydrolyzed at 37°C for 24 h. This amount represents at least a ten fold excess of enzyme when exposing fish to a 5% sec-butylbenzene solution. Samples were acidified and extracted three times using CH_2Cl_2. The three organic layers

obtained after the extraction of each sample were pooled, evaporated and redissolved in CH_2Cl_2 for GLC analyses of individual samples. The samples obtained from each type of exposure were pooled separately and analyzed by GLC-MS using the conditions described above.

RESULTS AND DISCUSSION

We have previously reported on the exposure of trout to n-alkylbenzenes (Hellou and King, 1987). In the case of n-butylbenzene, enzymatic hydrolysis of the metabolites present in the gall bladder bile using β-glucuronidase indicated the presence of 1-phenyl-1-butanol. The alcohol was identified by GLC-MS, where it showed a very simple fragmentation pattern with loss of a propyl moiety (Table 1, A). No other metabolites were detected in the chromatogram.

After oral exposure of trout to sec-butylbenzene, three alcohols were identified in the hydrolyzed bile extract. The major peaks observed in the mass spectra of these compounds are reported in Table 1, B. The major product is tentatively identified as the tertiary alcohol, 2-phenyl-2-butanol. The two minor metabolites could be a phenol and 3-phenyl-1-butanol. These were identified by comparing the obtained MS to that of commercially available alcohols and by analyzing the observed fragmentation pattern.

Exposure to sec-butylbenzene was attempted at three different concentrations, in order to find out if there is a correlation between the amount of chemical used in the exposure of fish and the type of metabolites present in the gall bladder bile. A 5%, a 0.5% and a 0.1% solution of sec-butylbenzene in olive oil were used. When the concentration of the solution was reduced from 5 to 0.5% (reduced by a factor of ten), the concentration of the metabolites was reduced by approximately a factor of four.* A further reduction, by a factor of five, of the concentration of the olive oil solution showed approximately the same decrease in the amount of metabolites. It should be mentioned that no metabolites could be detected when trout were exposed to a 0.01% solution. Our results tend to indicate that the lowest limit for the concentration of the solution used in the exposure is ~ 0.03%, which represents a 1 ppm solution, when using fish weighing around 150 g, and analyzing 100 µl of bile. An inverse relation between the size of the fish and the concentration of bile metabolites has also been observed during our experiments. Therefore, it would appear that a 1 or 2% hydrocarbon solution would give the optimum

*Comparison is done between the amount of glucuronides present in the gall bladder bile on the sixth day after exposure. Two variables are therefore eliminated: time after exposure and volume of bile.

Table 1. Major peaks observed in the MS of the metabolites obtained after exposure of trout to n-butylbenzene sec-butylbenzene and tert-butylbenzene

Metabolites from	Ratio[2]	MS peaks: m/z (relative intensities, %)
A) n-butylbenzene[2]	-	150(M^+, 20), 107(M^+-43, 100), 77(M^+-73, 10)
B) sec-butylbenzene	10	150(M^+50), 121(M^+-29, 100), 103(M^+-47, 25), 91(M^+-59, 30), 77(M^+-73, 35)
	1	150 (M^+, 5), 132(M^+-18, 20), 117(M^+-33, 40), 105(M^+-45, 100), 91(M^+-59, 30), 77(M^+-73, 20)
	1	150(M^+, 25), 135 (M^+-15, 100), 107(M^+-43, 30), 95(M^+-55, 15)
C) tert-butylbenzene	30	150(M^+, 80), 135(M^+-15, 100), 107 (M^+-43, 85), 91(M^+-59, 45), 77(M^+-73, 50)
	5	166(M^+, 20), 135(M^+-31, 100), 107(M^+-59, 60), 91(M^+-75, 15), 77(M^+-89, 20)
	1	166 (M^+, 45), 151(M^+-15, 100), 123(M^+-43, 25), 93(M^+-73, 10), 77(M^+-89, 10)
	1	150(M^+, 10), 119(M^+-31, 100), 91(M^+-59, 60)
	trace	164(M^+, 20), 119(M^+-45, 100), 91(M^+-73, 60), 77(M^+-87, 15)

[1]average relative ratio observed (GLC) after examination of individual bile samples.
[2]mass spectrum is not reported in Hellou and King, 1987

amount of bile metabolites in trout exposed to sec-butyl benzene. The most important result of this concentrations study is the presence of the same three glucuronides in the case of each exposure. It indicates that the present exposure to high concentrations of chemicals does not influence the type of metabolites produced.

When trout were exposed to tert-butyl benzene, five new peaks were detected by GLC, when comparing control to exposed fish. The major metabolite is a monohydroxylated derivative of tert-butyl benzene, while three other minor peaks observed in the chromatogram can be assigned to another monohydroxylated t-butylbenzene and to two diol derivatives of t-butyl-benzene. A carboxylic acid was also detected in trace amounts. The major peaks observed in the MS are reported in Table 1, C.

When the three isomers of butyl benzene were orally injected as a 1:1:1 mixture, the metabolites obtained from each hydrocarbon were compared. This experiment showed that the metabolite deriving from n-butylbenzene could not be detected in the hydrolyzed extract of the gall bladder bile, and that the major metabolites deriving from sec-butylbenzene and tert-butylbenzene were present in the same relative amount. Comparison of the metabolites formed was done on the sixth day after the beginning of the exposure because the authors have shown that the concentration of glucuronide metabolites is highest on that day, in the case of trout exposed to: no. 2 fuel oil (Hellou and Payne, 1987), and a mud-based oil (Hellou and King, 1987). This result also correlates with our prior observations (Hellou et al, 1986), where an n-alkyl moiety was not detected in the ^{13}C NMR of the mixture of metabolites obtained from the bile of cunners exposed to no. 2 fuel oil. The absence of 1-phenyl-1-butanol in the hydrolyzed bile extract after exposure of trout to the three butylbenzene isomers could indicate that the rate of metabolism or of uptake of the n-alkyl aromatic molecule is very different from that of sec-butylbenzene, tert-butylbenzene and the mixture of chemicals present in the above mentioned studies. Differences in the rate of accumulation or release of chemicals have been observed between cyclic and branched molecules in the aliphatic fraction of oil, in the case of clams (Clement et al, 1980); also between linear and cycloalkanes in the case of trout (Cravedi et al, 1983).

The results described above indicate that upon exposure of trout to three aromatic hydrocarbons of the same molecular weight, ring structure and with a single side chain, oxidation of the side chain will take place preferentially to that of the ring. In the case of the n-butybenzene isomer no phenolic type metabolite was detected. In the case of sec-butybenzene and tert-butylbenzene the phenols represented a minor fraction of the metabolites. These results are interesting since an n-alkyl moiety predominates in the aromatic fraction of petroleum (Gillet et al. 1981; Netzel et al. 1981; Qian et al. 1984) and previous studies of polycylic aromatic hydrocarbon (PAH) metabolites (for example: Collier et al. 1978; Gmuir and

Varanassi, 1982; Krahn et al. 1981, McLaren and Lech, 1984; Rouba et al. 1977) have concentrated mainly on unsubstituted or methyl substituted molecules. We have therefore shown that the type of glucuronides formed after exposure of fish to aromatic hydrocarbons is related to the side chain attached to the ring. Our work has indicated that an alkyl side chain on an aromatic molecule will be functionalized preferentially to the unsaturated ring.

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