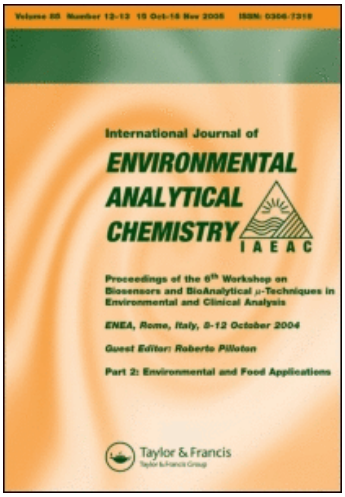

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Metabolic Fate of Aromatic Hydrocarbons in Aquatic Organisms:

Analysis of Metabolites by Thin-Layer Chromatography and High-Pressure Liquid Chromatography†

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Aquatic organisms convert aromatic hydrocarbons into a variety of conjugated and nonconjugated derivatives. Analytical techniques based on thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) were employed to separate, identify and quantitate individual metabolites from fish exposed to radiolabeled naphthalene and 2,6-dimethylnaphthalene. Significant differences in profiles of individual metabolites were found in relation to the type of biological sample analyzed. Liver of naphthalene-exposed salmonids contained eight polar derivatives, as shown by HPLC. Two nonconjugates (1-naphthol and 1,2-dihydro-1,2-dihydroxynaphthalene) and three conjugates (1-naphthyl glucuronic acid, 1-naphthyl sulfate and 1-naphthyl glucoside) were identified. HPLC revealed that brain of ^{3}H-naphthalene-exposed trout contained essentially the nonconjugated derivatives, 1-naphthol and 1,2-dihydro-1,2-dihydroxynaphthalene. TLC showed that the metabolities from trout urine were 1-naphthol, 1,2-dihydro-1,2-dihydroxynaphthalene, and 1-naphthyl glucuronic acid (99% of the total metabolites detected). Major components of the metabolite fractions of tissues and biological fluids were 1,2-dihydro-1,2-dihydroxy and glucuronic acid derivatives. Dihydrodiol derivatives arise from the corresponding arene oxides, some of which have been shown to be cytotoxic to certain mammalian systems.

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INTRODUCTION

An understanding of the bioconversion of aromatic hydrocarbons in aquatic organisms is of interest with respect to the impact of petroleum on marine environments.[1-3] Recent evidence has indicated that a variety of aquatic organisms contain enzymes capable of metabolizing aromatic hydrocarbons.[1,2] Both conjugated and nonconjugated derivatives are formed in organs, such as liver, and may be retained in tissues for at least several weeks after exposure.[4,5] Studies with mammalian systems imply that metabolite formation is linked to long-term cellular damage.[6] Accordingly, both the degree of conversion of the aromatic hydrocarbons and the specific structures formed in aquatic species are being studied in our laboratories.

Techniques of thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) employed in our laboratories for studying the metabolic fate of aromatic hydrocarbons are described. These methods elucidate individual metabolites in tissues from aquatic species exposed to radiolabeled and nonradiolabeled aromatic hydrocarbons. The application of the radiometric techniques to the analysis of tissue samples from organisms exposed to naphthalene and alkylated naphthalenes has revealed a variety of metabolites. These include relatively large amounts of 1,2-dihydro-1,2-dihydroxynaphthalene which is formed from the enzymatic hydration of the corresponding potentially toxic arene oxide.

EXPERIMENTAL

Tissue extraction

In studies with TLC, pooled tissues (1–10 g) from hydrocarbon-dosed animals (exposures performed as described earlier[4,5,7]) were homogenized and extracted with diethyl ether. The extracts (containing the nonconjugated derivatives) were concentrated under a stream of nitrogen. The residual tissue was then extracted again with dichloromethane:isopropyl alcohol:water (75:25:2; v:v:v) to remove conjugated aromatic hydrocarbon derivatives. These extracts were kept separate from the diethyl ether extracts and concentrated under reduced pressure.[4]

For HPLC, extractions, concentrations and separations of naphthalene metabolites were performed as follows:[7,8] pooled tissues (1–10 g) from naphthalene-dosed animals[4,5,7] were homogenized in distilled water. Proteins were precipitated by addition of acetone and solid sodium chloride to the homogenate. Metabolites were extracted from the homogenate with ethyl acetate, the extract was dried under nitrogen, and the resulting residue was redissolved in methanol.

Thin-layer chromatography

The concentrated extracts containing the nonconjugated metabolites were applied to 20 × 20 cm Merck silica gel TLC plates (EM Laboratories, Elmsford, New York). Plates were developed for 2–3 hr in *p*-dioxane:benzene:acetic acid (25:90:4; v:v:v; System B).[4] The concentrated extracts containing the conjugated derivatives were applied to Quantum LQD segmented TLC plates (Quantum Industries, Fairfield, New Jersey). These plates were more effective for the separation of conjugates than the plates used for the nonconjugates. Conjugates were separated during 3–4 hr with the upper solvent phase of 1-butanol:concentrated ammonium hydroxide:water (40:10:50; v:v:v; System A).

Colorimetric determinations of each compound were done by use of previously described spray reagents and procedures.[4] Rf values are given in Table I. Determinations of radiolabeled compounds were accomplished by scraping 1.0 cm bands from the TLC plates, suspending the scrapings in a mixture of 15 ml InstaGel Emulsifier (Packard Instrument Co., Downers Grove, Illinois) and 5 ml of 1% v/v HCl, and assaying for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer (Model 3255). Using these techniques, the amounts of compounds detected were 10–20 pg for ^{3}H-labeled compounds (2 Ci/mmole) and 2–10 ng for ^{14}C-labeled compounds (5 mCi/mmole).

High-pressure liquid chromatography

HPLC separations were performed using a Spectra-Physics (Model 3500, Santa Clara, California) high-pressure liquid chromatograph, a 1/2 m × 4 mm I.D. stainless steel column packed with 10 μm LiChrosorb RP-18 (EM Laboratories, Elmsford, New York) reverse-phase liquid chromatographic material, and a Fractomette 400 (Buchler Instruments, Fort Lee, New Jersey) fraction collector. The methanolic solution containing metabolites was applied to the column and eluted by a 60 min linear gradient elution from 5×10^{-4} M PO_4, pH 5, to 100% methanol at a rate of 1 ml/min. Eluant fractions (0.5 ml) were collected in silanized 13 × 100 mm glass culture tubes and transferred to 28 mm scintillation vials. Ten ml of InstaGel Emulsifier were added to each vial and the vials were counted in the scintillation spectrometer.

Identifications of metabolites were based on retention times of standards: 1-naphthol, 1-naphthyl glucuronic acid, 1-naphthyl glucopyranoside, and 1-naphthyl sulfate were obtained from Sigma Chemical Co., St. Louis, Missouri. Naphthalene was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, and 1,2-dihydro-1,2-dihydroxynaphthalene

was synthesized in our laboratories.[9] Concentrations of metabolites were calculated from the specific activity of the ^{3}H-naphthalene used and from extraction/concentration efficiencies of 0.25 ± 0.08, 0.86 ± 0.07, 0.75 ± 0.09, 0.92 ± 0.08, and 0.64 ± 0.20 ($\pm$s.d., $n=8$) for the glucuronide, sulfate, dihydrodiol, glucoside, and 1-naphthol, respectively.[8] Naphthalene recovery was erratic using this procedure, thus naphthalene was not analyzed quantitatively via HPLC. The data obtained indicate that 50–200 pg amounts of metabolites were detected via HPLC. The specific activity of the ^{3}H-naphthalene used in this study was 85 mCi/mmole.

RESULTS

TLC afforded an inexpensive means of resolving conjugated and nonconjugated metabolites of aromatic hydrocarbons, either in model mixtures

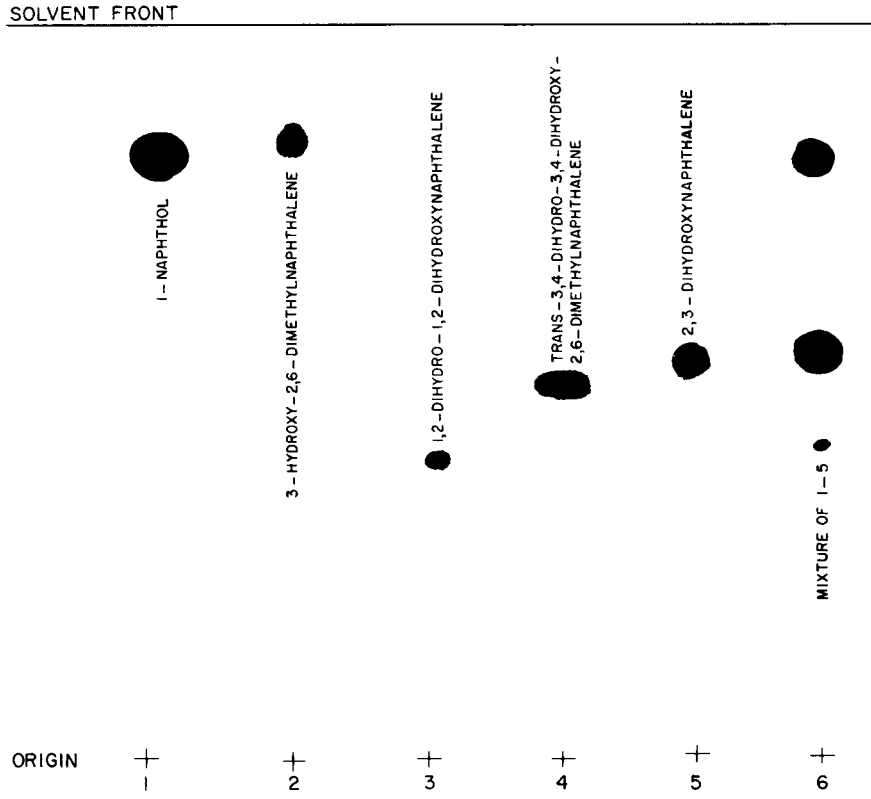

FIGURE 1 Thin-layer chromatography of hydroxylated derivatives of naphthalene and 2,6-dimethylnaphthalene. Experimental conditions given in text.

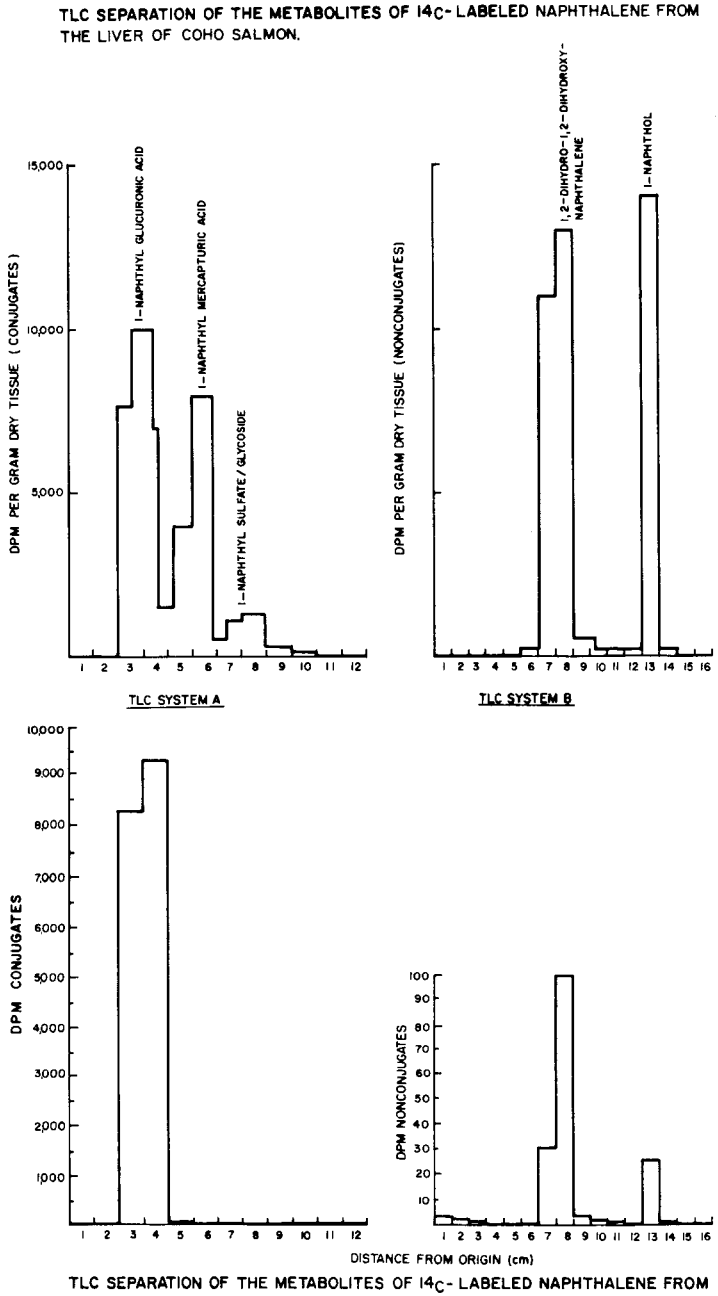

FIGURE 2 Thin-layer chromatographic separation of the metabolites of ^{14}C-naphthalene from the liver of coho salmon and the urine of rainbow trout. (a) Composition of the aromatic metabolites, 24 hr after receiving radiolabeled hydrocarbon (2.5 μCi) by intraperitoneal injection.[4] (b) Composition of the aromatic metabolites, collected from 90 to 140 hr after fish were force-fed 5.55 μCi of the radiolabeled hydrocarbon.

TABLE I

Metabolites of 2,6-dimethylnaphthalene in the urine of rats and in the gall bladder and liver (composite sample) of coho salmon[a]

Standard compound	Rf value standard colorimetric	Rf values of metabolites (mean ± s.d.)							
		Rat				Coho salmon			
		System A		System B		System A		System B	
		Radio-metric	Colori-metric	Radio-metric	Colori-metric	Radio-metric	Colori-metric	Radio-metric	Colori-metric
Trans-3,4-dihydro-3,4-dihydroxy-2,6-dimethylnaphthalene	0.56[b] (±0.03)			0.54[c]	0.54 (±0.01)			0.57 (±0.03)	
3-Hydroxy-2,6-dimethylnaphthalene	0.88[b] (±0.03)			0.85[c]	0.84 (±0.01)			0.86 (±0.03)	0.85 (±0.01)
1-Naphthyl-β-glucuronic acid	0.26[d] (±0.04)	0.24 (±0.02)	0.27 (±0.02)			0.23 (±0.01)			
1-Naphthyl mercapturic acid	0.38[d] (±0.01)	0.38 (±0.03)	0.38 (±0.03)			0.36 (±0.03)			
1-Naphthyl sulfate	0.58[d,e] (±0.03)	0.55 (±0.02)	0.57 (±0.01)			0.55 (±0.02)			
1-Naphthyl-α-glucoside	0.60[d,e] (±0.03)								

[a]Rats received 11.1 μCi of ^{3}H-labeled 2,6-dimethylnaphthalene and 50 mg of nonlabeled 2,6-dimethylnaphthalene (in separate experiments) by intraperitoneal injection. Fish received 8.5 μCi of ^{3}H-labeled 2,6-dimethylnaphthalene and 20 mg of nonlabeled 2,6-dimethylnaphthalene (in separate experiments) by force-feeding.

[b]System B: P-Dioxane:benzene:acetic acid (25:90:4, v:v:v) using Merck silica gel TLC plates.

[c]Single value.

[d]System A: Top layer from 1-butanol:conc. NH_4OH:H_2O (40:10:50, v:v:v) using Quantum LQD segmented TLC plates.

[e]1-Naphthyl sulfate and 1-naphthyl glucoside overlap in System A.

(Figure 1) or in samples isolated from tissues of animals exposed to radiolabeled hydrocarbons (Figure 2). The metabolites were identified in relation to marker compounds chromatographed on the same plate (Table I). Rf values and selective color reactions served to establish identities. With radiolabeled compounds, quantitation was achieved by assaying individual metabolites by liquid scintillation spectrometry. The complementary use of the two different solvent systems, A and B, provided an effective means for separating conjugates and nonconjugates from biological systems. The use of TLC for the isolation of metabolites from urine of rainbow trout (*Salmo gairdneri*) exposed to ^{14}C-naphthalene resulted in the identification of essentially one conjugated derivative, 1-naphthyl glucuronic acid, and two nonconjugated derivatives, 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol. Examples of separations of metabolites from the liver and urine of salmonids are given in Figure 2.

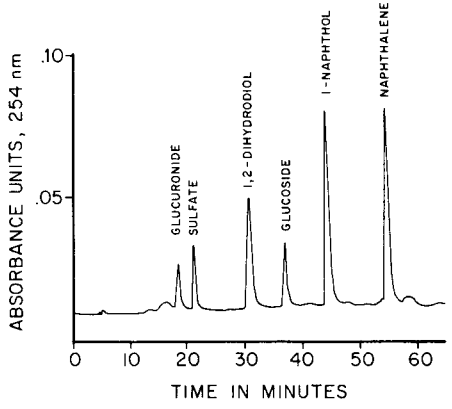

FIGURE 3 Elution profile of nonradiolabeled naphthalene and its metabolites. Experimental conditions given in text.

Solvent systems A and B were employed for the separation, identification, and quantitation of metabolites of 2,6-dimethylnaphthalene in the urine of exposed rats and in the gallbladder and liver of exposed coho salmon. The Rf values of metabolites of 2,6-dimethylnaphthalene (*trans*-3,4-dihydro-3,4-dihydroxy-2,6-dimethylnaphthalene and 3-hydroxy-2,6-dimethylnaphthalene), the only pure derivatives available, were established in solvent B by both colorimetric and radiometric techniques. The Rf value of 3,4-dihydro-3,4-dihydroxy-2,6-dimethylnaphthalene was different from the Rf value of the 1,2-dihydro-1,2-dihydroxynaphthalene; however,

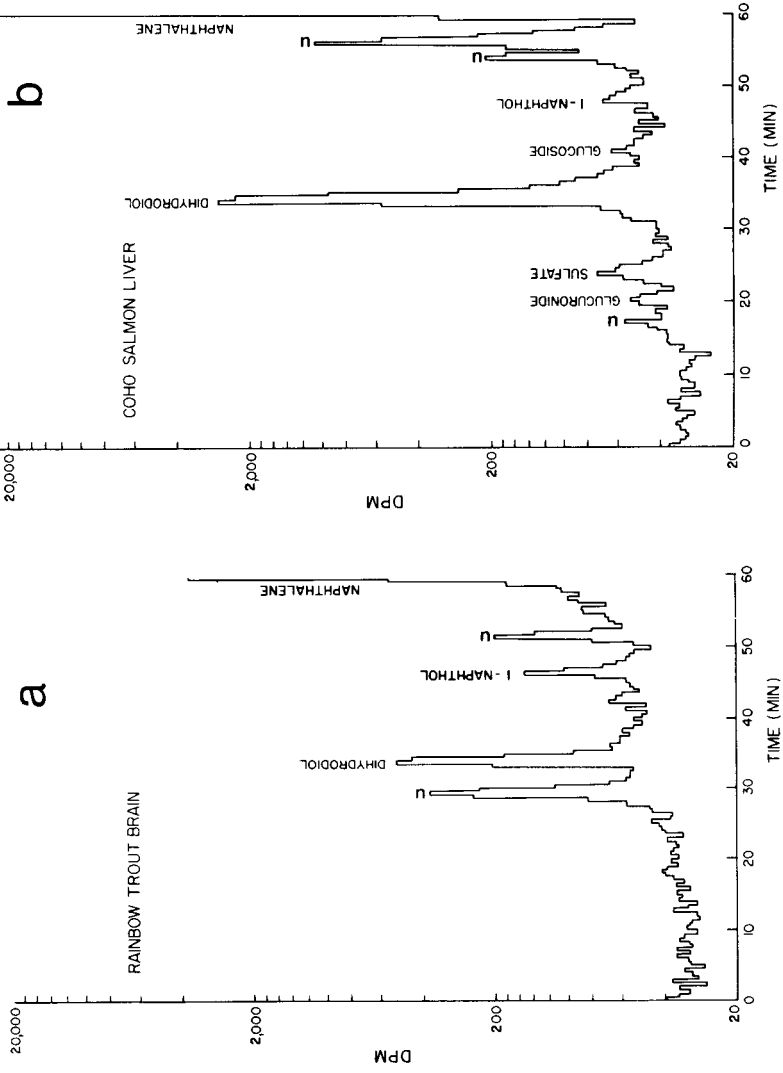

FIGURE 4 High-pressure liquid chromatography profiles of polar naphthalene metabolites in tissues of salmonids. (a) Brain tissue from nine rainbow trout, 16 hr after feeding of 120 μCi ^{3}H-naphthalene. (b) Liver tissue from four coho salmon, 16 hr after feeding of 75 μCi ^{3}H-naphthalene.[7] u-unknown.

the Rf value of 3-hydroxy-2,6-dimethylnaphthalene was virtually identical to that of 1-naphthol. On this basis 1-naphthyl-β-glucuronic acid, 1-naphthyl mercapturic acid, 1-naphthyl sulfate and 1-naphthyl-α-glucoside served as markers for the corresponding metabolites of 2,6-dimethylnaphthalene in the identification of radioactive fractions on thin-layer plates (Figure 1 and Table I). Each of these types of derivatives was also found in the urine of rats exposed to 2,6-dimethylnaphthalene (Table I). Comparative studies on the metabolite profiles of urine of trout exposed to 2,6-dimethylnaphthalene are presently being conducted in our laboratories.

TABLE II

Metabolites in brain of rainbow trout 16hr after feeding of ^{3}H-naphthalene

Peak #	Compounds†	P moles/g dry wt	% admin. dose ($\times 10^{-4}$)
I		3.9	0.39
II	Dihydrodiol‡	5.6	0.56
III	α-naphthol‡	1.5	0.15
IV		1.7	0.17

†Based on retention times of standards.
‡Corrected for extraction efficiency.

In the HPLC of metabolites of aromatic hydrocarbons, both conjugated and nonconjugated derivatives were separated in a single elution of about 60 minutes. Nonradioactive model mixtures of both classes of metabolites were separated by HPLC and quantitated via spectrophotometry at 254 nm (Figure 3). Naphthalene and derivatives, such as the glucuronide, sulfate, 1,2-dihydrodiol, glucoside and 1-naphthol were completely separated by the high-pressure techniques. The liver of coho salmon exposed to high specific activity ^{3}H-naphthalene was found to contain eight radioactive compounds more polar than naphthalene (Figure 4). Of these, the glucuronide, sulfate, dihydrodiol, glucoside, and 1-naphthol were identified.

In another study, rainbow trout were exposed to ^{3}H-naphthalene, and brain tissue was analyzed for metabolites by HPLC (Figure 4). No evidence was found for conjugated derivatives; however, four derivatives were isolated and, of these, the 1-naphthol and dihydrodiol were identified. Data on the amounts of each isolated derivative obtained from the brain are given in Table II.

DISCUSSION

Initial steps in the bioconversion of aromatic hydrocarbons take place in the endoplasmic reticulum of the cell. The enzymes, aryl hydrocarbon monooxygenases, operate in unison with the electron transport system (i.e., via cytochrome P-450). Oxygen from the electron transport system combines with the aromatic nucleus to form an epoxide which is subsequently converted to compounds such as mono- and dihydric alcohols.[2] Additional conversions occur which increase the hydrophilic nature of the initial oxidation products. These secondary reactions yield conjugated derivatives, such as mercapturic acids, glucuronides, sulfates, and glycosides. Conjugating enzymes, such as glutathione-*S*-epoxide transferase, mediate these reactions.

It is now well established that most aquatic animals, with certain possible exceptions (e.g., mussels, *Mytilus edulis*), contain the necessary enzyme systems for initiating both the first and second stage conversions.[1,2] Moreover, recent findings suggest that a spectrum of aromatic metabolites comparable to those found in exposed terrestrial animals are formed.[10] Thus far, however, only a few studies have attempted to delineate the structures of the individual metabolites formed in aquatic organisms.[1]

It has been shown[11] that three species of fish converted naphthalene to a compound tentatively identified as a dihydrodiol. It was further observed[12] that naphthalene was converted in the spider crab (*Maia squinado*) to a number of conjugated and nonconjugated hydroxy derivatives. Similarly, zooplankton were found to convert naphthalene to a variety of hydroxylated metabolites.[13] More recently, TLC was used to specifically identify several conjugated and nonconjugated derivatives from coho salmon exposed to ^{14}C-naphthalene.[4]

In another study,[5] English sole (*Parophrys vetulus*), rock sole (*Lepidopsetta bilineata*), and starry flounder (*Platichthys stellatus*) were exposed to ^{3}H-naphthalene through the diet. These workers found, using solvent partition techniques, that more than 80% of the radioactivity in the blood of all three species was associated with metabolic products after 168 hr. Thus, it was concluded that naphthalene was extensively metabolized. Highest concentrations of metabolites occurred in the gallbladder; hence, an assessment of the individual metabolites in the bile was undertaken. After 24 hr, TLC indicated that the major metabolite was 1-naphthyl glucuronide; smaller amounts of 1-naphthol, 1,2-dihydro-1,2-dihydroxynaphthalene, 1-naphthyl sulfate, and 1-naphthyl glucoside were also detected. It was also observed that the relative proportions of individual metabolites changed with time throughout the experiment.

Recently, it was demonstrated for the first time that HPLC could be

used for the resolution of naphthalene metabolites in marine organisms, specifically, the liver and gallbladder of ^{3}H-naphthalene-dosed coho salmon.[7] These workers quantitatively identified the glucuronide, sulfate, dihydrodiol, glycoside, and 1-naphthol derivatives, together with three additional polar compounds whose structures were not determined.

The present findings show that a variety of metabolic products of naphthalene and 2,6-dimethylnaphthalene are retained in the tissues and biological fluids of exposed fish; however, significant differences in the proportions of individual structures were found, depending upon whether the sample was taken from the urine or from sites such as brain or liver. Analyses of nonconjugated derivatives in urine taken from rainbow trout over a period of 50 hr revealed essentially only 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol. The only conjugate identified was 1-naphthyl glucuronic acid, which represented 99% of the total urine metabolites. The total recovered radioactivity represented 0.33% of the administered dose, and only trace amounts of the parent naphthalene were detected. Each of the metabolites identified in trout urine was found to be a major component of the metabolite fractions from tissues of the species examined. Findings showing that both the 1-naphthyl glucoside and 1-naphthyl sulfate are present in the bile of salmonids implies that substantial differences exist in metabolite profiles, depending on the route of excretion (i.e., biliary or renal).

TLC has shown that more complex profiles of conjugated and nonconjugated derivatives are obtained from the liver of salmonids in comparison to urine. Moreover, eight polar compounds were isolated by HPLC in the hepatic tissues; however, HPLC revealed primarily nonconjugated derivatives in the brain of mature rainbow trout exposed to high specific activity ^{3}H-naphthalene. Because trout brain contains virtually no detectable aryl hydrocarbon monooxygenase activity,[14] it appears that the conjugated derivatives are largely excluded from entering the brain of mature animals by the brain barrier systems. However, the occurrence of conjugated derivatives in the brain of naphthalene-exposed salmonid fingerlings has been shown,[4] suggesting that the brain barrier systems are not fully developed in immature fish.

The work described gives some examples of the application of TLC and HPLC to the determination of conjugated and nonconjugated derivatives of aromatic hydrocarbons in fish tissues. Studies of this type with aquatic organisms are still in the exploratory stage and considerable work needs to be done in the future to increase the resolution of metabolites, standardize procedures, and improve quantitation techniques. Moreover, some commercially available radiolabeled hydrocarbons are impure and/or undergo radiolytic conversion during handling; thus, strong emphasis

must be placed on prior purification of all hydrocarbons used in animal studies.

Acknowledgements

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