International Council for the Exploration of the Sea

C.M. 1978/E : 42
Marine Environment Quality Committee

ACCUMULATION AND METABOLISM OF PHENANTHRENE BY SAITHE (POLLACHIUS VIRENS)

by

Karsten H. Palmork[1], Jan Erik Solbakken[2] and Tore Neppelberg[1]

1) Institute of Marine Research, Nordnesparken 2, 5011 Bergen-Nordnes.

2) Institute of Fisheries Biology, University of Bergen, Nordnesparken 2, 5011 Bergen-Nordnes.

ABSTRACT

Phenanthrene labeled with carbon-14 was administered to saithe. Pollachius virens, in dry food and the radioactivity in the different tissues were measured at intervals using a Packard Tri-Carb scintillation-counter. Carbon-14 was rapidly accumulated in the liver and thereafter high concentrations was found in the gall bladder. Two weeks later most of the carbon-14 had disappeared.

The metabolites of phenanthrene were isolated from the gall bladder and the uring 36 hours after the administration of 25 mg. The analyses of the TMS-derivatives of the metabolites were performed using a computerized gas chromatograph mass spectrometer. Quantitative studies were carried out using massfragmentography. A total of seven metabolites were isolated and identified. The main metabolite found was 1.2-dihydro-1.2-dihydroxyphenanthrene, which constituted 80 - 90% of the total metabolic products. In the gall bladder 90% of the metabolites were conjugated, in the urine 67%.

RESUMÉ

(Accumulation et métabolisme de phenanthréne chez le Lieu Noir, Pollachius virens)
Phenanthrene étiquete avec carbone-14 a été administré au Lieu Noir, Pollachius virens, dans la nourriture seche et la radioactivité dans les tissue différents ont ete mesurés par intervalls en employant un compteur á scintillation - Packard Tri-Carb. Carbone-14 a été rapidement accumulé dans le foie et ensuite hautes concentrations ont été trouvées dans la vésicule biliaire. Après deux semaines la plupart du carbone-14 est disparu.

Les métabolites de phenanthréne ont été isolées de la vésicule biliaire et de l'urine 36 heures après l'administration de 25 mg. Utilisant la chromatographie en phase gazeuse sur capillaire de verre et des analyses en spectrometre de masse, nous avons analysé les TMS-dérivatives des metabolites. Des études quantitatives ont été accomplies par fragmentographie de masse. Une somme totale de sept métabolites a été isolée et identifiée. Le metabolite principal trouvé, a été 1.2-dihydro-1.2-dihydroxyphenanthrene, qui constituaient 80 - 90% de tous les produits métaboliques. Dans la vésicule biliaire, 90% des métabolites ont été conjugués, dans l'urine 67%.

INTRODUCTION

The polycyclic aromatic hydrocarbons (PAH's) a class of compounds to which phenanthrene belongs, have for some time been studied with considerable interest because of their possible carcinogenic effects. The main sources for PAH have been referred to as beeing petroleum refineries, petrochemical industries, and the burning of fossile fuels (coal and oil). In addition comes the sources aluminium smelters, ferro silicium-, iron and coke-works (Palmork, Wilhelmsen and Neppelberg, 1973). The increasing oil exploitation in the North Sea and the increasing use of oil within the riperian countries (with a population of approx. 250 mill.) results in the risk of more and more oil finding its way to the sea. Therefore it has been to an ever-increasing extent concern that oil should interfere with coastal and open water fishing. The mere fact that concern arises may harm the fisheries, for instance in leading to difficulties in marketing the catch (Anon, 1977).

The analyses of oil hydrocarbons in biota is a necessity but not sufficient in itself. In addition we ought to know more about the fate of the oil hydrocarbons, especially the polycyclic aromatic hydrocarbons. The study of metabolites in fish and marine organisms has only just begun and Varanasi and Malins (1977) has excellently reviewed the work done so far.

We have chosen the commercial important fish, saithe a lean fish, for our study of the fate of the polycyclic hydrocarbon, phenanthrene.

MATERIAL AND METHODS

Saithe of mixed sexes with an approx. mean weight of 150 grams and 330 grams were used for the ^{14}C-experiments and for metabolic studies respectively. The fish were kept in 260 liter tanks holding 5 fish each for the ^{14}C-experiment and 10 fish for the metabolic studies. The water ($7^{\circ}C$) was supplied from 120 m depth at a rate of 7 liters per minute. The fish were acclimatized for several weeks and starved for 3 days prior to the experiments.

^{14}C-phenanthrene (11.3 mCi/m mol) supplied from Amersham, England was dissolved in 5 ml of CS_2. Aliquotes of 20 µl, 15.8 µg, ^{14}C - phenanthrene were added to transparent gelatine capsule no. 2 (Park, Davies and Company, England) containing Tess Salmon food (dry pellets) no. 3 (from Skretting A/S, Norway).

To ensure rapid uptake of the content, approx. 1/4 of the capsule was removed (Fig. 1). The introduction of the capsules to the stomach was achieved using a modified plast syringe (Fig. 2). The fish were doped before the injection using 3 ml of saturated benzocain in ethanol per 10 l of sea water.

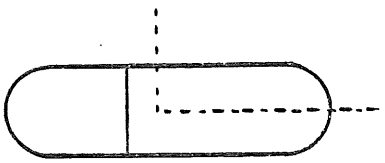


Fig. 1. Transparent gelatine capsule. The dotted line indicates the part which has been removed.

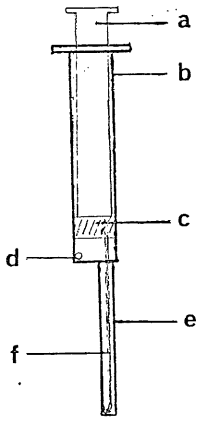


Fig. 2. Injection syringe for introduction of food capsules. a - piston, b - barrel, c - rubber end of piston d - hole for the removal of compressed air, e - plast tubing, f - electroplated string.

The activity was measured in liver, gall bladder and muscle tissue from 3 - 5 fish at different intervals after the administration of ^{14}C-phenanthrene. The tissue (100 mg) was dissolved in 1 ml of Soluene-350 (Packard) and counted after the addition of 10 ml scintillation cocktail, (Dimilume-30 Packard) on a Packard Tri-Carb scintillation counter (Model 2002, Packard Instrument Co. Inc. U.S.A.). ^{14}C-toluene (4.0×10^5 dpm/ml, New England Nuclear), was used as an internal standard.

For the metabolic studies 25 mg of phenanthrene (BDH, 29468 Chemicals, Ltd, England) was given according to the procedure described above. The urine and gall bladder were sampled 36 hours after the administration of phenanthrene and the samples from 10 fish were combined and diluted to 20 ml with distilled water.

The first half of the sample (10 ml), treated with Glusulase (sulphatase 35.126, glucuronidase 176.578, Endo Laboratories, Inc., U.S.A.) to liberate the conjugated metabolites, was used for the qualitative studies of the free fraction of the metabolites.

The quantitative measurements of the free metabolites were based on the untreated sample and the percentage of conjugated metabolites calculated.

The respective samples were extracted with 5 x 25 ml portions of ethyl acetate and the combined organic phase was subsequently dried with anhydrated sodium sulphate, decanted and evaporated down to 2 ml, and further evaporated to dryness in a small vial using a stream of dry nitrogen. (Strand and Scheline, 1975).

To finalize the isolated metabolites for analyses on GC/MS, 1 ml of Sylon HTP (Supelco, Inc.) was added to the dry sample in the vial in order to form the trimethylsilyl derivatives. α-Naphthol (Merck) was used as an internal standard.

The identification and quantification of the derivatized metabolites were performed on a gas chromatograph coupled to a mass spectrometer fully computerized (Finnigan GC/MS/DS Model 9000/3200 F/ 6100)

Conditions:

Column:	20 m x 0.33 mm ID capillary glas column SE-54 (H + G. Jaeggi, Trogen, Switzerland)
Inj. temp.:	260°C
Columntemp.:	100 - 250°C
Progr.:	6°C/min.
Carriergas:	Approx. 1.5 ml He/min.

For the identification of the isolated metabolites total ion chromatogram of the sample and mass spectra of the different metabolites were produced and compared with mass spectra of the synthesized metabolites.

One of the synthesized metabolites, 9,10-dihydro-9,10-dihydroxyphenanthrene, was a gift from Professor P. Sims, Chester Beatty, Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London S. W. 3, the others were synthesized in the laboratory: 1-Hydroxyphenanthrene (Langenbeck and Weissenborn. 1939. Bachmann and Boatner, 1936); 2.-and 3.-Hydroxyphenanthrene (Fieser, 1929); 9-Hydroxyphenanthrene (Moriconi, Wallenberger and O, Connor, 1959).

Sims (1970) showed that 9,10-dihydroxy-9,10-dihydroxyphenanthrene and 1.2-dihydro-1.2-dihydroxyphenanthrene were the main metabolites in urine from rats. Therefore 80 mg of phenanthrene, dissolved in ethyl propyl glycol, was given orally to male albino rat and the urine collected over a periode of 36 hours to isolate these metabolites. Total ion chromatograms and mass spectra of the TMS-derivatives of the urinary metabolites from the rat were used to confirm the identity of the two main metabolites in the urine and gall bladder from saithe.

The quantification of the different isolated metabolites from saithe was carried out using selected ion monitoring, (massfragmentography) and the internal standard α-naphthol.

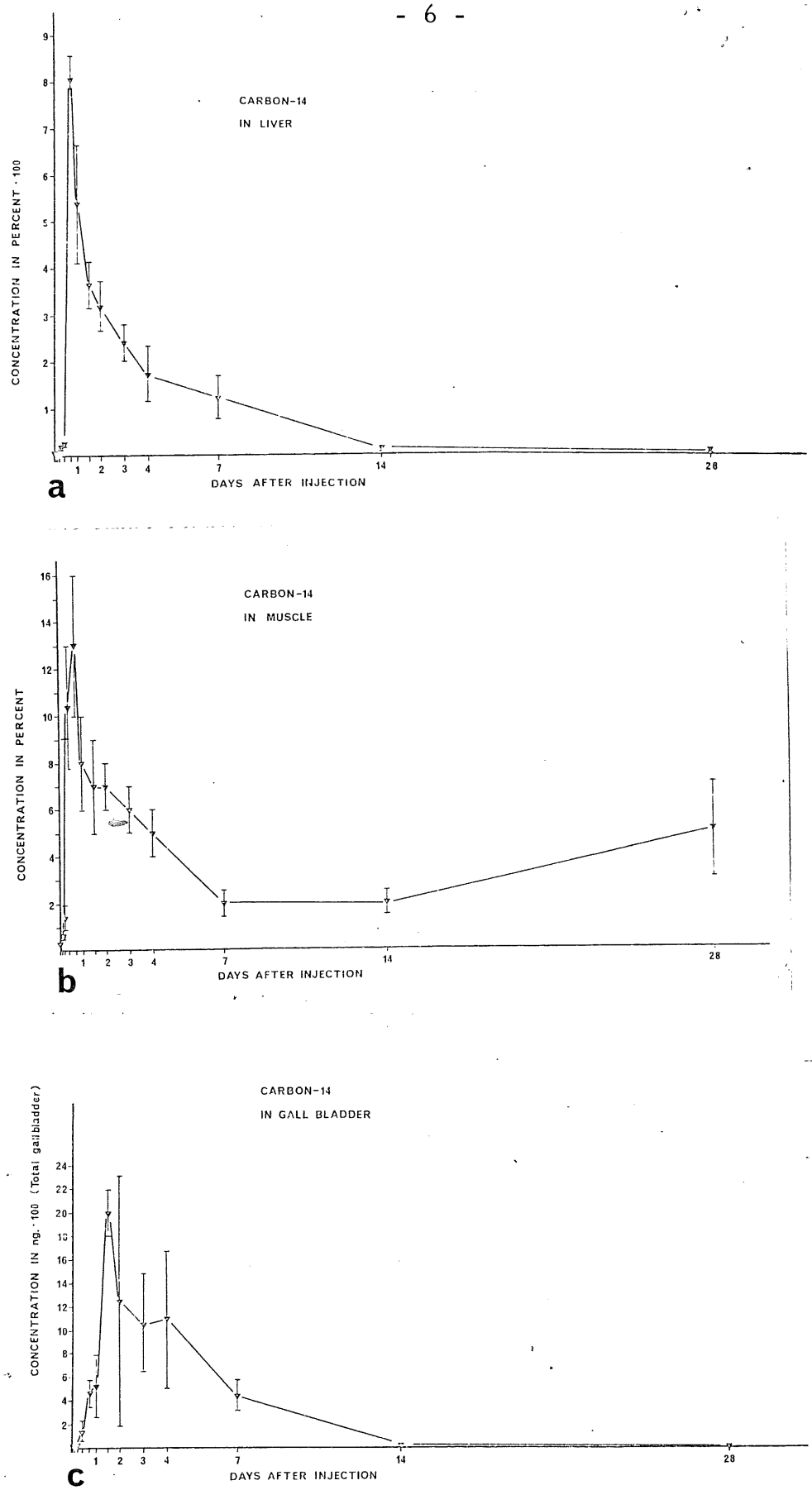


Fig. 3. Carbon-14 in tissue at different intervals after the introduction of ^{14}C-phenanthrene. For liver (a) and muscle (b) the concentration is expressed as percent of Carbon-14 in tissue/Carbon-14 given dose Gall bladder (c) is expressed as ng ^{14}C per gall bladder. Standard error is given for each value.

RESULTS AND DISCUSSION

Accumulation

The levels of Carbon-14 in liver, muscle and gall bladder from the termination of the feeding between 30 minutes and 28 days are shown in Fig. 3a - c. The uptake and discharge of Carbon-14 are expressed in percent (^{14}C in tissue/^{14}C-given dose). Since the amount of biliary fluid in the gall bladder depends on many factors; as for instance the elapse of time after food uptake, the level of Carbon-14 in gall bladder is expressed as ng ^{14}C per gall bladder. Standard error is calculated according to Elliott (1971).

Shortly after the introduction of the ^{14}C-phenanthrene traces of radioactivity were found in the liver (Fig. 3a), a few hours later an increase in the radioactivity was observed in the gall bladder (Fig. 3c). The Carbon-14 level in muscle tissue was much lower than in the liver and the gall bladder. The findings from the Carbon-14 experiments using saithe as lean fish, are in good agreement with results reported using other labeled polycyclic aromatic hydrocarbons (Lee et al., 1972, Roubal et al., 1977).

The measurements of Carbon-14 in accumulation and depuration studies using ^{14}C-labeled polycyclic aromatic hydrocarbons give us the radioactivity represented by the parent compound and the total formed metabolites containing Carbon-14 (Fig. 4).

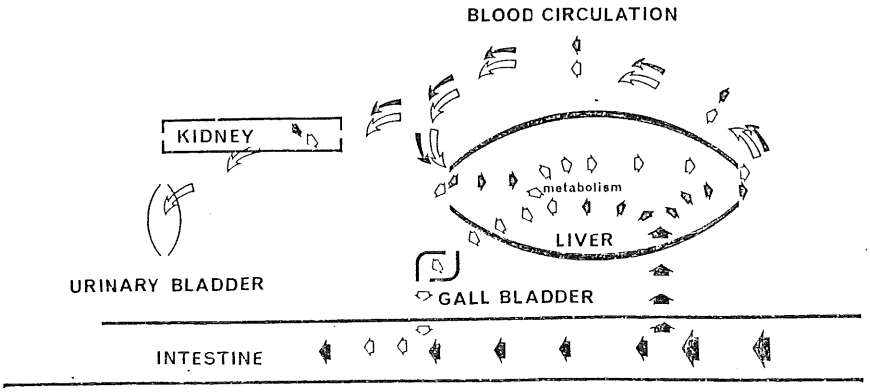


Fig. 4. Pathway for metabolism of phenanthrene
Black arrows: phenanthrene, white arrows: metabolites.

Metabolism.

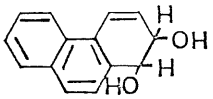
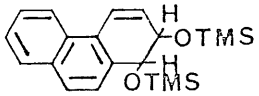
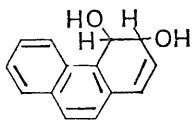
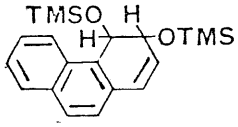
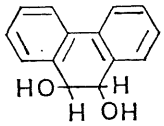
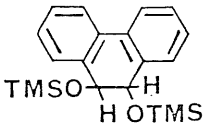
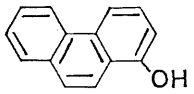
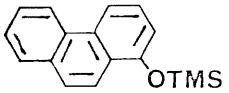
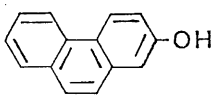
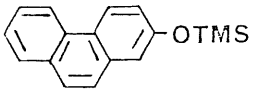
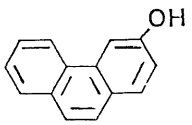
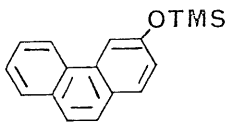
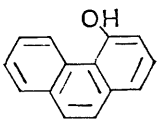
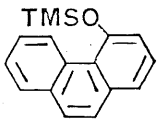
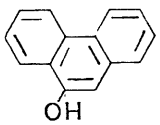
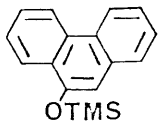
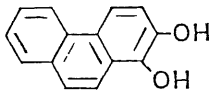
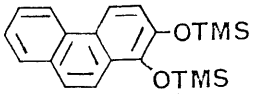
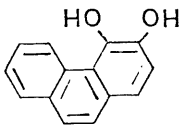
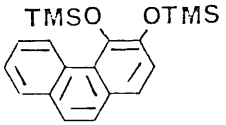
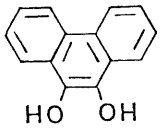
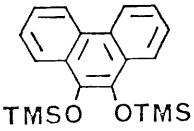
Table I shows the theoretical hydroxylated phenanthrene metabolites, their TMS-derivatives and the seven metabolites isolated and identified. Selected ion monitoring using M/e 354 indicates the presence of two more hydroxylated metabolites at an extremely low concentration. 3.4-Dihydro-3.4-dihydroxyphenanthrene found in rats by Boyland and Sims (1962), could not be detected in saithe, nor in rat.

The total ion chromatogram of the TMS-derivatized extract (Fig. 5) indicates the position and the quantity of the metabolites. The peak no. 7 in Fig. 5b, 1.2-dihydro-1.2-dihydroxyphenanthrene is the main metabolite in saithe. In rat the main metabolites are the peaks nos. 1 and 7 in Fig. 5b and 5c. Sims (1970) described the two main metabolites in rat and rat liver preparations to be the 9.10-dihydro-9.10-dihydroxyphenanthrene and 1.2-dihydro-1.2-dihydroxyphenanthrene. The confirmation of the metabolite, peak no. 1, was achieved by comparing its mass spectra with that of the synthesized compound (a gift from prof. Sims). The five monohydroxyphenanthrene metabolites, 1, 2, 3, 4 and 9, were identified by comparing their mass spectra with those of the respective synthesized metabolites.

The quantification of the metabolites were carried out applying selected ion monitoring of the ion M/e 266. This is the molecular ion for the TMS-derivatized monohydroxyphenanthrenes and also one of the fragments for the other TMS-derivatized metabolites. An example of selected ion monitoring is shown in Fig. 6. The peak area of the respective M/e 266 peaks nos. 1 to 7 is compared with the area of the internal standard, α-naphthol and the concentrations calculated.

Table II gives the values in µg/gram of the metabolites of phenanthrene from the gall bladder and urine. The main metabolite 1.2-dihydro-1.2-dihydroxyphenanthrene constituted 82% of the metabolites from urine and 90% from the gall bladder. It was expected that a greater part of the free metabolites should be found in the urine. Calculated from the values in Table II, which is based on samples taken 36 hours after the administration of phenanthrene, the free metabolites constitutes 33% and 10% of the urine and gall bladder metabolites respectively.

Table 1. The theoretical hydroxylated metabolites of phenanthrene and their respective TMS-derivatives. M/e-molecular ion. The identified metabolites are indicated by +

Name of metabolite	Chemical structure	TMS-derivatized metabolite	M/e	Identified metabolite
1.2-dihydro-1.2-dihydroxy phenanthrene			356	+
3.4-dihydro-3.4-dihydroxy phenanthrene			356	
9.10-dihydro-9.10-dihydroxy phenanthrene			356	+
1.-mono-hydroxy-phenanthrene			266	+
2.-mono-hydroxy-phenanthrene			266	+
3.-mono-hydroxy-phenanthrene			266	+
4.-mono-hydroxy-phenanthrene			266	+
9.-mono-hydroxy-phenanthrene			266	+
1.2-dihydroxy phenanthrene			354	
3.4-dihydroxy phenanthrene			354	
9.10-dihydroxy phenanthrene			354	

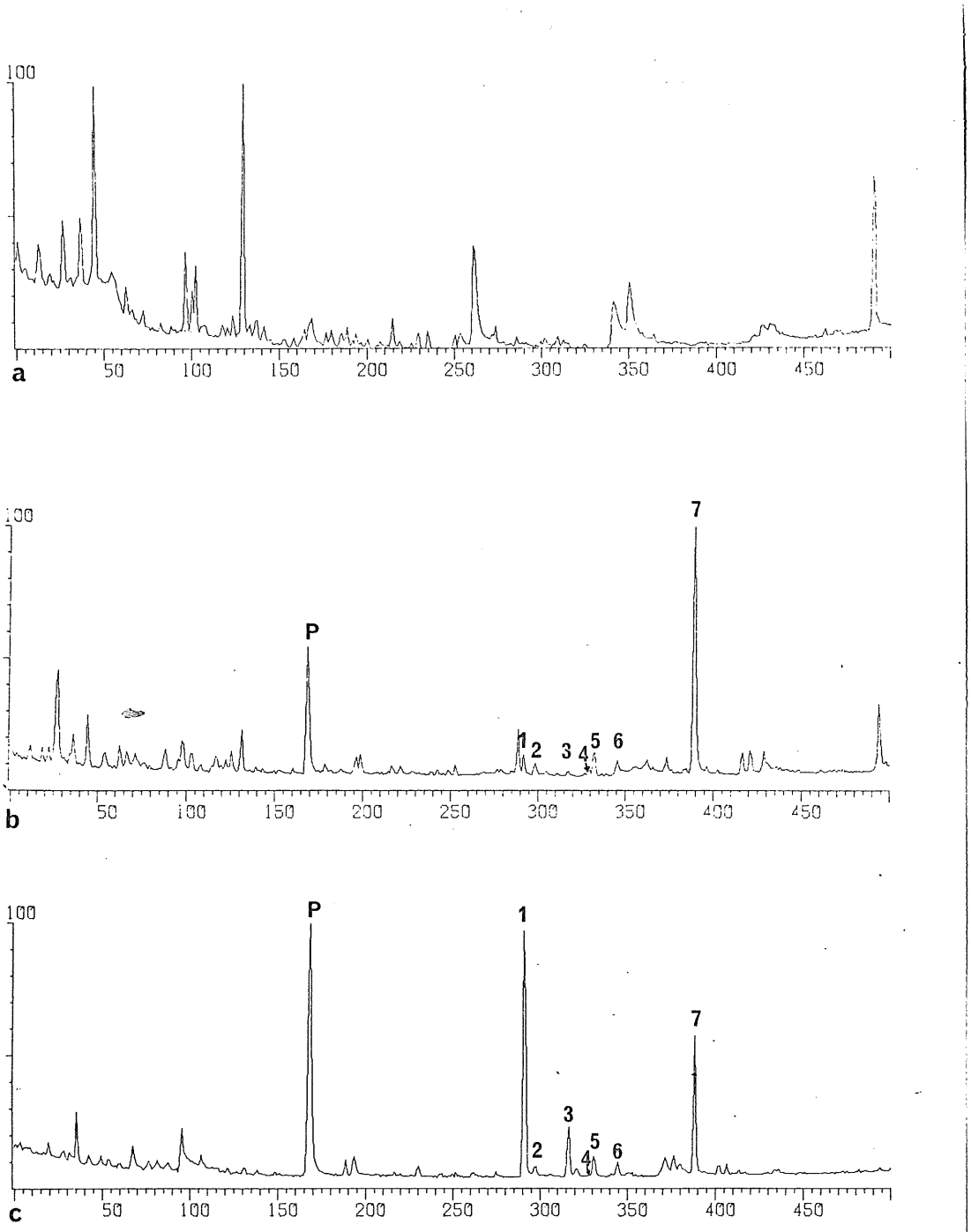


Fig. 5. Total ion chromatogram (TIC) of the TMS-derivatized extracts of urine. a) Blank, urine from untreated saithe. b) urine from 10 saithe, each given 25 mg of phenanthrene. c) urine from a rat given 80 mg of phenanthrene. P phenanthrene, nos. 1 to 7 see legend to Fig. 6.

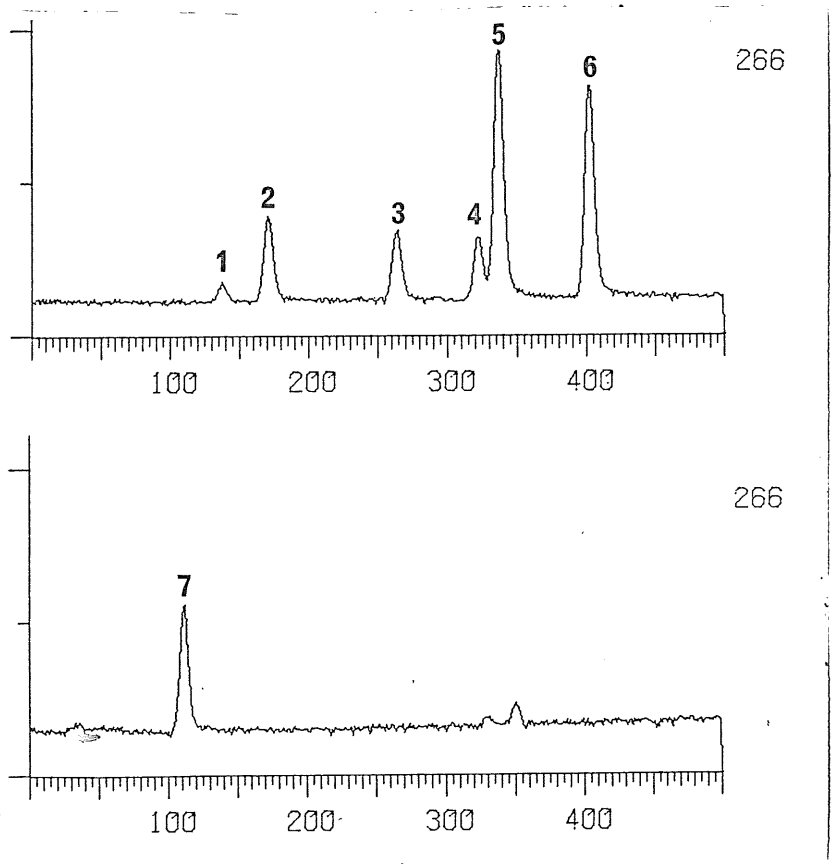


Fig 6. Selected ion monitoring, SIM, (massfragmentogram) of the molecular ion M/e 266:

1 = 9.10 dihydro-9.10-dihydroxyphenanthrene

2 = 4-monohydroxyphenanthrene

3 = 9- - " -

4 = 3- - " -

5 = 1- - " -

6 = 2- - " -

7 = 1.2-dihydro-1.2-dihydroxyphenanthrene

Table II. Metabolites in Glusulase treated and untreated samples from gall bladder and urine in µg/gram.

Name of metabolite	In gall bladder				In urine			
	Glusulase treated		untreated		Glusulase treated		untreated	
1.2-dihydro-1.2-dihydroxy phenanthrene	121.9	120.9	10.9	10.85	9.6	9.7	3.2	3.0
	120.0		10.8		9.8		2.8	
9.10-dihydro-9.10-dihydroxy phenanthrene	1.7	1.6	0.1	0.25	0.2	0.2	0.1	0.1
	1.5		0.4		0.2		0.1	
1.-mono-hydroxy-phenanthrene	1.5	1.5	0.4	0.35	0.2	0.2	0.1	0.1
	1.5		0.3		0.2		0.1	
2.-mono-hydroxy-phenanthrene	0.4	0.4	0.4	0.3	0.04	0.045	0.1	0.07
	0.4		0.2		0.05		0.04	
3.-mono-hydroxy-phenanthrene	0.8	0.7	0.3	0.25	0.1	0.1	0.03	0.025
	0.6		0.2		0.1		0.02	
4.-mono-hydroxy-phenanthrene	0.3	0.3	0.2	0.11	0.1	0.1	0.04	0.04
	0.3		0.02		0.1		0.04	
9.-mono-hydroxy-phenanthrene	0.2	0.25	0.1	0.1	0.1	0.1	0.1	0.1
	0.3		0.1		0.1		0.1	

Acknowledgements.

The authors want to thank Professor Ronald R. Scheline, Dept. of Pharmacology, School of Medicine, University of Bergen, for his suggestions for the isolation of metabolites, for the performing of the rat experiment and for contacting Professor Dr. Sims.

We also want to thank The Aquarium in Bergen for supplying us with saithe for our experiments.

REFERENCES

ANON, 1977. IMCO/FAO/UNESCO/WMO/WHO/IAEA/UN Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP), Impact of Oil on the Marine Environment. Rep. Stud. GESAMP, (6) : 250 p.

BACHMANN, W.E. and C.H. BOATNER, 1936. Phenanthrene derivatives. VI. The Preparation of 1-, 2- and 3-Phenanthryl Halides. J. Am. chem. Soc., 58 : 2194-2195.

BOYLAND, E. and P. SIMS, 1962. Metabolism of Polycyclic Compounds. Biochem. J., 84 : 564-571.

ELLIOTT, J.M. 1971. Some Methods for the Statistical Analysis of Samples of Bentic Invertebrates. Freshwater Biol. Ass. Scientific Publication No. 25.

FIESER, L.F. 1929. The Sulfonation of Phenanthrene. I. A New Monosulfonate. J. Am. chem. Soc., 51 : 2460-2486.

LANGENBECK, W. and K. WEISSENBORN, 1939. Über Organische Katalysatoren, XX. Mitteil. Ber. dt. chem. Ges., 72 : 724-727.

LEE, R.F., SAUERHEBER, R. and G.H. DOBBS, 1972. Uptake, Metabolism and Discharge of Polycyclic Aromatic Hydrocarbons by Marine Fish. Mar. Biol. 17 : 201-208

MORICONI, E.J., WALLENBERGER, F.T. and W.F. O'CONNOR, 1958. A New Synthesis of 9-Phenanthrol; Absorption Spectra of the Quinhydrone-Type Molecular Compound between 9-Phenanthrol and Phenanthrenequinone. J. org.Chem., 24 : 86-90.

PALMORK, K.H., WILHELMSEN, S. and T. NEPPELBERG, 1973. Report on the Contribution of Polycyclic Aromatic Hydrocarbons (PAH) to the Marine Environment from different Industries. Coun. Meet. int. Coun. Explor. Sea., 1973 (E:33) : 1-21.

ROUBAL, W.T., COLLIER, T.K. and D.C. MALINS, 1977. Accumulation and Metabolism of Carbon-14 labeled Benzen, Naphthalene and Anthracene by Young Coho Salmon (Oncorhynchus kisutch). Arch. Environ. Contam. and Toxicol., 5 : 513-529.

SIMS, P. 1970. Quantitative and Quantitative Studies on the Metabolism of a Series of Aromatic Hydrocarbons by Rat-liver Preparations. Biochem. Pharmac. 19 : 795-818.

STRAND, L.P. and R.R. SCHELINE, 1975. The Metabolism of Vanillin and Isovanillin in the Rat. Xenobiotica 5 : 49-63.

VARANASI, U. and D.C. MALINS. 1977. Metabolism of Petroleum Hydrocarbons: Accumulation and Biotransformation in Marine Organisms. P. 175-270 in MALINS, D.C. ed. Effects of Petroleum on Arctic and Subartic Marine Environments and Organisms. Vol. II. Biological Effects. Academic Press, Inc. New York San Francisco London 1977.