METABOLISM OF POLYCYCLIC HYDROCARBONS BY A HIGHLY ACTIVE ARYL HYDROCARBON HYDROXYLASE SYSTEM IN THE LIVER OF A TROUT SPECIES

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SUMMARY. In sharp contrast to earlier beliefs, some fish (trout) liver-microsomes are highly capable of metabolizing benzo(α)pyrene. The hydroxylating system is a typical monooxygenase system in many respects when compared with the mammalian system, needing oxygen and NADPH for full activity. The trout liver-microsomes convert benzo(α)pyrene to dihydrodiols and hydroxymetabolites at a rate 5 to 10 times higher than the male rat liver-microsomes, when measured per mg of microsomal protein. The trout liver-microsomes metabolize benzo(α)pyrene 15 to 30 times as fast as the male rat liver-microsomes if the activity is measured per unit of cytochrome P-450 and NADPH-cytochrome c reductase.

INTRODUCTION. On theoretical and experimental grounds it has been suggested that the activity of the so called drug-metabolizing enzymes change in a systematic way as one goes through the phylogenetic scale (1). More specifically, it has been proposed that aquatic organisms are less well equiped with drug-metabolizing enzymes than the terrestrial animals. Fish are said to excrete lipid-soluble compounds unchanged through their gills, but the higher animals convert lipid-soluble compounds - which they cannot excrete through their kidneys - to water-soluble metabolites before excretion. However, studies conducted in the recent years show that there is a drug-oxidizing monooxygenase system in fish liver-microsomes and it is cytochrome P-450 linked (2,3). Also recently Pedersen et al. in a preliminary report (4) describe the existence of 3,4-benzpyrene hydroxylase in the trout Salmo gairdneri. The relatively high levels of monooxygenase system components in trout liver stand in marked contrast to the

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above, widely accepted hypothesis of the phylogenetic development of the microsomal drug-oxidizing enzyme systems (5). In this paper we wish to report a rather surprising finding on the capacity of the trout liver-microsomes to convert a well known carcinogen, benzo(α)pyrene to a typical pattern of oxidized metabolites, including 3-hydroxybenzo(α)pyrene, several dihydrodiol derivatives and the 3,6-quinone of benzo(α)pyrene.

MATERIALS AND METHODS. The fish used in our experiments were hatchery reared Salmo trutta lacustris, a local lake trout (Oulujoki Pty Ltd, Montta, Finland). The trout were sexually immature about 1.5 years old and they were kept at least one week in our aquarium at 4-6 ^{o}C before using. The water of the aquarium was exchanged once every two days. Both, in the hatchery and in our aquarium Ewos Salmon Grower F 159 (Astra-Ewos AB, Södertälje, Sweden) fodder was used for feeding the fish at the rate of 10 gm per kilogram of fish per day. The rats used in the experiments were adult (about 250-300 gm) male Sprague-Dawley strain rats on a commercial pellet diet (Hankkija Co-op, Finland).

The livers were removed immediately after killing the animals by decapitation and homogenized in four volumes of 0.1 M K/Na phosphate buffer, pH 7.4, with a teflon glass Potter-Elvehjem homogenizer. The whole procedure was carried out in a cold room (0-4 ^{o}C). The homogenates were centrifuged at 1000 x g for 5 minutes, the supernatant obtained was centrifuged at 10 000 x g for 20 minutes. The 10 000 x g supernatant was centrifuged at 100 000 x g for 60 minutes and the pellet obtained (microsomes) was suspended in 0.1 M K/Na phosphate buffer so that one ml contained microsomes from one gram of liver. About 20 mg protein per gm of liver was recovered in the microsomal fraction.

The aryl hydrocarbon hydroxylase activity determinations were carried out according to the method of Nebert and Gelboin (6). The metabolites produced in the in vitro incubations were detected and quantitated according to the methods of Sims (7) and Borgen et al. (8). 100 nanomoles of tritiated benzo(α)pyrene (about 800 000 cpm; generally labelled from The Radiochemical Center, Ammersham) was incubated in the presence of a cofactor mixture (KCl, 50 mM; $MgCl_2$, 2.5 mM; Glucose-6-phosphate, 1.5 mM; NADP, 0.0625 mM; Glucose-6-phosphate dehydrogenase, 5 units) and fish liver-microsomes (0.25 ml corresponding to about 4 mg pro-

tein) in a total volume of 4 ml. All incubations with the rat liver-microsomes were carried out at 37°C, whereas with the trout liver-microsomes the incubations were carried out at 30°C (optimum temperature for trout is 29°C). After 15 min incubation the mixture was extracted twice with 6 ml of ethyl acetate, the extracts were combined and dried over Na_2SO_4. The ethyl acetate was evaporated under vacuum and the residue was dissolved in a small volume of ethyl acetate after which it was streaked onto a precoated Merck Sil G plate (thickness 0.25 mm) and chromatographed in benzene:ethanol (19:1, v/v). The plate was inspected under UV light for fluorescent bands which were marked, cut off and put into vials for liquid scintillation counting. As a reference standard we had 3-hydroxybenzo(α)pyrene (donated by A. McClean, London); with respect to other metabolites we had to refer to data given by Sims (7) and Borgen et al. (8).

RESULTS AND DISCUSSION. We have found that the benzo(α)pyrene metabolizing enzyme in the trout liver is a typical monooxygenase known to metabolize xenobiotics in the mammalian system. The quantity of cytochrome P-450 and NADPH-cytochrome c reductase is relatively high in trout liver, although lower than in the rat (table 1). On the other hand V_{max} is higher than for the rat and the K_m lower, when benzo(α)pyrene is used as a substrate (table 2). For full activity the hydroxylase requires oxygen and NADPH, the lack of which result in an almost total absence of activity. The system is activated by the soluble post-microsomal fraction of the liver and a small amount of albumin. The trout system was inhibited by SKF 525A and aminopyrine to a much lesser extent than

Table 1. Hepatic levels of cytochrome P-450 and NADPH-cytochrome c reductase activity in trout and rat

	Trout	Rat	Ratio Trout/Rat
Cytochrome P-450 (nmol/mg microsomal protein)	0.22	0.48	0.46
NADPH-cyt. c reductase (nmol cyt. c reduced/mg microsomal protein/min)	32	112	0.28

Cytochrome P-450 and NADPH-cyt. c reductase determinations were carried out in duplicate from three (for trout pooled from six fish) samples, by the methods of Omura and Sato (19) and Masters et al. (21) respectively.

Table 2. Liver-microsomal benzo(α)pyrene hydroxylase activity and its inhibition by selected inhibitors, in trout and rat

	Trout	Rat	Ratio Trout/Rat
Benzo(α)pyrene hydroxylase activity			
V_{max} (nmol/mg microsomal prot./min)	0.140	0.027	5.1
K_m (moles/l)	0.8×10^{-5}	2.0×10^{-5}	
% Activity remaining after addition of inhibitors			
- SKF 525A (200 μM)	40	17	
- aminopyrine (10 mM)	92	43	
- 7,8-benzoflavone (0.1 mM)	12	136	

Benzo(α)pyrene hydroxylase activity was measured fluorometrically (6) in duplicate from two samples. K_m was determined from a double reciprocal plot.

Table 3. Benzo(α)pyrene metabolites produced by trout and rat liver-microsomes

	Trout	Rat	Ratio Trout/Rat
Benzo(α)pyrene metabolites produced nanomoles in 15 min per			
mg of microsomal protein			
- dihydrodiols	2.12	0.25	8.1
- hydroxy	2.04	0.38	5.8
- quinone	0.44	0.35	1.4
nmole of cytochrome P-450			
- dihydrodiols	6.77	0.37	18.5
- hydroxy	6.61	0.50	13.1
- quinone	1.45	0.47	3.1
unit of cyt. c reductase			
- dihydrodiols	0.066	0.0023	28.7
- hydroxy	0.065	0.0032	20.3
- quinone	0.015	0.0030	5.0

Metabolites were separated on TLC and the fractions were assayed radiometrically.

rat, whereas with 7,8-benzoflavone the situation is the reverse (table 2). It has been shown with the mammalian system that SKF 525A and aminopyrine preferentially inhibit the phenobarbitone inducible cytochrome P-450, on the other hand 7,8-benzoflavone has strong inhibitory effect on the cytochrome P-448 catalysed system (9). However the Soret maximum of the reduced trout cytochrome complexed with carbon monoxide is approximately 450 nm.

The metabolite pattern of benzo(α)pyrene produced by trout liver-microsomes resembles that produced by rat liver-micro-

somes, but the amounts of metabolites are much higher (table 3). With fish liver-microsomes at least five strongly fluorescent bands could be seen which also corresponded to peaks of radioactivity. One of them was 3-hydroxybenzo(α)pyrene (co-chromatographed with authentic standard (R_f 0.50) and having typical excitation and emission wavelength characteristics), one presumably was benzo(α)pyrene-3,6-quinone (R_f 0.70, red fluorescence) and three were tentatively identified as dihydrodiol-metabolites (UV spectra; R_f values from 0.08 to 0.16; intense violet and blue-green fluorescence). The quantitative results are presented in table 3. When we relate the results to the microsomal protein content, the trout liver-microsomes produced about 5 to 10 times more dihydrodiols and hydroxymetabolite than the rat liver-microsomes. Furthermore when the metabolites produced were calculated per unit concentration of cytochrome P-450 or NADPH-cytochrome c reductase, trout liver produced about 15 to 30 times more dihydrodiols and hydroxymetabolites than rat liver.

Several recent findings point to the possible importance of this finding of very high aryl hydrocarbon hydroxylase activity in the trout. Polycyclic aromatic hydrocarbons are suspected to be an important factor in carcinogenesis (10). A specific mechanism for the initiation of cancer by polycyclic hydrocarbons was first proposed by Boyland (11), who suggested that during the metabolism of polycyclic hydrocarbons intermediary epoxides are formed which may combine covalently with cellular macromolecules. This hypothesis has recently been receiving considerable experimental support from the work of several investgators (12,13). It is also well established that the epoxidation of bromobenzene and other halogenated hydrocarbons may lead to considerable cellular injury (14). A common denominator in these events is a cytochrome P-450-linked monooxygenase system which catalyzes the epoxidation of aromatic and olefinic compounds (15). We have now shown that the prerequisite for the activation of polycyclic hydrocarbons and other xenobiotics, a cytochrome P-450-linked monooxygenase system, is present in trout liver-microsomes and is very efficient in converting benzo(α)pyrene to oxidized metabolites, probably via an epoxide intermediate.

From these results some prominent questions emerge. What is the origin of this high aryl hydrocarbon hydroxylase activity in the trout? Is it endogenous or induced? How does this aryl hydro-

carbon hydroxylase activity change in the environment of man-made pollutants? A very interesting question is whether this high aryl hydrocarbon hydroxylase activity plays an important role in the increased susceptibility of trout to aflatoxin-induced hepatocarcinogenesis. It has been shown that incubation of aflatoxin b_1 with microsomes from rat or trout liver produce metabolites mutagenic to certain bacteria (16,17). Our preliminary results: apparent lack of type I spectral interactions with the usual type I ligands (20), substrate specificity and studies with inhibitors (table 2) indicate that the trout liver monooxygenase system resembles the rodent liver P-448-linked monooxygenase system more than the P-450-linked system. Mannering (18) has suggested that the cytochrome P-448 may be an aberrant cytochrome present after exposure to polycyclic aromatic hydrocarbons. This leads to a very interesting and intriguing question - how can this view be reconciled with our present finding of the highly active benzo-(α)pyrene hydroxylase in livers of trout without an apparent exposure to polycyclic aryl hydrocarbons?

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REFERENCES

1. Brodie, B.B. and Maickel, R.P. (1962) Proc. 1st Int. Pharmac. Meet. 6, 299-324.
2. Dewaide, J.H. (1971) in Metabolism of Xenobiotics, Nijmegen.
3. Chan, T.M., Gillet, J.W. and Terriere, L.C. (1967) Comp. Biochem. Physiol. 20, 731-742.
4. Pedersen, M.G., Hershberger, W.K. and Juchau, M.R. (1974) Bull. Environ. Contam. Toxicol. 12, 481-486.
5. Ahokas, J.T., Pelkonen, O. and Kärki, N.T. (1974) Acta Pharmacol. Toxicol. 35 (supplement),14 (abstract).
6. Nebert, D.W. and Gelboin, H.V. (1968) J. Biol. Chem. 243, 6242-6249.
7. Sims, P. (1970) Biochem. Pharmac. 19, 795-818.
8. Borgen, A., Darvey, H., Costagnoli, N., Crocker, T.T., Rasmussen, R.E. and Wang, I.Y. (1973) J. Medicinal Chem. 16, 502-506.
9. Lu, A.Y.H. and West, S.B. (1972) Mol. Pharmacol. 8, 490-500.
10. Heidelberger, C. (1973) Fed. Proc.32, 2154-2161.
11. Boyland, E. (1950) Biochem. Soc. Symp. 5, 40.
12. Conney, A.H. (1973) New Engl. J. Med. 289, 971-973.
13. Jerina, D.M. (1974) Lloydia-J. Nat. Proc. 37, 212-218.
14. Gillette, J.R., Mitchell, J.R. and Brodie, B.B. (1974) Ann. Rev. Pharmacol. 14, 271-288.
15. Jerina, D.M. and Daly, J.W. (1974) Science 185, 573-582.

16. Garner, R.C., Miller, E.C. and Miller, J.A. (1972) Canc. Res. 32, 2058-2066.
17. Garner, R.C. and Wright, C.M. (1973) Br. J. Canc. 28, 544-551.
18. Mannering, G.J. (1971) Metabolism 20, 228-245.
19. Omura, H.T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-78.
20. Ahokas, J.T., Pelkonen, O. and Kärki, N.T. (1974) Second Congress of the Hungarian Pharmacological Soc. in press.
21. Masters, B.S.S., Williams, C.H. and Kamin, H. (1967) Methods in Enzymology, 10, p. 565, Estabrook, R.W. and Pullman, M.E. (eds.), Academic Press, New York.