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CHARACTERIZATION OF SOME MONOOXYGENASE ACTIVITIES AND SOLUBILIZATION OF HEPATIC CYTOCHROME P-450 IN TWO SPECIES OF FRESHWATER FISH, THE NASE (*CHONDROSTOMA NASUS*) AND THE ROACH (*RUTILUS RUTILUS*)*

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Abstract—1. Hepatic monooxygenase activities were studied in microsomal fractions from two species of freshwater fish, the nase (*Chondrostoma nasus*) and the European roach (*Rutilus rutilus*).
2. These activities were determined by using four substrates, 7-ethoxycoumarin, 7-ethoxyresorufin, benzo(a)pyrene, and 2,5-diphenyloxazole and were characterized according to pH, temperature, cofactors, and the differential effects of two inhibitors, metyrapone and α-naphthoflavone.
3. Solubilization of microsomes was achieved by the use of detergents, with a good recovery of the cytochrome P-450.

INTRODUCTION

Hepatic cytochrome P-450-dependent monooxygenases (MO) are involved in the metabolism of both endogenous substrates (such as steroids, fatty acids, bile acids) and exogenous substances (such as drugs, pesticides, polycyclic aromatic hydrocarbons; Estabrook *et al.*, 1979). The study of these enzymes in non-mammalian species is relevant to a number of questions, including the nature and the catalytic activities of the different forms of cytochrome P-450 present in liver and extrahepatic organs, and the effects of these enzyme systems in the activation and detoxication of pollutants.

Their existence in fish liver is now well established (Chambers and Yarbrough, 1976; James *et al.*, 1977; Bend and James, 1978; Lech and Bend, 1980). However, while MO activities of marine species and various trouts have received considerable attention, few detailed studies have been done on other freshwater species.

The present work was undertaken to investigate the MO activities in the liver of two species of freshwater fish, the nase (*Chondrostoma nasus*) and the roach (*Rutilus rutilus*) commonly found in the river Rhône. The selected substrates, 7-ethoxycoumarin, 7-ethoxyresorufin, benzo(a)pyrene, and 2,5-diphenyloxazole have been extensively used in mammals. We characterize here the *in vitro* metabolism of these substrates according to pH, temperature, cofactor requirements, and the inhibitory effects of two diagnostic inhibitors, metyrapone and α-naphthoflavone. Furthermore, this study examines the effects of ionic and non-ionic detergents that have been used for the solubilization of liver microsomes from different animal species.

MATERIALS AND METHODS

Animals

Fish were caught with a net and dissected live shortly after collection. Each liver was removed, wrapped in aluminium foil, and frozen in liquid nitrogen until processed for the preparation of microsomes. Adult male OFA rats (150–180 g) from IFFA-Credo (L'Arbesle, France) were used for comparison.

Preparation of microsomes

Fish and rat livers were homogenized in 0.15 M KCl, 50 mM phosphate buffer, pH 7.4, using a Potter–Elvehjem homogenizer with three passes of a motor-driven Teflon pestle. The tissue homogenate was centrifuged in a Beckman J-21C centrifuge for 15 min at 10,000 *g* at +4°C. The supernatant was then recentrifuged in a Beckman L8-55 ultracentrifuge for 60 min at 105,000 *g* at +4°C. The microsomal pellet was resuspended in 50 mM phosphate buffer, pH 7.4, containing 1 mM DTT, 1 mM EDTA, 20% glycerol, and stored in small aliquots at −80°C. When maintained at this temperature, there was no loss in monooxygenase activities over several months.

Chemicals

Glucose 6-phosphate (G 6-P), glucose 6-phosphate deshydrogenase (G 6-PDH), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and dithiothreitol (DTT) were purchased from Boehringer-Mannheim, France. 7-Ethoxycoumarin was synthesized by the method of Ullrich and Weber (1972). 7-Ethoxyresorufin (7-ethoxyphenoxazone) was synthesized by the method of Prough *et al.* (1978) from resorufin (7-hydroxyphenoxazone, Eastman Organic Chemicals, Rochester, NY). α-Naphthoflavone (7,8-benzoflavone) and metyrapone were from Aldrich, France. Emulgen 911 and 913, and Renex 690 were

*These data were presented, in part, at the 10th Drug Metabolism Workshop, July 1985, Guildford, UK.

Table 1. Components of the monooxygenase system and monooxygenase activities in hepatic microsomal fractions from nase and roach

	Nase	Roach
Protein* (mg/g liver).	21.2	18.8
Cytochrome P-450 (nmol/mg protein).	0.58	0.36
NADPH-cytochrome *c* reductase (nmol/mg protein/min).	40	30
7-ECOD (pmol/mg protein/min).	180	68
7-EROD (pmol/mg protein/min).	79	44
AHH (FU/mg protein/min).	125	69
PPOH (FU/mg protein/min).	0.183	0.123

obtained from Kao-Atlas, France. Sodium cholate and Lubrol PX were from Sigma (St Louis, USA). CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} was from Boehringer-Mannheim, France. Other analytical grade chemicals were commercially purchased.

Enzyme assays

The studied reactions were benzo(a)pyrene hydroxylase (AHH), 2,5-diphenyloxazole hydroxylase (PPOH), 7-ethoxycoumarin *O*-deethylase (7-ECOD), and 7-ethoxyresorufin *O*-deethylase (7-EROD). Activity was assayed in a final volume of 1 ml containing 0.1 M phosphate buffer, pH 7.4, 0.5 mM NADP, 5 mM G 6-P, 1 unit/ml G 6-PDH, microsomal protein, and substrate. The concentration of substrates were: benzo(a)pyrene (added in 40 μl methanol) 0.080 mM, 7-ethoxycoumarin (added in water) 0.1 mM, 7-ethoxyresorufin (added in 10 μl methoxyethanol) 1 μM, and 2,5-diphenyloxazole (added in 10 μl methanol) 100 μM. Unless otherwise stated, all activities were performed at environmental temperature (+10°C) during 1 min (7-EROD), 10 min (7-ECOD) or 15 min (AHH and PPOH). Reactions were stopped by 1 ml acetone (AHH and PPOH), 100 μl trichloracetic acid (20%; 7-ECOD) or 2 ml acetone (7-EROD). Hydroxylated metabolites of benzo(a)pyrene and 2,5-diphenyloxazole were extracted according to Nebert and Gelboin (1968) and assayed fluorometrically at excitation wavelength 395 nm and emission wavelength 522 nm (AHH), and excitation wavelength 345 nm and emission wavelength 510 nm (PPOH). Results were expressed in relative fluorescence units (FU) where 1 FU equals the fluorescence of a quinine sulfate solution (1 μg/ml) at these same wavelengths. The fluorescence of 7-hydroxycoumarin was measured (excitation wavelength: 380 nm; emission wavelength: 480 nm) after extraction of the product by ethyl acetate (4 ml) and mixing 1 ml of organic phase with 1 ml of ethanol and 1 ml of glycine buffer (pH 10.4) The fluorescence of resorufin was measured according to the method of Rifkind and Muschick (1983). Fluorometer was standardized by 7-hydroxycoumarin (7-ECOD) and resorufin (7-EROD).

NADPH-cytochrome *c* reductase activity was assayed spectrophotometrically by the rate of increase in absorbance at 550 nm (at +10°C) of cytochrome *c*. The reaction mixture consisted of 0.3 M phosphate buffer, pH 7.7, 0.1 mM NADPH, 0.1 mM EDTA, 1 mM KCN, and 50 μM cytochrome *c* (Strobel and Dignam, 1978). Cytochrome P-450 content was measured according to Estabrook and Werringloer (1978). Protein was determined by the method of Hartree (1972) with bovine serum albumin as a standard. All analyses were performed in duplicate. All reactions were linear with respect to time and protein concentration.

Solubilization of microsomes

It was achieved by using several ionic (CHAPS and sodium cholate) and non-ionic detergents (Lubrol PX, Renex 690, Emulgen 911 and 913). Microsomes were solubilized with each detergent (final concentration: 0.5%) in a medium containing 1 mM DTT, 0.1 mM phosphate buffer pH 7.4, 20% glycerol, and 1.6 mg of protein/ml at +4°C. The mixture was stirred for 30 min, then centrifuged at 105,000 *g* for 60 min. The content of cytochrome P-450 in the supernatant fraction and in the microsomes was determined as above.

RESULTS

The components of the MO system and the MO activities in nase and roach are shown in Table 1. The level of cytochrome P-450 and the associated activities were higher in nase than in roach.

The optimal pH for MO activity was 7.2 (AHH, both species), 7.0 and 7.4 (PPOH, nase and roach, respectively), 7.4 and 7.6 (7-ECOD, nase and roach, respectively), and 7.8 (7-EROD, both species) (Fig. 1). However, the activities measured at optimal pH represent only 100–125% of the corresponding activities measured at pH 7.4, thus we have routinely used the latter value for all determinations.

Temperature dependency showed two distinctly different patterns according to the substrate (Fig. 2). The highest MO activity for both species was found at 30°C for AHH, PPOH and 7-ECOD. In contrast, 7-EROD activity was maximal at 10°C for roach and 20°C for nase.

Cofactor requirements for MO activity were compared with liver microsomes from rat, nase and roach (Table 2). NADPH was an obligatory component for activity. Relative to NADPH, NADH was a poor source of electrons, and sustained the studied reactions at a very low rate, with the exception of rat liver 7-EROD. When both NADPH and NADH were present, some synergism was observed, especially for AHH and PPOH activities. Addition of Mg^{2+} ions does not modify 7-ECOD, AHH and PPOH activities, but strongly depresses rat liver 7-EROD activity.

α-Naphthoflavone and metyrapone are well-known inhibitors of MO activities. The only exceptions were rat liver AHH which was slightly activated by α-naphthoflavone, and nase liver AHH which was slightly activated by metyrapone (Fig. 3). The sensitivity of the four studied reactions toward inhibition by these two diagnostic inhibitors was quite different in the liver microsomal fraction of fish and rat, but no significant differences were found between roach and nase. Metyrapone proved to be a more potent inhibitor of reactions catalysed by rat microsomes as compared to fish, whereas α-naphthoflavone inhibited more strongly the reactions catalysed by the microsomes of fish compared to those of rat liver, especially at high concentrations of inhibitor.

Table 3 shows the effect of detergents on the solubilization and recovery of cytochrome P-450 from nase and roach liver microsomes. A better recovery was obtained with roach than with nase microsomes. It was also found that the non-ionic detergents and CHAPS were rather interchangeable. On the other hand, sodium cholate was less effective when used to solubilize microsomes from both species.

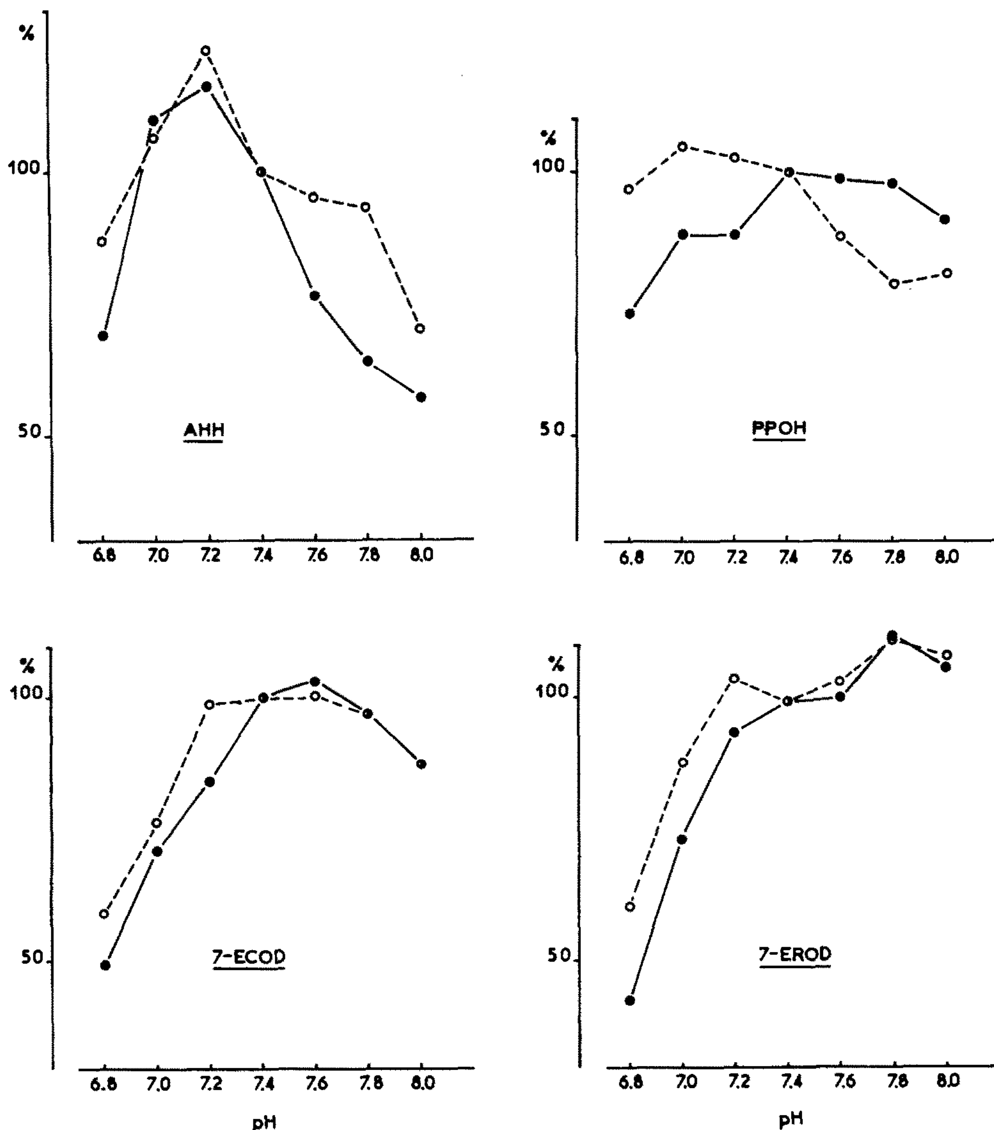

Fig. 1. pH-Dependency of 7-EROD, 7-ECOD, AHH, and PPOH in liver microsomes from nase (O---O) and roach (●——●). Activities are expressed as percentage of specific activity at pH 7.4.

DISCUSSION

The results presented here showed that liver microsomes from nase and roach were able to metabolize model xenobiotic compounds. Previous studies are rather limited, they include measurements of *N*-demethylation of aminopyrine and *p*-hydroxylation of aniline in roach (DeWaide and Henderson, 1970), hepatic cytochrome P-450 concentration in roach (Koivusaari *et al.*, 1980), cytochrome P-450, 7-ECOD, AHH and UDP-glucuronosyltransferase activities in roach (Lindström-Seppä *et al.*, 1981), and AHH in nase (Kezić *et al.*, 1983) and roach (Ahokas and Pelkonen, 1984). A lower cytochrome P-450 content (0.089 nmol/mg of protein) and 7-ECOD activity (0.019 nmol/mg of protein/min) were found in roach by Lindström-Seppä *et al.* (1981). These results emphasize the wide range of enzymatic activities found in wild species collected from different sites. However, when 7-ECOD activity is expressed on the basis of cytochrome P-450 concentration, the latter result (0.21 nmol/nmol cytochrome P-450/min) closely relates to our data (0.19 nmol/nmol cytochrome P-450/min).

The electron donor requirements are consistent with cytochrome P-450-dependent reactions which resemble that of fish and mammals. It is interesting to notice that NADH can be a useful electron donor for aminopyrine *N*-demethylase in scup (Stegeman *et al.*, 1979) and in trout (Koivusaari, 1984), but not for 7-EROD and 7-ECOD in trout (Koivusaari, 1984). The partial involvement of NADH in 7-EROD activity in rat was also found by Burke and Mayer (1974).

Some differences in pH dependence were seen with

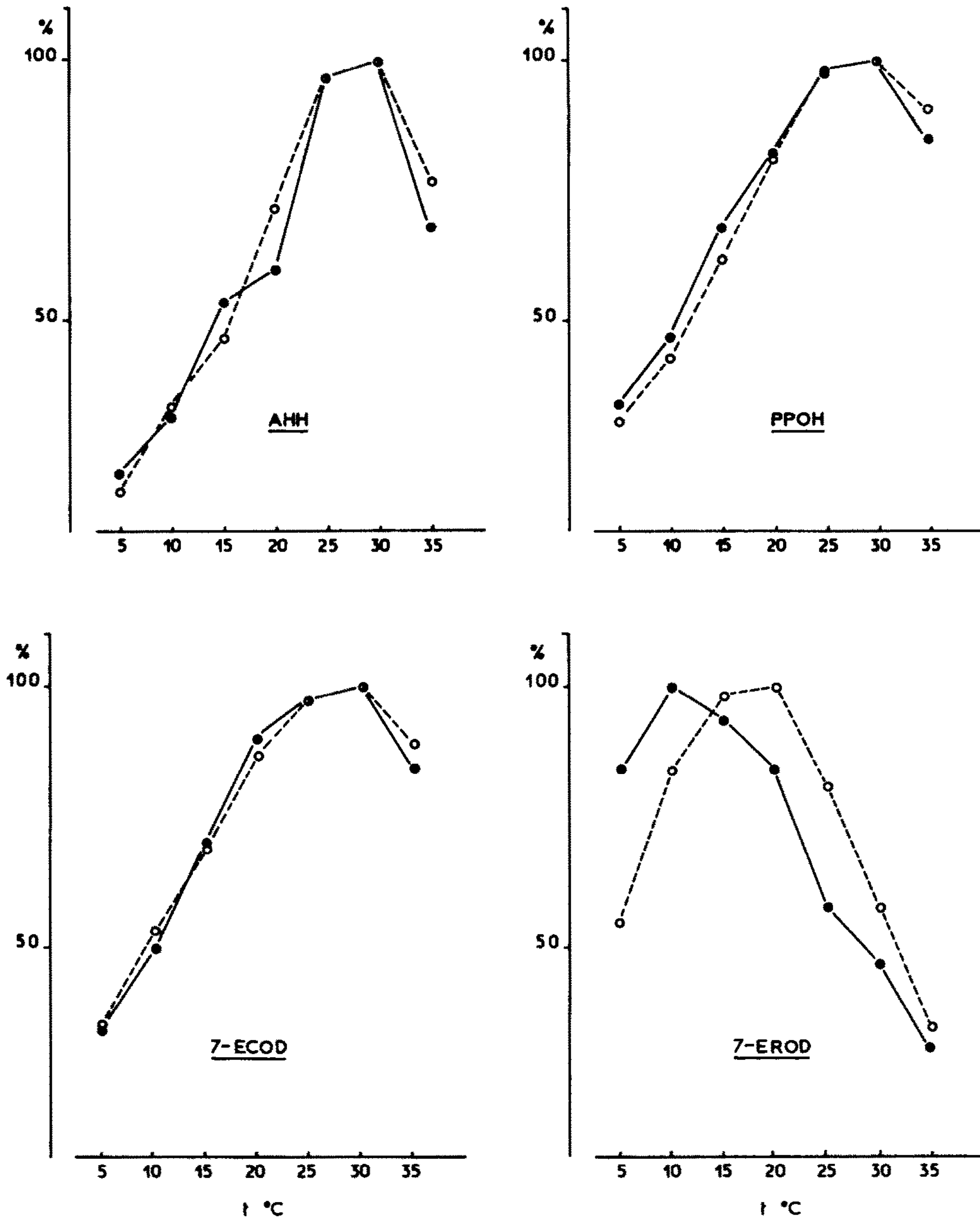

Fig. 2. Temperature dependency of 7-EROD, 7-ECOD, AHH, and PPOH in liver microsomes from nase (○---○) and roach (●——●). Activities are expressed as percentage of maximal activity.

fish microsomes using different species and different substrates. The most studied activity was AHH which showed an optimum at pH 7.4 with rainbow trout microsomes (Pedersen *et al.*, 1974) and a broad peak of activity between 6.8 and 8.0 with scup microsomes (Stegeman *et al.*, 1979). 7-EROD activity usually

Table 2. Cofactor requirements for monooxygenase activities in liver microsomes from rat, nase and roach*

Cofactor (1 mM)	7-ECOD			7-EROD			AHH			PPOH		
	Rat	Nase	Roach	Rat	Nase	Roach	Rat	Nase	Roach	Rat	Nase	Roach
0	0	0	0	0	2.5	0	0	0	0	0	0	0
NADPH	100	100	100	100	100	100	100	100	100	100	100	100
NADH	18	12	8	28	14	5	4	8	4	9	16	9
NADPH + NADH	115	108	112	107	102	102	123	130	109	130	117	139
NADPH + $MgCl_2$	105	100	102	60	94	94	101	108	95	101	106	122

*Microsomes were incubated with the above cofactors as indicated in Materials and Methods. Results are shown as a percentage of NADPH (generating system)-supported activity.

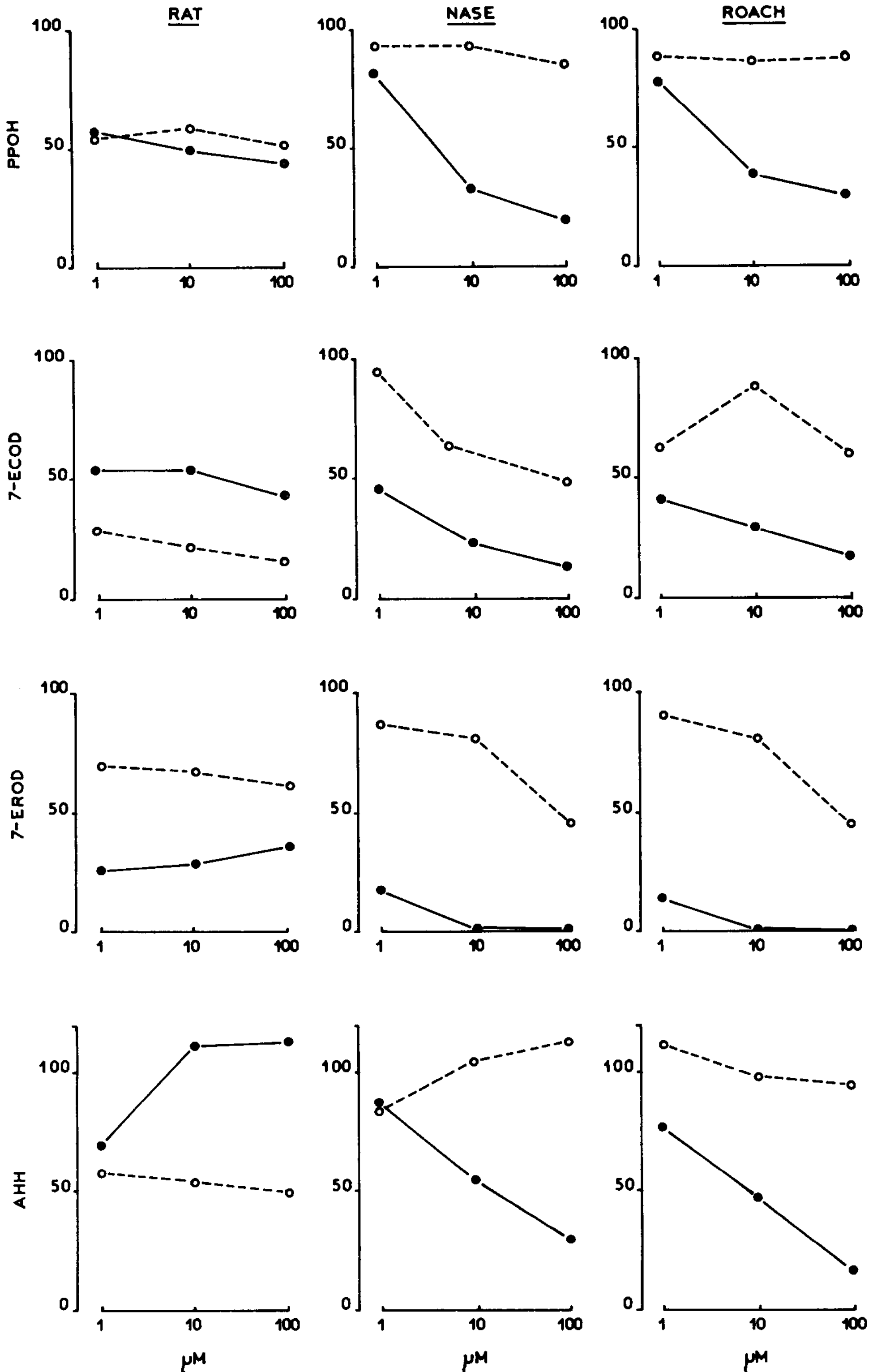

Fig. 3. *In vitro* inhibition of 7-EROD, 7-ECOD, AHH, and PPOH by metyrapone (○---○) and α-naphthoflavone (●——●) at various concentrations in liver microsomes from rat, nase, and roach. Inhibitors were added in 10 μl of DMSO. The results are expressed as percentage remaining enzyme activity compared to a DMSO-treated sample.

Table 3. Solubilization of cytochrome P-450 with various detergents from roach and nase liver microsomes

	Nase	Roach
Emulgen 911	83*	89
Emulgen 913	79	88
Cholate Na	40	29
CHAPS	76	88
Lubrol PX	70	97
Renex 690	84	94

*Percentage of recovery of cytochrome P-450 in supernatant (see Materials and Methods) relative to original microsomes. Each value represents the mean of two determinations.

shows a pH dependence with a peak rate between pH 7.8 and 8.0 in rat (Burke and Mayer, 1974) and fish (Klotz *et al.*, 1984).

The optimal incubation temperature of 30°C for AHH, PPOH and 7-ECOD activities from nase and roach is consistent with those reported for a variety of fish (DeWaide and Henderson, 1968; Pohl *et al.*, 1974; Elcombe and Lech, 1979; Schwen and Mannering, 1982). 7-EROD was a striking exception showing a distinct pattern with an optimal temperature of 20°C (nase) and even 10°C (roach). With trout microsomes, the maximum activity of 7-EROD was found at 20–25°C, but in the range of 10–15°C, close to the environmental temperature (12°C), the activity was still 90–95% of the maximum activity (Williams *et al.*, 1983). The exact causes for such different patterns should be found in the nature of the lipid components surrounding the active site of cytochrome P-450 (Gurumurthy and Mannering, 1985; Williams *et al.*, 1983; Hagar and Hazel, 1985). Obviously, these findings emphasize clearly the need for measuring MO activities in poikilothermic animals at environmental temperatures to obtain realistic values.

In nase and roach, inhibition of MO activities by metyrapone was less pronounced than in untreated rats. Previous studies have shown that this compound inhibited AHH activity in hepatic microsomes from control and phenobarbital-induced mice (Goujon *et al.*, 1972), but no inhibition was found in hepatic microsomes from carp (Melancon *et al.*, 1981) and rainbow trout (Elcombe and Lech, 1979). The inhibitory effect of α-naphthoflavone on hepatic microsomal AHH from nase and roach is similar to that observed in control and β-naphthoflavone-treated rainbow trout (Statham *et al.*, 1978; Elcombe and Lech, 1979), control lake trout (Ahokas *et al.*, 1975), control scup (Stegeman *et al.*, 1979), control and both polychlorobiphenyl- and β-naphthoflavone-induced carp (Melancon *et al.*, 1981) and polycyclic aromatic hydrocarbon-induced rat (Wiebel *et al.*, 1971). On the other hand, α-naphthoflavone stimulates AHH activity in microsomes from control rat (Wiebel *et al.*, 1971), control little skate (Bend *et al.*, 1977), control sheepshead (Bend and James, 1978). In mammalian species, it is generally believed that phenobarbital-inducible cytochromes P-450 are preferably inhibited by metyrapone, while polycyclic aromatic hydrocarbon-inducible cytochromes P-450 are preferably inhibited by α-naphthoflavone (Goujon *et al.*, 1972). Our data are in agreement with earlier studies, suggesting the presence of a "P-448-like" form of cytochrome P-450 in most species of fish.

The solubilization of microsomes is a prerequisite for purification and further characterization of cytochromes P-450. In fish, some problems have been reported with this step (Williams and Buhler, 1982; Klotz *et al.*, 1983). Emulgen 911 and 913 and various non-ionic detergents have been widely used in purification of mammalian enzymes with more than 90% recovery of cytochrome P-450 (Guengerich, 1982), a recovery of 96% was obtained with CHAPS (Williams and Buhler, 1982) in solubilizing trout microsomes, and a yield of 73% was obtained by Goksøyr (1985) by using CHAPS and sodium cholate to solubilize cod microsomes. Our results indicate a good recovery of cytochrome P-450 without appreciable contamination by cytochrome P-420 when nase and roach microsomes were solubilized by CHAPS, Emulgens and some other detergents.

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