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CHARACTERISTICS OF THE HEPATIC MONOOXYGENASE SYSTEM OF THE GOLDFISH (CARASSIUS AURATUS) AND ITS INDUCTION WITH β-NAPTHOFLAVONE

presented by

Jay William Gooch

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By

Jay William Gooch

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ABSTRACT

CHARACTERISTICS OF THE HEPATIC MONOOXYGENASE SYSTEM OF THE GOLDFISH (CARASSIUS AURATUS) AND ITS INDUCTION WITH β-NAPTHOFLAVONE

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Treatment of goldfish with β-napthoflavone (i.p.) resulted in induction of hepatic monooxygenase activity in a dose-dependent manner. Levels of 7-ethoxyresorufin-0-deethylase and mexacarbate NADPH dependent oxidative metabolism as well as cytochrome(s) P-450 were increased. Other substrates which have been associated with 3-methylcholanthrene type induction in mammals were unchanged as was δ-aminolevulinic acid synthetase. Enzyme activities were inhibited by carbon monoxide and piperonyl butoxide confirming their cytochrome P-450 nature. NADH was found to stimulate metabolism in a synergistic manner with NADPH. The pH dependent ethyl isocyanide binding characteristics were similar in control and induced preparations while mexacarbate binding to oxidized microsomes revealed a higher apparent affinity constant (K_s^{app}) as well as greater overall binding in induced preparations. SDS-gel electrophoresis revealed an increased band with a molecular weight of approximately 56,000.

To my Mother and Father

ACKNOWLEDGEMENTS

I would like to express by deepest gratitude to Professor Fumio Matsumura for taking an interest when things looked bleak and for continued encouragement and support through the course of this work and Dr. Howard Johnson for helping me put things together in the beginning.

I would also like to thank Dr. Burra V. Madhukar for his expertise and help, Dr. Don Garling for allowing me to maintain my space and Ms. Alice Ellis for her professional preparation of this manuscript. May your glow box always shine!

And to Ms. Cindi Garbus - a heart full of love for her patience and understanding.

This study was supported by the Michigan Agricultural Experiment Station and by research grant ES01963 from the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

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INTRODUCTION

The contamination of the aquatic environment by foreign organic chemicals is a widespread ongoing phenomenon. Between 1961 and 1975 over 465 million fish were reported killed by pollution overloads (Biernacki, 1978). The effects of this contamination range from the overt toxicity just mentioned to the bioaccumulation of harmful levels of toxic chemicals in edible species. The well known problem of the accumulation of polychlorinated biphenyls (PCB's) in Great Lakes fish species is an excellent example of the latter. Investigations into the mechanisms of biotransformation and its relationship to fate or toxicity are thus necessary to understand the behavior of chemicals in these organisms.

While initial investigators (Brodie and Maickel, 1962) failed to demonstrate the presence of enzymatic oxidations in fish, subsequent research has demonstrated the existence of a wide variety of biotransformation enzymes in various fish tissues (for review see Chambers and Yarbrough, 1976; Lech and Bend, 1980; Bend et al., 1980). The ability of fish to oxidize and conjugate exogenous as well as endogenous compounds is of great importance for the maintenance of normal metabolic function. Table 1 lists examples of the many types of reactions that have been seen in different fish species.

Presumably in an attempt to cope with fluctuating levels of chemical exposure, some of these enzyme systems are capable of being induced into higher activity. The most notable of these is the hepatic monooxygenase system. This system, located primarily in the endoplasmic reticulum (microsomes), functions

TABLE 1. Biotransformation reactions demonstrated in fish.

Reaction	Species	Compound	Reference
Glycine, glucuronide conjugation	Flounder, goosefish	Aminobenzoic acid	Huang and Collins, 1962
Glucuronide conjugation	Rainbow trout	3-trifluoromethyl-4 nitrophenol	Lech, 1973
	Rainbow trout	Pentachlorophenol	Glickman et al., 1977
Glutathione conjugation	Sheepshead	Styrene oxide	James and Little, 1981
Taurine conjugation	Flounder	2,4D	James and Bend, 1976
Sulfate conjugation	Goldfish	Pentachlorophenol	Akitake and Kobayashi, 1975
Hydrolysis	Catfish, bluegills	2,4D-esters	Rodgers and Stalling, 1972
	Rainbow trout	Diethylhexyl phthalate	Melancon and Lech, 1976
	Pinfish	Malathion	Cook and Moore, 1976
	Rainbow trout	Permethrin	Glickman and Lech, 1981
Acetylation	Dogfish shark	Ethyl m-aminobenzoate	Maren et al., 1968
	Rainbow trout	Ethyl m-aminobenzoate	Hunn et al., 1968
Oxidation	Mudsucker, sculpin	Naphthalene, benzo(a)pyrene	Lee et al., 1972
	Coho salmon	Naphthalene	Collier et al., 1978
	Rainbow trout	Methylnaphthalene	Malancon and Lech, 1978
	Carp	Rotenone	Fukami et al., 1969
	Bluegills	4-(2,4-DB)	Guttenmann and Lisk, 1965
	Mosquitofish	Aldrin, dieldrin	Yarbrough and Chambers, 1979
	Bluegill	Chlordane	Sudershan and Khan, 1980
	Flounder	2,6-dimethylnapthalene	Gruger et al., 1981
0-Dealkylation	Fathead minnow	p-Nitrophenylethers	Hansen et al., 1972
	Rainbow trout	Pentachloroanisole	Glickman et al., 1977
	Rainbow trout	Fenitrothion	Miyamoto et al., 1979
N-Dealkylation	Carp	Dinitramine	Olson et al., 1977
	Bluegill	Carbaryl	Chin et al., 1979

Updated from Lech and Bend, 1980.

via NADPH, cytochrome P-450 (named for its characteristic absorption at 450 nm when reduced and complexed with carbon monoxide), NADPH cytochrome P-450 reductase and uses oxygen to oxidize a wide variety of chemicals (for review see Kato, 1979; Parke, 1981; and Coon, 1978). Numerous studies in mammalian systems have shown that there are distinct increases in microsomal proteins and enzyme systems after chemical exposure and several scientists have made attempts to discern the complex relationship between the induction status of the system and toxicity or carcinogenesis (for review see Conney, 1967; Nebert and Jensen, 1979; Snyder and Remmer, 1979; Nebert, 1979; Parke, 1975).

Initially inducers of this system were categorized into two broad types; those of the phenobarbital (PB) or barbiturate type and those of the 3-methylcholanthrene (3-MC) or polycyclic aromatic hydrocarbon type depending on the nature of the enzyme activities induced. Also noted was that the 3-MC type of inducer caused the production of a new type of cytochrome called cytochrome P-448 (P_1-450). Today, the induction phenomenon has been shown to be considerably more complex (Snyder and Remmer, 1979; Madhukar and Matsumura, 1981).

While investigations into the nature of this system in fish have received much less attention, it is now well established that fish are also capable of increasing microsomal monooxygenase activities as a result of exposure to some of the xenobiotics that are known to cause induction in mammals (Pederson et al., 1974; Lidman et al., 1976; Gerhart and Carlson, 1978; Vodicinik et al., 1981). As a result of these investigations, it has been proposed that fish are incapable of responding to inducers of the phenobarbital-type, while 3-methylcholanthrene (3-MC) type compounds have been shown to cause high levels of induction (Ahokas et al., 1977; Ahokas, 1979). These workers also point to the similarity of fish liver systems to that of the P_1-450 system of mammals, although

cytochrome P-450 spectral maxima are at approximately 449-450 nm both before and after induction. James and Bend (1980) have isolated a partially purified cytochrome P-450 from the sheepshead (a marine teleost) which exhibits an absorbance maximum at 448 nm. That the system behaves in a manner analogous to the P_1-450 system of mammals may have important implications concerning the carcinogenic process in these organisms (Ahokas et al., 1977; Ahokas et al., 1979; Nebert and Jensen, 1979).

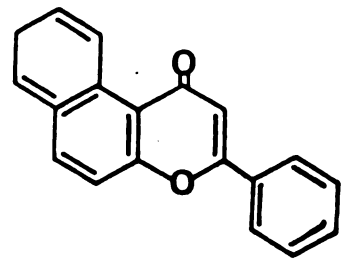
We have begun our studies with the goldfish for several reasons. First, the vast majority of work done on fish has been with coldwater species while there is a lack of data on warmwater species. Second, it has been shown that some of the warmwater fish are known to have relatively high metabolic capabilities. Indeed it has been generally recognized that warmwater fish are less sensitive to chemical pollution (Johnson, 1968). For example, work from this laboratory (Hinz and Matsumura, 1977) has demonstrated a greater capability of goldfish to degrade 2,5,2'-trichlorobiphenyl over that of rainbow trout or bullhead catfish. Third, goldfish are readily available in many locations even during the winter. In this study we have made an attempt to characterize the general nature of the goldfish monooxygenase system by using β-napthoflavone (a known 3-MC type inducer) as a model inducer. Special attention has been paid to compare similarities and differences between the goldfish system to other fish systems and to the mammalian systems.

MATERIALS AND METHODS

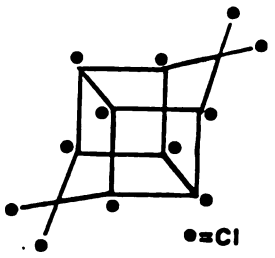
Materials

β-napthoflavone (5,6-benzoflavone), aminopyrine, and p-nitroanisole were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. 3-Methyl-monomethyl aminoazobenzene (3-MMAB) was kindly supplied by Dr. J. A. Miller, McArdle Laboratories, University of Wisconsin, Madison, Wisconsin. Benzphetamine was a gift from Dr. O'Connor, Upjohn Company, Kalamazoo, Michigan. Ethylmorphine was purchased from E. Merck Company, Rahway, New Jersey. [^{14}C] Mexacarbate (Zectran,^{R} 4-dimethylamino-3,5 [^{14}C] xylyl N-methylcarbamate) was provided by the Dow Chemical Company, Midland, Michigan. 7-Ethoxyresorufin was purchased from Pierce Chemical Company, Rockford, Illinois. Piperonyl butoxide was purchased from ICN, K & K Laboratories, Plainview, New York. Ethyl isocyanide was synthesized in this laboratory according to the method of Casanova et al. (1963). All biochemicals were purchased from Sigma Chemical Company, St. Louis, Missouri. Chemical structures of the inducers and substrates used in this study are shown in Figure 1.

Goldfish (Carassius auratus, Common Comet variety, 20-75 gm) were initially obtained from a local pet store whereas in later studies they were obtained from the Blue Ridge Fish Hatchery, Inc., Kernersville, North Carolina. Based on levels of cytochrome P-450, levels of 7-ethoxyresorufin induction and mexacarbate binding behavior, no obvious differences between groups were noted. Fish were kept at 21°C ± 1°C for at least one week prior to use. Fish were initially fed Goldfish Food Flakes (Wardley Products Company, Inc.,



β -NAPTHOFLAVONE



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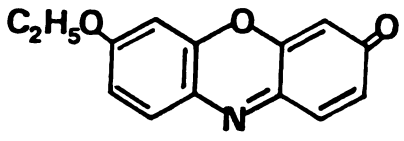
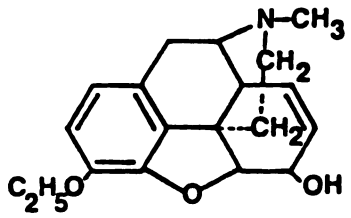
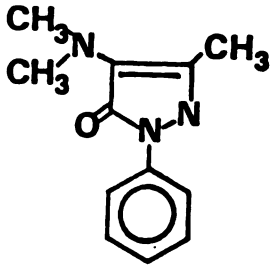


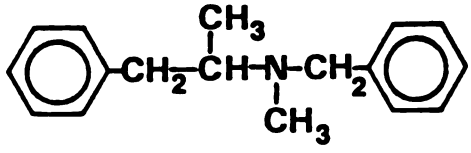
7-ETHOXYRESORUFIN



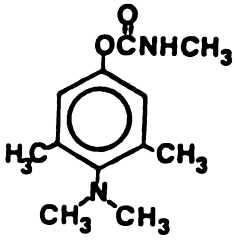
ETHYLMORPHINE



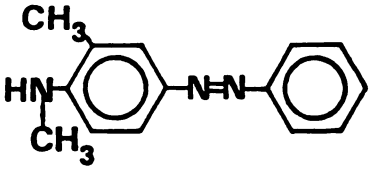
AMINOPYRINE



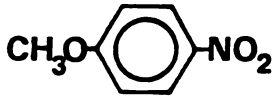
BENZPHETAMINE



MEXACARBATE



3-METHYL-MONOMETHYL-AMINOAZOBENZENE



ρ -NITROANISOLE

Figure 1. Chemical structures of inducers and substrates used in this study.

Secaucus, New Jersey) but were later fed Biodiet pellets (Bioproducts Inc., Warrenton, Oregon) once daily. The last feeding was approximately 24 hours prior to sacrifice.

Preparation of Microsomes

Fish were sacrificed by cervical dislocation, livers pooled (5 - 10 fish) and microsomes prepared as described by Elcombe and Lech (1979). This method involves homogenization in four volumes of 0.25 M sucrose using a motor-driven Potter-Elvehjem homogenizer followed by centrifugation at 8,500 g for 20 minutes in a Beckman L-2 ultracentrifuge. The supernatant was then centrifuged at 165,000 g for 60 minutes. The pellet thus obtained is resuspended in a volume of 0.154 M KCl equal to the volume of the original homogenate and recentrifuged at 165,000 g for 60 minutes. This pellet is then either resuspended in 150 mM potassium-phosphate buffer containing 50 mM sucrose (pH = 7.4) and used or quick frozen as a pellet in 150 mM phosphate buffer containing 50 mM sucrose, 20% glycerol and 1 mM dithiothreitol and stored in liquid nitrogen. Under this storage condition enzyme activity was stable for several weeks.

Enzyme Assays

All enzyme assays, with the exception of 7-ethoxyresorufin-0-deethylase, were carried out in 25 ml Erlenmeyer flasks in a shaking water bath at 29°C. Mixtures contained enzyme, substrate, 150 mM phosphate buffer (pH = 7.4) and in standard NADPH generating system consisting of 2 μ moles of NADP, 10 μ moles glucose-6-phosphate and four units glucose-6-phosphate dehydrogenase. 7-Ethoxyresorufin-0-deethylase was measured by a direct fluorometric procedure using a Varian model 634S UV/VIS spectrophotometer equipped with a fluorescence attachment. Excitation was at 560 nm while broad band emission below 575 nm was followed. Under these conditions approximately 20 picomoles of resorufin could be detected. p-Nitroanisole-0-demethylase was determined by

measuring the amount of p-nitrophenol formed, using a modified method of Bell and Ecobichon (1975) as described by Madhukar and Matsumura (1979). N-demethylation of 3-methylmonomethyl aminoazobenzene (3-MMAB) was measured as described by Madhukar and Matsumura (1979). Benzphetamine-N-demethylase, ethylmorphine-N-demethylase and aminopyrine-N-demethylase were determined by measuring the amount of formaldehyde formed using a modified NASH reagent (Nash, 1953). Oxidative degradation of mexacarbate was assayed by the method of Esaac and Matsumura (1979) with analysis of ^{14}C metabolic products using the TLC method of Benezet and Matsumura (1974).

δ-Aminolevulinic acid synthetase (δ-ALAS) was measured in whole liver homogenates by the method of Marver et al. (1966). This method involves homogenizing the liver in three volumes of 0.9% NaCl containing 0.5 mM EDTA and 10 mM Tris-HCl (pH = 7.4). The incubation mixture contained 0.5 ml of homogenate, 200 μ moles glycine, 20 μ moles EDTA, 150 μ moles Tris HCl in a final volume of 2.0 ml. The reaction was carried out for 60 minutes at 29°C in a shaking water bath. The reaction was stopped with 0.5 ml of 25% TCA and centrifuged. The supernatant was poured off and 0.7 ml of 1.0 M sodium acetate (pH = 4.6) + 50 μl of acetylacetone was added. The preparation was then heated in a boiling water bath for 15 minutes. δ-ALA was estimated using the methylene chloride extraction method of Poland and Glover (1973).

Binding Studies

Ethyl isocyanide (EtNC) binding at various pH's was accomplished by diluting a concentrated microsomal suspension in the standard buffer (150 mM phosphate, 50 mM sucrose) with 0.25 M buffer of the desired pH. pH's above 7.4 were achieved using a tris-HCl buffer. All pH's measured were final (i.e., after addition of 0.25 M buffer, sodium dithionite and EtNC [EtNC = 3.0 mM]).

Spectra were recorded within one minute after the addition of EtNC as it was noted that the peak ratio changed steadily with time.

Microsomes to be used for mexacarbate binding were suspended in 50 mM tris-HCl buffer, pH = 7.5. Substrate was dissolved in absolute ethanol and added to oxidized microsomes in the sample cuvette at various concentrations. Equivalent amounts of ethanol were added to the reference cuvette. Microsomal protein concentration was approximately 3.0 mg/ml. Data were then plotted according to the method of Ebel et al. (1978).

Cytochrome P-450 was measured by the method of Estabrook et al. (1972) using an extinction coefficient of 100 mM^{-1} cm^{-1}. This method measures the reduced carbon monoxide ligated cytochrome P-450 minus the oxidized CO ligated cytochrome. Cytochrome b_5 was measured by the method of Omura and Sato (1964). NADPH-cytochrome c reductase was determined at room temperature by the method of Williams and Kamin (1962) as modified by Masters et al. (1965). Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Electron Microscope Studies

Transmission electron micrographs of isolated hepatocytes were kindly done by Dr. Karen Baker, Coordinator of the Center for Electron Optics at the Pesticide Research Center, using standard electron microscopic techniques.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 1.5 mm slab gel apparatus (Bio-Rad, Model 221). The upper stacking gel (6-8 cm) contained 3% acrylamide while the lower resolving gel (26-28 cm) contained 7.5% acrylamide. A discontinuous buffer system similar to that described by Dent et al. (1978) was used with a constant current of 10 mamp. Staining for protein was done using Coomasie Blue R-250 in isopropanol:acetic acid:water (25:10:65 V/V). Destaining of the background was

achieved by repeated bathing in isopropanol:acetic acid:water (10:10:80 V/V). Staining for peroxidase activity was done via the 3,3',5,5'-tetramethyl benzidine method of Thomas et al. (1976).

RESULTS

Electron Microscope Analysis of Isolated Hepatocytes

Figures 2A and 2B show the appearance of control goldfish hepatocytes. Note the moderate distribution of both smooth and rough endoplasmic reticulum (SER and RER) and normal appearance of mitochondria and other subcellular organelles. Figures 2C and 2D show the hepatocytes of a goldfish treated with the enzyme inducer β-napthoflavone. Notable here is the proliferation of many RER with some apparent increases in SER. Correlation of ultrastructural changes in the endoplasmic reticulum with enzyme induction has been previously shown in mammals (Remmer and Merker, 1963) and also in marine fish (Schoor and Couch, 1979).

Studies on the Patterns of Induction by β-naphthoflavone

The time course of induction of 7-ethoxyresorufin-O-deethylation by a single 150 mg/kg dose of β-napthoflavone was studied. As shown in Figure 3, maximal level of induction was achieved between 72 and 96 hours. Although enzyme activities were highly variable, induction was already significant ($P < 0.01$) at 24 hours.

The dose-response relationship of β-naphthoflavone-induced O-deethylation changes was then studied. The results shown in Figure 4 indicate that there is a general increase in the response with increased dosage. From these studies a single 150 mg/kg dose followed by sacrifice at 96 hours was chosen as a standard induction treatment for further investigations on the nature of the goldfish monooxygenase system.

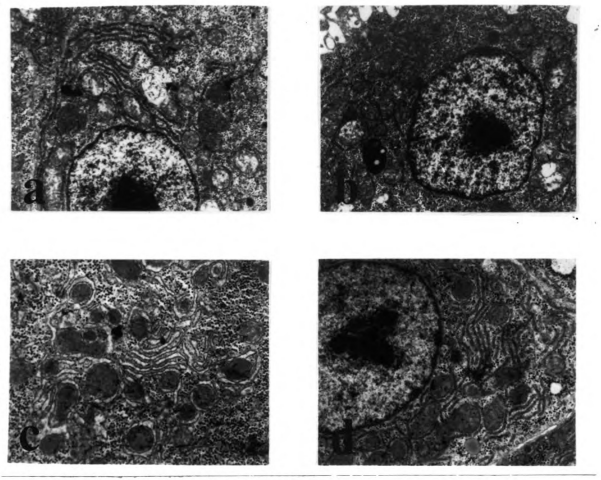


Figure 2. Transmission electron micrographs of isolated hepatocytes from control (a,b) and β-napthoflavone-treated (c,d) goldfish. Magnification 10,000 X.

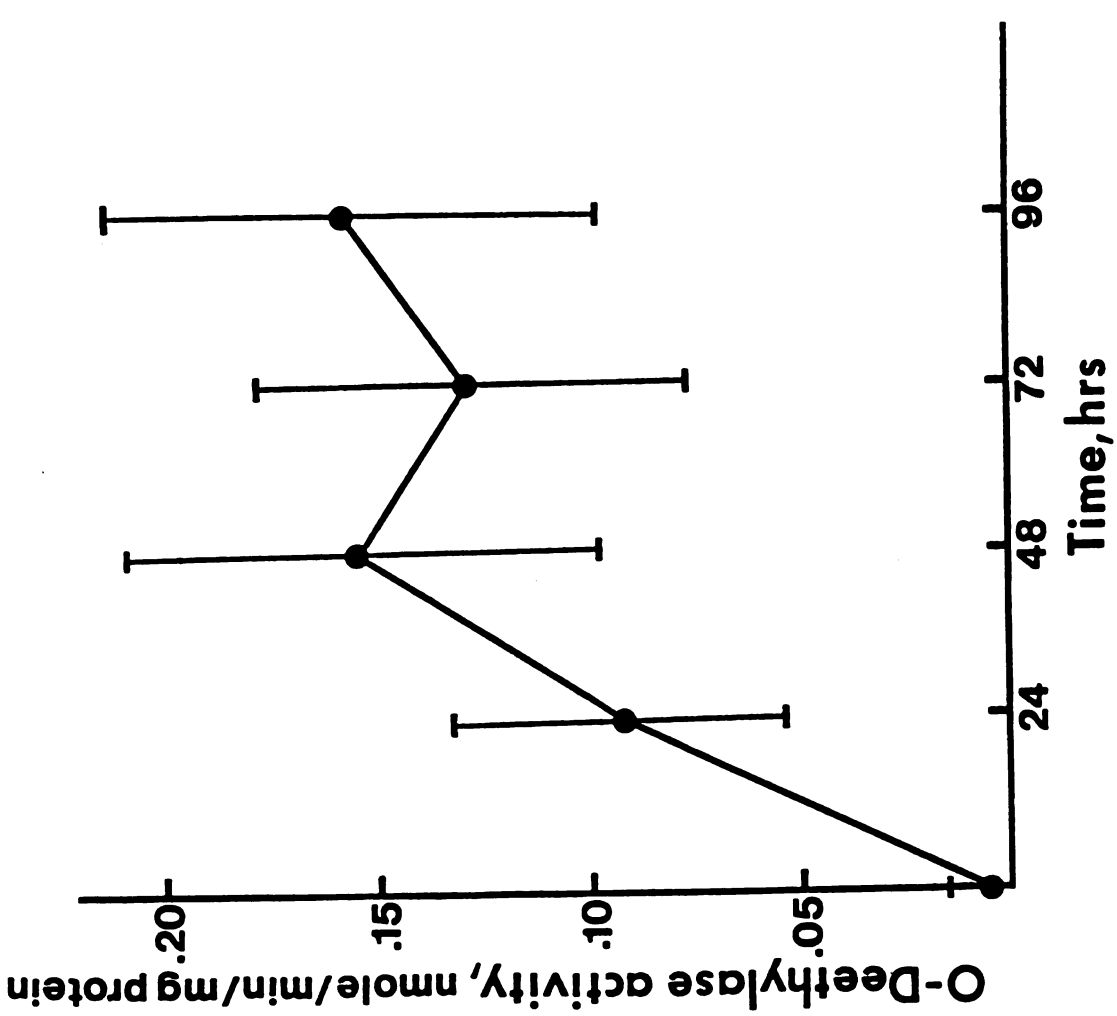


Figure 3. Time course of induction of 7-ethoxyresorufin-O-deethylase activity following a single i.p. dose of β-napthoflavone (100 mg/kg).

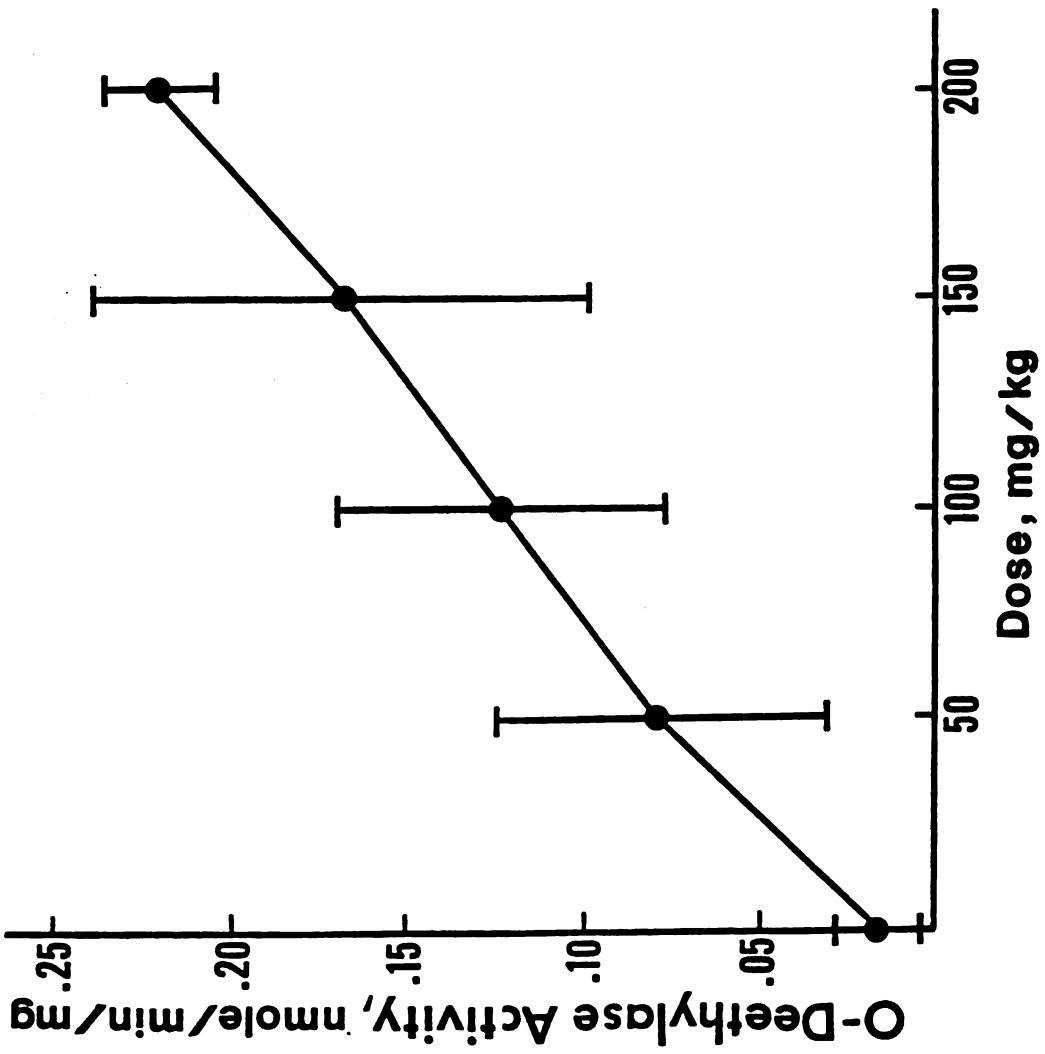


Figure 4. Dose-response curve for 7-ethoxyresorufin-O-deethylation induction. Fish received a single i.p. injection and were sacrificed 72 hours later.

Characterization of Induced Microsomal Monooxygenase Systems

The effect of β-napthoflavone pretreatment on various electron transfer components in the liver microsomes of the goldfish was investigated and the results are shown in Table 2. Under the standard test condition the cytochrome P-450 level increased nearly two-fold, while those for cytochrome b_5 and NADPH cytochrome c reductase were unaffected.

A variety of substrates which have previously been shown to indicate the characteristics of induced monooxygenase activities in higher animals has been tested on the β-naphthoflavone-induced liver microsomes of the goldfish. The results shown in Table 3 indicate that 7-ethoxyresorufin-0-deethylation activity, known as an indicator of enzyme activity for 3-MC type inducers (Burke and Mayer, 1974), was induced nearly eight-fold. Oxidative metabolism of mexacarbate, a carbamate insecticide, was also significantly increased. Metabolism of all other substrates was unaffected. The absence of induction of 3-MMAB N-demethylase activity is surprising in view of the reputation of this system to be useful in recognizing 3-MC type induction in higher animals (Madhukar and Matsumura, 1979).

Also shown in Table 3 is the effect of β-naphthoflavone on δ-aminolevulinic acid synthetase (δ-ALAS), the rate limiting enzyme in heme biosynthesis (Shemin and Russell, 1953). Mammalian induction studies often have shown increases in this enzyme activity apparently reflecting synthesis of new monooxygenase enzymes (Greim et al., 1970). In the case of the goldfish system, δ-ALAS activity was unchanged by β-naphthoflavone treatment.

To further examine the nature of the monooxygenase system of the goldfish, the effect of in vitro addition of inhibitors and cofactors on 7-ethoxyresorufin-0-deethylation and benzphetamine-N-demethylation was studied (Table 4). The 0-deethylation activity with 7-ethoxyresorufin was slightly less

TABLE 2. The effect of β-napthoflavone pretreatment on various parameters of the hepatic monoxoygenase system of the goldfish (Carassius auratus). Fish received a single 150 mg/kg dose in corn oil and were sacrified after 96 hours.

	CYT P-450[a]	CYT b_5[b]	NADPH-CYT C[c] REDUCTASE
Control	0.23 ± .09	.070 ± .02	51.01 ± 0.53
Treated	0.43 ± .08[d]	.074 ± .06	49.86 ± 3.55

[a]nmoles P-450/mg protein. Data are expressed as mean ± S.E.

[b]nmoles b_5/mg protein

[c]nmoles cyt c reduced/min/mg protein

[d]Significantly greater than control ($P < .05$)

TABLE 3. The effect of β-napthoflavone treatment on selected hepatic microsomal monooxygenase enzymes and on δ-ALA-synthetase.

Enzyme	Control	Treated
7-ethoxyresorufin-0-deethylase (nmol resorufin/min/mg protein)	0.015 ± .006	0.224 ± .092[a]
p-nitroanisole-0-demethylase (nmol p-nitrophenol/min/mg protein)	0.313 ± .004	0.358 ± .054
3-MMAB-N-demethylase[b] (nmol HCHO/min/mg protein)	0.787 ± .266	0.748 ± .109
Benzphetamine-N-demethylase (nmol HCHO/min/mg protein)	0.846 ± .445	0.605 ± .135
Ethylmorphine-N-demethylase (nmol HCHO/min/mg protein)	0.214 ± .104	0.257 ± .126
Aminopyrine-N-demethylase (nmol HCHO/min/mg protein)	0.853 ± .265	0.831 ± .211
Mexacarbate[c]	4.84 ± 2.29	11.20 ± 1.77[d]
δ-ALA Synthetase (nmol δ-ALA/g liver/hr)	12.72 ± 2.80	9.98 ± 2.36

[a]Significantly greater than control ($P < .02$). All data are expressed as mean ± S.E.

[b]3-methyl-4-monomethylaminoazobenzene

[c]radioassay - % of metabolites detected via TLC analysis (Benezet and Matsumura, 1974). The N-demethylated product represented approximately 1.81% and 3.26% in the control and treated preparations respectively.

[d]Significantly greater than control ($P < .005$).

TABLE 4. The effect of added cofactors and inhibitors in vitro on monooxygenase activity from control and β-napthoflavone treated goldfish.[a]

Treatment	7-Ethoxyresorufin-0-deethylase Control	7-Ethoxyresorufin-0-deethylase Treated	Benzphetamine-N-demethylase Control	Benzphetamine-N-demethylase Treated
Standard Mixture	100.0[b]	100.0	100.0	100.0
Carbon monoxide	20.0	18.5	0.0	0.0
Piperonyl butoxide (0.5 mM)	0.0	0.0	66.8	72.9
1/2 NADPH + 1.67 mM NADH	162.1	144.1	223.2	217.0
NADH alone (1.67 mM)	0.0	0.0	96.1	99.5

[a]All data are expressed as the percentage of activity compared to the standard incubation mixture containing an NADPH generating system (described in Materials & Methods). Injected fish received the standard treatment of β-NF (150 mg/kg) followed by sacrifice at 96 hours.

[b]For approximate specific activities refer to Table 3.

sensitive to carbon monoxide inhibition than was the benzphetamine-N-demethylase activity. 7-ethoxyresorufin activity was completely inhibited by 0.5 mM piperonyl butoxide. On the other hand, benzphetamine was less sensitive to the same level of inhibitor. Caution should be used in interpretation of this data since benzphetamine-N-demethylase reaction vessels contained considerably more protein (and hence P-450) and thus may require more piperonyl butoxide for complete inhibition. Addition of 1.67 mM NADH in the presence of one half of the normal NADPH generating system resulted in a significant enhancement of enzyme activities with both substrates. NADH (1.67 mM) alone was unable to support 7-ethyoxyresorufin-0-deethylase, while benzphetamine-N-demethylase was maintained at near normal levels.

Spectroscopic Examination of Induced Cytochrome P-450

Figure 5 shows the spectrum of ethyl isocyanide (EtNC) binding to reduced goldfish cytochrome P-450 as a function of pH. In accordance with the observations made in previous studies with higher animals (Dahl and Hodgson, 1978), the change in pH brought a profound change in the peak ratio. On the other hand, no clear-cut difference in the pH-induced spectral changes was observed between the treated and control cytochromes. It should be noted that with the methods employed here (described in Methods) a marked instability in the ratio of the two peaks was observed as time progressed. It was also noted that pHs above 7.5 caused a shift in the position of the 430 nm peak to the extent that at a pH of 8.0 the peak was shifted to approximately 436 nm. This phenomenon is demonstrated graphically in Figure 6. In order to maintain consistency the absorbance at 430 nm was adopted throughout in plotting the data for Figure 5.

Since no discernable differences were observed in binding to the heme iron moiety (as exemplified by EtNC), a suitable type I substrate was chosen to

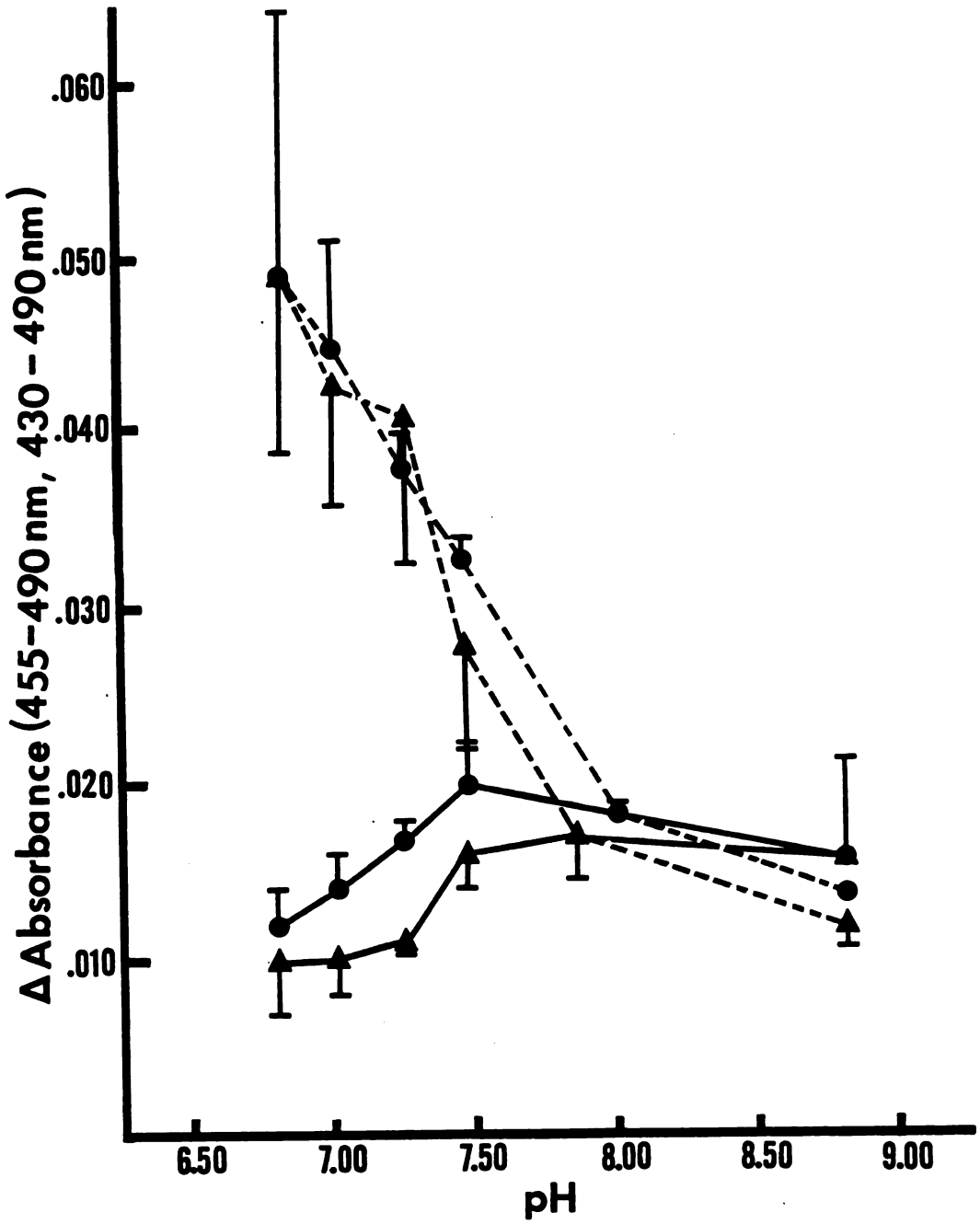


Figure 5. Ethyl isocyanide binding to sodium dithionite reduced goldfish microsomes as a function of pH from control (▲) and β-napthoflavone (●) induced preparations. Fish received the standard 150 mg/kg i.p. dose followed by sacrifice at 96 hours. Other aspects of the assay are described in Methods. Data is expressed as mean + S.E. The variability for the treated preparation at pH 8.75 was ± .003.

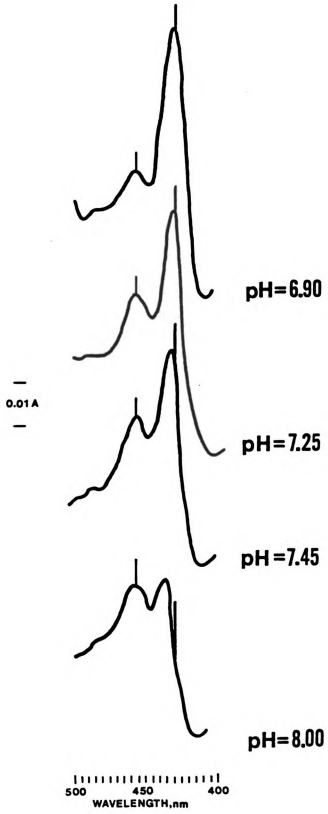


Figure 6. Ethyl isocyanide binding to reduced goldfish microsomes showing a gradual shift in the absorbance of the 430 nm peak with increasing pH.

examine the effect of β-naphthoflavone induction on binding to the protein moiety of cytochrome P-450. Preliminary tests established that mexacarbate is a suitable monooxygenase substrate for the goldfish system. This substrate has been previously shown to bind in a type I manner (Kulkarni et al., 1975).

Figure 7 demonstrates a typical type I spectrum obtained upon addition of mexacarbate to oxidized goldfish liver microsomes. This binding spectrum may be characterized by a fairly narrow absorption minimum at 420 nm followed by a broad maximum around 387 nm. A double reciprocal plot of the magnitude of the absorbance change as the microsomes were titrated with increasing amounts of mexacarbate is shown in Figure 8. An approximately 2.4-fold increase in the apparent affinity constant (K_s^{app}) over the control is evident. It should also be noted that the maximal (i.e., the point at which further addition of substrate does not increase the absorbance change) spectrum from the control was approximately one half that of the treated microsomes.

Gel-electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a valuable tool for examining the constitutive proteins from complex mixtures. Figure 9 shows the electrophoretic pattern of β-naphthoflavone-induced and non-induced goldfish liver microsomes. The appearance of an increased protein band at apparent molecular weight of 56,000 daltons is noted in the treated preparation. This is the general region where hemoprotein(s) P-450 are known to migrate and is also approximately the same molecular weight as the enhanced protein found in rainbow trout after β-napthoflavone induction (Elcombe and Lech, 1979). An effort to demonstrate the presence of heme in this band region by using peroxidase staining failed. Simultaneous electrophoresis of microsomes from rat liver, however, did reveal peroxidase activity indicating the marked species difference in this regard. Apparently all of the heme from the goldfish

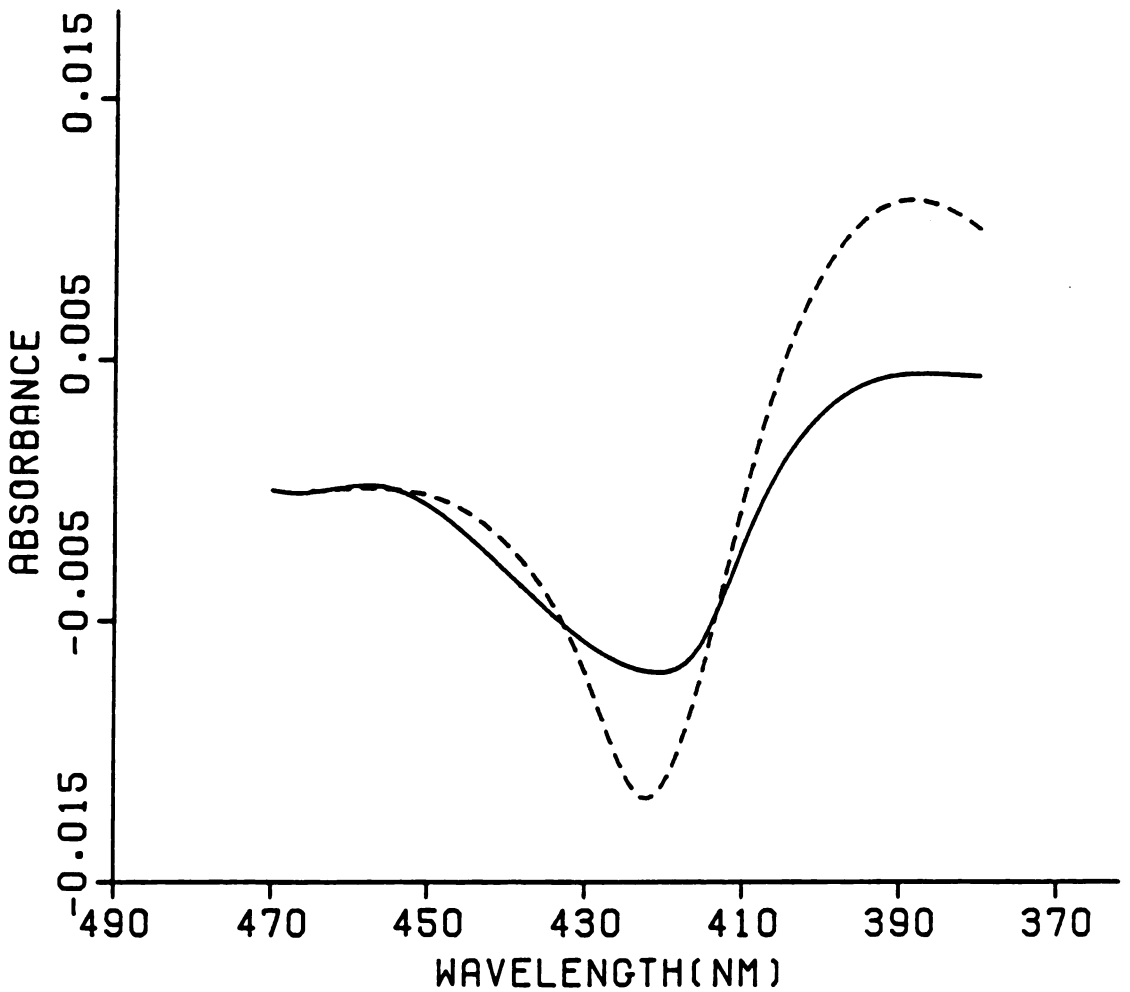


Figure 7. Computer-assisted representation of mexacarbate binding to oxidized goldfish liver microsomes from control (—) and β-napthoflavone induced (---) fish. [Mexacarbate] = 0.11 mM. Microsomal protein concentration was 2.8 mg/ml.

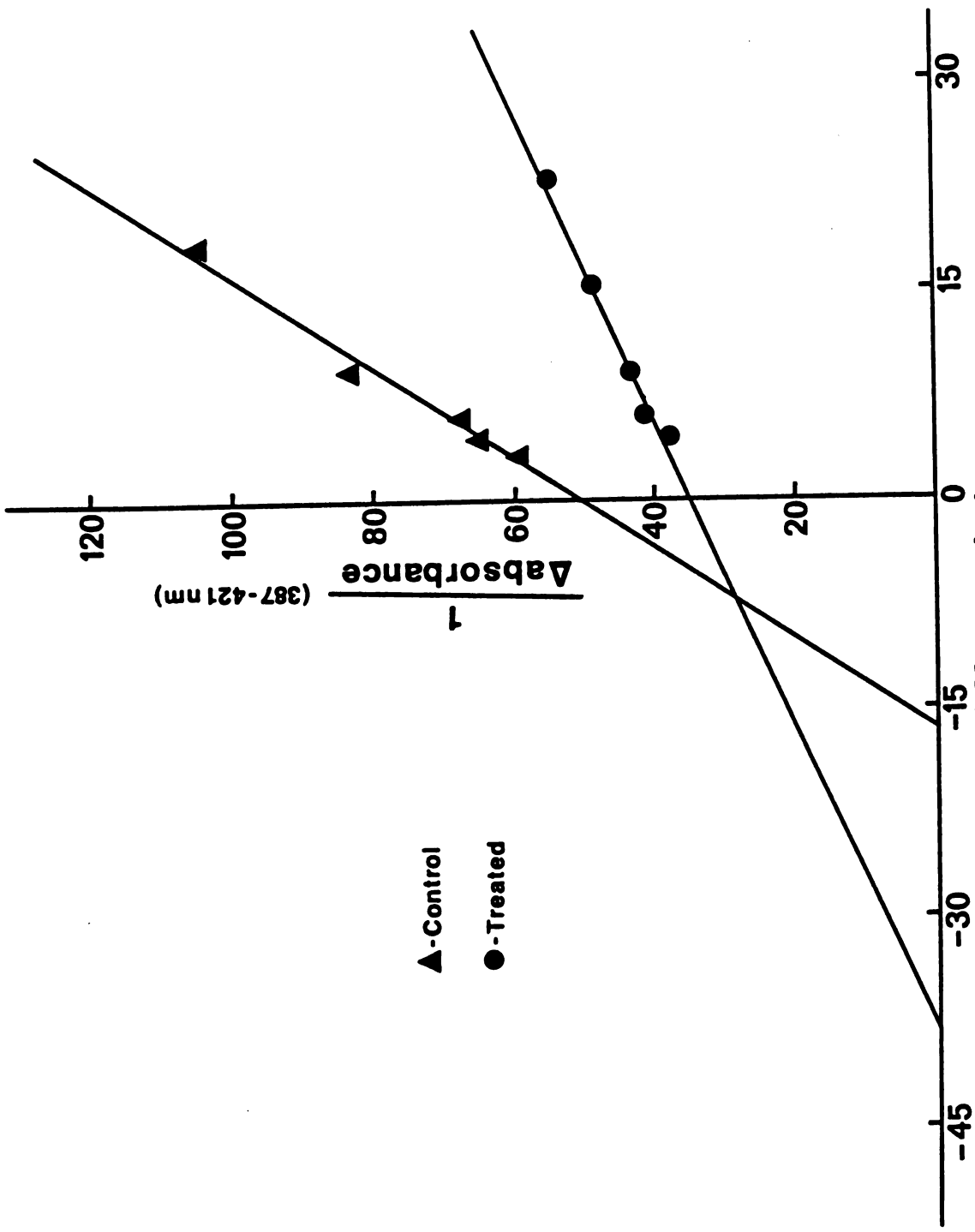


Figure 8. Double reciprocal plot of mexacarbate binding to goldfish microsomes. Binding was done in 50 mM tris buffer (pH 7.5) at a protein concentration of 2.9 mg/ml. The apparent affinity constants (K_s^{app}) were 6.05×10^{-5} M for control and 2.6×10^{-5} M for induced preparations.

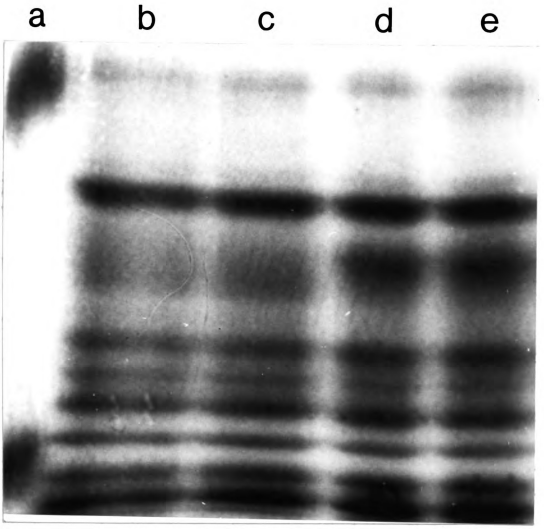


Figure 9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of hepatic microsomes from control (b,c) and β-napthoflavone-treated (d,e) goldfish. Well (a) contained standard proteins with molecular weights of 14,400, 21,500, 31,000, 45,000 (shown), 66,200 (shown) and 92,500. Wells contained 75 μg protein and the resolving gel was 7.5% acrylamide.

preparations had dissociated from the microsomes quite easily as peroxidase activity was noted at the migration front of the gel.

DISCUSSION

The results of this study indicate that goldfish possess a β-napthoflavone inducible monooxygenase system as has been seen in other marine and freshwater species (Elcombe and Lech, 1979; James and Bend, 1980). Dose response studies show a general linear relationship within the dose range tested, while time course experiments demonstrate maximal induction four to five days post-treatment.

Cytochrome P-450 levels of the uninduced goldfish were similar to those reported for rainbow trout (Elcombe and Lech, 1979), sheepshead (James and Bend, 1980), lake trout (Ahokas et al., 1977) and the common carp (Guiney et al., 1980). Significant induction, while variable, was readily achieved. Cytochrome b_5 levels were found to be generally greater than those reported for rainbow trout or brook trout (Stegeman and Chevion, 1980). Levels of NADPH cytochrome-c-reductase were also uninduced and compare favorably with other species of fish tested (James and Bend, 1980; Stanton and Khan, 1975; James et al., 1979). This enzyme, however, appears to be less active than in most mammalian systems (reviewed by Kato, 1979).

The substrates chosen for examination of inductive effects were meant to cover a variety of the types of cytochrome(s) P-450 found in mammalian systems (e.g., Madhukar and Matsumura, 1981). Some interesting differences were noted between goldfish and mammals, particularly with respect to 3-MMAB-N-demethylase and δ-ALA-synthetase. Both of these activities have been found to be inducible by 3-MC type inducers in mammalian systems (Madhukar and

Matsumura, 1981), while they are not induced in goldfish. Indeed, Madhukar and Matsumura (1979) showed nearly two-fold induction of 3-MMAB-N-demethylase activity following phenobarbital treatment of rats demonstrating that induction of 3-MMAB metabolism is not specific to 3-MC type induction. Curiously, mexacarbate oxidative metabolism, which proceeds primarily through a N-demethylation in our system (see footnote, Table 2), is apparently stimulated by β-napthoflavone induction. This is supported by the increase in the apparent affinity constant (K_s^{app}) based on mexacarbate binding to the microsomal preparation. Mexacarbate may prove to be a useful substrate when examining the induction of monooxygenase activity in fish.

δ-ALA-synthetase did not increase after β-napthoflavone induction even though SDS - PAGE indicates formation of an increased protein band in the hemoprotein region. There are at least two possible explanations for this: (1) there is a high enough titer of the porphyrine-heme pool to supply the new protein synthesized in the goldfish liver or (2) the increased band in the electrophoresis gel is not of a hemoprotein nature since it was not possible to detect peroxidase activity in this or any other region of the gel. Based on the increases in enzyme activity, binding of substrate and the work of others (Elcombe and Lech, 1979; Vodicinik et al., 1981), the former explanation appears to be more plausible.

The results in Table 4 apparently represent observations previously not investigated in fish liver monooxygenase systems. The synergism seen when NADH is added with NADPH for both of the substrates tested is quite similar to the phenomenon seen in rabbits (Cohen and Estabrook 1971). It is also interesting to note that benzphetamine-N-demethylation is supported by NADH nearly as well as with NADPH. Whether or not these effects are mediated through cytochrome b_5 in fish as they are in mammals (Staudt et al., 1974;

Kamataki and Kitagawa, 1977) remains to be seen. However, based on other qualitative similarities betwen the two systems, cytochrome b_5 involvement would appear to be likely.

Ethyl isocyanide binding to reduced microsomes has been used in the past (Sladek and Mannering, 1966; Imai and Siekevitz, 1971) to demonstrate differences in the binding behavior of microsomal preparations before and after induction. Generally an increase in the ratio of the absorbance at 455 and 430 nanometers has been observed after treatment with 3-MC type inducers. This effect has not been observed in rainbow trout (Elcombe and Lech, 1979). In a similar manner, induced rat liver microsomes show a shift in the pH dependent crossover point (the point at which the absorbance of the 455 nm and 430 nm peaks is equal) when compared to uninduced preparations. In contrast, we were unable to demonstrate any significant differences in the nature of the pH dependent binding between control and induced preparations. This observation is consistent with other observations in goldfish liver systems that the characteristics of control and induced preparations are qualitatively very similar.

The gradual shift in the absorbance of the 430 nm peak to 436 nm at higher pH's is an observation we have not seen described by any other authors. This could be due to a conversion of a portion of the cytochrome(s) P-450 to P-420 which is known to exhibit a single absorbance at 433 nm when complexed with ethyl isocyanide (Imai and Sato, 1967). However, the fact that the shift appears to be gradual as the pH is changed would seem to indicate that other phenomena are involved. Also, Imai and Sato (1967) report that the absorbance of the P-420–ethyl isocyanide complex is insensitive to changes in pH.

Substrate-induced spectral interactions with oxidized microsomes have been seen in virtually every system investigated (reviewed by Schenkman et al.,

1981). Those substrates that elicit a type I binding spectrum are believed to bind to the apoprotein moiety of the cytochrome P-450 enzyme. In this system the qualitative nature of the mexacarbate-induced type I difference spectrum was similar with both control and treated preparations. The fact that titration with substrate reveals differences in apparent affinity and also in the maximum inducible spectrum could be due to the synthesis of a different form of cytochrome P-450 capable of undergoing a low to high spin transition of the heme iron (Cinti et al., 1979) at lower substrate concentrations. Schenkman et al. (1981) suggest that this process could lead to an enhanced rate of cytochrome P-450 reduction which in turn would lead to increased rates of metabolism. Alternatively, it could be postulated that a change had occurred in the protein environment of a preexisting cytochrome P-450 enzyme which would affect the nature of the spin equilibrium surrounding that particular cytochrome P-450 enabling the low to high spin transition to occur more readily. Both of these possibilities could manifest themselves as the observed phenomenon.

Although SDS - PAGE shows an increased protein band of approximately 56,000 MW in microsomes from β-napthoflavone-treated fish, this alone is not sufficient to distinguish between the two possibilities.

Other investigations (Vodicinik et al., 1981; Bend et al., 1973; Addison et al., 1977) have demonstrated that fish are apparently refractive to inducers other than the 3-MC type. Apparently goldfish are also incapable of responding to mirex at 100 mg/kg. Preliminary data from this laboratory showed no changes in metabolism of ethylmorphine, benzphetamine, 7-ethoxyresorufin or mexacarbate 96 hours after a single injection. Also, no signficant changes in levels of cytochrome P-450 were noted.

In summary, the pattern of induction in the goldfish liver is much simpler than that found in mammalian livers. In the former, induction does not extend to

δ-ALAS, NADPH cytochrome c reductase, cytochrome b_5, monooxygenases to metabolize 3-MMAB, p-nitroanisole, ethylmorphine and aminopyrene. The induced monooxygenase system is qualitatively similar to the noninduced, control system. This makes the goldfish system particularly suitable for further study of inductive mechanisms without complex interactions from other factors.

LIST OF REFERENCES

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1. Addison, R. F., Zinck, M. E. and Willis, D. E. (1977). Mixed function oxidase enzymes in trout (Salvelinus fontinalis) liver: Absence of induction following feeding of p,p'-DDT or p,p'-DDE. Comp. Biochem. Physiol. 57C, 39-43.

2. Ahokas, J. T., Pelkonen, O. and Karki, N. T. (1977). Characterization of benzo[a]pyrene hydroxylase of trout liver. Cancer. Res. 37, 3737-3743.

3. Ahokas, J. T., Saarn, H., Nebert, D. W. and Pelkonen, O. (1979). The in vitro metabolism and covalent binding of benzo[a]pyrene to DNA catalyzed by trout liver microsomes. Chem.-Biol. Interact. 25, 103-111.

4. Akitake, H. and Kobayashi, K. (1975). Studies on the metabolism of chlorophenols in fish-III. Isolation and identification of a conjugated PCP excreted by goldfish. Bull. Japan. Soc. Sci. Fish 41, 321-327.

5. Bell, J. J. and Ecobichon, D. J. (1975). The development of kinetic parameters of hepatic drug-metabolizing enzymes in perinatal rats. Can. J. Biochem. 53, 433-437.

6. Bend, J. R. and James, M. O. (1978) Xenobiotic metabolism in marine and freshwater species. In Biochemical and Biophysical Perspectives in Marine Biology (D. C. Malins and J. R. Sargent, eds.), Vol. 4, pp. 126-188. Academic Press, New York.

7. Bend, J. R., James, M. O. and Pritchard, J. B. (1980). Aquatic toxicology. In Introduction to Environmental Toxicology (F. E. Guthrie and J. J. Perry, eds.), Elsevier-North Holland, New York.

8. Bend, J. R., Pohl, R. J. and Fouts, J. R. (1973). Further studies of the microsomal mixed-function oxidase system of the little skate, Raja erinacea, including its response to some xenobiotics. Bull. Mt. Desert Island Biol. Lab. 13, 9-13.

9. Benezet, H. J. and Matsumura, F. (1974). Factors influencing the metabolism of mexacarbate by microorganisms. J. Agr. Food Chem. 22, 427-430.

10. Biernacki, E. (1978). Fish kills caused by pollution. Fifteen-year summary, 1961-1975. U.S.E.P.A. (EPA-440/4-78-011). Office of Water Planning and Standards. 78 p.

11. Brodie, B. B. and Maickel, R. P. (1962). Comparative biochemistry of drug metabolism. Proc. Int. Pharmacol. Meet. Ist. 6, 299-324.

12. Buhler, D. R. and Rasmusson, M. E. (1968). The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25, 223-239.

13. Burke, M. D. and Mayer, R. T. (1974). Ethoxyresorufin: Direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methycholanthrene. Drug Metab. Disp. 2, 583-588.

14. Casanova, J., Schuster, R. E. and Weaver, W. D. (1963). Synthesis of aliphatic isocyanides. J. Chem. Soc. 1963, 4280-4281.

15. Chambers, J. E. and Yarbrough, J. D. (1976). Xenobiotic transformation systems in fishes. Comp. Biochem. Physiol. 55C, 77-84.

16. Chin, B. H., Sullivan, L. J. and Eldridge, J. E. (1979). In vitro metabolism of carbaryl by liver explants of bluegill, catfish, perch, goldfish and kissing gourami. J. Agr. Food Chem. 27, 1395-1298.

17. Cinti, D. L., Sligar, S. G., Gibson, G. G. and Schenkman, J. B. (1979). Temperature-dependent spin equilibrium of microsomal and solubilized cytochrome P-450 from rat liver. Biochemistry 18, 36-42.

18. Cohen, B. S. and Estabrook, R. W. (1971). Microsomal electron transport reactions III. Cooperative interactions between reduced diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide linked reactions. Arch. Biochem. Biophys. 143, 54-65.

19. Collier, T. K., Thomas, K. C., Malins, D. C. (1978). Influence of environmental temperature on disposition of dietary naphthalene in coho salmon: isolation and identification of individual metabolites. Comp. Biochem. Physiol. 61C, 23-.

20. Conney, A. H. (1967). Pharmacological implication of microsomal enzyme induction. Pharmac. Rev. 19, 317-366.

21. Cook, G. H. and Moore, J. C. (1976). Determination of malathion, malaoxon, and mono- and dicarboxylic acids of malathion in fish, oyster, and shrimp tissue. Agr. Food Chem. 24, 631-634.

22. Coon, M. J. (1978). Oxygen activation in the metabolism of lipids, drugs and carcinogens. Nutr. Rev. 36, 319-328.

23. Dahl, A. R. and Hodgson, E. (1978) The affinity of cytochrome P-450 for ethyl isocyanide: An explanation of double soret spectra. Chem. - Biol. Interact. 20, 171-180.

24. Dent, J. G., Elcombe, C. R., Netter, K. J. and Gibson, J. E. (1978). Rat hepatic microsomal cytochrome(s) P-450 induced by polybrominated biphenyls. Drug. Metab. Disp. 6, 96-101.

25. Ebel, R. E., O'Keefe, D. H., and Peterson, J. A. (1978). Substrate binding to hepatic microsomal cytochrome P-450: Influence of the microsomal membrane. J. Biol. Chem. 253, 3888-3897.

26. Elcombe, C. R. and Lech, J. J. (1979). Induction and characterization of hemoprotein(s) P-450 and monooxygenation in rainbow trout (Salmo gairdneri). Toxicol. Appl. Pharmacol. 49, 437-450.

27. Esaac, E. G. and Matsumura, F. (1979). Roles of flavoproteins in degradation of mexacarbate in rats. Pestic. Biochem. Physiol. 10, 67-78.

28. Estabrook, R. W., Peterson, J., Baron, J. and Hildebrandt, A. G. (1972). In Methods in Pharmacology (C. F. Chignell, ed.), Vol. 2, pp. 303-350. Meredith, New York.

29. Fukami, J. I., Shisido, T., Fukunaga, K. and Casida, J. E. (1969). Oxidative metabolism of rotenone in mammals, fish and insects and its relation to selective toxicity. J. Agr. Food. Chem. 17, 1217-1226.

30. Gerhart, E. H. and Carlson, R. M. (1978). Hepatic mixed-function oxidase activity in rainbow trout exposed to several polycyclic aromatic compounds. Environ. Res. 17, 284-295.

31. Glickman, A. H. and Lech, J. J. (1981). Hydrolysis of permethrin, a pyrethroid insecticide, by rainbow trout and mouse tissues in vitro: A comparative study. Toxicol. Appl. Pharm. 60, 186-192.

32. Glickman, A. H., Statham, C. N., Wu, A. and Lech, J. J. (1977). Studies on the uptake, metabolism, and disposition of pentachlorophenol and pentachloroanisole in rainbow trout. Toxicol. Appl. Pharmacol. 41, 649-658.

33. Greim, H., Schenkman, J. B., Klotzbucher, M. and Remmer, H. (1970). The influence of phenobarbital on the turnover of hepatic microsomal cytochrome b_5 and cytochrome P-450 hemes in the rat. Biochim. Biophys. Acta. 201, 20-25.

34. Gruger, E. H., Schnell, J. V., Fraser, P. S., Brown, D. W. and Malins, D. C. (1981). Metabolism of 2,6-dimethylnapthalene in starry flounder (Platichthys stellatus) exposed to napthalene and p-cresol. Aquat. Toxicol. 1, 37-48.

35. Guiney, P. D., Dickins, M. and Peterson, R. E. (1980). Paradoxical effects of cobaltous chloride treatment on 2-methylnapthalene disposition and hepatic cytochrome P-450 content in carp. Arch. Environ. Contam. Toxicol. 9, 579-590.

36. Gutenmann, W. H. and Lisk, D. J. (1965). Conversion of 4-(2,4-DB) herbicide to 2,4-D by bluegills. N. Y. Fish Game J. 12, 108-111.

37. Hansen, L. G., Kapoor, I. P. and Metcalf, R. L. (1972). Biochemistry of selective toxicity and biodegradability: comparative 0-dealkylation by aquatic organisms. Comp. Gen. Pharmacol. 3, 339-344.

38. Hinz, R. and Matsumura, F. (1977). Comparative metabolism of PCB isomers by three species of fish and the rat. Bull. Environ. Contam. Toxicol. 18, 631-639.

39. Huang, K. C. and Collins, S. F. (1962). Conjugation and excretion of aminobenzoic acid isomers in marine fishes. J. Cell. Comp. Physiol. 60, 49-52.

40. Hunn, J. B., Schoettger, R. A. and Willford, W. A. (1968). Turnover and urinary excretion of free and acetylated M.S. 222 by rainbow trout, Salmo giardneri. J. Fish. Res. Bd. Can. 25, 25-31.

41. Imai, Y. and Sato, R. (1967) Anomalous spectral interactions of reduced P-450 with ethylisocyanide and some other lipophilic ligands. J. Biochem. 62, 464-473.

42. Imai, Y. and Siekevitz, P. (1971). A comparison of some properties of microsomal cytochrome P-450 from normal, methylcholanthrene-, and phenobarbital-treated rats. Arch. Biochem. Biophys. 144, 143-159.

43. James, M. O. and Bend, J. R. (1976). Taurine conjugation of 2,4-dichlorophenoxyacetic acid in two marine species. Xenobiotica 6, 393-398.

44. James, M. O. and Little, P. J. (1981). Polyhalogenated biphenyls and phenobarbital: Evaluation as inducers of drug metabolizing enzymes in the sheepshead, Archosargus probatocephalus. Chem.-Biol. Int. 36, 229-248.

45. James, M. O., Khan, M. A. Q., and Bend, J. R. (1979). Hepatic microsomal mixed-function oxidase activities in several marine species common to coastal Florida. Comp. Biochem. Physiol. 62C, 155-164.

46. Johnson, D. W. (1968). Pesticides and fishes: A review of selected literature. Trans. Am. Fish Soc. 97, 398-424.

47. Kamataki, T. and Kitagawa, H. (1977). The involvement of cytochrome P-450 in the NADH dependent O-demethylation of p-nitroanisole in phenobarbital-treated rabbit liver microsomes. Biochem. Biophys. Res. Comm. 76, 1007-1013.

48. Kato, R. (1979). Characteristics and differences of the hepatic mixed function oxidases of different species. Pharmac. Ther. 6, 41-98.

49. Kulkarni, A. P., Mailman, R. B. and Hodgson, E. (1975). Cytochrome P-450 optical difference spectra of insecticides. A comparative study. J. Agric. Food Chem. 23, 179-183.

50. Lee, R. F., Sauerheber, R. and Dobbs, G. H. (1972). Uptake, metabolism and discharge of polycyclic aromatic hydrocarbons by marine fish. Mar. Biol. 17, 201-208.

51. Lech, J. J. (1973). Isolation and identification of 3-trifluoromethyl-4-nitrophenyl glucuronide from bile of rainbow trout exposed to 3-trifluoromethyl-4-nitrophenyl. Toxicol. Appl. Pharmacol. 24, 114-124.

52. Lech, J. J. and Bend, J. R. (1980). Relationship between biotransformation and the toxicity and fate of xenobiotic chemicals in fish. Environ. Health Perspect. 34, 115-131.

53. Lidman, U., Forlin, L., Molander, D. and Axelson, G. (1976). Induction of the drug metabolizing system in rainbow trout (Salmo gairdneri) liver by polychlorinated biphenyls (PCBs). Acta Pharmacol. Toxicol. 39, 262-272.

54. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

55. Madhukar, B. V. and Matsumura, F. (1979). Comparison of induction patterns of rat hepatic microsomal mixed-function oxidases by pesticides and related chemicals. Pestic. Biochem. Physiol. 11, 301-308.

56. Madhukar, B. V. and Matsumura, F. (1981). Differences in the nature of induction of mixed-function oxidase systems of the rat liver among phenobarbital, DDT, 3-methylcholanthrene, and TCDD. Toxicol. Appl. Pharmacol. 61, 109-118.

57. Maren, T. H., Broder, L. E. and Stenger, V. C. (1968). Metabolism of ethyl m-aminobenzoate (MS 222) in the dogfish, Squalus acanthias. Bull. Mt. Desert Isl. Biol. Lab. 8, 39-41.

58. Marver, H. S., Tschudy, D. P., Perlroth, M. G., Collins, A., and Hunter, G. Jr. (1966). The determination of aminoketones in biological fluids. Anal. Biochem. 14, 53-60.

59. Masters, B. S. S., Kamin, H., Gibson, Q. H., and Williams, C. H., Jr. (1965). Studies on the mechanism of microsomal triphosphopyridine nucleotide-cytochrome c reductase. J. Biol. Chem. 240, 921-.

60. Melancon, M. J., Jr. and Lech, J. J. (1976). Distribution and biliary excretion products of Di-2-ethylhexyl phthalate in rainbow trout. Drug Metab. Disp. 2, 112-118.

61. Melancon, M. J., Jr. and Lech, J. J. (1978). Distribution and elimination of naphthalene and 2-methylnaphthalene in rainbow trout during short and long term exposures. Arch. Environ. Contam. Toxicol. 7, 207-220.

62. Miyamoto, J., Takimoto, Y. and Mihara, K. (1979). Metabolism of organophosphorus insecticides in aquatic organisms, with special emphasis on fenitrothion. In Pesticide and Xenobiotic Metabolism in Aquatic Organisms. ACS Symposium Series, Khan, Lech, and Menn, Eds., American Chemical Society, Washington, D. C., pp. 3-20.

63. Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J. 55, 416-422.

64. Nebert, D. W. (1979). Multiple forms of inducible drug-metabolizing enzymes: A reasonable mechanism by which any organism can cope with adversity. Mol. Cell. Biochem. 27, 27-46.

65. Nebert, D. W. and Jensen, N. M. (1979). The Ah Locus: Genetic regulation of the metabolism of carcinogens, drugs, and other environmental chemicals by cytochrome P-450-mediated monooxygenases. In Critical Reviews in Biochemistry, pp. 401-438, CRC Press Inc., Ohio.

66. Olson, L. E., Allen, J. L. and Hogan, J. W. (1977). Biotransformation and elimination of the herbicide dinitramine in carp. J. Agr. Food Chem. 25, 554-556.

67. Omura, T. and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

68. Parke, D. V. (1975). Induction of the drug-metabolizing enzymes. In Enzyme Induction (D. V. Parke, Ed.), pp. 207-271. Plenum Press, London.

69. Pederson, M. G., Hershberger, W. K. and Juchau, M. R. (1974). Metabolism of 3,4-benzpyrene in rainbow trout (Salmo gairdneri). Bull. Environ. Contam. Toxicol. 12, 481-486.

70. Poland, A. and Glover, E. (1979). 2,3,7,8-tetrachlorodibenzo-p-dioxin: a potent inducer of δ-aminolevulinic acid synthetase. Science 179, 476-477.

71. Remmer, H. and Merker, H. J. (1963). Drug-induced changes in the liver endoplasmic reticulum - Association with drug metabolizing enzymes. Science 142, 1657-1658.

72. Rodgers, C. A. and Stalling, D. L. (1972). Dynamics of an ester of 2,4-D in organs of three fish species. Weed Sci. 20, 101-105.

73. Schenkman, J. B., Sligar, S. G. and Cinti, D. C. (1981). Substrate interaction with cytochrome P-450. Pharmac. Ther. 12, 43-71.

74. Schoor, J. A. and Schoor, W. P. (1979). Correlations of mixed-function oxidase activity with ultrastructural changes in the liver of a marine fish. Cancer Biochem. Biophys. 4, 95-103.

75. Sladek, H. E. and Mannering, G. J. (1966). Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene-treated rats. Biochem. Biophys. Res. Commun. 24, 668-674.

76. Snyder, R. and Remmer, H. (1979). Classes of hepatic microsomal mixed function oxidase inducers. Pharmacol. Ther. 7, 203-244.

77. Stanton, R. H. and Khan, M. A. Q. (1975). Components of the mixed-function oxidase of hepatic microsomes of fresh water fishes. Gen. Pharmac. 6, 289-294.

78. Staudt, H. Lichtenberger, F. and Ullrich, V. (1974). The role of NADH in uncoupled microsomal monooxygenations. Eur. J. Biochem. 46, 99-106.

79. Stegeman, J. J. and Chevion, M. (1980). Sex differences in cytochrome P-450 and mixed function oxygenase activity in gonadally mature trout. Biochem. Pharmac. 29, 553-558.

80. Sudershan, P. and Khan, M. A. Q. (1980). Metabolism of cis[^{14}C]chlordane and cis[^{14}C] photochlordane in bluegill fish. J. AGr. Food Chem. 28, 291-296.

81. Thomas P. E., Ryan, D. and Levin, W. (1976). An improved staining procedure for the detection of peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. 75, 168-176.

82. Vodicinik, M. J., Elcombe, C. E. and Lech, J. J. (1981). The effect of various types of inducing agents on hepatic microsomal monooxygenase activity in rainbow trout. Toxicol. Appl. Pharmacol. 59, 364-374.

83. Williams, C. H. and Kamin, H. (1962). Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver. J. Biol. Chem. 237, 587-595.

84. Yarbrough, J. D. and Chambers, J. E. (1979). The disposition and biotransformation of organochlorine insecticides in insecticide-resistant and susceptible mosquitofish. In Pesticide and Xenobiotic Metabolism in Aquatic Organisms, ACS Symposium Series, Khan, Lech, and Menn, Eds., American Chemical Society, Washington, D.C., pp. 145-149.