PURIFICATION AND CHARACTERIZATION OF THE HEPATIC MICROSOMAL MONOOXYGENASE SYSTEM FROM THE COASTAL MARINE FISH STENOTOMUS CHRYSOPS

by

Alan V. Klotz

B.A., Rice University

1978

Submitted to the Massachusetts Institute of Technology/
Woods Hole Oceanographic Institution
Joint Program in Biological Oceanography
August, 1983

in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Signature of Author:. ....
Massachusetts Institute of Technology/
Woods Hole Oceanographic Institution,
August, 1983

Certified by:. . ...
John J. Stegeman, Thesis Supervisor

Certified by:..... ....
Chris Walsh, Thesis Supervisor

Accepted by:.....
Chair, Joint Program in Biological Oceanography,
Massachusetts Institute of Technology/Woods Hole
Oceanographic Institution

PURIFICATION AND CHARACTERIZATION OF THE HEPATIC MICROSOMAL MONOOXYGENASE SYSTEM FROM THE COASTAL MARINE FISH STENOTOMUS CHRYSOPS

by

Alan V. Klotz

Submitted to the Massachusetts Institute of Technology/Woods Hole Oceanographic Institution Joint Program in Biological Oceanography on August 11, 1983 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Three cytochrome P-450 forms were highly purified (8-12 nmol/mg) from the hepatic microsomes of the untreated coastal marine fish Stenotomus chrysops (scup) by detergent solubilization and column chromatography. Scup cytochromes P-450A, P-450B and P-450E (labeled in order of elution from the first ion exchange column) had distinct spectroscopic properties, substrate profiles and minimum molecular weights on SDS-polyacrylamide gels (52.7, 45.9 and 54.3 K, respectively). The three purified cytochrome P-450 isozymes yielded different peptide maps when digested with a-chymotrypsin or S. aureus V8 protease. An additional hemoprotein fraction called cytochrome P-450 fraction D was also resolved and partially purified. This cytochrome P-450 preparation was characterized by a red shift in the CO-ligated, reduced difference spectrum with a chromophore near 451 nm. The scup NADPH-cytochrome P-450 reductase was purified to electrophoretic homogeneity (M_r = 82.6 K), had a specific activity of 45-60 U/mg with cytochrome c and contained both FAD and FMN.

Scup cytochrome P-450E (M_r = 54.3 K) is the major aryl hydrocarbon hydroxylating form in untreated hepatic microsomes as judged by both its abundance (30-50% of the total resolved cytochromes P-450) and catalytic activity with benzo[a]pyrene (turnover number = 0.9 nmol product/nmol P-450/min). Further, reconstituted cytochrome P-450E was inhibited 70-80% by 100 uM 7,8-benzoflavone in catalytic assays, similar to the 80-90% inhibition of benzo[a]pyrene hydroxylase in microsomal incubations. Analysis of benzo[a]pyrene products derived from reconstitutions of purified cytochrome P-450E revealed that greater than 50% of the oxidation occurred at benzo-ring positions, like the product profile observed in incubations with microsomes. The molecular weight of the purified cytochrome P-450E is identical to the major microsomal hemoprotein induced by 3-methylcholanthrene pretreatment, suggesting cytochrome P-450E is the major aromatic hydrocarbon-inducible cytochrome P-450 form in scup. Rabbit antisera raised

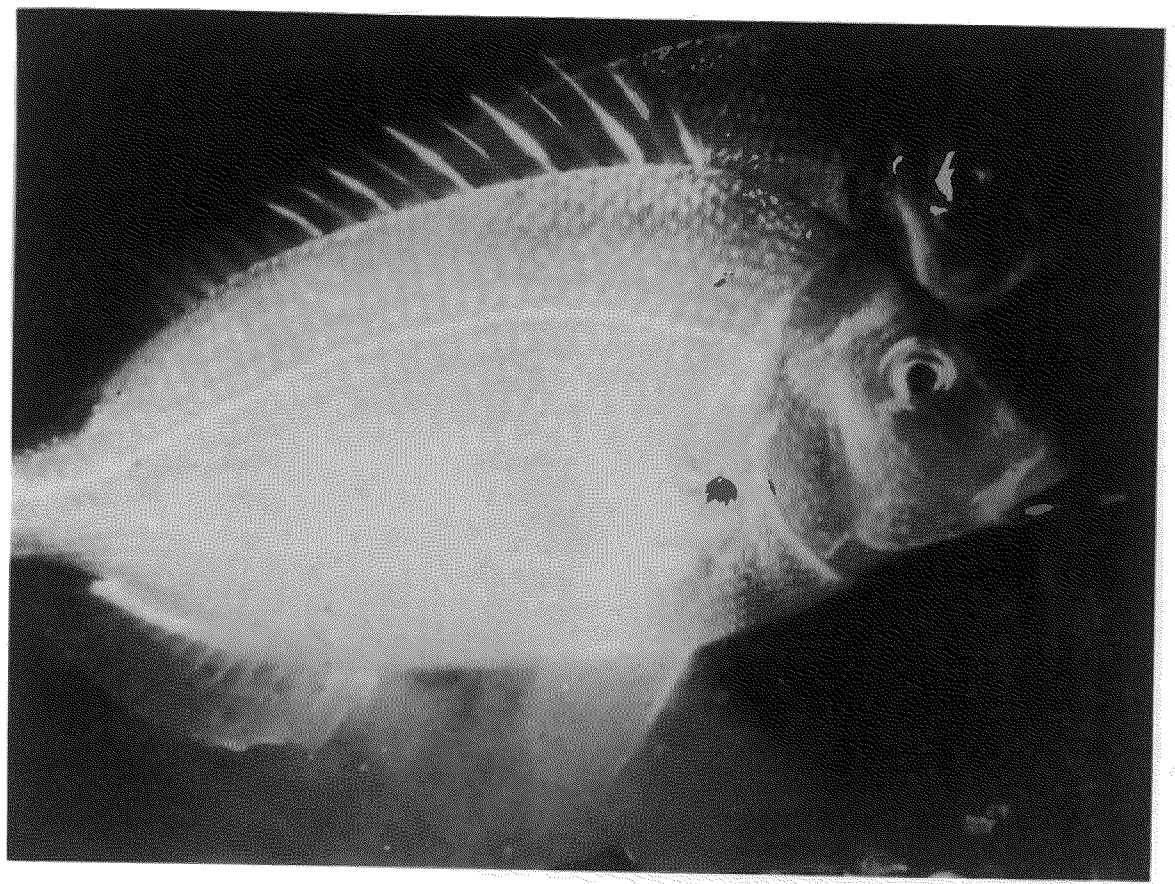
against purified scup cytochrome P-450E reacts in Ouchterlony diffusion analysis with cytochrome P-450E antigenic determinants in microsomes but not purified cytochrome P-450A. Further, the antisera cross-reacts with an apparent 3-methylcholanthrene-inducible cytochrome P-450 isozyme purified from trout liver (TLM-4a; Williams and Buhler, Comp. Biochem. Physiol. 75C: 25-32, 1983), giving a pattern of fusion without visible spurring in Ouchterlony analysis. These observations imply common antigenic determinants for the apparent major 3-methylcholanthrene-inducible cytochrome P-450 forms in trout and scup.

Monooxygenase reconstitution experiments indicated that purified scup cytochrome P-450A actively hydroxylated testosterone at the 6β position (turnover number = 0.8 nmol/min/nmol cytochrome P-450). Reconstituted cytochrome P-450B oxidized testosterone at two different sites tentatively identified as the 2-a and 15-a positions (total turnover number = 0.07 min^{-1}). Cytochrome P-450 fraction D produced several metabolites upon reconstitution (sum turnover number = 0.2 min^{-1}) including two chromatographically similar to 16a- and 16β-hydroxytestosterone. Reconstituted cytochrome P-450E was a poor catalyst of testosterone hydroxylase but the only detectable product was 6β-hydroxytestosterone (turnover number = 0.04 min^{-1}). However, besides benzo[a]pyrene, reconstituted cytochrome P-450E was active with 7-ethoxycoumarin, acetanilide and 7,8-benzoflavone as substrates.

Addition of purified scup cytochrome b_5 to monooxygenase reconstitutions had a stimulatory effect on some catalytic rates. The magnitude of the cytochrome b_5 stimulation depended on the P-450 isozyme and the substrate used in the reconstitution; cytochrome P-450A was generally influenced by the presence of cytochrome b_5. This rate stimulation was greater than the effect obtained with purified rabbit liver cytochrome b_5. In an extreme example, cytochrome P-450E was completely inactive in reconstitutions of 7-ethoxyresorufin O-deethylase (an activity associated in microsomes with aromatic hydrocarbon induction) in the presence or absence of rabbit cytochrome b_5 but the addition of scup cytochrome b_5 to the reconstitution led to an observed O-deethylation rate of 7.0 min^{-1}. It is uncertain whether these cytochrome b_5 effects are exhibited in microsomes or *in vivo* but the stimulation in reconstitutions appears to be important in the evaluation of catalytic activity with purified isozymes.

Thesis advisor: John J. Stegeman
Position: Associate Scientist, WHOI

Thesis advisor: Chris Walsh
Position: Professor of Chemistry and Biology, MIT



STENOTOMUS CHRYSOPS (LINNAEUS)

"There's some weird mechanism in the fish...I sure would like to get an answer."
Ronald O. Kagel, Director of Environmental Quality,
Dow Chemical, USA.

C&EN 61, 32 (1983).

"But ask the beasts... and the fish of the sea will declare to you."

Job 12, 7-8.

TABLE OF CONTENTS

List of Figures

List of Tables

ACKNOWLEDGEMENTS

I owe a great debt of gratitude to my thesis advisors, Chris Walsh and John Stegeman. Chris exposed me to the operation of "big science" in all its fullness and I can never repay him for that education. John triumphed in leading me to an understanding of the phrase "different but the same" as it applies in the comparison of biochemical systems; I believe I have taken that philosophical outlook to heart in this thesis research.

I have also acquired technical insights and specific methodologies from a host of aspiring scientists including Bob Binder, Bruce Branchaud, Hung-wen Liu, Bob Pascal, David Waxman and Bruce Woodin. Hung-wen Liu and Bob Pascal were particularly adept in the performance of ^{1}H-NMR spectroscopy. I would be remiss if I did not thank Dick Coburn, able captain of the RV Asterias, and the anonymous participants in our scup collecting voyages. My thesis research committee of Michael Marletta, John Stegeman, Craig Taylor, Stuart Wakeham and Chris Walsh were valuable as consultants in the process and Frank Carey was an able chairperson.

Financial support for my investigations was gleaned in part from a NSF Pre-doctoral Fellowship, the WHOI Education Office, WHOI Coastal Research Center grant 67.08, NSF grant OCE 80-18569 (J. S.) and NIH grant GM 21643 (C. W.). Finally, I thank my family, house-mates, and laboratory colleagues -- who have tolerated me for five short years.

ABBREVIATIONS AND OTHER TRIVIAL NAMES

AHH, aryl hydrocarbon (benzo[a]pyrene) hydroxylase

BP-OH, benzo[a]pyrene hydroxylase

DLPC, sonicated dilauroyl phosphatidylcholine

DOC, deoxycholate

DTT, dithiothreitol

ECOD, 7-ethoxycoumarin O-deethylase

EDTA, ethylene diamine tetraacetate

EROD, 7-ethoxyresorufin O-deethylase

FAD, flavin adenine dinucleotide

FMN, flavin mononucleotide

HEPES, 4-(2-hydroxyethyl)piperazine-ethanesulfonic acid

HPLC, high pressure liquid chromatography

kD, kilodaltons

3MC, 3(2Ø)-methylcholanthrene

NADH, reduced nicotinamide adenine dinucleotide

NADPH, reduced nicotinamide adenine dinucleotide phosphate

PAH, polycyclic aromatic hydrocarbon

PB, phenobarbital

PBS, phosphate buffered saline

PEG, polyethylene glycol

PMSF, phenylmethane sulfonyl fluoride

PTH-, phenylthiohydantoin-derivative

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TCA, trichloroacetic acid

TLC, thin-layer chromatography

Tris, tris(hydroxymethyl)-aminomethane

U, umol enzymatic product formed per min per mg protein

CHAPTER 1

HEPATIC MONOOXYGENASE METABOLISM AND THE CYTOCHROME P-450 SYSTEM

Xenobiotics such as pesticides, hydrocarbons and industrial wastes are distributed throughout the biosphere. The world oceans as the ultimate sink receive inputs from land runoff, atmospheric fallout, large scale dumping and spills of a chronic or acute nature. Anthropogenic contributions are significant both as point sources (Dunn and Stich, 1976) and via general atmospheric transport (Duce and Duursma, 1977; Neff, 1978). Polycyclic aromatic hydrocarbons (PAHs) are abundant in the aquatic environment (Giger and Blumer, 1974; LaFlamme and Hites, 1978) and it is clear that global transport occurs. Neff (1978) has estimated the annual flux into the aquatic environment at about 700 metric tons for benzo[a]pyrene and 230,000 metric tons for total PAH. Polychlorinated biphenyls are a second class of ubiquitous environmental contaminants (Risebrough and de Lappe, 1972; Kimbrough, 1974). Polychlorinated compounds are derived entirely from man's industrial activity, in contrast to PAHs which have varied origins. Many these diverse foreign compounds potentially present both a toxicological and mutagenic threat to aquatic inhabitants (Stegeman, 1981). While there are some data

suggesting a relationship between total environmental contamination and the incidence of tumors in fish (Brown et al., 1973; Mearns and Sherwood, 1973, Smith et al., 1979), the demonstration of a direct cause and effect relationship has been extremely difficult. Consequently the impact of these molecules on aquatic inhabitants is currently being investigated.

Foreign compounds are usually lipophilic and this property leads to facile transfer across cellular membranes. Significant accumulations of foreign compounds can occur within living tissues (Neff, 1978; Pancirov and Brown, 1977; Risebrough and de Lappe, 1972). One frequent response of organisms to the passive uptake of lipophiles involves enzymatically increasing the polarity of the unwanted compound by metabolism (Goldstein et al., 1974; Lu et al., 1976). Chemical modification by oxygen insertion leads to a more favorable partitioning of the derivative, further metabolism or conjugation, and potential attenuation of the molecule's biological activity. Many of the these metabolic oxidations are catalyzed by a multi-enzyme cytochrome P-450 monooxygenase system which is primarily located in the microsomal fraction (membranes derived from the endoplasmic reticulum, lysosomes and other cellular components) of hepatic tissues (Lu et al., 1976). Almost exclusively hepatic metabolism will be considered for the remainder of this thesis because liver is the major organ for xenobiotic metabolizing activities.

The observation that aquatic species possess these catalytic capacities is not novel (Creaven et al., 1965; Buhler and Rasmusson, 1968). However, subsequent work has suggested that certain untreated fish are very efficient at hydroxylating the model hydrocarbon substrate benzo[a]pyrene (Ahokas et al., 1975; Stegeman and Binder, 1979). The metabolic fate of PAHs such as benzo[a]pyrene is important in aquatic species because the cytochrome P-450 linked-monooxygenase processing of these compounds is involved in mutagenesis (Jerina and Daly, 1974; Sims and Grover, 1974). Prior PAH exposure enhances the capacities of the cytochrome P-450 complement in mammals to form mutagenic derivatives of benzo[a]pyrene by inducing cytochrome P-450 isozymes which preferentially form the ultimate mutagen (Rasmussen and Wang, 1974; Wood et al., 1976).

There are several major questions to address in aquatic toxicology. What is the <u>biochemical</u> nature of the benzo[a]pyrene hydroxylase activity in aquatic species i.e. how is the system in fish similar or different to that found in higher animals? What is the <u>biological</u> significance of the cytochrome P-450 monooxygenase system i.e. does it function in an adaptive manner, or is there an involvement between the metabolism of natural compounds and the foreign PAHs? It is recognized that PAHs and man-made contaminants are only one class of potential substrates for cytochrome P-450 monooxygenases <u>in vivo</u>. Naturally occurring compounds (or their relatives) such as flavones (Stegeman and Woodin,

1980) or safroles (Ryan et al., 1980) and endogenous molecules including fatty acids (Lu et al., 1969), bile acids (Danielsson and Sjovall, 1975), prostaglandins (Kupfer et al., 1978) and vitamin D_3 (Hansson et al., 1981) are also substrates *in vitro*.

Mammalian Hepatic Monooxygenase Systems

A scheme for cytochrome P-450-linked microsomal electron transport is presented in Figure 1-1 and the electron transfer components are summarized in Table 1-1. The terminal electron acceptor of this monooxygenase system is a cytochrome P-450 whose name is derived from its characteristic reduced, CO-difference spectrum (Omura and Sato, 1964). This cytochrome is a membrane-bound hemoprotein which coordinates the substrate and molecular oxygen at its active site. The cytochrome P-450 receives two electrons in single-electron transfer steps during the catalytic cycle, primarily from its membrane-bound attendant, the NADPH-dependent cytochrome P-450 reductase. The two-electron reduced [cytochrome P-450-0_2-substrate] complex fragments to produce water and an oxylide radical which inserts an oxygen atom into the substrate (White and Coon, 1980). Cytochrome P-450 formally catalyzes hydroxylations reflected as alkene epoxidations, O-, S- and N-dealkylations and C-, N- and S-oxidations.

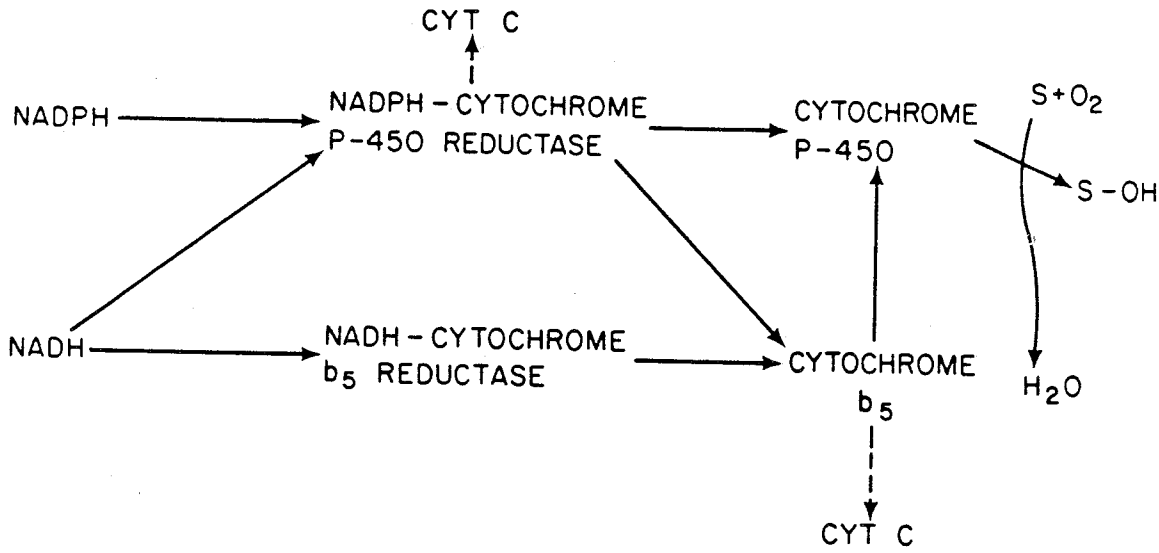


Figure 1-1. Pathways of hepatic microsomal electron transfer.

TABLE 1-1

Mammalian Liver
Microsomal Electron Transfer Components

Enzyme	Molecular Weight (kD)	Prosthetic Group	Reference
cytochrome b_5	16	1 Fe protoporphyrin IX	1
NADH-cytochrome b_5 reductase	33	1 FAD	2
cytochromes P-450	ca 50	1 Fe protoporphyrin IX	3
NADPH-cytochrome P-450 reductase	74-78	1 FAD & 1 FMN	4

kD, kilodaltons

References

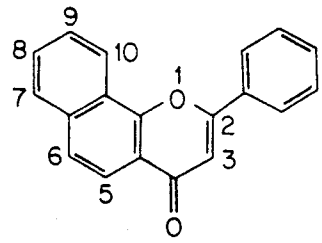
1. Strittmatter and Velick, 1956
2. Mihara and Sato, 1975
3. Lu and West, 1980; Johnson, 1979
4. Vermilion and Coon, 1978; French and Coon, 1979

The cytochrome P-450 reductase is a flavoprotein which normally accepts electrons from the reduced cofactor NADPH. Other in vitro electron transfer pathways include the reduction of NADPH-cytochrome P-450 reductase by high NADH concentrations (Noshiro et al., 1980), the reduction of cytochrome P-450 by cytochrome b_5 (West and Lu, 1977; Bonfils et al., 1981) and the reduction of cytochrome b_5 by the NADPH-cytochrome P-450 reductase (Enoch and Strittmatter, 1979). The physiological importance of these additional electron transfer pathways for xenobiotic oxidation is uncertain. Reconstitution studies suggest the NADH-mediated cytochrome b_5-dependent pathway for monooxygenase metabolism is a quantitatively minor contributor (ca 10%) to total benzo[a]pyrene oxidation potential (West and Lu, 1977). Cytochrome b_5 and the NADH-cytochrome b_5 reductase are clearly involved in a separate microsomal pathway for fatty acid desaturation (Oshino and Omura, 1973).

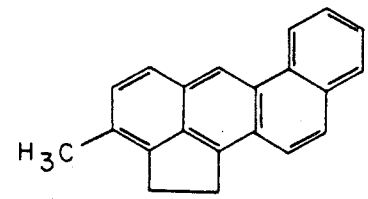
A short review of the replete mammalian literature will establish the framework for how the research problems are viewed in animals. One characteristic of the cytochrome P-450 monooxygenase system is its induction by a broad range of molecules. Early in the development of this field the use of inducers allowed researchers to infer the existence of multiple metabolic pathways (Conney, 1967). The variability in cytochrome P-450 monooxygenase metabolism is derived from the existence of multiple forms of cytochrome

P-450 (Lu and West, 1980). This isozyme family shares the same heme prosthetic group and certain other physical characteristics but has varied origins (Johnson, 1979). Responses to different inducers allow one to establish a simplified scheme for organizing the patterns of microsomal metabolism. Two broad classes of inducers have been distinguished. PAH inducers (Figure 1-2), e.g. 5,6-benzoflavone, benzo[a]pyrene, dimethylbenzanthracene, 3-methylcholanthrene (3MC) and many polychlorinated biphenyl isomers, elicit similar responses which shall be referred to as 3MC-type. The second major class of inducers are typically barbiturate-based drugs of which phenobarbital is the most commonly used and this will be called the PB-type. Other inducers such as pregnenolone 16a-carbonitrile (Lu et al., 1972b), ethanol (Koop et al., 1982), 2-acetylaminofluorene (Astrom et al., 1983) and isosafrole (Ryan et al., 1980) do not fall neatly into either of the pigeonholes outlined above.

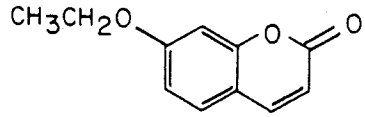
The typical 3MC-type response measured in microsomes is an increase in the cytochrome P-450 specific content, an increase in benzo[a]pyrene hydroxylase activity (also known as aryl hydrocarbon hydroxylase, AHH) with little change in N-demethylating activities, and a sensitivity of the benzo[a]pyrene hydroxylase activity to 7,8-benzoflavone inhibition (Wiebel et al., 1971). Several of these relevant aromatic hydrocarbon structures are displayed in Figure 1-2. Frequently, a spectroscopic shift of the CO-ligated,



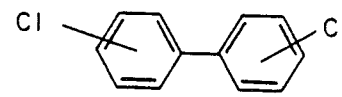
7,8-BENZOFLAVONE



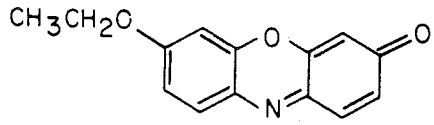
3-METHYLCHOLANTHRENE



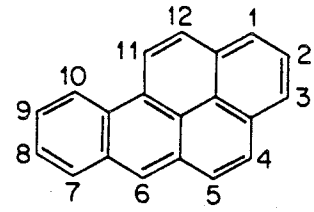
7-ETHOXYCOUMARIN



POLYCHLORINATED BIPHENYL



7-ETHOXYRESORUFIN



BENZO[α]PYRENE

Figure 1-2. Cardinal hydrocarbon structures.

TABLE 1-2

Purified Liver Microsomal Cytochromes P-450
From Two Favorite Mammalian Species*

Preparation	Molecular Weight (kD)	Preferred Substrate	Active Inducer	Comments	Reference
rabbit LM-1	48	benzphetamine progesterone	--		1,2
LM-2	48.7	benzphetamine	PB	major form	3
LM-3a	51	ethanol aniline	EtOH		4
LM-3b	52	aminopyrine testosterone	--	active steriod hydroxylation	5
LM-3c	53	p-nitroanisole?[&]	--		5
LM-4	55.3	acetanilide	3MC	major form	3,6
LM-6	57.5[#]	benzo[a]pyrene	3MC	minor form	1
LM-7[+]	60	benzo[a]pyrene	?		7

rat	P-450a	48	testosterone	--	active steroid hydroxylation	8
	P-450b	52	benzphetamine	PB	major form	8
	P-450c	56	benzo[a]pyrene acetanilide 7-ethoxyresorufin	3MC	major form	8,9
	P-450d	52	estradiol	3MC isosafrole	minor form	10,11
	P-450e	52.5	?	PB	highly similar to P-450b	11
	PCN	52	ethylmorphine?[@]	PCN		9
	UT-A	50.5	ethylmorphine testosterone	--	active steroid hydroxylation	9
	PB-C	47.5	ethylmorphine	PB		9

*Abbreviations: --, presumed to be constitutive and not very responsive to induction; EtOH, ethanol; kD, kilodaltons; 3MC, 3-methylcholanthrene-type inducers; PB, phenobarbital-type inducers; PCN, pregnenolone 16a-carbonitrile.

&While Koop et al. (1981) have found no cytochrome b_5 requirement for this activity, Sugiyama et al. (1980) have simultaneously reported an absolute requirement for cytochrome b_5 in reconstitutions of a purified cytochrome $P\text{-}450_{B1}$ which is otherwise highly similar to LM-3c. These data have not been recounciled in the literature but I assume that cytochromes $P\text{-}450_{B1}$ and LM-3c are the same form.

#The originally reported 60 kD molecular weight has since been renounced as an error (Johnson, 1979).

+LM-7 has never been highly purified, in part due to its instability, and consequently its precise nature is less completely defined.

@Elshourbaghy and Guzelian (1980) have reported immunochemical data to show that the PCN cytochrome P-450 isozyme is responsible for a majority of the potent ethylmorphine N-demethylase activity in PCN-induced rat liver microsomes. The reconstitutions of the purified PCN isozyme do not evidence this high catalytic activity for reasons that are not understood.

References

1. Johnson and Muller-Eberhard, 1977b
2. Dieter et al., 1982
3. Haugen and Coon, 1976
4. Koop et al., 1982
5. Koop et al., 1981
6. Johnson and Muller-Eberhard, 1977c
7. Guengerich, 1977
8. Ryan et al., 1979
9. Guengerich et al., 1982a
10. Ryan et al., 1980
11. Ryan et al., 1982

reduced cytochrome P-450 complex λ_{max} from ca 450 nm to 448 nm (Alvares et al., 1967) accompanies the induction events.

PB treatment induces a greater number of activities in mammalian liver including a proliferation of smooth endoplasmic reticulum, increased levels of cytochrome P-450, NADPH-cytochrome P-450 reductase and elevated cytochrome P-450-linked N-demethylation levels (Conney, 1967). Phenobarbital increases benzo[a]pyrene hydroxylase to a lesser extent than 3MC-type inducers and produces a catalytically different cytochrome P-450 complement with no spectroscopic shift, and with stimulation by 7,8-benzoflavone (Wiebel and Gelboin, 1975).

Certainly the cytochrome P-450 monooxygenase system is complex and precise details can best be unraveled in purified systems. As an aid to the ensuing discussion, Table 1-2 displays the salient features of cytochrome P-450 isozymes purified from two common laboratory animals. A minimum of seven distinct purified cytochrome P-450 forms have been characterized in rabbit liver (Haugen and Coon, 1976; Johnson and Muller-Eberhard, 1977b; Koop et al., 1981, 1982; Dieter et al., 1982). These cytochrome P-450 forms are enumerated according to their electrophoretic mobility on sodium dodecyl sulfate (SDS)-polyacrylamide denaturing gels with the caveat that measurements made in different laboratories frequently exhibit inconsistencies. A nomenclature introduced by Professor M. J. Coon and coworkers has

taken root in the field and is the reference frame for rabbits. Each rabbit cytochrome P-450 species is prefixed LM for liver microsomal and numbered in order of increasing apparent subunit molecular weight upon SDS-PAGE analysis. The substrate specificities for various cytochrome P-450 species overlap extensively (Guengerich, 1977) so the catalytic discriminations are only on a relative basis in most cases.

Analysis of the different reports on rabbit cytochrome P-450 isozymes supports several generalizations. LM-2 is the major form present in microsomes after phenobarbital induction. It has been purified to homogeneity and is the best characterized of all animal cytochrome P-450 preparations (Johnson, 1979). LM-4 is the major cytochrome P-450 induced in adults by 3MC-type inducers (Johnson and Muller-Eberhard, 1977a) and LM-4 has also been induced in adult rabbits by pretreatment with isosafrole (Delaforge et al., 1982). It has 7-ethoxyresorufin O-deethylase activity but is inefficient at aggregate benzo[a]pyrene hydroxylation (Johnson and Muller-Eberhard, 1977c). LM-6 is the minor induced form of cytochrome P-450 in adult microsomes upon 3MC-type treatment and is the major induced form found in neonates (Norman et al., 1978). It has high activities with both 7-ethoxyresorufin and benzo[a]pyrene which are sensitive to 7,8-benzoflavone inhibition (Norman et al., 1978). LM-2 is quite different from LM-4 and LM-6 in substrate specificity, spectroscopic characteristics,

antibody cross-reaction (Dean and Coon, 1977; Johnson and Muller-Eberhard, 1977b) and COOH- or NH_2-terminal sequence (Haugen and Coon, 1976; Black et al., 1982). These three isozymes are highly enriched in rabbit liver microsomes after a particular induction regime while the more mundane LMs 1 and 3a-c appear to be under a different type of regulation (Koop et al., 1981, 1982; Dieter et al., 1982) although LM-3a is induced by chronic alcohol exposure.

Rat liver microsomes are similarily wealthy in cytochrome P-450 isozymes. Guengerich (1982a) has characterized eight different forms purified from rat liver under various conditions. There is no established nomenclature in rats so reference is made wherever possible to the active inducer for the cytochrome P-450 isozyme in question. The major rat PB isozyme has a preference for benzphetamine and N,N-dimethylaniline N-demethylations (P-450b; Ryan et al., 1979). The major rat 3MC-type isozyme efficiently catalyzes benzo[a]pyrene hydroxylase, 7-ethoxyresorufin and 7-ethoxycoumarin O-deethylase (P-450c; Ryan et al., 1979). A rat cytochrome P-450 specifically induced by pregnenolone 16a-carbonitrile has also been purified and analyzed (Elshourbaghy and Guzelian, 1980). Lack of antibody cross-reactions indicate the pregnenolone 16a-carbonitrile form has antigenic determinants different from either the PB or 3MC isozymes and is a distinct polypeptide. An isosafrole-inducible cytochrome P-450 has also been purified and characterized (P-450d; Ryan et al., 1980). The isosafrole-

inducible form shares some antigenic determinants with the 3MC-inducible form but is distinct by several other criteria including subunit molecular weight, catalytic profile and peptide map. This isosafrole-inducible form is almost certainly identical to a minor aromatic hydrocarbon-inducible form identified by purification from rats induced with hexachlorobiphenyl (Goldstein et al., 1982; Parkinson et al., 1983). Amino-terminal sequence analysis indicates no homology with the major PB-, 3MC- or isosafrole-inducible cytochrome P-450 isozymes (Botelho et al., 1979; Botelho et al., 1982). Several "endogenous" cytochrome P-450 has also been resolved from microsomes after various treatments (Ryan et al., 1979; Cheng and Schenkman, 1982; Guengerich et al., 1982a; Waxman et al., 1983). These cytochrome P-450 forms have poor catalytic activity with many xenobiotic test substrates and often appear to be more active with steroid substrates (e.g. P-450a and UT-A; see Table 1-2).

The effort in purifying the mouse cytochrome P-450 complement has been less assiduous to date. Two research groups have reported a resolution of two fractions after different induction regimes. Fractions A_2 and C_2 have been purified from microsomes after PB treatment (Huang et al., 1976). Also two forms, labeled P-448 and P_I-450, have been resolved after 3MC induction (Negishi and Nebert, 1979). The subunit molecular weights were identical in the later case; however, distinctions arose in the λ_{max} and substrate discrimination. Cytochrome P_I-450 was twice as

efficient at metabolizing benzo[a]pyrene while cytochrome P-448 preferred acetanilide by a similar margin. Although these differences are not striking they were upheld by immunochemical criteria and peptide mapping.

Multiple cytochromes P-450 have been detected in other animals (e.g. Abe and Watanabe, 1983; Tsuji et al., 1980) including humans (ex. Guengerich et al., 1981). The potential number of cytochrome P-450 species in a given animal is a matter of contention. Some researchers prefer to view the diversity as parallel to immunoglobulins (Nebert, 1979). Technical difficulties in purifying the membrane proteins and the lack of stringent criteria for cytochrome P-450 homogeneity and identification make it difficult to evaluate the true extent of the diversity. Amino acid and nucleic acid sequence analysis suggest that, at least in some cases, the major isozyme induced in a given animal species is related to the analogous protein inducible under similar conditions in a different animal species (Heinemann and Ozols, 1983; Fuji-Kuryama et al., 1982; Botelho et al., 1979). However, within the same animal species the major cytochrome P-450 forms responding to different inducer types are more distantly related immunochemically (Thomas et al., 1976a, 1976b; Dean and Coon, 1977) and in NH_2-terminal sequence analysis (Botelho et al., 1979).

The field of cytochrome P-450 metabolism and induction is fascinating both because the complicated interactions of

compounds in their roles as potential substrates, inhibitors and inducers further confuse the *in vivo* implications, and because the processing of aromatic hydrocarbons is associated with mutagenesis. The formation of aromatic epoxides (arene oxides) is particularily deleterious (Jerina and Daly, 1974; Sims and Grover, 1974). In the case of benzo[a]pyrene the most mutagenic metabolite, a benzo-ring diol-epoxide, is preferentially formed by isozymes which are 3MC-inducible (Wood et al., 1976; Pezzuto et al., 1978). This illustrates a potential disadvantage to both metabolism and induction.

Monooxygenase Activities in Aquatic Species

When one turns to the aquatic sphere the number of reports is considerable but the picture is much more fragmented. Several comparative studies have established exhaustively that aquatic organisms have the complete cytochrome P-450 monooxygenase and conjugating system described in mammals (Buhler and Rasmusson, 1968; Bend et al., 1975). The enzymes are qualitatively similar by substrate profile (Pohl et al., 1974), subcellular localization (Stegeman et al., 1979), EPR spectrum (Chevion et al., 1977; Stegeman and Chevion, 1980) and tissue distribution (Stegeman et al., 1979). Marine fish will respond predictably in the laboratory to 3MC-type inducers (Elcombe and Lech, 1979; James and Bend, 1980) but show no

PB induction response (Bend et al., 1975; Elcombe and Lech, 1979; Balk et al., 1980). Fish respond to isosafrole (Vodicnik et al., 1981) and there are reports of an effect obtained with pregenolone 16a-carbonitrile (Hansson et al., 1980) in addition.

Elevated levels of AHH in fish are attributed on the basis of kinetic evidence to environmental induction in several cases (Payne, 1976; Stegeman, 1978; Kuralec et al., 1977; Stegeman et al., 1981). It has been suggested for several years that the cytochrome P-450 monooxygenase system (more specifically, AHH activity) should be a convenient monitor of environmental quality given its characteristic induction response (Payne, 1976). Nevertheless the utility of this catalytic activity applied as a diagnostic tool requires critical evaluation by a definition of baseline conditions and careful consideration of confounding variables (Stegeman, 1981).

Chemical induction experiments have demonstrated exhaustively that numerous marine fish species possess hepatic microsomal benzo[a]pyrene hydroxylase activity in the untreated state and yield a 10- to 35-fold induction upon 3MC-type treatment in the laboratory. In all but two cases the response to 3MC-type inducers is an increased benzo[a]pyrene hydroxylase activity with an enhanced sensitivity to 7,8-benzoflavone inhibition. No response was elicited in one exceptional case (Bend et al., 1975); this is attributed to pharmacokinetic difficulties with the

treatment route as the animal were inducible with other methods (James and Bend, 1980). The resulting benzo[a]-pyrene hydroxylase activity was stimulated by 7,8-benzo-flavone in the second atypical case (James and Bend, 1982) -- certainly this observation warrants further investigation.

The benzo[a]pyrene hydroxylase activity is extra-ordinarily high in the untreated state for two cases: European lake trout (Ahokas et al., 1975) and scup (Stegeman and Binder, 1979). This raises the possibility of environmental induction but is certainly tentative. One approach to verify the induced character of the benzo[a]-pyrene hydroxylase activity has been the application of the in vitro inhibitor 7,8-benzoflavone which in the case of mammalian liver appears to be a reliable indicator of 3MC-induced activities (Thorgeirsson et al., 1979). In untreated fish, however, the sensitivity of the benzo[a]-pyrene hydroxylase activity to 7,8-benzoflavone falls into two categories. Little skate (Bend et al., 1976), croaker (Stegeman et al., 1981) and a majority (greater than 80 %) of sheepshead (James et al., 1979) are stimulated by 100 uM 7,8-benzoflavone in a pattern identical to observations in mammals. But many aquatic species (including to date all fish with high levels of benzo[a]pyrene hydroxylase activity) are inhibited by 7,8-benzoflavone -- scup (Stegeman and Binder, 1979), rainbow trout (Elcombe and Lech, 1979), brook trout (Stegeman and Chevion, 1980) and the estuarine killifish (Stegeman, 1979). One formal

possibility for this second category is that endogenous fish cytochromes P-450, unlike the mammalian counterparts, are sensitive to 7,8-benzoflavone. Alternatively, these observations may reflect partial inductions in several species. Thus, some clarifying investigations are necessary before AHH activity levels may be used as an unambiguous criterion to evaluate potentially induced states in fish.

A closer examination of hepatic microsomal cytochrome P-450 monooxygenase characteristics from aquatic species is informative. Table 1-3 summarizes the few cases where cytochrome P-450 preparations which have been purified from aquatic species.

Little skate. Raja erinacea have a very low benzo[a]-pyrene hydroxylase activity which was stimulated in vitro three-fold by 100 uM 7,8-benzoflavone. Treatment with 3MC-type inducers produces up to a 35-fold induction of benzo[a]-pyrene hydroxylase activity (Bend et al., 1976) however the microsomal CO-ligated, reduced difference spectrum does not shift from 450 nm. Two cytochrome P-450 fractions have been resolved from dibenzanthracene-treated microsomes (Table 1-3). The so-called P-448 fraction had a turnover number of 0.8 nmol product/nmol P-450/min with benzo[a]pyrene upon reconstitution, is very sensitive to 7,8-benzoflavone inhibition and has a λ_{max} at 448 nm (Bend et al., 1979). A cytochrome P-451 fraction has also been separated in larger quantities (reported to be 2:1) which readily accounts for the lack of a wavelength shift in microsomes.

TABLE 1-3

Liver Microsomal Cytochromes P-450
Purified from Aquatic Species*

Species		nmol/mg	Molecular Weight (kD)	Preferred Substrate	Other Comments	Reference
little skate	P-448	1.6	--	BP	inhibited by ANF	1
	P-451	0.95	--	?	stimulated by ANF	2
spiny lobster	D1	14	52	progesterone	active steroid hydroxylation	3
rainbow trout	LM-2	10	54	?	activates aflatoxin	4,5
	LM-4a	11	57	BP	same as LM-4b?	4,6,7
	LM-4b	11	57	7-ER, BP	"major" BNF induced form	4,6,7

*Abbreviations: ANF, 7,8-benzoflavone; BNF, 5,6-benzoflavone; BP, benzo[a]-pyrene; 7-ER, 7-ethoxyresorufin; kD, kilodaltons.

References

1. Bend et al., 1979
2. Ball et al., 1979
3. James and Little, 1980; James and Shiverick, 1983
4. Williams and Buhler, 1983a
5. Williams and Buhler, 1983c
6. Williams and Buhler, 1982
7. Williams and Buhler, 1983b

This cytochrome P-451 fraction was relatively inactive toward benzo[a]pyrene with a turnover number of 0.08 min^{-1} upon reconstitution and was stimulated by 7,8-benzoflavone (Ball et al., 1979).

Trout. The levels of benzo[a]pyrene hydroxylase activity are very low in brook trout (Salvelinus fontinalis) and rainbow trout (Salmo gairdneri; Elcombe and Lech, 1979; Stegeman and Chevion, 1980). These observations make environmental induction a less attractive hypothesis despite a sensitivity to 7,8-benzoflavone inhibition. It is possible that 7,8-benzoflavone inhibition in these cases betrays an example of trace induction where an inhibitor-susceptible and aromatic hydrocarbon-inducible cytochrome P-450 form is present at very low levels. Such a possibility is difficult to exclude until the sensitivity of an immunochemical assay is available to distinguish cytochrome P-450 isozymes. Another species, European lake trout (Salmo trutta), however, has a much higher benzo[a]pyrene hydroxylase activity in the untreated state (Ahokas et al., 1975, 1977) yet the cytochrome P-450 difference spectrum peaks near 450 nm. These complications conspire against using a single catalytic activity as an unambiguous index of induction. Indeed, distinctions between untreated male and female trout in the levels of cytochrome P-450 and sensitivity to 7,8-benzoflavone suggest a hormone-related difference in the cytochrome P-450 monooxygenase complement (Stegeman and Chevion, 1980).

Treatment with 5,6-benzoflavone in rainbow trout causes the enrichment of a microsomal hemoprotein at 57,000 daltons from virtually undetectable levels in the control and a 40-fold increase in benzo[a]pyrene hydroxylase activity (Elcombe and Lech, 1979). Subsequently a cytochrome P-450 form has been purified and characterized from the microsomes of 5,6-benzoflavone treated rainbow trout (Williams and Buhler, 1982; 1983b). This cytochrome P-450 form which the investigators have more recently termed trout LM-4b (see Table 1-3 and Chapter 2, footnote 12) has a CO-ligated, reduced difference spectrum which is blue-shifted to near 447 nm, a molecular weight around 57,000 and is catalytically active with benzo[a]pyrene (1.1 nmol product/nmol cytochrome P-450/min) and inhibited by 7,8-benzoflavone upon reconstitution.

Croaker. Micropogon undulatus have a low benzo[a]pyrene hydroxylase activity which is induced 15-fold by pretreatment with benzo[a]pyrene concomitant with a 1 nm blue shift in the spectrum to 449 nm (Stegeman et al., 1981). Benzo[a]pyrene hydroxylase activity is slightly stimulated in the control state but upon induction the inhibition by 100 uM 7,8-benzoflavone is greater than 90%.

Estuarine marshminnow. Fundulus heteroclitus is one of the species believed to have suffered pollution-related induction (Stegeman, 1978). The differences between control and polluted sites are significant on an AHH activity basis but not by the criteria of catalytic efficiency (nmol

product per nmol cytochrome P-450) in this case. Generally, untreated Fundulus are quite sensitive to 7,8-benzoflavone inhibition but fish treated with benzo[a]pyrene showed a two-fold induction of benzo[a]pyrene hydroxylase activity and a slight wavelength shift toward 449 nm (Stegeman, 1979) indicating a capacity for further induction.

Winter flounder and sheepshead. Two species show a natural cline of benzo[a]pyrene hydroxylase activity and a concordant sensitivity to 7,8-benzoflavone. This pattern may be the consequence of partial induction in the field. Over 400 winter flounder (Pseudopleuronectes americanus) surveyed as individuals exhibited a dynamic range from high benzo[a]pyrene hydroxylase activity which was strongly inhibited by 7,8-benzoflavone to low benzo[a]pyrene hydroxylase activity which was stimulated by 7,8-benzoflavone (Foureman and Bend, 1982). Benzo[a]pyrene hydroxylase activity levels were also correlated with 7-ethoxyresorufin O-deethylase activity which in mammals is tightly linked with 3MC induction. Sheepshead (Archosargus probatocephalus) also have shown a similar range of individual natural variation (James et al., 1979; James and Bend, 1980). In a majority of cases benzo[a]pyrene hydroxylase activity was low and it was also stimulated by 7,8-benzoflavone. Treatment with 3MC then induces 16-fold higher benzo[a]pyrene hydroxylase activity which was inhibited by 7,8-benzoflavone (James et al., 1979) and a 1-2 nm spectral shift was observed (James and Bend, 1980).

Scup. Untreated freshly sampled populations of Stenotomus chrysops (formerly S. versicolor) have high levels of cytochrome P-450 and the amount of benzo[a]pyrene hydroxylase activity is remarkably elevated (Stegeman and Binder, 1979). Congruent with this observation the benzo[a]-pyrene hydroxylase activity is routinely sensitive to 7,8-benzoflavone inhibition. Measurements on over 100 field sampled individuals have uncovered no exceptions to the generalization of high benzo[a]pyrene hydroxylase activity and inhibition by 7,8-benzoflavone (Stegeman et al., 1981). Induction experiments have proven that in some scup the microsomal monooxygenase activity is responsive with a three-fold increase in benzo[a]pyrene hydroxylase activity, a greater elevation in 7-ethoxyresorufin O-deethylase activity and a wavelength shift to 448 nm (Stegeman et al., 1981). The induction experiment also revealed a 54,000 dalton hemoprotein associated with the hydrocarbon-treatment. However this induction pattern is not readily reproduced and it is believed that many untreated scup in the waters off Cape Cod have already expressed their full detectable induction response.

Thus, the results of induction experiments in several fish species indicate a common state is achieved with 3MC-type inducers. Aromatic hydrocarbon pretreatment produces an induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity, 7-ethoxyresorufin O-deethylase activity and an increased inhibition of benzo[a]pyrene

hydroxylase activity by 7,8-benzoflavone in hepatic microsomes (Elcombe and Lech, 1979; James and Bend, 1980). Untreated fish already possess this set of features in some cases i.e. elevated benzo[a]pyrene hydroxylase and 7-ethoxyresorufin O-deethylase activities which are both extremely sensitive to 7,8-benzoflavone. Aromatic hydrocarbon induction has also been associated with a blue-shift in the CO, reduced difference spectrum of the hepatic microsomal cytochrome P-450 or the increase of a particular microsomal hemoprotein (Elcombe and Lech, 1979; Stegeman et al., 1981). Such observations have been taken as evidence of a specific cytochrome P-450 isozyme induced by aromatic hydrocarbon pretreatment in these fish species.

Analysis of benzo[a]pyrene metabolites produced by fish hepatic microsomes indicates a strong preference for benzo-ring metabolism (ca 50 % of the total products) in scup (Tjessum and Stegeman, 1979), trout (Ahokas et al., 1979), Fundulus and winter flounder (Stegeman et al., 1983), starry flounder and coho salmon (Varanasi and Gmur, 1980). Little skate is one of few fish species examined where significant metabolism in other regions occurs (Bend et al., 1979). In general, regiospecific metabolism of benzo[a]pyrene at benzo-ring positions is associated with 3MC-type induction in mammals (Pezzuto et al., 1978) and this metabolism is related to the mutagenic action of benzo[a]pyrene (Wood et al., 1976). Benzo[a]pyrene metabolites produced by liver microsomes from scup or winter flounder are active in

bacterial mutagen assays and scup liver microsomes can activate several related PAHs including dimethylbenz-anthracene and dibenzanthracene (Stegeman et al., 1982b). These observations are consistent with active PAH metabolism in fish analogous to that found in mammals upon 3MC-type induction.

Some differences exist in patterns of induction in fish and mammals. While 3MC-type inducers do not generally affect NADPH-cytochrome P-450 reductase levels in mammals (Conney, 1967) significant induction of the NADPH-specific cytochrome c reductase in fish has been observed (Yarbrough and Chambers, 1978; Stegeman, 1979). Interestingly, benzo[a]pyrene also induces the levels of microsomal cytochrome b_5 three-fold in at least one fish species (Stegeman, 1981). While 3MC induction in mammals is also associated with elevated levels of the conjugating enzymes this is not the general case in fish. Epoxide hydrolase and glutathione-S-transferase activities are constant during otherwise successful inductions (Balk et al., 1980; James and Bend, 1980). The consequent toxicological risk has not been assessed but may be significant in light of mammalian models. Several studies have established that the major route of clearance for benzo[a]pyrene in fish is hydroxyl-ation and conjugation. Up to 90% of the absorbed dose was found in the liver or bile as glucuronide conjugates in one system (Varanasi and Gmur, 1981). Prior laboratory induction can increase the rates of metabolism and clearance

of another polycyclic aromatic hydrocarbon, 2-methylnaphthalene, (Statham et al., 1978) demonstrating that induction has effects on xenobiotic processing *in vivo*.

Both laboratory (James and Bend, 1980) and field observations (Kurelec et al., 1977) indicate that the effects of induction in fish are long-lasting. This strengthens the suggestion that certain characteristics may be attributable to environmental induction. However complications arise due to high natural variation within populations (James and Bend, 1980) and the possible dependence of benzo[a]pyrene hydroxylase activity on reproductive status (Stegeman et al., 1982b; Stegeman and Chevion, 1980) which preclude simple interpretations.

Scope of Thesis Research

The goal of research in the field of xenobiotic metabolism is to address the question of how foreign compounds affect natural populations. What is the capacity of marine organisms to metabolize xenobiotics and what parameters influence this? What are the metabolic roles of the cytochrome P-450 monooxygenase constitutents? What are the toxification/detoxification pathways?

It is clear that only the first half of one question has been adequately characterized in the literature -- there is a reasonable picture of what capacities exist *in vitro* and how they might relate *in vivo*. Yet, precise

descriptions are lacking for regulation of these complex patterns of metabolism. Until the various cytochrome P-450 isozymes have been more rigorously identified it is difficult to study their distribution and regulation. Further, the availability of purified cytochrome P-450 forms from lower animals will provide valuable information in the comparative biochemistry and interspecies relationships of this interesting family of isozymes.

The marine teleost fish Stenotomus chrysops (scup) was chosen at the onset for detailed investigations because it is a species which represents the premier research questions posed in marine systems. Fortunately, their microsomal activities have been extensively characterized (Stegeman and Binder, 1979; Stegeman et al., 1979; Stegeman et al., 1981) and the data set in Dr. Stegeman's laboratory representing collections since 1973 is an adequate base for my strategy of characterization by resolution, purification and reconstitution of the cytochrome P-450 monooxygenase components. As discussed above, the properties of microsomes prepared from untreated scup suggest that the feral population we are studying is partially induced. This makes the results particularly relevant as they can be related to effects observed in natural populations. My dissertation research has sought to specifically address three questions in scup:

1. What is the nature of the cytochrome(s) P-450 responsible for the high benzo[a]pyrene hydroxylase activity?

2. What is the catalytic spectrum of the remaining cytochrome P-450 forms?

3. How might these monooxygenase activities be related to induction?

A cautionary word on the strategy I have adopted is in order. The major focus of my research has been to purify the monooxygenase components in order to characterize the individual catalytic entities. Purification is the best method (in my opinion) to verify the conclusions about the nature of the catalysts responsible for microsomal metabolism because assessments of catalytic function in microsomal studies based on kinetic data are necessarily tentative. However, the results with a purified system can also be somewhat ambiguous. It is necessary to reconstitute activity after purification because the cytochrome P-450 monooxygenase system involves multiple cofactors and membrane-associated enzymes. The precise method of purification and reconstitution of catalytic activity can influence the subsequent studies. Consequently, it is advisable to consider data from both microsomal preparations and the reconstitutions before drawing conclusions.

Purification of membrane proteins requires solubilization of the components with detergents but the physical picture of events during this process is hazy (Helenius,

1975). Such treatment extracts the proteins out of the natural phospholipid vesicles into mixed detergent-lipid vesicles or micelles of a smaller size. The solubilized membrane proteins are then purified in a detergent environment by conventional techniques of column chromatography and selective precipitation. Solubilization facilitates the resolution of different hydrophobic proteins but interrupts the catalytically essential interactions between cytochrome P-450 and NADPH-cytochrome P-450 reductase. The two proteins must be returned to intimate association by the reconstitution in the absence of detergents in order to regain monooxygenase activity. While catalytic specificity resides with the particular cytochrome P-450 (Lu et al., 1972a), the cytochrome P-450 reductase and a lipid environment are required for activity (Lu et al., 1969). The lipid requirement is frequently not absolute since some detergents may play that role (Denk, 1979), however there is some specificity to the type of lipid used in reconstituted systems and diacylglyceroyl phosphatidylcholine has been identified as the most active component (Strobel et al., 1970).

Two reconstitution systems are widely reported in the literature. Most popular is the nonmembranous dilauroyl-glyceroyl phoshatidylcholine (DLPC) system where the monooxygenase components aggregate together with some associated phospholipid in a complex of approximately 800,000 daltons (French et al., 1980). Maximum turnover

rate can only be achieved with a steady state 1:1 molar ratio of cytochrome P-450 to NADPH-cytochrome P-450 reductase under these conditions (Miwa et al., 1979; French et al., 1980). This catalytically competent complex is readily formed with a K_d = 50 nM (Miwa et al., 1979) but is both an inefficient use of the NADPH-cytochrome P-450 reductase and extremely unnatural. The cytochrome P-450 is present in 15- to 20-fold molar excess of its reductase in mammalian microsomes (Peterson et al., 1976). Native scup hepatic microsomes contain cytochrome P-450 and NADPH-cytochrome P-450 reductase in the approximate molar ratio of 22:1. Supplementation of rat microsomes under various conditions with purified NADPH-cytochrome P-450 reductase indicates that either or both monooxygenase components can be rate-limiting. The nature of the limiting component and the magnitude of the effect depends on both the substrate and the experimental treatment of the microsomes (Miwa et al., 1978).

It would appear that an alternative membranous liposomal system would be the preferable medium for reconstitution. Egg yolk phosphatidylcholine has been used for this purpose to recreate optimal conditions where a 22-fold excess of cytochrome P-450 over NADPH-cytochrome P-450 reductase was still operating at v_{max} (Ingelman-Sundberg and Glaumann, 1977; Ingelman-Sundberg and Johansson, 1980). The catalytic rate and substrate specificity is the same as the DLPC system in most cases; more importantly, the requirement for

stoichiometric reductase levels is relieved. I have adopted the DLPC system due to its technical simplicity but I am cognizant of the potential pitfalls.

A subsidiary issue in reconstitution is the role of cytochrome b_5 in cytochrome P-450 monooxygenase catalysis. An absolute requirement for cytochrome b_5 in reconstitutions has been demonstrated only under a limited number of conditions with four substrates to date (Sugiyama et al., 1980; Kuwara and Omura, 1980; Coon et al., 1980; Vatsis et al., 1982). Cytochrome b_5 stimulates the rate of turnover but is not obligatory (Imai, 1981; Vatsis et al., 1982; Waxman and Walsh, 1982, 1983) in many other cases. It has been postulated that the reduction of cytochrome P-450 by the second electron is a partially rate-determining step in overall turnover. Cytochrome b_5 is more efficient at delivering the second equivalent than the NADPH-cytochrome P-450 reductase in this view (Hildebrand and Estabrook, 1971; Bonfils et al., 1981) although the <u>in vivo</u> meaning of this effect remains obscure. The cytochrome b_5 levels are frequently much lower in fish than mammals relative to cytochrome P-450. Further, the NADH synergism associated in mammalian microsomes with the beneficial effect of cytochrome b_5 is generally not evident in the few fish systems which have been examined. Initially it appeared to me that cytochrome b_5 was not an active participant in microsomal xenobiotic oxidation. However, microsomal aminopyrine N-demethylase

activity is efficiently supported by NADH in scup (Stegeman et al., 1979) suggesting the intermediacy of cytochrome b_5 in the microsomal electron transfer manifold and I have revised my earlier estimate.

The research described in this thesis is presented in the order one might follow when endeavoring to reproduce it. Chapter 2 details the solubilization, resolution and purification of the various scup microsomal monooxygenase components including the NADPH-cytochrome P-450 reductase, cytochrome b_5, cytochrome P-450A, cytochrome P-450B, cytochrome P-450 fraction D and cytochrome P-450E. The physical characterization of these enzymes is also contained in that chapter. Chapter 3 describes the reconstitution of these components in a DLPC system and assays of catalytic activity and product analysis. Chapter 4 synthesizes the conclusions from the foregoing chapters to present a summary of the questions which have been answered and the new research questions which have been unmasked. Finally, Chapter 5 (Appendix) contains several experiments which were indirectly related to the thesis research as outgrowths of queries that arose along the way.

CHAPTER 2

RESOLUTION AND PURIFICATION OF THE SCUP HEPATIC MICROSOMAL MONOOXYGENASE COMPONENTS

Introduction

It was necessary to purify and characterize the individual enzymes in order to study the scup monooxygenase components. The resolution of five hepatic microsomal cytochrome P-450-containing fractions is described in this chapter. Three cytochrome P-450 forms were highly purified from this milieu as well as the NADPH-cytochrome P-450 reductase and cytochrome b_5. All of these proteins were derived from untreated scup hepatic microsomes prepared from fish caught in local Woods Hole waters and held less than one week.

Chapter 3 will document the reconstituted monooxygenase activities of these resolved cytochromes P-450. Certain catalytic activities or enzymatic products are diagnostic of a given cytochrome P-450 form and thus, the contribution of various forms can be detected in impure fractions. However, monooxygenase catalytic activity was not a feasible method for routine evaluation of fractions during the course of purification since it requires an intervening column to remove detergent followed by reconstitution. Instead, physical

properties such as optical spectrum and molecular weight on SDS-PAGE have served as valid isozymic discriminators in this work.

Materials and Methods

Chemicals

Cholic acid was treated with activated charcoal in hot 90% ethanol-water and recrystallized from 50% aqueous ethanol. Stock solutions were prepared from 20% (w/v) cholic acid titrated with KOH to pH 7.5. Deoxycholic acid (Cal-Biochem) was prepared as a 10% (w/v) stock solution titrated with NaOH to pH 7.5. DEAE-Sephadex (DEAE-A25) and DEAE-cellulose (Whatman DE-52) were prepared by washing on a fritted glass funnel with 0.5 M ammonium sulfate, 0.5 M dibasic potassium phosphate, 0.5 M monobasic potassium phosphate, distilled water, the starting column buffer without detergent and the resin was titrated to the desired pH. CM-cellulose (Whatman CM-52) was prepared in an analogous fashion except the order of the phosphate buffer washes was reversed. Hydroxylapatite (Bio-Rad HTP) and Sephadex G-75 (Pharmacia superfine) gels were washed, poured and equilibrated according to the instructions of the manufacturer with the exception that the buffers were not degassed prior to use. Ion exchange resins were used only once in order to improve reproducibility. The biospecific 2',5'-ADP-Sepharose affinity column was prepared

by Dr. David R. Light, Department of Chemistry, MIT. All other reagents were the highest grade available commercially and were used without further purification. Purified trout hepatic cytochromes P-450 (TLM-4a and TLM-4b; Williams and Buhler, 1982; 1983b) were the generous gifts of Dr. David Williams and Professor Donald Buhler, Department of Agricultural Chemistry and Environmental Health Sciences Center, Oregon State University. Rat liver NADPH-cytochrome P-450 reductase was purified to electrophoretic homogeneity by standard methods (Waxman and Walsh, 1982).

General Methods

Protein concentration was estimated by a modified Lowry assay (Bensadoun and Weinstein, 1976) using a 1:1 bovine serum albumin:lysozyme (w/w) standard chosen for a balanced protein reaction from the BioRad product bulletin. Briefly, the protein sample was diluted to 1.0 ml with distilled water and 35 ul of 0.5% sodium deoxycholate carrier was added. The solution was vortexed, precipitated by the addition of 335 ul of 24% (w/v) TCA, again vortexed and incubated for ten minutes. Finally, the sample was centrifuged and the supernatant fraction was removed. A standard two ml Lowry assay was performed in the same tube on the pelleted residue and a linear response in A_{750} was observed between 2 and 20 ug protein under these conditions.

UV-visible spectra were obtained on a Perkin-Elmer model 554 spectrophotometer equipped with a recirculating water bath

at 18° C (for spectra of protein chromophores) or a Cary 118C dual beam spectrophotometer. Concentrations of hemoglobin, cytochromes P-450 and cytochrome b_5 were determined spectroscopically (Waterman, 1978; Stegeman et al., 1979) using an extinction coefficient of 91 mM^{-1} cm^{-1} (CO-ligated, reduced minus CO difference, $A_{450-490}$) for cytochrome P-450 and 185 mM^{-1} cm^{-1} (reduced minus oxidized difference, $A_{424-409}$) for cytochrome b_5. Partially purified cytochrome b_5 concentrations were measured spectroscopically after reduction with dithionite. Discontinuous sodium dodecyl sulfate polyacrylamide slab gel electrophoresis was performed (Laemmli, 1970) and gels were stained with Coomassie Brilliant Blue (Vesterberg, 1971). Gel scanning was performed on a Zeineh soft laser scanning densitometer (Biomed) in the laboratory of Professor Frank Solomon, Department of Biology, MIT. Apparent molecular weights were determined from a semilog plot of the molecular weight of the standards phosphorylase a (94K), bovine serum albumin (68K), pyruvate kinase (57K), glutamate dehydrogenase (53K), ovalbumin (43K), carbonic anhydrase (29K), soy bean trypsin inhibitor (21.5K), sperm whale myoglobin (17K) and lysozyme (14.4K) versus migration distance.

The scup NADPH-cytochrome P-450 reductase flavin cofactors were identified by modifications of literature methods (Mayhew and Massey, 1969; Light et al., 1981). Approximately 0.6 nmol purified enzyme was extracted on ice

with 5% TCA in 0.10 ml for ten minutes. The supernatant fraction was withdrawn after centrifugation and the pellet was washed with 50 ul of 5% TCA. The combined TCA extracts were neutralized with 60 ul 1M Na_2HPO_4. An aliquot of neutralized extract was separated with a DuPont 8800 HPLC on a Zorbax 25 cm C-18 reverse-phase column utilizing a 5-20% linear acetonitrile gradient in 50 mM NH_4^+ $HCOO^-$, pH 6.5, at 2 ml/min. The flavins were detected by absorbance at 436 nm and quantitated by peak area from a standard curve linear between 50 and 500 pmol flavin using a Hewlett-Packard 3390A integrator. FMN and FAD standard solutions were prepared in 0.35 M Na_2HPO_4/5% TCA, pH 6.0, according to their published extinction coefficients (FAD, 11.3 mM^{-1} cm^{-1} at 450 nm, 37 mM^{-1} cm^{-1} at 260 nm; FMN 12.2 mM^{-1} cm^{-1} at 450 nm, 27.1 mM^{-1} cm^{-1} at 260 nm; Dawson et al., 1969). The enzymic flavin identifications were confirmed by coinjection with authentic FAD and FMN.

NADPH-cytochrome P-450 (cytochrome c) reductase activity (EC 1.6.2.4) was routinely determined at 30° in 0.2 M KP_i, pH 7.7 (Stegeman et al., 1979). One unit (U) was defined as 1 umole cytochrome c reduced per minute under standard conditions. Apparent K_m values were determined by extrapolation from a Lineweaver-Burke plot of initial rates measured in triplicate at six substrate concentrations.

Enzyme Purification

The NADPH-cytochrome P-450 reductase was assayed spectrophotometrically based on its ability to reduce the non-physiological electron acceptor cytochrome c. The purity of this enzyme was evaluated by specific activity (umol substrate converted to product per min per mg protein), A_{277}/A_{456} (protein to flavin) ratio and appearance on SDS-PAGE. Cytochrome b_5 was assayed by its characteristic reduced minus oxidized difference spectrum and appearance on SDS-PAGE. Purity was evaluated by A_{413}/A_{295} (heme to protein) ratio and specific (heme) content. The cytochromes P-450 were detected by their characteristic CO-ligated, reduced difference spectrum and purified on the basis of their differing apparent molecular weights judged by SDS-PAGE and the λ_{max} of their CO, reduced difference spectrum. Purification was routinely evaluated by A_{416}/A_{295} (heme to protein) ratio, specific content and appearance on SDS-PAGE.

Scup hepatic microsomes were prepared from untreated fish (Stegeman et al., 1979) with the inclusion of a wash step utilizing 0.1 M $NaPP_i$, pH 7.4, containing 10 mM EDTA. The wash step was effective in removing extraneous proteins as judged by a ca 50% higher cytochrome P-450 specific content with no diminution in cytochrome P-450 recovery. Microsomes were frozen in liquid N_2 and stored in the same or a -70° C freezer until use. The hepatic monooxygenase components were purified at 0-4° C, whereas buffers were routinely prepared at

temperature and titrated to the indicated pH values (reported at room temperature) before adding the detergent component from a stock solution. The concentrations of glycerol and Emulgen in the buffers are given as v/v percentages while the PEG 6000, cholate and deoxycholate concentrations are w/v percentages. Phenylmethane sulfonyl fluoride (PMSF) was added as a solid just prior to the use of the solubilizing buffer. All columns were poured, equilibrated and run in accord with standard chromatographic procedures by gravity feed at rates of 10 to 50 ml/hr depending on the nature of the resin and column dimensions. A_{295} (protein), A_{413} (heme) and A_{416} (heme) measurements for column fractions were corrected by subtracting the A_{650}. The protein absorbance was measured at A_{295} to avoid the UV component of the Emulgen (λ_{max} = 276 nm) present in most of the column buffers.

A. NADPH-cytochrome P-450 reductase.

The scup hepatic microsomal NADPH-cytochrome P-450 reductase was purified by modifications of the standard chromatographic procedure on DEAE-A25 and 2',5'-ADP-Sepharose (Strobel and Dignam, 1978; Yasukochi and Masters, 1976). Microsomes were pelleted at 100,000g for 60 minutes and resuspended to 10 mg protein per ml in 0.2 M Tris-Cl, pH 7.7 containing 30% glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. The microsomal proteins were solubilized while stirring on ice by dropwise addition of aqueous 20% Emulgen 913 (Kao-Atlas, Japan) to a final 1.5% concentration. After twenty minutes

the solution was treated with 0.03% protamine sulfate, added dropwise from a 1.5% (w/v) aqueous stock, solution and stirring was continued for ten minutes. The soluble fraction was obtained by centrifugation at 100,000g for 90 minutes.

The solubilized enzyme activity was chromatographed on a DEAE-A25 ion exchange column[1] which had been equilibrated in several bed volumes of 50 mM HEPES, pH 7.7 containing 20% glycerol, 1 mM DTT, 1 mM EDTA and 0.15% Emulgen 913 (hence "column buffer"). The column was washed with column buffer to elute several red cloudy fractions containing cytochrome P-450[2] and was then developed with a linear gradient of 0-0.4 M KCl in four bed volumes of column buffer. The NADPH-dependent cytochrome c reducing activity eluted as a broad peak near the end of the gradient. The fractions containing the highest activity were pooled, supplemented with 2 uM FMN[3], concentrated several fold by ultrafiltration in an Amicon stirred cell (XM-50) and dialyzed overnight against fifteen volumes of columnn buffer without the detergent.

The retained fraction was chromatographed on an 8 ml bed volume 2',5'-ADP-Sepharose column equilibrated in the dialysis buffer. The column was washed with five volumes of 20 mM KP_i, pH 7.7 containing 20% glycerol, 1 mM DTT and 0.1% DOC (20 mM buffer); five column volumes of 100 mM KP_i, pH 7.7 containing 20% glycerol, 1 mM DTT and 0.1% DOC; and briefly with 20 mM buffer again. Finally, the enzyme activity was eluted with a linear 0 to 1.0 mM NADP in five bed volumes of 20 mM buffer. The peak activity was estimated to elute in a fraction

corresponding to about 0.13 mM NADP. The fractions containing the highest activity were pooled, concentrated by ultrafiltration and dialyzed against 250 volumes of 20 mM KP_i, pH 7.5 containing 20% glycerol, 0.1 mM EDTA and 0.05% DOC to remove NADP and DTT. The purified scup NADPH-cytochrome P-450 reductase was stored in the dialysis buffer at -70° C and the catalytic activity was stable for at least six months.

The overall scup cytochrome P-450 purification strategy was detergent solubilization of microsomes, fractionation with polyethylene glycol 6000 and resolution of the various cytochrome P-450 fractions by ion exchange chromatography on DEAE-cellulose. The resolved cytochrome P-450 forms were further purified by a combination of rechromatography on DEAE-cellulose, column chromatography on hydroxylapatite and CM-cellulose. Partially purified cytochrome P-450 samples were stable for at least six months when frozen in liquid N_2 and stored at -70° C.

Hepatic microsomes (typically 1 g microsomal protein) were suspended at 10 mg protein per ml in 0.2 M Tris-Cl, pH 7.4 containing 30% glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF (I_c = 0.25 adjusted with KCl) and solubilized while stirring by the dropwise addition of potassium cholate and Emulgen 911 to final concentrations of 0.5% and 0.2%, respectively. After 30 minutes the solution was centrifuged at 100,000g for 75 minutes and the pellet was discarded. The soluble fraction was then treated with PEG 6000 by the

dropwise addition of a 50% aqueous stock solution to a final concentration of 6% while stirring continuously. After fifteen minutes the cloudy solution was centrifuged 35,000g for fifteen minutes and the large grey pellet was discarded. The supernatant fraction was adjusted to a final concentration of 20% PEG 6000 by further addition while stirring and after fifteen minutes was centrifuged 100,000g for 90 minutes. The bright, clear orange supernatant fraction was discarded and the deep red pellet was dissolved in a minimum volume of 10 mM HEPES, pH 7.5 containing 20% glycerol, 1 mM DTT, 0.1 mM EDTA, 0.5% cholate and 0.2% Emulgen 911 (hence abbreviated buffer I).

The concentrated soluble cytochromes P-450 were chromatographed on a DEAE-cellulose column[4] which had been equilibrated with no more than one bed volume of buffer I. The column was washed with 20 mM KP_i, pH 7.5 containing 20% glycerol, 1 mM DTT, 0.1 mM EDTA, 0.5% cholate and 0.2% Emulgen 911 (buffer II) until no more visible color eluted (ca one bed volume) and then the column was developed with a linear gradient (4-5 bed volumes) of 0-0.5 M KCl[5] in buffer II. The KCl gradient was monitored with a YSI model 31 conductivity bridge. The five fractions containing cytochrome P-450 were labeled A-E in order of their elution from the DEAE-cellulose column. NADPH-dependent cytochrome c reductase activity was found to elute between cytochrome P-450E and cytochrome b_5. Complete resolution of cytochrome P-450A from hemoglobin was obtained in later experiments by an initial wash with buffer I

after loading (hemoglobin does not bind to the column in 10 mM HEPES).

B. Cytochrome P-450E.

Cytochrome P-450E was further purified by dialyzing the pooled DEAE-cellulose fractions against twenty volumes of buffer I (without detergent) overnight and chromatographing the retained fraction on a hydroxylapatite column[6] which had been equilibrated in buffer I including 0.2% Emulgen 911 but no cholate. The column was developed with five bed volumes of 20 mM KP_i, pH 7.5 containing 20% glycerol, 1 mM DTT, 0.1 mM EDTA and 0.2% Emulgen 911 (20 mM buffer III), five bed volumes of 50 mM buffer III and the cytochrome P-450 was eluted as a sharp peak with 100 mM buffer III.

C. Cytochrome P-450A.

Fractions containing cytochrome P-450A, derived from the first cytochrome P-450 peak of several separate DEAE-cellulose column resolutions, were pooled on the basis of a blue-shifted CO-ligated, reduced difference spectrum and a 53K polypeptide in SDS-PAGE analysis. The pool was dialyzed overnight against fifteen volumes of buffer I (without detergent) and chromatographed on a DEAE-cellulose column[4] which had been equilibrated in buffer I without detergent. The column was washed with buffer I containing 0.25% cholate and 0.1% Emulgen 911 and developed with buffer II. SDS-PAGE analysis indicated that the heme containing fractions which passed through the

column without binding also possessed a 53K polypeptide while the putative cytochrome P-450B (see below) and most other contaminants were bound[7]. The dilute unbound fractions, pooled on the basis of their A_{416}/A_{295} ratio and prominent 53K polypeptide on SDS-PAGE, were concentrated by ultrafiltration utilizing an Amicon XM-50 membrane and dialyzed overnight against twenty volumes of buffer I without detergent.

The retained fraction was chromatographed on a hydroxylapatite column[4] which had been equilibrated in the dialysis buffer. The column was washed with two bed volumes of 20 mM buffer III, four bed volumes of 50 mM buffer III and finally, the cytochrome P-450 was eluted with 100 mM buffer III. The peak fractions were pooled on the basis of their A_{416}/A_{295} ratio, concentrated by ultrafiltration with an XM-50 membrane and dialyzed overnight against 40 volumes of buffer I without detergent.

The retained fraction was rechromatographed on a hydroxylapatite column[4] which was developed with one bed volume of 20 mM buffer III, two bed volumes of 70 mM buffer III and the cytochrome P-450 was eluted as a sharp band with 150 mM buffer III. The peak fractions, pooled on the basis of their A_{416}/A_{295} ratio, were concentrated by ultrafiltration and dialyzed (with one change) for twenty-four hours against 40 volumes of 10 mM KP_i, pH 6.5 containing 20% glycerol, 1 mM DTT and 0.1 mM EDTA.

The retained fraction was chromatographed on a CM-cellulose column[4] which had been equilibrated in the

dialysis buffer. The column was washed briefly with 10 mM KP_i, pH 6.5 containing 20% glycerol, 1 mM DTT, 0.1 mM EDTA and 0.1% Emulgen 911 (10 mM buffer IV), followed by four bed volumes of 20 mM buffer IV and the cytochrome P-450 was eluted with 25 mM KCl in 20 mM buffer IV.

D. Cytochrome P-450B.

A pool of cytochrome P-450B, derived from a standard DEAE-cellulose column resolution and identified by its 46K polypeptide on SDS-PAGE and CO-ligated, reduced difference spectrum λ_{max} near 449 nm, was dialyzed overnight against twenty volumes of buffer I (without detergent) and chromatographed on a DEAE-cellulose column[4] which had been equilibrated in the dialysis buffer. The cytochrome P-450 was eluted as a sharp pink band with buffer I containing 0.5% cholate and 0.2% Emulgen 911. The peak fractions were pooled on the basis of their A_{416}/A_{295} ratio and dialyzed overnight against twenty volumes of buffer I (without detergent).

The retained fraction was chromatographed on a hydroxylapatite column[4] which had been equilibrated in buffer I containing 0.2% Emulgen 911. The column was washed briefly with the equilibration buffer, followed by two bed volumes of 20 mM buffer III and the cytochrome P-450 was eluted as a diffuse pink band with 50 mM buffer III. The peak fractions were collected and pooled on the basis of their A_{416}/A_{295} ratio.

E. Cytochrome P-450 Fraction D.

A pool of cytochrome P-450, isolated from a standard resolution on DEAE-cellulose and possessing a CO-ligated, reduced difference spectrum λ_{max} near 451 nm, was dialyzed overnight against fifteen volumes of buffer I without detergent and chromatographed on a DEAE-cellulose column[4] which had been equilibrated in one bed volume of buffer I. The column was briefly washed with buffer II and developed with a linear gradient of 0-0.4M KCl in six bed volumes of buffer II. The peak, spread over several fractions, was pooled on the basis of A_{416}/A_{295} ratio and red-shifted CO, reduced difference spectrum and was dialyzed overnight against twenty volumes of buffer I containing 10 mM mercaptoethanol but no detergent.

The retained fraction was chromatographed on a hydroxylapatite column[4] which had been equilibrated in buffer I containing 0.2% Emulgen 911. The column was washed with one bed volume of 20 mM buffer III, followed by two bed volumes of 50 mM buffer III and the cytochrome P-450 was eluted as a sharp band with 100 mM buffer III. The peak fractions were pooled on the basis of their A_{416}/A_{295} ratio.

F. Cytochrome b_5.

Scup cytochrome b_5 was purified by modifications of Strittmatter, et al. (1978) as described by Waxman and Walsh (1982). The fractions from several DEAE-cellulose column

resolutions which contained cytochrome b_5 were pooled, concentrated by ultrafiltration utilizing an Amicon PM-10 membrane and dialyzed overnight against fifteen volumes of 20 mM Tris-Ac, pH 8.1 containing 0.1 mM EDTA (buffer V). The retentate was chromatographed on a DEAE-cellulose column[6] which had been equilibrated with buffer V containing 0.4% DOC. After a brief wash with the equilibration buffer the cytochrome b_5 was eluted with a linear gradient of 0-0.2 M NaSCN (six column volumes) in buffer V containing 0.4% DOC. The peak fractions, pooled on the basis of their A_{413}/A_{295} ratio and appearance on SDS-PAGE, were concentrated by ultrafiltration and dialyzed overnight against fifteen volumes of buffer V.

The retained fraction was chromatographed on a hydroxylapatite column[8] which had been equilibrated in the dialysis buffer. The column was washed with one bed volume of buffer V, three bed volumes of buffer V containing 50 mM KCl and the remaining cytochrome b_5 was eluted with buffer V containing 50 mM KP_i and 0.4% DOC. The flow-thru fractions, and the peak which was eluted with 50 mM KP_i, were pooled, concentrated by ultrafiltration, and dialyzed overnight against 40 volumes of buffer V.

The retained fraction was rechromatographed on a DEAE-cellulose column[4] which had been equilibrated in the dialysis buffer. The column was washed with ten bed volumes of buffer V containing 0.1% DOC and developed with a linear

gradient of 0-0.2 M NaSCN (ten bed volumes) in buffer V. The cytochrome b_5 eluting as a sharp peak near the end of the gradient was dialyzed overnight against twenty volumes of buffer I without detergent.

The retained fraction was chromatographed on a hydroxylapatite column[4] which had been equilibrated in the dialysis buffer. The column was washed in five bed volumes of buffer I without detergent, five bed volumes of buffer III without detergent and finally 50 mM KP_i, pH 7.4 containing 20% glycerol, 1 mM DTT, 0.1 mM EDTA and 0.1% DOC. The peak cytochrome b_5 fractions eluted by the 50 mM KP_i wash were pooled, concentrated by ultrafiltration and dialyzed exhaustively against buffer I without detergent. A second cytochrome b_5 fraction which was eluted by buffer III (without detergent) contained residual Emulgen 911 judged by a high A_{280}. This fraction was concentrated by ultrafiltration and applied to a G-75 column (60 x 0.8 cm) which had been equilibrated in buffer V containing 0.4% DOC at a flow rate of 2 ml/min. The peak fractions, eluting in the void volume and free of Emulgen 911, were pooled and dialyzed exhaustively against buffer I without detergent. Cytochrome b_5 fractions were stored at -70° C in the dialysis buffer.

Nonionic Detergent Removal

Emulgen 911 was removed from the purified cytochrome P-450 preparations by repeating the hydroxylapatite chromatography but replacing the Emulgen 911 in the column

buffers with 0.4% cholate or 0.1% DOC. Briefly, the cytochrome P-450 sample was dialyzed overnight against twenty volumes of buffer I without detergent and chromatographed on a hydroxylapatite column[6] which had been equilibrated with buffer I containing 0.2% Emulgen 911. The column was washed with five bed volumes of 20 mM buffer III containing 0.4% cholate or 0.1% DOC until the column effluent fell below an A_{276} of 0.05 (typically ca three bed volumes). The column could be further washed with 50 mM buffer III containing 0.1% DOC and the cytochrome P-450 was finally eluted as a concentrated band with 200 mM buffer III containing 0.1% DOC. The peak cytochrome P-450 fractions were combined, frozen in liquid N_2 as small aliquots and stored at -70° C. Typical recoveries on this column chromatography step were 70% with little change in the specific content of the resulting preparation (see Figure 2-14, lanes e and f).

Protein Chemistry

A. Peptide mapping.

Proteolytic peptide mapping of the cytochrome P-450 forms was accomplished by the method of Cleveland et al. (1977) as modified by Waxman and Walsh (1983). The individual cytochrome P-450 samples were diluted to ca 0.14 mg protein per ml and dialyzed extensively against 0.125M Tris-Cl, pH 6.7 containing 15% glycerol. The samples were denatured by mixing 5 ul of 2.5% (w/v) SDS with 40 ul of dialyzed protein and incubation in a boiling water bath for one minute. Protease

(Staphylococcus aureus V8 protease, Miles or a-chymotrypsin, Worthington) was added in 5 ul of buffer to yield final concentrations of 5 and 20 ug S. aureus V8 protease per ml or 1 and 5 ug a-chymotrypsin per ml, and digestion was allowed to proceed for 90 min at 37° C. The digestion was stopped by the addition of 30 ul dye/denaturant (final concentrations: 10% 2-mercaptoethanol, 0.015% bromophenol blue and 0.5% SDS) and the samples were incubated in a boiling water bath for five minutes. The digestions were analyzed by SDS-PAGE on 13.5% polyacrylamide separating gels with a long (3.5 cm) stacking gel.

B. NH_2-terminal sequence analysis.

Purified cytochrome P-450E (8-9 nmol, ca 12 nmol/mg) was prepared for sequencing by co-precipitating the protein with 125 ug DOC per ml in 6% TCA. The supernatant fraction was removed by centrifugation after holding the sample on ice for 15 min and the pellet (acidic from the residual TCA) was washed three times with 4 ml ice cold acetone to remove the heme. The apoenzyme was lyophilized to dryness twice from 2 ml of 88% formic acid. The apoenzyme was applied to the spinning cup in 0.4 ml of 88% formic acid and the sample was run through a blank wash cycle in the absence of phenylisothiocyanate or heptafluorobutyric acid. The washed protein sample was subjected to sequential Edman degration on a Beckman 890C Automated Sequenator operated on a 0.1 M Quadrol single or double cleavage program (Brauer et al.,

1975) in the Protein Sequencing Facility maintained by Professor R. Sauer, Department of Biology, MIT.

PTH-norleucine (3.5 nmol) was added as an internal standard to the fractions and the 2-anilinothiazolinone-amino acid derivatives were converted to PTH-amino acids by incubation at 80° for ten minutes in 1 M HCl. The PTH-amino acid derivatives were extracted with ethyl acetate[9] and analyzed by a combination of gas-liquid chromatography on a Hewlett-Packard 5830A gas chromatograph using a 10% SP400 column under conditions which resolved the PTH derivatives of ala, gly, val, pro, ile and leu (Nial, 1973), and HPLC on a Waters RCM C-18 column which was effective for all organic-extracted derivatives (Sauer et al., 1981). Residue identification was based on HPLC retention time in comparison to authentic PTH-amino acid standards with confirmation by gas-liquid chromatography for most residues as noted above. PTH-gly was verified by a further 30 min acid conversion of the phenylthiocarbamyl (PTC)-gly which coeluted on HPLC with PTH-asp. Quantitation was based on HPLC detector response factors supplied at the Facility after taking extraction efficiencies into account as judged by recovery of the PTH-nL internal standard.

C. Immunochemical analysis.

Antibodies to cytochrome P-450E were raised in New Zealand white male rabbits by subcutaneous immunization. Pre-immune sera was collected for three weeks by bleeding from the ear artery. Immunogen was prepared by mixing equal

volumes of purified cytochrome P-450E (1.0 mg/ml in 100 mM buffer III containing 0.2% Emulgen 911; ca 10 nmol/mg) and Freund's complete adjuvant (Difco) in a double syringe apparatus. The emusified immunogen was judged to be stable by oozing a few drops into a beaker of water and noting that only the first drop dispersed. Each rabbit was primed by the subcutaneous injection of 500 ug protein (1 ml total volume) at four sites along the back. Four and six weeks after the prime each rabbit received a boost consisting of 40 ug protein diluted in 10 mM KP_i, pH 7.4 containing 150 mM NaCl (PBS) and mixed with an equal volume of Freund's incomplete adjuvant (Difco). The immunogen used for the boosts was found to be unstable unless a few drops of 2 M NaCl were added.

Seven days after the second boost the rabbits were bled (35-50 ml blood per rabbit) from the ear. The blood was allowed to clot at room temperature for one hour and sera was collected by centrifuging twice at 4000g for fifteen minutes. The crude IgG was precipitated with 40% saturated ammonium sulfate by addition of the solid salt while stirring and the pellet was collected by centrifuging 15,000g for fifteen minutes. The precipitate was redissolved in PBS and precipitated again with 40% saturated ammonium sulfate. This precipitate was collected by centrifugation, redissolved in a small volume of PBS and dialyzed extensively against the same buffer to remove excess ammonium sulfate. The retentate was clarified by brief centrifugation at 15,000g and stored at -20° C. Crude IgG protein concentrations were estimated by

application of an $E^{1\%}$ = 13 at 280 nm (Thomas et al., 1979).

Ouchterlony double diffusion analysis was performed as described by Thomas et al. (1976b). A solution was prepared containing 1 M glycine, 80 mM NaCl, 15 mM NaN_3, 0.2 mM EDTA and 10 mM KP_i in 24.5 ml at pH 7.4 (adjusted with NaOH) and 225 mg agarose (SeaKem, medium grade; final concentration 0.9%) was dissolved by heating on a steam bath until dissolution occurred. Emulgen 911 was added to 0.2% causing a cloudy appearance and the final solution (sufficient for a dozen slides) was placed in a 50° C water bath during the pouring operation. The molten agarose solution was layered onto clear glass microscope slides from a 10 ml pipette and adhered by capillary action. The gels were punched after cooling to ambient temperature and the slides were stored in a sealed box under a moist atmosphere.

A satisfactory punch was obtained from Professors Eisen or Steiner, Department of Biology, yielding a uniform pattern with a 1 mm diameter center well, six 1 mm diameter outer wells and 2 mm interwell distances. The agarose cores were removed by aspiration through a pasteur pipette to form the wells. Crude IgG (30 mg/ml) from each immunized rabbit was placed in the center well and concentrated purified cytochrome P-450 samples (5-15 uM) were placed in the outer wells. Dilute samples of purified scup cytochrome P-450A were concentrated prior to analysis by dehydration in a dialysis membrane using Aquacide as the absorbant. Reactions were

allowed to proceed overnight in a moist atmosphere and the precipitin lines were visualized directly or by staining with naphthol blue-black as described by Thomas et al. (1976b).

Results and Discussion

A. Scup hepatic microsomal NADPH-cytochrome P-450 reductase.

The NADPH-cytochrome P-450 reductase was efficiently solubilized by Emulgen 913 treatment and chromatographed on DEAE-A25 with reasonable purification although the activity eluted in an inconveniently large volume (Figure 2-1). An impressive purification occurred on the biospecific affinity column (Figure 2-2) affording virtually homogeneous enzyme in two column chromatography steps. A summary of the scup NADPH-cytochrome P-450 reductase purification is presented in Table 2-1. Two other purifications (not shown) have been effected with yields based on activity of 35% and 23% and final specific activities of 46.4 and 69.2 U/mg. The purified scup NADPH-cytochrome P-450 reductase migrated as a single polypeptide in SDS-PAGE experiments with a molecular weight of 82,600 $\pm$ 1500, distinct from the 77,700 $\pm$ 100 obtained for purified rat NADPH-cytochrome P-450 reductase (Figure 2-3; Yasukochi and Masters, 1976).

HPLC analysis (Figure 2-4) revealed that the purified scup NADPH-cytochrome P-450 reductase contained 1.0 FAD and 0.80 FMN per 82.6 kD and the UV-visible spectrum of the scup NADPH-cytochrome P-450 reductase indicated it was isolated as the one-electron reduced neutral blue semiquinone (Figure 2-5). Oxidation of the enzymic flavin with a slight excess of

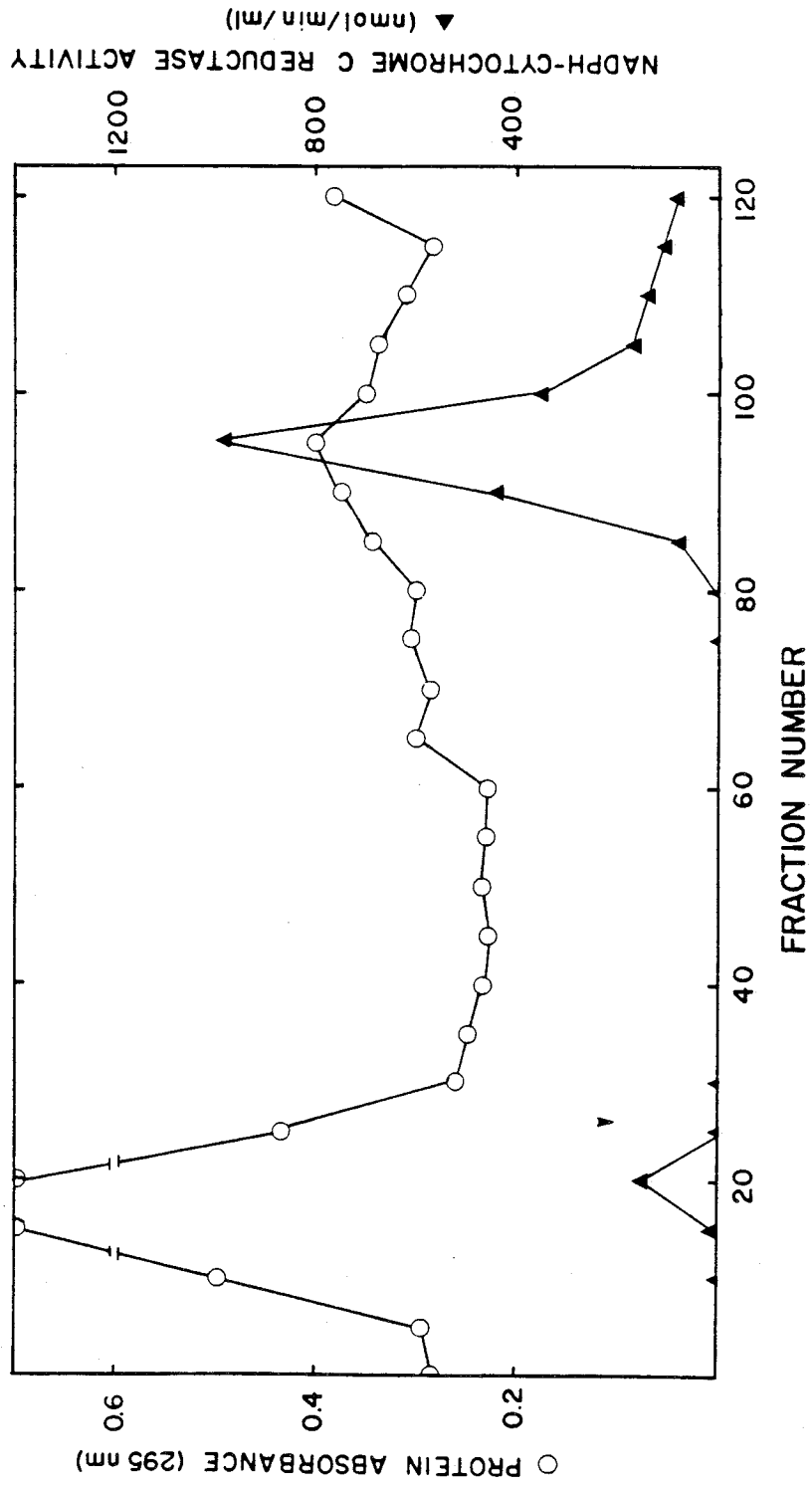

○ PROTEIN ABSORBANCE (295 nm)
0.2
0.4
0.6
▲ (nmol/min/ml)
NADPH-CYTOCHROME C REDUCTASE ACTIVITY
400
800
1200
20
40
60
80
100
120
FRACTION NUMBER

Figure 2-1. Scup NADPH-cytochrome P-450 reductase chromatography on DEAE-A25. Scup hepatic microsomal NADPH-cytochrome P-450 reductase was solubilized with Emulgen 913 and centrifuged as described in the methods section. The soluble protein (1200 mg) was chromatographed at 4° C on a DEAE-A25 column (350 ml bed volume) in 50 mM HEPES, pH 7.7, containing 20% glycerol, 1 mM DTT, 1 mM EDTA and 0.15% Emulgen 913. Approximately 14 ml fractions were collected throughout the chromatography. Fractions #13-21 flushed through the column and were pooled for cytochrome P-450 recovery. The column was developed in a 1400 ml linear gradient of 0-0.4 M KCl in the column buffer at fraction #25 (arrow). Fractions #88-110 contained the highest enzyme activity and were combined for further purification.

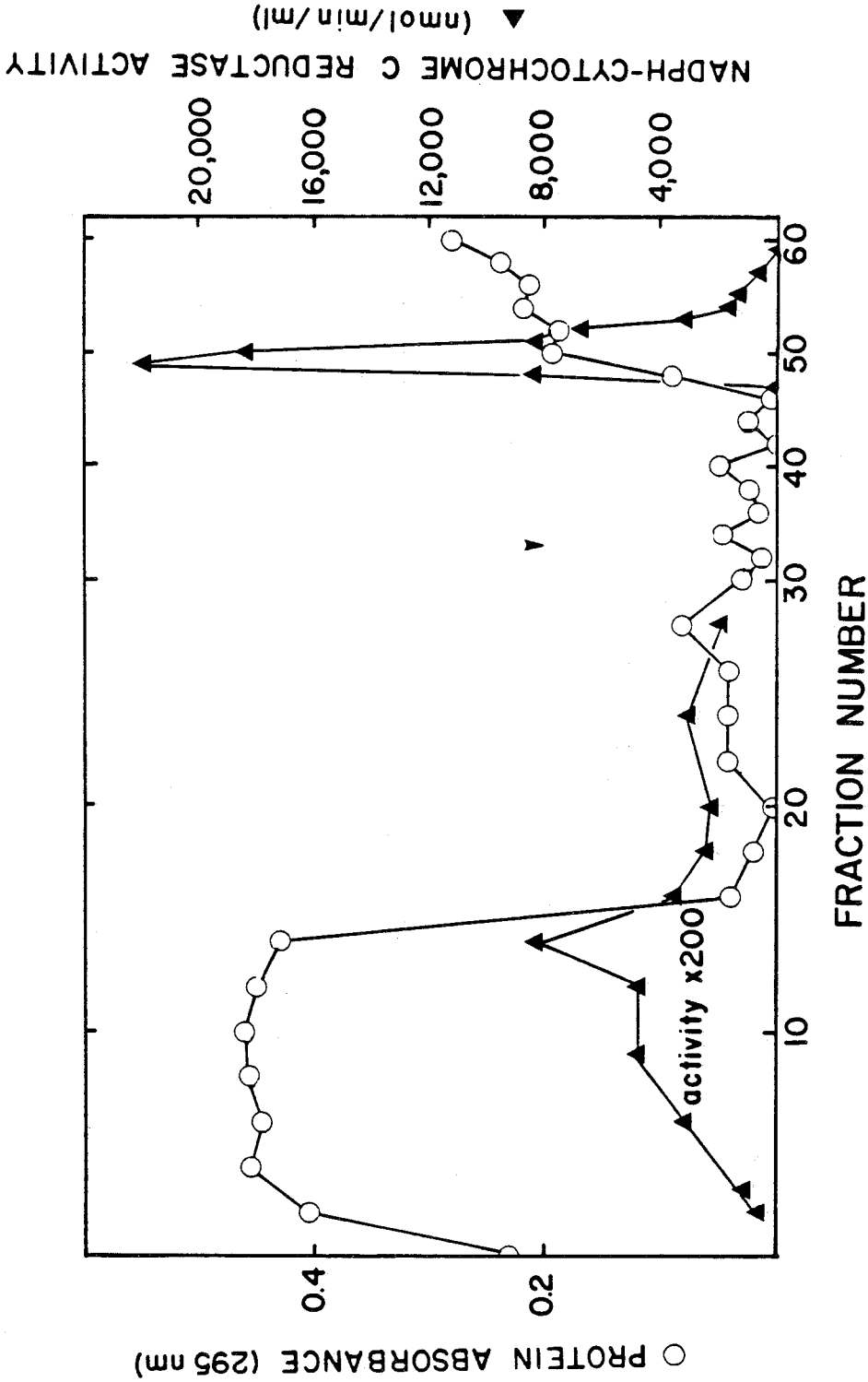

○ PROTEIN ABSORBANCE (295 nm)
0.4
0.2
activity x200
10
20
30
40
50
60
FRACTION NUMBER
20,000
16,000
12,000
8,000
4,000
NADPH-CYTOCHROME C REDUCTASE ACTIVITY
▲ (nmol/min/ml)

Figure 2-2. Scup NADPH-cytochrome P-450 reductase chromatography on 2'5'-ADP-sepharose. The peak enzyme activity obtained from DEAE-A25 chromatography (Figure 2-1) was concentrated and dialyzed against column buffer without detergent. The retained fraction was chromatographed at 4° C on a 2'5'-ADP-sepharose column (8 ml bed volume). The column was developed with the buffer schedule described in the methods section. The enzyme activity was eluted with a linear gradient of 0-1 mM NADP in 40 ml of 20 mM KP_i, pH 7.7, containing 20% glycerol, 1 mM DTT and 0.1% DOC at fraction #33 (arrow). The fractions (#48-55) containing the peak activity were pooled, concentrated and dialyzed to remove NADP. Approximate fraction volumes collected were: 16 ml (#1-14); 8 ml (#14-32); 1.35 ml (#33-60).

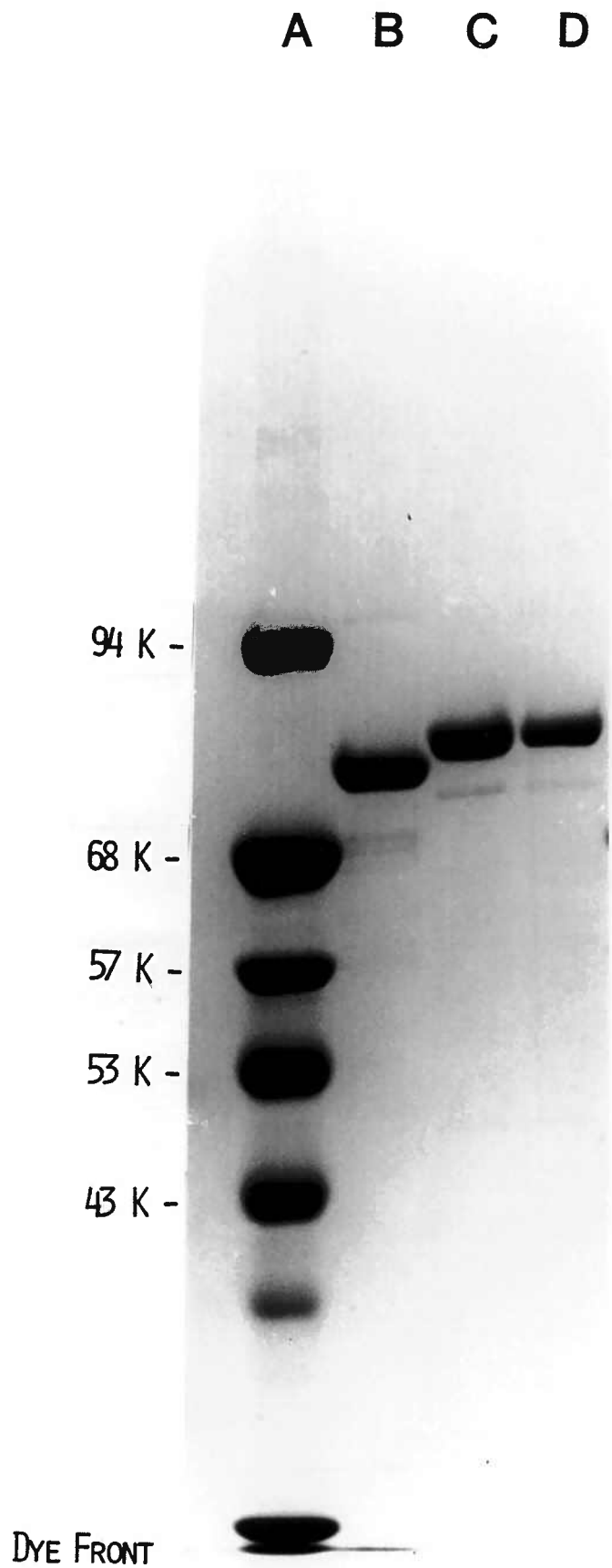

A
B
C
D
94 K -
68 K -
57 K -
53 K -
43 K -
DYE FRONT

Figure 2-3. SDS-PAGE of NADPH-cytochrome P-450 reductase purified from rat and scup hepatic microsomes. Discontinuous 7.5% polyacrylamide gel electrophoresis was performed by the method of Laemmli, 1970. Samples were loaded as follows: lane a, molecular weight standards (see methods), 5 ug each; lane b, 4 ug purified rat NADPH-cytochrome P-450 reductase; lane c, 5 ug purified scup NADPH-cytochrome P-450 reductase; lane d, 3 ug purified scup NADPH-cytochrome P-450 reductase.

TABLE 2-1

Purification of Scup NADPH-cytochrome P-450 Reductase

Step	Total Protein[a] (mg)	Total Activity[b] (Units)	Activity Recovery (%)	Specific Activity (U/mg)	Purification (fold)
Microsomes[c]	1285	256.1	--	0.199	---
100,000g supernatant	1196	256.6	100	0.216	1.1
DEAE-A25 chromatography[d]	104	156.2	61	1.50	7.5
2',5'-ADP-Sepharose[e]	1.2	54	21	44.8	225

[a]Protein was estimated by a modified Lowry assay.

[b]1 U equals 1 umole cytochrome c reduced per minute at 30°C in 0.2M KP_i, pH 7.7.

[c]This represents approximately 40 fish livers pooled.

[d]See Figure 2-1.

[e]See Figure 2-2.

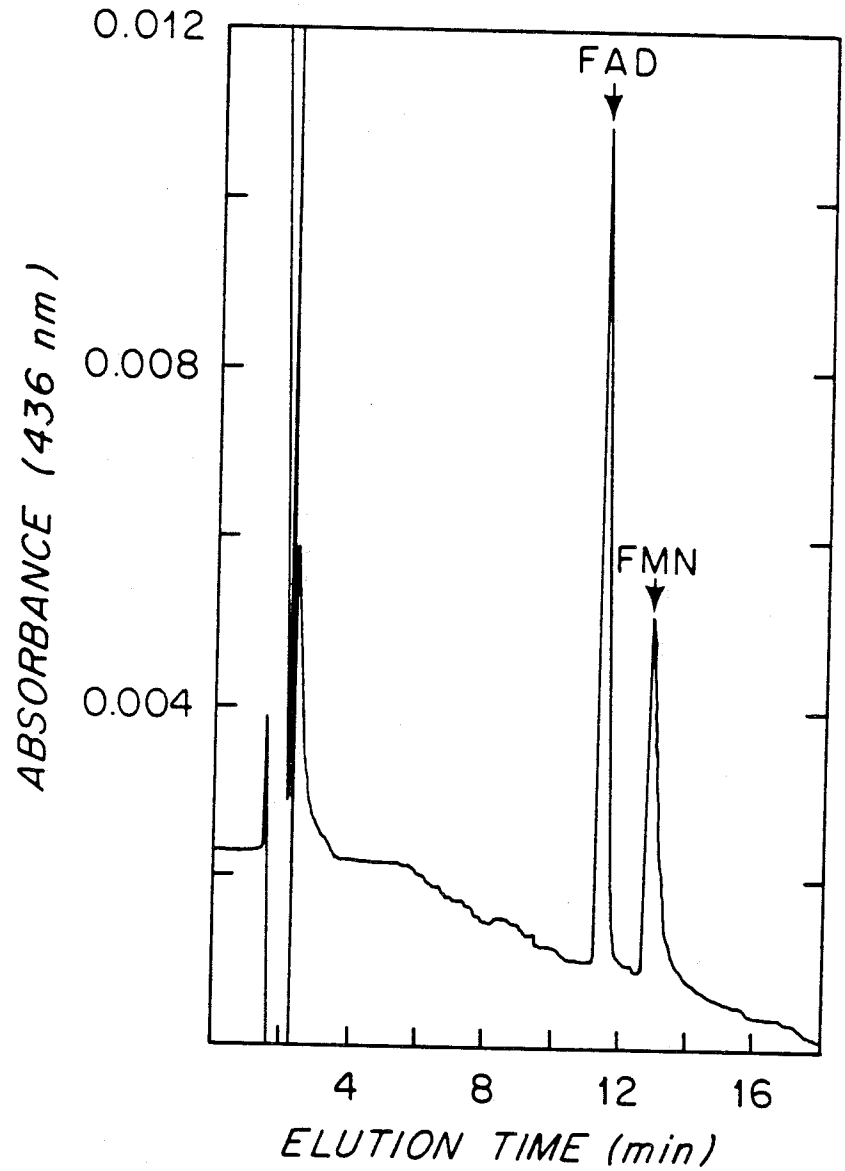


Figure 2-4. HPLC analysis of scup NADPH-cytochrome P-450 reductase flavin content. Approximately 0.6 nmol purified enzyme was extracted with 5% TCA as described in the methods section and an aliquot of the neutralized extract was injected onto a reverse-phase C-18 HPLC column at time zero. The flavins were separated with a 5-20% linear gradient of acetonitrile in 50 mM ammonium formate, pH 6.5, at 2 ml/min. The flavins were detected by their absorbance at 436 nm and identifications were confirmed in a separate chromatogram by coinjection with authentic standards.

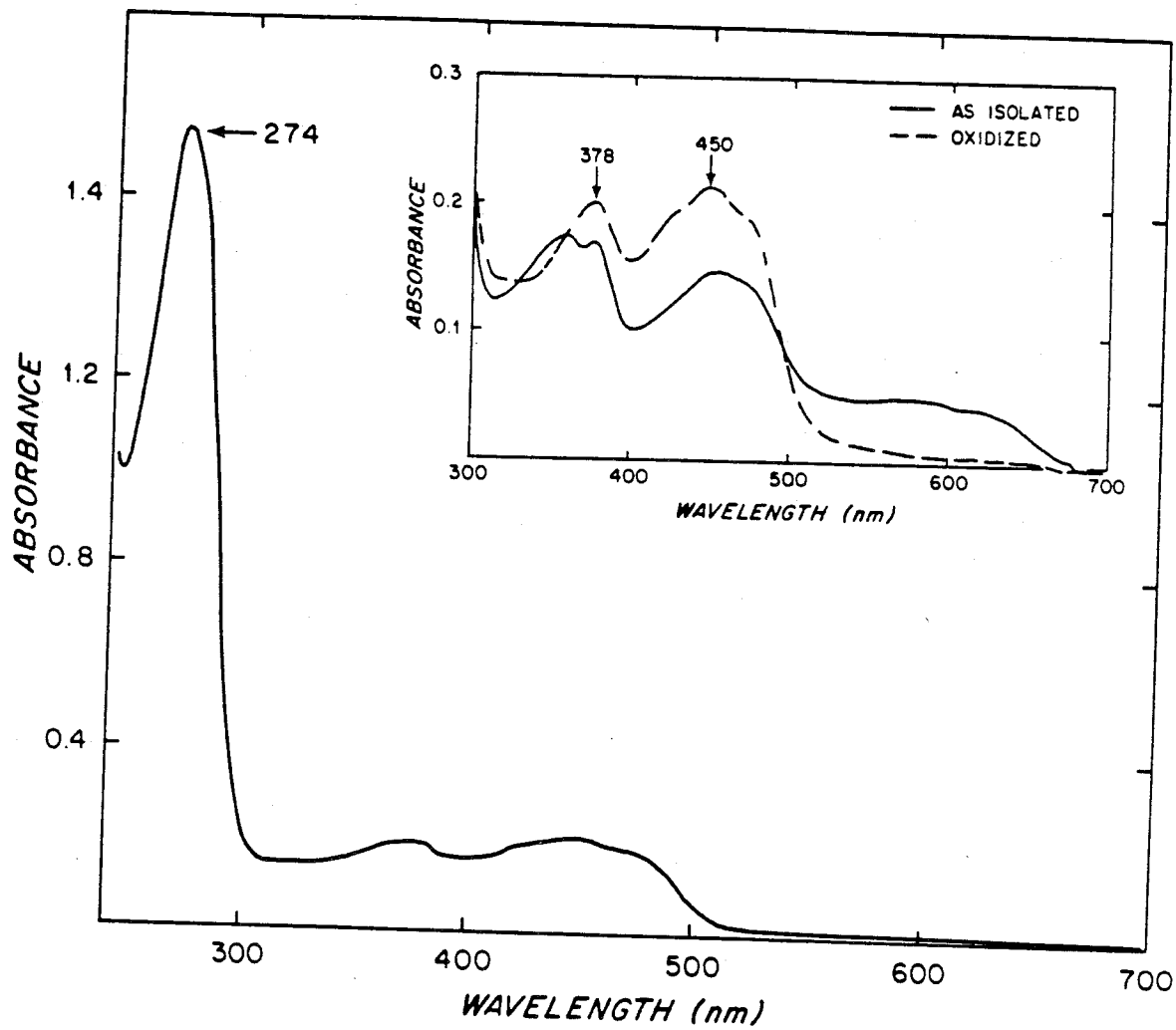


Figure 2-5. UV-visible spectrum of purified scup NADPH-cytochrome P-450 reductase. The 2'5'-ADP-sepharose eluate (10.6 uM) was dialyzed to remove NADP and scanned; inset, the visible (flavin) region expanded, before and after the addition of 25 uM potassium ferricyanide. The broad absorbance of the semiquinone charge-transfer complex was readily detected in the enzyme as isolated.

potassium ferricyanide yielded the typical oxidized flavin spectrum and an A_{277}/A_{450} = 8.0 which compares to 8.7 and 7.4 reported for rat (Vermilion and Coon, 1978) and rabbit (French and Coon, 1979), respectively. Application of the published extinction coefficients (Vermilion and Coon, 1978) for the neutral semiquinone (E_{585} = 2.4 mM^{-1} cm^{-1} per enzymic flavin) and fully oxidized forms (E_{456} = 10.6 mM^{-1} cm^{-1} per enzymic flavin) gave values which agreed (within 6%) for the enzymic flavin concentrations (20.6 nmol flavin per mg protein) supporting the notion of a one-electron reduced state for the scup enzyme as isolated. This supposition was validated in the laboratory of Professor William Orme-Johnson by the detection of a strong organic radical signal (g = 2.00) in the 77° K electron spin resonance spectrum of the enzyme (performed by N. R. Orme-Johnson; not shown). An isosbestic point between the one-electron reduced semiquinone and fully oxidized states was determined for the scup NADPH-cytochrome P-450 reductase as 498 nm with an extinction coefficient of 3.9 mM^{-1} cm^{-1} per enzymic flavin. The air-stable one-electron reduced, neutral semiquinone is a well known redox state for mammalian P-450 reductases although generally it is achieved by reduction with NADPH or dithionite (Vermilion and Coon, 1978). The isolation of the scup NADPH-cytochrome P-450 reductase in this form probably reflects the presence of thiols in the column buffers rather than novel properties for this enzyme species.

The apparent K_m of the purified scup NADPH-cytochrome

P-450 reductase was 24 uM for cytochrome c and 14 uM for NADPH, consonant with the values reported in microsomes of 21 and 13 uM, respectively (Stegeman et al., 1979). The turnover number with cytochrome c under standard assay conditions was 2200 min^{-1} per enzymic flavin while the uncoupled NADPH oxidase background[10] without the addition of an electron acceptor is 2.3 min^{-1} per enzymic flavin. A similar 0.2% oxidase background has been reported for the rat enzyme (Vermilion and Coon, 1978). Assays with scup NADPH-cytochrome P-450 reductase performed in 0.3 M KP_i, pH 7.7, at 30° C for direct comparison with the mammalian NADPH-cytochrome P-450 reductases resulted in 20-25% lower specific activities in part due to the loss of the FMN cofactor from the scup enzyme in high salt. Supplementation with FMN had a slightly stimulatory effect under high salt conditions but did not restore activity above the levels measured under standard assay conditions.

Scup hepatic microsomal cytochromes

Successful solubilization of scup microsomal cytochromes P-450 required carefully defined conditions. Scup cytochrome P-450 rapidly denatured to cytochrome P-420 when solubilized at room temperature by the method of Warner et al. (1978). This observation has been indirectly confirmed with other aquatic species (Williams and Buhler, 1982; James and Little, 1980) suggesting a general lability of cytochrome P-450 forms

in lower animals which probably reflects the differences in lipid composition or other temperature adaptations. Denaturation to cytochrome P-420 also occurred when the cholate concentration exceeded 0.6% or the Emulgen 911 concentration exceeded 0.4%. Numerous other detergents (including Triton X-100, deoxycholate, Tween 80, Triton B1956 and N101, Lubrol WX, Brij 35 and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) were tested individually or in concert and found unsatisfactory. Solubilization was ineffective at 4° C under low salt conditions unlike the case in spiny lobster where a curious red band is formed (James and Little, 1980). After more than a year of effort (involving 34 separate solubilization attempts under a variety of conditions and 20 DEAE column chromatographies) reproducible results were obtained at moderate protein concentrations (10 mg/ml) in 0.2M Tris-Cl at 4°C with a mixture of 0.5% cholate and 0.2% Emulgen 911. The combined action of ionic and nonionic detergent was particularly effective at protein concentrations chosen to insure complete solubilization. Greater than 90% of the cytochrome P-450 was solubilized after 30 min under these conditions, as judged by centrifugation at 100,000g for 75 min. The subsequent PEG fractionation was sensitive to the ionic strength of the solution so a constant I_c = 0.25 was adopted. While PEG precipitation did not drastically enhance the ensuing specific contents (judged by protein assays) it removed many non-protein impurities, markedly improved chromatographic resolution and served to concentrate the

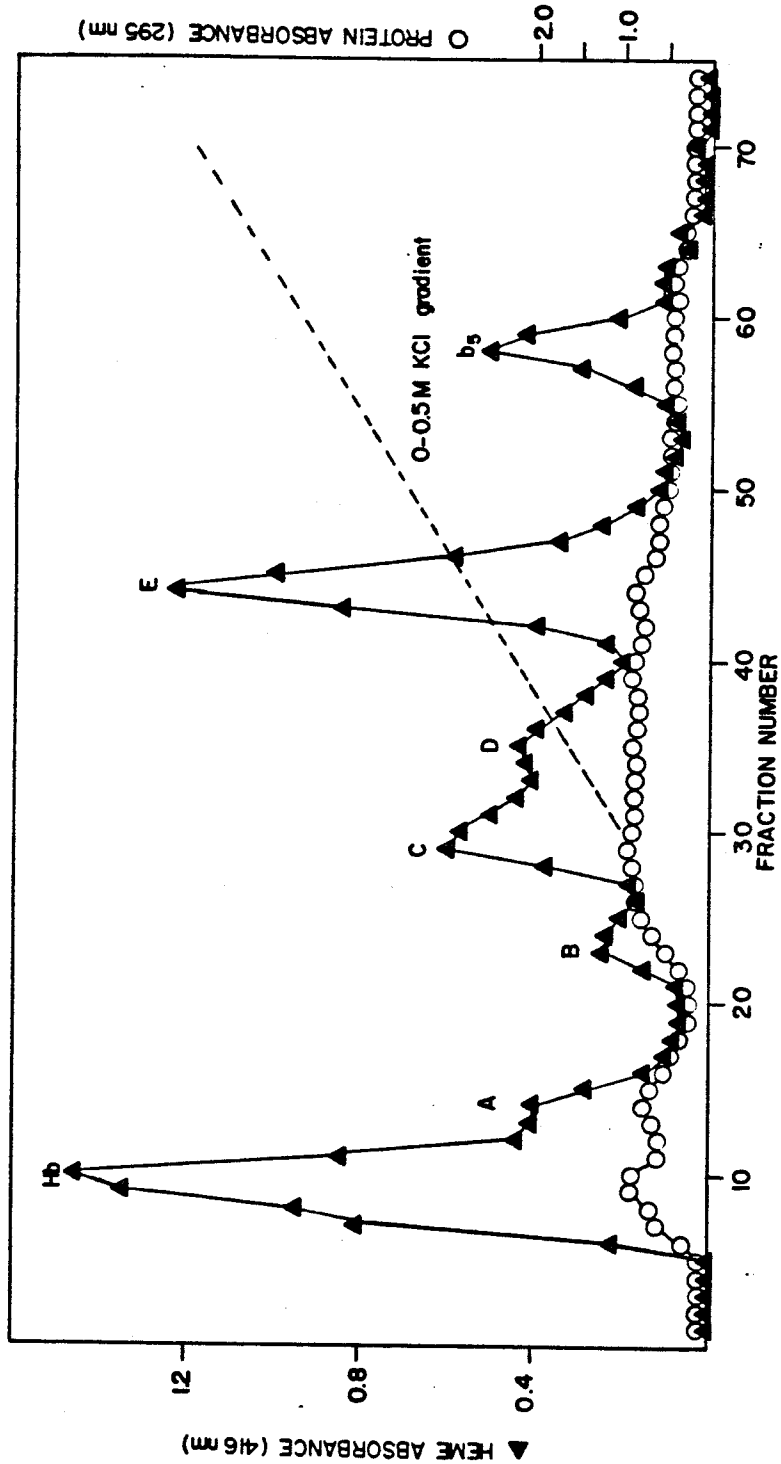
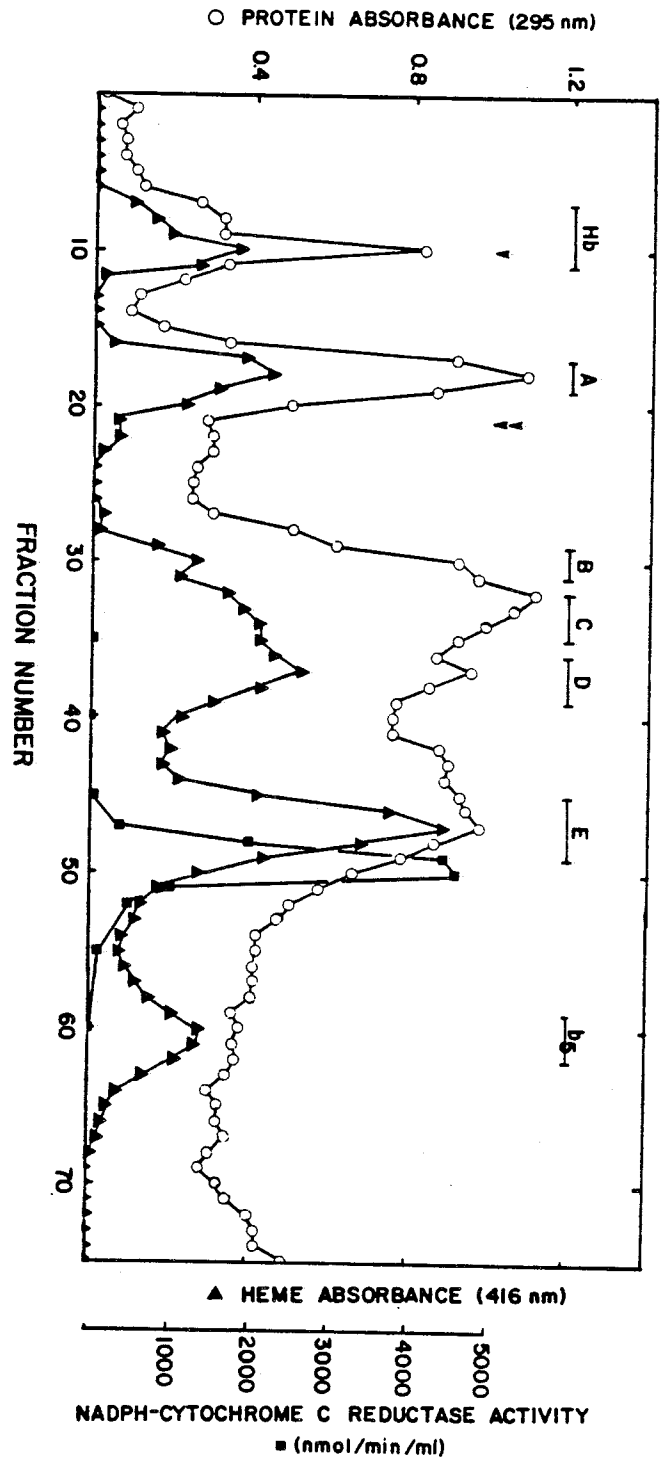

▲ HEME ABSORBANCE (416 nm)
O PROTEIN ABSORBANCE (295 nm)
0.4
0.8
1.2
1.0
2.0
10
20
30
40
50
60
70
FRACTION NUMBER
Hb
A
B
C
D
E
b_5
0-0.5 M KCl gradient

Figure 2-6. DEAE-cellulose ion exchange chromatography of hepatic microsomal cytochromes P-450 from untreated scup. 400 nmol of detergent-solubilized, PEG-fractionated cytochrome P-450 (see methods) were chromatographed at 4° C on a DEAE-cellulose column (80 ml bed volume) equilibrated in buffer I. The column was washed with buffer II to elute the hemoglobin and was developed with a 350 ml linear gradient of 0-0.5 M KCl in buffer II at fraction #12. Salt concentration was monitored by conductivity bridge. Cytochrome P-450-containing fractions were combined as follows: A (fractions #13-15); B (fractions #22-24); C (fractions #28-32); D (fractions #34-37); and E (fractions #42-47). Cytochrome b_5 eluted in fractions #56-60. Fractions of approximately 5.5 ml were collected throughout the chromatography. The total cytochrome P-450 recovery for the chromatography was greater than 90%.

Figure 2-7. DEAE-cellulose ion exchange chromatography of hepatic microsomal cytochromes P-450 from untreated scup. 450 nmol of detergent-solubilized, PEG-fractionated cytochrome P-450 (see methods) were chromatographed at 4° C on a DEAE-cellulose column (90 ml bed volume) equilibrated in buffer I. The column was washed with buffer I to elute hemoglobin (fractions #7-11); washed with buffer II (arrow) to elute cytochrome P-450 peak A (fractions #17-19); and developed with a 350 ml linear gradient of 0-0.5 M KCl in buffer II (double arrow). The remaining cytochrome P-450-containing fractions were combined as follows: B (fractions #29-31); C (fractions #32-35); D (fractions #36-39); and E (fractions #45-49). Additional protein fractions included the NADPH-cytochrome P-450 reductase (peak at fraction #50) and cytochrome b_5 (fractions #59-62). Fractions of approximately 7 ml were collected throughout the chromatography. The total cytochrome P-450 recovery for the chromatography was 73%.


O PROTEIN ABSORBANCE (295 nm)
0.4
0.8
1.2
Hb
A
B
C
D
E
b5
10
20
30
40
50
60
70
FRACTION NUMBER
▲ HEME ABSORBANCE (416 nm)
1000
2000
3000
4000
5000
NADPH-CYTOCHROME C REDUCTASE ACTIVITY
■ (nmol/min/ml)

cytochrome P-450 fraction in a low salt buffer. Recoveries in the 6-20% PEG pellet ranged from 43 to 76% but where typically 50% of the initial microsomal cytochrome P-450 (six experiments).

Hemoglobin, cytochrome b_5 and five pools containing cytochrome P-450 were resolved by DEAE-cellulose ion exchange chromatography (Figure 2-6) after detergent solubilization and PEG-fractionation of untreated scup hepatic microsomes using conditions modified from West et al. (1979) applied at 4° C. The five cytochrome P-450 pools (A-E; Figure 2-6) had CO, reduced difference spectrum absorbance maxima at 448, 449.5, 450.5, 451 and 447 nm, respectively. Recoveries of cytochrome P-450 on this column chromatography step ranged from 55 to 87% (six experiments) but were typically greater than 70%. The distribution of cytochrome P-450 in the identified pools also varied but cytochrome P-450E was consistently the largest fraction, representing 30-50% of the cytochrome P-450 chromatographed. The cytochrome P-450 present in the combination of pools A and B accounted for 15-20% of the cytochrome P-450 chromatographed. Cytochrome P-450 in pools C and D was poorly resolved and heterogeneous so that the extent of diversity was difficult to evaluate. NADPH-dependent cytochrome c reductase activity eluted between cytochrome P-450E and the cytochrome b_5 (see Figure 2-7).

B. Cytochrome P-450E.

The primary objective of this research project was to characterize the cytochrome responsible for the benzo[a]pyrene hydroxylase activity in microsomes. It was, therefore, rewarding to find that pool E (Figures 2-6 and 2-7) which was both the most highly purified and the most abundant cytochrome P-450 fraction[11] also contained a major polypeptide of 54 kD and a blue-shifted cytochrome P-450 chromophore. These latter properties are consistent with observations from hepatic microsomes isolated after scup had been induced with 3-methylcholanthrene (Stegeman et al., 1981) suggesting that cytochrome P-450E derived from these untreated (but not unexposed) fish would correspond to the major microsomal hemoprotein induced by 3-methylcholanthrene. Cytochrome P-450E was rechromatographed on DEAE-cellulose resulting in a single heme peak coincident with the sole protein peak but without further purification. Additional purification was achieved by column chromatography on hydroxylapatite (Figure 2-8) to a final specific content of 11.7 nmol heme per mg protein. SDS-PAGE analysis indicated that the final preparation (M_r = 54,300 $\pm$ 500) had no significant contaminants (Figure 2-9). A summary of the cytochrome P-450E purification is presented in Table 2-2.

UV-visible spectrum of cytochrome P-450E. Purified cytochrome P-450E (A_{416}/A_{295} = 2.2-2.4) had oxidized

absorbance maxima at 416.5, 532 and 568 nm. The hemoprotein absorbance maximum was 417.5 nm and the protein peak was observed at 278.5 nm (A_{417}/A_{280} = 1.17; Figure 2-10) after removal of the nonionic detergent (by exchange with deoxycholate). The CO, reduced difference spectrum revealed a typical cytochrome P-450 chromophore with a maximum at 447 nm. Cytochrome P-450E was calculated to have an oxidized heme extinction coefficient of E_{416} = 131 $\pm$ 7.5 mM^{-1} cm^{-1} from the difference spectrum and a protein chromophore of $E^{1\%}$ (295 nm) = 7.36 $\pm$ 0.82.

NH_2-terminal sequence analysis. It was interesting to investigate whether cytochrome P-450E shared sequence homology with its counterparts in higher animals because cytochrome P-450E was one of the first highly purified forms isolated from a lower animal. The cytochrome P-450E amino terminal sequence determination by Edman degradation is presented in Table 2-3. Three degradations were performed in order to confirm the first nine residues and to rule out ambiguity about the NH_2-terminal amino acid. The cytochrome P-450E amino terminal sequence has no homology with any known cytochrome P-450 form including the reported sequences for the major aromatic hydrocarbon-inducible cytochrome P-450 species in rat (P-450c; Botelho et al., 1979) or adult rabbit liver (LM-4; Coon and Black, personal communication) or minor aromatic hydrocarbon-inducible forms from rat (P-450d; Botelho et al., 1982) or adult rabbit liver (LM-6; Black et al.,

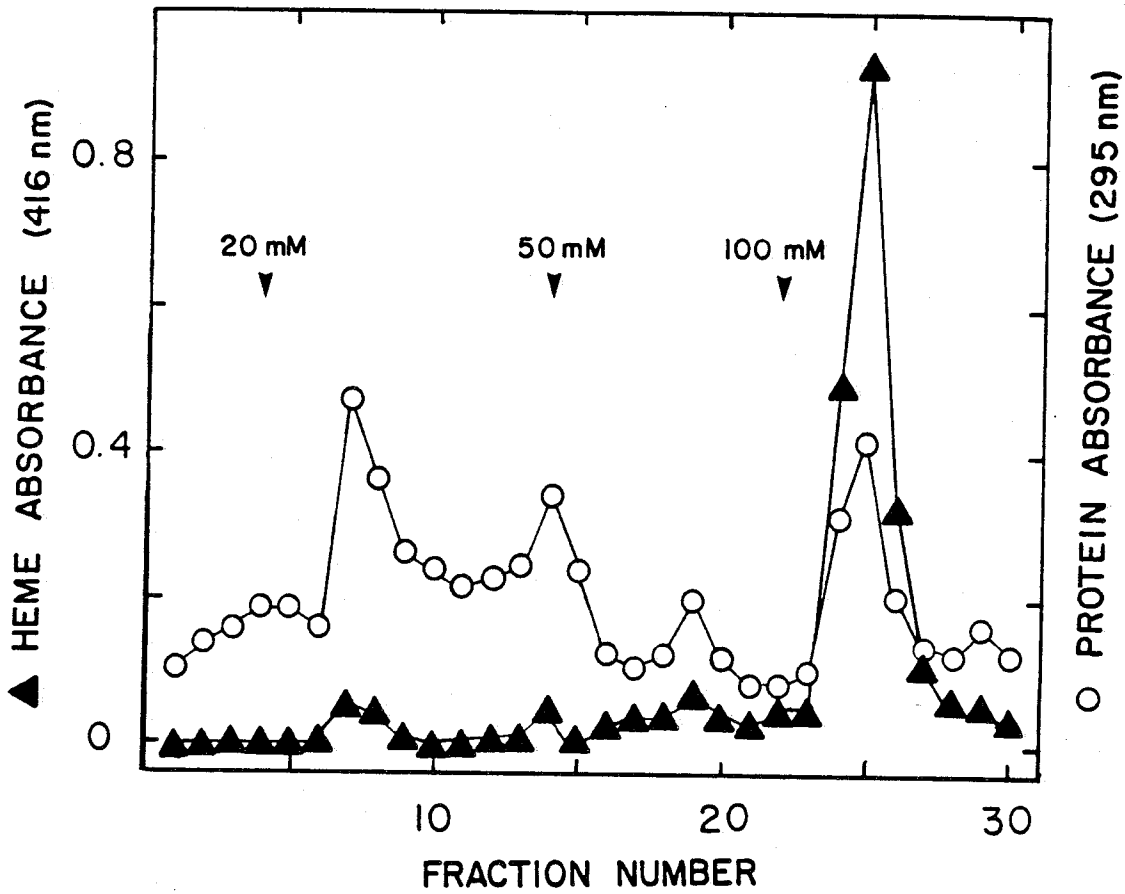


Figure 2-8. Hydroxylapatite chromatography of scup cytochrome P-450E. 54 nmol of peak cytochrome P-450E (Figure 2-6, fractions #43-45) were dialyzed and chromatographed on a hydroxylapatite column (10 ml bed volume) as described in the methods section. Fractions #24-26 were reserved on the basis of their A_{416}/A_{295} ratio and contained 39 nmol cytochrome P-450E (total recovery 73%).

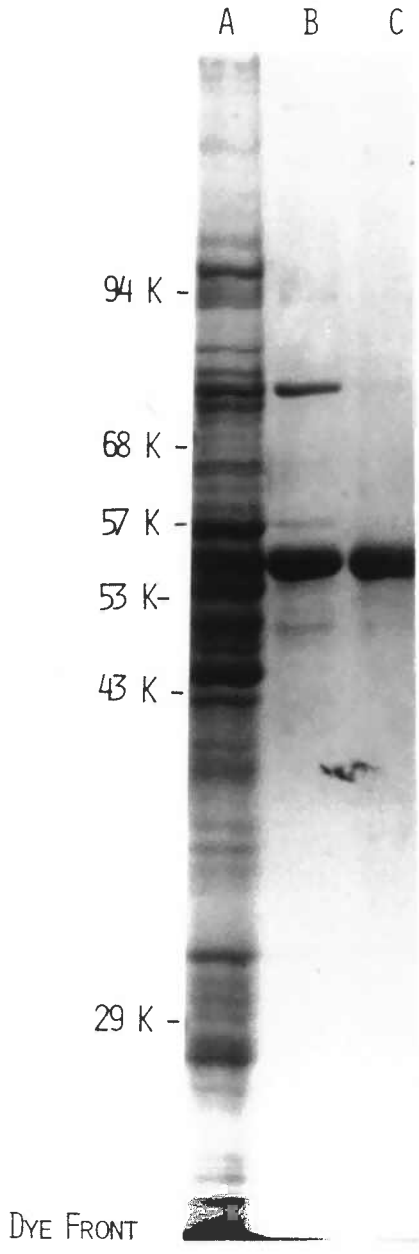

A
B
C
94 K -
68 K -
57 K -
53 K-
43 K -
29 K -
DYE FRONT

Figure 2-9. SDS-PAGE analysis of scup cytochrome P-450E purification. Discontinuous 9% polyacrylamide gel electrophoresis was performed by the method of Laemmli, 1970. Protein samples were loaded as follows: lane a, 30 ug microsomes; lane b, 7 ug DEAE-cellulose peak E (Figure 2-6, fractions #43-45); lane c, 5 ug hydroxylapatite peak (Figure 2-8, fraction #25).

TABLE 2-2

Purification of Scup Cytochrome P-450E[a]

Step	Total Cytochrome P-450 (nmol)	Specific Content (nmol/mg)	Step Recovery (%)
Microsomes[b]	782	0.84	--
Solubilization[c]	711	0.64	91
PEG fractionation, 6-20%	540	1.1	76
DEAE-cellulose[d], peak E (fr #43-45)	149	6.3	37
Hydroxylapatite[e], peak (fr #25)	21	11.7	39

[a]Cytochrome P-450 concentration was determined spectroscopically and protein concentration was determined by a modified Lowry assay.

[b]40 fish livers prepared as one pool.

[c]Cytochrome P-450 present in the supernatant fraction after detergent treatment and ultracentrifugation.

[d] 400 nmol PEG-fractionated cytochrome P-450 were chromatographed on DEAE-cellulose (Figure 2-6) and fractions 43-45 from pool E were combined. Fractions 42, 46 and 47 represented an additional 49 nmol cytochrome P-450 with a specific content of 4.3 nmol/mg.

[e] 54 nmol peak E cytochrome P-450 were chromatographed on hydroxylapatite (Figure 2-8). Fractions 24 and 26 representing an additional 17 nmol cytochrome P-450 had a slightly lower specific content of ca 10 nmol/mg. Thus 72% of the total applied cytochrome P-450 was recovered.

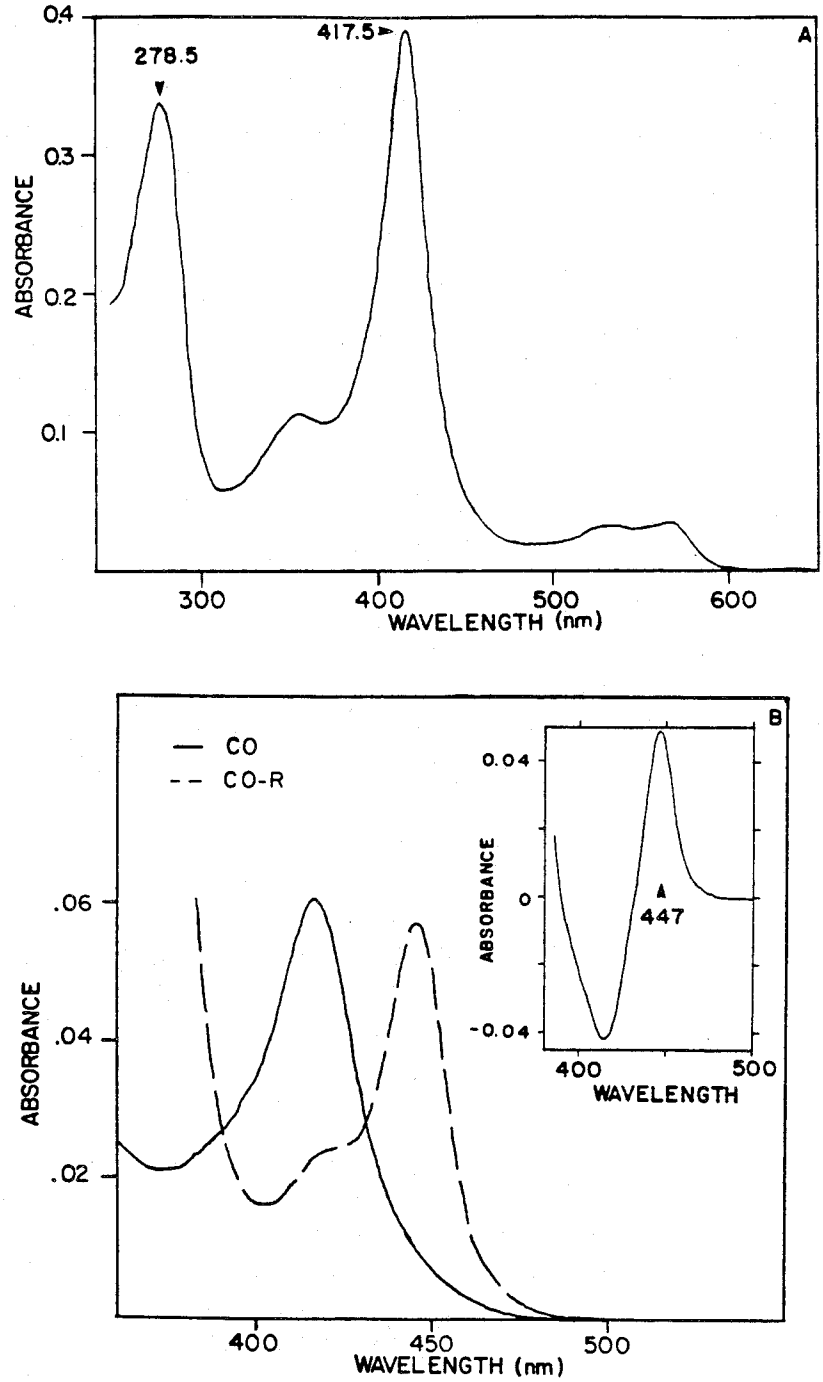


Figure 2-10. UV-visible spectrum of purified scup cytochrome P-450E. A, purified scup cytochrome P-450E (3 uM) after nonionic detergent removal (see methods). B, the direct spectrum of CO-ligated and CO-ligated, reduced forms of purified scup cytochrome P-450E (0.53 uM); inset, the CO-ligated, reduced difference spectrum.

1982). These sequences are summarized in Table 2-4.

C. Cytochrome P-450A.

Pool A, resolved on the first DEAE-cellulose ion exchange column (Figures 2-6 and 2-7), was interesting because it possessed a blue-shifted CO, reduced difference spectrum suggesting it might be involved in the metabolism of aromatic hydrocarbons (see Chapter 1). This tenuous hypothesis was strengthened by the observation that pool A eluted very early in the chromatography, similar to the report for a hexachlorobiphenyl-induced cytochrome P-450 form purified from rat liver microsomes (Goldstein et al., 1982).

Rechromatography of pool A on DEAE-cellulose revealed the presence of two cytochrome P-450 forms (Figure 2-11) having distinct CO, reduced difference spectra and different putative molecular weights. The peaks were labeled A and B in their order of elution from the column. The cytochrome P-450B contaminant in pool A appeared identical to fractions labeled B in Figures 2-6 and 2-7 as judged by reconstituted testosterone metabolism (see Ch. 3), SDS-PAGE analysis and UV-visible spectra (not shown). Cytochrome P-450A was then chromatographed twice on hydroxylapatite columns with mediocre recoveries and a notable lack of purification. Finally, I reasoned that poor binding to DEAE-cellulose might be complemented by good binding to CM-cellulose. Consequently, the cytochrome P-450A was chromatographed on a CM-cellulose column to achieve an acceptable preparation with 7.9 nmol heme

TABLE 2-3

Edman Degradation of Scup Cytochrome P-450E[a]

Cycle	Residue Identified	Yield (nmol) I[b]	II[f]	III[g]
1	Val	--[c]	6.3	3.7
2	Leu	4.5	9.4	3.5
3	Met	3.5	5.0	4.6
4	Ile	3.2	6.3	4.3
5	Leu	4.6	6.7[e]	3.4
6	Pro	1.9	--[e]	2.2
7	Val	6.2	--[e]	3.5
8	Ile	4.5[d]		4.3[h]
9	Gly	2.0[e]		2.2[e]
10	?	--		--
11	Val			4.1[e]
12	?			--
13	Val			3.1

[a]Purified cytochrome P-450E from three distinct preparations was degraded on a Beckman 890C Automated Sequenator using a 0.1 M Quadrol single or double cleavage program. The initial coupling yield was ca 50% and the repetitive yield thereafter was greater than 90%. Residue identification was by HPLC with confirmation by gas-liquid chromatography in this sequence for all residues except methionine. Quantitation was based on HPLC detector response factors.

[b]Approximately 10 nmol cytochrome P-450E was degraded by single cleavage with double coupling on the first cycle after an initial wash step.

[c]It appears that the NH_2-terminal residue was lost on the wash step for this sample because cycle 1 contained leucine in 3-fold excess of valine while a manual Edman analysis on an aliquot of the same sample indicated a 3-fold excess of valine over leucine.

[d]This cycle also contained a large peak coeluting with PTH-asp which was probably PTC-gly.

[e]No assignment was made since there was no unique amino acid derivative.

[f]Approximately 8 nmol cytochrome P-450E was degraded by the single cleavage program without an intervening double coupling or wash step. A PTH-nL (3.8 nmol) internal standard was included to assist in quantitation. Several cycles on this degradation had other significant PTH-amino acid derivatives suggesting the preparation was heterogeneous.

[g]After an initial wash step approximately 8 nmol cytochrome P-450E were degraded using a double cleavage program and double coupling on the first degradation cycle. A PTH-nL (3.5 nmol) internal standard was used. Contamination at the first two steps was less than 10% of the primary peak.

[h]Further conversion at 80°C for 30 min in 1 M HCl caused a destruction of the putative PTC-gly peak and an increase in the PTH-gly peak as monitored by HPLC.

TABLE 2-4

Comparison of Amino Terminal Sequences from
Cytochromes P-450 Induced by Aromatic Hydrocarbons

		1 5 10
Scup[a]	P-450E	H_2N-Val-Leu-Met-Ile-Leu-Pro-Val-Ile-Gly...
Rat[b]	P-450c	H_2N-Ile-Thr-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Glu...
	P-450d	H_2N-Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro...
Rabbit[c]	LM-4	H_2N-Ala-Met-Pro-Ala-Ala-Pro-Leu-Ser-Val-Thr...
	LM-6	H_2N-Ser-Asp-Val-Leu-Glu-Leu-Thr-Asp-Asp-Asn...

[a] See Table 2-3.

[b] Botelho et al., 1979; 1982.

[c] Black and Coon, personal communication; Black et al., 1982.

per mg protein. SDS-PAGE analysis indicated that the final cytochrome P-450A (M_r = 52,700 ± 300) had no significant contaminants (Figure 2-12). A summary of the cytochrome P-450A purification is presented in Table 2-5. With regard to future purifications of this isozyme, I would recommend that the cytochrome P-450A fraction resolved on the first DEAE-cellulose column be concentrated and rechromatographed on DEAE-cellulose (step II) to remove the cytochrome P-450B contaminant. This step can be followed directly by CM-cellulose column chromatography and a final chromatography on hydroxylapatite should then be propitious.

UV-visible spectrum of cytochrome P-450A. Purified cytochrome P-450A (A_{416}/A_{295} = 2.0) had oxidized absorbance maxima at 278, 417, 532 and 565 nm. The CO, reduced difference spectrum revealed a typical cytochrome P-450 chromophore with a maximum at 447.5 nm (Figure 2-13).

D. Cytochrome P-450B.

Fractions eluting from the first DEAE-cellulose column in the B region were further purified by rechromatography on DEAE-cellulose to remove traces of cytochrome P-450A cross-contamination. The cytochrome P-450B was finally chromatographed on a hydroxylapatite column to yield a highly purified enzyme containing 12.1 nmol heme per mg protein. SDS-PAGE analysis indicated a major polypeptide (M_r = 45,900 ± 900) and a trace low molecular weight contaminant (Figure

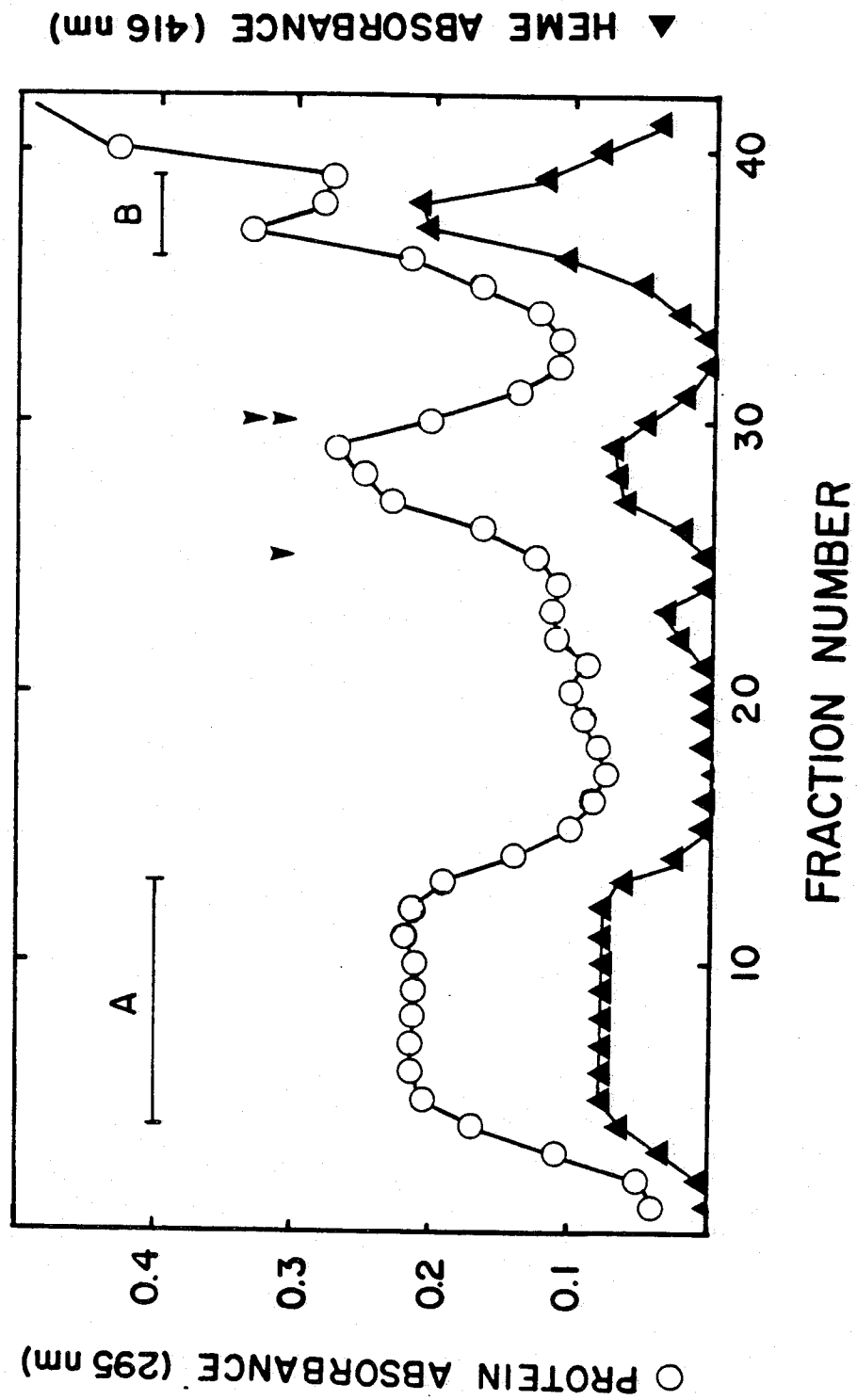

▲ HEME ABSORBANCE (416 nm)
○ PROTEIN ABSORBANCE (295 nm)
0.4
0.3
0.2
0.1
A
B
10
20
30
40
FRACTION NUMBER

Figure 2-11. DEAE-cellulose column resolution of scup cytochromes P-450A and P-450B. Fractions containing cytochrome P-450A (125 nmol), derived from several initial DEAE resolutions (see methods), were rechromatographed at 4° C on a DEAE-cellulose column (25 ml bed volume) in buffer I without detergent. The cytochrome P-450 fractions (#4-13; 40 nmol), containing a dominant 53 K polypeptide, were combined as cytochrome P-450A. The column was washed with buffer I containing 0.25% cholate and 0.1% Emulgen 911 at fraction #12; 35 mM KCl was added to the buffer wash (arrow); and a cytochrome P-450 peak was eluted with buffer II (double arrow). The cytochrome P-450 fractions which eluted (#36-39; 33 nmol) were labeled cytochrome P-450B on the basis of their SDS-PAGE migration and CO, reduced difference spectrum.

Figure 2-12. SDS-PAGE analysis of scup cytochrome P-450A purification. Discontinuous 9% polyacrylamide gel electrophoresis was performed by the method of Laemmli, 1970. Protein samples were loaded as follows: lane a, 20 ug microsomes; lane b, 24 ug 6-20% PEG fraction after solubilization; lane c, 8 ug DEAE column pool A (Table 2-5, step 1); lane d, 6 ug DEAE-cellulose column II peak (Figure 2-11, fractions #4-11; Table 2-5, step 2); lane e, 4 ug hydroxylapatite column II eluate (Table 2-5, step 4); lane f, 3 ug CM-cellulose eluate (Table 2-5, step 5). This SDS-PAGE experiment yields a calculated molecular weight of greater than 53 K for cytochrome P-450A, although the average for several experiments is 52.7 K.

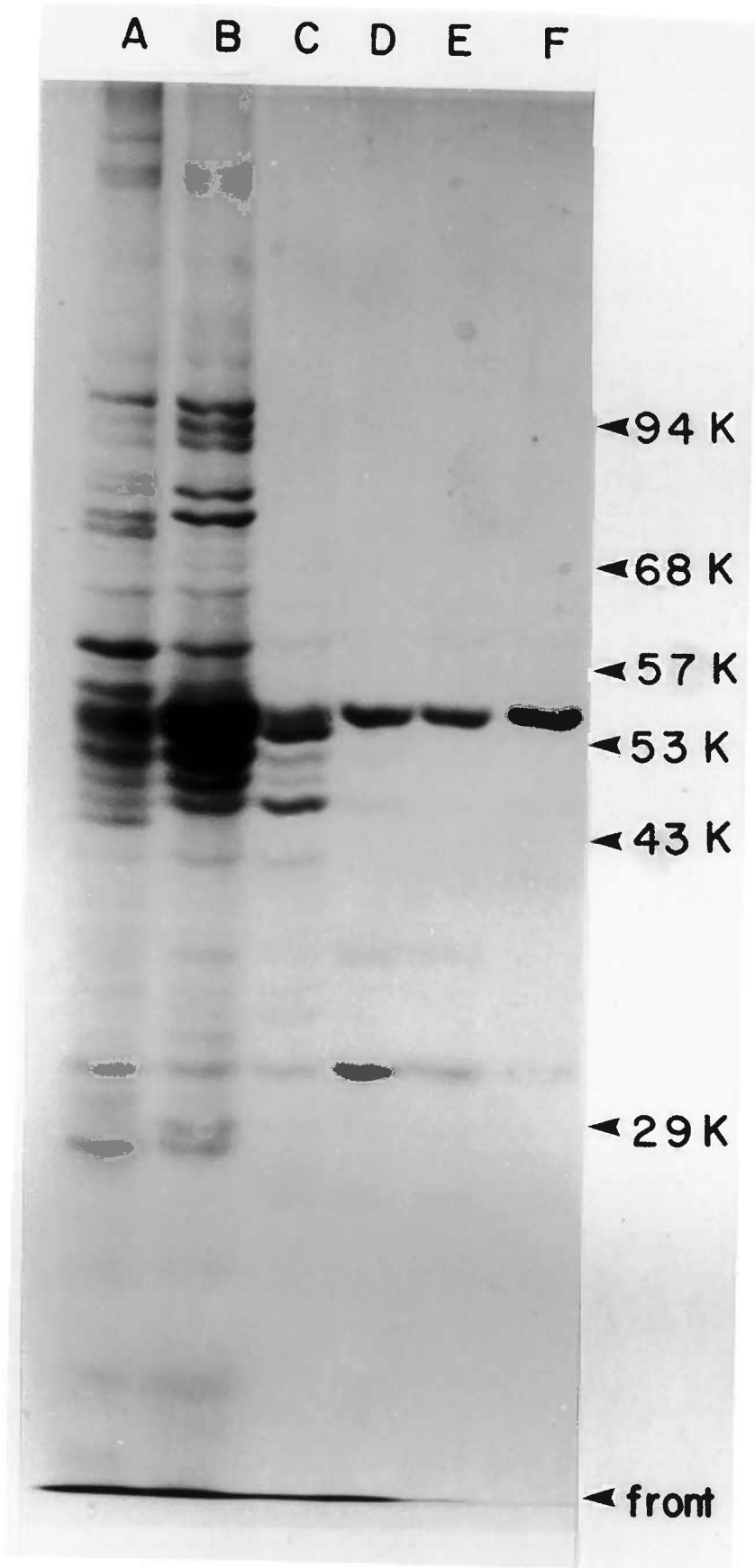

A
B
C
D
E
F
94 K
68 K
57 K
53 K
43 K
29 K
front

TABLE 2-5

Purification of Scup Cytochrome P-450A[a]

Step	Total Cytochrome P-450 (nmol)	Specific Content (nmol/mg)	Step Recovery (%)
1. DEAE column,[b] pool A	125	2.7	--
2. DEAE-cellulose,[c] column II	40	2.4	32
3. Hydroxylapatite, column I	24	3.2	62
4. Hydroxylapatite, column II	10	3.6	42
5. CM-cellulose[d] chromatography	4.4[e]	7.9	55

[a]Cytochrome P-450 concentration was determined spectroscopically and protein concentration was determined by a modified Lowry assay.

[b]Fractions from several separate DEAE-cellulose column resolutions were pooled based on their early elution; blue-shifted CO-ligated, reduced difference spectra; and 53K polypeptide.

[c]See Figure 2-11. Fractions were pooled on the basis of A_{416}/A_{295} ratio and appearance on SDS-PAGE. Fractions eluting in buffer II accounted for an additional 33 nmol cytochrome P-450 with a specific content of 3.8 nmol/mg.

[d]8 nmol cytochrome P-450A were chromatographed on the column.

[e]Estimated from an E_{416} = 140 mM^{-1} cm^{-1} determined at previous step. In an earlier preparation where no cytochrome P-420 was present the E_{416} was observed to be 110 mM^{-1} cm^{-1}. Application of this lower extinction coefficient would make the final cytochrome P-450A preparation 10 nmol/mg.

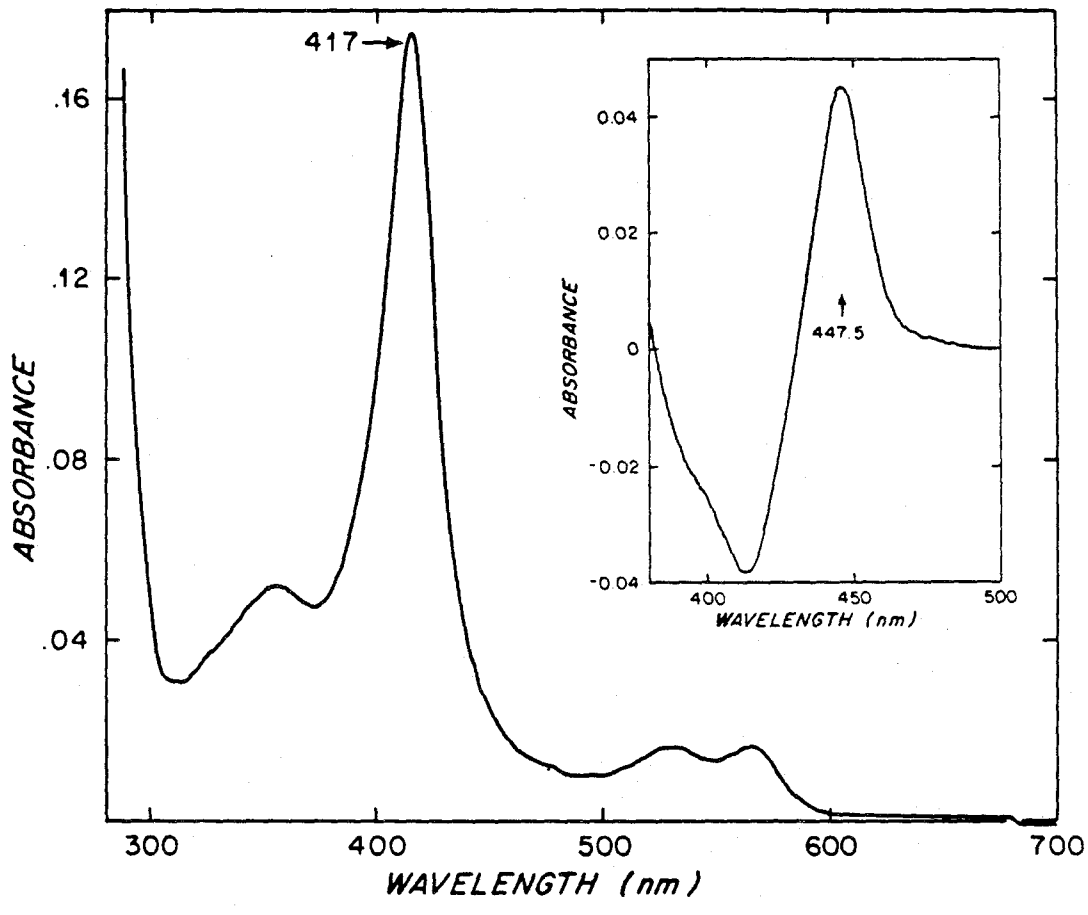


Figure 2-13. UV-visible spectrum of purified scup cytochrome P-450A. Purified scup cytochrome P-450A (1.3 uM) was scanned after CM-cellulose chromatography (Table 2-5, step 5); inset, the CO-ligated, reduced difference spectrum performed on an aliquot of purified cytochrome P-450A (0.5 uM).

2-14). Table 2-6 summarizes the purification of cytochrome P-450B.

UV-visible spectrum of cytochrome P-450B. Purified cytochrome P-450B (A_{416}/A_{295} = 2.4) had absorbance maxima at 278, 416, 532 and 567 nm. However, the nonionic detergent removal was probably incomplete as judged by broadening in the UV region around 276 nm (A_{416}/A_{280} = 0.80). The CO, reduced difference spectrum revealed a chromophore absorbing maximally at 449.5 nm (Figure 2-15). An oxidized heme extinction coefficient of E_{416} = 115 $\pm$ 6.4 mM^{-1} cm^{-1} was calculated from the difference spectrum and cytochrome P-450B had an $E^{1\%}$ (295 nm) = 5.87 $\pm$ 1.4 for the protein chromophore.

E. Cytochrome P-450 Fraction D.

The initial DEAE-cellulose ion exchange column revealed that the scup microsomal cytochrome P-450 complement also contained at least one red-shifted cytochrome P-450 chromophore in addition to the blue-shifted chromophores of cytochromes P-450A and P-450E. I elected to further purify the cytochrome P-450 fraction D proteins in light of the fact that several phenobarbital-inducible cytochrome P-450 forms in mammals exhibit a red-shifted spectrum (Haugen and Coon, 1976; Ryan et al., 1979), and pursuing the hypothesis that an analogue to phenobarbital-inducible forms might exist in scup hepatic microsomes. Rechromatography on DEAE-cellulose actually resulted in some net destruction of cytochrome P-450

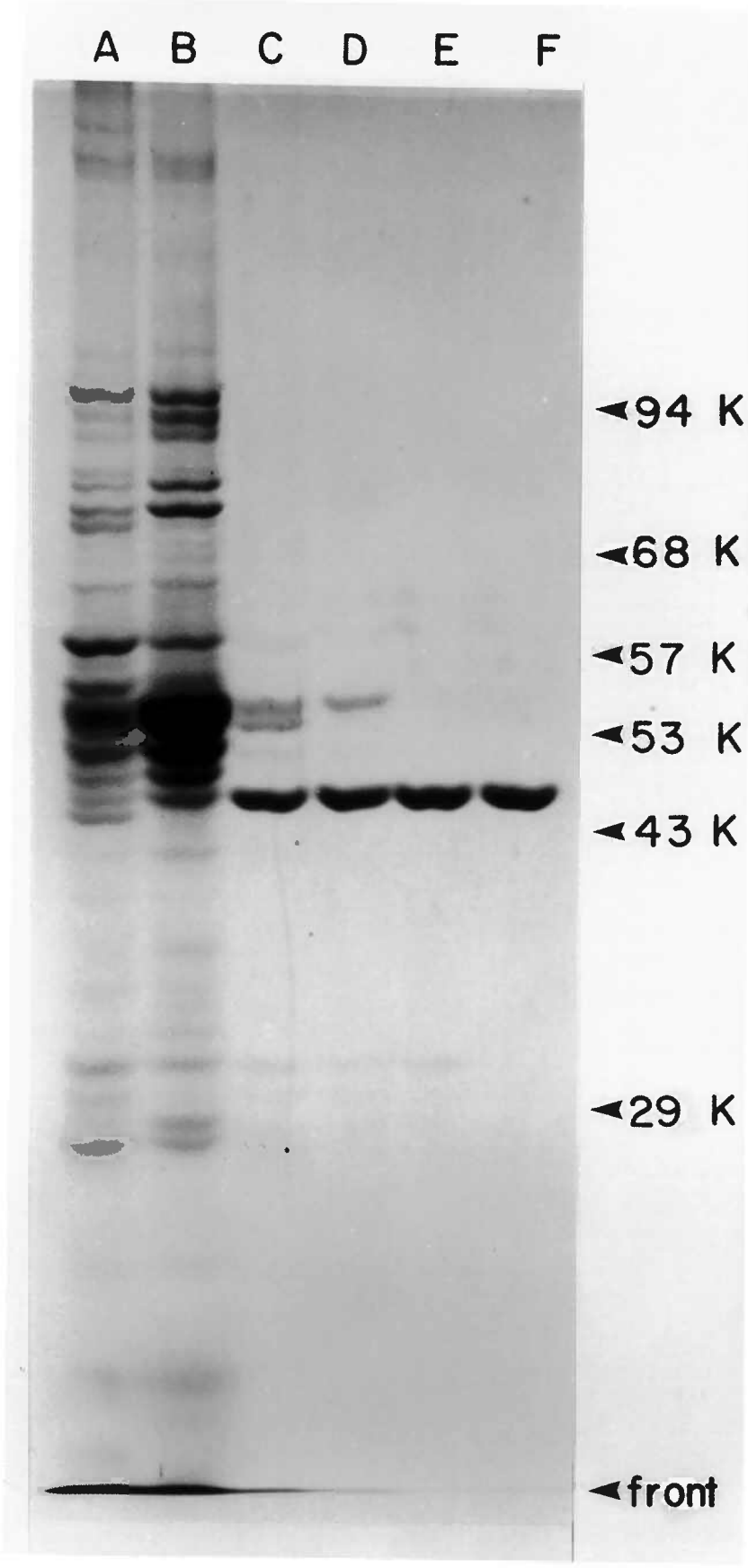

A
B
C
D
E
F
94 K
68 K
57 K
53 K
43 K
29 K
front

Figure 2-14. SDS-PAGE analysis of scup cytochrome P-450B purification. Discontinuous 9% polyacrylamide gel electrophoresis was performed by the method of Laemmli, 1970. Protein samples were loaded as follows (see Table 2-6): lane a, 20 ug microsomes; lane b, 24 ug 6-20% PEG fraction after solubilization; lane c, 6 ug DEAE column pool B; lane d, 3 ug DEAE-cellulose II eluate; lane e, 3 ug hydroxylapatite column eluate; lane f, 3 ug purified scup cytochrome P-450B after nonionic detergent removal by column chromatography.

TABLE 2-6

Purification of Scup Cytochrome P-450B[a]

Step	Total Cytochrome P-450 (nmol)	Specific Content (nmol/mg)	Step Recovery (%)
DEAE column,[b] pool B	80	4.6	--
DEAE-cellulose, column II	50	8.4	63
Hydroxylapatite[c] chromatography	14.5	12.1	36

[a]Cytochrome P-450 concentration was determined spectroscopically and protein concentration was determined by a modified Lowry assay.

[b]Identified as containing cytochrome P-450B based on 46K polypeptide and CO, reduced difference spectrum with absorbance maximum at 449 nm.

[c]40 nmol cytochrome P-450B were chromatographed. In addition to the peak, 15 nmol cytochrome P-450 were recovered in less pure fractions.

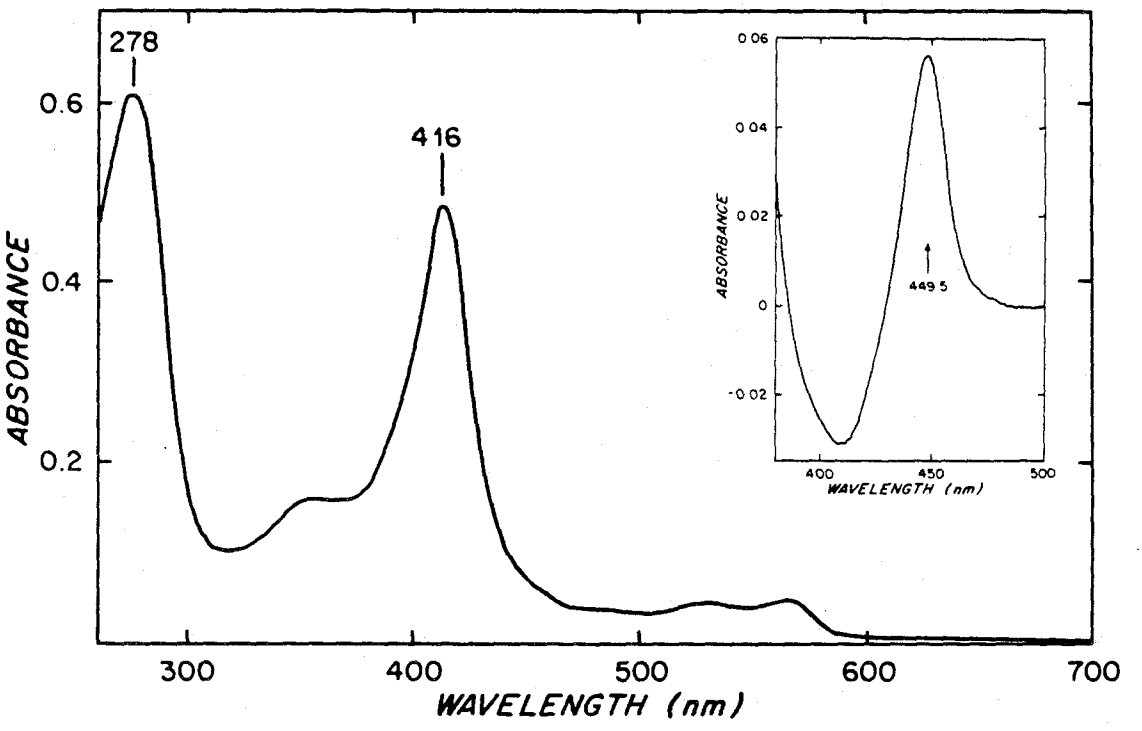


Figure 2-15. UV-visible spectrum of purified scup cytochrome P-450B. Purified scup cytochrome P-450B (4.3 uM) was scanned after nonionic detergent removal (see methods); inset, the CO-ligated, reduced difference spectrum performed on an aliquot of purified scup cytochrome P-450B (0.62 uM).

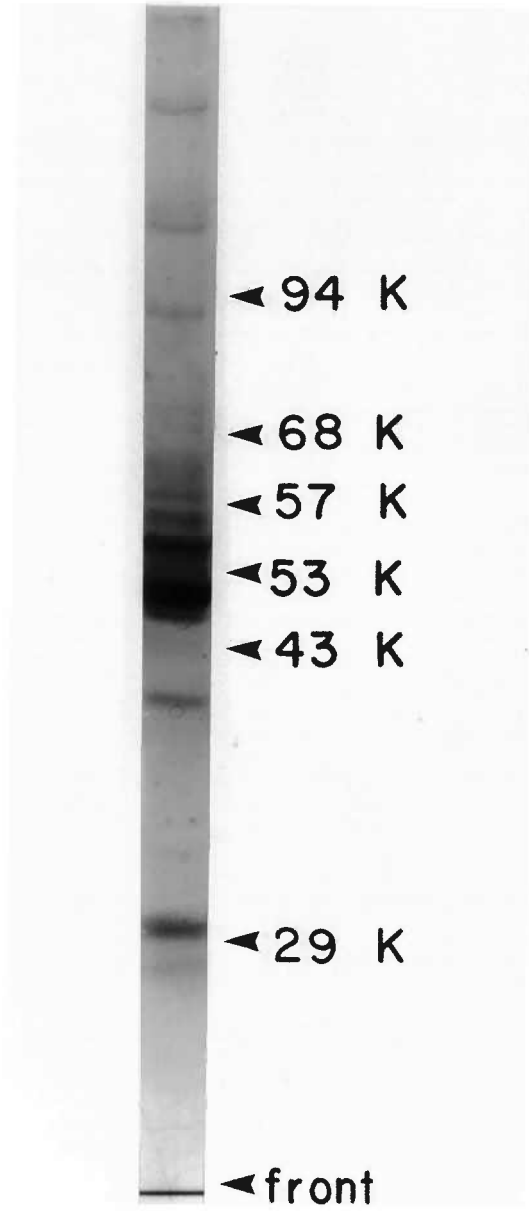

94 K
68 K
57 K
53 K
43 K
29 K
front

Figure 2-16. SDS-PAGE analysis of scup cytochrome P-450 fraction D. Discontinuous 9% polyacrylamide gel elctrophoresis was performed by the method of Laemmli, 1970. Partially purified cytochrome P-450 fraction D (2.8 nmol/mg) was prepared through the hydroxylapatite chromatography and nonionic detergent removal step and 8 ug protein was loaded on the gel. The major protein band is centered around 50.1 K.

TABLE 2-7

Purification of Scup Cytochrome P-450 Fraction D[a]

Step	Total Cytochrome P-450 (nmol)	Specific Content (nmol/mg)	Step Recovery (%)
DEAE chromatography,[b] pool D	70	1.2	--
DEAE-cellulose,[c] column II	33	0.90	47
Hydroxylapatite chromatography	14.5	2.8	44

[a]Cytochrome P-450 concentration was determined spectroscopically and protein concentration was determined by a modified Lowry assay.

[b]Pool D was identified by its CO-ligated, reduced chromophore which absorbed maximally above 450 nm.

[c]The peak was pooled on the basis of A_{416}/A_{295} ratio and CO, reduced difference λ_{max} near 451 nm. Fractions eluting earlier in the gradient comprised 22 nmol additional cytochrome P-450 with a lower CO, reduced difference spectrum λ_{max}. Fraction D evidenced significant apparent cytochrome P-420 in the spectrum which contributed to the low specific content.

as judged by the formation of increased cytochrome P-420 and a diminished specific content. A majority of the cytochrome P-450 eluted in one major peak suggesting that multiple chromatographic forms were not present in this region, unlike cytochromes P-450A and P-450B which are cross-contaminated. Some real purification was achieved by chromatography on a hydroxylapatite column and the resulting hemoprotein(s) fraction contained 2.8 nmol heme per mg protein. Clearly the proper stabilizing conditions for the purification of this fraction have not yet been established. The partial purification of cytochrome P-450 fraction D is summarized in Table 2-7.

SDS-PAGE analysis confirmed the suspicion that fraction D was a heterogeneous mix of proteins (Figure 2-16) precluding the assignment of a credible molecular weight to the chromophore. The UV-visible spectrum after detergent removal revealed absorbance peaks at 279, 419, 536 and 566 nm. The CO, reduced spectrum indicated the presence of a cytochrome P-450 chromophore which absorbed maximally near 451 nm and a nearly equal amount of a (non-reducible?) chromophore absorbing near 420 nm in the CO, reduced direct spectrum (not shown).

Comparison of cytochrome P-450 forms.

Table 2-8 summarizes the physical properties of the resolved cytochromes P-450. Brief inspection confirms the notion that the CO, reduced difference spectra and the

apparent molecular weights provide some discriminations although spectroscopic criteria alone are not compelling. Purified cytochromes P-450A, P-450B and P-450E are also separated by SDS-PAGE (Figure 2-17).

Although cytochromes P-450A and P-450E show slight differences in spectroscopic properties and apparent molecular weights the qualitative similarity in catalytic properties (discussed in Chapter 3) was a source of concern. Partial proteolytic analysis was performed in order to establish that each cytochrome P-450 represented a unique primary structure. Further, it was of interest to determine whether scup cytochrome P-450E and the analogous form (TLM-4b[12]), purified from 5,6-benzoflavone-treated rainbow trout by Drs. Williams and Buhler, are closely related. Incubation with S. aureus V8 protease (Figure 2-18) and a-chymotrypsin (Figure 2-19) clearly demonstrated that each of the four cytochrome P-450 forms analyzed was digested to a different pattern of polypeptides. The minimum conclusion from this experiment is that each of the four purified cytochromes P-450 is a unique primary structure.

Ouchterlony double diffusion analysis of antibodies raised in rabbits to purified scup cytochrome P-450E showed that the antibody cross-reacted with TLM-4a[12], yielding a line of fusion without visible spurring (Figure 2-20). Such a fusion pattern without spurring is commonly associated with immunochemical identity. The antibody raised against scup cytochrome P-450E did not recognize cytochrome P-450A but it

TABLE 2-8

Properties of Purified Emulgen-free Scup Cytochromes P-450[a]

Form	MW	UV-visible Absorbance Peaks (nm) CO, R difference	Oxidized direct			
E	54.3K	447	278.5	417.5	534	567
A	52.7	447.5	278	417	532	565
B	45.9	449.5	278	416	532	567
Fraction D	--[b]	451	279	419	536	566

[a]MW determined by SDS-PAGE.

[b]The preparation was heterogeneous.

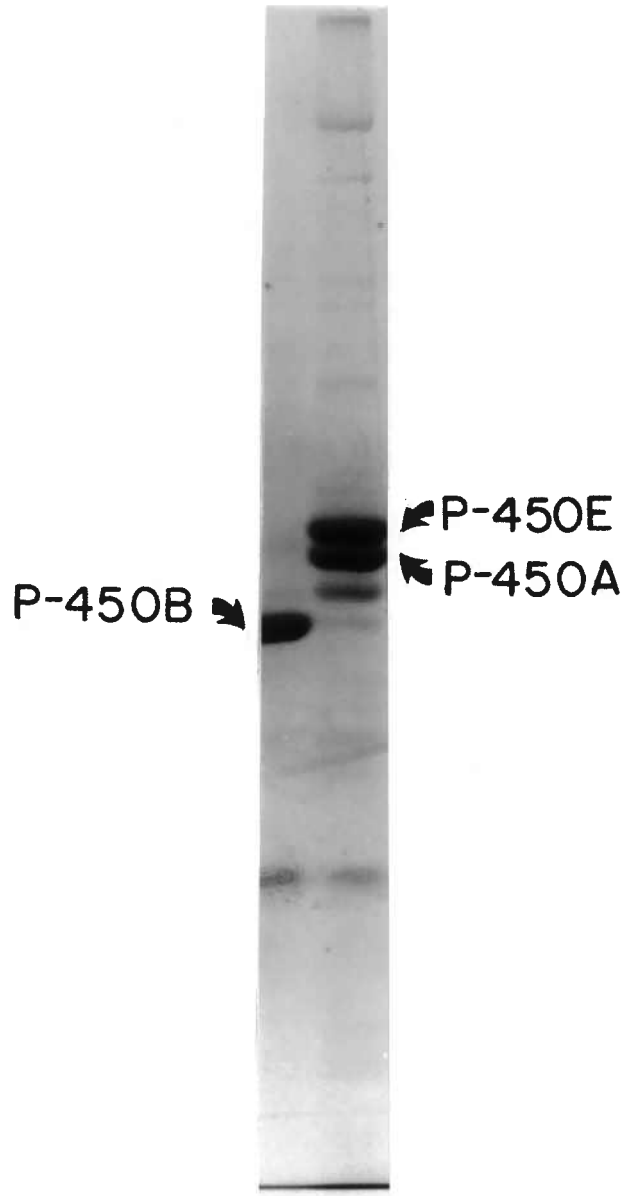

P-450E
P-450A
P-450B

Figure 2-17. SDS-PAGE analysis of purified scup cytochromes P-450A, P-450B and P-450E. Discontinuous 9% polyacrylamide gel electrophoresis was performed by the method of Laemmli, 1970. Samples of 3 ug protein for each cytochrome P-450 were loaded on the gel. The 50K protein band below cytochrome P-450A is a contaminant in that preparation of cytochrome P-450A but is apparently not a cytochrome P-450 form.

Figure 2-18. Cytochromes P-450 peptide mapping by partial proteolysis with S. aureus V8 protease. Digestions were performed in 0.25% SDS for 90 min at 37° C as described in the methods section. The digest were analyzed by discontinuous SDS-PAGE on a 13.5% separating gel. Protein samples (5 ug) were loaded in each lane after incubation (from left to right) with 0, 5 and 20 ug S. aureus V8 protease per ml. S. aureus V8 protease (20 ug/ml) alone was loaded in the lane marked V8 to indicate the origin of the protein doublet in adjacent lanes.

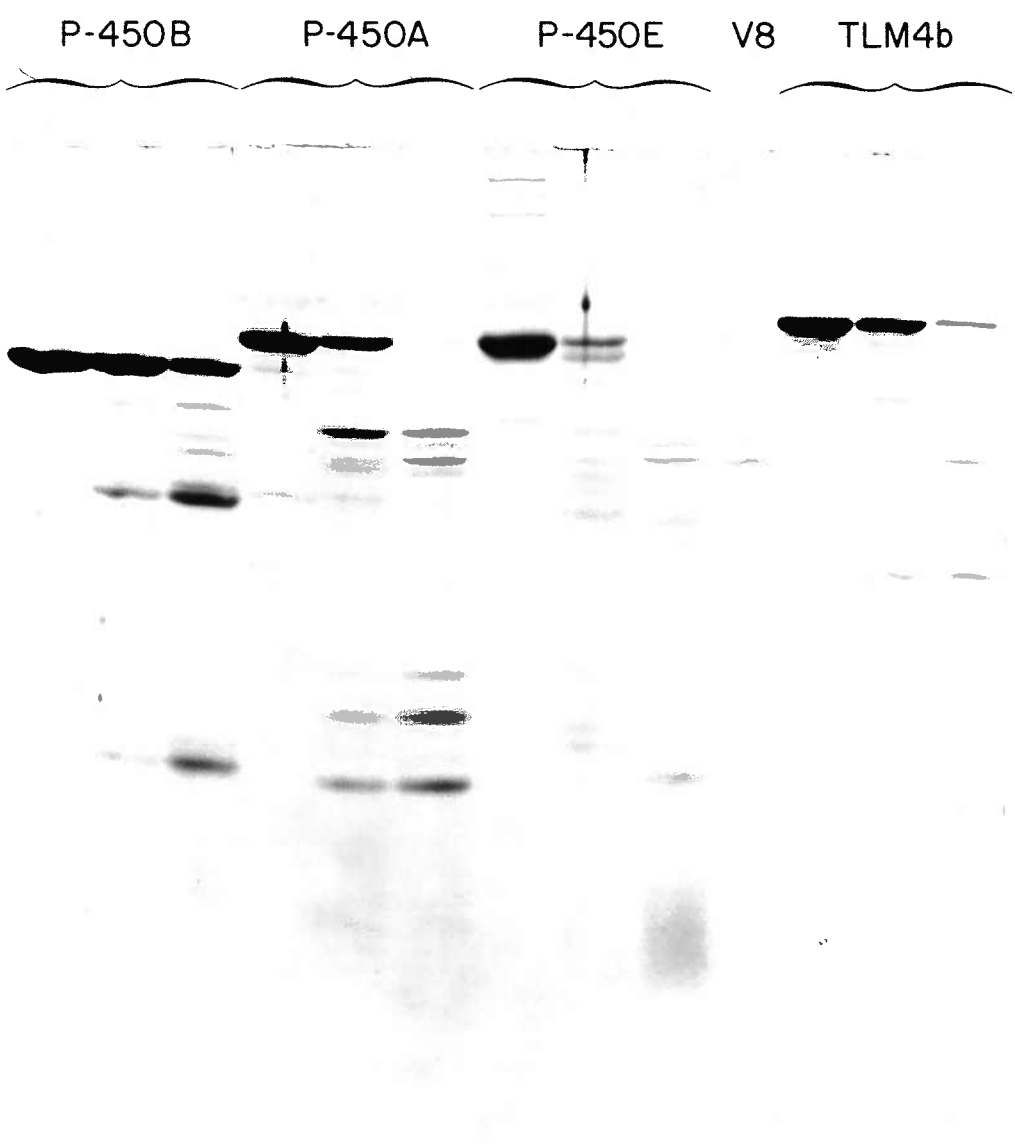
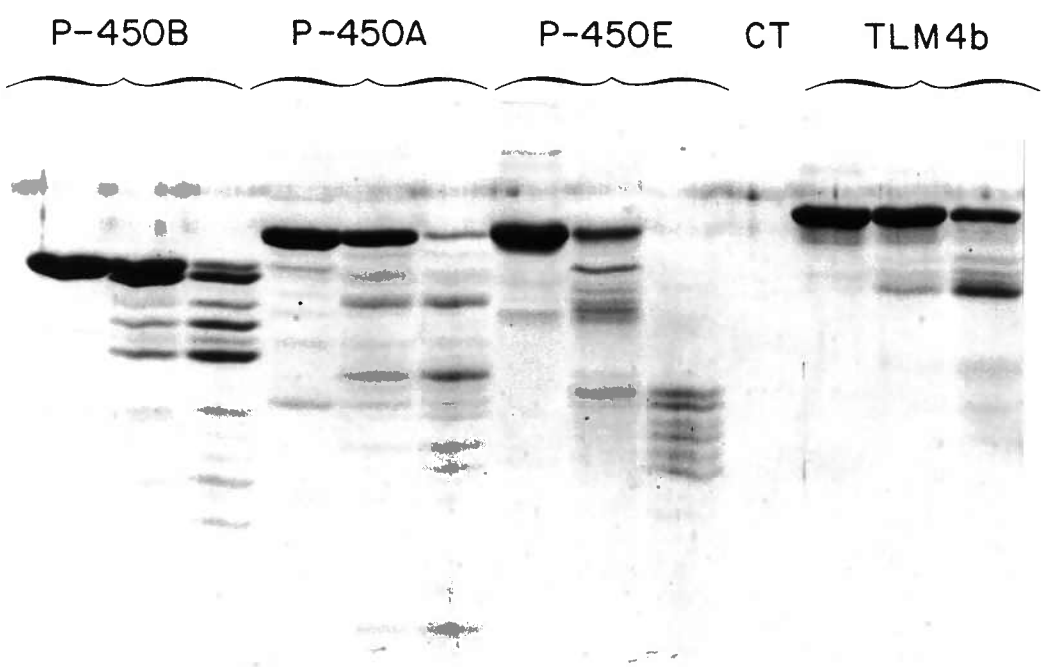

P-450B
P-450A
P-450E
V8
TLM4b

Figure 2-19. Cytochromes P-450 peptide mapping by partial proteolysis with α-chymotrypsin. Digestions were performed in 0.25% SDS for 90 min at 37° C as described in the methods section. The digests were analyzed by discontinuous SDS-PAGE on a 13.5% separating gel. Protein samples (5 ug) were loaded in each lane after incubation (from left to right) with 0, 1 and 5 ug α-chymotrypsin per ml. α-Chymotrypsin (5 ug/ml) alone was loaded in the lane marked CT to indicate the origin of one low molecular weight protein in adjacent lanes.


P-450B
P-450A
P-450E
CT
TLM4b

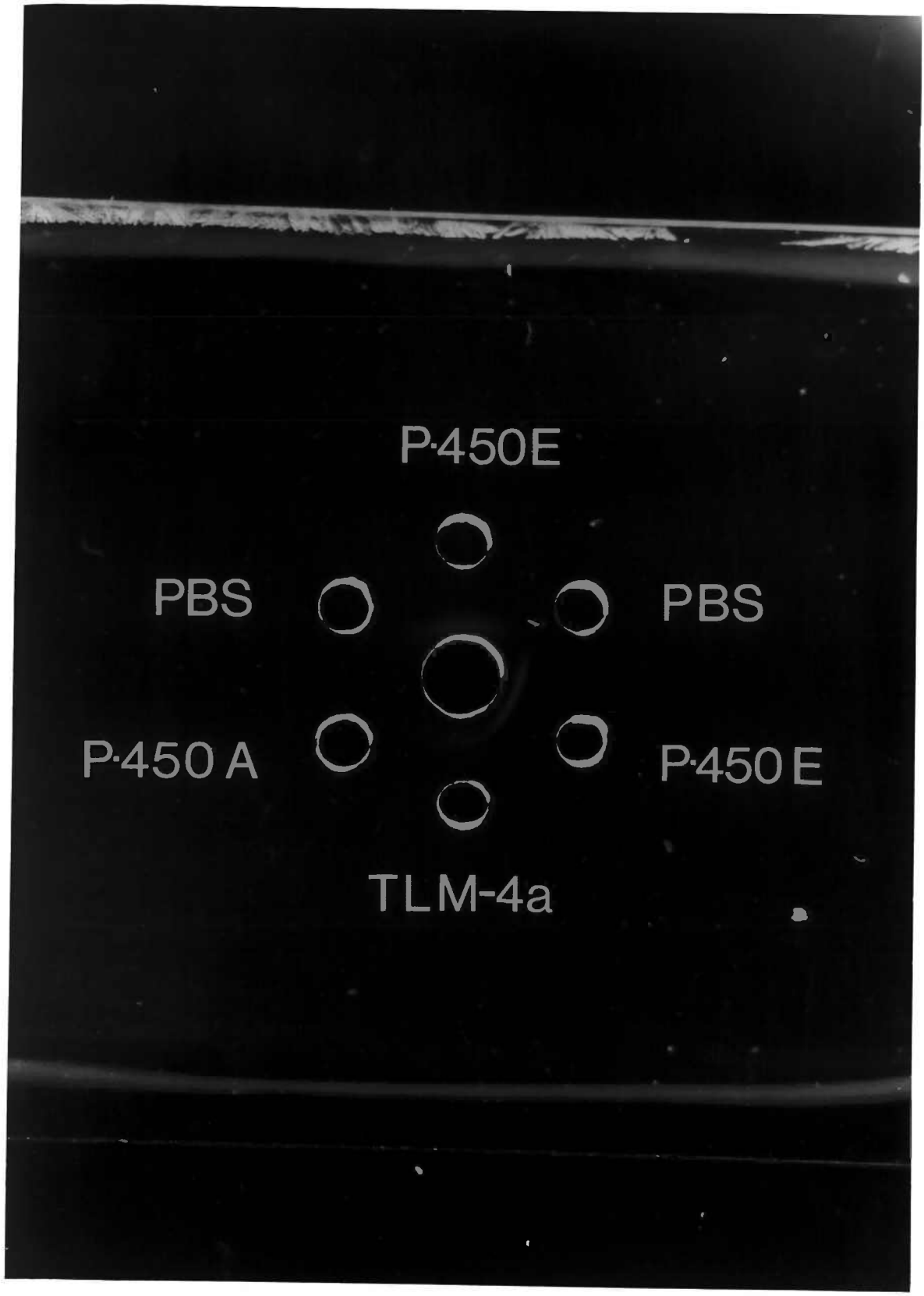

P-450E
PBS
PBS
P-450 A
P-450 E
TLM-4a

Figure 2-20. Ouchterlony double diffusion gel analysis of purified cytochromes P-450. Purified scup cytochrome P-450E (8-15 uM), purified scup cytochrome P-450A (3.2 uM) and purified trout liver cytochrome P-450 TLM-4a (5 uM) were placed in the outer wells of the 0.9% agarose gel and crude IgG (30 mg/ml) isolated from a rabbit immunized with purified scup cytochrome P-450E was placed in the center well. The proteins were allowed to diffuse overnight and the precipitin lines were visualized directly. The purified scup cytochrome P-450E in the top well was a sample of DEAE peak E (Figure 2-6, fractions #43-45) and the purified cytochrome P-450E in the lower right lane was the immunogen. PBS, phosphate-buffered saline.

did react with liver microsomal antigenic determinants which appear identical to purified cytochrome P-450E. The observation that scup cytochrome P-450E and trout cytochrome P-450 TLM-4 are immunochemically similar has been confirmed by other findings: antibody to trout cytochrome P-450 TLM-4b inhibited catalytic activity with reconstituted cytochrome P-450E and in scup microsomes (not shown); the antibody to trout TLM-4 cross-reacted with scup cytochrome P-450E with an identical pattern of fusion in Ouchterlony double diffusion analysis (Williams and Buhler, personal communication).

F. Cytochrome b_5.

Reports of cytochrome b_5 stimulating activity in assays reconstituting mammalian cytochromes P-450 (Vatsis et al., 1982; Waxman and Walsh, 1982) coupled with the low catalytic activites observed when scup cytochromes P-450 were reconstituted (Chapter 3) dictated the purification of scup cytochrome b_5 for tests in reconstitutions of catalytic activity. The fractionation of cytochrome b_5 was difficult to reconstruct in the early stages of the cytochrome P-450 purification since it could not be assayed reliably at the solubilization or PEG-fractionation step due to the interference of hemoglobin and uncoupling effects of detergents[13]. Approximately 25% of the microsomal cytochrome b_5 was recovered in the DEAE-column peak suggesting that significant losses occurred in the PEG-fractionation.

The cytochrome b_5 fractions pooled from several

DEAE-cellulose column resolutions all contained Emulgen 911. Rechromatography on DEAE-cellulose resulted in some purification but did not remove the Emulgen 911 despite extensive washing. Subsequent chromatography on hydroxylapatite resulted in partial binding but still without effective removal of Emulgen 911. Finally, rechromatography on DEAE-cellulose resulted in a significant purification of cytochrome b_5 with decreased Emulgen 911 levels. Hydroxylapatite chromatography yielded two fractions - one which was free of Emulgen 911 and one which was still contaminated. These two fractions appeared identical when subjected to SDS-PAGE analysis. The cytochrome b_5 fraction which still contained Emulgen 911 traces was efficiently rendered free of the unwanted detergent by gel filtration on a Sephadex G-75 column (Figure 2-21). The scup cytochrome b_5 purification is summarized in Table 2-9.

The final preparation was highly purified (28.6 nmol heme per mg protein) however SDS-PAGE revealed that it consisted of two polypeptides - a major band of 24.5 kD and a minor band at 20 kD (Figure 2-22). The reconstitution results with scup cytochrome b_5 reported in Chapter 3 made it essential to determine if the high molecular weight polypeptide was likely to be the cytochrome b_5. A gel scan of the lane bearing an aliquot from the G-75 column peak permitted the calculation that the two polypeptides were present in a 3:1 molar ratio or 79:29 mass ratio. Further calculations showed that while 72 pmol of heme were loaded onto the gel, the low molecular

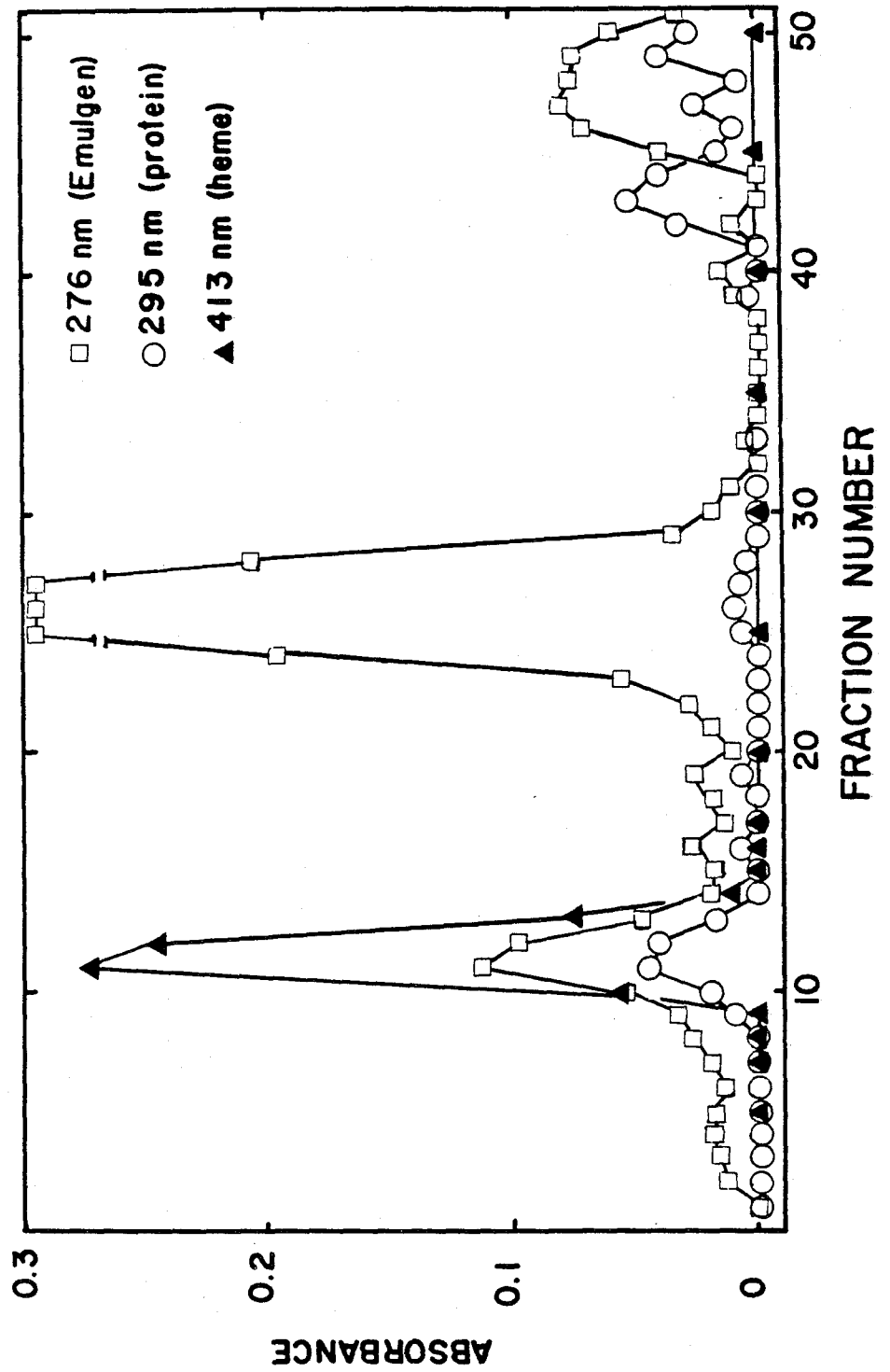

□276 nm (Emulgen)
○295 nm (protein)
▲413 nm (heme)
ABSORBANCE
0.3
0.2
0.1
0
10
20
30
40
50
FRACTION NUMBER

Figure 2-21. Scup cytochrome b_5 chromatography on G-75. Concentrated scup hepatic cytochrome b_5 (Table 2-9, step 5; 5 nmol) was chromatographed at 4° C on a Ø.8 x 6Ø cm Sephadex G-75 (superfine; 4Ø ml bed volume) column in buffer IV containing Ø.4% DOC. The chromatography was performed at 2 ml/hr and Ø.8 ml fractions were collected after 9.2 ml had passed through the column. Fractions #11 and 12 were combined as the peak cytochrome b_5. N.B. The cytochrome b_5 eluted near the void volume, well in advance of the partially included detergent micelles.

TABLE 2-9

Purification of Scup Cytochrome b_5[a]

Step	Total Cytochrome b_5 (nmol)	Specific Content (nmol/mg)	Step Recovery (%)
1. DEAE column, b_5 pool	180	3.0[b]	--
2. DEAE-cellulose II, chromatography	155[c]	4.8	86
3. Hydroxylapatite chromatography	63	5.7	41
4. DEAE-cellulose III, chromatography	17.1[c]	21.8	27
5. Hydroxylapatite chromatography	5.0[c,d]	23.2	29
6. G-75[e]	2.7	28.6	54

[a]Cytochrome b_5 concentration was determined spectroscopically and protein concentration was determined by modified Lowry assay.

[b]A typical specific content for microsomal cytochrome b_5 is 0.10-0.15 nmol/mg.

[c]Estimated from an experimentally determined $E_{413} = 145 \pm 5.6$ mM^{-1} cm^{-1} for the direct spectrum of the oxidized hemoprotein.

[d]Additionally, 6.2 nmol cytochrome b_5 (21 nmol/mg) was eluted by 50 mM KP_i buffer in later fractions and was free of Emulgen 911 contamination.

[e]See Figure 2-21.

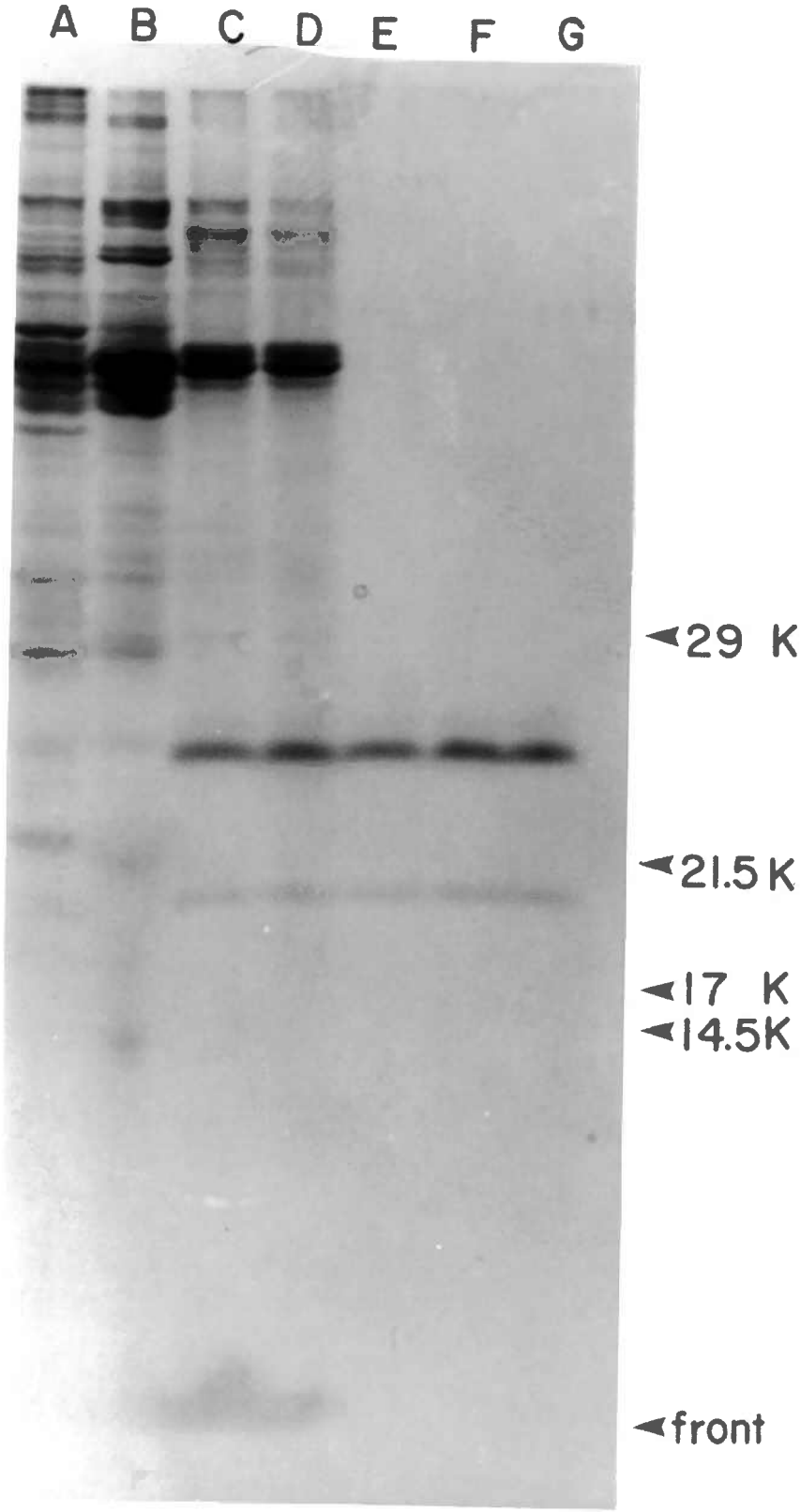

A
B
C
D
E
F
G
29 K
21.5K
17 K
14.5K
front

Figure 2-22. SDS-PAGE analysis of scup cytochrome b_5 purification. Discontinuous 12.5% polyacrylamide gel electrophoresis was performed by the method of Laemmli, 1970. Protein samples were loaded as follows: lane a, 20 ug microsomes; lane b, 24 ug 6-20% PEG fraction after solubilization; lane c, 20 ug DEAE column cytochrome b_5 pool (Table 2-9, step 1); lane d, 14 ug DEAE-cellulose column II eluate (Table 2-9, step 2); lane e, 3 ug DEAE-cellulose column III eluate (Table 2-9, step 4); lane f, 3 ug hydroxylapatite column eluate (Table 2-9, step 5); lane g, 3 ug G-75 column eluate (Table 2-9, step 6).

weight band represented 27 pmol protein while the high molecular weight band constituted 82 pmol protein. If one grants the reasonable assumption of one heme moiety per polypeptide, then it appears to this investigator that scup cytochrome b_5 is a 24.5 kD polypeptide. The nature of the 20 kD species is unknown for the present and it is certainly worth establishing in an unambiguous fashion whether scup cytochrome b_5 has a 24.5 K molecular weight. I would recommend that in future scup cytochrome b_5 purifications, the Emulgen be completely removed at an earlier stage to afford better chromatographic behavior. It would be useful to treat the crude cytochrome b_5 fraction with BioBeads (BioRad SM-2) prior to DEAE-cellulose chromatography (Table 2-9, step 2). The G-75 column was also effective in removing Emulgen if the detergent was present at only moderate levels and I would perform that chromatography after step 2. Hydroxylapatite chromatography and A-1.5m gel filtration should then provide very pure protein with a superior recovery.

UV-visible spectrum of cytochrome b_5. The oxidized spectrum of purified scup cytochrome b_5 (Figure 2-22) contained peaks at 276, 412.5, 530 and 558 nm (A_{413}/A_{295} = 5.2; A_{413}/A_{280} = 2.3). The spectroscopic ratio of A_{413}/A_{280} = 2.3 is near the 2.7-2.8 reported in mammals for apparently homogeneous preparations with specific contents of 32-36 nmol/mg (Strittmatter et al., 1978; Waxman and Walsh, 1982). The reduced difference spectrum had a peak at 423 nm and

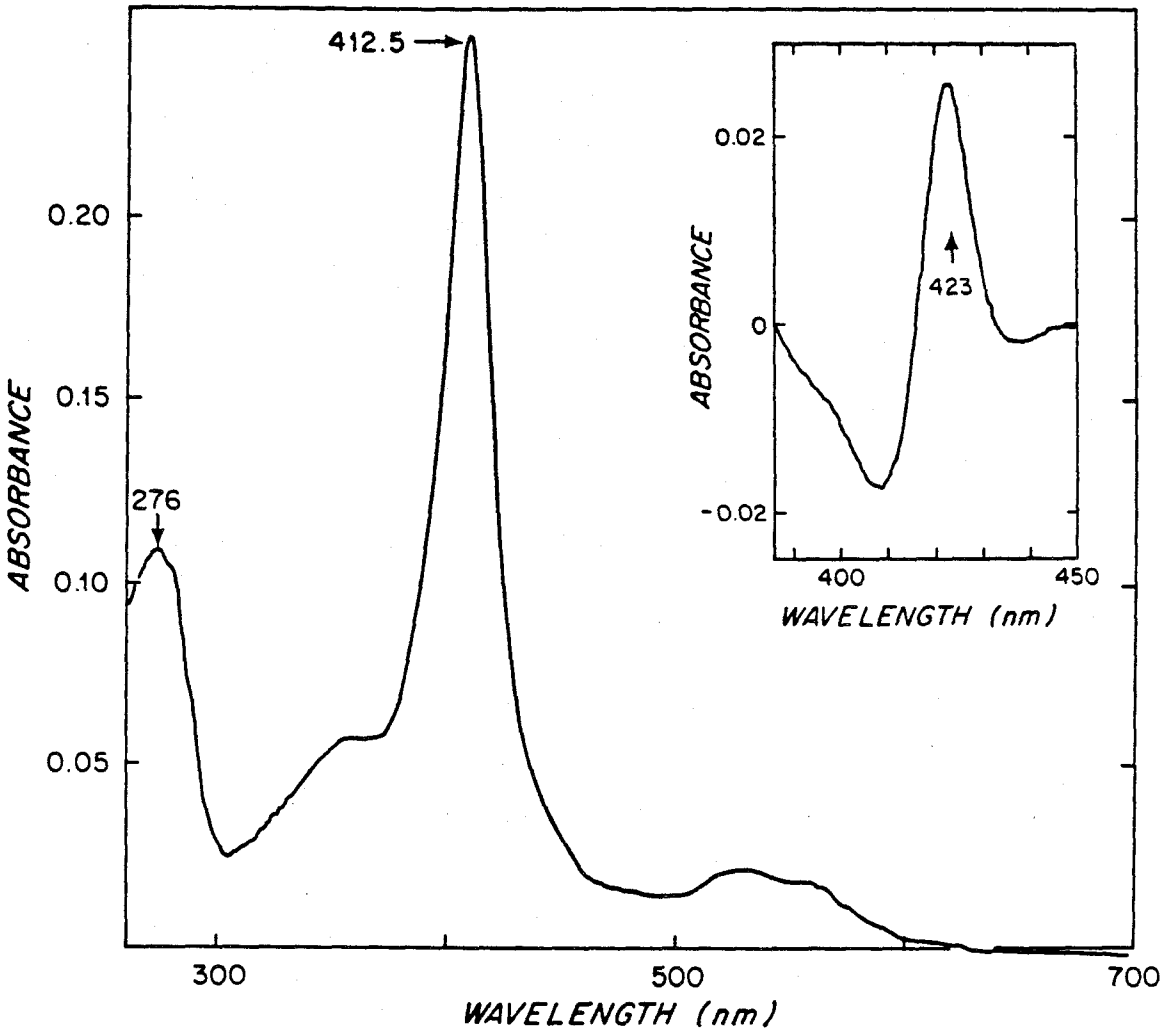


Figure 2-23. UV-visible spectrum of purified scup cytochrome b_5. Purified scup cytochrome b_5 (1.8 uM) was scanned after G-75 chromatography (Figure 2-21, fractions #11 and 12); inset, the dithionite-reduced difference spectrum performed on an aliquot (0.23 uM) of purified scup cytochrome b_5.

evidenced the typical cytochrome b_5 chromophore. An experimental extinction coefficient for the oxidized hemoprotein was calculated as $E_{413} = 145 \pm 5.6$ mM^{-1} cm^{-1} based on the reduced difference spectrum. This value does not agree extraordinarily well with the $E_{413} = 117$ mM^{-1} cm^{-1} reported for oxidized steer liver cytochrome b_5 (Strittmatter and Velick, 1956) and I have no account for the apparent difference.

CONCLUSIONS

The purification and characterization of the NADPH-cytochrome P-450 reductase from scup hepatic microsomes extends the supposition that cytochrome P-450 reductases from a variety of sources are structurally similar (Guengerich, 1978; Yasukochi et al., 1979; Black and Coon, 1982). The scup NADPH-cytochrome P-450 reductase flavin content of one FAD and one FMN per polypeptide is similar to the observations made with the reductases purified from the hepatic microsomes of little skate, Raja erinacea (Serabjit-Singh et al., 1981), rat (Yasukochi and Masters, 1976) and rabbits (French and Coon, 1979). In keeping with the comparable specific activities, the calculated turnover number of 2200 min^{-1} per enzymic flavin compares favorably with the 2015 min^{-1}, 2700 min^{-1} and 2790 min^{-1} observed with the rabbit (French and Coon, 1979), steer (Enoch and Strittmatter, 1979) and rat (Yasukochi and

Masters, 1976) enzymes, respectively. These results raise the possibility of structural homology between scup and mammalian NADPH-cytochrome P-450 reductases in light of their functional equivalence (Chapter 3) and could provide additional support for the claim that cytochrome P-450 reductases from a variety of sources share sequence homology (Guengerich, 1978; Black and Coon, 1982).

The strategy of rechromatographing individual peaks on DEAE-cellulose was useful as a confirmation that pool E represented a single chromatographic form while cytochromes P-450A and P-450B were both present at differing ratios in the first two pools. The latter finding must be taken as a caution in considering column resolution as a criterion for making distinctions between cytochrome P-450 forms. Clearly, only those chromatographic resolutions which are reproducible can have any force. In fact, aggregation phenomena and detergent artifacts appear to be common in the resolution of crude membrane fractions.

Criteria for cytochrome P-450 purity are subject to debate. Specific contents (nmol CO-reducible heme per mg protein) are reliable in distinguishing non-hemoprotein contamination but only to the extent that the protein determinations are accurate (not a trivial objection!) and assuming that the formation of apoenzyme or cytochrome P-420 is insignificant. Theoretical homogeneity based on specific content can be determined from the reciprocal of the molecular

weight -- approximately 20 nmol heme per mg protein for a 50 K polypeptide. Specific content is the touchstone of purity in the field of cytochromes P-450 since it is easy to measure but it is exiguous; multiple criteria are necessary to establish purity and distinguish cytochrome P-450 forms. SDS-PAGE analysis will resolve proteins which differ by ca 500 daltons but cytochromes P-450 can have identical molecular weights and all known microsomal cytochromes P-450 are in the 45-60K region. Even immunochemical evidence can be misleading since several pairs of distinct cytochrome P-450 forms can share determinants (rat P-450c and P-450d, Ryan et al., 1980; rat PB-4 and PB-5, Waxman and Walsh, 1982). Amino terminal sequence analysis would seem to be unambiguous in establishing a single polypeptide but after taking the 50% coupling yield into account the method loses some of its power. Considered as a group, however, these tests become much more convincing.

Scup cytochromes P-450A, P-450B and P-450E are distinct forms as judged by molecular weight, peptide mapping and CO, reduced difference spectra. The true extent of diversity in the scup microsomal cytochrome P-450 complement is not yet known. Probably a minimum of one unique cytochrome P-450 form is present in each of the five identified DEAE-cellulose column peaks (Figures 2-6 and 2-7). Peak A appears to be a composite of P-450A and P-450B, and peak B is primarily P-450B. The nature of peak C has not yet been disclosed but product analysis of testosterone hydroxylase reconstitutions (not shown) suggest it possesses unique activities. Fraction

C hemoproteins have not been substantially characterized and little more can be said about them. Peak D consists of at least one cytochrome P-450 which is red-shifted, and peak E is, by all criteria examined to date, exclusively cytochrome P-450E.

The absorbance maximum near 417 nm for the purified scup microsomal cytochromes P-450 indicates that these forms have been isolated in the low spin state (Jefcoate and Gaylor, 1969) in the presence or absence of nonionic detergent. This agrees with 1.6° K electron spin resonance spectroscopy on the microsomal cytochrome P-450 complement which detected no high spin heme iron (Chevion et al., 1977). The direct oxidized heme extinction coefficients of 131 and 115 mM^{-1} cm^{-1} at 416 nm for scup cytochromes P-450B and P-450E, respectively, calculated from the CO, reduced difference spectrum, agree with the same data for low spin mammalian cytochrome P-450 forms (110-117 mM^{-1} cm^{-1}; Haugen and Coon, 1976; Ryan et al., 1979). The slightly higher extinction coefficient for scup cytochrome P-450E probably reflects the proclivity to form cytochrome P-420 in the preparations; a characteristic observed in samples of cytochromes P-450E and fraction D, but not cytochromes P-450A or P-450B.

The general techniques for microsomal cytochrome P-450 solubilization, resolution and purification will hopefully be applicable to other aquatic species. It is my expectation that research questions such as sex-dependent differences in hepatic monooxygenases can be approached in other fish species

by minor modifications of these techniques. Moreover, additional interesting cytochrome P-450 forms can no doubt be "fished" out of scup hepatic microsomes. The application of antibodies raised against scup liver microsomal cytochrome P-450E will also be useful in probing for these determinants in extrahepatic tissues.

Chapter 3 will describe the results of reconstituting monooxygenase activity with the resolved cytochrome P-450 isozymes. These results demonstrate the power of purification techniques in dissecting isozymic contributions to metabolism in liver microsomes.

Footnotes

1. 3.5 mg protein was chromatographed per ml settled resin. The resin has an anomalously low protein binding capacity under these conditions and the vantage point of experience indicates that diligence must be exercised not to overload it.

2. A majority of this cytochrome P-450 could be recovered by PEG 6000 precipitation (vide infra).

3. The scup NADPH-cytochrome P-450 reductase seems to lose FMN (and consequently catalytic activity with cytochrome c) under high salt conditions and partially purified fractions frequently showed stimulation of activity in the presence of exogenous FMN. This FMN loss appears to occur particularly at the DEAE-A25 chromatography step and the fear was that apoenzyme might be unstable.

4. 5 nmol of cytochrome per ml of packed resin was chromatographed on this column.

5. More recently a 0-0.4 M KCl gradient was found to give an improved resolution of the cytochrome P-450 fractions while remaining effective in eluting cytochrome b_5.

6. 10 nmol of cytochrome per ml of packed resin was chromatographed on this column.

7. These absorbed proteins were eluted as heterogeneous fractions by the higher ionic strength of buffer II.

8. 20 nmole of cytochrome per ml of packed resin was chromatographed on this column.

9. PTH-arg and PTH-his remain in the aqueous phase under these extraction conditions and would appear as blanks in the analysis.

10. The oxidase background measures the rate of NADPH oxidation by the NADPH-cytochrome P-450 reductase in the absence of an added electron acceptor. It is assumed that O_2 is adventitiously being reduced to H_2O_2 and/or $^{\bullet}O_2^-$ at the enzyme flavin active site in order to balance the redox stoichiometry. The oxidase background is an important consideration in reconstitution experiments where high concentrations of the NADPH-cytochrome P-450 reductase are used. Under these conditions the enzyme is also producing reduced oxygen intermediates which may be responsible for nonenzymatic substrate oxidation or may affect the time-dependence of reconstituted monooxygenase turnover.

11. In the experiment documented in Figure 2-6 (see also Table 2-2) the cytochrome P-450E isolated in the eluted peak

was 30% of the initial cytochrome P-450 in the microsomes and most likely represented 50% of the microsomal cytochrome P-450 after a consideration of recoveries on the column. Pool E (fractions #42-47) represented 50% of the cytochrome P-450 chromatographed in Figure 2-6.

12. TLM-4a and TLM-4b are the current names for purified trout liver microsomal cytochrome P-450 forms originally described as peak g (aka DEc HA_3) and peak f (aka DEc HA_2), respectively (Williams amd Buhler, 1982; 1983b). TLM-4a and TLM-4b are indistinguishable by UV-visible spectrum, SDS-PAGE analysis, peptide mapping by partial proteolytic digestion, and catalytic activity (Williams and Buhler, 1982; 1983b). Antibodies prepared against either trout cytochrome P-450 form cross-react with the other form yielding apparent lines of identity in Ouchterlony diffusion analysis (Williams and Buhler, 1983a). Subsequent work by Williams and Buhler has revealed no credible differences between these two forms in the opinion of this investigator. The evidence for a distinction is based on a partial chromatographic resolution of two peaks, slight differences in amino acid content and a difference in the inhibition curve at low concentrations when rabbit antibody raised against TLM-4b is added to reconstitutions of TLM-4a- or TLM-4b-dependent benzo[a]pyrene hydroxylase activity. The discriminating reader will perceive that I treat TLM-4a and TLM-4b as identical in my analyses comparing TLM-4 with scup cytochrome P-450E.

13. The spectrophotometric assay requires the generation of a reduced minus oxidized difference spectrum. Ferrous cytochrome b_5 is generated by chemical reduction with dithionite or enzymatic reduction in the presence of NADH which requires the mediation of NADH-cytochrome b_5 reductase (see Table 1-2). Hemoglobin undergoes a spectroscopic shift (formation of deoxy Hb) in the presence of dithionite and interferes with the spectroscopic observation of cytochrome b_5 reduction. The enzymatic cytochrome b_5/cytochrome b_5 reductase redox couple is disrupted by detergent which obviates the use of this method for fractions containing high detergent concentrations.

CHAPTER 3

RECONSTITUTION OF MONOOXYGENASE ACTIVITIES MEDIATED BY PURIFIED SCUP CYTOCHROME P-450 FORMS

Introduction

The assay of catalytic activity with purified cytochrome P-450 isozymes requires the reconstitution of the monooxygenase system in a lipid environment. The NADPH-cytochrome P-450 reductase is required for accepting electrons from NADPH in a single two-electron transfer step and reducing the cytochrome P-450 complex by delivering the electrons in one-electron transfer steps to the cytochrome P-450 heme active site. Both the cytochrome P-450 and its reductase are lipophilic and their interactions are optimal in a hydrophobic environment. The reconstitution events are generally performed for technical convenience in an artificial lipid system by mixing stoichiometric amounts of the two redox catalysts together in the presence of sonicated DLPC, NADPH and occasionally, additional proteins such as cytochrome b_5. Detergents, which are necessary during the purification stages, frequently interfere at the reconstitution step by preventing the formation of the catalytically active 1:1

complex of cytochrome P-450 and NADPH-cytochrome P-450 reductase (see Chapter 1).

High concentrations of the NADPH-cytochrome P-450 reductase flavoenzyme in the presence of NADPH can lead to the production of reduced oxygen intermediates like superoxide (see Chapter 2 and footnote 10 therein). The cytochrome P-450 activated oxygen complex is unstable and may also decay to hydrogen peroxide without coupled substrate oxidation (Nordblom and Coon, 1977). Hydrogen peroxide and superoxide are both deleterious to proteins and may participate in nonenzymatic oxidation reactions. These types of problems make reconstitution results more challenging to interpret and require caution in the applications of this system.

Another facet of catalytic activity analysis is the problem of which substrates should be tested. Cytochromes P-450 are responsible for a variety of reactions and hepatic microsomal forms are notorious for broad, overlapping substrate profiles (cf. Guengerich, 1977; Guengerich et al., 1982a). It is necessary to test a battery of compounds because few substrates appear satisfactory in meeting the criteria of being isozyme-specific. I have used the patterns of microsomal metabolism associated with aromatic hydrocarbon induction in fish as a starting point in the analysis of the catalytic activities possessed by the resolved cytochrome P-450 forms. As noted in Chapter 1, data from both fish and mammalian hepatic microsomal assays implicate benzo[a]pyrene hydroxylase and 7-ethoxyresorufin O-deethylase activities

along with inhibition by 7,8-benzoflavone as properties linked to an aromatic hydrocarbon-induced state.

It was essential to determine appropriate conditions for reconstitution assays including the nature of the lipid requirements and the restrictions of NADPH-cytochrome P-450 reductase purified from mammalian sources before these monooxygenase activities could be investigated. The latter was a crucial point because reconstitutions require stoichiometric amounts (or molar excesses) of NADPH-cytochrome P-450 reductase. Casual inspection of Table 2-1 will confirm that scup livers are not a prodigious source of the enzyme, and consequently rat NADPH-cytochrome P-450 reductase was substituted wherever possible. Experiments with scup NADPH-cytochrome P-450 reductase did not reveal any gross differences except as noted (vide infra) with cytochrome P-450A. A second tacit assumption was that incubation conditions could be properly applied to different substrate assays or the reconstitution of other cytochrome P-450 forms after being developed in microsomes and further optimized by reconstitutions of cytochrome P-450E assessed with 7-ethoxycoumarin O-deethylase assays. This logic, unfortunately, has broken down recently and great care is necessary in evaluating the data.

Despite all of the difficulties mentioned above it is possible to press on and be enlightened by the diverse catalytic activities. This chapter will describe the results of several catalytic assays in both microsomes and

reconstitutions of multiple scup cytochrome P-450 forms, based on analysis of hydroxylated products. Product analysis is the best method to reliably follow these monooxygenase reactions but there are few continuous assays available. A simple, continuous spectrophotometric assay of 7-ethoxyresorufin O-deethylase activity which is superior to the more tedious spectrofluorimetric method will also be detailed in this chapter. This spectrophotometric assay has subsequently been utilized to reliably assay monooxygenase activity in the hepatic microsomes of several other fish species.

Material and Methods

Chemicals. 6a-OH, 6 β-OH, 7a-OH, 14a-OH, 15a-OH, 16a-OH and 16 β-OH testosterone standards were obtained from the Medical Research Council Steroid Reference Collection. HPLC solvents and authentic benzo[a]pyrene metabolite standards were obtained from standard sources (Stegeman et al., 1982c; Tjessum and Stegeman, 1979). Resorufin (practical grade) was obtained from Matheson Coleman and Bell, Norwood, OH. 7-Hydroxycoumarin (95%; Aldrich) was recrystallized once from water. Other substrates and product standards were the highest quality commercially available (Aldrich Chemical Co. wherever possible).

Synthesis and purification.

7-Ethoxyresorufin (more correctly 7-ethylresorufin) was prepared by the method of Prough et al. (1978) with several modifications. Briefly, the sodium salt of resorufin was prepared by dissolving 1.2 g crude resorufin (5.6 mmol) in 15 ml anhydrous dimethylformamide containing 2 g anhydrous Na_2CO_3 (19 mmol). After dissolution occurred, 4 ml ethyl iodide (50 mmol) were passed slowly through a 1 ml alumina column and then introduced into the solution. The reaction was refluxed at 45°C for twenty-four hours and stopped by the addition of 50 ml water. The crude product was extracted into methylene chloride, washed extensively with 0.1 M $NaHCO_3$ and evaporated to dryness. The washed extract was redissolved in a small volume of $CHCl_3/CH_3CN$, 9:1 (v/v; solvent A) and chromatographed in the same on a 2 x 30 cm silica gel 60 (E. Merck) column. The orange fractions which eluted with an R_f of approximately 0.3 were individually spotted for TLC (silica G) in solvent A. The fractions judged free of other chromophores were pooled, evaporated to dryness and recrystallized in acetone. The 7-ethylresorufin obtained (161 mg[1]; 12% yield) was judged greater than 95% pure by a combination of TLC, HPLC, 90 MHz ^{1}H-NMR and elemental analysis (Galbraith Laboratories, Inc., Knoxville, TN).

The resorufin (R_f = 0.40) purchased commercially was contaminated with resazurin (R_f = 0.2) as judged by TLC (silica G) in $CH_3CN/CHCl_3/CH_3OH$, 9:1:1 (v/v; solvent B).

Consequently, it was derivatized by acetylation, purified as 7-acetylresorufin (acetoxyresorufin), deblocked and recrystallized. The procedure was as follows: 1 g resorufin was dissolved in 140 ml dimethylformamide/pyridine/acetic anhydride, 5:1:1 (v/v) and allowed to react overnight at room temperature. The reaction mixture was evaporated to dryness, redissolved in chloroform, washed with 0.1M $NaHCO_3$ then water and again evaporated to dryness. The crude acetylresorufin was dissolved in a small volume of solvent A and chromatographed in the same on a 2 x 50 cm silica gel 60 column. The orange fractions which eluted with an R_f of approximately 0.3 were individually spotted on TLC plates. The pure fractions were combined, evaporated to dryness and recrystallized from acetone. The acetylresorufin (250 mg) was judged greater than 95% pure by TLC, 90 MHz ^{1}H-NMR and elemental analysis.

The acetylresorufin was hydrolyzed in methanol by addition of 20 mM NaOH and the resorufin was collected as a precipitate after adjusting the pH to 1 with HCl. The precipitate was recrystallized in hot dimethylformamide (warning: this step requires caution as DMF is moderately flammable). The resorufin was judged greater than 95% pure by a combination of TLC, HPLC and elemental analysis.

General Methods.

UV-visible spectra were obtained on a Perkin-Elmer model 554 or Cary 118C dual beam spectrophotometer. Fluorescence

measurements were determined with Perkin-Elmer model MPF-4 or LS-3 recording spectrofluorimeters or a Turner 110 fluorimeter. Liquid chromatography was performed on a DuPont 8800 or Waters Associates HPLC equipped with a 25 cm Zorbax ODS or uBondapak C-18 reverse-phase column, a 254 nm UV detector and a Hewlett-Packard 3390A integrator. Radioactivity was measured by scintillation counting in a Beckman LS-100C liquid scintillation spectrometer. ^{1}H-NMR spectra were determined on a 90 MHz Jeol FT-NMR or a 250 MHz Varian FT-NMR spectrometer. Melting points (uncorrected) were measured on a Fisher melting point apparatus by heating at 5°C/min.

Extinction coefficients for resorufin and 7-ethoxyresorufin were determined from a known mass by appropriate dilution into 0.1 M Tris, pH 8.0 containing 0.1 M NaCl. Resorufin was titrated in 0.2 M NaCl (adjusted to pH 11 with NaOH) using 0.1 M HCl and the protonation of the phenolate was followed by the loss of A_{572}.

Apparent K_m and K_i values were obtained by extrapolation from standard double reciprocal plots of concentration vs. initial rate measured at five substrate concentrations in duplicate.

Enzymes.

Purified rat liver microsomal epoxide hydrolase was the generous gift of Dr. A. Y. H. Lu, Merck, Sharp and Dohme Research Laboratories, Rahway, NJ. Purified rat liver

microsomal cytochrome P-450 β-NF-B (Guengerich et al., 1982a) was the generous gift of Dr. F. P. Guengerich, Department of Biochemistry and Center of Environmental Toxicology, Vanderbilt University, Nashville, TN. Purified rat liver microsomal cytochrome P-450 PB-4 and cytochrome b_5 (Waxman and Walsh, 1982) were the gracious gifts of Dr. D. J. Waxman, Department of Chemistry, MIT. Purified rabbit liver microsomal cytochrome b_5 was kindly provided by Dr. Y. Takagaki, Department of Chemistry, MIT.

Scup hepatic microsomes were prepared from untreated fish and stored as described in Chapter 2. Rat hepatic microsomal NADPH-cytochrome P-450 reductase (35 U/mg) was purified to electrophoretic homogeneity (Figure 2-3) as previously described (Waxman and Walsh, 1982). Scup hepatic microsomal NADPH-cytochrome P-450 reductase was purified as described in Chapter 2 and standard reductase solutions were prepared by dilution using an enzyme-bound oxidized flavin extinction coefficient of 10.6 mM^{-1} cm^{-1} per flavin (Vermilion and Coon, 1978) after fully oxidizing the enzyme by addition of potassium ferricyanide. The purified NADPH-cytochrome P-450 reductase standard solutions (2 nmol/ml) were stored in 0.1M KP_i, pH 7.8 at -20°C and the catalytic activity was stable for at least three months.

Purified scup hepatic microsomal cytochromes P-450A, P-450B, P-450E and fraction D were prepared and Emulgen 911 was removed as detailed in Chapter 2. Quantitation for the purpose of catalytic assays was based on the CO, reduced

difference spectrum near 450 nm. Catalytic assays were performed in many cases utilizing a preparation of cytochrome P-450A which had a lower specific content (6.2 nmol/mg) and a significant contaminant near 50K on SDS-PAGE (see Figure 2-17) but an impeccable UV-visible spectrum. This preparation of cytochrome P-450A was similar to the more highly purified sample both qualitatively and quantitatively in reductase requirement and cytochrome b_5 stimulation in either a 7-ethoxycoumarin O-deethylase or testosterone hydroxylase assay, suggesting that the contaminant was not contributing to the featured activities.

Purified scup hepatic microsomal cytochrome b_5 was prepared as detailed in Chapter 2. Solutions were standardized by the reduced minus oxidized difference spectrum prior to dilution and were stable in buffer at -20°C for at least three months. The detergent-free fraction obtained in step 5 (Table 2-9) was used after extensive dialysis as the source of scup cytochrome b_5 for most reconstitution assays. The G-75 eluate (Table 2-9, step 6) was similar in its appearance on SDS-PAGE and catalytic stimulating properties however the total sample after dialysis was insufficient for a complete analysis in reconstitution assays.

Assays of reconstituted monooxygenase activity

Acetanilide was prepared in a stock solution of buffer. Other substrates were added to incubations from 20-100X concentrated stock solutions prepared in methanol or acetone.

DLPC stock solutions (2 mg/ml) were prepared in 0.1 M KP_i, pH 7.8 containing 0.1 mM EDTA, sonicated to optical clarity prior to use and an aliquot was diluted to 100 ug/ml for a working solution.

Reconstitutions were performed at room temperature by mixing the purified NADPH-cytochrome P-450 reductase, purified cytochrome P-450 and DLPC, followed by dilution with buffer containing the substrate and incubation at 30°C for three minutes while shaking. The reactions were then initiated by the addition of NADPH. Control incubations were carried out in parallel, typically with boiled protein samples but occasionally utilizing assay tubes lacking NADPH or cytochrome P-450. Blank values differed little under the various conditions but boiled protein controls were generally adopted as the most prudent method. Optimal lipid concentrations of 5 ug/ml (cytochrome P-450E) or 20 ug/ml (cytochromes P-450A, P-450B and fraction D) were chosen from titrations in either 7-ethoxycoumarin O-deethylase or testosterone hydroxylase assays. Cytochrome P-450A was routinely reconstituted with scup NADPH-cytochrome P-450 reductase while the other forms were generally reconstituted with rat NADPH-cytochrome P-450 reductase. Unless noted otherwise the monooxygenase assays were performed for 20-30 minutes in a final volume of 0.4 ml 0.1 M KP_i, pH 7.8 at 30°C to make conditions more uniform in comparing substrates.

A. 7-Ethoxycoumarin O-deethylase (ECOD).

ECOD assays were performed at 30°C as described earlier (Waxman et al., 1982). Briefly, duplicate incubations were performed for the desired time period and halted by the addition of 60 ul 2 M HCl. The reaction was extracted with 0.9 ml $CHCl_3$ and the aqueous phase was aspirated away. Finally, 0.6 ml of the $CHCl_3$ phase was transferred to a fresh tube containing 2 ml of 30 mM $Na_2B_4O_7$, pH 9.2 and the fluorescence of the aqueous phase was measured by excitation at 370 nm and monitoring the emission at 455 nm. Quantitation was based on the fluorescence of a 7-hydroxycoumarin standard which was linear between 20 and 500 pmol under identical conditions.

The standard reconstituted system contained 20 pmol purified cytochrome P-450, 30 pmol purified NADPH-cytochrome P-450 reductase, DLPC concentrations appropriate to the cytochrome P-450 form (5 ug/ml for cytochrome P-450E; 20 ug/ml for cytochromes P-450A, P-450B and fraction D), 0.5 mM 7-ethoxycoumarin (Aldrich) and 0.5 mM NADPH. The activity of reconstituted cytochrome P-450E was linear for at least 30 minutes at 30°C under these conditions[2].

The catalytic activities of scup cytochrome P-450 forms were measured in some other experiments using 30 pmol purified cytochrome P-450 and 45 pmol purified NADPH-cytochrome P-450 reductase incubated under otherwise standard conditions. In experiments titrating NADPH-cytochrome P-450 reductase into a

reconstituted system with rat cytochrome P-450 PB-4 or scup cytochrome P-450E to investigate the properties of reductase isolated from different sources, 12 pmol purified cytochrome P-450 and 1 mM 7-ethoxycoumarin were employed under standard assay conditions.

B. Benzo[a]pyrene hydroxylase (BP-OH).

Routine catalytic assay of aryl hydrocarbon hydroxylase (BP-OH) activity was performed radiometrically in a final 50 ul volume (Binder and Stegeman, 1980). The [^{3}H]benzo[a]pyrene substrate was purified by dissolving in hexane and washing three times with 0.15M KOH in 85% dimethylsulfoxide, followed by a water wash or prepared by HPLC utilizing a acetonitrile/-water gradient as described below. The resulting purified substrate (extracted in hexane) was evaporated to dryness and prepared as a stock solution in acetone. Specific radioactivity was determined by liquid scintillation counting of a known benzo[a]pyrene mass or measuring the concentration of the stock solution spectrophotometrically ($E_{296.5}$ = 57.5 mM^{-1} cm^{-1} in methanol; CRC Handbook of Chemistry and Physics) and counting an aliquot. All manipulations were performed under red light to minimize photo-oxidation. The reconstituted system contained 8-16 pmol purified cytochrome P-450, 1.5-2 equivalents purified NADPH-cytochrome P-450 reductase, 5 or 20 ug DLPC/ml as appropriate, 0.5 mM NADPH and 60 uM [G-^{3}H]benzo[a]pyrene (40-160 uC_i/umol). Complete incubations were measured in triplicate while control

reactions lacking cofactor were performed in duplicate. Reactions were stopped with 100 ul of 0.15 M KOH in 85% dimethylsulfoxide and extracted three times with 0.5 ml of hexane. Base-soluble radioactivity was quantitated by counting a 60 ul aliquot of the aqueous layer in 3 ml of Aquasol (New England Nuclear) which had been acidified with 20 ul of 0.6 M HCl. Scintillation counting efficiency was determined with an [^{3}H]toluene internal standard.

Assays were performed with an increased protein concentration in a final volume of 0.2 ml for benzo[a]pyrene product analysis. In some incubations for product analysis, 2 ug (ca 40 pmol) of purified rat epoxide hydrolase was included to trap nascent epoxides. The rat epoxide hydrolase possessed no benzo[a]pyrene hydroxylase activity in reconstitutions with rat NADPH-cytochrome P-450 reductase under identical conditions in routine catalytic assays. The reactions were stopped with one volume of ice cold acetone at the end of the incubation, anthraquinone (ca 0.5 ug) or 6β-hydroxy-testosterone was added as an internal standard, and the reaction mixtures were extracted twice with two volumes of ethyl acetate. The combined organic extracts were dried under a stream of N_2 and redissolved in a small volume of pure acetonitrile or methanol. Two HPLC solvent systems were employed for product separations for the two experiments described below. Labeled products were separated by HPLC in the traditional acetonitrile/water system on a reverse-phase C-18 column using a gradient system of 40-60% acetonitrile (20

min), 60-85% acetonitrile (20 min) and 85-88% acetonitrile (3 min) in water at 35°C and a 2 ml/min flow rate. The order of elution was 9,10-diol; 4,5-diol; 7,8-diol; anthraquinone (internal standard); 1,6-quinone; 3,6-quinone; 6,12-quinone; phenol I (including 9-OH); phenol II (including 3-OH) and benzo[a]pyrene under these conditions (Stegeman and Kaplan, 1981). The benzo[a]pyrene products were identified by retention time and quantitated by UV absorbance or liquid scintillation counting, with unlabeled anthraquinone used as the internal standard. A second HPLC solvent system, methanol-ethanol/water, was applied to improve the resolution of the 4,5-/7,8-dihydrodiols and phenols I/II. Labeled products were separated using a gradient of 50-60% B (15 min), 60-75% (25 min) and 75% B (8 min) at 35° C and 1.5 ml/min flow where the strong solvent, B, was methanol/ethanol, 2:1 (v/v; Burke et al., 1977b). The order of metabolite elution was identical to the acetonitrile/water system but anthraquinone could not be used as an internal standard because it eluted near the 7,8-dihydrodiol. Instead, 6β-hydroxytestosterone, eluting prior to the 9,10-dihydrodiol, was adopted as an internal standard. The benzo[a]pyrene products were identified by co-injection with unlabeled standards and quantitated by liquid scintillation counting the collected peaks. Both data sets were corrected for extraction efficiencies previously determined for individual metabolites (diols, 83%; quinones, 73%; phenols, 70%; Stegeman et al., 1982c).

C. 7,8-Benzoflavone hydroxylase.

Oxidation of 7,8-benzoflavone was detected by the HPLC method of Stegeman and Woodin (1980). The reconstitution conditions utilized 40 pmol purified cytochrome P-450, 60 pmol NADPH-cytochrome P-450 reductase, 5 or 20 ug DLPC per ml as appropriate, 100 uM 7,8-benzoflavone (Aldrich) and 0.5 mM NADPH in a final volume of 0.4 ml. Some incubations were augmented with 2 ug (ca 40 pmol) purified rat epoxide hydrolase to trap nascent arene oxides. The reactions were stopped with one volume of ice cold acetone and extracted twice with two volumes of ethyl acetate. The combined organic extracts were dried under a stream of N_2 or under reduced pressure and resuspended in a small volume of pure acetonitrile. The products were separated by HPLC on a reverse-phase C-18 column utilizing a 30 min linear gradient of 40-70% acetonitrile in water at a 2 ml/min flow rate. Products were quantitated by peak areas using a Hewlett-Packard 3390A integrator and activities were expressed in arbitrary integration units/min/nmol cytochrome P-450 because no product standards were available for quantitation[3].

D. Acetanilide hydroxylase.

Acetanilide hydroxylase activity was measured with a reconstituted system containing 40 pmol purified cytochrome P-450, 60 pmol purified NADPH-cytochrome P-450 reductase, DLPC at 5 or 20 ug/ml as appropriate, 4 mM acetanilide (Aldrich)

and 0.5 mM NADPH in a final volume of 1 ml. Hydroxylated products were extracted (Koop et al., 1981) by quenching the reaction with 2 ml ice cold ethyl ether, addition of 0.1 g NaCl (a saturating quantity) to each tube, removing the organic phase and re-extracting the aqueous layer with 1 ml ethyl ether. The combined ether extracts were evaporated to dryness, redissolved in the HPLC mobile phase and analyzed by HPLC. Products were separated isocratically on a reverse-phase column in an 85% H_2O/15% CH_3CN solvent system, identified by retention time and quantitated by reference to a standard curve generated with authentic 4-hydroxyacetanilide (aka 4-acetamidophenol; Aldrich) using a Hewlett-Packard 3390A integrator. The retention times for 4-hydroxyacetanilide, 3-hydroxyacetanilide and the parent compound were 2.8, 4.1 and 10.5 min, respectively, under these HPLC conditions at 2 ml/min[4]. This solvent system was superior to the CH_3OH/H_2O, 2:1 (v/v) mobile phase (Guenther et al., 1979) used in earlier experiments by virtue of its improved peak height/area ratio, diminished back pressure, sharper resolution of 3-hydroxy-acetanilide and total time per injection.

E. 7-Ethoxyresorufin O-deethylase (EROD).

7-Ethoxyresorufin O-deethylase activity was measured at room temperature by either continuous spectrofluorimetry (Prough et al., 1978) or utilizing the continuous spectrophotometric assay developed as an improvment. A saturated stock solution (ca 400 uM) of 7-ethoxyresorufin in

methanol was prepared fresh daily by dissolving a crystal of 7-ethoxyresorufin in hot methanol, allowing the solution to cool to room temperature and filtering. The concentration was verified by dilution into buffer and measuring the A_{482} (E_{482} = 22.5 mM^{-1} cm^{-1}; Prough et al., 1978). The reconstituted system contained 40 pmol purified cytochrome P-450, 60 pmol purified NADPH-cytochrome P-450 reductase, 2 uM 7-ethoxyresorufin, 5 or 20 ug DLPC per ml as appropriate and 0.5 mM NADPH in either 1 ml (for the spectrophotometer) or 2 ml (for the spectrofluorimeter) of 0.1 M KP_i, pH 7.8. Microsomal assays were also performed in 0.1 M Tris-Cl, pH 8.0 containing 0.1 M NaCl without any great difference in observed rate. Resorufin product formation was followed fluorimetrically by emission at 585 nm when excited at 530 nm or spectrophotometrically by exploiting the potent resorufin visible chromophore at 572 nm (E_{572} = 73 mM^{-1} cm^{-1}). The visible assay was much more convenient as it did not require daily calibration and was not subject to quenching artifacts. The visible extinction coefficient was reliably applicable at pH 7.5 and higher, regardless of buffer and salt conditions. Further, in parallel reactions the same rate was measured for incubations in either the fluorimeter or spectrophotometer.

F. Testosterone hydroxylase.

Testosterone hydroxylase activity was measured by either an HPLC (Stegeman et al., 1982a) or TLC (Waxman et al., 1983) method. The reconstitution contained 30-40 pmol purified

cytochrome P-450, 1.5 equivalents purified NADPH-cytochrome P-450 reductase, 5 or 20 ug DLPC per ml as appropriate, 0.5 mM NADPH and 25 uM [4-^{14}C]testosterone (52 uC$_i$/umol) or 100 uM [1,2,6,7,16,17-^{3}H]testosterone (25 uC$_i$/umol) in a final volume of 0.4 ml 0.1 M KP$_i$, pH 7.8. The reactions were quenched with one volume of ice cold acetone, extracted twice with 2 ml of ethyl acetate and the combined organic extracts were evaporated to dryness.

Prior to extraction for HPLC analysis each sample was spiked with unlabeled 17-epitestosterone as an internal standard. The extracts were resuspended in a small volume of pure methanol and analyzed on a C-18 reverse-phase column. Testosterone metabolites were separated with a gradient of 0-30% solvent D (20 min), 30-80% solvent D (30 min) and 100% solvent D (30 min) at a flow of 1 ml/min where the weak solvent (C) was composed of 45% CH_3OH/55% H_2O (v/v) and the strong solvent (D) was composed of 55% CH_3OH/10% CH_3CN/35% H_2O (v/v). The order of elution from the HPLC column was 6a-OH, 15a-OH, 7a-OH, 6β-OH, 14a-OH, 16a-OH, 11a-OH, 16β-OH, 11β-OH and 2β-OH testosterone; androstendione; testosterone; and 17-epitestosterone under these conditions. All standards were resolved to baseline except the 15a/7a-OH and 2β/11β-OH testosterone pairs. Products were identified by relative to standards and quantitated by liquid scintillation counting, with the aid of the unlabeled internal standard.

When TLC analysis was contemplated, the [^{14}C]testosterone was purified by spotting on a 20 x 20 cm silica plate (0.2 mm

thick, aluminum-backed; E. Merck F-254) and chromatographed twice in the same dimension in CH_2Cl_2/acetone, 4:1 (v/v; solvent E). The UV-active spotted was cut out of the plate, eluted with ethyl acetate, filtered through glass wool, dried down and resuspended in benzene/ethanol, 9:1 (v/v). Stock solutions of testosterone were calibrated by UV absorbance (E_{241} = 17 mM^{-1} cm^{-1} in methanol; CRC Handbook of Chemistry and Physics) or liquid scintillation counting. An appropriate aliquot of the [^{14}C]testosterone solution was evaporated in each assay tube prior to reconstitution of the monooxygenase system, and at time zero a small aliquot of the assay mixture was withdrawn and liquid scintillation counted to establish an accurate substrate concentration. The dried organic extracts from the incubation were resuspended in a small volume of ethyl acetate and spotted on 20 x 20 cm silica plates. Unlabeled steriod standards were spotted in adjacent lanes and the plates were developed once in solvent E, air dried for 30 min and then developed in the same dimension with $CHCl_3$/ethyl acetate/ethanol, 4:1:0.7 (v/v; solvent F). The metabolites 15a-OH, 16a-OH, 14a/7a-OH, 6a-OH, 6β-OH, 16β-OH, 2β-OH and 2a-OH testosterone were resolved under these conditions. The 14a/7a-OH testosterone pair were poorly resolved from each other and 15a-OH testerone is known to migrate near 11a-OH testosterone (Waxman et al., 1983) however they could be resolved in other TLC solvent systems. The [^{14}C]-labeled testosterone products and parent compound were visualized by autoradiography involving the exposure of film (Kodak X-OMAT

AR) for 48-72 hours. The radioactive bands were cut out for direct quantitation in Liquiscint (National Diagnostics) by liquid scintillation counting or were eluted in ethyl acetate for further chromatographic characterization. Product formation was calculated for each metabolite as a percentage of the total radioactivity in the lane for the purpose of normalization (because no internal standard was used), and then converted to absolute values based on the initial substrate concentration in the incubation.

Products were tentatively identified by R_f in comparison to the standards in adjacent lanes on the same plate. Identification was supported by additional TLC experiments mixing the radiolabeled metabolites with unlabeled steriod standards and observing the coincidence of radioactivity derived from the unknown enzymatic metabolite with the UV absorbance of the carrier steroid standard. Enzymatic 6β-OH, 16a-OH and 16 β-OH testosterone metabolite identifications were pursued by TLC in both benzene/acetone, 2:3 (v/v; two developments in the same dimension), and solvent system E (two developments in the same dimension) from incubations of reconstituted cytochrome P-450A and cytochrome P-450 fraction D. The enzymatic 15a-OH (cytochrome P-450B) and 6β-OH (cytochrome P-450E) testosterone metabolite identifications were supported by TLC development twice in the same dimension with solvent F. HPLC further corroborated the identitification of 6β-OH testosterone metabolites from scup cytochromes P-450A and P-450E as well as 16a-OH testosterone

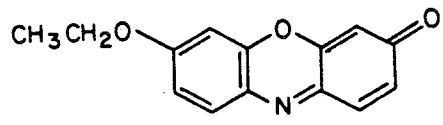
metabolites produced by reconstituted scup cytochrome P-450 fraction D.

Microsomal incubations were performed for each activity assay in parallel to the monooxygenase reconstitutions under identical substrate, cofactor and buffer conditions utilizing 0.01-0.1 nmol scup microsomal cytochrome P-450. Purified cytochrome b_5 was added to some reconstituted monooxygenase assays at concentrations stoichiometric with the molar amount of purified cytochrome P-450 present. The scup cytochrome b_5 possessed no monooxygenase activity when reconstituted with NADPH-cytochrome P-450 reductase and assayed for ECOD, EROD or BP-OH.

Practical limits of detection in the reconstitution assays were: ECOD, 10 pmol 7-hydroxycoumarin; acetanilide hydroxylase, 50 pmol individual hydroxyacetanilide; BP-OH, 1.5 pmol polar product; testosterone hydroxylase, 5 pmol individual product; EROD, 5 pmol resorufin product/min by UV or 1 pmol resorufin product/min by fluorescence.

Results and Discussion

Characterization of 7-ethylresorufin (7-ethoxyresorufin).



7-Ethoxyresorufin

The synthetic product migrated as a single vivid orange spot on TLC with an R_f = Ø.32 in solvent system A. The orange crystals melted at 228-229°C (literature 231-232°C; Prough et al., 1978). 7-Ethylresorufin migrated as a single peak on reverse-phase HPLC in H_2O/CH_3CN, 3:1 (v/v), run isocratically. The 9Ø MHz ^{1}H-NMR was performed in $CDCl_3$ and revealed chemical shifts of δ1.48 (t, 3H, J = 6.8 Hz), δ4.15 (q, 2H, J = 6.8 Hz), δ5.3Ø (s, 1H), δ6.32 (d, 1H, J = 1.9 Hz), δ6.87 (m, 2H), δ7.43 (d, 1H, J = 9.8 Hz) and δ7.7Ø (d, 1H, J = 9.8 Hz) ppm relative to the tetramethylsilane (TMS) internal standard. The elemental analysis was satisfactory (Table 3-1). The UV-visible spectrum of 7-ethylresorufin in buffer is displayed in Figure 3-1. The calculated extinction coefficient at 482 nm was 22.3 ± Ø.89 mM^{-1} cm^{-1} (literature value = 22.5 mM^{-1} cm^{-1}; Prough et al., 1978). 7-Ethylresorufin was weakly fluorescent with a λ_{max} (excitation) = 493 nm and λ_{max} (emission) = 58Ø nm.

TABLE 3-1

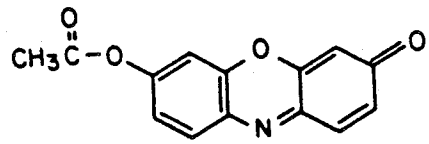
Elemental Analyses[a]

compound		weight percentage C	H	N	O
7-Ethylresorufin[b]:	calculated	69.70	4.60	5.81	19.90
	found	69.35	4.72	5.63	19.91
7-Acetylresorufin:	calculated	65.88	3.55	5.49	25.08
	found	65.59	3.63	5.44	25.29
Resorufin:	calculated	67.44	3.48	6.79	22.34
	found	67.60	3.31	6.57	22.52

[a]Quantitative microanalysis was performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

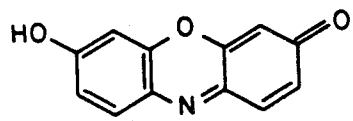
[b]7-ethylresorufin is the rigorous chemical name for the compound popularly called 7-ethoxyresorufin.

Derivatization and purification of resorufin.



7-Acetylresorufin

The light orange flakes of acetylresorufin had a melting point of 229-229.5°C and an R_f = Ø.25 on TLC in solvent system A. The 9Ø MHz ^{1}H-NMR spectrum was performed in $CDCl_3$ and contained peaks at δ 2.35 (s, 3H), δ 5.3Ø (s, 1H), δ 6.34 (d, 1H, J = 2 Hz), δ 6.87 (dd, 1H, J = 9, 2 Hz), δ 7.17 (dd, 1H, J = 9, 2 Hz), δ 7.44 (d, 1H, J = 9.8 Hz) and δ 7.8Ø (d, 1H, J = 9.8 Hz) ppm with respect to the tetramethylsilane (TMS) internal standard. The elemental analysis was satisfactory (Table 3-1).



Resorufin

The resorufin which was obtained from this derivative was a fine black powder and decomposed above 3ØØ°C in the melting point apparatus. It was judged free of Cl^- ions by a negative Beilstein test. The compound migrated as a single pink spot on silica TLC with an R_f = Ø.4Ø in solvent system B. The 25Ø MHz ^{1}H-NMR spectrum of a saturated solution in CD_3OD/NaOD had peaks at δ 6.34 (d, 2H, J = 2.2 Hz), δ 6.7Ø (dd, 2H, J = 7.1, 2.4 Hz) and δ 7.48 (d, 2H, J = 9.7 Hz) ppm with respect to the

sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) internal standard. The compound migrated as a single peak on reverse-phase HPLC in an isocratic H_2O/CH_3CN, 85:15 (v/v) solvent system. The elemental analysis was also satisfactory (Table 3-1). The UV-visible spectrum in buffer is depicted in Figure 3-1. The calculated extinction coefficient at 572 nm was 73.2 $\pm$ 2.7 mM^{-1} cm^{-1} which is at variance with the literature value (E_{572} = 40 mM^{-1} cm^{-1}; Prough et al., 1978) presumably reflecting my efforts to purify the resorufin. Resorufin has a pK_b = 6.1 (most likely the phenolate) below which the chromophore at 572 nm is lost. Fluorescence spectrophotometry revealed a λ_{max} (emission) = 585 nm and a λ_{max} (excitation) near 575 nm. The fluorescence excitation spectrum is congruent with the UV-visible absorbance spectrum. Excitation at 510 or 530 nm yielded 30% and 50%, respectively, of the maximum fluorescence elicited by excitation at 575 nm.

Monooxygenase Assays

A. Microsomal 7-ethoxyresorufin O-deethylase assays.

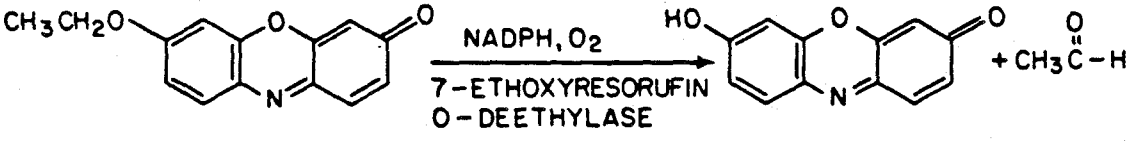


Figure 3-1 highlights the clear distinction between the absorbance spectra of 7-ethoxyresorufin and resorufin at 572 nm. I pursued the 7-ethoxyresorufin O-deethylase assay in the

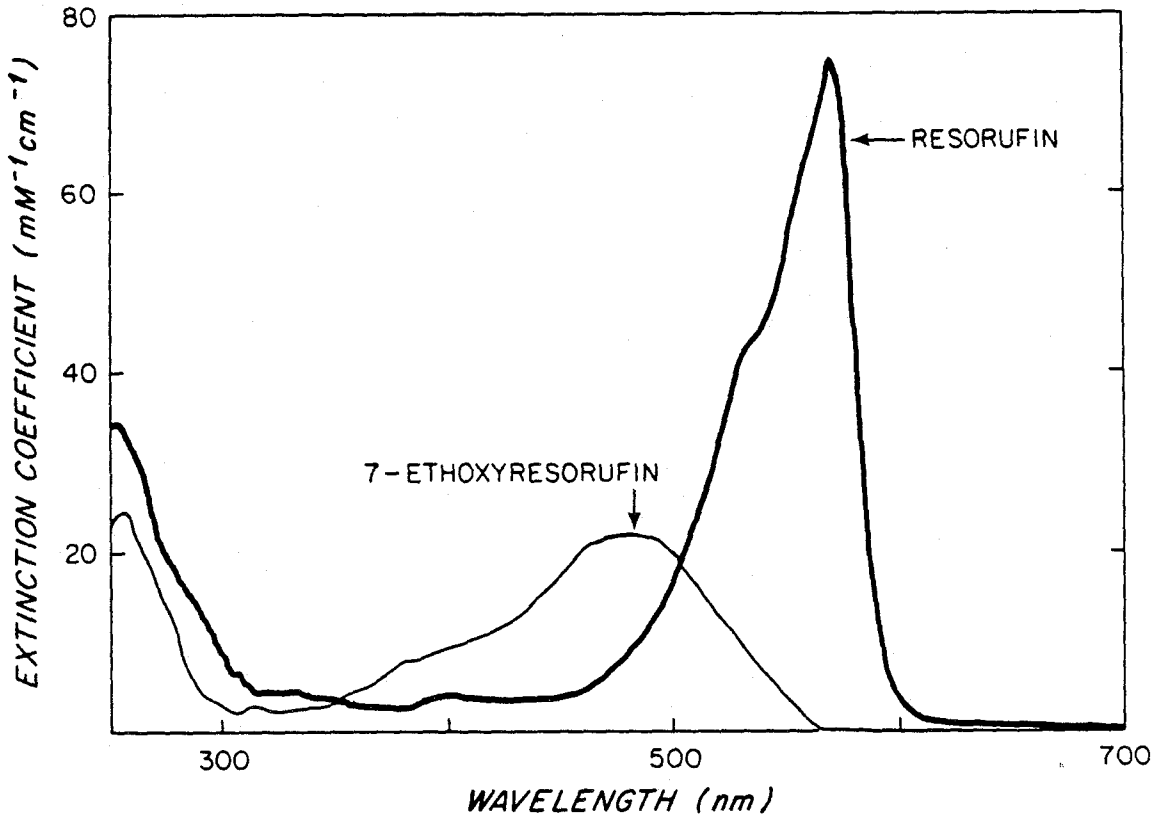


Figure 3-1. UV-visible spectrum of resorufin and 7-ethoxy-resorufin. Resorufin (1.6 uM) and 7-ethoxyresorufin (8.4 uM) were dissolved in 0.1 M Tris-Cl, pH 8.0, containing 0.1 M NaCl and scanned.

visible spectrophotometer in view of the vaunted extinction coefficient for resorufin. Addition of a limiting 7-ethoxyresorufin quantity to a complete microsomal incubation elicited a typical resorufin visible spectrum with an A_{572} equivalent to 85% of the amount predicted assuming E_{572} = 73 mM^{-1} cm^{-1}. These data were interpreted as a confirmation of the experimentally determined extinction coefficient. The assay conditions at pH 8.0 and I_c = 0.2 were optimal for untreated scup hepatic microsomes and turnover numbers of 3-5 nmol product/nmol cytochrome P-450/min were routinely observed with no background activity. Microsomal EROD activity had a sharp pH dependence with a peak at 7.8 to 8.0. The rate of resorufin formation is linear over a range of 5-150 ug protein/ml[5] and generally first order in time until substrate exhaustion begins to occur. The apparent microsomal K_m for 7-ethoxyresorufin was 0.24 uM and 7,8-benzoflavone was an extremely potent competitive-type inhibitor with an apparent K_I = 0.080 uM.

Given the continuous nature of this monooxygenase assay it was possible to determine the NADPH coupling in microsomes under 7-ethoxyresorufin turnover conditions:

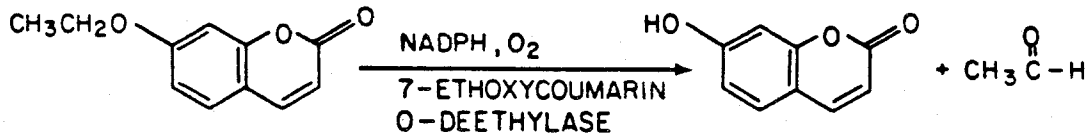
NADPH oxidation (+ 7-ER) = 54.6 nmol/min/mg
NADPH oxidation (- 7-ER) = 5.9
net = 48.7 nmol/min/mg

7-ER oxidation = 3.5 nmol/min/mg

3.5/48.7 = 7.2% coupling

I have not yet uncovered any major pitfalls in the visible 7-ethoxyresorufin O-deethylase assay. For instance, resorufin is not an inhibitor in scup microsomes as has been reported in other systems (Burke et al., 1977a) nor does it appear to be reduced by the NADPH-cytochrome P-450 reductase (Mayer et al., 1977). While NADPH-cytochrome P-450 reductase may treat 7-ethoxyresorufin as a substrate, I have found no clear effect on O-deethylase catalysis and hence it is unclear whether "iminoquinone" reduction is a transient on the reaction pathway, as suggested by others (Warner and Neims, 1979). Two observations are technically important. First, 7-ethoxyresorufin at 5 uM concentrations was inhibitory so the substrate was generally kept at 2 uM which corresponds to less than 0.2 A_{572} equivalents of resorufin product. Second, stock solutions of 7-ethoxyresorufin stored on the benchtop in methanol at room temperature degraded within 24 hours to multiple UV-active products (detectable by TLC) which may be inhibitory. Consequently, it is best to make fresh substrate solutions daily. It can be calculated that 10 pmol of resorufin product corresponds to 0.0015 OD units in 0.5 ml using the extinction coefficient of 73 mM^{-1} cm^{-1} at 572 nm. Assuming this amount is produced over a period of ten minutes the conservative limit of detection for the visible assay is 1 pmol/min.

B. 7-Ethoxycoumarin O-deethylase activity.



Microsomal ECOD activity had a broad optimum near pH = 7.8 and was inhibited 60-70% by 100 uM 7,8-benzoflavone. A standard substrate concentration of 0.5 mM 7-ethoxycoumarin was adopted although the microsomal apparent K_m is 18 uM. Reconstituted cytochrome P-450E monooxygenase activity was readily assayed with 7-ethoxycoumarin as a substrate. Titration of DLPC into the reconstituted system evoked a 3-fold stimulation of monooxygenase activity at 5 ug/ml and a diminution of activity at higher lipid concentrations (Figure 3-2). The apparent K_m for 7-ethoxycoumarin was 0.27 mM at optimal lipid concentrations suggesting that the observed decrease in monooxygenase activity at higher lipid concentrations might be due to an increase in the apparent K_m of the substrate with elevated lipid concentrations. The ECOD activity of reconstituted cytochrome P-450E was inhibited 70-80% by 100 uM 7,8-benzoflavone, like the effect in microsomes.

The electron transfer efficiencies of purified scup and rat NADPH-cytochrome P-450 reductase were compared by titrating purified scup cytochrome P-450E (Figure 3-3A) or purified rat cytochrome P-450 PB-4 (Figure 3-3B) with each of the two NADPH-cytochrome P-450 reductases. These titration

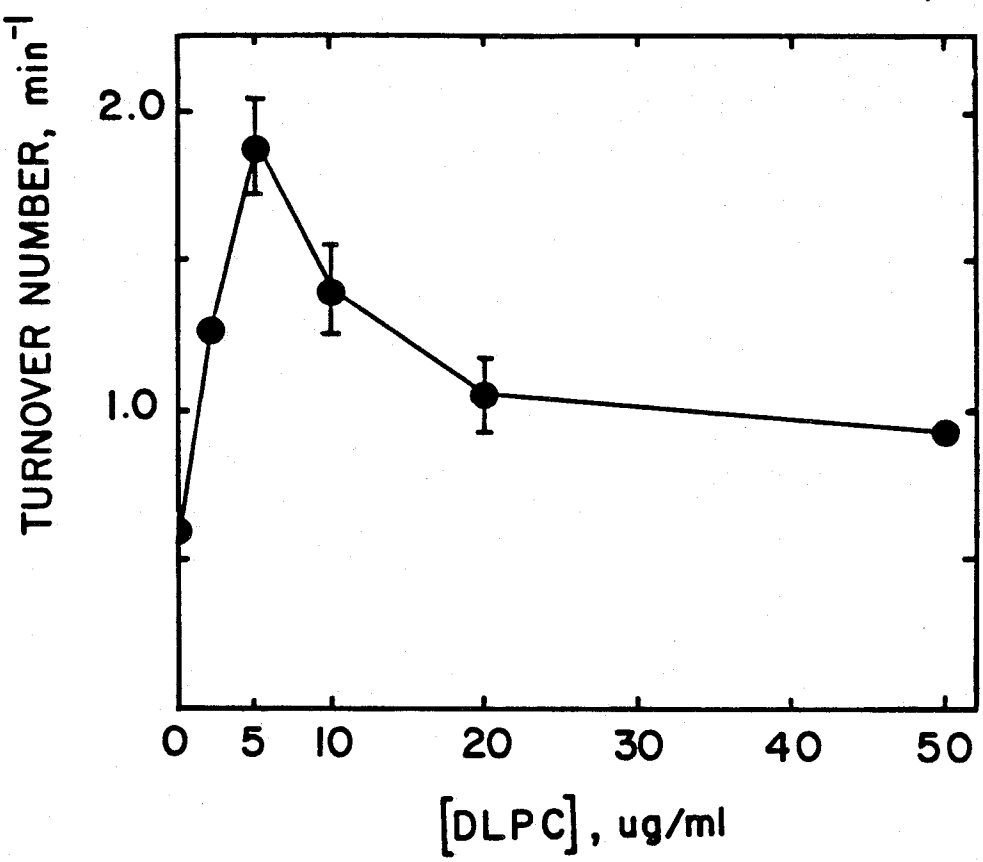


Figure 3-2. DLPC titration into cytochrome P-450E monooxygenase reconstitutions of ECOD. The standard catalytic assay (see methods section) was performed for 30 min at 30° C utilizing 20 pmol purified cytochrome P-450E, 30 pmol purified rat NADPH-cytochrome P-450 reductase and variable concentrations of DLPC. The vertical bars display the range for duplicate incubations.

curves, yielding comparable rates with a given cytochrome P-450 form, indicate the two NADPH-cytochrome P-450 reductases are functionally equivalent in reconstitution experiments. Subsequently, rat NADPH-cytochrome P-450 reductase was routinely used for reconstitutions of scup cytochromes P-450 in place of scup NADPH-cytochrome P-450 reductase due to the greater availability of the enzyme purified from rats. The exception to this rule occurred with purified scup cytochrome P-450A; NADPH-cytochrome P-450 reductase titration experiments with this form indicated that the rat NADPH-cytochrome P-450 reductase was 5-fold less effective in the reconstitution of monooxygenase activity (Figure 3-4). This result appears to be attributable to a slower electron transfer rate rather than a decreased association constant for the binary protein complex because saturation occurs at the same molar ratios for both scup and rat NADPH-cytochrome P-450 reductase. While detailed studies were not pursued with purified scup cytochrome P-450B or fraction D, preliminary experiments indicated that rat NADPH-cytochrome P-450 reductase was a perfectly competent reductant for these cytochrome P-450 forms.

Cytochromes P-450 catalyze a variety of oxidations (noted in Chapter 1) and therefore I tested several substrates in order to characterize the purified scup cytochrome P-450 forms. I have focused on activities associated in microsomes with the aromatic hydrocarbon-induced state in fish because I

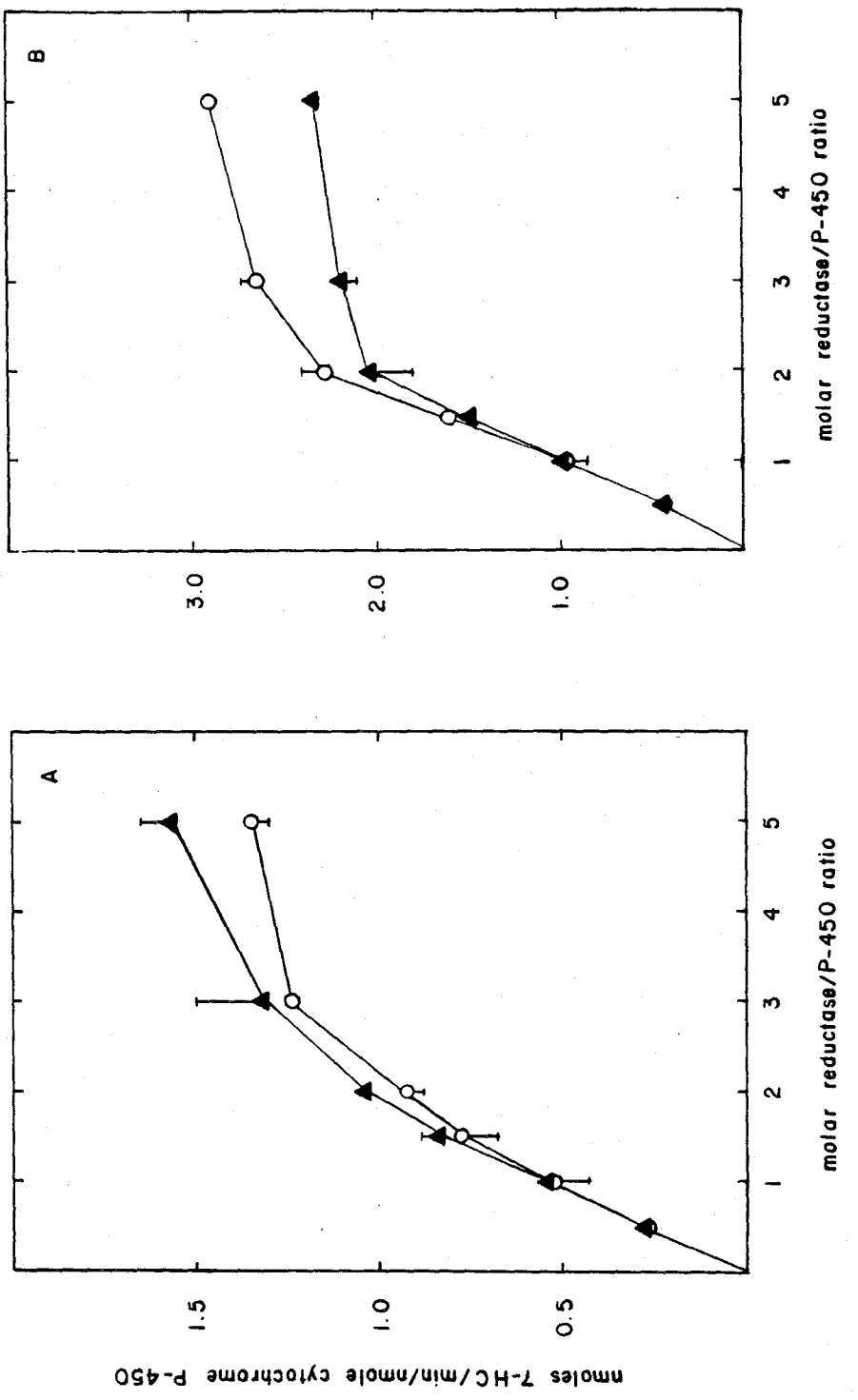

A
B
nmoles 7-HC/min/nmole cytochrome P-450
molar reductase/P-450 ratio
molar reductase/P-450 ratio
0.5
1.0
1.5
1.0
2.0
3.0
1
2
3
4
5

Figure 3-3. Reconstituted cytochrome P-450 titration with purified rat and scup NADPH-cytochrome P-450 reductases. The standard ECOD assay (see methods section) was performed for 20 min at 30° C utilizing 5 ug DLPC/ml, 12 pmol purified cytochrome P-450 and variable purified NADPH-cytochrome P-450 reductase. Vertical bars indicate the range of duplicate incubations. A, purified scup cytochrome P-450E titrated with purified rat (○) and scup (▲) NADPH-cytochrome P-450 reductase. B, purified rat cytochrome P-450 PB-4 titrated with purified rat (○) and scup (▲) NADPH-cytochrome P-450 reductase.

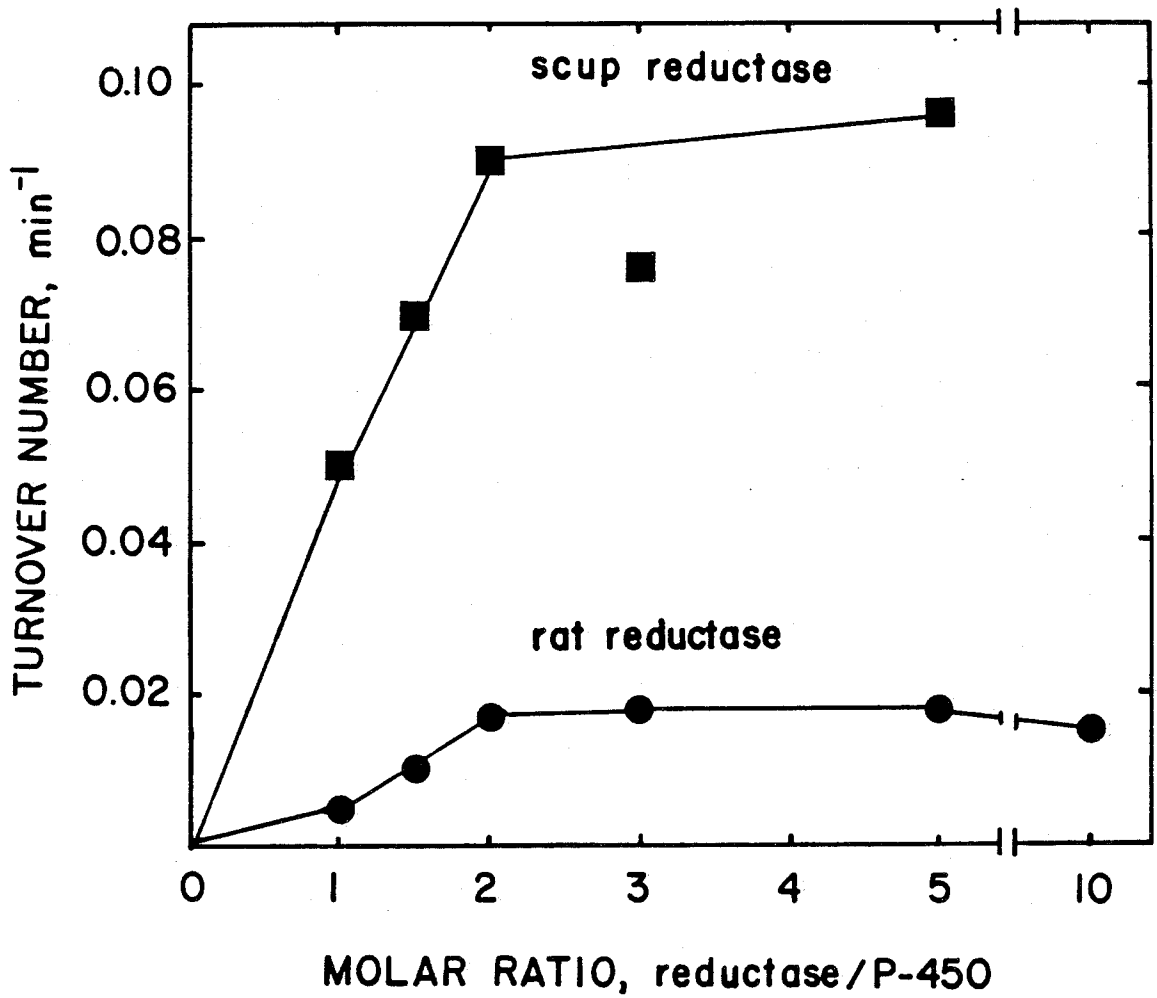
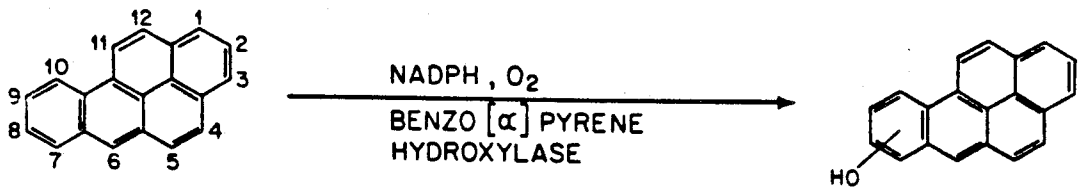


Figure 3-4. Reconstituted cytochrome P-450A titration with purified rat and scup NADPH-cytochrome P-450 reductases. The standard ECOD assay (see methods section) was performed for 30 min at 30° C utilizing 20 ug DLPC/ml, 30 pmol purified cytochrome P-450A and variable purified scup (■) or rat (●) NADPH-cytochrome P-450 reductase. The values represent single incubations for each reconstitution due to the shortage of purified scup NADPH-cytochrome P-450 reductase.

wished to know which form(s) possessed aryl hydrocarbon hydroxylase activity. Reconstitution conditions developed in 7-ethoxycoumarin O-deethylase assays of cytochrome P-450E monooxygenase activity were applied to the other substrate assays without further optimization.

C. Benzo[a]pyrene hydroxylase activity.



Reconstituted cytochrome P-450E was the most active form with benzo[a]pyrene in catalytic assays (Table 3-2). Although this oxidation was 3-fold less than the turnover rate observed in microsomes, it remains an order of magnitude greater than the limits of detection for activity in the reconstitutions of the other forms. The BP-OH activity of reconstituted cytochrome P-450E was inhibited 70-80% in the presence of 100 uM 7,8-benzoflavone like the 80-90% inhibition observed in untreated scup microsomes (Stegeman and Binder, 1979; Stegeman et al., 1981). Reconstitution experiments with impure fractions after resolution on DEAE-cellulose support the notion that a predominant fraction of the BP-OH activity is associated with peak E and that the other forms are an order of magnitude less active with benzo[a]pyrene (not shown).

TABLE 3-2

Scup Cytochrome P-450 Catalytic Activity[a]

	nmol product/nmol cytochrome P-450/min				
		Reconstituted Forms			
Substrate	Microsomes	A	B	E	Fraction D
7-Ethoxycoumarin	1.5 ± .3	0.10	0.04	2.2 ± .3	0.055
Benzo[a]pyrene	1.7 ± .6	ND[b]	ND[c]	0.56 ± .04	ND[c]
7,8-Benzoflavone[d]	100	4[e]	ND[f]	24	2[e]
Acetanilide[g]	1.7 ± .2	ND[h]	ND[b]	0.43 ± .07	ND[b]
7-Ethoxyresorufin	4.3 ± 1.6	ND[i]	--	ND[i]	--
Testosterone[j]	1.1	0.8	0.07	0.04	0.20

-- = not determined

[a]Assays were performed as described in materials and methods sections. Standard reconstitutions were performed with 20 ug DLPC/ml and 1.5-2 equivalents rat NADPH-cytochrome P-450 reductase with two exceptions: cytochrome P-450E was reconstituted with 5 ug DLPC/ml; cytochrome P-450A was reconstituted with scup NADPH-cytochrome P-450 reductase. Error ranges express the standard deviations when three or more experiments were performed. Otherwise the values represent the averages of duplicate or triplicate incubations.

[b]Not detectable where the limit of detection was 0.05 turnovers per min.

[c]Not detectable where the limit of detection was 0.02 turnovers per min.

[d]Rates reported as a percentage relative to values determined for total product in microsomes. 100% corresponds to about 10 turnovers per min based on initial rates of substrate disappearance in microsomal incubations. Cytochromes P-450A and P-450E were reconstituted in duplicate; the other values represent single incubations.

[e]These rates were so low as to be unreliable as direct enzymatic products and could be due to uncoupled oxidation.

[f]Not detectable where the limit of detection was approximately 2% of the microsomal rate.

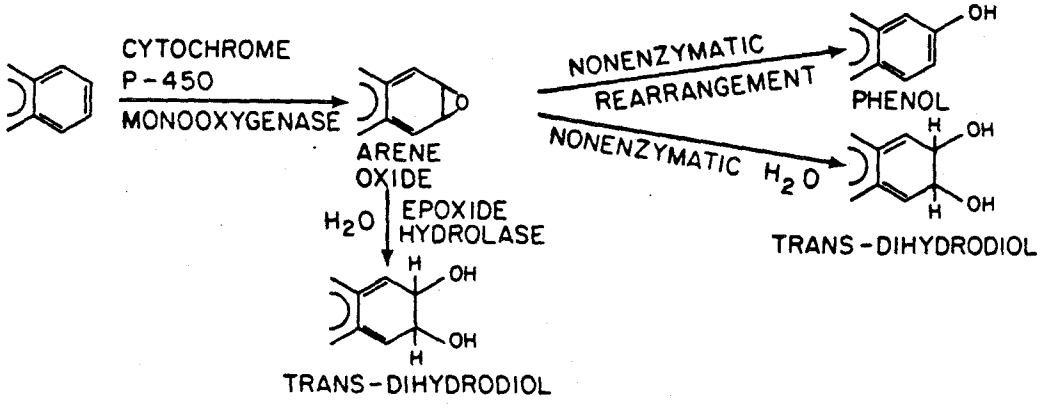
[g]Values for reconstituted cytochromes P-450B and fraction D represent single incubations; the other rates were derived from duplicate incubations.

[h]Not detectable where the limit of detection was 0.1 turnovers per min.

[i]Not detectable where the limit of detection was 0.015 turnovers per min.

[j]Reconstitutions were performed with stoichiometric levels of purified scup liver cytochrome b_5. Total product is represented by the turnover numbers.

BP-OH product profile. The hepatic microsomal oxidation of benzo[a]pyrene at benzo-ring positions is associated with the aromatic hydrocarbon-induced state in mammals but is common with untreated fish species including scup (Stegeman, 1981). Benzo-ring dihydrodiols are major metabolites isolated from incubations with scup microsmes and have been unambiguously identified by mass spectrometry (Stegeman et al., 1983). The HPLC analysis of benzo[a]pyrene metabolites produced by reconstituted cytochrome P-450E (Table 3-3, Figure 3-5) supplemented with purified rat epoxide hydrolase revealed a marked selection for oxidation at benzo-ring positions (7,8- and 9,10-dihydrodiols) as observed in scup hepatic microsomes (Stegeman et al., 1981; 1983) which contain endogenous microsomal epoxide hydrolase. The BP-OH product profile in the presence of epoxide hydrolase is informative because many of the benzo[a]pyrene phenols coelute in the standard HPLC separation (Tjessum and Stegeman, 1979).



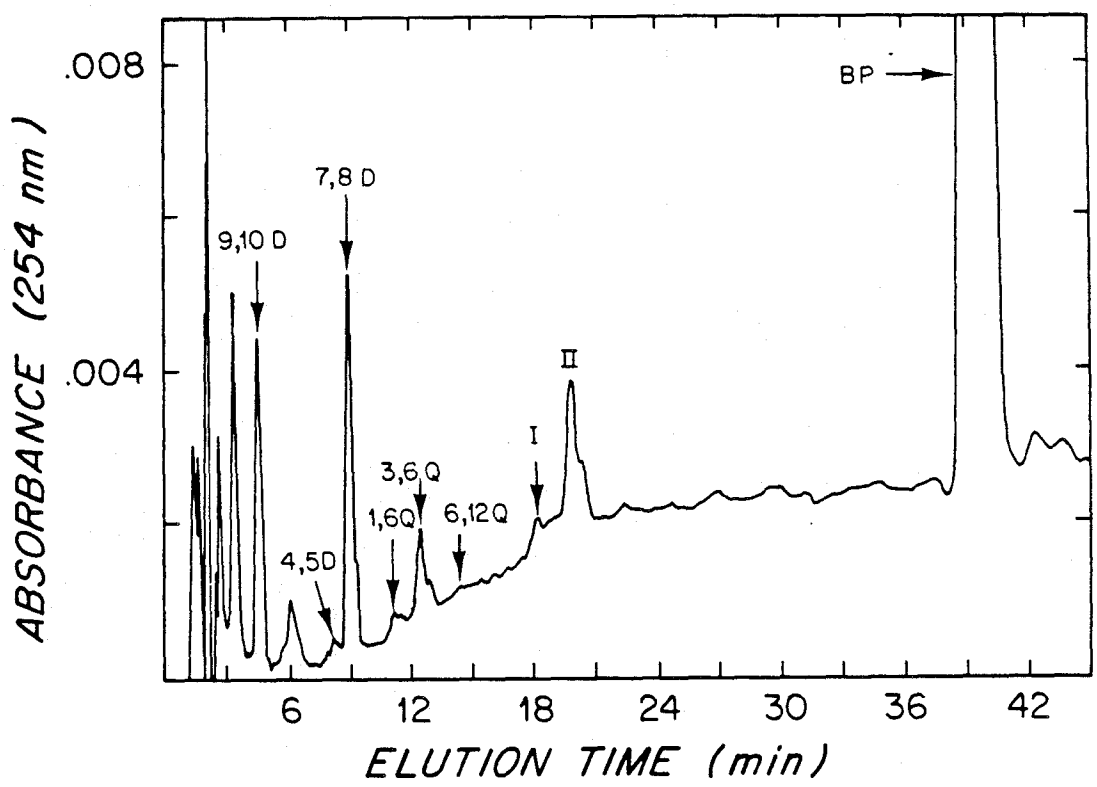


Figure 3-5. HPLC separation of benzo[a]pyrene metabolites formed by reconstituted cytochrome P-450E in the presence of purified rat epoxide hydrolase. The incubation was performed with 40 pmol purified scup cytochrome P-450E, 80 pmol purified scup NADPH-cytochrome P-450 reductase, 40 pmol purified scup cytochrome b_5, 40 pmol purified rat epoxide hydrolase, 5 ug DLPC per ml, 60 uM benzo[a]pyrene and 0.5 mM NADPH for 20 min at 30° C. The reaction was stopped with acetone and extracted with ethyl acetate (see methods section). The organic extract was evaporated to dryness and resuspended in pure methanol. Benzo[a]pyrene and its metabolites were separated by the methanol-ethanol/water gradient (described in the methods section) after injection onto a reverse-phase C-18 HPLC column at time zero. Products were detected by absorbance at 254 nm and identified (see also Table 3-3) by retention time in comparison to authentic standards.

The peak immediately preceding the 9,10-diol is 6β-hydroxytestosterone which was used as an internal for this experiment. The broad peak eluting near 6 min was an unidentified compound which appeared to be derived from benzo[a]pyrene as judged by radioactivity. I and II refer to phenol peaks I and II as detailed in Table 3-3. N.B. Benzo[a]pyrene-4,5-diol and 6,12-quinone were present in fleeting quantities if at all.

TABLE 3-3

Benzo[a]pyrene Metabolites Formed by Purified Reconstituted Scup Cytochrome P-450E[a]

Metabolite: Experiment I	pmol/nmol P-450/min (% of total) P-450E	P-450E + EH
9,10-dihydrodiol	17 (8)	124 (42)
4,5-dihydrodiol	ND (--)	ND (--)
7,8-dihydrodiol	14 (7)	100 (34)
1,6-quinone	14 (7)	ND (--)
3,6-quinone	141 (69)	62 (21)
6,12-quinone	ND (--)	ND (--)
phenol I (9-OH)	9 (5)	8 (3)
phenol II (1-,3-,7-OH)	9 (5)	ND (--)
TOTAL	204	292

Metabolite: Experiment II	Microsomes	P-450E	P-450E + EH[b]
9,10-dihydrodiol	222 (26)	53 (12)	239 (28)
4,5-dihydrodiol	ND (--)	ND (--)	ND (--)
7,8-dihydrodiol	188 (22)	46 (10)	226 (27)
1,6-quinone	127 (15)	35 (8)	49 (6)
3,6-quinone	132 (16)	77 (17)	130 (15)
6,12-quinone	ND (--)	ND (--)	ND (--)
phenol I (9-OH)	103 (12)	54 (12)	68 (8)
phenol II (1-,3-,7-OH)	75 (9)	177 (40)	136 (16)
TOTAL	847	442	848

ND, not detectable where the limits of detection were estimated as 2 pmol/nmol cytochrome P-450/min.

[a]Incubations were performed for 20 min at 30° C in 0.1 M KP_i, pH 7.8. In experiment I the reconstituted system contained 40 pmol scup cytochrome P-450E (specific content = 9 nmol/mg), 60 pmol rat NADPH-cytochrome P-450 reductase and 2 ug rat epoxide hydrolase (EH) where indicated. Products were identified on reverse-phase HPLC by retention time in the acetonitrile/water solvent system and quantitated by UV absorbance or liquid scintillation counting. In experiment II the reconstituted system contained 40 pmol scup cytochrome P-450E (specific content = 6 nmol/mg), 80 pmol scup NADPH-cytochrome P-450 reductase, 40 pmol scup cytochrome b_5 and 2 ug rat epoxide hydrolase (EH) where indicated. The microsomal incubation similarly contained 40 pmol cytochrome P-450. Products were identified on reverse-phase HPLC in the methanol-ethanol/water solvent system by coelution with unlabeled standards and quantitated by liquid scintillation counting.

[b]The results of two incubations were averaged.

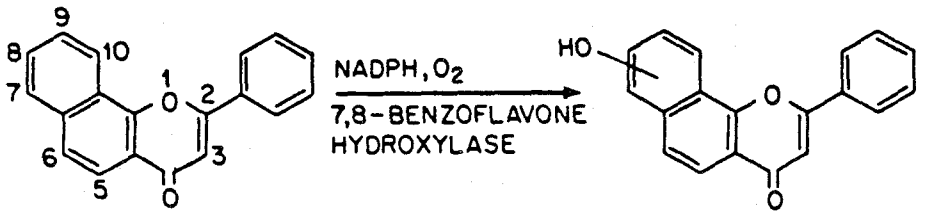
The marked elevation of the 7,8- and 9,10-dihydrodiols in the presence of purified epoxide hydrolase clearly illustrates that in both experiments cytochrome P-450E was forming an abundance of benzo-ring arene oxides which could be hydrated to dihydrodiols, or could rearrange nonenzymatically to phenols and may oxidize further to quinones (Table 3-3). The two experiments summarized in Table 3-3 were performed with different cytochrome P-450E preparations reconstituted without and with purified scup cytochrome b_5 and the incubations differed vastly in the total amount of ethyl acetate-extractable products. The reconstituted systems produced primarily quinones or phenols in the absence of epoxide hydrolase for both experiments. The effect of added epoxide hydrolase on the appearance of dihydrodiols indicates that at least the cytochrome P-450E preparation utilized in experiment I was relatively free of epoxide hydrolase contamination.

The amplification of total ethyl acetate-extractable products in the presence of epoxide hydrolase is not matched by a similar effect in routine catalytic assays where total base-soluble products were not altered by the presence of epoxide hydrolase. This discrepancy suggests that the extraction of benzo[a]pyrene metabolites is variable or being influenced by factors such as the concentration of protein. Experiment II is also puzzling in that epoxide hydrolase did not produce dihydrodiols at the great expense of phenols and quinones, as might be expected (and is reflected in Experiment I). This observation also suggests that product recovery was

problematic. Nonetheless, the regio-selective pattern of benzo-ring oxidation by reconstituted cytochrome P-450E is unambiguous in both experiments.

The major difference in BP-OH products between scup microsomes and reconstituted cytochrome P-450E (other than the depressed dihydrodiols without epoxide hydrolase) is the higher ratio of quinones to phenols produced in one reconstitution. The experimental conditions under which phenols will be oxidized to quinones are elusive to me and it is not obvious why one or the other product will predominate. This may be due to the low protein concentration or low rates of product formation in the reconstitution experiments; an increased quinone/phenol ratio is a trend observed with microsomal incubations under low protein concentrations (Stegeman et al., 1983) although these conditions may also be synonymous with poor recovery of phenols. Yet, a similar pattern of prominent quinones has been observed with reconstitutions of the major aromatic hydrocarbon-inducible cytochrome P-450 purified from rat (Holder et al., 1974) and adult rabbit livers (LM-4; Wiebel et al., 1975) in the absence of epoxide hydrolase. Benzo-ring dihydrodiols increased roughly ten-fold to 36% of the total hydroxylated products when epoxide hydrolase was added to the rat cytochrome P-450 reconstitution (Holder et al., 1974).

D. 7,8-Benzoflavone hydroxylase activity.



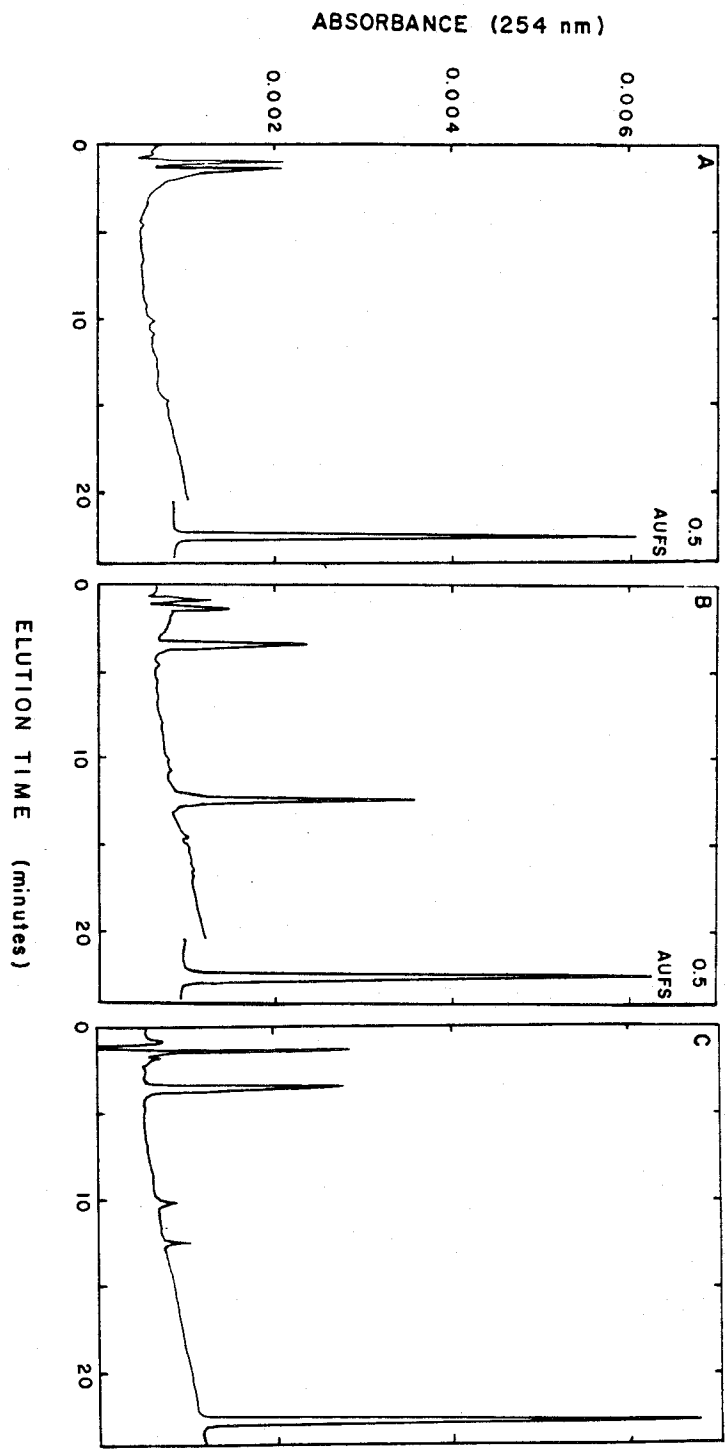
7,8-Benzoflavone may exert its inhibitory influence by virtue of being an alternative substrate (Stegeman and Woodin, 1980). The initial rate of substrate disappearance in scup microsomes suggests it is an excellent substrate with a turnover number near 10 nmol product/nmol cytochrome P-450/min (Stegeman and Woodin, unpublished observations). Further, the kinetics of 7,8-benzoflavone inhibition in microsomal EROD assays was the competitive type (vide supra). Consistent with the observation of metabolism in microsomes, I have found that reconstituted cytochrome P-450E oxidizes 7,8-benzoflavone to the same major metabolites produced by microsomes (Figure 3-6) -- a dihydrodiol (R_t = 3.3 min) and a putative phenol (R_t = 12.2 min). The tentative identification of the 12.2 min peak as a phenol was based on the increase of this peak with microsomal incubations in the presence of the epoxide hydrolase inhibitor, trichloropropylene oxide (Stegeman and Woodin, 1980) and the diminution of the peak by the addition of epoxide hydrolase to monooxygenase reconstitutions with cytochrome P-450E.

The rate of product formation in microsomes was 21 and 3 units/min/nmol cytochrome P-450 for the diol and phenol,

respectively (Figure 3-6). The formation rates were 1.0 and 1.5 without epoxide hydrolase or 1.4 and 0.82 in the presence of epoxide hydrolase for the diol and putative phenol, respectively, in the monooxygenase system reconstituted with purified cytochrome P-450E. Thus, in the reconstituted system, the diol/phenol ratio was increased from 0.67 to 1.6 by the addition of epoxide hydrolase. The reasons for the presence of significant diol in the reconstitutions without epoxide hydrolase, or the difference in ratio of diol to phenol produced by microsomes and the epoxide hydrolase-supplemented reconstitutions, are not known.

More detailed studies of 7,8-benzoflavone metabolism in rat liver microsomes have led to the identification of a primary metabolite (40%), a 7,8-dihydrodiol, and a stable 5,6-oxide (40%) plus additional phenols and unidentified products (Nesnow and Bergman, 1980; Vyas et al., 1983). The major aromatic hydrocarbon-inducible rat liver cytochrome P-450 (P-450c; Table 1-2) forms a great deal of the 7,8-dihydrodiol when reconstituted in the presence of epoxide hydrolase. However, the 5,6-oxide is chemically stable and a very poor substrate for rat liver epoxide hydrolase (Vyas et al., 1983). Hence, only limited quantities of 5,6-dihydrodiol are formed by rat liver microsomal incubations or the reconstituted rat cytochrome P-450c monooxygenase system. It seems likely that the major product is the 7,8-dihydrodiol in scup microsomes and reconstitutions of scup cytochrome P-450E, in analogy to the observations with the mammalian

Figure 3-6. HPLC analysis of 7,8-benzoflavone metabolism by scup hepatic microsomes and purified, reconstituted scup cytochrome P-450E. Reconstitutions and microsomal incubations were performed (see methods section) for 20 min at 30° C utilizing 40 pmol cytochrome P-450. The reaction was stopped with acetone and extracted with ethyl acetate. The organic extract was evaporated to dryness and resuspended in pure methanol. Oxidation products were separated by reverse-phase HPLC on a C-18 column (see methods section). Injection was at time zero in the elution program and a 254 nm absorbance detector was used. The peaks eluting at 3.3, 12.2 and 22.4 min correspond to the dihydrodiol and putative phenol resulting from the oxidation of 7,8-benzoflavone, and the parent compound, respectively. A, boiled cytochrome P-450E reconstitution; B, reconstituted cytochrome P-450E; C, microsomal incubation.


ABSORBANCE (254 nm)
0.002
0.004
0.006
A
B
C
0.5 AUFS
0.5 AUFS
0
10
20
ELUTION TIME (minutes)

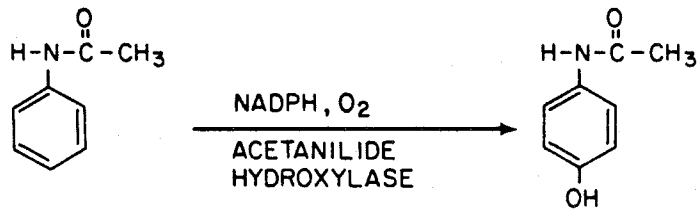
monooxygenase system. The complexity of 7,8-benzoflavone oxidation revealed in mammalian systems (Vyas et al., 1983) suggests several minor products could be co-chromatographing in the putative phenol region (Figure 3-6). The stable 5,6-oxide, if it were formed by scup cytochromes P-450, would certainly elute near the putative phenol peak.

Monooxygenase reconstitution of scup cytochromes P-450A, P-450B and fraction D are much less active with 7,8-benzoflavone as a substrate (Table 3-2). The low rate measured for fraction D may represent nonenzymatic oxidation as a result of reduced oxygen species formed by uncoupled oxidation of NADPH. Cytochrome P-450B is truly inactive under these conditions. Therefore, scup cytochrome P-450E appears to possess most of the 7,8-benzoflavone hydroxylating activity. 7,8-Benzoflavone is a much easier substrate than benzo[a]pyrene with which to work because it is commercially available in a very pure state and it is not light-sensitive. It might supplant benzo[a]pyrene as a rapid sensitive assay of aryl hydrocarbon hydroxylase activity in some monooxygenase systems when the tritium labeled substrate is widely available.

E. Acetanilide hydroxylase activity.

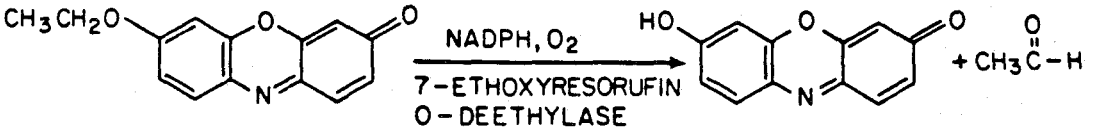
The literature on mammalian monooxygenase metabolism contains several reports documenting the induction of acetanilide hydroxylase activity by aromatic hydrocarbon-pretreatment (Atlas and Nebert, 1976; Nebert and Jensen,

1979). It is also alleged that in mammals acetanilide hydroxylase activity is more closely associated with a blue-shifted cytochrome P-450 form which is less active with benzo[a]pyrene than another particular form, the so-called P_I-450 (Nebert et al., 1981). The various resolved scup cytochrome P-450 forms were reconstituted and assayed for acetanilide hydroxylase in order to test the validity of this claim in a fish species.



As might be expected given the association with aromatic hydrocarbon metabolism, acetanilide was a substrate in scup microsomes with an apparent K_m = 0.8 mM and optimal activity at pH = 7.8. Microsomal acetanilide hydroxylase activity was inhibited 70% by 100 uM 7,8-benzoflavone suggesting this activity was associated with aromatic hydrocarbon-inducible forms. Only purified cytochrome P-450E was active with acetanilide in reconstitution experiments (Table 3-2), although purified cytochrome P-450A may possess a low but relevant activity (the limit of detection was unsatisfactorily high for this incubation). However, these data do not support the hypothesized distinction between the cytochrome P-450 forms on the basis of benzo[a]pyrene and acetanilide hydroxylase activity (cf. Negishi and Nebert, 1979).

F. 7-Ethoxyresorufin O-deethylase activity.

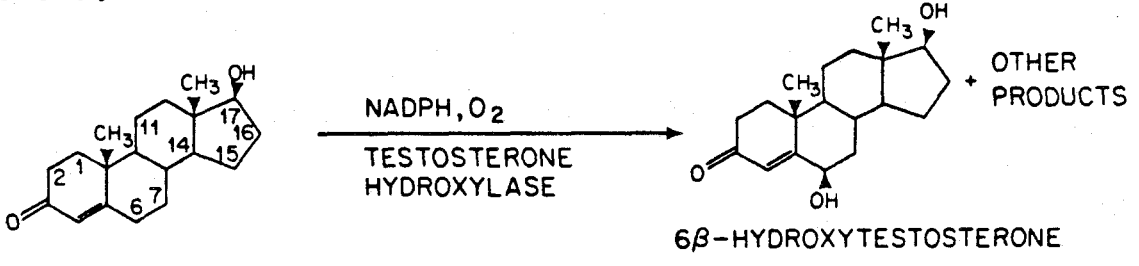


Earlier experiments had amply demonstrated that microsomal EROD activity was induced by aromatic hydrocarbon pretreatment in some scup (Stegeman et al., 1981). The extreme sensitivity of the microsomal activity to 7,8-benzoflavone inhibition coupled with the metabolism of the latter by reconstituted cytochrome P-450E intimated that cytochrome P-450E would possess EROD activity. Yet, neither reconstituted cytochrome P-450E nor P-450A was active in EROD activity assays (Table 3-2) when reconstituted with either scup or rat NADPH-cytochrome P-450 reductase in either the spectrophotometric or spectrofluorimetric assay. Additional experiments (not shown) with resolved partially purified scup cytochrome P-450 forms did not detect activity in DEAE-cellulose peaks A-E (Chapter 2) after reconstitution. Against the possibility that the reconstitution assays of EROD activity were being improperly attempted, the positive control of purified rat liver cytochrome P-450 β-NF-B (Guengerich et al., 1982a; Table 1-2) was reconstituted under identical conditions and yielded normal EROD activity (2 nmol product/nmol cytochrome P-450/min) in my hands. The scup monooxygenase reconstitutions were also demonstrated to have

no inhibitory effect on scup microsomal EROD activity, buttressing the notion that the reconstitution conditions were not grossly flawed.

G. Testosterone hydroxylase activity.

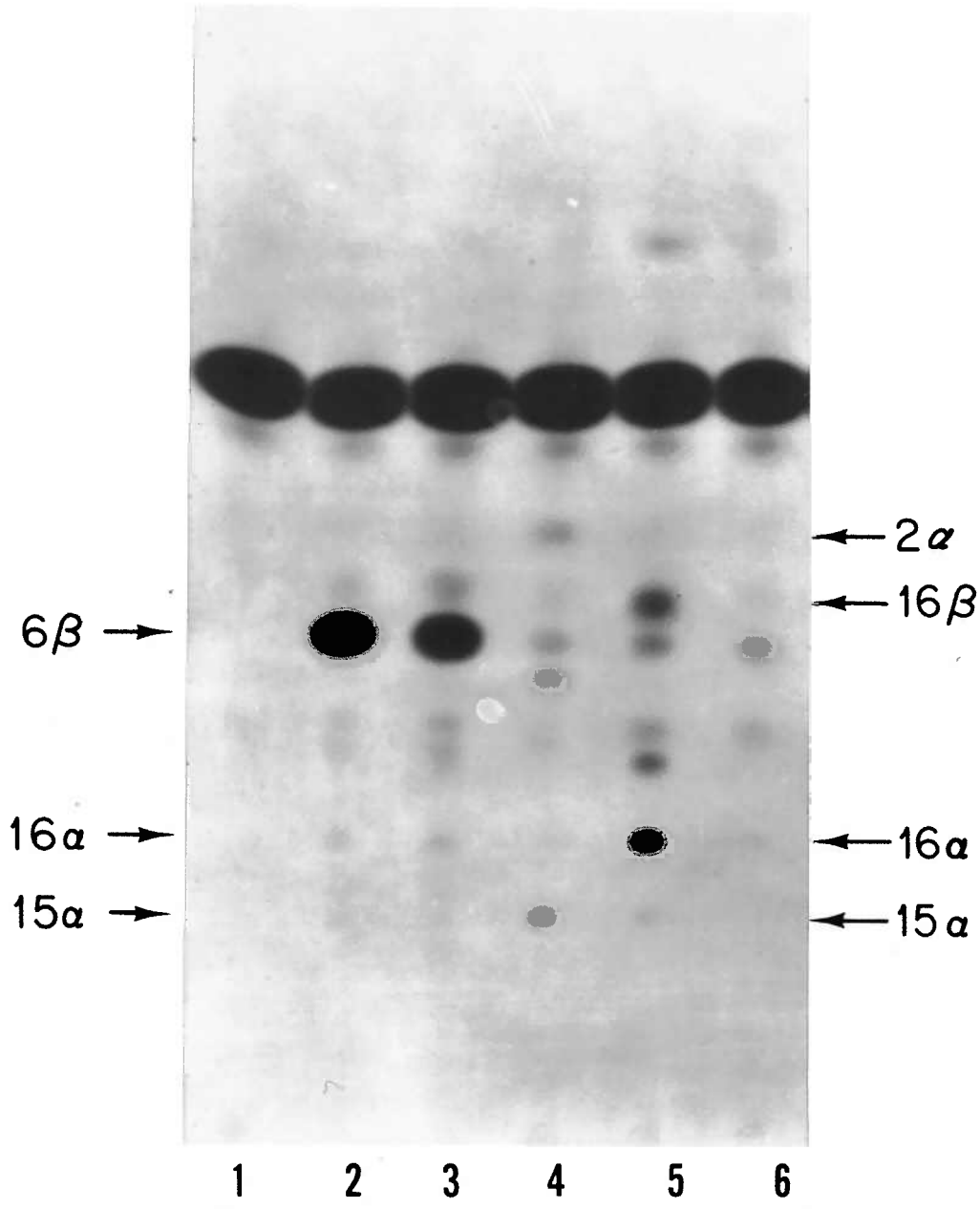
The scup cytochrome P-450 forms other than P-450E had been virtually inactive in all the catalytic assays described to this point. In part this was not too surprising because the assays were chosen for their relevance to aryl hydrocarbon hydroxylase activity. It was imperative, however, to demonstrate that the other discrete scup cytochrome P-450 forms had credible catalytic activities. Testosterone hydroxylase assays were selected for this purpose because the possibility of multiple hydroxylated products affords great flexibility in assaying metabolism by various cytochrome P-450 forms.



6β-HYDROXYTESTOSTERONE

Scup hepatic microsomes produce numerous testosterone metabolites although 6 β-OH testosterone is approximately half of the total (not shown). Reconstitutions of purified cytochrome P-450 forms and assay of catalytic activity with testosterone revealed some kinetic differences between the purified forms (Table 3-2). Reconstituted cytochrome P-450E was the least active form, supporting my assertion that

Figure 3-7. TLC separation of testosterone metabolites produced by reconstituted scup cytochromes P-450. Incubations were performed for 30 min at 30° C with 25 uM [4-^{14}C]-testosterone, 0.5 mM NADPH, 30 pmol purified scup cytochrome P-450, 30 pmol purified scup cytochrome b_5, 45 pmol purified scup NADPH-cytochrome P-450 reductase and DLPC (as appropriate). The reactions were stopped with acetone, extracted with ethyl acetate, the organic extracts were an chromatographed on a 20 x 20 cm silica TLC plate with development in solvent E followed by development in solvent F, and autoradiography was performed as described in the methods section. The labels indicate the position of hydroxylated testosterone standards chromatographed in adjacent lanes. The organic extracts form incubations were spotted as follows: lane 1, testosterone alone (no protein); lanes 2 and 3, reconstituted purified cytochrome P-450A (two separate preparations); lane 4, reconstituted purified cytochrome P-450B; lane 5, reconstituted cytochrome P-450 fraction D; lane 6, reconstituted purified cytochrome P-450E.


6β
16α
15α
2α
16β
16α
15α
1
2
3
4
5
6

cytochrome P-450E is directed toward aromatic hydrocarbons. TLC analysis of the testosoterone metabolites provided clear distinctions between cytochromes P-450A, P-450B and fraction D (Figure 3-7). Reconstituted cytochrome P-450A almost exclusively produced a single metabolite which was chromatographically identical to 6β-OH testosterone. Reconstituted cytochrome P-450E formed a chromatographically identical metabolite in much more limited quantities. Reconstituted cytochrome P-450B produced several metabolites including two which were chromatographically similar to 15a-OH and 2a-OH testosterone (see methods). Rechromatography of the putative 2a-OH testosterone metabolite, isolated from incubations with reconstituted cytochrome P-450B, resulted in some degradation of the radiolabeled compound to a species with a larger R_f in solvent F. Moreover, the primary radioactive spot migrated slightly in advance of the carrier. These results cast doubts on the identity of this metabolite as 2a-OH testosterone and no standards are available with an equivalent R_f. Cytochrome P-450 fraction D reconstitutions formed a complex suite of products although two metabolites, comigrating with 16β-OH and 16a-OH testosterone, were prominent. Finally, although cytochrome P-450 fraction C has received almost no attention, preliminary reconstitution experiments indicated that in addition to other products, it formed metabolites which chromatographed in the poorly resolved 7a/14a-hydroxytestosterone region.

Separation and identification of testosterone metabolites is a challenging task and the results must be viewed with some caution. Standards are not available for all possible metabolites so that identification by liquid chromatography must be considered tentative. High resolution mass spectrometry for structural confirmation has not yet been performed on the metabolites isolated from reconstitutions or microsomal incubations with the exception that 6β-OH testosterone has been confirmed as a major metabolite isolated from scup microsomal incubations. The gross product profile certainly is useful for distinguishing cytochrome P-450 forms in the interim. For example, the most polar metabolite (putative 15a-OH testosterone) formed by reconstituted cytochrome P-450B appears to be unique to that scup cytochrome P-450 isozyme.

Monooxygenase reconstitutions in the presence of cytochrome b_5.

The relatively low catalytic activities observed with the scup cytochrome P-450 reconstitutions were a source of some concern. Moreover, the inability to locate EROD activity in any of the cytochrome P-450 reconstitutions was particularly troublesome. Several different reconstitution conditions were evaluated with the view to optimize the ensuing catalytic activity. Variations included utilization of Tris, HEPES or MOPS assay buffers, inclusion of 20% glycerol, 40 ug DOC/ml or 0.5 mg bovine serum albumin per ml in the assay buffer, or

reconstitution with dimyristoylphosphatidyl choline. None of these permutations enhanced the catalytic activity (ECOD or BP-OH) with reconstituted cytochrome P-450E above levels in the standard assays as described.

Rabbit cytochrome b_5 also had little effect in cytochrome P-450E reconstitutions. However, the literature precedents (cited in Chapters 1 and 2) were so encouraging that I felt compelled to purify scup cytochrome b_5 for the purpose of testing its effects in the scup reconstitutions. A survey of scup cytochrome P-450 reconstitutions, analyzing for ECOD activity in the presence of rabbit or scup cytochrome b_5, demonstrated profound stimulations depending on reconstitution conditions (Table 3-4). Reconstituted cytochrome P-450A-dependent monooxygenase activity had the striking requirement for scup NADPH-cytochrome P-450 reductase noted earlier (Figure 3-4) and was stimulated 2- to 4-fold by the supplementation with either cytochrome b_5. Scup cytochrome b_5 yielded the same or greater stimulation in comparison to rabbit cytochrome b_5 for all reconstitutions. Because the absolute numbers are very low with reconstituted cytochrome P-450B it is difficult to know whether the stimulation is real and if so, precisely what the stimulation factor is. Unfortunately, the more accessible cytochrome P-450E manifested none of these effects and was an inadequate model for studying the nature of the cytochrome b_5 stimulation.

The activity differences in reconstitutions of purified cytochrome P-450A with NADPH-cytochrome P-450 reductase and cytochrome b_5 isolated from scup or mammalian liver were sufficiently surprising as to justify additional experiments to establish the nature of this catalytic effect[6]. Titration of scup cytochrome b_5 into a reconstitution of cytochrome P-450A with either rat or scup NADPH-cytochrome P-450 reductase yielded a hyperbolic increase in ECOD activity with saturation near one cytochrome b_5 molar equivalent per cytochrome P-450A but preserving the 5- to 6-fold difference between reconstitutions with scup and rat NADPH-cytochrome P-450 reductase (Figure 3-8). Additionally, comparison of scup and rabbit cytochrome b_5 confirms the catalytic distinction with saturating quantities of cytochrome b_5 and this stimulation does not occur in the absence of lipid (Figure 3-9). These data, taken together, argue for a protein complex of equimolar cytochromes P-450 and b_5 whose catalytic competence is lipid-dependent. Both of these properties have been observed in other systems (Sugiyama et al., 1980; Vatsis et al., 1982; Waxman and Walsh, 1983) and this asserts that scup cytochrome b_5 (rather than some trace contaminant) is responsible for the striking effect.

Because cytochrome P-450A showed such a large cytochrome b_5 stimulation it appeared that this could account for the absence of EROD activity in the standard reconstitutions. However, cytochrome P-450A, when reconstituted with scup NADPH-cytochrome P-450 reductase and scup cytochrome b_5,

TABLE 3-4

Cytochrome b_5 Stimulation of ECOD Activity in Monooxygenase Reconstitutions[a]

Reconstitution Conditions	Stimulation Factor[b]		
	No Additions	+Rabbit b_5	+Scup b_5
P-450A: rat reductase	1	2.7	5.6
scup reductase	5.5	11	20
P-450B: rat reductase	1	1.3	1.4
scup reductase	1.3	1.4	1.7
P-450E: rat reductase	1	0.90	0.92
scup reductase	0.91	1.0	1.1
Fraction D: rat reductase	1	2.2	2.9
scup reductase	0.94	3.5	3.8

[a]Single 30 min incubations were performed at 30°C utilizing 20-30 pmol cytochrome P-450, 1.5 equivalents purified NADPH-cytochrome P-450 reductase, and 1.0 equivalents purified cytochrome b_5 (as indicated) at 5 ug DLPC/ml (P-450E) or 20 ug DLPC/ml (P-450A, P-450B, fraction D). Fluorimetric product analysis was performed as described in materials and methods sections.

[b]The rate is displayed as a multiple of the monooxygenase activity observed with rat NADPH-cytochrome P-450 reductase without further additions. The absolute rates in the reconstitution with scup NADPH-cytochrome P-450 reductase and scup cytochrome b_5 were (turnovers per min): cytochrome P-450A, 0.42; cytochrome P-450B, 0.068; cytochrome P-450E, 2.2; fraction D, 0.21.

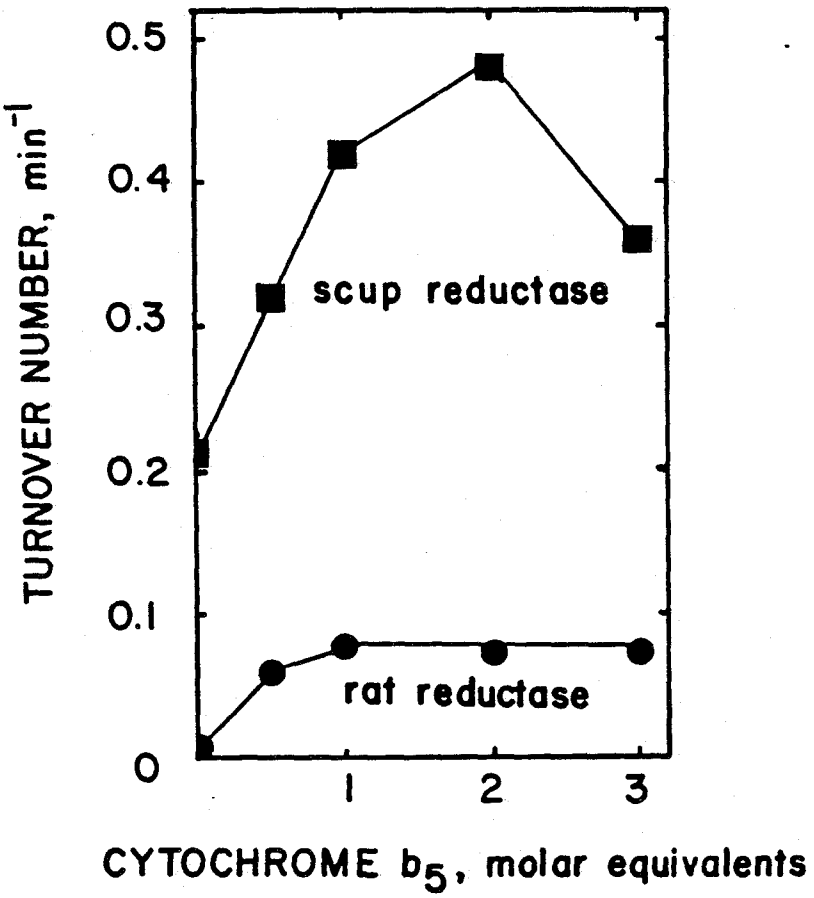


Figure 3-8. Scup cytochrome b_5 stimulation of reconstituted scup cytochrome P-450A ECOD: titrations in the presence of purified rat and scup NADPH-cytochrome P-450 reductase. The standard ECOD assay was performed (see methods section) for 30 min at 30° C utilizing 20 ug DLPC/ml, 30 pmol purified scup cytochrome P-450A, 60 pmol purified scup (■) or rat (●) NADPH-cytochrome P-450 reductase and variable purified scup cytochrome b_5. The values represent single incubations for each reconstitution due to the shortage of purified scup cytochrome b_5 and NADPH-cytochrome P-450 reductase.

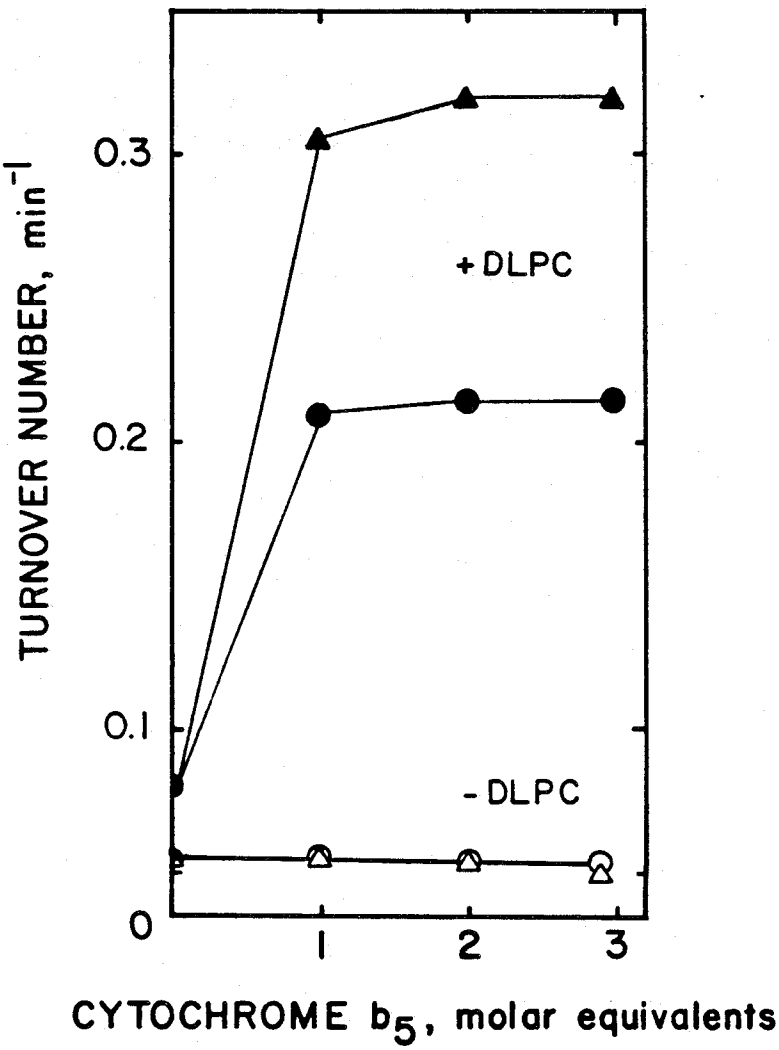


Figure 3-9. Cytochrome b_5 stimulation of reconstituted scup cytochrome P-450A ECOD: titrations in the presence and absence of lipid. The standard ECOD assay was performed (see methods section) for 30 min at 30° C utilizing 0 or 20 ug DLPC/ml, 20 pmol purified scup cytochrome P-450A, 30 pmol purified scup cytochrome P-450 reductase and variable purified scup (▲,△) or rabbit (●,○) cytochrome b_5. The filled symbols represent reconstitutions in the presence of 20 ug DLPC/ml and the empty symbols represent reconstitutions in the absence of DLPC. The values represent single incubations for each reconstitution due to the shortage of purified scup cytochrome b_5 and NADPH-cytochrome P-450 reductase.

remained inactive with 7-ethoxyresorufin. Much to my surprise, cytochrome P-450ØE was extraordinarily active (turnover number = 7.1 min^{-1}) when reconstituted under identical conditions. This activity appears to be entirely dependent on scup cytochrome b_5 as cytochrome P-450ØE reconstitutions with scup NADPH-cytochrome P-450 reductase alone are inactive, and reconstitutions supplemented with rabbit cytochrome b_5 are also catalytically incompetent.

Stimulation was observed for both cytochrome P-450ØE and cytochrome P-450A in a limited number of BP-OH assays with reconstitutions containing scup cytochrome b_5. The apparent turnover numbers were Ø.9Ø and Ø.16 min^{-1} for cytochrome P-450ØE and P-450A, respectively, under the new conditions. These various stimulations are summarized in Table 3-5. The data from Tables 3-4 and 3-5 persuasively argue that cytochrome b_5 stimulations depend on both the cytochrome P-450 form reconstituted and the substrate chosen. N.B. In the extreme cases for each, cytochrome P-450A is stimulated 7Ø-fold in the hydroxylation of testosterone and cytochrome P-450ØE is stimulated 4ØØ-fold above the limit of detection for 7-ethoxyresorufin O-deethylation. This makes extrapolation between different activity assays a dangerous exercise.

TABLE 3-5

Assays of Reconstituted Monooxygenase Activity Supplemented with Purified Scup Cytochrome b_5 [a]

Substrate	nmol product/nmol cytochrome P-450/min	
	P-450A	P-450E
Benzo[a]pyrene	0.16 (4X)	0.90 (2X)
7-Ethoxyresorufin	ND[b]	7.1 (400X)
7-Ethoxycoumarin[b]	0.42 (20X)	2.2 (1.1X)
Testosterone[c]	0.80 (70X)	0.04 (ca 1.2X)

[a]Reconstitutions were performed with 1.5-2 equivalents scup NADPH-cytochrome P-450 reductase and 1.0 equivalents scup cytochropme b_5 under otherwise standard conditions. The 7-ethoxyresorufin O-deethylase assay was performed at 30°C in this case rather than room temperature. Values in parenthesis represent the approximate stimulation factor relative to reconstitution with rat NADPH-cytochrome P-450 reductase and no supplementing cytochrome b_5.

[b]Data abstracted from Table 3-4.

[c]Data abstracted from Table 3-2.

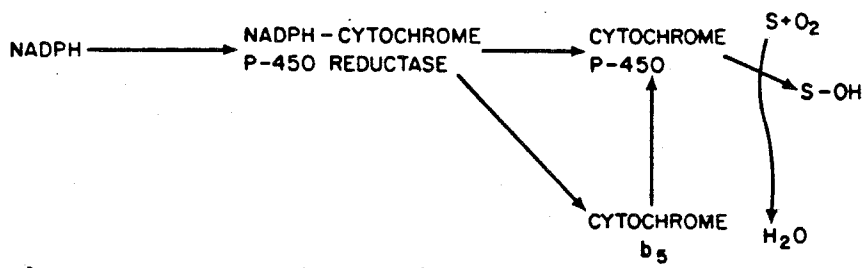
CONCLUSIONS

The choice of monooxygenase reconstitution in the non-membraneous DLPC system can potentially cause artifacts. One study (Ingelman-Sundberg and Johnson, 1980) has suggested that DLPC and an egg yolk phosphatidylcholine vesicle system yield similar substrate specificities for reconstituted rabbit cytochrome P-450 LM-2. However, incorporation into microsomal phospholipid vesicles slightly altered the patterns of metabolism. In the same study, rabbit cytochrome P-450 LM-2 was generally more active in the DLPC system rather than vesicles but cytochromes P-450 LM-3 and LM-4 were often more active in vesicles. One advantage of vesicles is the relief of a stoichiometric requirement for NADPH-cytochrome P-450 reductase. Saturation of monooxygenase activity occurs near one cytochrome P-450 reductase molecule per twenty cytochrome P-450 molecules, like the molar ratio found in microsomes. Clearly there is a need for further investigations into the physical nature of the reconstitution system.

The catalytic activity profile of the four reconstituted scup cytochrome P-450 forms supports my expectation that cytochrome P-450E is relatively efficient in the metabolism of aromatic hydrocarbons. The standard substrates benzo[a]-pyrene, acetanilide and 7-ethoxyresorufin are all preferentially hydroxylated by cytochrome P-450E, in contrast to the much lower activities portrayed by reconstitutions of cytochromes P-450A, P-450B and fraction D. The ability of

reconstituted cytochrome P-450E to metabolize 7,8-benzoflavone is consistent with the benzo[a]pyrene hydroxylase inhibition by 7,8-benzoflavone in both hepatic microsomes and the reconstituted cytochrome P-450E system. The suite of benzo[a]pyrene metabolites formed by reconstituted cytochrome P-450E is highlighted by a regioselective pattern of benzo-ring oxidation similar to the product profile observed in microsomal incubations (Stegeman et al., 1981). I shall argue in Chapter 4 that purified cytochrome P-450E is both the form responsible for the prominent benzo[a]pyrene hydroxylating activity in scup microsomes, and the major cytochrome P-450 form induced by aromatic hydrocarbon pretreatment. The data summarized above are relevant to that claim.

The stimulation of reconstituted monooxygenase activity by cytochrome b_5 remains a controversial field. Because the NADH-cytochrome b_5 reductase (EC 1.6.2.2) was not present in the reconstitution, if the stimulation involves electron transfer steps then the cytochrome b_5 must be receiving an electron from the NADPH-cytochrome P-450 reductase and interacting with one or more states of the cytochrome P-450.



Such redox events are known in mammalian systems and the

cytochrome b_5 stimulation is proposed to originate from the ability of cytochrome b_5 to transfer the second electron to the ferrous cytochrome P-450-substrate-oxygen complex. Addition of the second electron is a partially rate-determining step in some cytochrome P-450 systems (Gunsalus et al., 1973; Bonfils et al., 1981), and hence if cytochrome b_5 can accomplish the transfer at a rate with the ability of NADPH-cytochrome P-450 reductase to reduce the same complex then an acceleration of overall turnover will ensue. The incorporation of cytochrome b_5 into the monooxygenase reconstitution decreased H_2O_2 formation concomitant with increased catalytic activity in one study (Ingelman-Sundberg and Johnson, 1980). This improved coupling has been interpreted to reflect facile delivery of the second electron to the oxyferrous cytochrome P-450-substrate complex by cytochrome b_5.

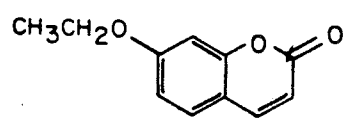
Further experimental evidence in favor of this model in the scup monooxygenase reconstitutions awaits the purification of additional scup cytochrome b_5. However, several specific predictions can be tested as they have been in mammalian systems (Sugiyama et al., 1980; Vatsis et al., 1982; Waxman and Walsh, 1983). First, the model makes redox capability a prerequisite for effective stimulation. The prediction is that reconstitutions supplemented with apo-cytochrome b_5 will not manifest improved monooxygenase activity. Second, the model implicates a ternary stoichiometric complex (with cytochrome P-450 and NADPH-cytochrome P-450 reductase) whose

formation is mediated by hydrophobic interactions. Cytochrome b_5 reduction by NADPH-cytochrome P-450 reductase is dependent on the presence of lipid (Enoch and Strittmatter, 1979). The prediction is that proteolytic removal of the hydrophobic "membrane binding" segment from cytochrome b_5 will destroy hydrophobic interactions and abolish the stimulation of monooxygenase activity.

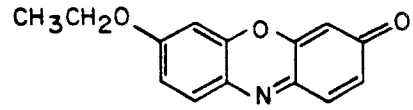
I have no data on the question of whether cytochrome b_5 involvement occurs in scup hepatic microsomes or *in vivo*. This is an area worthy address; the first half of the question can almost certainly be approached by titrating purified scup cytochrome b_5 into hepatic microsomal incubations.

The absolute dependence of EROD activity on scup cytochrome b_5 is astonishing but not unique. Reconstituted rabbit cytochrome P-450 LM-2 monooxygenase metabolism was dependent on cytochrome b_5 in the case of prostaglandin (Vatsis et al., 1982), *p*-nitrophenetole (Kuwahara and Omura, 1980) and acetanilide (Coon et al., 1980) hydroxylation. The metabolism of *p*-nitroanisole by reconstituted rabbit cytochrome P-450 LM-3c also required cytochrome b_5 (Sugiyama et al., 1980) and the cytochrome P-450 had a cytochrome b_5 binding constant sufficiently low to enable purification on an affinity column of immobilized cytochrome b_5 (Miki et al., 1980). The differences in cytochrome b_5 stimulation observed when reconstituted cytochrome P-450E was assayed with various substrates (Table 3-5) is difficult to rationalize. Witness that 7-ethoxycoumarin and 7-ethoxyresorufin are both

O-deethylated (same chemical reaction on a similar nucleus) but with enormous differences in cytochrome b_5 stimulation.



7-ETHOXYCOUMARIN



7-ETHOXYRESORUFIN

However, there is precedent for this type of perverse behavior with the reconstitution of rabbit cytochrome P-450 LM-4 in prostaglandin monooxygenase assays where the cytochrome b_5 stimulation varied for a series of similar prostaglandin substrates (Vatsis et al., 1982). The curious behavior outlined here may account for the report of a pregnenolone 16a-carbonitrile-induced rat cytochrome P-450 form (Elshourbagy and Guzelian, 1980) which lost its characteristic ethylmorphine N-demethylase activity upon purification. I suspect that rat cytochrome b_5 could be a participant in the monooxygenase metabolism of that substrate by that form.

It would be prudent to reassess the reconstituted monooxygenase activity of cytochrome P-450A with 7,8-benzoflavone and acetanilide in light of the elevated BP-OH activity with cytochrome P-450A reconstitutions when augmented with scup cytochrome b_5. Cytochrome P-450A is potentially a minor form induced by aromatic hydrocarbon pretreatment; as such it probably would possess activity (albeit low) with other substrates associated with aryl hydrocarbon hydroxylase activity. The inclusion of cytochrome b_5 may well raise the activity of reconstituted cytochrome

P-450A above the limits of detection in several substrate assays.

Chapter 4 will summarize the progress this thesis research has made toward providing answers to the three questions which I posed for investigation in Chapter 1. Further, there are new questions, as always, which have been raised by these contributions and these are entertained.

FOOTNOTES

1. The fruits of working part-time for two days had a street value of $8000 at Pierce Chemical Co.'s asking price ($50 per mg). As an added bonus the synthetic material was pure, unlike the sample purchased from Pierce.

2. A different experiment with 10 pmol scup cytochrome P-450E and 15 pmol rat NADPH-cytochrome P-450 reductase indicated that the activity was linear for at least 60 min.

3. These rates do not correspond to the units of Stegeman and Woodin (1980) which were measured planimetrically.

4. 2-Hydroxyacetanilide (R_t = 6-7 min) appears to be unstable in the acetonitrile/water solvent system and chromatographs poorly. However, other experiments using the traditional methanol/water system show that neither microsomes nor the reconstituted forms produced detectable 2-hydroxy- or 3-hydroxyacetanilide, as noted in the results section. These observations nominate 3-hydroxyacetanilide as a useful internal standard although it was not used as such in this study.

5. Significant rate deviations were recorded at microsomal protein concentrations below 5 ug/ml.

6. No difference was found in preliminary reconstitution experiments between purified rabbit and rat liver microsomal cytochrome b_5.

CHAPTER 4

SUMMARY:

THE HEPATIC MONOOXYGENASE SYSTEM OF SCUP

The original intention of this research project was to purify and characterize the monooxygenase components in the liver microsomes of a marine fish. I planned to investigate: the nature of the cytochrome(s) P-450 which catalyzed BP-OH; the catalytic activities of the remaining isozymes; and how these activities might be related to induction in the field (see Chapter 1, scope of thesis research). The rationale was to gain biochemical insight into aryl hydrocarbon hydroxylating activities which are relevant to the environmental toxicology of coastal marine populations exposed to hydrocarbon pollutants. The comparative biochemical aspects of the monooxygenase characterization were attractive because at the outset no monooxygenase from a lower animal had been dissected thoroughly. In support of the third objective, this study also sought to more rigorously establish the likelyhood of environmental induction in the field by direct immunoquantitation if possible. Unfortunately, this final goal has not yet been fully realized. The means for the task are now available but not the time.

Resolution and purification of scup hepatic microsomal cytochromes P-450 clearly demonstrates the existence of four cytochrome P-450 forms; probably there are several additional isozymes present. The extent of microsomal cytochrome P-450 diversity expressed in a given species is still controversial although there is convincing evidence for at least seven forms in rabbit liver (Koop et al., 1981; 1982), and eight forms in rat liver (Guengerich et al., 1982a), as summarized in Table 1-2. The cytochrome P-450 forms which I find (speaking as a biologist) to be "interesting" in fish at the moment, are those with identified functions i.e. pollutant metabolism and steroid hydroxylation. Naturally, I do not wish to exclude the metabolism of other xenobiotics (flavones, safroles) or endogenous molecules (vitamin D, bile acids, prostaglandins, fatty acids) but so little is known in these areas that it is hardly worth speculation.

I believe that with regard to aromatic hydrocarbon metabolism in scup hepatic microsomes the picture is now quite clear from this work. Cytochrome P-450E is the isozyme principally responsible for the aryl hydrocarbon (benzo[a]-pyrene) hydroxylase activity in liver microsomes. Three lines of evidence support this assessment:

1. The ability of reconstituted cytochrome P-450E to metabolize benzo[a]pyrene and 7,8-benzoflavone (Table 3-2; Figure 3-6) is consistent with the benzo[a]pyrene hydroxylase inhibition by 7,8-benzoflavone observed in

microsomes. Cytochrome P-450E is a more efficient catalyst of BP-OH than cytochrome P-450A, P-450B and fraction D by a factor of five-fold or more. Further, I estimated that cytochrome P-450E constitititutes 30-50% of the hepatic microsomal cytochrome P-450 complement in field samples (Table 2-2 and Figure 2-6).

2. The suite of benzo[a]pyrene metabolites formed by reconstituted cytochrome P-450E (Table 3-3 and Figure 3-5) shows a regioselective pattern of benzo-ring oxidation similar to the product profile observed in microsomal incubations (Stegeman et al., 1981). However, while the major benzo[a]pyrene metabolizing form must necessarily be similar to microsomes in BP-OH product profile, this does not preclude other scup cytochrome P-450 isozymes from producing the same pattern.

3. The 54,300 molecular weight of the purified isozyme (Figure 2-9) corresponds to that of the microsomal hemoprotein associated with BP-OH activity in analysis of individual scup sampled in the field (Stegeman et al., 1981).

Thus I make the claim that by the criteria of both abundance and catalytic efficiency purified scup cytochrome P-450E is the major if not sole aryl hydrocarbon hydroxylase activity in this teleost. The purification work also supports the

correlation of a 54K hemoprotein with BP-OH activity in liver microsomes (Stegeman et al., 1981).

Cytochrome P-450E appears to also be the major aromatic hydrocarbon-inducible form in scup hepatic microsomes. The evidence for this claim is indirect because I have not purified cytochrome P-450E from chemically induced scup livers. Nonetheless, I believe the data are convincing and no great leap of faith is required. Cytochrome P-450E is identical in molecular weight to the major scup microsomal hemoprotein (54K) induced by 3-methylcholanthrene pretreatment (Stegeman et al., 1981). The catalytic activities of reconstituted cytochrome P-450E (Table 3-2) with benzo[a]pyrene, 7-ethoxyresorufin, 7-ethoxycoumarin and inhibition by 7,8-benzoflavone are entirely consistent with the properties of hepatic microsomes isolated from chemically induced scup (Stegeman et al., 1981) and other fish species (Elcombe and Lech, 1979; James and Bend, 1980). Further, the cytochrome P-450E-carbon monoxide, reduced difference spectrum with a maximum at 447 nm (Figure 2-10) readily accounts for the blue-shift observed in the microsomal cytochrome P-450 difference spectrum after aromatic hydrocarbon induction in several fish species including scup (James and Bend, 1980; Stegeman et al., 1981). Finally, a datum I find most compelling is the immunochemical similarity between scup cytochrome P-450E and the trout aromatic hydrocarbon-inducible cytochrome P-450 TLM-4. Unlike scup cytochrome P-450E, trout cytochrome P-450 TLM-4 is nearly undetectable in untreated

liver microsomes and the induction is clearcut[1]. The immunochemical studies with antibodies raised against both scup cytochrome P-450E and trout cytochrome P-450 TLM-4 implicate cytochrome P-450E as the major aromatic hydrocarbon-inducible form in scup. The fact that both antigens cross-react in apparent lines of identity with either antibody, while some other fish cytochromes P-450 (scup cytochrome P-450A, Figure 2-20; trout cytochrome P-450 TLM-2, Williams and Buhler, 1983a) are inactive, provides strong evidence for structural similarities between scup cytochrome P-450E and trout cytochrome P-450 TLM-4.

If scup cytochrome P-450E is the major hydrocarbon-inducible form, then the abundance (30-50% of the total microsomal cytochrome P-450) in field samples supports our growing belief that feral scup exhibit activity levels rivalling the maximally induced state. Studies exploiting immunochemical techniques have demonstrated that after intraperitoneal injection of 3-methylcholanthrene the major aromatic hydrocarbon-induced cytochrome P-450 form comprises 66-90% of the Long-Evans or Sprague-Dawley rat hepatic microsomal cytochrome P-450 complement (Thomas et al., 1979; Pickett et al., 1981; Guengerich et al., 1982b). This form was elevated from 3% of the microsomal cytochrome P-450 in untreated Sprague-Dawley rats while a more modest induction of cytochrome P-450 LM-4 (from 22%) to 59% in New Zealand white rabbits has been reported using an indirect immunoperoxidase

method (Guengerich et al., 1982b).

Although I believe the scup populations I sampled were environmentally induced, it is not yet possible to identify the nature of the inducer. Coastal fish contain a plethora of potential inducers including polychlorinated biphenyls. Tissue residues of PCBs in scup sampled near New Bedford Harbor, approximately 15 miles from my major collection site, are 2-10 ppm wet weight (J. Farrington, personal communication). This level of contamination could be responsible for the apparent induction of cytochrome P-450-linked activities in this species as it has been sufficient in some other fish systems (Binder, 1982).

The catalytic activities of reconstituted cytochrome P-450E relative to the other monooxygenase reconstitutions show that cytochrome P-450E possesses all the enzymatic activities associated with aryl hydrocarbon hydroxylase activity including BP-OH, EROD, acetanilide hydroxylase and 7,8-benzoflavone hydroxylase (Table 3-2). For the present, the data suggest that the EROD activity is restricted to cytochrome P-450E while the bulk of these other activities are also associated with cytochrome P-450E.

Early work analyzing individual scup livers for microsomal monooxygenase characteristics (Stegeman et al., 1981) had indicated EROD might not be directly associated with BP-OH or levels of the 54K microsomal hemoprotein. This suggested to us that, while EROD activity was clearly linked

with the aromatic hydrocarbon-induced state, the majority of this activity might be mediated by a minor aromatic hydrocarbon-inducible cytochrome P-450 form. The dramatic cytochrome b_5 dependence of EROD activity in reconstitutions of purified scup cytochrome P-450E (Table 3-5) provides a ready explanation for the apparent dissociation between EROD and BP-OH microsomal monooxygenase activities. It is plausible that cytochrome b_5 is a partially rate-determining component for EROD activity in microsomes under some conditions. The differences in the apparent degree of EROD and BP-OH activity induction (James and Bend, 1980; Stegeman et al., 1981), assuming a single cytochrome P-450 catalyst, could be a reflection of cytochrome b_5 involvement in microsomal monooxygenase metabolism. Although cytochrome b_5 was not assayed in the earlier study with scup there is a precedent for cytochrome b_5 induction (three-fold) by aromatic hydrocarbon pretreatment in croaker (Stegeman, 1981).

Scup cytochrome P-450E is similar catalytically to its isozyme equivalent in trout liver microsomes. Cytochrome P-450 TLM-4[2] has been purified from 5,6-benzoflavone-treated rainbow trout (Salmo gairdneri) to a specific content of 11 nmol/mg (Williams and Buhler, 1982). Reconstituted trout cytochrome P-450 TLM-4 hydroxylated benzo[a]pyrene with a turnover number over 1 min^{-1} and was inhibited ca 70% by 100 uM 7,8-benzoflavone (Williams and Buhler, 1983b). EROD activity with reconstituted trout cytochrome P-450 TLM-4 was 2.3 min^{-1} (Williams and Buhler, 1983b) yet I suspect it is

also problematic and cytochrome b_5-dependent. The trout cytochrome P-450 TLM-4 difference spectrum was blue-shifted to near 447 nm like scup cytochrome P-450E.

Purified trout cytochrome P-450 TLM-4 had a molecular weight near 57,000 (like the major aromatic hydrocarbon-inducible microsomal hemoprotein in trout; Williams and Buhler, 1982; Elcombe and Lech, 1979) suggesting it is somewhat different from scup cytochrome P-450E. These differences were confirmed by peptide mapping in comparison to the purified scup cytochrome P-450 forms (Figures 2-18 and 2-19). The differences in primary structure of the scup and trout cytochrome P-450 forms contrasts with the immunochemical similarities found to exist (Figure 2-20). This immunochemical identity was rewarding in view of reports that rabbit and rat cytochromes P-450 cross-react poorly in Ouchterlony gels (Thomas et al., 1976a; 1976b), because scup (order Perciformes) and trout (order Salmoniformes) are distantly related fish species. These observations with antibodies to scup cytochrome P-450E then hold out the potential for using immunochemical probes of these determinants for studies in other fish species.

Several applications for antibody directed against scup cytochrome P-450E come to mind. First, field studies in feral scup to quantitate levels of cytochrome P-450E can more directly investigate correlations of BP-OH with tissue PCB residues or other indicators of exposure. Second, more

extensive investigations of Fundulus sp. populations at chemically defined environmental sites can be pursued. Fundulus sp. offer the advantages of a restricted geographical range and the opportunity for laboratory breeding or rearing studies to confirm hypotheses and control for genetic components to variability. Third, cytochrome P-450E determinants could be surveyed in deep sea fish (Stegeman, 1983) or other species of interest such as winter flounder (Stegeman and Woodin, 1983). Nor is the list of experimental applications limited to the ideas above.

While scup cytochrome P-450E had the greatest aryl hydrocarbon hydroxylase activity of the four reconstituted forms, the nature of cytochrome P-450A has been an enigma to me. Cytochrome P-450A had a catalytic profile identical to cytochrome P-450E but exhibited only about 20% the activity with the exception of testosterone hydroxylase activity. The similarities in UV-visible spectrum and denatured molecular weight were sufficient to raise the spectre that cytochrome P-450A might be a modified form or derivative of cytochrome P-450E. In order to discredit that proposal I established that cytochromes P-450E and P-450A have distinct primary structures as judged by proteolytic peptide mapping (Figures 2-18 and 2-19). Further, rabbit antisera to cytochrome P-450E did not cross-react with cytochrome P-450A implying that extensive immunochemical similarities do not exist between the two scup isozymes[3].

An appealing correlation with the literature emerged after I had established that cytochromes P-450A and P-450E were genuinely different cytochrome P-450 forms. A second aromatic hydrocarbon-inducible cytochrome P-450 exists in rats (Goldstein et al., 1982; Guengerich et al., 1982a; Parkinson et al., 1983) which is also induced by isosafrole (Ryan et al., 1980; see P-450d, Table 1-2). One property of this so-called minor inducible form is elution from DEAE-cellulose under low salt conditions analogous to the observations with scup cytochrome P-450A. Further, the rat minor inducible form has a blue-shifted CO, reduced difference spectrum and a molecular weight (52-53K) slightly lower than the major cytochrome P-450 induced by 3-methylcholanthrene pretreatment. Monooxygenase reconstitutions of purified isosafrole-induced rat cytochrome P-450d have low but real BP-OH or ECOD activity, and proficiently hydroxylated testosterone at the 6β position (Ryan et al., 1980). These physicochemical properties are shared by scup cytochrome P-450A and justify the hypothesis that cytochrome P-450A is a minor form induced by aromatic hydrocarbons in scup liver in strict analogy to the minor inducible form in mammals. While I have never treated scup with isosafrole, the compound is an active inducer in rainbow trout (Vodicnik et al., 1981).

The physicochemical and catalytic data on the scup NADPH-cytochrome P-450 reductase are all consistent with the notion that both the structure and function of this enzyme are highly

conserved over broad taxonomic lines. It would be useful to perform peptide mapping on scup and mammalian NADPH-cytochrome P-450 reductases to probe for sequence homologies as a test of these theories. The reasons for this possible functional conservation are not known precisely but they could be rationalized as reflecting the lack of a direct catalytic responsibility for monooxygenase substrate specificity. A few minimal structural requirements would seem to exist if the only physiological role for the NADPH-cytochrome P-450 reductase is the function as an attendant, for the purpose of relaying reducing equivalents from NADPH to the hemoprotein. The cytochrome P-450 reductase must possess the binding sites for NADPH and both flavins and the appropriate domains for interaction with cytochromes P-450 and membrane lipids. It is tempting to speculate that all microsomal NADPH-cytochrome P-450 reductases characterized to date, represent this optimized minimum, from which stems their perceived conservation of structure and function. The mechanism of enzymatic cytochrome P-450 reduction is understood in some detail from studies in rat and rabbit liver systems (Oprian et al., 1979; Vermilion et al., 1981; Iyanagi et al., 1981). These mammalian NADPH-cytochrome P-450 reductases deliver two electrons in single electron transfer steps at nearly equal mid-point potentials during the catalytic cycle. It can be argued that these similar mid-point potentials represent the complete optimization of function for this enzyme. The postulate of a single function for the NADPH-cytochrome P-450

reductase may be an over simplification; other physiological functions may exist for the enzyme in liver or other tissues. This array of potential activities includes reduction of quinones in hepatic tissue and participation in the production of activated oxygen intermediates in circulating leukocytes.

The literature is not yet rotund with the characterizations of microsomal cytochromes P-450 isolated from aquatic species (see Table 1-3). Seminal work in little skate, Raja erinacea, demonstrated the existence of multiple cytochrome P-450 forms in a fish species (Bend et al., 1973; 1979). One of these partially purified cytochrome P-450 fractions possessed aryl hydrocarbon (benzo[a]pyrene) hydroxylase when reconstituted. A highly purified preparation (14 nmol/mg) from spiny lobster, Panulirus argus, has been characterized (James and Little, 1980). This cytochrome P-450 D1 fraction was active in the 16a hydroxylation of progesterone (30 min^{-1}) and testosterone (2 min^{-1}; James and Shiverick, 1983) when reconstituted with pig liver NADPH-cytochrome P-450 reductase. These data suggest a role in steroid metabolism[4] for spiny lobster cytochrome P-450 fraction D1 although the cytochrome P-450 form was inactive in the hydroxylation of the arthropod molting hormone ecdysone. More recently multiple cytochrome P-450 forms have been resolved from 5,6-benzoflavone-treated rainbow trout, Salmo gairdneri (Williams and Buhler, 1982; 1983b). In addition to cytochrome P-450 TLM-4 (discussed earlier in this thesis, see footnote 2), a cytochrome P-450 TLM-2 has been resolved which

is physically, immunochemically and catalytically distinct from isozyme TLM-4 (Williams and Buhler, 1983a). Elaborate characterization for cytochrome P-450 TLM-2 has not yet been published although it is reported to activate aflatoxin to mutagenic species (Williams and Buhler, 1983c).

Many open questions remain in aquatic molecular toxicology. These research problems should prove amenable to approaches outlined herein, based on a reliable technique to resolve multiple cytochrome P-450 isozymes from untreated fish. One emphasis of research in the future will certainly involve a definition of the interactions between steroids and xenobiotics through influences of induction, inhibition and patterns of metabolism (Colby, 1980). The reproductive status of an individual fish probably impacts on its ability to clear foreign compounds and may determine the ultimate toxicological effect of pollutants. Conversely, the inducing effects of aromatic hydrocarbons may represent a destabilizing factor in the steroid balance of an individual at a critical stage in its development. These complex phenomena are under current investigation (e.g. Hansson et al., 1980; Stegeman et al., 1982a). Surely the importance of hepatic monooxygenase systems transcends their role in the activation of foreign compounds and further progress can be expected in the hydroxylation of endogenous substrates.

An important question in the toxicology of aquatic organisms is the complete absence of an induction response to phenobarbital treatment in hepatic microsomal studies (Bend et

al., 1973; Elcombe and Lech, 1979; Balk et al., 1980). A variety of PB-type inducers are also ineffective (reviewed by Stegeman, 1981). This raises the questions of whether the structural gene is missing in aquatic organisms, or whether the mechanism of induction might be fundamentally different or absent. The availability of cDNA clones derived from transcripts of mammalian cytochrome P-450 genes affords one experimental tool to probe this question. Screening with a mammalian cDNA for homologies in a fish genome could provide a clue to whether the PB-inducible cytochrome P-450 gene equivalent is present in fish genomic DNA. Alternatively, the purification of fish cytochrome P-450 forms may yet yield an isozyme with catalytic properties similar to the major PB-inducible cytochrome P-450 form in mammals i.e. potent aflatoxin activation[5], high N-demethylation activity and/or sensitivity to turnover-dependent inactivation by barbiturates (Waxman and Walsh, 1982). To this end I have sought among the resolved scup cytochrome P-450 forms to reconstitute the monooxygenase activity for aminopyrine N-demethylation without notable success. This activity exists in scup liver microsomes and no doubt is present in some of the reconstitutions but at a level beneath current reliable limits of detection.

The role of microsomal monooxygenase activities in extrahepatic tissues is intriguing. These studies are generally limited in scope until the experimental methodology has been practiced in liver, because microsomes are less

abundant in extrahepatic tissues and monooxygenase activities are typically low. One exception to this generalization is the high benzphetamine N-demethylation activity of sheepshead and black drum gill microsomes (James et al., 1979). Induction of monooxygenase activities in the extrahepatic tissues of aquatic species is also a sadly neglected field with few reports (i.e. James and Bend, 1980). Scup heart microsomes present another fascinating problem -- the levels of cytochrome P-450 are relatively high (0.2 nmol/mg) but the monooxygenase activities measured to date have been very low (Stegeman et al., 1982c). We are now in a position to supplement heart microsomes with purified scup NADPH-cytochrome P-450 reductase and/or purified scup cytochrome b_5 in order to discover potentially limiting monooxygenase components. Further, the application of antibodies to scup cytochrome P-450E may reveal the expression of these cytochrome P-450 antigenic determinants in heart or other extrahepatic tissues. Thus, this thesis project rather than merely closing a chapter of research, has opened a new volume of research problems to be investigated by other sturdy experimentalists.

Footnotes

1. Recently immunochemical data was presented by Williams and Buhler (Second International Symposium on Responses of Marine Organisms to Pollutants) showing that trout cytochrome P-450 TLM-4 was induced 100-fold by aromatic hydrocarbon pretreatment.

2. See footnote 12, Chapter 2 for an explanation and reconciliation of the nomenclature. TLM-2 is a 54K cytochrome P-450 which is inactive with benzo[a]pyrene when reconstituted. At the Second International Symposium on Responses of Marine Organisms to Pollutants held in Woods Hole on April 27-29, 1983 it was agreed to refer to scup cytochrome P-450E and the 57K cytochrome P-450 purified from trout liver (TLM-4b) as the apparent major 3-methylcholanthrene-inducible forms in these species. It is anticipated that this concession will ease some potential confusions until a proper standardization can be reached.

3. There are several caveats to this assertion. First, the concentration of cytochrome P-450A in the Ouchterlony gel (Figure 2-20) may have been insufficient to form an impressive precipitin line if the recognition reaction occurred; although other experiments with increased concentrations of P-450A argue against that possibility. Second, the Ouchterlony was performed in 0.2% Emulgen 911 and there are reports (Thomas et al., 1983) that purified isosafrole-induced rat liver cytochrome P-450d reacts poorly in the presence of Emulgen.

4. The physiological meaning of testosterone metabolism by hepatic microsomal monooxygenases is believed to involve the inactivation of the hormone. I view testosterone as a model steriod nucleus with the sites of oxidation potentially applicable to other steriod molecules. The idea that sites of oxidation by a cytochrome P-450 form are common to multiple steroid substrates is supported by the study with spiny lobster cytochrome P-450 fraction D1 (James and Shiverick, 1983) and other investigations (Waxman et al., 1983). Moreover, the multiple products derived from testosterone are useful to identify and distinguish various cytochrome P-450 isozymes by what some investigators characterize as a testosterone hydroxylase "fingerprint."

5. Aflatoxin is a potent hepatocarcinogen in trout (Lee et al., 1968) and this species is particularly sensitive to AFB_1 carcinogenicity. Scup microsomes are not proficient at the activation of aflatoxin to mutagenic derivatives (Stegeman et al., 1982b) although this would be consistent with aflatoxin metabolism at sites other than the 2,3 double bond (J. J. Stegeman, personal communication) which is involved in formation of the ultimate mutagen.

CHAPTER 5

APPENDIX

I. Monoclonal Antibody Probes

Park et al. (1982) have described several IgG-producing hybridoma clones raised against the major aromatic hydrocarbon (3MC)-inducible cytochrome P-450 purified from rat liver. The ascites fluids isolated from mice bearing these implants were reactive with the 3MC-P-450 antigen either in its purified form or in aromatic hydrocarbon-induced rat liver microsomes but not in untreated or phenobarbital-induced rat liver microsomes. The BP-OH or ECOD activities of 3MC-induced rat liver microsomes were inhibited 70-80% by appropriate dilutions of these monoclonal antibodies (Park et al., 1982). I performed assays for antibody inhibition of reconstituted catalytic activity with purified scup cytochrome P-450E. My studies sought to determine whether these rat liver 3MC-inducible cytochrome P-450 antigenic determinants were present in purified scup liver cytochrome P-450E in support of Dr. Stegeman's investigations with untreated scup and 3MC-induced trout liver microsomes.

5,6-Benzoflavone-induced rat liver microsomes were the generous gift of Professor M. Marletta, Department of Nutrition and Food Science, MIT. We obtained two active

clones (1-7-1p2 or 1-7-1p3 and 1-8-1p2 or 1-8-1-2p1Ø) raised against the 3MC-inducible rat liver cytochrome P-45Ø and a control clone (NBS 1-48-5p28) from Dr. H. V. Gelboin, NIH. The effects of these antibodies on monooxygenase metabolism was assessed using the ECOD and BP-OH catalytic assays. Standard assay conditions were chosen (see Chapter 3) utilizing 2Ø pmol cytochrome P-45Ø (ECOD) or 8 pmol cytochrome P-45Ø (BP-OH). For reconstitution experiments 1.5 equivalents of purified rat NADPH-cytochrome P-45Ø reductase and 5 ug DLPC per ml were included. Incubations were held for 15 min at room temperature with antibody followed by NADPH addition for initiation of the catalytic assay at 3Ø$^{\circ}$ for 3Ø minutes. ECOD assays were performed in duplicate and boiled blank values were subtracted. Complete BP-OH assays were performed in triplicate and duplicate boiled blank values were subtracted.

The results of these antibody inhibition studies are summarized in Tables 5-1 through 5-4. Scup hepatic microsomal monooxygenase activity was uneffected by antibody concentrations 1Ø- to 1ØØ-fold higher than the levels effective in aromatic hydrocarbon-induced rat liver microsomes. The lack of inhibition in the purified reconstituted scup cytochrome P-45ØE monooxygenase system confirmed that these antigenic determinants were not merely latent in scup hepatic microsomes. It was concluded that at least some antigenic determinants which are important to catalytic activity in rat 3MC-cytochrome P-45Ø are absent from the the cytochrome P-45Ø equivalent in scup.

TABLE 5-1

Effect of Monoclonal Antibodies on the Microsomal ECOD Activity of 5,6-Benzoflavone-treated Rat Livers[a]

Reaction Conditions	% , no Ab		
	I	II	III
complete (no additions)	100 (3.2)	100 (4.9)	100 (4.6)
0.1 ug NBS (control)	100	104	102
0.1 ug 1-7-1p2		84	62
0.1 ug 1-7-1p3	67		68
0.1 ug 1-8-1p2		52	45
0.1 ug 1-8-1-2p10	52		
1.0 ug NBS (control)	100		
1.0 ug 1-7-1p3	48		
1.0 ug 1-8-1-2p10	48		
10 ug NBS (control)		94	106
10 ug 1-7-1p2		46	42
10 ug 1-7-1p3			51
10 ug 1-8-1-2p10			47

[a]The results of three experiments are displayed. The concentration of antibody is expressed as ug protein per pmol cytochrome P-450. The absolute rate in nmol product/nmol cytochrome P-450/min is given in parenthesis beside the control for each experiment.

TABLE 5-2

Effect of Monoclonal Antibodies on the ECOD Activity of Feral Scup Liver Microsomes[a]

Reaction Conditions	% , no Ab I	% , no Ab II
complete (no additions)	100 (1.0)	100 (1.4)
0.1 ug NBS (control)	103	107
0.1 ug 1-7-1p2	97	107
0.1 ug 1-7-1p3		96
0.1 ug 1-8-1p2		96
0.1 ug 1-8-1-2p10	103	
1.0 ug NBS (control)	105	
1.0 ug 1-7-1p2		104
1.0 ug 1-7-1p3	108	
1.0 ug 1-8-1-2p10	100	
10 ug NBS (control)		111
10 ug 1-7-1p3		111
10 ug 1-8-1p2		100

[a]The results of two separate experiments are displayed. The concentration of antibody is expressed as ug protein per pmol cytochrome P-450. The absolute rate in nmol product/nmol cytochrome P-450/min is given in parenthesis beside the control for each experiment.

TABLE 5-3

Effect of Monoclonal Antibodies on the ECOD Activity of Reconstituted Scup Cytochrome P-450E[a]

Reaction Conditions	% , no Ab	
	I	II
complete (no additions)	100 (2.2)	100 (2.2)
0.1 ug NBS (control)	100	95
0.1 ug 1-7-1p2		100
0.1 ug 1-7-1p3	99	100
0.1 ug 1-8-1p2		98
0.1 ug 1-8-1-2p10	99	
1.0 ug NBS (control)	98	
1.0 ug 1-7-1p2		98
1.0 ug 1-7-1p3	101	
1.0 ug 1-8-1-2p10	101	
10 ug NBS (control)		44
10 ug 1-7-1p3		39
10 ug 1-8-1p2		41

[a]The results of two experiments are displayed. The concentration of antibody is expressed as ug protein per pmol cytochrome P-450. The absolute rate in nmol product/nmol cytochrome P-450/min is given in parenthesis beside the control for each experiment.

TABLE 5-4

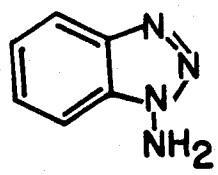
Effect of Monoclonal Antibodies on the BP-OH Activity of Reconstituted Scup Cytochrome P-450E[a]

Reaction Conditions	%, no Ab
complete (no additions)	100 (0.29)
0.1 ug NBS (control)	102
0.1 ug 1-7-1p2	104
0.1 ug 1-7-1p3	111
0.1 ug 1-8-1-2p10	101
1.0 ug NBS (control)	101
1.0 ug 1-7-1p2	93
1.0 ug 1-7-1p3	94
1.0 ug 1-8-1-2p10	104

[a]The concentration of antibody is expressed as ug protein per pmol cytochrome P-450. The absolute rate in nmol product/nmol cytochrome P-450/min is given in parenthesis beside the control for the experiment.

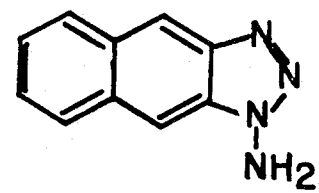
II. Suicide Inactivation of Scup Microsomal Cytochromes P-450

A suicide substrate is an innocuous molecule which is recognized by an enzyme as a substrate. During the process of enzymatic turnover the suicide substrate forms a highly reactive intermediate which inactivates the enzyme typically by covalent modification (reviewed by Walsh, 1982). Recently several additional compounds have been reported to behave as suicide substrates for microsomal monooxygenases (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano et al., 1981; Reichhart et al., 1982). The compounds which were appealing to me were structures which offered some possible specificity for the cytochromes P-450 which metabolized aromatic hydrocarbons. The two compounds below, obtained from Dr. P. R. Ortiz de Montellano, Department of Pharmaceutical Chemistry, UCSF, fit this description:



1-aminobenzotriazole

(ABT)



2,3-naphthoaminotriazole

(NAT)

The proposed mechanism of cytochrome P-450 inactivation involves hydroxylation of the exocyclic nitrogen in both

cases, followed by decomposition to two molecules of N_2 and a benzyne or naphthyne moiety. The latter compound is highly reactive and is believed to insert into the porphyrin ring system of the hemoprotein (Ortiz de Montellano and Mathews, 1981).

The loss of scup microsomal cytochrome P-450 upon incubation with inhibitor and NADPH was detectable spectroscopically. The CO-reducible cytochrome P-450 was assayed after 15 min at 30° C in 0.1 M Tris-Cl, pH 7.5, containing 20% glycerol and 0.5 mM NADPH (see Chapter 2).

	Cytochrome P-450
microsomes	20.2 nmol/ml
microsomes + NADPH + MeOH	18.9
microsomes + NADPH + 100 uM ABT	16.2 = 83%
microsomes + NADPH + 100 uM NAT	12.7 = 65%

The time-dependent loss of catalytic activity was followed by performing an incubation with scup microsomes, NADPH and the inactivator. An aliquot was withdrawn at various time points for routine catalytic assay (Chapter 3). The results of these incubations followed by assay of residual microsomal EROD or ECOD activity are summarized in Tables 5-5 and 5-6.

TABLE 5-5

Time-dependent Loss of Scup Microsomal 7-Ethoxyresorufin O-Deethylase Activity[a]

	% Residual Activity					
	[NAT], uM					
time	0	4	5	6.6	10	20
0 min	99[b]	96	110	83	111	96
5	107	37	46	32	26	17
10	107	23	28	18	12	9.6
15	98	13	17	11	7.8	4.3
20	90	12	12	8.7	6.5	2.9
25	96	10	11	7.5	5.2	
30	93	7.9	9.6	5.3		
	[ABT], uM					
	0	50	66	100	200	400
0 min	100[c]	103	88	102	82	73
5	91	73	73	87	61	56
10	94	58	59	52	45	42
15	76	60	53	34	24	31
20	86	50	39	32	22	23
25	87	46	37	29	19	18
30	94	35	35	22	10	

[a]Microsomes (1.2 uM cytochrome P-450) were incubated at 25° C with 0.5 mM NADPH in 0.5 ml of 0.1 M Tris-0.1 M NaCl, pH 8.0. An aliquot was withdrawn (final dilution 1:20) at the indicated times and assayed for activity at 25° C with 2 uM 7-ethoxyresorufin and 0.5 mM NADPH by the standard continuous spectrophotometric assay.

[b]The control rate was 5.9 nmol product/nmol cytochrome P-450/min.

[c]The control rate was 6.1 nmol product/nmol cytochrome P-450/min.

TABLE 5-6

Time-dependent Loss of Scup Microsomal 7-Ethoxycoumarin O-deethylase Activity[a]

	% Residual Activity				
			[NAT], uM		
time	0	5	10	20	33
0 min	100[b]	100*	100*	100*	100*
5	99	81	47	37	34
10	104	59	33	30	27
15	100	39	23	24	17
			[ABT], uM		
	0	50	66	100	200
0 min	100[c]	104	95	92	93
5	97	94	77	75	70
10	98	73	63	60	53
15	102	67	54	45	43

[a]Microsomes (0.8 uM cytochrome P-450) were incubated at 25°C with 0.5 mM NADPH in 0.5 ml of 0.1 M Tris-0.1 M NaCl, pH 8.0. An aliquot was withdrawn at the indicated times for a twenty minute standard catalytic assay (final dilution 1:16) in 0.4 ml with 0.5 mM NADPH and 0.5 mM 7-ethoxycoumarin.

[b]The control rate was 1.7 nmol product/nmol cytochrome P-450/min.

[c]The control rate was 1.5 nmol product/nmol cytochrome P-450/min.

*Arbitrarily set at 100% assuming competitive inhibition accounts for the lowered activity from the control incubation. The absolute rate of the zero time control in the presence of 33 uM NAT was 70% of the rate in the absence of NAT.

My colleagues, Dr. Stegeman and Bruce Woodin, have also established that NAT is a potent inactivator of microsomal BP-OH in preliminary experiments. I quote their data (Stegeman and Woodin, personal communication) here because it is cogent. The standard radiometric BP-OH assay was performed in 50 ul at 30° C using a five minute pre-incubation with several concentrations of NAT. The control was concurrent addition of NAT and benzo[a]pyrene in the presence of NADPH.

[NAT], uM	relative rate (%)
0	100
1	74
10	44
100	4
100, control	43

Three assumptions were made in the treatment of my kinetic data: 1. Because the semi-log plots of residual activity vs. time are not linear the the killing rate for the first 5 minutes was chosen for calculating k_{obs} from the extrapolated half-life (where $k_{obs} = 0.693/t_{0.5}$); 2. The control activity losses were assumed to be negligible and were ignored; 3. The killing process was assumed to cease upon dilution for catalytic assay. With these major qualifications the replots of $(k_{obs})^{-1}$ vs $[I]^{-1}$ (Figures 5-1 and 5-2) yielded the following:

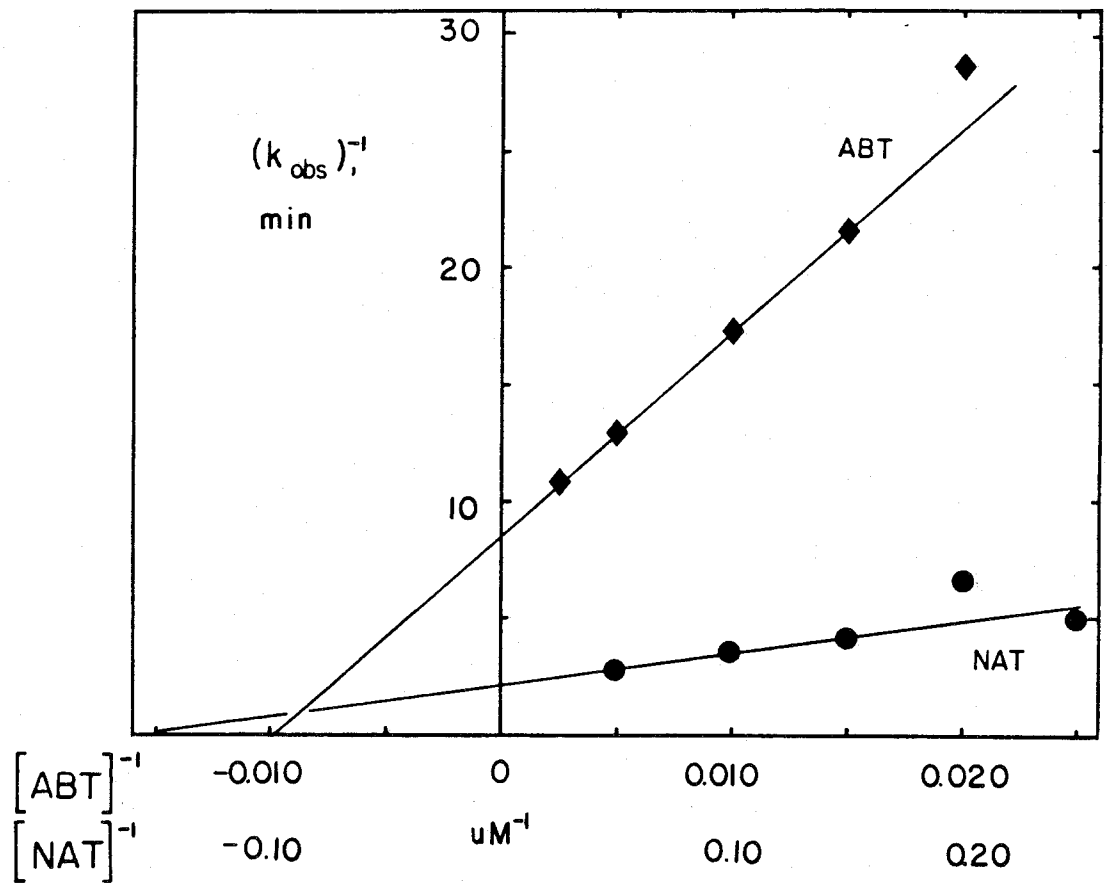


Figure 5-1. Replot of microsomal loss of EROD activity in the presence of inactivators. A k_{obs} was calculated from the residual EROD activity after a five minute incubation with NAT (●) or ABT (◆) as described within. The double reciprocal plot was generated from incubations with various concentrations of inactivator.

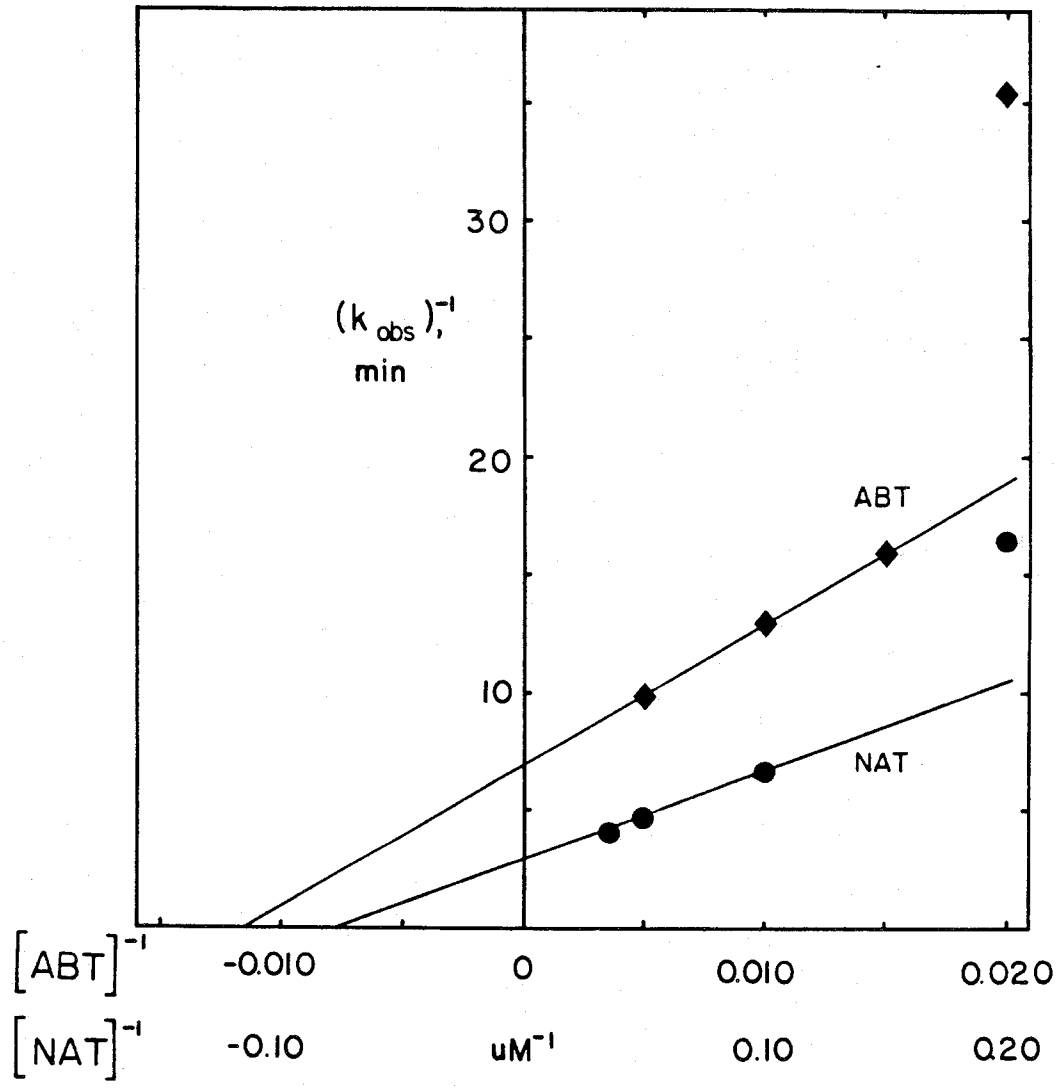


Figure 5-2. Replot of microsomal loss of ECOD activty in the presence of inactivators. A k_{obs} was calculated from the residual ECOD activity after a five minute incubation with NAT (●) or ABT (◆) as described within. The double reciprocal plot was generated from incubations with various concentrations of inactivator.

	EROD	ECOD
NAT, K_i	7 uM	12 uM
k_{inact}	0.47 min^{-1}	0.32 min^{-1}
ABT, K_i	100 uM	75 uM
k_{inact}	0.12 min^{-1}	0.14 min^{-1}

While these experiments were preliminary and the data was subject to massive errors, it appeared that microsomal EROD and ECOD activities were inactivated with identical kinetics and that NAT was much more effective in this process, based on both binding affinity and inactivation rate. Further, the microsomal BP-OH activity was clearly susceptible to the same type of inactivation. The observation that the total microsomal cytochrome P-450 loss was much less than the concomitant activity loss (at 100 uM NAT the EROD activity was totally abolished within five minutes) and the low inactivator K_i holds out the possibility of some inactivation specificity.

The inactivation susceptibility of microsomal monooxygenase activities which are also associated with reconstituted cytochrome P-450E suggested that cytochrome P-450E was a target for NAT (as I would hope). The reconstituted system was subjected to a similar kinetic analysis of catalytic activity loss in order to test this hypothesis. Recognizing the nonlinear time-dependence of inactivation, I chose the effective five minute pre-incubation time period and tested the dependence of inactivation on the NAT concentration. It was not possible to assay dilutions of the reconstituted monooxygenase system after incubation with

inactivator for technical reasons. The results, as obtained, are summarized in Table 5-7.

TABLE 5-7

NAT-dependent Loss of Reconstituted Scup Cytochrome P-450E 7-Ethoxycoumarin O-Deethylase Activity[a]

[NAT], uM	relative rate (%)
0	66
5	55
10	39
20	36
33	28

[a]Cytochrome P-450E was reconstituted in the standard ECOD assay with 20 pmol cytochrome P-450, 30 pmol purified rat NADPH-cytochrome P-450 reductase, 5 ug sonicated DLPC per ml, 0.5 mM NADPH and variable NAT in methanol in 0.395 ml of 0.1 M KP_i, pH 7.8. The reconstitutions were incubated for 5 minutes at 30° C and 5 ul of 7-ethoxycoumarin was added to a final concentration of 0.63 mM for a further ten minute catalytic assay. As a control all assays were also performed with inactivator and substrate added concurrently to account for inhibition which is assumed to be independent of NAT turnover. The relative rate is the ratio of activity with and without a five minute pre-incubation with inactivator. The time zero control reconstitution had a rate of 2 nmol product/nmol cytochrome P-450/min.

The dreaded auto-inactivation appeared to plague these incubations at 30° C making the abstraction of kinetic parameters tenuous. However, if the auto-inactivation background was ignored with the assumption that this was an artifact under non-turnover conditions in the presence of NADPH (the reconstituted system is thermally stable at 30° C for at least twenty minutes and normally the ECOD activity is linear for 60 min) then estimates of k_{cat} and K_i were 0.23 min^{-1} and 4 uM, respectively. The astonishing agreement with the kinetic parameters obtained in microsomes is probably fortuitous.

REFERENCES

Abe, T. and Watanabe, M. (1983). Purification and characterization of three forms of microsomal cytochrome P-450 in liver from 3-methylcholanthrene-treated guinea pigs. Mol. Pharmacol. 23: 258-264.

Ahokas, J. T., Pelkonen, P. and Karki, N. T. (1975). Metabolism of polycyclic hydrocarbons by a highly active aryl hydrocarbon hydroxylase system in the liver of a trout species. Biochem. Biophys. Res. Comm. 63: 635-641.

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

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

Alvares, A. P., Schilling, G., Levin, W. and Kuntzman, R. (1967). Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methyl-cholanthrene. Biochem. Biophys. Res. Comm. 29: 521-526.

Astrom, A., Meijer, J. and DePierre, J. W. (1983). Characterization of microsomal cytochrome P-450 species induced in rat liver by 2-acetylaminofluorene. Cancer Res. 43: 342-348.

Atlas, S. A. and Nebert, D. W. (1976). Genetic association of increases in naphthalene, acetanilide and biphenyl hydroxylations with inducible aryl hydrocarbon hydroxylase in mice. Arch. Biochem. Biophys. 175: 495-506.

Balk, L., Meijer, J., Seidegard, J., Morgenstein, R. and DePierre, J. W. (1980). Initial characterization of drug-metabolizing systems in the liver of the northern pike, Esox lucius. Drug Metab. Disp. 8: 98-103.

Ball, L. M., Elmamlouk, T. H. and Bend, J. R. (1979). Metabolism of benzo[a]pyrene in little skate mixed-function oxidase systems. In "Microsomes and Drug Oxidations and Chemical Carcinogenesis" (Coon, M. J. et al, eds.), pp 1203-1206, Academic Press, New York.

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 xenobiotics. Bull. Mt. Desert Island Biol. Lab. 13: 9-13.

Bend, J. R., James, M. O. and Dansette, P. M. (1975). In vitro metabolism of xenobiotics in some marine animals. Ann. N. Y. Acad. Sci. 298: 505-521.

Bend, J. R., Hall, P. and Foureman, G. L. (1976). Comparison of benzo[a]pyrene hydroxylase (aryl hydrocarbon hydroxylase, AHH) activities in hepatic microsomes from untreated and 1,2,3,4-dibenzanthracene (DBA)-induced little skate (Raja erinacea). Bull. Mt. Desert Island Biol. Lab. 16: 3-5.

Bend, J. R., Ball, L. M., Elmamlouk, T. H., James, M. O. and Philpot, R. M. (1979). Microsomal mixed-function oxidation in untreated and polycyclic aromatic hydrocarbon-treated fish. In "Pesticide and Xenobiotic Metabolism in Aquatic Organisms" (Khan, M. A. Q., Lech, J. J. and Menn, J. J., eds.), pp 297-318, Am. Chem. Soc., Washington, D. C.

Bensadoun, A. and Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. Anal. Biochem. 70: 241-250.

Binder, R. L. (1982). Xenobiotic monooxygenase activity and the response to inducers of cytochrome P-450 during embryonic and larval development in fish. Massachusetts Institute of Technology/Woods Hole Oceanographic Institution Joint Program in Biological Oceanography, Ph.D. thesis, 263 p, WHOI-82-17.

Binder, R. L. and Stegeman, J. J. (1980). Induction of aryl hydrocarbon hydroxylase activities in embryos of an estuarine fish. Biochem. Pharm. 29: 949-951.

Black, S. D. and Coon, M. J. (1982). Structural features of liver microsomal NADPH-cytochrome P-450 reductase. J. Biol. Chem. 257: 5929-5938.

Black, S. D., Tarr, G. E. and Coon, M. J. (1982). Evidence from studies on primary structure that rabbit hepatic NADPH-cytochrome P-450 reductase and isozymes 2 and 4 of cytochrome P-450 represent unique gene products. In "Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications," (Heitanen, E., Laitinen, M. and Hanninen, O., eds.), pp 277-281, Elsevier Biomedical Press, New York.

Bonfils, C., Balny, C. and Maurel, P. (1981). Direct evidence for electron transfer from ferrous cytochrome b_5 to the oxyferrous intermediate of liver microsomal cytochrome P-450 LM-2. J. Biol. Chem. 256: 9457-9465.

Botelho, L. H., Ryan, D. E. and Levin, W. (1979). Amino acid compositions and partial amino acid sequences of three

highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital or 3-methylcholanthrene. J. Biol. Chem. 254: 5635-5640.

Botelho, L. H., Ryan, D. E., Yuan, P-M., Kutny, R., Shively, J. E. and Levin, W. (1982). Amino-terminal and carboxy-terminal sequence of hepatic microsomal cytochrome $P\text{-}450_d$, a unique hemoprotein from rats treated with isosafrole. Biochemistry 21: 1152-1155.

Brauer, A. W., Margolies, M. N. and Haber, E. (1975). The application of 0.1M Quadrol to the microsequence of proteins and the sequence of tryptic peptides. Biochemistry 14: 3029-3035.

Brown, E. R., Hazdra, J. J., Keith, L., Greenspan, I., Kwapinski, J. B. G. and Beamer, P. (1973). Frequency of fish tumors found in a polluted watershed as compared to nonpolluted Canadian waters. Cancer Res. 33: 189-198.

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

Burke, M. D., Prough, R. A. and Mayer, R. T. (1977a). Characteristics of a microsomal cytochrome P-448-mediated reaction. Drug Metab. Disp. 5: 1-8.

Burke, M. D., Vadi, H., Jenstrom, B. and Orrenius, S. (1977b). Metabolism of benzo[a]pyrene with isolated hepatocytes and the formation and degradation of DNA-binding derivatives. J. Biol. Chem. 252: 6424-6431.

Cheng, K-C. and Schenkman, J. B. (1982). Purification and characterization of two constitutive forms of rat liver microsomal cytochrome P-450. J. Biol. Chem. 257: 2378-2385.

Chevion, M., Stegeman, J. J., Peisach, J. and Blumberg, W. E. (1977). Electron paramagnetic resonance studies on hepatic microsomal cytochrome P-450 from a marine teleost fish. Life Sci. 20: 895-900.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252: 1102-1106.

Colby, H. D. (1980). Regulation of hepatic drug and steroid metabolism by androgens and estrogens. In "Advances in Sex Hormone Research," (Thomas, J. A. and Singhal, R. L., eds.), vol 4, pp 27-71, Urban and Schwartenberg.

Conney, A. (1967). Pharmacological implications of microsomal enzyme induction. Pharm. Rev. 19: 317-366.

Coon, M. J., Koop, D. R., Persson, A. V. and Morgan, E. T. (1980). Inducible and constitutive isozymes of microsomal cytochrome P-450. In "Biochemistry, Biophysics and Regulation of Cytochrome P-450," (Gustafsson, J-A., Carlstedt-Duke, J., Mode, A. and Rafter, J., eds.), pp 7-15, Elsevier, New York.

Creaven, P. J., Parke, D. V. and Williams, R. T. (1965). A fluorimetric study of the hydroxylation of biphenyl in vitro by liver preparations of various species. Biochem. J. 96: 879-885.

Danielsson, H. and Sjovall, J. (1975). Bile acid metabolism. Ann. Rev. Biochem. 44: 233-253.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. (1969). "Data for Biochemical Research." Second edition, Oxford University Press, London, 654 p.

Dean, W. L. and Coon, M. J. (1977). Immunochemical studies on two electrophoretically homogeneous forms of rabbit liver microsomal cytochrome P-450: $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$. J. Biol. Chem. 252: 3255-3261.

Delaforge, M., Koop, D. R. and Coon, M. J. (1982). Role of isosafrole as a complexing agent and inducer of cytochrome P-450 LM-4 in rabbit liver microsomes. Biochem. Biophys. Res. Comm. 108: 59-65.

Denk, H. (1979). Effect of detergents on the mixed function oxidase and other microsomal enzymes. Pharm. Ther. 6: 551-577.

Dieter, H. H., Muller-Eberhard, U. and Johnson, E. F. (1982). Identification of rabbit microsomal cytochrome P-450 isozyme, form 1, as a hepatic progesterone 21-hydroxylase. Biochem. Biophys. Res. Comm. 105: 515-520.

Duce, R. A. and Duursma, E. K. (1977). Inputs of organic matter to the ocean. Mar. Chem. 5: 319-339.

Dunn, B. P. and Stich, H. F. (1976). Monitoring procedures for chemical carcinogens in coastal waters. J. Fish Res. Bd. Can. 33: 2040-2046.

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

Elshourbaghy, N. A. and Guzelian, P. S. (1980). Separation, purification and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone 16a-carbonitrile. J. Biol. Chem. 255: 1279-1285.

Enoch, H. G. and P. Strittmatter (1979). Cytochrome b_5 reduction by NADPH-cytochrome P-450 reductase. J. Biol. Chem. 254:8976-8981.

Foureman, G. L. and Bend, J. R. (1982). Variation of certain hepatic monooxygenase activities in feral winter flounder (Pseudopleuronectes americanus) from Maine: apparent association with induction by environmental exposure to PAH-type compounds. In "Cytochrome P-450, Biochemistry, Biophjysics and Environmental Implications," (Heitanen, E., Laitinen, M. and Hanninen, O., eds.), pp 233-244, Elsevier Biomedical Press, New York.

French, J. S. and Coon, M. J. (1979). Properties of NADPH-cytochrome P-450 reductase purified from rabbit liver microsomes. Arch. Biochem. Biophys. 195: 565-577.

French, J. S., Guengerich, F. P. and Coon, M. J. (1980). Interactions of cytochrome P-450, NADPH-cytochrome P-450 reductase, phospholipid and substrate in the reconstituted liver microsomal enzyme system. J. Biol. Chem. 255: 4112-4119.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K. and Muramatsu, M. (1982). Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. Proc. Nat. Acad. Sci. (USA) 79: 2793-2797.

Giger, W. and Blumer, M. (1974). Polycyclic aromatic hydrocarbons in the environment: isolation and characterization by chromatography, visible, ultraviolet and mass spectrometry. Anal. Chem. 46: 1663-1671.

Goldstein, A., L. Aronow and S. M. Kalman (1974). "Principles of Drug Action: the basis of pharmacology." Second edition, John Wiley and Sons, New York.

Goldstein, J. A., Linko, P., Luster, M. I. and Sundheimer, D. W. (1982). Purification and characterization of a second form of hepatic cytochrome P-448 from rats treated with a pure polychlorinated biphenyl isomer. J. Biol. Chem. 257: 2702-2707.

Guengerich, F. P. (1977). Separation and purification of multiple forms of microsomal cytochrome P-450. J. Biol. Chem. 252: 3970-3979.

Guengerich, F. P. (1978). Comparison of highly purified microsomal cytochromes P-450 and NADPH-cytochrome P-450 reductases by peptide mapping. Biochem. Biophys. Res. Comm. 82: 820-827.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V. and Kaminsky, L. S. (1982a). Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry 21: 6019-6030.

Guengerich, F. P., Wang, P. and Davidson, N. K. (1982b). Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biochemistry 21: 1698-1706.

Guenther, T. M., Negishi, M. and Nebert, D. W. (1979). Separation of acetanilide and its hydroxylated metabolites and quantitative determination of "acetanilide 4-hydroxylase activity" by high-pressure liquid chromatography. Anal. Biochem. 96: 201-207.

Gunsalus, I. C., Meeks, J. R. and Lipscomb, J. D. (1973). Cytochrome $P\text{-}450_{cam}$ substrate and effector interactions. Ann. N. Y. Acad. Sci. 212: 107-121.

Hansson, R., Holmberg, I. and Wikvall, K. (1981). 25-Hydroxylation of vitamin D_3 and side chain hydroxylations of 5β-cholestane-3a,7a,12a-triol by purified rabbit and rat liver microsomal cytochromes P-450. J. Biol. Chem. 256: 4345-4349.

Hansson, T., Rafter, J. and Gustafsson, J-A. (1980). Effects of some common inducers on the hepatic microsomal metabolism of androstenedione in rainbow trout with special reference to cytochrome P-450-dependent enzymes. Biochem. Pharmacol. 29: 583-587.

Haugen, D. A. and Coon, M. J. (1976). Properties of electrophoretically homogeneous phenobarbital-inducible and -naphthoflavone-inducible forms of liver microsomal cytochrome P-450. J. Biol. Chem. 251: 7929-7939.

Heineman, F. S. and Ozols, J. (1983). The complete amino acid sequence of rabbit phenobarbital-induced liver microsomal cytrochrome P-450. J. Biol. Chem. 258: 4195-4201.

Helenius, A. (1975). Solubilization of membranes by detergents. Biochem. Biophys. Acta 415: 29-79.

Hildebrandt, A. and Estabrook, R. A. (1971). Evidence for the participation of cytochrome b_5 in hepatic microsomal mixed function oxidation reactions. Arch. Biochem. Biophys. 143: 66-79.

Holder, G., Yagi, H., Dansette, P., Jerina, D. M., Levin, W., Lu, A. Y. H. and Conney, A. H. (1974). Effects of inducers and epoxide hydrase on the metabolism of benzo[a]pyrene by liver microsomes and a reconstituted system: analysis by high pressure liquid chromatography. Proc. Nat. Acad. Sci. (USA) 71: 4356-4360.

Huang, M.-T., West, S. B. and Lu, A. Y. H. (1976). Separation, purification and properties of multiple forms of cytochrome P-450 from the liver microsomes of phenobarbital-treated mice. J. Biol. Chem. 251: 4659-4665.

Imai, Y. (1981). The roles of cytochrome b_5 in reconstituted monooxygenase systems containing various forms of hepatic microsomal cytochrome P-450. J. Biochem. 89: 351-362.

Ingelman-Sundberg, M. and Glauman, H. (1977). Reconstitution of the liver microsomal hydroxylase system in liposomes. FEBS Lett. 78: 72-78.

Ingelman-Sundberg, M. and Johansson, I. (1980). Catalytic properties of purified rabbit liver microsomal cytochrome P-450 in reconstituted phospholipid vesicles. Biochem. 19: 4004-4011.

Iyanagi, I., Makino, R. and Anan, F. K. (1981). Studies on the microsomal mixed-function oxidase system: mechanism of action of hepatic NADPH-cytochrome P-450 reductase. Biochemistry 20: 1722-1730.

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.

James, M. O. and Bend, J. R. (1980). Polycyclic aromatic hydrocarbon induction of cytochrome P-450-dependent mixed-function oxidases in marine fish. Toxicol. Appl. Pharm. 54: 117-133.

James, M. O. and Little, P. J. (1980). Characterization of cytochrome P-450 dependent mixed-function oxidation in spiny lobster, Panulirus argus. In "Biochemistry, Biophysics and Regulation of Cytochrome P-450" (Gustafsson, J-A., Carlstedt-Duke, J., Mode, A. and Rafter, J., eds.), pp 113-120, Elsevier, New York.

James, M. O. and Bend, J. R. (1982). Effect of polynuclear aromatic hydrocarbons and polyhalogenated biphenyls on hepatic mixed-function oxidase activity in marine fish. In "Carcinogenic Aromatic Polynuclear Aromatic Hydrocarbons in the Marine Environment," (Richards, N., ed.), Proceedings of Conference, Pensacola, Florida, August, 1978, U. S. EPA, Washington, D. C.

James, M. O. and Shiverick, K. T. (1983). Steroid oxidation in spiny lobster hepatopancreas. Fed. Proc., Fed. Am. Soc. Exp. Biol. 42: 913.

Jerina, D. M. and Daly, J. W. (1974). Arene oxides: a new aspect of drug metabolism. Science 185: 573-582.

Johnson, E. F. (1979). Multiple forms of cytochrome P-450: criteria and significance. Reviews in Biochemical Toxicology (Hodgson, E., Bend, J. R. and Philpot, R. M., eds.), vol I, pp 1-26. Elsevier, North Holland.

Johnson, E. F. and Muller-Eberhard, U. (1977a). Resolution of two forms of cytochrome P-450 from the liver microsomes of rabbits treated with 2,3,7,8-tetrachloro-dibenzo-p-dioxin. J. Biol. Chem. 252: 2839-2845.

Johnson, E. F. and Muller-Eberhard, U. (1977b). Multiple forms of cytochrome P-450: resolution and purification of rabbit liver aryl hydrocarbon hydroxylase. Biochem. Biophys. Res. Comm. 76: 644-651

Johnson, E. F. and Muller-Eberhard, U. (1977c). Purification of the major cytochrome P-450 of liver microsomes from rabbits treated with 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). Biochem. Biophys. Res. Comm. 76: 652-659.

Kimbrough, R.D. (1974). The toxicity of polychlorinated polycyclic compounds and related chemicals. CRC Critical Reviews in Toxicology, vol 2, pp 445-498.

Koop, D. R., Persson, A. V. and Coon, M. J. (1981). Properties of electrophoretically homogeneous constitutive forms of microsomal cytochrome P-450. J. Biol. Chem. 256: 10704-10711.

Koop, D. R., Morgan, E. T., Tarr, G. E. and Coon, M. J. (1982). Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. J. Biol. Chem. 257: 8472-8480.

Kupfer, D., Navarro, J. and Piccolo, D. E. (1978). Hydroxylation of prostaglandins A_1 and E_1 by liver

microsomal monooxygenase. J. Biol. Chem. 253: 2804-2811.

Kurelec, B., Britivic, S., Muller, W. E. G. and Zahn, R. K. (1977). Benzo[a]pyrene monooxygenase induction in marine fish - molecular response to oil pollution. Mar. Biol. 44: 211-216.

Kuwahara, S. and Omura, T. (1980). Different requirement for cytochrome b_5 in NADPH-supported O-deethylation of p-nitrophenetol catalyzed by two types of microsomal cytochrome P-450. Biochem. Biophys. Res. Comm. 96: 1562-1568.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

LaFlamme, R. E. and Hites, R. A. (1978). The global distribution of polycyclic aromatic hydrocarbons in recent sediments. Geochim. Cosmochim. Acta 42: 289-303.

Lee, D. J., Wales, J. H., Ayres, J. L. and Sinnhuber, R. O. (1968). Synergism between cyclopropenoid fatty acids and chemical carcinogens in rainbow trout (Salmo gairdnerii). Cancer Res. 28: 2312-2318.

Light, D. R., Walsh, C. and Marletta, M. A. (1980). Analytical and preparative high-performance liquid chromatography separation of flavin and flavin analog coenzymes. Anal. Biochem. 109: 87-93.

Lu, A. Y. H., Junk, K. W. and Coon, M. J. (1969). Resolution of the cytochrome P-450 containing w-hydroxylation system of liver microsomes into three components. J. Biol. Chem. 244: 3714-3722.

Lu, A. Y. H., Kuntzman, R., West, S., Jacobson, M. and Conney, A. H. (1972a). Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds and endogenous substrates. J. Biol. Chem. 247: 1727-1734.

Lu, A. Y. H., Somogyi, A., West, S., Kuntzman, R. and Conney, A. H. (1972b). Pregnenolone-16a-carbonitrile: a new type of inducer of drug metabolizing enzymes. Arch. Biochem. Biophys. 152: 457-462.

Lu, A. Y. H., R. Kuntzman and A. H. Conney (1976). The liver microsomal hydroxylation enzyme system. In "Frontiers of Gastrointestinal Research," (van der Reis, L. ed.), vol II, S. Karger AG, Basel.

Lu, A. Y. H. and West, S. B. (1980). Multiplicity of

mammalian cytochromes P-450. Pharm. Rev. 31: 277-295.

Mayer, R. T., Jermyn, J. W., Burke, M. D. and Prough, R. A. (1977). Methoxyresorufin as a substrate for the fluorometric assay of insect microsmal O-dealkylases. Pest. Biochem. Physiol. 7: 349-354.

Mayhew, S. G. and Massey, V. (1969). Purification and characterization of flavodoxin from Peptostreptococcus elsdenii. J. Biol. Chem. 244: 794-802.

Mearns, A. J. and Sherwood, M. J. (1973). Distribution of neoplasms and other diseases in marine fishes relative to the discharge of waste water. Ann. N. Y. Acad. Sci. 298: 210-224.

Mihara, K. and Sato, R. (1975). Purification and properties of the intact form of NADH-cytochrome b_5 reductase from rabbit liver microsomes. J. Biochem. 78: 1057-1073.

Miki, N., Sugiyama, T. and Yamano, T. (1980). Purification and characterization of cytochrome P-450 with high affinity for cytochrome b_5. J. Biochem. 88: 307-316.

Miwa, G. T., West, S. B. and Lu, A. Y. H. (1978). Studies on the rate-limiting enzyme component in the microsomal monooxygenase system. J. Biol. Chem. 253: 1921-1929.

Miwa, G. T., West, S. B., Huang, M-T. and Lu, A. Y. H. (1979). Studies on the association of cytochrome P-450 and NADPH-cytochrome c reductase during catalysis in a reconstituted hydroxylating system. J. Biol. Chem. 254: 5695-5700.

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.

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. CRC Critical Reviews in Biochemistry, vol 6, pp 401-437.

Nebert, D. W., Eisen, H. J., Negishi, M., Lang, M. A. and Hjelmeland, L. M. (1981). Genetic mechanisms controlling the induction of polysubstrate monooxygenase (P-450) activities. Ann. Rev. Pharmacol. Toxicol. 21: 431-462.

Neff, J. M. (1978). "Polycyclic Aromatic Hydrocarbons." Applied Science Publisher, Ltd., London, 262 p.

Negishi, M. and Nebert, D. W. (1979). Structural gene products of the Ah locus. J. Biol. Chem. 254: 11015-11023.

Nesnow, S. and Bergman, H. (1981). Metabolism of α-naphthoflavone by rat liver microsomes. Cancer Res. 41: 2621-2626.

Nial, H. D. (1973). Automated Edman degradation: the protein sequenator. Meth. Enzymol. 27: 942-1010.

Nordblom, G. D. and Coon, M. J. (1977). Hydrogen peroxide formation and stoichiometry of hydroxylation reactions catalyzed by highly purified liver microsomal cytochrome P-450. Arch. Biochem. Biophys. 180: 343-347.

Norman, R. L., Johnson, E. F. and Muller-Eberhard, U. (1978). Identification of the major cytochrome P-450 form transplacentally induced in neonatal rabbits by 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Biol. Chem. 253: 8640-8647.

Noshiro, M., Harada, N. and Omura, T. (1980). Immunochemical study on the route of electron transfer from NADH and NADPH to cytochrome P-450 of liver microsomes. J. Biochem. 88: 1521-1535.

Omura, T. and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. J. Biol. Chem. 239: 2370-2378.

Oprian, D. D., Vatsis, K. P. and Coon, M. J. (1979). Kinetics of reduction of cytochrome P-450 LM_4 in a reconstituted liver microsomal enzyme system. J. Biol. Chem. 254: 8895-8902.

Ortiz de Montellano, P. R. and Mathews, J. M. (1981). utocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. Biochem. J. 195: 761-764.

Ortiz de Montellano, P. R., Mico, B. A., Mathews, J. M., Kunze, K. L., Miwa, G. T. and Lu, A. Y. H. (1981). Selective inactivation of cytochrome P-450 isozymes by suicide substrates. Arch. Biochem. Biophys. 210: 717-728.

Oshino, N. and Omura, T. (1973). Immunochemical evidence for the participation of cytochrome b_5 in microsomal stearyl-CoA desaturase reaction. Arch. Biochem. Biophys. 157: 395-404.

Pancirov, R. J. and Brown, R. A. (1977). Polynuclear aromatic hydrocarbons in marine tissues. Environ. Sci. Tech. 11: 989-992.

Park, S. S., Fujino, T., West, D., Guengerich, F. P. and Gelboin, H. V. (1982). Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. Cancer Res. 42: 1798-1808.

Parkinson, A., Safe, S.H., Robertson, L.W., Thomas, P.E., Ryan, D.E., Reik, L.M. and Levin, W. (1983). Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. J. Biol. Chem. 258: 5967-5976.

Payne, J. F. (1976). Field evaluation of benzopyrene hydroxylase induction as a monitor for marine petroleum pollution. Science 191: 945-946.

Peterson, J. A., Ebel, R. E., O'Keefe, D. H., Matsubara, T. and Estabrook, R. W. (1976). Temperature dependence of cytochrome P-450 reduction. J. Biol. Chem. 251: 4010-4016.

Pezzuto, J. M., Yang, C. S., Yang, S. K., McCourt, D. W. and Gelboin, H. V. (1978). Metabolism of benzo[a]pyrene and (-)trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene by rat liver nuclei and microsomes. Cancer Res. 38: 1241-1245.

Pickett, C. B., Jeter, R. L., Morin, J. and Lu, A. Y. H. (1981). Electroimmunochemical quantitation of cytochrome P-450, cytochrome P-448 and epoxide hydrolase in rat liver microsomes. J. Biol. Chem. 256: 8815-8820.

Pohl, R. J., Bend, J. R., Guarino, A. M. and Fouts, J. R. (1974). Hepatic microsomal mixed-oxidase activity of several marine species from coastal Maine. Drug Metab. Disp. 2: 545-555.

Prough, R. A., Burke, M. D. and Mayer, R. T. (1978). Direct fluorometric methods for measuring mixed-function oxidase activity. Meth. Enzymol. 52: 372-377.

Rasmussen, R. E. and Wang, I. Y. (1974). Dependence of specific metabolism of benzo[a]pyrene on the inducer of hydroxylase activity. Cancer Res. 34: 2290-2295.

Reichhart, D., Simon, A., Mathews, J. M. and Ortiz de Montellano, P. R. (1982). Autocatalytic destruction of plant cytochrome P-450 enzymes: selective inactivation of cinnamic acid 4-hydroxylase from Helianthus tuberosus by 1-aminobenzotriazole. Arch. Biochem. Biophys. 216: 522-529.

Risebrough R. W. and de Lappe, B. (1972). Accumulation of polychlorinated biphenyls in ecosystems. Environ. Health Persp. 1: 39-45.

Ryan, D. E., Thomas, P. E., Korzeniowski, D. and Levin, W. (1979). Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. J. Biol. Chem. 254: 1365-1374.

Ryan, D. E., Thomas, P. E. and Levin, W. (1980). Hepatic microsomal cytochrome P-450 from rats treated with isosafrole. J. Biol. Chem. 255: 7941-7955.

Ryan, D. E., Thomas, P. E. and Levin, W. (1982). Purification and characterization of a minor form of hepatic microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls. Arch. Biochem. Biophys. 216: 272-288.

Sauer, R. T., Pan, J., Hopper, P., Hehir, K., Brown, J. and Poteete, A. R. (1981). Primary structure of the phage P22 repressor and its gene c2. Biochemistry 20: 3591-3598.

Serabjit-Singh, C. J., Slaughter, S. R., Pohl, R. J., Fouts, J. R. and Philpot, R. M. (1981). Characterization of hepatic NADPH-cytochrome P-450 reductase purified from little skate (Raja erinacea). Fed. Proc., Fed. Am. Soc. Exp. Biol. 40: 697.

Sims, P. and Grover, P. L. (1974). Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. Adv. Cancer Res. 20: 165-274.

Smith, C.E., Peck, T.H., Klauda, R.J. and McLauren, J.B. (1979). Hepatomas in Atlantic tomcod Microgadus tomcod (Walbaum) collected in the Hudson river estuary in New York. J. Fish Dis. 2: 313-319.

Statham, C. N., Elcombe, C. R., Szyijka, S. P. and Lech, J. J. (1978). Effect of polycyclic aromatic hydrocarbons on hepatic microsomal enzymes and disposition of methyl-naphthalene in rainbow trout in vivo. Xenobiotica 8: 65-71

Stegeman, J. J. (1978). Influence of environmental contamination on cytochrome P-450 mixed-function oxygenases in fish: implications for recovery in the Wild Harbor marsh. J. Fish. Res. Board Can. 35: 668-674.

Stegeman, J. J. (1979). Temperature influence on basal activity and induction of mixed function oxygenase activity in Fundulus heteroclitus. J. Fish Res. Bd. Can. 36: 1400-1405.

Stegeman, J. J. (1981). PAH and their metabolism in the marine environment. In "Polycyclic Hydrocarbons and Cancer," (Gelboin, H. V. and Ts'O, P. O. P., eds.), vol 3, pp 1-60, Academic Press, New York.

Stegeman, J. J. (1983). Hepatic microsomal mono-oxygenase activity and the biotransformation of hydrocarbons in deep benthic fish from the western North Atlantic. Can J. Fish Aq. Sci., in press.

Stegeman, J. J. and Binder, R. L. (1979). High benzo[a]pyrene hydroxylase activity in the marine fish Stenotomus versicolor. Biochem. Pharm. 28: 1686-1688.

Stegeman, J. J., Binder, R. L. and Orren, A. (1979). Hepatic and extrahepatic microsomal electron transport components and mixed-function oxygenases in the marine fish Stenotomus versicolor. Biochem. Pharm. 28: 3431-3439.

Stegeman, J. J. and Chevion, M. (1980). Sex differences in cytochrome P-450 and mixed-function oxygenase activity in gonadally mature trout. Biochem. Pharm. 28: 553-558.

Stegeman, J. J. and Woodin, B. R. (1980). The metabolism of α-naphthoflavone (7, 8-benzoflavone) by hepatic microsomes from the marine fish Stenotomus versicolor. Biochem. Biophys. Res. Comm. 95: 328-333.

Stegeman, J. J. and Kaplan, H. B. (1981). Mixed-function oxygenase activity and benzo[a]pyrene metabolism in the barnacle Balanus eburneus (Crustacea, Cirripedia). Comp. Biochem. Physiol. 68C: 55-61.

Stegeman, J. J., Klotz, A. V., Woodin, B. R. and Pajor, A. M. (1981). Induction of hepatic cytochrome P-450 in fish and the indication of environmental induction in scup (Stenotomus chrysops). Aquat. Toxicol. 1: 197-212.

Stegeman, J. J., Pajor, A. M. and Thomas. P. (1982a). Influence of estradiol and testosterone on cytochrome P-450 and monooxygenase activity in immature brook trout, Salvelinus fontinalis. Biochem. Pharmacol. 31: 3979-3989.

Stegeman, J. J., Skopek, T. J. and Thilly, W. G. (1982b). Bioactivation of polynuclear aromatic hydrocarbons to cytotoxic and mutagenic products by marine fish. In "Carcinogenic Polynuclear Aromatic Hydrocarbons in the Marine Environment" (N. Richards, ed.). Proceedings of Conference, Pensacola, Florida, August 1978. U. S. EPA, Washington, D. C.

Stegeman, J. J., Woodin, B. R., Klotz, A. V., Wolke, R.

E. and Orme-Johnson, N. R. (1982c). Cytochrome P-450 and monooxygenase activity in cardiac microsomes from the fish Stenotomus chrysops. Mol. Pharmacol. 21: 517-526.

Stegeman, J. J. and Woodin, B. R. (1983). Differential regulation of hepatic xenobiotic and steroid metabolism in marine teleost species. Marine. Envi. Res., in press.

Stegeman, J. J., Woodin, B. R. and Binder, R. L. (1983). Patterns of benzo[a]pyrene metabolism by varied species, organs and developmental stages of fish. J. Nat. Cancer Inst., in press.

Strittmatter, P. and Velick, S. F. (1956). The isolation and properties of microsomal cytochrome. J. Biol. Chem. 221: 253-264.

Strittmatter, P., Fleming, P., Connors, M. and Corcoran, D. (1978). Purification of cytochrome b_5. Meth. Enzymol. 52: 97-101.

Strobel, H. W., Lu, A. Y. H., Heidema, J. and Coon, M. J. (1970). Phosphatidylcholine requirements in the enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon and drug hydroxylation. J. Biol. Chem. 245: 4851-4856.

Strobel, H. W. and J. D. Dignam (1978). Purification and properties of NADPH-cytochrome P-450 reductase. Meth. Enzymol. 52: 89-96.

Sugiyama, T., Miki, N. and Yamano, T. (1980). NADH- and NADPH-dependent reconstituted p-nitroanisole 0-demethylation system containing cytochrome P-450 with high affinity for cytochrome b_5. J. Biochem. 87: 1457-1467.

Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B. and Kawalek, J. (1976a). Immunochemical evidence for six forms of rat liver cytochrome P-450 obtained using antibodies against purified rat liver cytochromes P-450 and P-448. Mol. Pharmacol. 12: 746-758.

Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. and Levin, W. (1976b). Multiple forms of rat liver cytochrome P-450. J. Biol. Chem. 251: 1385-1391.

Thomas, P. E., Korzeniowski, D., Ryan, D. and Levin, W. (1979). Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. Arch. Biochem. Biophys. 192: 524-532.

Thomas, P. E., Reik, L. M., Ryan, D. E. and Levin, W.

(1983). Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochromes P-450_c and P-450_d, by structurally diverse xenobiotics. J. Biol. Chem. 258: 4590-4598.

Thorgeirsson, S. S., Atlas, S. A., Boobis, A. R. and Felton, J. S. (1979). Species differences in the substrate specificity of hepatic cytochrome P-448 from polycyclic hydrocarbon-treated animals. Biochem. Pharmacol. 28: 217-226.

Tsuji, H., Muta, E. and Ullrich, V. (1980). Separation and purification of liver microsomal monooxygenases from induced and untreated pigs. Hoppe-Seyler's Z. Physiol. Chem. 361: 681-696.

Tjessum, K. and Stegeman, J. J. (1979). Improvement of reverse-phase HPLC resolution of benzo[a]pyrene metabolites using organic amines: application to metabolites produced by fish. Anal. Biochem. 99: 129-135.

Varanasi, U. and Gmur, D. J. (1980). Metabolic activation and covalent binding of benzo[a]pyrene to deoxyribonucleic acid catalyzed by liver enzymes of marine fish. Biochem. Pharmacol. 29: 753-761.

Varanasi, U. and Gmur, D. J. (1981). Hydrocarbons and metabolites in English sole (Parophrys vetulus) exposed simultaneously to [^{3}H]benzo[a]pyrene and [^{14}C]-naphthalene in oil-contaminated sediment. Aquatic Tox. 1: 49-67.

Vatsis, K. P., Theoharides, A. D., Kupfer, D. and Coon, M. J. (1982). Hydroxylation of prostaglandins by inducible isozymes of rabbit liver microsomal cytochrome P-450. J. Biol. Chem. 257: 11221-11229.

Vermilion, J. L. and Coon, M. J. (1978). Purified liver microsomal NADPH-cytochrome P-450 reductase. J. Biol. Chem. 253: 2694-2704.

Vermilion, J. L., Ballou, D. P., Massey, V. and Coon, M. J. (1981). Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase. J. Biol. Chem. 256: 266-277.

Vesterberg, O. (1971). Staining of protein zones after isoelectric focusing in polyacrylamide gels. Biochem. Biophys. Acta 243: 345-348.

Vodicnik, M. J., Elcombe, C. R. 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.

Vyas, K. P., Shibata, T., Highet, R. J., Yeh, H. J., Thomas, P. E., Ryan, D. E., Levin, W. and Jerina, D. M. (1983). Metabolism of α-naphthoflavone and β-naphthflavone by rat liver microsomes and highly purified reconstituted cytochrome P-450 systems. J. Biol. Chem. 258: 5649-5659.

Walsh, C. (1982). Suicide substrates: mechanism-based enzyme inactivators. Tetrahedron 38: 871-909.

Warner, M., LaMarca, M. V. and Neims, A. H. (1978). Chromatographic and electrophoretic heterogeneity of the cytochromes P-450 solubilized from untreated rat liver. Drug Metab. Dispos. 6: 353-362.

Warner, M. and Neims, A. H. (1979). Multiple forms of ethoxyresorufin O-deethylase and benzphetamine N-demethylase in solubilized and partially resolved rat liver cytochromes P-450. Drug Metab. Dispos. 7: 188-193.

Waterman, M. R. (1978). Spectral characterization of human hemoglobin and its derivatives. Meth. Enzymol. 52: 456-463.

Waxman, D. J., Light, D. R. and Walsh, C. (1982). Chiral sulfoxidations catalyzed by rat liver cytochromes P-450. Biochemistry 21: 2499-2507.

Waxman, D.J. and Walsh, C. (1982). Phenobarbital-induced rat liver cytochrome P-450. J. Biol. Chem. 257: 10446-10457.

Waxman, D. J. and Walsh, C. (1983). Cytochrome P-450 PB-1 from phenobarbital-induced rat liver: purification, characterization and interactions with metyrapone and cytochrome b_5. Biochemistry, in press.

Waxman, D. J., Ko, A. and Walsh, C. (1983). Regio-selectivity of steroid hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. J. Biol. Chem., in press.

West, S. B. and Lu, A. Y. H. (1977). Liver microsomal electron transport systems. Arch. Biochem. Biophys. 182: 369-378.

West, S. B., Huang, M-T., Miwa, G. T. and Lu, A. Y. H. (1979). A simple and rapid procedure for the purification of phenobarbital-inducible cytochrome P-450 from rat liver microsomes. Arch. Biochem. Biophys. 193: 42-50.

White, R. E. and Coon, M. J. (1980). Oxygen activation

by cytochrome P-450. Ann. Rev. Biochem. 49: 315-356.

Wiebel, F. J., Leutz, J. C., Diamond, L. and Gelboin, H. V. (1971). Aryl hydrocarbon (benzo[a]pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and organic solvents. Arch. Biochem. Biophys. 144: 78-86.

Wiebel, F. J. and Gelboin, H. V. (1975). Aryl hydrocarbon (benzo[a]pyrene) hydroxylases in liver from rats of different age, sex and nutritional status. Biochem. Pharm. 24: 1511-1515.

Wiebel, F. J., Selkirk, J. K., Gelboin, H. V., Haugen, D. A., van der Hoeven, T. A. and Coon, M. J. (1975). Position-specific oxygenation of benzo[a]pyrene by different forms of purified cytochrome P-450 from rabbit liver. Proc. Nat. Acad. Sci. (USA) 72: 3917-3920.

Williams, D. E. and Buhler, D. R. (1982). Purification of cytochromes P-448 from β-naphthoflavone-treated rainbow trout. Biochem. Biophys Acta 717: 398-404.

Williams, D. E. and Buhler, D. R. (1983a). Multiple forms of cytochrome P-448 and P-450 purified from β-naphthoflavone-fed rainbow trout. Fed. Proc., Fed. Am. Soc. Exp. Biol. 42: 910.

Williams, D. E. and Buhler, D. R. (1983b). Comparative properties of purified cytochrome P-448 from β-naphthoflavone treated rats and rainbow trout. Comp. Biochem. Physiol. 75C: 25-32.

Williams, D. E. and Buhler, D. E. (1983c). A purified form of cytochrome P-450 from rainbow trout liver with high activity towards conversion of aflatoxin B_1 to aflatoxin B_1-2,3-epoxide. Cancer Res., in press.

Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M. and Conney, A. H. (1976). Metabolism of benzo[a]pyrene and benzo[a]pyrene derivatives to mutagenic products by highly purified hepatic microsomal enzymes. J. Biol. Chem. 251: 4882-4890.

Yarbrough, J. D. and Chambers, J. E. (1978). Crude oil effects on microsomal mixed-function oxidase system components in the striped mullet (Mugil cephalus). Life Sci. 21: 1095-1100.

Yasukochi, Y. and Masters, B. S. S. (1976). Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. 251: 5337-5344.

Yasukochi, Y., Okita, R. T. and Masters, B. S. S. (1979). Reaction cross-specificities between NADPH-cytochrome c (P-450) reductases and cytochromes P-450 of pig liver and kidney microsomes. In "Flavins and Flavoproteins" (Yagi, K. and Yamano, T., eds.) pp 711-717, University Park Press, Baltimore.