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Title: PURIFICATION, CHARACTERIZATION AND COMPARATIVE PROPERTIES OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE ENZYMES FROM RAT AND RAINBOW TROUT

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Utilizing new techniques for solubilization and initial purification, cytochromes P-450 and NADPH-cytochrome P-450 reductase were purified from liver microsomes of β-naphthoflavone-treated rainbow trout. The properties of the trout enzymes were compared to rat cytochromes P-450 and P-448 and NADPH-cytochrome P-450 reductase using a number of criteria for distinguishing multiple isozyme forms.

A minimum of four to five cytochrome P-450 forms were purified from β-naphthoflavone-treated trout. The major form was similar to cytochrome P-448 from β-naphthoflavone-treated rat with respect to spectral properties, activity and regioselectivity towards benzo(a)-pyrene, and sensitivity to *in vitro* inhibitors. However, rat and trout cytochrome P-448 differed in substrate specificity, molecular weight and did not share identical antigenic determinants. Rat and trout cytochrome P-448 were quite different from P-450 from phenobarbital-treated rats using all of the above criteria for distinguishing multiple forms.

A single form of NADPH-cytochrome P-450 reductase was also

purified from β-naphthoflavone-treated rainbow trout. Comparison with NADPH-cytochrome P-450 reductase purified from phenobarbital-treated rat, showed that the two enzymes differed markedly in spectral properties, molecular weight, amino acid and flavin composition and peptide profiles following limited proteolysis. Cytochrome c reduction by the trout enzyme was inhibited by antibody to rat NADPH-cytochrome P-450 reductase, but not to the same extent as was the rat enzyme. No precipitin lines between trout NADPH-cytochrome P-450 reductase and rat antibody were observed on Ouchterlony plates.

Comparison of ethoxyresorufin-O-deethylase temperature profiles with various combinations of trout and rat cytochrome P-448, NADPH-cytochrome P-450 reductase and lipid, in membranous and non-membranous reconstitution systems, demonstrated that the lower temperature optimum of trout microsomes could only be reproduced when all three trout components were incorporated into liposomes. These results suggest that it is the structural organization of the mixed-function oxidase enzymes and lipid within trout microsomes which are responsible for the lower temperature optimum compared to mammalian mixed-function oxidase reactions.

PURIFICATION, CHARACTERIZATION AND COMPARATIVE PROPERTIES OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE ENZYMES FROM RAT AND RAINBOW TROUT

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Typed by Dolores Williams for David Edward Williams

DEDICATION

This thesis is dedicated to my two sons, Anthony Michael and David Ryan Williams. They've given me more joy in my life than completing a thousand degrees. They, and my wife, will always come first.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PURIFICATION, CHARACTERIZATION AND COMPARATIVE PROPERTIES OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE ENZYMES FROM RAT AND RAINBOW TROUT

INTRODUCTION

The microsomal cytochrome P-450 dependent mixed-function oxidase (mfo) system has been found in mammals, birds, amphibians, marine invertebrates, fish, insects and even plants (1-7). Substrates of the mfo system include endogenous compounds such as fatty acids (8), steroids (9), prostaglandins (10), and cholesterol (11) as well as a vast number of chemically diverse exogenous chemicals, or xenobiotics (12). The types of reactions are also quite diverse and include aliphatic or aryl hydroxylation, N-hydroxylation, O-dealkylation, N-dealkylation, S-oxidation, arene oxide formation, dechlorination and deamination (13). Action of the mfo system usually results in a metabolite more polar and excretably than the parent compound. This is especially true if the metabolite (e.g., epoxide or hydroxyl group) can be further acted upon by Phase II conjugation enzymes such as UDP-glucuronyl transferase (14), epoxide hydrolase (15) or glutathione transferase (16).

In some cases action by the mfo system produces a more toxic compound. Examples of such activated metabolites included those formed from benzo(a)pyrene (17), aflatoxin (18), carbon tetrachloride (19), safrole (20) and 2-acetylaminofluorene (21).

MFO activity is highest in liver (vertebrates) but also can be demonstrated in extrahepatic tissues such as kidney, intestine, skin, lung (mammals) and gill (fish) (22). The tissue distribution of the mfo system at "portals of entry" into the organism is consistent with

its role in the detoxification of xenobiotics.

Even before cytochrome P-450 had ever been purified, recognition of the great number and chemical diversity of substrates and the manner in which enzymatic activity could be selectively regulated by prior administration of different types of inducers, led to speculation that cytochrome P-450 must exist in multiple forms (1).

Subsequently, an intense effort over the last 10-15 years has resulted in the isolation, purification and characterization of P-450s from a number of mammalian sources (23-26). Most work has been done with either rat or rabbit liver following pretreatment with either phenobarbital (PB) or 3-methylcholanthrene (3-MC). These two classic inducers had been known for years to drastically alter microsomal mfo activity in quite different manners. PB significantly elevates P-450, NADPH-P-450 reductase, cytochrome b_5 and epoxide hydrolase and causes hypertrophy due to extensive proliferation of the endoplasmic reticulum (27). Other compounds which appear to be "PB-type" inducers include non-coplanar polyhalogenated biphenyls (28), DDT, kepone and mirex (29). The molecular mechanism(s) by which PB produces its inductive effect is unknown, although there are some theories (30-32). Treatment with PB does result in a large increase in mRNA which, when transcribed, produces a product identical to the main P-450 form <u>in vivo</u> following PB induction (33).

Much more is known about the mechanism by which 3-MC type inducers (TCDD, coplanar polyhalogenated biphenyls, β-naphthoflavone and polycyclic aromatic hydrocarbons) exert their effects. Induction by these compounds differs from PB type induction in that P-450 levels are not increased as much, and cytochrome P-450 reductase,

cytochrome b_5, UDP-glucuronyl transferase and glutathione transferase are not increased at all. Furthermore, 3-MC induction in mammals is characterized by a hypochromic shift in the CO-reduced difference spectra to 447-448 nm. Hence, this induced enzyme has been termed cytochrome P-448 (25). This research was advanced by the finding that there were genetically responsive and non-responsive strains of inbred mice (34). Genetic studies pioneered by Dan Nebert established that aryl hydrocarbon (Ah) responsiveness is inherited as an autosomal dominant trait termed the Ah locus (35,36). This locus is proposed to consist of a minimum of six alleles and two loci with structural, regulatory and perhaps temporal genes. Structural genes code for P-450(s) while the main regulatory gene product is a cytosolic receptor protein. This receptor protein is thought to function in a manner similar to steroid hormone receptors (37). In this model, the steroid (or inducer) enters the cell by passive diffusion and is bound by the receptor. This binding is characterized by a high affinity, low capacity and is highly specific. Following binding, the steroid (inducer)-receptor complex is translocated into the nucleous, binds to specific sites in the genome and enhances the transcription of select genes.

The existence of such a receptor for Ah inducers was discovered by Alan Poland (38). Relative binding affinities of a series of TCDD-cogeners correlated nicely with their effectiveness as Ah inducers (39). PB and other "non-3-MC like" inducers did not compete with TCDD or 3-MC for receptor binding sites.

Early attempts at isolating and purifying the components of the microsomal mfo system showed that two enzymes and a lipid fraction

were needed to successfully reconstitute an enzymatically active system (40). The two enzymes were cytochrome P-450 and NADPH-cytochrome P-450 reductase.

Purification of Cytochrome P-450 and the Existence of Multiple Forms

Cytochrome P-450 from rat liver has been purified from a number of laboratories (41-45). The characteristics of the various forms purified from untreated rats or following treatment with various inducers confirm the existence of multiple forms. Guengerich (25) postulated the probable existence of 6-9 forms in the rat.

Purification of P-450 from rabbit (46-48) has yielded similar results. There appear to be a total of 6-8 forms in the rabbit liver.

Most of the work to date has been done with PB or 3-MC induced animals. Purification of constituitive hepatic or any non-hepatic P-450 has proved to be more difficult, however, some recent successes have been achieved (49-52).

The methods used to date for P-450 purification are varied. Most laboratories utilize either sodium cholate or a non-ionic detergent for solubilizing the microsomal membrane. The solubilized P-450 is subsequently purified by a series of chromatography steps. The most common procedures involve the use of hydrophobic (n-aminooctyl agarose), anion exchange (DEAE), cation exchange (CM-cellulose) and absorption chromatography (hydroxylapatite). Aminooctyl agarose is quite effective at absorbing P-450 from solutions containing ionic detergents. Following washing, the P-450 can be eluted with non-ionic detergent, such as, Lubrol, Emulgen or Triton N101 (52,53).

Aminooctyl agarose was originally proposed as an affinity column matrix as octylamine binds to P-450, however, since the gel retains NADPH-P-450 reductase, epoxide hydrolase and cytochrome b_5 under conditions in which P-450 is eluted, the column is probably functioning as a hydrophobic column (53).

More recently, Anthony Lu's laboratory has developed a technique which appears to resemble, more closely, affinity chromatography. The ligand employed is tryptamine, linked to Sepharose through a six carbon spacer (54). This column is capable of purifying microsomal P-450 from PB treated rats to a high specific content with high yield, in a single step.

The ion exchange and hydroxylapatite columns are used to separate the multiple P-450 forms. The P-450s are absorbed onto the columns at low ionic strength and the column eluted with stepwise or linear gradients of NaCl or phosphate (25).

As increasing numbers of P-450 isozymes were purified, it became increasingly necessary to develop methods for establishing homogeneity and distinguishing multiple P-450 forms.

The degree of purity has mainly been determined by the specific content (nmoles P-450 per mg protein) which, for a form with molecular weight of 50,000, should be close to 20 and electrophoretic homogeneity on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). More recently, mono-specific antibodies have been used to confirm the homogeneity of purified P-450s.

There are a number of techniques for distinguishing properties of multiple P-450 forms. These properties include substrate specificity and regioselectivity, molecular weight, amino acid composition,

partial amino acid sequencing, peptide patterns on SDS-PAGE after limited proteolysis, sensitivity to *in vitro* inhibitors, ethylisocyanide difference spectra and immunochemical properties (24,25). The use of monoclonal antibodies may prove to be the best test for determining P-450 homogeneity and distinguishing multiple forms (55). Antibodies prepared in the conventional manner have shown varying degrees of cross-reactivity (56).

Purification and Properties of NADPH-Cytochrome P-450 Reductase

NADPH-P-450 reductase is a flavoprotein which serves to transfer electrons from NADPH to P-450. Unlike P-450, the reductase exists as a single enzyme (57). The molecular weight of detergent extracted reductase is 72-80,000, while that of protease or lipase extracted reductase is 6,500-12,500 daltons lower (58-60), suggesting that the reductase is anchored in the microsomal membrane by a hydrophobic tail.

Purification of NADPH-P-450 reductase has been made relatively simple by the use of an affinity column, 2',5'-ADP agarose (61). Following washings, the reductase is eluted with either 2'-AMP or NADP. It appears that some preliminary purification step is necessary before the reductase will bind effectively to the column (62).

The degree of enzyme purity is determined by the specific activity using cytochrome c as the electron acceptor (53). One unit (U) is defined as 1 μmole of cytochrome c reducted per minute. Purified NADPH-P-450 reductase has a specific activity of 40-60 U per mg protein when assayed by the method of Imai (53).

The properties of purified mammalian reductase have been

recently reviewed by Masters (57). Highly purified reductase from mammals contains one mole of both FAD and FMN per mole of enzyme. As is the case with P-450, purification of NADPH-P-450 reductase from mammalian non-hepatic tissues or from non-mammalian species has proved difficult, although some success has been achieved (51,63-65). The problem is that the enzyme is quite susceptible to proteolysis especially when being purified from tissues from which one would expect a high content of protease, such as the hepatopancreas of the spiny lobster (66). Therefore, it is recommended that a protease inhibitor, such as phenylmethylsulfonylfluoride (PMSF) be present throughout purification.

Reaction Sequence of the MFO System

A schematic representation of the multi-step reaction sequence is shown in Figure 1. In step one the substrate is bound to the ferric form of P-450. The binding of the substrate is accompanied by a change in the heme configuration from a low to a high spin state and the occurrence of a Type I pattern in the optical absorbance spectrum. In the second step, one electron is transferred from NADPH to P-450 through its reductase, to reduce the heme to the ferrous state. This form may combine with either O_2 or CO in the sixth ligand position.

In the presence of O_2, the oxycytochrome P-450-substrate complex is rapidly formed (step three), and the bound oxygen becomes "activated". Two forms with equivalent electronic valence states are thought to exist, the ferrous P-450 combined with substrate and molecular oxygen and the ferric substrate form in which the oxygen is

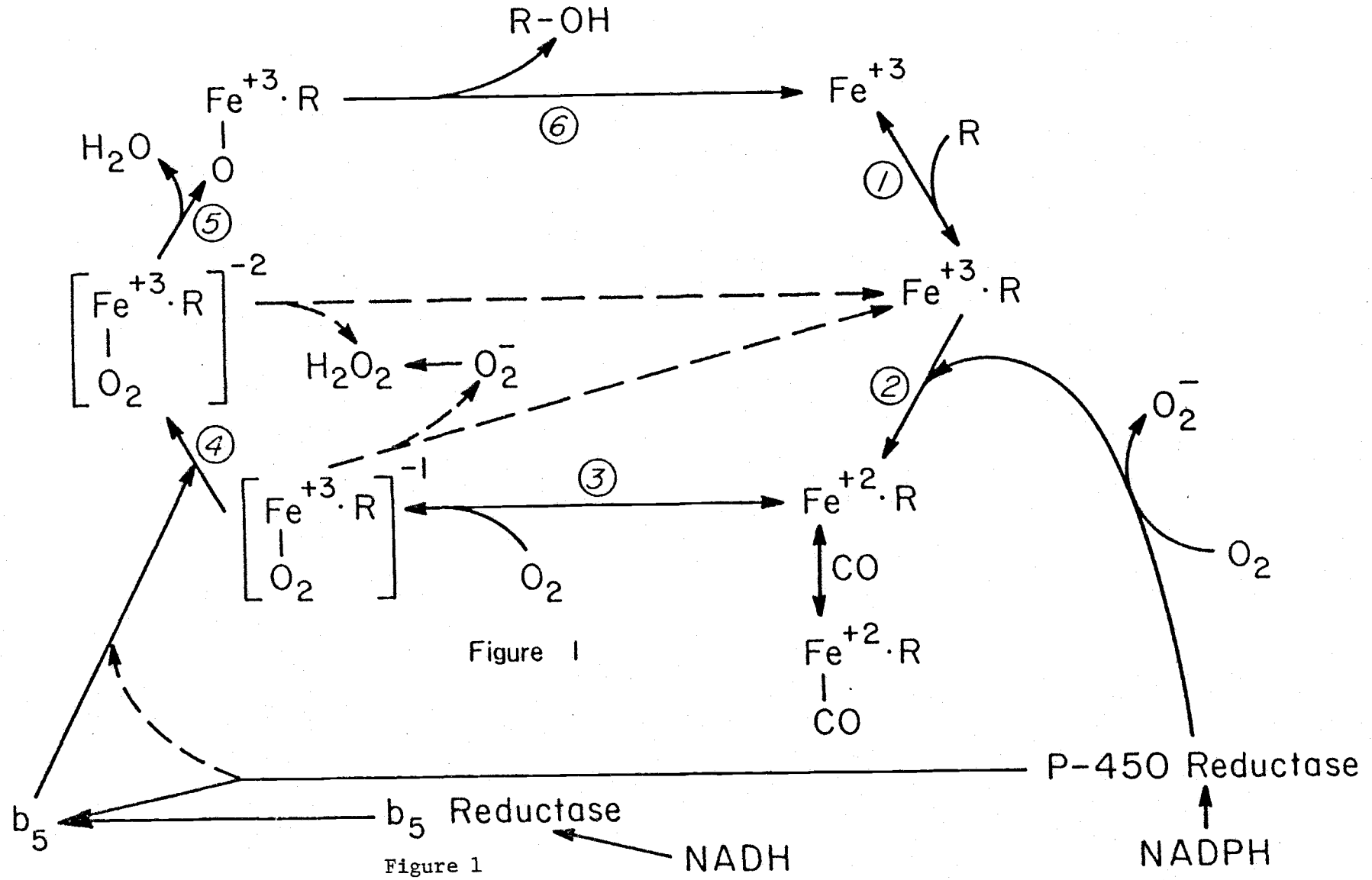


Figure 1

Figure 1

activated to a superoxide anion. The latter form is capable of decaying to regenerate the ferric substrate form with the release of superoxide anion (or ultimately hydrogen peroxide). Peroxide forming "uncoupling" of the mfo system could have destructive effects on the heme moiety of P-450 and also could initiate lipid peroxidation.

The oxycytochrome P-450 substrate complex is further reduced (step four) to peroxycytochrome P-450 in which the ferric heme has two electrons and can exist in a number of forms. If protonated, the peroxide anion could dissociate with the formation of H_2O_2 and the ferric substrate complex again producing an uncoupling effect. The source of the electron in the second reduction is still controversial; evidence for the participation of both NADPH-cytochrome b_5 reductase and NADPH-cytochrome P-450 reductase has been presented.

In the fifth step, peroxycytochrome P-450 is thought to lose a molecule of H_2O, generating the highly reactive oxene P-450 which reacts with substrate producing the oxidized product (step six) and releasing the free ferric P-450 which can now combine with new substrate and repeat the cycle.

Early studies on the kinetics of the mfo reaction and the interaction between P-450, reductase, substrate and lipid was done with microsomal membranes (67,68). Such studies are made difficult by the heterogeneity of such a system.

More recently, acquisition of the purified enzymes has provided the opportunity for study utilizing a more well defined system. For example, one can vary the amounts of P-450, reductase, lipid or substrate and calculate K_ms or effects on reaction rate. Such studies have shown that: 1) P-450 and reductase form a 1:1 binary complex

(69); 2) binding of substrate lowers the K_m of P-450 for its substrate (70) and binding of substrate or lipid to P-450 seems to stabilize the protein and convert a higher percentage of the heme to the high spin state (71). Furthermore, other enzymes or effects can be added to determine their effect on mfo function. For instance, addition of cytochrome b_5 to reconstituted systems has recently been shown to markedly increase the turnover of some substrates (72-74). Also, the effect of varying ratios of epoxide hydrolase to P-448 on the mutagenicity of benzo(a)pyrene has been examined (75).

Although such studies have proved interesting, their validity as models for the functioning microsomal mfo system has been questioned. The reconstituted studies cited above are non-vesicular as the lipid added is at a concentration below the critical micelle concentration (CMC). Such results with soluble enzymes may not accurately reflect their organization and function in a membranous environment. In order to correct this, some recent studies have used artificial membranes. The purified P-450, NADPH-P-450 reductase and certain lipids are incorporated into vesicles or liposomes (76-78). There is some evidence suggesting that the properties of these differ from non-vesicle reconstitution systems. For instance, cytochrome P-450 incorporated into liposomes was found to be less thermolabile than the soluble P-450 (79,80).

MFO Activity and Regulation in Fish

Twenty years ago, initial studies suggested that fish did not possess mfo activity (81). Subsequent work has not only established the existence of these enzyme systems in fish (3) but also

demonstrated some rather unique properties. For instance whereas mammals respond to PB treatment with a large proliferation of hepatic smooth endoplasmic reticulum accompanied by increases in selected P-450 forms and NADPH-P-450 reductase, fish appear refractory to such treatment (3,82-84). This lack of response extends to other PB type inducers such as DDT and non-coplanar polyhalogenated biphenyl isomers (85,86). In contrast, fish are very responsive to induction by polycyclic aromatic hydrocarbons (PAHs), 3-MC or β-naphthoflavone (BNF) (87,88). The P-450 of both untreated (89) and PAH or BNF treated trout (87,88) have many characteristics in common with mammalian P-448, including spectral properties, substrate specificity and sensitivity to inhibitors in vitro. However, the hypochromic shift in the microsomal CO difference spectra has consistently been reported to be absent in these fish; such observations have lead to its classification as a P_1-450 (90). A number of recent reports, however, using fish hepatic microsomes (91,82), or utilizing partially purified P-450 (93), have suggested the existence of a P-448 formed in PAH treated fish.

Regardless of how this P-450(s) should be classified, it has been demonstrated, using benzo(a)pyrene (BP) metabolite profiles and covalent DNA binding (94,95), that trout P-450 is very similar to rat 3-MC or BNF induced P-448 in the transformation and activation of BP to mutagenic and carcinogenic metabolites.

The mfo activity of certain fish populations towards BP, has been found to vary quite widely (5). This activity has been correlated to environmental levels of PAH pollutants (96) and it has further been suggested that BP hydroxylase assays of fish could be

used as monitors of PAH pollution (97,98). There is also some evidence to suggest that genetics may also play a factor in background mfo activity of certain strains or species of fish (99).

The importance of endogenous BP hydroxylase activity in fish, and the degree to which it can be induced by commonly encountered environmental levels of PAH can be seen in the example of English sole and Starry flounder from Puget Sound (100-102).

Another interesting difference between mammalian and fish microsomal mfo activity is the temperature optima. The optimum temperature for mammals is 35-45°C, while that for fish is much lower (20-30°C). This lower optima is consistent with the poikiothermic nature of fish. Certain warm water fish have been found to have higher temperature optima than cold water fish such as trout (103,104). The lower temperature optima seen in trout could be due to either: 1) a lower thermal stability of P-450 or NADPH-P-450 reductase; 2) a factor present in fish microsomes which inactivates either enzyme at elevated temperature; 3) different fluidity properties of fish and mammalian microsomal membranes which decrease fish mfo activity at higher temperatures; or 4) differences in the structural arrangement of P-450, reductase and lipid within the trout or mammalian microsomal membrane.

Definitive studies regarding the first possibility require the isolation and purification of fish P-450 and P-450-reductase. Recently, John Stegeman's group at Woods Hole have purified P-450 from the marine fish, Scup (92). To date, no studies have been done comparing its thermolability with mammalian P-450. In a recent report, Serabjit-Singh et al. (64,105), have suggested that hepatic

NADPH-cytochrome c reductase purified from the marine elasmobranch, little skate, is more thermolabile than a purified mammalian (rabbit) reductase. These studies were done using a non-vesicle reconstitution system and rabbit P-450. Conclusions from this study on the thermolability properties of little skate reductase should not be regarded as definitive due to the possibility that such a non-vesicle system may not serve as an adequate model for microsomes. The second possibility, that is a factor being present in fish microsomes which inactivates P-450 or P-450-reductase at higher temperatures, could be related to the relatively high content of polyunsaturated lipids in fish microsomal membranes (106-108). These lipids would be capable of propagating lipid peroxidation, with subsequent destruction of the heme moiety of P-450 (109). The third possibility concerns the differences in fluidity between fish and mammalian microsomal membranes at different temperatures. Fish have a higher proportion of polyunsaturated lipid, lower levels of cholesterol and a higher phosphatidyl choline to phosphatidyl ethanolamine ratio than do mammalian microsomes membranes (106). All of these factors contribute to an increase in fluidity at any given temperature (110,111). Many studies which have examined the effect of membrane fluidity on an enzyme reaction rate have seen marked changes in reaction rate with increases in fluidity (111). Arrhenius plots, which are plots of the log of the reaction rate vs. the inverse of temperature, exhibit changes in slope, referred to as break points, which are thought to represent liquid-gel transitions of the membrane lipids. Such studies have been utilized to investigate the fourth possibility mentioned, that is, the structural organization of P-450, NADPH-P-450

reductase and lipid within the microsomal membrane. Results to date have been equivocal. Some investigators observe microsomal or liposomal Arrhenius break points for the mfo system (112-116) while others do not (117,118). Not surprisingly, there is also no agreement on the molecular model of the microsomal mfo system. Some feel it is a fairly random distribution (119,120) while others have proposed a semi-rigid or rigid clustering of P-450 molecules around a single reductase (121-124). Increases in membrane fluidity with increasing temperature might tend to increase mfo activity for random distributed P-450 and reductase, while a cluster of enzymes may begin to diffuse apart.

Studies with purified non-mammalian P-450 and NADPH-P-450 reductase are still in their infancy. There is much useful information that can be obtained with respect to evolutionary and comparative properties. Many of the factors which influence mammalian mfo activity in mammals (diet, sex, age, etc.) may be important in the functioning of the mfo system throughout the life of the fish. Some interesting studies (125-130) have already appeared. The answers to others, such as how does mfo activity relate to other factors, such as cortisol or thyroxine levels (both mfo substrates) which are thought to regulate parr-smolt transformation and migration to the sea in salmon (131,132)? In the same vein, further studies with respect to the effect of PAHs and related mfo inducing pollutants on the general health of fish populations is needed. The importance of such studies from an environmental, economical and human health aspect are great. A more detailed understanding of the fish mfo system would also seem necessary, as some fish are being proposed as animal

models for the action of mfo-activated carcinogens (133). Finally, the rather unique properties of the response of fish to inducers may prove to be useful for elucidation of the molecular mechanism(s) involved in induction, especially by PB. Comparative work with mammalian enzymes and lipid in reconstituted vesicles may also provide clues to how mfo components are arranged and interact in the microsomal membrane.

For the reasons above (and others) the work presented here was initiated to obtain the first fish P-450 and NADPH-P-450 reductase purified to homogeneity. We chose rainbow trout because of their availability and the fact that they are, by far, the most commonly used experimental fish. For comparative purposes, it was also necessary to obtain purified P-450 and reductase from a mammalian species. We chose male Sprague-Dawley rats for the same reason cited above.

The following thesis, presents a technique developed in our laboratory for obtaining purified P-450 and NADPH-P-450 reductase from BNF-treated rainbow trout. These purified enzymes are then compared to their mammalian counterparts by some currently used techniques for distinguishing multiple isozyme forms. Finally, various combinations of rat and trout P-450, NADPH-P-450 reductase and microsomal phospholipid were examined in vesicle and non-vesicle reconstitution systems and compared to microsomes from which they were purified to obtain information on the best model for the trout microsomal mfo system.

MATERIALS AND METHODS

Unlabeled BP, BNF, 3-dimethylaminopropylamine, 1,3-propane sultone and ethylchloroformate were purchased from Aldrich Chemical Co. Pfaltz and Bauer, Inc. was the source of α-naphthoflavone (ANF) and SKF-525A was from Smith Kline and French Laboratories. Radioactively labeled [^{14}C]-BP and [^{14}C]-testosterone were from Amersham. Resorufin and ethoxyresorufin were from Spectrum Chemical Mfg. Co. and Pierce Chemical Co., respectively. Hydroxylapatite was obtained from Clarkson Chemical Co. Hydroxylated testosterone standards were obtained from Steraloids, Inc. Ultrafiltration equipment and Diaflo (PM 30 or PM 10) membranes were from Amicon. All equipment and chemicals used for SDS-PAGE were from Bio-Rad. Antibodies directed against rat P-450, P-448 and NADPH-cytochrome P-450 reductase were generously supplied by Dr. F. Peter Guengerich of Vanderbilt University, Nashville, Tennessee. The BP metabolites (three dihydrodiols, two quinones and six phenols) were kindly provided by Dr. Donald M. Jerina of NIH, Bethesda, MD. The substrate benzphetamine, was a gift from Dr. Paul O'Connell of Upjohn Co., Kalamazoo, MI. All other chemicals and column materials were obtained through Sigma Chemical Co.

Preparation of Detergents and Tryptamine-Sepharose 4B

3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) was synthesized as described by Hjelmeland (134) and co-chromatographed, on TLC, with authentic CHAPS from Cal Biochem. Lubrol PX was purified by the procedure of Chang and Bock (135) to

eliminate oxidizing contaminants. Tryptamine was coupled to 6-aminohexanoic acid-Sepharose 4B using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and the degree of substitution (5 μmol/ml gel) estimated from the extinction coefficient of tryptamine (log ε = 3.78) at 282 nm.

Treatment of Fish and Preparation of Microsomes

Fifty rainbow trout (Salmo gairdneri), Mt. Shasta strain), average weight 250 g, were injected intraperitoneally with BNF (100 mg/kg) suspended in corn oil (100 mg/ml). The trout, starved beginning 24 hr prior to injection, were maintained in flowing well water at 12°C for 72 hr after injection, and then killed by a blow to the head. The livers were weighed, placed in ice-cold buffer A (0.1 M Tris-acetate pH 7.4, 0.1 M KCl, 1 mM EDTA, 20 μM butylated hydroxytoluene (BHT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF)) and minced, rinsed and homogenized in four volumes of buffer A. The microsomal pellet was obtained by centrifugation at 10,000 x g for 30 min followed by 100,000 x g for 90 min. All procedures involved in the preparation and solubilization of microsomes and the purification of cytochrome P-450 and NADPH-P-450 reductase were performed on ice or in a 4°C room and using buffers containing BHT and PMSF. The microsomes were resuspended to a protein concentration of about 20 mg/ml in buffer B (0.1 M potassium phosphate, pH 7.25, 20% glycerol and 1 mM EDTA), flushed with nitrogen and stored at -70°C for a period of about two weeks.

Solubilization and Purification of Trout P-450s and NADPH-P-450 Reductase

After thawing, the microsomes were diluted 2-fold with buffer B and CHAPS added dropwise to a final concentration of 1%. The solution was stirred for 30 min and then centrifuged at 100,000 x g for 90 min. The supernatant was diluted 10-fold and loaded onto a tryptamine-Sepharose 4B column (2 x 20 cm) which was sequentially washed with buffer B containing 0.2% CHAPS and 0.05% Lubrol PX (250 ml), 0.5% CHAPS and 0.2% Lubrol PX (150 ml) followed by 0.5% CHAPS and 1% Lubrol PX (400 ml). Cytochrome P-450 was eluted with buffer B containing 0.5% cholate and 0.2% Lubrol PX. Fractions (20 ml) were collected and A_{417} and A_{280} determined. The P-450-containing fractions were pooled, concentrated and dialyzed first with 20% glycerol, 1 mM EDTA (2 hr), followed by the same solution containing 10 mM potassium phosphate pH 7.7, 0.2% cholate and 0.1% Lubrol PX (buffer C). The sample was applied to a DEAE-Sepharose column (2 x 40 cm), washed with 400 ml of buffer C and eluted with a linear gradient of 0-0.125 M NaCl in buffer C (500 ml). Fractions (10 ml) were analyzed for A_{417}, A_{280} and conductivity (Radiometer, Copenhagen). Fractions from the two main P-450 peaks were separately pooled, concentrated, dialyzed twice with buffer C and loaded onto two hydroxylapatite columns (1.2 x 6 cm), washed with buffer C containing 0.1 mM EDTA (50 ml) and then eluted with a linear gradient of 10-125 mM potassium phosphate in buffer C (200 ml). Fractions (5 ml) were assayed for A_{417}, A_{280} and conductivity. The P-450 containing fractions from each peak were pooled, treated with Amberlite XAD-4, concentrated, dialyzed with

buffer D (10 mM Tris-HCl pH 7.4, 20% glycerol, 1 mM EDTA) for 16 hr and frozen at -70°C in small aliquots.

Trout NADPH-P-450 reductase eluted in two fractions on tryptamine-Sepharose. About half the activity eluted in the void volume and the remainder in a peak following the P-450. Either fraction could then be further purified following concentration and dialysis, by 2',5'-ADP agarose affinity chromatography. The affinity column was washed and the trout reductase eluted, dialyzed and concentrated as described for the mammalian enzyme (136). Small aliquots of the enzyme were stored at -70°C.

Purification of Cytochromes P-448, P-450 and NADPH-P-450 Reductase from Rat

P-448 from 3-MC treated rats and P-450 and NADPH-P-450 reductase from PB-induced rats were purified as described by Guengerich and Martin (136) to specific contents of 11.6 nmol/mg, 14.4 nmol/mg and 32 U/mg, respectively.

Analysis of Rat and Trout NADPH-P-450 Reductase Amino Acid and Flavin Composition and Peptide Patterns Following Proteolysis

Rat and trout NADPH-P-450 reductase were dialyzed against distilled water overnight and then hydrolyzed for 22 hrs at 110°C in 6 M HCl in sealed evacuated tubes. The hydrolysates were analyzed in a Beckman Model 120B amino acid analyzer updated to a 6 cm single column system. Cystine was oxidized in DMSO and determined as cysteic acid and tryptophan content was assayed fluorometrically (137). The FAD and FMN content were analyzed fluorometrically by a slight

modification of the method of Faeder and Siegel (138). The trout reductase was analyzed following dialysis with either no added FAD or FMN or a 150-fold excess of both flavins. Peptide maps of rat and trout reductase on SDS-PAGE following proteolysis, were obtained using the procedure of Cleveland et al. (139) as modified by Guengerich (140).

Reconstitution of MFO Activity Towards Various Substrates

[^{14}C]-BP hydroxylase was reconstituted by preincubating either purified trout P-450, rat P-448 or P-450 (0.1 nmol) with rat NADPH-P-450 reductase (0.1 nmol) and L-α-dilauroylglyceryl-3-phosphorylcholine (DLPC) (20 µg) for 10 min at room temperature in 1 ml of 50 mM Tris-HCl pH 7.5, containing 5 mM KCl, 1 mM $MgCl_2$ and 0.1 mM EDTA. Substrate ([^{14}C]-BP, 2 µCi/µmol) was added in 5 µl acetone to a final concentration of 60-80 µM. Inhibitors ANF, metyrapone or SKF-525A were added just prior to the addition of [^{14}C]-BP. Flasks not containing inhibitors received 5 µl of acetone. Incubation was for 20 min at either 29° or 37°C. The reaction was initiated by the addition of NADPH to 1 mM. Blanks contained all components except NADPH. [^{14}C]-BP hydroxylase was quantitated by a radioactive procedure (141) and the metabolite profile by HPLC (142) with individual metabolites identified by coelution with nonlabeled standards. For the study on the inhibition of [^{14}C]-BP hydroxylase by antibodies, rabbit antibodies directed against rat P-448 (BNF-B_2 IgG) and P-450 (PB-B_2 IgG) were preincubated with trout P-450s or rat P-448 and reconstituted and assayed as described above.

Reconstitution of [^{14}C]-testosterone hydroxylase was performed

as described above for [^{14}C]-BP. The reaction was terminated and substrate and products extracted with methylene chloride:methanol (2:1) and analyzed by HPLC. [^{14}C]-Testosterone metabolites were eluted from a Zorbax silica gel column (4.6 mm x 25 cm) using a non-linear gradient of isopropanol in isooctane:methylene chloride (10:1) at 15°C in a Spectra Physics 8000 liquid chromatograph at a flow rate of 2 ml/min. Fractions (1 ml) were collected and analyzed for radioactivity. Tentative identification of 6β, 16α and 7α[^{14}C]-hydroxy-testosterone was by coelution with unlabeled standards. This procedure is superior to TLC or paper chromatography methods (9) and similar in resolving power to that obtained by a reverse phase HPLC system recently published (143).

Ethoxycoumarin-O-deethylase and ethoxyresorufin-O-deethylase were assayed fluorometrically (144). Trout P-450s, rat P-448 or P-450 (0.1 nmol) were preincubated with rat NADPH-P-450 reductase (0.1 nmol) and DLPC (20 μg) for 5 min at either 22.5°C (trout) or 37°C (rat) and the reaction initiated by the addition of NADPH to 0.1 mM and assayed at the same temperature.

Reconstitution of ethoxyresorufin-O-deethylase, using various combinations of rat and trout P-448, NADPH-P-450 reductase and lipid were performed as described above or with liposomes prepared by a cholate dialysis procedure (145). The effect of temperature on the reaction rate was analyzed with a Perkin-Elmer 650-10S fluoroscence spectrophotometer equipped with a constant temperature regulator. Microsomes or reconstituted enzymes were preincubated for 2 min at the temperature at which they were to be assayed; substrate and buffer were then added to a volume of 1 ml and incubation continued for

another two min before the reaction was initiated with NADPH.

The rate of N-demethylation of benzphetamine was by determination of formaldehyde with Nash Reagent (146). The various P-450s (0.1 nmol) were preincubated with rat NADPH-P-450 reductase (0.1 nmol) and DLPC (20 µg) for 5 min at room temperature. Benzphetamine-HCl was added to a final concentration of 1 mM, the volume taken to 1 ml with 50 mM potassium phosphate pH 7.7, and the reaction initiated by the addition of NADPH to a final concentration of 0.5 mM. The reaction was allowed to proceed at either 29° or 37°C for 10 min.

Procedure for SDS-PAGE

SDS-PAGE was done utilizing slab gels (15 cm long, 1.5 mm thick) by the method of Laemmli (147). On some gels the modifications by Guengerich (41) were used. The separating gels were 7.5-10% acrylamide and were run under constant power (1.5 W/gel during stacking and then 10 W/gel). Gels were fixed and stained by shaking overnight in 45% ethanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250 and then destained with 10% ethanol, 7.5% acetic acid. Molecular weights were determined by comparing the relative mobility of unknowns to the logarithms of standards with known molecular weights.

Other Procedures and Assays

Trout microsomal P-450 was assayed by the procedure of Estabrook *et al.* (148) to avoid hemoglobin interference. Purified trout and mammalian P-450s were also assayed by the method of Omura and Sato (149). Routine assays were done on a Cary 219 but more precise determinations of spectral properties were performed on an Aminco

DW-2a.

NADPH-P-450 reductase was assayed using cytochrome c as substrate as described by Imai (53), except at room temperature.

Protein was determined by the method of Lowry et al. (150) with bovine serum albumin as the standard. Phospholipid was extracted from rat and trout microsomes as described by Bligh and Dyer (151). Double precipitin reactions were analyzed using Ouchterlony plates as described previously by Guengerich et al. (152,153).

RESULTS

Purification of Trout Cytochromes P-450

Solubilization of BNF-induced rainbow trout microsomes with CHAPS resulted in a greater than 90% yield of P-450. In the presence of CHAPS, trout P-450 bound quite tightly to tryptamine-Sepharose 4B (Figure 2) as concentration of Lubrol PX as high as 1% could not elute the protein. Only when sodium cholate was substituted for CHAPS was elution of P-450 achieved. The yield and increase in purity with tryptamine-Sepharose (Table 1) was not as effective as reported previously for PB-induced rat P-450 (54), indicating that the chromatographic condition used here may not have been optimal. Chromatography of partially purified P-450 on DEAE-Sepharose (Figure 3) produced two main fractions (peaks b and c), with specific contents of 4.7 and 5.7. These fractions were further resolved on hydroxylapatite (Figure 4) and a total of four P-450 peaks obtained. Fraction b produced only one P-450 peak on hydroxylapatite and the specific content (Table 1) was still relatively low. Fraction c was resolved into three peaks. The high specific content and a A_{417}/A_{280} ratio near unity suggested that the last two peaks eluted from hydroxylapatite chromatography of peak c were nearly homogenous. The degree of purity was confirmed by SDS-PAGE (Figure 5). Only one band (55,000 daltons) can be seen in the molecular weight range (45,000-60,000 daltons) of P-450, but there appears to be two minor contaminants of higher molecular weight in each sample, possibly dimers of P-450. The latter two hydroxylapatite peaks from fraction c, peaks f and g, could not be resolved using this electrophoresis

Figure 2. Tryptamine-Sepharose 4B Chromatography of Solubilized Cytochromes P-450 from β-Naphthoflavone-Induced Rainbow Trout.

Solubilized P-450 (865 nmol/2240 mg protein) in 0.1% CHAPS (1000 ml) was loaded onto a tryptamine-Sepharose 4B column (2 x 20 cm) and then washed (CHAPS and Lubrol PX) and eluted (cholate and Lubrol PX). The collection of fractions was not begun until after all the sample had been applied. Flow rate was about 30 ml/hr.

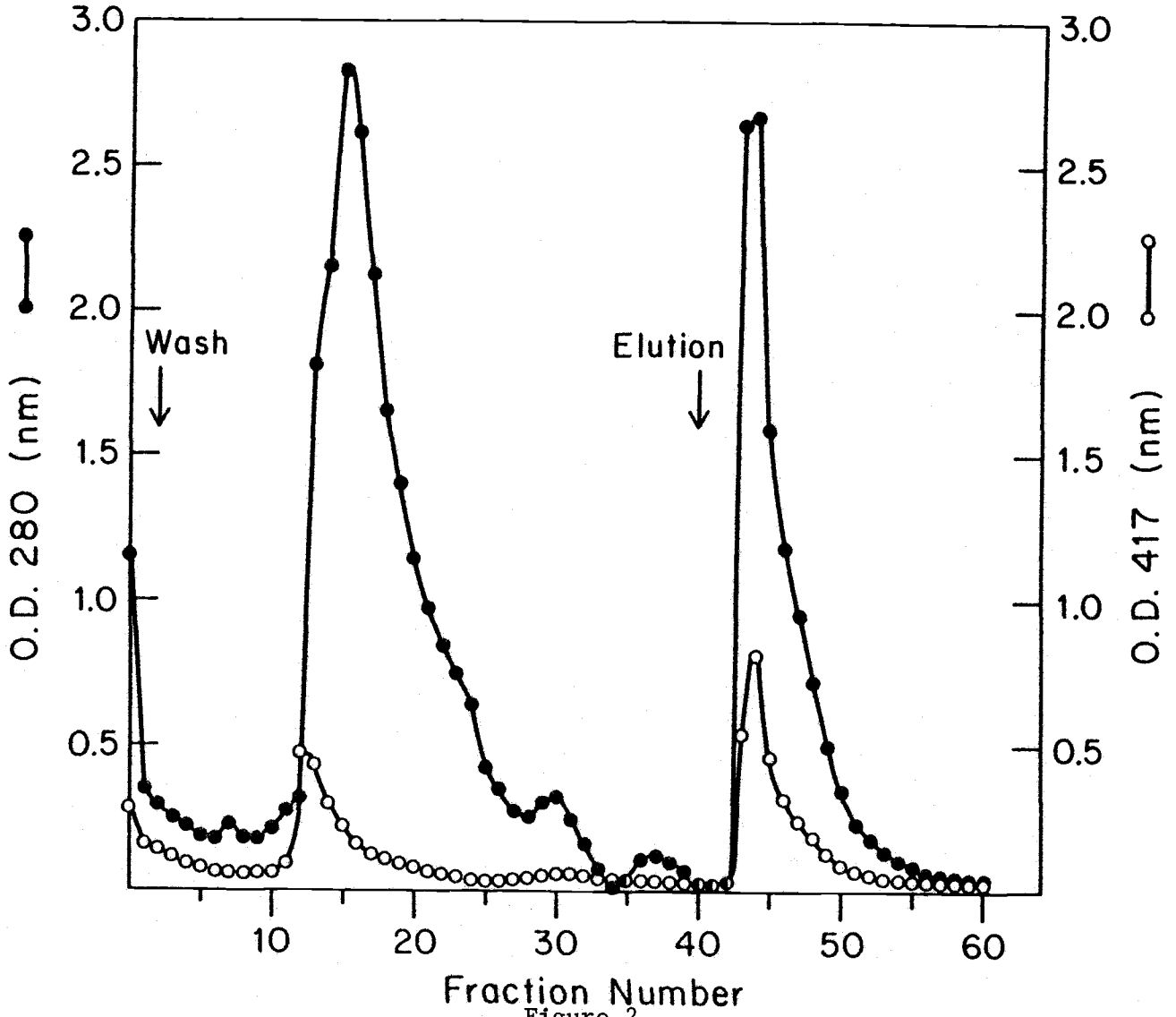

3.0
2.5
2.0
1.5
1.0
0.5
O.D. 280 (nm)
O.D. 417 (nm)
Wash
Elution
10
20
30
40
50
60
Fraction Number
Figure 2

TABLE 1

Specific Content and Yield of BNF Induced Trout Fractions During Purification

Fraction	P-450 (nmoles)	Protein (mg)	Specific content	Yield (%)	Purification (fold)
Microsomes	969	2758	0.35	100	1.0
Solubilized	880	2280	0.39	91	1.1
Tryptamine-Sepharose Eluent	386	214	1.80	40	5.2
DEAE-Sepharose Eluent					
fraction b	68	14.5	4.69	7	13.4
fraction c	202	35.5	5.68	21	16.2
Hydroxylapatite Eluent of					
b--fraction d	25	4.7	5.32	3	15.2
Hydroxylapatite Eluent of					
c--fraction e	15	6.9	2.11	2	6.0
fraction f	73	6.6	11.0	8	31.5
fraction g	15	1.1	13.7	2	39.2

Figure 3. DEAE-Sepharose Chromatography of Trout P-450 Partially Purified by Tryptamine-Sepharose 4B.

Approximately 370 nmol of partially purified P-450 (206 mg protein, 30 ml) was loaded onto a DEAE-Sepharose (2 x 40 cm) anion exchange column. The sample was washed with 400 ml equilibration buffer and eluted with a linear NaCl gradient of 0-125 mM (500 ml). Flow rate was about 20 ml/hr.

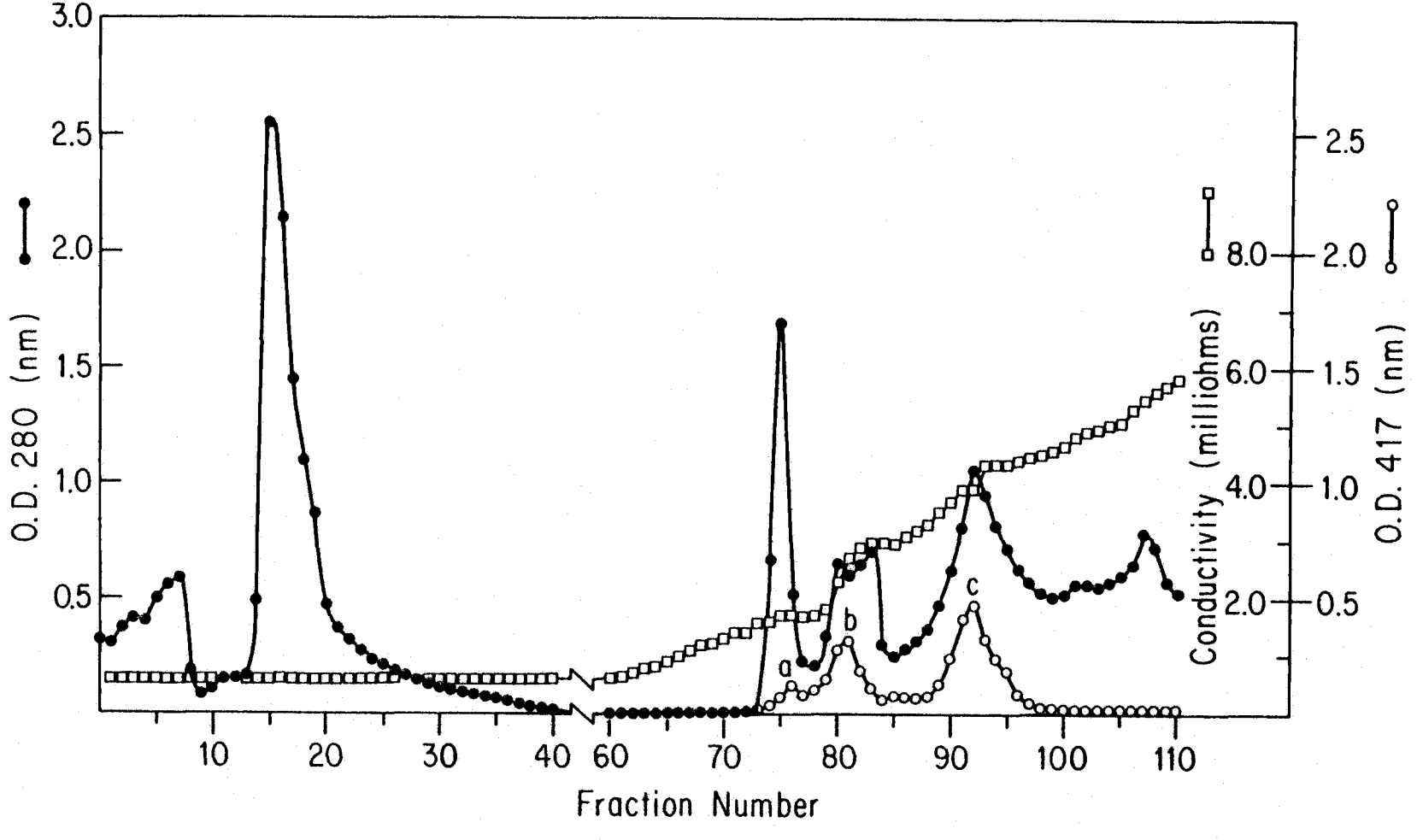

3.0
2.5
2.0
1.5
1.0
0.5
O.D. 280 (nm)
2.5
2.0
1.5
1.0
0.5
O.D. 417 (nm)
8.0
6.0
4.0
2.0
Conductivity (milliohms)
a
b
c
10
20
30
40
60
70
80
90
100
110
Fraction Number

Figure 3

Figure 4. Hydroxylapatite Chromatography of DEAE-Sepharose Peaks b and c.

Top panel: Approximately 50 nmol of P-450 from DEAE b (10.6 mg protein) was loaded onto a hydroxylapatite column (1.2 x 6 cm) previously equilibrated with 10 mM potassium phosphate, 20% glycerol, 0.1 mM EDTA. The column was washed with 50 ml equilibration buffer and eluted with a linear gradient of 10-250 mM phosphate (200 ml). Bottom panel: Approximately 150 nmol P-450 from DEAE c (26.3 mg protein) was loaded onto a hydroxylapatite column (1.2 x 10 cm), washed and eluted as described above.

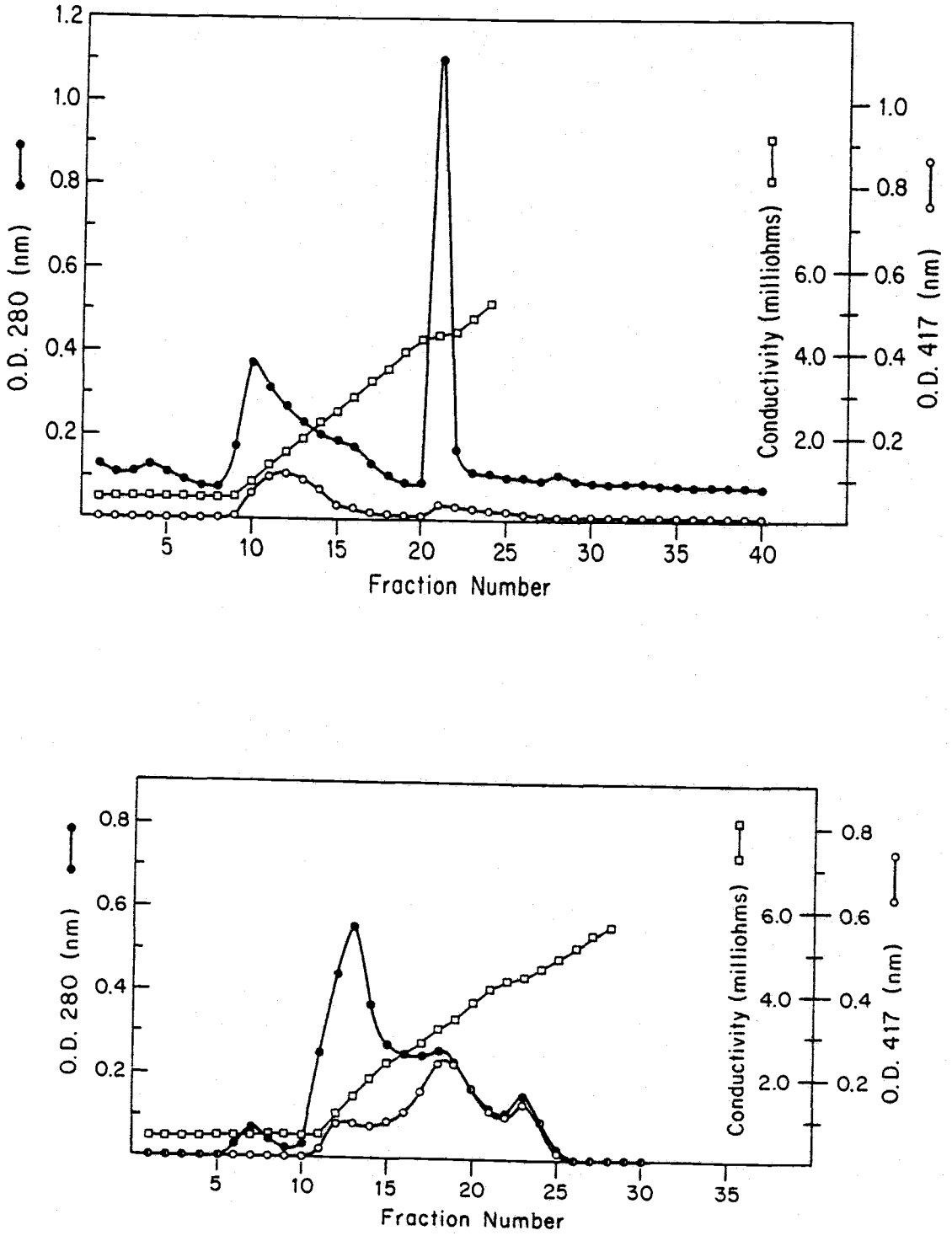

O.D. 280 (nm)
Conductivity (milliohms)
O.D. 417 (nm)
Fraction Number
O.D. 280 (nm)
Conductivity (milliohms)
O.D. 417 (nm)
Fraction Number

Figure 4

Figure 5. SDS-PAGE of Trout P-450 Fractions at Each Stage During Purification Procedure.

Samples were prepared by boiling in 1% SDS for 4 minutes. The samples are from right to left: microsomes, 50 µg; tryptamine-Sepharose 4B fraction, 3.5 µg; DEAE peak b, 4.5 µg; DEAE peak c, 10 µg; hydroxylapatite fraction d, 3 µg; hydroxylapatite fraction e, 8 µg; hydroxylapatite fraction f, 4 µg; hydroxylapatite fraction g, 2 µg and hydroxylapatite fraction f (1.5 µg) and g (1.0 µg).

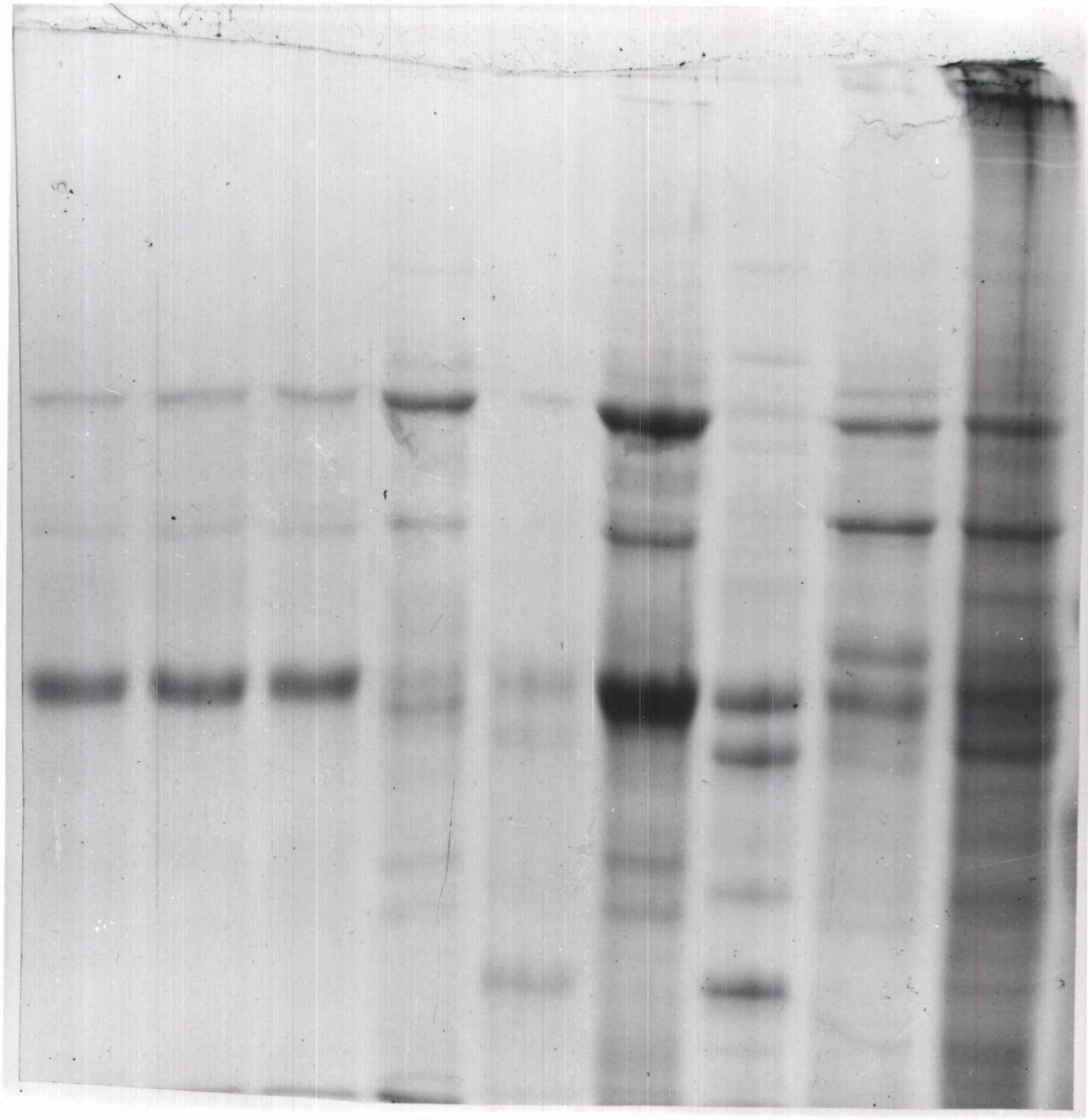


Figure 5

system.

Comparative Properties of Purified Trout P-450s and Rat P-448 and P-450

Spectral Properties. Absorption spectra (oxidized, reduced and CO-reduced) were determined for all four purified trout P-450 forms and compared to the purified rat PB and 3-MC induced forms. The Soret band of the absolute oxidized spectra has a λ_{max} of 415.5-418.2 nm for all trout and rat P-450s, suggesting that all forms isolated were in the low spin state (Table 2). Other wavelength peaks are consistent with values previously reported for purified P-450 and P-448 from rats (42). The Soret λ_{max} of the CO-reduced difference spectra for fractions f and g from BNF-induced trout (447.9 and 447.7 nm) were much closer to P-448 from 3-MC rat than PB rat P-450; thus, BNF-treated rainbow trout possess at least two P-448 type P-450s. The entire oxidized, reduced and CO-difference spectra of the main trout P-448 form (f) and rat P-448 are shown in Figure 6. As can be seen, the entire spectra for both forms are quite similar.

Substrate Specificity. The substrate specificities of the various trout P-450s and rat P-448 and P-450 were examined with the substrates [^{14}C]-BP, ethoxycoumarin, ethoxyresorufin, benzphetamine and [^{14}C]-testosterone (Table 3). From studies with mammals, it appears that BP and ethoxyresorufin are metabolized by P-448 (154). Ethoxycoumarin has been reported to be metabolized by both PB induced P-450 and by P-448 (42), whereas benzphetamine is metabolized at a high rate only by PB-induced-P-450. Testosterone is preferentially hydroxylated at the 6β, 7α and 16α positions by P-448, a constituitive

TABLE 2

Comparative Spectral Properties of Purified Trout P-450 Fractions and Rat P-450 and P-448

	λ_{max} (nm)		
Cytochrome	Oxidized	Reduced	CO-Reduced
Rat P-448	417.5, 533.0, 569.5	414.2, 537.5	447.3, 544.5, 557.1
Trout d	416.8, 531.3, 566.9	413.5, 529.9, 554.7	448.4, 541.8, 570.6
Trout e*	415.5	413.8	450.2
Trout f	418.1, 534.9, 568.1	416.5, 544.5	447.9, 546.0, 570.9
Trout g	418.2, 537.5, 569,7	417.7, 545.2	447.7, 543.3, 569.8
Rat P-450	418.0, 532.5, 568.9	421.3, 532.5, 557.1	450.5, 544.5, 573.5

* λ_{max} at longer wavelengths not determined due to low degree of purity.

Figure 6. Absolute Spectra of Purified Rat and Trout P-448.

Top panel: P-448, purified from BNF treated rats, was diluted with 0.1 M potassium phosphate buffer (pH 7.4), containing 20% glycerol to 1 nmol/ml. Oxidized (solid line) and reduced (dashed line) spectra and CO-difference spectrum (dotted line) were recorded from a single sample using an Aminco DW-2a Spectrophotometer calibrated just prior to use with a holmium oxide filter. Botton panel: Oxidized and reduced spectra and the CO-difference spectrum of P-448 (f) from BNF treated trout were recorded as described above except that the concentration of P-448 was 0.7 nmol/ml.

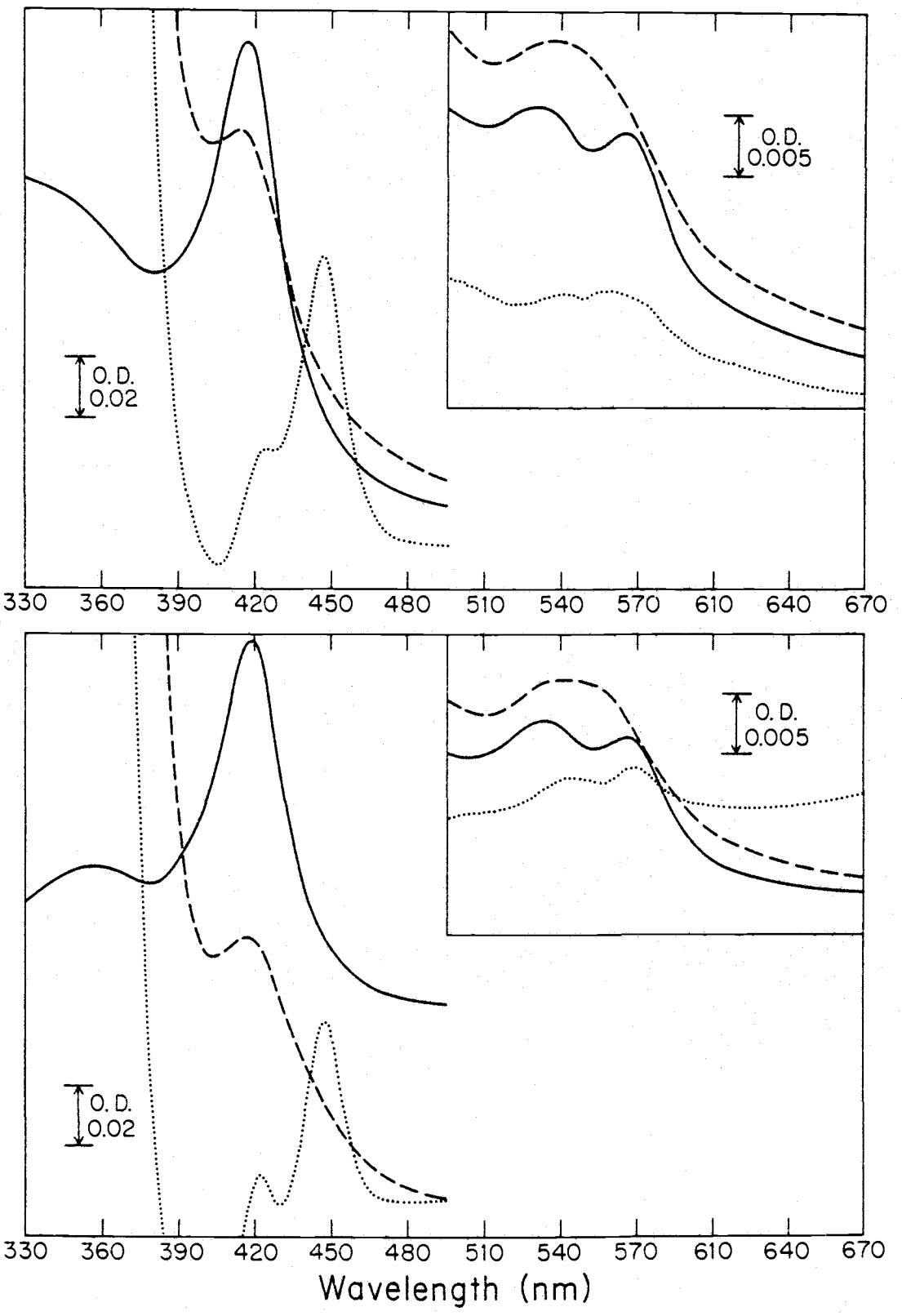

O.D.
0.02
O.D.
0.005
330
360
390
420
450
480
510
540
570
610
640
670
O.D.
0.02
O.D.
0.005
330
360
390
420
450
480
510
540
570
610
640
670
Wavelength (nm)

Figure 6

TABLE 3

Substrate Specificity of Purified Trout P-450s and Rat P-448 and P-450*

	Enzyme activity (nmoles/min/nmole P-450)						
				^{14}C-Testosterone			
Cytochrome	^{14}C-BP	Ethoxyresorufin	Ethoxycoumarin	6β	7α	16α	Benzphetamine
Rat P-448	5.94	6.54	10.2	0.12	0.007	0.013	⩽5
Trout a	⩽0.01	⩽0.02	⩽0.05	⩽0.003	⩽0.003	⩽0.003	⩽5
Trout d	⩽0.01	⩽0.02	⩽0.05	0.003	⩽0.003	⩽0.003	⩽5
Trout e	0.02	0.74	⩽0.05	0.011	⩽0.003	⩽0.003	⩽5
Trout f	1.10	2.30	0.20	0.012	⩽0.003	⩽0.003	⩽5
Trout g†	1.60						
Rat P-450	0.38	0.07	1.60	0.17	0.10	3.22	260

* Reconstitution of enzyme activity towards each substrate was performed either at 37°C (rat) or 29°C (trout) as described in Materials and Methods. Results are means of duplicate assays.

† Insufficient amounts of g were available for a complete analysis.

P-450 and PB induced P-450, respectively (42).

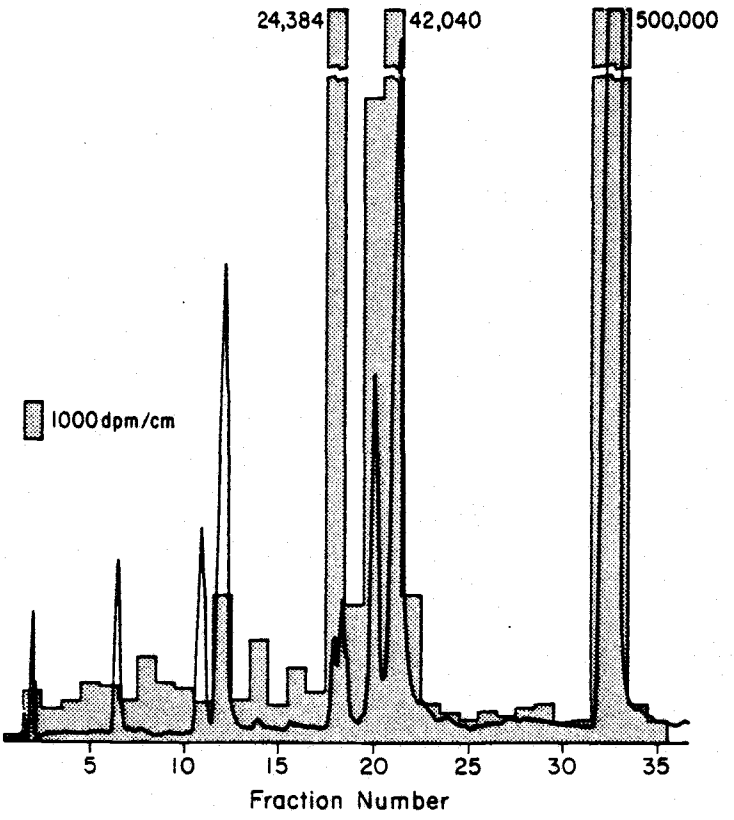
Trout P-448 (f) specificity differed from both rat P-448 and P-450. The trout P-448 had its highest turnover with ethoxyresorufin but exhibited low activity towards ethoxycoumarin. Both substrates are metabolized quite effectively by rat P-448, whereas, rat P-450 had only moderate activity towards ethoxycoumarin and ethoxyresorufin-O-deethylase was barely detectable. Rat P-450 demethylated benzphetamine and hydroxylated [^{14}C]-testosterone at the 16α position at high rates. These activities were not detectable with trout P-448. Rat P-448 also had no detectable benzphetamine demethylase activity, but there was a low level of 16α-testosterone hydroxylation. The 6β hydroxylated testosterone was the main product formed by rat P-448 and was the only metabolite detected after incubation with trout P-448.

Regioselectivity of [^{14}C]-BP Hydroxylase by Trout P-448 and Rat P-448 and P-450

The regioselectivity of trout P-448 (f) towards [^{14}C]-BP was compared to rat P-448 and P-450 by HPLC (Figure 7) and the metabolic profile of all three compared to the microsomes from which they were obtained (Table 4). The relative proportion of the various dihydrodiols, quinones and phenols was similar between microsomes from BNF treated rats and trout with two exceptions. Relative formation of 4,5-dihydrodiol was about 10-fold less and total quinones about 2.5-fold less in trout microsomes. Ratios of metabolites using reconstituted, purified trout and rat P-448s were similar with the only noticeable difference being a greater degree of 7,8-dihydrodiol

Figure 7. HPLC of [^{14}C]-BP Metabolites Produced by Reconstituted Rat and Trout P-448.

Top panel: [^{14}C]-BP hydroxylase activity using P-448 purified from BNF treated rats, was assayed in a reconstituted system containing rat NADPH-cytochrome c reductase (0.25 U) and DLPC (20 µg) in 1 ml of buffer as described in Materials and Methods. After 20 minutes at 37°C, the reaction was terminated with 2.5 ml of methylene chloride:methanol (2:1) and the substrate and products extracted twice into the organic phase. Each sample was then spiked with unlabeled BP metabolite standards. Following evaporation under N_2, the residue was redissolved in 50 µl of methanol and injected onto a Zorbax ODS (4.6 mm I.D. x 25 cm) column and the metabolites eluted using a 35 minute, non-linear gradient of acetonitrile (40-85%) in water. The column temperature was 40°C (Spectra Physics 8000) and the flow rate 2 ml/min. Total metabolites (labeled and unlabeled) were monitored at 254 nm (solid line) with an Altex u.v. monitor. Radioactivity (histograms) was determined by collecting 1 minute fractions directly into scintillation vials, adding 10 ml of scintillant and counting on a Packard Tri-Carb model 3375 using the automatic standardization to determine efficiency. Botton panel: P-448 (f) purified from BNF treated trout was reconstituted and assayed for the [^{14}C]-BP metabolite profile as described above, except that the temperature was 20°C and 0.5 minute fractions were collected for radioactive determination.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42,040
500,000
1000dpm/cm
5
10
15
20
25
30
35
Fraction Number

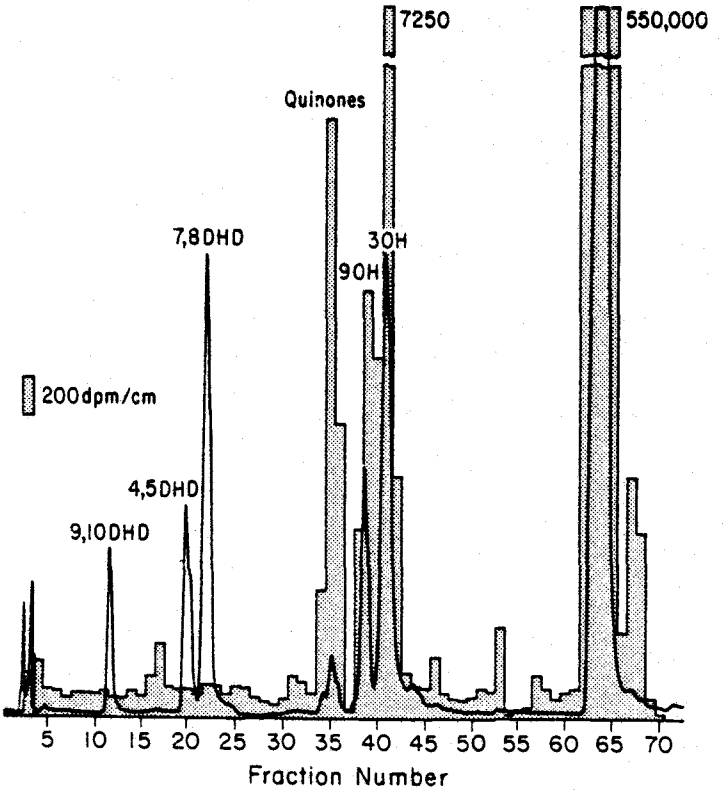

7250
550,000
Quinones
7,8DHD
30H
90H
200dpm/cm
4,5DHD
9,10DHD
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Fraction Number

Figure 7

TABLE 4

Comparative [^{14}C]-BP Metabolite Profile of BNF Rat and Trout Microsomes, PB Rat Microsomes, and Purified Rat P-450 and P-448 and Trout P-448*

	9,10 DHD†	4,5 DHD	7,8 DHD	Quinones	9OH	3OH
Rat BNF P-448	30.4 (0.7±0.3)	54.9 (1.5±1.0)	151.3 (3.9±0.8)	896.4 (23.2± 2.9)	507.3 (12.8±2.3)	1345.5 (33.5± 6.5)
Rat BNF microsomes	(18.2)	(4.0)	(16.3)	(16.4)	(4.1)	(36.1)
Trout BNF P-448	7.3 (0.8±0.1)	13.3 (2.2±1.8)	7.9 (1.0±0.3)	155.1 (23.9± 4.3)	123.0 (16.4±2.9)	313.2 (37.2± 9.3)
Trout BNF microsomes	(20.5)	(0.4)	(20.6)	(6.4)	(3.4)	(38.2)
PB P-450	0	7.5 (2.4±0.2)	2.9 (0.9±0.1)	58.7 (16.9±12.3)	1.2 (0.3±0.5)	90.2 (30.5±10.5)
Rat PB microsomes	(5.1)	(14.9)	(2.0)	(29.4)	(2.8)	(21.9)

* [^{14}C]-BP metabolites formed in pmoles/min/nmole P-448. Values in parentheses represent the percentage of total metabolites. Assays were carried out at either 37°C (rat) or 29°C (trout) as described in Materials and Methods. Results for the P-450 and P-448s are the mean and standard deviation of triplicates. Results from the corresponding microsomes are from a single determination.

† Abbreviations used are: 9,10 DHD (BP-9,10-dihydrodiol); 4,5 DHD (BP-4,5-dihydrodiol); 7,8 DHD (BP-7,8-dihydrodiol; Quinones (BP-3,6-quinone and BP-1,6-quinone); 9OH (9-hydroxy-BP); and 3OH (3-hydroxy-BP).

formation by rat P-448. Rat P-450 from PB treated animals had a lower activity towards [^{14}C]-BP and formed very little of the 9-phenol and no detectable 9,10-dihydrodiol. Very little dihydrodiol formation was observed with any of the purified P-448s and P-450, which is consistent with the lack of epoxide hydrolase contamination. The decrease in dihydrodiol formation (relative to the epoxide hydrolase containing microsomes) with purified rat or trout P-448 was accompanied by increases in quinone and 9-phenol formation with little or no change in the rate of formation of the 3-phenol, whereas, 9-phenol formation by the P-450 actually decreased and the 3-phenol increased.

In Vitro Inhibition of Rat and Trout P-448 Catalyzed [^{14}C]-BP Hydroxylase by Chemicals and Antibodies

The main fraction obtained from BNF-treated rainbow trout P-448 (f) had high activity towards [^{14}C]-BP which was significantly inhibited by ANF but not by SKF-525A or metyrapone (Table 5). Rat P-448, assayed at 37°C, exhibited a higher turnover and sensitivity to ANF, whereas, rat PB induced P-450 had a lower turnover number and was actually activated by ANF. When sodium cholate was used in place of DLPC as the lipid and both enzymes were assayed at 30°C, rat and trout P-448 had almost identical turnover numbers and sensitivity to ANF (data not shown).

Although purified rat and trout P-448 were similar with respect to spectral properties and activity towards [^{14}C]-BP, inhibition of reconstituted BP-hydroxylase by rabbit antibodies directed against rat P-450 and P-448 (Figure 8) suggested that they do not share

TABLE 5

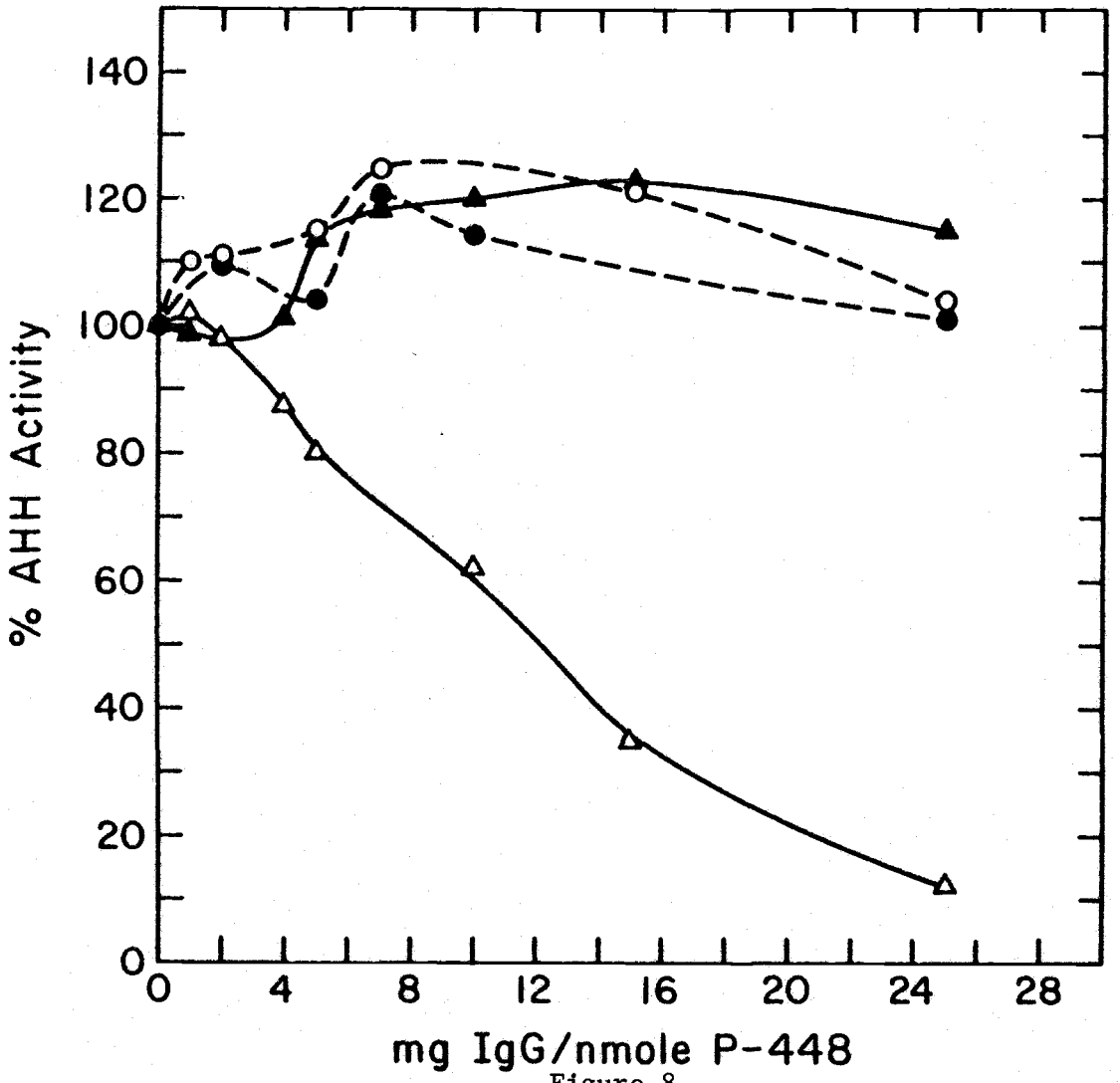
Comparative Metabolism of [^{14}C]-Benzo(a)pyrene by Purified Trout P-450 Fractions and Rat P-450 and P-448 in a Reconstituted System*

Cytochrome	Activity (pmoles ^{14}C-BP metabolized/min/nmole)	Inhibition (% of control) SKF-525A (100 μM)	metyrapone (100 μM)	ANF (100 μM)
Rat P-448	5070	95	86	13
Trout P-448	880	111	87	34
Rat P-450	95	107	78	281

* AHH activity was reconstituted at 29°C as described in Materials and Methods. Values are means of two determinations.

Figure 8. Effect of Antibodies Directed Against Rat P-450 and P-448 on Reconstituted [^{14}C]-BP Hydroxylase Catalyzed by Rat and Trout P-448.

P-448 (0.1 nmol) from rat or trout was preincubated with various amounts of either anti-PB-B_2 (P-450) or anti-BNF-B_2 (P-448) IgG in 0.25 ml of 50 mM Tris HCl buffer (pH 7.5) containing 5 mM KCl and 0.1 mM EDTA. The samples were incubated at 20°C for 10 minutes following which were added rat NADPH-P-450 reductase (0.2 U), DLPC (20 µg) and the above buffer containing 12 mM $MgCl_2$ to 1 ml. The addition of substrate, initiation and termination of the reaction and quantitation of products was as described in Materials and Methods. Open circles indicate trout P-448 incubated with anti-BNF-B_2; solid circles, trout P-448 and anti-PB-B_2; open triangles, rat P-448 and anti-BNF-B_2 and solid triangles, rat P-448 and anti-PB-B_2. Symbols represent the mean of duplicate assays.


140
120
100
80
60
40
20
0
% AHH Activity
0
4
8
12
16
20
24
28
mg IgG/nmole P-448
Figure 8

common antigenic determinants. Concentrations of rat BNF B_2 IgG which inhibited rat P-448 [^{14}C]-BP hydroxylase by 90% did not effect trout P-448. Neither form was inhibited by rat PB B_2 IgG.

This lack of immunological cross reactivity between rat and trout enzymes was confirmed by double precipitin analysis on Ouchterlony plates (Figure 9). Both rat P-448 and P-450 formed precipitin lines with their respective antibodies, while no trout form reacted with either antibody.

SDS-PAGE of Trout P-448 and Rat P-448 and P-450

Purified rat P-448 and P-450 and trout P-448 could be distinguished by their molecular weights as determined by SDS-PAGE. The bands were resolved when run separately or together and the molecular weights were calculated as 59,000, 57,000 and 54,500 for trout P-448, rat P-448 and rat P-450, respectively (Figure 10). These calculated molecular weights for rat BNF P-448 and PB-P-450 are slightly higher than most published values (26). The molecular weight here is 4,000 higher than cited above (Figure 5) using a different electrophoretic system, and is 2,000 higher than reported by Elcombe and Lech (82) for the main band increased in rainbow trout microsomes following BNF induction. The PB P-450 appeared homogenous, but both the rat and trout BNF P-448 had a faint band with a molecular weight of 1,000-2,000 below the main band. Because of the lack of complete homogeneity, amino acid compositions or peptide profiles after limited proteolysis were not done.

Figure 9. Ouchterlony Double Diffusion Precipitin Analysis with Purified P-450s and Anti-Rat P-450 and P-448 IgG.

Center wells contained 0.6 mg anti-BNF-B IgG (left) and anti-PB-B IgG (right). Top wells containing 2 µg of rat P-448 (left) and P-450 (right). The remaining wells contained various purified trout P-450s. The plates were left at room temperature in a moist environment for 48 hr. The gels were stained with Coomassie blue.

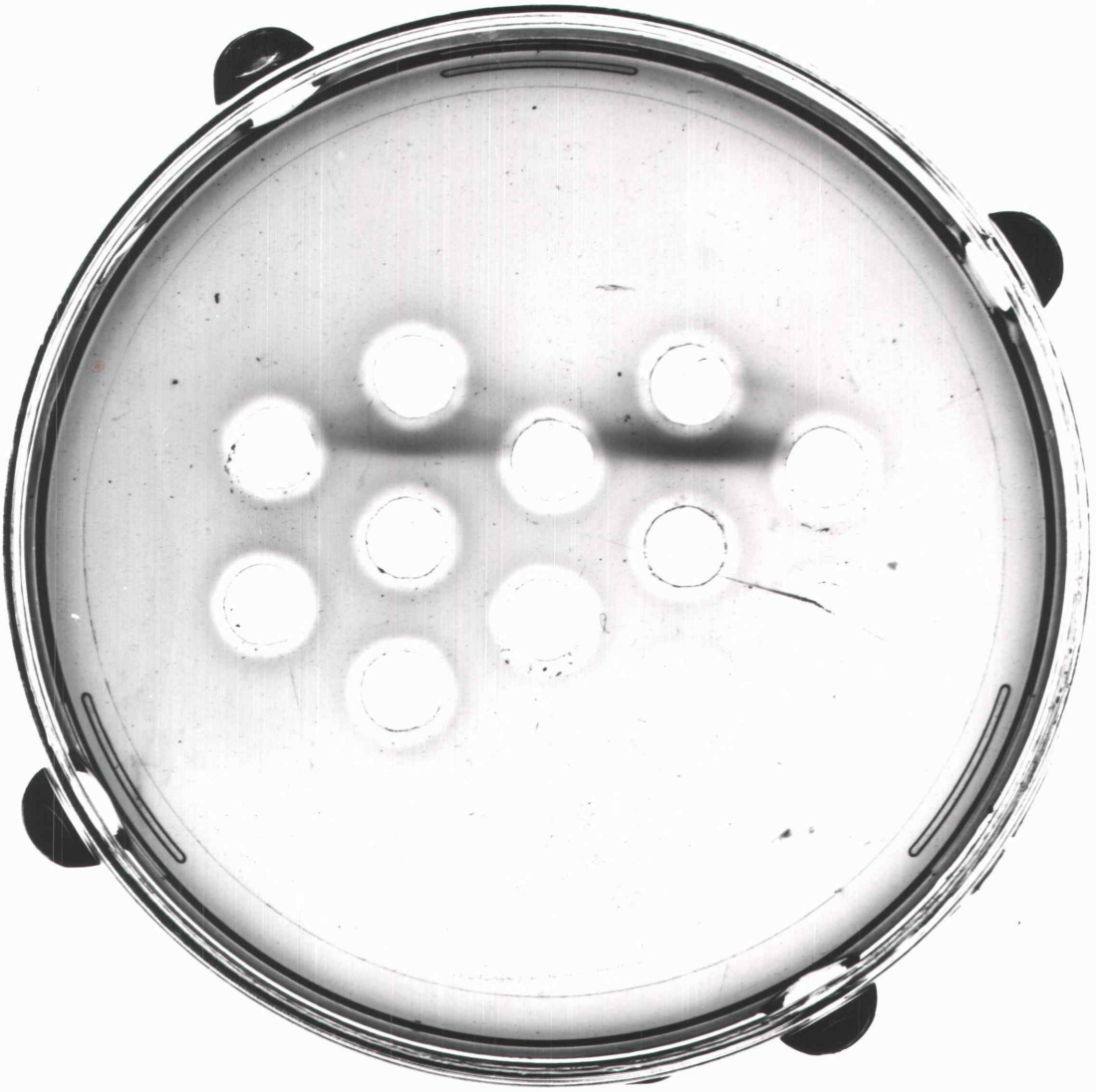


Figure 9

Figure 10. SDS-PAGE of Purified Rat and Trout P-448.

Samples, containing 0.25% SDS were immersed in boiling water for 4 minutes and electrophorized at 30 mAMP/gel (about 5 hr) using a Protean Slab gel connected to a Model 1420-A constant power supply (both from Bio-Rad). Gels were fixed and stained by shaking overnight in 45% ethanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250 and destained with 10% ethanol, 7.5% acetic acid. Molecular weights were calculated by comparing the relative mobility of unknowns to the logarithms of four standards with molecular weights of 66,000, 53,000, 45,000 and 40,000 daltons. Samples were, from left to right, molecular weight standards (6 µg bovine serum albumin and ovalbumin and 2.5 µg glutamate dehydrogenase and aldolase); rat P-450 (1 µg); trout P-448 (1 µg); rat P-448 (1 µg); rat P-450, P-448 and trout P-448 (1 µg each); molecular weights standards; rat P-450 (2.5 µg); trout P-448 (2.5 µg); rat P-448 (2.5 µg); rat P-450, P-448 and trout P-448 (2.5 µg each).

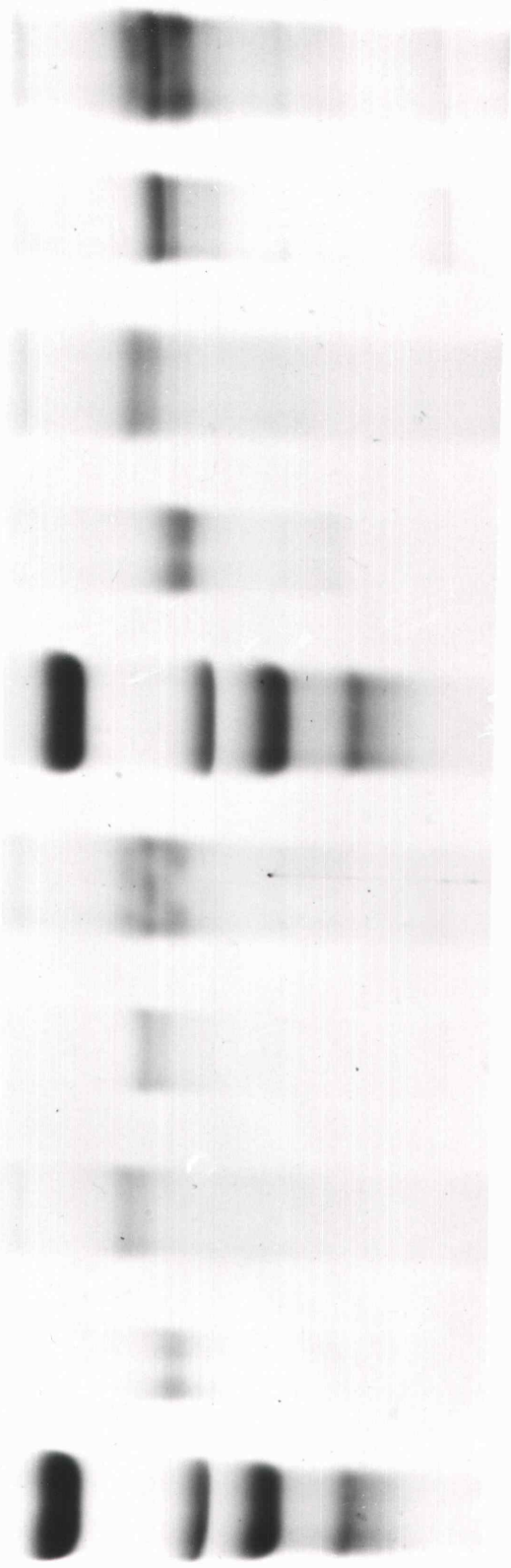


Figure 10

Purification and Comparative Properties of NADPH-P-450 Reductase from Rainbow Trout and Rat

Purification and SDS-PAGE of Trout NADPH-P-450 Reductase.

NADPH-P-450 reductase was purified over 1,200-fold from BNF-induced rainbow trout microsomes, in two steps, to a specific activity of 36.9 with a final yield of 18% (Table 6). Both rat and trout reductase appeared homogenous on SDS-PAGE (Figure 11), with molecular weights of 75,000 (rat) and 77,000 (trout) when run separately. However, the two enzymes could not be resolved when run together. The molecular weights reported here are in good agreement with those obtained for highly purified reductase from other sources (57).

Amino Acid and Flavin Composition and Spectral Properties of Purified Rat and Trout Reductase

The A_{380}/A_{455} ratio was 0.87 and 1.12 for rat and trout reductase, respectively. This value for the rat enzyme is identical to that previously reported by Guengerich and Martin (136). The A_{280}/A_{455} ratios were 9.4 (rat) and 11.5 (trout). For comparison, a ratio of 7.4 has been reported for rat (136) and Serabjit-Singh *et al.* observed a value of 11.3 for the little skate reductase (64).

The flavin composition of rat reductase was 0.97 mol of FAD and 0.92 mol of FMN per mol of reductase, whereas, trout reductase contained 1.06 mol of FAD, but only 0.76 mol of FMN per mol of reductase.

The amino acid composition (Table 7) of rat NADPH-P-450 reductase obtained in this study agrees well with results from a different

TABLE 6

Purification of NADPH-P-450 Reductase from BNF-Treated Rainbow Trout

Fraction	Total protein* (mg)	Total activity† (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Fold purification	Yield (%)
Microsomes	872	26.1	0.03	1.0	100
CHAPS extract	412	34.1	0.07	2.2	131
Tryptamine-Sepharose-4B	24.0	20.9	0.87	29	80
2,5-ADP Agarose	0.13	4.8	36.9	1230	18

* Protein was determined by the method of Lowry et al. with bovine serum albumin as the standard.

† NADPH-cytochrome c reduction (550 nm) was measured at 20°C in buffer containing 0.35 M potassium phosphate, pH 7.5, 0.05 mM cytochrome c and 0.1 mM NADPH.

Figure 11. SDS-PAGE of Purified Rat and Trout NADPH-P-450 Reductase.

Samples (from left to right) were, molecular weight standards (bovine serum albumin, 68,000; glutamate dehydrogenase, 53,000; ovalbumin, 45,000; aldolase, 40,000) (1 µg each); rat reductase (2 µg); trout reductase (1 µg); rat (2 µg) and trout (1 µg) reductase; molecular weight standards; rat reductase (1 µg), trout reductase (0.5 µg); rat (1 µg) and trout (0.5 µg) reductase; molecular weight standards.

Figure II

TABLE 7

Amino Acid Composition of Rat and Rainbow Trout NADPH-P-450 Reductase

Amino acid	No. of residues per reductase		
	Trout*	Rat*	Rat†
Lysine	44	34	36
Histidine	14	22	21
Arginine	32	35	35
Cystine	11	4	7
Aspartate	79	61	62
Threonine	44	33	36
Serine	37	39	45
Glutamate	84	79	82
Proline	30	30	31
Glycine	60	48	48
Alanine	50	52	50
Valine	36	42	44
Methionine	11	15	16
Isoleucine	25	22	27
Leucine	64	66	62
Tyrosine	26	38	32
Phenylalanine	24	31	29
Tryptophan	8	8	10
Total	679	659	673

* Amino acid composition determined as described in Materials and Methods.

† Data taken from Vermilion and Coon (155). Their reductase was isolated from PB induced rat. Tryptophan was determined by base hydrolysis.

laboratory (155) using the same strain of rat (difference index, 3.2) (156). The lower number of total amino acid residues is due to the fact that Vermilion and Coon used a molecular weight of 76,000, while 75,000 was used in this study. Rainbow trout NADPH-P-450 reductase consisted of 20 more amino acid residues than the rat reductase. Other differences, most notably a higher relative amount of lysine, threonine, glycine and cystine and lower amounts of histidine, and tyrosine in the trout, were observed. The difference index between rat and trout reductase was 8.5.

Peptide Patterns of Rat and Trout NADPH-P-450 Reductase Following Proteolysis

Partial proteolysis, followed by SDS-PAGE of the peptide fragments, is a useful tool for distinguishing isozymes and has been used as such with P-450 isozymes and NADPH-P-450 reductase from various tissues and species. Peptide maps from rat and trout reductase, obtained following limited proteolysis with either Staph. aureous VIII protease, α-chymotrypsin or papain (Figure 12) are quite dissimilar, especially with papain. Such a finding is consistent with other interspecies comparisons and suggests differences in primary protein structure.

Immunological Comparison of Rat and Trout NADPH-P-450 Reductase

When rat and trout reductase were preincubated with rabbit antibody directed against the rat enzyme and then analyzed for cytochrome c reductase activity, both enzymes were inhibited, the rat form being more sensitive to a given amount of antibody (Figure 13). However,

Figure 12. SDS-PAGE of Rat and Trout NADPH-P-450 Reductase Following Proteolysis.

Samples are, from left to right, trout reductase and Staph. aureous V8 protease; Staph. aureous V8 protease and rat reductase; Staph. aureous V8 protease only; trout reductase and α-chymotrypsin; rat reductase and α-chymotrypsin; α-chymotrypsin only; trout reductase and papain; rat reductase and papain and papain only. Samples (15 μg) were treated at 100°C for 2 minutes in buffer containing SDS. After cooling, protease was added (0.5 μg for Staph. aureous V8 protease and α-chymotrypsin and 0.05 μg for papain) and digestion allowed to proceed for 30 minutes at 37°C. Following addition of 2-mercaptoethanol and SDS to 2%, samples were again treated at 100°C for 2 minutes. Electrophoresis, staining and destaining were performed as described in Materials and Methods except that the separating gel was 15% acrylamide.

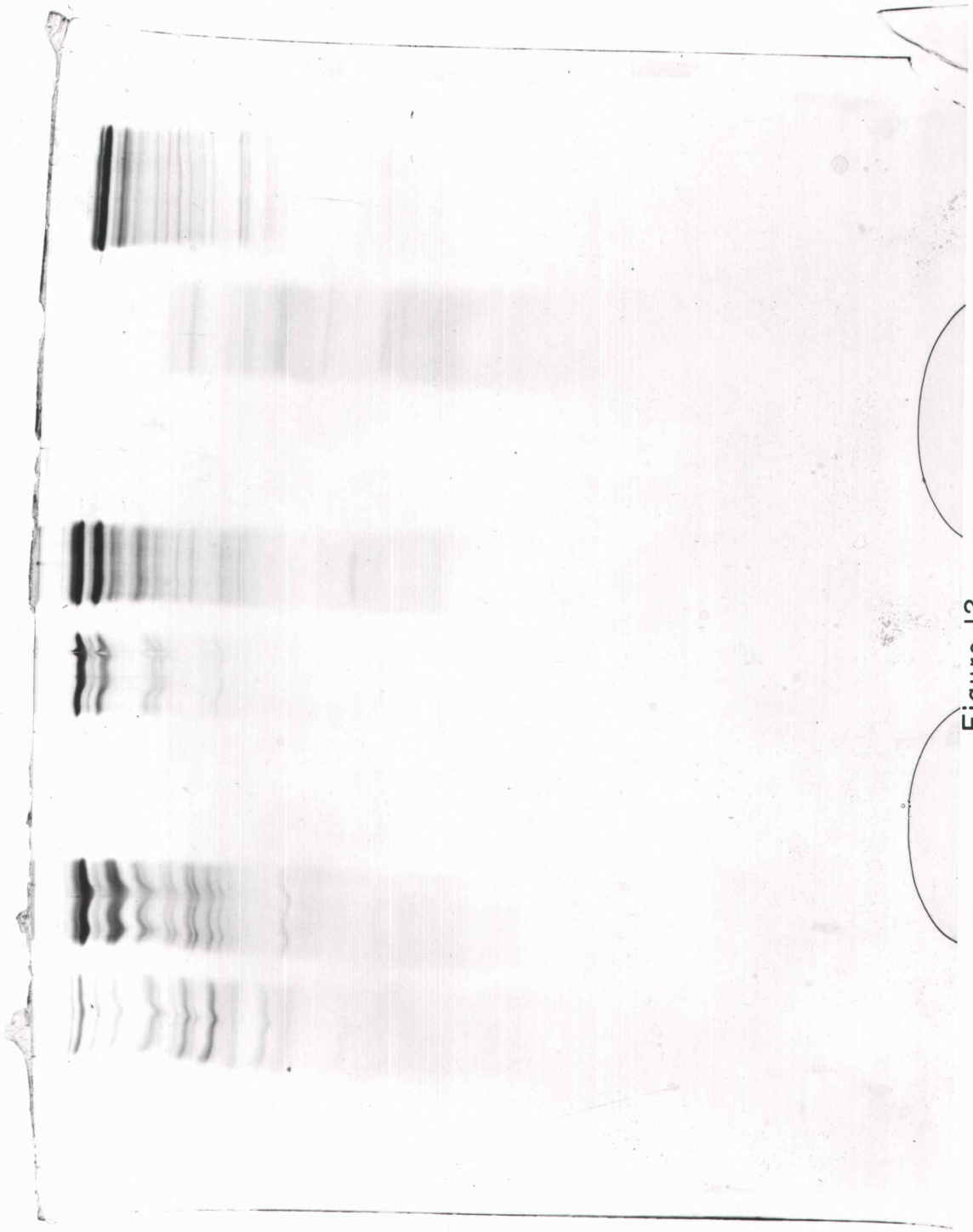


Figure 12

Figure 13. Inhibition of Rat (Open Circles) and Trout NADPH-Cytochrome c Reductase (Closed Circles) by Rat Antibody.

The reductase (0.2 μg) was incubated for 10 minutes with rat antibody, then, buffer containing cytochrome c was added and the reaction initiated by the addition of NADPH. The Δ550 nm/min in the absence of antibody (100% activity) was 0.16 for rat and 0.20 for trout (20°C).

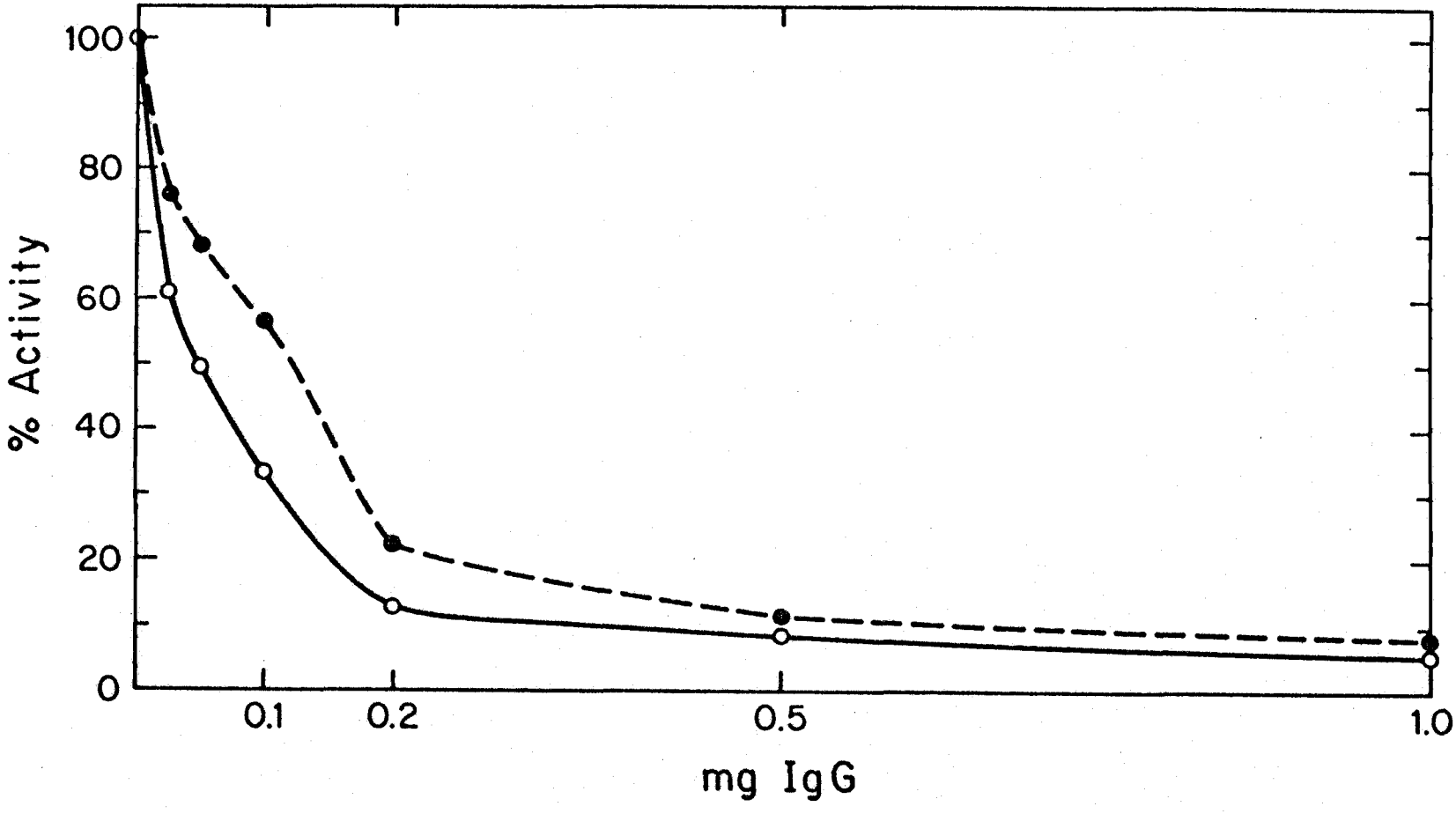


Figure 13

analysis of immunochemical cross reactivity by double diffusion precipitin formation on Ouchterlony plates showed no reaction between the trout NADPH-P-450 reductase and the rat antibody (Figure 14). A previous study comparing rat and rabbit reductase gave similar results; that is, inhibition of rabbit NADPH-cytochrome c reductase activity by rat antibody with no precipitin line formation on Ouchterlony plates (153). This suggests that, immunologically, the rat and trout reductase are at least as similar as rat and rabbit reductase.

Temperature Profiles of EROD Activity in Rat and Trout Microsomes and Reconstituted Systems

The temperature optima for ethoxyresorufin-O-deethylase (EROD) with trout and rat microsomes (Figure 15, top) was 20-25°C and 40°C, respectively. Acquisition of both purified trout P-448 and NADPH-P-450 reductase prompted us to investigate which of these mfo enzyme components might be responsible for the lower temperature optima seen in trout microsomes.

Reconstitution of rat P-448 with trout NADPH-P-450 reductase gave a EROD profile identical to that obtained with rat P-448 and rat reductase (Figure 15, bottom). In fact on a molar basis, trout NADPH-P-450 reductase and rat P-448 had a slightly higher EROD turnover than when rat reductase was used. Therefore, it seems unlikely that they trout NADPH-P-450 reductase is the thermolabile component.

Trout P-448, reconstituted with either rat or trout NADPH-P-450 reductase again, produced identical EROD temperature profiles (Figure 15, bottom). The temperature optima with trout P-448 (30-35°C) was

Figure 14. Double Diffusion Precipitin Analysis of Rat and Trout NADPH-P-450 Reductase on Ouchterlony Plates.

Ouchterlony plates were prepared as described by Guengerich et al. (152,153). The center well contained rabbit anti-rat NADPH-P-450 reductase IgG (0.6 mg). Surrounding wells, clockwise starting from the top, contained buffer, rat NADPH-P-450 reductase (2.4 µg); trout reductase (2.4 µg); buffer, rat reductase (2.4 µg); trout reductase (2.4 µg). Gels were allowed to develop at room temperature for 48 hrs and then were photographed directly.

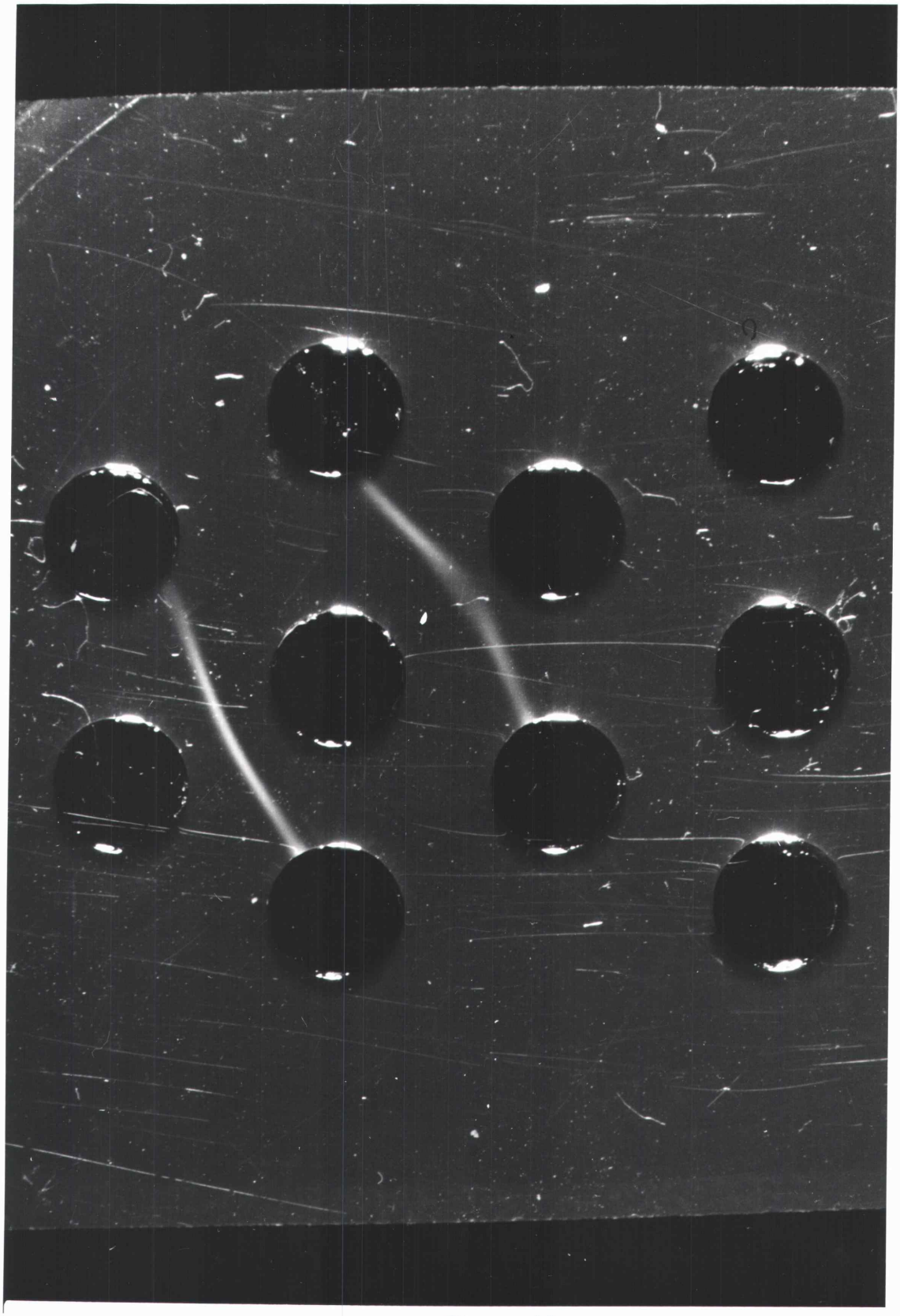


Figure 14

Figure 15. Temperature Profiles of EROD Activity from BNF-Treated Microsomes and Reconstituted Enzymes from Rat and Trout.

The microsomes and reconstituted systems contained 0.1 nmol of P-450. The reconstituted systems also contained 0.1 nmol of reductase and 20 µg of lipid. The microsomes or reconstituted systems were preincubated for 4 minutes at the temperature at which they were assayed. Following the addition of ethoxyresorufin and buffer, the reaction was initiated with NADPH. The top panel depicts the results with trout (closed circles) and rat (open circles) microsomes (solid lines) and liposomes (broken lines). Maximum activity was 2.11 and 9.88 nmol per minute per nmol P-448 for trout and rat microsomes, respectively, and 2.75 and 3.17 for trout and rat liposomes, respectively. Bottom panel: Solid lines represent rat P-448 reconstituted with either trout reductase (closed circles) or rat reductase (open circles). Maximum activity was 4.30 with trout reductase and 3.39 with rat reductase. Dashed lines depict trout P-448 and either trout reductase (closed circles) or rat reductase (open circles). Maximum activities were 0.96 with trout reductase and 0.64 with rat reductase.

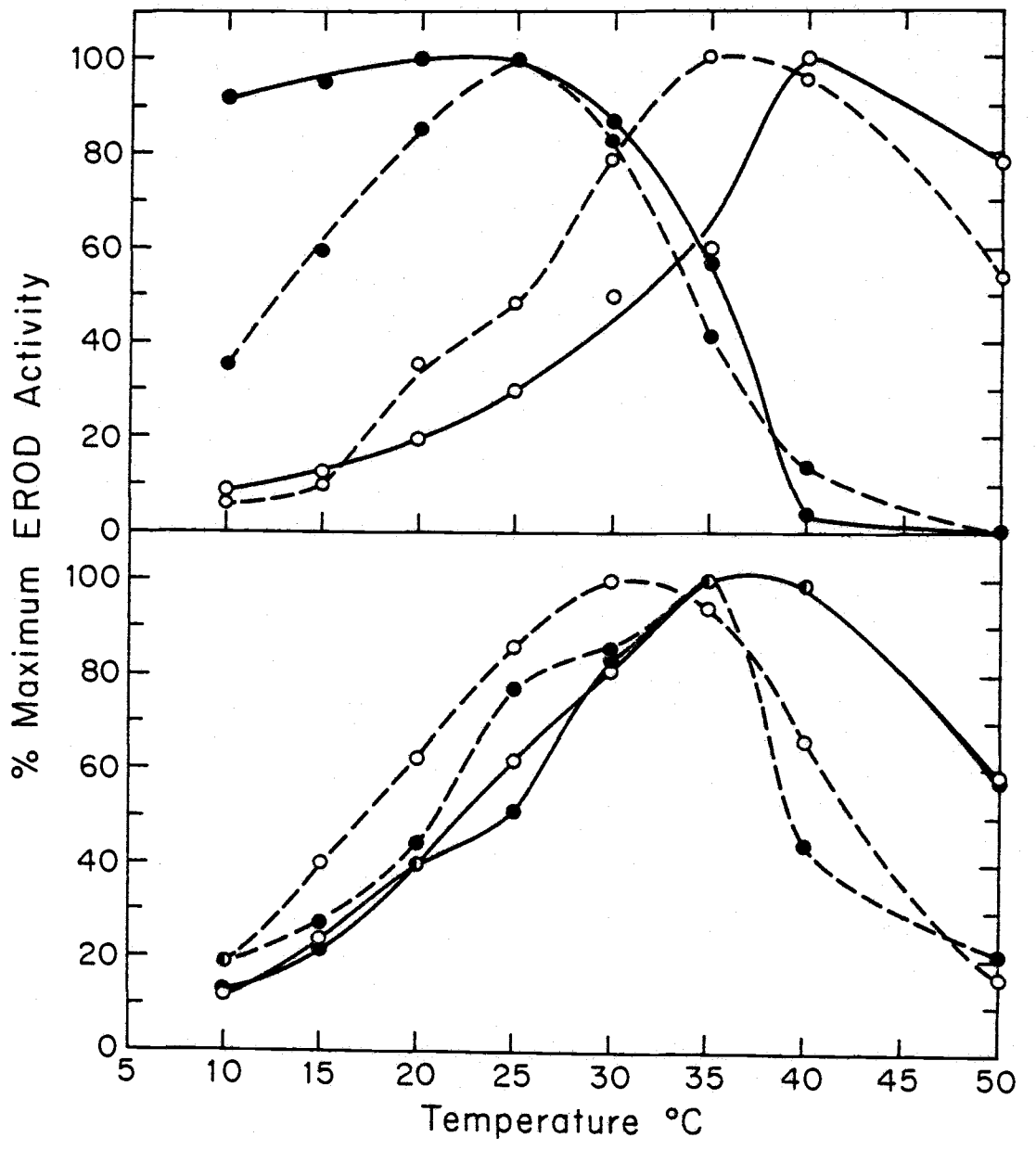

% Maximum EROD Activity
100
80
60
40
20
0
5
10
15
20
25
30
35
40
45
50
Temperature °C

Figure 15

slightly lower than with rat P-448, but still resembled rat microsomes more than trout microsomes, as can be seen by the relatively higher degree of activity at 40-50°C with reconstituted trout enzymes.

The above results were obtained with DLPC as the lipid. The lower mfo temperature optima observed with trout microsomes could be due to some properties of the trout microsomal lipid. However, when trout P-448 and trout NADPH-P-450 reductase were reconstituted with either rat or trout microsomal phospholipids, the temperature profiles were nearly identical to those obtained with DLPC as the lipid (data not shown). Interestingly, the EROD activity, using trout P-448 and either reductase or phospholipid, was only about one-third of the activity as when DLPC was the lipid. Furthermore, when over 12 different phosphatidylcholines, varying with respect to length and degree of unsaturation of the fatty acyl chain, were tested for their ability to reconstitute trout EROD activity, only DLPC increased the activity significantly over that obtained in the absence of lipid (Table 8). In addition, four detergents (one anionic, two neutral and one zwitterionic) were tested and all were found to have either no effect or to inhibit EROD activity compared to reconstitution with no added lipid. Therefore, the results, using a non-vesicle reconstitution system, suggest that none of the three trout mfo components are responsible for the lower EROD temperature optima seen in trout microsomes.

It is possible that the studies conducted above, using a non-membranous system may not be an accurate model of the thermolabile properties of P-450 and NADPH-P-450 reductase. Indeed, some studies

TABLE 8

Effect of Various Synthetic and Extracted Phosphatidylcholines (PCs) and Detergents on Reconstituted Ethoxyresorufin-O-Deethylase (EROD) Activity with Trout P-448

Lipid or Detergent*	EROD† nmol/min/nmol P-448	Activity (%)
None	1.10	30
Dilauryl PC	3.64	100
Dimyristoyl PC	1.47	40
Dipalmitoyl PC	1.10	30
Distearyl PC	0.65	18
Dioleoyl PC	0.80	22
β-oleoyl-γ-palmitoyl PC	0.94	26
Soybean PC	0.88	24
Egg yolk PC	1.02	28
Hydrogenated egg yolk PC	0.84	26
Bovine liver PC	1.18	32
Rat liver PC	1.35	37
Trout liver PC	1.35	37
Cholate	1.18	32
Triton N101	0.72	20
Lubrol PX	0.50	14
CHAPS	0.42	12

* All lipid and detergent fractions were sonicated just prior to use and added to final concentrations of 32 μM.

† Ethoxyresorufin-O-deethylase was assayed fluorometrically at 25°C in a reconstituted system containing trout P-448 (0.05 nmol), rat NADPH-P-450 reductase (0.12 μmol min^{-1}) and lipid or detergent which was preincubated for 2 min at 25°C following which buffer was added to 1 ml. The substrate concentration was 0.5 μM and the reaction initiated by the addition of NADPH to 0.1 mM. Values shown are means of duplicate assays.

have suggested that the heme spin state and the thermolability properties of mammalian P-450 are altered by incorporation into artificial membranes. In order to investigate this possibility, the above EROD temperature profiles, using various combinations of rat and trout enzymes and lipid, were repeated using vesicles prepared by the cholate dialysis method. The profile using rat P-450, NADPH-P-450 reductase and microsomal lipid in vesicles (Figure 15, top) was very similar to the non-vesicle profile. Compared to rat microsomes, the temperature activity curve using purified rat mfo components, in either a vesicle or non-vesicle system, is shifted to lower temperatures.

When trout P-448, NADPH-P-450 reductase and lipid were reconstituted in vesicles (Figure 15, top) the EROD temperature profile was quite similar to the trout microsomes except in the lower (10-15°C) range. This result contrasted to the temperature profile of these same mfo components in a non-vesicle system. All other combination of rat and trout mfo components, reconstituted in vesicles, gave profiles similar to non-vesicle results. Therefore, it appears, that only the combination of trout P-448, trout NADPH-P-450 reductase and trout lipid reconstituted into vesicles can mimic the lower temperature optima for EROD activity seen in trout microsomes.

DISCUSSION

Purification of Trout P-450s

In this thesis, two recent developments in the field of mammalian P-450 purification, solubilization with CHAPS and tryptamine-Sepharose affinity chromatography, have been utilized to purify multiple forms of P-450 from BNF-treated rainbow trout. Three forms were resolved on DEAE-Sepharose. Further fractionation of two of these peaks produced four forms. Thus, it appears that in these trout, there may be at least five distinct P-450 isozymes. Two of these forms (f and g) were obtained with a high specific content and were nearly homogenous on SDS-PAGE. Preliminary attempts at solubilization and purification of untreated trout P-450, utilizing conventional mammalian procedures, were not successful. There are a number of explanations for this finding. For instance, a number of nonionic detergents, commonly used for P-450 purification, have been shown recently to contain oxidizing contaminants (135,157), which could act upon polyunsaturated lipids from the microsomal membrane, generating reactive lipid peroxides with subsequent destruction of the P-450 heme moeity. Fish contain relatively high amounts of polyunsaturated lipids and may be especially sensitive to such degradation. Alternatively, P-450 from BNF-treated trout may be more stable than the endogenous form(s). Lastly, fish P-450 appears to be more sensitive to conversion to P-420 by sodium cholate during solubilization than mammalian P-450 (data not presented).

In order to compare the properties of the main form to rat P-450 and P-448, these forms were purified from PB and BNF-treated

rats, respectively.

Comparative Properties of Trout P-448 and Rat P-448 and P-450

As mentioned in the Introduction, there are numerous techniques currently being employed for distinguishing multiple P-450 forms. In this study, rat and trout P-450s were compared by molecular weight on SDS-PAGE, spectral properties, substrate specificity and regioselectivity, the effect of *in vitro* inhibitors and immunochemical cross-reactivity.

The main trout form (f) had a λ_{max} in the CO-reduced spectrum (447.9 nm) closer to that of rat P-448 (447.3 nm) than rat P-450 (450.5 nm). Therefore, trout are similar to mammals, in that the main form purified following BNF induction, is a "P-448-type" of P-450. These results are consistent with those of Bend *et al.* (93) in which a P-451 and a P-447 were resolved on DEAE from little skate microsomes. The relative amount of the P-447 form was enhanced by pretreatment with benzanthracene.

Evaluation of substrate specificity demonstrated that trout P-448 (form f) had no detectable activity towards the two substrates usually associated with PB induced P-450, testosterone 16α-hydroxylase and benzphetamine-N-demethylase. Conversely, the fish enzyme was similar to rat P-448, with its highest relative activities towards ^{14}C-BP and ethoxyresorufin. However, the trout P-448 had relatively low activity towards ethoxycoumarin, which was an excellent substrate for rat P-448. No attempt was made to optimize conditions of temperature, time, concentration of substrate, reductase, cofactors, pH, lipid or type of reconstitution system (vesicle or

non-vesicle) used for any of these reactions, and it is possible that such an effort could alter some of the results obtained above.

Further similarities between rat and trout P-448 were observed upon examination of the regioselectivity towards ^{14}C-BP. Both forms had high activity towards ^{14}C-BP and exhibited a preference for metabolism at the non-K region of BP (9-OH, 7,8-diol and 9,10-diol) while rat P-450 had lower overall activity and formed mainly K region metabolites (3-OH and 4,5-diol). These results are consistent with previous analysis using purified mammalian P-448 and P-450 (158,159) and fish microsomes (92,95,100,102). This evidence suggests that, like rat PAH-induced P-448, trout P-448 has a preference for metabolizing BP to activated carcinogenic and mutagenic metabolites, the most potent of which is the 7,8-diol, 9,10-epoxide (17). In addition to the main trout P-448 (f), form g was also active towards ^{14}C-BP but the regioselectivity was not determined. The relative amounts of these P-448s in trout may be regulated by the same variables (sex, age, hormonal status, diet and environmental chemicals) as is rat P-448, and may determine the susceptibility of these animals to the carcinogenic effects of BP type pollutants.

When rat and trout P-448s (f and g) ^{14}C-BP hydroxylase activity was examined in the presence of various inhibitors, both were found to be sensitive to ANF but not SKF-525A or metyrapone, whereas, the low activity exhibited with rat P-450 was actually enhanced by ANF. Previous findings with these inhibitors has shown that ANF selectively inhibits PAH-induced P-448 BP hydroxylase but, in some cases, actually increases BP hydroxylase in untreated fish (160) or rat (161,162). SKF-525A and metyrapone are more active towards

PB-induced P-450 with the latter being somewhat more selective. In this study, only two purified trout forms (f and g) were active towards BP, and both of these forms were inhibited by ANF. As mentioned above, ANF activates BP hydroxylase in some untreated fish. This suggests the possibility that untreated fish may have an endogenous P-450 with some activity towards BP (ANF activated), but after induction with a PAH type inducer, a new P-448 type of enzyme is synthesized (ANF sensitive) and the endogenous form is decreased. Previous studies with P-448 induction in mammals have shown that, along with the induced synthesis of P-450 or P-448, the relative amount of other forms are reduced (163,164).

Although rat and trout P-448 were similar with respect to substrate specificity and regioselectivity, sensitivity to inhibitors and spectral properties, inhibition of ^{14}C-BP hydroxylase by rabbit antibodies directed against rat P-448 and Ouchterlony double diffision precipitin analysis, show that they do not possess identical antigenic determinants.

Determination of molecular weight on SDS-PAGE further demonstrated structural differences between rat and trout P-448, the latter have a molecular weight about 2,000 daltons higher than the former and 4-5,000 higher than rat P-450.

Purification and Comparative Properties of Trout NADPH-P-450 Reductase

NADPH-P-450 reductase was purified from BNF treated rainbow trout microsomes, to a high specific activity and apparent homogeneity. Properties of purified reductase were compared to enzyme

purified from PB treated rats by SDS-PAGE, peptide mapping following proteolysis, spectral properties, amino acid and flavin composition, immunochemical cross-reactivity and activity in reconstituted systems.

The molecular weight of the trout reductase (77,000), as calculated from SDS-PAGE, was 2,000 daltons higher than the rat enzyme (75,000). Serabjit-Singh et al. (64) reported that reductase purified from the marine elasmobranch, little skate, had a molecular weight of 74,000.

The A_{380}/A_{455} (1.12) and A_{280}/A_{455} (11.5) ratios for trout were both higher than the corresponding values for rat. Little skate reductase had a A_{380}/A_{455} value of 11.3. Even with inclusion of FMN in the 2',5'-ADP agarose elution and final dialysis buffers, analysis of the flavin composition of trout reductase showed that this enzyme was partially depleted of FMN (0.76 moles per mole of reductase). When FMN was omitted from these buffers, the levels were depleted even further, to 0.48 moles per mol of reductase. In spite of lower amounts of FMN, trout reductase was slightly more effective (on a molar basis) than rat reductase in reducing cytochrome c or rat P-448.

Differences between the trout and rat reductase amino acid composition were also observed, especially with regard to lysine, histidine, threonine, cystine and tyrosine.

Differences in peptide patterns on SDS-PAGE following limited proteolysis provided further confirmation that rat and trout NADPH-P-450 reductase have different primary structures. Such differences were also observed between rabbit and little skate reductase (64).

Immunologically, rabbit antibody against rat NADPH-P-450 reductase was quite effective in inhibiting trout NADPH-cytochrome c reductase activity but did not form a precipitin line with trout enzyme on Ouchterlony plates. Again, these results are similar to the comparison of rabbit and little skate reductases (64). Antibodies to the rabbit enzyme were capable of partial inhibition of little skate NADPH-cytochrome c reduction.

Thermolability Properties of Purified Rat and Trout Enzymes in Reconstituted Systems

Acquisition of both purified trout P-448 and NADPH-P-450 reductase prompted the investigation of which of these enzyme components was responsible for the lower mfo temperature optimum seen in trout microsomes compared to rat microsomes. Reconstitution of rat P-448 with rat or trout reductase suggests that trout NADPH-P-450 reductase is not the temperature sensitive component, as the EROD temperature profiles using either reductase were almost identical. These results contrast with the results of Serabjit-Singh et al. (64), in which NADPH-P-450 reductase from little skate was only 50% as effective at 30°C as rabbit reductase in reconstituting rabbit P-450-catalyzed benzphetamine-N-demethylation. At 37°C, the little skate reductase was only 25% as effective as rabbit reductase.

Trout P-448, reconstituted with either rat or trout reductase, has a slightly lower temperature optima (30-35°C), but the activity was still much higher than trout microsomes, especially at 40° and 50°C. Therefore, trout P-448 is probably not the temperature sensitive component.

Another possibility for the temperature sensitive component of trout microsomes is the phospholipid fraction. Trout have been shown to contain high proportions of polyunsaturated fatty acids, which could make the trout microsomes more susceptible to lipid peroxidation and destruction of mfo activity. However, when total phospholipids were extracted from rat and trout microsomes and used to reconstitute EROD activity, in place of DLPC, no change in the temperature profile was seen. Therefore, the phospholipid is probably not the temperature sensitive component, although the reconstituted systems contained traces of EDTA (about 10 μM) which could, possibly, have prevented peroxidation of added phospholipids.

If none of the individual components of the trout mfo system were responsible for the lower temperature optimum in microsomes, this suggests that there may be some structural organization of P-450, reductase and lipid within trout microsomes that is responsible for its lower temperature profile, which can not be reproduced using individual components in a non-vesicle system. Therefore, the above experiments on the EROD temperature profiles, using various combinations of either rat or trout P-448, reductase or microsomal lipid (in the same ratio) were repeated using vesicles prepared by the cholate dialysis method. Only the combination of trout P-448, reductase and lipid could mimic the lower EROD temperature optima seen in trout microsomes. This suggests that incorporation of the trout mfo components into a membranous environment either alters the physical-chemical properties of the enzymes or there is some structural organization in liposomes and microsomes that confers the lower temperature optima. Although incorporation of P-450 into membranes has been

shown to alter its thermal stability, this does not explain why it is only when trout P-448 is incorporated into membranes containing trout reductase and lipid, that its thermal properties are altered. It seems more likely that trout mfo components form some kind of structurally functional unit. It is interesting that trout liposomes used here resembled trout microsomes even though the ratios of mfo components were quite different. The P-450:reductase ratio in vesicles was 1:1, while in trout microsomes the ratio is about 1:0.03. More definitive data could be obtained on the mfo organization in trout by construction of liposomes with varying amounts of P-450, reductase and various lipids. The physical-chemical properties of such systems could be examined by construction of Arrenhius plots (112), viscosity as determined by spin labelling (110), cross-linking (165), saturation transfer epr spectroscopy (124), or rotational diffusion from the decay of absorption anisotropy after photolysis of the heme:CO complex by a vertically polarized laser flash (166,167).

The molecular organization of the mammalian mfo system is not known. Some evidence suggests a fairly random distribution (119,120) while others have proposed a semi-rigid or rigid clustering of P-450 molecules around a single reductase (121-124,166,167). Increases in membrane fluidity with increasing temperature might tend to increase mfo activity for randomly distributed P-450 and reductase, while a cluster of enzymes may begin to diffuse apart.

Studies on the fatty acid hydroxylase, desaturase and elongation activities of trout P-450, reductase and, perhaps, cytochrome b_5 may prove very interesting. It has been shown that rainbow trout

hepatocytes acclimate to lower temperature by fatty acid desaturation and elongation (108). What role might the mfo system play in this process and could this be related to the inability of fish to respond to mfo induction at low temperatures (125)? Paine (31) has proposed that lipid peroxides (from unsaturated fatty acids) may control the activity of the P-450 system. Further investigations on the structural organization and functional interaction of mfo enzymes and lipid in trout microsomes and possible relationships between lipid composition, membrane fluidity, mfo activity and P-450 induction may prove useful as a model for studying the mammalian system.

In summary, cytochromes P-450 from BNF treated rainbow trout have been purified and compared to rat P-448 and P-450 by a number of criteria for distinguishing multiple P-450 forms. The main form obtained from trout appeared to be a P-448 type of P-450 and had some characteristics in common with rat P-448, both of which were readily distinguishable from rat PB induced P-450. Trout and rat P-448, however, were not identical with respect to M_r, substrate specificity or immunochemical properties. Preparation of antibodies against purified trout P-448, combined with immunochemical techniques for quantitating relative amounts of certain P-450s in microsomes should enable one to investigate the effect of variables such as sex, age, hormonal status and exposure to environmental pollutants on the relative amount of this P-448. Such knowledge may enable prediction of the relative risk of fish population, under certain conditions, to the carcinogenic effects of PAH type pollutants.

Purified NADPH-P-450 reductase from trout differed from the rat enzyme in spectral and immunochemical properties, flavin and

amino acid composition and primary structure. Yet, the trout enzyme was even slightly more effective than the rat reductase in reconstituting rat P-448 EROD activity or reducing cytochrome c.

Finally, reconstitution experiments demonstrated that only the combination of all three trout mfo components into liposomes could reproduce the lower temperature optima characteristic of most fish microsomal mfo activities. These results not only expand the knowledge of comparative properties of purified P-450 and NADPH-P-450 reductase into non-mammalian species, but also suggest that fish may be a useful model for investigation of the structural organization of the mfo system and the molecular events involved in the induction of certain forms of P-450.

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