This is to certify that the

dissertation entitled

INFLUENCE OF POLYHALOGENATED AROMATIC HYDROCARBONS ON THE INDUCTION, ACTIVITY, AND STABILIZATION OF CYTOCHROME P450

presented by

Richard Voorman

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Steven D. Aust
Major professor

Date May 29, 1987

Influence of Polyhalogenated Aromatic Hydrocarbons on the Induction, Activity, and Stabilization of Cytochrome P450

By

Richard Voorman

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1987

ABSTRACT

INFLUENCE OF POLYHALOGENATED AROMATIC HYDROCARBONS ON THE INDUCTION, ACTIVITY, AND STABILIZATION OF CYTOCHROME P450

By

Richard Voorman

In the course of experiments evaluating the metabolism of polybrominated biphenyls by cytochrome P450 isozymes induced by 3,4,5,3',4',5'-hexabromobiphenyl (HBB), it was discovered that the inducer remained closely associated with cytochrome P450d. Subsequent purification of cytochromes from HBB treated rats revealed a 0.5:1 association of HBB to cytochrome P450d but virtually none with cytochrome P450c or cytochrome b5. Immunochemical quantitation of cytochrome P450d in the same microsomes yielded a ratio of P450d:HBB that approached unity. Measurement of cytochrome P450d estradiol 2-hydroxylase indicated non-competitive or mixed type inhibition caused by HBB at a concentration of 10-1000 nM. Inhibition was specific to cytochrome P450d since estradiol 2-hydroxylase catalyzed by cytochrome P450h was unaffected by HBB. Compounds which approximated the dimensions and planarity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypic Ah receptor ligand, were also inhibitors of cytochrome P450d. TCDD was evaluated as a tight-binding inhibitor of cytochrome P450 and found to have of Ki of 8 nM. Estradiol 2-hydroxylase was inhibited by 3,4,5,3',4',5'-hexachlorobiphenyl (HCB) despite a 20 fold

increase in specific content of cytochrome P450d caused by HCB treatment. Thus it appears that these compounds bind to the active site of cytochrome P450d in a specific and essentially irreversible yet non-covalent manner and therefore produce what appears to be non-competitive inhibition of the enzyme.

The ability of HCB and isosafrole to stabilize cytochrome P450d, and thus indirectly influence regulation of the enzyme, was evaluated by treating rats with a dose of TCDD sufficient to produce maximum induction of cytochromes P450c and P450d via the Ah receptor, yet insufficient to bind to the enzyme. Subsequent treatment of these animals with HCB or isosafrole and a radio-labeled amino acid, revealed a significant increase in cytochrome P450d specific content relative to cytochrome P450c and significant retention of the radiolabel in P450d relative to rats treated only with TCDD. This suggests that the ligands are also regulating cytochrome P450d by another mechanism in addition to the Ah receptor, probably enzyme stabilization.

To my wife, Mary, for her love,
support, and patience

ACKNOWLEDGMENTS

First of all, I thank my advisor and mentor, Steven D. Aust, for his guidance, patience, and encouragement, for believing in me when I didn't believe in myself, for all the helpful criticism, and for all the good times we've had over the years. Thanks are also due to the members of my guidance committee, W.C. Deal, S. Ferguson-Miller, R.A. Roth, and J.T. Watson for their time, attention, and suggestions during the course of this research. The help of John Bumpus and Lee Morehouse was especially appreciated in the purification of the cytochrome P450 and P450-reductase. I am especially indebted to Elizabeth Pulsford for her assistance in purification of P450 isozymes and development of the ELISA as well as numerous experiments. For helpful discussions, cooperation, lots of fun (and some grief), my grateful appreciation goes to my laboratory co-workers including Bob Powers, Bob Leedle, Dennis Miller, Dave Reif, and Barry Brock. I am grateful to Dennis Miller and Anne Aust for their work on the influence of P450d in the Ames mutagenesis assay; a subject which lent significance to my work. Last, and most important, I want to express my thanks and heartfelt appreciation to my wife, Mary, for her patience, encouragement, endurance, and continued love and support; without her help, I could not have completed my degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter II

Chapter III

PREFACE

This dissertation is organized into three chapters which are preceeded by a general review of the literature. Each section includes its own set of references and the chapters are written in the form common to most scientific literature, including an abstract, introduction to the topic, methods and materials, results, and discussion. Chapter I is a slightly expanded version of a paper that has been accepted for publication in Toxicology and Applied Pharmacology. Chapter II is being submitted for publication in the Journal of Biological Chemistry, and Chapter III will be submitted to Archives of Biochemistry and Biophysics.

The work proceeds largely from an observation made during experiments on the in vitro metabolism of certain polybrominated biphenyl (PBB) congeners by isozymes of cytochrome P450. The isozymes had been derived from rats which had been treated with a PBB congener to induce synthesis of cytochromes P450c and P450d and it was noted, during analysis of metabolites, that the inducing PBB was associated with cytochrome P450d. Verification and characterization of this interaction is the focus of Chapter I. Experiments described in Chapter I suggested that certain compounds might bind very tightly to cytochrome P450d; this subject is examined in Chapter II using one inhibitor and purified cytochrome P450d. Chapter III examines the effects of two structurally diverse chemicals, isosafrole and

hexachlorobiphenyl, on the catalytic activity and stabilization of cytochrome P450d in rat liver.

In summary, I have found that certain polyhalogenated aromatic hydrocarbons which can serve as ligands for the Ah receptor (a cytosolic protein with high affinity for certain aromatic hydrocarbons) and induce synthesis of cytochromes P450c and P450d can also bind specifically to cytochrome P450d and inhibit its catalytic activity. The binding is essentially irreversible, although noncovalent; it may be similar to the well-described interaction of isosafrole with cytochrome P450d. Furthermore it is possible that the ligands may influence the turnover of cytochrome P450d by inhibiting its degradation.

May 28, 1987

LITERATURE REVIEW

INTRODUCTION

Heme proteins have held a great fascination for biochemists, probably owing to the convenience of a visible protein and the knowledge that the catalytic center is likely centered on heme, a prosthetic group well disposed to spectroscopic examination. The heme protein, cytochrome P450, is found in the mitochondria and endoplasmic reticulum of many tissues in most organisms, from bacteria to humans. Its discovery proceeded largely from work done at the Johnson Research Foundation in Philadelphia (under the direction of B. Chance) on the kinetics and spectroscopy of cytochrome b5 from rat liver microsomes (Chance and Williams, 1954). Klingenberg (1958) noted that there was considerably more pyridine hemochromogen in rat liver microsomes than could be accounted for by cytochrome b5 and, independently with Garfinkel (1958), published the first spectrum of the carbon monoxide binding pigment, which when reduced and complexed with CO yields a peak at 450 nm. Omura and Sato (1962) reported on the protoheme nature of the enzyme and coined the "provisional" term, cytochrome P450, a trivial name which has nevertheless stood the test of time. Because of the unstable nature of the cytochrome in aqueous solutions and the difficulty of purifying membrane-bound proteins, it was several years before the enzyme was partially purified by Lu et al. (1969). Soon after the initial isolation it became

apparent that there were multiple forms of cytochrome P450 and that some of these were inducible by drugs or xenobiotics (Sladek and Mannering, 1966; Welton and Aust, 1974; Thomas et al., 1976). The requirement of heme for the catalytic ability of cytochrome P450 was established by Estabrook et al. (1963) and Cooper et al. (1965) when they published the photochemical action spectra of photo-reversible CO binding and photo-inhibitable substrate metabolism.

Cytochrome P450, a single polypeptide with a molecular weight of 48-56 Kdal, forms the catalytic portion of the microsomal electron transport chain (Figure 1).

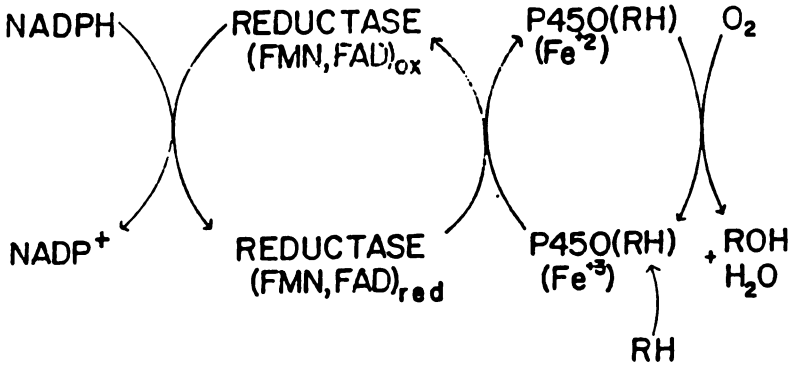


Figure 1. Microsomal electron transport chain.

The most common reaction carried out by cytochrome P450 (and there are many exceptions) is the hydroxylation or monooxygenation of a hydrophobic substrates with a stoichiometry as shown below:

H+ + RH + NADPH + O2 -----> ROH + NADP + H2O

The activation energy barriers are formidable since the molecule of dioxygen must undergo heterolytic cleavage with

incorporation of one atom into the carbon-hydrogen bond, and reduction of the other atom by two electrons to water. Electrons from NADPH are supplied to the enzyme via the membrane-bound flavoprotein, NADPH-cytochrome P450 reductase. Since the electron transport chain resides in the membrane, there is an essential phospholipid component in the interaction between the P450 enzyme and NADPH-P450-reductase, and electron transport.

The term mixed-function oxidase is often applied to cytochrome P450, a reference to the reduction of oxygen to water (oxidase) and oxygenation of the substrate. A substantial problem of nomenclature has developed over the years, such that most names given the enzyme are improper. The enzyme is not a true cytochrome since its primary function involves substrate binding and oxygen activation rather than electron transfer. The wavelength description should include the heme a-band rather than the reduced CO absorption band in the Soret region; even the "450" is incorrect as this can change between 447 and 453 depending on the isozyme. The problem is further compounded in the naming of isozymes, which, according to rules of the Enzyme Commission, should be designated by electrophoretic mobility. Yet isozymes of cytochrome P450 all have Mr values in the range of 48-56 Kdal, and consequently some isozymes are indistinguishable by SDS-PAGE. It has been suggested that isozymes be designated by substrate and position of attack;

however most cytochrome P450 isozymes have variable substrate specificity and no known endogenous substrate.

In this work the terminology of Ryan et al. (1982) will be used in reference to rat liver microsomal cytochrome P450; in that system isozymes are simply designated by a letter suffix in alphabetical order of first isolation. Cytochrome P450a is a constitutive enzyme of rat liver, cytochrome P450b is induced by phenobarbital, cytochromes P450c and P450d are induced by methylcholanthrene. The number of isozymes by this system now extends to cytochrome P450j (Ryan et al., 1985).

CHEMISTRY AND STRUCTURE OF CYTOCHROME P450

The chemistry and structure of the active site of cytochrome P450 have been the focus of most structural investigations. Cytochrome P450 is a b-type cytochrome; that is, its heme consists of iron protoporphyrin-IX bound rather loosely in the active site by a combination of hydrophobic and coulombic forces on the porphyrin ring and a coordinate-covalent bond to the penta- or hexacoordinate iron atom. The metal ion is held in place by four nitrogen ligands in the porphyrin ring and a fifth axial bond to a thiolate, probably from a cysteine in the polypeptide. A potential sixth axial coordination site above the plane of the porphyrin ring, the dioxygen binding site, may be either empty or filled with an occupying ligand such as water or a hydroxyl contributed by

the protein. Crystallographic data are lacking for most P450's since they are membrane-bound proteins and resist crystallization. However Poulos (1986) has recently carried out a structural analysis of the soluble camphor hydroxylating cytochrome P450 from Pseudomonas putida to a resolution of 2.20 A. The substrate-free form shows a hexacoordinate iron with sulfur from Cys-357 on one axial ligand and water or hydroxide as the other axial ligand. The substrate pocket and heme appear to be buried deep inside the enzyme, accessible only by a channel, quite unlike the classic model of an enzyme with active site envisioned as a cleft or depression on the surface. In the substrate-free form the pocket is filled with hydrogen bonded solvent molecules. The substrate, camphor, displaces the solvent molecules and the sixth axial iron ligand and although it does not cause a significant conformational change in protein structure it does bring about large decreases in thermal motions of several regions, probably restricting access to the active site. Upon substrate binding, the heme is converted to a high spin state by displacement of the sixth ligand with concomitant redox potential shift from -300 mV to -173 mV, thus allowing for reduction of heme iron. It is likely that mammalian microsomal cytochrome P450's are structurally similar to the bacterial enzyme since the molecular weights are similar and the sequences share enough homology that they were likely derived from the same ancesteral gene.

SUBSTRATES AND SUBSTRATE SPECIFICITY

The definition of isozyme is used loosely in reference to substrates for cytochrome P450, since isozymes of one enzyme family are confined to one type of reaction and usually on the same substrate; for example the isozymes of lactate dehydrogenase. However the isozymes of cytochrome P450 carry out a variety of reactions on a large number of unrelated substrates.

Steroid Hydroxylases

Among cytochrome P450 isozymes which metabolize steroid hormones, the stringency of substrate binding is very high and the reactions very specific. Waxman (1986) has isolated rat hepatic cholesterol 7a-hydroxylase which is highly specific for that reaction and appears the first and rate-limiting step in conversion of cholesterol into bile acids.

Cytochrome P450 isozymes involved in steroidogenesis are found in both the mitochondria and endoplasmic reticulum of the adrenal gland. The mitochondrial electron transport system consists of the flavoprotein, NADPH-adrenodoxin reductase, and adrenodoxin, an iron-sulfur protein, both located loosely on the matrix side of the inner mitochondrial membrane, while the cytochrome P450 is membrane bound (Takemori and Kominami, 1984). Mitochondrial cytochrome P450scc catalyses side-chain-cleavage of cholesterol, the rate limiting step in steroidogenesis, to form the steroid

nucleus, pregnenolone. This in turn is metabolized in the endoplasmic reticulum by cytochrome P450 17a,lyase. Recently it has been shown that both 17a hydroxylase activity, leading to the formation of cortisol, and 17,20-lyase, which along with the former activity leads to the formation of androstenedione, both reside in the same cytochrome P450 (Zuber et al., 1986). The regulation of these two activities remains to be elucidated. Cytochrome P450-11b, a mitochondrial enzyme, catalyzes the 11b-hydroxylation of either 11-deoxycorticosterone or 11-deoxycortisol leading to the formation of aldosterone and cortisol, respectively.

Liver mitochondria contain a cytochrome P450 dependent on electrons from NADPH-ferredoxin reductase and ferridoxin, to catalyze the 26-hydroxylation of a bile acid intermediate in the formation of cholic acid (Atsuta and Okuda, 1977).

Fatty Acid Hydroxylases

Cytochrome P450 participates in the metabolism of fatty acids by carrying out w, w-1, and w-2 hydroxylation of medium and long chain fatty acids and some prostaglandins. Evidence for physiologic hydroxylation of fatty acids was presented by Das et al. (1968) when they showed that w-hydroxylation of laurate by rat liver microsomes occurred with a Km of 30 uM. This activity also occurs in kidney cortex (Jakobsson et al. 1970) and lung microsomes (Ichihara, 1969). Metabolism of prostaglandins occurs in liver, kidney cortex, adrenals, and

lung microsomes, albeit with species specificity in extrahepatic tissues. It is difficult to determine if these reactions are physiologically relevant. Kupfer et al. (1979) showed that inducers of cytochrome P450 affected these reactions. Although the same group has shown that the position of hydroxylation is dependent on the particular prostaglandin and P450 isozyme, the reaction appears to be relatively fortuitous and non-specific. Prostaglandin E1 is hydroxylated by rabbit cytochrome P450 isozyme 6 (Holm et al., 1985); however the Km is 140 uM and the Vmax is 2.1/min, not very sensitive for a hormone like compound.

Adverse Functions of Cytochrome P450

Although cytochrome P450 appears to function primarily as an oxygenase it can, under the proper conditions, function as a reductase. Certain compounds with highly oxidized substituents such as carbon tetrachloride or halothane can compete with oxygen for electrons from cytochrome P450 and undergo reductive metabolism by cytochrome P450. The facility with which a carbon halogen bond is reduced is dependent on the halogen substituent and the overall structure of the molecule. Carbon tetrachloride can undergo a one electron reduction with loss of chloride to yield the trichloromethyl radical (MacDonald, 1982). This in turn can initiate a radical chain reaction resulting in lipid peroxidation and, given enough substrate, severe liver damage. The anesthetic halothane, is normally metabolized

oxidatively by cytochrome P450. However under conditions of extremely low oxygen partial pressure it can compete with oxygen and be reductively metabolized, resulting in severe membrane damage produced by the halothane-radical initiated chain reaction (Ahr et al., 1982).

Non-Mammalian Cytochrome P450

In plants, cytochrome P450 carries out reactions analogous to those of adrenal steroidogenesis. Murphy and West (1969) showed that the formation of kaurenol in the biosynthesis of gibberllin in cucumber seed endosperm was a cytochrome P450 dependent reaction. Although a detoxification mechanism based on cytochrome P450 might exist in plants, this seems unlikely since plants can simply sequester toxic compounds in vacuoles (Hendry, 1986).

Recently, an unusual cytochrome P450 was isolated from Bacillus megabacterium (Narhi and Fulco, 1986). It is a soluble, catalytically self-sufficient single polypeptide (119 Kdal) which contains a mol each of FMN, FAD, and heme--the reductase apparently resides on the same polypeptide as the cytochrome P450 (microsomal P450 is about 52 kdal and the reductase is 79 kdal). This enzyme has an extremely high activity for fatty acid hydroxylation, 4600/min for palmitate and is inducible by xenobiotics. Since it is soluble and catalytically self-sufficient, it will no doubt provide an excellent model for cytochrome P450 chemistry.

Two narrow-specificity cytochrome P450 isozymes have been isolated from yeast microsomes (Kappeli, 1986). Study of cytochrome P450 in yeasts could be useful, owing to the similarity with mammalian P450 (being microsomal) and the wealth of information on yeast genetics and biochemistry.

Xenobiotic metabolism

The most notable activity of cytochrome P450 is the metabolism of xenobiotics. In this capacity it is a protective mechanism, working in analogy to the immune system but on small non-polar molecules which would reside in lipid membranes and continue to exert a pharmacologic effect were it not for their metabolism by this enzyme. As such, cytochrome P450 hydroxylates the xenobiotic thus making it amenable to conjugation with glucuronide or sulfate followed by excretion of the water soluble conjugate. It is striking that, unlike most enzymes which are substrate-specific and operate only on one or a few substrates of structural similarity, cytochrome P450 can act on a number of structurally diverse substrates. Moreover, the enzymatic activity of certain isozymes is enhanced following administration of certain chemicals to the organism. This inducing activity may result in increased excretion of the agent or, in a few cases, enhanced toxicity of the inducer or other agents. The type and magnitude of induction response is dependent on the inducer and dose.

Hepatic metabolism of xenobiotics was initially studied from the standpoint of both drug inactivation, as in the metabolism of narcotic analgesics (Brodie, 1956), and activation of procarcinogens, as in the demethylation of 3-methyl-4-monomethylaminoazobenzene to render it an hepatic carcinogen (Brown et al., 1954). The latter work led to the discovery that treatment of animals with 3-methylcholanthrene resulted in increased demethylation and increased hepatocarcinogenicity. In a similar fashion it was discovered by Remmer (1961) and Conney and Burns (1959) that barbiturates could stimulate drug metabolism and could decrease hexobarbital sleeping time in animals. It is now known that hundreds of compounds are capable of inducing cytochrome P450 and being metabolized by it; however, unlike the immune system, cytochrome P450 copes with this variation by low substrate specificity rather than by highly specific gene products.

Early work suggested that there were two major forms of cytochrome P450 induced by the prototypic compounds phenobarbital and 3-methylcholanthrene, the latter compound induces enzyme accompanied by an absorption shift to 448 nm and is frequently referred to as cytochrome P448. We now know that cytochrome P450 is composed of many isozymes: phenobarbital induces primarily cytochromes P450 b and e (Ryan et al., 1982), while 3-methylcholanthrene induces cytochrome P450c,d and perhaps a third form (Seidel and Shires, 1986). Cytochromes P450a,f,g, h, and i are

significantly expressed in untreated rats but their levels may rise or fall depending on the inducer (Ryan et al, 1985). Cytochrome P450h is specific to male rats while cytochrome P450i appears to be specific to female rats. The discovery of inducers proceeded largely because they produce significant changes in the total specific content of cytochrome P450. However certain inducers, such as isoniazid which induces cytochrome P450j (Ryan et al., 1985), induce synthesis of one isozyme without significantly altering the total specific content of cytochrome P450.

The isozymic composition of cytochrome P450 in a microsomal sample can be differentiated by immunochemical, enzymatic, or spectrophotometric methods. The latter method is least specific owing to small spectral differences between isozymes but is helpful in regard to certain isozymes (such as the high spin peak and isosafrole complex of cytochrome P450d) and in combination with other methods. Enzymatic charcterization is frequently and successfully used to determine isozyme composition of a microsomal sample. Although there are hundreds of substrates for cytochrome P450, some substrates are restricted to a particular isozyme. In other words, the turnover number for the substrate of one isozyme maybe an order of magnitude or more greater compared to other isozymes. In this regard a few substrates are of note. Benzphetamine, aminopyrine, and pentoxyresorufin are typical diagnostic substrates for dealkylase activity of cytochromes P450 b/e induced by phenobarbital. The enzyme

carries out this activity by hydroxylating the substrate's O or N linked alkyl group which then leaves as the corresponding aldehyde. The so called arylhydrocarbon hydroxylase activity of cytochrome P450c is very specifically measured with benzo-(a)-pyrene or ethoxyresorufin. Cytochrome P450a is specific to testosterone 7a-hydroxylation. As will be discussed later in this thesis, estradiol 2-hydroxylation is carried out most efficiently by the male specific cytochrome P450h and by the inducible cytochrome P450d.

Immunochemical methods are useful in comparing and quantitating isozymes and provide the most direct and potentially most specific probe. The problem is that some isozymes exhibit so much sequence homology that polyclonal antibodies show significant cross reactivity. Cytochromes P450b and P450e cannot be immunochemically quantitated individually because a monospecific polyclonal antibody can not be prepared.

EFFECT OF POLYHALOGENATED AROMATIC HYDROCARBONS ON CYTOCHROME P450

The mechanism of toxicity of the halogenated aromatic hydrocarbons has attracted a great deal of attention over the past 20 or 30 years. These compounds are intriguing because they are chemically rather inert, generally recalcitrant to metabolism, and cause a delayed, although dose-dependent toxicity, the mechanism of which is still not clear. Members

of this group include hexachlorobenzene, polychlorinated- and polybrominated biphenyls and chlorinated dibenzodioxins and dibenzofurans. Most notable among the group is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; see Figure 2). A minor contaminant of herbicide manufacture, it is the most toxic member of the group having an LD50 in the rat of 100 nmol/kg. Poland and Glover (1973) showed that it was a highly effective inducer of cytochrome P450 arylhydrocarbon hydroxylase and that toxicity was highly correlated with enzyme induction. Based on the correlation of toxicity to steric structure of TCDD isomers and diminished toxicity and enzyme inducibility in certain inbred mouse strains, a cytosolic receptor protein was discovered for which TCDD serves as the tightest fitting ligand known. This protein is frequently referred to as the Ah receptor in reference to its connection to arylhydrocarbon hydroxylase (cytochrome P450c). Thus induction of cytochromes P450c and d is thought to be initiated by interaction of appropriate ligands with the receptor. Enzyme induction has been a hallmark of exposure to toxic polyhalogenated aromatic hydrocarbons; but there appears to be no direct relationship between the activity of induced cytochrome P450's and the ensuing toxicity.

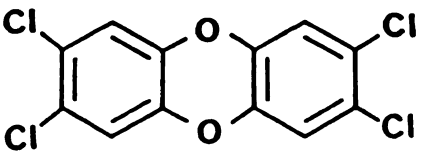
The polyhalogenated aromatic hydrocarbons have been divided into 2 or 3 groups based on biological response. One group is relatively non-toxic, does not bind to the Ah receptor and, at high doses, induces cytochrome P450b typically induced by phenobarbital. An example of this group

is 2,4,5,2',4',5'-hexachlorobiphenyl (Figure 2). The second group, which can be toxic if not metabolized, can serve as ligands for the Ah receptor and induces cytochrome P450c and d. An example of this group is 3,4,5,3',4',5'-hexachlorobiphenyl (Figure 2). A third group is composed of members which show toxicity at high doses, but which induce cytochrome P450 isozymes typically induced by both phenobarbital and methylcholanthrene, so called mixed-type inducers (Dannan et al., 1982); e.g.,2,4,5,3',4',5'-hexachlorobiphenyl (Figure 2). Expression of these characteristics depend on the position and number of halogen substituents, resulting in variable affinity for the Ah receptor and thus appears to depend on the ability of the compound to fit within certain steric restrictions of size and planarity (Poland and Knutson, 1982).

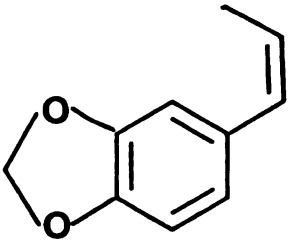
LIGANDS AND INHIBITORS OF CYTOCHROME P450

Methylenedioxyphenyl (MDP) Compounds

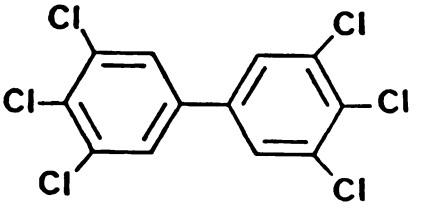
This group of compounds is unusual in that they both induce and inhibit cytochrome P450. Piperonyl butoxide is used as an insecticide synergist and functioned in this capacity by inhibiting insectide inactivation by cytochrome P450 (Casida et al., 1966). When used in vitro, piperonyl butoxide can inhibit many microsomal cytochrome P450 reactions including acetaminophen metabolism and aniline hydroxylation (Mitchell et al., 1973). On the other hand,



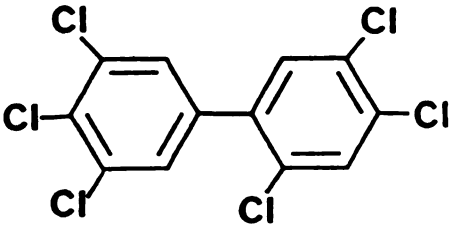
2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD)



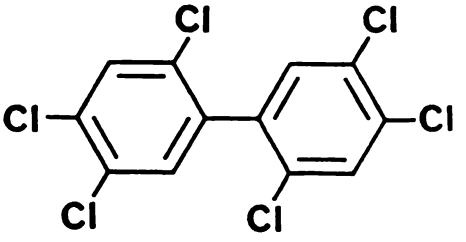
ISOSAFROLE



3,4,5,3',4',5'-HEXACHLORO-BIPHENYL (HCB)



2,4,5,3',4',5'-HEXACHLORO-BIPHENYL



2,4,5,2',4',5-HEXACHLORO-BIPHENYL

Figure 2. Representative aromatic hydrocarbons.

administration of piperonyl butoxide to mice results in increased metabolism of hexobarbital and increased cytochrome P450 levels (Matthews et al., 1970). Thus, although it can inhibit cytochrome P450, it also has the capacity to induce cytochrome P450 and increase xenobiotic metabolism as well, suggesting that MDP compounds can be displaced by some substrates. The inhibition produced by MDP compounds is of a dual nature consisting of both competitive inhibition, resulting from competition of the MDP compound with the substrate for the enzyme, and non-competitive inhibition, due to formation of a nearly irreversible complex with the enzyme (Philpot and Hodgson, 1972). The latter activity is apparently the result of suicide inhibition, where the enzyme, in an attempt to metabolize the MDP compound, activates it to a reactive metabolite which forms a covalent bond to the enzyme forming a metabolite-intermediate complex. Safrole is a constituent of sassafras oil and its isomer, isosafrole (Figure 2), is one of the most intensely studied MDP compounds. The isosafrole metabolite-intermediate complex is manifested optically in the Soret region by absorption peaks at 426 and 455 nm, in the dithionite-reduced difference spectrum, owing to its interaction with heme. The interaction of the metabolite with heme is relatively weak since it can be displaced by a variety of ligands (Dickens et al., 1979). It is thought that metabolism proceeds by hydroxylation of the methylene group yielding an unstable product analogous to an ortho-ester (Anders et al., 1984)

which rearranges and produces either a catechol (loosing carbon monoxide) or a carbene. Carbenes are known to interact with ferrous heme and it has been shown that a carbene derived from 1,3-benzodioxole forms a complex with ferrous-tetraphenylporphyrin and produces a Soret region spectrum very much like that observed for the cytochrome P450 metabolite-intermediate complex (Mansuy et al., 1979). Mechanistically, it appears that cytochrome P450 metabolizes MDP compounds by hydroxylation; the product then rearranges to the carbene, and binds to heme iron, inhibiting or inactivating the enzyme. The stability of the MDP-carbene-P450 complex probably varies with different isozymes of cytochrome P450 since the complex formed in vitro with rabbit cytochrome P450 isozymes tends to degrade over time (Delaforge et al., 1982) whereas in rats it is more stable (Ryan et al., 1980). This probably results from a property of the active site of cytochrome P450 where the substrate might be held in place by various hydrophobic interactions in addition to ligation to heme iron. Indeed, more carbon monoxide is formed upon incubation of isosafrole with microsomes from phenobarbital treated rats than those from 3-methylcholanthrene treated rats even though isosafrole can form the metabolite intermediate complex with both (Yu et al., 1980). Substitution with one or two methyl groups on the methylene carbon prevents induction of cytochrome P450 in treated rats and also prohibits formation of the metabolite-intermediate complex in vitro (Cook and Hodgson, 1983).

Aromatic Hydrocarbons

The naturally occurring flavonoids have been extensively studied as inhibitors of cytochrome P450. In particular 5,6- and 7,8-benzoflavone have been widely used as inhibitors of cytochrome P450c (referred to as P448 in the early literature), and were frequently used diagnostically to indicate a P448 activity. Some have recently been shown to be very potent inhibitors not only of cytochrome P450c (Sousa and Marletta, 1985) but also of the substrate-specific estrogen synthase or aromatase (Kellis and Vickery, 1984).

Cytochrome P448-1 (equivalent to cytochrome P450d) isolated from 3-methylcholanthrene treated rabbits contained up to 0.88 mol 3-methylcholanthrene per mol P450 and was apparently tightly bound such that 3-methylcholanthrene fluorescence was quenched (Imai et al., 1980). This and related compounds were shown to bind in vitro to P450 in such a way that circular dichroism spectra were significantly changed upon addition of ligand.

REGULATION OF CYTOCHROME P450

Catalytic activity of cytochrome P450 in the cell is directly proportional to the amount of polypeptide present, thus changes in the level or activity of cytochrome P450 are thought to be mediated at the level of transcription. Specific chemicals or hormones selectively increase synthesis

of one or more forms of cytochrome P450 through presumably specific, though largely unknown, mechanisms.

Steroidogenic cytochromes P450 appear to be under the control of cAMP which in turn is under the control of either ACTH or FSH depending on the cell type (reviewed in Whitlock, 1986). Intracellular regulation by cAMP is probably indirect via another receptor protein which then interacts with DNA.

Hepatic microsomal cytochrome P450p, while expressed constitutively at significant levels, is induced by pregnenolone-16a-carbonitrile and glucocorticoid-like compounds such as dexamethasone (Gonzalez et al, 1985). The level of this enzyme is increased to very high levels in response to certain macrolide antibiotics. These compounds form a stable metabolic-intermediate complex with the reduced form of the enzyme, similar to isosafrole and cytochrome P450d, and are presumed to increase the level of the enzyme by inhibiting its degradation (Watkins et al., 1986).

The control of isozyme induction by phenobarbital remains a mystery. To date there has been no evidence of a receptor for phenobarbital-induced cytochrome P450, in contrast to the steroid-and aryl hydrocarbon induced enzymes. Indirect induction by accumulation of an unknown endogenous substrate was recently tested by development of a specific suicide substrate for cytochrome P450b. However, although the substrate fully inhibited the enzyme, there was no increase in cytochrome P450b mRNA or protein (Ortiz de Montellano and Costa, 1986).

As mentioned earlier, cytochromes P450c and P450d appear to be controlled by the Ah receptor, a cytosolic protein for which a number of aromatic hydrocarbons have an unusually high affinity. Polypeptide and mRNA for cytochrome P450c are virtually undetectable in untreated rats whereas cytochrome P450d is expressed to a level of about 5% of total P450 in microsomes from untreated rats (Ryan et al., 1982). Both isozymes are induced 20 to 80 fold in rats. Evidence suggests that unmodified substrate binds to the receptor, translocates to the nucleus, interacts with a regulatory segment of DNA and tiggers production of mRNA for P450c and P450d or their species equivalent (Whitlock, 1986). Isosafrole induces primarily cytochrome P450d and some P450c and although increased levels of mRNA were detected in response to isosafrole (Kawajiri et al., 1984) it does not appear to serve as a ligand for the Ah receptor (Cook and Hodgson, 1985). It produces synergistic induction of cytochrome P450d with the Ah receptor ligand, 3-methylcholanthrene (Thomas et al., 1983), and evidence suggests that it might have a stabilizing effect on cytochrome P450d (Steward et al., 1985).

REFERENCES

Atsuta, Y., Okuda, Y. (1978). Isolation of rat liver mitochondrial ferredoxin and its reductase active in the 5b-cholestane-3a,12a-triol 26-hydroxylase. J. Biol. Chem. 253, 4653-4658.

Ahr, H.J., King, L.J., Nastainczyk, W., Ullrich, V. (1982). The mechanism of reductive dehalogenation of halothane by liver cytochrome P450. Biochem. Pharmacol. 31, 383-392.

Anders, M.W., Sunram, J.M., Wilkinson, C.F. (1984). Mechanism of the metabolism of 1,3-benzodioxoles to carbon monoxide. Biochem. Pharmac. 33, 577-580

Brodie, B.B. (1956). Pathways of drug metabolism. J. Pharm. Pharmac. 8, 1-17.

Brown, R.R., Miller, J.A., Miller, E.C. (1954). The metabolism of methylated aminoazo dyes IV. Dietary factors enhancing demethylation in vitro. J. Biol. Chem. 209, 211-222.

Casida, J.E., Engel, J.L., Essac, E.G., Kamienski, F.X., Kuwatsuka S. (1966). Methylene-14C-dioxyphenyl compounds: Metabolism in relation to their synergistic action. Science 153, 1130-1133.

Chance, B., Williams, G.R. (1954). Kinetics of cytochrome b5 in rat liver microsomes. J. Biol. Chem. 209, 945-951.

Conney, A.H., Burns, J.J. (1959). Stimulatory effect of foreign compounds on ascorbic acid biosynthesis and on drug-metabolizing enzymes. Nature 184, 363-364.

Cook, J.C., Hodgson, E. (1983). Induction of cytochrome P450 by methylenedioxyphenyl compounds: importance of the methylene carbon. Toxicol. Appl. Pharmacol. 68, 131-139.

Cook, J.C., Hodgson, E. (1985). The induction of cytochrome P450 by isosafrole and related methylenedioxyphenyl compounds. Chem-Biol. Interact. 54, 299-315.

Cooper, D.Y., Levin, S., Narisimhulu, S., Rosenthal, O., Estabrook, R.W. (1965). Photochemical action spectrum of the termical oxidase of mixed function oxidase4 systems. Science 147, 400-402.

Dannan, G.A., Guengerich, F.P., Kaminsky, L.S., Aust, S.D. (1983). Regulation of cytochrome P450: immunochemical quantitation of eight isozymes in liver micrsomes of rats treated with polybrominated biphenyl congeners. J. Biol. Chem. 258, 1282-1288.

Dannan, G.A., Sleight, S.D., Aust, S.D. (1982). Toxicity and microsomal enzyme induction effects of several polybrominated biphenyls of Firemaster. Fund. Appl. Toxicol. 2, 313-321.

Das, M.L., Orrenius, S., Ernster, L. (1968). On the fatty acid and hydrocarbon hydroxylation in rat liver microsomes. Eur. J. Biochem. 4, 519-523.

Delaforge, M., Koop, D.R., Coon, M.J. (1982). Role of isosafrole as complexing agent and inducer of P450LM4 in rabbit liver microsomes. Biochem. Biophys. Res. Comm. 108, 59-65.

Dickens, M., Elcombe, C.R., Moloney, S.J., Netter, K.J., Bridges, J.W. (1979). Further studies on the dissociation of the isosafrole metabolite-cytochrome P450 complex. Biochem. Pharmac. 28, 231-238.

Estabrook, R.W., Cooper, D.Y., Rosenthal, O. (1963). The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex. Biochem. Z. 338, 741-755.

Garfinkel, D. (1958). Studies on pig liver microsomes I. Enzymatic and pigment composition of different microsomal fractions. Arch. Biochem. Biophys. 77, 493-509.

Gonzalez, F.J., Nebert, D.W., Hardwick, J.P., Kasper, C.B. (1985). Complete cDNA and protein sequence of a pregnenolone 16a-carbonitrile-induced cytochrome P450. J. Biol. Chem. 260, 7435-7441.

Hendry, G. (1986). Why do plants have cytochrome P450? Detoxification versus defence. New Phytol. 102, 239-247.

Hodgson, E., Philpot, R.W. (1975). Interaction of methylenedioxyphenyl (1,3-benzdioxole) compounds with enzymes and their effects on mammals. Drug Metab. Revs. 3, 231-301.

Holm, K.A., Koop, D.R., Coon, M.J., Theoharides, A.D., Kupfer, D. (1985). w-1 and w-2 Hydroxylation of prostaglandins by rabbit hepatic microsomal cytochrome P450 isozyme 6. Arch. Biochem. Biophys. 243, 135-143.

Ichihara. K., Kusunose. E., Kusunose. M. (1969). Some properties and distribution of the w-hydroxylation system of medium-chain fatty acids. Biochem. Biophys. Acta. 176, 704-712.

Imai, Y., Hashimoto-Yutsudo, C., Satake, H., Girardin, A., Sato, R. (1980). Multiple forms of cytochrome P450 purified from liver microsomes of phenobarbital- and 3-methylcholanthrene-pre-treated rabbits I. Resolution,

Purification, and molecular properties. J. Biochem. 88, 489-503.

Imai, Y. (1982). Interaction of polycycli hydrocarbons with cytochrome P450. I. Specific binding of various hydrocarbons to P448 1. J. Biochem. 92, 57-66.

Jakobsson, S., Thor, H., Orrenius, S. (1970). Fatty acid inducible cytochrone P454 of rat kidney microsomes. Biochem. Biophys. Res. Comm. 39, 1073-1080.

Kappeli, O. (1986). Cytochromes P450 of yeasts. Microbiol. Rev. 50, 244-258.

Klingenberg, M. (1958). Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75, 376-386.

Kupfer, D., Miranda, G.K., Navarro, J., Piccolo, D.E., Theoharides, A.D. (1979). Effect of inducers of and inhibtors of monooxygenase on the hydroxylation of prostaglandins in the guinea. Evidence for several monooxygenases catalyzing w- and (w-1)-hydroxylation. J. Biol. Chem. 254, 10405-10414.

Lu, A.Y.H., Junk, K., Coon, M.J. (1969). Resolution of the cytochrome P450-containing w-hydroxylation system of liver microsomes into three components. J. Biol. Chem. 244, 3714-3721.

MacDonald, T. (1982). Chemical mechanism of halocarbon metabolism. Crit. Rev. Toxicol. 11, 85-120.

Matthews, H.B., Skrinjaric-Spoijar, M., Casida, J.E. (1970). Insecticide synergist interactions with cytochrome P450 in mouse liver microsomes Life Sci. 9, 1039-1048.

Michell, J.R., Jollow, D.J., Potter, W.Z., Davis, C.D., Gillette, J.R., Brodie, B.B. (1973). Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J. Pharmac. Exp. Therap. 187, 185-194.

Murphy, P.J., West, C.A. (1969). The role of mixed function oxidases in kaurene metabolism in Echinocystis macrocarpa Greene endosperm. Arch. Biochem. Biophys. 133, 395-407.

Narhi, L.O., Fulco, A.J. (1986). Characterization of catalytically self-sufficient 119,000-dalton cytochrome P450 monooxygenase induced by barbiturates in Bacillus megabacterium. J. Biol. Chem. 261, 7160-7169.

Omura, T., Sanders, E., Estabrook, R.W., Cooper, D.Y., Rosenthal, O. (1966). Isolation from adrenal cortex of a non-heme iron protein and a flavoprotein functional as a reductase triphosphopyridine nucleotide cytochrome P450 reductase. Arch. Biochem. Biophys. 117, 660-673.

Omura, T., Sato, R. (1962). A new cytochrome in liver microsomes. J. Biol. Chem. 237, 1375-1376.

Ortiz de Montellano, P.R., Costa, A.K. (1986). Dissociation of cytochrome P450 inactivation and induction. Arch. Biochem. Biophys. 251, 514-524.

Philpot, R.M., Hodgson, E. (1972). The effect of piperonyl butoxide concentration on the formation of cytochrome P450 difference spectra in hepatic microsomes from mice. Mol. Pharmacol. 8, 204-214.

Poland, A., Glover, E. (1973). Chlorinated dibenzo-p-dioxins: Potent inducers of delta-aminolevulinic acid synthase and aryl hydrocarbon hydroxylase II. A study in structure-activity relationship. Mol. Pharmac. 9, 736-747.

Poland, A., Knutson, J.C. (1982). 2,3,7,8-tetrachlorocibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol. 22, 517-554.

Poulos, T.L., Finzel, B.C., Howard, A.J. (1986). Crystal structure of substrate-free Pseudomonas putida cytochrome P450. Biochemistry 25, 5314-5322.

Suhara, K. (1978). Cytochrome P450-containing oxygenase systems: bacterial systems In: Sato, R., Omura, T. Cytochrome P450. Kodansha Academic Press 184-194.

Remmer, H. (1961). Drugs as activators of drug enzymes. In: Proceedings fo the First International Pharmacological Meeting: Vol 6, Metabolic factors controlling duration of drug action. Brodie, B.B., Erdos, E.G. (eds) Macmillan, New York

Ryan, D.E., Ramanathan, L., Iida, S., Thomas, P., Haniu, M., Shively, J.E., Lieber, C.S., Levin, W. (1985). Characterization of a major form of rat hepatic microsomal cytochrome P450 induced by isoniazid. J. Biol. Chem. 260, 6385-6393.

Ryan, D.E., Thomas, P.E., Levin, W. (1980). Hepatic microsomal cytochrome P450 from rats treated with isosafrole: purification and characterization of four enzymic forms. J. Biol. Chem. 255, 7941-7955.

Ryan, D.E., Thomas, P.E., Reik, L.M., Levin, W. (1982). Purification, characterization and regulation of five rat hepatic microsomal cytochrome P450 isozymes. Xenobiotica 12, 727-744.

Seidel, S.L., Shires, T.K. (1986). Purification and characterization of a previously unreproted form of cytochrome P448 from the liver of 3-methylcholanthrene-pretreated rats. Biochem. J. 235, 859-868.

Sladek, N.E,, Mannering, G.J. (1966). Evidence for a new P450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Comm. 24, 668-674.

Steward, A.R., Wrighton, S.A., Pasco, D.S., Fagan, J.B., Li, D., Guzelian, P.S. (1985). Synthesis and degradation of 3-methylcholanthrene-inducible cytochromes P450 and their mRNAs in primary monolayer cultures of adult hepatocytes. Arch. Biochem. Biophys. 241, 494-508.

Takemori, S. (1978). Cytochrome P450-containing oxygenase systems: Adrenocortical mitochondrial systems. In: Sato, R., Omura, T. Cytochrome P450. Kodansha Academic Press 164-184.

Takemori, S., Kominami, S. (1984). The role of cytochromes P-450 in adrenal steroidogenesis. Trends Biochem. Sci. 9, 393-394.

Thomas, P.E., Lu, A.Y.H., Ryan, D., West, S.B., Kawalek, J., Levin, W. (1976). Multiple forms of rat liver cytochrome P450. Immunochemical evidence with antibody against cytochrome P448. J. Biol. Chem. 251, 1385-1391.

Thomas, P.E., Reik, L.M., Ryan, D.E., Levin, W. (1983). Induction of two immunochemically related rat liver cytochrome P450 isozymes, cytochromes P450c and P450d, by structurally diverse xenobiotics. J. Biol. Chem. 258, 4590-4598.

Watkins, P.B., Wrighton. S.A., Schuetz, E.G., Maurel, P., Guzelian, P.S. (1986). Macrolide antibiotics inhibit the degradation of the glucorticoid-responsive cytochrome P450p in rat hepatocytes in vivo and in primary monolayer culture. J. Biol. Chem. 261, 6264-6271.

Waxman, D.J. (1986). Rat hepatic cholesterol 7a-hydroxylase: biochemical properties and comparison to constitutive and xenobiotic inducible cytochrome P450 enzymes. Arch. Biochem. Biophys. 247, 335-345.

Welton, A.F., Aust, S.D. (1974). Multiplicity of cytochrome P450 hemoproteins in rat liver microsomes. Biochem. Biophys. Res. Comm. 56, 898-906.

Whitlock, J.P.,Jr. (1986). The regulation of cytochrome P450 gene expression. Ann. Rev. Pharmacol. Toxicol. 26, 333-369.

Yu, L.S., Wilkinson, C.F., Anders, M.W. (1980). Generation of carbon monoxide during the microsomal metabolism of methylenedioxyphenyl compounds. Biochem. Pharmac. 29, 1113-1122.

Zuber, M.X., Simpson, E.R., Waterman, M.R. (1986). Expression of bovine 17a-hydroxylase cytochrome P450 cDNA in nonsteroidogenic (COS 1) cells Science 234, 1258-1261.

Zuber, M.X., Maliyaki, J.E., Okamura, T., Simpson, E.R., Waterman, M.R. (1986). Bovine adrenocortical cytochrome P450-17a: regulation of gene expression by ACTH and elucidation of primary sequence. J. Biol. Chem. 261, 2475-2482.

CHAPTER I

SPECIFIC BINDING OF POLYHALOGENATED AROMATIC HYDROCARBON INDUCERS OF CYTOCHROME P450d TO THE CYTOCHROME AND INHIBITION OF ITS ESTRADIOL 2-HYDROXYLASE ACTIVITY

ABSTRACT

Treatment of male Sprague-Dawley rats with 3,4,5,3',4',5'-hexabromobiphenyl (HBB) at 10 μmol/kg followed by purification of hepatic microsomal cytochrome P450d revealed that HBB remained specifically bound to P450d throughout purification. Binding was noncovalent since HBB was removed by extraction with dichloromethane. Although HBB induced both cytochrome P450c and P450d, specific immunoprecipitation of these isozymes from HBB treated rats showed that HBB was associated only with cytochrome P450d. Quantitation of HBB and cytochrome P450d in microsomes from HBB treated rats suggested a 0.9:1 ratio of HBB to cytochrome P450d. Five other halogenated aromatic hydrocarbon inducers of cytochrome P450d, bearing steric similarity to HBB (including 2,3,7,8-tetrachlorodibenzo-p-dioxin), were associated with cytochrome P450d when used to induce cytochrome P450d in rats. HBB inhibited estradiol 2-hydroxylase activity of purified cytochrome P450d in a non-competitive manner with an I_{50} of 38 nM for 50 nM P450d whereas its non-coplanar isomer, 2,4,5,2',4',5'-hexabromobiphenyl, had an I_{50} over 700 fold higher. Thus certain polyhalogenated aromatic hydrocarbons with the capacity to induce cytochrome P450d also bind to the cytochrome when used as inducing agents and inhibit catalytic activity of the cytochrome.

INTRODUCTION

Cytochrome P450 represents a family of isozymes that catalyze the monooxygenation of numerous substrates, including drugs and some physiologic substrates. The broad substrate specificity of the system and its involvement in toxicity and carcinogenicity has led to the resolution and characterization of the different forms of cytochrome P450 in several species, including humans. The concentrations of some cytochrome P450 isozymes increase dramatically in various tissues in response to treatment of animals with certain chemicals. Cytochromes P450c and P450d (cytochrome P448 isozymes) are induced by many toxic xenobiotics such as 3-methylcholanthrene and certain polyhalogenated aromatic hydrocarbons (PHAH) (Parkinson et al., 1983). These isozymes appear to be coordinately induced but differentially regulated, and although they share some sequence homology they have different physical characteristics (Reik et al., 1982).

Many compounds are able to inhibit specific cytochrome P450 isozymes by binding to active sites in either a covalent or non-covalent manner (Walsh, 1984; Sousa and Marletta, 1985). For example, metyrapone inhibits metabolism catalyzed by the phenobarbital inducible cytochrome P450 isozymes (Hildebrandt et al., 1969) while α-naphthoflavone inhibits the MC inducible isozymes (Weibal et al., 1971). The binding of both inhibitors and substrates is typically and conveniently measured by

spectral changes in the enzyme brought about by low to high spin state transitions of the heme iron; cytochrome P450d, however, exists largely in a high spin state (White and Coon, 1982).

Isosafrole induces cytochrome P450d and a metabolite of Isosafrole is bound to the cytochrome giving rise to a characteristic metabolite-inhibitor complex of the reduced cytochrome with a Soret peak at 455 nm (Ryan et al., 1980). This is probably a result of metabolite coordination to the 6th axial position on ferrous heme (Mansuy et al., 1979). Methylcholanthrene induces rabbit cytochrome P450 LM_4 (a high spin isozyme analogous to P450d) and binds tightly to the cytochrome P450 LM_4 such that Methylcholanthrene fluorescence is quenched (Imai et al., 1980).

We found that 3,4,5,3'4'5'-hexabromobiphenyl (HBB) and five sterically similar compounds were associated with cytochrome P450d isolated from the livers of rats which had been treated with the chemicals to induce cytochrome P450d. The binding cannot be measured spectrally, but the compounds inhibit estradiol 2-hydroxylase activity catalyzed by the cytochrome.

METHODS AND MATERIALS

Materials: 17β-[2-^{3}H]Estradiol was purchased from New England Nuclear (Boston, MA). 2',5'-ADP-Sepharose was purchased from Pharmacia (Piscataway, NJ). ω-Amino-n-octyl-Sepharose (AO-Sepharose) was prepared by

coupling 1,8-diaminooctane (Sigma, St. Louis, MO) to Sepharose 4B following cyanogen bromide activation as described (March et al., 1974). Cholic acid (Sigma) was decolorized with charcoal, recrystallized from 50% ethanol, converted to the sodium salt and used as a 20% solution (w/v based on cholic acid) for AO-Sepharose chromatography. A 20% solution of sodium cholate (w/v) not recrystallized was used for the remaining chromatography steps. Hydroxylapatite (Hypatite C) was purchased from Clarkson Chemical (Williamsport, PA). Protein A-Sepharose was obtained from Sigma and DEAE-cellulose (DE-52) was obtained from Whatman (Clifton, NJ). Goat anti-rabbit-peroxidase complex was purchased from Cappel Labs (Malvern, PA). Crude 3,4,5,3',4',5'-hexabromobiphenyl was obtained from Ultra Scientific, Inc. (Hope, RI) and purified by alumina column chromatography (Millis, 1984). 2,4,5, 2'4'5'- and 2,4,5,3',4',5'-Hexabromobiphenyl were purified from Firemaster BP-6 (lot 6224-A, Michigan Chemical Corp., St. Louis, MI) as described by Dannan et al., 1982. 3,4,5,3',4'-Pentachlorobiphenyl was kindly supplied by Dr. Stephen Safe (Texas A & M University, College Park, TX) and 3,4,5,3',4',5'-hexachlorobiphenyl was purchased from Pathfinder Labs (St. Louis, MO). TCDD was generously donated by Dow Chemical Co. (Midland, MI). Emulgen 911 was obtained from Kao Corporation (Tokyo). The bicinchoninic acid protein assay reagent was purchased from Pierce Chemical (Rockford, IL).

Animal Treatment and Microsomal Preparation: Male Sprague-Dawley rats (Charles River, Portage, MI) weighing 150-250 g were treated by oral gavage with the various PHAH dissolved in corn oil and killed 3 days later; or with ISF (150 mg/kg/day) for 3 days and killed 24 hours following last dose. Rats were starved overnight and killed by decapitation following CO_2 anesthesia; livers were homogenized in 1.15% KCl, 10 mM EDTA, pH 7.4 (1:4, w/v). Microsomes were prepared by differential centrifugation, (20,000 xg for 30 min, followed by 100,000 xg for 90 min) washed with 100 mM sodium pyrophosphate, pH 7.4, containing 0.1 mM EDTA, pelleted at 100,000 xg for 90 min, and suspended in 20 mM potassium phosphate, pH 7.4, containing 20% glycerol and 0.1 mM EDTA and stored at -20°C.

Purification of Cytochromes: Cytochrome b_5 was purified according to published methods (Imai, 1976; Spatz and Strittmatter, 1971) to specific content of 32 nmol/mg protein. Epoxide hydrolase was purified to electrophoretic homogeneity as described (Guengerich and Martin, 1980). Cytochromes P450c and P450d were purified by a combination of procedures used by others (Guengerich et al., 1982; Astrom and DePierre, 1985; Imai et al., 1980). Unless otherwise noted, all manipulations were done at 4°C. Microsomes were diluted to 1.5 mg protein/ml in 100 mM potassium phosphate, pH 7.4, containing 20% glycerol and 1

mM EDTA (100mM PGE). Recrystallized cholate was added slowly to 0.8% (w/v). Approximately 3000 nmol of cytochrome P450 were loaded on a 2.5 x 40 cm AO-Sepharose column which had been previously equilibrated with three bed volumes of 100mM PGE containing 0.6% cholate. The column was washed with 2 bed volumes of 100 mM PGE 0.4% cholate and eluted with 100 mM PGE containing 0.3% cholate and 0.1% Lubrol PX (Figure 1). Peak fractions of cytochrome P450 were pooled, dialyzed against 20% glycerol followed by dialysis against 10 mM potassium phosphate, pH 7.6, containing 20% glycerol and 0.1 mM EDTA, (designated 10 mM PGE) with 0.2% sodium cholate and 0.1% Lubrol PX. The cytochrome P450 was loaded on a DEAE-cellulose column (2.5 x 15 cm) which had been previously equilibrated with 10 mM PGE containing 0.5% cholate and 0.2% Lubrol PX. A small band of mixed hemoproteins was eluted from the column with 2 bed volumes of equilibration buffer and a major band enriched in P450d migrated halfway down the column. A 400 ml linear gradient of 0-200 mM NaCl containing the same buffer was applied to elute cytochrome P450d followed by cytochrome P450c (Figure 2). The cytochrome P450-enriched fractions were dialyzed against 30 mM potassium phosphate, pH 7.25, containing 20% glycerol, 0.1 mM EDTA, 0.2% Lubrol PX and separately applied to hydroxylapatite columns (2.5 x 10 cm) equilibrated with the same buffer. After washing with 2 bed volumes of this buffer, cytochrome P450c was eluted with the same buffer but containing 90 mM phosphate while P450d eluted with the same

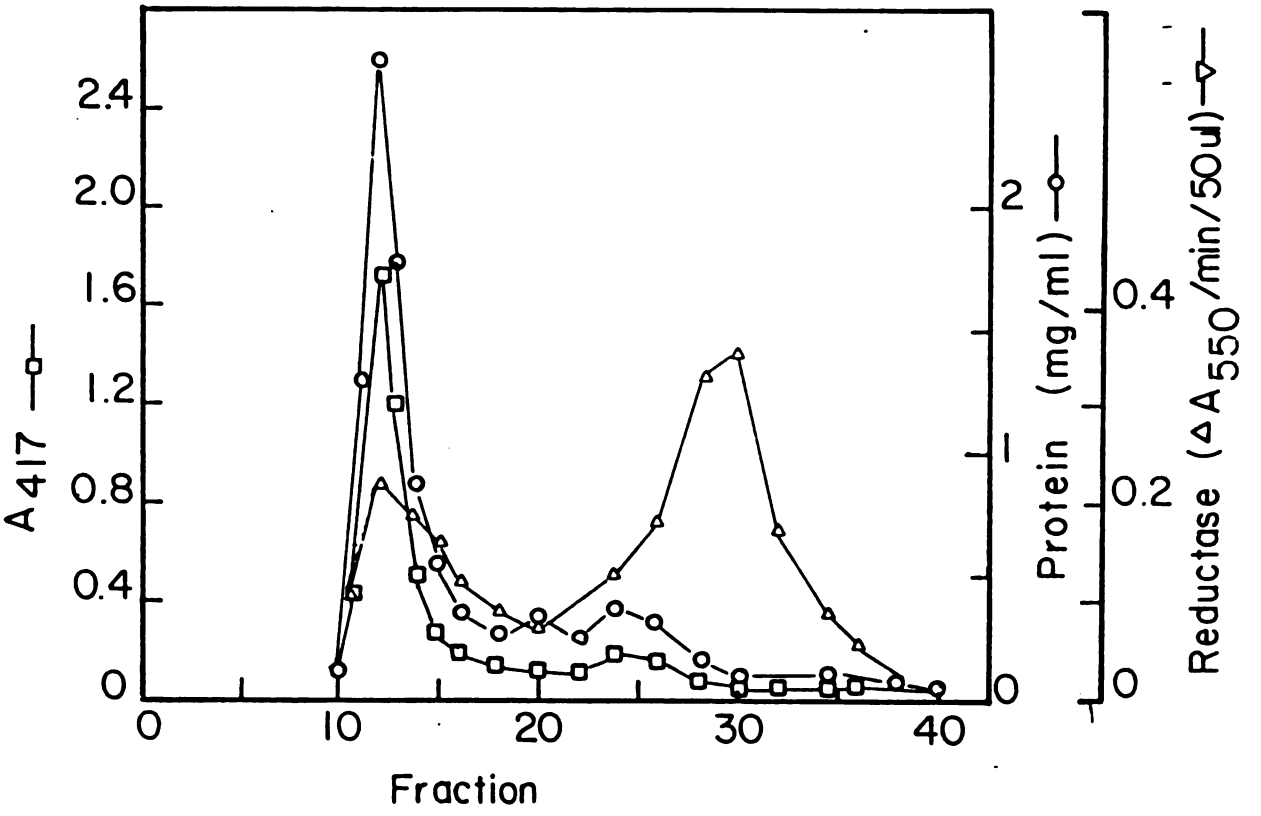


Figure 1. Aminooctyl Sepharose (AO-Sepharose) chromatography of cholate-solubilized rat liver microsomes from isosafrole-treated rats. Microsomes were prepared and loaded onto column as described in "Methods." Following column washing, cytochrome P450 was eluted with 100 mM potassium phosphate, pH 7.4, containing 20% glycerol, 1 mM EDTA, 0.3% cholate and 0.1% Lubrol PX. 20 ml fractions were collected. Cytochrome P450 was monitored by A_{417}; cytochrome P450 reductase was monitored by reduction of cytochrome C by ΔA_{550}. Protein was determined by the BCA method (see "Methods").

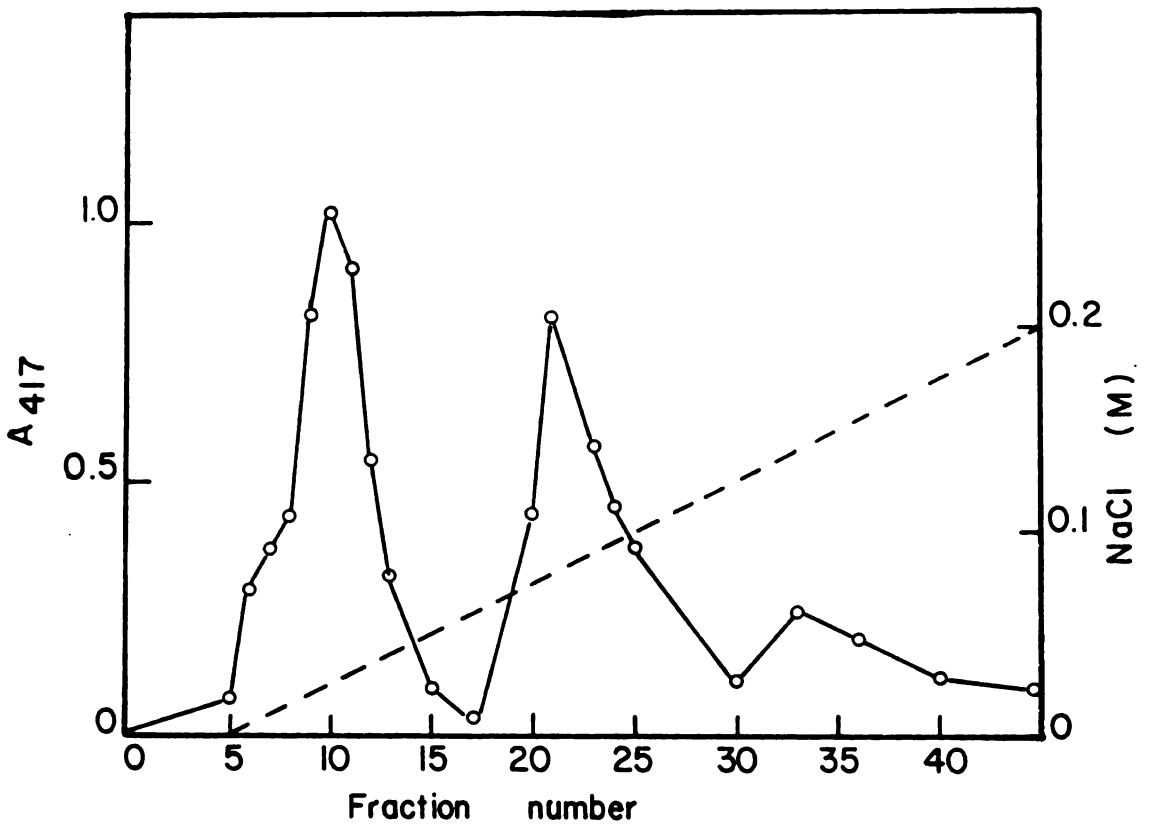


Figure 2. DEAE-chromatography of the cytochrome P450 fraction from an AO-Sepharose column. The dialyzed sample was loaded onto a DEAE-cellulose column, washed and eluted with a gradient of 0-200 mM NaCl in 10 mM PGE containing 0.5% cholate, 0.2% Lubrol PX. 7 ml fractions were collected.

buffer containing 180 mM phosphate and 0.2% cholate. Rechromatography of the peak fractions on either hydroxylapatite or DEAE-cellulose resulted in an electrophoretically homogeneous protein solution with specific content of 12-18 nmol/mg protein (Figure 3). Detergent was removed from purified cytochrome P450 by adsorbing cytochrome P450 on a small (1.5 x 5 cm) hydroxylapatite column equilibrated with 30 mM potassium phosphate, pH 7.25 and containing 20% glycerol, 0.1 mM EDTA, 0.2% cholate and washing with ten bed volumes of this buffer to remove Lubrol PX (as measured by the method of Garewal, 1973). Cytochrome P450 was eluted from the column with 300 mM potassium phosphate, pH 7.25, containing 20% glycerol, 0.1 mM EDTA, and 0.6% cholate,dialyzed against 100 mM PGE, and stored at -70°C.

Butanol (250 mM) was used to displace the isosafrole metabolite from cytochrome P450d by a method similar to that described by Fisher et al. (1981). White and Coon (1982) showed that oxygen was likely the bonding atom of the axial ligand of heme iron trans to thiolate and furthermore showed that oxygen of n-butanol could coordinate to heme of cytochrome $P450_{LM4}$, a high spin isozyme in the rabbit, and convert it to low spin. Thus, it seemed likely that such a compound might displace the isosafrole metabolite; the oxygen of butanol displacing the metabolite coordination to heme and the hydrophobic tail of butanol aiding in displacement of the metabolite from the active site. The

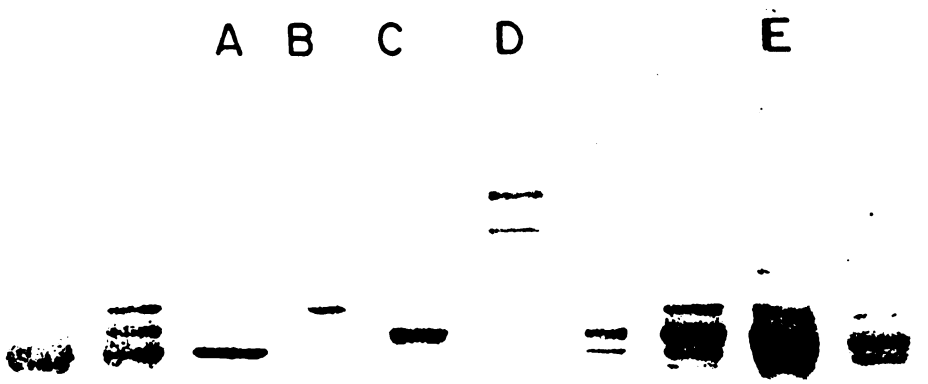


Figure 3. SDS-polyacrylamide gel electrophoretogram. Slab gel (15 x 20 cm x 0.75 mm) was prepared, run, and stained according to the method of Laemmli (1970) except that arylamide concentration was 7.5%. (A) epoxide hydrase; (B) cytochrome P450c; (C) cytochrome P450d; (D) detergent solubilized reductase (upper band), protease solubilized reductase (lower band); (E) partially pure cytochrome P450 from AO-Sepharose fraction.

metabolite was usually displaced before purification of P450d by incubating n-butanol with the microsomes at 37°C for 30 min prior to addition of cholate and passage through AO-Sepharose. If metabolite-free microsomes were needed, the butanol treated microsomes were passed through a 1.5 x 30 cm Sepharose CL-6B column in 100 mM PGE (Figure 4).

Assay Methods: Catalytic activity of cytochrome P450 was measured under conditions in which product formation was proportional to enzyme concentration and incubation time. Metabolic activity was reconstituted using cytochrome P450, NADPH-cytochrome P450 reductase, and dilauroylphosphatidylcholine at a 1:2:200 molar ratio. These components were preincubated 3-5 min at high concentration before addition of remaining components. NADPH-cytochrome P450 reductase was prepared by affinity chromatography on 2',5'-ADP-Sepharose by the methods of Dignam and Strobel (1977) and Yasukochi and Masters (1976). Dilauroyl-phosphatidylcholine was prepared as a 1 mM suspension in 50 mM potassium phosphate, pH 7.4 containing 0.1 mM EDTA and sonicated before use. Bovine gamma globulin (2.5 mg/ml) was included as a carrier. NADPH was used to start the reactions and was generated with a system consisting of (final concentration): 0.5 mM $NADP^+$, 5 mM isocitrate, 5 mM $MgCl_2$, 5 μM $MnCl_2$ and 0.4 unit isocitrate dehydrogenase. This system was pre-incubated at 37°C for 5

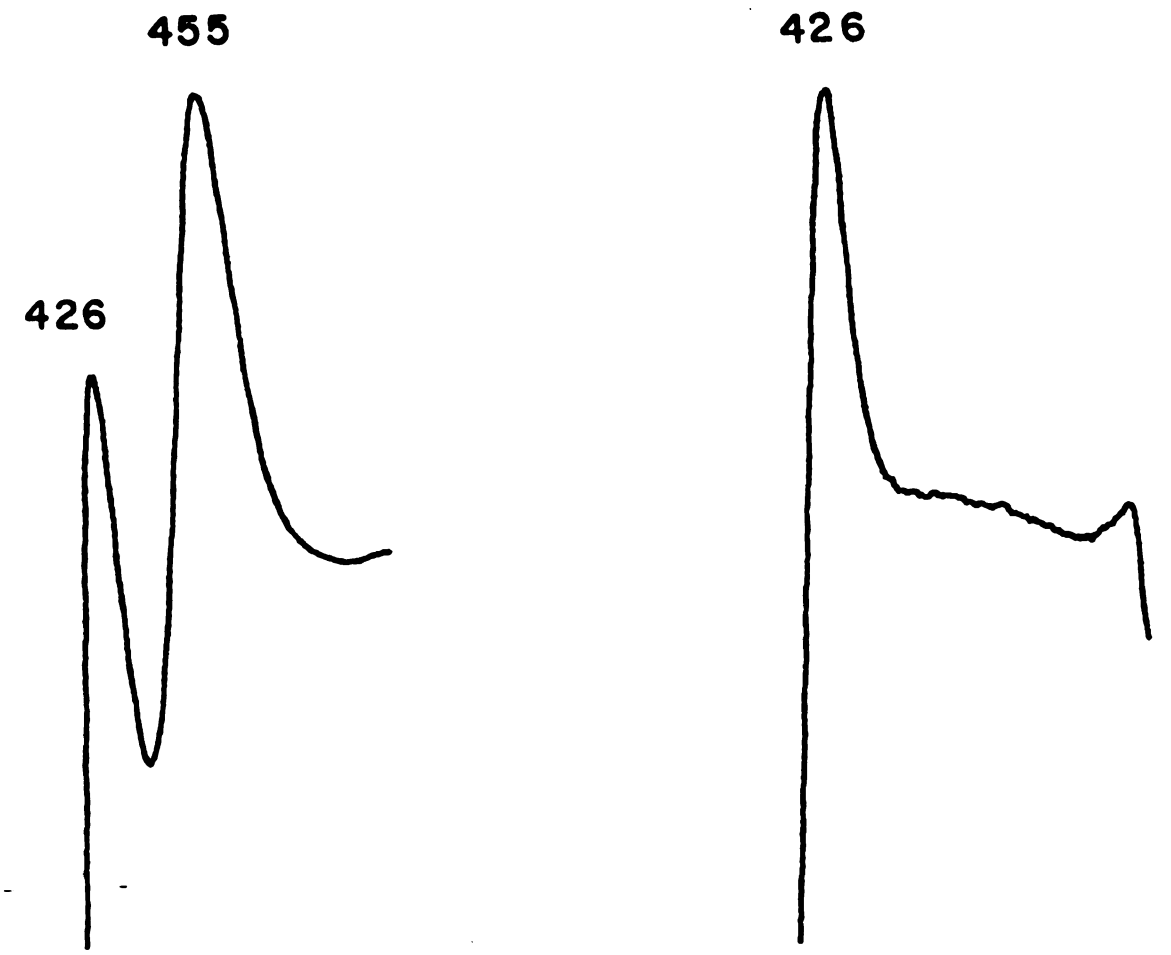


Figure 4. Difference spectrum of dithionite-reduced microsomes from isosafrole treated rats. Microsomes (10 mg protein) were incubated without (A) and with (B) 250 mM n-butanol at 37° for 30 min followed by chromatography on Sepharose CL-6B. An aliquot (1 mg) was divided between two cuvets, the suspension in the sample cuvet was reduced with a few crystals of sodium dithionite and the absorption recorded between 490 and 410 nm. The 455 nm peak is due to the isosafrole metabolite complex with cytochrome P450; the 426 nm peak is cytochrome b_5.

min to achieve a steady state level of NADPH before addition as a 100 µl aliquot to the assay mixture.

Estradiol 2-hydroxylase was measured by the method of Numazawa et al. (1980) as modified by Ryan et al. (1982). Protein was determined by the bicinchoninic acid micromethod (Redinbaugh and Turley, 1986) standardized with bovine serum albumin. Cytochrome P450 concentration was determined based on an extinction coefficient of 91 mM^{-1} cm^{-1} for the CO difference spectrum of dithionite-reduced samples. PHAH were extracted from cytochrome P450 by preparing a solution of cytochrome P450 (0.1 nmol P450 or 50 µg microsomal protein) in 200 µl 0.5 N KOH and extracting 3 times with 800 µl dichloromethane each time. PHAH were transferred to hexane and quantitated by gas chromatography with a 3% OV-1 column and electron capture detector.

Immunochemical Methods: Antibodies were raised in New Zealand rabbits by injecting 300 µg antigen (emulsified in Freund's complete adjuvant) intradermally in 15-20 sites along the back. This was followed by monthly subcutaneous injections of 100 µg antigen using incomplete adjuvant. Rabbits were bled 1 week after boosting and the titer was checked by Ouchterlony double diffusion. Serum immunoglobulins (IgG) were prepared by affinity chromatography on protein A-Sepharose (Hjelm et al., 1972). An indirect, non-competitive ELISA for cytochrome P450d was developed based on the method of Paye et al. (1984).

Antigen was diluted in 50 mM sodium bicarbonate, pH 9.6, 2% sodium cholate, 0.02% azide and 50 µl/well added to triplicate wells of a microtiter plate. Plates were incubated 3 hr at 37°C or over night at 4°C. Antigen solution was replaced with 200 µl 0.1% gelatin in 10 mM potassium phosphate, pH 7.4, 0.15 M NaCl, 0.02% azide and incubated 30 min at 37°C. Plates were then rinsed with tap water with a microtiter plate washer, and IgG was added in 0.1 M potassium phosphate, pH 7.4, containing 0.4 M KCl, 0.4 mM EDTA, 0.5% cholate, 0.1% Emulgen 911, and 0.02% azide (IgG-buffer) containing 1% BSA, at 10 µg/ml and 100 µl per well for 1.5 hr at 4°C. Plates were washed and 100 µl goat-anti-rabbit peroxidase conjugate diluted 1:2000 in IgG-buffer containing 1% BSA (without azide) added per well and incubated 30 min at 37°C. After thoroughly washing the plate, 100 µl color reagent (2 mM o-phenylendiamine HCl, 2.5 mM H_2O_2 in 0.1 M sodium citrate, pH 5.0) was added. Color development was stopped with 50 µl 4N H_2SO_4 after 5 min and absorbance read at 490 nm using a Biotech ElA Model 307 plate reader (Burlington, VT). Response to cytochrome P450d was linear from 0.02 to 0.4 pmol per well. This allowed quantitation of microsomal cytochrome P450d in and 0.05 to 0.4 µg microsomal protein per well depending on specific content of P450d (Figure 5). Cross reactivity of anti-P450d with cytochrome P450c was minimized by adsorption of the cross reacting antibodies onto cytochrome P450c coupled to CNBr-activated Sepharose (Reik et al., 1982).

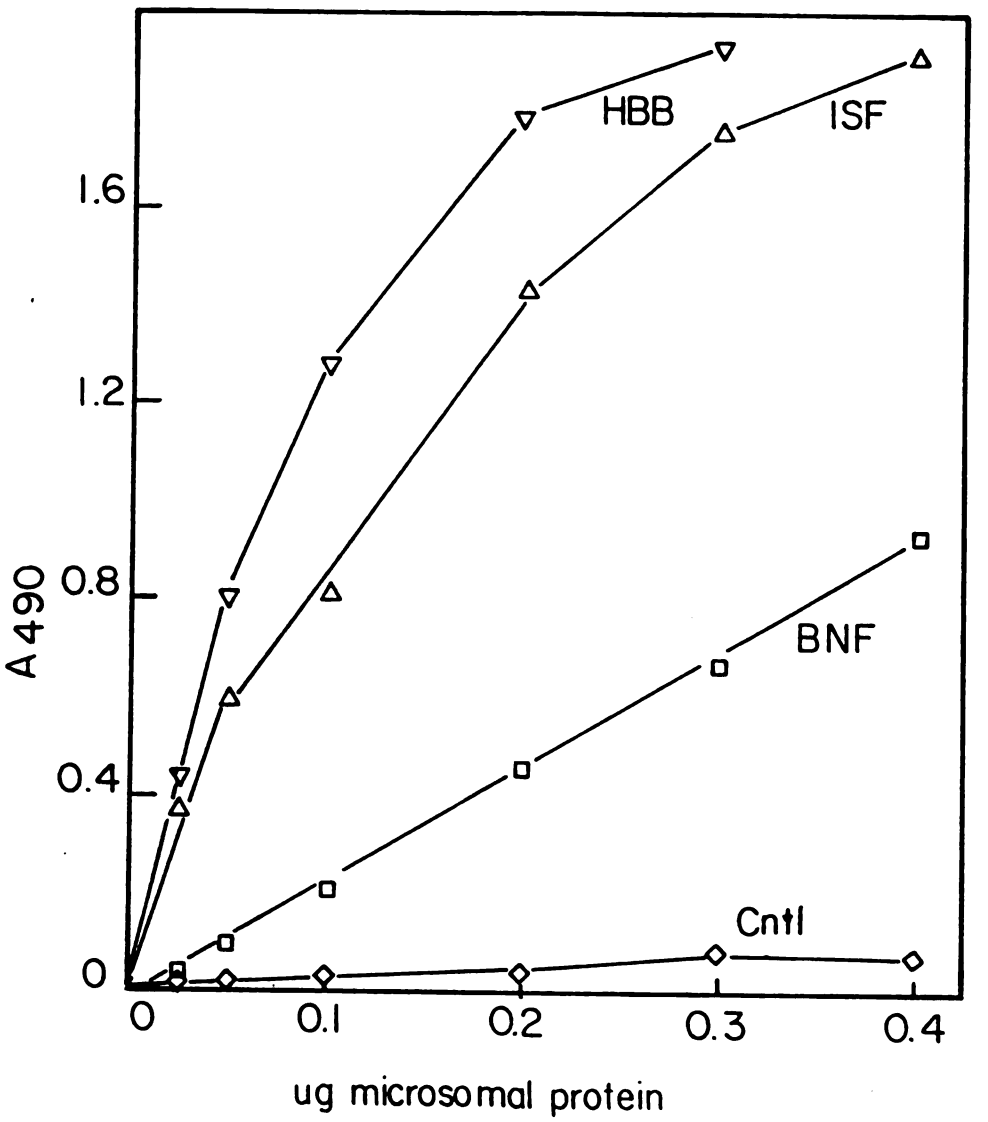


Figure 5. Detection of cytochrome P450d by ELISA. Microtiter plates prepared as described in "Methods." Microsomes from untreated rats (◇); microsomes from B-naphthoflavone treated rats (□); microsomes from isosafrole treated rats (△); microsomes from HBB treated rats (▽).

For immunoprecipitation, microsomes were solubilized in IgG-buffer at 5 mg/ml. Ten microliters of solubilized microsomes were added to 200 µl of the same buffer containing 1% (w/v) BSA and appropriate amount of specific IgG and incubated overnight at 4°C. The immunoprecipitate was washed 3 times with IgG-buffer and assayed for PHAH content.

RESULTS

Association of Ligands with Cytochrome P450d

When HBB was used to induce cytochrome P450 in rats, followed by purification of cytochromes b_5, P450c and P450d, HBB was found to be selectively associated with cytochrome P450d (Table 1). The association was apparently non-covalent since HBB could be extracted from the cytochrome with dichloromethane and co-chromatographed with an HBB standard. However, HBB was tightly coupled to the cytochrome since it was not fully removed even though the microsomal cytochrome P450 was solubilized with detergents and had been passed through four different columns.

Since it was still possible that HBB simply co-chromatographed with cytochrome P450d, cytochromes P450c and P450d and epoxide hydrase were immunoprecipitated from microsomes of HBB treated rats (Table 2) and the precipitate assayed for HBB. In this case, HBB was significantly associated only with the immunoprecipitate of cytochrome

Table 1. Association of HBB with microsomal cytochromes purified from HBB treated rats. Male Sprague-Dawley rats were treated with HBB (10 μmol/kg), microsomal cytochromes were isolated and assayed for HBB as described in "Methods".

Cytochrome	mol HBB/mol cytochrome
b_5	<0.01
$P450_c$	0.01
P450d	0.49

Table 2. Association of HBB with immunoprecipitated microsomal proteins. Detergent solubilized microsomes (50 µg protein) from HBB treated rats were treated with the IgG's shown. The immunoprecipitates were assayed for HBB as described in "Methods".

IgG	Amt. IgG added (mg)	HBB in immuno-precipitate (pmol)
pre-immune	1.0	1.7
anti-P450c	0.1	1.9
anti-P450c	1.0	2.6
anti-P450d	0.1	2.6
anti-P450d	1.0	19.3
anti-EH	1.0	2.3

P450d. The amount of immunoreactive cytochrome P450d was measured in microsomes from HBB treated rats by ELISA and compared to the total microsomal content of HBB (Table 3). The ratio of HBB to cytochrome P450d was near unity suggesting a 1:1 association of enzyme and ligand.

Five other halogenated aromatic hydrocarbons bearing steric similarity to HBB were tested for their ability to form a similar stable complex with cytochrome P450d. Rats were injected with these compounds, liver microsomes isolated 72 hr later, and cytochrome P450d was immunoprecipitated. The results, shown in Table 4, reveal that all of the compounds tested were associated with the cytochrome P450d immunoprecipitate. Control experiments with purified cytochrome P450d with HBB bound at 0.9 mol/mol cytochrome P450d showed that 42% precipitation occurred using antigen-antibody ratios similar to those used to obtain the data in Table 4. Thus it is not surprising that in most cases we could not achieve complete immunoprecipitation of the ligand-P450 complex. It should be noted the 2,4,5,3',4',5'-hexabromobiphenyl is unlike HBB in that it contains one bromine located ortho to the biphenyl bridge and is energetically restricted from assuming a coplanar configuration. In this respect it is only a moderate inducer of cytochrome P450 (Dannan et al., 1983) and moreover, this attribute probably results in the diminished association with cytochrome P450d. Although 3,4,3'4'-tetrachlorobiphenyl is metabolized by cytochrome

Table 3. Correlation of microsomal HBB content with immunologically measured (ELISA) content of microsomal cytochrome P450d. Rats were treated with HBB (10 μmol/kg), and hepatic microsomes were prepared and assayed for HBB and cytochrome P450d as described in "Methods".

Microsomal preparation	P450d (nmol/mg)	HBB (nmol/mg)	mol HBB/mol P450
A	1.41	1.27	0.901
B	2.11	2.10	0.995
C	1.67	1.46	0.874

Table 4. Association of five polyhalogenated aromatic hydrocarbons with immunoprecipitated cytochrome P450d. Animals were treated with the chemicals and hepatic microsomes prepared as described in "Methods". An aliquot of microsomes was extracted and assayed for total ligand content, another aliquot was solubilized and diluted to 5 mg protein/ml; 25 µg microsomal protein was incubated overnight with 1 mg anti-P450d IgG in 200 ul immunoprecipitation buffer. The resulting precipitate was washed, solubilized with 0.5 N KOH, extracted, and the ligand in the immunoprecipitate was quantitated by gas chromatography.

Inducer	DOSE (µmol/kg)	Content of Inducer (pmol/mg protein)		Percent Inducer In ippt.
		Micro-somes	P450 Immuno-precipitate	
3,4,5,3',4',5'-hexachlorobiphenyl	10	714	373	52
2,3,7,8-tetrachloro-dibenzodioxin	1	195	218	112
3,4,3',4'-tetra-chlorobiphenyl	100	388	102	26
3,4,5,3',4'-penta-chlorobiphenyl	6	753	450	60
2,4,5,3',4'5'-hexa-bromobiphenyl	10	384	43	11

P450c but not P450d (Voorman et al., 1984) it was still associated with cytochrome P450d although the recovery was low probably due to metabolism by P450c.

Influence of Cytochrome P450d Ligands on Enzymatic Activity of Cytochrome P450d

Estradiol 2-hydroxylase (E2H) is one of the few well characterized activities associated with cytochrome P450d (Astrom and DePierre, 1985) and was studied in the following experiments to measure the ability of HBB and other potential cytochrome P450d ligands to inhibit the catalytic activity of the cytochrome. Cytochrome P450d was isolated from isosafrole treated rats and the isosafrole metabolite removed from the cytochrome (see Methods). E2H activity in this preparation was compared to activity of a cytochrome P450d preparation from HBB treated rats which contained 0.8 mol HBB/mol cytochrome P450d (Figure 6). The cytochrome P450d-HBB complex exhibited non-competitive inhibition compared to the ligand free cytochrome P450d. The Km's of the two preparations were nearly equal while the V_{max} of the inhibited reaction was 23% of uninhibited cytochrome P450. Non-competitive inhibition indicates that HBB was either not bound at the active site or had a very slow dissociation rate from the active site. In another experiment HBB was added to ligand free cytochrome P450d and E2H was measured (Figure 7). Distinct inhibition was observed at 10 nM HBB and increasing HBB concentrations revealed that HBB caused

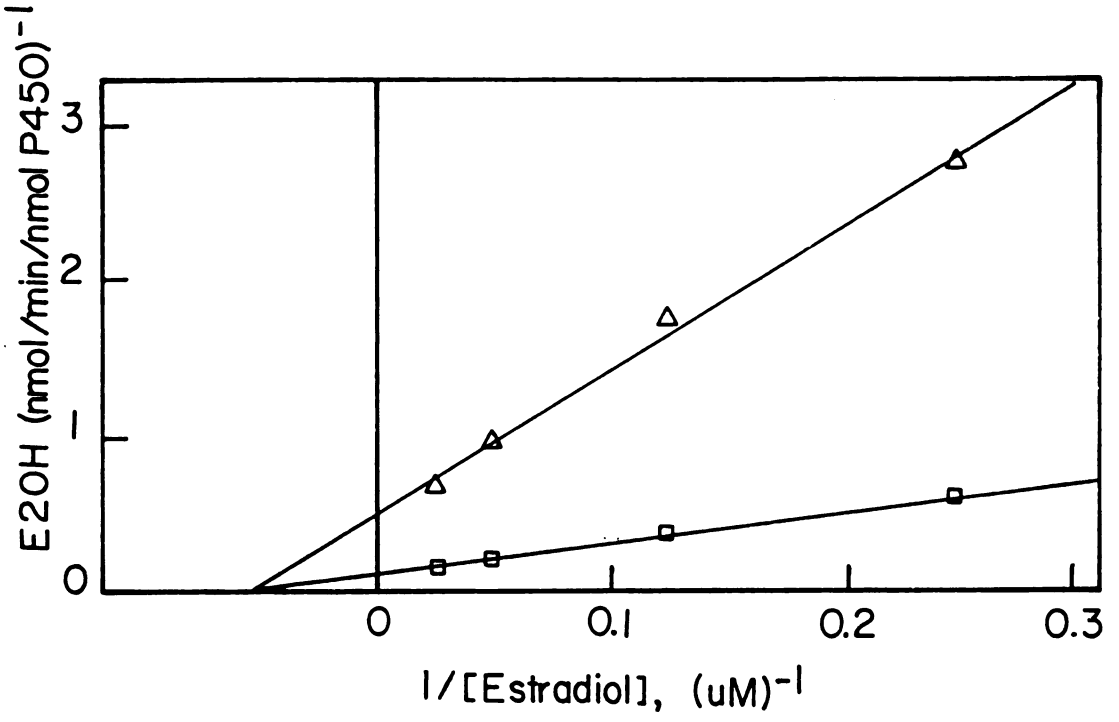


Figure 6. Lineweaver-Burk plot of estradiol 2-hydroxylase (E2H) activity in two cytochrome P450d preparations. Purification and reconstitution of cytochrome P450d and measurement of E2H were as described in "Methods." Cytochrome P450d was used at 50 nM in 1 ml incubation; E2H activity measured following 10 minutes incubation. Cytochrome P450d isolated from isosafrole treated rats where the isosafrole metabolite was displaced from cytochrome P450d, K_m = 19.1 μM, V_{max} = 8.7 min^{-1} (□); cytochrome P450d isolated from HBB treated rats (10 μmol/kg) with 0.8 mol HBB/mol cytochrome P450d, K_m = 17.3 μM, V_{max} = 2.0 min^{-1} (Δ).

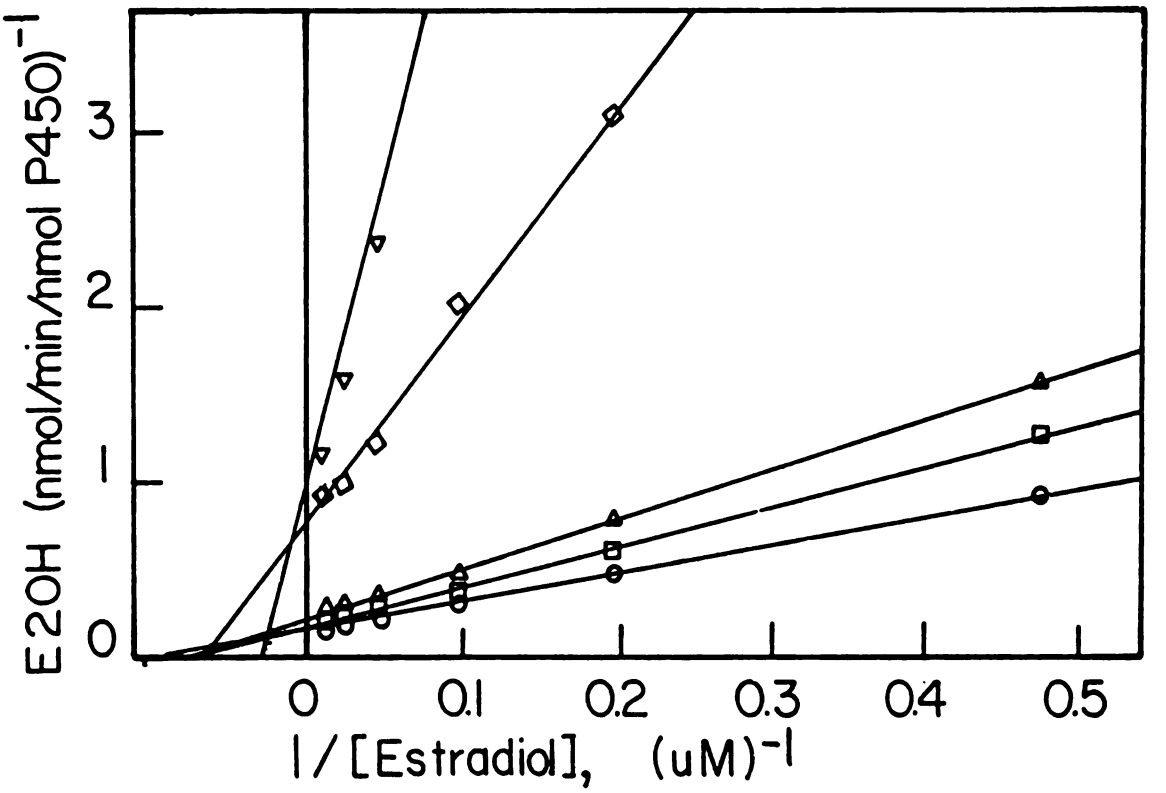


Figure 7. Lineweaver-Burk plot of effect of HBB on cytochrome P450d catalyzed estradiol 2-hydroxylase. Ligand-free cytochrome P450d prepared and reconstituted at 50 nM cytochrome P450d. HBB was added in 10 µl methanol and the reaction started following 5 min preincubation. The concentrations of HBB were 0 (O); 10 (□); 25 (Δ); 100 (◇); and 1000 (▽) nM. Apparent Km values ranged from 9.4 - 41.6 µM and apparent V_{max} values ranged from 6.2 - 1.1 min^{-1}.

both competitive and non-competitive inhibition. These data indicate that HBB interferes with E2H activity and is probably bound at or near the active site of cytochrome P450d.

Cytochrome P450h, a constitutive P450, has E2H activity similar to P450d (Astrom and DePierre, 1985). Since it is possible that HBB somehow interferes with estradiol itself, or perhaps with the reductase binding site on cytochrome P450, the ability of HBB to inhibit cytochrome P450h-catalyzed E2H was tested by incubating HBB with microsomes from untreated mature male rats. Although E2H activity in these microsomes was similar to that in microsomes from HBB treated animals no significant change in kinetic constants was observed even when HBB was added at concentrations up to 4 μM (Figure 8).

Further examination of steric restrictions on the binding site of cytochrome P450d was done using three isomers of hexabromobiphenyl, differing only in the degree of bromination at carbons ortho to the phenyl:phenyl bridge. The isomers were tested for their ability to inhibit E2H activity in a reconstituted system using ligand-free cytochrome P450d. The results, shown in Figure 9, revealed that the ability of the polybrominated biphenyl congener to inhibit cytochrome P450d decreased with increased ortho bromination of the congener. A fully planar, nonhalogenated hydrocarbon, methylcholanthrene was also found to be a strong inhibitor of P450d. Methylcholanthrene was shown

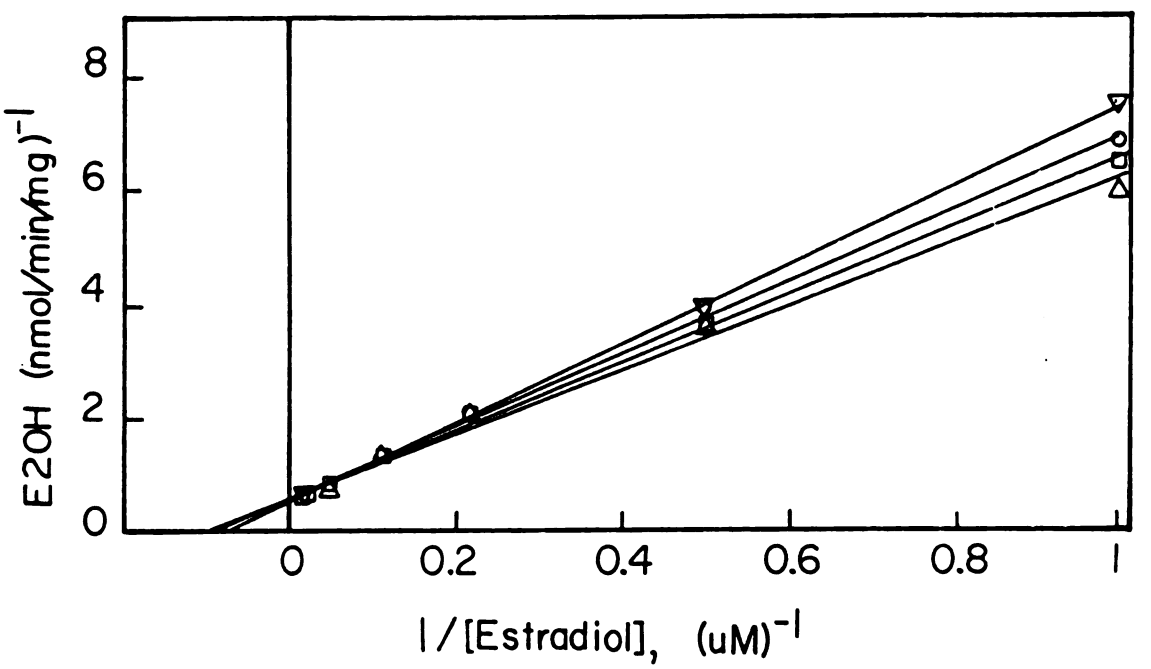


Figure 8. Lineweaver-Burk plot of effect of HBB on hepatic microsomal estradiol 2-hydroxylase activity in untreated rats. Microsomes were isolated from untreated rats and estradiol 2-hydroxylase was measured using 50 µg protein/ml with HBB added at: 0 (O); 0.1 (□); 1.0 (△) and 4 (▽) µM.

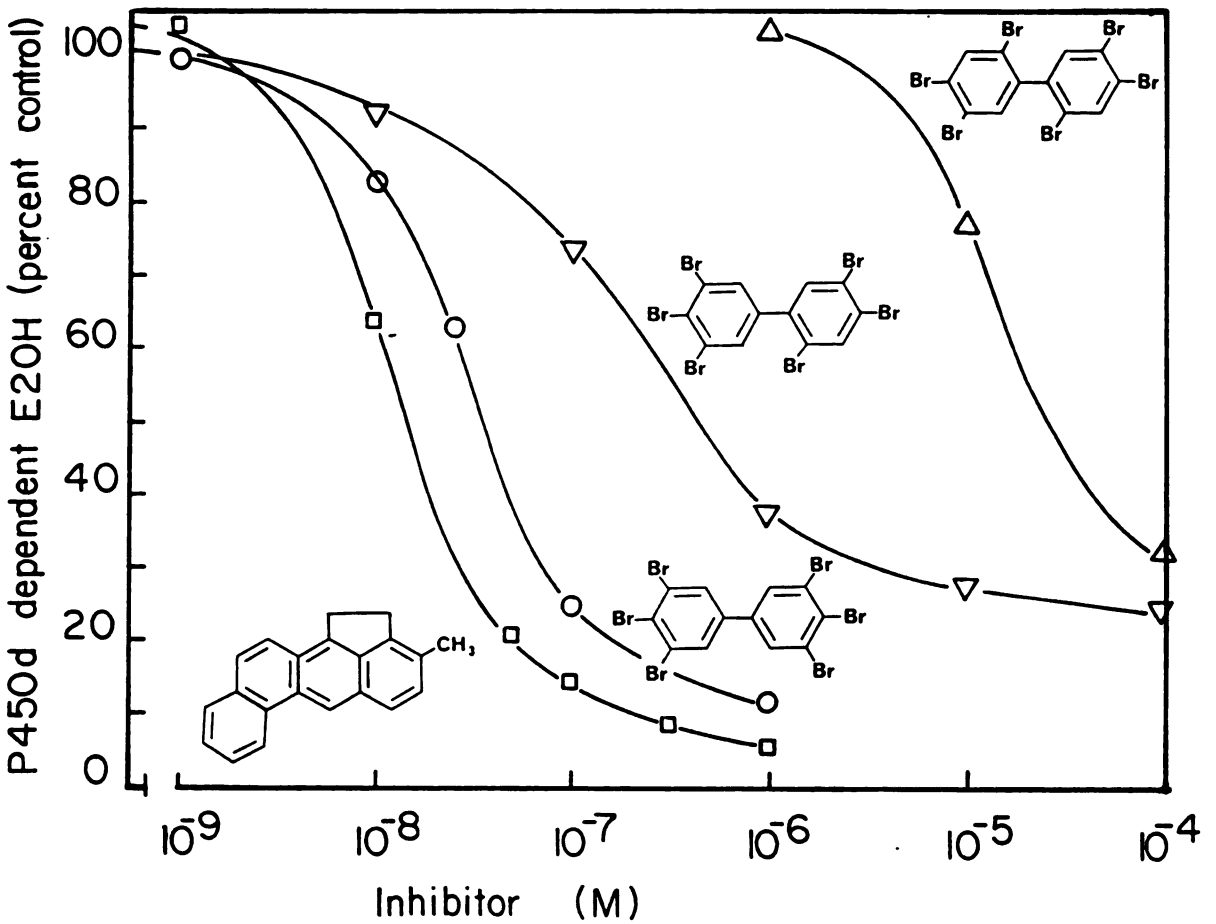


Figure 9. Determination of inhibitory concentrations of selected compounds on E2H activity of cytochrome P450d. E2H was measured at 50 nM cytochrome P450d in 1 ml buffer. Inhibitors were added in 10 1 methanol. Methylcholanthrene (□); HBB (O); 2,4,5,3',4',5'-hexabromobiphenyl (▽); 2,4,5,2',4',5'-hexabromobiphenyl (△).

earlier to be a ligand for cytochrome P448, an isozyme isolated from methylcholanthrene-treated rabbits and analogous to cytochrome P450d (Imai et al., 1980).

DISCUSSION

Our experiments reveal that, when rats were dosed with 10 μmol HBB/kg, HBB was selectively bound to cytochrome P450d. It was not bound to cytochrome P450c or cytochrome b_5 and appeared to be associated with cytochrome P450d in a 1:1 ratio. The binding was non-covalent although apparently essentially irreversible since HBB was easily extracted with dichloromethane but not removed by hydrophobic or ion exchange chromatography. Enzymic activity of cytochrome P450d was clearly inhibited by HBB in what appeared to be a non-competitive or mixed inhibition of cytochrome P450d at concentrations less than the concentration of enzyme. Two isomers of HBB which are more restricted from co-planarity of the biphenyl rings by the presence of ortho bromines inhibited catalytic activity to a significantly lesser degree, indicating structural specificity for inhibition. Implicit in these results are that HBB and other polyhalogenated aromatic hydrocarbons that induce cytochrome P450d are bound non-covalently to cytochrome P450d following induction and that the dissociation rate is so slow that the reaction is essentially irreversible.

Isosafrole induces cytochrome P450d and an isosafrole metabolite forms a stable complex with cytochrome P450 heme

(Ryan et al., 1980). The isosafrole metabolite can be displaced from cytochrome P450 by n-butanol (see Methods) and other compounds (Dickins et al., 1979), as determined by loss of the 455 nm ferrocytochrome P450 chromophore and recovery of metabolism of selected substrates. When cytochrome P450d isolated from HBB treated rats was subjected to similar treatment, HBB was not displaced from cytochrome P450d (data not shown). There was no change in the catalytic capacity of cytochrome P450d following butanol treatment and chromatography and HBB could still be extracted from cytochrome P450d. Despite the inability of n-butanol to remove HBB from cytochrome P450d it seems likely that HBB binds to cytochrome P450d in a manner like the isosafrole metabolite. This assertion is supported by the fact that both compounds preferentially induce and bind to cytochrome P450d. Although isosafrole forms the cytochrome P450-isosafrole metabolite complex in vitro with several cytochrome P450 isozymes (Ryan et al., 1980) we found that when cytochrome P450c,d, and h were isolated from isosafrole treated rats, the isosafrole-complex was observed only on cytochrome P450d (unpublished). Isosafrole inhibits cytochrome P450d catalyzed estradiol 2-hydroxylase like HBB although it is impossible to determine if the isosafrole alone, and not the metabolite, inhibits the hydroxylase activity since the metabolite forms upon addition of NADPH. It is thought that the cytochrome P450-isosafrole metabolite complex is formed when the methylene carbon of isosafrole is

oxidized to a carbene and this coordinates to the sixth axial position of heme iron (Mansuy et al., 1979). This reaction does not occur with HBB since no change in the Soret spectrum was observed with cytochrome P450-HBB and CO was not inhibited from binding to cytochrome P450 by HBB as it was by isosafrole (data not shown).

It is likely that HBB binds in close proximity to the heme group of cytochrome P450d. Experiments with rabbit cytochrome P450 LM_4 (a high spin cytochrome P450 analogous to cytochrome P450d) indicated that methylcholanthrene, pyrene, and similar aromatic hydrocarbons bind to it in such a way that heme circular dichroism absorption bands were shifted (Imai, 1982). Moreover, cytochrome P450 LM_4-bound pyrene was metabolized when activity was reconstituted with reductase and NADPH, and metabolism was inhibited when CO was added. HBB was metabolized little if at all by microsomal cytochrome P450 (Mills et al., 1985), therefore metabolism of HBB would be unlikely even if bound in the active site of cytochrome P450d.

The significance of the phenomenon we have observed is not clear. Certainly, the very tight binding of HBB would affect the metabolism of an endogenous substrate, but a physiologic function of cytochrome P450d has yet to be identified. Uncoupling of cytochrome P450 electron transport by certain substrates has been proposed (Kuthan and Ullrich, 1982; Sousa and Marletta, 1985). Presumably a ferrous-oxygen complex is formed following substrate binding

and iron reduction. This complex could be: a) released as O_2^-, b) reduced by one more electron and released as H_2O_2, or c) undergo homolytic cleavage to release H_2O with subsequent 2 electron reduction of the $Fe^{+3}=O$ complex to release another H_2O. Although superoxide and peroxide anions would be expected to be deletorious to the cell, damage would occur only if their production overwhelmed the protective activities of superoxide dismutase and catalase. We have not determined the ability of HBB to uncouple cytochrome P450 electron transport, although we have shown in a preliminary account that cytochrome P450d has a slightly greater oxidase activity than either cytochromes P450c or P450b (Morehouse, 1984).

Recently it has been shown that isosafrole (and perhaps some PCB's) stabilized cytochrome P450 in cultured rat hepatocytes and partially prevented cytochrome P450d degradation (Steward et al. 1985). That is, the bound ligand might directly affect regulation of cytochrome P450d levels by restricting turnover of the enzyme. Although cytochromes P450c and P450d appear to be induced via the Ah receptor, they are differentially regulated depending on the inducer (Fagan et al., 1986) and there is evidence for non-Ah receptor mediated induction of P450d (Cook and Hodgson, 1985; Linko et al., 1986). Isosafrole is one of the most effective inducers of cytochrome P450d, inducing it to considerably higher levels than cytochrome P450c. Cytochrome P450d is the major cytochrome P450 induced by HBB

and by 3,4,5,3',4',5'-hexachlorobiphenyl (Parkinson et al., 1983). In Tables 1 and 2 we showed that HBB was tightly bound to cytochrome P450d analogous to isosafrole and that nearly all the cytochrome P450d could be complexed with HBB. Thus it seems likely that HBB could affect the turnover of cytochrome P450d in a manner analogous to isosafrole. Such a stabilizing effect is not unprecedented. Cytochrome P450p appears to be stabilized by a metabolite complex of troleandomycin, and this cytochrome P450 is elevated to extremely high levels in rats following treatment with troleandomycin, probably owing to decreased degradation of the cytochrome P450p (Watkins et al., 1986).

REFERENCES

Astrom, A., and De Pierre, J.W. (1985). Metabolism of 2-acetyl-amino fluorene by eight different forms of cytochrome P450 isolated from rat liver. Carcinogenesis 6, 113-120.

Cook, J.C., and Hodgson, E. (1985) The induction of cytochrome P450 by isosafrole and related methylenedioxyphenyl compounds. Chem-Biol. Interactions 54, 299-315.

Dannan, G.A., Guengerich, F.P., Kaminsky, L.S., and Aust, S.D. (1983). Regulation of cytochrome P450: Immunochemical quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. J. Biol. Chem. 258,1282-1288.

Dannan, G.A., Mileski, G.J., and Aust, S.D. (1982). Purification of polybrominated biphenyl congeners. J. Toxicol. Environ. Health 9, 423-438.

Dickins, M., Elcombe, C.R., Moloney, S.J., Netter, K.J., and Bridges, J.W. (1979). Further studies on the dissociation of the isosafrole metabolite-cytochrome P450 complex. Biochem. Pharmacol. 28, 231-238.

Dignam, J.B., and Strobel, H.W. (1977). NADPH-cytochrome P450 reductase from rat liver: Purification by affinity chromatography and characterization. Biochemistry, 16, 1116-1122.

Fagan, J.B., Pastewka, J.V., Chalberg, S.C., Gozukara, E., Guengerich, F.P., and Gelboin, H.V. (1986). Non-coordinate regulation of the mRNAs encoding cytochromes P450 BNF/MC-B and P450 ISF/BNF-G. Arch. Biochem. Biophys. 244, 261-272.

Fisher, G.J., Fukushima, H., and Gaylor, J.L. (1981). Isolation, purification, and properties of a unique form of cytochrome P450 in microsomes of isosafrole-treated rats. J. Biol. Chem. 256, 4388-4394.

Garewal, H.S. (1973). A procedure for the estimation of microgram quantities of triton X-100. Anal. Biochem. 54, 319-324.

Guengerich, F.P., and Martin, M.V. (1980). Purification of cytochrome P450, NADPH-cytochrome P450 reductase, and epoxide hydrase from a single preparation of rat liver microsomes. Arch. Biochem. Biophys. 205, 365-379.

Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, M.V. and Kaminsky, L.S. (1982). Purification and characterization of liver microsomal cytochrome P450: Electrophoretic,

spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or B-naphthoflavone. Biochemistry, 21, 6019-6030.

Hildebrandt, A.G., Leibman, K.C., and Estabrook, R.W. (1969). Metyrapone interaction with hepatic micrsomal cytochrome P450 from rats treated with phenobarbital. Biochem. Biophys. Res. Comm. 37, 477-485.

Hjelm, H., Hjelm, K., and Sjoquist, J. (1972). Protein A from Staphylococcus aureus. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. FEBS Lett. 28, 73-76.

Imai, Y. (1976). The use of 8-aminooctyl Sepharose for the separation of some components of the hepatic microsomal electron transfer system. J. Biochem. 80, 267-276.

Imai, Y. (1982). Interaction of polycyclic hydrocarbons with cytochrome P450. I. Specific binding of various hydrocarbons to P448. J. Biochem. 92, 57-66.

Imai, Y., Hoshimoto-Yutsudo, C., Satoke, H., Girardin, A., and Sato, R. (1980). Multiple forms of cytochrome P450 purified from liver microsomes of phenobarbital- and 3-methylcholanthrene-pretreated rabbits. I. Resolution, purification, and molecular properties. J. Biochem. 88, 489-503.

Kuthan, H., and Ullrich, V. (1982). Oxidase and oxygenase function of the microsomal cytochrome P450 monooxygenase system. Eur. J. Biochem. 126, 583-588.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-682.

Linko, P., Yeowell, H.N., Gasiewicz, T.A., and Goldstein, J.A. (1986). Induction of cytochrome P450 isozymes by hexachlorobenzene in rats and aromatic hydrocarbon (Ah) - responsive mice. J. Biochem. Toxicol. 1, 95-107.

Mansuy, D., Battioni, J.P., Chottard, J.C., and Ullrich, V. (1979) Preparation of a porphyrin-iron-carbene model for the cytochrome P450 complexes obtained upon metabolic oxidation of the insecticide synergists of the 1,3-benzodioxole series. J. Am. Chem. Soc. 101, 3971-3973.

March, S.C., Parikh, I., and Cictrecasas, P. (1974). A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60, 149-152.

Millis, C.D. (1984). Studies on the chemical and pharmacotoxicological properties of polybrominated biphenyls. M.S. Thesis, Michgan State University.

Mills, R.A., Millis, C.D., Dannan, G.A., Guengerich, F.P., and Aust, S.D. (1985). Studies on the structure - activity relationships for the metabolism of polybrominated biphenyls by rat liver microsomes. Toxicol. Appl. Pharamacol. 78, 88-95.

Morehouse, L.A., Bumpus, J.A., Voorman, R., and Aust, S.D. (1984). Superoxide production by reconstituted cytochrome P450-dependent mixed function oxidases. Fed. Proc. 43, 355.

Numazawa, M., Kiyono, Y., and Nambara, T. (1980). A simple radiometric assay for estradiol 2-hydroxylase activity. Anal. Biochem. 104, 290-295.

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 P450 isozymes and epoxide hydrase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats: A study of structure-activity relationships. J. Biol. Chem. 258, 5967-5976.

Paye, M., Beaune, P., Kremers, P., Guengerich, F.P., Letawe-Goujon, F., and Gielen, J. (1984). Quantification of two cytochrome P450 isoenzymes by an enzyme-linked immunosorbent assay (ELISA). Biochem. Biophys. Res. Commun. 122, 137-142.

Redinbaugh, M.G., and Turley, R.B. (1986). Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal. Biochem. 153, 267-271.

Reik, L.M., Levin, W., Ryan, D.E., and Thomas, P.E. (1982). Immunochemical relatedness of rat hepatic microsomal cytochromes P450c and P450d. J. Biol. Chem. 257, 3950-3957.

Ryan, D.E., Thomas, P.E., and Levin, W. (1980). Microsomal cytochrome P450 from rats treated with isosafrole: Purification and characterization of four enzymic forms. 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 P450 from rats treated with polychlorinated biphenyls. Arch. Biochem. Biphys. 216, 272-288.

Sousa, R.L., and Marletta, M.A. (1985). Inhibition of cytochrome P450 activity in rat liver microsomes by the naturally occurring flavonoid, quercetin. Arch. Biochem. Biophys. 240, 345-357.

Spatz, L., and Strittmatter, P. (1971). A form of cytochrome b_5 that contains an additional hydrophobic sequence of 40 amino acid residues. Proc. Nat. Acad. Sci. 68, 1042-1046.

Steward, A.R., Wrighton, S.A., Pasco, D.S., Fagan, J.B., Li, D., and Guzelian, P.S. (1985). Synthesis and degradation of 3-methylcholanthrene-inducible cytochromes P450 and their mRNAs in primary monolayer cultures of adult rat hepatocytes. Arch. Biochem. Biophys. 241, 494-508.

Voorman, R., Mills, R.A., Bumpus, J.A., Morehouse, L.A., and Aust, S.D. (1984) Metabolism of PBB congeners by reconstituted monoxygenase systems. The Toxicologist 4, 374.

Walsh, C.T. (1984). Suicide substrates, mechanism-based enzyme inactivators: Recent developments. Ann. Rev. Biochem. 53, 493-535.

Watkins, P.B., Wrighton, S.A., Schaertz, E.G., Maurel, P., and Guzelian, P. (1986). Macrolide antibiotics inhibit the degradation of the glucocorticoid responsive cytochrome P450p in rat hepatocytes in vivo and in primary monolayer cultures. J. Biol. Chem. 261, 6264-6271.

White, R.E., and Coon, M.J. (1982). Heme ligand replacement reactions of cytochrome P450. J. Biol. Chem. 257, 3073-3083.

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.

Yasukochi, Y., and Masters, B.S.S. (1976). Some properties of a detergent solubilized NADPH-cytochrome c (cytochrome P450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. 251, 5337-5334.

CHAPTER II

TCDD (2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN) IS A TIGHT BINDING INHIBITOR OF CYTOCHROME P450d

ABSTRACT

Cytochrome P450c and P450d are induced in rat liver endoplasmic reticulum upon treatment of the animal with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or similar compounds thought to exert their effect through the Ah locus. Isosafrole induces cytochromes P450b, c, and d and forms a displaceble metabolite-intermediate complex with cytochrome P450d. Based on experiments which indicated that compounds which induce cytochrome P450d, also bind to the enzyme, we tested the ability of TCDD to inhibit reconstituted cytochrome P450d-dependent estradiol 2-hydroxylase, using ligand-free cytochrome P450d from isosafrole treated rats. TCDD was a mixed-type inhibitor of estradiol 2-hydroxylase changing the apparent K_m from 9.6 to 24.5 μM and Vmax from 6.7 to 1.2 min^{-1}. Since maximum inhibition of estradiol 2-hydroxylase occurred at TCDD concentrations comparable to the concentration of enzyme, a modified form of steady state kinetics was used. Cytochrome P450d estradiol 2-hydroxylase was inhibited by nearly equimolar concentrations of TCDD. Using $I_{50} = Et/2 + K_i$ (Et = total enzyme concentration), we showed that TCDD inhibited cytochrome P450d estradiol 2-hydroxylase with $K_i = 8$ nM. Association of TCDD with P450d was rapid; maximum inhibition occurred within two minutes of inhibitor addition. It seems likely that TCDD is a non-covalent, tight binding competitive inhibitor of cytochrome P450d.

INTRODUCTION

Cytochrome P450 catalyses the hydroxylation of many hydrophobic compounds, facilitating their excretion and thus playing a key role in the metabolism of many xenobiotics and several endogenous compounds. Cytochrome P450d is one of many isozymes of cytochrome P450 in the rat liver and is induced coordinately with cytochrome P450c by chemicals thought to exert their effect through the ah receptor (Whitlock, 1986). Enzymatic activities of cytochrome P450c have been well characterized using the hydroxylation of benzopyrene or the deethylation of ethoxyresorufin. Recently it has been shown that cytochrome P450d can catalyze acetanilide 4-hydroxylase and estradiol 2-hydroxylase (Tuteja et al., 1985; Reik et al., 1982; Astrom et al., 1985).

Interestingly, cytochrome P450d is induced by certain methylenedioxyphenyl compounds such as isosafrole which are then metabolized to form a stable inhibitory complex with cytochrome P450d (Philpot and Hodgson, 1972; Fisher et al., 1981; Ryan et al., 1980). Polycyclic aromatic hydrocarbons are known to form stable complexes with cytochrome P450LM4, an analogous isozyme in the rabbit (Imai et al, 1980), and a toxic pentachlorodibenzofuran was shown (Kuroki et al., 1986) to reside largely on a cytochrome P450 equivalent to P450d. We have recently shown that certain polyhalogenated aromatic hydrocarbons can form a stable complex with

cytochrome P450d when the compounds are used as inducers of cytochrome P450d and that the bound ligand can inhibit the metabolic activity of cytochrome P450d (Voorman and Aust, 1987).

The substrate specificity of cytochrome P450 is, with the exception of the steroid hydroxylases, relatively broad and non-specific and thus most substrates tend to have low turnover numbers and high Km's. Similarly, many compounds can inhibit cytochrome P450 and tend to function as classical competitive or non-competitive inhibitors where both inhibitor and substrate exist in vast excess relative to enzyme concentration. Certain enzyme inhibitors function at a concentration similar to the enzyme and can not be evaluated by Michaelis-Menton enzyme kinetic analysis which presupposes steady state conditions with an infinite excess of both substrate and inhibitor (Morrison, 1969). In this report we show that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) functions as a tight binding inhibitor of cytochrome P450d and have used a modification of steady state kinetics to evaluate the inhibition of cytochrome P450d dependent estradiol 2-hydroxylase by TCDD.

METHODS AND MATERIALS

Materials

2[^{3}H]-Estradiol was obtained from New England Nuclear (Boston, MA). TCDD was generously supplied by Dow Chemical (Midland, MI). Isosafrole was purchased from Fluka

(Ronkonkoma, NY) and used without further purification. Dilauroylphosphatidylcholine was purchased from Sigma (St. Louis, MO).

Animals

Male Sprague-Dawley rats (Charles River, Portage, MI) weighing 150-250 g were treated with isosafrole dissolved in corn oil at 150 mg/kg/day for 3 days and killed on the 4th day by decapitation following CO_2 anesthesia. Liver microsomes were isolated and cytochrome P450d was prepared as described (Voorman and Aust, 1987) using a combination of published methods (Guengerich et al. 1982; Astrom and DePierre, 1985; Imai, 1980) to a specific content of 12 to 18 nmol/mg protein. To prepare ligand-free cytochrome P450d, the microsomal preparation was incubated with 250 mM n-butanol at 37°C for 30 min, in order to displace the isosafrole metabolite from cytochrome P450d prior to the first chromatography step (Voorman+Aust, 1987).

Enzyme Assays

Estradiol 2-hydroxylase activity was measured under conditions where product formation was proportional to enzyme concentration and incubation time. Catalytic activity of cytochrome P450d was reconstituted by incubating cytochrome P450d, NADPH-cytochrome P450 reductase, and dilauroylphosphatidyl-choline at a 1:2:200 molar ratio for several minutes before addition of remaining reagents.

NADPH cytochrome P450 reductase was prepared by affinity chromatography according to the methods of Dignam and Stroebel (1977) and Yasukochi and Masters (1976). Dilauroylphosphatidylcholine was prepared as 1 mM suspension in 50 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA and sonicated before use. Final volume was brought to 1 ml with 50 mM potassium phosphate, pH 7.4 containing 0.1 mM EDTA and included 2.5 mg bovine gamma globulin as a carrier. The reaction was started by addition of either substrate or NADPH generating system (0.5 µmol $NADP^+$, 5 µmol isocitrate, 0.4 unit isocitratate dehydrogenase). Estradiol 2-hydroxylase was measured by the method of Numazawa et al. (1980) as modified by Ryan et al. (1982). TCDD was dissolved in methanol and added to the enzyme solution as a 10 ul aliquot.

RESULTS

In an earlier report we showed that various inducers of cytochrome P450d formed a complex with the enzyme which was stable throughout purification of the enzyme and, in the case of the halogenated aromatic hydrocarbons, could not be readily removed from the cytochrome (Voorman and Aust, 1987). Isosafrole is also a potent inducer of cytochrome P450d but the isosafrole metabolite is readily displaced from cytochrome P450d by n-butanol and can be removed by chromatography. The removal of the isosafrole metabolite

from our preparation of cytochrome P450d was confirmed by both the loss of the 455 nm peak of the reduced difference spectrum (Fig. 1) and by a 210% increase in estradiol 2-hydroxylase activity.

The addition of TCDD to the hydroxylase assay resulted in an inconsistent pattern of estradiol 2-hydroxylase inhibition when the kinetic data were displayed in double reciprocal plot (Fig. 2). Significant variation occurred in both slopes and intercepts (calculated by the weighted linear regression method of Cornish-Bowden (1979) using the Wilman3 computer program kindly suppled by C. Suelter) at different inhibitor concentrations making interpretation of the inhibition mechanism difficult. Considerable inhibition occurred at 35 nM TCDD and maximum inhibition at 65 nM when the concentration of cytochrome P450d was 50 nM. Although an accurate Ki could not be calculated from these data, if non-competitive inhibition was assumed, a Ki of approximately 30 nM was obtained. According to Morrison (1969), since the inhibitor exhibits a Ki comparable to the enzyme concentration it should be considered a tight binding inhibitor and thus steady state conditions do not prevail with respect to the inhibitor and a modified form of steady state kinetics is required. That is, formation of the enzyme-inhibitor complex significantly depletes the pool of free inhibitor, and thus alters the concentration of free inhibitor, a condition not allowed for steady state kinetics.

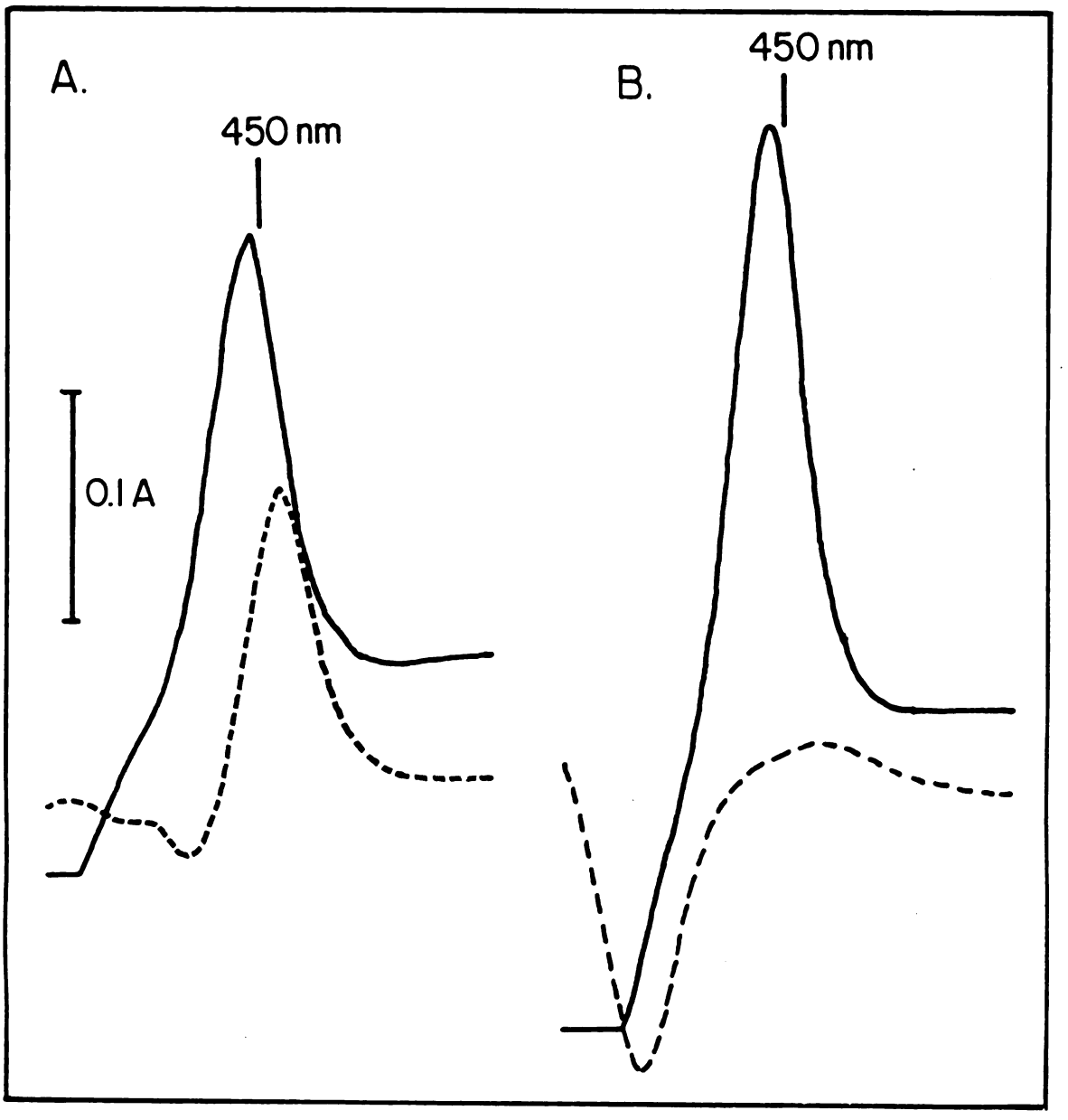


Figure 1. Difference spectra of two dithionite-reduced cytochrome P450d preparations. A. Cytochrome P450d purified from isosafrole treated rats. Sample cuvet reduced with dithionite (- - -); both cuvets treated with dithionite and CO bubbled into sample cuvet (———). B. Same as A but microsomes were incubated with 250 mM n-butanol prior to the first chromatography step. Each sample was scanned from 500 to 400 nm.

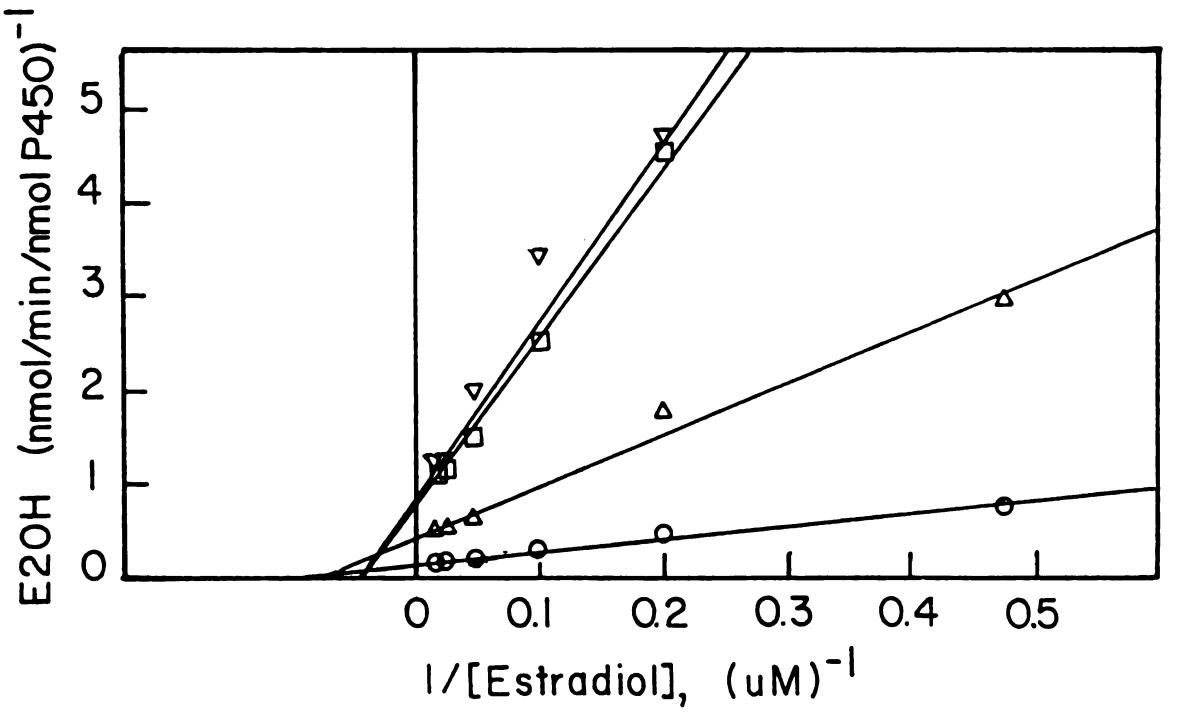
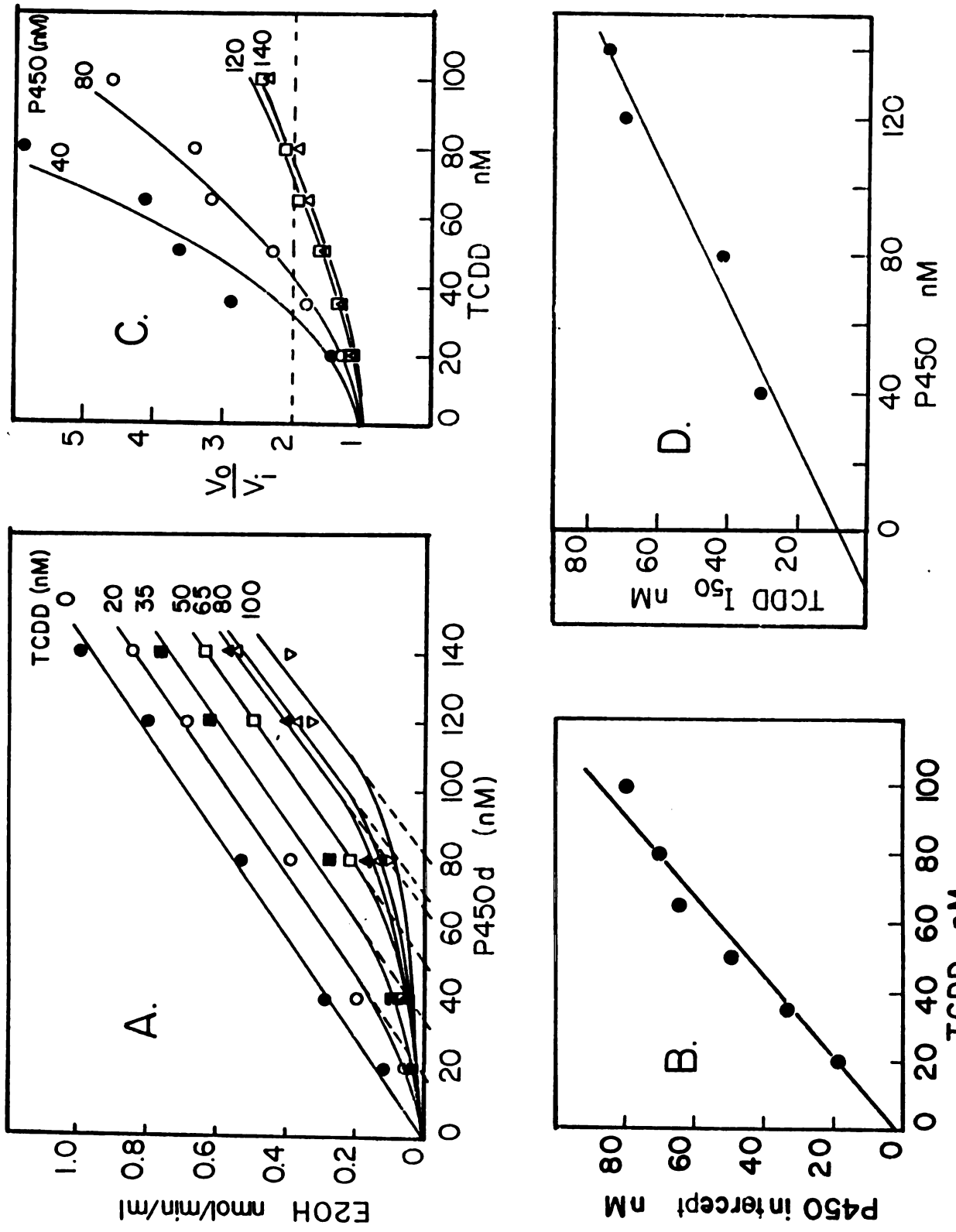


Figure 2. Lineweaver-Burk plot of effect of TCDD on cytochrome P450d estradiol 2-hydroxylase. Enzyme activity was reconstituted as described in "Methods" using 50 nM cytochrome P450d. TCDD was added to solution in 10 ul methanol followed by estradiol in 10 µl methanol; tubes were incubated 3 min at 37° before starting reaction with NADPH. Activity was determined after a 10 min incubation. Concentrations of TCDD were: 0 (O), 36 (Δ); 65 (□); and 135 (▽) nM. K_m ranged from 9.6 to 24.50 µM and Vmax from 6.7 to 1.2 min^{-1}.

Using the methods set forth by Ackermann and Potter (1949) and as described by Cha (1975), various concentrations of cytochrome P450d were titrated with various concentrations of the inhibitor and, after a 10 min pre-incubation, assayed for estradiol 2-hydroxylase activity. The data are displayed in the form of an Ackermann-Potter plot where velocity is plotted against total enzyme concentration (Fig. 3a). It can be seen that at sufficient inhibitor concentration virtually all of the enzyme activity was inhibited and only when the enzyme concentration exceeded the concentration of the inhibitor was velocity directly proportional to enzyme concentration. The intercepts of the extended linear portion of each curve should represent the amount of enzyme complexed with TCDD for each titration curve. When the intercepts were plotted against respective concentrations of TCDD (Fig. 3b) a linear curve was obtained. The slope of the curve (0.84) indicated that cytochrome P450d was inhibited (complexed) by TCDD with nearly 1:1 stoichiometry.

By combining the method of Henderson (1972), for depletion of free enzyme and inhibitor due to binding, with those of Cheng and Prusoff (1973) and Chou (1974), who derived detailed analyses for the relation of I_{50} to inhibition constants, Cha (1975) showed that I_{50} could be used to estimate the Ki for a tight binding inhibitor using the relationship $I_{50} = Et/2 + Ki$ where Et = total enzyme concentration. The I_{50}'s for TCDD inhibition of E2H were

Figure 3. Titration of cytochrome P450d estradiol 2-hydroxylase activity with TCDD. Enzyme activity was reconstituted as described in "Methods." TCDD was preincubated 10 min with enzyme and substrate, reaction was started with NADPH and stopped after 5 min. (A) Plot of velocity against cytochrome P450d concentration at various levels of TCDD (Ackermann-Potter plot). (B) Abscissa intercepts from (A) were plotted against respective TCDD concentrations, slope = 0.84. (C) I_{50}'s were estimated by plotting the velocity of the control reaction (V_0) and the inhibited reaction (V_i) against cytochrome P450d at various TCDD concentrations using the data from (A). (D) The estimated I_{50}'s were plotted against respective cytochrome P450d concentrations and the K_i for TCDD was calculated to be 8 nM by $I_{50} = E_t/2 + K_i$.


A.
E2OH nmol/min/ml
P450d (nM)
TCDD (nM)
0
20
35
50
65
80
100
B.
P450 intercept nM
TCDD nM
C.
V0/Vi
TCDD nM
P450(nM)
40
80
120
140
D.
TCDD I50 nM
P450 nM

estimated by plotting the ratio of uninhibited to inhibited E2H activities against TCDD concentration for a series of fixed enzyme concentrations (Fig. 3c). When Vo/Vi = 2 the reaction was inhibited 50%. By the aformentioned equation, a plot of I_{50} against enzyme concentration will yield the Ki at the ordinate intercept and -2Ki at the abscissa intercept. The data in Figure 3d revealed an average Ki of 8 nM TCDD.

Since TCDD binds very tightly to cytochrome P450d, evaluation of the association rate is of interest. Preliminary experiments showed that inhibition was maximum following a 10 minute pre-incubation of inhibitor and enzyme. E2H activity was measured under conditions when either the enzyme was pre-incubated with inhibitor and reaction started by substrate addition or by addition of inhibitor immediately following substrate solution (Fig. 4). Association of the inhibitor with the enzyme was rapid, apparently occurring within the first two minutes following inhibitor addition. In a similar experiment inhibitor was added several minutes after start of the reaction (Fig. 4 inset) which again resulted in an immediate (<2 min) maximum change in reaction velocity.

DISCUSSION

Based on earlier work (Voorman and Aust, 1987) and the present report, it seems likely that compounds which have a

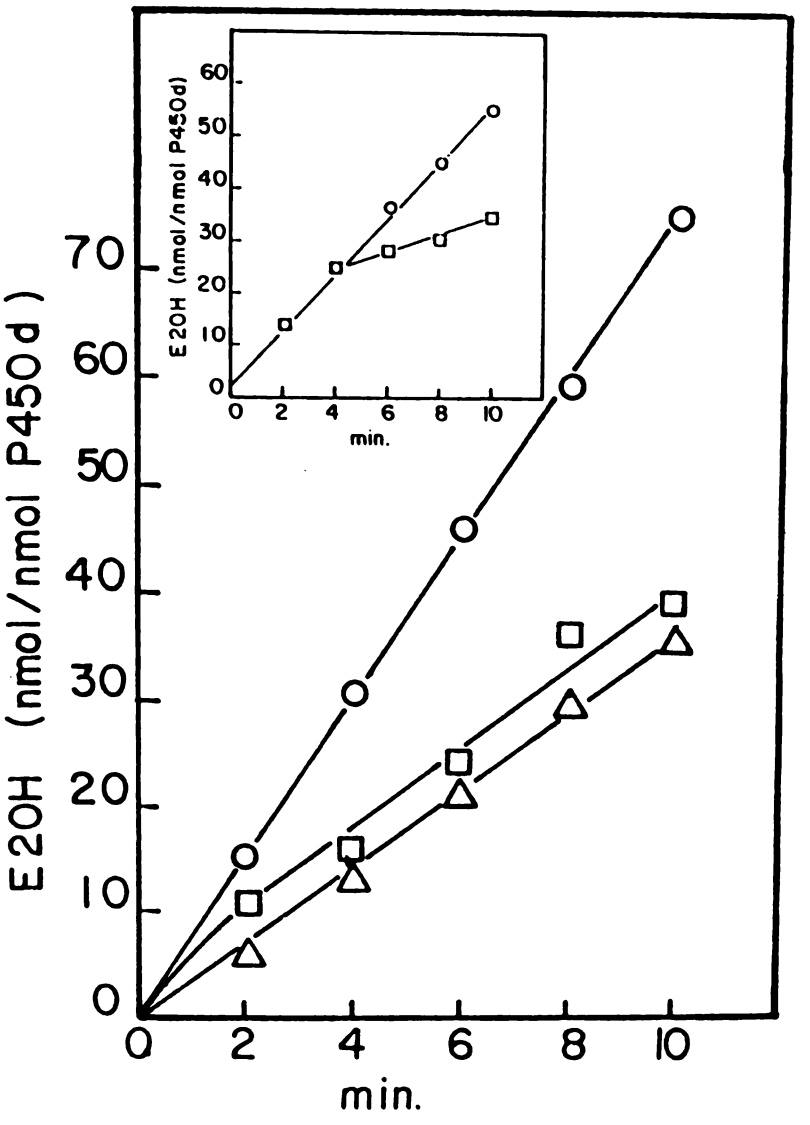


Figure 4. Effect of TCDD on estradiol 2-hydroxylase activity versus time. Enzyme activity reconstituted as described in "Methods." Using 50 nM cytochrome P450d (O) NADPH was added and reaction started by addition of substrate, aliquots of mixture were quenched at two minute intervals and product formation measured. (□) as above but 18nM TCDD added immediately following substrate addition. (Δ) as above but TCDD incubated with sample 5 min at 37° prior to NADPH and substrate addition. Inset. Reaction started by addition of substrate. (O) no TCDD; (□) 36 nM TCDD added to mixture at 4 min time point.

structure approximating that of TCDD are likely to induce and very effectively inhibit cytochrome P450d. The present work was undertaken to evaluate the affinity of cytochrome P450d for TCDD. Indeed, we have shown that TCDD can act as a titrating inhibitor of cytochrome P450d. That is, the inhibitor tends to bind fully to the enzyme when inhibitor concentration is below that of the enzyme. Thus, addition of the enzyme to a comparable solution of inhibitor will significantly deplete the pool of free inhibitor. We used a modified form of steady state kinetics which presupposes non-steady state conditions with respect to the concentration of inhibitor and, by estimation of I_{50} at infinitely low enzyme concentration, calculated a K_I of 8 nM.

The significance of this observation is not clear. It is difficult to envision any role it could play in TCDD toxicity: the K_I for inhibition is an order of magnitude higher than the K_D for TCDD binding to the Ah receptor (Poland et al., 1976) and the concentration of TCDD required to cause toxicity (10-100 nmol/kg) is below the level which would saturate the binding capacity of cytochrome P450d. Nevertheless we have shown that when TCDD is given at 1 µmol/kg it is significantly associated with cytochrome P450d (Voorman and Aust, 1987). Others have shown that high concentrations of pentachlorodibenzofuran in the liver following treatment with that compound are associated with cytochrome P450d (Kuroki et al., 1986).

In vitro experiments with TCDD and similar hydrophobic compounds pose a problem in that their distribution in a lipid suspension, such as occurs with microsomes or reconstituted cytochrome P450d, will result in micro heterogenieity as TCDD would be found only in the lipid phase. In this respect, one could argue that the inhibitor would be directed to the active site of cytochrome P450d since the active site is a very hydrophobic domain and cytochrome P450d itself would be found in the lipid phase, thus enhancing the binding of TCDD to the cytochrome. Although these factors would contribute to the rapid association of TCDD with cytochrome P450, there is considerable structural specificity for inhibition of cytochrome P450d that appears to be limited by steric constraints rather than hydrophobicity. We found that isomers of hexabromobiphenyl had inhibition constants that varied over 700 fold depending on chemical structure (Voorman and Aust, 1987).

Compounds which exhibit tight binding inhibition are typically substrate or transition state analogues. It is difficult to make any inferences about the structure of TCDD in this regard since so little is known about either the active site of cytochrome P450d or the substrate(s) for the enzyme. Johnson *et al.* (1986) have developed a mechanism based inhibitor of rabbit cytochrome P450 1, an enzyme with high progesterone 21-hydroxylase activity wherein an amino group on the 17β side chain of pregnenolone

can coordinate with heme iron. Kellis and Vickery (1984) have shown that certain naturally occurring flavanoids can inhibit human placental aromatase (a cytochrome P450 isozyme) with a very low I_{50}. Recently the same group (Kellis *et al.*, 1987) showed that two steroid analogs with oxirane or thiirane side groups bound not only to the substrate binding site, but coordinated to heme iron and showed K_i's of 2-10 nM.

It is believed that the halogens of carbon tetrachloride ligate directly to heme iron of cytochrome P450, in place of oxygen, and that the chemical is reductively metabolized by cytochrome P450 (Nastainczyk *et al.*, 1982). The halogens of TCDD could interact specifically with heme iron in a similar manner. TCDD was metabolized *in vivo* and *in vitro* (Sawahata *et al.*, 1982; Ramsey et al., 1982) although metabolic transformation occurred very slowly. Thus the binding and subsequent slow metabolism of TCDD by cytochrome P450d remains a possibility. Ligation to heme seems unlikely however, since no spectral perturbations were observed when hexabromobiphenyl was bound to cytochrome P450d and CO was not inhibited from binding the reduced cytochrome (unpublished observations). It is most likely that TCDD simply fits the substrate binding site very well but is not susceptible to metabolism. The inhibitor would in this case be competitive, but since binding is tight and the off-rate so slow, the inhibitor

inactivates the enzyme and behaves as a non-competitive inhibitor. In effect it acts like a suicide substrate except that a covalent bond is not formed between enzyme and substrate.

REFERENCES

Ackermann, W.W., and Potter, V.R. (1949) Enzyme inhibition in relation to chemotherapy. Proc. Soc. Exptl. Biol. Med. 72, 1-9.

Astrom, A., and DePierre, J.W. (1985) Metabolism of 2-acetyl-aminofluorene by eight different forms of cytochrome P450 isolated from rat liver. Carcinogensis 6, 113-120.

Cha, S. (1975) Tight binding inhibitors-I; Kinetic behavior. Biochem. Pharmacol. 24, 2177-2185.

Cheng, Y.C., and Prusoff, W.H. (1973) Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmac. 22, 3099-3108.

Chou, T.C. (1974) Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms, and different types and mechanisms of inhibition. Molec. Pharmac. 10, 235-247.

Cornish-Bowden, A. (1979) Fundamentals of enzyme Kinetics. Butterworths, London.

Dignam J.B., and Strobel, H.W. (1977) NADPH-cytochrome P450 reductase from rat liver: Purification by affinity chromatography and characterization. Biochem. 16, 1116-1123.

Fisher, G.J., Fukushima, H., and Gaylor, J.L. (1981) Isolation, purification, and properties of a unique form of cytochrome P450 in microsomes of isosafrole-treated rats. J. Biol. Chem. 256, 4388-4394.

Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, M.V., and Kaminsky, L.S. (1982) Purification and characterization of liver microsomal cytochrome P450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or B-Naptho flarone. Biochem. 21, 6019-6030.

Henderson, P.J.F. (1972) A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors. Biochem. J. 127, 321-333.

Imai, Y., Hashimoto-Yutsudo, C., Satoke, H., Girardin, A., and Sato, R. (1980) Multiple forms of cytochrome P450 purified from liver microsomes of phenobarbital- and 3-methylcholanthrene-pretreated rabbits. I. Resolution, purification, and molecular properties. J. Biochem. 88, 489-503.

Johnson, E.F., Schwab, G.E., Singh, J., and Vickery, L.E. (1986) Active site-directed inhibition of rabbit cytochrome P450 by amino substituted steroids. J. Biol. Chem. 261, 10204-10209.

Kellis, J.T., Jr., Childers, W.E., Robinson, C.H., and Vickery, L.E. (1987) Inhibition of aromatase cytochrome P450 by 10-oxirane and 10-thiirane substituted androgens: Implications for the structure of the active site. J. Biol. Chem. 262, 4421-4426.

Kellis, J.T., and Vickery, L.E. (1984) Inhibition of human estrogen synthetase (aromatase) by flavone. Science 225, 1032-1034.

Kuroki, J., Koga, N., Yoshimura, H. (1986) High affinity of 2,3,4,7,8-pentachlorodibenzofuran to cytochrome P450 in the hepatic microsomes of rats. Chemosphere 15, 731-738.

Morrison, J.F. (1969) Kinetics of the reversible inhibition of enzyme-catalyzed reactions by tight-binding inhibitors. Biochem. Biophys. Acta 185, 269-286.

Morrison, J.F. (1982) The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions. Trends Biochem. Sci. 7, 102-106.

Nastainczyk, W., Ahr, H.J., and Ullrich, V. (1982) The reductive metabolism of halogenated alkanes by liver microsomal cytochrome P450. Biochem. Pharmacol. 31, 391-396.

Numazawa, M., Kiyono, Y., and Nambara, T. (1980) A simple radiometric assay for estradiol 2-hydroxylase activity. Anal. Biochem. 104, 290-295.

Philpot, R.M., Hodgson, E. (1972) The effect of piperonyl butoxide concentration on the formation of cytochrome P450 difference spectra in hepatic microsomes from mice. Mol. Pharmac. 8, 204-214.

Poland, A., Glover, E., Kende, A.S. (1976) Stereospecificity high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol: evidence that the binding species is receptor for induction of arlyl hydrocarbon hydroxylase. J. Biol. Chem. 251, 4936-4946.

Ramsey, J.C., Hefner, J.G., Karbowski, R.J., Braun, W.H., and Gehring, P.J. (1982) The in vivo biotransformation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the rat. Toxicol. Appl. Pharmacol. 65, 180-184.

Reik, L.M., Levin, W., Ryan, D.E., and Thomas, P.E. (1982) Immunochemical relatedness of rat hepatic microsomal cytochromes P450c and P450d. J. Biol. Chem. 257, 3950-3957.

Ryan, D.E., Thomas, P.E., and Levin, W. (1980) Hepatic microsomal cytochrome P450 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 P450 from rats treated with polychlorinated biphenyls. Arch. Biochem. Biophys. 216, 272-288.

Sawahata, T., Osoh, J.R., and Neal, R.A. (1982) Identification of metabolites of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) formed on incubation with isolated rat hepatocytes. Biochem. Biophys. Res. Comm. 105, 341-346.

Tuteja, N., Gonzalez, F.J., Nebert, D.W. (1985) Developmental and tissue-specific differential regulation of the mouse dioxin-inducible P_1-450 and P_3-450 genes. Developmental Biology 112, 177-184.

Voorman, R., and Aust, S.D. (1987) Specific binding of polyhalogenated aromatic hydrocarbon inducers of cytochrome P450d to the cytochrome and inhibition of its estradiol 2-hydroxylase activity. Toxicol. Appl. Pharmacol., in press.

Whitlock, J.P., Jr. (1986) The regulation of cytochrome P450 gene expression. Ann. Rev. Pharmacol. Toxicol. 26, 333-369.

Wroblewski, V.J., and Olson, J.R. (1985) Hepatic metabolism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the rat and guinea pig. Toxicol. Appl. Pharmacol. 81, 231-240.

Yasukochi, Y. (1976) Some properties of a detergent solubilized NADPH cytochrome c (cytochrome P450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. 251, 5337-5344.

CHAPTER III

INDUCERS OF CYTOCHROME P450d: INFLUENCE ON MICROSOMAL *IN VIVO* CATALYTIC ACTIVITY AND DIFFERENTIAL REGULATION BY ENZYME STABILIZATION

ABSTRACT

The *in vivo* significance of the interaction of isosafrole, 3,4,5,3',4',5'-hexabromo-, and hexachlorobiphenyl on cytochrome P450d was evaluated enzymatically by determination of microsomal estradiol 2-hydroxylase activity. Displacement of the isosafrole metabolite from microsomes derived from isosafrole treated rats resulted in a 160% increase in estradiol 2-hydroxylase. The increase was fully removed by incubation with 1 μM HBB. Although isosafrole is capable of forming a complex with many differernt cytochrome P450 isozymes, it apparently binds significantly only to cytochrome P450d *in vivo* as was demonstrated by measuring the enzymatic activity of microsomal cytochrome P450d, P450c and P450d from isosafrole treated rats. When estradiol 2-hydroxylase was measured in rats treated with increasing doses of HCB, there was a gradual decrease in enzyme activity despite a 20-fold increase in cytochrome P450d over the dose range.

The ability of cytochrome P450d ligands to stabilize the enzyme was investigated in two ways. First, cytochromes P450c and P450d were immunochemically measured in microsomes from rats treated with TCDD, at a dose to maximally induce total cytochrome P450, followed by a single dose of a potential enzyme stabilizing inducer. The specific content of cytochrome P450d was significantly increased when isosafrole or HCB was the second inducer but not when 3-methylcholanthrene was the second inducer. Second, the

relative turnover of cytochrome P450d was measured by the dual label technique. Following TCDD treatment, microsomal protein was labeled *in vivo* with ^{3}H-leucine, the second inducer was given and protein was again labeled 3 days later with ^{14}C-leucine. Immunoprecipitation by cytochrome P450d from the various treatment groups revealed a higher ratio of $^{3}H/^{14}C$ in the ISF- and HCB-treated rats relative to TCDD (control) treated rats suggesting that isosafrole and HCB were able to retard the degradation of cytochrome P450d, presumably by virtue of being tightly bound to the active site.

INTRODUCTION

Cytochrome P450 is a family of monooxygenases which activate dioxygen to hydroxylate a variety of structurally diverse substrates. These may be bound to the enzyme in either a highly specific manner, as in the case of adrenal steroids, or a relatively non-specific manner, as apparently happens with many xenobiotics. Certain cytochrome P450 isozymes are induced in respone to presumed xenobiotic substrates. Induction of cytochromes P450c and P450d is thought to be controlled via the Ah receptor, a cytosolic protein for which certain aromatic hydrocarbons have an unusually high affinity (Nebert and Gonzalez, 1985). Recently, it has been shown that certain compounds which induce cytochrome P450 also bind to the enzyme and inhibit its catalytic activity (Voorman and Aust, 1987). Isosafrole is a potent inducer of cytochrome P450d and is metabolized by the enzyme to form a metabolite-intermediate complex which inhibits the catalytic activity of cytochrome P450d (Ryan *et al.*, 1980; Murray *et al.*, 1986). It has been suggested that isosafrole does not induce cytochrome P450d via the Ah receptor but regulates cytochrome P450d synthesis in another manner (Cook and Hodgson, 1985). Steward *et al.* (1985) has shown that isosafrole can prolong the half-life of cytochrome P450d in primary cultures of rat hepatocytes. However, in rat liver microsomes there was no evidence for a change in the half life of cytochrome P450d relative to

other cytochrome P450s induced by 5,6-benzoflavone (Shiraki and Guengerich, 1984).

In this report it is shown that isosafrole and 3,4,5,3',4',5'-hexachlorobiphenyl (HCB) induce and inhibit cytochrome P450d in the rat liver. Both isosafrole and HCB have an additive inducing effect on cytochrome P450d beyond that maximally induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The additive effect is probably due to a prolonged half life of the enzyme resulting from stabilization of the enzyme by the bound inducer.

METHODS AND MATERIALS

Chemicals

Isosafrole was obtained from Fluka (Ronkonkoma, NY), 3,4,5,3',4',5'-hexachlorobiphenyl was purchased from Pathfinder Laboratories (St. Louis, MO); 2-[^{3}H]-estradiol, was obtained from New England Nuclear (Boston, MA). TCDD was generously supplied by Dow Chemical Company (Midland, MI). 3,4,5,3',4',5'-Hexabromobiphenyl was obtained from Ultra Scientific, Inc. (Hope, RI) and purified by alumina column chromatography (Millis, 1984). L-[4,5-^{3}H]Leucine (sp. act. 146 Ci/mmol), L-[U-^{14}C]leucine (sp. act. 342 mCi/mmol) and NCS tissue solubilizer were obtained from Amersham (Arlington Heights, IL). Goat anti-rabbit peroxidase complex was purchased from Capped Labs (Malvern, PA).

Animal Treatment

Male Sprague-Dawley rats (Charles River, Portage, MI) were treated with the inducing chemical dissolved in corn oil by oral gavage as described in figure legends. Rats were starved overnight and killed by decapitation following CO_2 anesthesia; livers were homogenized in 1.15% KCl, 10 mM EDTA, pH 7.4 (1:4, w/v). Microsomes were prepared by differential centrifugation, washed with 100 mM sodium pyrophosphate, pH 7.4, containing 0.1 mM EDTA and suspended in 20 mM potassium phosphate, pH 7.4, containing 20% glycerol, and 0.1 mM EDTA and stored at -20°C. For the pulse labeling studies, animals were treated with a single dose of TCDD followed three days later by an ip dose of ^{3}H-leucine (300 uCi/ml in 0.9% NaCl) at 3 mCi/kg. Three hours after labeling, animals were dosed by oral gavage with the second inducer. Exactly three days after the ^{3}H-leucine dose, animals were treated with ^{14}C-leucine (50 µCi/ml in 0.9% NaCl) at 500 µCi/kg and killed 4 hr later. Microsomes were prepared as described above.

Assays

Ethoxyresorufin-O-dealkylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) were measured by a modified method of Pohl and Fouts (1980). Samples (5-50 µg microsomal protein) were prepared for a final volume of 1 ml in 100 mM potassium phosphate, 0.1 mM EDTA, pH 7.8 buffer.

Substrate was added in 10 µl methanol (standardized by E_{482} = 22.5 mM^{-1} cm^{-1}) and following 3 min incubation at 37°C, the reaction was started by addition of an NADPH generating system. The reaction was terminated after 3 min by addition of 2 ml 50% methanol. Following centrifugation, resorufin fluorescence of the supernatant was measured at 585 nm using excitation at 530 nm and compared to a resorufin standard in buffer (standardized by E_{472} = 73 mM^{-1} cm^{-1}). Estradiol-2-hydroxylase was measured by the method of Numazawa (1980) as modified by Ryan (1982). Protein was determined by the bicinchoninic acid (Pierce Chemical, Rockford, IL) micromethod (Redinbaugh, 1986) and stardardized with bovine serum albumin.

Cytochrome P450c and P450d were quantified by ELISA as described (Voorman and Aust, 1987). Immunoprecipitation of cytochrome P450d was done by suspending 50-200 µg microsomal protein in 300 µl 0.1 M potassium phosphate, pH 7.4, containing 0.4 M KCl, 0.4 mM EDTA, 0.5% cholate, 0.1% Emulgen 911, 0.02% azide and 1% BSA and adding 100-300 µl anti-serum against cytochrome P450d. This mixture was incubated overnight at 4°C, and spun in a centrifuge. The immunoprecipitate was washed once with the buffer just described and once with water. The pellet was dissolved in NCS tissue solubilizer, added to scintillation cocktail and $^{3}H/^{14}C$ determined in a Beckman LS 5801 liquid scintillation counter.

Total cytochrome P450 content was measured by absorption at ~450 nm of the dithionite-reduced CO difference spectrum using $E_{450-490}$ = 91 mM^{-1} cm^{-1}. The isosafrole complex was measured spectrophotometrically by reducing the sample cuvet with dithionite and measuring $E_{455-490}$ =75 mM^{-1} cm^{-1}.

RESULTS

Effect of Isosafrole on Estradiol 2-Hydroxylase

Previously it was shown that HCB, when incubated with purified cytochrome P450d, could inhibit cytochrome P450d estradiol 2-hydroxylase by forming a tightly bound complex with cytochrome P450d. When microsomes from isosafrole treated rats were incubated with n-butanol and chromatographed on Sepharose Cl-6B to remove the isosafrole metabolite and n-butanol there was a 160% increase in estradiol 2-hydroxylase activity relative to non-butanol treated microsomes (Figure 1). The estradiol 2-hydroxylase activity in the non-butanol treated sample is likely due to other constitutive isozymes of cytchrome P450 since another potent inhibitor of cytochrome P450d, HBB, was able to titrate out the estradiol 2-hydroxylase due to cytochrome P450d. This suggests that HBB is able to inhibit cytochrome P450d to the same degree as isosafrole even though it has a considerably different structure.

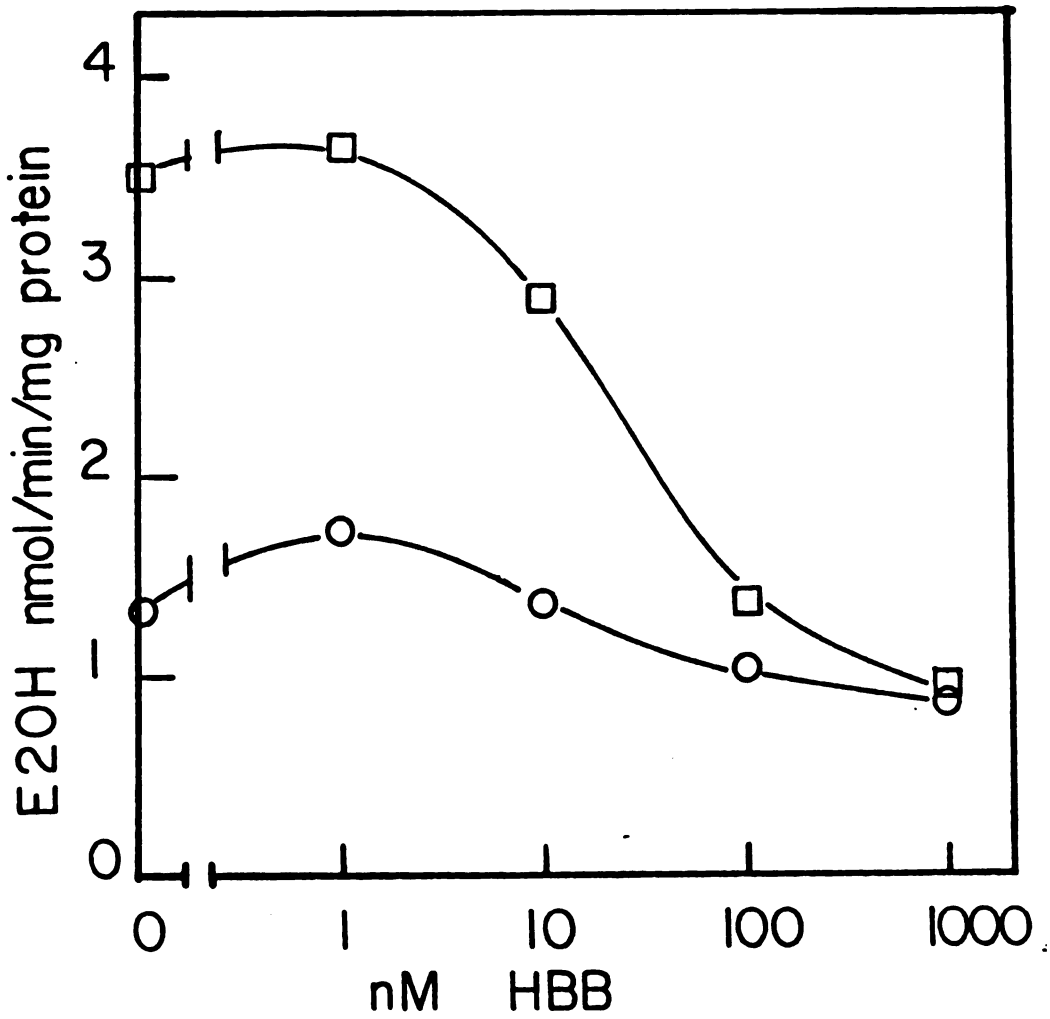


Figure 1. Effect of HBB on microsomal estradiol 2-hydroxylase from isosafrole treated rats. Male Sprague-Dawley rats (150-250 gm) were treated with isosafrole (150 mg/kg) by oral gavage once a day for three days and killed on the fourth day following overnight starvation. Hepatic microsomes were isolated, and an aliquot was treated with 250 mM n-butanol at 37° for 30 min followed by chromatography to remove the isosafrole metabolite. Estradiol 2-hydroxylase in 50 µg protein was measured with the isosafrole metabolite intermediate complex still bound (-o-) or following displacement and removal of the metabolite (-□-).

The effect of isosafrole on other microsomal cytochrome P450 isozymes was evaluated using relatively specific substrates for cytochrome P450b, c, and d, each isozyme known to be induced by isosafrole (Table 1). In each case there was a significant increase in enzymatic activity following isosafrole displacement by butanol, however the magnitude of increase varied depending on substrate and isozyme contribution. Estradiol 2-hydroxylase, measures both P450d and P450h (Astrom & Depierre, 1985), therefore, the 47% increase in activity is due to cytochrome P450d. The smaller increase in activity compared to Figure 1 is probably due to a lower specific content of cytochrome P450d. Ethoxyresorufin-o-dealkylase is catalyzed almost exclusively by cytochrome P450c (Astrom & DePierre, 1985). Thus, the high rates observed in each treatment group for this activity are in all likelihood due to cytochrome P450c, apparently largely uncomplexed with isosafrole prior to treatment with butanol. The 30% increase in ethoxyresorufin- o-dealkylase following isosafrole displacement is probably due not only to the freeing of some cytochrome P450c but also to cytochrome P450d which has about 10% the ethoxyresorufin-o-dealkylase activity of cytochrome P450c. Pentoxyresorufin-o-dealkylase, specific for cytochrome P450b (Burke et al., 1985), showed 31% increase in activity following displacement of the isosafrole metabolite.

TABLE 1. Catalytic activity of microsomes from isosafrole treated rats. Rats were treated for 3 days with isosafrole (150 mg/kg/day); microsomes were isolated and either used without further treatment or chromatographed with or without preincubation in 250 mM n-butanol. Conditions for enzyme assays were as described in Methods. Numbers in parenthesis are standard deviations (n=3).

	Activity (nmol/min/mg protein)		
		Chromatographed	
	Untreated	Control	+BuOH
Estradiol 2-hydroxylase	1.29 (0.06)	1.48 (0.11)	2.18* (0.24)
Ethoxyresorufin-O-deethylase	8.29 (0.28)	8.70 (0.42)	11.3* (1.1)
Pentoxyresorufin-O-depentylase	0.68 (0.03)	0.64 (0.07)	0.84* (0.05)

*Significantly different from controls by Duncan's multiple range test at 5% level.

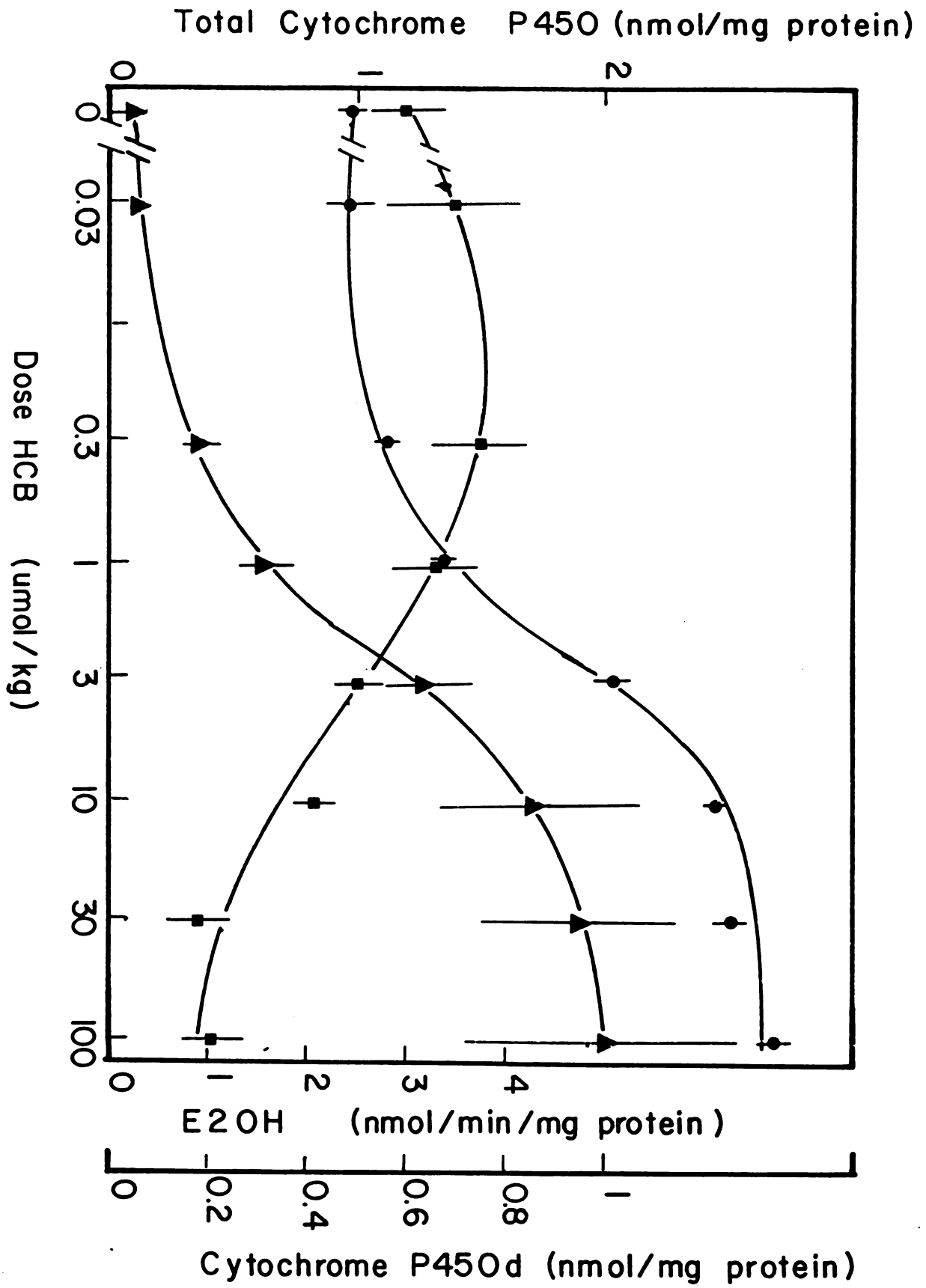
Effect of HCB on Microsomal Estradiol 2-Hydroxylase

Although an endogenous substrate for cytochrome P450d is not known, it was of interest to assess the effect of inducers which bind to P450d on the metabolic activity of P450d. Thus, estradiol 2-hydroxylase was evaluated over a log-dose range of HCB (Figure 2). It can be seen that, in general, estradiol 2-hydroxylase activity decreased with increasing dose of HCB despite the 20 fold increase in level of cytochrome P450d and 2.5-fold increase in total cytochrome P450. The activity of estradiol 2-hydroxylase at the control dose (no HCB) is probably due to other cytochrome P450 isozymes including cytochrome P450h. The decrease in enzyme activity to below the control level could be due to a repression of cytochrome P450h synthesis since it was shown that levels of this isozyme decrease in response to certain polybrominated biphenyls (Dannan et al., 1982). We showed earlier that the bromo analogue of HCB did not inhibit cytochrome P450h (Voorman & Aust, 1987).

Effect of HCB and Isosafrole on Regulation of Cytochrome P45d

Cytochrome P450c and P450d are induced in the rat by TCDD in a dose-dependent fashion with maximum induction occurring at 10 nmol TCDD/kg body weight (Fig. 3). Like many inducers of cytochrome P450, induction was maximal three days following dosage and did not change significantly over the 12-day period (Figure 4). The effect of isosafrole

Figure 2. Effect of 3,4,5,3',4',5'-hexachlorobiphenyl on cytochrome P450 specific content and estradiol 2-hydroxylase. Male Sprague-Dawley rats (~175 gm) were treated with HCB by oral gavage at the doses shown and killed three days later following overnight starvation. Liver microsomal were isolated and assay for total cytochrome P450 specific content (o-o) and specific content of cytochrome P450d(Δ-Δ). Estradiol 2-hydroxylase was measured with 50 μg protein (□-□).


Total Cytochrome P450 (nmol/mg protein)
0
1
2
Dose HCB (umol/kg)
0
0.03
0.3
1
3
10
30
100
0
1
2
3
4
E2OH (nmol/min/mg protein)
0
0.2
0.4
0.6
0.8
1
Cytochrome P450d (nmol/mg protein)

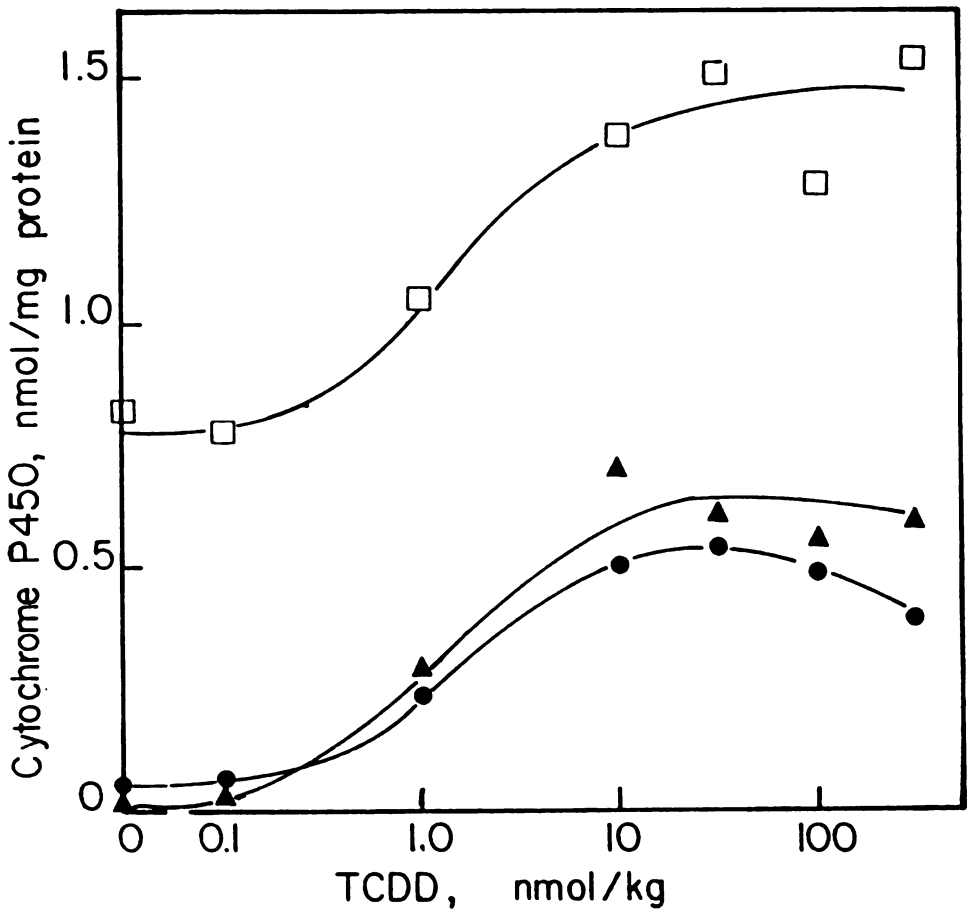


Figure 3. Effect of TCDD on cytochrome P450 specific content. Male Sprague-Dawley rats (~125 gm) were treated with a single dose of TCDD at the levels shown. Animals, three per treatment group, were killed 12 days later, hepatic microsomes were isolated, and specific content of cytochrome P450 was determined. Total cytochrome P450 (□-□); cytochrome P450c (Δ-Δ); cytochrome P450d (o-o).

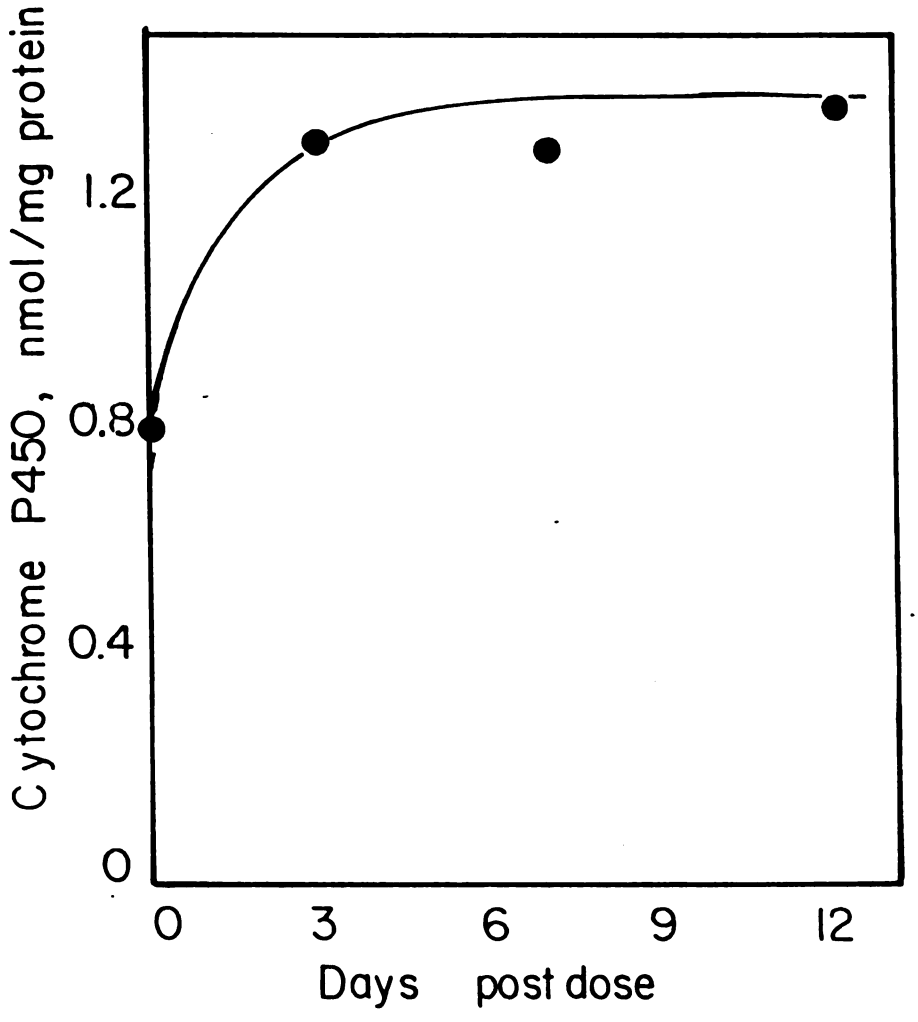


Figure 4. Effect of TCDD on cytochrome P450 specific content over time following single dose of TCDD. Rats were given a single dose of TCDD (10 nmol/kg) as described in Figure 3, and killed 3, 7, and 12 day following dosage. Microsomes were prepared and cytochrome P450 measured as described in "Methods."

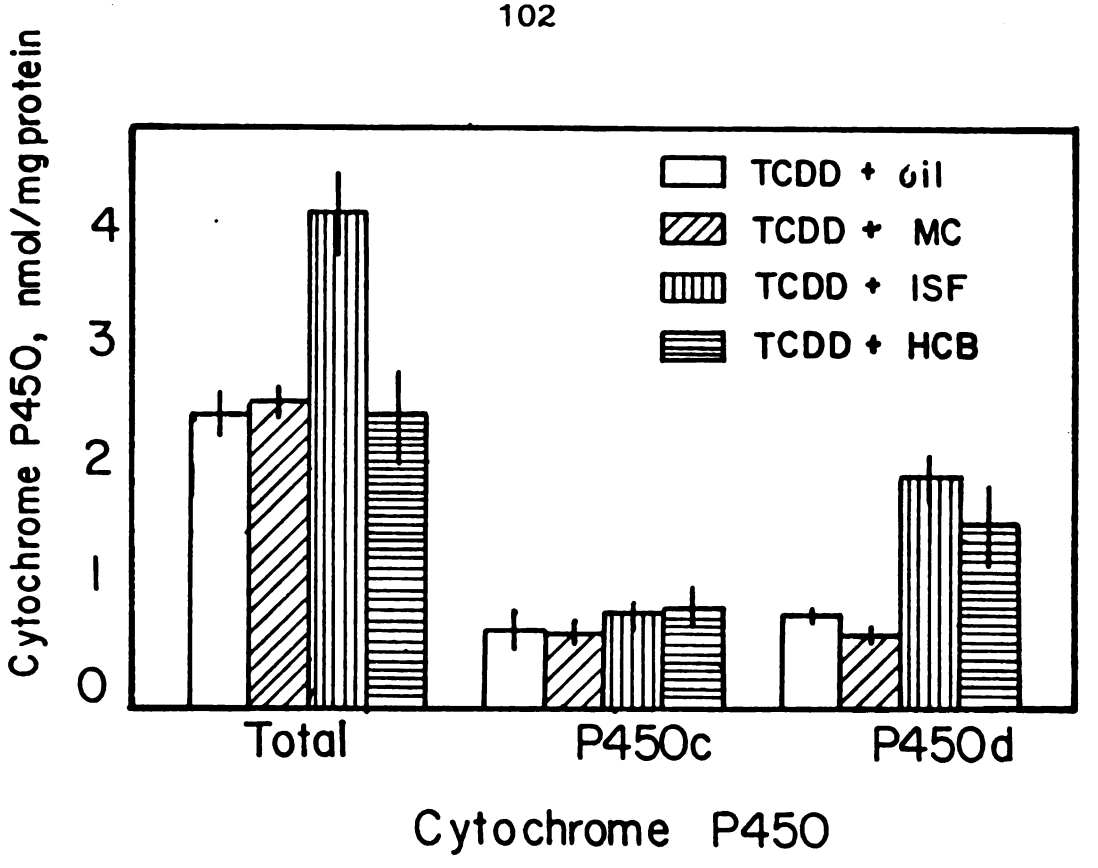


Figure 5. Effect of TCDD plus 3-methylcholanthrene, isosafrole, and HCB on levels of cytochrome P450. Male Sprague-Dawley rats (~150 gm) were treated with TCDD (10 nmol/kg) by oral gavage; three days later the previously treated rats were given a single dose of a second inducer or corn oil control (3 rats per treatment group). Rats were killed 24 hr later following overnight starvation and liver microsomes were prepared. Total cytochrome P450 represents that determined spectrally by the dithionite-reduced CO difference spectrum and, in the case of isosafrole, also includes the metabolite-intermediate complex. Cytochromes P450c and P450d were quantitated immunochemically as described in "Methods."

and HCB on the regulation of cytochrome P450c and P450d was examined in rats previously treated with TCDD (Figure 5). Rats given corn oil or 3-methylcholanthrene showed no significant difference in the level of cytochrome P450c or P450d. However, rats given isosafrole or HCB in addition to the earlier dose of TCDD showed a marked increase in cytochrome P450d content, but not cytochrome P450c. Rats were treated with HCB at a concentration to produce maximum induction of cytochrome P450d (see Figure 2). Quantitation of microsomal HCB content and cytochrome P450d content in the 100 nmol/kg group in the dose-response experiment (Figure 2) revealed a 0.7:1 association HCB to P450d. Microsomes from the isosafrole-treated animals clearly showed the presence of the isosafrole metabolite-intermediate complex when spectrally assayed. The isosafrole-treated rats had significantly increased specific content of total cytochrome P450 (including measurement of the metabolite-intermediate complex) but the HCB-treated rats did not. The lower total P450 content in the latter group could be due to the presence of apo-cytochrome P450.

The stabilizing effect of inducing ligands on cytochrome P450d was investigated by labeling cytochrome P450d, following its induction by TCDD, using the Schimke method (Arias et al., 1969). It seemed likely that if certain ligands could stabilize cytochrome P450d, then labeling of the cytochrome P450d pool, followed by addition

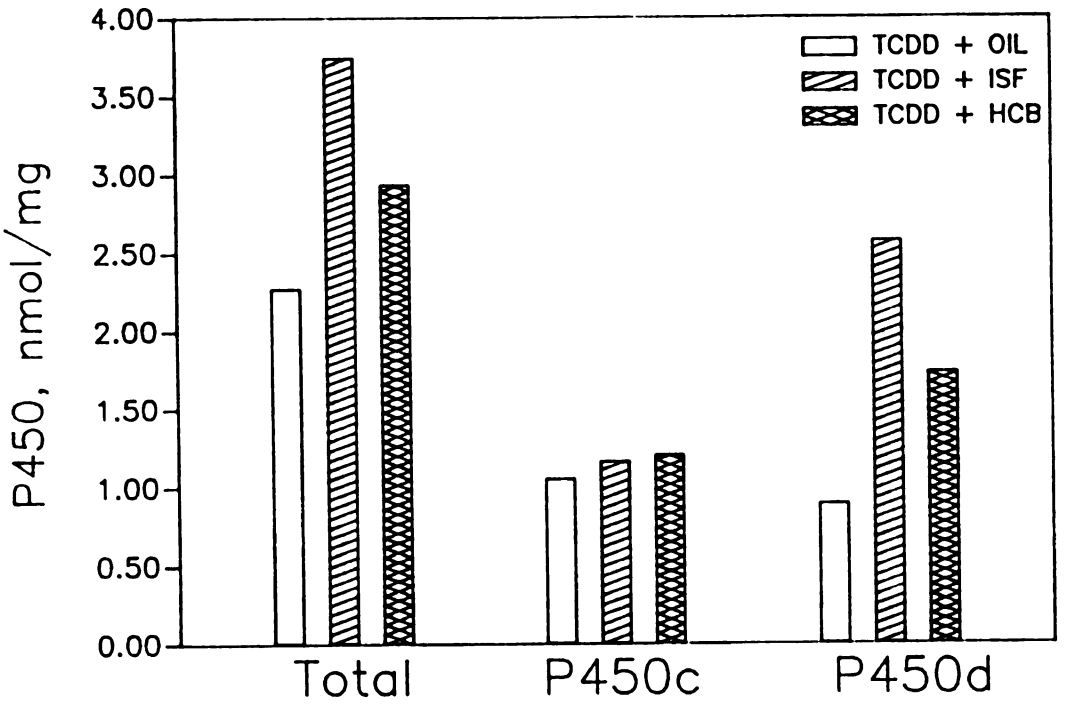


Figure 6. Effect of isosafrole and HCB on cytochrome P450 specific content following treatment with TCDD. Treatments were as described in Table 2. Cytochrome P450 was measured as described in Figure 5.

of the stabilizing ligand, should result in an increased half life of the labeled cytochrome P450d. The levels of cytochrome P450c and P450d were similar to those of the last experiment, except that now the increased level of cytochrome P450d in the HCB-treated group is reflected in the total specific content of cytochrome P450 (Figure 6). Significant differences in the incorporation of radioactivity into cytochrome P450d were apparent when the immunoprecipitation was carried out based on either P450d or protein concentration (Table 2). By virtue of the increased ratio of $^{3}H/^{14}C$ in the isosafrole- or HCB treated rats, it seems likely that these compounds increase the half-life of cytochrome P450d in the rat.

DISCUSSION

Our results suggest that even though isosafrole can form the metabolite-intermediate complex *in vitro* with several cytochrome P450 isozymes (Ryan *et al.*, 1980), the binding and inhibition were significant only with regard to cytochrome P450d. Apparently HBB, a structurally-dissimilar chemical, was able to inhibit estradiol 2-hydroxylase to the same degree as isosafrole. It has been suggested that isosafrole also binds significantly to and inhibits cytochrome P450b (Murray *et al.*, 1986) as determined by a four-fold increase in androstenedione 16β-hydroxylation following isosafrole displacement. The results in Table I

Table 2. Effect of inducers on the turnover of cytochrome P450. Male Sprague-Dawley rats (50-60 gm) were treated with TCDD (10 nmol/kg). Three days later, animals were treated (2 per group) with ^{3}H-leucine, followed immediately by corn oil, isosafrole (150 mg/kg) or HCB (100 µmol/kg). 72 hours after the ^{3}H-leucine, all animals were administered ^{14}C-leucine and killed 4 hr later. Hepatic microsomes were prepared as described in "Methods." Radioactivity in total microsomal protein was measured by solubilization of 200-400 µg protein in NCS tissue solubilizer and counting ^{3}H and ^{14}C. Cytochromes P450d and P450c were immunoprecipitated from 50-200 µg protein. The precipitate was washed, solubilized, and assayed for ^{3}H and ^{14}C.

	Microsomes dpm/mg protein			P450d dpm in ippt.			P450c dpm in ippt.		
Treatment	^{3}H	^{14}C	Ratio	^{3}H	^{14}C	Ratio	^{3}H	^{14}C	Ratio
TCDD + oil	22,706	14,780	1.53	827	545	1.52	714	334	2.14
TCDD + Isosafrole	27,375	14,171	1.94	1397	540	2.60	646	298	2.17
TCDD + HCB	26,578	13,630	1.95	1358	496	2.74	541	222	2.43

agree with this in part; however, the magnitude of increase (30%) was not as great as previously observed by Murray et al. (1986). The results agree with a previous report indicating that isosafrole forms a stable complex only with cytochrome P450d (Ryan et al., 1980). Also, we found that the isosafrole metabolite was detected only in cytochrome P450d but not cytochromes P450c or P450h purified from isosafrole treated rats (data not shown).

Hexachlorobiphenyl induces cytochrome P450 in a dose-dependent manner and, in accordance with earlier results, binds to and apparently inhibits cytochrome P450d estradiol 2-hydroxylase. A recent report suggests that NADPH-cytochrome P450-reductase can become limiting in cytochrome P450d catalyzed reactions (Graham et al., 1987). Although this phenomenon could have occurred in our experiment, there should still have been an increase estradiol 2-hydroxylase as was observed upon displacement of isosafrole from cytochrome P450d in Figure 1.

It has been known for some time that cytochromes P450c and P450d are induced coordinately by numerous xenobiotics. The genetic studies using the mouse (Gonzalez et al., 1984) suggest that induction of both proteins is controlled from the Ah receptor at the level of transcription. The receptor has been found in five tested mammalian species and has significant homology across these species (Gasiewicz and Rucci, 1984). In addition, cytochrome P450c and P450d have been identified in variety of species including humans and

also share significant homology across species (Thomas et al., 1984; Jaiswal et al., 1985; Quattrochi et al., 1985). Other data, however, suggest that the regulation of the two isozymes is differential, depending on the inducer (Thomas et al., 1983; Dannan et al., 1983; Parkinson et al., 1983). That is, the relative levels of the two isozymes can vary considerably in response to structurally diverse chemicals.

Other factors, both cis-acting (on the gene) and trans-acting (receptor level) have been suggested as controlling gene expression (Jones et al., 1985), but demonstration of inducer interaction with these factors resulting in altered gene expression appears limited to the Ah receptor.

The induction of cytochrome P450 elicited by isosafrole is an enigma. This compound has a low degree of structural similarity to the high affinity ligands of the Ah receptor, yet it is a very potent inducer of cytochrome P450d. Although it has been assumed that it could serve a ligand for the Ah receptor (the methylene dioxyphenyl structure is aromatic in nature), recent data suggest that it is not a competitive ligand (Cook and Hodgson, 1985) and thus, casts doubt on its ability to regulate cytochrome P450d synthesis through the Ah receptor. Moreover, other experiments suggest that isosafrole may act in an additive, or even synergistic, manner with the Ah ligand 3-methylcholanthrene (Fennel et al., 1979; Thomas et al., 1983). In these experiments, animals were treated for one day with isosafrole following three days treatment with

3-methylcholanthrene. This resulted in greater levels of cytochrome P450d than obtained with three days treatment of isosafrole or methylcholanthrene above. Implicit in these results is that isosafrole might have an effect on cytochrome P450d beyond that controlled by the Ah receptor, assuming, of course, that methylcholanthrene elicited the maximum response obtainable through the Ah receptor. Certain polychlorinated and polybrominated biphenyls are also potent inducers of cytochrome P450d, and are unusual in that they induce cytochrome P450d to greater levels than cytochrome P450c (Parkinson et al., 1983; Ozawa et al., 1979). Several of these inducers can form stable complexes with cytochrome P450d (Voorman and Aust 1987; Kuroki et al., 1986). It seemed possible that compounds which induce and bind to cytochrome P450d might also stabilize the enzyme against degradation and prolong its half life. To test this possibility, TCDD was used to induce cytochrome P450d followed by treatment with potential enzyme-stabilizing ligands. The dose of TCDD was sufficient to induce maximum levels of cytochrome P450d, and presumably saturate the Ah receptor, yet the dose was low enough that there was approximately a hundred-fold excess of hepatic cytochrome P450d over the total body burden of TCDD. Therefore, even though TCDD can bind to cytochrome P450d, it could not stabilize a significant part of the pool of cytochrome P450d. TCDD treatment will result in prolonged occupation of the Ah receptor and continued induction of cytochrome P450d. Thus,

an additional ligand for Ah receptor should not have an additive inducing effect with TCDD. Indeed, 3-methylcholanthrene, a ligand for the Ah receptor had no additional inducing effect when given to rats following TCDD treatment. This was in contrast to the additive induction of cytochrome P450d by both isosafrole and HCB suggesting control of cytochrome P450d beyond the level of the Ah receptor.

To test the possibility that the stabilizing ligands might effect the turnover or half life of the protein, the double label technique of Schimke was used to measure in vivo protein degradation. The double label technique allows measurement of protein degradation in a single animal, eliminating significant inter-animal variation and also measures relative rather than absolute rates of change (Arias et al., 1969). There was greater retention of the first label [^{3}H] relative to the second label [^{14}C] in the isosafrole- and HCB-treated rats. These differences in ratio were apparent in the specific activity of total microsomal protein, and the differences were magnified upon precipitation of cytochrome P450d.

Primary cultures of rat hepatocytes have the capacity to synthesize cytochrome P450c but, for unknown reasons, not cytochrome P450d (Steward et al., 1985). When hepatocytes were isolated from rats previously treated with agents to induce cytochrome P450d, the level of the enzyme decreased to zero over three days. However, if isosafrole was added

to the culture medium degradation was significantly retarded, indicating stabilization of cytochrome P450d by isosafrole. The macrolide antibiotic, tricetyloleandomycin (TAO), greatly increases levels of cytochrome P450p, a glucocorticoid responsive enzyme (Watkins et al., 1986). TAO forms a metabolic intermediate complex with P450p and when given with dexamethasone, increases levels of cytochrome P450p about three fold over that maximally induced by dexamethasome alone. Measurement of cytochrome P450p degradation by both in vivo and in vitro pulse labeling, revealed a significant decrease in that rate of cytochrome P450p degradation in response to TAO treatments.

The mechanism by which these compounds could inhibit cytochrome P450 degradation is unknown. Substrate-stabilization of enzymes has been demonstrated with arginase and tryptophane pyrrolase (Schimke 1964; Schimke et al., 1965). It is possible that degradation of cytochrome P450 is controlled in part by heme oxygenase (Sadler et al., 1986). Cytochrome P450 and its heme turn over at the same rate (Parkinson et al., 1983a). Thus, a substrate (or inhibitor) that prevents access to heme or prohibits its removal, could control degradation of the enzyme.

REFERENCES

Arias, I.M., Doyle, D. and Schimke, R. (1969) Studies on the synthesis and degradation of proteins of the endoplasmic reticulum of rat liver. J. Biol. Chem. 244:3303-3315.

Astrom, A., and DePierre, J.W. (1985) Metabolism of 2-acetyl-aminofluorene by eight different forms of cytochrome P450 isolated from rat liver. Carcinogenesis 6:113-120.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., and Mayer, R.T. (1985) Ethoxy-, pentoxy- and benzyl-oxyphenazones and homologues: a series of substrates to distinguish between different induced cytochromes P450. Biochem. Pharmacol. 34:3337-3345.

Cook, J.C., and Hodgson, E. (1985). The induction of cytochrome P450 by isosafrole and related methylene dioxyphenyl compounds. Chem.-Biol. Interactions 54:299-315.

Dannan, G.A., Gungerich, F.P., Kaminsky, L.S. and Aust, S.D. (1983) Regulation of cytochrome P450. Immunochemical quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. J. Biol. Chem. 258:1282-1288.

Fennell, T.R., Dickens, M., and Bridges, J.W. (1979) Interaction of isosafrole *in vivo* with rat hepatic microsomal cytochrome P450 following treatment with phenobarbitone or 20-methylcholanthrene. Biochem. Pharmac. 28:1427-1429.

Gasiewicz, T.A. and Rucci, G. (1984) Cytosolic receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Evidence for a homologous nature among various mammalian species. Mol. Pharmac. 26:90-98.

Gonzalez, F.J., Tukey, R.H. and Nebert, D.W. (1984) Structural gene products of the Ah locus. Transcriptional regulation of cytochrome P_1-450 and P_3-450 mRNA levels by 3-methylcholanthrene. Mol. Pharmac. 26:117-121.

Graham, M.J., Lucier, G.W., Rickenbacher, U. and Goldstein, J.A. (1987) Induction of rat hepatic microsomal estradiol 2-hydroxylase (E2-OHase) activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Fed. Proc. 46:361.

Jaiswal, A.K., Gonzales, F.J. and Nebert, D.W. (1985) Human dioxin-inducible cytochrome P_1-450: complementary DNA and amino acid sequence. Science 228:80-83.

Jones, P.B.C., Galeazzi, D.R., Fisher, J.M., and Whitlock, J.P., Jr. (1985) Control of cytochrome P_1-450 gene expression by dioxin. Science 227:1499-1502.

Kuroki, J., Koga, N., Yoshimura, H. (1986) High affinity of 2,3,4,7,8-pentachlorodibenzofuran to cytochrome P450 in the hepatic microsomes of rats. Chemosphere 15:731-738.

Millis, C.D. (1984) Studies on the chemical and pharmacotoxicological properties of polybrominated biphenyls. M.S. Thesis, Michigan State University.

Murray, M., Zaluzny, L., and Farrell, G.C. (1986) Selective reactivation of steroid hydroxylases following dissociation of the isosafrole metabolite complex with rat hepatic cytochrome P450. Arch. Biochem. Biophys. 251:471-478.

Nebert, D.W. and Gonzales, F.J. (1985) Cytochrome P450 gene expression and regulation. Trends Pharmacol. Sci. 6:160-164.

Numazawa, M., Kiyono, Y., and Nambara, T. (1980) A simple radiometric assay for estradiol 2-hydroxylase activity. Anal. Biochem. 104:290-295.

Ozawa, N., Yoshihara, S., Kawano, K., Okada, Y., and Yoshimura, H. (1979) 3,4,5,3',4'-pentachlorobiphenyl as a useful inducer for purification of rat liver microsomal cytochrome P448. Biochem. Biophys. Res. Commun. 91:1140-1147.

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 P450 isozymes and epoxide hydrase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. J. Biol. Chem. 288:5967-5976.

Parkinson, A., Thomas, P.E., Ryan, D.E. and Levin, W. (1983a) The in vivo turnover of rat liver microsomal epoxide hydrase and both the apoprotein and heme moieties of specific cytochrome P450 isozymes. Arch. Biochem. Biophys. 225:216-233.

Pohl, R.J. and Fouts, J.R. (1980) A rapid method for assaying the metabolism of 7-ethoxyresorutin by microsomal subcellular fractions. Anal. Biochem. 107:150-155.

Quattrochi, L.C., Okino, S.T., Usha, R.P., and Tukey, R.H. (1985) Cloning and isolation of human cytochrome P450 cDNAs homologous to dioxin-inducible rabbit mRNAs encoding P450 4 and P450 6. DNA 4:395-400.

Redinbaugh, M.G. and Turley, R.B. (1986) Adaptation of the bicinchoninic acid protein assay for use with microtiter

plates and sucrose gradient fractions. Anal. Biochem. 153:267-271.

Rose, J.Q., Ramsey, J.C., Wentzler, T.H., Hummel, R.A., and Gehring, P.J. (1976) The fate of 2,3,7,8-tetrachlorodibenzo-p-dioxin following single and repeated oral doses to the rat. Toxicol. Appl. Pharmacol. 36:209-226.

Ryan, D.E., Thomas, P.E., and Lewin, W. (1980) Microsomal cytochrome P450 from rats treated with isosafrole. Purification and characterization of four enzymic forms. 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 P450 from rats treated with polychlorinated biphenyls. Arch. Biochem. Biophys. 216:272-288.

Sadler, E.M., Reddy, V.R., and Piper, W.N. (1986) Increased rat testicular heme oxygenase activity associated with depressed microsomal heme and cytochrome P450 levels after repeated administration of human chorionic gonadotropin. Arch. Biochem. Biophys. 249:382-387.

Schimke, R.T. (1964) The importance of both synthesis and degradation in the control of arginase levels in rat liver. J. Biol. Chem. 239:3808-3817.

Schimke, R.T., Sweeney, E.W., and Berlin, C.M. (1965) The roles of synthesis and degradation in the control of rat liver tryptophan pyrrolase. J. Biol. Chem. 240:322-331.

Shiraki, H. and Guengerich, E.P. (1984) Turnover of membrane proteins: kinetics of induction of rat liver microsomal cytochrome P450, NADPH-cytochrome P450 reductase, and epoxide hydrolase. Arch. Biochem. Biophys. 235:86-96.

Steward, A.R., Wrighton, S.A., Pasco, D.S., Fagan, J.B., Li, D., and Guzelian, P.S. (1985) Synthesis and degradation of 3-methylcholanthrene-inducible cytochromes P450 and their mRNAs in primary monolayer cultures of adult rat hepatocytes. Arch. Biochem. Biophys. 241:494-508.

Thomas, P.E., Reik, L.M., Ryan, D.E., and Levin, W. (1983) Induction of two immunochemically related rat liver cytochrome P450 isozymes, cytochromes P450c and P450d by structurally diverse xenobiotics. J. Biol. Chem. 258:4590-4598.

Thomas, P.E., Reidy, J., Reik, L.M., Ryan, D.E., Koop, D.R., and Levin, W. (1984) Use of monoclonal antibody probes against rat hepatic cytochromes P450c and P450d to detect

immunochemically related isozymes in liver microsomes from different species. Arch. Biochem. Biophys. 235:239-253.

Voorman, R., and Aust, S.D. (1987) Specific binding of polyhalogenated aromatic hydrocarbon inducers of cytochrome P450d to the cytochrome and inhibition of its estradiol 2-hydroxylase activity. Toxicol. Appl. Pharmacol., in press.

Watkins, P.B., Wrighton, S.A., Schuetz, E.G., Maurel, P. and Guzelian, P. (1986) Macrolide antibiotics inhibit the degradation of the glucocorticoid responsive cytochrome P450p in rat hepatocytes in vivo and in primary monolayer culture. J. Biol. Chem. 261:6264-6271.

CONCLUSION

The focus of this thesis has been the interaction of planar polyhalogenated aromatic hydrocarbons with cytochrome P450d. My experiments revealed a specific, non-covalent, yet essentially irreversible association of HBB and sterically similar structures with cytochrome P450d. The ligands apparently bind to the active site of cytochrome P450d since its estradiol 2-hydroxylase activity was inhibited. Indeed, TCDD functioned as a tight-binding inhibitor having a Ki = 8 nM.

Furthermore I showed that HCB and isosafrole, both capable of forming a stable complex with cytochrome P450d, could increase the hepatic content of P450d beyond that maximially induced through the Ah receptor. The increased level of cytochrome P450d was shown to due to reduced turnover of the enzyme, presumably a result of enzyme stabilization by the ligands. It has been known for many years that isosafrole forms a stable complex with cytochrome P450d. My results suggest that HCB and other polyhalogenated aromatic hydrocarbons capable of binding to the Ah receptor can bind to cytochrome P450d in a manner similar to isosafrole. The reason for this binding is not clear. The inhibitors might be functioning as psuedo-substrates or transition-state analogues. Likewise the mechanism of turnover inhibition is not clear. It might be due to blockage of access to P450 heme; removal of heme might be a controlling step in P450 turnover.

Thus although this work has answered some questions it has also raised others. Much work is being done on the receptor-mediated mechanism of cytochrome P450 induction and much is already known about protein synthesis. However, very little is known about the mechanisms controlling protein degradation; it is in this context that further research on cytochrome P450 regulation could be informative and rewarding.